

The Fermentation of Sucrose by *Aerobacter aerogenes*

By G. G. FREEMAN, *Imperial Chemical Industries Limited, Explosives Division, Research Department, Stevenston, Ayrshire*

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The production of 2:3-butylene glycol as a bacterial metabolic product from carbohydrate was first observed by Harden & Walpole (1906) and Walpole (1911), but received comparatively little further attention until the second world war when the formation of this product as an intermediate for butadiene, synthetic rubber and other purposes was investigated in considerable detail. Recent work in Canada and the U.S.A. (Adams & Stanier, 1945; Perlman, 1944; Stanier & Adams, 1944; Ward, Pettijohn & Coghill, 1945), has established the characteristics of three distinct fermentations yielding 2:3-butylene glycol: (a) the *Aerobacter aerogenes* fermentation of hexoses yielding a mixture of *d*- and *meso*-isomers of the glycol, in which the latter predominates as the main non-gaseous product, (b) the *Aerobacillus polymyxa* fermentation of starches and dilute sugar solutions which yields *l*-2:3-butylene glycol and ethanol, and (c) the *Aeromonas hydrophila* fermentation in which an *l*- and *meso*-mixture of 2:3-butylene glycol isomers, ethanol, and lactic acid are the main non-gaseous products. The object of the present investigation was to study the production of *d*- and *meso*-2:3-butylene glycol from *Aerobacter aerogenes* fermentations of sucrose as the basis for a technical method using molasses. The factors influencing rate of fermentation and yield of product have been investigated with a view to decreasing the time necessary for complete fermentation and simplifying the culture medium.

EXPERIMENTAL

Composition of media

Although the early workers used complex nutrients such as peptone as a basis for the culture media it is now well known that *A. aerogenes* grows well on simple media containing citrate or glucose as carbon source and nitrates or ammonium salts as source of nitrogen (Koser, 1924; Lodge & Hinshelwood, 1944). The basal medium used in the present work was based on the formulae of Koser (1924) and of Czapek (as modified by Dox (1910)) and had the following composition:

	g.
Sucrose	100
Dipotassium hydrogen phosphate (K_2HPO_4)	2.5
Ammonium sulphate	4.0
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.1
Potassium chloride	0.5
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.01
Water to	1000 ml.

The final media contained an excess of calcium carbonate, usually 12 g., as neutralizing agent. The salts were dissolved in water, the reaction adjusted to pH 7.6 and the solution diluted to 200 ml. The sucrose was dissolved in 800 ml. water. The salts and sucrose solutions were separately sterilized by autoclaving. The calcium carbonate was sterilized by dry heat. The three components of the medium were mixed aseptically immediately before inoculation; the reaction at this stage was usually pH 6.5-7.0.

General bacteriological methods

Stock strains and subcultures of *A. aerogenes* were stored on nutrient agar slopes. They were sub-cultured weekly, incubated for 24-48 hr. at 30° and kept until required at 2-5°. To prepare inocula for fermentations the organism was obtained in an actively dividing condition by transfer of a loopful of culture to 50-100 ml. of a liquid medium which was incubated at 35° for 16-18 hr.; one vol. of this culture was then transferred to five vol. of medium and a vol. of this dilution used such that the total volume of diluted inoculum amounted to 3% of the total volume of fermentation mash. The inoculum culture was incubated at 35° for 7-8 hr. before addition to the main fermentation.

The following strains of *A. aerogenes* were used: B.199 (Northern Regional Research Laboratory of U.S. Department of Agriculture, Peoria, N.R.R.L.B.199); 38 (N.C.T.C.No.418); 39 (Dr G.A. Ledingham, National Research Council, Ottawa No.5); 44 (Dr M. J. Johnson, University of Wisconsin, College of Agriculture, M5 A1).

Methods of analysis and expression of the fermentation results

The course of fermentation was followed by determination of the rate of decrease of sugar content and of production of 2:3-butylene glycol. The methods of analysis are described by Freeman & Morrison (1946). In addition to the rate of removal of sugar and of production of the glycol, completeness of sugar fermentation and yield of the product in terms of sugar initially present and of sugar fermented have also been used as criteria. In the tables the sugar contents have been expressed as g./l. of invert sugar. At the completion of incubation the sugar fermented (i.e. the difference between the initial and final invert sugar concentrations) was

Investigation of variables affecting rate of fermentation and 2:3-butylene glycol production

The variables which affect the rate of fermentation and glycol yield were systematically investigated, including composition of the medium, concentration of substrate, pH, temperature and aeration.

Inoculum

The effect of variation of the volume of inoculum added to the main fermentation mixture was investigated over the range of 0.6–12% (v/v). There was little variation in the rate of sugar conversion and 2:3-butylene glycol yield throughout the range of inoculum concentration from 0.6–12% (v/v). There was, however, some indication that highest glycol yields (though slightly slower fermentation) were obtained with smaller inocula. This may be due to the production of a greater number of generations in the fermentation medium giving a better acclimatized organism and a more vigorous culture. An inoculum level of 3% was chosen for subsequent work but the results showed that this might be decreased to 0.6% or even lower without appreciable diminution in the rate of fermentation. The use, for the inoculum, of either a synthetic medium based on the fermentation medium described, a glucose + salts + corn steep liquor medium, or a beer wort + calcium carbonate medium, had no significant effect on the rate of fermentation. The use of a synthetic medium for this purpose is recommended.

Organic nutrients such as yeast extract

Although *A. aerogenes* is apparently able to synthesize all its necessary growth factors, the effect of addition of such factors on the rate of fermentation was investigated. The effect of addition of yeast extract clearly showed that such additional organic nutrients played no part in the fermentation process and were unnecessary. Similar negative results were obtained with yeast autolysate and malt rootlet extract.

Optimal concentration of salts

Ammonium sulphate. In the early work on *A. aerogenes* fermentations peptone was used as the nitrogenous nutrient (Harden & Walpole, 1906). More recent work has shown that amino-acids (Lodge & Hinshelwood, 1944), urea and ammonium salts (Verhave, 1928) are satisfactory for this purpose. In the present work, ammonium sulphate was used as the sole source of nitrogen. In preliminary experiments to determine the optimal concentration an excess of the salt was added and by difference the amount of ammonia assimilated by the bacteria at the completion of fermentation was determined. This was found to be about 2.6 g./l. expressed as ammonium sulphate. The effect of

a series of ammonium sulphate concentrations between zero and 10 g./l. on rate of fermentation and glycol yield was determined. The rate of fermentation and glycol yield were practically independent of ammonium sulphate concentration in the range 4–10 g./l. In subsequent experiments an initial ammonium sulphate concentration of 4 g./l. was used.

Dipotassium hydrogen phosphate. By similar means to that described above the optimal K_2HPO_4 concentration was found to be 2.5 g./l.; increase of phosphate concentration to 10 g./l. did not influence the characteristics of fermentation. In the absence of added phosphate (other than the traces introduced with the inoculum) fermentation was greatly suppressed; only 40% conversion of sucrose took place in 24 hr. and the glycol yield was only about 30% of the maximum possible.

Determination of optimal pH

The products of the *A. aerogenes* fermentation of hexoses include 2:3-butylene glycol, acetoin (acetyl-methylcarbinol), ethanol, lactic acid and formic acid (Ledingham & Adams, 1944). Lactic acid is the main acidic product; as described below it has been obtained to the extent of 5–10% of the hexose fermented. It is necessary to neutralize this lactic acid as it is formed in order to maintain an optimal pH. This may be done either by continuous addition of soluble alkali or by maintaining an excess of calcium carbonate in equilibrium with the fermenting liquor. The effect of pH on rate of fermentation and glycol production was investigated in three ways; by variation in the amount of calcium carbonate suspension, by maintenance of a constant pH by addition of soluble alkali and by a washed suspension technique in which no bacterial growth took place.

Variation of amount of calcium carbonate suspension. A series of fermentations was carried out in agitated 100 ml. mashers in which the calcium carbonate contents were varied from 0–12 g./l. The pH of the fermenting mixtures was recorded at intervals (glass electrode). The results showed that complete conversion was obtained in 41 hr. or less with $CaCO_3$ from 3–12 g./l. The initial rates of sucrose conversion and 2:3-butylene glycol formation were greatest with the highest amounts of calcium carbonate but did not differ greatly between 9 and 12 g./l. The mean pH values during the period of vigorous fermentation were pH 5.78, 5.52, 5.40, 5.03 and 4.08 for calcium carbonate additions of 12, 9, 6, 3 and 0 g./l. respectively, and the corresponding yields of 2:3-butylene glycol in terms of sugar fermented were 35.8, 36.5, 39.3, 41.3 and 41.0% respectively. Whilst fermentation was most rapid in the pH range 5.4–6.0 the glycol yield was at a maximum at pH 5.03–5.4.

Washed suspension technique. Preliminary experiments with washed suspensions of 17 hr. cultures of *A. aerogenes* B. 199 showed that on addition of 0.5–2.0% solutions of glucose at 35°, the latter was fermented with the production of 2:3-butylene glycol as the main non-gaseous metabolic product. When the bacterial concentration was less than 1.30 g. dry weight/l. in 0.5% glucose, concentration of enzyme system was the limiting factor controlling

aerated at a rate of 100 l. air/hr.; incubation temperature, 35°; organism, *A. aerogenes* B. 199. A calomel-glass electrode cell was built into the fermentation vessel and connected directly to the pH meter so that determinations could readily be made without the necessity of sampling. The fermenting liquor was gently stirred to maintain the bacterial growth in suspension and to avoid high local alkalinity on addition of the NaOH. The pH was determined at 15–30 min. intervals during the initial 32 hr. of fermentation and the reaction restored to pH 6.5 or 5.5, respectively,

Table 2. *Determination of optimal pH by washed suspension technique*

(The following mixtures were prepared: 10 ml. bacterial suspension (concentration: 10.9 g. dried cells/l.) + 5 ml. 20% glucose solution + 35 ml. 0.5M-phosphate buffer at the following pH: (1) 5.97; (2) 6.47; (3) 7.00; (4) 7.50. Initial glucose concentration: 18.31 g./l. Incubation at 35°.)

Initial pH	Mean pH during fermentation	Glucose after 22 hr. (g./l.)	Glucose fermented (by difference) (g./l.)	Yield of 2:3-butylene glycol in 22 hr. (g./l.)	Yield as % of sugar fermented
5.97	4.86	8.32	9.99	4.32	43.3
6.47	5.14	3.93	14.38	5.70	39.6
7.00	5.50	2.78	15.53	4.75	30.6
7.50	5.57	1.53	16.78	5.95	35.5

rate of fermentation and the rate of reaction was a linear function of time until the bulk of the glucose had been converted. The rate of fermentation, under these conditions, was proportional to the concentration of bacterial suspension.

The organism was grown on either a glucose + peptone + Lemco medium or on a glucose-containing synthetic medium buffered at pH 6.8 with 0.05M-phosphate + citrate buffer at 35° for 17–20 hr. During growth the reaction fell to pH 5.5. The bacterial cells were separated by centrifuging, washed with buffer (pH 6.8) and resuspended in buffer to give a washed suspension containing the equivalent of 10.9 g. dried cells/l. This was used to prepare the suspensions used to obtain the results recorded in Table 2.

The buffering power of the medium was insufficient to overcome the acidity of the lactic acid produced and a marked fall of pH took place particularly in the early stages of the reaction. The quantity of glucose fermented was greatest at a mean pH of 5.57 and fell progressively with the more acid media. The yield of 2:3-butylene glycol in terms of glucose fermented was greatest at a mean pH of 4.8 (86.6% of theory) decreasing to 61.2–71.0% of theory at pH 5.50–5.57.

Control of pH by addition of soluble alkali. Control of pH during fermentation by stepwise addition of soluble alkali (sodium hydroxide or ammonia) had the great advantage over the use of an excess of calcium carbonate that there was more accurate control of reaction at any chosen point in the range pH 4.5–8.

Two fermentations of sucrose (2.5 l. scale) were conducted in which the pH was maintained at (a) 6.0–6.5, and (b) 5.5, by stepwise addition of 2.5N-NaOH. The solutions were

by addition of a suitable volume of the alkali. Determinations of sucrose and 2:3-butylene glycol were made at 3 hr. intervals.

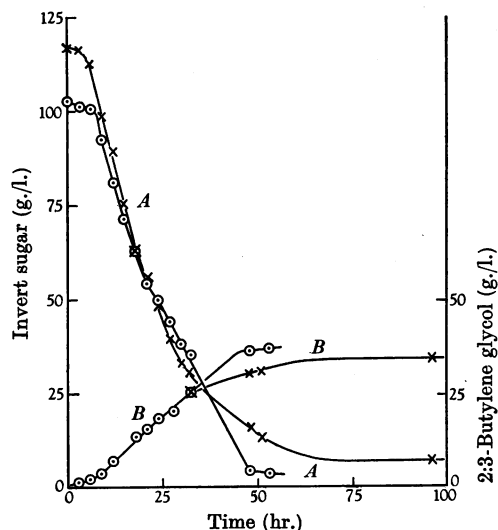


Fig. 1. pH control by stepwise addition of sodium hydroxide. Fermentations of sucrose by *A. aerogenes* B.199 (2.5 l. scale) were carried out in which the pH was maintained at (I) 6.0–6.5 and (II) 5.5, by stepwise addition of 2.5N-sodium hydroxide at 15–30 min. intervals. Incubation temperature, 35°; aeration, 100 l. air/hr. ×—× pH 6.0–6.5; ○—○ pH 5.5. A, invert sugar fermentation; B, 2:3-butylene glycol production.

The results (Fig. 1) confirmed the existence of a lag phase of fermentation of about 6 hr. duration. The rates of fermentation and 2:3-butylene glycol production were slower than in similar fermenta-

tions in the presence of calcium carbonate (cf. F. 27 (b), Table 1), but this was probably due to the unavoidable width of the pH steps between successive alkali additions rather than to any stimulating effect of calcium ions. At pH 6.0-6.5 the initial rate of fermentation (sucrose conversion and glycol production) was 25-30% greater than at pH 5.5, but the 2:3-butylene glycol yield was 22% higher at the lower pH value. Lactic acid formation was favoured by increase of pH. Thus in the experiment at pH 6.0-6.5 the alkali required for neutralization was equivalent to 18.95% formation of lactic acid in terms of sugar fermented, while in the experiment at pH 5.5 the corresponding yield of lactic acid was only 10.62%. The sum of 2:3-butylene glycol and lactic acid yields in these experiments, viz. 50.7 and 49.3% was approximately constant. The fermented liquors were very opalescent owing to the bacterial growth, part of which flocculated.

Effect of initial carbohydrate concentration

The influence of the initial sucrose concentration on the rate of sugar conversion and 2:3-butylene glycol yield was investigated in the range of 5-20% sucrose concentration.

In each case 100 ml. of medium was agitated in a 350 ml. flask at 35°. The amounts of calcium carbonate added were kept constant at 12 g./l. Under conditions of free aeration provided by means of a cotton-wool plug in the flask, acetoin began to replace 2:3-butylene glycol as the product of the reaction after the sugar concentration had fallen to less than 1%. To minimize acetoin formation the cotton-wool plug was replaced by a rubber stopper connected by a short length of glass tubing to a Bunsen valve when the carbohydrate concentration had fallen to about 1%. By this means free evolution of carbon dioxide from the flask was possible but ingress of air was greatly impeded. There was an increase in the duration of the initial lag phase of sugar fermentation with increasing carbohydrate concentration. With initial sucrose concentrations of 5 and 10% the lag periods were 5-6 hr. At 15 and 20% initial sucrose concentrations they had increased to 9-10 hr. and 15-16 hr. respectively. The yield of 2:3-butylene glycol was at a maximum (87.4% of theory) with an initial sugar concentration of 15%. The slope of the curves in the rapid, linear phase of fermentation (Fig. 2) increased slightly with increasing initial sugar concentration, but owing to the longer lag phase and greater duration of the linear period the overall time of fermentation increased with increasing initial sugar concentration. Fermentation was complete in 20, 25, 35 and c. 47 hr. with initial sucrose concentrations of 5, 10, 15 and 20% respectively.

According to Fulmer, Christensen & Kendall (1933) the optimal initial sucrose concentration is 8%, but under the conditions used by these workers fermentation was very slow, requiring 4 days and 23 days for completion at initial sucrose concentrations of 1 and 10% respectively.

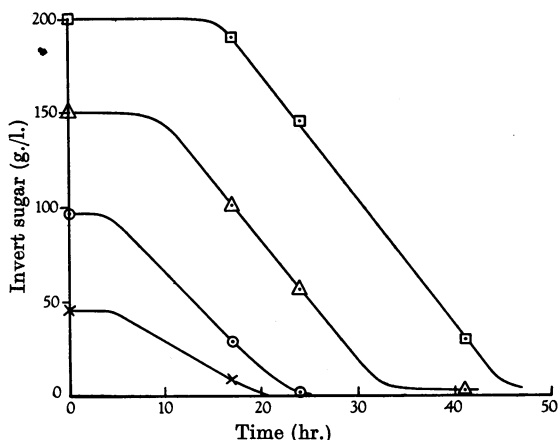


Fig. 2. Effect of initial carbohydrate concentration. 100 ml. quantities of medium containing a series of different initial sucrose concentrations in the range of 5-20% were fermented by *A. aerogenes* B. 199 at 35°. Aeration was restricted after the sugar concentration had fallen below 1%. A constant quantity (equivalent to 12 g./l.) of CaCO₃ was added to each fermentation. Invert sugar concentration initially: 45.6 g./l. x-x; 97.2 g./l. o-o; 149.7 g./l. Δ-Δ; 200.4 g./l. □-□.

Determination of optimal temperature

The rates of sucrose fermentation and 2:3-butylene glycol production in *A. aerogenes* B. 199 fermentations were determined at intervals in the temperature range 25-40°. The fermentations were conducted with 100 ml. of medium in 350 ml. flasks with agitation. The temperature was maintained by immersing the flasks in a thermostatically controlled water bath, with a variation of less than ±0.1° of the stated temperatures. In series I (Table 3) the fermentation vessels were closed only with cotton-wool plugs which allowed free aeration; in series II free aeration was allowed in the initial stages of fermentation during which a high concentration of sugar was present, and after 17 hr. the plugs were replaced by Bunsen valves as described above. The results (Table 3) show that:

(a) Practically complete sugar conversion was obtained under aerobic conditions with temperatures in the range 30-37.5° in a 41 hr. fermentation. Outside this temperature range fermentation was much slower and was not complete in 41 hr. With restricted aeration much lower conversions were obtained in the same period, but maximum conversion was again obtained at 35° (89.2%).

Table 3. *Determination of optimal temperature*
(100 ml. volumes of medium inoculated with *A. aerogenes* B. 199 were agitated in 350 ml. flasks, in thermostatically controlled baths at a series of temperatures in the range 25–40°.)

Exp. no.	Temperature	Initial invert sugar (g./l.)	Conversion after 41 hr. (%)	Initial rate of sugar conversion (g./l./24 hr.)	Initial rate of glycol formation (g./l./24 hr.)	Final 2:3-butylene glycol (g./l.)	Glycol yield (% sugar fermented)	pH at 41 hr.
				I. With cultures freely aerated				
46 I	25.0	110.9	68.2	47.1	14.0	23.8	31.4	6.04
46 A	30.0	116.3	96.6	66.3	18.3	37.8	33.7	6.53
46 E	30.0	109.1	93.5	65.7	20.2	36.7	36.0	5.86
46 G	32.5	115.4	93.3	73.5	20.2	36.0	33.4	6.27
41	35.0	111.5	100	105.6	35.6	39.5	35.8	6.91
46 H	37.5	112.1	87.5	57.6	16.4	31.9	32.5	5.69
46 F	40.0*	110.1	73.8	25.5	5.2	25.6	31.5	5.40
				II. With restricted aeration				
46 I	25.0	110.9	62.3	47.4	12.6	19.5	28.2	5.82
46 A	30.0	116.3	68.4	58.1	14.3	22.9	28.8	6.20
46 G	32.5	115.4	80.1	66.9	17.8	27.4	29.7	6.07
41	35.0	111.5	89.2	87.4	28.0	32.6	32.8	5.54
46 H	37.5	112.1	47.6	41.3	12.3	15.7	29.5	5.73

* Lag phase lasted about 17 hr.

(b) The initial rates of sucrose conversion and 2:3-butylene glycol production, both under aerobic and 'anaerobic' conditions showed sharp maxima at 35°. There was a very steep fall with increasing temperature and a slower fall of these rates towards lower temperatures. The optimal temperature was 35.0°. According to *Bergey's Manual* (1940) the optimal temperature for *growth* of this organism is about 30°.

(c) Variation in temperature had comparatively little effect on yield of 2:3-butylene glycol in terms of sucrose fermented but there was a definite maximum at 35°.

Effect of different strains of Aerobacter aerogenes

Within the species *A. aerogenes* there exists a large number of different strains which, though morphologically identical, differ in their rates of fermentation and yields of metabolic products. Reversible and irreversible changes in a given strain may also be induced by acclimatization to new growth media. Lodge & Hinshelwood (1944) showed how the organism could adapt itself to certain changes in source of carbohydrate and nitrogenous nutrients and Perlman (1944) obtained higher yields of 2:3-butylene glycol and faster fermentation of wood hydrolysates with acclimatized strains than with the parent organism.

A number of strains obtained from other workers have been compared in fermentations of sucrose at 35°. A few strains have also been isolated from natural sources such as grains, plants, faecal matter and soil; those whose production of acetoin was demonstrated by the Voges-Proskauer reaction were submitted to fermentation tests to determine production of 2:3-butylene glycol. So far only a number of disappointingly slow fermenters have been obtained which gave only 23–25% of 2:3-butylene glycol in terms of sugar fermented. Strains N.C.T.C. 418, M5 A 1/L and S were fast fermenters with which practically complete conversion of sucrose was obtained in 40.5 hr. with high glycol yields. They were, however, all inferior to the B. 199 strain. The Wisconsin strain M5 A 1 as received was separated into two forms M5 A 1/L and S, characterized by large and small colonies on nutrient agar, respectively. There was an appreciable difference of rate of fermentation between these strains, the former being the more rapid fermenter.

Effect of aeration on fermentation

Kluyver, Donker & Hooft (1925) first showed that the presence of hydrogen acceptors, including oxygen, stimulated the production of acetoin and 2:3-butylene glycol in yeast fermentations; this principle was applied to *A. aerogenes* fermentations by Verhave (1928). According to Scheffer (1936) the accelerating effect of aeration on 2:3-butylene

glycol production is not due to a specific physiological action of oxygen but to a diminution in the concentration of carbon dioxide and he claimed that the use of other gases such as hydrogen or nitrogen or a reduction of pressure had a similar effect.

Our experiments have confirmed the accelerating effect of aeration and shown that higher yields of

complete conversion of sugar was not reached until 91 hr., although 95.9% of the initial sugar had been fermented in 51 hr. and 98.9% in 67 hr. The yield of 2:3-butylene glycol was 32% higher under aerated conditions than under unaerated; in the former it reached 39.1% (i.e. 78.2% of theory) and in the latter 29.6% (59.2% of theory).

Table 4. *Effect of aeration on fermentation*

(Cultures of *A. aerogenes* B. 199 were allowed to ferment at 35° with and without aeration.)

Time (hr.)	Changes during 'anaerobic' fermentation 2.5 l. scale. Exp. 38		Changes during fermentation of 2.5 l. culture with aeration (150 l. of air/hr.). Exp. 27	
	Invert sugar (g./l.)	2:3-Butylene glycol (g./l.)	Invert sugar (g./l.)	2:3-Butylene glycol (g./l.)
0	115.0	—	111.0	—
3.5	110.0	2.2	—	—
8	—	—	97.1	4.05
19	76.5	10.7	—	—
23	55.8	15.5	—	—
24	(53.2)	(16.7)	12.2	37.3
27	45.3	20.4	—	*
43	12.6	28.5	—	—
51	4.7	30.8	—	—
67	1.2	33.2	—	—
72	0.44	33.8	0.0	43.4
91	0.0	34.0	—	—
116	0.0	34.0	—	—
2:3-Butylene glycol as % of total sugar		29.6		39.1
2:3-Butylene glycol as % of theoretical		59.2		78.2

* Aeration and stirring arrested.

2:3-butylene glycol in terms of sugar fermented are obtained in aerated than in unaerated cultures. This effect was evident in the series of experiments recorded in Table 3, but the full effect of aeration was somewhat masked as in series II the aeration was restricted only after the first 17 hr. of fermentation.

In order to determine the effect of unaerated conditions throughout the period of fermentation a stirred sucrose-containing medium (2.5 l.) was fermented at 35°, using strain B. 199. Egress of carbon dioxide from the flask was allowed by means of a glass tube (5 mm. bore) tightly closed with a cotton-wool plug. A similar culture was aerated at 150 l. air/hr. (Table 4). From interpolated values in exp. 38 (in parentheses in Table 4) the rates of sucrose attenuation and 2:3-butylene glycol production during the linear phase of the reaction under aerobic conditions (exp. 27) were 1.6 and 2.2 times respectively, the corresponding rates under unaerated conditions. Under both sets of conditions complete fermentation was obtained; in the aerated culture the reaction was shown graphically to be complete in 30–32 hr. and in the unaerated culture

Products of the Aerobacter aerogenes fermentation

Characterization of the d- and meso-2:3-butylene glycol mixture

In order to establish the nature of the glycol produced in this work, samples were isolated as described by Freeman & Morrison (1947) and their properties determined. The pure product after fractional distillation *in vacuo* had the properties recorded in Table 7 which agree satisfactorily with the literature data for *d*- and *meso*-mixtures of the glycol.

The differences of m.p., b.p. and refractive index exhibited by the three preparations of 2:3-butylene glycol are due to the different proportions of the *d*- and *meso*-isomers present in the mixtures. The product obtained in the present work appears to contain more of the *d*-isomer than the glycols isolated by Harden & Walpole (1906) or Morell & Auerheimer (1944). Its content of the *d*-isomer is calculated from the optical rotation data to be approximately 14%.

2:3-Butylene glycol diphenylurethane and dibenzoate were prepared by treatment with phenylisocyanate and benzoyl chloride, respectively.

The diphenylurethane was obtained as a colourless crystalline solid, m.p. 197° (corr.) (authentic value 199°) and gave on analysis C, 65.89, 65.60; H, 5.78, 6.16; N, 8.47, 8.54. $C_{18}H_{20}O_4N_2$ requires C, 65.85; H, 6.16; N, 8.54%. The dibenzoate was a colourless crystalline solid, m.p. 76-77° (literature value, 77°). The dibenzoate gave an equivalent on hydrolysis with ethanolic KOH of 145 (theory 149).

During the crystallization of the pure diphenylurethane, traces of other urethanes were obtained but all attempts to isolate an isomeric diphenylurethane melting at 151° as reported by Walpole (1911) were unsuccessful. Similarly, only a single dibenzoate was isolated. It was concluded that the main non-gaseous product of *A. aerogenes* fermentation of sucrose was a *d*- and *meso*-mixture of 2:3-butylene glycol isomers in which the *meso*-compound greatly predominated.

Acetoin

The formation of 2:3-butylene glycol in bacterial fermentation appears to take place through the intermediate stages of pyruvic acid, acetaldehyde, acetoin and finally reduction of the latter to 2:3-butylene glycol. Under aerobic conditions, the

glycol is oxidized by *A. aerogenes* yielding acetoin, or diacetyl if the aeration is excessive (Stanier & Fratkin, 1944). Our results have confirmed the production of acetoin and shown that even under aerobic conditions appreciable oxidation of 2:3-butylene glycol to acetoin does not take place until the sucrose concentration has decreased to 1% or less. In experiments under aerobic conditions (particularly those in which the cultures were agitated in cotton-wool plugged vessels) appreciable quantities of acetoin were produced when the sugar conversion was allowed to proceed to completion. In these cases acetoin was determined by the method of Langlykke & Peterson (1937) and suitable corrections were made to the reducing sugar and 2:3-butylene glycol values as described by Freeman & Morrison (1946).

Acetoin production in fermentations of sucrose under different conditions of aeration are summarized in Table 5. Under anaerobic conditions (exp. 38) no acetoin was present after 116 hr. fermentation, despite the fact that over 95% of the sugars were fermented in the first 51 hr. When 100 ml. of culture were freely aerated throughout the fermentation period of 40.5 hr., acetoin production reached 27 g./l., i.e. 24.2% of hexose fermented (exps. 41/4 and 5). When the aeration was partly restricted after 16 hr. (43.8% unfermented sucrose

Table 5. *Acetoin production by Aerobacter aerogenes B. 199 in aerated cultures fermented under various conditions*

Conditions	Time of fermentation (hr.)	Exp. no.	Acetoin (g./l.)	Unfermented sugar as invert sugar (g./l.)	2:3-Butylene glycol (g./l.)	Acetoin (% of sugar fermented)	2:3-Butylene glycol (% of sugar fermented)
Anaerobic 2.5 l. culture	116	38	Nil	Nil	34.0	Nil	29.6
100 ml. culture, aeration partly restricted by Bunsen valve plug after 16 hr. when 44% unfermented sugar present	40.5	41/1	3.35	12.1	30.6	3.4	30.8
		41/2	3.80	0.5	32.3	3.42	29.1
		41/3	2.96	9.7	29.7	2.91	29.2
(Mean of above)			3.37	7.4	30.9	3.2	29.7
As above but freely aerated for total 40.5 hr. period	40.5	41/4	27.0	0.0	12.1	24.2	10.9
		41/5	27.0	0.0	13.1	24.2	11.7
		(Mean of above)	27.0	0.0	12.6	24.2	11.3
Fermentation of glucose, 12.5 l. culture. Aeration 100 l./hr. until 46 hr. when 0.89% sugar unfermented	91	44	0.9	6.8	29.2	0.84	27.1
				(as glucose)			
Varying initial sucrose concentration. 100 ml. cultures, aeration partly restricted when following amounts of unfermented invert sugar were present (as g./l.):							
1. 8.9 (17 hr.)	65	45/1	4.5	Nil (45.6)	10.7	9.9	23.4
2. 2.4 (24 hr.)	65	45/2	9.7	Nil (97.2)	30.3	10.0	31.1
3. 3.6 (41 hr.)	65	45/3	9.5	Nil (149.7)	55.9	6.3	37.4
4. 3.0 (49 hr.)	65	45/4	4.7	3.6 (200.4)	77.4	2.4	39.3

Initial sugar concentration in parentheses

as invert sugar present) the corresponding acetoin yields were only 3.4 g./l. (3.2 % of sugar fermented). Similar evidence of acetoin production under aerobic conditions when the sucrose concentration had fallen to 1 % or less is presented in Table 6 (exps. 44 and 45). Acetoin production takes place during the third, slow phase of fermentation described above.

1945) 2:3-butylene glycol (85 % of theory), acetoin (3 % of theory) and ethanol (4 % of the theoretical based on glucose fermented). Similar results have been obtained in the present work (Table 6) but under certain conditions, particularly with un-aerated cultures, high yields of ethanol (12-14 % of the sugar fermented) and of lactic acid (5-10 %) were obtained.

Table 6. Yields of non-volatile metabolic products of fermentation of sugars by *Aerobacter aerogenes* under various conditions (as % of sugar fermented)

Products	Conditions of fermentation			
	Un-aerated fermentation of sucrose 2.5 l. culture 91 hr. (Exp. 38)	Glucose fermentation with aeration at 100 l./hr. 12.5 l. culture 91 hr. (Exp. 44)	Sucrose fermentation with aeration 100 ml. culture. Initial concentration of sucrose 149.7 g./l. (Exp. 45/3)	As 45/3 but initial sucrose concentration 200.4 g./l. (Exp. 45/4)
2:3-Butylene glycol	29.6	27.1	37.4	39.3
Acetoin	Nil	0.8	6.3	2.4
Lactic acid	5.6	9.8	—	—
Ethanol	13.6	12.1	—	—
Total non-gaseous products	48.8	49.8	—	—

Table 7. Properties of *d*- and *meso*-mixtures of 2:3-butylene glycol isomers isolated from *Aerobacter aerogenes* fermentations

	Product from <i>A. aerogenes</i> B. 199 fermentations	<i>d</i> - and <i>meso</i> -mixtures of 2:3-butylene glycol isomers	
		(a) Harden & Walpole (1906)	(b) Morell & Auerheimer (1944)
m.p.	11-12°	14°	25°
b.p.	183.0°	180°	180-182°
Refractive index:			
$n_D^{21.5}$	1.4371	1.4360 (27°)	1.4381 (18°)
$[\alpha]_{5461}^{20}$	+2.27	—	—
$[\alpha]_D^{23}$	+1.80	+0.82 (25°)	+1.06
Density 20°/4°	1.002	—	1.000 (25°)

Other products

The main non-gaseous products of the *A. aerogenes* fermentation are 2:3-butylene glycol, acetoin, ethanol, formic acid and lactic acid (Adams & Stanier, 1945). Acid-hydrolyzed starch mashes fermented by *A. aerogenes* yielded (Ward *et al.*

SUMMARY

1. The following factors affecting the conversion of sucrose to 2:3-butylene glycol by *Aerobacter aerogenes* have been investigated: pH, sucrose concentration, optimal temperature, aeration, and addition of organic nutrients.

2. The pH optimum for production of the glycol was 5.0-5.5 and the optimal temperature, 35°.

3. Rapid fermentation and high 2:3-butylene glycol yields were obtained with initial sucrose concentrations in the range 5-20 %. Maximum yield of the product (87.4 % of theory) was obtained with an initial sucrose concentration of 15 %.

4. In aerated stirred cultures containing 10 % sucrose and 1.2 % calcium carbonate as neutralizing agent, rapid fermentation began after a lag phase of 5-7 hr. and was complete in c. 24 hr.

5. The main non-gaseous products were *d*- and *meso*-2:3-butylene glycol, ethanol, and lactic acid. Acetoin was not formed under anaerobic conditions, but replaced the glycol as the major product when aeration was continued after the carbohydrate concentration had fallen to 1 % or less.

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The Turnover of Phosphate in the Pineal Body Compared with that in Other Parts of the Brain

BY U. BORELL AND Å. ÖRSTRÖM, *B. A. Hjorth's Department for Metabolic Research, Wenner-Gren's Institute, Stockholm*

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The metabolism of the brain has been investigated by various methods. Chute & Smyth (1939) and Broman & Jacobsohn (1941) have shown that the oxygen consumption of isolated perfused cat brain amounts to about 100–300 ml. O₂/100 g. of brain/hr. An approximate calculation of the Q_{O_2} results in values of between 4 and 12 ($Q_{O_2} = \mu\text{l./hr./mg. dry weight.}$) Another method of determining the metabolism of the brain consists in measuring, in the intact brain *in situ*, the composition of the arterial blood flowing into and the venous blood flowing from the brain. This method is however less suitable for obtaining absolute values, because the velocity of the blood streaming through the brain must be evaluated. This meets with certain difficulties.

In the method most usual at present, metabolism is measured in surviving brain slices by the Warburg procedure. The Q_{O_2} values thus found for cortex slices have usually varied between 5 and 10. This method also shows that the metabolism of the brain is chiefly associated with carbohydrates. Pyruvic acid is one of the most important metabolic products. The oxidation of this substance in the brain has been studied by, for example, Simola & Alapenso (1943) and Long (1943). A number of enzymic reactions takes place in the brain, but no scheme for the carbohydrate metabolism in this tissue has yet been established (Evans, 1944).

Transphosphorylation in the brain has also been studied by this method. Macfarlane & Weil-Malherbe (1941) thought that the breakdown of glucose in the brain takes place with the aid of a cycle of phosphate transfers. This supposition was based on the observation that inorganic phosphate in slices of rat brain undergoes no change during

anaerobic glycolysis, whereas the pyrophosphate is reduced to one-third of its original value. Borell & Örström (1945) showed later that radioactive phosphate chiefly accumulates as hydrolyzable acid-soluble phosphate esters, which supports the above supposition.

The employment of P³² constitutes an advance in technique. This method allows us to measure, in very small parts of the brain, the velocity at which the marked P atom participates in the metabolism. The phosphorus participates, however, not only in the carbohydrate metabolism, but also in that of the phospholipids and nucleic acids. These latter processes in the brain have been studied by several workers (Chargaff, 1939; Chargaff, Olson & Partington, 1940; Hevesy & Hahn, 1940; Hevesy & Ottesen, 1943; Hevesy, 1940).

The investigations of Hevesy & Hahn (1940) show that a strikingly low metabolism is found in the brain, when measured by means of radioactive P; this we are in a position to confirm. Their subsequent work furnished further evidence for the view of Hevesy & Ottesen (1943) that there is only a low metabolism in the brain. These authors isolated deoxyribonucleic acid from the brain and the metabolism proved to be very low even in this quantitatively considerable fraction.

In our investigations we attempted to divide the brain into sections comparatively well-defined anatomically, and to compare their phosphate metabolism by means of radioactive P. A very unexpected result was obtained, in that the pineal body was found to be the most active portion of the brain (Borell & Örström, 1945). These problems will be dealt with further in this work.