

Cholera Toxins: Purification and Preliminary Characterization of Ileal Loop Reactive Type 2 Toxin

WILLIAM H. COLEMAN, JASBIR KAUR,¹ MARIAN E. IWERT, GEORGE J. KASAI,
AND WILLIAM BURROWS

Department of Microbiology, University of Chicago, Chicago, Illinois 60637

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Details for the preparation and partial purification of culture supernatant fluids of *Vibrio cholerae* (*V. comma*) 569B which retain rabbit ileal loop fluid-accumulating activity are presented. These preparations were fractionated on Sephadex G-200 and on diethylaminoethyl-Sephadex. On the latter, two fractions were obtained by elution with a linear sodium chloride gradient. The fraction designated "fraction I" retains the toxic activity as demonstrated in the rabbit ileal loop model. Chemical and immunological properties of this active fraction are described.

The cholera toxins designated type 2 are characterized by their heat-lability (95% inactivation at 56 C in 15 min), retention on dialysis, and presence in cell-free liquid culture supernatant fluids and in the intracellular substance, but not cell wall, of the cholera vibrio. Toxic activity is demonstrable as a toxin-induced water and ion movement from the tissues into the lumen of the bowel in three animal models, viz., the infant rabbit (9) and the dog (4) after inoculation by the intragastric route or directly into the small bowel on laparotomy, and in the ligated loop of the lower ileum in the adult rabbit by intraluminal inoculation (8). In the first two, a profuse diarrhea is produced; in the last, there is an accumulation of fluid in the lumen which closely resembles the human cholera stool in composition (16). Present evidence suggests that the same toxic substance is active in all three models (W. Burrows, Cholera toxins, *Ann. Rev. Microbiol. in press*).

The reaction of the ileal loop is overtly indistinguishable, whether it is produced by infection or cell-free toxin. Quantitative studies have shown that the effect of infection may be accounted for the amount of toxin present (3). Quantitation of the reaction has also allowed the titration of neutralizing antibody as well as of toxin (14). Other toxic effects, notably, an associated apparent alteration in bowel permeability to allow the absorption of Boivin antigen (13), may be produced when crude preparations such

as ultrasonic lysate of vibrios are used; however, the effects produced by water and ion movement are overriding. A relation of this toxic activity to the pathogenesis of the human disease is suggested by the neutralizing antibody response of patients (14) and by the occurrence of high anti-toxin titers found in late convalescent sera in the sera of asymptomatic carriers of the infection (*unpublished data*).

The present communication is concerned with the preparation of this toxin in an apparently high state of purity and a description of some characteristics of this purified material.

MATERIALS AND METHODS

Toxin preparations. The strain of *Vibrio cholerae* (*V. comma*) used in the experiments described here was the toxigenic 569B Inaba serotype strain obtained originally from N. K. Dutta. Whole-cell lysate was prepared from an 18-hr growth of the vibrios on 3% peptone-agar (Difco), washed off in sterile deionized water, and the washed suspension was disrupted by ultrasonic treatment as described elsewhere (3). The preparations were freeze-dried, because the toxic activity in this crude form is stable to this treatment, and the unit of toxicity (3) was 130 μ g.

Peptone-water supernatant fluids (PSUP) were prepared from 6.5-hr cultures, in a medium containing 3% dialysate of peptone (pH 8), incubated at 37 C as agitated cultures. The culture was chilled, the vibrios removed by centrifugation at $4,340 \times g$ for 30 min at 0 C, and the supernatant fluid was filtered through membrane filters (0.42 μ m; Millipore Corp., Bedford, Mass.). PSUP was concentrated 12- to 15-fold by flash evaporation at a temperature of ca. 12 C, and exhaustively dialyzed. Such preparations contain no detectable endotoxin, no unaltered culture medium, and no toxic activity affecting active sodium transport.

¹ Research Officer, Indian Council of Medical Research; Research Fellow of the Office of International Research, National Institutes of Health, Public Health Service.

Toxin titration. Toxin was titrated in the rabbit ileal loop as previously described (14). After a rough titration to establish the dilution range, toxin was titrated by using four dilutions in at least four rabbits, i.e., every dilution was tested in quadruplicate, to give a close approximation of toxin titer. When more precise titers were required, additional animal titrations were performed.

Chromatography. Chromatographic separations were carried out by using Sephadex products and columns (Pharmacia Fine Chemicals, Inc., New Market, N.J.). Materials were fractionated in Sephadex G-200 in columns (1.5×18.0 cm) by elution in 0.05 M NaCl at an average flow rate of 3 ml/hr. Diethylaminoethyl (DEAE)-Sephadex A50 columns were eluted with 300 ml of linear gradient NaCl to 0.5 M. Gel beds were maintained at 4°C in jacketed columns. Elution was monitored by ultraviolet absorption or protein analysis (or both) and, in the case of labeled preparations, by radioactivity.

Thin-layer gel filtration was according to the procedures of Andrews (1), using Sephadex G-75 Superfine equilibrated in 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-HCl buffer, pH 7.5. Plates were spread to a thickness of 0.5 mm and eluted with the same buffer at an angle of 30° for 22 hr at room temperature. The standards employed were bovine serum albumin (Armour Pharmaceuticals, Kankakee, Ill.), lysozyme (Worthington Biochemicals, Freehold, N.J.), and ribonuclease (Pentex, Inc., Kankakee, Ill.). Standards were located by staining with amido black. The material under investigation stained poorly with amido black or Oil Red O and was located by radioisotope labeling.

Labeling. PSUP was labeled with tritium by the inclusion of L-alanine-3-³H (New England Nuclear Corp., Boston, Mass.) in amounts of 0.5 mc/liter of peptone dialysate medium. Radioactivity was measured in counting vials containing 10 ml of a dioxane-based scintillation fluid (2) in a Packard liquid scintillation counter. All counts were corrected for background.

Analytical methods. Column eluates were usually monitored by absorption at 280 nm in a Beckman model DU spectrophotometer, but also for protein by the Folin-Ciocalteu method (11). Protein content of fractions was measured by this method, standardized against crystalline bovine serum albumin (Armour Pharmaceuticals, Kankakee, Ill.), and calculated from total nitrogen by the micro-Kjeldahl method (15). Amino acid analyses were carried out by the quantitative ninhydrin procedure of Cocking and Yemm (6). Carbohydrate was measured by the anthrone method (20) with glucose as a standard. Phosphate analyses were by the ammonium molybdate procedure (7).

Lipid was extracted with chloroform-methanol, 2:1 (v/v), by homogenization and in a Soxhlet apparatus. In the former, each 100 mg of freeze-dried fraction was extracted with 60 ml of the solvent mixture (5) in a Sorvall homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.) at 10,000 rev/min for 30 min in a stainless-steel container immersed in ice. The solvent phase was separated by centrifugation at

3,000 rev/min at 4°C. The extraction was repeated twice, the extracts combined and washed according to Folch (10), and the solvents removed by evaporation under a stream of N₂. Extraction in the Soxhlet apparatus was carried out at the same weight-volume ratio for 5 hr, repeated a second time, and the combined extracts treated as above. The characterization of lipids by gas-liquid chromatography will be reported elsewhere. However, for the rough approximation of total lipid, the extracts were taken up in a known volume of chloroform and applied in duplicate on strips of Whatman no. 1 filter paper. Alternate spots of methyl oleate were similarly applied in amounts of 5, 10, 20, 40, and 80 μ liters each. After drying, the paper was stained with Oil Red O or Sudan black B for 20 hr and washed free from excess dye with 60% ethyl alcohol. The lipid stains were prepared according to Grabar (12). After drying at room temperature, the stained spots were cut out and the color was extracted with isoamyl alcohol for 16 hr. The color was read in a Klett-Summerson colorimeter, with the no. 54 filter for Oil Red O and no. 66 for Sudan black B.

Immunological methods. Immunoelectrophoresis was carried out on glass slides (19) in agarose-gel (Bausch Lomb, Inc., Rochester, N.Y.) suspended in veronal buffer, ionic strength 0.025, pH 8.6, with thimerosal added as a preservative at a final concentration of 1:100,000. After equilibration at room temperature, antigen solutions in wells (1- to 2-mm diameter) were electrophoresed at 48 to 52 ma and 200 v, or 8 v/cm, for 60 min through the completed circuit containing veronal buffer at ionic strength 0.10, pH 8.6. After electrophoresis, 100 μ liters of hyperimmune antiserum prepared in a rabbit against whole-cell lysate antigens was placed into a 1- to 2-mm longitudinal trough cut perpendicular to and equidistant between the antigen wells, and the reaction was allowed to develop for 48 hr at room temperature. After rinsing out the excess reactants, the preparation was dried at room temperature, stained with 0.25% thiazine red R (Allied Chemical Corp., New York, N.Y.) in methanol-acetic acid-water (45:10:45, v/v), and destained with the same solvent. Gel double-diffusion reactions were also carried out on glass slides in agarose in 0.40% NaCl, at approximately pH 7.0, with 1:100,000 thimerosal (17, 18). Immune precipitates were developed, washed, stained, and destained, as above.

RESULTS

Preliminary experiments had shown that the vibrios grew well in the dialysate of peptone, but not in the nondialyzable residue. The vibrios were grown as agitated cultures in 250 ml of medium in 1-liter flasks, giving a medium depth of about 2.5 cm. The rates of toxin production and growth were studied to arrive at a maximal toxin production with minimal contamination with vibrio autolysate. With an inoculum of 10¹⁰ agar-grown vibrios from 18-hr cultures, the optimal incubation time was found to be about 7.5 hr (Fig. 1).

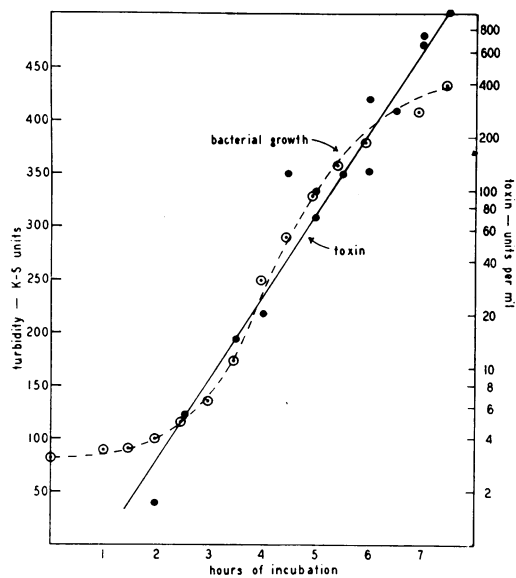


FIG. 1. Toxin production during growth of *V. cholerae* 569B. Toxin was titrated in the rabbit ileal loop model. Growth was at 37C on a reciprocal shaker, turbidity of bacterial growth in Klett-Summerson units; units of toxin per milliliter.

It was not possible to reduce the incubation time by increasing this kind of inoculum, but it could be reduced to 6.5 hr by using the same number of vibrios in the exponential growth phase in 4-hr maximally aerated cultures in the dialysate medium. Dialysis of supernatant fluids of such cultures removed unaltered culture medium and toxic activity inhibiting active sodium transport, and the resulting dialysands contained no detectable polysaccharide. This indicates that autolysis of cells is not extensive in these cultures.

Chromatographic separation. The elution pattern of whole-cell lysate, undialyzed PSUP, and dialyzed PSUP is illustrated in Fig. 2. The toxic activity was present in the 10 ml between 15 and 25 ml of the eluate. Whole-cell lysate is complex in composition, and the toxic fraction is admixed with carbohydrate. In undialyzed PSUP concentrated 15-fold from the original volume of culture supernatant fluid, there was no carbohydrate detectable by the anthrone method, i.e., $< 0.02 \mu\text{g/ml}$. The considerable amount of protein indicated by the Folin method of analysis is an artifact in that it consisted largely of amino acids demonstrable by the ninhydrin reaction and possibly also low molecular weight polypeptides. The toxic activity of dialyzed PSUP is eluted as a single protein peak which was shown to be uncontaminated with amino acids.

Dialyzed PSUP and the toxic fraction eluted

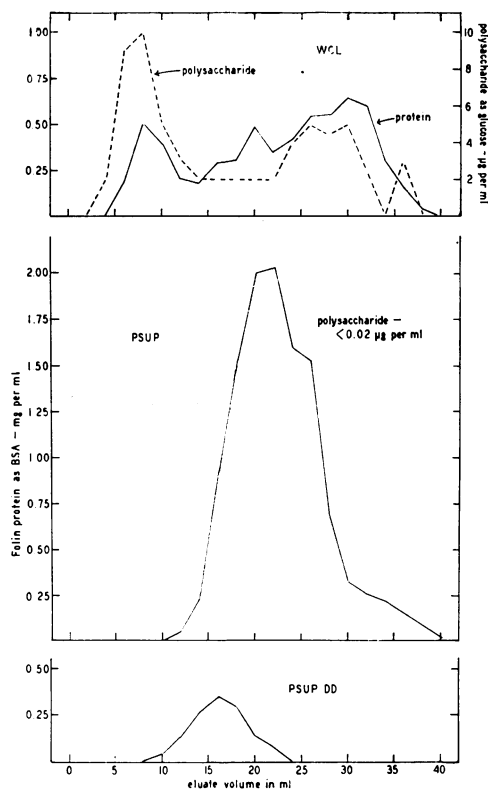


FIG. 2. Fractionation of toxin preparations of *V. cholerae* 569B with Sephadex G-200. Elution was with 0.05 M NaCl . Whole-cell lysate fractionation is represented in the top graph; peptone supernatant fractionation is in the center graph; dialyzed peptone supernatant fractionation is shown at the bottom.

from Sephadex G-200 were examined by immunoelectrophoresis and gel diffusion against hyperimmune rabbit serum. The results obtained by immunoelectrophoresis of dialyzed PSUP are shown in Fig. 3 in comparison with those given by whole-cell lysate at the same dry-weight concentration. Whole-cell lysate contained a multiplicity of antigens demonstrable in this way—not less than 17—by using antigen solutions containing approximately 25 mg of the freeze-dried material per ml, whereas 15-fold concentrated PSUP showed 3 antigenic components differing in electrophoretic mobility. The two major components, designated I and II in Fig. 3, were found to be present in the single protein peak of dialyzed PSUP as eluted from Sephadex G-200. It is notable that I was sufficiently positive that it migrated to the cathode under the conditions employed here.

The indicated difference in charge in the two antigenic components suggested the possibility

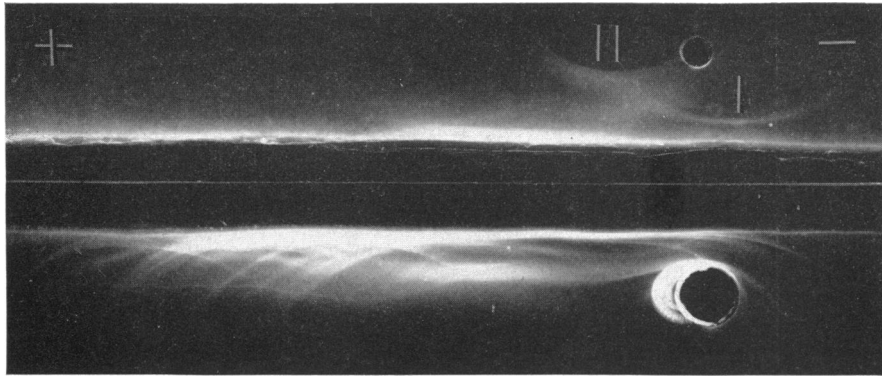


FIG. 3. Immunoelectrophoresis of *V. cholerae* 569B whole-cell lysate and PSUP. Top well contains whole-cell lysate and the bottom well contains PSUP. Center well contains antiserum to whole-cell lysate.

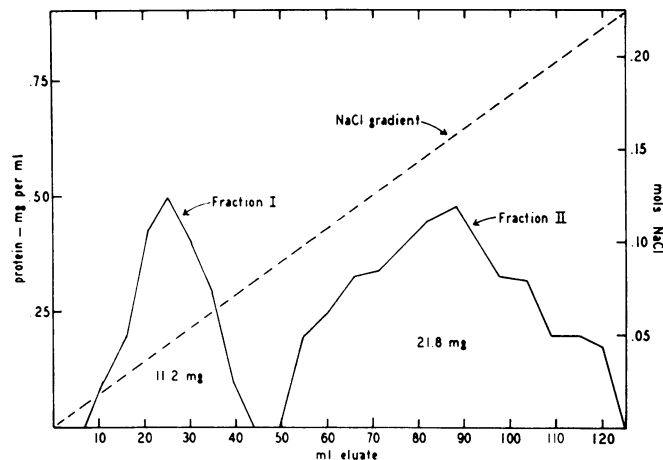


FIG. 4. Fractionation of dialyzed PSUP with DEAE Sephadex. Elution was with a linear NaCl gradient indicated by the hyphenated line.

of ready separation by ion-exchange chromatography. Accordingly, this material was chromatographed on DEAE-Sephadex. Gradient electrolyte elution gave the results illustrated in Fig. 4; these data are based on Folin protein rather than the more commonly used ultraviolet monitoring. The single protein fraction from Sephadex G-200 was cleanly separated into two fractions, here designated fraction I and fraction II, in the order of their elution; these correspond to the precipitin lines marked I and II in Fig. 3. All of the toxic activity occurred in fraction I; fraction II appeared to be completely atoxic in the ileal loop.

It was shown by double gel diffusion that fraction I gave an identity reaction with the more rapidly diffusing antigen present in PSUP, fraction II gave an identity reaction with the more slowly diffusing antigen, and the two antigens, both as separated fractions and as

occurring together in PSUP, gave nonidentity reactions. Further, the precipitin line given by fraction I was present in toxic preparations of PSUP and was not detectable in nontoxic or slightly toxic preparations.

In Fig. 4, the relative proportion of fraction I to fraction II is approximately 1:2 when measured as Folin protein. This ratio was found to vary with the titratable toxicity of PSUP by bioassay, the amount of fraction I being directly related to toxicity. When fraction I was small in amount, there appeared to be more fraction II. In these preparations, the latter was associated with a light tan color and PSUP, generally of relatively less toxicity, was darker in color.

For preparative purposes, PSUP was fractionated directly on DEAE-Sephadex without preliminary separation on Sephadex G-200. It was unnecessary to elute fraction I in an electrolyte gradient because it could be eluted directly

in deionized water. After such elution, as monitored by ultraviolet absorption, the column was washed with 0.5 M NaCl, and fraction II was eluted in 0.5 M NaCl.

Analysis of fractions I and II. When analyzed by the Folin-Ciocalteu method, fraction I was found to have a total protein content of 55 to 60%, and fraction II contained 90% Folin protein. When fraction I was analyzed for total nitrogen, it was found to contain 70% of calculated protein. The amino acid composition of the hydrolysates of fractions I and II is shown in Table 1; no tryptophan or tyrosine were found in alkaline hydrolysates of either fraction. The absence of methionine and the paucity of aromatic amino acids in fraction I are notable; perhaps the latter accounts, in large part, for the discrepancy in total protein values for fraction I obtained by the Folin-Ciocalteu and total nitrogen methods.

Although no carbohydrate could be found in culture supernatant fluids or other dilute preparations, carbohydrate was detected in lyophilized preparations. Fraction I contained 2.7% total carbohydrate and fraction II contained 2.9% total carbohydrate. Stainable lipid was extractable in chloroform-methanol from both fractions.

TABLE 1. Amino acid composition of fractions I and II^a

| Amino acid | Fraction I | Fraction II |
|-------------------------|---------------------|---------------------|
| | $\mu\text{mole/mg}$ | $\mu\text{mole/mg}$ |
| Alanine | 0.071 | 0.567 |
| Arginine | 0.011 | 0.182 |
| Aspartic acid | 0.074 | 0.080 |
| Cysteic acid | | |
| Glutamic acid | 0.071 | 0.131 |
| Glycine | 0.228 | 1.29 |
| Histidine | 0.010 | 0.16 |
| Hydroxyproline | 0.145 | tr |
| Isoleucine | 0.011 | 0.084 |
| Leucine | 0.020 | 0.158 |
| Lysine | 0.044 | 0.140 |
| Methionine | tr | 0.018 |
| Phenylalanine | tr | 0.087 |
| Proline | 0.055 | 0.512 |
| Serine | 0.078 | 0.097 |
| Threonine | 0.024 | 0.070 |
| Tryptophan ^b | tr | 0.113 |
| Tyrosine ^b | tr | 0.023 |
| Valine | 0.026 | 0.153 |

^a Samples were hydrolyzed with 6 N H₂SO₄ in sealed evacuated ampoules under reflux conditions (110 C) for 20 hr.

^b Data are from hydrolysis in 5 N Ba(OH)₂ for 22 hr at 110 C in sealed evacuated ampoules. Minimal molecular weights calculated are 7,740 for fraction I and 24,500 for fraction II from these data.

By approximation as equivalents of oleic acid, fraction I was found to contain 23 to 34%, and fraction II, 9 to 15%, of extractable lipid. These amounts, although of questionable reliability, are of the same order as those obtained by difference, i.e., 42% for fraction I and 7% for fraction II. In view of the presence of appreciable amounts of lipid in both fractions, it was of interest that no phosphorus (less than 0.005%) was found in fraction I. Less than 0.05% phosphorus was found in fraction II.

Molecular weight. Estimations of molecular weight by thin-layer gel filtration were carried out with ³H-labeled preparations; representative results are shown in Fig. 5. Fraction I migrated at a rate similar to that of lysozyme, giving a molecular weight of 10,000 to 14,000, whereas the major component of fraction II gave a molecular weight of 25,000 to 30,000. The presence of both fractions in PSUP is apparent. These data are in agreement with the calculated minimum molecular weights from the amino acid analysis shown in Table 1, when it is considered that the

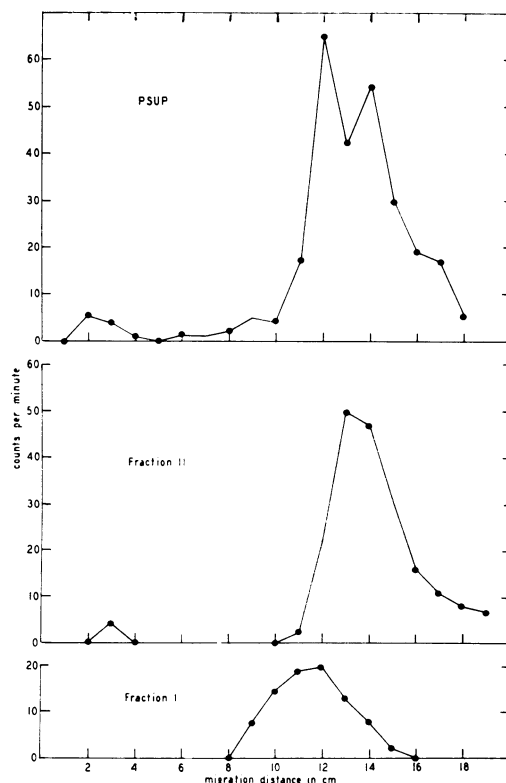


FIG. 5. Thin-layer gel chromatography of tritiated PSUP, fraction I and fraction II of *V. cholerae* 569B. Radioactivity is expressed in counts per minute per square centimeter of chromatogram.

protein moiety of fraction I is perhaps 65 to 70% and that of fraction II 90%.

Biological activity. As indicated above, toxicity demonstrable in the ileal loop appeared to be confined exclusively to fraction I. The unit of activity of this material was about 10 μ g, whereas fraction II produced no reaction in amounts as great as 10 mg. In the purified form, the toxic activity was relatively unstable, showing a half-life of about 2 weeks in solution in the refrigerator, and half the activity was lost immediately on freeze-drying. This instability was in contrast to the stability of the activity in whole-cell lysate to freeze-drying; in the dry state, the activity of whole-cell lysate was stable for at least 2 years when stored in a refrigerator under nitrogen.

Immunological activity appeared to be fully retained in the purified form. Fraction I specifically combined with antibody, not only in the gel precipitation reaction, but also with neutralizing antibody as assayed in the ileal loop. In the latter system, it combined unit for unit with antitoxin standardized against whole-cell lysate toxin.

DISCUSSION

The substance responsible for toxin-induced water and ion movement from the tissues into the lumen of the bowel in the rabbit ileal loop model appeared to be a lipoprotein complex having a molecular weight similar to that of lysozyme, e.g., about 12,000. The protein moiety contained relatively large amounts of glycine but no methionine and was generally deficient in aromatic amino acids. The total protein content of the complex as determined by the Folin-Ciocalteu method was, therefore, too low, and that calculated from total nitrogen analysis is probably more realistic. The total carbohydrate present (3%) is sufficiently small that, if it includes amino sugars, the calculated protein would not be markedly affected. A tentative estimate of about 65% protein would appear to be reasonable.

The amount of lipid present in the complex is perhaps less certain, and there is no assurance that its extraction by chloroform-methanol is complete. Estimates of the lipid content of the complex based on difference and oleic acid equivalent of extractable lipid indicate a total content of extractable lipid of about 30%. The absence of detectable phosphorus clearly indicates that the lipid moiety does not include phospholipid, and more detailed study to be reported separately has shown that the lipid of fraction I is glycerol-based.

The toxic activity separable here as fraction I

is clearly different from the nontoxic fraction II in relative content of protein and lipid. The protein moiety is distinctive in amino acid composition, and other studies on the lipid have shown that it is serine-based. The complex also differs from fraction I in immunological specificity and in having about twice its molecular weight.

The data presented here indicate that both fractions are relatively homogeneous, but there is no assurance that toxicity may not be a property of some fragment of the fraction I complex. Whether such an active component would retain the complete antigenicity of fraction I is a matter of speculation. In addition, one may speculate that a possible role of fraction II is a source of biologically inactive fraction I.

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