Oligomeric Structure of Cholera Toxin: Characteristics of the H and L Subunits

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SUMMARY

Structural analysis of cholera toxin by sodium dodecylsulphate polyacrylamide electrophoresis demonstrated two types of non-covalently linked subunits, heavy (H) and light (L), with respective molecular weights of 28 000 and 8000 to 9000. The H:L protein ratio was 1:2, indicating that the toxin of molecular weight 84 000 consists of 1 H and 6 or 7 L subunits, linked into an aggregate with non-covalent bonds. Cholera toxinoid, a natural toxin derivative, contained only the L subunits of the toxin. Reduction and alkylation cleaved the H but not the L subunit. The specific cleavage of the H subunit by reduction appeared to yield identical half-molecules; the smaller peptide seemed to originate from non-specific degradation. The H subunit also differed from L subunits by having a higher affinity for labelling with radioactive iodine and by precipitating below pH 3.5.

In immunodiffusion studies the toxin possessed antigenic determinants shared with the toxinoid as well as toxin-specific determinants. Comparative analyses with purified subunit preparations revealed that the toxinoid-shared determinants reside in the L-type of subunit and the toxin-specific ones in the H subunit.

By precipitation-in-gel, binding to ganglioside-coated tubes, and sodium dodecyl sulphate polyacrylamide electrophoresis it was demonstrated that the ability of toxin to attach to the apparent receptor ganglioside, G_{M1} , is similar to that of cholera toxinoid, and is due to the G_{M1} -binding ability of the L subunits. The toxin H subunit did not react with the G_{M1} ganglioside.

The results support our previous structural model for cholera toxin, and explain the antigenic and receptor-binding properties of the toxin in terms of component subunits.

INTRODUCTION

Strains of *Vibrio cholerae* of different serotypes (Inaba or Ogawa) produce immunologically identical diarrhoeogenic toxin (Holmgren, Lönnroth & Ouchterlony, 1971). This exo-enterotoxin has been isolated as a protein with molecular weight 84 000 (Finkelstein & LoSpalluto, 1969; Richardson, Evans & Feeley, 1970; LoSpalluto & Finkelstein, 1972). The isolation procedure also provides a 'natural' toxinoid, cholera toxinoid, with molecular weight 56 000 (Finkelstein & LoSpalluto, 1969). The toxin consists of non-covalently linked subunits of different size, heavy (H) and light (L), whereas in the toxinoid only the L subunit has been demonstrated (Holmgren, Lönnroth, Ouchterlony & Svennerholm, 1972; Lönnroth & Holmgren, 1973). However, somewhat surprisingly, cholera toxin does not differ immunologically from cholera toxinoid despite the difference in their subunit composition (Finkelstein & LoSpalluto, 1969; Lönnroth & Holmgren, 1973).

Both the toxin and the cholera toxinoid attach to intestinal mucosa (Peterson, LoSpalluto & Finkelstein, 1972) and the observation that bowel segments incubated with cholera toxinoid before challenge with toxin do not respond to the toxin indicates that both

proteins bind to the same tissue sites (Pierce, 1973; Holmgren, 1973*a*). Moreover, detailed analyses with various non-intestinal target cells have demonstrated that the toxin and the toxoid bind almost identically to mammalian cell membranes (Cuatrecasas, 1973*a*; Holmgren, Lindholm & Lönnroth, 1974*a*). Recent studies have indicated that a defined ganglioside, G_{M1} , functions as tissue receptor for cholera toxin (Holmgren, Lönnroth & Svennerholm, 1973*a, b*; Cuatrecasas, 1973*b*; King & van Heyningen, 1973). The oligosaccharide portion of the ganglioside appears to determine the specificity of the attachment, but the sphingosine portion is also important for the strength of binding with toxin (Holmgren *et al.* 1973*b*; Holmgren, Månsson & Svennerholm, 1974*b*). Cholera toxin also binds specifically to this ganglioside (Holmgren *et al.* 1973*a, b*).

In the present investigation the subunits of cholera toxin are characterized with regard to chemical properties, immunological relationships and G_{M1} ganglioside-binding ability. Additional data on the subunit structure of this important diarrhoeogenic bacterial toxin are presented, and the submolecular basis for the antigenic and receptor-binding properties of the toxin is elucidated.

METHODS

Culture filtrate. Lyophilized culture filtrate of *V. cholerae*, serotype Inaba, Lot No. 4493 G, was provided by the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A.

Cholera toxin. Isolated, highly purified cholera toxin, cholera toxin, prepared under contract for the National Institute of Allergy and Infectious Diseases by R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Texas, U.S.A. (Finkelstein & LoSpalluto, 1970) was used (Batch Nos. 1071 and 0572). The freeze-dried material was dissolved in sterile tris-EDTA buffer pH 7.5 (0.005 M-tris, 0.001 M- Na_2 EDTA, 0.0003 M- NaN_3 and 0.2 M- $NaCl$) to give a solution containing either 2 or 5 mg/ml, dispensed in small volumes and stored at $-20^\circ C$ until used.

Natural cholera toxoid. The 'natural' highly purified cholera toxoid, cholera toxin, prepared by R. A. Finkelstein, was dispensed and stored in the same manner as the toxin but at a concentration of 1 mg/ml (Finkelstein & LoSpalluto, 1970).

Ganglioside G_{M1} . The monosialosylganglioside G_{M1} was isolated from human brain by Svennerholm (1972).

Reference proteins and their molecular weights. Fresh human serum, human IgG (mol. wt 150000), human serum transferrin (mol. wt 88000), human serum albumin (mol. wt 68000), ovalbumin (mol. wt 46000), ribonuclease A from bovine pancreas (mol. wt 14500), cytochrome *c* from equine heart (mol. wt 12400), and chymotrypsin (mol. wt 10000) and insulin (mol. wt 6000) from bovine pancreas were used as references in the sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis and gel filtration runs.

Antisera. Antisera to the purified cholera toxin and toxoid were prepared in rabbits. Each animal received two or three subcutaneous injections each of 30 μg protein. The interval between injections was usually 3 weeks, but in a few animals there was a 4 month interval between the second and third injections. The antisera were obtained from cardiac puncture bleeding performed 1 or 2 weeks after the last injection.

Rabbit antisera to culture filtrates (crude toxin) of *V. cholerae*, serotypes Inaba or Ogawa, were produced as previously described (Holmgren *et al.* 1971).

Radioiodination. Cholera toxin, toxoid and toxin fractions were labelled with ^{125}I by the chloramine T method (Hunter & Greenwood, 1962). Carrier-free $Na^{125}I$ (specific activity > 14 mCi/ μg) was purchased from The Radiochemical Centre, Amersham, Buckingham-

shire. To 50 μg of the cholera protein in a volume of 10 μl was added 5 μl Na^{125}I (= 0.5 mCi) and usually 2.5, but occasionally 5, 10 or 20 μl chloramine T (10 mg/ml in distilled water; Merck, Darmstadt, West Germany.) After 1 min at 23 °C the reaction was stopped by adding 5 μl (or when more chloramine T had been used 7.5, 15 and 25 μl , respectively) of sodium metabisulphite, 10 mg/ml in phosphate buffered saline (PBS) pH 7.2 (0.05 M- Na_2PO_4 , 0.14 M- NaCl). Fifty μl 0.1 M-KI in tris-gelatin buffer pH 7.5 (0.1 M-tris, 0.2 % gelatin) was then added and the sample filtered through a 5×70 mm Sephadex G-50 column pre-equilibrated and eluted with tris-gelatin buffer. The protein fraction was collected and stored in samples at -20 °C.

Fractionation of toxin. Fractionation of toxin for preparative purposes was performed by incubation of the toxin in acid buffer followed by gel filtration or centrifugation. The procedures are fully described under Results.

Reduction and alkylation. The standard procedure for reduction of protein disulphide bridges was as follows. The protein was incubated with varying concentrations of freshly dissolved 2-mercaptoethanol (ME) at 23 °C for 90 min in 0.2 M-tris-HCl buffer pH 8.2. The reduced protein was then alkylated with 0.1 M-iodoacetamide in an ice bath for 30 min, desalted by filtration through a Sephadex G-25 column and freeze-dried.

On specified occasions reduction was performed with ME or dithiothreitol (DTT) in PBS pH 7.2, or in a buffer consisting of 2 % (w/v) SDS, 8 M-urea and 0.05 M-sodium phosphate, pH 7.2. When the latter buffer was used the alkylation was done at 23 °C to prevent cold precipitation of the SDS. In these modified procedures the reduced and alkylated protein was tested after a further five-fold dilution with the SDS-urea buffer without gel filtration or lyophilization.

SDS polyacrylamide electrophoresis. This was used to characterize the subunits in cholera toxin, toxoid and toxin fractions. The procedure and the densitometric quantitation after staining of the separated protein components has been described previously (Lönnroth & Holmgren, 1973). Radioactive material subjected to SDS polyacrylamide electrophoresis was quantitated by cutting the gel in 2.5 or 5 mm slices after the run and measuring their radioactivity in a Packard gamma counter.

Precipitation-in-gel. A microplate method was used for double diffusion-in-gel analyses of the ability of antisera or ganglioside G_{M1} to precipitate cholera toxin, toxoid or toxin subunits (Holmgren *et al.* 1973*b*).

Ganglioside sorbent immunoassay (GSIA). This technique was fully described by Holmgren (1973*a*). Briefly, G_{M1} ganglioside was incubated in 11×70 mm disposable polystyrene test tubes (Nunc, Roskilde, Denmark) at 37 °C for 4 to 5 h, which led to attachment of ganglioside to the tube wall without loss of its ability to bind cholera toxin. The coated tubes were then incubated with toxin, toxoid or subunit preparation to permit binding to the ganglioside. Non-attached material was removed by repeated washing. The bound toxin or toxoid was quantified by incubation with antitoxin antiserum, followed after washing by incubation with anti-IgG antibody conjugated with alkaline phosphatase, and finally, after further washing, by incubation with substrate for this enzyme (Holmgren & Svennerholm, 1973). If toxin or antigenically related material had become attached to the ganglioside-coated tubes, it bound antitoxin antibodies which in the subsequent reaction bound the enzyme-conjugated anti-immunoglobulin. When substrate for the enzyme was added a colour change developed which was proportional to the amount of toxin or related material bound to G_{M1} . The colour was measured spectrophotometrically at 400 nm and expressed as extinction change per 100 min ($E_{100 \text{ min}}$). Non-specific reactions were negligible.

Ganglioside sorbent radioassay (GSRA). Non-labelled toxin, toxoid or toxin subunits

Fig. 1. SDS polyacrylamide electrophoresis of (a) cholera toxin, (b) cholera toxinoid (c) toxin fraction 'H', and (d) toxin fraction 'L'. The presence of two types of subunits in the toxin, L (mol. wt 8000) and H (mol. wt 28000), the presence of only L-sized subunit in the toxinoid, and the isolation of L and H subunits by gel filtration of toxin in acid glycine buffer are apparent.

in tris-gelatin buffer were incubated in G_{M1} -coated tubes at 23 °C for 2 h on a roller drum, the tubes were washed three times with PBS containing 0.05 % Tween 20 (PBS-Tween), and then radiolabelled toxin in tris-gelatin buffer was added and the tubes incubated at 24 °C for another 2 h. After the incubation the tubes were washed 3 or 4 times with PBS-Tween and the radioactivity of the tubes measured in a gamma counter. This method, GSRA-inhibition, measures the binding to G_{M1} of the non-labelled cholera proteins as a function of their inhibition of the attachment of the added iodotoxin.

Toxicity assay. The toxic potency of cholera toxin preparations was determined by the rabbit intradermal assay of Craig (1965). This technique was also used to test consumption of toxin-neutralizing G_{M1} ganglioside by cholera toxinoid (Holmgren *et al.* 1973b).

RESULTS

Comparative studies of cholera toxin and cholera toxinoid subunits

Characterization by SDS polyacrylamide electrophoresis. The isolated toxin and toxinoid were studied by SDS polyacrylamide electrophoresis. When preincubation of the proteins in the 2 % SDS-urea buffer was performed at 55 °C, SDS polyacrylamide electrophoresis demonstrated an almost complete dissociation of the toxin into the two differently sized subunits H and L, and the toxinoid into L subunits alone (Fig. 1). At lower preincubation temperatures or SDS concentrations part of the L chains of both proteins remained aggregated and therefore migrated slowly in the gel. Based on comparisons with reference proteins ranging in molecular weight from 88000 to 6000 tested in the same plate, the positions of the subunit protein spots indicate that the molecular weights of the H and L subunits are about 28000 and 8000 to 9000, respectively. Densitometric measurements of the protein-stained spots gave a mean value of 2.0 for the L:H spot ratio, indicating that cholera toxin, mol. wt 84000, contains 1 H and 6 or 7 L subunits.

Toxin and toxinoid which had been radioiodinated were also analysed by SDS polyacrylamide electrophoresis. With toxinoid virtually all the radioactivity was found in a position corresponding to that of the L subunit. With toxin the distribution of radioactivity between

Table 1. *Influence of the amount of chloramine T on the radioiodine labelling of cholera toxin*

Chloramine T (μ g)	Yield* (%)	Toxicity† (blueing doses/ng)	Radioactivity in subunit‡ (μ Ci)	
			H	L
200	16	0.2	51	29
100	13	0.2	24	41
50	22	0.4	87	23
25	22	3	85	25

* Refers to proportion of added radioactivity precipitable with 10 % trichloroacetic acid.

† Mean values of rabbit intradermal assays where the mean activity of non-labelled toxin was 4 blueing doses/ng.

‡ Calculated from the distribution of radioactivity in H and L subunit positions in SDS polyacrylamide electrophoresis tests and expressed as μ Ci in the subunits of 50 μ g toxin.

the H and L subunit positions depended on the amount of chloramine T used in the radio-labelling procedure as illustrated in the experiment presented in Table 1. High concentrations of chloramine T gave a low yield of radioactive H subunit and resulted in parallel destruction of toxic activity (Table 1). Therefore, in the experiments to be described the lowest amount of chloramine T (25 μ g) was used.

Immunological studies. The immunochemical properties of cholera toxin and cholera-genoid toxoid were compared using double diffusion-in-gel immunoprecipitation. When tested against two different antisera to culture filtrate or against three different antisera to the toxoid, the toxin and the toxoid gave a single precipitation line which fused in an 'identity reaction' (Fig. 2*a, b*).

The pattern was the same with two out of four different antisera to isolated toxin, but different with the other two. With the latter antisera the toxoid still gave a single line but the isolated toxin gave two precipitates, one identifying with the 'toxoid line' and the other showing no relation to this precipitate (Fig. 2*c*). Both lines of the isolated toxin coalesced with the single toxin precipitate formed with culture filtrate, which indicates that both lines represent antigen factors present in the native toxin molecule (Fig. 2*d*).

Studies of ganglioside-binding ability. It was important to learn whether the ability of cholera toxin to bind to the ganglioside G_{M1} resided in the L or the H type of subunit or in both. Precipitation-in-gel analyses, and competitive binding studies evaluated by skin toxicity assays verified our previous observation that cholera-genoid toxoid binds to G_{M1} with an efficiency little different from that of the toxin (Holmgren *et al.* 1973*b*). Since the toxoid may be regarded as an aggregate of L chains exclusively, this indicated that the L type of subunit can bind to G_{M1} . This concept was further supported by quantitative binding studies of toxin and toxoid to G_{M1} -coated tubes (Fig. 3). Two techniques were used, GSIA, where the G_{M1} -coated tubes were incubated with different concentrations of the cholera proteins and the bound toxin or toxoid quantitated by immunoassay, and GSRA-inhibition where, after the incubation, the amount of G_{M1} -bound material was calculated from the extent of its inhibition of the binding of 125 I-labelled toxin to the tubes. In both systems the shapes of the binding curves of toxin and toxoid were similar, but the toxoid showed significant binding at a slightly lower concentration than the toxin (Fig. 3).

The influence of G_{M1} ganglioside on the behaviour of toxin and toxoid in SDS polyacrylamide electrophoresis was also studied since this could elucidate the effect of the ganglioside on the dissociated cholera protein subunits. It was found that the conditions

Fig. 2. Immunodiffusion studies of cholera toxin, cholera toxin toxoid and *V. cholerae* culture filtrate with antisera to these preparations. For comments see text.

regularly used to dissociate cholera toxin into its subunit constituents, i.e. incubation at 55 °C for 30 min in 2 % (w/v) SDS with 8 M-urea, could be modified to 53 °C for 15 min in 0.1 % SDS alone without loss of dissociation and separation of the H from the L subunits, although the disaggregation of the L subunit complex was incomplete. However, when toxin had been premixed with an equal concentration (w/v) of G_{M1} ganglioside the pattern

Fig. 3. Binding to ganglioside G_{M1}-coated plastic tubes of (●) cholera toxin and (○) cholera-genoid toxoid compared by (a) GSIA and (b) GSRA-inhibition. Each point is the mean value of three experiments performed in duplicate.

was different. In 0.1 % SDS the spots of dissociated as well as aggregated L subunit completely disappeared but the H subunit spot developed with unchanged protein staining intensity. In 1 % (w/v) SDS protein-staining material appeared in a position between that of the unaffected H subunit spot and that expected for the L subunit spot, with 'tailing' towards the H subunit region. In 4 % (w/v) SDS with 8 M-urea, the G_{M1}-mixed toxin was qualitatively identical to the PBS-mixed toxin in the SDS polyacrylamide electrophoresis tests.

Based on the information from the above experiments, titrations of the influence of G_{M1} ganglioside on the L subunit of toxin and toxoid were performed. The toxin was mixed with

Fig. 4. Evaluation by SDS polyacrylamide electrophoresis of the influence of G_{M1} ganglioside on the subunits of toxin and cholera toxin. The L subunit spot of both proteins is eliminated by the ganglioside, as is the spot of slowly-migrating L subunit complex, whereas the toxin H subunit spot is not specifically influenced by G_{M1} .

different concentrations of G_{M1} and after 1 h at 4 °C, SDS was added to a final concentration of 0.1 % and the sample heat-treated and examined by SDS polyacrylamide electrophoresis. Controls consisting of toxin mixed with PBS were likewise treated and examined in parallel. One of several similar experiments is illustrated in Fig. 4. The G_{M1} ganglioside had no specific effect on the migration of the toxin H subunit spot and the protein-staining intensity of the H subunit spot was usually unchanged by the ganglioside. In contrast, a specific pronounced effect on the L subunit was noted. As repeatedly determined by two- or threefold serial dilutions of G_{M1} , a ratio of ganglioside to toxin or toxoid of 1:10 or higher prevented the formation of the L subunit spot, whereas lower ratios gave increasingly less inhibition and a ratio of 1:60 or lower resulted in no inhibition of this spot. This means that a G_{M1} to toxin or toxoid molar ratio of about 1:5 effectively prevented the migration of the L sub-units.

Comparative studies of toxin H and L subunit fractions

Preparation. In accordance with previous studies (Lönnroth & Holmgren, 1973) incubation of toxin in 0.1 M-glycine-HCl buffer at pH 3.55 for 30 min followed by gel filtration through a 15 × 600 mm Sephadex G-100 column in this buffer at 20 °C resulted in dissociation and separation of toxin H and L subunits. When analysed with SDS polyacrylamide electrophoresis a fast-filtering fraction ('H') was found to contain almost pure H subunit and a slow-filtering fraction ('L') almost pure L subunit (Fig. 1). Fractions with intermediate filtration rates contained both subunits. Portions of fraction 'H' and fraction 'L' were also labelled with ^{125}I (using 25 μg chloramine T), and the iodinated proteins again tested by SDS polyacrylamide electrophoresis. These labelled fractions 'H' and 'L' migrated at the same rate as their unlabelled counterparts.

It was found that if the toxin (3 mg/ml) was incubated in glycine-HCl buffer pH 3.30, a

Fig. 5. Immunodiffusion analysis of cholera toxin (upper middle well) and purified preparations of L (upper left) and H (upper right) subunits with antiserum to toxin (lower wells). The upper dense toxin line, which identifies with choleragenoid toxoid (see Fig. 2c), gives a reaction of identity with the L subunit preparation and the fainter toxin-specific line (Fig. 2c) an identity reaction with the H subunit.

visible precipitation took place. The precipitate was sedimented by centrifugation, washed with the acid buffer and redissolved in 0.1 M-glycine-NaOH, pH 10. The redissolved sediment, and the supernatant (fractions 'sed' and 'sup') were tested by SDS polyacrylamide electrophoresis. Fraction 'sed' contained almost exclusively (> 95%) protein migrating as H subunit, and fraction 'sup' only protein which migrated as L subunit.

Immunological studies. Immunodiffusion analyses of the purified subunits in fractions 'H' and 'L' showed that the H and L subunits of cholera toxin constitute immunologically distinct entities. The antisera to isolated toxin which gave two lines with the toxin caused precipitation of both the purified L subunit and H subunit preparations (fractions 'L' and 'H'). The L chain precipitate identified with the precipitate of toxin which had shown identity with toxoid, whereas the H subunit precipitate identified with the 'toxin line' not shared with toxoid (Fig. 5). The antisera to toxoid as well as those anti-toxin antisera which only gave one precipitate with toxin reacted with the L subunit fraction of toxin but not with the H subunit fraction.

Fractions 'sup' and 'sed' were also tested by the double diffusion-in-gel technique against anti-toxin antiserum reacting with both the L and H subunits. With fraction 'sup' a sharp precipitation line, which identified with the toxoid-shared L-chain line of toxin was formed, and with fraction 'sed' a faint precipitate developed which identified with the H-chain line of the toxin. In addition, trace amounts of precipitinogenic material of L-chain type was demonstrated in fraction 'sed'.

Studies of ganglioside-binding ability. Fractions 'H' and 'L' and fractions 'sed' and 'sup' were tested by means of the double diffusion-in-gel technique for reactivity with pure G_{M1} ganglioside. Sharp precipitation lines were formed with the 'L' and 'sup' fractions which 'identified' with the precipitation line formed between G_{M1} and toxin or toxoid. No reaction with G_{M1} could be demonstrated for fractions 'H' and 'sed'. In comparative analyses the ganglioside-L subunit precipitate showed signs of fusion with the previously-described L chain immunoprecipitate between antisera and toxin, toxoid or L subunit preparation. The antibody precipitate gave a 'spur reaction' over the ganglioside precipitate.

The G_{M1} -binding ability of the purified H and L subunit preparations was also studied using G_{M1} -coated test tubes. When examined by both immunoassay (GSIA) and radioassay (GSRA-inhibition) fraction 'L' showed a G_{M1} -binding capacity which resembled that of intact toxin or toxoid (Table 2). Fraction 'H' was virtually without G_{M1} -binding ability,

Table 2. *Binding to G_{M1} ganglioside of L but not H subunit of toxin*

Cholera protein	Concentration required for 50 % binding to G _{M1} (ng/ml)	
	GSIA	GSRA-inhibition
Toxin	22	21
Toxoid	8	8
Fraction 'L'	14	22
Fraction 'H'	1000	890

since a reaction was only demonstrated at concentrations where the effect was probably caused by contaminating L subunit material (Table 2).

G_{M1}-coated tubes for purification of H subunit. The consistent finding in the various types of experiments described that it is the L subunit of cholera toxin which is binding to G_{M1} ganglioside, whereas the H subunit is non-reactive with the ganglioside, suggested the possibility of using G_{M1}-coated tubes to eliminate contaminating L subunit in preparations of H subunit. A procedure was developed which gave improved isolation of electrophoretically and immunologically pure H subunit: a 200 μ l sample of toxin (5 mg/ml) is mixed with 100 μ l of 1 M-glycine-HCl (pH 3.8) and incubated at room temperature for 30 min; it is then subjected to filtration through a 15 $\times$ 600 mm Sephadex G-100 column using 0.1 M-glycine-HCl (pH 3.8) as eluting buffer. The higher pH employed in this modified fractionation procedure gives a much better yield of the labile H subunit but less complete separation of this subunit from L-chain material. The fractions are dialysed against neutral volatile buffer (0.05 M-ammonium acetate pH 6.5) and portions lyophilized and examined for their subunit content by means of SDS polyacrylamide electrophoresis. Fast-filtering fractions which are rich in H subunit (> 50 %) are passed through a series of tubes coated with G_{M1} ganglioside, with a 15 min incubation period at room temperature in each tube. Usually 15 tubes are sufficient to remove all L subunit, but if activity is demonstrated in the last tube by GSIA after the passage, the preparation is transferred through additional tubes.

This procedure of selective removal of L subunit material from toxin fractions has been repeated many times and found to be a convenient method for isolating H subunit, which is pure as tested both by SDS polyacrylamide electrophoresis and immunodiffusion tests.

Influence of reducing agents on cholera toxin studied by SDS polyacrylamide electrophoresis

Protein-staining components. Reduction of cholera toxin by ME or DTT followed by alkylation with iodoacetamide had different effects on the H and L subunits as evaluated by SDS polyacrylamide electrophoresis and protein-staining of the plates. The reducing agents aided the dissociation of the L subunit complex by SDS in a manner similar to that described for heat treatment, but did not cleave the dissociated L subunits. By contrast, the H subunit spot disappeared on reduction, and a proportional increase of a new spot was observed indicating cleavage of disulphide-linked H subunit components (Fig. 6a). The position of this spot denoted protein of molecular weight 18000 when compared with the non-reduced reference proteins, but this is probably too high a value since the further uncoiling of proteins by reduction has been reported to lower their mobility in SDS polyacrylamide electrophoresis (Griffith, 1972). The previously reported linear relationship between this conversion of the H subunit and the logarithm for the concentration of ME

Fig. 6. Influence of reduction and alkylation on the H and L subunits of cholera toxin as tested by SDS polyacrylamide electrophoresis and protein staining of the gel. (a) Comparison of unreduced toxin and of toxin reduced with different concentrations of ME. A ME-concentration-dependent split of the H subunit, mol. wt 28 000, to protein of mol. wt 18 000 is evident. The L subunit spot is qualitatively unchanged by the reduction. (b) Two-dimensional SDS polyacrylamide electrophoretic analysis. SDS-urea treated cholera toxin was applied in the basin (S) and run from left to right which separated the H and L subunits as denoted (first separation). Thereafter the gel was soaked at 23 °C for 1 h in electrode buffer added with 0.2 M-ME, and a second run performed at right angles giving a separation of the reduced subcomponents. For reference purposes the positions of protein spots obtained with toxin rested in parallel in the second separation are indicated (for comments see text).

Table 3. *Radioactivity of cholera toxin components after separation by SDS polyacrylamide electrophoresis of reduced and unreduced ¹²⁵I-toxin*

Samples of ¹²⁵I-toxin, 20 µg/ml, were incubated with reducing agent or with volume adjusting PBS alone at 23 °C for 90 min, and then alkylated in an ice bath with 0.1 M-iodoacetamide. After five-fold dilution with SDS-urea buffer followed by heating at 55 °C for 15 min, 10 µl of each sample were separated by SDS polyacrylamide electrophoresis, and the gel serially sliced in 2.5 mm sections which were analysed for radioactivity. From the stained protein spots of cholera toxin and the reference proteins tested in parallel in the plate, the molecular weights of the radioactive components were calculated. The radioactivity of each component peak is expressed as a percentage of the total activity for the gel slices of the entire separation; the recovery was very similar for the reduced and unreduced samples. Four different concentrations of each reducing agent were tested, but since all gave very similar results the values for each agent are quoted as mean ± S.E.M. for the four tests. Experiments not shown in the Table, where reduction-alkylation was performed in the SDS-urea buffer, gave similar data.

Reducing agent	Percentage of activity positioned as having mol. wt. of:			
	> 50000	28000	18000	8000
None	7.3 ± 0.3	63.1 ± 1.0	6.5 ± 0.4	21.7 ± 1.3
Dithiothreitol (20–60 mM)	2.3 ± 0.6	5.5 ± 0.3	63.7 ± 1.0	27.8 ± 0.9
2-Mercaptoethanol (200–1000 mM)	0.9 ± 0.2	4.7 ± 0.6	63.8 ± 0.5	28.7 ± 0.3

(Lönnroth & Holmgren, 1973) was confirmed; a parallel shift towards approximately fivefold lower ME concentrations was seen in the presence of 2 % SDS–8 M-urea.

Preliminary studies by Cuatrecasas, Parikh & Hollenberg (1973) and ourselves had shown smaller fragments of reduced isolated H subunit similar in size to L subunit, which suggested the possibility that the reductive cleavage of the H subunit resulted in two differently sized peptides. This was examined in three experimental systems. The H subunit was isolated by eluting an appropriate section of SDS polyacrylamide gel in which cholera toxin had been separated. After dialysis against distilled water and lyophilization, the material was again tested by SDS polyacrylamide electrophoresis both before and after reduction with ME. Reduced samples showed the expected ME concentration-dependent conversion of the 28000 dalton material of non-reduced samples to the '18000 dalton' component; L-sized material appeared in unpredictable amounts in both unreduced and reduced samples.

Another experimental approach was to separate toxin by SDS polyacrylamide electrophoresis, soak the gel in ME–SDS and perform a second electrophoretic run at right angles (see legend to Fig. 6*b*). This gave excellent separation of component proteins. Densitometry on the stained plate showed a protein ratio between the true L subunit spot and the '18000

Fig. 7. Influence of reduction and alkylation on isolated ¹²⁵I-labelled cholera toxin components as tested by SDS polyacrylamide electrophoresis. (a) Radiolabelled H subunit was isolated by elution with PBS of appropriate gel slices from the SDS polyacrylamide electrophoretic separations of unreduced ¹²⁵I-toxin described in Table 3. After dialysis against distilled water at 4 °C for 20 h, two equal portions were lyophilized, redissolved in 2 % SDS–8 M-urea buffer and 2 % SDS–8 M-urea 0.5 M-ME buffer, respectively, and incubated at 23 °C for 90 min. Iodoacetamide (0.1 M) was added, and after incubation at 23 °C for 30 min and 56 °C for 15 min, 15 µl of the samples were separated by SDS polyacrylamide electrophoresis. The radioactivity of each gel slice is expressed as a percentage of the total activity for the separation. Recovery for the reduced sample was four-fifths of that for the unreduced portion. The solid arrows indicate the positions of reference cholera toxin H and L subunits, and the broken arrow the position of '18000 dalton' component of reduced reference toxin tested in parallel. The hatched area shows the position and relative radioactivity of the gel slices of Table 3 chosen for elution. (b) Similar isolation, treatment and testing of '18000 dalton' peptide from the separations of DTT-reduced and ME-reduced ¹²⁵I-toxin presented in Table 3. Arrows as in (a). Recovery for reduced samples was two-fifths to three-fifths of that for the unreduced portions. For comments see text.

Fig. 7(a) and (b). For legend see opposite.

dalton' component spot of 2.0, and together these spots constituted more than 90 % of the stained protein (Fig. 6*b*). This L:'18000' ratio is identical with the L:H subunit ratio of unreduced toxin, which indicates a stoichiometric conversion of the H to the '18000 dalton' peptide. Only a trace of L-sized fragment of the H subunit was observed using this mild separation technique (Fig. 6*b*).

Finally, experiments were performed in which toxin was premixed with G_{M1} ganglioside, reduced and tested by SDS polyacrylamide electrophoresis under the conditions described for the experiment illustrated by Fig. 4. In no instance was any L-sized protein seen with reduced toxin when the G_{M1} concentration was sufficient to eliminate completely the L subunit spot of unreduced G_{M1} -mixed toxin tested in parallel. The ME concentration-dependent conversion of the H subunit to the '18000 dalton' component took place both in the presence and absence of G_{M1} .

Radioactive components. Reduction-alkylation studies were undertaken also with ^{125}I -toxin, labelled predominantly in the H subunit. Table 3 presents a quantitative evaluation of SDS polyacrylamide electrophoretic changes in toxin components caused by reduction with DTT or ME. With both agents the conversion of the H subunit into '18000 dalton' peptide was almost quantitative, and a small increase of radioactivity in the 'L subunit region' compared with unreduced samples was associated with a decrease of aggregated radioactive material (> 50000 daltons).

Experiments with toxin components isolated by elution from appropriate gel sections after SDS polyacrylamide electrophoretic separation of unreduced or reduced radioactive toxin gave further information on the interrelationships of 28000 dalton-H subunit and 'smaller conversion products' (Fig. 7). Of the H subunit, less than half of the radioactivity migrated as intact 28000 dalton component in SDS polyacrylamide electrophoresis after dialysis and freeze-drying; of the remainder, one-third had a mobility denoting a molecular weight of 18000 and two-thirds a migration rate similar to that of L subunits (Fig. 7*a*). After reduction all 28000 dalton material had been replaced by increased activity in the 18000 dalton area, but with no increase of smaller-sized material (Fig. 7*a*). Radioactive 18000 dalton' components of ^{125}I -toxin obtained by reduction with DTT and ME respectively were likewise isolated and examined in parallel (Fig. 7*b*). With both preparations, in the absence of reducing agent, part of the material had a migration suggesting recombination into intact H subunit and part of it a mobility indicating a split. Reduction with DTT or ME removed the H subunit-like peptide, increased the '18000-dalton' component and decreased the smaller-sized material (Fig. 7*b*).

DISCUSSION

The present investigation demonstrates that the H and L subunits of the toxin are immunologically distinct, apparently non-related entities. This was evident from the immunodiffusion analyses where purified subunit preparations were tested against antisera to isolated toxin. The precipitating reactivity of these immune sera with both types of subunits explains why they gave two precipitation lines with the isolated toxin but only one of these lines with choleragenoid toxoid. The precipitate shared between toxin and toxoid represented the antigenic determinants of L subunit, and the toxin-specific precipitate represented H subunit which had probably dissociated from the toxin. Both of these toxin immunoprecipitates gave reactions of identity with the single precipitate formed between the double-reactive antitoxin antisera and culture filtrate of *V. cholerae*. This suggests that the 'native' toxin in the filtrate is less prone than the isolated protein to release its

H subunit, a process which appears to take place particularly when isolated toxin is diluted to the low concentrations used in the immunodiffusion experiments. Our observation that all immune sera against crude as well as isolated toxin contained precipitating antibodies to the L subunit determinants, but only certain antisera to isolated toxin contained antibodies precipitating with the H subunit, may explain why previous attempts to demonstrate antigenic differences between toxin and cholera toxinoid have failed.

It is clear, as shown by three different techniques – precipitation-in-gel, binding to G_{M1} -coated tubes in two modifications, and SDS polyacrylamide electrophoresis – that it is the L type of subunit which binds to the ganglioside G_{M1} , whereas the H subunit does not do so. Since the G_{M1} ganglioside in all probability functions as tissue receptor for cholera toxin, the results confirm the previous suggestion (Holmgren *et al.* 1973*b*) that the membrane-combining site(s) of the toxin is located in the L, and not the H, subunit. Further support for this concept has been provided by the recent demonstration that purified L subunit but not H subunit binds to mammalian cell membranes (Holmgren *et al.* 1974*a*).

In their original report on the subunit structure of cholera toxin, Lönnroth & Holmgren (1973) proposed that the toxin (mol. wt 84000) consisted of one H subunit and several, probably about seven, L subunits. This model was based on the molecular weights of 28000 for the H subunit and approximately 8000 for the L subunit calculated from SDS polyacrylamide electrophoresis tests together with a protein staining intensity ratio of 1:2 for the H:L subunit spots in these experiments, data which suggest a molar ratio of 1:7 for H:L. This protein ratio is supported by the present study, where also the increased number of reference proteins tested, including low molecular weight proteins like chymotrypsin and insulin, give additional validity to the molecular weight values we have reported for the H and L subunits. The dissociation of H and L subunits in SDS-urea or in acid-glycine buffer indicates that only non-covalent bonds link the subunits together; apparently the L subunits are held together more strongly than is the H subunit to the L subunit complex, e.g. heating is required for the complete dissociation of the L subunits in SDS.

Cuatrecasas, Parikh & Hollenberg (1973) and van Heyningen (1974) have presented results which agree with the general features of the subunit structure we have proposed for cholera toxin. Apparently the A and C peptides of Cuatrecasas *et al.* (1973) correspond to aggregated and dissociated L subunits, respectively, and the B peptide to the H subunit; van Heyningen (1974) designates the H and L subunits A and B respectively. In both of these studies the observation (Lönnroth & Holmgren, 1973) that the H but not the dissociated L subunit is cleaved by reduction and alkylation has been confirmed. However, whereas our data (Lönnroth & Holmgren, 1973) suggested that the H subunit was split quantitatively into peptide migrating in SDS polyacrylamide electrophoresis, with a rate denoting a molecular weight of 18000 (probably representing a cleavage into half-molecules), Cuatrecasas *et al.* (1973) and van Heyningen (1974) found evidence for a split into two differently sized peptides. The extensive re-examination of this problem in the present investigation supports our previous concept that the specific effect of reduction and alkylation is a quantitative conversion of the 28000 dalton H subunit into equally sized, probably identical, peptides. These peptides would have a molecular weight of 14000, but since there is evidence for the retardation of reduced compared with unreduced peptides in SDS polyacrylamide electrophoresis (Griffith, 1972) the denoted molecular weight of 18000 for the conversion product of reduced H subunit is compatible with this interpretation. The pertinent observations favouring this view are: (i) the quantitative conversion of H subunit to the '18000 dalton' peptide by reduction, seen in the experiment where SDS electrophoresis was performed in two directions and in the experiments with radioactive toxin; (ii) the spontaneous

aggregation of isolated radioactive '18000 dalton' peptide into material behaving like intact H subunit; (iii) the appearance in the SDS polyacrylamide electrophoresis gels of only the '18000 dalton' peptide when reduced toxin was tested in mixtures with L subunit-removing G_{M1} ganglioside. The data presented may also explain the origin of L-sized material formed by treatment of H subunit preparations with reducing agents. When H subunit is isolated by SDS polyacrylamide electrophoresis, it appears prone to non-specific degradation. H subunit isolated by G_{M1} absorption may contain aggregated L subunit, the dissociation of which in SDS is supported by the reducing agents.

The observation that cholera toxin is an oligomeric protein containing different subunits has helped to elucidate the toxin's biological activities (Lönnroth & Holmgren, 1973; Holmgren *et al.* 1973*b*, 1974*a*; Cuatrecasas *et al.* 1973; van Heyningen, 1974). We hope that the further characterization of the component subunits in the present study will add to this understanding and also provide a fruitful approach to a finer structural analysis.

A condensed presentation of the data of this report was given at the 9th Joint U.S.-Japan Cholera Conference, Grand Canyon, U.S.A., October 1973 (Holmgren, 1973*b*). We thank Dr R. A. Finkelstein for a gift of cholera toxin and for preparing the isolated toxin we received from the National Institute of Allergy and Infectious Diseases. We also thank Dr L. Svennerholm for generous gifts of G_{M1} ganglioside. The skilled technical assistance of Gun Wallerström and Ulla Westerberg-Berndtsson is gratefully acknowledged. The study was supported by grants from the Swedish Medical Research Council (Nos. B74-16X-3382-04 and B74-40P-3591-03), the Medical Faculty of the University of Göteborg, and the Walter, Ellen and Lennart Hesselman Foundation for Scientific Research.

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