Development of a Purified Cholera Toxoid

II. Preparation of a Stable, Antigenic Toxoid by Reaction of Purified Toxin with Glutaraldehyde

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Evidence is presented which confirms that cholera toxoids obtained by reaction of purified toxin with Formalin possess the ability to partially reactivate both in vivo and in vitro. At the same time, conditions are presented for the preparation of stable, antigenic cholera toxoids by reaction of purified toxin with glutaraldehyde. Treatment of purified cholera toxin with approximately 200 mol of glutaraldehyde per mol of toxin at pH 7.8 reproducibly resulted in the preparation of toxoids which: (i) possessed less than 20 bluing doses per 100 μg; (ii) did not reactivate in vivo or in vitro; (iii) precipitated with, and neutralized antitoxin; (iv) elevated prolonged serum antitoxin in immunized rabbits; (v) protected immunized guinea pigs against toxin skin challenge; and (vi) lent themselves to enhanced antigenicity by means of an in situ adjuvant system which may be suitable for man. Acrylamide gel electrophoresis and molecular sieve chromatography of a series of glutaraldehyde-derived toxoids suggested that the reaction products consisted of monomeric and polymeric species and that the proportion of higher-molecular-weight species was determined by the relative concentrations of toxin and glutaraldehyde. The results suggested a relationship between complete and irreversible elimination of toxicity and the formation of higher-molecular-weight toxoids.

Like other microbial toxins, cholera toxin cannot be used for immunization in its active form because of its potent biological activity. If given orally to experimental animals or man, it may cause the classical diarrheagenic outpouring of fluid in the gut (4, 11, 14, 34), and, if given parenterally, it will, at the very least, cause reactions at the site of inoculation (10, 13, 24). For purposes of immunization, then, it is necessary to convert cholera toxin into a stable inactive form (toxoid) which can still produce neutralizing antitoxin in experimental animals and man.

By analogy with diphtheria and tetanus toxoids, purified cholera toxin was detoxified chemically with Formalin. Although complete or nearly complete elimination of detectable toxicity was generally achieved under the conditions used, Craig (personal communication) observed that mice injected intravenously with one such toxoid suffered weight loss or delayed death, or both—events which were usually attributed to toxin. Subsequently, it was confirmed in a number of laboratories that another Formalin toxoid (Wyeth lot 00101) exhibited

reactivation both in vivo (24; J. P. Craig, W. F. Verwey, R. A. Finkelstein, personal communications) and in vitro (A. Bernstein, personal communication; R. Rappaport, unpublished results). Furthermore, an examination of numerous Formalin toxoids prepared under a variety of conditions showed that in vitro reactivation could be eliminated only under conditions which severely altered immunogenicity (A. Bernstein and R. Northrup, personal communications).

On the basis of these observations as well as considerable evidence that some diphtheria (2, 21, 32, 35, 36, 38) and tetanus (1) Formalin toxoids also exhibit reversion to toxicity, it was considered important to explore new methods for detoxification. In order to eliminate the problem of reactivation, it was reasoned that an irreversible chemical modification which eliminated toxicity without adversely affecting antigenicity was required. Glutaraldehyde, a bifunctional dialdehyde, was selected as a feasible reagent to explore since the stability of reaction products obtained by treatment of various proteins with glutaraldehyde has been documented

by a number of investigators (3, 16, 17, 26, 29, 30) and since plausible reaction mechanisms have been proposed (17, 20, 33). The conditions surrounding the preparation of stable, antigenic cholera toxoids by reaction of purified toxin with glutaraldehyde, as well as a preliminary analysis of the reaction products, are the subject of this investigation.

MATERIALS AND METHODS

Production and purification of toxin. Purified toxin was obtained by production and purification procedures previously described (31).

Measurement of toxin potency. Toxin content of various toxin and toxoid preparations was estimated by the rabbit bluing dose (BD) titration method for vascular permeability activity devised by Craig (6, 8, 9). Unless specified otherwise, two New Zealand albino rabbits were injected intracutaneously with duplicate or triplicate 0.1-ml volumes of serial twofold dilutions of a sample, and 18 h later the rabbits were injected intravenously with 1% Evans blue dye as previously described (31). The results are given as BD_7/ml, determined from the reciprocal of the sample dilution which yielded a 7-mm bluing lesion in rabbit skin.

Measurement of antitoxin combining power. Toxoid units, a measure of antitoxin combining power, were determined as previously described (31) according to the method of Craig (9). One toxoid unit was defined as that amount of toxoid capable of binding 1 unit of antitoxin (AU) (9), and the number of toxoid units was regarded as a measure of the amount of antigen in various toxoid preparations.

In vitro reversion test. Samples of toxoid (2 to 5 ml) in 0.067 M phosphate-buffered saline (PBS), pH 7.8, were incubated in the presence of 0.01% thimerosal at 37 C for 2 weeks before being assayed for toxin activity by the BD titration method described above. Control samples were held at 4 C for 2 weeks and assayed in parallel with the samples incubated at 37 C.

In vivo reversion test. Toxin activity and the development of toxin activity associated with various toxoid preparations was monitored for a period of 2 weeks by an abbreviated rabbit induration test similar to the one developed by Craig (personal communication; 10), with the exception that only induration diameter was recorded. (Craig includes thickness of palpable swelling in estimating the amount of reversion and also monitors induration for approximately 30 days.) Serial twofold dilutions of toxin or toxoid were injected intracutaneously in duplicate on the clipped backs of two New Zealand albino rabbits, and the diameters of induration were recorded daily for a period of 14 days. The data are presented as a plot of the rise or fall in induration diameter with time.

Titration of levels of antitoxin in immune rabbit sera. The amount of antitoxin in the sera of rabbits immunized with various preparations of toxoid was estimated by the intracutaneous method in rabbits according to the method of Craig (6, 8, 10), with the exceptions that: (i) sera were inactivated undiluted;

(ii) serial twofold dilutions of sera (in 0.067 M phosphate buffer with 0.01% gelatin, pH 7.4) were incubated with an equal volume of previously standardized toxin containing 1 limit-of-bluing (Lb) dose/ml (9); and (iii) the provisional standard cholera antitoxin (Swiss Serum and Vaccine Institute standard antitoxin containing 4,470 antitoxin units per ml) was tested at 2, 1, 0.5, and 0.25 AU/ml. Bluing was performed as previously described (31). The results are presented in AU per milliliter, determined from the reciprocal of the serum dilution which, in the presence of an equal volume of toxin containing 1 Lb dose/ml, yielded a 4-mm bluing lesion in rabbit skin.

Guinea pig immunogenicity test. Immunogenicity of certain toxoids was estimated on the basis of the magnitude of suppression of bluing which occurred when immunized guinea pigs were challenged intracutaneously with graded doses of toxin (7, 9, 12). Groups of guinea pigs (six animals per group) were immunized intramuscularly with 1 ml containing approximately 1 μg of a selected toxoid. Three animals from each group were given an identical booster shot 4 weeks after the primary inoculation. Three guinea pigs and an equal number of unimmunized guinea pigs were challenged by intradermal injection of appropriate serial twofold dilutions of toxin 4 weeks after primary immunization; another set of animals were similarly challenged 2 weeks after secondary immunization. The factor of suppression was calculated from the difference in the dilution of toxin challenge required to produce an 8-mm bluing lesion in immunized versus unimmunized guinea pigs.

Ultraviolet absorption spectra. Ultraviolet spectra of selected toxoids in 0.067 M PBS, pH 7.8, were determined by using a Beckman DU spectrophotometer and 1-cm quartz cuvettes.

Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was performed as previously described (31). Electrophoresis was terminated when the tracking dye reached the bottom of the gel.

Agarose chromatography. Chromatography was performed at room temperature with Bio-Gel A-0.5 M (Bio-Rad, Richmond, Calif.) and a 2.5- by 45-cm column (K25/45, Pharmacia, Piscataway, N.J.). Samples containing 2.5 to 5.0 mg of protein in 2 to 3 ml were applied to the column, and the chromatogram was developed by using 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, as the elution buffer. Fractions (4.0 ml) were collected automatically in a refrigerated fraction collector (Buchler Instruments, Fort Lee, N.J.), and each fraction was monitored for optical density at 280 nm by using a Beckman DU spectrophotometer.

Immunodiffusion. Standard double-diffusion tests were performed in IDF-I cells (Cordis Corp., Miami, Fla.) by using Wyeth goat no. 1 antitoxin (31). For quantitative measurement of toxin or toxoid antigen, radial immunodiffusion was performed as previously described (31) by using a 1:480 final dilution of Wyeth goat no. 1 antitoxin.

Protein determinations. Protein was determined either by the spectrophotometric method of Warburg and Christian (39) or by the Lowry modification of the Folin-Ciocalteau method (23) using crystalline bovine serum albumin as standard.

RESULTS

Reaction of toxin with glutaraldehyde. Assuming a molecular weight of 100,000 for monomeric toxin molecules, the concentration of glutaraldehyde was initially varied between 50 and 800 mol/mol of toxin to establish optimal conditions for inactivation. (After the completion of these studies, the molecular weight for cholera toxin was reported to be 84,000 [22].) The reactions were carried out with a range of toxin concentrations, 125 to 1,070 μg/ml, at 30 C in 0.067 M PBS, pH 7.8, for various time intervals. All reactions were terminated by two sequential dialyses against 100 volumes of PBS, pH 7.8. When the concentration of toxin was above 600 μg/ml, the reaction products tended to become insoluble with increasing concentrations of glutaraldehyde (if not during the reaction, then sometimes during dialysis or subsequent storage at 4 C). When the concentration of toxin was within the range 125 to 600 μg/ml and the concentration of glutaraldehyde was varied between 50 and 400 mol/mol of toxin, the reaction products were soluble and exhibited a straw color, the intensity of which increased with increasing concentrations of glutaraldehyde and with time. In addition, when such reactions were carried out as a function of pH in the range between pH 6.2 and 8.2, vascular permeability assays showed that detoxification was optimal between pH 7.8 and 8.2. Under these conditions, no significant change in pH was observed during incubation of the reaction mixtures at 30 C for 72 h.

Under conditions which produced soluble reaction products, inactivation kinetics exhibited a two-phase reaction: an initial rapid inactivation of vascular permeability activity followed by a slower rate of inactivation which leveled off during subsequent incubation. For the case in which 200 mol of glutaraldehyde per mol of toxin (580 μg/ml) was used, a 3-log reduction in toxicity occurred within 6 h, followed by a slower 2-log reduction which reached equilibrium at about 60 h (Fig. 1). For a particular concentration of toxin, the magnitude of the initial inactivation and the time at which inactivation reached equilibrium at pH 7.8 were dependent on the ratio of moles of glutaraldehyde to moles of toxin.

When the concentration of toxin was between 400 and 600 μg/ml, the level of residual toxicity (measured after incubation for 72 h at 30 C) decreased approximately 10-fold with each twofold increase in the concentration of glutaraldehyde between 50 and 200 mol/mol of toxin, whereas the level of residual toxicity approached the borderline of detectability as the
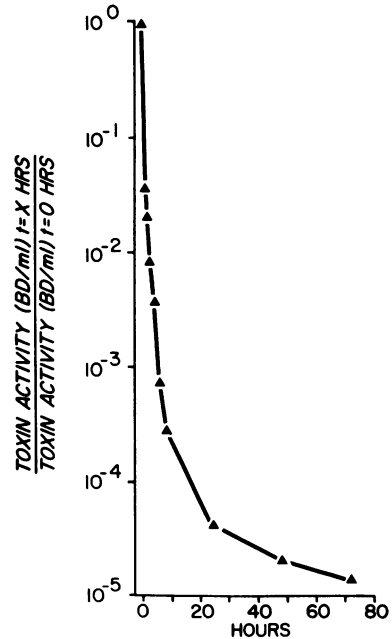

FIG. 1. *Inactivation curve for detoxification of 580 μg of purified cholera toxin per ml by 200 mol of glutaraldehyde per mol of toxin in 0.067 M phosphate-buffered saline, pH 7.8.*

concentration of glutaraldehyde was increased from 200 to 400 mol/mol of toxin (Table 1, column 1). In the range of 400 mol of glutaraldehyde per mol of toxin or above, complete elimination of detectable toxicity could be achieved, but with some risk of obtaining either insoluble reaction products or adversely affecting the ability of the antigen to stimulate circulating antitoxin in immunized rabbits (Table 2).

Under conditions in which reaction products retained residual toxicity, prolonged incubation for periods of up to 6 days did not result in any further reduction of activity. To determine whether or not this result could be explained by consumption of the reagent during the course of the reaction, untreated toxin was added to a glutaraldehyde-toxin (200:1, mol/mol) reaction mixture at some point after equilibrium had been reached. Compared with a toxin control which was incubated in PBS under the same conditions, the activity of toxin added to the reaction mixture was significantly reduced after incubation for 5 h (Table 3). This result suggested that those molecules which had not undergone detoxification were either inaccessible or resistant to the reagent under the conditions used.

TABLE 1. *Stability of glutaraldehyde toxoids*

Type of toxoid	Mol of reagent/ mol of toxin	BD_7[a] at weeks post-detoxification						
		4 C						37 C
		0	2	4	6.5	8	24	2
Glutaraldehyde.......	50:1	3,260		4,157	35,630	33,240	25,600	33,240
Glutaraldehyde	100:1	452		1,420	3,261		1,280	1,748
Glutaraldehyde								
Prepn A	200:1	46		57	35	<40	40	57
Prepn B	200:1	51				92		74
Glutaraldehyde								
Prepn A	400:1	28		28	70	<20	<20	≤20
Prepn B	400:1	22				<20		<20
Formalin								
Prepn A	16,000:1	<20	<20		<20	<20	20	>5,120
Prepn B	16,000:1	<20	<20			135		6,085

[a] Determined as described in Materials and Methods.

TABLE 2. *Relationship between extent of detoxification and ability of antigen to stimulate circulating antitoxin in immunized rabbits*

Antigen	Mol of glutaraldehyde/ mol of toxin	Residual toxicity $(BD/100 \mu g)$[a]	Immunization schedule (weeks)	Dose per inoculation (μg)	AU/ml[b]		
					2 weeks	4.5 weeks	6 weeks
Glutaraldehyde toxoid (soluble)	208:1	21	0, 4.5	60	12 (3) (9–14)	53 (3) (50–60)	909 (3) (699–1,243)
Glutaraldehyde toxoid (soluble)	417:1	<20	0, 4.5	60	<5 (3)	NT[c]	379 (3) (274–445)
Glutaraldehyde toxoid (turbid suspension)	5,000:1	0	0, 4.5	60	<5 (3)	NT	79 (3) (53–197)

[a] BD_7, determined as described in Materials and Methods.

[b] AU, Antitoxin units; determined as described in Materials and Methods. Numbers indicate geometric averages; single number in parentheses indicates number of rabbits; two numbers in parentheses indicates range of values.

[c] NT, Not tested.

TABLE 3. *Prolonged inactivation of toxin by glutaraldehyde and reactivity of the equilibrium reaction mixture towards freshly added toxin*

Sample	Incubation time at 30 C (h)	BD_7[a]
Toxin, 500 µg/ml + 200 mol glutaraldehyde (per mol of toxin)	0	820,750
	6	1,260
	23	368
	30	211
	48	106
	53	29
	72	28
	144	22
Equal vol of 53-h reaction mixture from above + toxin (500 µg/ml)	0	430,300
	5	<20,000
Equal volumes of toxin (500 µg/ml) + PBS buffer	0	415,700
	5	278,600

[a] Determined as described in Materials and Methods.

In vitro stability studies. Samples of purified toxin (420 µg/ml) were incubated with 50, 100, 200, and 400 mol of glutaraldehyde per mol of toxin, respectively, at 30 C in PBS, pH 7.8, for 72 h. A sample of the same toxin preparation was incubated with 0.2% Formalin (16,000 mol/ mol of toxin) under identical conditions. After incubation and dialysis, samples of each reaction mixture were removed for in vitro reversion tests, and the bulk solutions were stored at 4 C. Activity assays showed that toxoids prepared by reaction of toxin with 200 or 400 mol of glutaraldehyde per mol of toxin, respectively, did not reactivate after incubation at 37 C for 2 weeks or at 4 C for 6 months (Table 1). Toxoids prepared by reaction of toxin with 50 or 100 mol of glutaraldehyde per mol of toxin, however, exhibited increases in activity of 10-fold and 4-fold, respectively, after incubation at 37 C for 2 weeks and at some time during incubation at 4 C. Formalin toxoid, on the other hand, exhibited at least a 300-fold increase in activity after

incubation at 37 C, while remaining essentially stable at 4 C.

In vivo stability studies. Based on the results of in vitro stability studies, selected toxoids were examined for their ability to reactivate in vivo by using the abbreviated rabbit induration test previously described. Defining reactivation on the basis of the delayed appearance of induration, the data showed that glutaraldehyde toxoids prepared with 200 or more mol of glutaraldehyde (per mol of toxin) did not reactivate in vivo throughout a 14-day observation period, whereas a representative Formalin toxoid did exhibit in vivo reactivation under the same conditions (Fig. 2). The induration profile produced by toxin alone showed that induration associated with toxoids, whether initial or delayed, was due to the presence of toxin.

Ultraviolet absorption spectra. Since Habeeb and Hiramoto (17) demonstrated a shift in ultraviolet absorption from 280 to 265 nm for glutaraldehyde-modified bovine serum albumin and ovalbumin, respectively, the ultraviolet spectra of selected toxoids were examined in order to detect changes in the chromophoric groups accompanying reaction with glutaraldehyde. Toxoids prepared by reaction of toxin with 50, 100, 200, and 400 mol of glutaraldehyde

per mol of toxin, respectively, exhibited a shift in their ultraviolet absorption maxima from 280 nm towards 260 nm, the extent of which increased with increasing concentrations of glutaraldehyde (Fig. 3). There were also correlative increases in the value of $E_{1\,mg}^{1\,cm}$, indicating some change in the chromophoric groups. It is noteworthy that there appeared to be a relationship between the extent of the ultraviolet shift (and the magnitude of the increase in $E_{1\,mg}^{1\,cm}$) and the extent of detoxification (Table 1, column 1; Fig. 3), since toxoids prepared with 200 or 400 mol of glutaraldehyde per mol of toxin, which were inactivated to nearly the same extent, exhibited nearly identical ultraviolet absorption spectra. Toxoids, which were not inactivated to the same extent (50 and 100 mol of glutaraldehyde per mol of toxin), however, exhibited a significant difference in the extent of the ultraviolet shift relative to each other as well as relative to the more completely detoxified toxoids.

Acrylamide gel electrophoresis of reaction products. Toxoids comparable to those described above were examined by acrylamide gel electrophoresis. The results showed that: (i) with the exception of the toxoid prepared with 50 mol of glutaraldehyde per mol of toxin, the glutaraldehyde toxoids consisted of multiple species with significantly different electropho-
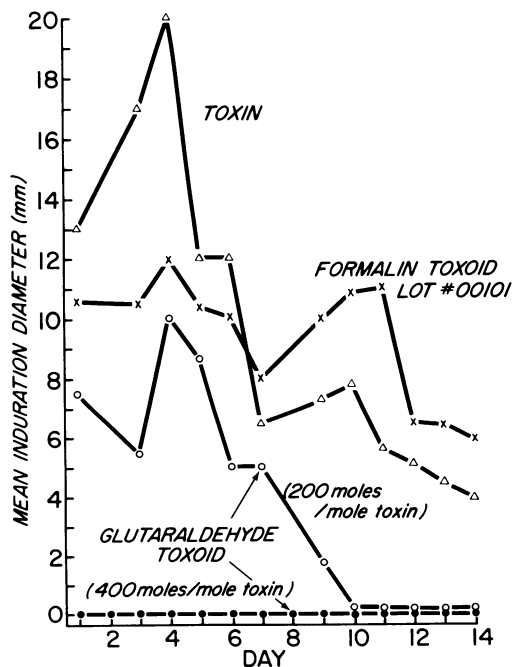

FIG. 2. *Time course of rabbit induration response after intracutaneous injection of toxin or toxoid. Volume of injection was 0.1 ml, and each sample contained 30 µg/ml.*
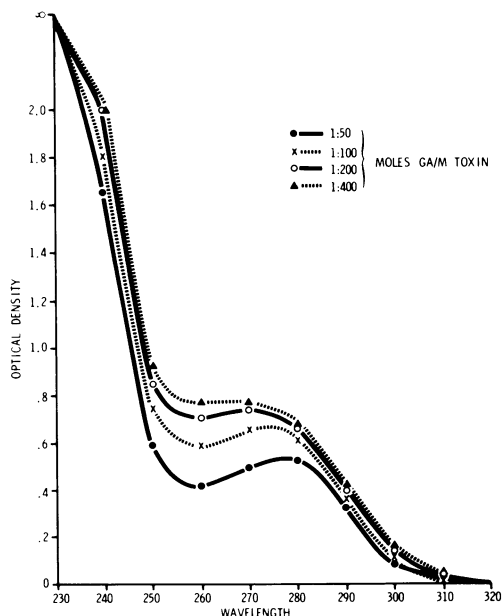

FIG. 3. *Ultraviolet absorption spectra of various glutaraldehyde toxoids in 0.067 M phosphate-buffered saline, pH 7.8.*

retic mobilities; and (ii) the distribution of electrophoretic species shifted towards the slower-moving components as the concentration of glutaraldehyde was increased (Fig. 4). Under the same electrophoretic conditions, the majority of Formalin toxoid molecules co-migrated with the most rapidly moving component of the glutaraldehyde toxoids. (A small percentage of Formalin toxoid molecules sometimes co-migrated with the second most rapidly moving species associated with the glutaraldehyde-toxoids.)

Agarose chromatography of reaction products. To determine whether or not the multiple electrophoretic species associated with the glutaraldehyde toxoids were related by charge or by molecular size, a series of toxoids comparable to those described above (Fig. 4) were fractionated by chromatography on Bio-Gel A-0.5 M. The resulting optical density profiles (Fig. 5) for each of the toxoids showed that the electrophoretic heterogeneity associated with them was due to a distribution of molecular sizes and that the proportion of higher-molecular-weight species increased with increasing concentrations of glutaraldehyde. As in the case of the ultraviolet spectra (Fig. 3), there appeared to be less difference (in the molecular weight distribution) between the toxoids produced with 200 and 400 mol of glutaraldehyde per mol of toxin than there was between the toxoids produced with 50 and 100 mol of glutaraldehyde per mol of toxin. Also, a comparison of the data in Fig. 5 with that in Table 1 (column 1) suggested a correlation between the

FIG. 4. *Acrylamide gel electrophoresis patterns of Formalin and glutaraldehyde toxoids. From left to right: Formalin toxoid; glutaraldehyde toxoids prepared with 50, 100, 200, and 400 mol of glutaraldehyde per mol of toxin, respectively.*

degree of detoxification and the formation of higher-molecular-weight toxoids.

Acrylamide gel electrophoresis of selected fractions from one of the chromatograms (200:1, mol/mol; Fig. 5D) corroborated that the different species were of different molecular weights since the various electrophoretic species migrated on the gels in reverse order to their elution from the column (Fig. 6).

In addition, it should be noted that the series of toxoids used in the chromatography experiments appeared to contain a larger proportion of higher-molecular-weight species than did the toxoids depicted in Fig. 4 (compare staining patterns of acrylamide gels depicted in Fig. 4 and 5). The only known difference between the two sets of toxoids was that the set used for chromatography was prepared 4 to 6 weeks before analysis by chromatography and electrophoresis, whereas the other set of toxoids (Fig. 4) was analyzed by electrophoresis directly after preparation. These results suggest the possibility that some proportion of the reaction products may tend to aggregate or even polymerize (via an exposed aldehyde on the bifunctional glutaraldehyde molecule) during either concentration or prolonged liquid storage at 4 C. None of the toxoids, however, exhibited any insoluble reaction products under these conditions, and residual toxicity levels remained effectively unchanged for at least 6 months (see Table 1).

Immunodiffusion properties. Toxoids prepared with 50, 100, 200, and 400 mol of glutaraldehyde per mol of toxin, respectively, were tested against Wyeth goat no. 1 antitoxin (31) along with Formalin toxoid and parent toxin. The results showed that, although all of the glutaraldehyde toxoids formed precipitin lines with antitoxin, the distance of antigen migration and the sharpness of the precipitin lines decreased as the molar ratio of glutaraldehyde to toxin was increased (Fig. 7). Occasionally, a slight spur was observed on the precipitin line formed by toxin and antitoxin as it merged with the precipitin line formed by the 400:1 (mol/mol) glutaraldehyde toxoid and antitoxin (barely visible in Fig. 7). These observations were consistent with the results showing an increase in molecular weight heterogeneity (Fig. 5) and an alteration in antigenicity (Table 2) with increasing concentrations of glutaraldehyde.

On the basis of toxoid unit determinations, glutaraldehyde toxoids prepared with 200 mol of glutaraldehyde per mol of toxin exhibited about a 50% reduction in antitoxin combining power relative to Formalin toxoids (Table 4). This observation indicated that some determinant
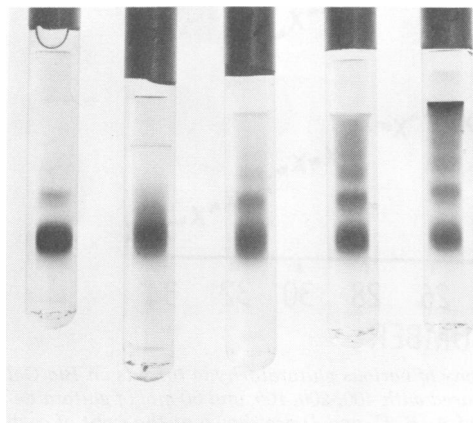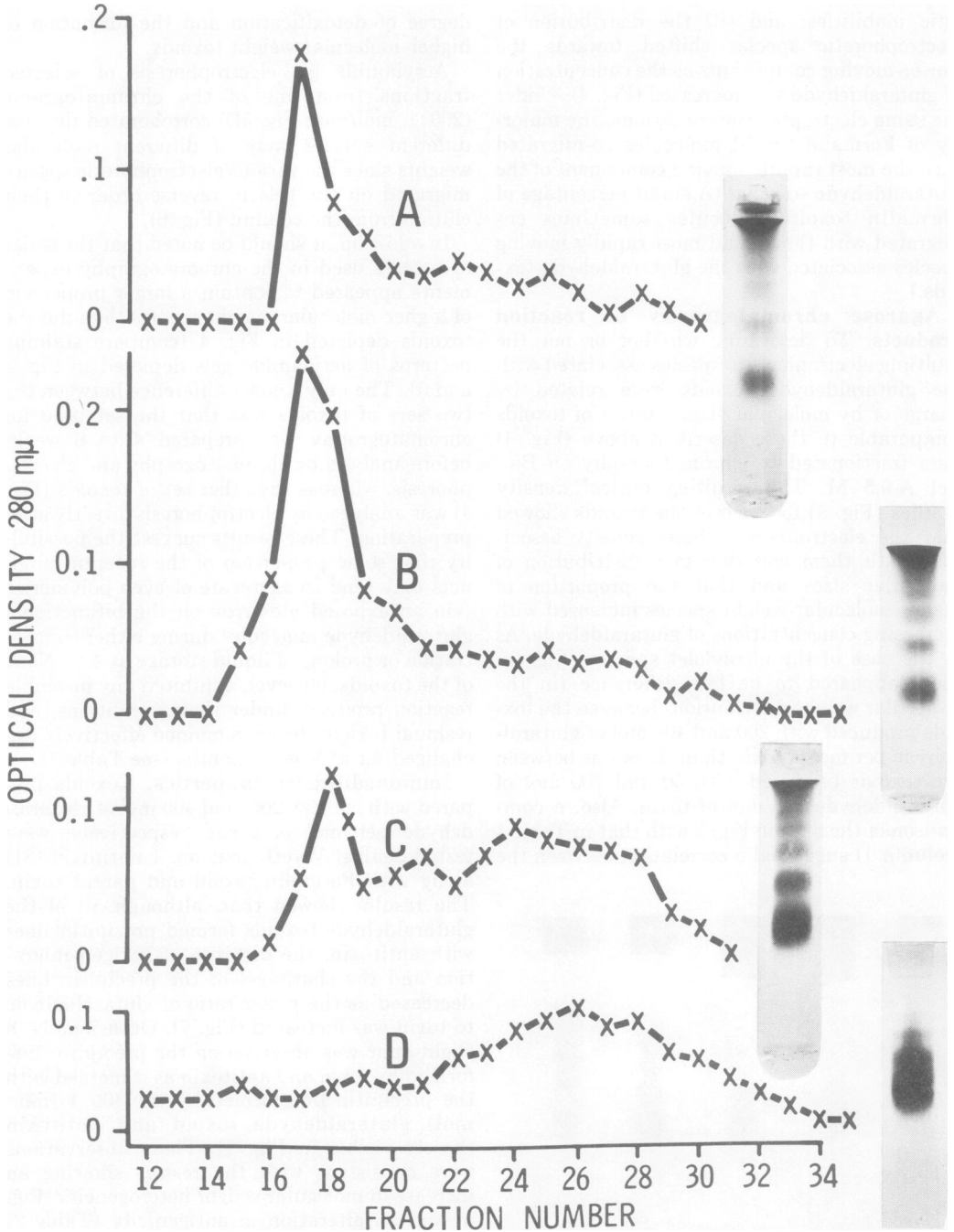
FIG. 5. *Optical density profiles resulting from chromatography of various glutaraldehyde toxoids on Bio-Gel A-0.5 M. A, B, C, and D represent glutaraldehyde toxoids prepared with 400, 200, 100, and 50 mol of glutaraldehyde per mol of toxin, respectively. Acrylamide gel patterns of A, B, C, and D are shown at the right of each chromatogram.*

groups on the glutaraldehyde toxoid antigen were either altered or masked. Nevertheless, rabbit and goat antisera prepared against such toxoids were highly effective in neutralizing

toxin in rabbit skin vascular permeability assays and they formed a discrete precipitin line of identity when tested against toxin and glutaraldehyde toxoid in double-diffusion tests.
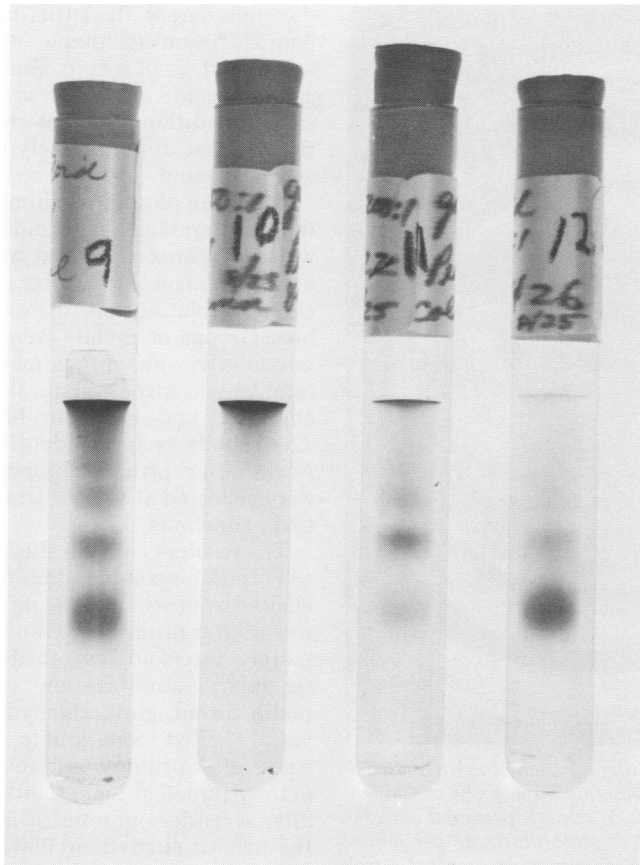
FIG. 6. *Acrylamide gel electrophoresis patterns of toxoid prepared with 200 mol of glutaraldehyde per mol of toxin and selected fractions from Bio-Gel A-0.5 M chromatography (see Fig. 5, profile B). From left to right: glutaraldehyde toxoid (200:1); and Bio-Gel fractions 17, 21–22, and 25–26, respectively.*

TABLE 4. *Antitoxin combining power of Formalin and glutaraldehyde toxoids*

	Toxoid units/μg[a]	
Prepn	Formalin toxoid	Glutar-aldehyde toxoid
A	18.4	9.5[b]
B	20.5	8.5[b]
C	19.6	10.8

[a] Toxoid units were determined as described in Materials and Methods, using Swiss Serum and Vaccine Institute antitoxin with an assigned value of 4,470 AU/ml.

[b] Data provided by J. P. Craig, Downstate Medical School, Brooklyn, N.Y.

Antigenicity. On the basis of their stability, toxoids prepared by reaction of toxin with 200 mol of glutaraldehyde per mol of toxin were selected for antigenicity studies. The ability of these toxoids to elicit circulating antitoxin in rabbits was compared with Formalin toxoid (0.2% Formalin or approximately 16,000 mol per mol of toxin) prepared from the same parent toxin. In addition, the effect of adjuvant on the antigenicity of glutaraldehyde toxoid was also investigated. For this purpose, it was necessary to devise an effective adjuvant system (which would ultimately be suitable for man) because both Formalin and glutaraldehyde toxoids either did not bind to or were rapidly released from preformed aluminum gels at or near physiological pH (R. Rappaport, unpublished data). For example, no adjuvant effect was observed in rabbit immunization studies comparing the antigenicity of Formalin toxoid with Formalin toxoid bound to an aluminum gel (Reheis F-5000) at pH 5.5 (Table 5). In vitro studies (radial immunodiffusion and protein determinations) corroborated that approximately 70% of the antigen was bound to the gel at pH 5.5, but that the bulk of it was released upon adjustment of the gel suspension to pH 7.5
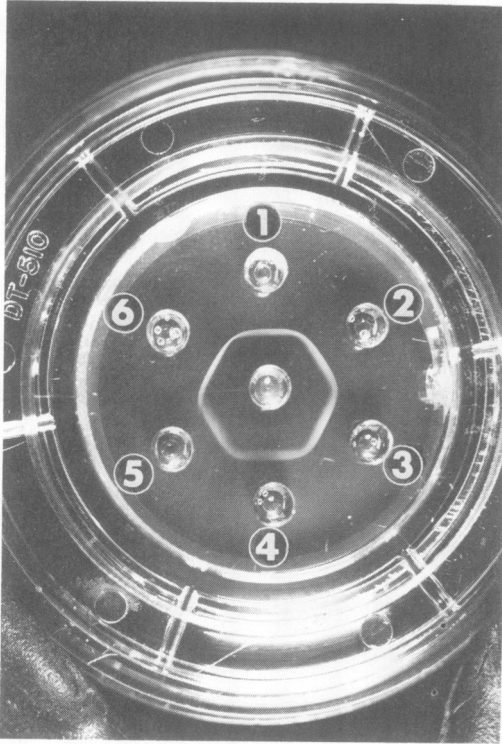
FIG. 7. *Double immunodiffusion of toxin and various toxoids against antitoxin. Outer wells contained: (1) Formalin toxoid; (2–5) toxoids prepared with 50, 100, 200, and 400 mol of glutaraldehyde per mol of toxin, respectively; and (6) parent toxin. Center well contained polyvalent antitoxin (Wyeth goat no. 1 antitoxin).*

(Fig. 8). Although less antigen was bound above pH 5.5, similar adsorption and release (at pH 7.5) properties were observed (Fig. 8).

To promote binding of the negatively charged toxoids to aluminum gels, protamine sulfate was used. It was reasoned that the presence of a preponderance of basic amino acids in the protamines might favor an interaction between them and the acidic toxoids and that such a complex might be more readily bound by aluminum gels at or near physiological pH.

Aluminum phosphate gels were prepared in situ by using aluminum chloride (with or without protamine) as the diluent for lyophilized PBS buffer (pH 7.8) and toxoid antigen. A turbid suspension was formed instantaneously when 500 μg of dried glutaraldehyde toxoid (plus PBS salts) was rehydrated with 5 ml of a clear aqueous solution containing 0.5 mg of protamine sulfate per ml (Schwartz/Mann, Orangeburg, N.Y.) and 3.75 to 5 mg of aluminum chloride per ml. Upon removal of the precipitate by centrifugation, no antigen could be detected in

the supernatant fluid (pH 6.1) by a radial immunodiffusion technique which could detect less than 1 μg of toxoid. Similar analysis of adjuvant-toxoid mixtures prepared under the same conditions, except without protamine, showed that approximately 40% of the antigen was not bound.

Using the protamine-aluminum adjuvant system, glutaraldehyde toxoids (200:1, mol/mol) were compared with and without adjuvant to Formalin toxoid on the basis of their ability to elicit circulating antitoxin in immunized rabbits. Groups of rabbits were immunized intramuscularly with approximately 100 μg of glutaraldehyde toxoid, glutaraldehyde toxoid plus adjuvant, and Formalin toxoid, respectively. The rabbits received identical booster shots 4 weeks after primary immunization, and sera were collected at various time intervals for antitoxin titrations.

The results (Table 6) showed that: (i) relative to Formalin toxoid, glutaraldehyde toxoid alone elicited approximately eightfold less antitoxin 4 weeks after primary immunization and approximately threefold less antitoxin 2 weeks after secondary immunization; (ii) relative to Formalin toxoid, glutaraldehyde toxoid plus adjuvant elicited comparable antitoxin titers 4 weeks after primary immunization and approximately threefold higher antitoxin titers 2 weeks after secondary immunization; and (iii) each of the toxoids elicited circulating antitoxin which
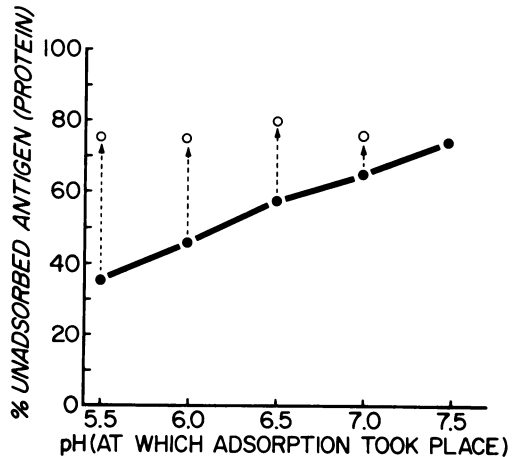

FIG. 8. *Adsorption (and release) of Formalin toxoid, lot 00101, to (and from) Reheis F-5000 aluminum gel as a function of pH. The concentration of toxoid was 120 μg/ml and the final concentration of gel was 0.2%. Closed circles indicate the percentage of protein antigen remaining in the supernatant after adsorption to the gel at the specified pH. Open circles indicate the percentage of protein antigen in the supernatant after adjustment of the gel suspensions to pH 7.5.*

TABLE 5. *Rabbit antitoxin response to Formalin toxoid administered with and without aluminum gel adjuvant*

Antigen	Immuni-zation schedule (weeks)	Dose per inoculation (µg)	AU/ml[a] at weeks post-immunization						
			0	2	4	6	8	12	16
Formalin toxoid, lot 00101, pH 7.8	0, 4	41	<2 (5)	47 (5)	233 (5)	649 (5)	421 (5)	123 (5)	67 (5)
Formalin toxoid, lot 00101, absorbed to Reheis F-5000 gel, at pH 5.5	0, 4	41	<2 (5)	33 (5)	109 (5)	484 (5)	236 (5)	78 (5)	46 (5)

[a] Determined as described in Materials and Methods. Numbers indicate geometric mean titers. Numbers in parentheses indicate the number of rabbits.

TABLE 6. *Rabbit serum antitoxin titers after immunization with Formalin or glutaraldehyde toxoids*

Immunogen	Immuni-zation schedule (weeks)	Dose per inocu-lation (µg)	AU/ml[a] at weeks post-immunization						
			0	4	6	10	12	16	24
Glutaraldehyde toxoid (200:1)[b]	0, 4	100	1.3 (7)	23 (7) (8–47)	242 (6) (145–722)	46 (6) (10–153)	27 (6) (7–124)	27 (6) (8–108)	14 (6) (2–52)
Glutaraldehyde toxoid (200:1) + adjuvant[c]	0, 4	100	1.5 (7)	147 (7) (56–259)	2031 (7) (764–2,850)	402 (7) (137–1,024)	164 (6) (52–652)	139 (5) (97–724)	90 (5) (26–388)
Formalin toxoid (16,000:1)	0, 4	100	1.7 (7)	198 (7) (107–291)	706 (7) (476–1,256)	204 (7) (74–389)	169 (7) (66–477)	113 (7) (79–338)	73 (5) (30–239)

[a] Determined as described in Materials and Methods. Numbers indicate mean values; single number in parentheses indicates number of animals; two numbers in parentheses indicates range of values.
[b] Molar ratio, reagent to toxin.
[c] Protamine-aluminum phosphate, prepared as described in Results.

TABLE 7. *Inhibition of skin bluing reaction in guinea pigs after immunization with Formalin or glutaraldehyde toxoids*

Immunogen	Immuni-zation schedule (weeks)	Dose per inoculation (µg)	Time of challenge (weeks)	Log toxin dilution producing 8-mm bluing lesion	Fold reduction in reaction
None (unimmunized)			4	>4.1	1
			6	4.1	1
Glutaraldehyde toxoid (200:1)[a]	0, 4	0.73	4	>4.1	~1
			6	>4.1	<1
Glutaraldehyde toxoid (200:1) + adjuvant[b]	0, 4	0.73	4	3.5	>4
			6	2.0	126
Formalin toxoid (lot 00101)	0, 4	1.07	4	3.85	>1.8
			6	2.4	50

[a] Moles of glutaraldehyde per mole of toxin.
[b] Protamine-aluminum phosphate, prepared as described in Results.

persisted for at least 6 months. The level of persisting antitoxin was nearly equivalent for Formalin toxoid and glutaraldehyde toxoid plus adjuvant. Relative to these toxoids, the level of persisting antitoxin elicited by glutaraldehyde toxoid alone was approximately five- to sixfold less.

Immunogenicity. Groups of guinea pigs were immunized with approximately 1 µg of For-malin toxoid, glutaraldehyde toxoid, and glu-taraldehyde toxoid plus adjuvant, respectively, according to the schedule previously described. The Formalin toxoid was Wyeth lot 00101. The results showed that relative to unimmunized controls, guinea pigs immunized with glutaral-dehyde toxoid plus adjuvant exhibited about a fourfold suppression of bluing response 4 weeks after primary immunization and a 126-fold

suppression of bluing response 2 weeks after secondary immunization, whereas, at the immunogen dose used (0.7 µg), guinea pigs immunized with glutaraldehyde toxoid alone did not exhibit any suppression of bluing relative to controls (Table 7). In the same test, guinea pigs immunized with approximately 1 µg of Formalin toxoid exhibited about a twofold suppression of bluing 4 weeks after primary immunization and a 50-fold suppression of bluing 2 weeks after secondary immunization (Table 7).

DISCUSSION

The purpose of this investigation was to develop a reliable method for preparing stable antigenic toxoids for ultimate use in human immunization programs. In view of the instability of cholera Formalin toxoids as observed in this laboratory, a series of new toxoids prepared by reaction of purified toxin with glutaraldehyde was evaluated in terms of three criteria: (i) residual toxicity; (ii) in vitro and in vivo stability; and (iii) ability to elicit neutralizing antitoxin in animal models.

In establishing optimal conditions for detoxification, the availability of a sensitive biological assay (the rabbit skin vascular permeability test [6, 8, 9]), capable of detecting as little as 0.1 to 0.5 ng of toxin, permitted the determination of trace amounts of residual toxicity. By using this assay to monitor the reaction between toxin and glutaraldehyde, it was observed that the extent of detoxification was determined by the relative concentrations of each component. When conditions were such that the concentration of toxin was between 400 and 600 µg/ml and the molar ratio of glutaraldehyde to toxin was between 200:1 and 400:1, soluble reaction products were obtained and nearly complete elimination of toxicity was achieved. Under these conditions, the glutaraldehyde toxoids were characterized by a molecular heterogeneity which was absent from Formalin toxoids. The results of electrophoresis and molecular sieve chromatography, in fact, suggested a relationship between the extent of detoxification and the formation of higher-molecular-weight toxoids, since levels of residual toxicity decreased and molecular heterogeneity increased as the molar ratio of glutaraldehyde to toxin was elevated from 50:1 to 400:1.

In the absence of prior knowledge about the immunogenic properties of glutaraldehyde toxoids, the selection of reaction conditions which favored the production of a toxoid with minimal detectable toxicity appeared to be a reliable approach to preventing structural modification of the toxoid molecules (such as might occur if extensive reaction between toxin and glutaral-

dehyde were allowed to take place). Because rabbit immunization studies showed that toxoids prepared with 400 or more mol of glutaraldehyde per mol of toxin were less antigenic than toxoids prepared with 200 mol of glutaraldehyde (Table 2), the latter toxoids were selected for further study. Although these toxoids exhibited slight residual toxicity, it should be noted that such toxicity was generally detectable only when the concentration of toxoid was at least fivefold greater than the maximal dose anticipated for animal or human immunization (100 µg). Quantitatively, the aforementioned (200 molar) glutaraldehyde toxoids contained no more than 20 BD_7 per 100 µg, or, on the basis of data reported by Craig (9), approximately 0.025 to 0.05 Lb doses (1 to 2 ng). This amount of toxin contained in 100 µg of toxoid did not produce any observable adverse reactions in numerous rabbits immunized parenterally, in guinea pigs injected intraperitoneally, or in mice inoculated intravenously. Furthermore, Craig et al. (10) have recently demonstrated that this amount of toxin can be safely injected intracutaneously into man.

In evaluating the stability of toxoids obtained by reaction of toxin with various molar ratios of glutaraldehyde, it was observed that significant levels of residual toxicity remained associated with toxoids prepared with 50 or 100 mol of glutaraldehyde per mol of toxin. Since these toxoids were not sufficiently detoxified to warrant their use as immunogens, their stability in vivo was not carefully investigated. Questions pertaining to their ability to reactivate under physiological conditions, therefore, remain unresolved. In vitro reversion studies, however, suggested that these toxoids were not completely stable since vascular permeability assays performed at different times after incubation at 4 or 37 C showed increases in toxicity of as much as 10-fold (see Table 1). Although Craig (9) has pointed out that BD titers from different rabbit pairs can vary by as much as 16-fold, appropriate controls indicated that the results could not be explained by variation in animal susceptibility.

Assuming that this evidence does, in fact, represent legitimate in vitro reactivation, it implies that either one step of the reaction between toxin and glutaraldehyde is reversible or that there is a subpopulation of toxin molecules which reacts differently from the majority of molecules. On the basis of the present findings, it is probable that the most likely candidates for reactivation are among those species which, like Formalin toxoid, have not undergone intermolecular cross-linking, since the glutaraldehyde toxoid which exhibited the

least amount of polymerization exhibited the most reactivation (10-fold). The observation that the extent of reactivation exhibited by this toxoid was approximately 30-fold less than that exhibited by Formalin toxoid (300-fold) (see Table 1), under conditions where the molar concentration of Formalin was 320 times greater than glutaraldehyde, indicated a difference in the respective reaction mechanisms. Further, on the basis of the in vitro reversion data (Table 1), it was estimated that reactivation, when it occurred, was limited to between 0.1 and 1.0% of the molecules for both glutaraldehyde and Formalin toxoids. It is possible that these molecules differ chemically from the majority of the toxin molecules and that they represent a subpopulation more resistant to detoxification. The existence of such a population was, in fact, suggested by the inactivation curve (following the reaction of 580 μg/ml of toxin with 200 mol of glutaraldehyde) which departed from linearity after a 3-log reduction in toxicity (see Fig. 1).

In contrast to toxoids with significant residual toxicity, the stability of toxoids with minimal toxicity, such as those prepared with 200 or 400 mol of glutaraldehyde per mol of toxin, was studied intensively. These toxoids reproducibly did not exhibit any in vitro reactivation when tested at intervals during storage for at least 6 months. Further, such toxoids did not exhibit any reactivation in rabbit in vivo reversion tests as they were performed in this laboratory, or in in vivo reversion tests performed independently in rabbits and mice by Craig (personal communication), or in monkeys by R. Northrup and F. Chisari (personal communication).

Having satisfied the two criteria of low residual toxicity and stability, glutaraldehyde toxoids prepared with 200 mol of glutaraldehyde per mol of toxin were selected for evaluation of the third criterion—the ability to elicit antitoxin or protection against toxin challenge in animal models. In the present study, glutaraldehyde toxoid (with and without adjuvant) was compared with Formalin toxoid since the antigenicity of the latter toxoid has been demonstrated repeatedly (7, 12, 15, 18, 28).

From the standpoint of both magnitude and duration of circulating antitoxin in immunized rabbits, the results showed that glutaraldehyde toxoid plus adjuvant was as antigenic as Formalin toxoid and approximately six times more antigenic than glutaraldehyde toxoid alone. It was also observed that, irrespective of whether it was administered with or without adjuvant, glutaraldehyde toxoid reproducibly elicited a sharper anamnestic response than did Formalin toxoid. In addition, long-term surveillance of

rabbits immunized twice with 100 μg of glutaraldehyde toxoid plus adjuvant (with a 6-week interval between inoculations) showed that measurable antitoxin persisted for at least 48 weeks and that the antitoxin titers were raised more than 100-fold 2 weeks after a booster inoculation (R. Rappaport, unpublished data). Provided that circulating antitoxin is a measure of protection, these findings indicate that a vaccine composed of a toxoid antigen may, unlike the classical cholera vibrio vaccine, offer long-term immunity.

In terms of the ability to protect immunized guinea pigs against toxin skin challenge, glutaraldehyde toxoid plus adjuvant was at least as effective as Formalin toxoid. It is noteworthy that the comparative immune response elicited by these toxoids in guinea pigs was similar to the response observed in rabbits, even though the sensitivity of the guinea pig test allowed the antigen dose to be reduced 100-fold. The observation that glutaraldehyde toxoid alone (0.73-μg dose) was ineffective in eliciting suppression of toxin challenge was regarded as evidence that less than 1 μg was incapable of evoking an effective primary (or secondary) immune response in the guinea pig. (Recent results [not presented] have demonstrated that 1 μg of glutaraldehyde toxoid [without adjuvant] is capable of evoking a primary [and secondary] immune response in this system.)

In both the rabbit and guinea pig models, the enhancement of the immune response produced by the addition of adjuvant to glutaraldehyde toxoid not only reflected the effectiveness of the adjuvant system but also demonstrated the inherent antigenicity of the toxoid. The fact that glutaraldehyde toxoid alone was not nearly as effective an antigen as Formalin toxoid is most probably related to the observation that Formalin toxoid reactivated in vivo, whereas glutaraldehyde toxoid did not. This phenomenon means that comparative antigenicity studies such as those presently described are, in effect, measuring the relative antigenicity of toxoid versus toxoid plus toxin.

On the basis of in vitro reversion data, and assuming a comparable degree of reversion in vivo, the amount of toxin released by 100 μg of Formalin toxoid was estimated to be on the order of 2 to 5 Lb doses. This amount of toxin represents approximately 100 times more than the amount of residual toxin associated with glutaraldehyde toxoid, and it appears either to be released gradually or to exert its biological effect gradually (over a period of days) at the site of inoculation (see Fig. 2).

Since it has been postulated that cholera toxin, which produces prolonged stimulation of

cyclic adenosine monophosphate-mediated processes in various tissues (27, 37, 40), may enhance antibody response either by activating cyclic adenosine monophosphate-mediated mechanisms in immunocompetent cells (5) or by stimulating cellular infiltration at the site of inoculation (24, 25, 28), it is reasonable to assume that the immune response to toxoids which reactivate in vivo (or which otherwise contain significant amounts of toxin) may be enhanced by the presence of toxin. The observation that rabbit vibriocidal antibody titers elicited by trace amounts of somatic antigen in representative toxin preparations (31) were reduced to insignificant levels when toxin was detoxified by glutaraldehyde instead of by Formalin further substantiates this view. Provided that glutaraldehyde did not have a direct effect on the lipopolysaccharide contaminant, these results suggested that toxin liberated by reactivation could act as an adjuvant not only for toxoid but for other antigens as well. In the light of these observations, it is interesting to speculate that the reason Formalin toxoid did not elicit an anamnestic response (in rabbits) equivalent to that produced by glutaraldehyde toxoid may be that the biological effect of (reactivated) toxin was partially neutralized by pre-existing (primary) antitoxin.

Finally, since the studies reported here demonstrate only that stable glutaraldehyde toxoids elicit neutralizing antitoxin when administered parenterally to rabbits and guinea pigs, it remains to be determined whether these toxoids, like Formalin toxoids (15, 18, 28), will be effective in protecting various animal models against intraintestinal challenge with toxin or viable Vibrio cholerae. In particular, it remains to be determined whether any intraintestinal immunity developed by such toxoids is mediated by secretory or humoral antibody (or both) and, correlatively, which route of immunization, oral or parenteral (or both), best promotes this type of immunity. The availability of a purified cholera toxoid which does not reactivate and which does not otherwise exhibit toxic side effects should provide a suitable antigen for studying some of the fundamental problems associated with antitoxic immunity and its relationship to prevention of diarrheal disease in man.

ACKNOWLEDGMENTS

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