Microbiology. — *On the fermentation of some dibasic C_4-acids by Aerobacter aerogenes.* By H. ALBERT BARKER. (Communicated by Prof. A. J. KLUYVER).

(Communicated at the meeting of April 25, 1936).

1. *Introduction.* Already in 1863 PASTEUR gave convincing proof that it was possible to bring about a fermentation of calcium tartrate and he also made some observations regarding the causative organism. Afterwards GAUTIER, FITZ and HOPPE SEYLER studied the chemical conversions involved in this fermentation but since these investigators worked with impure cultures, no great significance can be attached to their results. In 1897 GRIMBERT and FIQUET published a rather thorough qualitative investigation of the fermentation of tartrates by a bacterium called by them *Bacillus tartricus,* and in 1907 a study of a closely related organism, *Aerobacter tartarivorum,* was performed by NIJDAM. [1]) The latter has given a detailed description of the bacterium isolated by him and has also identified the various products formed not only in the fermentation of tartaric acid but also in that of several other related compounds. In recent years there seem to have been no further investigations in this direction. The interesting results obtained by QUASTEL [2]), to which we shall refer later, are only of indirect importance, since this author did not study the anaerobic but only the aerobic breakdown of succinic and fumaric acids.

From this survey of the literature it appears that until now no quantitative data regarding the fermentation of tartaric and other dibasic C_4-acids have been reported. It seemed, therefore, worthwhile to fill this gap, especially since it is evident from the studies mentioned above that the fermentation in question is not caused by a very specific organism. On the contrary it appears that the bacteria which have been isolated until now are identical with or closely related to *Aerobacter aerogenes* (Escherich) Beijerinck. With a view to the fact that the fermentation of carbohydrates by this species has been studied in great detail (HARDEN, SCHEFFER), it seemed particularly interesting to attempt to find out the way in which C_4-compounds are broken down anaerobically by the organism. For in the fermentation of hexoses everything points to a primary splitting of the substrate into two C_3-compounds which after decarboxylation yield C_2-compounds. C_4-compounds only occur in this

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1) H. W. M. NIJDAM, Aerobacter tartarivorum. Diss. Leiden (1907). The older literature will be found in this publication.

2) J. H. QUASTEL, Biochem. J. **18**, 365 (1924).

fermentation as products derived from the condensation of two C_2-compounds.

Even when we survey the whole of our knowledge regarding bacterial fermentation of carbohydrates, practically no instances are found of a break down of a C_4-compound. A single exception is the formation of acetone from acetoacetic acid. For these reasons a closer study of the fermentation of tartaric and related dibasic C_4-acids seemed also to be of some theoretical interest.

2. *Isolation and identification of the organism.* The tartaric acid fermenting organism was isolated from garden soil by an enrichment culture in the following medium made up with tapwater:

$(NH_4)_2$ tartrate (d)	1 %
K_2HPO_4	0.02 %
$MgSO_4$	0.01 %

The culture was incubated at 37° C. in an anaerobic bottle. After 3 days, when abundant development of bacteria had occurred with gas production, a transfer was made to the same medium. After another three days the second culture was plated out upon an agar medium of the same composition. This was incubated anaerobically. The colonies which developed were almost all of one type. The organism was apparently a member of the coli-aerogenes group and consequently the final isolation was carried out upon aerobic peptone-agar plates. When a pure culture of the organism was inoculated into the above mineral medium with tartaric acid, it caused an active fermentation with moderate evolution of gas.

As already mentioned, the organism isolated undoubtedly belongs to the coli-aerogenes group, since it is a facultatively anaerobic, non-spore forming rod which ferments glucose and lactose with acid and gas production. It can further be identified as belonging to the genus *Aerobacter* because 2—3 butylene glycol was shown to be one of the quantitatively important products of the fermentation of glucose. The organism is immotile, does not liquefy gelatin, and ferments glycerol and starch in the presence of peptone water, and therefore may be considered to belong to the species *Aerobacter aerogenes*. It seems to be identical in all important characters with NIJDAM's *Aerobacter tartarivorum*. The recognition of *A. tartarivorum* as a separate species does not appear to be desirable in as much as we have observed that several typical *A. aerogenes* strains from the pure culture collection are capable of fermenting tartaric acid; this property therefore is not distinctive for NIJDAM's bacterium.

3. *General remarks concerning the experiments made.* As for the the conditions under which the experiments were made the following remarks will suffice.

Although the isolation of our strain of *A. aerogenes* was carried out

at 37° C., preliminary experiments showed that it developed and fermented much more rapidly at 30° C. All subsequent cultures were therefore incubated at the latter temperature.

The cultures in which the fermentation products were estimated quantitatively were incubated under strictly anaerobic conditions. The media contained only inorganic salts (ammonia-N) in addition to the organic acid to be fermented and this was present in a concentration of 1.0—1.5 %. Higher concentrations were found not to be completely fermented. The substrate in a 1 % solution is completely decomposed in 5—6 days at 30° C. All media were adjusted to an initial p_H of 7.0—7.5. The fermentation of the three organic acids studied proceeds in such a way that no significant change of p_H occurs.

Since the substrates were practically completely fermented and the products formed appeared to be the usual products of carbohydrate fermentation, we can refrain from describing the analytical methods employed.

The fermentability of *d*-tartaric acid, *d*- and *l*-malic acids, fumaric acid, maleic acid and succinic acid in mineral media was tested. It appeared that only *d*-tartaric, *l*-malic and fumaric acids were decomposed. The results of the quantitative experiments made it seem desirable to deal first with the decomposition of fumaric acid.

4. *Fermentation of fumaric acid.* Data for the fermentation of fumaric acid are given in Table I.

Columns 1 and 2 are self-explanatory. Column 3 gives the percentage

TABLE I.

The fermentation of fumaric acid by Aerobacter aerogenes.

(Composition of medium: Na_2 fumarate 1.44%, $(NH_4)_2SO_4$ 0.05%, K_2HPO_4 0.03%, $MgSO_4$ 0.01% in tapwater).

Substance	gm.	% of carbon	Millimols observed	Millimols per 100 of substrate fermented	Milli-equivalents of "available hydrogen"	% "available hydrogen"
Substrate						
Initial fumaric acid	11.70					
Final fumaric acid	None					
Fermented fumaric acid	11.70	100	101	100	1212	100
Products recovered						
Carbon dioxide	2.90	16.3	65.9	65.3	—	—
Hydrogen	0.0095	—	4.7	4.7	9.4	0.8
Formic acid	2.15	11.6	46.8	46.4	93.6	7.7
Acetic acid	2.76	22.9	46.0	45.6	365	30.4
Succinic acid	5.42	45.5	45.9	45.5	643	53.0
Ethyl alcohol	0.276	1.5	6.0	5.9	70.8	5.8
Total		97.8				97.7

of the carbon of the substrate which is recovered in each product. In column 4 are given the quantities of substrate and fermentation products expressed in millimols actually observed. Column 5 expresses the same results in terms of 100 millimols of substrate decomposed, i.e., the figures represent the number of molecules of each product formed per 100 molecules of substrate fermented. In column 6 the milli-equivalents of "available hydrogen" present in the observed quantities of each compound have been given. By "available hydrogen" is meant the number of atoms of hydrogen which have to be removed from a molecule of a given compound in order to obtain complete dehydrogenation to carbon dioxide. The milli-equivalents mentioned in column 6 are therefore obtained by multiplying the millimols observed (column 4) with the specific factor of each compound. To cite an example: the specific factor of acetic acid is 8, since we have the equation $CH_3 . COOH + 2 H_2O \rightarrow 2 CO_2 + 8 H$; hence for the 45.6 millimols of $CH_3 . COOH$ found, the milli-equivalents of "available hydrogen" amount to $8 \times 45.6 = 365$. In column 7 the amounts of "available hydrogen" are expressed as percentages of the amount present in the fermented substrate.

The usefulness of these calculations follows directly from the necessity that in all fermentation experiments an oxidation-reduction balance must be taken into consideration in addition to the carbon balance. It is clear that in an ideal balance both the total amount of carbon and the total amount of "available hydrogen" of the substrate must be recovered in the products of the fermentation. Any shortage in the balances indicates, of course, errors in the analysis or failure to take into account all products formed. Any discrepancy between the two balances means a difference in the state of reduction of the missing product and the substrate. Since in all our analyses the amount of substrate converted into bacterial substance has not been determined, it is self-evident that there will always be a slight deficiency in both balances.

Considering the figures in Table I we may first conclude that in this fermentation the substrate fermented can be most satisfactorily accounted for by the products recovered.

The large amount of succinic acid and its obvious relation to fumaric acid makes it at once probable that it is formed by direct reduction of the substrate. This implies that another part of the substrate has been oxidized. The question then arises how this oxidation has proceeded. The occurrence of formic acid and acetic acid in approximately equimolecular quantities suggests that these acids have been derived from pyruvic acid [1]). This offers a strong indication that malic, oxalacetic and pyruvic acids occur as intermediates in the oxidation of fumaric acid as has also been postulated by QUASTEL (l.c.) in his study on the aerobic breakdown of fumaric acid.

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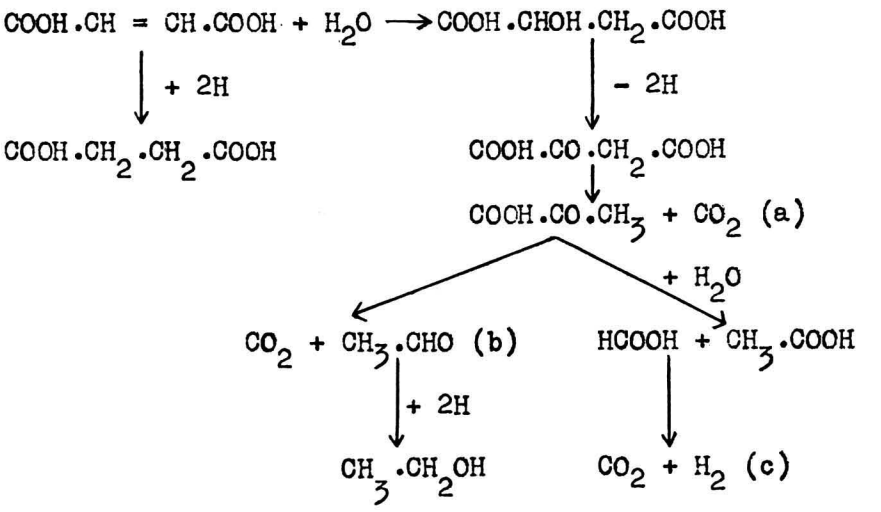
[1]) Cf. C. NEUBERG, Biochem. Zeitschr. **67**, 90 (1914).

These considerations lead to the following scheme:



The small quantity of hydrogen observed may well originate from a partial splitting of the formic acid, i.e., in the same way as has been made acceptable for the formation of hydrogen in the fermentation of carbohydrates by bacteria of the colon group[1]). The ethyl alcohol may owe its origin to a side reaction of the pyruvic acid leading primarily to carbon dioxide and acetaldehyde, which is then reduced under simultaneous dehydrogenation of another molecule of fumaric acid (malic acid).

It is obvious that the scheme given implies that several quantitative relations between the various fermentation products must be fulfilled.

1. The sum of the molecular quantities of hydrogen and formic acid ($4.7 + 46.4 = 51.1$) must equal that of acetic acid (45.6).

2. Since carbonic acid is assumed to be formed in three different reactions (denoted as (a), (b) and (c) in the scheme), the following relation must also hold. Each molecule of fumaric acid which is not reduced to succinic acid will yield a molecule of carbon dioxide (a); further, there will be extra carbon dioxide formed for each molecule of ethyl alcohol (b) and of hydrogen (c) formed. Therefore we must have the relation that the molecular quantity of the substrate minus that of the succinic acid ($100—45.5 = 54.5$) must equal the total amount of carbon dioxide less the amount of ethyl alcohol and of hydrogen ($65.3—5.9—4.7 = 54.7$).

3. Another feature of the proposed scheme is that compounds with two carbon atoms never arise directly from a splitting of the C_4-substrate, but always after successive splitting off of two compounds with one carbon atom (either carbon dioxide or formic acid). This means that the following relation should also hold. The sum of the molecular quantities of acetic

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[1]) Cf. A. TASMAN and A. W. POT, Biochem. J. **29**, 1749 (1935); A. TASMAN, Biochem. J. **29**, 2446 (1935).

acid and ethyl alcohol ($45.6 + 5.9 = 51.5$) will equal half the sum of the molecular quantities of carbon dioxide and formic acid

$$\left(\frac{65.3 + 46.4}{2} = 55.8\right).$$

Taking into account the inevitable experimental errors in the complicated analysis performed, the agreement between the experimental results and the requirements of the scheme may be deemed to be quite satisfactory. As for relation 1, we may add that in a second experiment the agreement between the sum of the quantities of hydrogen and formic acid ($6.0 + 46.3 = 52.3$) and the quantity of acetic acid (50.8) is still better. In this second experiment also the other relations deduced from the scheme were fulfilled.

From these results the conclusion seems justified that the scheme given represents in all essential respects the actual mechanism of the fermentation.

5. *The fermentation of l-malic acid.* From the presumed occurrence of malic acid as an intermediate in the fermentation of fumaric acid it should follow that malic acid will be decomposed by the bacterium in question according to the same scheme as given for the fermentation of fumaric acid, at least in so far as the organism will also be able to reduce this substrate to succinic acid. The results of the *l*-malic acid fermentation are given in Table II.

TABLE II.

The fermentation of l-malic acid by Aerobacter aerogenes.
(Composition of medium: Na *l*-malate 1.08%, $(NH_4)_2SO_4$ 0.05%, K_2HPO_4 0.03%, $MgSO_4$ 0.01% in tapwater).

Substance	gm.	% of carbon	Millimols observed	Millimols per 100 of substrate fermented	Milli-equivalents of "available hydrogen"	% "available hydrogen"
Substrate						
Initial malic acid	12.03					
Final malic acid	0.31					
Fermented malic acid	11.72	100	87.5	100	1049	100
Products recovered						
Carbon dioxide	2.24	14.5	51.0	58.3	—	—
Hydrogen	None					
Formic acid	1.75	10.9	38.1	43.6	76.2	7.26
Acetic acid	2.38	22.6	39.7	45.3	318	30.3
Succinic acid	4.58	44.3	38.8	44.3	544	51.9
Ethyl alcohol	Not estimated					
Total		92.3				89.5

The only qualitative deviation from the fermentation of fumaric acid is the absence of hydrogen. Several additional experiments confirmed the fact that hydrogen never is formed in the fermentation of malic acid by the particular strain of *A. aerogenes* used. No explanation can be offered for this fact.

As for the quantitative requirements of the scheme, we can apply the same tests as previously.

1. The molecular quantity of formic acid (43.5), no hydrogen being present, must equal that of acetic acid (45.3).

2. The molecular quantity of the substrate minus that of the succinic acid (100—44.3 = 55.7) must equal the total amount of carbon dioxide less the amount of ethyl alcohol (58.3—5.0 [1]) = 53.3).

3. The sum of the molecular quantities of acetic acid and ethyl alcohol (45.3 + 5.0 [1]) = 50.3) must equal half the sum of the molecular quantities of carbon dioxide and formic acid $\left(\frac{58.3 + 43.5}{2} = 50.9\right)$.

The evidence obtained from this fermentation balance offers strong support to the correctness of the scheme already proposed on the basis of the results of the fumaric acid fermentation.

6. *The fermentation of d-tartaric acid.* Because of the successful outcome of the interpretation of the two foregoing fermentations it was tempting to investigate whether a similar scheme could be applied in the fermentation of tartaric acid. The results of such a fermentation are given in Table III.

Tartaric acid being more oxidized than the two substrates previously considered and having the same state of oxidation as oxalacetic acid, one might expect that in this fermentation no succinic acid at all would have been formed. Experience shows, however, that an appreciable quantity is still produced, although much less than in the foregoing fermentations. This necessitates a simultaneous dehydrogenation process in some other part of the scheme. Obviously the hydrogen required for the succinic acid production can only arise from a dehydrogenation of acetaldehyde derived from the pyruvic acid formed as an intermediate. Since the amount of acetaldehyde formed in the previous fermentations would be insufficient to provide the necessary hydrogen, we have to assume that in the oxidized medium of a tartrate fermentation the splitting of the pyruvic acid into acetaldehyde and carbon dioxide will be favoured as compared with the conversion into acetic and formic acids. The acetaldehyde when acting as a hydrogen donator will be expected to be converted either into acetic acid or into other compounds of the same state of oxidation.

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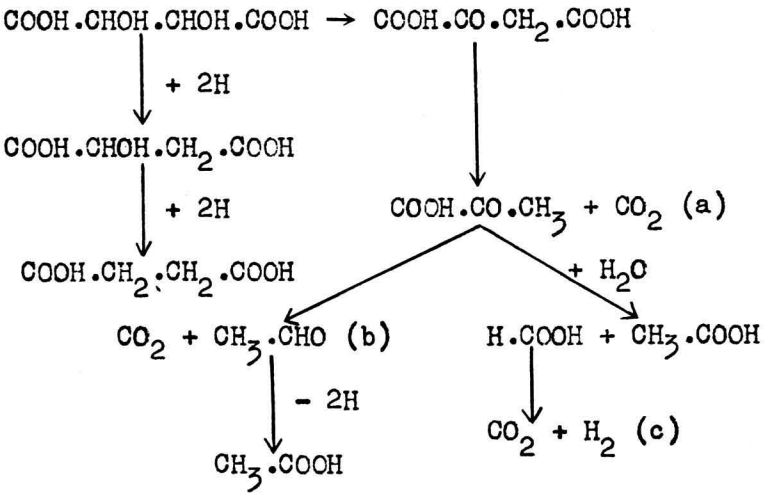
[1]) In this particular experiment the amount of ethyl alcohol was not determined, although qualitative tests showed its presence in small amounts. The figure given must be considered as a rough estimation based on the results of other experiments.

TABLE III.

The fermentation of d-tartaric acid by Aerobacter aerogenes.
(Composition of medium: NH_4 *d*-tartrate 1.21%, K_2HPO_4 0.03%, $MgSO_4$ 0.01% in tapwater).

Substance	gm.	% of carbon	Millimols observed	Millimols per 100 of substrate fermented	Milli-equivalents of "available hydrogen"	% "available hydrogen"
Substrate						
Initial tartaric acid	11.00					
Final tartaric acid	0.16					
Fermented tartaric acid	10.84	100	72.2	100	722	100
Products recovered						
Carbon dioxide	3.99	31.4	90.6	125.5	—	—
Hydrogen	0.0066	—	3.3	4.6	6.6	0.91
Formic acid	1.484	11.13	32.3	44.8	64.6	8.95
Acetic acid	3.00	34.6	50.0	69.3	400	55.5
Succinic acid	1.40	16.4	11.9	16.5	167	23.2
Ethyl alcohol	0.049	0.72	1.07	1.48	12.8	1.77
Acetyl methyl carbinol	Trace					
Total		94.2				90.3

These considerations lead to the following scheme:



Since in this fermentation acetic acid originates from two independent reactions, it is clear that here there should no longer be equality between the molecular quantities of formic and acetic acids. However, in support

of the scheme the remaining two tests can be applied, although somewhat modified.

1. The molecular quantity of the substrate minus that of the succinic acid ($100—16.5 = 83.5$) should equal the total amount of carbon dioxide (125.5) less the amount produced in the reactions (b) and (c). Carbon dioxide formed in (b) is measured by the amount of ethyl alcohol (1.5) and of acetic acid (69.3) less the amount of formic acid (44.8) and hydrogen (4.6). The carbon dioxide formed in the reaction (c) may be taken equal to the hydrogen produced (4.6). Therefore 83.5 should equal $125.5—4.6—69.3+44.8+4.6+1.5 = 99.5$.

2. The sum of the molecular quantities of acetic acid and ethyl alcohol ($69.3 + 1.5 = 70.8$) should equal half the sum of the molecular quantities of carbon dioxide and formic acid $\left(\frac{44.8 + 125.5}{2} = 85.1\right)$.

3. A third consequence of this particular scheme is that the molecular quantity of succinic acid (17.5) should equal half the total amount of acetic acid (69.3) less the formic acid (44.8) and hydrogen (4.6) and less the amount of ethyl alcohol (1.5). Therefore 17.5 should equal $\frac{69.3—44.8—4.6—1.5}{2} = 9.2$.

It cannot be denied that the agreement between the requirements of the scheme and the actual amounts observed is not very satisfactory. This cannot be due to analytical errors since three further experiments yielded essentially the same results. The agreement would be better if the reduction of the tartaric acid would not have yielded succinic acid but would have proceeded only as far as malic acid. A careful examination of the nonvolatile acids failed, however, to detect more than traces of malic acid. Therefore the lack of agreement can be best explained by supposing that there is a shortage in oxidation products of acetaldehyde. This also seems to be indicated by the fact that on the basis of the carbon dioxide of reaction (b), which can be calculated, more conversion products of acetaldehyde should be expected. Notwithstanding the slight quantitative deviation from expectation, it seems reasonable to accept this scheme as representing the main lines of tartaric acid fermentation.

Conclusion.

In the foregoing sections arguments have been given in favour of the view that the fermentation by *Aerobacter aerogenes* of the dibasic C_4-acids studied proceeds by an initial oxidation-reduction between two molecules of the substrate followed by a decomposition of the oxidized molecule into much the same products as are obtained in the fermentation of carbohydrates. The characteristic feature of this breakdown is that the C_4-compound is always first converted into a C_3-compound, the latter being decomposed, as in carbohydrate fermentation, into a C_2- and a C_1-com-

pound. Although C_2-compounds are most common amongst the fermentation products, they never seem to be derived from a direct splitting of a C_4-molecule.

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Embryology. — *Gebiss- und Zahnentwicklung bei der Irisforelle (Salmo irideus).* V. *Gaumen und Schlundkopf.* Von B. VAN DER EYKEN. (Communicated by Prof. M. W WOERDEMAN.)

(Communicated at the meeting of April 25, 1936).

a. *Gaumen* [s. Fig. 1] :

Etwas später als die Zwischen- und Oberkieferzähne fangen die Gaumenzähne mit ihrer Entwicklung an und das erste Zahnelement in unserer Präparatenserie findet sich in der Larve *H* [1]), wo beiderseits auf dem Palatinum eine kleine Papille sich angelegt hat.

Im Stadium *N* sind links und rechts schon je drei Keime vorhanden, von denen der mittlere der älteste ist und ein Scherbchen Dentin besitzt; rechts im Präparate liegt, mesial von diesem Zahne, eine schön gestaltete aber dentinlose Papille, während distal noch eine kleine Papille folgt. Links ist auch der mittlere Zahn der älteste, wegen einer Beschädigung der betreffenden Schnitte ist es aber unmöglich zu entscheiden, ob der mesiale oder der distale Keim in Alter folgt, wahrscheinlich ist die mesiale Anlage die jüngste.

Das nächst ältere Stadium ist **47**, wo der linke Gaumen drei Zähne besitzt und der rechte vier. Links hat sich wieder der mittlere Keim zuerst angelegt und zeigt eine allerdings dünne Dentinscherbe; die mesiale Anlage ist eine kleine aber deutliche Papille, während distal eine sehr junge Papille folgt, welche noch sehr an der Oberfläche liegt und eine flache Form zeigt. Von den Keimen auf dem rechten Gaumen ist der meist distale der jüngste und wird durch eine sehr junge und flache Papille dargestellt. Die mesial folgende Anlage ist die älteste, während sich noch mehr mesialwärts eine Papille befindet, welche sich als Nummer zwei angelegt haben muss. Unmittelbar lateral an diesen Keim anschliessend sehen wir eine kleine Epithelausbuchtung, in welcher einige Bindegewebszellen liegen, es ist aber nicht sicher, ob wir diese Erscheinung als eine andere Papille aufzufassen haben, weil in andern Präparaten auf dieser Stelle öfters gleichartige Gebilde sichtbar sind, welche zweifellos keine Zahn-

[1]) Für Einzelheiten betreffs Grösze und Alter der Larven und Weise der Untersuchung sei auf die Proceedings **38**, No. 8, 1935 verwiesen. S. auch diese Proceed. **38**, No. 10, 1935, **39**, No. 2 und 4, 1936.