surface; the net rate of movement down the column may therefore be expected to be comparable with that of leucine. Finally, histidine and the other diamino-acids will be completely ionized and firmly held by the resin at this pH.

Fractions III, IV and V consist of acids whose pK values are so close that a separation depending on this factor would not be expected, and according to the argument given in the second section we should look for an explanation in terms of adsorption affinity. The molecular weights of the acids concerned are:

Fraction

III Glycine, 75; alanine, 89

IV Valine, 117

V Leucine, isoleucine, 131; methionine, 149

The results do, therefore, seem to provide real evidence that, in the absence of significant pK differences, separations based on quite small differences in chain length and adsorption affinity can be carried out on ion-exchange columns.

Partridge (1949) found that tyrosine and phenylalanine showed anomalous behaviour, and had to be removed in a preliminary operation. This is in keeping with the strong adsorption of all aromatic compounds, a factor which seems at present to set the most important limitation to the use of ion-exchange resins.

SUMMARY

The theory of the separation of weak electrolytes by ion-exchange resins is discussed and illustrated by reference to recent work with the amino-acids.

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**Direct Transformation of Fumarate to Oxaloacetate,
without Intermediate Formation of Malate, by
Clostridium saccharobutyricum, Strain GR 4**

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Annau, Banga, Gösz, Huszák, Laki, Straub & Szent-Györgyi (1935), on the basis of differences between fumarate and malate oxidation, thought that fumarate might be oxidized without intermediate formation of malate. Later, Laki (1936) abandoned this view. However, Kalckar (1939) showed that kidney extracts can dehydrogenate an addition product of fumarate and phosphate with formation of phosphoenoloxaloacetate. Lipmann (1941) assumed that the earlier view of Annau *et al.* (1935) was in part correct, and that the differences between the oxidation of the two substrates could probably be explained by the co-existence of two different paths of fumarate breakdown, the first occurring via malate, the second leading directly to oxaloacetate through a phosphorylated derivative of fumarate. This hypothesis is supported by the observation of Lipmann (1942) that, in a similar manner, the transformation of crotonate to aceto-

acetate occurred in rabbit kidney without intermediate formation of β -hydroxybutyrate.

Clifton (1942), studying the fermentation of C_4 -dicarboxylic acids by washed suspensions of *Clostridium tetani*, found that fumarate is partly fermented to butyrate and acetate and partly to lactate, malate and ethanol. Malate and succinate were not fermented. Pickett (1943), with the same organism, showed that if incubation is continued for 4 days, malate is slowly fermented with production of acetate and butyrate, ethanol, carbon dioxide and succinate. The latter was not fermented. Cohen, Nisman & Cohen-Bazire (1948) have shown that citrate and α -ketoglutarate are slowly fermented to butyrate and acetate by strain GR 4 of *Cl. saccharobutyricum*. It is very unlikely that these two compounds are broken down via the C_4 -dicarboxylic acids system, as it will be shown that succinate is not fermented by this organism.

Further work with this organism, reported in the present paper, shows that it is improbable that the breakdown of fumarate occurs via malate, and evidence is presented in support of an alternative scheme.

EXPERIMENTAL

Organism

The organism, named according to Prévot (1948), is a variety of *Cl. butyricum* Prazmowski. Prévot, who has given us this strain, names it *Cl. butyricum*, var. *saccharobutyricum* Schattenfroh & Grassberger. The strain GR4 of this organism, which has been isolated from river mud, is a small thin rod, with subterminal spores, motile, Gram-positive; its dimensions vary from 3–12 × 0.8–1 μ. Deep agar colonies appear as dense cottony masses. Gelatin is liquefied after 7 days. Milk is coagulated in 5 days; the clot is further fragmented, but not digested. Indole is formed. All the sugars tested were fermented except galactose, arabinose, dulcitol and inulin. Butyrate, acetate and lactate were formed when the organism was grown on VF broth (peptic digest of muscle and liver) containing 0.2% glucose. Acetone and acetylmethylcarbinol were not formed under these conditions. Cohen & Cohen-Bazire (1948*a, b*) have separated the butyric bacteria into two biochemical types according to their ability, in washed suspension, to ferment pyruvate with production of (a) acetate and butyrate ('normal' strains), or (b) acetate only ('deficient' strains). The GR4 strain is 'normal'; lactate is also formed, but in the present work attention is confined to the volatile acids.

Preparation of cell suspensions

The organism was grown on VF broth, of which 1 l. was inoculated with 5 ml. of an 18 hr. subculture and incubated for 20 hr. at 37°. The cells from 1 l. of culture were centrifuged out, washed twice with 15 ml. of previously de-aerated saline and finally resuspended in approx. 25 ml. of saline. All operations were carried out with sterile precautions. A quantity of suspension (3 ml.), corresponding to approx. 8 mg. bacterial N, was taken for each experimental tube. The yield of cells varied only slightly from one culture to another.

Procedure and methods

Chemicals. These were obtained from the sources indicated. Oxaloacetic acid (prepared as described by Fenton & Jones, 1900); succinic acid (Merck); fumaric acid, malic acid, hydroxylamine hydrochloride and sodium arsenite (Pro-labo); sodium pyruvate (Hofmann-la Roche); congo red (R.A.L.). Solutions of the acids and of hydroxylamine hydrochloride were neutralized before use.

Fermentations. All operations up to the completion of the fermentation were carried out with sterile precautions in view of the long period of incubation (24 hr.). Substrates and inhibitors were sterilized by filtration (L_2 Chamberland candles); water and buffer by autoclaving. Substrates, bacterial suspension, phosphate buffer (pH 7, final conc. 0.033 M) were measured into test tubes and brought to a total volume of 20 ml. with water. The tubes were evacuated, sealed and incubated for 24 hr. at 37°. The total contents of each tube were then acidified with 5 ml. of 40% (w/v) tartaric acid and steam distilled; 130 ml. of distillate were

collected. A portion (20 ml.) of the distillate was used for the determination of total volatile acids by titration with 0.1 N-NaOH. The remaining 110 ml. were used for the determination of the nature and proportions of these acids by the Duclaux (1895) method. Our Duclaux calibration curves, performed with redistilled acids, differed little from the original Duclaux curves.

The method of Raynaud & Gros (1947) was used for the determination of NH_3 in the experiments in which NH_4OH was used. In the same experiments, one-dimensional paper chromatography (Whatman no. 1 paper; solvent, 95% phenol) was used for the detection of aspartic acid (Consden, Gordon & Martin, 1944).

RESULTS

Comparative rate of fermentation of succinate, fumarate, malate, oxaloacetate and pyruvate

The results recorded in Table 1 show that volatile acids are produced from these substrates at a rate which decreases in the order: pyruvate > oxaloacetate > fumarate > malate. Succinate is not

Table 1. *Fermentation of succinate, fumarate, malate, oxaloacetate and pyruvate*

(Each tube, sealed *in vacuo*, contained 4000 μmol. of substrate and 8 mg. bacterial N. Total vol. 20 ml.; pH 7; 37°. Incubation 24 hr.)

Exp. no.	Substrate	Total volatile acid (μmol.)	Acetic acid (μmol.)	Butyric acid (μmol.)
1	Fumarate	780	478	302
	Malate	390	312	78
	Pyruvate	936	468	468
2	Succinate	0	0	0
	Fumarate	562	315	247
	Malate	351	281	70
	Pyruvate	940	470	470
3	Succinate	0	0	0
	Fumarate	655	406	249
	Oxaloacetate	842	505	237
	Pyruvate	611	409	202
4	Succinate	0	0	0
	Fumarate	390	260	130
	Malate	250	200	50
	Malate (4 days)	671	505	134
5	Oxaloacetate	1014	639	375
	Fumarate	562	264	298
	Oxaloacetate	795	452	328
	Pyruvate	2184	1245	939
6	Fumarate	702	435	267
	Malate	499	375	124
	Oxaloacetate	780	460	320
	Pyruvate	1638	868	770

attacked; the organism had no succinic dehydrogenase activity. With malate the relative proportion of butyric and acetic acids differs markedly from that obtained from the other substrates; much less butyric acid is formed from malate. These results

make it very improbable that the classical reaction sequence

occurs in these fermentations. They suggest rather a direct conversion of fumarate to oxaloacetate, the latter then giving rise to pyruvate.

Non-inhibition of fumarate fermentation by congo red

Quastel (1931, 1936-7) found, both with whole cells of *Escherichia coli* and isolated enzyme preparations, that this dye (at a concentration of 1.2×10^{-5} M) inhibits fumarase. Table 2 shows that the fermentation of fumarate is not affected by congo red. This is consistent with the view that the conversion of fumarate to malate is not a stage in the fermentation of the former by *Cl. saccharobutyricum* GR 4. The dye is also without effect in the fermentation of malate, oxaloacetate, pyruvate and citrate.

Table 2. *Effect of congo red on fermentation of fumarate*

(Conditions: see Table 1. Substrate: fumarate, 4000 μ mol.)

Congo red Concentration (M)	Volatile acidity (μ mol.)	Nature of acids
(a) 0	780	$\frac{1}{2} < \frac{\text{Butyric}}{\text{Acetic}} < \frac{1}{1}$
(b) 1.2×10^{-5}	780	
(c) 2.4×10^{-5}	749	
(d) 4.8×10^{-5}	733	
(e) 2.4×10^{-4}	749	

Effect of hydroxylamine on the fermentation of C₄-dicarboxylic acids

A further difference in the behaviour of malate, compared with fumarate and oxaloacetate, was observed in experiments in which an attempt was made to inhibit the fermentation of these substances by adding equimolar quantities of hydroxylamine. It was thought that the chain of reactions would be blocked at the oxaloacetate stage by the fixation of this keto acid as an oxime. It was found, however, that, with this organism, oxaloacetate and fumarate but not malate, could act as hydrogen donor for the reduction of hydroxylamine to ammonia. After 24 hr. incubation the tubes containing hydroxylamine plus fumarate or oxaloacetate had become deep yellow; on opening there was a strong smell of ammonia. Furthermore, the steam distillate from

such tubes had an odour of acetaldehyde. This was identified by preparation of the dimedone derivative (m.p. 139°). In the case of hydroxylamine plus malate there was no formation of yellow colour, ammonia or acetaldehyde.

Experiments were carried out in which both total volatile acid and ammonia were estimated after the action of the organism on all three dicarboxylic acids in the presence and absence of hydroxylamine (Table 3). It will be seen that ammonia is not formed from hydroxylamine in the presence of malate, but only with fumarate or oxaloacetate. In all three cases total volatile acid is diminished by 30-100% in the presence of hydroxylamine. The rate of reduction of hydroxylamine to ammonia is apparently independent of the proportion of the dicarboxylic acid converted to volatile acids.

Table 3. *Effect of hydroxylamine on fermentation of C₄-dicarboxylic acids*

(Conditions: see Table 1. Neutralized $\text{NH}_2\text{OH} \cdot \text{HCl}$: 4000 μ mol. C₄ acid: 4000 μ mol.)

Exp. no.	Substrate	Volatile acidity (μ mol.)	NH ₃ (μ mol.)
2	Fumarate	562	0
	Hydroxylamine	0	0
	Fumarate + hydroxylamine	280	1850
3	Fumarate	655	0
	Hydroxylamine	0	0
	Fumarate + hydroxylamine	390	1880
4	Fumarate	390	0
	Hydroxylamine	0	0
	Fumarate + hydroxylamine	120	1800
	Oxaloacetate	1014	0
	Oxaloacetate + hydroxylamine	312	960
5	Fumarate	562	0
	Fumarate + hydroxylamine	0	2000
	Oxaloacetate	795	0
	Oxaloacetate + hydroxylamine	78	1040
7	Fumarate	—	0
	Fumarate + hydroxylamine	—	1600
	Malate	—	0
	Malate + hydroxylamine	—	0
8	Fumarate	350	0
	Fumarate + hydroxylamine	—	1000
	Malate	—	0
	Malate + hydroxylamine	—	28

In the case of fumarate the production of ammonia can be explained in two different ways:

- (a) $\text{COOH} \cdot \text{CH} \cdot \text{CH} \cdot \text{COOH} + \text{H}_2\text{O} \rightarrow \text{COOH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH} + \text{H}_2$, $\text{NH}_2\text{OH} + \text{H}_2 \rightarrow \text{NH}_3 + \text{H}_2\text{O}$;
 (b) $\text{COOH} \cdot \text{CH} \cdot \text{CH} \cdot \text{COOH} + \text{H}_2\text{O} \rightarrow \text{COOH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH} + \text{H}_2$,
 $\text{COOH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH} + \text{NH}_2\text{OH} \rightarrow \text{COOH} \cdot \text{C}(\text{:NOH}) \cdot \text{CH}_2 \cdot \text{COOH} + \text{H}_2\text{O}$,
 $\text{COOH} \cdot \text{C}(\text{:NOH}) \cdot \text{CH}_2 \cdot \text{COOH} + 4\text{H} \rightarrow \text{COOH} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{COOH} + \text{H}_2\text{O}$,
 $\text{COOH} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{COOH} + \text{H}_2\text{O} \rightarrow \text{COOH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH} + \text{NH}_3 + \text{H}_2$.

These two series of equations represent the same overall reaction:

- (c) $\text{COOH} \cdot \text{CH} \cdot \text{CH} \cdot \text{COOH} + \text{NH}_2\text{OH} \rightarrow \text{COOH} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH} + \text{NH}_3$.

Scheme (b) is supported by the fact that *Cl. saccharobutyricum* GR4 rapidly deaminates aspartic acid (Cohen, Nisman & Cohen-Bazire, 1948). Furthermore, aspartic acid, and no other amino-acid, can be detected by paper chromatography of the contents of tubes containing hydroxylamine and fumarate or oxaloacetate (Table 4). However, it is also possible that aspartic acid arises from a secondary reductive amination of oxaloacetate

It is, therefore, not yet possible to decide which of the suggested mechanisms is correct. Furthermore, Nisman (1948) has shown that washed suspensions of our strain can carry out the direct amination of fumarate in presence of ammonia. Such a reaction may also complicate the overall phenomenon. This problem is being further investigated.

It will also be seen from Table 4 that no aspartate was detected in the tubes containing hydroxylamine plus malate. Since it has already been shown that malate cannot act as hydrogen donor for the reduction of hydroxylamine, the totally different behaviour of malate again indicates that it cannot be an intermediate in the fermentation of fumarate.

Table 4. *One-dimensional filter paper chromatography*

(Solvent: 95% phenol. Paper: Whatman no. 1. Ninhydrin: 1% butanol solution.)

Substrate	R_F
(a) Fumarate-NH ₂ OH	0.10
None (suspension)	—
Aspartate (blank) (10 ⁻³ M)	0.10
(b) Fumarate-NH ₂ OH	0.11
Aspartate (blank)	0.11
Suspension	—
(c) Fumarate-NH ₂ OH	0.11
Oxaloacetate-NH ₂ OH	0.11
Aspartate (blank)	0.12
Suspension	—
Fumarate	—
Oxaloacetate	—
(d) Fumarate-NH ₂ OH	0.12
Malate-NH ₂ OH	—
Aspartate (blank)	0.12
Suspension	—
Fumarate	—
Malate	—
(e) Fumarate-NH ₂ OH	0.12
Malate-NH ₂ OH	—
Fumarate	—
Malate	—

Effect of arsenite on the fermentation of C₄-dicarboxylic acids

Cohen-Bazire, Cohen, Nisman & Raynaud (1948) have shown that the fermentation of pyruvate in the presence of 10⁻³M-sodium arsenite leads to the pro-

duction of acetate only; production of butyrate was completely inhibited and total volatile acid was decreased by approx. 30%. The effect of arsenite on the fermentation of fumarate, malate and oxaloacetate was therefore tested (Table 5). Inhibition of the same type as with pyruvate was obtained. Taken in conjunction with the experiments of Table 1 these results are consistent with the following reaction sequence:

The results with arsenite do not exclude the possibility that pyruvate is also an intermediate in the fermentation of malate. If this is the case, then, in view of the other results already given, malate must be converted to pyruvate by a mechanism not involving oxaloacetate.

Table 5. *Effect of arsenite on fermentation of C₄-dicarboxylic acids*

(Conditions: see Table 1. Dicarboxylic acids: 4000 μmol. Sodium arsenite, final concentration: 10⁻³M.)

Substrate	Volatile acidity (μmol.)	Acetic acid (μmol.)	Butyric acid (μmol.)
Pyruvate	1820	910	910
Pyruvate + arsenite	1560	1560	0
Oxaloacetate	663	330	303
Oxaloacetate + arsenite	598	544	58
Fumarate	858	572	286
Fumarate + arsenite	510	464	46
Malate	473	379	94
Malate + arsenite	390	390	0

DISCUSSION

The present results give experimental support to the hypothesis (Lipmann, 1941) that there may be direct transformation of fumarate to oxaloacetate without intermediary formation of malate. Clifton (1942), working with *Cl. tetani*, found that malate was not fermented, but Pickett (1943) in experiments with the same strain in which incubation was prolonged to 4 days showed that it was attacked very slowly. With *Cl. saccharobutyricum* GR4, malate is attacked at a somewhat higher rate than with *Cl. tetani*, although less rapidly than fumarate and oxaloacetate. This observation, and the different butyric acid/acetic acid ratio found for malate compared with fumarate excludes malate as an intermediate in fumarate fermentation. Evidence supporting this conclusion was obtained in the experiments with congo red and hydroxylamine.

It has also been found, incidentally, that the test organism reduces hydroxylamine to ammonia in the presence of fumarate or oxaloacetate; oximinol-succinic acid and aspartic acid are possibly intermediates in this reaction. Although the exact

mechanism is not yet certain, the phenomenon is interesting since many butyric acid bacteria have been described as nitrogen fixers, and hydroxylamine or ammonia have been proposed for other organisms as primary products of nitrogen fixation. The present experiments indicate that the hydroxylamine theory of Virtanen & Laine (1939, see also Virtanen, 1947), and the ammonia theory of Burris & Wilson (1945, 1946; see also Wilson & Burris, 1947), are not necessarily incompatible, since both substances give rise to aspartic acid with either fumarate or oxaloacetate. Woods (1938) has shown that hydroxylamine is reduced by molecular hydrogen to ammonia in the presence of suspensions of *Cl. welchii*. It is now shown that such reduction can also occur with other hydrogen donors; this point will be further studied. Another type of reaction between hydroxylamine and fumarate leading to hydroxyaspartic acid, which is not further utilized, has been reported by Jacobsohn & Soares (1936-7) for suspensions of *Esch. coli*.

SUMMARY

1. A study has been made of the comparative rates of fermentation of fumarate, malate, oxaloacetate and pyruvate by cell suspensions of *Clostridium saccharobutyricum* GR 4. The relative proportions of butyric and acetic acids formed were also measured.

2. The results indicate the following sequence of reactions

Malate is not an intermediate in the transformation of fumarate to oxaloacetate. This is supported by experiments with various inhibitors.

3. The organism reduces hydroxylamine to ammonia in the presence of fumarate or oxaloacetate.

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The Fate of Certain Organic Acids and Amides in the Rabbit

8. TOLUIC ACIDS AND AMIDES

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The metabolism of the toluic acids and their amides was investigated primarily in order to determine the effect of a nuclear substituted methyl group upon the hydrolysis of the carbamyl group *in vivo*. It was also of interest to compare the excretion products of the acids with those of the xylenes (Bray, Humphris & Thorpe, unpublished results) from which they are

derived *in vivo*. Further, since the toluamides provide examples of compounds with two different potential 'centres for conjugation' (cf. Bray, Ryman & Thorpe, 1948), the metabolic fates of these compounds could be compared with those of the xylenes, in which the two centres are identical in character.