DIALYSIS-CULTURE PRODUCTION OF *VIBRIO CHOLERAE* EXOTOXIN AND ITS PRECIPITATION WITH ZINC ACETATE

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PLATE XIX

CHOLERA toxin has been isolated in purified form by methods involving preliminary concentration, followed by a combination of gel-filtration and chromatographic separation (Coleman *et al.*, 1968; Finkelstein and LoSpalluto, 1969, 1970; Richardson, Evans and Feeley, 1970). An initial separation and partial purification of the toxin from culture filtrate has been effected more simply by absorption on aluminium-hydroxide gels (Spyrides and Feeley, 1970). I have been able to simplify this step further and obtain a purer product by precipitation with zinc acetate. A preliminary account of this method, which should be equally suitable for experimental work and large-scale production, is presented here.

MATERIALS AND METHODS

Vibrio cholerae, strain 569B, was used for toxin production in a medium basically similar to the dialysed peptone of Coleman *et al.*, but in dialysis-culture flasks (Schultz and Gerhardt, 1969). The 500-ml conical culture flask contained 120 ml of 0.5 per cent. NaCl and a dialysis bag (20 × 2.5 cm) filled with 15 ml of 30 per cent. Difco Peptone in 0.5 per cent. NaCl, pH 7.6. This complete set-up was autoclaved at 121°C for 20 min. and samples of peptone were then taken from the flask for growing 4-hr starter-cultures which were used to inoculate the flasks by loop with approximately 1×10^7 cells. The flasks were incubated for 18 hr at 30°C on a rotary shaker. After the addition of 0.01 per cent. merthiolate, cells were removed from the culture by centrifugation in the cold at 9000 *g* for 20 min., and the toxic supernate was adjusted to pH 6.7 before storage at 4°C. Proteolytic activity in the supernate was insignificant at pH values of less than 7.0, and the toxic activity was stable under these conditions for at least 2 mth.

Toxin, or its formalised anatoxin, was precipitated from the supernate by a modification of the methods used for the purification of staphylococcal α -haemolysin (Madoff and Weinstein, 1962); 0.5-M zinc acetate was added to the supernate in the cold to a final concentration of 10 to 20 mM, maintaining the pH above 6.4 and finally adjusting it to 6.7. The resulting suspension was left for 1 hr before centrifuging off the precipitate in the cold at 3000 *g* for 10 min.; active toxin could actually be recovered from such a suspension after several days. The sediment was stored as a suspension in 1/40th the culture volume of 0.85 per cent. NaCl, and was used directly for the production of antitoxic sera in rabbits by weekly intramuscular injections of 0.5 ml. Active toxin was rapidly recovered from the suspension by dialysing in the cold against 13 per cent. di-sodium ethylenediaminetetra-acetate, pH 6.4, until dissolved, and then against 100 vol. 0.85 per cent. NaCl. The recovered toxin was stable for at least 1 mth at 4°C.

Yield and specific activity were measured by radial diffusion (RD) assay with antitoxic

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FIGURE.—Disk-electrophoresis of cholera toxin (protein stain). (a) Aquacide-concentrated supernate, batch 1: 0.5 mg protein; 60-min. run; (b) zinc-precipitated toxin, batch 1: 0.08 mg protein; 60-min. run; (c) zinc-precipitated toxin, batch 3: 0.15 mg protein; 70-min. run; (d) zinc-precipitated toxin, batch 4: 0.08 mg protein; 45-min. run.

sera incorporated in 1 per cent. Ionagar (Oxoid) in 0.1-M NaCl, buffered to pH 8.0 with 0.077-M tris(hydroxymethyl)aminomethane. The anticholera serum of Dr R. A. Finkelstein was used for comparison (Finkelstein, 1970). Readings were taken after 48 hr at room temperature (25–30°C). The cube of the radius of the ring of precipitation formed (R^3), was found to be proportional to antigen concentration and this unit was used for calculations. Toxicity was assayed in rabbits by the skin permeability test (PF; Craig, 1966) and some samples were assayed for ileal-loop toxicity (Bhattacharyya and Narayanaswami, 1971). The protein content of the culture supernate and the zinc-precipitated toxin was estimated by the method of Lowry, with bovine albumin fraction V (Sigma) as standard (Lowry *et al.*, 1951).

The purified toxin was analysed by disk-electrophoresis in polyacrylamide gel following the procedures described in the Shandon Manual (Shandon Scientific Co., 1969). The high gel concentration of 15 per cent. was used to restrict zone diffusion and to increase definition. Untreated culture supernate, concentrated ten-fold with Aquacide 1 (Calbiochem), was used for comparison. Electrophoresed gels were split and incorporated in agar plates for immunodiffusion analysis, or they were fixed and stained with amido black.

RESULTS

The growth of *V. cholerae* in peptone dialysis-culture differed from the usual flask procedure employing the pre-dialysed peptone medium of Coleman *et al.* The cell-division phase of the growth cycle was linear rather than exponential; it was of longer duration and the transition to the stationary phase was slower. Cell density and toxin concentration were increased more than two-fold, the latter reaching a level detectable by immunodiffusion.

The efficacy of the zinc-acetate precipitation for the purification of enterotoxin is illustrated in the table. The immunological and biological data for three culture batches are consistent, with high yields and substantial degrees of purification. From the relatively small volumes used, a 30- to 40-fold concentration was obtained.

After electrophoresis of the recovered material at pH 9.5 for 1 hr at a current of 3.75 mamp. per tube, the toxin zone moved 5 mm from the origin towards the anode, and was identified by immunoprecipitation with Finkelstein's reference antiserum. The immunoprecipitating and protein-staining zone of the zinc-precipitated toxin coincided with the toxin zone of the crude supernate. The zinc-precipitated toxin was contaminated in trace amounts with some of the nine other components discernible in the untreated culture supernatant (the figure). These contaminating traces could not be completely removed by washing the precipitated toxin or by treating it with trypsin. All the components of the culture supernate moved to the anode at pH 9.5; there was no movement at acid pH.

Immunodiffusion demonstrated that two major and three minor antigenic components were separated by electrophoresis of the crude supernate, of which one other than the toxin antigen persisted minimally in the zinc-precipitated toxin. Finkelstein's antiserum also reacted weakly with this contaminant. In two of the five immuno-electrophoretic runs carried out, the zinc-precipitated toxin zone reacted with Finkelstein's antiserum as two overlapping but unrelated components of equal intensity. In reversed RD assay with culture supernate incorporated in the agar, and antisera distributed in the wells, splitting of the precipitation rings was observed in the overlapping diffusion areas of

TABLE
Specific activity and yield of cholera toxin obtained by precipitation with zinc acetate

Culture batch	Culture volume used (ml)	Final zinc-acetate concentration used for precipitation (mM)	Material* tested	Toxin concentration factor after recovery (Z/S)	Result of assay by the stated procedure					
					Radial diffusion†			Skin permeability		
					Activity per μ g	Increase in activity (Z/S)	Percentage yield	Activity per μ g	Increase in activity (Z/S)	Percentage yield
1	80	20	S Z	... 30	0.03 0.20	... $\times 7$	... 70	150 1000	... $\times 7$	... 70
2	200	10	S Z S	... 40 ...	0.018 0.202 0.016	... $\times 11.2$...	... 59 ...	83 900 60	... $\times 11$...	... 60 ...
3	140	10	Z Z _w	40 ...	0.153 0.188	$\times 9.1$ $\times 11.7$	70 ...	1000‡ ...	$\times 16$...	70 ...

* S = Culture supernate; Z = toxin recovered from the zinc acetate precipitate; Z_w = toxin recovered from precipitate that had been washed once with buffered saline.

† Three per cent. antitoxic serum (equivalent to 0.47 per cent. Finkelstein's reference antiserum) was used for the assay of S.

‡ Ileal loop activity of this preparation was 13 units per μ g—an increase of 16-fold over S.

adjacent wells, indicating two distinct antigens of differing solubilities in the region of antibody excess.

Electrophoretic analysis and double-diffusion immunoassay showed that toxin precipitated from undialysed peptone culture, or from culture supernatants treated with 0.12 per cent. formaldehyde was comparable in quality. Purity of the formaldehyde-treated toxin was considerably reduced when the precipitation was performed at room temperature (25°C).

DISCUSSION

The advantages of dialysis culture for the production of bacteria and their extracellular products at the experimental and industrial level have been discussed in the review of Schultz and Gerhardt (1969) who found that, compared with results obtained with conventional flask culture, product concentration could be increased 11-fold, and the volume to be handled was correspondingly reduced. In the relatively crude version of dialysis culture described in the present work, the diffusion of nutrients from the bag was rate-limiting, and this resulted in the altered growth-cycle dynamics typical of the system. At the experimental level the procedure is simple and economical; it gives a stable culture and allows a less critical harvest time. The increased toxin yield permits immunoassay without prior concentration of the supernate, and it is reasonable to anticipate that a further increase in toxin production might be achieved by improvement of cultural conditions. The investigations of Finkelstein and LoSpalluto (1970) and Richardson, Evans and Feeley (1970) indicate that less complex nitrogen and energy sources give better toxin yields than peptone-based media.

Zinc-acetate precipitation of toxin from culture supernates is comparable in principle and method with the aluminium-hydroxide gel absorption technique of Spyrides and Feeley (1970) and the dextran sulphate-calcium chloride precipitation of Richardson and his co-workers. Of these precipitation methods, the zinc-acetate procedure appears to be the simplest, most rapid and suitable for bulk processing. The aluminium hydroxide gel procedure involves some difficulty in the preparation of the gel, and the dextran-sulphate-calcium chloride procedure requires prior dialysis of the culture supernate. Richardson *et al.* obtained yields of 30 and 44 per cent. from two-litre lots by dextran-sulphate precipitation with only a 1.6-fold increase in specific activity, as measured by PF assay in guinea-pigs. Spyrides and Feeley reported 56 and 95 per cent. yields from 4-l lots by PF assay in rabbits. By the zinc-acetate procedure in the present study, toxin could be precipitated from small volumes with a recovery of 70 per cent., in a single step and with increases of specific activities of 9 and 12-fold by RD and PF assay respectively.

Richardson and his colleagues further purified their dextran-sulphate product by ammonium-sulphate precipitation, gel filtration and DEAE-Sephadex chromatography to achieve total increase in purity of 12.5-fold. Another highly purified toxin has been prepared by Finkelstein and LoSpalluto by repeated membrane-filtration concentration and Sephadex-filtration. The toxic activities of these

pure preparations have variously been reported as 300 to 6000 PF units per μg , and 5–30 ileal loop units per μg . However Greenough, Pierce and Vaughan (1970) simultaneously assayed samples of both these preparations for PF and ileal loop activity in the rabbit; PF activities were 700 and 300 units per μg and ileal loop activities were 25 and 29 units per μg for the Richardson and the Finkelstein toxins respectively. The activities of my preparation were of the order of 1000 PF units and 13 ileal loop units per μg .

On the basis of RD assay, the activity of my preparation was of the order of 0.2 units per μg . However, Finkelstein found that this assay was biased in favour of toxoid, the content of which in my preparations is unknown. Finkelstein's own data indicate RD activities of 0.23 for the toxin and 0.53 for the toxoid, which were separately isolated from fermenter cultures in the ratio of four to six by the procedures used (Finkelstein, 1970; Finkelstein and LoSpalluto).

On the basis of this comparison of biological and RD activity data, the zinc acetate-precipitated toxin appears to be about 50 per cent. pure. It should be emphasised that the different parameters involved and the large errors inherent in bio-assay make such a comparison tentative, but the results indicate that zinc-acetate precipitation is an effective means of by-passing several of the concentration and purification steps necessary in existing procedures for the preparation of pure toxin.

The PF and ileal-loop toxic activities have been chromatographically separated (Lewis and Freeman, 1969). My immuno-electrophoretic observations made with Finkelstein's reference antitoxin serum, and corroborated by RD assay, imply that not only my toxin preparation but also that of Finkelstein and LoSpalluto comprises a complex of two molecular and antigenic species with almost identical physico-chemical properties. Further work is necessary to confirm this finding and to examine the possibility that the two antigens may be the skin-permeability and enterotoxic factors.

SUMMARY

Methods are described for the dialysis culture and subsequent concentration and purification of *Vibrio cholerae* enterotoxin, or its formalised toxoid (anatoxin), by precipitation with zinc acetate. The techniques are simple and rapid, and are likely to be suitable for research and large-scale production. An 11-fold increase in specific activity, a 67 per cent. yield, and a 40-fold concentration were achieved in a single precipitation step. Immuno-electrophoretic data indicate that the purified toxin may be a complex of two different antigenic components.

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