Nutritional Studies with the *Clostridium botulinum* Group

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SUMMARY: The factors which influence the initiation of growth from small inocula of *Clostridium parabolulinum* type A in the defined medium previously described were re-examined. CO₂ was found essential for rapid and regular growth from small inocula; it could be partially replaced under conditions of strict CO₂ depletion by an unidentified factor present in enzymic hydrolysate of casein or yeast extract.

The growth requirements of the various serological types of *C. botulinum* group were studied and compared with those of *C. parabolulinum* type A. It was found that the various organisms can be classed into two nutritional groups: (a) those which gave rapid and heavy growth in a chemically defined medium as described for type A, with some variations in respect of vitamin requirements: here belong *C. parabolulinum* type A (12 strains tested), type B (9 strains) and *C. sporogenes* (10 strains); (b) organisms which failed to grow in the defined medium or in a partially-defined medium (containing acid hydrolysate of casein) supplemented with all known B-vitamins: this group comprised organisms of the type C (2 strains) type D (4 strains) and type E (5 strains, of which 3 grew in a partially defined medium).

The similarity in nutritional requirements of *Clostridium sporogenes* to *C. parabolulinum* types A and B and the similarity in cultural and biochemical properties make *C. sporogenes* indistinguishable from, and perhaps identical with, non-toxicogenic strains of *C. parabolulinum* types A and B.

In a previous communication (Mager, Kindler & Grossowicz, 1954) the nutritional requirements of *Clostridium parabolulinum* type A were described. The present paper deals with factors which determine the growth of *C. parabolulinum* type A from small inocula. In addition, the growth requirements of other *C. botulinum* types and *C. sporogenes* were studied and compared with those of *C. parabolulinum* type A.

METHODS

The general procedure and the growth assays were carried out as described (Mager *et al.* 1954). Mercaptoacetate (0.05%) and cysteine (0.025%) were present as reducing agents.

Organisms. The 43 strains used in this study were obtained from the following sources: National Collection of Type Cultures (NCTC) England (11 strains); American Type Culture Collection (ATCC) U.S.A. (16 strains); U.S. Depart-

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ment of Agriculture (7 strains); N.Y. Department of Health (NYDH) (3 strains); Department of Bacteriology, the Hebrew University, Jerusalem (1 strain); Dr K. F. Meyer, University of California (3 strains); Dr D. A. Boroff, State Research Hospital, Galesburg, Ill. (1 strain), Dr J. Keppie, Microbiological Research Department, Porton, Wiltshire, England (1 strain).

RESULTS

Effect of CO₂ on growth of Clostridium parabotulinum type A

In the described medium excellent growth was obtained regularly with large inocula (0.1 ml. of 24–48 hr. culture containing *c.* 0.5×10^8 organisms). On the other hand, growth was irregular and the lag period was very much prolonged when smaller inocula were used (1×10^5 organisms or less). It therefore became apparent that some factor is required for growth initiation. Since it was shown by Gladstone, Fildes & Richardson (1935), with a rigid technique, that CO₂ exerts a controlling effect on the initiation of growth of both aerobic and anaerobic bacteria, the effect of CO₂ was tested. The medium was acidified to pH 2, boiled for 10–15 min. to remove CO₂ and the pH value readjusted to 7.4 with freshly distilled ammonia solution. After autoclaving, the medium was rapidly cooled, inoculated, and incubated anaerobically in a McIntosh and Fildes jar. Under these conditions no growth, or irregular growth, was obtained when small inocula were used, whereas with large inocula growth was unimpaired. This was in contrast to the results obtained in a medium which contained an enzymic casein hydrolysate where no adverse effect of CO₂-removal by acidification etc., was noticed (Table 1). Since acidification (in order to remove bound CO₂) of the enzymic casein hydrolysate medium (ECH

Table 1. *Effect of size of inoculum on growth of Clostridium parabotulinum type A in CO₂-free medium*

The partially-defined medium contained (mg./10 ml.): glucose 50; Casamino acids vitamin-free (Difco) 50; L-tryptophan, 0.05; L-arginine 30; DL-phenylalanine 20; L-tyrosine 2.5; L-cysteine 2.5; Na-mercaptoacetate 5. Salts A 0.1 ml. and salts B 0.01 ml. (Snell, Strong & Peterson 1937). Biotin, 0.005 µg; thiamine 4 µg; *p*-aminobenzoic acid, 0.1 µg. The ECH medium was the partially-defined medium (as above) supplemented with 0.5 % Enzymic Casein Hydrolysate (Difco).

Dilution of inoculum (undiluted = <i>c.</i> 5×10^8 organisms)	Relative degree of growth after 24 hr. (% transmission)	
	Partially defined medium (%)	ECH medium (%)
10 ⁻¹	18	17
10 ⁻²	18	17
10 ⁻³	35	18
10 ⁻⁴	80	18
10 ⁻⁵	95	18
10 ⁻⁶	100	18
10 ⁻⁷	100	20
10 ⁻⁸	100	25

medium) did not alter the results, it became obvious that the enzymic casein hydrolysate contained material capable of replacing CO_2 .

In order to determine the nature of the CO_2 replacing factor (CRF) a number of substances known as products of CO_2 fixation were tried. The following compounds proved active: nucleic acids, guanine, xanthine and adenine (Table 2).

Table 2. *Effect of various substances on growth of Clostridium parabotulinum from small inocula in a CO_2 -deficient medium*

CO_2 was removed by evacuation of the McIntosh and Fildes jar the traces of residual CO_2 being absorbed with concentrated KOH. The inoculum was 5×10^8 organisms of *C. parabotulinum* type A strain 1.

Substances added to the partially defined medium (Table 1) (mg./10 ml.)		CO ₂ removed by			
		Evacuation		Evacuation + KOH	
		Period of growth (hr.)			
		24	72	24	72
		Relative degree of growth (% transmission)			
No addition		100	95	100	100
Sodium bicarbonate*	2.0	20	80†	20	65†
Enzymic casein hydrolysate (Difco)	50.0	18	85	18	90
Enzymic casein hydrolysate (Difco)	10.0	30	40	63	32
Enzymic casein vitamin-free	50.0	76	22	100	88
Yeast extract (Difco)	50.0	18	72	17	65
Ribonucleic acid	1.0	75	18	100	100
Deoxyribonucleic acid	1.0	80	17	100	100
Guanine	0.1	76	20	100	100
Xanthine	0.1	78	18	100	100
Adenine	0.1	75	19	100	100
Uracil	0.1	100	96	100	100
Thymine	0.1	100	95	100	100
Hypoxanthine	0.1	100	98	100	100
Malate*	50.0	100	92	100	100
Pyruvate*	50.0	100	96	100	100
Citrate	50.0	100	95	100	100
Acetate	50.0	100	95	100	100
Formate	50.0	100	93	100	100
Succinate	50.0	100	97	100	100
α-Keto-glutarate*	50.0	100	92	100	100
Oxalacetate*	50.0	70	20	45	25

* Sterilized by Seitz filtration and added aseptically to the partially defined medium.

† The decrease of turbidity on continued incubation was due to autolysis.

Among the Krebs-cycle intermediates only oxalacetate possessed CRF-activity. However, when traces of CO_2 were removed from the jar by concentrated KOH, only bicarbonate, oxalacetate, enzymic casein hydrolysate and yeast extract enabled growth from small inocula to occur (Table 2). It cannot be ascertained, however, whether the effect of oxalacetate may not have been due to CO_2 released by spontaneous decarboxylation.

Sources and properties of the CRF. Besides casein hydrolysate good sources of CRF were yeast extract (Difco), proteose-peptone (Difco) and a Seitz-filtrate of an *Escherichia coli* culture grown in a casein hydrolysate (hydrolysed with acid) medium. The active substance of the enzymic casein hydrolysate was heat stable and was not destroyed by heating for 1 hr. at 120° in 5 N-HCl or N-NaOH. The CRF was removed from the enzymic casein hydrolysate by repeated treatment with Norite A at pH 3.0; some CRF activity was obtained by continuous extraction with ethyl acetate or acetone.

Stimulatory effect of non-essential amino acids. The rapid growth regularly obtained in the presence of CO₂ allowed re-evaluation of the importance for growth of the non-essential amino acids when relatively small inocula were employed. With an inoculum of 1×10^5 organisms, maximal growth was obtained after 16–24 hr. of incubation in a medium composed of a mixture of 19 amino acids (see Table 3).

Table 3. *Effect of threonine, histidine, glycine and serine on growth of Clostridium parabotulinum type A*

Hall strain (ATCC 3502): inoculum, 1×10^5 cells.

	Period of growth (hr.)			
	17	24	41	65
	Relative degree of growth (% transmission)			
19 amino acids*	41	35	32	43
8 amino acids†	95	90	87	82
8 amino acids + glycine + histidine	87	34	36	67
8 amino acids + serine + histidine	84	46	47	50

* Nineteen amino acids in defined medium (Mager *et al.* 1954).

† Eight amino acids: cysteine + the following 7 essential amino acids: L-arginine, DL-isoleucine, DL-leucine, DL-methionine, DL-phenylalanine, L-tryptophan and L-tyrosine.

In a medium containing the 8 essential amino acids (L-arginine, DL-isoleucine, DL-leucine, DL-methionine, DL-phenylalanine, DL-threonine, L-tryptophan, L-tyrosine) and cysteine as a reducing agent, rapid growth was obtained with a large inoculum (5×10^5 organisms). However, with a small inoculum (1×10^5 organisms) no growth appeared at all or it was delayed for 3–4 days. This lag was substantially decreased by the addition of glycine and histidine to the above mixture of essential amino acids. Addition of glycine abolished the requirement for threonine, glycine becoming essential in the threonineless medium; serine replaced glycine. These findings are illustrated in Table 3 with 1 out of the 6 strains tested. Among the various strains of type A, some preferred glycine whereas others responded better to serine.

As reported previously, the demand for excessive amounts of arginine in a casein hydrolysate medium could be satisfied partially by ornithine and lysine (Mager *et al.* 1954). In an arginine-free medium, however, neither ornithine nor lysine was able to support growth. Thus, ornithine and lysine showed a sparing effect on arginine requirement. Citrulline, on the other hand, was as effective as arginine in promoting growth.

Nutrition of Clostridium parabolinum type B

All the 8 strains tested grew in the defined medium (19 amino acids, vitamins, salts), which was found adequate for cultivation of *Clostridium parabolinum* type A. A closer comparison in regard to vitamin and amino acid requirements did not reveal any significant differences between type B and type A strains. In respect of vitamin requirements the same pattern of variability was found as with type A strains. Thus, all the type B strains required biotin and *p*-aminobenzoic acid (*p*-AB). Two out of 8 strains showed a dependence on pyridoxine or pyridoxamine. Thiamine was stimulatory for some strains.

In general the rate of growth of type B strains was somewhat slower than that of type A strains; however, no differences in either physico-chemical (optimal pH value and temperature) or metabolic properties (utilization of glucose, salicin etc.) were found.

Nutrition of Clostridium sporogenes

Clostridium sporogenes appears to be closely related to *C. parabolinum* type A in cultural, biochemical and serological properties (Hadley, 1927). The nutritional requirements of this organism were studied by Knight & Fildes (1933), Fildes & Richardson (1935), and Shull, Thoma & Peterson (1949). The later investigators established both the vitamin and amino acid requirements of this organism but made no distinction between essential and stimulatory amino acids.

In view of the observed interplay between non-essential and essential amino acids in *Clostridium parabolinum* type A (see above) the possibility of distinguishing between *C. parabolinum* and *C. sporogenes* by a study of amino acid interrelationships was explored. All the 10 strains tested grew well in the defined medium (Mager *et al.* 1954). The same 8 amino acids were found to be essential for *C. sporogenes* and for *C. parabolinum* types A and B. Moreover, serine or glycine was able to replace threonine. In respect of arginine requirement, the strains of both species behaved alike (Shull *et al.* 1949). All the strains of *C. sporogenes* required biotin and *p*-AB whilst nicotinic acid and thiamine were found stimulatory each for one strain only (strains NTCC 534 and ATCC 7955 respectively). Similarly, no biochemical differences were found which would aid in distinguishing non-toxicogenic strains of *C. parabolinum* from *C. sporogenes*.

Nutrition of Clostridium botulinum types C, D and E

Type C strains. Two toxigenic and two non-toxigenic strains were examined. The two non-toxigenic strains were successfully cultivated in the same chemically defined medium as that described for types A and B. These organisms, however, showed typical R-appearance (rhizoid colonies on agar and clumping in liquid media) and were devoid of toxicity for mice and chickens and therefore indistinguishable from *C. sporogenes*. Hence it seems doubtful whether

these strains are true varieties of the C group as labelled (strains 462 and 6060 of the ATCC). The toxigenic strains (one from Dr K. F. Meyer and the other from Dr D. A. Boroff) did not grow in the defined or partially-defined media, even on enrichment with a mixture of all the known B vitamins (see legend to Table 4.)

Table 4. *Vitamin requirements of Clostridium botulinum type E*

Basal medium (mg./ml.); Casamino acids vitamin-free (Difco), 100; glucose, 50; L-tryptophan, 0.5; L-cysteine, 2.5; Na mercaptoacetate, 5; Na bicarbonate, 2. Salts A, B (Snell *et al.* 1937) 0.1 ml. and 0.01 ml./10 ml. medium, respectively.

Strains* (no.)	Vitamins added (μ g.) to 10 ml. basal medium	Period of growth (hr.)	
		24	48
		Relative degree of growth (% transmission)	
1, 2	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxamine 0.05	69	30
	Biotin 0.005, thiamine 4, folic acid 0.1	100	100
	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxine 5	95	90
	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxine 500	67	39
	— thiamine 4, folic acid 0.1, pyridoxamine 0.05	100	100
	Biotin 0.005, — folic acid 0.1, pyridoxamine 0.05	95	90
	Biotin 0.005, thiamine 4, — pyridoxamine 0.05	100	100
	Biotin 0.005, thiamine 4, <i>p</i> -AB 0.1, pyridoxamine 0.05	100	100
	Biotin 0.005, thiamine 4, CF 0.01, pyridoxamine 0.05	70	29
	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxamine 0.05, ECH 0.1 %	23	15
3	Pantothenic acid 10, nicotinic acid 10	42	48
	Pantothenic acid 10	95	90
	Nicotinic acid 10	92	95
	Pantothenic acid 10, nicotinic acid 10, ECH† 0.1 %	16	20
4, 5	Mixture of 19 growth factors‡	100	100

* Strain 1=ATCC 9565; strain 2=NYDH 36208; strain 3=NCTC 8266; strain 4=ATCC 9564 and strain 5=obtained through the courtesy of Dr K. F. Meyer.

† ECH=enzymic casein hydrolysate (Difco).

‡ Nineteen growth factors (μ g./10 ml.): biotin, 0.005; thiamine, 4; folic acid, 0.1; *p*-aminobenzoic acid (*p*-AB) 0.1; citrovorum factor (CF), 0.1; choline, 25; Ca pantothenate, 10; pyridoxine 5; pyridoxamine 5; pyridoxal HCl 5; pyridoxal phosphate 5; nicotinic acid, 10; nicotinamide, 10; riboflavin, 5; inositol, 400; vitamin B₁₂, 0.5; biocytin, 0.1; α -lipoic acid, 0.01.

Type D strains. Three strains were tested: none grew in the defined medium or in the medium enriched with all the known B vitamins.

Type E strains. Five strains were studied; all were toxigenic, but the toxic culture filtrate (growth in cooked meat broth) was of low titre. Two strains (ATCC 9564 and another obtained through the courtesy of Dr K. F. Meyer) did not grow in the defined or in the partially-defined media even on supplementation with various accessory growth factors (Table 4). However, addition of enzymic casein hydrolysate produced heavy growth of these strains.

The remaining 3 strains gave luxuriant growth in the partially-defined medium. One of them (NCTC 8266) required pantothenic and nicotinic acids,

being the only one out of 43 strains of *Clostridium botulinum* tested which did not need biotin for growth. The other 2 strains of this group (ATCC 9565 and NYDH 36208) required biotin, thiamine, pyridoxamine and folic acid or citrovorum factor. Folic acid could not be replaced by *p*-aminobenzoic acid. On the other hand, pyridoxal was as effective as pyridoxamine, whilst pyridoxine was much less effective (*c.* 0.1 %). The results are summarized in Table 4.

Replacement of the acid hydrolysate of casein in the partially defined medium by a mixture of 19 amino acids resulted in only slightly inferior growth. The essentiality of different amino acids however was not determined.

DISCUSSION

Like many other micro-organisms (Fildes & Richardson, 1935) the parobotulinum group exhibits dependence on a CO₂ supply for the initiation of growth. The growth-initiating effect of CO₂ is not yet fully understood. CO₂ is known to be required for the synthesis of purines and various intermediates of the Krebs cycle (Pappenheimer & Hottle, 1940; Lwoff & Monod, 1947). However, none of these compounds, singly or in combination, was able to replace CO₂ when rigid conditions for CO₂-removal were applied. Under conditions of CO₂ depletion an unidentified factor present in enzymic casein digests was able to replace CO₂. This factor (CRF) is similar in properties to that required by *Escherichia coli* and *Aerobacter aerogenes* as described by Wong & Ajl (1953). Whether CRF is a product of CO₂ fixation or is a catalyst is not clear.

The distinction between essential and non-essential amino acids cannot always be rigidly drawn. Change of growth conditions, e.g. decreasing the size of inoculum, may necessitate the inclusion of an amino acid which is dispensable when a large inoculum is used. This possibility is exemplified by the requirement of *Clostridium parobotulinum* types A and B and *C. sporogenes*, for glycine (or serine) + histidine in a medium deficient in threonine. These interrelations suggest the existence of a biosynthetic pathway leading from serine or glycine to threonine. The interconversion between serine and glycine has been established in various micro-organisms (Sakami, 1955). Histidine may contribute to the synthesis of threonine, possibly by serving as a formyl (C-1) donor (Sprinson & Rittenberg, 1952; Toporek, Miller & Bale, 1952).

Summing up the experimental results it may be concluded that the various botulinum types fall into two nutritional groups: (a) strains which can be grown in a defined medium as described, notwithstanding differences in the vitamin requirements among the various strains; (b) strains which have a different nutritional pattern in respect of amino acids and of accessory growth factors. The first comprises strains of *Clostridium parobotulinum* types A and B. Evidently the serological and chemical differences between the two types of toxins are not reflected in the nutritional requirements of the organisms. The claim of Roessler & Brewer (1946) that *C. parobotulinum* type B differs from type A strains in requiring histidine instead of threonine, was not confirmed for the strains studied by us. *C. sporogenes* from a nutritional viewpoint appears indistinguishable from *C. parobotulinum* types A and B.

In view of the limited number of strains studied, it is difficult to decide to what extent the dispensability of thiamine (9 out of 10 strains of *Clostridium sporogenes* did not require thiamine) may serve as a guide for classifying a strain as *C. sporogenes* or as a non-toxigenic variant of *C. parabotulinum*. However, all the strains of *C. sporogenes* may be classified tentatively at least together with *C. parabotulinum* types A and B in a 'parabotulinum group'.

This paper forms part of a Ph.D. Thesis presented by S. H. Kindler to the Hebrew University, Jerusalem. The helpfulness of the investigators mentioned in providing us with the various cultures is hereby gratefully acknowledged.

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(Received 3 May 1956)