THE UTILIZATION OF AMINO ACIDS AND OF GLUCOSE BY CLOSTRIDIUM BOTULINUM[1]

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Knowledge of the metabolism of *Clostridium botulinum* is scanty. Wagner, Meyer and Dozier (1925) observed a considerable accumulation of ammonia, amino acids and volatile acids in 2 per cent peptone cultures of *C. botulinum*, the volatile acid being identified as a mixture of valeric, butyric and acetic acids. Decreased ammonia production and the development of an acid reaction was observed in glucose-broth. Anderson (1924) reported the gaseous products from broth to be a mixture of CO_2 and H_2 with traces of N_2 and H_2S. A CO_2/H_2 ratio of 18.3 was observed in 2-per-cent peptone solution, while ratios of 5.7 and 3.8 were observed in broth containing 1 per cent formate or glucose.

Knight (1936) states that the simplest medium which will support the growth of *C. botulinum* contains glucose and the amino acids proline, glycine, leucine, alanine, lysine and cystine together probably with traces of tryptophan and of the "sporogenes" growth factor. He suggests that *C. botulinum* may obtain its energy by means of the "Stickland reaction," a coupled oxido-reduction reaction between pairs of different amino acids. This suggestion has been verified by Clifton (1939) who found that glycine and proline act as hydrogen acceptors, while alanine and leucine act as hydrogen donators in a manner similar to that reported by Stickland (1934) for *Clostridium sporogenes*. These

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amino acids could not be utilized directly as by *Clostridium tetanomorphum* (Woods and Clifton, 1937) or by an unidentified anaerobe studied by Barker (1937). Studies on *C. botulinum* have been extended to include not only the products of amino acid utilization, but also certain of the products formed from nutrient broth and from glucose by actively growing cultures or by washed cells.

EXPERIMENTAL

One strain (E-44) of Type B *C. botulinum* and one strain (E-43) of Type A, obtained from the National Canners Laboratory, were used. Plain broth containing 0.1 per cent yeast extract (Difco), or this medium plus 1 per cent glucose, was inoculated with 0.5 ml. of a beef-brain culture and incubated anaerobically at 37°C. Eight hundred milliliters of 20-hour cultures were centrifuged, the cells washed in saline and finally suspended in distilled water or phosphate buffer. The suspensions were immediately deaerated by evacuation or by a current of O_2-free N_2 or $-H_2$. All tests were carried out at 37°C.

The usual Thunberg tube method was used to detect hydrogen donators, methylene blue being the indicator. Carbon dioxide was determined by absorption of the gas in standard $Ba(OH)_2$ solution, correction being made for the initial and final bound CO_2. In other experiments, CO_2 and H_2 were determined by the Warburg technic, both in phosphate buffer and in bicarbonate buffer, the latter in equilibrium with either N_2 or H_2 gas containing 5 per cent CO_2.

Glucose was determined by the method of Shaffer and Somogyi (1933); alcohol by distillation from alkaline solution and subsequent oxidation with bichromate for 1 hour at 85°C. in strongly acid solution, the amount of bichromate reduced being determined by titration with dilute sodium thiosulfate; volatile acids by steam distillation; and ammonia by the method of Parnas as described by Niederl and Niederl (1938). Lactic acid was determined by a modification of Friedman and Graeser's method (1933), more reproducible results being obtained by steam distillation of the acetaldehyde than by straight distillation.

THE UTILIZATION OF AMINO ACIDS

Since Burrows (1933) obtained growth of *C. botulinum* in an inorganic medium plus alanine, leucine, lysine, proline, cystine, glycine and glucose, these substances were studied separately to determine how they are utilized by this organism. Of the amino acids (with the exception of cystine, which may be regarded as "essential" in limited amounts for synthetic purposes), only alanine and leucine reduce methylene blue readily in the presence of *C. botulinum*. Glycine and proline do not serve as H-donators; however, they may act as H-acceptors from alanine or leucine as reported by Clifton (1939) for Type B *C. botulinum*. Lysine does not appear to be utilized to any extent either as a H-donator or acceptor.

The above amino acids, together with valine and glutamic acid, are not markedly attacked when present singly with washed cells *in vacuo* or in an atmosphere of N_2. However, a slow rate of H_2 consumption is observed when either glycine or proline is employed as the substrate in an atmosphere of H_2, a Q_{H_2} (H_2 taken up per mgm. dry bacteria per hour) in the neighborhood of 4 being observed. Hoogerheide and Kocholaty (1938) observed a similar, although apparently more rapid, uptake of H_2 with *C. sporogenes* in the presence of glycine, proline and certain other substrates.

Direct reactions between pairs of amino acids are most conveniently followed by measuring the ammonia liberated in the presence of glycine or proline. Typical results with glycine as the H-acceptor are reported in table 1, 1 ml. of M/10 substrate, 1 ml. M/5 glycine or water and 2 ml. of a suspension of *C. botulinum* (E-44) in phosphate buffer of pH 7.4 being incubated for 20 hours at 37°C. in evacuated Thunberg tubes.

An absolutely sharp distinction cannot be maintained between H-donating and H-accepting amino acids, amino acids such as leucine and particularly serine apparently undergoing an intermolecular oxido-reduction, one molecule being oxidized, another reduced. This apparent fermentation of certain amino acids by *C. botulinum* is being studied in more detail.

The kinetics of the oxidation of alanine by glycine and by

TABLE 1

Ammonia production from amino acids by washed cells of type B, C. botulinum

SUBSTRATE	ML. M/100 NH_3	
	Substrate only	Substrate + glycine
Control suspension	0.20	
Glycine	1.74	
d-Alanine	1.76	12.74
l-Proline	1.68	1.96
l-Leucine	2.25	15.62
dl-Serine	9.46	12.36
d-Glutamic acid	0.58	3.86
dl-Lysine	0.75	2.92
dl-Lysine + d-alanine	2.00	

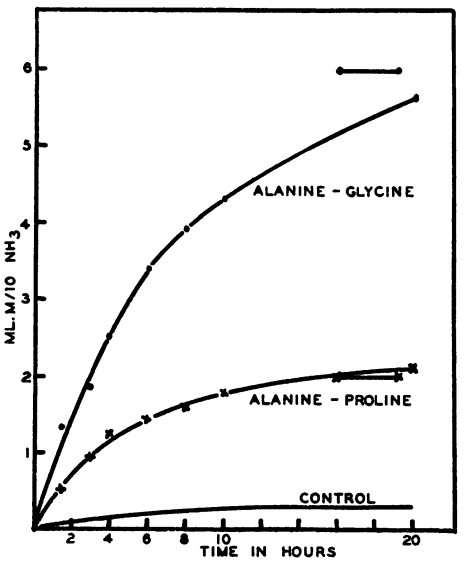

FIG. 1. Kinetics of deamination during the oxidation of 1 ml. of M/5 d-alanine by washed cells of type B *C. botulinum* in the presence of 2 ml. of M/5 glycine or l-proline at 37°C in M/15 phosphate buffer, pH 7.4. Heavy horizontal lines represent NH_3 production postulated by equations 1 and 2.

proline was studied by determining the ammonia produced at different intervals of time. Typical results are presented in figure 1.

Clifton (1939) suggested that alanine is oxidized to acetic acid

by loss of 4 atoms of hydrogen which are taken up by glycine or proline. Quantitative data indicate that the main course of the reactions may be represented as

$$CH_3 \cdot CHNH_2 \cdot COOH + 2CH_2NH_2COOH + 2H_2O = 3CH_3COOH + CO_2 + 3NH_3 \quad (1)$$

and

$$CH_3 \cdot CHNH_2 \cdot COOH + 2CH_2 \cdot (CH_2)_2 \cdot \overset{\ulcorner\!\!-\!\!NH\!\!-\!\!\urcorner}{CH} \cdot COOH + 2H_2O = CH_3COOH + CO_2 + NH_3 + 2CH_2NH_2 \cdot (CH_2)_3 \cdot COOH \quad (2)$$

The volatile acid obtained from a number of experiments was combined, concentrated and identified as acetic acid by Duclaux

TABLE 2

Ammonia production in 20 hours during the oxidation of d-alanine by washed cells of type A C. botulinum at 37°C. with glycine or l-proline as the H-acceptor

ML. SUBSTRATE, M/10		ML. BACTERIAL SUSPENSION IN M/7.5 PHOSPHATE BUFFER, pH 7.4	WATER	ML. M/100 NH_3 PRODUCED
H-donator	H-acceptor			
			ml.	
		3	3	0.72
1 Alanine		3	2	0.90
	2 Glycine	3	1	1.14
	2 Proline	3	1	0.75
1 Alanine	2 Glycine	3		10.17
1 Alanine	2 Proline	3		9.27
1 Proline	1 Glycine	3	1	0.90

distillation, by the lanthanum-iodine test and as the quinine salt. The quinine acetate, prepared according to the procedure of Phelps and Palmer (1917), melted at 123–4°C. as compared with their figure of 124–6°. The postulated reduction product of proline, δ-amino-n-valeric acid, has been identified as its α-naphthyl isocyanate derivative according to the procedure employed by Stickland (1934), the α-naphthylcarbamido-n-valeric acid so prepared decomposing at 187–190°C. and melting at 228°.

Results similar to those reported above were obtained when Type A *C. botulinum* (E-43) was employed as the test organism. Typical results on the oxidation of alanine as determined by

ammonia production in the presence of glycine or proline, are presented in table 2.

FERMENTATION OF GLUCOSE

As *C. botulinum* does not grow readily in Burrow's synthetic medium or in low concentrations of broth or of yeast extract plus glucose, ordinary broth and glucose-broth cultures were analyzed for ammonia, volatile acids and lactic acid, these being the main products reported by Wagner, Meyer and Dozier (1925). No

TABLE 3

Products of metabolism per 100 ml. of cultures of types A and B, C. botulinum

AGE OF CULTURE	TYPE OF CULTURE	pH OF MEDIUM	ML. M/10 NH_3	ML. M/10 VOLATILE ACIDS	LACTIC ACID
Broth + 0.1 per cent yeast extract					
days					*mgm.*
1	B	7.0	14.7	3.6	
2	B	7.1	24.2	11.8	
7	B	6.7	40.5	27.2	21
7	A	6.9	41.9	28.0	27
Broth + 0.1 per cent yeast extract + 1.0 per cent glucose					
1	B	6.2	16.9	10.0	
2	B	5.8	19.8	13.4	
7	B	5.5	21.5	24.0	69
7	A	5.6	20.8	22.7	90

analyses were made for amino acids in the present study. Typical results are presented in table 3.

The volatile acids produced in broth and in glucose-broth were tentatively identified by Duclaux distillation as a mixture of acetic and butyric acids in an approximate ratio of 2:1. Valeric acid could not be detected in cultures of either Type A or B *C. botulinum*. Since glucose disappeared from the medium the cultures were also analyzed for succinic acid, acetyl methyl carbinol, 2:3 butylene glycol, acetone and alcohols. A positive test for alcohol was obtained with the glucose-broth. The alcohol was identified as ethyl alcohol by the iodoform test and by the fact that only acetic acid was produced on oxidation with bichro-

mate in strongly acid solution. In a number of cultures of different ages the mols of ethyl alcohol produced per mol of glucose fermented varied between 1.1 and 1.3.

Between 30 and 50 per cent of the glucose in 1 per cent glucose-broth was fermented. Since the amounts of free ammonia produced tended to be less in the presence of glucose (although growth appeared to be more abundant) and the pH shifted to approximately 5.5, the medium was buffered with 1 per cent $CaCO_3$. After 7 days the pH of the broth cultures was 6.7 and that of the broth-glucose 6.3. Seventy per cent of the glucose was fermented. Analyses of the cultures gave the results recorded in table 4.

TABLE 4

Products of metabolism (ml. M/10) per 100 ml. of cultures of type B, C. botulinum buffered with 1 per cent $CaCO_3$

	BROTH	GLUCOSE-BROTH
NH_3	44.8	19.9
Volatile acids	37.7	37.2
Total acids*	44.5	53.6
Lactic acid	5.3	9.4
Ethyl alcohol	16.0 mgm.	233.0 mgm.

* Total acids determined in an ether extract.

Ethyl alcohol and carbon dioxide are the main products of glucose fermentation by washed cells in an atmosphere of either H_2 or N_2. Typical results are reported in table 5, negative results being obtained for other alcohols, acetone, acetyl methyl carbinol and 2:3 butylene glycol. Neither could glycerol be an end-product of fermentation as preliminary studies show that it is fermented, the chief end-products being CO_2 and ethyl alcohol.

That the alcohol is ethyl alcohol was again demonstrated by the iodoform test and by the fact that the acid formed on oxidation gave a positive lanthanum-iodine reaction for acetate and its behavior on Duclaux distillation corresponded to that of acetic acid. Similar results were obtained with type A *C. botulinum.*

This fermentation was studied in more detail on a micro scale, CO_2 and H_2 production in both phosphate and bicarbonate buf-

fers being determined by the Warburg technic. A rapid stream of O_2-free N_2 or $-H_2$ or O_2-free 5 per cent CO_2 in N_2 or H_2 was

TABLE 5

Products of glucose fermentation by washed cells of type B, C. botulinum in M/15 phosphate buffer, pH 7.2

		MILLIMOLS	MOLS/MOL GLUCOSE FERMENTED
Initial glucose	540 mgm.		
Final glucose	157 mgm.		
Glucose fermented	383 mgm.	2.13	
CO_2	102 mgm.	2.32	1.09
Ethyl alcohol	111 mgm.	2.41	1.13
Volatile acid	12.8 ml. M/10	1.28	0.60
Lactic acid	66 mgm.	0.73	0.34

TABLE 6

The fermentation of glucose by washed cells of type B, C. botulinum

REACTANTS (ML.)	VESSEL NUMBER				
	1	2	3	4	5
Bacterial suspension	0.8	0.8	0.8	0.8	0.8
Glucose M/5	0.2	0.2	0.2	0.2	0.2
Phosphate buffer M/7.5, pH 7.0	1.0	1.0	1.0		
Na bicarbonate				1.0	1.0
20 per cent KOH	0.2				
10 per cent H_2SO_4		0.2*	0.2†	0.2*	0.2†
Gas	N_2	N_2	N_2	N_2CO_2	N_2CO_2
μl H_2 produced	55				
μl Initial bound CO_2		30		370	
μl Final bound CO_2			350		222
μl CO_2 liberated			928		1758
μl CO_2 from fermentation			1248		1610
μl CO_2 from acid produced					148
Mgm. ethyl alcohol	2.05	0	2.06	0	2.19

* Acid tipped at zero time.

† Acid tipped at end of fermentation.

passed through the Warburg vessels for 10 minutes and the system was equilibrated for a further 15 minutes before tipping

in the substrate. The protocol and results of a typical experiment are given in table 6.

In the majority of experiments the H_2 production was no greater than that reported in table 6 and in many instances even less. Most of the H_2 was liberated in the first 20 to 30 minutes

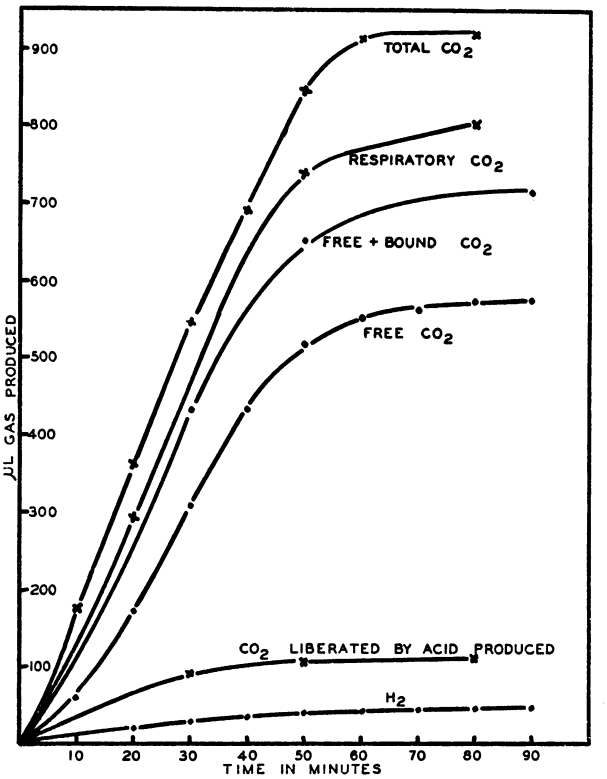

FIG. 2. CO_2 and H_2 production during the fermentation of 2 mls. M/100 glucose at pH 7.0 and 37°C. by washed cells of type B *C. botulinum*. ·——· phosphate buffer, +——+ bicarbonate buffer.

while marked CO_2 production generally continued for 120 to 150 minutes. H_2 may therefore be neglected as a principal product of the fermentation. Control experiments without substrate present gave negligible values for CO_2 and H_2 and were therefore neglected in the calculations. The observed values for Q_{CO_2} ranged between 20 and 30. The rate of fermentation

and the amounts of CO_2 produced were essentially the same over the pH range studied, 5.8 to 7.8. Typical results on CO_2 and H_2 production are presented in figure 2, while the results of a number of experiments are summarized in table 7.

In all cases greater amounts of CO_2 were produced in bicarbonate buffer than in phosphate buffer of the same pH. The results obtained by the Warburg technic are probably more reliable than those obtained on a larger scale since the fermentation of a few milligrams of sugar approaches completion in 2 hours, while an incubation period of 20 hours was employed with the macro technic, concentration of bacteria available being a limit-

TABLE 7

Mols products per mol of glucose fermented by washed cells of C. botulinum

	N_2	5% CO_2 in N_2	H_2	5% CO_2 in H_2
Type B				
CO_2	1.39–1.52	1.79–1.87	1.24–1.51	1.69–1.86
Ethyl alcohol	1.12–1.38	1.19–1.38	1.05–1.29	1.27–1.43
Acid produced		0.17–0.26		0.21–0.23
Type A				
CO_2	1.60	1.77	1.66	1.87
Ethyl alcohol	1.10	1.49	1.41	1.32
Acid produced		0.12		0.12

ing factor. In the Warburg experiments, the mols of CO_2 and alcohol produced per mol of sugar fermented are somewhat higher than in the macro-experiments, while the mols of acid produced are lower, possibly due to side reactions in the latter experiments.

DISCUSSION

Since *C. botulinum* does not grow readily in Burrow's (1933) synthetic medium an attempt was made to determine the nature of the energy-providing reactions by studying the utilization of the constituent amino acids and glucose separately. Glycine, alanine, proline and lysine are not utilized to an appreciable extent when present singly in suspensions of *C. botulinum* exposed

to an atmosphere of N_2, while leucine appears to be attacked directly although at a slow rate. When the studies are carried out in an atmosphere of H_2, a slow rate of H_2 uptake may be observed in the presence of glycine or proline, but not with alanine, leucine or lysine.

In general these amino acids are utilized by washed cells of Types A or B *C. botulinum* by means of the "Stickland reaction," although as pointed out by Hoogerheide and Kocholaty (1938) an absolutely sharp distinction cannot be maintained between H-donating and H-accepting amino acids, substrates such as leucine apparently undergoing an intermolecular oxido-reaction which is being studied in more detail. It has been demonstrated that both types of *C. botulinum* oxidize alanine in the presence of a suitable H-acceptor to acetic acid, CO_2 and NH_3. Quantitative data, and the identification of acetic acid, indicate that the oxidation may be represented as

$$CH_3CHNH_2COOH - 4H + 2H_2O = CH_3COOH + NH_3 + CO_2 \quad (3)$$

the oxidation probably taking place in two steps with pyruvic acid as the intermediate compound. It has also been demonstrated that glycine is reductively deaminated to acetic acid while proline is reduced without deamination to δ-amino-n-valeric acid. Thus, it appears that Types A and B *C. botulinum* obtain energy from amino acids in a manner similar to that reported for *C. sporogenes* by Stickland (1934, 1935), Woods (1936) and Hoogerheide and Kocholaty (1938).

The main products in broth cultures of *C. botulinum* are acetic and butyric acids together with ammonia, carbon dioxide and smaller amounts of hydrogen and lactic acids. δ-amino-n-valeric acid is probably another end-product, but no attempt was made to isolate this substance. Wagner, Meyer and Dozier (1925) reported the volatile acids to be a mixture of valeric, butyric and acetic acids. No evidence of valeric acid production was obtained in this study but the possibility exists that certain strains may be able to deaminate δ-amino-n-valeric acid. The acetic acid probably arises from the oxidation of amino acids such as alanine or the reduction of glycine, while the source of the butyric acid is as yet uncertain.

The addition of glucose to broth leads to the formation of increased amounts of carbon dioxide, hydrogen and lactic acid together with the production of ethyl alcohol. It has been shown that the fermentation of glucose by types A and B *C. botulinum* differs from the majority of bacterial fermentations in that ethyl alcohol and carbon dioxide are the main products of the fermentation, smaller amounts of acetic and lactic acids and hydrogen also being formed. The source of the hydrogen remains obscure, several experiments indicating that formate is not attacked by washed suspensions of this organism. Approximately 25 per cent of the glucose disappearing from fermentations carried out by washed cells has not been accounted for, and may be employed in synthesis or present as unidentified neutral products. Therefore, no scheme of fermentation is presented although whatever the other products may be, the fermentation is primarily alcoholic in character.

SUMMARY

Amino acids are utilized by Types A and B *Clostridium botulinum* primarily by means of the "Stickland reaction" while glucose is fermented directly, ethyl alcohol and carbon dioxide being the chief products of its dissimilation.

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