been demonstrated that there occurs an inverse relationship between antibody production and DNA-synthesizing capacity of immune cells(17). With this in mind, a recent study has shown that once the processes proceeding toward antibody production are underway, the immune cell is unreactive to the interference of PHA and no alteration of antibody production occurs. The data presented in the present study are consistent with this view. PHA was effective only when administered prior to grafting, no increase in survival was present if PHA was given 24 hours after grafting. It appears likely that the cells responsible for graft rejection are already committed and thereby unreactive to the effects of PHA. That PHA had no effect upon "second set" rejection is again indicative of a lack of reactivity by PHA upon immune cells committed to graft rejection. The exact mechanism of graft rejection in the animals recovering from PHA administration remains to be determined.

Summary. The effect of phytohemagglutinin (PHA) on skin allograft survival in mice of two strains, DBA/2 and C57Bl/6J has been studied. The administration of PHA prior to grafting significantly increased graft survival time in both strains. No increase in survival was observed if PHA was given 24 hours after grafting. Likewise no differences were observed in second set skin graft survival between PHA treated mice and untreated controls. No toxic effects of PHA were noted in any of the treated mice at the dosages and times of administration used.

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Immunological Reactivity of Trypsinized *Clostridium botulinum* Type E Toxin.* (32543)

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Clostridium botulinum produces several antigenic types of neurotoxin(1). Lethality for mice after i.p. injection of the food poisoning toxin produced by pure cultures of type E strains is usually low when compared to that of type A or B cultures. Potency of type E toxin is increased, however, upon proper treatment with proteolytic enzymes such as trypsin(2,3).

The activation phenomenon with type E toxin must be considered in the possible

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use of *in vitro* toxin-antitoxin reaction for assay of the toxin. If the serological method is to show the necessary correlation to mouse toxicity, increased lethality resulting from activation must be accompanied by a corresponding increase in immunological reactivity.

Literature on this subject is limited. The highest dilution of type E toxin which agglutinated erythrocytes sensitized with homologous antitoxin was the same for activated and non-activated toxin, although lethalities differed by 100-fold. Based on a limited number of animals, about the same amount of antitoxin was required to protect mice injected with identical volumes of toxin prior to and after activation(4). Activated toxin was selected in challenging mice immunized with type E toxoids because "nonactivated toxin did not give a true picture of the antigenic value of the toxoid"(5).

Materials and methods. C. botulinum type E strains were grown in trypticase-peptone-glucose broth(6) for 3-4 days at 30 C. Cells were removed by centrifugation; supernatant fluids sterilized by passing through Millipore or Seitz filters were used as sources of type E toxin. One preparation of Alaska E43 toxin was obtained by a cellophane dialysis sac procedure(7).

Toxin samples were kept frozen in small aliquots to avoid repeated freezing and thawing. Toxin was activated by incubating with final concentration of 0.01% crystalline trypsin at 37 C for 30 min(3). Soy bean trypsin inhibitor was added to final concentration of 2000 μg/ml immediately after trypsinization to prevent continued action of the enzyme.

Toxicity was calculated as LD_{50}/ml of toxin from deaths resulting from i.p. injections of dilutions of toxin into groups of 8-10 white mice of 18-24 g(8). Each mouse received 0.5 ml of toxin diluted with gelatin-phosphate buffer(3). Stability of a given toxin preparation used over several weeks was confirmed by retitration.

Equine type E antitoxin obtained from Microbiological Research Establishment, Porton, England, was reconstituted to contain 1500 International units (IU)/ml. Rabbit type E antitoxin was prepared in the authors' laboratory with trypsinized toxoid supplied by Dr. G. G. Wright (U. S. Army Biological Lab., Ft. Detrick, Md.) and had about 60 IU/ml.

Mouse protection tests were used to determine the change in immunological reactivity of toxin following trypsinization. Selected LD_{50}'s of toxin were mixed with dilutions of antitoxin and incubated at 37 C for 30 minutes. Each mixture was injected i.p. into 8-10 mice in 0.5 ml volumes and units of antitoxin required to protect half of the animals calculated(8).

In vitro serological reactivity of toxins was measured by the Ouchterlony gel-diffusion plate procedure(9). Ten ml of melted 1% Ionagar (Colab, Chicago Heights, Ill.) in 0.2 M Tris or phosphate buffer of pH 7.2, 0.85% NaCl, and 1:50,000 merthiolate were poured into Petri dishes and solidified. A #4 cork borer (8 mm diameter) was used to dig 6 equidistant wells in a circle around a central well, giving wells 14 mm apart (center to center). The peripheral wells were charged with 0.05 ml of toxin, the center wells with a like volume of type E antitoxin. Plates were held at 25 C in a closed container and examined for lines of precipitate for 2-4 days.

Results. All 6 type E strains tested gave a single antigen-antibody precipitin line when 0.05 ml of undiluted culture supernatant fluid was made to react with 1-2 IU of the antiserums. The precipitate is due to type E toxin. A total of 95 cultures, obtained in other experiments and expected to have high type E toxin titer if it was present in the sample, were tested in parallel for presence of toxin by mouse inoculation and by gel-diffusion. Complete agreement was obtained by the 2 procedures with these samples, about 50% of which contained type E toxin.

Highest dilutions of the toxic preparations capable of producing the immune precipitin line were determined. Sensitivity of the procedure was calculated from these dilutions and expressed as LD_{50}/well needed to obtain a visible line (Table I). Each well received 0.05 ml of diluted toxin.

Sensitivity of the gel-diffusion procedure decreased when trypsinized toxin was used.

TABLE I. Sensitivity of Ouchterlony Immunodiffusion Procedure in Detection of Untrypsinized Type E Toxin.

Type E strain	LD_{50}/ml	Dilution of toxin giving precipitin line	LD_{50}/well detected
Can	46 (30– 72)*	1:2 but not 1:3	1.2
Alaska E43	694 (526–916)	1:3 " " 1:4	11.6
066B	180 (130–250)	1:5 " " 1:6	1.8
1304	264 (182–382)	1:5 " " 1:6	2.6
Minneapolis	536 (358–804)	1:4 " " 1:5	6.7
Beluga	558 (430–728)	1:4 " " 1:5	7.0

* LD_{50} with 95% confidence limits.

TABLE II. Effect of Tryptic Activation of Type E Toxin on Mouse Toxicity and Sensitivity of Gel-Diffusion Reaction.

Toxin		LD_{50}/ml	Dilution of toxin giving precipitin line	LD_{50}/well detected
Alaska E43:	Not activated	1,380 (1,100–1,700)*	1:14 but not 1:16	4.6
	Activated	243,000 (170,000–270,000)	1:12 " " 1:14	1012
Minneapolis:	Not activated	194 (134–278)	1:8 " " 1:10	1.2
	Activated	46,000 (36,000–58,000)	1:8 " " 1:10	287
Beluga:	Not activated	200 (151–260)	1:6 " " 1:7	1.7
	Activated	50,000 (30,000–62,000)	1:6 " " 1:7	312

* LD_{50} with 95% confidence limits.

Trypsinization resulted in about 200-fold increase of mouse lethality but the highest dilution giving the immune precipitate line remained essentially unchanged (Table II).

Lack of correlation between the increase in toxicity and immunological reactivity of the trypsinized toxin shown in the Ouchterlony procedure was also found in mouse protection experiments with Porton type E antitoxin (Table III). Increase in lethality of toxin for mice by trypsin treatment is given as the activation factor.

TABLE III. Neutralization of Mouse Toxicity of Activated and Non-Activated Type E Toxin by Porton Equine Type E Antitoxin.*

Toxin challenge LD_{50}/mouse	IU of antitoxin/mouse to protect 50% of mice
Alaska E43 (activation factor: × 176):	
5 of non-trypsinized	0.0117 (0.0104–0.0131)†
5 " trypsinized	0.0013 (0.0011–0.0016)
50 " non-trypsinized	0.184 (0.173 –0.196)
870 " trypsinized	0.0429 (0.0384–0.0482)
Minneapolis (activation factor: × 237):	
10 of non-trypsinized	0.110 (0.107 –0.112)
10 " trypsinized	0.0039 (0.0031–0.0049)
50 " non-trypsinized	0.532 (0.481 –0.588)
2370 " trypsinized	0.214 (0.202 –0.226)

* Non-activated toxoid used as immunizing antigen.
† 95% confidence limits.

The amount of culture supernatant fluid of the Minneapolis strain having 10 LD_{50} is convertible into 2370 LD_{50} by trypsin. Toxicity is 237 times greater but a 2-fold increase in amount of antitoxin gives the same level of protection to mice. When the same number of LD_{50}'s is used for challenge, significantly less antitoxin is needed for protection against activated than against non-activated toxin.

Trypsin and trypsin inhibitor, of themselves, did not affect the titrations. Trypsin inhibitor and trypsin were added in sequence to Alaska E43 toxin to give the same concentrations as in the activated toxin. Protection of half of the mice challenged with 5 LD_{50} of this toxic mixture resulted with 0.0107 IU (0.0097-0.0120), an amount comparable to 0.0117 IU needed when mice were challenged with 5 LD_{50} of the original toxin without these additions (Table 3).

Porton type E antitoxin is obtained from a horse immunized with an antigen which has not been trypsinized. The possibility that antitoxin obtained in rabbits with trypsinized toxoid might give different results was studied

TABLE IV. Neutralization of Mouse Toxicity of Activated and Non-Activated Type E Toxin (Alaska E43) by Rabbit Type E Antitoxin.*

Toxin challenge LD_{50}/mouse	IU of antitoxin/mouse to protect 50% of mice
5 of non-trypsinized	0.0117 (0.0100–0.0137)†
5 " trypsinized	0.0009 (0.0007–0.0011)
50 " non-trypsinized	0.225 (0.220 –0.232)
870 " trypsinized	0.0451 (0.0419–0.0483)

* Immunized with trypsinized antigen.
† 95% confidence limits.

(Table IV). Potency of rabbit antiserum was calculated from parallel titrations(10) of rabbit and standard Porton antiserums in mice injected with 5 LD_{50} of the untreated Alaska E43 toxin. Antitoxin units required for the other challenges were based on the IU/ml of rabbit serum obtained by this comparison.

Results with antitoxins prepared against the two forms of toxoid were essentially the same. With both antiserums the amount of antitoxin giving the desired level of protection shows a correlation to the amount of toxin in its original form rather than to mouse toxicity. Absolute correlation was not obtained. Data of Table IV show that protection against 5 LD_{50}/mouse of activated toxin required 0.0009/0.0117 or 0.077 the amount of antitoxin needed for same LD_{50} of toxin without activation. Theoretically, 5/176 or 0.028 times should have sufficed. Similarly, instead of being 0.0451/0.0117 or 3.85 times larger, mice challenged with 870 LD_{50} of trypsinized toxin should have been protected by the same amount of antitoxin as those injected with 5 LD_{50} of untrypsinized toxin. These differences are probably due to avidity of antitoxin (10) and toxoid formation during trypsinization.

Discussion. That different sites of type E toxin molecule are responsible for the pharmacological and antigenic reactions is indicated by the role of toxoid in active immunization and in serological reactions(11). Further evidence is given by the present results which show that tryptic activation of the toxin involves a structural change wherein additional toxic receptors are made reactive without a concomitant increase in antitoxin combining sites.

Whether the increased toxicity results from an unmasking process(12) or the formation of smaller molecular units(13), all toxic molecules must have at least one antigenic site to account for antitoxin protection. Direct neutralization of the toxophores does not occur but the protection could be explained if the toxin-antitoxin complex is removed from the circulation by the blood clearing mechanisms or if the steric configuration of the complex prevented the necessary alignment of the toxin at the neuromuscular junction(14).

Potency of type E antitoxin expressed as numbers of lethal units of activated toxin neutralized by a volume of antiserum can be misleading. Such values are dependent on the degree of activation. Effectiveness of antitoxin is different for parenteral challenges with trypsinized or untrypsinized toxin(5).

Since the number of antigenic sites is not increased, the usual practice of trypsinizing toxin for toxoid preparation can be questioned. The variable extent of toxin activation expected in mixed cultures containing *C. botulinum* type E makes it unlikely that *in vitro* serological methods for the assay of the food poisoning toxin in such samples would give a correlation to mouse toxicity.

Summary. The number of LD_{50}'s of *C. botulinum* type E toxin necessary to produce a toxin-antitoxin precipitin line in the Ouchterlony gel-diffusion test is much greater for trypsin-activated than for non-activated toxin. For the same lethal challenge dose, less antitoxin is needed to protect mice against toxin after trypsinization than before the toxin is activated. Antitoxin units required for animal protection are related to the amount of toxin which is activated rather than to mouse toxicity obtained by trypsinization. The data indicate that tryptic activation of type E toxin results in appearance of more toxic groupings without a concomitant increase in the number of immunologically reactive sites.

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Changes in Pancreatic Enzymes as a Function of Diet in the Chick.* (32544)

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The phenomena of enzyme induction and repression are very well established in microorganisms, yet for vertebrates and mammals these have not been thoroughly investigated. The ratio of pancreatic enzymes is variable among species(5,9,12,13). Determinations of enzymes in homogenates of pancreata from rats fed either starch-rich or casein-rich diet show that specific activities of amylase and chymotrypsin reflect the starch and casein content of the diet(15). In chicks, the pancreata enlarge when unheated soybean meal is consumed. These pancreata show depletion of zymogen granules to a greater extent than the corresponding pancreata from chicks fed autoclaved soybean meal diet. This histological evidence was confirmed by chemical analysis(1). We investigated the rate of synthesis of enzymes by the pancreas of chickens after an adaptation period of 4 weeks on either unheated or autoclaved soybean meal.

Experimental. Two groups of chicks were fed either unheated or autoclaved soybean meal for 4 weeks. The composition of the basal diet was essentially that used by Dal Borgo(4). After this adaptation period, the chicks were fasted; 3 birds of each group were sacrificed at 0, 6, 12, 16, and 22 hours after the start of fast. After 16 hours of fasting, each group was divided into 2 subgroups, one of which was fed the same diet as before the fast, and the other the alternate diet. Three chicks from each of the 4 subgroups were sacrificed after 2 and 6 hours on the diet. The pancreata were excised and weighed (after removal of extraneous tissue), then stored at −15°C.

Trypsin and chymotrypsin were assayed using p-toluene-sulphonyl-L-arginine-methyl ester (TAME) and N-Benzoyl-L-tyrosine ethyl ester (BTEE), respectively(7). Amylase activity was determined by the 3,5,-dinitrosalicylate method(3). Protein in the pancreatic homogenates was determined by measuring the absorbancy at 280 and 260 mμ to correct for nucleic acids(10).

A portion of the pancreas was homogenized in H_2O for determination of amylase activity, while another part of the same pancreas was homogenized in 0.25 M sucrose for analysis of trypsin and chymotrypsin. The homogenote was diluted 10-fold with a 0.05 M phosphate buffer at pH 7.8 for chymotrypsin determination. Trypsinogen was converted to trypsin by activation with 4.0 mg enterokinase per 100 mg of tissue for 2 hours at 28°C. Crystalline trypsin, 0.01 mg/mg of protein, was found adequate for activating chymotrypsinogen to chymotrypsin at 5°C in 2 hours. Purified enterokinase (1-123R) and

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