Cholera Toxins: Immunogenicity of the Rabbit Ileal Loop Toxin and Related Antigens

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A method of assay of immunogenic potency of the cholera gut toxin is described; it is based on the relation of dose of antigen to neutralizing antibody titer produced in the rabbit under defined conditions and allows quantification of immunogenicity as immunogenic units per milligram of protein. Evidence, based on immunogenicity and rabbit ileal loop toxicity, is presented which indicates that the positively charged fraction of liquid-culture supernatant fluid eluted in deionized water from diethylaminoethyl Sephadex, or in electrolyte from carboxymethyl Sephadex, is a complex made up of a nonantigenic toxic moiety, a nontoxic protein component which elicits the formation of toxin-neutralizing antibody, and an inactive fraction. The complex may also be dissociated in high-salt concentrations with apparent recombination of the toxic moiety with a nondialyzable constituent of peptone to give a negatively charged complex. The immunogenic component is found in nontoxic supernatant fluids of cultures grown at pH 6.5 or in media deficient in peptone. It is also present in the nontoxic fraction eluted from diethylaminoethyl Sephadex in electrolyte or in deionized water from carboxymethyl Sephadex. When separated from the positively charged toxic moiety, the net charge of the antigen is reduced as shown by immunoelectrophoresis. On primary fractionation, the antigen may be associated with a minor antigenic component of the negatively charged complex containing a major antigen eliciting vibriocidal antibody formation, but antisera to the antigen preparations, either in this form or freed of antigenic contamination by recycling, do not contain vibriocidal antibody. It is suggested that this antigen be designated the T (toxin) antigen, and the antigen producing vibriocidal antibody the V antigen. These two antigens would appear to represent the major antigenic specificities associated with the antitoxic and antibacterial elements of the immune response to infection.

The rabbit ileal loop toxin produced by Vibrio cholerae has been shown to stimulate the production of neutralizing antibody, or antitoxin, and a method of titration against a standard freeze-dried whole-cell lysate (WCL) toxin has been described (7). The toxic activity was separated as an apparently homogeneous peak from peptone dialysate liquid-culture supernatant fluid by elution from diethylaminoethyl (DEAE) A50 Sephadex in deionized water by Coleman et al. (2) as a lipoprotein complex and was differentiated from a second nontoxic fraction eluted in 0.5 M NaCl. As described by them, the two fractions were separable antigenically as single precipitin lines demonstrable by gel diffusion and immunoelectrophoresis. The second, nontoxic, fraction was subsequently found to be antigenically heterogeneous, giving a main heavy precipitin line and three or four light or secondary lines after gel diffusion. Of these antigens, the predominant one was found to give an identity reaction with antigenically homogeneous vibrio lipopolysaccharide (LPS) after gel diffusion, and to stimulate the formation of vibriocidal antibody in the rabbit (8).

After further study, the toxic fraction of Coleman et al. (2) was also found to be heterogeneous. Using purified preparations from which extractable lipid had been removed by quantitative precipitation of the toxic activity with ethyl alcohol-ether in the cold, the toxic material was found to give two precipitin lines after gel diffusion and immunoelectrophoresis. Other studies, directed toward purification of the toxic activity and to be reported elsewhere, led, in addition, to the hypothesis that the toxic activity as it is formed by the vibrios occurs as a dissociable complex. This report is concerned with the dissociation of the toxic moiety from its specific antigenicity and with the preparation of a nontoxic
antigen which elicits the formation of toxin-neutralizing antibody.

**MATERIALS AND METHODS**

**Vibrio strain.** The strain of *V. cholerae* used in this study was the Dutta rabbit-passed toxigenic Inaba 569B. It was received from N. K. Dutta of the Haffkine Institute, Bombay, India, and stored in liquid nitrogen in the frozen state. The seed cultures were the third transplant from the Dutta strain as received.

**Preparation of the toxic complex.** Liquid peptone culture supernatant fluid (PSUP) was prepared essentially as described elsewhere (2, 8). The vibrios were grown as agitated cultures in 3% peptone (Difco) or peptone dialysate (pH 7.2 to 7.8) in volumes of 200 ml in 1,000-ml Erlenmeyer flasks at 37°C. Maximal toxic activity was obtained at pH 7.5 in culture media containing 0.5% NaCl. After incubation for 7.5 hr, the vibrios were removed by centrifugation at 104,000 \( \times g \) for 30 min, and the supernatant fluid was passed twice through 0.45-μm membrane filters (Nalgene). The product was cell-free unless the filters were overloaded. The filtrate was concentrated 8- to 10-fold by flash evaporation to 10 to 12°C or against polyethylene glycol (Carbowax 20M, Union Carbide Corp., New York, N.Y.), filtered again, and dialyzed exhaustively against deionized water or 0.001 M phosphate buffer at pH 7.5.

**Radioactive labeling.** Labeled PSUP was prepared by the inclusion of 0.1 mc/liter of uniformly labeled \(^{14}\)C-L-alanine (Tracerlab, Waltham, Mass.) in the culture medium.

**Precipitation with \((\text{NH}_4)_2\text{SO}_4.** The toxic complex was precipitated from unconcentrated PSUP at 90% saturation with \((\text{NH}_4)_2\text{SO}_4\) to give about an 80% recovery. Precipitation was carried out in the cold by the addition of the calculated amount of the dry salt in small increments with constant stirring and maintenance of the pH at 7.5 by the occasional addition of NaOH. This procedure required about 40 min. After completion of the addition of \((\text{NH}_4)_2\text{SO}_4\), the material was allowed to stand for 3 hr or overnight in the cold room. The precipitate was skimmed off and dissolved in deionized water, and the \((\text{NH}_4)_2\text{SO}_4\) was removed by dialysis.

**Chromatography.** Chromatographic separations were carried out in Sephadex columns (2.5 by 45 cm) using DEAE A50, DEAE A25, carboxymethyl (CM) 50 and CM 25 Sephadex (Pharmacia, Inc., New Market, N.J.). The toxic complex was eluted from the DEAE Sephadex in deionized water and from the CM Sephadex in 0.5 M NaCl at an average flow rate of 20 ml/hr. The elution was monitored by ultraviolet (UV) absorption at 280 nm with an LKB 8300A Ulvacord II (LKB Produkter AB, Stockholm, Sweden) and by analysis for protein by the Folin-Ciocalteau method (10). Preparations containing radioactive label were also monitored by radioactivity of the fractions as counted in a liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

**Ethyl alcohol-ether precipitation.** A mixture of ethyl alcohol and diethyl ether in a ratio of 3:2 was prepared from redistilled solvents and chilled overnight in the freezer compartment of the refrigerator. The toxic complex, as the fraction I of Coleman et al. (2), was eluted in deionized water from DEAE A50 Sephadex, and the volume was adjusted to contain 10 to 12 mg of protein per ml. The toxic activity was precipitated by the dropwise addition of the chilled aqueous solution to the solvent mixture to a final proportion of 10 mg of protein to 30 ml of solvent mixture. The flask was shaken at 4°C for 3.5 hr. The precipitate was removed by centrifugation at 104,000 \( \times g \) for 15 min, washed once with cold ether, dried, and stored in vacuo in the refrigerator.

This preparation gave a main precipitin line after gel diffusion appearing within 24 hr, and a faint secondary line which developed later. The main precipitin line was that of the antigenic specificity of the toxic complex. This preparation was used as a reference for the identification of the toxic complex antigen in other preparations by the identity reaction in immunodiffusion.

**Immunological methods.** Specific precipitation by double diffusion according to Ouchterlony was carried out as described previously (8), using antigen in concentrations of 25 to 30 mg/ml. Immunelectrophoresis was carried out on glass slides in buffered agar solution containing 1% agarose in veronal buffer (pH 8.4; ionic strength 0.025), 0.01% calcium lactate, and 1:10,000 thimerosal. Antigen wells, 3 mm in diameter, were cut with an LKB 6819A cutter; serum troughs were cut with an LKB 6814A cutter. Antigen was dissolved to 25 to 30 mg/ml and applied in volumes of 10 μliters. The antigen solutions were electrophoresed in veronal buffer (pH 8.4; ionic strength 0.1) containing 0.038% calcium lactate for 1 hr at 52 ma and 200 v. Anti-WCL serum was put in troughs in amounts of 100 μliters, and the precipitin lines were developed for 48 hr and stained with thiazine red R.

**Toxin and antitoxin titration.** Toxin and antitoxin were titrated in the rabbit ideal loop and expressed as units of toxin and of antitoxin as described elsewhere (7).

**RESULTS**

**Immunogenic potency.** Preliminary experiments indicated that the antitoxin response of rabbits given graded doses of the toxin, in crude or purified form, was regularly related to the size of the dose of antigen. This was taken to suggest that the immunogenicity of toxin antigen, in the sense of stimulating the formation of neutralizing antibody, might be quantified on such a basis.

Rabbits were immunized with graded doses of antigen, using four animals for each dose. Serum pools were prepared for each dose group and titrated for antitoxin by bioassay (7), using not less than four animals for each titration. It was found that when the antigen was given in a single dose, the antitoxin titration curves tended to be flat, in contrast to the slope observed when antitoxin was titrated in hyperimmune rabbit or human patients' sera (7) or in human carrier or monkey sera (unpublished observations); however,
if the total amount of antigen was given in two doses, the antitoxin titration curves closely resembled those obtained with other antisera.

Titration of sera taken at weekly intervals showed that peak antitoxin titer was reached at 3 to 4 weeks postinoculation, and then declined. The route of inoculation appeared to be unimportant; substantially the same results were obtained when antigen was given by the intraperitoneal (ip), subcutaneous, intramuscular, or intravenous routes. On the basis of these observations, the procedure of inoculating of the antigen in two doses ip 4 days apart and bleeding 3 weeks after the first inoculation was adopted.

Initially, antigen dosage was measured as units of toxic activity (1). Over a dose range of 2 to 20 units, the relation between dose of antigen and log antitoxin titer was linear. However, when a series of toxic preparations was tested discrepancies became apparent, particularly between fresh and aged material. It has been observed earlier that the half-life of the toxic activity in the form of PSUP stored in the refrigerator was about 3.5 weeks. Some, though not all, aged preparations showed greater immunogenicity than expected on the basis of toxicity, suggesting a less rapid decay of the former than the latter. The lack of correlation between the two became complete with the isolation of nontoxic antigen. Therefore, the use of toxicity was abandoned, and antigen dosage was measured as Folin protein.

Without the preliminary orientation provided by toxicity titrations, it was necessary to extend the dose range beyond the antigen equivalent of 20 units of toxin. On the semilog plot noted above, the dose-response relation tended to flatten beyond a dose of 20 units of toxin, but linearity was essentially restored in higher dose ranges on log-log plot, allowing titration of immunogenicity by interpolation over a wider dose range of antigen. Figure 1 illustrates such titrations for which four graded doses of antigen were used. The two lots of PSUP shown were freshly prepared but they differed markedly in toxicity, PSUP 112 containing 100 units/ml and PSUP 113,500 units/ml. The correlation of immunogenicity with toxicity in such fresh preparations is apparent. The applicability of this method of assay of immunogenicity to purified materials as well is illustrated by the results obtained with fraction I prepared according to the method of Coleman et al. (2).

A numerical measure of relative immunogenicity may be arrived at as the point at which lines, such as those shown in Fig. 1, intercept a designated antitoxin level. For present purposes, this antitoxin level has been taken as 100 units/ml, and the interpolated amount of antigen, as

Folin protein, required to give an antibody response of 100 units/ml of neutralizing antibody is defined as the immunogenic unit. Using the data shown in Fig. 1, the immunogenic unit of fraction I is 44 μg, that of PSUP 113 is 70 μg, and that of PSUP 112 is 720 μg. The relative immunogenicity of these preparations may be expressed as 22.7, 14.3, and 1.4 immunogenic units per mg of protein, respectively.

Dissociation of toxin and antigen. Evidence suggesting that the gut toxin complex might be dissociated was initially observed in connection with (NH₄)₂SO₄ precipitation of the toxic activity in purification studies described in preliminary form by one of us (W. Burrows, 8th Int. Congr. Trop. Med. Malaria, 7–15 Sept., 1968, Teheran).

As described by Coleman et al. (2), concentration of the raw culture supernatant fluid may be effected by flash evaporation in the cold, and it was subsequently observed that such supernatant fluids may also be concentrated by treatment with polyethylene glycol or by precipitation of the toxic activity with 90% saturated (NH₄)₂SO₄. When PSUP prepared from cultures in either peptone dialysate or peptone medium was concentrated by flash evaporation or treatment with polyethylene glycol, the first fraction to be eluted from DEAE A50 Sephadex in electrolyte gradient or in deionized water contained the toxic activity.

When (NH₄)₂SO₄ precipitation was applied to supernatant fluids of cultures in peptone dialysate medium, subsequent elution from DEAE A50 Sephadex showed the toxicity to be also in the first fraction. But when supernatant
fluid from cultures in peptone medium was precipitated with \((\text{NH}_4)_2\text{SO}_4\), the first fraction to be eluted from DEAE A50 Sephadex was nontoxic, and the toxicity was eluted in electrolyte as a part of the fraction II of Coleman et al. (2). The results of this kind of preparative procedure are illustrated in Fig. 2. On examination of the nontoxic fraction by gel diffusion, it was found to give a single precipitin line which gave an identity reaction with the toxin antigen line of the ethyl alcohol-ether-precipitated reference preparation. It was also found to be immunogenic in the rabbit and, as assayed by the immunogenic potency test, to contain 13.1 immunogenic units per mg.

In view of these observations, the fractionation procedure of Coleman et al. (2) on DEAE A50 Sephadex was reviewed. A small shoulder of the leading edge of their fraction I, initially considered to be an artifact, was found to recur consistently. When this portion of the fraction was separated, it was found to contain a major part of the toxic activity, and both this subfraction and the remainder of fraction I gave two precipitin lines on gel diffusion, one of which was identical with the toxin line of the reference preparation. Each subfraction was obviously contaminated with the other because of the considerable overlap of their trailing and leading edges, and as well with antigen found in fraction II.

Considerably better resolution was obtained when DEAE A25 Sephadex was used. The material eluted in deionized water was resolved into three peaks (Fig. 3) as subfractions 1, 2, and 3. Subfraction 1 contained the major portion of the toxicity; subfraction 2 contained only very small amounts of toxicity, and subfraction 3 contained little or no detectable toxicity. The ratio of the toxicity of subfractions 1 to 2 was usually on the order of 10:1 to 12:1. This preparation of PSUP had been labeled with \(^{14}\text{C}\), and the elution pattern was monitored by radioactivity as well as by protein and UV absorption as shown. It is evident that the major portion of the label occurred in subfraction 2 and substantially none in subfraction 3.

Subfraction 1 usually, though not invariably, gave two precipitin lines on gel diffusion and subfraction 2 gave a single line, whereas subfraction 3 gave no precipitin line. One line, or the single line if only one was present, of subfraction 1 gave an interference reaction (Ouchterlony type IV) with the line of subfraction 2, as illustrated in Fig. 4, and with the toxin line of the ethyl alcohol-ether-precipitated reference preparation (not shown). On examination of the immunogenicity of subfractions 1 and 2, it was found that both stimulated the formation of neutralizing antibody and that subfraction 2 was the more active in this respect. Of the preparations shown in Fig. 3, subfraction 1 contained 5.5 and subfraction 2 19.2 immunogenic units per mg. Similar studies of other preparations of relatively
fractionation on the vascular
munogenicity, namely, subfraction
lap of the anticipated; i.e.,
former gave recycled
through II of Coleman
PSUP, showed even greater differences in im-
munogenicity, namely, subfraction 1, 3.2, and
subfraction 2, 33.3 immunogenic units per mg.
In view of the report of Lewis and Freeman (9)
that the vascular permeability factor (PF) of
Craig (3) is separable from the gut toxin by fractionation
on QAE Sephadex and on DEAE
Sephadex, the PF activity of subfractions 1 and
2 was assayed. In confirmation of their results,
it was found that PF activity occurred largely
in the material eluted in electrolyte.

It is evident from Fig. 3 that the separation
of subfractions 1 and 2 is imperfect, with some overlap
of the trailing and leading edges, respectively.
Other studies had shown that the fractions I and
II of Coleman et al. (2) could also be separated
on CM Sephadex, but in reverse order as might be anticipated; i.e., fraction II was eluted in deionized water and fraction I in 0.5 M NaCl.
Subfraction 1 from DEAE A25 Sephadex was
recycled through CM 25 Sephadex and was sharply
separated into two fractions; an immunogenic nontoxic fraction eluted in electrolyte. This separation is illustrated in Fig. 5. The
former gave a single line on gel diffusion which showed identity with the reference toxin line, whereas the latter gave no precipitin lines. The
former was also highly immunogenic and was found to contain 31.2 immunogenic units per mg.
In effect, these procedures apparently separated
the specific immunogenic antigen from the toxic
activity, the former having little or no detectable
toxicity. The data given in Fig. 5 also illustrate
the apparent fragmentation of both toxic and
antigenic fractions repeatedly observed on
recycling through either anionic or cationic Sephadex.

Of the various subsidiary precipitin lines
given by fraction II of Coleman et al. (2), as
described by Kaur and Burrows (8), one was
found on reexamination to be the toxin antigen
line. The occurrence of the toxin antigenicity
in this fraction could also be demonstrated by its separation from PSUP by primary fractiona-
tion on CM Sephadex with elution in deionized
water, and by recycling it on DEAE A25 Sepha-
dex. The nontoxic antigen was eluted in
deionized water and was found to give the toxin antigen precipitin line and an identity reaction
with the reference preparation on gel diffusion;
it was immunogenic, containing 12.0 immuno-
genic units per mg.

The foregoing observations suggest that the
net charge of the toxin antigen may be altered.
The net positive charge of the complex as it
occurs in PSUP was shown by Coleman et al.
(2) to be sufficient for the toxin antigen to move
toward the negative pole from the point of
application on immunoelectrophoresis at pH 8.4,
and for it to be eluted from DEAE Sephadex in

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**Fig. 4.** Immunodiffusion pattern of subfractions 1 and 2 separated on DEAE A25 Sephadex, showing the interference reaction between the two. Well 1, sub-
fraction 1; well 2, subfraction 2; well 3, subfraction 3;
well 4, a second lot of subfraction 1; well 5, subfraction
2 in half concentration; well 6, blank.

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**Fig. 5.** Separation of nontoxic antigen from the
toxic moiety in subfraction 1 from DEAE A25 Sepha-
dex by recycling on CM 25 Sephadex. The net charge
of the antigen has been sufficiently reduced that it
elutes in deionized water, whereas the toxic moiety re-
tains net positive charge and is eluted in electrolyte.
deionized water. Whereas the retained net positive charge was sufficient to give this elution pattern, on recycling in the CM-DEAE sequence the partial purification effected resulted in a product sufficiently reduced in positive charge that the precipitating antigen moved toward the positive pole from the point of application on immunoelectrophoresis (Fig. 6). As described above, an even greater reduction in net positive charge, sufficient to allow elution from CM Sephadex in deionized water, was produced by recycling in the DEAE-CM sequence. Throughout all such fractionations, however, the toxic moiety consistently retained a net positive charge as judged by elution patterns.

Antigen in nontoxic PSUP. The production of toxin in peptone or peptone dialysate medium has not been completely consistent in this laboratory; there have been occasional occurrences of lots of very low (<20 units/ml) or undetectable toxicity. Although, as noted above, immunogenicity tended to parallel toxicity in fresh toxic preparations, preliminary studies had suggested that specific antigenicity might be present in the absence of detectable toxicity. Accordingly, a nontoxic PSUP was fractionated on DEAE Sephadex. The single peak eluted in deionized water was found to give the toxin antigen precipitin line on gel diffusion and, when titrated for immunogenicity, to contain 13.0 immunogenic units per mg, a potency of the same order as that of the nontoxic antigen separated from toxic peptone PSUP by (NH₄)₂SO₄ precipitation. Another preparation showing very low toxicity (10 units/ml) was similarly fractionated to give a nontoxic antigen of still higher immunogenic potency containing 33.3 immunogenic units per mg.

Although factors favoring the regular production of PSUP of high toxicity are not fully understood, the foregoing observations suggested the independence of the formation of antigen and of toxic activity, with the production, under appropriate conditions, of the former in the absence of the latter. It has been reported by De et al. (4) that gut-reactive toxin formation is affected by the pH and by the kind and concentration of peptone in the culture medium. It has also been observed in this laboratory that a slightly alkaline reaction (pH 7.2 to 7.8) was required for toxin production. According to these workers, a concentration of 3 to 5% peptone is required, with little or no toxin being produced in 1% peptone media. Dutta et al. (5) and Oza and Dutta (11) were able to grow toxic vibrios in 3% peptone. In this laboratory, PSUP is prepared from 3% peptone, or peptone dialysate, and increasing the peptone or peptone dialysate concentration to 5% has not increased toxicity.

When the concentration of peptone was reduced to 1%, the resulting PSUP was found to be of low toxicity (<12 units/ml). On fractionation on DEAE Sephadex, a single fraction was eluted in deionized water which contained no detectable toxicity but which gave the toxin antigen precipitin line on gel diffusion and proved to be immunogenically highly active. The yield was relatively low (70 mg/liter) as compared with 190 mg of antigen per liter prepared by (NH₄)₂SO₄ precipitation of toxic peptone PSUP.

When the pH of 3% peptone was adjusted to 6.5, there was no apparent inhibition of growth, but the supernatant fluids contained no detectable toxicity, confirming the observations of De et al. (4). Preparations of this kind were fractionated on DEAE Sephadex with the elution of a single peak in deionized water (Fig. 7). This material was obtained in good yields, 150 to 180 mg/liter. It gave the toxin antigen precipitin line on gel diffusion and was immunogenic, containing 10 to 15 immunogenic units per mg.

In sum, the nontoxic antigen could be prepared from toxic PSUP by its separation from the toxic activity by (NH₄)₂SO₄ precipitation followed by fractionation, or by fractionation on cationic or anionic Sephadex with or without recycling. It could also be prepared from PSUP of very low or undetectable toxicity produced by reducing the peptone concentration to 1% or the pH to 6.5. The immunological identity of the antigen prepared by these several methods was established by the demonstration of the identity reaction given by them on gel diffusion (Fig. 8) and was further substantiated by their common

**Fig. 6. Reduction in net positive charge of subfractions 1 and 2 from DEAE A25 Sephadex from that of the toxic complex as it occurs in PSUP. Top well, subfraction 1; bottom well, subfraction 2; trough, anti-WCL serum. Both now move toward the positive pole from the point of application, the nontoxic antigen having the greater net negative charge.**
removed by centrifugation-filtration through a modified sintered glass tube at 1,085 × g for 10 min. The filtrate was treated a second time with fresh Sephadex and the process was repeated. The yield, based on this kind of fractionation of toxic PSUP, was about 70%, similar to that obtained by column fractionation. When applied to nontoxic PSUP, the product was of substantially the same immunogenic potency as that obtained by single-column fractionation.

**Stability of the antigen.** The toxic activity is well known to be unstable in partially purified form, and the antigen proved to be unstable also but to a lesser degree. On storage in solution in the refrigerator, the immunogenicity had disappeared in 3 months and such preparations no longer gave a characteristic precipitin line on gel diffusion (Fig. 8). Although about 50% of the immunogenicity was lost immediately on freeze-drying, the ability to form precipitin lines on gel diffusion was substantially unimpaired, and the remaining immunogenic activity was

ability to stimulate the formation of neutralizing antibody.

The yields and relative immunogenic potencies of representative preparations are shown in Table 1. It is clear that the toxic antigen preparations are less active than the nontoxic preparations by a factor of about three. Of the former group, the least immunogenic is subfraction I from which antigen was partially removed from the toxic activity by fractionation on DEAE A25 Sephadex. The latter group may be divided into those preparations having an antigen content of 10 to 15 immunogenic units per mg, and those containing >30 immunogenic units per mg. The higher potency nontoxic preparations are the more highly purified materials obtained as subfraction 2 or by recycling in the DEAE-CM sequence. Similar purification was not effected by recycling in the CM-DEAE sequence, an observation consistent with the above noted failure to reduce the net positive charge of the antigenic component in this way.

**Batch preparation of antigen.** The separation of the antigen-containing fraction of nontoxic PSUP could also be effected by a batch method. DEAE A50 Sephadex hydrated in deionized water was added to an equal volume of PSUP with stirring and was allowed to stand in the refrigerator for 2 hr with occasional stirring. The Sephadex was

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**Fig. 7.** Separation of immunogenic antigen from nontoxic pH 6.5 PSUP by elution in deionized water from DEAE A25 Sephadex. Its relative homogeneity in comparison with the elution pattern of toxic PSUP shown in Fig. 3 is apparent.

**Fig. 8.** Immunodiffusion identity reaction given by the nontoxic immunogenic antigen prepared by several methods. Well 1, ethyl alcohol-ether-precipitated reference preparation; well 2, antigen prepared from low toxic PSUP by fractionation on DEAE Sephadex and stored for 3 months in solution in the refrigerator; well 3, antigen separated from nontoxic pH 6.5 PSUP on DEAE Sephadex; well 4, antigen separated from the (NH₄)₂SO₄ precipitate of toxic peptone PSUP on DEAE Sephadex; well 5, same as well 4 but a different lot; well 6, antigen separated from nontoxic pH 6.5 PSUP by (NH₄)₂SO₄ precipitation and elution from DEAE Sephadex in deionized water. The center well contains anti-WCL serum.
stable, persisting for more than a year when stored in a dry state in the refrigerator.

**DISCUSSION**

The separation of antigen associated with the gut toxin is of limited significance if only its ability to combine with one of the antibodies present in antiserum to the vibrio or to a mixture of its metabolic products is known; i.e., its specificity is necessarily established by its ability to elicit the formation of specific neutralizing antibody demonstrable by bioassay. It appeared to us, therefore, that the development of an assay of immunogenic potency, based on such formation of neutralizing antibody, was an essential feature of the study of such antigenicity. The regular relation between dose and antibody response over appropriate dose ranges has been known for many years, and has been most recently studied by Holt (6) with diphtheria toxin. A similar situation was found to obtain with the cholera gut toxin, as described elsewhere in preliminary form (Symp. Cholera. U.S.-Japan Coop. Med. Sci. Program, Palo Alto, 26–28 July 1967, p. 151–153), to provide the basis for the immunogenic potency test described here. The definition of the end point, or immunogenic unit, as the interpolated amount of antigen required to produce, under the prescribed conditions, an antitoxin level of 100 units/ml is arbitrary. This titer corresponds to the serum antitoxin level found in human patients at about 4 days post-onset of the disease (7).

Using this immunogenic unit, the more highly purified preparations of antigen had an immunogenic potency of 30 to 35 immunogenic units per mg, whereas less-purified preparations, such as the entire fraction eluted in deionized water from DEAE A50 Sephadex, contained 10 to 15 immunogenic units per mg. Disregarding possible inactivation of antigenicity during the preparative process and possible adjuvant activity of incompletely removed reagents, relative immunogenicity may be considered to be indicative of relative purity of the antigen. Thus, the toxin antigen preparations were of less immunogenic potency than the nontoxic antigens, presumably a consequence of contamination of the latter with nonantigenic toxin.

The data presented here led to, and are consistent with, the working hypothesis that the gut toxin of the cholera vibrio occurs as a complex from which the toxic moiety and the protein carrying the specific antigenicity may be separated from one another and from the inactive material represented here as subfraction 3 from DEAE A25 Sephadex fractionation. The protein moiety may be analogous to the "choleraenoid" described by Finkelstein and Lospalluto in preliminary reports (Bacteriol. Proc., p. 75, 1969; Fed. Proc., p. 633, 1969) as giving gel diffusion reactions with anticholera sera absorbed with intact vibrios, although it was not shown to elicit the formation of toxin-neutralizing antibody.

That the toxicity is not the specific antigenic determinant of the complex is indicated by the 5- to 10-fold difference in immunogenicity between even the incompletely separated toxic and nontoxic but immunogenic subfractions separable on DEAE Sephadex. On further purification by recycling through CM Sephadex, or by recycling through DEAE Sephadex following primary fraction on CM Sephadex, the toxic moiety gave no evidence of antigenicity, as in gel diffusion reactions. Although such observations are consistent with the assumption that the toxic moiety does not contribute to specific antigenicity, it is also possible that it is too small to give a visible precipitate or to otherwise function as an antigen; this point is a matter of continued study.

The nature of the naturally occurring toxic complex is as yet a matter of speculation. It apparently may be dissociated in high-salt concentrations, as in the \( \text{(NH}_4\text{)}_2\text{SO}_4 \) precipitation, to allow the toxic moiety to combine with an undialyzable component of peptone sufficiently
negatively charged that the new complex requires electrolyte for elution from DEAE Sephadex. In the absence of such a peptone component (e.g., when the culture is in peptone diaysate medium), if the complex is so dissociated it is presumably recombines with the protein moiety, with which it was originally associated. This, and other evidence not directly relevant to the present study, suggests that the toxic moiety may be relatively reactive and, perhaps in part because of its net positive charge, tend to combine with components of the culture medium, leading to apparent differences in its properties as produced in different culture media. However this may be, the protein antigen of the complex as produced in peptone media and presumably present as such in the vibrios since WCL toxin is specifically neutralized by antiserum to it, behaves as if it had a net negative charge similar to, for example, that of serum globulins.

The terminology of fraction I and fraction II introduced by Coleman et al. (2) was derived from the order of their elution from DEAE Sephadex in linear electrolyte gradient. This becomes confusing, not only because the elution sequence is reversed from the CM Sephadex but also because the toxic and antigenic moieties are subfractions of fraction I, and the antigen occurs in fraction II as well as in fraction I. For purposes of clarification, it is suggested that the antigen characterized by giving the toxin precipitin line on gel diffusion and eliciting the formation of toxin-neutralizing antibody be designated the T, or toxin, antigen. Similarly the predominant antigen of the fraction II of Coleman et al. (2) shown by Kaur and Burrows (8) to give a precipitin identity with vibrio lipopolysaccharide and stimulate the formation of vibriocidal antibody may be designated the V antigen (i.e., that concerned with vibriocidal antibody).

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