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Title: Growth Characteristics of Type E Clostridium botulinum
in the Temperature Range 34 to 50 F.

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This report supplements our more detailed Annual Report dated April 30, 1967 and summarizes the results of work done for the period May 1, 1967 through October 15, 1967. A condensation of the complete data and a discussion of the progress made during this interim is as follows:

Phase VII Toxin Assay Procedures

The only method currently available to detect the presence of C1. botulinum toxins is the mouse bioassay test. Improvements in the mouse toxin assay have been explored. Also, other methods which might be faster and more sensitive than the conventional mouse test have been sought. Work on the Immunodiffusion technique in Dr. H. Sugiyama's laboratory at the Food Research Institute has appeared encouraging. Using his technique, the possible applicability of the plate double diffusion Immunodiffusion test to detect toxin in incubated fresh fish fillets inoculated with Type E spores is being investigated in our laboratory.

Preliminary studies with Type E antisera of equine origin have not been encouraging. The fact that Dr. Sugiyama is employing Type E antiserum which was produced in rabbits perhaps accounts for his success with the double diffusion method.

It is known that antibodies produced in horses against proteins often form weak and readily reversible combinations with their antigens. Such horse protein antibodies are called "H" or "flocculating" antibodies. In contrast,

antibodies produced in rabbits against proteins usually form less reversible combinations with their antigens. Such antibodies are designated "R" or "precipitating" antibodies (Crowle, 1961). With the "H" type of antibodies, inhibition or reversal of the formation of visible precipitin bands is readily obtained by an excess of either antigen or antibody. However, with "R" type of antibodies, an antibody excess may cause only partial inhibition of visible band formation and have little effect on dissociation of antigen-antibody aggregates, although in such a system, an excess of antigen may prevent the formation of distinguishable precipitin bands.

Using the plate double diffusion technique, the reactions of Beluga and Minneapolis toxins against antiserum from the Pasteur Institute were tested. Visible precipitation bands failed to develop. When these same toxins were tested against Porton antiserum of equine origin, weak precipitation bands were seen. Additional work with antiserum of rabbit origin is planned.

Ref. A. J. Crowle, 1961. Immunodiffusion. Academic Press, New York
p. 14.

Phase XVII Toxin Development in Fresh Marine Products.

An inoculated pack experiment on fresh cod was conducted according to the procedures described for haddock in our annual report dated April 30, 1967. The purpose of these inoculated pack experiments is to determine the

relationship between the earliest time of Ci. botulinum Type E toxin production and the maximal expected product storage life. Consumer recognition of unquestionable spoilage markedly ahead of possible toxin production would give a considerable margin of public health safety for low-dose irradiated marine food products.

The protocol recommended by the Ad Hoc committee on botulism to the AEC was followed in the inoculated cod experiment. Estimates of the maximal storage life values, or "X" values, were determined by an untrained consumer type panel at the Bureau of Commercial Fisheries Laboratory at Gloucester. Acceptance or rejection of a sample by the panel was solely on the basis of odor.

Triplicate samples of cod were inoculated per variable with 10^6 , 10^4 , or 10^2 spores of the Beluga strain. The 10^6 inoculum level was preheated at 140 F for 15 minutes to reduce the possibility of toxin carry-over. The lower inocula levels were not preheated. After inoculation, samples were irradiated with 0.1 and 0.2 Mrad and then incubated aerobically at 50, 46, 42 and 38 F along with inoculated unirradiated control samples. During incubation, samples were examined for odor, pH, total aerobic plate count, and the presence of Type E toxin at fractions and multiples of X.

The toxin assay results for 50 and 46 F and for 42 and 38 F incubation are shown in Tables 17-1 and 17-2. The tables are arranged to show incubation temperature, irradiation dose, the fraction or multiple of X, and the toxin assay result. The absence of Type E toxin was confirmed without

trypsin digestion and with trypsin digestion by the acetate procedure previously described. The data at 46 F and below appear quite encouraging. However, the appearance of toxin at X in one of three samples inoculated with 10^4 spores and irradiated at 0.2 Mrad is somewhat disturbing, since no toxin was detected in three comparable replicates inoculated with 10^6 spores. A similar situation can be seen at 42 F with 0.1 Mrad irradiation at a period 1 1/2 times the expected product storage life. Such results are infrequent and likely indicate that if additional replicates had been used, some samples may have shown toxin at the higher inoculum level.

The toxin assay data for cod, as for haddock, appear less favorable at 50 F than at lower temperatures. Toxin was detected at X with 0.1 and 0.2 Mrad irradiated samples, although toxin was notably absent in similar unirradiated controls. The failure to detect toxin in the unirradiated control samples may merely reflect the shorter incubation time used. Therefore, the occurrence of toxin with irradiation might be the result of only longer incubation rather than any direct effect of irradiation. Additional experiments are planned to clarify this point. Also it is conceivable that with unirradiated samples growth and toxin production is delayed or inhibited by the competitive microflora which is eliminated by low-dose irradiation.

In evaluating the 50 F data, the rigorous criterion used in establishing the X-values must be remembered, that being complete rejection of a sample based on odor by an untrained panel. Samples often appear unacceptable due to mold and slime growth before such samples are rejected according

to their odor. It seems likely that the potential consumer would consider both odor and appearance in evaluating the quality of irradiated food products. From the practical standpoint, if the BCF panel had considered appearance in addition to odor this might have caused a reduction in the storage life estimates for the irradiated product and given a more favorable picture at 50 F with respect to the botulinum problem. It is apparent that further studies on both cod and haddock are necessary before definite conclusions can be made about the botulinum hazard with these products.

Phase XVIII Outgrowth Properties of Clostridium botulinum Type C in Marine Products

Crude Type C cultures were obtained from Dr. B. Q. Ward of the University of Florida. The cultures were encountered during a survey of the Gulf and Atlantic Coasts for Ci. botulinum Type E. An attempt is being made in our laboratory to isolate pure Type C cultures from these grossly contaminated cultures and from materials taken during the survey.

So far our attempts to isolate pure Type C cultures have not been successful, although the presence of Type C toxin is clearly evident. As might be expected, the level of toxin is low and interference of non-specific toxicities frequent. The non-specific toxicities encountered here are distinctly different from those seen in the inoculated pack experiments on haddock and cod which were described in our annual report, April 30, 1967. In the present work, mice injected with undiluted

cultures invariably show a violent tremor and die within 2-5 minutes. Mice protected with Type C antitoxin die as soon as the unprotected mice. Dilution of such cultures 1:10 and injection of 0.5 ml quantities rarely produces death. It appears probable that these non-specific deaths are caused by some toxic amine rather than an infection as was experienced in the inoculated pack experiments.

It seems reasonable to expect that the possibility of isolating pure Type C cultures from the materials of marine origin would be greatly improved if the organism can be induced to sporulate. Unfortunately, little is known about the sporulation requirements of C. botulinum Type C. Therefore, our initial efforts have been to develop a suitable sporulation medium using two known Type C cultures, namely 468 C and 571 C. Originally, both cultures were in the K. F. Meyer collection. Various broth formulations which have been used to sporulate clostridia have been examined. These media included liver infusion, beef infusion, Trypticase, Peptone-glucose medium (TPG), TPG plus yeast extract, the lactalysate medium of Dr. Wayne Jensen, PE-2 medium, NCA Sporulation Medium (Fisher), Difco Cooked Meat Medium, and Difco Egg - Meat Medium. While growth was initiated in most of these media, sporulation was not apparent. The Cooked Meat and Egg-Meat media showed about 1-5% sporulation after 5 days of incubation and suggested that by proper supplementation satisfactory spore yields might be achieved. The addition of 0.5% glucose, 1% yeast extract, and 1.0% $(\text{NH}_4)_2 \text{SO}_4$ greatly increased sporulation. The inclusion of these three adjuncts increased the spore yield from less than one million to 40-50 million spores per ml of culture medium. Studies are in progress to determine the optimal concentration of glucose, yeast extract,

and $(\text{NH}_4)_2 \text{SO}_4$ for sporulation of Type C. Then, this medium will be used for the crude Type C cultures in an attempt to facilitate isolation of pure cultures.

Phase XIX Outgrowth Properties at Low Temperature of Non-Proteolytic Clostridium Botulinum Types B and F in Marine Products.

Introduction:

Since non-proteolytic Type B and Type F cultures have been isolated from surveys conducted on the West Coast by Dr. Eklund there is a reasonable presumption that they might be present in marine products subjected to low dose irradiation. Therefore, it is intended to conduct studies on the outgrowth properties at low temperature in marine products of spores of these two types. Pure cultures of the non-proteolytic strains 17B and 202F have been obtained from Dr. Eklund for this work.

Sporulation and Spore Suspensions:

Eklund has used a cooked meat medium for growth and sporulation of the non-proteolytic strains. This is composed of Beef infusion plus the following ingredients per liter: NaCl 5 gm, $\text{K}_2 \text{HPO}_4$ 0.8 gm, Peptone 5 gm, Yeast Extract 10 gm, Trypticase 10 gm, glucose 2 gm. This is combined with the ground infused beef in either tubes or bottles. Since this medium was not available when the cultures were received, a modified cooked meat medium was used temporarily. This was

prepared by dissolving the constituents added to the beef infusion and adding Difco Egg Meat in the proportion of 1 gm to 20 ml in tubes or 5 gm to 200 ml in bottles. Subsequently, a lot of Beef Infusion was prepared and with the cooked meat used to prepare the Eklund cooked meat medium.

Transfers from the original cultures grew well in the modified cooked meat medium and showed sporulation at 85 F. A small lot of spore suspension was prepared with each of these cultures in a bottle of medium at 85 F. Other small spore crops of each culture were prepared in the Eklund cooked meat medium at 85 F and at 70 F. The results in terms of refractile spore yield per ml of medium are shown in Table 19-1. The yield of refractile spores of these strains at 85 F in the modified cooked meat medium was disappointly low as compared to the yields obtained in TPG medium with Type E strains. Also at 85 F the refractile spore yields in the Eklund cooked meat medium were no better. However, in the Eklund cooked meat medium at 70 F the refractile spore yield of 17 B increased about 5.9 fold and the 202 F about 11 fold. In each case the refractile spores appeared to form about 95 % of the total population. It is intended for the present at least to prepare spore suspensions of these strains in Eklund cooked meat medium at 70 F.

Using the first suspensions prepared in the modified cooked meat medium experiments were conducted to determine the medium and conditions which would yield the maximum viable count. The results of these experiments are shown in Table 19-2. The viable counts were conducted in 16 x 125 mm screw cap tubes with all media containing 1.5% agar and 0.1% Na-thioglycolate. In general, maximum colony count was reached in 3-4 days

at 85 F and in 6-7 days at 70 F.

From Table 19-2, it may be observed that maximum viable spore counts were obtained at 70 F in Beef Infusion agar, 5% Peptone agar plus 1% Yeast Extract and TP agar plus 1% Yeast Extract. At 85 F the viable count in each of these three media appears lower. In the only test conducted on preheating using 5% Peptone-Yeast Extract at 85 F for viable count a preheat of 15 minutes at 140 F does not appreciably affect the viable count while 15 minutes at 176 F eliminates the small number of spores present. In the dilution used for counting there were theoretically 150 refractile spores per ml of 17B and 80 refractile spores per ml of 202F. Therefore, the viable count as percent of the refractile count for 17B is 23% and for 202F is 18%. These percentages are considerably lower than that found with Type E suspensions.

It is intended that the viable spore counts on these two strains will be conducted in Beef Infusion agar at 70 F until further comparative studies may completely justify replacement with either of the two samples of media.

Future Work

A continuation of the work presented in this report is anticipated. Accompanying this report is a proposal for studies to be conducted during the contract year January 15, 1968 - January 14, 1969.

TABLE 17-1

Toxin assay results on cod with incubation at 50 and 46 F (Spores produced at 50 F).

TEMP°F.	MRAD	INC (DAYS)	X-VALUE	PRESENCE OF TOXIN ¹			
				INOC	10 ⁶	10 ⁴	10 ²
50	0	6	1			-	-
		9	1 1/2			-	-
	0.1	13	1			+	+
		18	1 1/2			+	+
	0.2	18	1			+	+
		27	1 1/2			+	+
46	0	6	3/4		-	-	
		8	1		-	-	
		13	1 1/2		-	-	
	0.1	14	3/4		-	-	
		19	1		++	-	
		26	1 1/2		++	-	
	0.2	17	3/4		-	-	
		24	1		-	++	
		35	1 1/2		+	-	

¹ + Means toxin detected; - Means no toxin detected in three replicate samples.

* Toxin present in one out of three replicate samples.

TABLE 17-2

Toxin assay results on cod with incubation at 42 and 38 F (Spores produced at 50 F).

TEMP° F.	MRAD	INC (DAYS)	X-VALUE	PRESENCE OF TOXIN ¹	
				INOC 10 ⁶	10 ⁴
42	0	7	3/4	-	-
		9	1	-	-
		13	1 1/2	-	-
	0.1	19	3/4	-	-
		26	1	-	-
		39	1 1/2	-	++
	0.2	24	3/4	++	-
		31	1	++	-
		46	1 1/2	-	-
38	0	8	3/4	-	-
		10	1	-	-
		16	1 1/2	-	-
	0.1	25	3/4	-	-
		33	1	-	-
		48	1 1/2	-	-
	0.2	27	3/4	-	-
		38	1	-	-
		55	1 1/2	-	-

¹ + Means toxin detected; - means no toxin detected in three replicate samples.

* Toxin present in one out of three replicate samples.

TABLE 19-1

Refractile spore yield per ml of medium of strains 17B and 202F.

MEDIUM	TEMP°F.	TIME DAYS	REFRACTILE SPORES/ml X 10 ⁶	
			17B	202F
Modified CM	85	5	19	9
Eklund CM	85	5	18	6
Eklund CM	70	7	107	66

TABLE 19-2

Viable spore count of suspensions of strains 17B and 202F.

MEDIUM	TEMP° F.	PREHEAT	VIALE COUNT/ml OIL SUSPENSION	
			17B	202F
5% P	85	0	1.2 (ng)	0
5% P + Y.E.	85	0	25 (ng)	9 (ng)
5% P + Y.E.	85	15'/140 F	22 (ng)	6 (ng)
5% P + Y.E.	85	15'/176 F	0	0
5% P + Y.E.	70	0	34 (g)	10 (ng)
TP + YE	85	0	18 (g)	9 (g)
TP + YE	70	0	34 (g)	10 (ng)
TP + 0.2% G	70	0	4 (ng)	5 (ng)
TPG + Y.E.	70	0	14 (gt)	10 (ng)
BI	85	0	16 (g)	2 (gt)
BI	70	0	34 (ng)	14 (ng)

Y.E. 1% Yeast Extract
P Peptone
TP 5% Trypticase + 0.5% Peptone
G Glucose
BI Beef Infusion

ng = no gas

g = slight gas

gt = excessive gas