Protein Reagent Modification of Cholera Toxin: Characterization of Effects on Antigenic, Receptor-binding and Toxic Properties

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(Received 6 March 1975; revised 12 June 1975)

SUMMARY

The effects of protein modification procedures on the biologically most important properties of cholera toxin, i.e. the toxic activity, the G_{M1} receptor-binding capacity and the antigenic (antibody-fixing) properties, have been studied quantitatively using microgram amounts or less of toxin protein.

Most of the 24 group-specific reagents used had either no inhibitory effect on the toxic or the combination of G_{M1} -binding and antibody-fixing properties of cholera toxin, or they had a concomitant inhibitory effect on these activities. Separate testing of G_{M1} - and antibody-binding revealed a close, but not absolute, structural association between these properties. Amino group reactive substances were particularly effective in decreasing the G_{M1} -binding activity, while leucine aminopeptidase had no effect. This suggests that lysine residues may be involved in binding toxin to the acidic G_{M1} receptor.

Sodium dodecylsulphate and mercaptoethanol, which caused dissociation of the subunits of cholera toxin as indicated by polyacrylamide gel electrophoresis, abolished toxicity without inhibiting the concomitant G_{M1} - and antibody-binding properties of the toxin. Similar differential effects were also obtained with three reagents which did not seem to change the aggregation state of the toxin. These substances all had specificity for arginine, suggesting that arginyl residues of the toxin molecule may be involved in a 'toxic site' distinct from the receptor-binding site(s). A selective effect on the toxic site was also found by treating the toxin with carboxypeptidase or trypsin in the presence of urea; in the absence of urea no enzymic effect on any toxin property was noted.

INTRODUCTION

Recent research has shed much light on the biochemical aspects of cholera. The cholera-genic agent has been identified as an antigenic, neutral protein exotoxin with a molecular weight of 84000, and has been obtained in a pure form (Finkelstein & Lo Spalluto, 1970; Lo Spalluto & Finkelstein, 1972). The activity of the toxin seems to be due to its ability to activate adenylate cyclase in the small bowel mucosa, leading to a dramatic increase of intestinal electrolyte and fluid secretion which results in profuse diarrhoea (Field *et al.* 1972; Schafer *et al.* 1970; Sharp & Hynie, 1970; Pierce, Greenough & Carpenter, 1971). When added to other tissues the toxin causes various effects all of which, however, appear to be secondary to enhanced formation of cyclic 3',5'-adenosine monophosphate in the responding cells (Pierce *et al.* 1971). The local development of increased vascular permeability in the skin of rabbits following intradermal injection of cholera toxin is the basis for a widely employed assay of toxicity (Craig, 1975).

Rapid tight binding to the outer membrane of target cells precedes the biological action

of the toxin (Cuatrecasas, 1973*a*; Holmgren, Lindholm & Lönnroth, 1974) and it is probable that the membrane binding site is the ganglioside G_{M1} (monosialosyl-*N*-tetra-glycosylceramide) (Holmgren, Lönnroth & Svennerholm, 1973; Cuatrecasas, 1973*b*; King & van Heyningen, 1973). In a functional characterization of the toxin it is important to find out how its structure determines both receptor-binding and toxic properties. The relationship between structure and antigenicity is also of obvious importance in protective immunity and immunoprophylaxis. One heavy (H) and about seven light (L) subunits linked non-covalently make up a toxin molecule (Lönnroth & Holmgren, 1973; Cuatrecasas, Parikh & Hollenberg, 1973; van Heyningen, 1974; Holmgren & Lönnroth, 1975). The L-type of subunit is responsible for the cell-binding and G_{M1} -ganglioside-binding ability of the toxin and also for most of its antigenicity (Holmgren *et al.* 1974; Holmgren & Lönnroth, 1975). The H subunit, which is also antigenic, is necessary for the toxicity and probably represents the portion of the toxin which, directly or via an intermediate reaction, activates cellular adenylate cyclase (Lönnroth & Holmgren, 1973; Cuatrecasas *et al.* 1973; Holmgren *et al.* 1974). Other authors have used a different subunit nomenclature: Cuatrecasas *et al.* (1973) name the aggregated L subunits of cholera toxin 'A', the dissociated L subunits 'C', and the H subunit 'B'; van Heyningen (1974) calls the L and H subunits 'B' and 'A', respectively.

We have applied residue-specific protein modification procedures, commonly used in enzymology, to prepare numerous derivatives of the toxin. Methods have been devised which permit the quantitative study of these modifications on the toxic, receptor-binding and antigenic properties, using only micrograms or less of the cholera toxin derivatives. A preliminary report has been published (Lönnroth & Holmgren, 1974).

METHODS

Cholera toxin. Highly purified cholera toxin, prepared from *Vibrio cholerae* strain 569B by R. A. Finkelstein, Dallas, Texas (Finkelstein & Lo Spalluto, 1970), was obtained from the National Institutes of Allergy and Infectious Diseases, Bethesda, Maryland, U.S.A. The freeze-dried material was dissolved in sterile tris-EDTA buffer, pH 7.5 (0.05 M-tris, 0.001 M- Na_2 -EDTA, 0.003 M- NaN_3 , 0.2 M- $NaCl$) to give a solution containing 5 mg/ml which was dispensed in small volumes and stored at $-70^\circ C$ until used.

^{125}I -labelled cholera toxin. Cholera toxin was 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, Buckinghamshire. To 10 μl of cholera toxin (5 mg/ml in tris-EDTA buffer) was added 5 μl $Na^{125}I$ ($= 0.5$ mCi) and 2.5 μl chloramine T (10 mg/ml in distilled water; Merck, Darmstadt, Germany). After 1 min at $23^\circ C$ the reaction was stopped by adding 5 μl sodium metabisulphite [10 mg/ml in phosphate buffered saline (PBS; 0.05 M- Na_2PO_4 , 0.14 M- $NaCl$, pH 7.2)]. Fifty μl 0.1 M-KI in tris-gelatin (TG) buffer (0.1 M-tris, 0.2 %, w/v, gelatin, pH 7.5) was then added and the sample filtered through a 5×70 mm Sephadex G-50 column pre-equilibrated and eluted with the TG buffer. The protein fraction was collected and portions stored at $-70^\circ C$.

Fresh ^{125}I -toxin preparations were made each month, and for each preparation dilution experiments were done to ensure that the radioiodination had not influenced the cell-binding (Holmgren *et al.* 1974) or the G_{M1} -binding properties (see below), and that skin toxicity (see below) was virtually unaffected.

Vibrio cholerae culture filtrate. Culture filtrate from *V. cholerae* strain 569B was obtained from the National Institutes of Allergy and Infectious Diseases, Bethesda (Lot 4493 G).

Anti-cholera toxin. Antiserum to cholera toxin was prepared in a rabbit. Three injections

Table 1. *Reagents and procedures for group-specific modification of cholera toxin*

Reagent	Source	Incubation conditions*	Reference
Acetylimidazole	Sigma	10 mM in 0.1 M-phosphate pH 7.0	Riordan & Vallee (1967 <i>b</i>)
5-Amino-1 H-tetrazole	Merck	10 mM in 0.1 M-borate pH 9.2	Horinishi <i>et al.</i> (1964)
Bromosuccinimide	Sigma	0.01–10 mM in 0.2 M-acetate pH 6.0	Ramachandran & Witkop (1967)
2,3-Butanedione	Merck	0.1–10 mM in phosphate pH 7.0 (3 h)	Huang & Tang (1972)
1,2-Cyclohexanedione	Pierce	0.1–10 mM in 0.04 M-sodium hydroxide	Toi <i>et al.</i> (1967)
4-Diazobenzenesulphonic acid	Pfaltz & Bauer	0.1–10 mM in 0.1 M-borate pH 9.2	Ishikura <i>et al.</i> (1959)
1,5-Difluoro-2,4-dinitro- benzene	Sigma	10 mM in 0.1 M-phosphate– 5 % ethanol pH 7.0†	Wold (1967)
4,4-Difluoro-3,3-dinitro- diphenylsulphone	Pierce	10 mM in 0.1 M borate–10 % acetone pH 9.2†	Wold (1967)
Diisopropylfluorophosphate	Koch–Light	1–10 mM in 0.05 M-tris– 5 % isopropanol pH 7.5†	Cohen <i>et al.</i> (1967)
Ethyl-3,3-dimethyl-amino- propylcarbodiimide	Sigma	10 mM in 0.15 M-NaCl pH 4.3	Hoare & Koshland (1966)
Ethyl-3,3-dimethyl-amino- propylcarbodiimide and glycine- <i>O</i> -methylester	Sigma	As above, with 50 mM-glycine- <i>O</i> -methylester	Hoare & Koshland (1966)
<i>N</i> -ethylmaleimide	Sigma	10 mM in 0.1 M-phosphate pH 7.0	Riordan & Vallee (1967 <i>a</i>)
Glutaraldehyde, grade IV	Sigma	0.1–10 mM in 0.1 M-phosphate pH 7.0	Wold (1967)
Glyoxal	Sigma	10 mM in 0.2 M-ethyl- morphiline pH 9.2	Takahashi (1968)
2-Hydroxy-5-nitro-benzyl bromide	Sigma	10 mM in 0.1 M-phosphate– 5 % acetone pH 7.0 (1 min)†	Horton & Koshland (1967)
Iodoacetamide	Merck	10–100 mM in 0.2 M-tris pH 8.2	Gurd (1967)
2-Mercaptoethanol	Merck	1–100 mM in 0.2 M-tris pH 8.2	Hirs (1967)
2-Mercaptoethanol; iodoacetamide	Merck	100 mM-2-mercaptoethanol. Thereafter 10 mM iodo- acetamide for 15 min at 0 °C	Hirs (1967)
2-Methoxy-5-nitro-benzyl bromide	Sigma	10 mM in 0.1 M-phosphate– 5 % acetone pH 7.0†	Horton & Koshland (1967)
S-Methylisothiurea sulphate	Pfaltz & Bauer	10 mM pH 11.0, 4 °C (24 h)	Hughes, Saroff & Carney (1949)
Phenanthrenequinone	Merck	0.001–10 mM in 0.04 M- NaOH, 5 % ethanol†	Yamada & Itano (1966)
Phenylglyoxal	Koch–Light	10 mM in 0.2 M-ethyl- morphiline pH 8.0	Takahashi (1968)
Picrylsulphonic acid	Sigma	0.1–10 mM in 0.1 M-borate pH 9.2	Okuyama & Satake (1960)
Succinic anhydride	Sigma	10 mM in 0.1 M-phosphate pH 7.0	Klotz (1967)
Tetranitromethane	Sigma	10 mM in 0.2 M-tris–5 % ethanol pH 8.2†	Riordan, Sokolovsky & Vallee (1966)

* Unless specified incubation was performed at 23 °C for 1 hour.

† The reagent was first dissolved in the organic solvent and then added to the buffered toxin solution.

of 30 μg of toxin were given subcutaneously at intervals of 3 weeks; the blood was collected two weeks after the last injection. The serum contained precipitating antibodies against the L as well as the H subunits of the toxin as shown by double diffusion-in-gel analyses (Holmgren & Lönnroth, 1975).

Ganglioside G_{M1} . The ganglioside G_{M1} was purified from human brain by L. Svennerholm, Göteborg, Sweden (Svennerholm, 1972).

Protein modification. Table 1 presents the amino acid 'specific' protein modification reagents used, their sources, and the incubation conditions employed for toxin treatment. A 12 μM (1 mg/ml) solution of cholera toxin was prepared by dilution in distilled water and was further diluted 1:10 in the appropriate buffer containing the protein-modifying reagent. The mixture, total volume 50 μl , was incubated as specified for each reagent (Table 1). The standard reagent concentration was 10 μM , but if an effect on any of the toxin properties studied was observed then additional toxin derivatives were prepared using other reagent concentrations. The reaction was stopped and the reactants separated by filtration through a 5×70 mm Sephadex G-15 column. The protein fraction was collected and diluted in TG buffer to a final concentration of 0.024 μM (2 $\mu\text{g}/\text{ml}$) cholera protein. It was divided into small portions which were stored at -20°C until tested at multiple concentrations. The control test for each reaction consisted of incubating toxin under the appropriate conditions but without the modifying reagent. The control was gel-filtered, diluted and stored like the test preparation.

Lyophilized *V. cholerae* culture filtrate was dissolved to 200 mg/ml in distilled water, dialysed against 0.05 M-NaCl and concentrated to give a solution containing 20 mg bacterial protein/ml. This preparation, which contained approximately 0.2 mg cholera toxin/ml, was treated with chemical reagents as described for the purified toxin.

Enzymic treatment. Three enzymes (Sigma) were used: leucine aminopeptidase type III (86 u/mg) at a final concentration of 0.67 mg/ml, carboxypeptidase A DFP (47 u/mg) at 7 mg/ml, and trypsin type III (100 u/mg) at 1 mg/ml. Each enzyme was incubated with 3.8 μM -cholera toxin (0.33 mg/ml) at 37°C for 4 h in 0.1 M-N-ethylmorpholine-HCl, 0.001 M-MgCl₂, pH 8.4, with or without 2 M-urea. The reaction was stopped by a 250-fold dilution of the sample in 0.2 M-ammonium formate pH 4.5, containing 0.2 % (w/v) gelatin. The solution was immediately divided into small portions which were stored at -70°C . Three controls were prepared for each test: (i) a portion of the enzyme and toxin mixture taken at zero time, (ii) the enzyme, and (iii) the toxin incubated separately under the same conditions as the test sample.

Toxicity assay. The toxic activities of the toxin derivatives and of their control preparations were determined by testing multiple concentrations with the rabbit intradermal assay (Craig, 1965). To minimize errors due to the different sensitivities of individual rabbits, treated and control toxin were always compared in the same animal. The concentration required for a blueing reaction 7 mm in diameter (one blueing dose) was calculated for each preparation and the effect on toxicity of each chemical treatment expressed as the ratio of control to test values. A ratio of 1.0 meant no effect and a ratio of 0.0 a complete abolition of toxicity.

Combined assay of G_{M1} - and antibody-binding. The G_{M1} sorbent immunoassay technique (Holmgren, 1973) measures the *in vitro* binding capacity of cholera toxin to polystyrene-attached G_{M1} ganglioside, combined with the ability of the bound toxin to bind subsequently added specific antibody; the principal steps are shown in Fig. 1(a). Multiple concentrations of treated and control toxin preparations were assayed and the values expressed as extinction change per 100 min, plotted against the concentration. The concentration C_0 of the control

Fig. 1. Reaction steps in the combined and separate assays of cholera toxin binding to G_{M1} ganglioside or specific antibody *in vitro*: (a) ganglioside sorbent immunoassay for measurement of combined G_{M1} - and antibody-binding; (b) competitive ganglioside sorbent radioassay for measurement of G_{M1} -binding; (c) enzyme-linked immunoassay inhibition for estimation of antibody-binding. Hatched area is the inside of plastic tube, and 'toxin' is toxin derivative prepared by protein modification. The anti-rabbit IgG (antigamma) was prepared in a goat.

Fig. 2. *In vitro* assays of control cholera toxin and of toxin derivatives prepared by treatment with different concentrations of picrylsulphonic acid: ●, 0 mM (control toxin); ○, 0.1 mM; △, 1 mM; □, 10 mM. Dotted lines and the arrows show how the concentration C_0 of untreated toxin and the concentration C_t of one of the toxin derivatives are calculated. These concentrations are used to express the relative activity of the toxin derivative compared with the control preparation: (a) relative G_{M1} + antibody-binding activity ($= C_t/C_0$); (b) relative G_{M1} -binding activity ($= C_0/C_t$); (c) relative antibody-binding activity ($= C_t/C_0$).

preparation which would give a half-maximal extinction change was calculated. The extinction value for the treated preparation at this concentration was transposed to the control preparation curve, see Fig. 2(a). C_t is the concentration of untreated toxin that gives the same extinction value as treated toxin at concentration C_0 . The relative G_{M1} + antibody-binding activity of the treated preparation is expressed by the ratio C_t/C_0 , where a ratio of 1.0 means no effect by the modification procedure and 0.0 a complete abolition of one or both of the properties tested.

Assay of G_{M1} -binding. G_{M1} -binding was measured with a G_{M1} -sorbent radioassay, in which unlabelled treated toxin competed with ^{125}I -toxin for binding to solid-phase G_{M1} ganglioside (Fig. 1b). As described for the G_{M1} -sorbent immunoassay, but with a 0.2 ml volume, the G_{M1} ganglioside was coated to the inside of plastic tubes and the non-bound fraction washed

away with PBS containing 0.05 % Tween 20 (PBS-Tween). In a total volume of 0.2 ml, treated toxin in various amounts was added followed by 10 μ l of a fixed amount (0.002 μ g) of 125 I-toxin. The tubes were incubated at 23 °C for 2 h in a roller drum, washed 3 $\times$ 3 min with PBS-Tween and tested for radioactivity in a gamma scintillator (Packard Instrument Corp.). The values for percentage of bound radioactivity were plotted against total toxin concentration. The curve intercepts with the background level were used to indicate the concentration of control (C_0) or test toxin preparations (C_t) required for saturation of the G_{M1} receptors (Fig. 2b). The relative G_{M1} -binding activity of the test protein is expressed as the ratio C_0/C_t , where 1.0 means unchanged activity as compared with the control preparation and 0.0 means no activity.

Assay of antibody-binding. An inhibition technique was used. Mixtures of different concentrations of treated or control toxin preparation and a specific dilution of antitoxin antiserum in PBS-Tween were incubated in a total volume of 0.5 ml, at 4 °C overnight in glass tubes. These mixtures (absorbed antiserum) were transferred to plastic tubes which had been coated with toxin (Fig. 1c), and the tubes incubated in a roller drum at 23 °C for 5 h (Holmgren & Svennerholm, 1973). This allowed binding of the antitoxin antibodies to the solid-phase toxin, unless the combining sites of the antibodies had already been occupied by the toxin during the preincubation. After washing the tubes with PBS-Tween, bound antibodies were measured by reacting them, first with enzyme-conjugated anti-IgG and then, after washing, with substrate for the enzyme in the same manner as in the G_{M1} -sorbent immunoassay. Extinction values were plotted against concentration of treated and control toxin preparation and the inhibitory concentrations C_0 and C_t were derived as shown in Fig. 2(c) and described for the G_{M1} -sorbent immunoassay. The relative antibody-binding activity of treated as compared with control toxin preparation is given as the ratio C_t/C_0 where 1.0 means intact and 0.0 abolished activity.

Electrophoresis in polyacrylamide. Two types of electrophoresis in polyacrylamide gel were used: conventional (p.g.e.) and with added sodium dodecyl sulphate, SDS (SDS-p.g.e.). Our p.g.e. method was similar to that of Finkelstein, Fujita & Lo Spalluto (1971) except that we used flat beds (plates), and pre-electrophoresis was performed for 2 h before application of the samples. The SDS-p.g.e. technique in flat beds has been described previously (Lönnroth & Holmgren, 1973). Before SDS-p.g.e., the proteins were incubated with 2% SDS, 8 M-urea and 0.04 M-iodoacetamide at 53 °C for 15 min.

RESULTS

Sensitivity and accuracy of methods

The rabbit skin test is an established quantitative assay of cholera toxin activity (Craig, 1965). The overall mean concentration of unmodified toxin required for 1 blueing dose was 0.002 μ g/ml. In the three *in vitro* methods for measuring the receptor-binding and/or the antigenic properties of cholera toxin or toxin derivatives, the mean concentrations of unmodified toxin giving the indicated activity were 0.044 μ g/ml for the combined G_{M1} + antibody-binding immunoassay, 0.80 μ g/ml for the G_{M1} -binding radioassay, and 0.18 μ g/ml for the antibody-fixation immuno-inhibition assay.

The variation in the determinations of relative activity was less than 1.2-fold for the G_{M1} + antibody-binding activity and less than 1.5-fold for the other activities ($P < 0.05$). Over twenty of the toxin derivatives were prepared more than once and their relative activities were all within the experimental limits of the test methods, indicating a high degree of reproducibility.

Table 2. *Effects of chemicals on the activities of cholera toxin*

Here, 0.0 indicates a relative activity below 0.05.

	Concentration (mm)	Toxicity	Binding activity		
			G _{M1} + antibody	G _{M1}	Antibody
A. Amino group reagents					
Picrylsulphonic acid	10	0.0	0.0	0.0	0.3
	1	0.1	0.4	0.0	0.7
	0.1	1.0	0.8	0.6	1.1
	0.01	1.0	1.0	0.8	1.3
Succinic anhydride	10	0.1	0.2	—	0.7
S-methylisothiurea sulphate	10	0.5	0.3	—	0.5
B. Cysteine and cysteine reagents					
N-ethylmaleimide	10	1.0	1.3	0.7	1.0
Iodoacetamide	10	1.0	1.2	1.5	0.6
2-Mercaptoethanol	100	0.0	0.8	1.4	0.2
	10	0.1	1.1	1.5	0.6
	1	1.0	1.1	1.9	0.7
2-Mercaptoethanol + iodoacetamide	100	0.0	1.4	0.6	0.8
	10				
C. Arginyl reagents					
Phenylglyoxal	10	1.0	1.8	—	—
Glyoxal	10	1.0	0.9	—	—
1,2-Cyclohexanedione	10	0.0	1.5	1.2	1.0
	1	0.1	3.5	1.3	3.7
	0.1	1.0	3.5	1.3	2.0
2,3-Butanedione	10	0.0	0.0	0.0	0.4
	1	0.0	—	1.0	0.2
	0.1	1.0	—	1.0	—
Phenanthrenequinone	10	0.0	0.0	—	0.4
	1	0.0	0.3	0.1	0.5
	0.1	0.0	0.8	1.0	0.7
	0.001	1.0	1.0	—	1.0
D. Aromatic amino acid reagents					
2-Hydroxy-5-nitrobenzyl bromide	10	1.0	2.2	—	2.8
2-Methoxy-5-nitrobenzyl bromide	10	1.0	1.0	—	—
Acetylimidazole	10	1.0	1.0	—	—
Bromosuccinimide	10	0.0	0.0	0.0	—
	1	0.0	0.0	0.0	—
	0.1	0.0	0.0	0.0	—
	0.01	1.0	2.0	1.0	—
5-Amino-1H-tetrazole	10	1.0	0.7	—	—
4-Diazobenzenesulphonic acid	10	0.0	0.0	—	0.1
	1	0.0	0.0	0.0	0.2
	0.1	1.0	1.3	1.0	1.5
Tetranitromethane	10	2.0	5.2	—	—
E. Other group-specific reagents					
Ethyl-3,3-dimethyl-amino- propylcarbodiimide	10	1.0	1.1	—	—
As above with (50 mm) glycine-O- methylester		1.0	0.7	1.0	1.0

Table 2. *Cont.*

		Binding activity			
	Concentration (mM)	Toxicity	G _{M1} + antibody	G _{M1}	Antibody
E. Other group-specific reagents (<i>cont.</i>)					
Glutaraldehyde	10	0.1	0.3	0.0	0.1
	1	1.0	1.1	0.7	0.9
	0.1	1.0	1.0	1.1	0.9
1,5-Difluoro-2,4-dinitrobenzene	10	0.1	0.3	0.1	—
4,4-Difluoro-3,3-dinitro-phenylsulphone	10	1.0	1.0	—	—
Diisopropylfluorophosphate	10	0.0	0.3	0.0	0.0
	1	1.0	0.8	1.0	1.0
F. Various reagents					
Leucine aminopeptidase*	Without urea	1.0	0.5	1.0	1.0
	With urea	1.0	0.7	—	—
Carboxypeptidase*	Without urea	1.0	0.8	1.0	0.7
	With urea	0.0	0.9	1.0	—
Trypsin*	Without urea	1.0	—	—	—
	With urea	0.0	3.3	1.0	—
Sodium hydroxide	1000	0.0	0.1	0.0	0.0
	100	0.0	0.2	0.2	0.0
	40	0.2	0.4	0.8	0.1
	10	1.0	5.2	7.1	0.7
Sodium dodecylsulphate	100	0.0	0.7	0.1	0.9
	10	0.0	0.8	1.4	0.8
	1	0.0	0.8	1.0	1.2

* The relative activities of the enzyme-treated preparation are based on comparison with those of the control preparation (i) described in Methods; controls (i) and (iii) gave identical values and control (ii) had no activity in the tests.

The activities of the various control toxin preparations were compared with those of purified toxin diluted directly in TG buffer. Of the buffers employed to modify the protein, only the 0.04 M-sodium hydroxide used for 1,2-cyclohexanedione and phenanthrenequinone had by itself any effect on the toxin's reactivity in the various tests (see Table 2 F).

Chemical modification of cholera toxin

Amino-group specific reagents. Reagents which modify amino groups in proteins do so by attaching covalently to free amino groups of lysine and to the *N*-terminal amino acid(s). Different concentrations of picrylsulphonic acid caused a concomitant reduction of toxicity and G_{M1}-binding activity of cholera toxin, whereas the antibody-fixing activity was slightly more resistant (Table 2 A and Fig. 2). Succinic anhydride and, to a lesser extent, S-methyl-isothiouraea, had similar effects (Table 2 A).

Cysteine and cystine residue specific reagents. Toxin was treated with chemicals that react with free sulphhydryl groups on cysteine or with cystine disulphide bridges (Table 2 B). *N*-ethylmaleimide and iodoacetamide, which both attach covalently to free sulphhydryl groups, did not affect any of the measured properties of the toxin. In contrast, the disulphide bridge splitting reagent 2-mercaptoethanol (alone or with iodoacetamide) decreased the toxic activity, and at higher concentrations destroyed it completely, without changing the G_{M1}- and antibody-binding properties to the same extent.

Arginyl specific reagents. Treatment of toxin with arginyl specific reagents had differential

effects on the properties measured (Table 2C). Phenylglyoxal and glyoxal had no effect, whereas the other three reagents used that also contained a diketene structure inhibited the toxicity without decreasing other properties concomitantly. The most divergent effects were caused by 1,2-cyclohexanedione, which inhibited toxicity but enhanced the toxin's reactivity in the combined G_{M1} -antibody-binding assay. The latter effect may have been due to an elevated antibody-fixing capacity with the G_{M1} -binding ability remaining unchanged (Table 2C). Enhanced antibody-binding ability was seen after treatment with several reagents of various specificities. 2,3-Butanedione (1 mM) produced the most pronounced differences between the G_{M1} -binding and the antibody-fixing properties of the toxin: the former property was not affected whereas the immunological reactivity was decreased. Phenanthrenequinone was the most potent detoxifying reagent of all, but affected the G_{M1} - and antibody-binding qualities only at high concentration.

Reagents reacting with aromatic amino acids. Toxin was treated with reagents which are more or less specific for aromatic amino acids (Table 2D). The tryptophan-specific chemicals 2-hydroxy-5-nitrobenzyl bromide and 2-methoxy-5-nitrobenzyl bromide, also named Koshland's reagents 1 and 2, decreased neither toxicity nor the G_{M1} -antibody-binding properties of the toxin. Acetylimidazole which is a tyrosine-specific reagent, 5-amino-1-H-tetrazole which modifies tyrosine and histidine, and tetranitromethane which binds to tyrosine and cysteine residues behaved similarly. The latter reagent was repeatedly found to potentiate both the toxicity and the G_{M1} -antibody-binding capacity. Bromosuccinimide (reactive with tryptophan > tyrosine > histidine at pH 6.0) and 4-diazobenzenesulphonic acid (reactive with tyrosine and histidine) destroyed both the toxicity and the G_{M1} -antibody-binding capacity.

Other group-specific reagents. Toxin derivatives were prepared by treatment with a variety of reagents with different specificities (Table 2E). Carbodiimides alter, specifically, amino acids containing a free carboxyl group, i.e. glutamic and aspartic acid and the carboxy-terminal amino acid. Ethyl-3,3-dimethyl-aminopropylcarbodiimide, which was tested with and without glycine-*O*-methyl ester, did not affect either the toxicity or the G_{M1} -antibody-binding capacity. Three bifunctional reagents were tested: glutaraldehyde and 1,5-difluoro-2,4-dinitrobenzene, both of which decreased the toxic, G_{M1} - and antibody-binding activities, and 4,4-difluoro-3,3-dinitrodiphenylsulphone which had no effect on any of these properties. Diisopropylfluorophosphate is a widely used serine esterase inhibitor in enzymology; it has a reported specificity for the serine group responsible for esterase activity, but at higher concentration it also has an affinity for tyrosine and glutamine residues (Cohen, Oosterbaan & Berends, 1967). Only at these higher concentrations (10 mM) of diisopropylfluorophosphate was there any effect on the cholera toxin; toxicity was abolished, together with G_{M1} - and antibody-binding capacities. This indicates that the toxin itself does not act as a serine esterase, since the toxic activity then would probably have been much more labile than the two other properties.

Other types of reagents. The toxin was treated with two exopeptidases, leucine aminopeptidase and carboxypeptidase A, which digest protein from the amino and carboxy terminal ends respectively, and with the endopeptidase trypsin (Table 2F). None of these enzymes had any influence, even at high concentrations, on the toxic or the G_{M1} - or antibody-fixing activities of the toxin when tested in ordinary buffer. However, in the presence of 2 M-urea both carboxypeptidase A and trypsin abolished toxicity without affecting G_{M1} - and antibody-fixing properties.

Sodium hydroxide (0.04 to 1 M) also inhibited both toxicity and G_{M1} -antibody-binding capacity. When G_{M1} -binding and antibody-fixation were tested separately it appeared that

Fig. 3. Polyacrylamide gel electrophoresis of control toxin and toxin treated with various concentrations of (a) 2-mercaptoethanol, (b) SDS and (c) picrylsulphonic acid. Anode at top.

the most pronounced effect was on antigenicity. With 0.01 M-sodium hydroxide a selective increase of G_{M1} -binding capacity of the toxin was produced.

Sodium dodecylsulphate has previously been used for disaggregation of the toxin into its subunits (Lönnroth & Holmgren, 1973). It abolished toxicity but had little effect on the G_{M1} - and antibody-binding activities of the toxin.

Behaviour of modified toxin in polyacrylamide electrophoresis

SDS-p.g.e. separates the non-covalently linked subunits of cholera toxin into two protein bands representing the fast-migrating L subunit, molecular weight about 8000, and the slower-moving H subunit, molecular weight 28000 (Lönnroth & Holmgren, 1973). Any change in migration speed after treatment would imply that covalent bonds had been generated or broken between or in the peptides. In p.g.e. the toxin migrates as a single band with a relatively slow mobility. Separated H subunit and L subunit complexes, corresponding to choleragenoid, migrate faster and slower, respectively, than the intact toxin (unpublished). A changed banding pattern in p.g.e. after treatment would suggest a change in aggregation state and/or in the net charge of the toxin.

A number of toxin derivatives were examined both by electrophoresis and in the toxicity assay, to test whether effects on toxic activity induced by the chemical treatment were due to changes in the aggregation state of the toxin. The effect of SDS modification on toxicity was paralleled by a changed migration in p.g.e.; the single band of untreated toxin was replaced by a faster-moving component probably representing H subunit negatively charged by the SDS groups, and a slower material which migrated like aggregated L subunit (Fig. 3) 2-Mercaptoethanol also changed toxicity and the behaviour in p.g.e. concomitantly (Table 3). A similar pattern as described for SDS-treated derivatives was seen except that the fast-moving component, devoid of the negative SDS-groups, had the same migration rate as isolated H subunit, and the slower component showed more tailing (Fig. 3). In SDS-p.g.e. the effect was visible only at higher concentrations of 2-mercaptoethanol than those which

Table 3. *Effect of modification on cholera toxin studied by polyacrylamide gel electrophoresis with and without SDS*

Reagent	Concentration (mM)	Effect on		
		SDS-p.g.e.*	P.g.e.†	Toxicity
SDS	10		+	+
	1		+	+
	0.1		—	—
2-Mercaptoethanol	100	+	+	+
	10	—	(+)	+
	1	—	—	—
Bromosuccinimide	1	—	—	+
Picrylsulphonic acid	1	+	+	+
	0.1		+	—
	0.01		(+)	—
1,2-Cyclohexanedione	1	—	—	+
Phenanthrenequinone	10	+		+
	0.1	—	—	+

* Polyacrylamide gel electrophoresis in buffer, pH 7.0, containing SDS.

† Polyacrylamide gel electrophoresis in tris-glycine buffer.

diminished the toxicity. At these higher concentrations a split of the H subunit into probable half-molecules takes place (Lönnroth & Holmgren, 1973; Holmgren & Lönnroth, 1975).

Bromosuccinimide (1 mM, pH 6.0), which has been reported to cleave peptides at acid pH (Ramachandran & Witkop, 1967), had no visible effect on the behaviour of the toxin in any of the electrophoretic tests, despite a total inhibitory influence on all the functional properties studied (Table 3). 1,2-Cyclohexanedione (1 mM) also did not affect the electrophoretic patterns, although differential effects were seen on toxicity and G_{M1} + antibody-binding. Increasing concentrations of picrylsulphonic acid progressively increased the mobility of the toxin in p.g.e., which probably was due to electroneutralization of positively charged lysine groups (Fig. 3). The aggregation state of toxin treated with this reagent seemed unaffected in the p.g.e. analyses; in SDS-p.g.e. the proportion of L and H subunit material was unchanged but the L subunit had a slower mobility than usual. At high concentration, phenanthrenequinone had an effect similar to that of 2-mercaptoethanol in SDS-p.g.e., i.e. it cleaved the H subunit, but it also abolished toxicity at lower concentrations, which had no effect on the behaviour of the toxin in either p.g.e. or SDS-p.g.e.

Modification of V. cholerae culture filtrate

Preliminary studies with *V. cholerae* culture filtrate showed that the *in vitro* assays of the G_{M1} receptor-binding and the antibody-fixing properties of purified toxin could be used with similar accuracy for evaluating the crude material (Holmgren, 1973; Holmgren & Svennerholm, 1973). Although dialysed and concentrated *V. cholerae* culture filtrate contained large amounts of other bacterial proteins, after treatment with four reagents (*N*-ethylmaleimide, phenylglyoxal, picrylsulphonic acid and 1,2-cyclohexanedione, all at 10 mM) which had different residue specificity and different effects on the tested properties of purified cholera toxin, the derivatives gave the same results as those produced with treated purified toxin.

DISCUSSION

The techniques described here, which can be applied to small quantities of purified or crude toxin preparations, may be useful for comparative studies between cholera toxin and other bacterial enterotoxins. Some of the treatments decreased the ability of cholera toxin to bind *in vitro* to the ganglioside G_{M1} and caused a concomitant reduction in toxicity. These effects were not unexpected, since recent studies strongly indicate that the G_{M1} ganglioside functions as membrane receptor for the toxin in various target cells (Holmgren *et al.* 1973; King & van Heyningen, 1973; Cuatrecasas, 1973*b*; Holmgren *et al.* 1975). The toxicity, however, was also destroyed by a few reagents which had no influence on the G_{M1} -binding ability. This supports the previous finding that structures which are not involved in cell-binding are required for the expression of toxic activity. The H subunit of cholera toxin is needed for toxic activity but is not binding either to G_{M1} ganglioside *in vitro* or to target cells (Lönnroth & Holmgren, 1973; Cuatrecasas *et al.* 1973; Holmgren *et al.* 1974; van Heyningen, 1974; Holmgren & Lönnroth, 1975). It seems likely, therefore, that those reagents which had a selective effect on the toxic activity affected principally the H subunit in a manner which prevented it inducing the presumed key event in the toxic action, i.e. activation of membrane adenylate cyclase. The decrease in toxicity caused by reagents such as 2-mercaptoethanol and SDS illustrates the importance of assembled subunits for biological activity (Lönnroth & Holmgren, 1973). Two of the other three reagents which selectively destroyed the toxicity did not, at the concentrations used, affect the quaternary structure of the toxin as studied by gel electrophoresis. This suggests that the loss in biological activity of the toxin was caused by a critical modification *in situ* of the H subunit. Holmgren *et al.* (1974) and Holmgren & Lönnroth (1975) found that all of the receptor-binding as well as most of the antigenic properties of the toxin reside in the same type of subunit. This finding is supported by the present results from the separate testing of G_{M1} -binding and antibody-fixation of toxin derivatives. Most reagents had a concomitant effect on these properties, indicating a close structural association between the receptor-binding and the antigenic sites. A few reagents however, like 2-mercaptoethanol (100 mM), picrylsulphonic acid (1 mM) or 2,3-butanedione (1 mM), affected these properties differently.

This information on the gross relationships between the toxic, receptor-binding and antigenic sites of cholera toxin, is supplemented by information on the type of residues contributing to the structural arrangement of these sites. However, such interpretations are preliminary, since with the limited amounts of toxin available it has not been possible to assess the efficiency of the chemical treatments in terms of specificity and extent of residue modification. Amino group reagents such as picrylsulphonic acid, succinic anhydride, S-methylisothiouraea, glutaraldehyde, and 1,5-difluoro-2,4 dinitrobenzene destroyed the properties of toxicity and receptor + antibody-binding concomitantly. Since leucine aminopeptidase, which digests proteins from the amino terminal end, did not affect any of these features of the toxin, with or without urea, even at high concentration, it is probably the modification of lysine groups which is responsible for the observed changes in toxin properties induced by amino group reagents. The G_{M1} -binding quality seemed to be more sensitive than the antibody-binding capacity, which suggests that lysine groups are involved in the binding of toxin to the acidic G_{M1} ganglioside receptor. This interpretation is consistent with data on the amino acid composition of cholera toxin, and on a 'natural' cholera toxoid, choleragenoid (Finkelstein, 1973). The latter protein has the same composition as the toxin except for lacking the 'toxic' H subunit (Lönnroth & Holmgren, 1973). Therefore, the reported finding that choleragenoid contains 80 % of the lysine residues of the toxin demon-

strates that lysine residues are particularly frequent in the receptor-binding L subunit aggregate portion of the toxin. Three types of toxin derivatives prepared by reagents which are specific for arginine lost toxicity but retained intact G_{M1} - and antibody-binding qualities. Since arginine is much more abundant in the toxin than in cholera toxinoid (Finkelstein, 1973), i.e. it is present predominantly in the 'toxic' subunit H, and since tests with two of our derivatives showed that the chemical modification had not changed the aggregation state of the toxin, we believe that arginine residues of the toxin may be involved in the 'toxic site' inducing activation of target cell adenylate cyclase.

Reagents with the same reported specificity sometimes had quite different effects on cholera toxin. Variation in reactivity of a functional group is a common observation in protein modification studies, and is usually explained by differences in accessibility of the functional groups to the reagents (Cohen, 1968). Altered accessibility of the site of proteolysis could also explain the observed difference in effect of the two proteolytic enzymes trypsin and carboxypeptidase A in the presence and absence of urea.

Cholera is the prototype for the acute bacterial diarrhoeal diseases named enterotoxic enteropathies (Craig, 1972). The techniques described here, which require only small quantities of toxin, may be useful for comparative studies between cholera toxin and other bacterial enterotoxins. Toxoids for immunoprophylaxis against these diseases are urgently required (Feeley, 1974). A formalinized cholera toxoid was tested but was unsuitable due to reversion to toxicity (Northrup & Chisari, 1972), and glutaraldehyde treatment reduced antigenicity as well as toxicity (Rappaport *et al.* 1974). The present study has indicated that selective chemical treatments of cholera toxin supported by sensitive methods of detecting the biological results of such treatments might be useful in the search for efficacious toxoid preparations.

We are grateful to Ms Christina Norrman and Ms Gun Wallerström for skilful technical assistance, to Dr C. Miller, Bethesda, for providing the purified cholera toxin and to Dr L. Svennerholm, Göteborg, for generous gifts of pure G_{M1} ganglioside. The work was supported by grants from the Swedish Medical Research Council (project no. 16X-3383), the W.H.O., the Medical Faculty at the University of Göteborg, and the Ellen, Walter and Lennart Hesselman Foundation.

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