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Volatile Acid Production from Threonine, Valine, Leucine and Isoleucine by Clostridia

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Abstract. The amounts of the volatile acids produced from threonine, valine, leucine and isoleucine by growing cultures of clostridia have been measured. The species used were *Clostridium sporogenes; C. caloritolerans; C. botulinum* proteolytic type A; *C. botulinum* proteolytic type B; *C. botulinum* proteolytic type F; *C. botulinum* proteolytic type G; *C. putrificum; C. difficile; C. ghoni; C. bifermentans; C. sordellii; C. mangenoti; C. cadaveris; C. lituseburense; C. propionicum; C. sticklandii; C. scatologenes; C. subterminale; C. putrefaciens; C. histolyticum; C. tetanomorphum; C. limosum; C. lentoputrescens; C. tetani; C. malenomenatum; C. cochlearium; C. sporospheroides.* Most of the species tested gave increased yields of propionic acid when grown in the threonine medium; in addition, some species resembled *C. propionicum* and produced *n*-butyric acid when grown in this medium. *C. histolyticum* produced only acetic acid in the basal medium; all seven strains of this species produced more acetic acid when grown in the threonine medium than in the basal medium. Species which oxidize valine to *iso*-butyric acid also oxidize leucine to 3-methyl butyric acid and isoleucine to 2-methylbutyric acid. The *iso*-caproic fraction produced by some species is shown to be derived from leucine. The identitity of the branched-chain acids produced by *C. sporogenes* has been confirmed by gas liquid chromatography/mass spectrometry.

Key words: Clostridia – Threonine – Valine – Leucine – Isoleucine – Propionic acid – *iso*-butyric acid – *n*-butyric acid – 2-methyl butyric acid – 3-methyl butyric acid – 4-methyl valeric acid.

Abbreviations. GLC = gas liquid chromatography; RCM = reinforced clostridial medium; VFA = volatile fatty acid

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Stickland (1934) demonstrated that washed suspensions of *Clostridium sporogenes* oxidized aliphatic amino acids with either glycine, proline or hydroxyproline as electron acceptor. Subsequently, Cohen-Bazire et al. (1948) obtained evidence that a number of clostridia, including *C. sporogenes*, oxidized valine to *iso*-butyric acid, leucine to 3-methyl butyric acid and isoleucine to 2-methyl butyric acid. At the same time Saissac et al. (1948) showed that addition of glucose to the medium reduced the amounts of the branched-chain VFAs produced by these organisms. None of the techniques available in the 1940's separated the isomeric VFAs and the unequivocal identification of the individual VFAs in and the quantitative analysis of mixtures was very difficult. The development of the GLC by James and Martin (1951) greatly improved matters but, even today, the quantitative separation of 3-methyl butyric acid from 2-methyl butyric acid is not readily achieved.

Moore et al. (1966), Lewis et al. (1967) and Werner (1972) have made extensive use of GLC techniques for the identification of the VFAs produced by the clostridia. *Clostridium sporogenes* and related organisms were shown to produce acetic acid, propionic acid, *n*-butyric acid and *iso*-butyric acid and components with retention volumes corresponding to *iso*-valeric acid and *iso*-caproic acid. The columns used did not separate 3-methyl butyric acid from 2-methyl butyric acid, the products of oxidation of leucine and isoleucine respectively, and thus it is impossible to decide from the chromatographic data whether the various organisms oxidize leucine or isoleucine or both. Because the retention volume does not give unequivocal identification, the identity of the *iso*-caproic acid component has not been established by chemically acceptable evidence.

This paper describes the volatile fatty acids produced by species of clostridia growing in a medium containing a protein hydrolysate and yeast extract as a

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source of growth factors. The effect of supplementing this medium with either threonine, valine, leucine or isoleucine on volatile acid production is also described.

Materials and Methods

Organisms. The reference strains of clostridia listed by Smith and Hobbs (1974) were used together with strains from the Institute's collection. The strains used were *Clostridium bifermentans* NCIB 10716; *C. botulinum* proteolytic type A, NCIB 10640; *C. botulinum* proteolytic type B, NCIB 10657; *C. botulinum* proteolytic type F, NCIB 10658; *C. botulinum* proteolytic type G, NCIB 10714; *C. cadaveris*, NCIB 10676; *C. cochlearium*, ATCC 17787; *C. difficile*, NCIB 10666; *C. ghoni*, NCIB 10636; *C. histolyticum*, NCIB 503; *C. lentoputrescens*, NCIB 10629; *C. limosum*, NCIB 10638; *C. lituseburense*, NCIB 10637; *C. malenomenatum*, ATCC 25776; *C. mangenoti*, NCIB 10639; *C. propionicum*, NCIB 10656; *C. putrefaciens*, NCIB 9836; *C. putrificum*, NCIB 10677; *C. sordellii*, NCIB 10717; *C. sporogenes*, NCIB 10696; *C. sporosphaeroides*, NCIB 10672; *C. sticklandii*, NCIB 10654; *C. subterminale*, NCIB 9384; *C. tetani*, NCIB 10628; in addition to these reference strains the following strains were also used: *C. caloritolerans*, NCIB 9360; *C. scatologenes*, NCIB 8855; *C. tetanomorphum*, NCTC 500; *C. histolyticum*, CN 3300, CN 3267, CN 950, IP 10, IP 4105, N 78/36A.

Media and Cultural Conditions. Stock cultures were maintained in cooked meat medium. The basal growth medium used to examine the production of VFAs contained: BBL trypticase 2% (w/v); Difco yeast extract, 0.2% (w/v); sodium thioglycollate 0.05% (w/v). L-threonine, L-valine, L-leucine and L-isoleucine were added to the basal medium to give a final concentration of 20 µmoles/ml. All media were adjusted to pH 7.2–7.4 and dispensed in 20 ml amounts in 28 ml screw-capped bottles and autoclaved for 15 min at 121 °C. Immediately before use the bottles were heated for 15 min in a boiling water bath and then rapidly cooled and inoculated with 0.5 ml of 16 h culture of the organism grown in RCM (Hirsch and Grinsted, 1954). All cultures were incubated at 37 °C.

Fatty Acid Analysis. The VFAs were analysed by GLC. Samples (2.0 ml) were acidified and steam distilled in the apparatus of Markham (1942). The distillates (100 ml) were made slightly alkaline with 0.1 *N*-NaOH and taken to dryness under a stream of air in a

Table 1. Effect of threonine on volatile acid production by *Clostridium* spp. The basal medium was supplemented with 20 µmoles threonine/ml as indicated

Species	Strain	Medium	Products formed µmole/ml							
			acetic[a]	propionic	*iso*-butyric	*n*-butyric	*iso*-valeric	*n*-valeric	*iso*-caproic	*n*-caproic
C. histolyticum	NCIB 503	basal	48	0	0	0	0	0	0	0
		threonine	67	0	0	0	0	0	0	0
C. tetanomorphum	NCTC 500	*basal*	*9.8*	*2.0*	*0*	*7.8*	*0*	*0*	*0*	*0*
		threonine	2.4	9.0	0	8.7	0	0	0	0
C. limosum	NCIB 10638	basal	43	3.6	0	9.8	0	0	0	0
		threonine	44	8.3	0	11	0	0	0	0
C. lentoputrescens	NCIB 10629	basal	13.3	2.5	0	7.7	0	0	0	0
		threonine	0.9	18	0	8.7	0	0	0	0
C. tetani	NCIB 10628	basal	8.4	2.4	0	6.4	0	0	0	0
		threonine	2.0	12	0	7.5	0	0	0	0
C. malenomenatum	ATCC 25776	basal	14	2.4	0	3.4	0	0	0	0
		threonine	16	5.1	0	4.4	0	0	0	0
C. cochlearium	ATCC 17787	basal	6.3	2.4	0	6.5	0	0	0	0
		threonine	1.4	13	0	7.0	0	0	0	0
C. sporospheroides	NCIB 10672	basal	9.3	1.4	0	1.8	0	0	0	0
		threonine	11	1.4	0	1.5	0	0	0	0
C. sporogenes	NCIB 10696	basal	11	4.2	8.6	2.0	13	0	5.9	0
		threonine	9.6	15	9.2	7.3	12	0	6.0	0
C. caloritolerans	NCIB 9360	basal	17	4.0	9.3	1.2	16	0	5.5	0
		threonine	7.8	10	7.3	2.5	11	0	3.9	0
C. botulinum A	NCIB 10640	basal	2.4	2.7	6.1	3.8	12	0	4.6	0
		threonine	− 0.7	13	6.7	6.8	12	0	3.7	0
C. botulinum B	NCIB 10657	basal	10	1.8	3.4	1.7	9.8	0	1.7	0
		threonine	2.3	8.2	5.2	3.4	12	0	1.7	0
C. botulinum F	NCIB 10658	basal	− 1.3	2.1	5.4	3.7	15	0	0	0
		threonine	− 4.7	10	5.8	5.0	14	0	0	0
C. botulinum G	NCIB 10714	basal	38	0.3	1.8	8.6	7.1	0	0	0
		threonine	34	0.4	1.8	8.7	7.0	0	0	0

Table 1 (continued)

Species	Strain	Medium	Products formed μmole/ml							
			acetic[a]	propionic	*iso*-butyric	*n*-butyric	*iso*-valeric	*n*-valeric	*iso*-caproic	*n*-caproic
C. putrificum	NCIB 10677	basal	14	2.5	7.5	0.6	16	0	2.3	0
		threonine	23	16	8.1	2.3	14	0	3.9	0
C. difficile	NCIB 10666	basal	6.6	1.5	3.6	4.0	3.8	0.2	6.8	0
		threonine	1.0	12	3.4	9.2	3.8	0.6	5.1	0
C. ghoni	NCIB 10636	basal	21	4.3	2.5	13	5.5	0	2.6	0
		threonine	2.6	11	2.7	12	5.1	0	2.7	0
C. bifermentans	NCIB 10716	basal	7.0	8.1	4.9	0	9.1	0	6.2	0
		threonine	6.6	17	4.3	0	7.2	0	5.8	0
C. sordellii	NCIB 10717	basal	16.5	5.5	8.1	0	11	0	8.0	0
		threonine	8.8	13	3.1	0	7.2	0	4.1	0
C. mangenoti	NCIB 10639	basal	5.0	2.0	4.1	0.7	5.6	0	5.8	0
		threonine	1.6	13	3.6	1.0	6.5	0	4.7	0
C. cadaveris	NCIB 10676	basal	39	0.9	0.8	10	1.6	0	0	0
		threonine	53	0.6	0.7	12	1.6	0	0	0
C. lituseburense	NCIB 10637	basal	11	3.7	0.8	4.9	3.0	0	0	0
		threonine	2.6	16	1.2	5.4	3.6	0	0	0
C. propionicum	NCIB 10656	basal	3.0	6.6	2.0	1.6	8.7	0	0	0
		threonine	3.6	12	2.0	6.4	8.0	0	0	0
C. sticklandii	NCIB 10654	basal	6.6	3.5	1.7	10	5.3	0	0	0
		threonine	12	3.5	1.4	9.4	4.8	0	0	0
C. scatologenes	NCIB 8855	basal	15	3.0	4.1	4.7	11	0.2	0	0.2
		threonine	20	20	4.2	4.5	11	3.4	0	0.2
C. subterminale	NCIB 9384	basal	44	0	3.8	12	13	0	0	0
		threonine	56	0	3.8	10	13	0	0	0
C. putrefaciens	NCIB 9836	basal	18	0	1.4	3.9	7.3	0	0	0
		threonine	17	0	1.4	4.6	6.1	0	0	0

[a] The uninoculated medium used contained 21 μmoles/ml acetic acid except in the case of the experiments with *Clostridium botulinum* types A, B, F and G where the acetic acid content was 25 μmoles/ml; no significant quantities of other volatile acids were found. Values for acetic acid produced have been corrected by subtraction of the corresponding blank values

boiling water bath; the process was completed in a test tube in which the sample was stored. Immediately before analysis the mixture of sodium salts was dissolved in 0.4 ml 0.5 M-H_3PO_4 and samples of 1 μl injected into the GLC, a Pye Series 104 fitted with a flame ionisation detector. The glass column, 152 cm × 6.33 mm, was packed with 20% Carbowax 20 M on Diatomite C-AW 100–120 mesh (J. J.'s Ltd., King's Lynn, Norfolk, U.K.) and operated isothermally at 150 °C with argon as the gas phase, flow rate 30 ml min^{-1}. The method has limitations. Thus, formic acid is not detected by the flame ionization detector and the column does not separate 2-methyl butyric acid from 3-methyl butyric acid which appear as a single peak and which we refer to as the *iso*-valeric fraction. Acetic acid, propionic acid, *iso*-butyric acid, *n*-butyric acid, the *iso*-valeric fraction, *n*-valeric acid, the *iso*-caproic fraction and *n*-caproic acid emerge in that order and are well separated from one another. The amounts present in a mixture were calculated from the peak areas in the usual way.

Results and Discussion

Table 1 shows the amounts of the VFAs produced by species of clostridia grown in the basal medium and in this medium supplemented with threonine (20 μmoles ml^{-1}). The essential features of the basal medium are that amino acids are the major energy sources and that the only carbohydrate is that provided by 0.2% (w/v) yeast extract. It was important to minimize the amount of carbohydrate in the medium because of its distorting effect on amino acid metabolism reported by Saissac et al. (1948) and which we have confirmed. It will be seen from Table 1 that there are three distinct patterns of fatty acid production in the basal medium; first, where acetic acid is the only VFA; second, where acetic acid, *n*-butyric acid and some propionic acid are produced; third, where a more complex mixture of VFAs is produced including the branched-chain acids. These results confirm and extend those published by Moore et al. (1966), Lewis et al. (1967) and Werner (1972).

Guillaume et al. (1956) showed that *Clostridium histolyticum* produced only acetic acid when grown in

protein hydrolysates and that washed suspensions of the organism fermented glycine in a manner similar to *Diplococcus glycinophyllus* (Cardon and Barker, 1947), reaction (1).

$$4CH_2NH_2COOH + 2H_2O \rightarrow 3CH_3COOH + 4NH_3 + 2CO_2 \quad (1)$$

Mead (1971) showed that growing cultures use arginine, serine and threonine as well as glycine and presumably these too are converted to acetic acid. Table 1 shows that when *C. histolyticum* is grown in the threonine medium it produces more acetic acid than it does when grown in the basal medium. Six other strains of *C. histolyticum* were subsequently examined and all produced extra acetic acid when grown in the threonine medium. A mechanism for the conversion of threonine to acetic acid, proposed by Barker (1961), involved the threonine aldolase followed by an oxido-reduction reaction between the products, acetaldehyde and glycine – reactions (2) and (3).

$$CH_3CHOH\,CHNH_2COOH \rightarrow CH_3CHO + CH_2NH_2COOH \quad (2)$$

$$CH_3CHO + CH_2NH_2COOH + H_2O \rightarrow 2CH_3COOH + NH_3 \quad (3)$$

Whilst there is no evidence that *C. histolyticum* contains threonine aldolase, Dainty and Peel (1970) have established that another species, *C. pasteurianum*, contains this enzyme which Dainty (1970) purified and studied in some detail.

Seven species, *Clostridium tetanomorphum*, *C. limosum*, *C. lentoputrescens*, *C. tetani*, *C. malenomenatum*, *C. cochlearium* and *C. sporospheroides*, when grown upon the basal medium produced acetic and *n*-butyric acids with some propionic acid. Of these seven species all save *C. sporospheroides* made extra propionic acid when grown in the threonine medium. *C. sporospheroides* is also exceptional in so far as it ferments glutamic acid by the hydroxyglutarate pathway, whereas the others use the methyl-aspartate pathway (Buckel and Barker, 1974; Buckel, private communication to SRE) and it does not convert tyrosine to phenol whereas the other six species do (Elsden et al., 1976). The mechanism of propionate formation from threonine probably involves the sequential action of the threonine dehydrase and the oxo acid dehydrogenase – reactions (4) and (5).

$$CH_3CHOH\,CHNH_2COOH \rightarrow CH_3CH_2C{:}OCOOH + NH_3 \quad (4)$$

$$CH_3CH_2C{:}OCOOH + H_2O \rightarrow CH_3CH_2COOH + 2H + CO_2 \quad (5)$$

The remaining organisms, nineteen strains of sixteen species, produced branched-chain VFAs when grown in the basal medium (see Table 1). Two species, *Clostridium subterminale* and *C. putrefaciens*, do not produce propionic acid and the former, but not the latter, produces extra acetic when grown in the threonine medium, vide *C. histolyticum*. Three species, *C. botulinum* G, *C. cadaveris* and *C. sticklandii*, produced some propionic acid when grown in the basal medium. The amounts produced, small in the cases of *C. botulinum* G and *C. cadaveris*, were not increased by growth in the threonine medium.

All species save *Clostridium bifermentans* and *C. sordellii* produce *n*-butyric acid in the basal medium but, in contrast to the glutamic acid-fermenters, neither the sources of the *n*-butyric acid nor the mechanisms by which it is formed have been established with certainty. Table 1 shows that *C. propionicum*, *C. sporogenes*, *C. caloritolerans*, *C. botulinum* A, *C. botulinum* B (proteolytic), *C. botulinum* F (proteolytic), *C. putrificum* and *C. difficile* produce more *n*-butyric acid in the threonine medium than they do when grown in the basal medium – the increases are at least 50%. Cardon and Barker (1947) showed that *C. propionicum* fermented threonine according to reaction (6).

$$3CH_3CHOH\,CHNH_2COOH + H_2O \rightarrow CH_3CH_2CH_2COOH + 2\,CH_3CH_2COOH + 2\,CO_2 + 3\,NH_3 \quad (6)$$

Barker and Wilken (1948) produced evidence that butyrate was not formed from threonine by the conventional mechanism, i.e. via acetyl CoA, and their results suggested that the carbon skeleton of threonine remained intact during the process. It could well be that the species of clostridia considered here also convert threonine to *n*-butyric acid in a similar manner but we have no evidence of this.

Stickland (1934) and Cohen-Bazir et al. (1948) showed that *Clostridium sporogenes* and similar organisms oxidized some monocarboxylic amino acids according to reaction (7).

$$RCH\,NH_2COOH + 2H_2O \rightarrow RCOOH + CO_2 + NH_3 + 4H \quad (7)$$

but, as was pointed out by Elsden et al. (1976), the mechanism of these oxidations has not been established with certainty. In the case of alanine, valine, leucine and isoleucine, the evidence suggests that the end products are acetic acid, *iso*-butyric acid, 3-methyl butyric acid and 2-methyl butyric acid respectively.

Table 2 shows the effect of valine, leucine and isoleucine on the production of branched-chain VFAs; it will be seen that *C. cadaveris* formed much smaller amounts of these fatty acids and gave a very small response to the added amino acids and also to threonine

Table 2. Effect of valine, leucine and isoleucine on volatile acid production by *Clostridium* spp. The basal medium was supplemented with 20 μmoles/ml valine, or leucine or isoleucine as indicated

Species	Strain	Medium	Products formed μmoles/ml							
			acetic[a]	propionic	*iso*-butyric	*n*-butyric	*iso*-valeric	*n*-valeric	*iso*-caproic	*n*-caproic
C. sporogenes	NCIB	basal	18	4.6	12	2.2	17	0	5.4	0
	10696	valine	17	2.6	30	1.8	12	0	7.0	0
		leucine	23	3.3	7.8	2.1	31	0	20	0
		isoleucine	23	3.6	6.4	1.8	30	0	8.2	0
C. caloritolerans	NCIB	basal	22	3.2	6.1	3.8	12	0	4.6	0
	9360	valine	21	2.2	13	2.6	7.7	0	5.1	0
		leucine	20	2.4	3.8	3.4	20	0	9.1	0
		isoleucine	19	2.2	2.9	2.6	19	0	4.9	0
C. botulinum A	NCIB	basal	2.4	2.7	6.1	3.8	12	0	4.6	0
	10640	valine	− 0.9	1.4	13	2.6	7.7	0	5.1	0
		leucine	0	2.1	3.8	3.4	20	0	9.1	0
		isoleucine	− 1.7	1.5	2.9	2.6	19	0	4.9	0
C. botulinum B	NCIB	basal	10	1.8	3.4	1.7	9.8	0	1.7	0
	10657	valine	4	0.8	9.6	1.1	7.8	0	1.1	0
		leucine	5.1	1.0	1.9	1.3	15	0	2.5	0
		isoleucine	2.6	0.9	2.1	1.1	17	0	1.2	0
C. botulinum F	NCIB	basal	− 1.3	2.1	5.4	3.7	15	0	0	0
	10658	valine	− 2.0	1.4	13	2.6	11	0	0	0
		leucine	− 2.1	1.4	2.9	2.6	21	0	0	0
		isoleucine	− 2.0	1.2	3.0	2.1	22	0	0	0
C. botulinum G	NCIB	basal	38	0.3	1.8	8.6	7.1	0	0	0
	10714	valine	39	0.4	5.3	8.7	5.1	0	0	0
		leucine	30	0	1.4	8.2	11	0	0	0
		isoleucine	34	0.2	1.1	8.3	9.3	0	0	0
C. putrificum	NCIB	basal	8.7	2.4	6.4	0.6	12	0	2.3	0
	10677	valine	4.5	1.5	13	0.6	10	0	1.5	0
		leucine	5.9	1.4	3.0	0.5	18	0	2.3	0
		isoleucine	4.3	1.4	3.6	0.4	21	0	2.5	0
C. difficile	NCIB	basal	12	1.6	5.0	7.0	4.8	1.0	8.2	0
	10666	valine	4.2	0.6	30	5.4	3.8	1.0	9.6	0
		leucine	11	2.0	5.0	4.6	12	0.6	28	0
		isoleucine	3.3	0.6	3.6	5.4	28	0.6	10	0
C. ghoni	NCIB	basal	30	4.2	3.0	20	7.8	0	3.2	0
	10636	valine	26	3.0	8.4	18	5.0	0	2.6	0
		leucine	31	4.2	2.6	19	11	0	6.8	0
		isoleucine	28	3.2	1.6	18	14	0	3.0	0
C. bifermentans	NCIB	basal	30	12	5.4	0	9.0	0	7.6	0
	10716	valine	29	11	13	0	7.0	0	9.2	0
		leucine	24	13	3.8	0	15	0	17	0
		isoleucine	21	9.0	2.4	0	18	0	7.8	0
C. sordellii	NCIB	basal	30	4.6	10	0	12	0	9.6	0
	10717	valine	26	3.2	27	0	9.0	0	9.6	0
		leucine	28	3.6	9.2	0	26	0	21	0
		isoleucine	20	3.0	6.4	0	37	0	9.2	0
C. mangenoti	NCIB	basal	18	2.0	5.0	0.4	6.8	0	6.2	0
	10639	valine	17	1.4	17	0.4	4.6	0	7.6	0
		leucine	22	4.0	7.0	0.4	17	0	27	0
		isoleucine	16	1.4	2.0	0.4	19	0	6.6	0
C. cadaveris	NCIB	basal	27	0.8	0.9	7.8	1.2	0	0	0
	10676	valine	32	0.8	1.4	8.9	0.9	0	0	0
		leucine	30	0.8	0.3	9.2	0.7	0	0	0
		isoleucine	27	0.5	0.3	8.8	2.0	0	0	0

Table 2 (continued)

Species	Strain	Medium	Products formed μmoles/ml							
			acetic[a]	propionic	*iso*-butyric	*n*-butyric	*iso*-valeric	*n*-valeric	*iso*-caproic	*n*-caproic
C. lituseburense	NCIB 10637	basal	26	4.0	0.8	8.4	4.4	0	0	0
		valine	27	4.0	3.6	8.0	2.8	0	0	0
		leucine	27	4.4	0.4	8.4	6.6	0	0	0
		isoleucine	26	4.0	0.4	8.2	7.4	0	0	0
C. propionicum	NCIB 10656	basal	34	14	3.6	2.2	11	0	0	0
		valine	26	18	20	3.0	8.8	0	0	0
		leucine	25	19	2.2	3.2	30	0	0	0
		isoleucine	32	18	1.8	3.2	22	0	0	0
C. sticklandii	NCIB 10654	basal	22	3.5	1.5	11	5.6	0	0	0
		valine	19	3.0	6.0	10	3.8	0	0	0
		leucine	16	2.7	1.0	10	9.7	0	0	0
		isoleucine	17	2.2	1.2	11	9.4	0	0	0
C. scatologenes	NCIB 8855	basal	35	4.8	6.0	8.2	16	1.6	0	0.8
		valine	39	4.2	35	9.4	14	0.4	0	0.8
		leucine	28	3.6	5.4	8.0	31	0.8	0	0.8
		isoleucine	29	3.6	5.6	8.6	41	0.8	0	0.8
C. subterminale	NCIB 9384	basal	35	0	4.0	13	13	0	0	0
		valine	30	0	14	11	12	0	0	0
		leucine	23	0	2.4	9.4	21	0	0	0
		isoleucine	21	0	2.4	8.2	28	0	0	0
C. putrefaciens	NCIB 9836	basal	22	0	0.5	1.4	2.6	0	0	0
		valine	24	0	2.0	1.8	1.5	0	0	0
		leucine	22	0	0.2	1.6	4.9	0	0	0
		isoleucine	23	0	0.2	1.7	4.0	0	0	0

[a] The uninoculated medium contained 25 μmoles acetic acid/ml. All values have been corrected for this

Table 3. Mass spectra of volatile acids from cultures of *C. sporogenes*

Reference compounds		*m/e*, five most intense ions				
2-methyl propionic acid	(isobutyric acid)	43 (100)[a]	41 (46)	73 (20)	28 (19)	45 (16)
3-methyl butyric acid		60 (100)	43 (61)	41 (54)	27 (33)	45 (31)
2-methyl butyric acid		74 (100)	57 (64)	29 (62)	41 (53)	27 (32)
3-methyl valeric acid		60 (100)	41 (30)	87 (22)	29 (19)	73 (18)
4-methyl valeric acid		57 (100)	74 (67)	73 (56)	41 (50)	55 (48)
Products						
iso-butyric fraction[b]	(basal)	43 (100)	41 (46)	73 (32)	27 (28)	45 (14)
iso-butyric fraction	(+ valine)	43 (100)	41 (37)	73 (20)	45 (16)	42 (9)
iso-valeric fraction	(basal)	60 (100)	41 (51)	74 (42)	57 (34)	43 (25)
iso-valeric fraction	(+ leucine)	60 (100)	43 (47)	87 (43)	41 (36)	74 (23)
iso-valeric fraction	(+ isoleucine)	74 (100)	57 (72)	29 (62)	41 (53)	60 (32)
iso-caproic fraction	(basal)	57 (100)	74 (63)	73 (50)	29 (41)	55 (39)
iso-caproic fraction	(+ leucine)	57 (100)	74 (60)	73 (55)	41 (37)	55 (36)
iso-caproic fraction	(+ isoleucine)	57 (100)	74 (74)	73 (62)	41 (52)	55 (50)

[a] The figures in parenthesis are the intensities of the peaks expressed as a percentage of the intensity of the base peak which = 100

[b] The nature of the medium is given in parenthesis

(see Table 1). In the case of the other 18 strains, addition of valine to the basal medium resulted in an increase in the amount of *iso*-butyric acid formed suggesting that they oxidize valine to *iso*-butyric acid. Growth in both the leucine medium and the isoleucine medium gave an increased yield of the *iso*-valeric fraction indicating that these organisms oxidize both leucine and isoleucine to the corresponding fatty acids. Since the basal medium contains both leucine and isoleucine the *iso*-valeric fraction is probably a mixture of these two isomers. Only growth in the leucine medium increased the yield of the *iso*-caproic acid fraction.

In order to establish the identities of the branched-chain fatty acids, the products formed by *Clostridium sporogenes* were examined by GLC/mass spectrometry. The output from the chromatograph entered the mass spectrometer – an AEI MS902 – via a membrane separator. Table 3 gives the *m/e* values of the five most intense ions of the reference compounds and of the branched-chain VFAs produced in the various media; the relative intensities of the five peaks expressed as a percentage of the base peak are given in parenthesis. The results confirm that *iso*-butyric acid is produced from valine. The spectrum of the *iso*-valeric fraction from the basal medium is that expected of a mixture of 2-methyl valeric acid and 3-methyl valeric acid. It will be seen that the spectrum of the *iso*-valeric fraction from the leucine medium resembles that of 3-methyl butyric acid and that the spectrum of the same fraction from the isoleucine medium is more closely allied to that of 2-methyl butyric acid than to 3-methyl butyric acid. These mass spectrometer data were further analysed by our colleague, Mr. R. Self, who reported that the proportions of 3-methyl butyric acid to 2-methyl valeric acid in the *iso*-valeric fraction were: basal medium 2:1; leucine medium 10:1; isoleucine medium 1:3.

Table 3 also gives the spectra of the *iso*-caproic fractions from the three media along with those of the reference compounds 3-methyl valeric acid and 4-methyl valeric acid, the reduction products of isoleucine and leucine respectively. It will be seen that the spectra of the three *iso*-caproic fractions correspond to that of 4-methyl valeric acid in keeping with the observation that leucine, but not isoleucine, increased the amount of the *iso*-caproic acid fraction. The results in Tables 2 and 3 confirm the suggestion of Werner (1972) that leucine can be reduced to 4-methyl valeric acid and it follows that leucine must now be considered not only as an electron donor in the Stickland reaction (Stickland, 1934), but also as an electron acceptor.

Table 2 shows that *Clostridium botulinum* types A and B, but not types F and G, make *iso*-caproic acid. Moss et al. (1970) studied a number of strains of type F and found that, amongst the proteolytic types, the production of *iso*-caproic acid was variable and depended upon both the growth medium used and on the strain. Mayhew and Gorbach (1975) also reported that two strains of *C. botulinum* type F produce *iso*-caproic acid. With the exception of *C. propionicum*, which was not tested, all the species found by Elsden et al. (1976) to reduce phenylalanine to phenyl propionic acid also reduce leucine to *iso*-caproic acid. In this context it is of interest that the two strains of *C. botulinum* type F which Mayhew and Gorbach (1975) showed make *iso*-caproic acid also produced a compound which Dr. Mayhew (private communication to SRE) has now shown to be phenyl propionic acid.

Of the amino acids examined in this paper, threonine is the most versatile. Evidence is produced of its oxidation to propionic acid, its reduction to *n*-butyric acid and its conversion to acetate possibly through the action of threonine aldolase. In addition to this, Stadtman (1954) has shown that extracts of dried cells of *Clostridium sticklandii* reduce threonine to 2-amino butyric acid. Mead (1971) showed that a number of clostridia produced substantial amounts of 2-amino-butyric acid when grown upon protein hydrolysates and obtained direct evidence that threonine was the precursor in the case of *C. sporogenes*, *C. caloritolerans*, *C. bifermentans* and *C. difficile*. The enzymic mechanism of this reaction is not known; indeed, as will be apparent from this paper, the mechanism of threonine catabolism has not been studied in any detail. Such a study would be useful for the characterisation of the proteolytic clostridia.

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