ed the cleavage of sea urchin blastomeres, and was generally toxic to many invertebrates. Fishes were highly susceptible to ostracitoxin, and all but a few (*10*) were rapidly killed by immersion in sea water containing this toxin.

Intraperitoneal injections of ostracitoxin into white mice quickly caused ataxia, labored breathing, coma, and death. However, the minimum lethal dose of crude ostracitoxin to mice was quite high (0.2 mg/g of mouse) precluding the use of mice for bioassay. Sublethal injections into mice caused symptoms of poisoning, but recovery was complete, unlike ostracitoxin's irreversible action on fish.

Ostracitoxin caused hemolysis of vertebrate erythrocytes in vitro at concentrations as low as 1.0 part per million. A marked agglutination reaction preceded hemolysis when ostracitoxin was added to fish erythrocytes in citrated or heparinized saline; such agglutination was not observed with human or mouse blood, but rabbit erythrocytes were agglutinated as readily as those of fish. The minimum effective concentration for agglutination of fish erythrocytes of the skipjack tuna (*Katsuwonus pelamis*) was 1 : 20,000. Agglutination in vitro also preceded hemolysis in boxfish erythrocytes, providing further evidence of the toxicity of ostracitoxin to boxfish.

Ostracitoxin is clearly unique among all known fish toxins. Its ichthyotoxic property alone—that is, its property of poisoning fish immersed in ostracitoxin solutions—distinguishes it from tetrodotoxin and ciguatera toxin. In this respect ostracitoxin closely resembles certain red tide, sea cucumber, and starfish toxins (*11*). The many similarities between ostracitoxin and holothurin A, and the clearly saponin-like properties (*12*) of crude ostracitoxin, indicated that the boxfish toxin might be a steroid saponin (*7*). However, preliminary studies now in progress with the pure toxin indicate that ostracitoxin is not a saponin (*13*).

Although ostracitoxin has been identified only in the stress secretions of the boxfish, *O. lentiginosus*, preliminary studies showed that the mucous secretions of three other Hawaiian trunkfishes, *Lactoria fornasini, L. diaphanus*, and *Rhynchostracion* sp. were ichthyotoxic and hemolytic. Furthermore, a Red Sea boxfish (*2*) and an Atlantic trunkfish (*4*) have already been reported to be poisonous to fish. It appears that ostracitoxin-like poisons may be as characteristic of the Ostraciontidae as tetrodotoxin is of the Tetraodontidae and Salamandridae. Such a biogenetic finding may be of systematic importance within the trunkfish group and suborder Balistoidae.

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9. Assay concentrations between 10 and 100 parts per million, fatal to reef fishes within 10 minutes, had no effect on boxfish. The minimum lethal dose to boxfish could not be readily determined because distressed boxfish continually added toxic secretions to their aquarium water.
10. Besides the boxfish, the pearl fish, *Carapus homei*, an inquiline in the cloaca of certain sea cucumbers and starfishes, was as resistant to ostracitoxin as it was to crude holothurin, the steroid saponin of the sea cucumber.
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12. Like the saponins, crude ostracitoxin is toxic to fish and aquatic organisms and to living systems in general; it is heat stable, detoxified by cholesterol, and cannot be dialyzed. It also resembles the saponins in foaming ability and solubilities.
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14. I thank the Hawaii Marine Laboratory, University of Hawaii, and the Honolulu Biological Laboratory, U.S. Bureau of Commercial Fisheries, for use of their facilities. This research was part of a doctoral dissertation and was supported in part by a grant from the Hawaii Marine Laboratory. This is contribution No. 209, Hawaii Marine Laboratory, University of Hawaii.

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Tuberculin Reactivity of a Carbohydrate Component of Unheated BCG Culture Filtrate

Abstract. *Carbohydrate and protein fractions from the filtrate of a culture of* Mycobacterium bovis *strain BCG have equal activity in sensitive guinea pigs. The sequential action of two proteolytic enzymes caused little alteration in the reactivity of the carbohydrate but almost completely eliminated the reactivity of the protein.*

Carbohydrate components of mycobacterial culture filtrates have usually been considered incapable of eliciting a tuberculin reaction in sensitive animals (*1*). Our previous study (*2*), however, indicated that a carbohydrate fraction, GA, isolated from unheated filtrates of cultures of *Mycobacterium bovis* strain BCG may induce a tuberculin reaction indistinguishable from that induced by protein fractions. It also has been reported recently that a lipopolysaccharide derived from *M. tuberculosis* may have tuberculin activity (*3*).

Because the carbohydrate fraction, GA, contained approximately 10 percent protein, a question arose over the role of protein in the tuberculin activity of the carbohydrate. This report describes the results of proteolytic digestion of fraction GA and establishes that the carbohydrate is, in fact, capable of inducing a tuberculin reaction in sensitive guinea pigs indistinguishable in intensity, appearance, and time of development from that induced by a protein fraction FB or by PPD-S (*4*).

Solutions of FB and GA (25 mg/ml) were prepared in 0.03*M* phosphate buffer, *p*H 7.5, and ethanol was added to a concentration of 4 percent. To 2.0 ml portions of each of these solutions 0.8 mg of Pronase, a prote-

Table 1. Yields and nitrogen analyses of enzyme and nonenzyme treated components isolated by Sephadex G-25 chromatography.

Substance	Yield* (mg)	Nitrogen (%)
FBS	27.8	10.4
FBES	6.3	6.8
GAS	22.2	1.1
GAES	32.9	0.4

* Yield is based on only that portion of the eluate that was salt-free.

olytic enzyme preparation from *Streptomyces griseus* (*5*), was added and the mixtures were incubated for 20 hours at 37°C. Crystalline trypsin (0.8 mg) was then added and these mixtures were incubated for 22 hours at 37°C and 30 minutes at 56°C. The resulting solutions were designated FBE and GAE. As controls, portions of the FB and GA solutions, without added enzymes, were treated in an identical manner. In addition, each enzyme was tested for proteolytic activity prior to use.

Solutions of FB, FBE, GA, and GAE were injected intradermally into normal guinea pigs to establish that they were nontoxic. Similar injections were made into guinea pigs that had been sensitized with heat-killed BCG organisms, but that had not been previously skin-tested. The enzyme solutions alone did not induce a reaction in sensitized animals. All dilutions were made with phosphate buffered saline, *p*H 7.2. The reaction sites, barely perceptible at 3 hours, had reached maximum intensity at 24 hours when the observations were recorded and did not disappear for several days. PPD-S injected simultaneously into the same animals induced identical responses.

Biopsies of the reaction sites and histologic examination by Ruth Kirschstein of the Laboratory of Pathology, Division of Biologics Standards, indicated no differences in the guinea pig response to GA, FB, or PPD-S. There was a light infiltrate of lymphocytes and macrophages in the upper dermis, particularly around capillaries. In the subcutaneous fat, there was a dense infiltrate of polymorphonuclear leukocytes and macrophages which extended into the muscle just beneath the fat. This reaction is typical of a tuberculin hypersensitivity reaction as described by Rich (*6*).

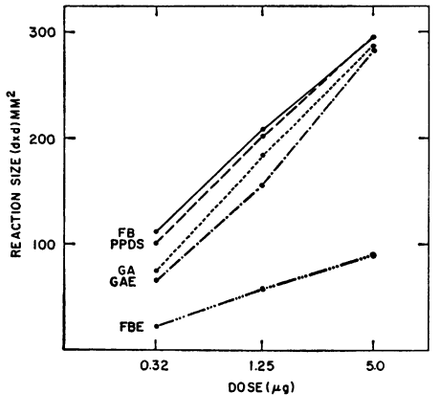

Fig. 1. Dose-response curves of PPD-S and fractions before (GA and FB) and after (GAE and FBE) enzyme treatment.

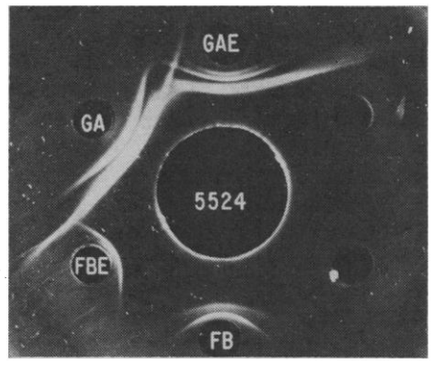

Fig. 2. Agar gel precipitation of fractions before and after enzyme treatment; rabbit antiserum 5524 was used. The concentration of antigens was 10 mg/ml.

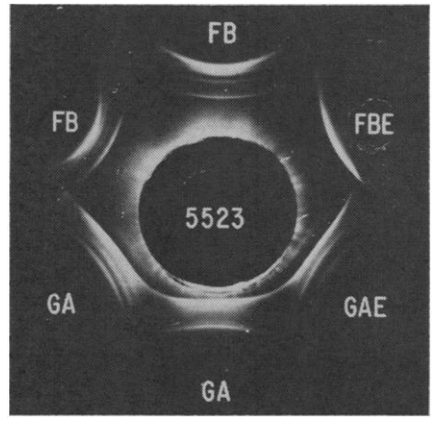

Fig. 3. Agar gel precipitation of fractions before and after enzyme treatment; rabbit antiserum 5523 was used. The concentration of antigens was 10 mg/ml.

The reactions were read at 24 hours as the product of the longest and shortest diameters of erythema and dose-response curves were prepared (Fig. 1) from the average values obtained from 4 to 8 guinea pigs. From Fig. 1 a calculation can be made of the quantity of each of the four substances required to produce a 100 mm^2 reaction (extrapolations of the curves were made where necessary). The ratios of these values, GA/GAE (0.44 μg/0.53 μg) = 0.83 and FB/FBE (0.26 μg/6.6 μg) = 0.04, reveal that enzyme digestion reduced the reactivity of GA to 83 percent of the original, whereas the reactivity of FB was reduced to 4 percent of its original value.

Gel diffusion analyses showed the nature of some of these changes. Reactions were carried out in 1 percent agar containing 1 percent sodium azide at 4°C for 5 to 10 days. The reactions between rabbit antiserum 5524, prepared against fraction G (*7*), and the enzyme and nonenzyme treated solutions are shown in Fig. 2. The enzyme treatment resulted in the elimination of one of at least four band-producing antigens from GA. This antiserum showed only two bands with FB and the loss of one of these from enzyme action. Figure 3 shows results obtained with rabbit antiserum 5523, prepared against all of the nondialyzable culture filtrate components (*7*); FB showed many bands but enzyme treatment, which yielded FBE, eliminated all but one. This antiserum revealed no significant differences between GA and GAE.

Equal volumes of all four solutions were passed through 30- by 1.5-cm columns of Sephadex G-25 equilibrated with water. In order to obtain salt-free antigens, only that portion of the effluent was collected that failed to show a precipitate when a sample was tested with a 10 percent solution of $AgNO_3$. Each of the four salt-free effluents was freeze-dried to yield FBS, FBES, GAS, and GAES in solid form. From Table 1 it may be seen that the yields of FBS, GAS, and GAES were similar but that the yield of FBES was markedly lower than that of the other fractions. This is consistent with the fact that FB was largely protein and hence digested to small fragments which were retarded on the Sephadex G-25 column. Analyses indicated that GAES contained 0.4 percent nitrogen; a portion of this nitrogen undoubtedly was due to some enzyme protein of sufficiently high molecular weight to elute with the salt-free tuberculin fractions.

Although enzymatic digestion resulted in a reduction of the already very low nitrogen content of GA there was no corresponding loss of skin reactivity, illustrating the lack of correlation between nitrogen content and skin reactivity. It is concluded, therefore, that in sensitized guinea pigs the tuberculin reaction is elicited by both carbohydrate and protein components of unheated BCG culture filtrate, as well as by PPD-S.

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6 July 1964

Phytohemagglutinin Elicitation of Specific Anamnestic Immune Response in vitro

Abstract. *Fragments of lymph nodes from rabbits hyperimmunized with human chorionic gonadotropin or bovine plasma albumin were cultivated in organ culture for 1 month. An anamnestic response was elicited in vitro by the sensitizing antigen. Phytohemagglutinin also produced such a response. Throughout cultivation the cultures showed excellent preservation of morphological integrity and of capacity to produce antibody.*

Nonspecific induction of a secondary response was taken as a matter of fact by early workers in the field of immunology (*1*). Later, it was concluded that only specific or cross-reacting antigen could elicit such a response (*2*). We used phytohemagglutinin P (PHA) (*3*) as a mitotic stimulant to elicit an anamnestic response in vitro; our results suggest that nonspecific elicitation may occur under these circumstances. This study attempted to determine whether physical presence of the antigen is necessary for elicitation and maintenance of the anamnestic response.

Adult female albino rabbits were used; each received intravenous injections totaling 2000 to 3000 international units (IU) of human chorionic gonadotropin (HCG) (*4*) or 25 mg of bovine plasma albumin (BSA) (*3*). Three to 6 months after the last injection, the peripheral lymph nodes were removed, pooled, and cut into fragments of 1 mm^3 for cultivation. All animals showed circulating antibody titers of 1 to 1280 to 1 to 20,480 at the time of death. An organ culture system requiring the use of glass beads and filter paper (*5*) was employed. The ratio of tissue wet weight to medium-volume was adjusted to 15 to 20 mg/ml. The culture medium was Eagle's minimum essential medium, supplemented with 20 percent normal rabbit serum and 100 IU of penicillin per millilter; it was renewed every 1 to 3 days. The cultures were treated in vitro with the antigen or PHA for 2 to 3 hours on day 0. Throughout the month of cultivation, antibody production of the fragments was followed by determining the antibody titer in the medium by means of the passive hemagglutination method (*6*).

Parallel morphological studies were carried out with respect to the mitotic activity and histology of the cultural fragments. To determine mitotic activity, fragments were incubated for 24 hours in a medium containing tritiated thymidine labeled on the methyl group (1 to 2 μc/ml; specific activity, 6.7 c/m*M*), fixed, embedded in paraffin, serially sectioned at 5 μ, and processed through the radioautographic procedure, using the dipping technique (*5*, *7*). Kodak Nuclear Track Emulsion NTB-3 was used, with an exposure time of 10 to 14 days. With every 40th to 50th section of each fragment, an area of 0.16 mm^2 (microscopic field, × 430) showing the greatest degree of labeling was selected, and the number of cells containing ten or more grains per nucleus counted. Counts from four to five fragments for each group of cultures were then averaged to express their mitotic activity. Methyl green–pyronin stain was employed for routine histological examination, and Feulgen's stain used for radioautographs.

Throughout the month of cultivation, the fragments showed excellent preservation of morphology and of functional capacity to produce antibody. The morphological preservation was reflected not only in the maintenance of topographical integrity but also in the multiplication and differentiation of those cells generally considered to participate in antibody formation, for example, hemocytoblasts and immature and mature plasmocytes (*8*). The untreated control cultures often showed a low antibody titer during the first several days, with a subsequent steady decrease in titer to an undetectable level. On the the other hand, cultures that were exposed to the antigen for 2 hours on day 0 responded by producing a classical anamnestic antibody response. The antibody titers were low or undetectable during the first 3 to 6 days, reached a peak for 3 to 9 days between days 9 and 27, and then decreased but remained high for the remainder of the cultivation period (Table 1, Fig. 1). With HCG-sensitized nodes, the minimal concentration that elicited such an anamnestic response in vitro was between 1 and 0.1 IU/ml. The smallest concentration of BSA to stimulate a secondary response has been reported as about 1×10^{-9} g/ml (*9*). When the cultures were treated with PHA instead of the sensitizing antigen under the same conditions, fragments of lymph nodes from four out of seven rabbits responded by producing an anamnestic response (three from five HCG-sensitized rabbits, and one from two rabbits sensitized with BSA). Table 1 shows

Table 1. Titers of antibody to HCG in cultures of HCG-sensitized lymph node fragments following stimulation by HCG (20 IU of Follutein per milliliter) or PHA (600 μg/ml) on day 0; duplicate cultures for each group.

Days in culture	Not stimulated	Stimulated with	
		HCG	PHA
	Reciprocal HCG antibody titer		
0 to 1	40	5	80
	40	5	80
1 to 2	20	40	160
	20	40	160
3 to 4	20	80	160
	20	80	160
4 to 5	20	80	160
	20	80	160
5 to 6	20	40	160
	20	40	160
6 to 7	10	80	160
	10	80	160
7 to 8	5	80	160
	5	80	160
8 to 9	10	160	80
	10	160	80
11 to 14	160	2560	320
	80	2560	160
14 to 17	160	2560	320
	80	2560	160
17 to 20	80	1280	320
	40	1280	80
20 to 23	160	1280	320
	40	1280	80
23 to 26	40	1280	160
	20	640	40

Table 2. Number of cells incorporating tritiated thymidine (per 0.16 mm^2 per day) in cultures of HCG-sensitized lymph node fragments following stimulation by HCG (20 IU of Follutein per milliliter) or PHA (600 μg/ml) on day 0. Average counts of five fragments for each group.

Days in culture	Not stimulated	Stimulated with	
		HCG	PHA
0 to 1	31	82	81
4 to 5	57	208	111
8 to 9	44	142	65