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The End Products of the Metabolism of Aromatic Amino Acids by Clostridia

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Abstract. The end products of the metabolism of phenylalanine, tyrosine and tryptophan by growing cultures of clostridia have been identified. The species used were *Clostridium aminovalericum; C. bifermentans; C. botulinum* proteolytic type A; *C. botulinum* proteolytic type B; *C. cochlearium; C. difficile; C. ghoni; C. histolyticum; C. lentoputrescens; C. limosum; C. lituseburense; C. malenomenatum; C. mangenoti; C. propionicum; C. putrefaciens; C. sordellii; C. sporogenes; C. sporosphaeroides; C. sticklandii; C. subterminale; C. tetani; C. tetanomorphum.* The mixture of aromatic compounds formed, which depended upon the species, included phenyl acetic acid, phenyl propionic acid, phenyl lactic acid, phenol, *p*-cresol, *p*-hydroxy phenyl acetic acid, *p*-hydroxy phenyl propionic acid, indole, indole acetic acid and indole propionic acid.

Key words: Clostridia – Phenylalanine – Tyrosine – Tryptophan – Metabolism.

Mead (1971) made a qualitative and quantitative study of the amino acids utilized by growing cultures of clostridia. The species selected for study had in common the ability to grow in a medium containing only amino acids, growth factors and minerals; these organisms were thus able to use amino acids as their source of both carbon and energy for growth. A feature of the results was the large number of species which catabolized one or more of the aromatic amino acids, phenylalanine, tyrosine and tryptophan. Moss et al. (1970) showed that certain species, including *Clostridium sporogenes*, produced 3-(phenyl)propionic acid from phenylalanine but, apart from this, there has been no systematic study of the metabolism of aromatic acids by growing cultures of clostridia. This paper is an attempt to rectify this omission.

Abbreviation. GLC = gas liquid chromatography.

MATERIALS AND METHODS

Organisms. The species of clostridia studied were selected on the basis of their ability to grow on the amino acid/growth factor medium used by Mead (1971). The reference strains of clostridia listed by Smith and Hobbs (1974) were used together with strains provided by colleagues from other laboratories and from the Institute's collection. The reference species were: *Clostridium aminovalericum* NCIB 10631; *C. bifermentans* NCIB 10716; *C. botulinum* proteolytic type A NCIB 10640; *C. botulinum* proteolytic type B NCIB 10657; *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. sordellii* NCIB 10717; *C. sporogenes* NCIB 10696; *C. sporosphaeroides* NCIB 10672; *C. sticklandii* NCIB 10654; *C. subterminale* NCIB 9834; *C. tetani* NCIB 10628.

The other strains used were: *C. bifermentans* NCIB 2912, NCIB 1341; *C. botulinum* proteolytic type A NCTC 887; *C. botulinum* proteolytic type B NCTC 751; *C. caloritolerans* NCIB 9360; *C. difficile* 871 (from the collection of L. S. McClung), N1, N3, ATCC 9689, ATCC 17857, ATCC 17858 (all five strains from the collection of the late C. L. Oakley); *C. sordellii* NCTC 6972; *C. sporogenes* NCIB 532, NCIB 8053, NCIB 8243, NCIB 9381, TC4, TC98, MRI14 (from the collection of T. C. Roberts); *C. tetani* NCTC 540, NCTC 2933, NCTC 2937, NCTC 2938, NCTC 5404, NCTC 9567, NCTC 9573, CN879, CN1341; *C. tetanomorphum* NCTC 288, NCTC 500, NCTC 2909, ATCC 3606, 252 and 292 (both from the collection of L. S. McClung).

Reagents. L-Phenylalanine, L-tyrosine, L-tryptophan, 2-(phenyl) acetic acid, 3-(phenyl) propionic acid, 3-(phenyl) lactic acid, 2-(*p*-OH phenyl) acetic acid, 3-(*p*-OH phenyl) propionic acid, 3-(*p*-OH phenyl) lactic acid, 2-(3′-indolyl) acetic acid, 3-(3′-indolyl) propionic acid, 3-(3′-indolyl) lactic acid, phenyl, *p*-cresol, indole and skatole were obtained either from Koch-Light Laboratories Ltd., Colnbrook, Bucks, or from Sigma Chemical Company, London; these compounds will be referred to by their trivial names hereafter.

Media. Stock cultures were maintained in cooked meat medium. The growth medium used to examine the end products of aromatic

amino acid metabolism contained: BBL trypticase 3.0% (w/v); Oxoid yeast extract 0.5% (w/v); sodium thioglycollate 0.03% (w/v); L-phenylalanine 0.2% (w/v); L-tyrosine 0.2% (w/v); L-tryptophan 0.2% (w/v); the pH was adjusted to pH 7.2. The free aromatic amino acids, which supplemented those present in the trypticase, were added to ensure the formation of sufficient end products for the tests used. The medium was dispensed in 25 ml lots in 28 ml screw capped bottles and autoclaved for 15 min at 15 lbs/in^2.

Growth Conditions. Immediately before inoculation bottles of medium were heated for 15 min in a boiling water bath and cooled rapidly. The inoculum consisted of 0.5 ml from a culture grown in the meat medium. Cultures were incubated for 3 days at 37° C with the exception of *Clostridium putrefaciens* which was incubated for 3 days at 30° C.

Sample Preparation. At the of 3 days 10 ml samples of the growth medium were mixed with 1.0 ml of 3.5 *M*-perchloric acid and the insoluble material removed by centrifugation. The supernatants were neutralised with 1 ml of 3.5 *N*-KOH, cooled in ice and centrifuged again; the deproteinized supernatants were stored at −18° C until required. If the samples were to be examined by thin layer chromatography (TLC), 10 ml of the protein-free supernatant was transferred to a stoppered test tube, acidified and extracted with 2 ml ether. The ether phase was separated, removed and stored in a screw capped vial at −18° C until required.

Strecker's celite column technique, described by Knight (1962), was used for the quantitative extraction of the aromatic end products from growth media. The procedure for phenol, *p*-cresol, indole and skatole was as follows: 2 ml of the neutral supernatant and 2 ml saturated NaCl were mixed with 10 g ether-washed celite in an evaporating dish. The mixture was then transferred quantitatively to a chromatography tube (2.5 cm internal diameter) and gently tamped. The column was developed with dry diethyl ether and 30 ml of eluate gave a quantitative extraction – some phenyl acetic and phenyl propionic acid are also extracted at pH 7.0. On GLC phenyl acetic acid is well separated from the other compounds in this extract but phenyl propionic acid has a retention volume similar to that of indole (Table 1). If the presence of both compounds is suspected then a portion of the extract is methylated, vide infra, when the methyl ester is well separated from indole. The ether was removed in a stream of nitrogen and the residue taken up in 100 µl diethyl ether for analysis.

The acid end products were extracted from the neutralised protein free extracts as follows: 2 ml of the solution and 2 ml 2 *N*-H_2SO_4 saturated with $MgSO_4 \cdot 7\,H_2O$ were mixed with 10 g ether-washed celite, packet into a 2.5 cm column and eluted with 50 ml diethyl ether. The ether was removed as above and the sample redissolved in approx. 1 ml diethyl ether. The acids were then methylated by the addition of a few drops of ethereal diazomethane followed immediately by the removal of both the excess diazomethane and the ether on a rotary evaporator. The methyl esters remaining were dissolved in 100 µl diethyl ether for GLC analysis.

Gas-Liquid Chromatography. A stainless steel column, 50 cm × 6.35 mm and packed with Diatomite CQ, 90–100 mesh (JJ's Ltd., King's Lynn, Norfolk, UK) which contained 5% OV17 as the liquid phase. The column was operated isothermally at 140° C and 200° C with an argon gas phase and a flame ionisation detector; the flow rates were: 140° C, 60 ml min^{-1}; 200° C, 45 ml min^{-1}. Table 1 gives the retention volumes of the compounds studied.

Thin Layer Chromatography. Plates of silica gel H, 0.25 mm thick were conditioned for 2 h at 110° C. Two solvents were used: (A) contained light petroleum (60–80°), diethyl ether and acetic acid (100:100:2) and (B) contained light petroleum (60–80°), diethyl ether and acetic acid (100:100:1). Because of the various mixtures of products which occur it is desirable to analyse samples of an ether extract with both solvents. Indole compounds were located by spraying with 0.06 *M*-*p*-dimethyl amino benzaldehyde in 1.0 *N*-HCl. Phenolic compounds were located by spraying with the nitraniline reagent, Smith et al. (1969); the aromatic acids were located by spraying with bromocresol green, Nordmann and Nordmann (1969). R_f values are given in Table 2.

Table 1. GLC Retention volumes

	Retention volume (ml)	
	140° C	200° C
Phenol	112	–
p-Cresol	177	–
Indole	864	–
Skatole	1300	–
Phenyl acetic acid	516	–
Phenyl propionic acid	831	–
Phenyl acetic acid methyl ester	359	–
Phenyl propionic acid methyl ester	593	–
Phenyl lactic acid methyl ester	1093	–
p-HO phenyl acetic acid methyl ester	–	212
p-HO phenyl propionic acid methyl ester	–	302
Indole acetic acid methyl ester	–	964
Indole propionic acid methyl ester	–	1367

Stainless steel column (150 cm × 6.35 mm) packed with diatomite CQ, 90–100 mesh coated with 5% OV 17; gas phase argon. Column operated isothermally; gas flow rate 60 ml/min at 140° C and 45 ml/min at 200° C.

Table 2. Chromatography of aromatic compounds

Compounds	R_f value in solvent	
	A	B
Phenol	0.78	0.75
p-Cresol	0.80	0.81
Indole	0.78	0.84
Skatole	0.86	0.88
Phenyl acetic acid	0.51	0.67
Phenyl propionic acid	0.57	0.78
Phenyl lactic acid	0.33	0.44
p-OH phenyl acetic acid	0.18	0.20
p-HO phenyl propionic acid	0.31	0.25
p-HO phenyl lactic acid	0.08	0.06
Indole acetic acid	0.33	0.20
Indole propionic acid	0.41	0.24
Indole lactic acid	0.08	0.06

Plates of silica gel H, 0.25 mm thick and conditioned for 2 h at 110° C. Solvent A = 60–80° light petroleum, 100; diethyl ether, 100; acetic acid, 2. Solvent B = 60–80° light petroleum, 100; diethyl ether, 100; acetic acid, 1.

RESULTS

The amounts of the products formed from the aromatic amino acids by the representative strains studied

Table 3. Aromatic end products

Species	Strain	Products formed μmoles/ml									
		Phenyl acetic	Phenyl propionic	Phenyl lactic	Phenol	HO-phenyl acetic	HO-phenyl propionic	*p*-Cresol	Indole	Indole acetic	Indole propionic
Clostridium sporogenes	NCIB 10696	0	13.1	0	0	0	7.4	0	0	0	2.3
Clostridium botulinum A	NCIB 10640	0	13.0	0	0	0	8.0	0	0	0	1.6
Clostridium botulinum B	NCIB 10657	0	11.8	0	0	0	8.2	0	0	0	2.4
Clostridium caloritolerans[a]	NCIB 9360	0	11.2	0	0	0	3.8	0	0	0	1.6
Clostridium mangenoti	NCIB 10639	0	2.3	2.3	0	0	0	0	0	0	0
Clostridium ghoni	NCIB 10636	0	6.4	4.9	0	0	0	0	0.4	0	0
Clostridium bifermentans	NCIB 10716	0.8	9.8	2.3	0	0	0	0	0.6	0	0
Clostridium sordellii	NCIB 10717	0.8	5.9	0.5	0	0	0	0	1.2	0	0
Clostridium difficile	NCIB 10666	4.8	0.8	0	0	0.5	0	2.3	0	0.6	0
Clostridium sticklandii	NCIB 10654	2.6	0	0	0	0.8	0	0	0	0.5	0
Clostridium lituseburense	NCIB 10637	0.6	0	0	0	0.6	0	0	0	0.2	0
Clostridium subterminale	NCIB 9384	0.5	0	0	0	0.7	0	0	0	0.2	0
Clostridium putrefaciens	NCIB 9836	0.5	0	0	0	0.4	0	0	0	0.2	0
Clostridium propionicum	NCIB 10656	0.1	0	0	0	0.1	0	0	0	0	0
Clostridium malenomenatum	ATCC 25776	0	0	0	0.3	0	0	0	0.4	0	0
Clostridium limosum	NCIB 10638	0	0	0	2.6	0	0	0	9.8	0	0
Clostridium lentoputrescens	NCIB 10629	0	0	0	0.5	0	0	0	3.3	0	0
Clostridium tetani	NCIB 10628	0	0	0	1.9	0	0	0	3.9	0	0
Clostridium tetanomorphum[a]	NCTC 500	0	0	0	5.0	0	0	0	0.6	0	0
Clostridium cochlearium	ATCC 17787	0	0	0	3.5	0	0	0	0	0	0
Clostridium histolyticum	NCIB 503	0	0	0	0	0	0	0	0	0	0
Clostridium aminovalericum	NCIB 10631	0	0	0	0	0	0	0	0	0	0
Clostridium sporospheroides	NCIB 10672	0	0	0	0	0	0	0	0	0	0

[a] These strains are not included in the reference strains of Smith and Hobbs (1974).

are shown in Table 3. The trypticase in the medium contained unknown amounts of the three aromatic amino acids and to ensure an adequate supply of each the medium was supplemented with L-phenylalanine (12.1 μmoles ml^{-1}); L-tyrosine (11 μmoles ml^{-1}); L-tryptophan (9.8 μmoles ml^{-1}). It follows, therefore, that the theoretical maximal yields of the products must be greater than the above by amounts equivalent to the aromatic amino acids present in the trypticase preparation. The quantitative results (Table 3) show that the species which produce phenyl propionic acid are the most active in metabolizing the aromatic amino acids. With the exception that 6 out of 10 strains of *Clostridium tetani*, viz. CN 879 and 540, 2933, 5404, 9567, 9573 from the NCTC, and one out of 6 strains of *C. tetanomorphum*, viz. NCTC 2909, did not make indole in amounts detectable by our methods, the responses of all the strains of each species examined were similar to those of the representative strains. Further, inspection of the GLC charts of the qualitative GLC analyses indicated that the amounts of the products made by the reference strains were of the same order as those produced by all the strains of any particular species.

In general, the end products were identified by their behaviour on GLC but, in the case of 5 species, *Clostridium bifermentans* (10716), *C. sporogenes* (10696), *C. ghoni* (10636), *C. difficile* (10666) and *C. limosum* (10638), the end products were examined by GLC/mass spectrometry using an AEI MS 902 mass spectrometer (the mass spectrometry data are available on request).

The GLC/mass spectrometer analysis confirmed that *Clostridium sporogenes* produces phenyl propionic acid, *p*-OH phenyl propionic acid and indole propionic acid. Table 3 shows that *C. ghoni*, *C. bifermentans*, *C. sordellii* and *C. mangenoti* produce phenyl propionic acid and phenyl lactic acid; in addition the first three produce indole and *C. bifermentans* produces phenyl acetic acid. The products formed by *C. ghoni* and *C. bifermentans* were confirmed by GLC/mass spectrometry. The 7 strains of *C. difficile* examined produced *p*-cresol, which was confirmed by GLC/mass spectrometry; the only other species we have found which makes this compound is *C. scatologenes*.

Inspection of Table 3 shows that a number of species—all of which ferment glutamic acid and histidine—produce phenol and this was characteristic

of all the strains of these species used in this work. The production of indole was more variable. The formation of phenol and indole by *Clostridium limosum* was confirmed by GLC/mass spectrometry.

DISCUSSION

In this study of the catabolism of the aromatic amino acids by clostridia the chemical changes observed were restricted to the aliphatic side chains; this contrasts with the aerobic processes where hydroxylation followed by ring fission also occurs. Possible reactions leading to the formation of the end products are given below where *R* is the aromatic nucleus.

$$R\mathrm{CH_2CHNH_2COOH} + \mathrm{H_2O} \rightarrow R\mathrm{H} + \mathrm{CH_3C{:}OCOOH} + \mathrm{NH_3} \quad (1)$$

$$R\mathrm{CH_2CHNH_2COOH} \rightarrow R\mathrm{CH{:}CHCOOH} + \mathrm{NH_3} \quad (2)$$

$$R\mathrm{CH_2CHNH_2COOH} + \mathrm{NAD} + \mathrm{H_2O} \rightarrow R\mathrm{CH_2C{:}OCOOH} + \mathrm{NADH_2} + \mathrm{NH_3} \quad (3)$$

$$R\mathrm{CH_2CHNH_2COOH} + \text{2-oxoglutarate} \rightarrow R\mathrm{CH_2C{:}OCOOH} + \text{glutamate} \quad (4)$$

$$R\mathrm{CH_2C{:}OCOOH} + \mathrm{NADH_2} \rightarrow R\mathrm{CH_2CHOHCOOH} + \mathrm{NAD} \quad (5)$$

$$R\mathrm{CH_2CHOHCOOH} \rightarrow R\mathrm{CH{:}CHCOOH} + \mathrm{H_2O} \quad (6)$$

$$R\mathrm{CH{:}CHCOOH} + 2\,\mathrm{H} \rightarrow R\mathrm{CH_2CH_2COOH} \quad (7)$$

$$R\mathrm{CH_2C{:}OCOOH} + \mathrm{H_2O} \rightarrow R\mathrm{CH_2COOH} + \mathrm{CO_2} + 2\,\mathrm{H} \quad (8)$$

$$R\mathrm{CH_2COOH} \rightarrow R\mathrm{CH_3} + \mathrm{CO_2} \quad (9)$$

Save in the case of phenol production, there is relatively little information on the nature of the enzyme reactions involved.

In reaction (1) the side-chain is removed with the formation of pyruvic acid, ammonia and the aromatic residue. Phenol was produced by all strains of *Clostridium malenominatum*, *C. limosum*, *C. lentoputrescens*, *C. tetani*, *C. tetanomorphum* and *C. cochlearium*. These organisms also have in common the ability to ferment glutamic acid and histidine. Woods and Clifton (1937) showed that washed suspensions of *C. tetanomorphum* fermented tyrosine with the formation of ammonia, carbon dioxide and hydrogen, and later Brot et al. (1965) showed that the products were phenol, ammonia and pyruvic acid; the latter was further metabolized to carbon dioxide and hydrogen (c.f. Woods and Clifton, 1937). The enzyme tyrosine phenol lyase was purified and shown to contain pyridoxal phosphate (Brot et al., 1965). The corresponding enzyme from *Escherichia intermedia* was later obtained crystalline by Kumagi et al. (1970). Indole was produced by 8 species. The mechanism of its formation by clostridia has not been established. Although tryptophanase was not detected in 9 strains of an unnamed species of clostridium (Nakozawa et al., 1972), it seems reasonable, none the less, to assume that this is the enzyme responsible.

The reduction of the side-chain of the substrate to the corresponding substituted propionic acid was first reported by Hoogerheide and Kocholaty (1938) who showed that washed suspensions of *Clostridium sporogenes* incubated under hydrogen in the presence of tryptophan formed indole propionic acid; they also observed that hydrogen was slowly consumed in the presence of tyrosine. Boezi and De Moss (1959) confirmed that *C. sporogenes* produced indole propionic acid from tryptophan and more recently Moss et al. (1970) found that phenyl propionic acid was produced by growing cultures of *C. sporogenes*, *C. botulinum* (type A and proteolytic strains of type B, D and F), *C. bifermentans*, *C. sordellii* and *C. caproicum*. Suspensions of *C. sporogenes* incubated anaerobically with phenylalanine produced phenyl propionic acid. Cinnamic acid accumulated in the early stages of the reaction and then disappeared with the formation of an equivalent amount of phenyl propionic acid, suggesting that cinnamic acid is an intermediary in the reaction. Hoogerheide and Kocholaty (1938) did not identify the reduction product of tyrosine but our results show that this is *p*-OH phenyl propionic acid.

There are two possible pathways for the reduction of the side chains. The first entails an initial desaturation (reaction 2) by an "ammonia lyase" enzyme similar to those found in plants (c.f. Koukol and Conn, 1961; Camm and Towers, 1973), followed by a reduction, reaction (7). In the alternative pathway, the initial formation of the 2-oxo acid occurs by either reaction (3) or (4); this step is followed by reactions (5), (6) and (7) in that order. There is no evidence for the occurrence of the first pathway; on the other hand, there is evidence that *Clostridium sporogenes* converts tryptophan to indole propionic acid by the second pathway (see Boezi and De Moss, 1959; O'Neill and De Moss, 1968; Jean and De Moss, 1968).

Neither Kocholaty and Hoogerheide (1938), using the hydrogenase system, nor Stickland (1934), using reduced viologen dyes, obtained evidence that *Clostridium sporogenes* reduced phenylalanine; on the other hand, they found that this amino acid was slowly oxidised in the presence of methylene blue. The cause of this discrepancy between their in vitro experiments and the growth experiments of Moss et al. (1970) and those reported here is not known. Of the organisms

making phenyl propionic acid, four, namely *C. mangenoti*, *C. ghoni*, *C. bifermentans* and *C. sordellii*, also make phenyl lactic acid which, on the De Moss hypothesis, is an intermediate in the formation of phenyl propionic acid.

Clostridium difficile, judged by the variety of its end products, is the most versatile of the organisms examined. However, its attack on the aromatic amino acids is predominantly oxidative converting all three to the corresponding acetic acids; the amount of phenyl propionic acid produced is small. The finding that *C. difficile* produced *p*-cresol was a surprise. In the preliminary experiments a non-specific colour test was used to detect phenol and a positive result led us to believe that *C. difficile* produced phenol from tyrosine. Since *C. difficile* had no other biochemical characters in common with the six species which produced phenol the compound was examined in more detail. It was identified by GLC/mass spectrometry as *p*-cresol. There have been, from time to time, papers reporting the anaerobic formation of *p*-cresol from tyrosine; thus Stone et al. (1949) demonstrated the formation of *p*-cresol from tyrosine by an anaerobe and obtained evidence that *p*-hydroxyphenyl acetic acid was the precursor. In this context, it is relevant that *C. difficile* makes *p*-hydroxyphenyl acetic acid. The comparable production of skatole from indole acetic acid by rumen bacteria has been reported recently by Yokoyama and Carlson (1974).

We informed the late Professor Oakley, who had provided us with cultures of *Clostridium difficile*, that this organism made *p*-cresol. Oakley and Hafiz (see Hafiz, 1974) showed that *C. difficile* had a remarkable tolerance for *p*-cresol, that cultures would grow in media containing up to 0.4% and that addition of 0.2% *p*-cresol to the RCA and RCM media of Hirsch and Grinstead (1954) gave media which were highly selective for *C. difficile* and permitted the direct isolation of this organism.

The results in Table 3 show that some of the end products of the metabolism of aromatic acids are common to a number of species. Thus, those organisms able to reduce phenylalanine to phenyl propionic acid – first reported by Moss et al. (1970) – also reduce proline to 5-amino valeric acid (c.f. Mead, 1971); however, it should be noted that the converse is not true. The formation of phenyl propionic acid, in most cases in substantial amounts, indicates that phenylalanine must now be considered as a significant electron acceptor in the Stickland reaction.

Clostridium sporogenes, *C. botulinum* type A and *C. botulinum* proteolytic type B are unique in that they reduce the side-chains of all three aromatic amino acids to the corresponding substituted propionic acids. In addition, these organisms also have in common morphological characters, the amino acids they use during growth (Mead, 1971), and the ability to reduce uracil to dihydrouracil (Hilton et al., 1975). Further, Lee and Riemann (1970) showed that their DNA's were 50–100% homologous to the DNA of their reference strain of *C. botulinum* (but see also Johnson and Francis, 1975). In the light of these common properties the case for continuing to consider them as discrete species merits attention. Moss et al. (1970) also showed that *C. botulinum*, proteolytic types D and F also produce phenyl propionic acid and it would be of interest to examine their metabolism of tyrosine and tryptophan; unfortunately we have not been in a position to do these experiments.

Of the remaining organisms which make phenyl propionic acid, *Clostridium ghoni* and *C. mangenoti* also produce phenyl lactic acid and the former also makes indole. *C. bifermentans*, *C. sordellii* and *C. difficile* produce both phenyl acetic acid and phenyl propionic acid. The first two species, which have a number of characters in common, also make phenyl lactic acid and indole.

Clostridium sticklandii, *C. lituseburense*, *C. subterminale* and *C. putrefaciens* oxidize all three of the aromatic amino acids to the substituted acetic acids, but *C. sticklandii* differs from the other three by reducing proline to 5-amino valeric acid. *C. propionicum* is also of interest. For, whilst it reduces alanine to propionic acid, probably via acrylyl Co-enzyme A (Stadtman and Vagelos, 1958), it does not reduce any of the aromatic amino acids; instead, it oxidizes phenylalanine and tyrosine to the corresponding substituted acetic acids.

Clostridium tetani, *C. tetanomorphum* and *C. cochlearium* have in common the fact that they ferment glutamic acid, via the methyl aspartic pathway, to acetic and butyric acids, Buckel and Barker (1974); they also ferment histidine. We have found that *C. malenomenatum*, *C. limosum* and *C. lentoputrescens* also ferment glutamic acid and histidine but the pathway has not so far been examined. Table 3 shows that these six species produce phenol from tyrosine. *C. cochlearium* did not produce indole, nor did the following 6 strains of *C. tetani:* CN 879, NCTC 540, NCTC 2933, NCTC 5404, NCTC 9567; NCTC 9573; in addition, one strain of *C. tetanomorphum* did not produce indole.

It is generally accepted that, compared with other groups of bacteria, only a limited number of the biochemical characters are used to describe the clostridia. The comparative studies of the utilization of amino acids by Mead (1971), and of the metabolism of glutamic acid by Buckel and Barker (1974), suggested that there is here a fruitful field of work for

those seeking to describe the proteolytic clostridia in more precise terms. The results in this paper support this view. At the same time it must be emphasized that our experiments were concerned solely with end products of the metabolism of the aromatic amino acids and not with the pathways by which these products are made. It is obvious that the identification of the enzymes concerned in these pathways is essential for a rational description of an organism because the number of enzymes involved reflects the genetic make-up of an organism.

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