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Stanier, R. Y.; Adams, G. A.

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The Nature of the *Aeromonas* Fermentation

By R. Y. STANIER and G. A. ADAMS, *Division of Applied Biology, National Research Council, Ottawa, Canada*

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The genus *Aeromonas* was established by Kluyver & van Niel (1936) for pseudomonads fermenting carbohydrates with the production of carbon dioxide and hydrogen. Since von Wolzogen Kühr (1932) had previously shown that a member of this group, *Pseudomonas fermentans*, gave a positive Voges-Proskauer reaction, the existence of a butylene glycol fermentation in the genus was suspected, although the absence of exact data on the products of fermentation left this point unsettled.

During the course of an intensive investigation on the bacteriological production of 2:3-butylene glycol (Adams & Stanier, 1944), it seemed desirable to make a somewhat closer study of this little-known *Aeromonas* fermentation. The recent discovery of polar flagellation in *P. oleus hydrophilus* (Kulp & Borden, 1942; Guthrie & Hitchner, 1943), taken in conjunction with its fermentative abilities, mark this organism as an *Aeromonas* species, and since cultures were readily available it was chosen as a representative of the group.

MATERIALS AND METHODS

Through the kindness of Dr W. L. Kulp and Dr E. R. Hitchner we obtained a number of strains of *Aeromonas hydrophila*. Preliminary analyses having shown that all these organisms were capable of producing substantial amounts of ethanol and 2:3-butylene glycol from sugars, a strain with particularly vigorous fermentative properties was selected for more detailed quantitative investigations.

The basal medium employed consisted of yeast extract 1·25 g., $CaCO_3$ 1·0 g., $MgSO_4$ 0·025 g., 1·0 M-phosphate buffer (pH 7·4) 0·65 ml., tap water 250 ml. The carbohydrates used were sterilized separately in concentrated aqueous solution and added to the basal medium just before inoculation in amounts giving a final concentration of 1–2%. Fermentations were carried out at 30° and allowed to run for 3–4 days. During this period a slow stream of oxygen-free nitrogen was passed through the flasks. At the end of the fermentation, an excess of HCl was added to dissolve the residual $CaCO_3$, and aeration with nitrogen was continued for several hours to sweep out all the CO_2.

Carbon dioxide was determined gravimetrically. Hydrogen was continuously oxidized by passage over heated copper oxide and determined as water. After completion of the fermentation, the fermented solution was neutralized and made up to 500 ml. A measured fraction was made alkaline and extracted continuously with ether in order to remove butylene glycol and acetoin. The ether extract was evaporated, and the residue taken up in water. Butylene glycol was determined by an unpublished method developed in the Department of Biochemistry at the University of Wisconsin. The butylene glycol is oxidized with periodic acid, and the excess periodate determined iodometrically in neutral solution. Acetoin was estimated by the method of Langlykke & Peterson (1937). After extraction of the alkaline solution with ether, it was made acid and re-extracted with ether to remove the organic acids, which were taken up in water again after removal of the ether. The acid extract was steam distilled and the volatile portion estimated as acetic acid by titration. The non-volatile fraction contained lactic and succinic acids. The former was determined by the method of Boyland (1928), the latter by the formation of the silver salt. Pyruvic acid, when present, was withheld from going into the ether acid extract by saturating the solution with sodium bisulphite. Ethanol was determined by dichromate oxidation, following fractional distillation of a measured fraction of the fermentation solution.

Pyruvic acid was determined manometrically by ceric sulphate oxidation (Krebs & Johnson, 1937). Xylose estimations were carried out by the copper reduction method of Schoorl (1929). Glucose was determined by the Hanes (1929) modification of the Hagedorn-Jensen procedure.

RESULTS

Carbon balances were obtained for the fermentations of glucose, xylose and pyruvic acid. An attempt was also made to study the fermentation of mannitol but, largely owing to the slow and incomplete attack on this compound, a satisfactory balance was not obtained.

Fermentation of glucose. The products of glucose fermentation by *A. hydrophila* are shown in Table 1. The main organic ones are always butylene glycol, ethanol and lactic acid; in addition, smaller amounts of acetoin, acetic acid and succinic acid are found. Fluctuations in the quantitative relationships between the various products may occur from one experiment to the next; thus, CO_2 production can vary from 140 to 170 mol. per 100 mol. of glucose fermented, with concomitant differences in the relative amounts of C_2 and C_3 compounds. The ratio of butylene glycol to ethanol can also fluctuate over quite a wide range, although it is usually about 1 : 1 on a molecular basis.

In order to establish beyond doubt the nature of the glycol produced in this fermentation, a small sample was isolated and characterized. About 15 l. of a fermented yeast extract-glucose medium were

Table 1. *Fermentation of glucose by* Aeromonas hydrophila

Glucose fermented: 5·490 g.

Products	Weight (g.)	Number of mol./100 mol. of glucose fermented: Product	Carbon	Calculated CO_2	Calculated H_2	Oxidation values: +	Oxidation values: −
2:3-Butylene glycol	1·502	54·7	218·8	109·4	54·7	—	164·1
Acetoin	0·047	1·7	6·8	3·4	3·4	—	3·4
Ethanol	0·730	52·0	104·0	52·0	—	—	104·0
Acetic acid	0·085	4·6	9·2	4·6	9·2	—	—
Lactic acid	0·639	23·3	69·9	—	—	—	—
Succinic acid	0·130	3·6	14·4	−3·6	−3·6	3·6	—
CO_2	2·230	166·2	166·2	—	—	332·4	—
H_2	0·035	57·5	—	—	—	—	57·7
Total	—	—	589·3	165·8	63·7	336·0	329·2

Carbon recovery ... ... 98·2%
CO_2 calculated/CO_2 observed ... 0·997
H_2 calculated/H_2 observed ... 1·108
O/R index ... ... 1·021

evaporated to a volume of 4 l. in an open steam kettle, saturated with anhydrous K_2CO_3, and extracted with twice the volume of butanol. Most of the butanol was removed by distillation through a short Vigreux column, the residual extract was distilled under reduced pressure in a Stedman column and the pure glycol fraction recovered. Oxidation of a small sample of this glycol with bromine and $FeCl_3$ gave a good yield of diacetyl, which was characterized by the melting-point of its bis-phenyl hydrazone. The production of diacetyl on oxidation proved conclusively that the glycol was 2:3-butylene glycol. Judging from its optical rotation (−0·97°), it consists of a mixture of the *l*- and meso-forms with a preponderance of the latter. Thus it differs both from the 2:3-butylene glycol produced in the *Aerobacter* fermentation, which is a *d*-meso mixture, and from that produced in the *Aerobacillus* fermentation, which is the pure *l*-isomer.

Fermentation of xylose. Table 2 presents the carbon balance for a xylose fermentation by *A. hydrophila*. In this particular experiment the butylene glycol-ethanol ratio was somewhat less than 1:1, but this is not characteristic; ratios of 1:1 or over have been obtained in other similar experiments. Thus there are no significant differences, either qualitative or quantitative, between the end-products of pentose and hexose fermentation by *A. hydrophila*. This indicates very strongly that there must be a resynthesis from the xylose (or from the C_2 fragments of the xylose) of a C_6 compound which is subsequently decomposed in a manner similar to that operative in glucose dissimilation. Unless one is willing to assume the production of CO_2 from a C_2 compound—a reaction unknown in fermentative processes—a simple calculation shows the necessity for this assumption. Were the end-products of pentose fermentation derived directly from the fragments of a C_3-C_2 split, the CO_2 production could not exceed 100 mol./100 mol. of pentose fermented, since no CO_2 could be derived from the C_2 fraction. Furthermore, the production of C_3 compounds will reduce this possible maximum of CO_2 production by an amount equivalent to the number of mols of C_3 compound formed. In the experiment shown in Table 2, the observed CO_2

Table 2. *Fermentation of xylose by* Aeromonas hydrophila

Xylose fermented: 4·010 g.

Products	Weight (g.)	Number of mol./100 mol. of xylose fermented: Product	Carbon	Calculated CO_2	Calculated H_2	Oxidation values: +	Oxidation values: −
2:3-Butylene glycol	0·939	39·0	156·0	78·0	39·0	—	117·0
Acetoin	0·061	2·6	10·4	5·2	5·2	—	5·2
Ethanol	0·600	48·9	97·8	48·9	—	—	97·8
Acetic acid	0·150	9·3	18·6	9·3	18·6	—	—
Lactic acid	0·491	20·4	61·2	—	—	—	—
Succinic acid	0·036	1·1	4·4	−1·1	−1·1	1·1	—
CO_2	1·584	134·7	134·7	—	—	269·4	—
H_2	0·029	53·9	—	—	—	—	53·9
Total	—	—	483·1	140·3	61·7	270·5	273·9

Carbon recovery ... ... 96·6%
CO_2 calculated/CO_2 observed ... 1·041
H_2 calculated/H_2 observed ... 1·126
O/R index ... ... 0·988

Table 3. *Fermentation of pyruvic acid by* Aeromonas hydrophila

Pyruvic acid fermented: 3·530 g.

	Weight (g.)	Number of mol./100 mol. of pyruvic acid fermented					
		Product	Carbon	Calculated CO_2	Calculated H_2	Oxidation +	Oxidation −
Pyruvic acid fermented	3·530	—	—	—	—	—	100
Products							
2:3-Butylene glycol	0·125	3·5	14·0	7·0	−3·5	—	10·5
Acetoin	0·021	0·5	2·0	1·0	—	—	1·0
Ethanol	0·038	2·1	4·2	2·1	−2·1	—	4·2
Acetic acid	1·800	74·8	149·6	74·8	74·8	—	—
Lactic acid	0·776	21·5	64·5	—	−21·5	—	—
CO_2	1·479	83·8	83·8	—	—	167·6	—
H_2	0·035	43·6	—	—	—	—	43·6
Total	—	—	318·1	84·9	47·7	167·6	159·3

Carbon recovery ... ... 106·3%
CO_2 calculated/CO_2 observed ... 1·013
H_2 calculated/H_2 observed ... 1·102
O/R index ... ... 1·052

production amounted to 135 mol./100 mol. of xylose fermented: i.e. 35 mol. more than the possible maximum from a C_3-C_2 split and 58 mol. more than the amount to be expected after taking into account the lactic and succinic acid formed. It would be interesting to determine the effect of *A. hydrophila* on glycol aldehyde, the C_2 compound which one might expect to result from the primary fission of the pentose molecule.

Fermentation of pyruvic acid. Aeromonas hydrophila causes a vigorous fermentation of pyruvic acid. The balance sheet for this fermentation is shown in Table 3. Surprisingly enough, the production of butylene glycol, acetoin and ethanol is largely suppressed, and the chief organic end-products are acetic and lactic acids. Approximately three molecules of pyruvic acid are oxidized to acetic acid for every one reduced to lactic acid, and consequently a considerable volume of hydrogen is produced. The three main reactions appear to be:

$$3CH_3.CO.COOH + 3H_2O \rightarrow 3CH_3.COOH + 3CO_2 + 6H,$$
$$CH_3.CO.COOH + 2H \rightarrow CH_3.CHOH.COOH,$$
$$4H \rightarrow 2H_2.$$

Thus the dismutation of pyruvic acid by *A. hydrophila* belongs to the type demonstrated for heterofermentative lactic acid bacteria (Nelson & Werkman, 1936) and for staphylococci (Krebs, 1937), with the difference that in the latter fermentations there is an equimolecular production of the two acids and consequently no hydrogen formation.

The dissimilation of pyruvic acid by *A. hydrophila* differs notably from that which occurs in the other two bacterial groups where one finds a butylene glycol fermentation. When growing anaerobically on pyruvic acid, *Aerobacillus polymyxa* produces principally acetoin and acetic acid (Adams & Stanier, 1944), while under similar conditions *Aerobacter aerogenes* gives rise to butylene glycol and acetic acid (Mickelson, Reynolds & Werkman, 1936).

DISCUSSION

These investigations have definitely established the existence of a butylene glycol fermentation in a bacterial group where it was previously not known. Thus there are at present three morphologically widely separated bacterial families—the Enterobacteriaceae (*Aerobacter* and *Serratia*), the Bacillaceae (*Aerobacillus*) and the Pseudomonadaceae (*Aeromonas*) in which a butylene glycol fermentation occurs. In each family there are also organisms which carry out entirely different carbohydrate fermentations; in the Pseudomonadaceae, the alcoholic fermentation of *Zymomonas lindneri*: in the Enterobacteriaceae, the mixed acid fermentation of *Escherichia coli* and other species: and in the Bacillaceae a wide variety of dissimilatory processes (acetone-butanol, acetone-ethanol and other fermentations) performed by various organisms.

This absence of correlation between morphological and biochemical characters is of considerable interest from the standpoint of comparative biochemistry. It suggests very strongly that the present fermentative mechanisms for the dissimilation of carbohydrates have developed independently along similar lines in different branches of the bacteria. This hypothesis is supported by the slight but significant differences which characterize a particular fermentation as carried out by organisms belonging to different morphological groups. A good example is the differences in optical rotation of the butylene glycols produced by *Aerobacter*, *Aeromonas* and *Aerobacillus*.

SUMMARY

1. *Aeromonas hydrophila*, a typical representative of the genus *Aeromonas* Kluyver and van Niel, causes a butylene glycol fermentation of carbohydrates. The end-products from glucose are 2:3-

butylene glycol, ethanol, acetic and lactic acids, CO_2 and H_2, with traces of acetoin and succinic acid. The 2:3-butylene glycol is a mixture of the *l*- and meso-forms.

2. The products of xylose fermentation are very similar, both quantitatively and qualitatively, to those of glucose fermentation. This is regarded as evidence against a simple C_3-C_2 split of the pentose molecule.

3. The main products of the fermentation of pyruvic acid are lactic and acetic acids, CO_2 and H_2. Approximately three molecules of pyruvic acid are oxidized to acetic acid for every one reduced to lactic acid.

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Experiments on the Methylation and Acetylation of Wool, Silk Fibroin, Collagen and Gelatin

By S. BLACKBURN and H. PHILLIPS, *The Wool Industries Research Association, Torridon, Leeds*

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In a previous paper (Blackburn, Carter & Phillips, 1941) it was shown that wool and silk fibroin could be *O*-methylated either by contact with methyl bromide or iodide at room temperatures, or with methyl sulphate in buffers within the pH range 2·5–8·5. With both proteins the number of CH_3 groups introduced by repeated treatment with methyl sulphate was greater than could be accommodated on the free carboxyl groups of glutamic and aspartic acids, and it was suggested that certain 'activated' peptide linkages methylated as well as the free carboxyl groups of the salt-linkages. CH_3I and CH_3Br introduced fewer CH_3 groups into wool and silk fibroin than did methyl sulphate.

The present paper describes the extension of this work to the methylation of collagen and gelatin by similar methods, and the methylation with methyl sulphate and CH_3Br of wool which has been chemically modified by alkali, acid, nitrous acid and formaldehyde. In addition, the simultaneous acetylation and methylation of wool, silk fibroin and collagen by the combined action of methanol and acetic anhydride has been investigated.

METHODS

Methylation of collagen and gelatin with methyl sulphate and methyl halides

The collagen used in these investigations was a limed hide which had been delimed with acetic acid and then dehydrated in acetone. The gelatin was Coignet's Gold Label. The methylation procedure was similar to that described for wool and silk (Blackburn *et al.* 1941), with certain modifications to suit the properties of collagen and gelatin. Both proteins were cut into small pieces and acetate buffer was used for the methylations with methyl sulphate. At the end of each period of methylation about 1 g. of collagen was removed, washed in three changes of water and then shaken mechanically with water (25 ml.) for 1 hr. It was then dehydrated with acetone, air-dried and conditioned.* The moisture content of the collagen, the physical properties of which appeared to be unaltered, was determined by Barritt & King's method (1926). During methylation, the gelatin absorbed water and swelled greatly. The resulting gel and solution was dialyzed in cellophan against water for 3 days, the water being changed frequently. The resulting gelatinous mass was dried *in vacuo* below 60°.

Before methylation with either CH_3Br or CH_3I, the collagen was allowed to reach equilibrium with a borate buffer of pH 8, and then dehydrated with acetone and conditioned. The gelatin was treated dry as bought. After methylation, both proteins were rinsed in several changes of benzene and then conditioned. When proteins are methylated by either CH_3Br or CH_3I, one halogen anion is liberated for each CH_3 group introduced. To provide an additional measure of their degree of methylation, the halogen content of some of the methylated collagens and gelatins was therefore determined by the open Carius method (Peters & Van Slyke, 1932). The methylated gelatin

* All proteins described in this paper as conditioned had been exposed to a controlled atmosphere of 21° and 70% R.H. until they attained a constant weight. Although all the analyses were made on the conditioned proteins, the results are recorded as percentages of the anhydrous material.