

## STUDIES ON THE LUMINOUS BACTERIA

### II. SOME OBSERVATIONS ON THE ANAEROBIC METABOLISM OF FACULTATIVELY ANAEROBIC SPECIES

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In view of the fact that all of the strains of luminous bacteria used in the present series of investigations (Doudoroff, 1942) were found to be capable of developing anaerobically in suitable media, it seemed desirable to study the fermentative dissimilations carried out by these organisms, in the hope of contributing to a better understanding of this group of bacteria from the physiological, taxonomic and ecological points of view. The problem seemed to be of particular interest because the luminous bacteria are morphologically related to a number of facultative anaerobes unquestionably belonging to the family Pseudomonadaceae, and at present scattered through the genera *Pseudomonas*, *Phytomonas*, *Flavobacterium*, and *Achromobacter*. The literature offers little information on the types of anaerobic metabolism to be found in such polarly flagellated organisms beyond the routine observations on acid and gas production with various carbohydrate substrates.

#### EXPERIMENTAL

Glucose was a satisfactory fermentable substrate for the growth of all the species studied in peptone-containing media under anaerobic conditions. The anaerobic development of *Photobacterium phosphoreum* and *Photobacterium fischeri* was poor in peptone media without sugar; *Photobacterium splendidum*, *Photobacterium sepiacae*, and *Achromobacter harveyi* grew somewhat better, but still to a considerably lesser extent than with glucose. The addition of glycerol to peptone media did not increase the development of any of the species.

In glucose-peptone medium (consisting of  $m/30$  Sørensen  $KH_2PO_4$ - $Na_2HPO_4$  buffer at pH 7.2 or 7.5 with 3% NaCl, 1% Bacto-Peptone and 0.5–1.0% glucose by weight) all strains produced acid, usually lowering the pH value to about 5.5, and only one species, *P. phosphoreum*, produced visible gas. Upon analysis of the products of fermentation, it appeared that except for minor differences, all of the species showed essentially the same type of sugar dissimilation, similar to the "mixed acid" fermentations characteristic of the Enterobacteriaceae. The principal products found were formic, acetic, lactic and succinic acids, ethyl alcohol,  $CO_2$ , and, in the case of *P. phosphoreum*, hydrogen.

Quantitative or semi-quantitative determinations of the products of fermentation were made with growing cultures of three strains of *P. phosphoreum* (Strains #2, #12, "Delft Strain") and with all of the other species under investigation. Sugar determinations were made with the ferricyanide reduction method de-

scribed by Hassid (1937). None of the major products of dissimilation were found to interfere with such determinations. Alcohol was distilled from the neutralized culture medium, redistilled with alkaline HgO (Friedman and Klaas, 1932) and quantitatively estimated by the reduction of acid dichromate. It was identified after oxidation to acetic acid (Duclaux distillation and sodium uranyl acetate). The organic acids were extracted from the culture medium after treatment with tungstic acid by liquid ether extraction and then separated into the steam-volatile and non-volatile fractions. Formic acid was identified microchemically as the cerous or lead salt and its amount computed from the weight of calomel formed by reduction of HgCl<sub>2</sub>. Acetic acid was recognized as sodium uranyl acetate and estimated by subtraction of the amount of formic acid from the total volatile acid value. The correctness of the ratio so obtained was checked by Duclaux distillation of the volatile acid fraction. Lactic acid was identified microchemically as zinc lactate or by decomposition to acetaldehyde. Its quantity was determined by the method of Hartmann and Hillig (1933). Succinic acid was qualitatively and quantitatively determined by the use of succinic dehydrogenase preparations in a Warburg respirometer. Hydrogen was identified by quantitative combustion and both hydrogen and CO<sub>2</sub> were determined as free and dissolved gases with the help of the Van Slyke apparatus. Acetylmethylcarbinol was recognized through various modifications of the Voges-Proskauer test, carried out with distillates of the culture medium boiled with FeCl<sub>3</sub>. 2,3-butylene glycol was identified and determined by the method of Knipphorst and Kruisheer (1937) with special precautions to avoid possible errors due to the presence of acetylmethylcarbinol.<sup>1</sup>

*Fermentation of glucose by P. fischeri, P. splendidum, P. sepiac and A. hareyi*

In glucose-peptone medium, the strains tested produced chiefly formic and acetic acids and ethyl alcohol. All produced some succinic acid as well as small amounts of CO<sub>2</sub>. The principal difference observed among the various species appeared to be in the relative amounts of lactic acid formed. *P. fischeri* produced lactic acid in appreciable quantities, *P. harveyi* formed less, while *P. splendidum* and *P. sepiac* produced very little, if any, from sugar. Minute amounts of acetylmethylcarbinol could be detected in cultures of *P. fischeri* and *P. splendidum*, and slight traces with the other species, but no 2,3-butylene glycol was ever found among the products of fermentation, nor was hydrogen ever detected.

A fermentation balance obtained in an experiment with *P. fischeri* is presented in table 1. In the first column the figures represent the amounts of the various products of fermentation corrected only for blank determinations made with the uninoculated medium. In the second column, the quantities have been further corrected by subtraction of values obtained in a parallel experiment, in which the bacteria were allowed to grow in the peptone medium without added sugar.

<sup>1</sup> I wish to thank Mr. J. R. Gilliland for his kind cooperation in making the determinations of 2,3-butylene glycol, and in establishing the validity of the tests in the presence of the various constituents of the fermentation mixtures.

Carbon and "available hydrogen" balances based on the determinations (Barker, 1936) are included, as are the ratios of one-carbon to two-carbon derivatives found among the dissimilation products. In view of the generally accepted scheme for the formation of  $C_1$  and  $C_2$  compounds in sugar fermentation by the cleavage of three-carbon intermediates, the ratio of their derivatives serves as a valuable index to the validity of fermentation balances by comparison with the theoretical value of 1.0. Formic acid and  $CO_2$  are the chief  $C_1$  end products,

TABLE 1

*Products of glucose dissimilation by P. fischeri and P. phosphoreum in glucose-peptone medium*

Quantities of glucose utilized and of products recovered given in millimols, per 75 ml. of culture medium.

	P. FISCHERI*		P. PHOSPHOREUM†	
	A	B	A	B
Glucose utilized.....	0.850	0.850	0.694	0.694
Found:				
Hydrogen.....	0.00	0.00	0.380	0.380
$CO_2$ .....	0.094	0.077	0.510	0.334
Formic acid.....	0.840	0.825	0.662	0.650
Acetic acid.....	0.602	0.484	0.428	0.304
Ethyl alcohol.....	0.540	0.540	0.560	0.560
Lactic acid.....	0.565	0.482	0.480	0.285
Succinic acid.....	0.084	0.062	0.062	0.042
Acetylmethylcarbinol.....	Trace	Trace	Trace	Trace
2,3-butylene glycol.....	0.000	0.000	0.004	0.004
Carbon recovered.....	103.3%	91.1%	113.6%	90.1%
Available hydrogen recovered.....	102.6%	91.4%	111.3%	89.7%
Ratio $C_1/C_2$ derivatives‡.....	0.82	0.88	1.24	1.18

Column A—amounts of fermentation products, corrected by subtraction of values obtained with uninoculated medium.

Column B—same, corrected by subtraction of values obtained with bacterial cultures in sugar-free medium.

\* Medium initially at pH 7.5, containing 0.75% glucose.

† Medium initially at pH 7.2, containing 0.5% glucose.

‡ 2,3-butylene glycol included with 2-carbon compounds; succinic acid with one-carbon derivatives (see text).

while alcohol and acetic acid are the principal  $C_2$  compounds. For the computation of  $C_1/C_2$  ratio, succinic acid has been included with the  $C_1$  derivatives in view of the accumulating evidence for the formation of succinic acid through the reduction of  $CO_2$  and its addition to a three-carbon compound (Wood, C. W., 1941). That succinic acid may be produced in a similar manner by luminous bacteria is indicated by experimental findings with *P. phosphoreum* to be discussed further.

The small amounts of the products recovered make it difficult to place too much emphasis on the carbon and hydrogen balances, especially because of the large amount of peptone in the medium, which not only necessitated substantial corrections in some of the determinations, but made it impossible to conclude just how much of the products recovered had actually originated from the sugar. Small amounts of unidentified non-volatile acid were produced in media with, as well as without, sugar. From comparison of the values corrected for metabolites produced in sugar-free peptone medium (Column B) with those not so corrected (Column A) it would seem that the former give a slightly better agreement, but that the corrections are, most probably, justified only in part, or for some of the compounds rather than others. The good agreement of the carbon with the hydrogen balance may be more or less fortuitous, especially since unidentified products were certainly formed, and since the  $C_1/C_2$  ratio was at best below 0.9.

In fermentations with *P. splendidum*, *P. sepiae* and *A. harveyi*, which grow to a somewhat greater extent without sugar, greater amounts of unidentified acidic products occurred, and it was even more difficult to decide how much of the recognized compounds arose from the dissimilation of sugar. Thus, the very small quantities of lactic acid produced by *P. sepiae* and *P. splendidum* in the presence of glucose were almost matched by the amounts formed in sugar-free peptone medium. Further experiments with resting cells, or with the use of synthetic media, may well be worth while for the elucidation of this problem.

#### *Fermentations with P. phosphoreum*

*Fermentation of glucose.* In addition to the compounds occurring in the anaerobic dissimilation of sugar by *P. fischeri*, hydrogen was produced by all the available strains of *P. phosphoreum*. It appeared only after the cultures were allowed to develop for some time and an appreciable amount of formic acid had accumulated.  $CO_2$  appeared in relatively greater quantities than with *P. fischeri*. Some, though apparently not all, strains produced minute amounts of 2,3-butylene glycol; this compound seemed to appear in the later stages of fermentation, recalling the interesting observations by Mickelson and Werkman (1938), and Silverman and Werkman (1941) on the influence of the reaction of the culture medium on the production of acetylmethylcarbinol by *Aerobacter*.

Fermentation balances obtained with Strain #2 growing anaerobically in a glucose-peptone medium are presented in Table I. As with *P. fischeri*, subtraction of values obtained in cultures without glucose from the quantities of fermentation products recovered gives more reasonable balances, but the same uncertainty as to the validity of the corrections remains. The ratio of one-carbon to two-carbon derivatives is rather high, being in the neighborhood of 1.2. In the computation, 2,3-butylene glycol was treated as a  $C_2$ , and succinic acid as a  $C_1$  derivative. Although the inclusion of succinic acid with the  $C_2$  rather than with the  $C_1$  derivatives would reduce the ratio to less than 1.1, this did not seem justified in view of the considerations set forth earlier and of experiments with resting cell suspensions to be reported further. It must be remembered that the dissimilation of the other constituents of the medium may be so altered by the

presence of sugar as to make the corrections applied to the analyses of the fermentation mixture too high or too low. Thus, if less acetic and more formic acid were produced from peptone in the presence than in the absence of glucose, the  $C_1/C_2$  ratio would be materially reduced.

Some experiments were carried out with washed resting cells harvested from young cultures grown under semi-aerobic conditions in peptone-glycerol medium, the Warburg manometric technique being employed for the measurement of acid and gas production. Suspensions in phosphate buffers in a nitrogen atmosphere and in bicarbonate solutions with nitrogen and  $CO_2$  in the gas phase were allowed to ferment known quantities of sugar to completion. Small amounts of  $CO_2$  were produced as well as from  $2\frac{1}{2}$  to  $3\frac{1}{2}$  equivalents of acid per mol of glucose under such conditions. The  $CO_2$  production was greatest in the early stages of the fermentation, decreasing or disappearing later. In fact, on several occasions, there was some evidence of a disappearance of minute amounts of  $CO_2$  from the system in the later stages. No hydrogen production was observed in these experiments, possibly due to the small quantity of sugar decomposed.

Partial analysis of a sugar fermentation by washed resting cells in phosphate-free bicarbonate medium gave no evidence of the presence of lactic acid, but showed considerable amounts of succinic, as well as formic acids. This suggests that succinic acid may arise in place of lactic acid, possibly through the reduction of  $CO_2$ . (Wood *et al.* 1941). It was hoped that the addition of  $CaCO_3$  to the medium in which cultures were allowed to develop would, in the same manner, favor the production of succinic acid, but an experiment to test this possibility failed to show any significant difference in the course of sugar dissimilation with and without  $CaCO_3$ , except for a slight inhibition of development and fermentation by the added chalk.

#### *Decomposition of formic acid*

Although it seems almost certain that formic hydrogenlyase is responsible for the formation of hydrogen and part of the  $CO_2$ , its presence was not demonstrated by the usual methods employing resting cell suspensions. This may have been due to the great difficulty of obtaining active cells. However, the addition of small amounts of formate to fermenting anaerobic cultures did lead to a corresponding increase in gas production.

#### *Decomposition of pyruvic acid*

Formic and acetic acids,  $CO_2$ , and hydrogen were identified as products of fermentation of pyruvic acid by growing cells of *P. phosphoreum*. As might be expected, no alcohol was found. Traces of acetylmethylcarbinol were detected, but no attempt was made to determine lactic and succinic acids.

#### *Decomposition of alanine and fumaric acid*

Since *P. phosphoreum* can develop to some extent anaerobically in peptone media without any added fermentable substance, and can utilize some amino acids (e.g., alanine) as chief carbon sources for aerobic growth, it appeared of in-

terest to test for the occurrence of the "Stickland reaction" with cultures of this species. Washed cell suspensions of Strain # 2 were incubated anaerobically in Thunberg tubes in the presence of alanine together with some likely organic hydrogen acceptors, and qualitative tests for ammonia were made to determine whether a decomposition of alanine or other added amino acids had occurred. No ammonia was produced either from alanine alone or in mixtures of alanine with glycine, proline, pyruvate, or glucose. However, if alanine and fumarate were added together, a considerable evolution of ammonia resulted. It seems therefore, that although the classical "Stickland reaction" was not carried out under these conditions, alanine could be oxidized with fumaric acid acting as hydrogen acceptor, itself probably becoming reduced to succinic acid. Excellent anaerobic development could be obtained with fumarate, but not with succinate, added to peptone media. It seems probable that fumaric acid can be fermented (Barker, 1936) although no analyses were made with cultures so obtained. It is also possible that fumarate acts as an oxidizing agent for amino acids present in the peptone.

#### *Autofermentation*

The endogenous fermentation by resting cells could be shown manometrically to give rise to acidic products and small amounts of CO<sub>2</sub>. Little if any ammonia was liberated, particularly if the cells were taken from young vigorous cultures. This may be taken as indirect evidence of the carbohydrate nature of the reserve products stored by the cells.

It is a well-known fact that many of the luminous bacteria produce acid when growing in a glycerol-containing medium under aerobic conditions. Aerobic development of *P. phosphoreum* in synthetic medium with glycerol as chief carbon source and methionine as nutrilitate was accompanied by slight acid production and the presence of formic acid could be demonstrated in such cultures. Yet, no acid production could be detected in the respiration of glycerol by resting cells in a Warburg respirometer, and no fermentation of glycerol by cell suspensions or growing cultures was observed. No evidence for a "glycerohydrogenlyase" (Nakamura, 1940) was found. Whether the acid production is due to an autofermentation of reserve products in organisms which find themselves in the deoxygenated deep strata of the medium, or to an incomplete respiration of the glycerol to triose, followed by a fermentation of the latter, is impossible to decide at present.

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#### SUMMARY AND CONCLUSIONS

1. All of the facultatively anaerobic species of luminous bacteria investigated showed essentially the same general "mixed acid" type of anaerobic sugar dissimilation.
2. Among the products formed were formic, acetic, lactic and succinic acids,

alcohol, CO<sub>2</sub>, acetylmethylcarbinol, and, in fermentations with *Photobacterium phosphoreum*, hydrogen and occasionally 2,3-butylene glycol.

3. *Photobacterium fischeri*, *Photobacterium splendidum*, *Photobacterium sepiac*, and *Achromobacter harveyi* produced no hydrogen, and differed among themselves mainly in the extent of lactic acid production.

4. Some experiments with washed cell suspensions and growing cultures of *P. phosphoreum*, dealing with the anaerobic metabolism of glucose, formic, and pyruvic acids and alanine in the presence of fumaric acid are discussed.

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