GROWTH OF *CLOSTRIDIUM BOTULINUM* TYPE E (IWANAI) IN SEMI-SYNTHETIC MEDIUM

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INTRODUCTORY

The cultivation of clostridia in chemically defined media was first attempted by KNIGHT & FILDES (*Cl. sporogenes*). Since 1933, defined media for number of other anaerobic spore-forming bacteria have been reported by many workers. Further, investigations of the growth requirements of *Cl. botulinum* were studied by BURROWS, FILDES, LAMANNA & LEWIS, ROESSLER & BREWER, MAGER, KINDLER & GROSSWICZ and other workers. Generally, the nutritional requirements of *Cl. botulinum* are widely various; so that, for each strain, complete elucidation has not been attained except for a few.

As the first step to study the mechanism of toxin production by *Cl. botulinum* type E (Iwanai), identified by NAKAMURA et al. as the cause of botulism in Hokkaido, the present writers attempted to cultivate it in semi-synthetic media containing casamino acids, vitamins, inorganic salts and others.

MATERIALS AND METHODS

Media All media were derived from the following stock solutions.

Vitamin solns: Biotin soln. (100 γ/dl), thiamine soln. (10 mg/dl), riboflavine soln. (10 mg/dl), folic acid soln. (5 mg/dl), pyridoxine soln. (10 mg/dl), Ca-pantothenate soln. (10 mg/dl), p-aminobenzoic acid soln. (10 mg/dl), nicotinamide soln. (10 mg/dl), inositol soln. (500 mg/dl), β-alanine soln. (10 mg/dl), pimeric acid soln. (10 mg/dl), RNA soln. (200 mg/dl), DNA soln. (200 mg/dl), choline soln. (25 mg/dl), adenine soln. (200 mg/dl).

Amino acid solns: Casamino acids soln. (0.5 g/dl), cysteine-HCl soln. (2.5 g/dl), tryptophan soln. (0.5 g/dl).

Salts solns: Solution A 0.1 M phosphate buffer pH 7.4, solution B $MgSO_4 \cdot 7H_2O$ 2g, $FeSO_4 \cdot 7H_2O$ 0.1 g, $MnSO_4 \cdot 3H_2O$ 0.1 g, in 100 ml of redistilled water.

Glucose soln. (20 g/dl).

Sodium thioglycollate soln. (5 g/dl).

Suitable volumes of these solutions were mixed and pH was adjusted to 7.4. The medium was dispensed into test tubes plugged with cotton wool, then, paraffin liquid was added.

Inoculum Twenty-four hours cultures in liver-broth at 32°C were centrifuged and

the precipitate was washed 3 times with physiological saline containing 0.05% sodium thioglycollate, then diluted to original volume with the same solution. Each 0.25 ml of this suspension was placed in test tubes containing 10 ml of medium.

Estimation of growth Growth was estimated by measurement of turbidity with an electrophotometer at 530 mμ and expressed in terms of percentage transmission.

Assay of toxin Toxin production was tested after growth for 5 days in culture medium and expressed in terms of MLD for mice (intraperitoneally).

RESULTS

Attempts to grow *Cl. botulinum* type E (Iwanai) in media containing casamino acids vitamin free (Difco), instead of liver broth, were successful, when cysteine, tryptophan, thioglycollate, glucose, salts and 15 vitamins prepared were added. Even when only one of them was lacking, growth if any, was irregular and scanty. In order to determine the optimum concentration of these materials for growth, the following medium was used as the standard.

Casamino acids soln.		3.0 ml	
Tryptophan soln.		0.1	
Cysteine-HCl soln.		0.1	
Na-thioglycollate soln.		0.1	
Glucose soln.		0.2	
Salts soln. A		0.1	
Salts soln. B		0.1	
Vitamin solns.	Each	0.1	(Total 1.5 ml)

Made up to 10 ml with redistilled water.

Results are shown in tables 1~7.

From above results, the standard medium for the following experiments was established as follows.

Casamino acids soln.		3.0 ml	
Tryptophan soln.		0.12	
Cysteine-HCl soln.		0.12	
Na-thioglycollate soln.		0.12	
Glucose soln.		0.20	
Salts soln. A		0.10	
Salts soln. B		0.10	
Vitamin solns.	Each	0.10	(Total 1.50 ml)

Made up to 10 ml with redistilled water.

In this medium, growth of micro-organisms was about 70 per cent of that in liver-broth.

To determine the vitamins having an important effect for growth, 15 vitamins were divided into 3 groups and tested. In table 8 are shown the effects of these groups.

As in table 8, all three groups were effective to some extent. Then, in each group effective factors were investigated. It was found that biotin, thiamine, nicotinamide, folic acid and Ca-pantothenate in group 1, pyridoxine in group 2, DNA (or adenine) in group 3. were effective.

TABLE 1. *Tryptophan Requirement*

CONC. mg/10 ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0.0	85	78	68
0.3	51	42	38
0.6	24	25	23
0.9	27	24	25
1.2	28	31	32

TABLE 2. *Cysteine-HCl Requirement*

CONC. mg/10 ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0.0	83	69	53
1.5	53	45	41
3.0	24	26	23
4.5	24	23	22
6.0	23	22	22

TABLE 3. *Glucose Requirement*

CONC. mg/10ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0.0	100	98	98
10.0	59	48	45
20.0	36	32	32
30.0	24	24	24
40.0	23	22	24

TABLE 4. *Salts Solution A Requirement*

ADDED ml/10ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0.0	30	33	33
0.1	24	24	24
0.2	23	24	21

TABLE 5. *Salts Solution B Requirement*

ADDED ml/10ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0.0	91	95	78
0.1	24	24	24
0.2	28	29	28

TABLE 6. *Thioglycolate Requirement*

CONC. mg/10ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0.0	93	52	45
3.0	72	46	36
6.0	25	24	23
9.0	33	26	26
12.0	58	32	25

TABLE 7. *Casamino Acids Requirement*

CONC. mg/10 ml medium	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
0	100	100	100
50	75	60	49
100	45	37	32
150	27	24	22
200	26	27	24
250	25	28	26
300	29	30	28

To investigate the effect of each vitamin which influenced growth, one vitamin at a time was omitted from medium containing initially 6 vitamins (DNA was omitted, because lack of DNA had no great effect). The results of these experiments are summarized in table 9.

From the results it can be seen, that of the 6 vitamins added, biotin and nicotinamide were required essentially, thiamine, pyridoxine and Ca-pantothenate effected growth to some extent, and folic acid seemed to be a stimulus because omission of it brought delay of lag phase for growth above 48 hours. Other vitamins; p-aminobenzoic acid, choline,

TABLE 8. *Effects of Three Groups of Vitamins on Growth of Cl. botulinum Type E* (Iwanai)

VITAMINS ADDED	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
Groups 1, 2 and 3	26	24	23
Groups 1 and 2	43	32	31
Group 1	72	59	60
Groups 2 and 3	100	100	98
Group 3	100	100	100
Groups 1 and 3	62	61	60
Group 2	100	100	100
LAMANNA*	100	83	79
ROESSLER**	100	80	77
KINDLER***	100	100	100

Notes: Each 0.1 ml of vitamin stock solutions was added in 10 ml of medium.
Group 1. biotin, thiamine, p-aminobenzoic acid, Ca-pantothenate, nicotinamide, folic acid.
Group 2. choline, pyridoxine, riboflavine, inositol, β-alanine.
Group 3. RNA, DNA, adenine, pimeric acid.

* Biotin, thiamine, Ca-pantothenate, folic acid, choline and pyridoxine were added. These vitamins were required for growth of *Cl. botulinum* type A (Hall).

** Biotin, p-aminobenzoic acid, nicotinamide, riboflavine, DNA and RNA were added. These vitamins were required for growth of *Cl. botulinum* type A.

*** Biotin, thiamine and p-aminobenzoic acid were added. These vitamins were required for growth of *Cl. botulinum* type A.

TABLE 9. *Effect of Vitamins on Growth of Cl. botulinum Type E* (Iwanai)

VITAMINS ADDED	% TRANSMISSION AFTER		
	24 hrs.	48 hrs.	120 hrs.
Six vitamins	38	35	30
Vitamin omitted from mixture of 6			
Biotin	98	97	95
Thiamine	70	64	62
Ca-Pantothenate	53	37	33
Nicotinamide	100	98	92
Folic acid	100	100	42
Pyridoxine	72	63	60

riboflavine, inositol, pimeric acid and β-alanine had no effects, further, attempts to replace each of the six vitamins influenced with them could not succeed.

Titre of toxin produced in the semi-synthetic medium was low as compared with yields in more complex media. For example, the usual titre in liver-broth varied between 500~2,000 MLD/ml, whereas in the semi-synthetic medium the maximum titre obtained was usually below 50 MLD/ml.

DISCUSSION

Cl. botulinum type E (Iwanai) can be cultivated in semi-synthetic medium containing casamino acids as the nitrogen source and its requirements for growth are almost identical with those of other clostridia except in the matter of vitamins. The results for vitamin requirements show that biotin and nicotinamide are essential, and that thiamine, pyridoxine, Ca-pantothenate and folic acid are necessary for sufficient growth. But whether they are essential or not can not be detected perfectly, because in these experiments casamino acids vitamin free (Difco) was used as nitrogen source. According to the observation by MAGER et al., (1954), *Cl. botulinum* type A required p-aminobenzoic acid essentially in perfect synthetic medium, but did not in semi-synthetic medium—included casamino acids vitamin free Difco. Accordingly, for vitamin requirements of *Cl. botulinum* type E (Iwanai), re-investigation using a chemically defined medium is desirable.

This organism has an absolute requirement for glucose as indicated in table 3 (of course glucose may be replaced with certain carbohydrates or its derivatives). According to the report by MAGER et al. for type A, in a medium containing 2.5% casein hydrolysate supplemented with arginine, phenylalanine and tyrosine, omission of glucose resulted in only slightly inferior growth, while in an unsupplemented medium, growth was much poorer when glucose was absent. But, in any case, a certain carbohydrate or its derivatives would be the best as the energy source.

The titre of toxin produced in semi-synthetic medium was considerably low as compared with yields in natural media. For the cause leading to such results, many cases may be considered. It is unreasonable to assume that a certain substance inhibiting the production of toxin may exist in semi-synthetic medium, because the addition of concentrated semi-synthetic medium in natural media results in little effect on yield of toxin. Consequently, in semi-synthetic medium a certain factor (factors) regulating the production of toxin may be lacking, or a certain factor concerned with both growth and toxin production exists in natural media, which can be replaced by any factor included in semi-synthetic medium for growth, but not for toxin production. Toxin of *Cl. botulinum* type E (Iwanai) can be found not only in culture filtrates, but also in bacterial cells. Accordingly,

it is considered that toxin may be synthesized in bacterial cells and appear in culture medium as a result of some certain process. In view of this supposition, the mechanism of toxin production should be studied for two phases. It is interesting to ascertain whether the above described factors regulating the yield of toxin are related to any phase, whether to synthesis or to liberation (the writers are not sure, if this term is appropriate or not) or both.

Summary

As the first step to study the mechanism of toxin production by *Cl. botulinum* type E (Iwanai), semi-snythetic medium containing casamino acids as nitrogen source was prepared. For sufficient growth, at least casamino acids, tryptophan, cysteine, thioglycollate, glucose, inorganic salts, nicotinamide, biotin, pyridoxine, thiamine, folic acid and Ca-pantothenate were necessary. In this medium, the growth of micro-organisms reached to about 70 per cent as in liver-broth, while the production of toxin was only below 1/10 as compared with yields in liver-broth.

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