CHANGES IN TOXICITY OF *CLOSTRIDIUM BOTULINUM* TYPE E TOXIN BY CHEMICAL MODIFICATION AND ENZYMATIC CLEAVAGE

by

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ABSTRACT

The single residue of cysteine in Cl. botulinum type E strain Iwanai toxin has been linked with toxicity, by chemical modification using p-chloromercuribenzoate.

A peptide containing the cysteine residue has been isolated by exhaustive tryptic digestion of the toxin molecule tagged with N-(4-dimethylamino-3,5-dinitrophenyl) maleimide, and subsequent gel filtration with Sephadex G-25 and descending paper chromatography.

The toxic peptide of trypsin-activated toxin was isolated by fractionation through a composite Sephadex G-75 and G-50 column.

By chymotryptic and tryptic digestion of the toxin at pH 5.8, a toxic fragment has been isolated by gel filtration with Sephadex G-25.

On the basis of quantitative amino acid analyses, the molecular weights of the intact toxin, the trypsin-activated toxin and the chymotrypsin-trypsin fragmented toxin have been estimated to be 14,000-16,000, 10,000-12,000 and 4,000-6,000 respectively.

Although the mechanism of tryptic activation was found to involve chiefly the removal of at least 18 amino acid residues from the N-terminus of the toxin molecule, the manner of reduction by cleavage has not been determined for the chymotrypsin-trypsin fragmented toxin.

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INTRODUCTION

Cl. botulinum type E produces a toxic protein which can be obtained in a highly purified state and its molecular weight has been determined. It is serologically distinct from other types of botulinus toxins, which indicate differences in their molecular structures. However, they share a common mode of action which allegedly involves the inhibition of acetylcholine release mechanism at nerve terminals. Exactly how the toxin molecule participates in the interaction is unknown.

The common mode of action of the various types of botulinus toxins suggests the possible existence of a common, biologically active portion in the toxin molecules. This investigation attempts to characterise the biologically active portion of type E toxin. Attempts were made to determine which reactive side chains of the amino acid residues in the toxin molecule are associated with biological activity, (ie. which amino acid residues are involved in the active site), by means of chemical modification. Having established the above, attempts were then made to isolate a relatively small peptide from that predetermined region of the toxin molecule. Previous studies have suggested that the phenomenon of tryptic activation of type E toxin is due to molecular fragmentation of the toxin molecule by trypsin giving rise to a smaller, more active peptide. Attempts were made to confirm the above hypothesis. The latter's confirmation led to further attempts of fragmentation by means of other proteolytic enzymes and the

subsequent isolation of a low molecular weight, toxic fragment (peptide) from type E toxin. The structure of such a toxic peptide may be characterised with relative ease, and in turn may yield the key to the understanding of the toxicity of botulinus toxins.

Specific methods available for the chemical modification of the reactive side chains of amino acid residues are limited. Therefore, either the single histidine or cysteine residue in the toxin molecule, as revealed by quantitative amino acid analysis, became the obvious target for chemical modification studies. Moreover, the observations that pretreatment of the dialysis tubing with versene prevents the inactivation of the toxin during the process of dialysis, and mercaptoethanol stabilizes the toxin, strongly suggest that the single cysteine residue was the obvious choice for chemical modification. Because the natural route of toxigenesis is <u>per os</u>, the toxin molecules must be somewhat resistant to proteolytic enzymes encountered in the digestive tract. In other words, even though the toxin molecule has been reduced in molecular size through cleavage by proteolytic enzymes, the diminished fragment must remain biologically active for intestinal absorption. Hence, the proteolytic enzymes were the fragmentation agents of choice for the toxin molecule.

I. CHEMICAL MODIFICATION

A. Introductory remarks

Botulinus toxins may be considered as molecules consisting of single polypeptide chains due to the lack of contrary evidence. The length of the polypeptide chain and the primary structure vary among the types of botulinus toxins. The amount of cross-links and coiling is dependent on the primary structure. Chemical bonds of varying strengths responsible for the secondary and tertiary structures can be subjected to modification. Denaturation or inactivation may be defined as modification of any of these chemical bonds giving rise to a polypeptide chain which differed in stereochemical conformation from the biologically active molecule. The degree of denaturation can be assayed from the biological activity, and manipulated by chemical substitution or conversion of the diverse reactive side chains of the amino acid residues in the polypeptide. In theory, it is possible to modify chemically any amino acid residue with a side chain exhibiting a distinctive type of reactivity; but in practice, only a few suitable chemicals are available and well established. During the recent years, a number of proteins have been successfully modified by chemical means that resulted in their loss of biological activity. This has provided an approach for locating groups of amino acid residues in proteins which may be referred to as the active centres (sites).

B. Chemical modification of botulinus toxins

The lack of data on type E toxin necessitates a review of the chemical studies undertaken on type A toxin. In order to understand what the chemical studies have revealed, the physical and chemical nature of type A toxin must be considered. Type A toxin can be obtained in crystalline form (Lamanna et al., 1946). The elemental and amino acid analyses as performed by Buehler et al., (1947) have shown it to be a simple protein. It has a calculated molecular weight of 900,000 on the basis of physical measurements (Putnam et al., 1948). Lamanna (1948) found that crystalline type A toxin has both toxic and hemagglutinating properties and drew attention to the lack of identity between the toxic and hemagglutinating factors (Lamanna and Lowenthal, 1951; Lowenthal and Lamanna, 1951). The dissociation of crystalline type A toxin into subunits under the proper conditions of pH and ionic strength has been demonstrated by Wagman and Bateman (1951, 1953) and by Wagman (1954). Moreover, the highly purified type A toxin as isolated by Gerwing et al., (1965) has a molecular weight of 12,200 as calculated on the basis of physical measurements served to point out emphatically that the heterogeneity of crystalline type A toxin is highly probable.

In spite of the heterogeneous nature of the crystalline type A toxin, various investigators have conducted chemical and physical studies on it. When Boroff (1959) found experimentally that detoxification was coincidentally

accompanied by the loss of fluorescence, he postulated that toxicity was linked with fluorescence in the toxin molecule. The above hypothesis was invalidated by Schantz et al., (1961) who demonstrated that the fluorescence was retained when the toxin molecule was detoxified by 6M urea. Weil et al., (1957) found that toxicity was destroyed by photooxidation in the presence of methylene blue. Having confirmed this using the same method, Boroff and DasGupta (1964) related toxicity to the tryptophan residues in the toxin molecule. More recently, Boroff and DasGupta (1965) used 2-hydroxy-5-nitrobenzyl bromide (HNBB), a "more or less" specific reagent for tryptophan residues and have claimed that toxicity is directly linked to the tryptophan residues. Schantz and Spero (1957) reacted the toxin with ketene and found that toxicity was lost when the free $-NH_2$ groups were reacted. They further claimed that the phenolic -OH and free -SH groups are not associated with toxicity. Spero and Schantz (1957) found that deamination of the toxin with nitrous acid caused rapid detoxification. Spero (1958) found that the decrease in toxicity due to an increase in pH is associated with the ionisation of a small number of epsilon $-NH_2$ groups of the lysine residues. Thus the toxicity of botulinus toxins is linked with the reactive side chains of tryptophan and lysine residues as revealed by chemical studies on type A crystalline toxin. Since the latter has some 477 residues of lysine and 82 residues of tryptophan (Buehler et al., 1947), and the chemical reagents

employed are not exclusively specific for reaction with lysine or tryptophan, the significance of the findings based on these chemical studies is rather vague and dubious. Furthermore, studies relating molecular structure to biological activity can have fundamental significance only if the material to be tested is of a high degree of homogeneity. The biological evaluation of "impure" materials is likely to yield misleading results.

C. Methods of chemical modification

Gerwing et al., (1964) reported that type E toxin can be obtained in a highly purified state and that the pretreatment of the dialysis tubing by versene prevents the inactivation of the toxin during the process of dialysis. The latter fact hints at the possibility that the cysteine residues exist in the toxin in the reduced form. Further substantiation of such a possibility can be derived from the stabilizing effect of the toxin by mercaptoethanol which can be used to reduce or cleave the disulfide bonds in proteins (Thompson and O'Donnell, 1961). Photooxidation in the presence of methylene blue (Weil et al., 1957) or alkylation by iodoacetate are the methods of choice for a preliminary study on the toxicity of type E toxin. Photooxidation in the presence of methylene blue is a pH-, and to a certain extent, temperature-dependent reaction, and will modify tryptophan, histidine, methionine, tyrosine and cysteine residues without breaking the peptide bonds.

Because the reaction conditions in photooxidation are difficult to control and the apparatus involved complicated, alkylation by iodoacetate was chosen in this study.

Both iodoacetic acid and its derivative, iodoacetamide have been used successfully for the chemical modification of proteins. Goddard and Michaelis (1935) and Sela et al., (1959) have shown that alkylation by these reagents can be restricted to -SH groups at a pH of about 8. Observed differences in the reactivity between the two reagents are due to the negative charge on the iodoacetate ion. Yankeelov and Koshland (1961) found that phosphoglucomutase reacts with one mole of iodoacetate with a 40% loss of activity but the action of iodoacetamide is considerably more rapid. The mechanism of alkylation by iodoacetate is best illustrated with the various data obtained from inactivation of ribonuclease. Zittle (1946) observed that ribonuclease could be slowly inactivated by iodoacetate and attributed the inactivation to the alkylation of -SH groups. Ribonuclease has been shown by Hirs et al., (1958) not to contain any free -SH groups. Thorough examination by Gundlach et al., (1959) demonstrated that the alkylation of ribonuclease was a pH dependent reaction. In the absence of -SH groups and under well controlled pH, alkylation can be confined to the reactive side chains of certain amino acid residues; ie. at pH 5-6, the imidazole group of histidine; at pH 2-3, the thioether sulphur of methionine and at pH 8-10, the epsilon $-NH_2$ group of lysine and the thioether

sulphur of methionine. Moore et al., (1958) investigated the different methods of reducing the disulfide bonds in proteins and examined the methods of converting the resultant cysteine residues to stable derivatives. They found that sodium borohydride was effective in the reduction of ribonuclease and chymotrypsinogen; and iodoacetate was capable of forming the carboxymethylated product at pH 8.5. The completeness and the specificity of the reaction were checked by acid hydrolysis followed by quantitative ion-exchange chromatography (Spackman et al., 1958). Botulinus toxins are inactivated at alkaline pHs, thus thwarting any attempt to evaluate precisely the inactivation of -SH groups in the toxin molecule by iodoacetate.

D. Modification of -SH groups

The importance of the role of sulphur in proteins has been recognised and emphasised recently (Benesch et al., 1959; Boyer, 1959; Cecil and McPhee, 1959; and Cecil, 1963). Relatively little is known about the function of the methionine residues in proteins. Recent studies with ribonuclease (Vithayathil and Richards, 1960a,b and 1961a,b) and with myoglobin (Kendrew et al., 1961) suggested that the possible role of methionine is structural; ie. the formation of hydrophobic bonds. It is sufficient to say that the S-S group, when present, plays a major role in stabilizing the three dimensional structures of proteins. The estimation of the number of -SH groups in

the toxin molecule is a prerequisite to the evaluation of the group's function. The formation of highly undissociated mercaptides by silver salts, mercuric salts and organic mercury derivatives (RHgX) provides the basis for several estimation methods. Silver salts are stable, easily purified and their concentrations can be easily determined by amperometric titrations (Benesch and Benesch, 1948; Benesch et al., 1955). However the resultant silver mercaptides have a strong tendency to bind additional silver ions, thus often giving rise to misleading positive errors (Burton, 1958). Mercuric ions are more specific than silver ions in reaction with -SH groups and the complexes of mercuric mercaptides with mercuric ions are less stable than the corresponding silver compounds (Cecil and McPhee, 1959). Simple thiols react to form mercaptides of the type $(RS)_2Hg$, whereas proteins often give the half-mercaptides Prot.SHgX. In the reaction of mercuric ions and a given protein, the type of mercaptide formation will depend on whether or not it is sterically possible for two -SH groups in the protein molecule to react with one mercuric ion. Denatured proteins normally form $(Prot.S)_2Hg$ (Allison and Cecil, 1958). Organic mercury derivatives (RHgX) have a comparable specificity for -SH groups to that of mercuric ions and have the added advantage of being univalent. The R group can either be aliphatic or aromatic. The latter is preferred as it is more soluble in water and less poisonous. Among the mercaptide-forming reagents, organic mercury compounds and

notably p-chloromercuribenzoic acid (Hellerman, 1937; Hellerman et al., 1943) have long been the compounds of choice for the detection of -SH groups in biological systems because of their selectivity and the stability of the resulting mercaptides. The use of these compounds as quantitative reagents was realised by Boyer (1954) who used the increase in absorption that occurs at 250 mμ when PCMB reacts with the -SH groups. The technique employed titration of the mercurial with the -SH compound until there was no further change in differential absorption. The method has the advantage in that the measurements indicate the amount of mercaptide formed rather than the uptake of the mercurial.

Two types of -SH groups may be recognized from their reactivity toward the -SH reagents. The reactive -SH group has the same reactivity as do the simple thiols. The unreactive -SH groups are those which show a low reactivity in the native protein but normal reactivity after the protein has been denatured. The high reactivity of the reactive -SH groups has led investigators to postulate their participation in the active sites of enzymes, and there are a large number of enzymes that can be inactivated by heavy metal reagents and reactivated by thiols (Boyer, 1959). In very few cases, the -SH groups have been found to form part of the active site.

Since the introduction of Sanger's reagent for N-terminal amino acid residue determination, the value of specific colored reagents in protein chemistry has been recognised.

Witter and Tuppy (1960) established the specificity and reaction mechanism of the yellow -SH group reagent, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide. Another reagent, N-(2,4-dinitroanilino)maleimide has been synthesised and was used by Clark-Walker and Robinson in 1961. More recently, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide has been used to label an essential -SH group of rabbit glyceraldehyde-3-phosphate dehydrogenase (Gold and Segal, 1964). In each case cited, the labelling with the colored -SH reagent led to successful isolation and subsequent characterisation of a small peptide.

II. ENZYMATIC CLEAVAGE

A. Introductory remarks

Even though chemical methods can provide an approach leading to the isolation of a relatively small peptide from a biologically active protein molecule, such a peptide may not constitute the entire active centre because the reactive side chains of amino acid residues which are adjacent in sequence need not necessarily be adjacent sterically. Nevertheless, protein chemists to-day have recognised the relationship between biological activity and the reactive side chains of a few amino acid residues in the protein molecule. Even if the reactive side chain of a specific amino acid residue in a given protein molecule having been chemically modified resulted in the loss of biological activity, this does not imply that the other amino acid residues in the molecule are functionless. It may be argued that only a certain number of these other amino acid residues are necessary for the maintenance of the stereochemical conformation of the molecule. How many and which amino acid residues in a particular protein molecule fulfill this structural function must be known before such an argument can be entertained. Chemical methods are available for specific fragmentation of protein molecules (Thompson, 1960), but the loss of biological activity may be due to the drastic reaction conditions rather than the fragmentation process itself. Thus, enzymatic degradation of protein molecules may provide the means of

yielding a small, biologically active peptide.

B. Peptic digestion

Since the natural route of toxigenesis by botulinus toxins is per os, the toxin molecule may be reduced in molecular size as it passes down the digestive tract. Moreover, this smaller moiety must retain its toxicity for intestinal absorption. The toxin molecule is probably subjected to slight digestion on encountering the gastric proteolytic enzyme, pepsin. Wagman (1963) isolated a toxic, dialysable subunit with a molecular weight of 3,800 from a peptic digest of type A toxin which was previously treated at a pH of about 9. Bovey and Yanari (1960) and Herriott (1962) have reviewed studies on pepsin in detail. In essence, pepsin is an endopeptidase which catalyses the hydrolysis of a wide variety of peptide bonds but those between adjacent aromatic amino acid residues are especially sensitive. The presence of an amino group adjacent to the sensitive bond inhibits enzymatic hydrolysis. The optimum pH is near 2.0 but at this acid environment it is subjected to autolysis. Maximal stability is at pH 5.0-5.5, and the enzyme is rapidly denatured at pH of about 6.0. Dolman (1964) reported that type E toxin when digested by pepsin at pH 2.5 rapidly declined in toxicity; whereas at pH 5.5 there is no detectable effect on its potency except under prolonged digestion. The significance of these results is very difficult to assess

as the same author made no effort to isolate and compare the toxic fragment after peptic digestion at these pHs.

C. Tryptic digestion

In the intestine, the toxin molecule is subjected to further digestion by other endopeptidases as well as exopeptidases. The extent of digestion by the latter is difficult to speculate on, but significant digestion by endopeptidases prior to intestinal absorption is highly probable. The specificity of trypsin's attack on peptide bonds to which an arginine or lysine residue has contributed to the carboxyl group has been well established with synthetic and other highly purified protein substrates (Neurath and Schwert, 1950). The optimal pH range for trypsin activity is 7-9 (Green and Neurath, 1954) but the enzyme also readily undergoes autolysis at this pH range. It has maximal stability at pH 2.3 at 30 C. (Neurath and Schwert, 1950). Type E toxin is not readily inactivated by trypsin but its toxicity may be activated or potentiated (Duff et al., 1956). This phenomenon has been both speculated on and investigated recently by two independent groups. The Japanese workers, Sakaguchi et al., (1959, 1961) have speculated about a toxin "precursor" molecule whose toxic moiety was masked by a group associated with nucleic acid. They believed that this masking or inhibiting group is cleaved from the toxic moiety in tryptic digestion and the "activated" toxin molecule is

is released. They further claimed that both the "precursor" and the "activated" toxin have molecular weights of approximately 200,000 (Sakaguchi et al., 1964). The Canadian investigators, Gerwing et al., (1961, 1962) showed that their partially purified type E toxin, both before and after trypsin treatment, was devoid of nucleic acid, and also postulated that the process of tryptic activation involves some degree of molecular fragmentation resulting in the formation of a smaller, more active molecule.

D. Chymotryptic digestion

In the intestine, the toxin molecule must encounter the other endopeptidase, chymotrypsin. Even though tryptic activation is an *in vitro* phenomenon, it has been established that highly purified preparations of trypsin are contaminated with a trace amount of chymotrypsin (Inagami and Sturtevant, 1960). Chymotrypsin acts on a variety of peptide and ester linkages. The specificity of action has been discussed by Desnuelle (1960). The enzyme preferentially attacks peptide bonds which involve tyrosine and phenylalanine. It has been reported that trypsin and chymotrypsin cause extensive degradation in type A toxin (Coleman, 1954; Halliwell, 1954; Meyer and Lamanna, 1959); however no information is available on chymotryptic treatment of type E toxin.

MATERIALS AND METHODS

Culture

Cl. botulinum type E, strain Iwanai was used throughout this study. This non-proteolytic, gas producing strain was found to be consistently toxigenic.

Toxin production

The type of medium, growth conditions, purification procedure and potency assay as described by Gerwing et al., (1961, 1964) were used. An average batch of toxin so obtained contained 30-50 mg protein with potencies ranging from 5,000-10,000 MLD per ml. The total volume was in the region of 100 ml. Whenever subsequent procedures required changes in pH and salt concentration of the toxin preparations, the latter were dialysed against the appropriate buffers or distilled water. In the same context, the toxin preparations were concentrated by the addition of saturated ammonium sulphate solution to a final volume of 60% and allowed to stand at 4 C for 4-6 hours. The precipitate thus formed was collected by centrifugation at 5,000 X g and resuspended in the desired amount of 0.01M acetate buffer pH 4.5.

Treatment of dialysis tubing

Before use, all dialysis tubings (Visking Company) were boiled for 5 minutes in a 0.01M solution of ethylenediaminetetraacetic acid (Versene) adjusted to pH 7.0, and then washed in distilled water, except where otherwise

stated.

Quantitative assay

The toxin was assayed with the Folin-Ciocalteu phenol reagent based on the modification of Lowry et al., (1951). For comparison, the absorbance at 280 mμ of 3.0 ml of 1:4, 1:3, 1:2 diluted and undiluted samples of a single "desalted" toxin preparation were measured with a Beckman model DU spectrophotometer. The toxin samples were then freeze-dried and their dry weights determined. The optical densities were correlated with the dry weights.

Potency assay

Randomly bred Swiss white mice weighing approximately 20 gm ± 5 were injected intraperitoneally with 0.05, 0.1, 0.2 and 0.3 ml of serial decimal dilutions of the toxic samples. Dilutions were made in sterile physiological saline. A single mouse per dilution was used whenever an indication of the potency of the toxic sample was required. For accurate potency assays, groups of 5 mice per dilution were used. The MLD was taken as the highest dilution by which death occurred within 48 hours.

Acid hydrolysis procedure

Toxin samples of known concentration (0.2-0.5 μM) were dissolved in 6N HCl in ampoules which were then sealed under vacuum. Hydrolysis was carried out for either 8 or 18 hours

at 105 C, according to the requirements of the respective procedures. Samples to be used for amino acid analysis were washed thrice in distilled water alternately with flash evaporation at 40 C.

Performic acid oxidation

Performic acid was prepared by adding 1.0 ml of 30% H_2O_2 to 9.0 ml of 88% formic acid. The mixture was allowed to stand at room temperature for 1.0 hour and was then cooled to 0 C. Dried toxin samples of known concentration to be analyzed for cysteine and methionine residue content were oxidized with 2.0 ml of performic acid at 0 C for 18 hours. Excess performic acid was removed by the addition of 0.3 ml of 48% HBr and the mixture was subjected to freeze-drying and hydrolysis as described above.

Amino acid analysis

Quantitative amino acid analyses were carried out on a Spinco automatic amino acid analyser according to the methods as described by Spackman et al.(1958).

N-terminal amino acid determinations

The labelling of the N-terminal amino acid residues with Sanger's reagent (FDNB), acid hydrolysis and paper chromatographic identification as described by Fraenkel-Conrat et al. (1955) were employed.

Inactivation by iodoacetate

The reaction was carried out at pH 2.5 and 5.5. In both cases, approximately one mg of toxin was used. For pH 2.5, the toxin was adjusted to the required pH with 0.01 N HCl; for pH 5.5 - the toxin was pre-dialysed against 0.01 M acetate buffer at pH 5.5. The toxin samples so treated were halved. Excess iodoacetate (obtained from the Department of Chemistry, U.B.C.) dissolved in distilled water was added to one half; the same volume of distilled water was added to the other half for control. These were allowed to stand at 25 C and the biological activity of 0.1 ml aliquots were taken and assayed at timed intervals.

Inactivation by p-chloromercuribenzoate (PCMB)

The reaction of PCMB (Calbiochem) with toxin was followed spectrophotometrically at pH 7.0 by a procedure modified from that as described by Boyer (1954) using cysteine as the standard. PCMB was dissolved in the minimal amount of 0.001 N NaOH and made up to a final volume of 10 ml with 0.1 M phosphate buffer pH 7.0 (1,000 γM PCMB per ml). Aliquots were taken and diluted to graded γ molar concentrations. The latter were added to toxin samples of constant, known amounts (15-20 γM per ml). Toxin samples were taken from a single batch of pure toxin in each run. The reaction was carried out at 25 C. The extinction at 250 mμ of the reaction

mixtures was measured with a Beckman model DU spectrophotometer immediately after the additions and thorough mixing of PCMB; and subsequently at 1,2 and 3 hours. Blanks comprised of the respective graded excess of PCMB solution mixed with phosphate buffer in the same volume as the toxin were used to zero the spectrophotometer prior to each measurement of the reaction mixtures. Spectrophotometric readings of the reaction mixtures were corrected by the toxin's absorption at 250 mμ. Toxicity was assayed when the reaction mixtures showed no further change in differential absorption. Controls were made up of toxin mixed with the phosphate buffer.

Chromatographic techniques

Sephadex dextran gel (Pharmacia Fine Chemicals, Uppsala, Sweden) of different types were used. The dextran gels were hydrated in the appropriate buffers or distilled water overnight at 25 C. Fines were discarded by repeated decantations. The evenly sedimented dextran gels were then packed into their respective columns:

For the isolation of a DDPS-peptide, Sephadex G-25 (fine) dextran gel in distilled water was packed into a column 100 X 1 cm. The gel packed to a height of 93 cm was washed with 100 ml of distilled water and settled to a final height of 80 cm. Flow rate was not regulated.

For the fractionation of a trypsin-activated toxin,

Sephadex G-75 dextran gel in 0.05 M acetate buffer pH 4.5 was first packed into a column 110 X 0.8 cm, the gel bed was washed with 200 ml of the same buffer and settled to a height of 42 cm. Similarily prepared Sephadex G-50 (fine) dextran gel was then supraimposed to a final height of 105 cm. The whole composite gel bed was washed with 500 ml of the same buffer. Flow rate was regulated to 7.5 ml per hour.

For the analysis of chymotrypsin-trypsin fragmented toxin, Sephadex G-25 (fine) dextran gel in 0.05 M acetate buffer pH 4.5 was packed into a column 180 X 0.8 cm. The gel bed was washed with 200 ml of the same buffer and settled to a height of 160 cm. Flow rate was regulated to 15 ml per hour.

Collection of all eluted material as 2.5 ml fractions was accomplished in a model V-10 Fraction Collector (Gilson Medical Electronics, Middleton, Wis.).

All eluted fractions were analysed for the presence of 280 mμ absorbance. In the isolation of a DDPS-peptide, the absorbance at 440 mμ was also analysed. In the isolation of a trypsin-activated toxin, the presence of ninhydrin positive material was analysed thus:

Ninhydrin (5% in acetone) was added in a 1:1 ratio to the samples to be tested (usually 1.0 ml of each). The tubes were heated for 15 minutes at 100 C then immediately cooled. The reaction tubes were diluted 3:2 with 95% ethanol and read at 570 mμ with a Beckman B spectrophotometer.

In the isolation of a chymotrypsin-trypsin fragmented toxin, the presence of ninhydrin positive material was analysed according to the method described by Morris (1961):

2 gm of ninhydrin was added to 10 ml distilled water and 6.0 ml 0.1 M acetate buffer pH 5.0 in 170 ml 95% ethanol. This ninhydrin solution was then made up to a final volume of 200 ml with 95% ethanol, with 2.0 ml 0.1 M $CdCl_2$ added prior to use. 5.0 ml of the ninhydrin reagent was added to the samples (0.5 ml) to be tested. The reaction tubes were heated for 30 minutes at 100 C and then cooled. The extinction at 500 mμ of each tube was then measured with a Spectronic 20 (Bausch & Lomb Inc., Rochester 2, New York).

Absorbance at 225 mμ by the chymotrypsin-trypsin fragmented toxin was also tested.

Preparative paper chromatographic technique

Whatman's #1 paper was pre-washed with Partridge's solvent, the upper phase of a mixture of n-butanol, glacial acetic acid and water (4:1:5, V/V/V). Having dried the paper at 40 C overnight, the sample was spotted as a narrow band at the origin. The paper equilibrated for half an hour was run with the same solvent as the descending mobile phase.

Elution from chromatographic paper

The desired material, detected by its yellow colour, was cut out of the paper chromatogram and wrapped in a piece of tin foil. Sufficient amount of eluting agent, distilled water was allowed to impregnate the paper by ascending capillary action. The whole bundle was secured by means of a rubber band onto an acid cleaned broth tube and spun for 5 minutes at approximately 1,000 rpm in a clinical centrifuge. The eluted sample was collected from the bottom of the broth tube. Usually the procedure was repeated for complete elution.

Isolation of a DDPS-peptide

20-25 mg of toxin were treated with 0.05 ml of mercaptoethanol and allowed to stand at 37 C overnight. The pH of the toxic sample was adjusted to 7.2 with 1.0% $NaHCO_3$ and an excess of N-(4-dimethylamino-3,5-dinitrophenyl)maleimide or DDPM (obtained from Dr. G.H. Dixon, Department of Biochemistry, U.B.C.) which was dissolved in methylcellosolve, added. The labelling was allowed to take place at 25 C, followed by exhaustive dialysis of the material against distilled water. The dialysis tubing was not pre-treated with versene. The DDPM-labelled toxin was then digested by 1.0% trypsin by weight (2 X crystallised, Nutritional Biochemicals Corporation) overnight at pH 7.5 and 37 C. The tryptic digest was then eluted with distilled water through a

Sephadex G-25 (fine) dextran gel column. The retarded materials were pooled, re-trypsinized under the same conditions as before and again eluted with distilled water through the same Sephadex column. The most retarded, yellow coloured material was collected, concentrated by flash evaporation at 40 C, and separated chromatographically with pre-washed Whatman's #1 paper using Partridge's solvent as the descending mobile phase. The DDPS-peptide separated itself from the other ninhydrin positive peptides by running close to the solvent front. The amino acid content as well as the N-terminal amino acid residue of the yellow DDPS-peptide, eluted off the paper chromatogram with distilled water, were analysed as previously described.

Trypsin activation

Trypsin (2 X crystallised, Nutritional Biochemicals Corporation) dissolved in 1 M ammonium acetate buffer pH 5.8 was added to the toxin in an approximate micromolar ratio of 1:120 (assuming the molecular weight of the toxin to be 18,000 and that of trypsin to be 24,000). The pH of the preparation was adjusted to the required level, which was either 5.8 or 7.5. The mixtures were incubated at 37 C for 24 hour period to determine when maximal activation occurred and to delineate the degradation curve. At various time intervals (5,15, and 30 minutes; 1,2,3,4,5,6,9 and 24 hours), samples were removed

for titration. They were ten fold diluted in 0.05 M acetate buffer pH 4.0 to inhibit further tryptic digestion.

Chymotrypsin-trypsin degradation

Alpha chymotrypsin (3 X crystallised, Nutritional Biochemicals Corporation) dissolved in 1.0 M ammonium acetate buffer pH 5.8 was added to the toxin in an approximate micromolar ratio of 1:60 (assuming the molecular weight of the toxin to be 18,000 and that of the chymotrypsin to be 25,000). The mixture was incubated overnight at 37 C, after which an aliquot of 0.1 ml was taken and its potency assayed. Likewise, chymotrypsin was again added and incubated for a further 12 hours. Trypsin was added thrice to the chymotryptic digest in the manner as described in trypsin activation, at 5 hour intervals. Prior to each addition, the potency of the digest was assayed.

Isolation of the trypsin activated toxin

5 hour, trypsin-treated sample containing 3-5 mg toxic material in 10 ml quantities were added to the composite Sephadex G-75 and G-50 column. The toxic material was eluted with 0.05 M acetate buffer pH 4.5.

Isolation of the chymotrypsin-trypsin fragmented toxin

5 hours after the second addition of trypsin, the digest *in toto*, containing 5.5 mg toxic material in a volume of 20 ml was added to the Sephadex G-25 column. The same method of elution as above was used. The amino acid contents and the N-terminal amino acid residues of the isolated trypsin activated toxin and the chymotrypsin-trypsin fragmented toxin were determined.

RESULTS AND DISCUSSION

Quantitative assay

It was not possible to conduct the whole study with a single batch of toxin. The quantity of toxin produced varies from batch to batch. This variance directly affects the potency of the toxin. To standardize the quantity of toxin produced each time implies the standardization of one or more of the following: growth conditions, quality of the media, inoculation and harvesting procedures and contamination by other organisms. For quantitative comparison and stoichiometric evaluation of any chemical reaction, it is necessary to know the concentrations of the reactants participating in the reaction. Hence a rapid and convenient means of quantity assay for the toxin has to be devised. The amount of a given protein is usually determined indirectly by the Micro-Kjeldahl method of total nitrogen, or by the Lowry method of total protein; the Biuret and ninhydrin reactions have also been used (Kabat and Meyer, 1961). Each method cited has limitations and disadvantages. The total nitrogen determination method does not discriminate between nitrogen of the toxin and that originating from the ammonium sulphate used in the toxin purification process. The ammonium sulphate could not be dialysed free completely. The methods for total protein determination involve comparison of colour development between the standard and the protein reacted with the respective reagents. The standard can be any suitable homogeneous protein preparation containing aromatic amino

acid residues, especially tyrosine and tryptophan; for example egg albumin is one of the advocated standards as it is considered to be a representative protein.

The method of protein estimation with the Folin-Ciocalteu phenol reagent is the method of choice as it has been found to be expedient for the estimation of soluble protein with an accuracy range of 10 to 100 ug. The method is based on the colorimetric measurement of the blue colour produced by the addition of the phenol reagent to an alkaline solution of protein. The colour intensity produced by a given amount of protein is chiefly a function of its tyrosine and tryptophan content. However, other factors are known to play a role, especially the length of time to which the protein is exposed to the alkali prior to the addition of the phenol reagent, and the presence of -SH and other reducing groups. Shoa-Chia and Goldstein (1960) found that any peptide bond will yield some colour, but certain amino acid sequences, not necessarily containing aromatic residues, which are more chromogenic than others largely contribute to the colour yield of the protein. Unlike the method of total nitrogen determination, contaminating ammonium sulphate gives no interference.

Proteins show characteristic absorption at 270-290 mμ with a maximum at about 280 mμ. This absorption has been attributed chiefly to the contents of aromatic amino acid residues, especially tyrosine and tryptophan (Smith, 1929).

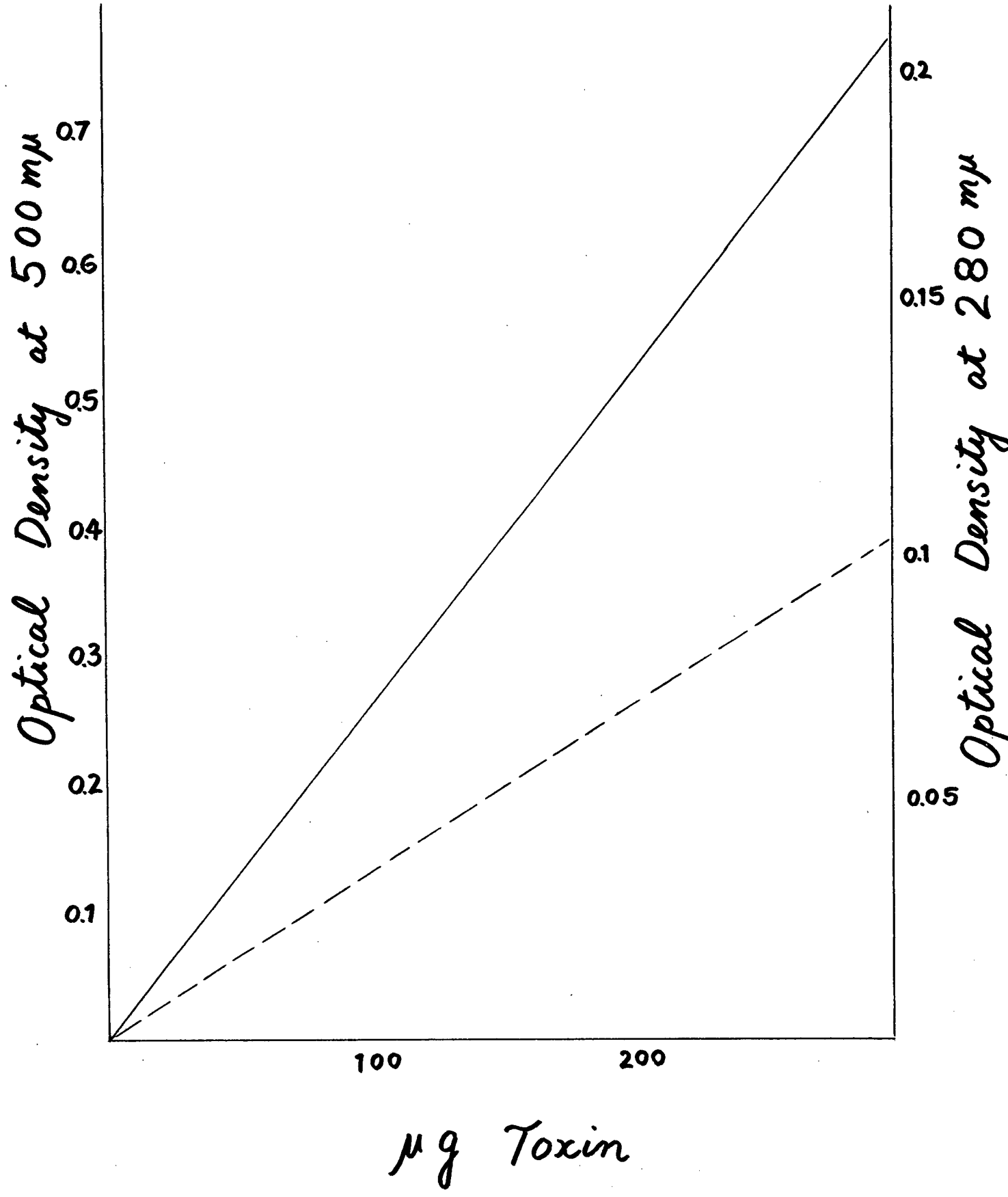
FIGURE 1

Quantitative assay of toxin.

Symbols:

_____ Lowry determination at 500 mμ

----- Spectrophotometric determination at 280 mμ


Optical Density at 500 mμ
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density at 280 mμ
0.2
0.15
0.1
0.05
100
200
μg Toxin

The toxin absorbs strongly at 280 mμ. The correlation of the toxin's absorption at this ultraviolet region with its dry weight may provide a comparison and check for its quantitative assay.

In figure 1, the quantitative assay for the toxin with the two different methods are compared and related to the optical densities of different amounts of toxin at 280 mμ. At any given optical density at 280 mμ for a particular sample of toxin, the Lowry method indicated 50% less toxin than that by the spectrophotometric method. The high result obtained in the latter method is probably due partially to the contaminating, undialyzable salt which is significant in μg amounts, and also to the inaccuracies of the weighing procedure. On the other hand, the low result obtained in the Lowry method cannot be regarded as absolutely accurate. The accuracy of this method is chiefly a function of the resemblance between the standard, egg albumin and the protein to be estimated, the toxin. Considering the inaccuracies inherent in both the methods employed and the use of the calculated molecular weight of the toxin for converting the toxin into gram molar quantities; it was decided to use the results as obtained by the more reliable Lowry method for subsequent work with the toxin.

Inactivation by iodoacetate

In the absence of -SH groups, the confinement of alkylation by iodoacetate of the reactive side chains of certain amino acid residues, by the change of pH, was found to be effective. However, when -SH groups are present in the protein, restriction of the alkylation reaction is complicated by the concurrent alkylation of -SH groups at the different pHs. When the toxin was reacted with iodoacetate, the toxicity decreased by approximately 50% at both pH 2.5 and 5.5. Should -SH groups be absent, alkylation would be confined to the thioether S of the methionine residues at pH 2.5; and to the imidazole group of histidine at pH 5.5. If the single histidine residue is associated with toxicity, ie. in the region of the active centre of the molecule, then its alkylation should yield more than the observed 50% inactivation. On the other hand, if the -SH groups of cysteine residues were alkylated but at a greatly reduced rate, as would be expected at these pHs, then the observed inactivation may be mainly due to the alkylation of -SH groups.

Inactivation by PCMB

Repeated determination of the number of cysteine residues in the toxin molecule by quantitative amino acid analysis of performic acid oxidized and subsequent acid hydrolysed samples of the toxin, has revealed that cysteine

TABLE 1

Reaction of Toxin with PCMB at pH 7.0

Molar ratio of PCMB to toxin	Moles mercaptide formation per mole of toxin	Percent inactivation
3:1	0.48	50
5:1	0.68	50-75
10:1	0.80	75
20:1	0.88	80

occurs as a single residue. The chemical modification by PCMB of the -SH group in the toxin molecule is not only specific but the reaction may be followed quantitatively. Preliminary experiments have shown that an excess of PCMB was necessary for mercaptide formation between the reactants. This may be due to the masking of the -SH group by the unknown secondary and tertiary structures of the toxin. If the intention was the quantitative assay of cysteine in the toxin, then denaturation or partial enzymatic digestion of the toxin prior to the reaction with PCMB becomes a prerequisite step. However, the object was to determine the extent of inactivation of the toxin when its cysteine residue has been specifically modified. Only 80% inactivation was observed when the toxin was reacted with approximately 20 fold molar excess of PCMB. The mercaptide formation between the toxin and the PCMB was never 100% when compared with that of the standard, cysteine and PCMB. Parallel results can be seen in table 1 between the extent of mercaptide formation and the percent inactivation of the toxin. Inaccuracies are due to the errors inherent in the procedures of the spectrophotometric measurements, of the biological assay and of the initial quantitative assay of the toxin. Considering the comparative inaccuracies between the methods of spectrophotometric determination and biological assay, the results showed remarkable concordance.

The experiment demonstrated that the reactive -SH

group of the cysteine residue, when specifically modified by the thiol reagent PCMB, resulted in the loss of biological activity of the toxin. Possible interpretations of the results are that the -SH group is essential and that the cysteine residue is located in the active centre of the molecule. Such interpretations imply that the -SH group is intimately related to the mode of action of the toxin. However, no data confirming this speculation are as yet available. Bearing in mind that the cysteine was tagged with a relatively large group and that the amino acid residues adjacent in sequence need not necessarily be adjacent in space, it is also possible that the cysteine residue may be located very near to the active centre of the toxin molecule rather than in it.

Isolation of a DDPS-peptide

Having established a relationship between the cysteine residue with the biological activity of the toxin molecule, labelling of the cysteine residue should facilitate the isolation of a small peptide from that predetermined region of the toxin molecule. To ensure the labelling of all the toxin molecules with the -SH group specific, yellow colored reagent, DDPM, mercaptoethanol was added prior to the labelling to reduce the cysteine residues. Excess DDPM and labelled molecules of mercaptoethanol were mostly eliminated by dialysis before subjecting the labelled toxin to tryptic digestion. Since the toxin has nine residues of lysine and three of arginine, as revealed by quantitative amino acid analysis (table 2), exhaustive tryptic digestion theoretically should give rise to 13 peptides. Because of the specificity and reproducibility of cleavage, trypsin is the enzymatic agent of choice for the fragmentation of the labelled toxin.

For the preliminary fractionation of the tryptic digest which was water soluble, gel filtration through a Sephadex G-25 (fine) column was chosen, employing distilled water as the eluent. Wheaton and Bauman (1953) have described in detail the weak ion exchange properties and the absorption effects which are pronounced in tightly cross-linked gels such as G-25. The theoretical basis, practical considerations and experimental technique of fractionation of proteins, peptides and amino acids by gel filtration have been

FIGURE 2a

Fractionation of the once-trypsinized DDPM-labelled toxin by Sephadex G-25

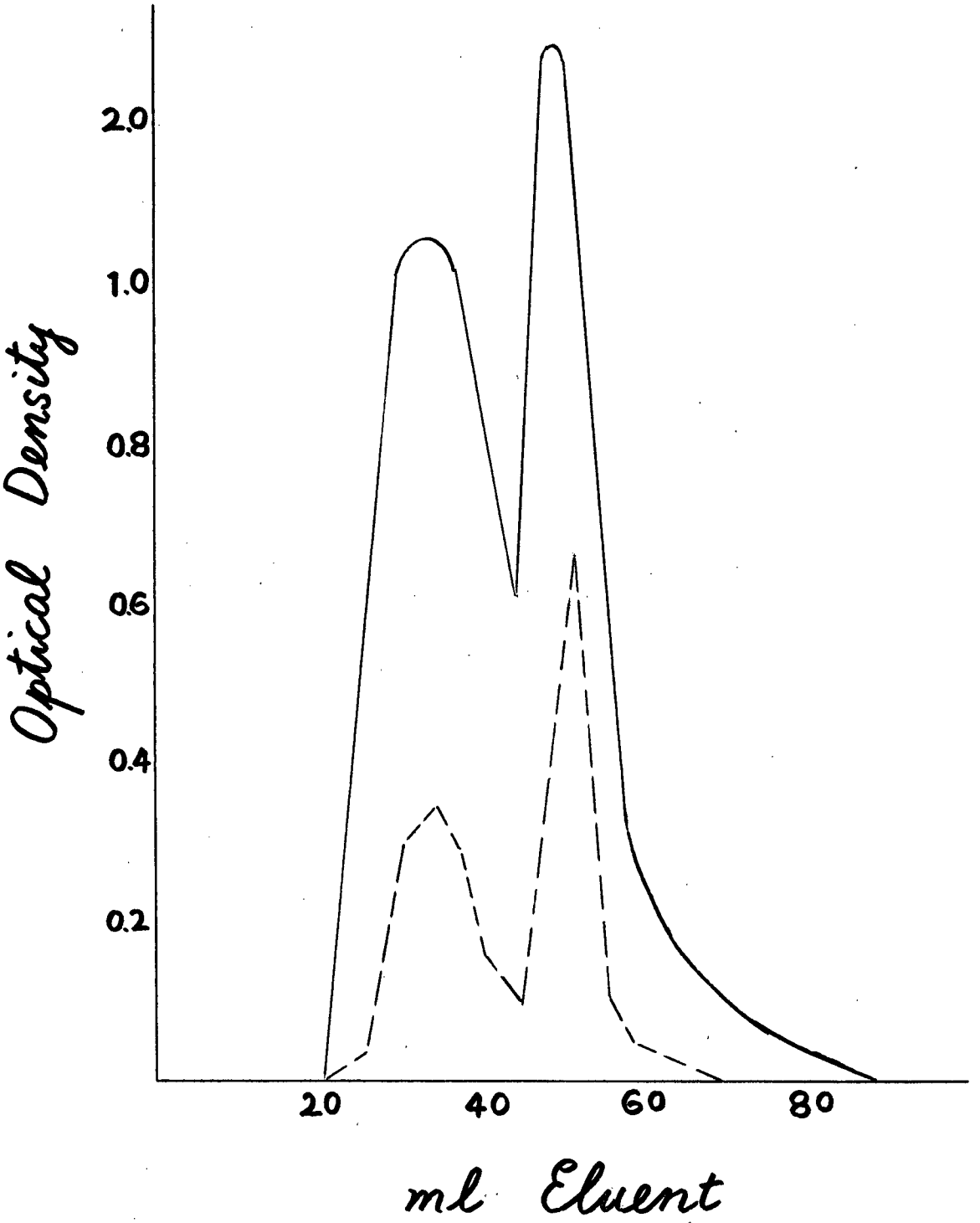
FIGURE 2b

Fractionation of the twice-trypsinized DDPM-labelled toxin by Sephadex G-25

Symbols:

_____ Absorbance at 280 mμ

----- Absorbance at 440 mμ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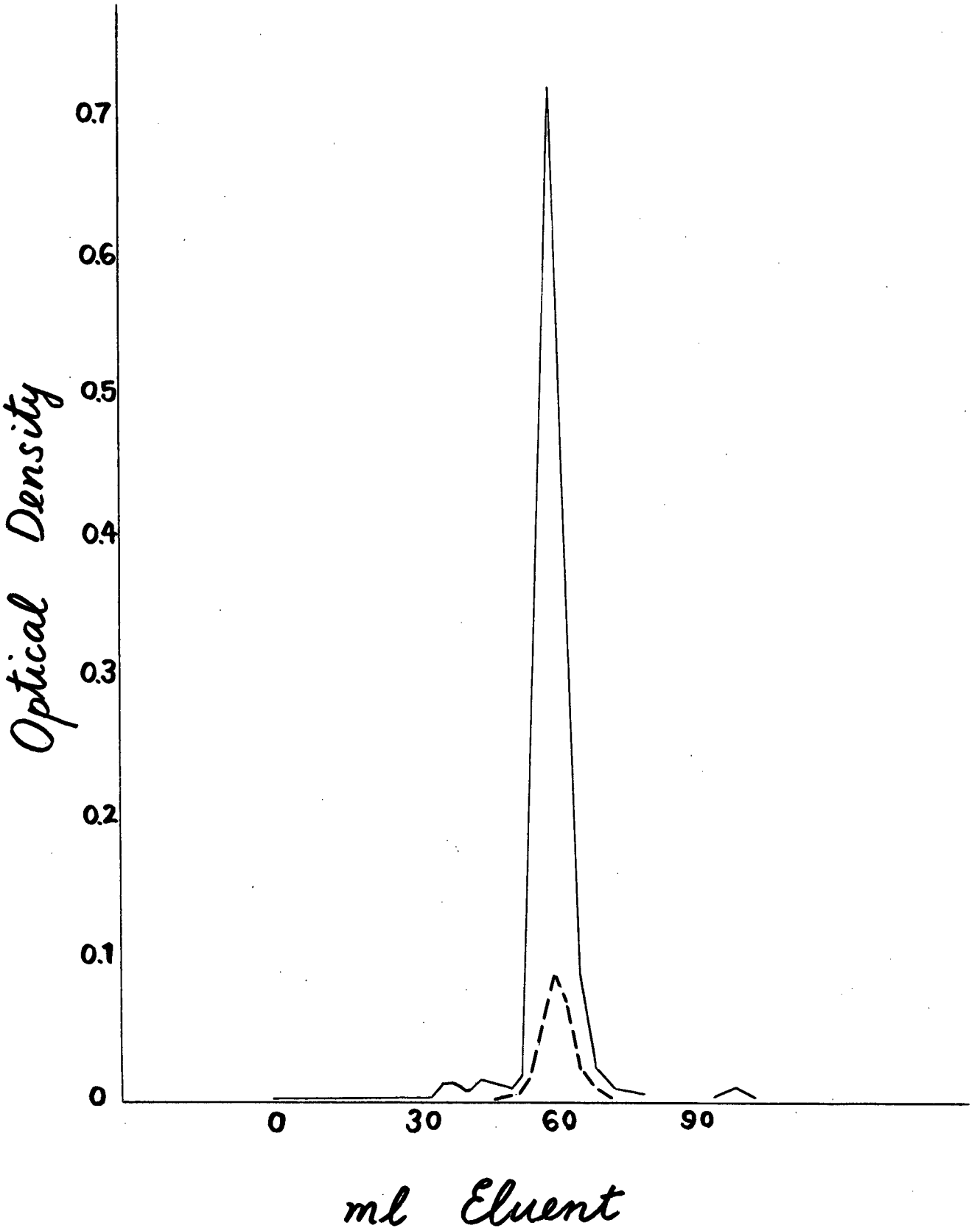
2.0
1.0
0.8
0.6
0.4
0.2
20
40
60
80
ml Eluent


Optical Density
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
30
60
90
ml Eluent

discussed by Gelotte (1964). The process of fractionation of the once trypsin-digested toxin by gel filtration is illustrated in figure 2a. The retention of the eluted yellow material indicated that fragmentation of the labelled toxin had occurred. The two peaks indicated that not all the molecules had been cleaved to the same extent. Redigestion by trypsin of the pooled, eluted yellow material should cleave labelled fragments of various sizes into more uniform fragments of similar size. This is evident in figure 2b, a typical elution profile of fractionation of the twice tryptic digested labelled fragments. The two figures, 2a and 2b, illustrate the efficacy of cleavage by trypsin and the fractionation of the digests by gel filtration using distilled water as the eluent.

Further fractionation of the gel filtered, labelled preparation was performed by descending paper chromatography. The DDPS-peptide ran very near to the descending solvent front away from at least four other ninhydrin positive peptides which migrated to a position on the paper nearer the origin rather than the solvent front.

The DDPS-peptide so obtained was analysed in terms of its amino acid content and N-terminal residue. At least two factors complicated the quantitative amino acid analysis of the peptide. When the toxin was labelled with DDPM, the carbonyl group in the adduct tends to condense with the amino group at alkaline pH to form a cyclic compound. The latter breaks down into at least two moieties during acid

hydrolysis. However, repeated analyses gave results which indicated that the peptide has the probable amino acid content as shown in table 2. The homogeneity of the peptide was indicated by the presence of only one N-terminal residue, alanine. The latter was found by examination of the 8- and 18-hour hydrolysates of the dinitrophenylated peptide sample. Because the peptide is a product of trypsin digestion, the C-terminal residue may be either arginine or lysine. Quantitative amino acid analysis ruled out the former. Determination of the C-terminal residue by carboxypeptidase B which is specific for the further degradation of products of tryptic degradation did not give clear cut, conclusive results but merely indicated that lysine is the probable C-terminal amino acid residue. The amino acid sequence or the primary structure of the isolated dodeca-peptide has not been established.

Tryptic activation

Previous studies (Gerwing et al., 1961, 1962) suggested that tryptic activation of type E toxin involved some degree of fragmentation of the toxin molecule, but the mechanism has not been established.

In order to isolate the trypsin-activated toxic fragment, it is essential to determine the conditions for maximal activation of the toxin by trypsinization. The pH range for the activity of trypsin and the lability of the toxin at alkaline pHs set the parameters for the experiment. A pH of 7.5 was selected because it is in the region of optimal activity for trypsin although considerable inactivation of the toxin occurs. On the other hand, at pH 5.8 the toxin inactivation is minimised and the tryptic activation, although retarded, reaches a higher maximum potency.

Reproducible results were obtained when the toxin was treated with trypsin at these pHs. Figure 3a illustrates the degree of potentiation of toxicity and the subsequent degradation of the toxin under the *in vitro* conditions of treatment by trypsin. At pH 7.5, a sample of toxin with a potency of 10,000 MLD per ml was activated in approximately 15 minutes to a maximal titre of 200,000 MLD per ml with a subsequent rapid decline in toxicity. At pH 5.8, the activation process required about 5 hours and the maximal titre observed was 300,000 MLD per ml. The subsequent decline in toxicity was gradual.

Trypsin preferentially attacks peptide bonds to which

FIGURE 3a

Effect of pH and time on the tryptic digestion of type E toxin

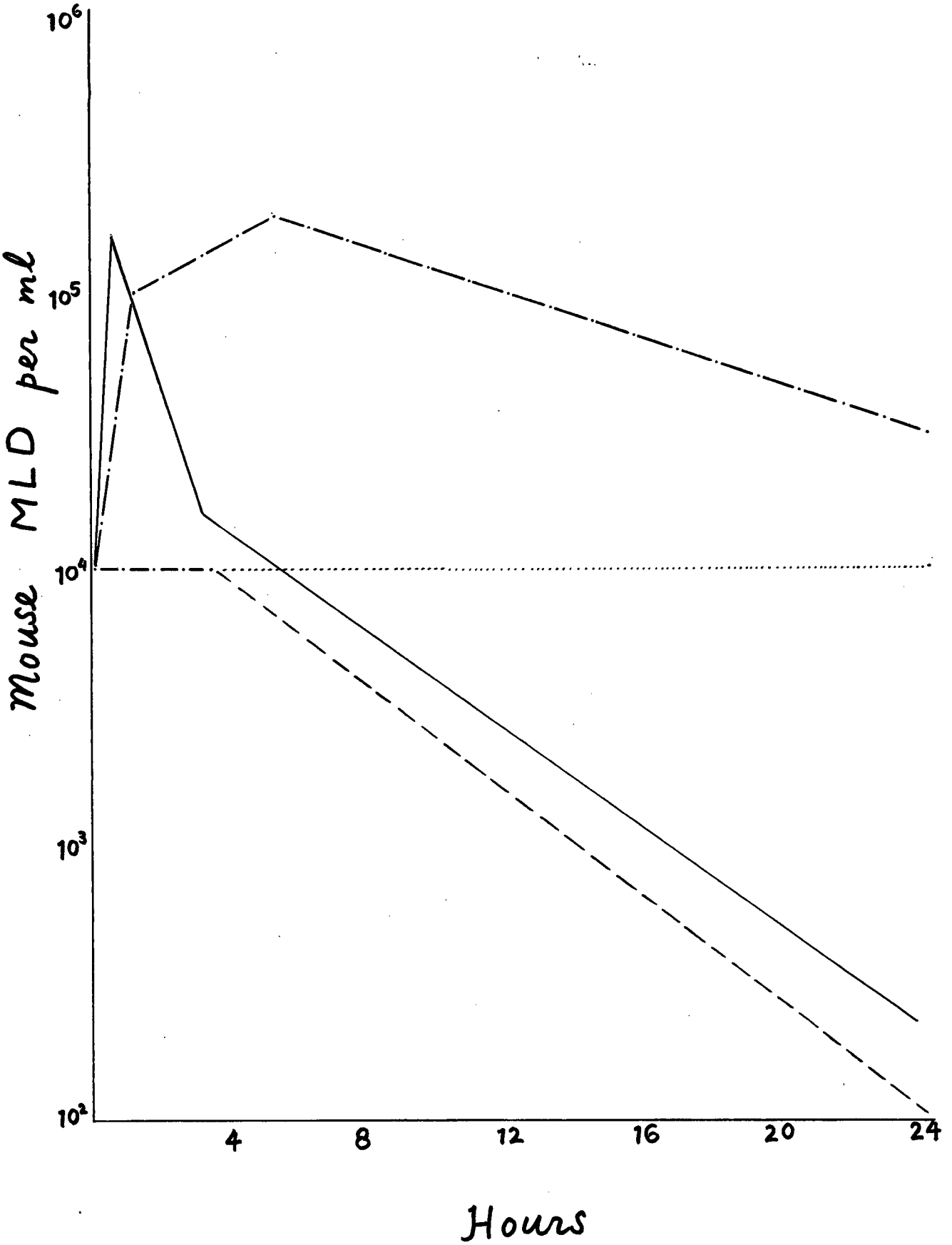
Symbols:

----- Toxin control at pH 7.5

_____ Toxin and trypsin at pH 7.5

..... Toxin control at pH 5.8

-·-·- Toxin and trypsin at pH 5.8


10^6
10^5
10^4
10^3
10^2
Mouse MLD per ml
4
8
12
16
20
24
Hours

an arginine or lysine residue has contributed to the carboxyl group. Depending on the amino acid sequence and the tertiary structure of the substrate, some of these bonds may become partially or totally resistant (Thompson, 1960). Apart from this, the process of enzymatic cleavage is random in nature. Not all the toxin molecules may be cleaved to the same extent simultaneously, and some may even escape cleavage by trypsin.

At pH 7.5, the rapid potentiation in toxicity of the toxin may be attributed to the near optimal activity of the trypsin which was sufficient in 15 minutes to reduce enough of the toxin molecules into smaller, more toxic fragments giving a titre of 200,000 MLD per ml. Thereafter, progressive digestion continued to cleave more of the lysyl and arginyl bonds, some of which are more intimately involved with the active site of the molecule. The instability of the toxin at pH 7.5 may coincidentally contribute to the subsequent rapid decline in toxicity. At pH 5.8, tryptic activity is retarded and cleavage of the toxin molecule was at first confined to the most readily cleaves sites. Sufficient number of toxin molecules are transformed into smaller, more toxic fragments only after a relatively longer period of 5 hours. The more potent titre of 300,000 MLD per ml indicates that probably more toxin molecules have been similarily reduced in molecular size. The subsequent, gradual decline in toxicity may be due to the progressive cleavage of some of the partially

FIGURE 3b

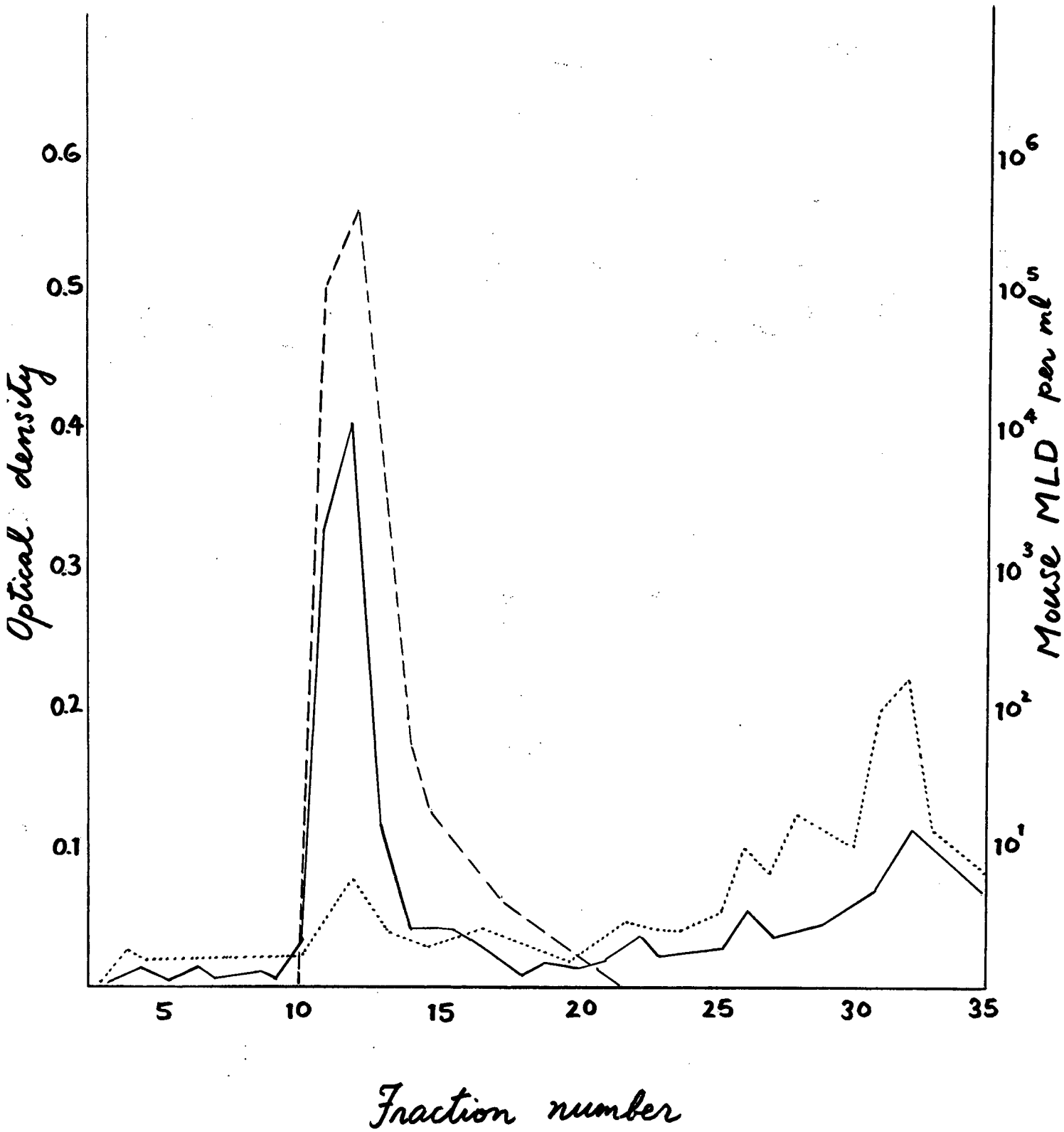
Fractionation of the trypsin-activated toxin by Sephadex G-75 and G-50

Symbols:

_____ Absorbance at 280 mμ

..... Ninhydrin reaction read at 570 mμ

- - - Mouse MLD per ml


0.6
0.5
0.4
0.3
0.2
0.1
Optical density
10^6
10^5
10^4
10^3
10^2
10^1
Mouse MLD per ml
5
10
15
20
25
30
35
Fraction number

resistant bonds. The latter may have become susceptible with the change in tertiary structure. The fragmentation process after maximal activation becomes too complicated and involved for further interpretation.

Toxin samples were activated for 5 hours at pH 5.8 prior to their fractionation by a composite Sephadex column. Because the molecular size of the trypsin-activated fragment to be isolated is unknown, a column of Sephadex G-50 dextran gel superimposed upon G-75 with a diameter to height ratio of 1:130 was used. Sephadex G-50 has an approximate exclusion limit of 10,000 and Sephadex G-75 - 50,000. Should the activated fragment be too large for retention in the G-50 portion of the column, then some measure of fractionation may be achieved by the G-75 portion.

The successful fractionation of the tryptic digest is illustrated by figure 3b (a representative elution profile). The activated toxin can be isolated from fractions 10-15. Quantitative amino acid analyses of the toxin molecule and the activated toxin (table 2) indicated that the latter has at least 18 amino acid residues fewer than the intact toxin molecule (even though the content of tryptophan and methionine residues have not been determined for either). The presence of a single N-terminal amino acid residue, arginine was taken as indirect evidence for the homogeneity of the isolated peptide. The N-terminal residue determination was performed by the examination of

8- and 18- hour hydrolysates, as well as the aqueous phase of the dinitrophenylated peptide samples by the paper chromatographic systems as previously described (Fraenkel-Conrat et al., 1955). The presence of the N-terminal arginine residue was further confirmed by the Sakaguchi stain reaction. Closer inspection of table 2 revealed that the trypsin-activated toxin has two lysine and one arginine residues fewer than the intact toxin. Perhaps, at maximal activation the remaining 7 lysyl and 2 arginyl bonds displayed relative resistance to cleavage by trypsin at pH 5.8.

Within the limitations of the experimental procedures employed, the results indicate that there is a lack of identity between the N-terminal amino acid residues and a difference in the amino acid contents, in the intact and activated toxin molecules. These results strongly suggested that the process of tryptic activation involves the fragmentation of the intact toxin giving rise to a smaller, more active molecule.

Chymotrypsin-trypsin fragmentation

The trypsin activation experiment demonstrated that under certain *in vitro* conditions, the toxin molecule may be fragmented without the loss of toxicity. The cross contamination of trypsin and chymotrypsin preparation has been established (Inagami and Sturtevant, 1960). In other words, even highly purified preparations of trypsin are contaminated by chymotrypsin, and vice versa. Moreover, under *in vivo* conditions, the two pancreatic endopeptidases should work synergistically. These considerations led to the formulation of the hypothesis that the toxin may be further fragmented by the two endopeptidases under controlled, *in vitro* conditions.

Chymotrypsin, like trypsin, also acts optimally on protein substrates in the pH range 7.0-9.0. Preliminary experiments indicated that prolonged exposure of the toxin to chymotrypsin at pH 5.8 and 37 C resulted in no detectable loss of toxicity. The degradation curve, figure 4a, showed that there was no decrease in potency when the toxin was exposed to two additions of chymotrypsin over a period of 24 hours, at pH 5.8 and 37 C. Since chymotryptic activity is retarded at pH 5.8, perhaps only very susceptible peptide bonds, which are not involved in the active site of the toxin molecule, are cleaved.

It has been previously established that trypsin activation at pH 5.8 requires approximately 5 hours. Figure 4a showed that there was a potentiation of toxicity in the

FIGURE 4a

Effect of chymotryptic-tryptic digestion on the toxicity of type E toxin at pH 5.8

Symbols:

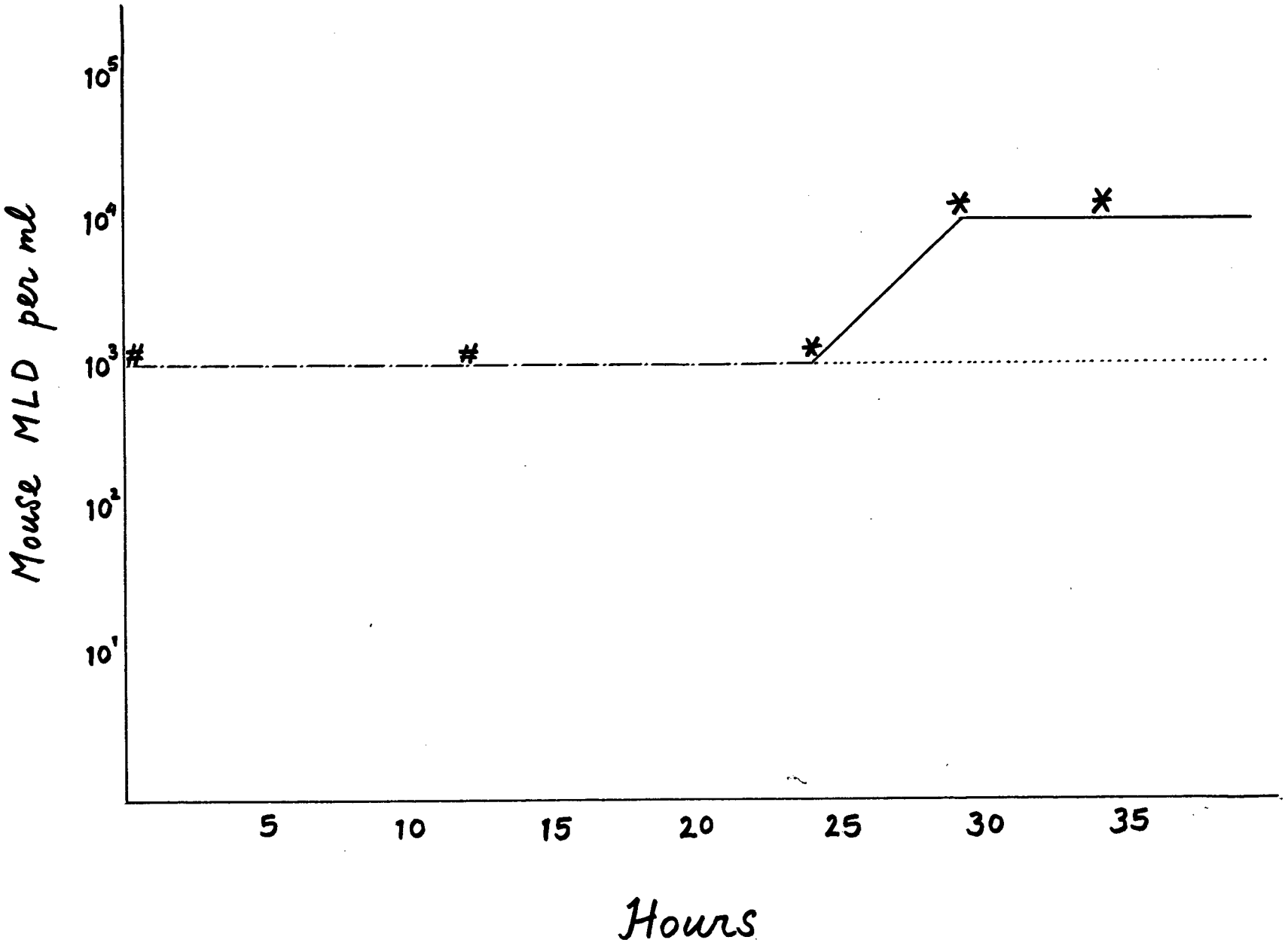
-·-·- Chymotryptic digestion

_____ Tryptic digestion

..... Toxin control at pH 5.8

\# Addition of chymotrypsin

* Addition of trypsin


Mouse MLD per ml
10^5
10^4
10^3
10^2
10^1
#
#
*
*
*
5
10
15
20
25
30
35
Hours

chymotryptic digest 5 hours after the first addition of trypsin. Subsequent additions of trypsin failed to further potentiate or decrease the toxicity. The absence of a subsequent decline in toxicity as observed in tryptic activation, is open to speculation. Possibly the chymotryptic digest gave rise to a toxic polypeptide whose conformation (or tertiary structure) permits only limited fragmentation by trypsin. This postulation seems to be physiologically sound.

Following fractionation of the chymotryptic-tryptic digest, the presence of ninhydrin positive, 280 and 225 mμ absorbing material in each fraction was analyzed. Realizing that the ninhydrin reaction used in the tryptic activation experiment is at best only semi-quantitative, the quantitative method as described by Morris (1961) was adopted. Because chymotrypsin preferentially cleaves peptide bonds involving aromatic amino acid residues, detection of the presence of 280 mμ absorbing material as a quantitative index of the fragmented toxin becomes inadequate. At shorter wavelengths in the ultraviolet region, notably between 190-240 mμ, the absorbance exhibited by aromatic amino acid residues is overlapped with that by histidine, methionine, cystine and cysteine, as well as the peptide bond itself (Edsall, 1963). It was arbitrarily conceived that analysis of the presence of 225 mμ absorbing material may serve as a useful, additional detection method.

The chymotrypsin-trypsin fragmented toxin, taken from

FIGURE 4b

Fractionation of the chymotrypsin-trypsin fragmented toxin by Sephadex G-25

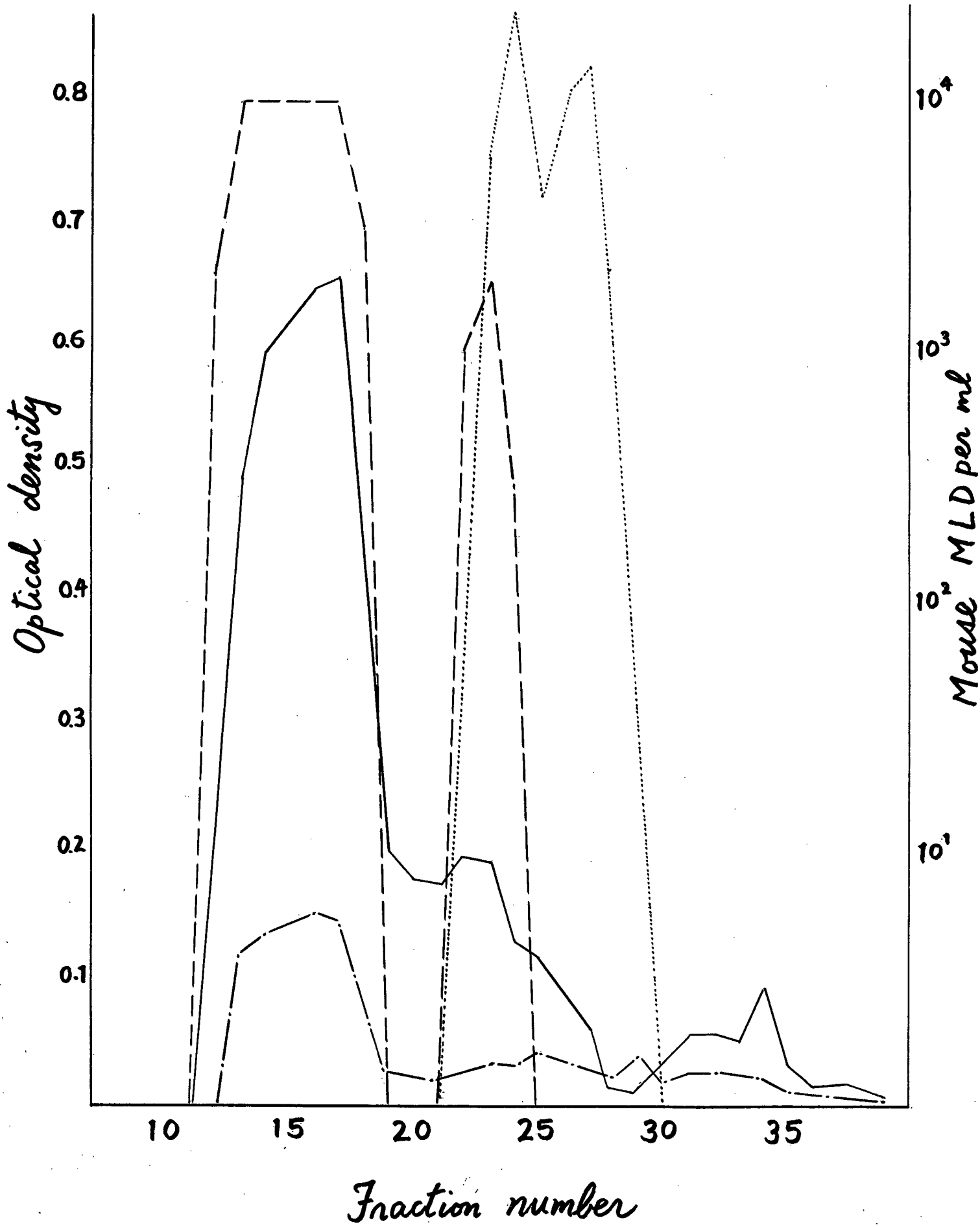
Symbols:

_____ Absorbance at 225 mμ

-·-·- Absorbance at 280 mμ

----- Mouse MLD per ml

..... Ninhydrin reaction read at 500 mμ


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical density
10^4
10^3
10^2
10^1
Mouse MLD per ml
10
15
20
25
30
35
Fraction number

the first peak (fractions 12-18) as shown in figure 4b, was further characterized. Closer examination of the elution profile of the chymotryptic-tryptic digest (figure 4b) revealed that other fractions which are more retarded, also exhibit biological activity. As previously mentioned, retardation or retention in gel filtration indicates moieties of smaller molecular size. No attempt was made to further characterize these smaller toxic fragments because of their probable heterogeneous nature, as indicated by the elution profile.

Again the presence of a single N-terminal amino acid residue, tentatively identified as valine, was taken as indirect evidence for the homogeneity of the chymotrypsin-trypsin fragmented toxin. Quantitative amino acid analysis revealed that there was a considerable reduction of amino acid residues as compared with the intact toxin and the trypsin-activated toxin (table 2). Even though the quantitative amino acid analysis for the chymotrypsin-trypsin fragmented toxin was incomplete, the reduction in molecular size was obvious.

TABLE 2

Type E toxin, trypsin-activated toxin and chymotrypsin-trypsin fragmented toxin compared in terms of amino acid contents, N-terminal residues and comparative toxicities

Amino acid	Intact toxin	Trypsin-activated toxin	Chymotrypsin-trypsin fragmented toxin	DDPS-peptide
Cysteic acid	1	1	1	1
Aspartic acid	16	15	6	1
Threonine	7	7	3	
Serine	8	8	4	3
Glutamic acid	11	11	5	1
Proline	8	8	3	
Glycine	12	8	6	3
Alanine	6	4	4	2
Valine	6	4	1	
Isoleucine	10	6	4	
Leucine	10	8	4	
Tyrosine	4	4	1	
Phenylalanine	5	5	2	
Lysine	9	7	*	1
Histidine	1	1	*	
Arginine	3	2	*	
Tryptophan	*	*	*	
Methionine	*	*	*	
N-terminal	gly	arg	val	ala
Comparative toxicity	toxic	30-100 fold	3-10 fold	non-toxic
Molecular weight	14,000-16,000	10,000-12,000	4,000-6,000	1,100

* not determined

GENERAL DISCUSSION & CONCLUSIONS

The investigation can be divided into two parts. First, chemical modification studies were employed to relate biological activity with the various amino acid residues in the toxin molecule. Linking the first part to the second was the isolation of a DDPS-peptide from the toxin. In the second part, fragmentation of the toxin by proteolytic enzymes was attempted in order to isolate a toxic peptide of the smallest possible molecular size.

The random nature of the process of chemical modification is illustrated by alkylation with iodoacetate. Even if the alkylation reaction was confined to the reactive side chain of a particular amino acid residue by the manipulation of pH, concurrent alkylation of -SH groups at different pHs made it very difficult to assess the extent of modification of the toxin molecules, and to relate the latter to the loss of biological activity. The data obtained merely indicated that the single cysteine residue may be associated with toxicity. On the other hand, modification of the cysteine residue with the -SH specific reagent, PCMB may be followed quantitatively. The amount of mercaptide formation which corresponded to the percent inactivation of the toxin indicated that the cysteine residue must be located at the active site of the toxin, or very near it. Schantz and Spero (1957) reported that PCMB inactivated type A toxin by 30%; but they failed to relate the biological

activity of the toxin to the cysteine residue. Studies in progress (unpublished data) have revealed that PCMB inactivated the highly purified type A toxin obtained by Gerwing et al., (1965) by at least 90% at pH 7.0. The same toxin has been shown to be devoid of tryptophan. The latter contradicts the erroneous claim by Boroff's group that the tryptophan residues are related to the toxicity of type A toxin (Boroff and DasGupta, 1965).

It is believed that there is only one configuration for the active site of each type of biologically active molecules (Ram et al., 1962). The rest of the biologically active molecule (contributing structure), not included in the active site may differ among the various types and species. Studies in progress (unpublished data) have demonstrated that both types A and B toxins contain a peptide with the same amino acid composition as the DDPS-peptide of type E toxin. If the isolated DDPS-peptides form the active sites of botulinus toxins, then their amino acid sequences should be more or less identical. Moreover, maximal cleavage of the contributing structures of botulinus toxins should yield a toxic peptide which must include those amino acid residues as in the DDPS-peptide. The validity of the claim that the isolated dodecapeptide forms the active site of botulinus toxins must await confirmation. The simplicity and specificity of the procedure for isolating the DDPS-peptide from type E toxin compared favorably with that

employed by Segal and Gold (1964) who fragmented their DDPM-labelled preparation (rabbit glyceraldehyde-3-phosphate dehydrogenase) with pepsin, and isolated a hexapeptide by means of electrophoresis at different pHs coupled with descending paper chromatography.

The molecular weight of type E toxin, calculated on the basis of physical measurements, was reported to be 18,600 (Gerwing et al., 1964), whereas calculated from the number of amino acid residues, the molecular weight appeared to be between 14,000-16,000. Considering the errors inherent in physical measurements and in quantitative amino acid analysis, these calculated molecular weights for type E toxin can be regarded as in good agreement. Thus any fragmentation or cleavage of the contributing structure of the toxin molecule, must yield a fragment with a reduced molecular weight.

According to Sakaguchi et al.,(1964), both the toxin "precursor" and the trypsin-activated toxin have molecular weights of approximately 200,000. The results (table 2) clearly disputed the Sakaguchis' claim and further demonstrated that the process of tryptic activation probably involves a removal of at least 18 amino acid residues from the N-terminus of the toxin molecule. The activated toxin has a molecular weight of 10,000-12,000, as calculated on the basis of the number of amino acid residues. Besides demonstrating that cleaving away part of the contributing structure of the toxin molecule resulted in a

change in biological activity, the tryptic activation experiment also showed that the toxin was not inactivated by limited enzymatic hydrolysis. Incidentally, the biological activity of the trypsin-activated toxin became comparable to that of type A toxin which has a molecular weight of 12,200.

With a combination of chymotrypsin and trypsin, it was possible to obtain a fragment of lesser molecular size (4,000-6,000 in molecular weight) with maintenance of biological activity. Perhaps, further manipulation of the conditions in the process of chymotryptic-tryptic fragmentation and fractionation with Sephadex G-10 dextran gel, an even smaller peptide with biological activity may be isolated.

In conclusion, the cysteine residue has been linked to the toxicity of type E toxin by chemical modification with PCMB. A cysteine-containing peptide has been isolated from the active site region of the toxin molecule. By means of limited, enzymatic hydrolysis, a toxic fragment of 4,000-6,000 in molecular weight was isolated from type E toxin.

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