THE BACTERIAL FORMATION OF ACETYLMETHYLCARBINOL AND 2:3-BUTYLENE GLYCOL

by

FRANK C. HAPPOLD AND C. P. SPENCER
Department of Biochemistry, University of Leeds (England)

Acetylmethylcarbinol and 2:3 butylene glycol have frequently been reported as products of the microbiological fermentation of glucose and numerous other carbohydrates. Conflicting reports whether or not *Aerobacter aerogenes* can form acetylmethylcarbinol from pyruvic acid as the sole carbon source have been made (WHETHAM[1]; BARRITT[2]). The studies of WERKMAN and his associates (REYNOLDS, JACOBSSON AND WERKMAN[3]; MICHELSON AND WERKMAN[4]) with *Aerobacter indologenes* seemed to suggest that acetylmethylcarbinol and 2:3 butylene glycol resulted from the condensation of two molecules of acetaldehyde. Subsequent isotopic studies to some extent confirmed these views (SLADE AND WERKMAN[5]) which were in accordance with the findings of NEUBERG and his associates obtained with yeast. (NEUBERG AND LIEBERMANN[6]; NEUBERG AND OHLE[7]; NEUBERG AND REINFURTH[8]; NEUBERG AND MAY[9]; NEUBERG AND ROSENTHAL[10]). A later series of studies by DIRSCHERL[11,12,13] led to the postulation of a different method of intervention of acetaldehyde, but supported its role as a possible precursor of acetylmethylcarbinol and 2:3 butylene glycol. Nevertheless, a satisfactory microbiological synthesis of acetylmethylcarbinol from acetaldehyde alone (*i.e.* in the absence of the concurrent fermentation of pyruvic acid) was not achieved and the position was further complicated by the preparation of a cell free enzyme from *A. aerogenes*. This system catalysed the decarboxylation of pyruvic acid with concurrent formation of acetylmethylcarbinol but was unable to utilize acetaldehyde either alone or during the concurrent decarboxylation of pyruvic acid. (SILVERMAN AND WERKMAN[14]).

Data were required regarding the nature of the enzymic reactions leading to the formation of acetylmethylcarbinol and 2:3 butylene glycol. Since the cell free juice of SILVERMAN AND WERKMAN did not contain the complete enzyme system for the formation of both the carbinol and the glycol, whole washed cells were used. The use of these in experiments of reasonable size necessitated the use of a micro-method of estimation of mixtures of acetylmethylcarbinol and 2:3 butylene glycol, and the development of a suitable method became the primary problem. The method of NEUBERG AND STRAUSS[15] for the estimation of diacetyl was adapted for this purpose.

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EXPERIMENTAL METHODS

Estimation Procedures

The estimation of acetylmethylcarbinol and 2:3 butylene glycol

Reagents

Unless otherwise stated A.R. chemicals were used throughout. A commercial specimen of diacetyl was purified by fractionation. The product had a yellow colour and boiled at 88.5°. Acetylmethylcarbinol was synthesised by the reduction of diacetyl (VON PECHMANN[16]). The crystalline product was further purified by grinding it to a powder and suspending for one hour in a large volume of ether with constant agitation. After filtration and drying, a product was obtained which was white and odourless. It was stored in sealed glass tubes, solutions being made up immediately before use. A purified sample of 2:3 butylene glycol was obtained from a commercial specimen by fractionation. The product boiled at 179° and was practically colourless. Sodium ethylate solution was made up as required by allowing 3 g of metallic sodium to dissolve in 1 l of absolute alcohol.

The Estimation of Diacetyl

A method applicable to amounts of diacetyl up to 100 μg was required. We have adapted the method of NEUBERG AND STRAUSS to our purpose. The procedure laid down below includes slight changes of technique which enable the accurate estimation of the larger quantities of diacetyl.

A sample of the protein-free filtrate containing not more than 100 μg of diacetyl was added to 5 ml of a (200 μg per ml) solution of 2:4-dinitrophenylhydrazine contained in a thick glass graduated centrifuge tube. The tube was tightly stoppered with a rubber bung and heated on a boiling water bath for 60 minutes. After cooling a knife point of kaolin was added and the precipitated diacetyl bis(2:4-dinitrophenyl)-hydrazone sedimented on the centrifuge. The supernatant was discarded and the sediment washed successively with 5 ml of 2 *N* HCl and two 5 ml portions of distilled water. After pouring off the washings, 3 ml of sodium ethylate were added and throughly mixed, the suspension being allowed to stand for five minutes before centrifuging. The coloured eluate was decanted from the kaolin into a dry 25 ml stoppered graduated cylinder, and the sediment further eluted with 3 ml quantities of sodium ethylate until the eluates were colourless. The combined eluates were diluted to 12 or 25 ml depending on the amount of diacetyl in the original sample. The colour was compared with that of a blank determination using a Spekker Photoelectric Absorptiometer with Ilford Colour Filters No. 605.

The colour formed accurately obeys BEER's Law over the concentration range used and is discernable with amounts of diacetyl as low as 0.5 μg. Two calibration curves were prepared, one for the range 0 to 10 μg and one for the range 0 to 100 μg of diacetyl, the final volumes being 12 ml in the case of the lower and 25 ml in the case of the higher range. These calibration curves were used in all further work and are reproduced as Fig. 1.

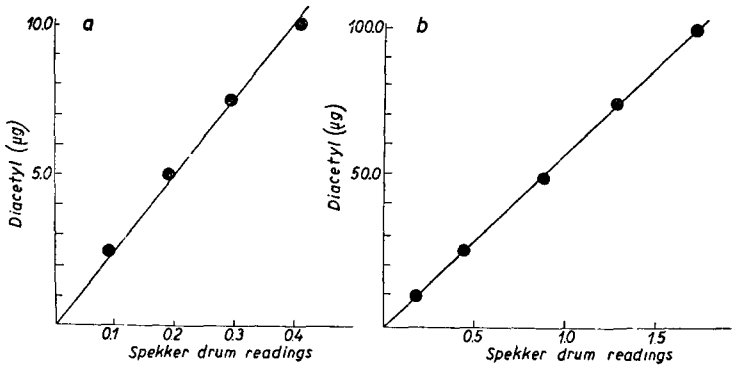

Fig. 1. Calibration curves for the estimation of diacetyl. a. Range 0 to 10 μg diacetyl (final dilution 12 ml); b. Range 0 to 100 μg diacetyl (final dilution 25 ml). Colour intensities compared with Spekker Photoelectric Absorptiometer using 1 cm cuvettes and Ilford Colour Filters No. 605

The coloured solutions are reasonably stable if stored at 0° in the absence of an excess of air. At room temperature a precipitate appears after some time giving rise to a slight turbidity but, when necessary, solutions may be stored in the refrigerator for 12 hours without serious error.

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The Estimation of Acetylmethylcarbinol

The estimation of acetylmethylcarbinol depends upon its oxidation to diacetyl and subsequent estimation of the latter, the diacetyl being separated from the oxidation mixture by distillation. An all glass apparatus of the size and type shown in Fig. 2 was found to give quantitative recovery of known amounts of diacetyl after distillation and trapping in the stock hydrazine solution. The carbinol can be oxidised to the diketone by heating with ferric chloride in acid solution, but as NEUBERG points out, an excess of ferric chloride leads to destruction of the diacetyl, and the ferric chloride concentration must be kept as low as is compatible with complete oxidation of the carbinol. The addition of 2 ml of 50% (W/V) ferric chloride and 1 ml of 18 *N* sulphuric acid to the sample diluted to 9 ml produces an almost quantitative yield of diacetyl from acetylmethylcarbinol and makes possible the direct estimation of the latter as diacetyl, eliminating the necessity of a separate calibration curve. The procedure laid down below was adopted as standard for the estimation of acetylmethylcarbinol.

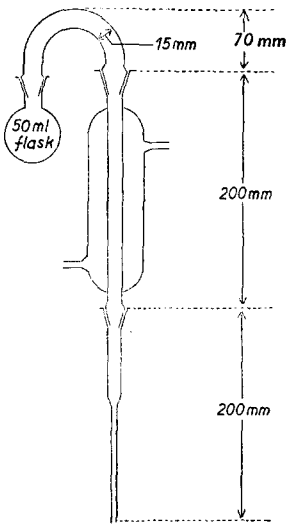

Fig. 2. Distillation apparatus for the estimation of acetylmethylcarbinol and 2:3-butylene glycol

A sample of the protein free filtrate containing not more than 100 μg of acetylmethylcarbinol was pipetted into a 6" × 1" pyrex test tube and the volume adjusted to 9 ml with distilled water. 1 ml of 18 *N* sulphuric acid and 2 ml of 50% (W/V) ferric chloride were added and the tube tightly stoppered with a rubber bung and heated on a boiling water bath for 15 minutes. After cooling, the contents of the tube were transferred quantitatively to the flask of the distillation apparatus, the tube being washed out with three successive 1 ml portions of distilled water. A small piece of porous pot was added and the oxidation mixture and washings distilled into 5 ml of a (200 μg per ml) solution of 2:4 dinitrophenylhydrazine contained in a thick glass graduated centrifuge tube. The delivery tube was arranged to reach the bottom of the hydrazine solution, 10 ml of distillate being collected. This distillation took about five minutes. The flask and tube were detached from the top of the condenser and the condenser and delivery tube washed with a small quantity of distilled water. The distillate and washings were then estimated for diacetyl as previously described.

The Estimation of 2:3-Butylene Glycol

Bromine has been used by several previous workers to oxidise 2:3-butylene glycol to diacetyl, the reaction being affected by light (TOMIYASU[17]; MATIGNON, MOREAU AND DODE[18]). Quantitative oxidation of the glycol to the diketone was obtained by conducting the reaction in two distinct steps, the glycol was first oxidised to the carbinol by heating with bromine in the dark followed, after removal of the excess bromine, by oxidation and estimation of the carbinol by the normal procedure. Because of the increased volume of the oxidation mixture at the latter stage it was necessary to increase the amount of 18 *N* sulphuric acid added. The normal addendum of ferric chloride was quite adequate. The procedure laid down below was adopted as standard for the estimation of the glycol as diacetyl; it eliminates the necessity of a separate calibration curve.

A neutral sample of the protein free filtrate containing not more than 100 μg of 2:3-butylene glycol was pipetted into a 6" × 1" pyrex test tube, the volume adjusted to 9 ml with distilled water, and 4 ml of saturated bromine water added. The tube was immediately stoppered with a rubber bung and heated on a boiling water bath for 3 minutes in the dark. It was then removed and rapidly cooled by plunging into a beaker of water at room temperature in which it was allowed to stand, also in the dark, for 15 minutes. After cooling, 3 ml of 20% (W/V) ferrous sulphate were added to remove the excess bromine, and after the addition of 2 ml of 50% (W/V) ferric chloride and 1.5 ml 18 *N* sulphuric acid, the acetylmethylcarbinol in the mixture was estimated.

Protein Precipitation

The protein free filtrate for the estimation of the glycol must be neutral and protein precipitation is best accomplished by the use of exactly equivalent amounts of zinc sulphate and sodium hydroxide. Alternatively, 10% trichloracetic acid may be used if the filtrate is neutralized before estimating the glycol.

Accuracy of the Method

The accuracy of the method was tested by assaying known amounts of the two compounds by the procedures outlined above. The mean recovery of acetylmethylcarbinol as diacetyl over a series of 10 estimations was 98% with a maximum variation of $\pm$ 2.5%. A similar series with 2:3-butylene glycol gave a mean recovery figure of 97% with a maximum variation of $\pm$ 3.5%.

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Estimation of Mixtures of Acetylmethylcarbinol and 2:3 Butylene Glycol

Any carbinol present will be oxidised during the estimation of the glycol. The method can, therefore, be applied to mixtures of the two, the carbinol being estimated separately and the glycol determined by difference. Assays of mixtures of known composition gave recovery figures for acetylmethylcarbinol of 97% and an equivalent figure of 94% for the glycol. Diacetyl cannot be estimated directly when the carbinol is present by an analogous method since acetylmethylcarbinol is sufficiently rapidly oxidised to diacetyl (during the heating period) to interfere with the estimation of the diacetyl as such.

Interfering Substances

Acetaldehyde, ethyl alcohol, lactic acid, formic acid, pyruvic acid, and acetic acid do not interfere with the estimation of the carbinol or the glycol. Interference from glucose (owing to the formation of furfural or hydroxymethyl-furfural) may be eliminated by elution of the mixed precipitates of the hydrazones with 5% aqueous methyl alcohol before addition of the sodium ethylate (NEUBERG AND STRAUSS[15]).

The Estimation of Pyruvic Acid

Pyruvic acid was estimated as the 2:4-dinitrophenylhydrazone after the specific extraction procedure of FRIEDEMANN AND HAUGEN[19]. The colour intensities were read with a Spekker Photoelectric Absorptiometer with Ilford Colour Filters No. 604.

The Estimation of Glucose

Glucose was determined by the colorimetric method of HASELWOOD AND STROOKMAN according to the directions of KING[20]. Although acetylmethylcarbinol will reduce the alkaline copper reagent, the reaction being quantitative after 30 minutes heating, the glucose and carbinol are additive in their effect, and it is thus possible to correct for the effect of the amount of carbinol which is known to be present; no other products of glucose metabolism by washed cells of *A. aerogenes* interfere with the estimation of glucose by this method. The colour intensities were measured with a Spekker Photoelectric Absorptiometer using Ilford Colour Filters No. 601.

Preparation and Methods of Use of Washed Cell Suspensions of A. aerogenes

A. aerogenes (laboratory strain) was subcultured fortnightly on ordinary agar slopes. For the preparation of washed cell suspensions, the organism was grown in bulk in 10 l quantities of a medium of the following composition: 1.0% glucose, 0.3% peptone, 0.8% dipotassium hydrogen phosphate, 10% tap water. The tap water and phosphate were autoclaved separately, the components being mixed aseptically after sterilization. The bulk medium was inoculated with the whole of the growth from a 24 hour 1% glucose-agar slope culture of the organism and incubated at 37° for 20 to 24 hours. Before harvesting the cells, the presence of acetylmethylcarbinol in the medium was checked by means of the VOSGES-PROSKAUER Reaction. The cells were harvested in the form of a paste using an Alfa Laval Centrifugal Oil Separator. The cell paste was washed by twice suspending in 5 l of distilled water and reharvesting by the same procedure. The average yield of cell paste was about 20 g (wet weight). The cell paste from the final washing was resuspended in distilled water and diluted to a convenient volume for use. Suspensions of viable cells prepared in this way were non poliferating, free from nutrients, and endogenous acetylmethylcarbinol formation. The activity of these suspensions was retained for several days if stored at 0°.

When using cell suspensions for metabolic studies control experiments were always conducted. The results which follow have been corrected for these controls, which were normally negligible or very small. All experiments were conducted in $M/15$ phosphate buffer at p_H 6.0 and at 37°. The usual precautions were taken to ensure the attainment of thermal equilibrium before the cells were added to the reaction mixtures. Samples for analysis were pipetted into a protein precipitant and the cells removed by centrifugation.

Pyruvic acid was added in the form of the sodium salt. This was synthesised from tartaric acid (GILMAN AND BLATT[21]), the pyruvic acid being purified by fractionation under reduced pressure and carefully neutralized with sodium bicarbonate to a p_H of 4.0. The sodium salt was precipitated from this saturated solution by pouring into 8 volumes of acetone and allowing to stand in the refrigerator overnight. Solutions of sodium pyruvate were stored in a frozen condition.

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EXPERIMENTAL RESULTS

The Production of Acetylmethylcarbinol and 2:3 Butylene glycol from Glucose and Pyruvic Acid

BARRITT[2] claimed equal production of acetylmethylcarbinol from identical (percentage) concentrations of glucose and pyruvic acid in growing peptone broth cultures of *A. aerogenes*. His methods were only semi-quantitative and contained no data regarding 2:3-butylene glycol formation. Using washed cell suspensions of the organism, gross differences in the production of the two four-carbon compounds became apparent (Fig. 3). With either substrate the rate of acetylmethylcarbinol production was comparable over the lower substrate concentration ranges only (Fig. 4) and varied with substrate concentration up to a critical value.

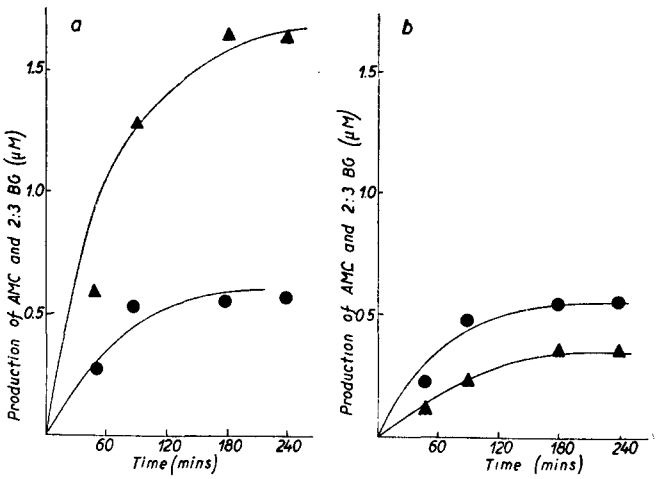

Fig. 3. Production of acetylmethylcarbinol (AMC) and 2:3-butylene glycol (2:3 BG) by washed cell suspension of *A. aerogenes* from equimolar amounts of (a) glucose and (b) pyruvate.
—●——●— AMC. —▲——▲— 2:3 BG

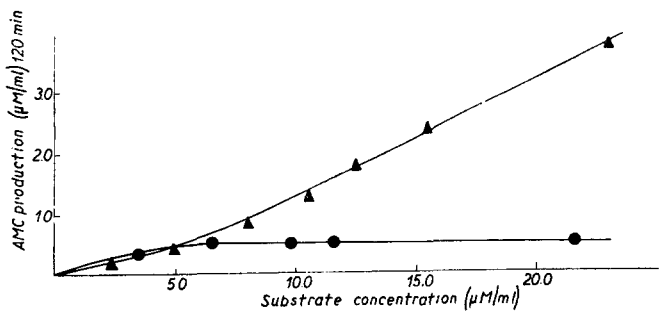

Fig. 4. Effect of substrate concentration on AMC production by washed cell suspension of *A. aerogenes* from glucose —●——●— and pyruvate —▲——▲—

At higher substrate concentrations the rate continued to increase with increasing pyruvate concentration, but with glucose substrates the rate showed no further increases. A similar effect existed in the case of 2:3-butylene glycol formation. The addition

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of pyruvate to glucose dissimilations was next investigated to see if the observed effect with glucose was due to pyruvate formation being the rate determining step in the series of reactions leading from glucose to acetylmethylcarbinol. The results obtained (Fig. 5) showed that the increase in both carbinol and glycol formation was greater than would be expected if the pyruvate was merely acting by increasing the concentration of an intermediate. Furthermore, since the equilibrium position had been reached it was clear that the increase was more than could be accounted for by the sum of the separate effects of the glucose and pyruvate. The results suggested that it was the concurrent dissimilation of small amounts of glucose with the pyruvate which caused a disproportionate increase in the formation of acetylmethylcarbinol from pyruvate.

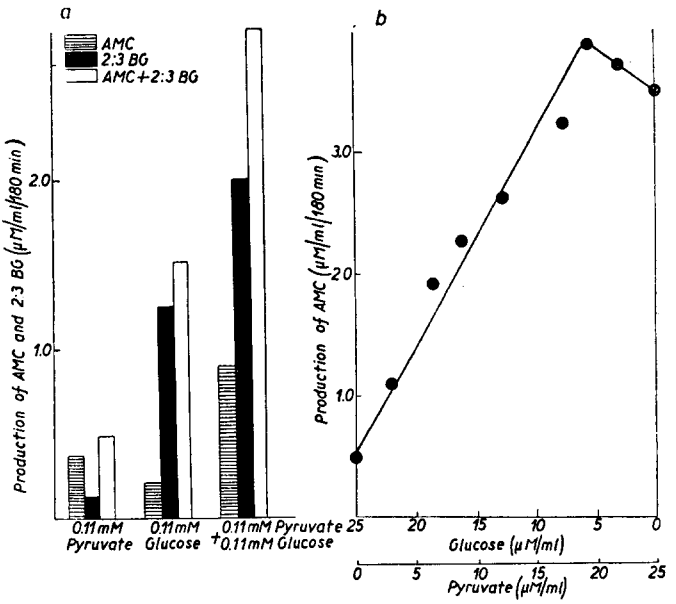

Fig. 5. a. Production of AMC and 2:3 BG from pyruvate, glucose and identical amounts of glucose + pyruvate; b. Effect of the relative proportions of glucose and pyruvate on AMC production by washed cell suspensions of *A. aerogenes* from mixed glucose-pyruvate substrates

An investigation of the rate of substrate disappearance in simple and mixed glucose-pyruvate dissimilations showed that both substrates were utilized together, though at a much reduced rate than when present alone (Fig. 6). Samples taken during these experiments, about four hours after inoculation and some time after complete substrate disappearance, suggested that further changes in the composition of the carbinol-glycol mixture were occurring. Extended observations up to 8 hours after inoculation furnished the results shown in Fig. 7. The salient feature observed when these and the previous experiments are considered together may be summarised as below.

Glucose Substrates

During the dissimilation of about the first two thirds of the substrate the glycol was the major product, its rate of formation being more than twice that of the carbinol. During the utilization of the remaining glucose, the rate of acetylmethylcarbinol and 2:3-butylene glycol formation fell, the effect being greatest with the carbinol. Complete

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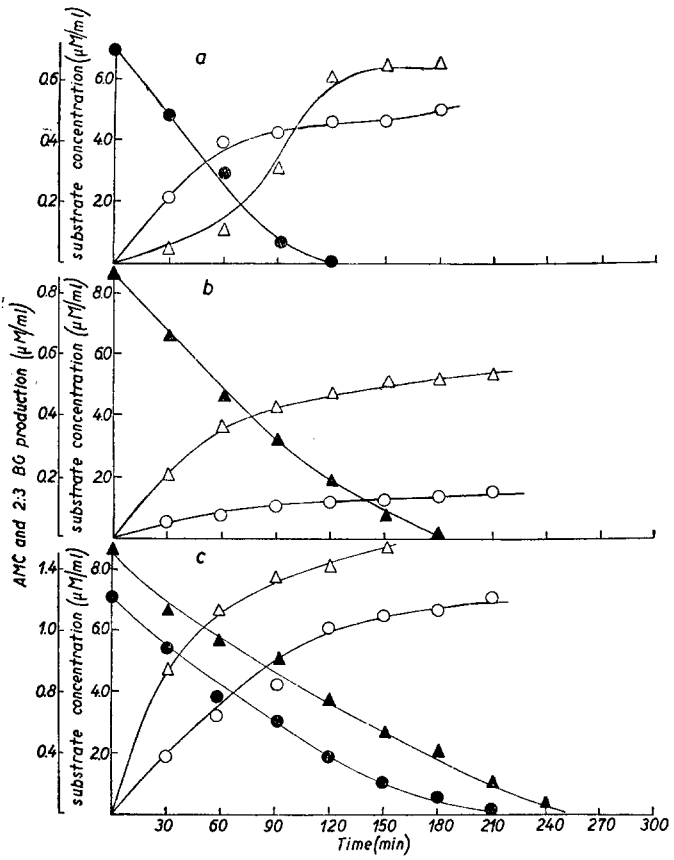

Fig. 6. Progress curves for substrate utilization, AMC and 2:3-butylene glycol production by washed cell suspensions of *A. aerogenes*. a. Pyruvate —●——●—; b. Glucose —▲——▲—; c. Pyruvate + Glucose. —o——o— AMC. —△——△— 2:3 BG

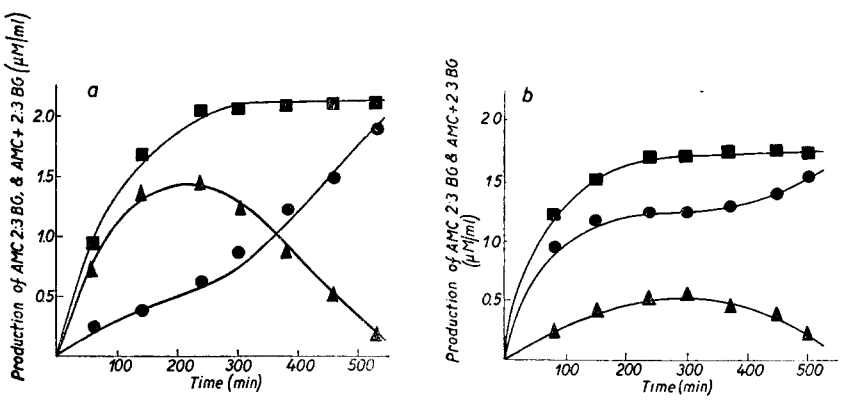

Fig. 7. Progress curves for AMC and 2:3 BG production by washed cell suspensions of *A. aerogenes* from (a) glucose and (b) pyruvate. —●——●— AMC. —▲——▲— 2:3 BG. —■——■— AMC + 2:3 BG

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glucose disappearance was marked by a second increase in the rate of formation of the carbinol, the rate of glycol formation continuing to decrease and becoming zero some time after complete glucose utilization. After the rate of glycol formation had become zero and the carbinol + glycol level had become maximal, rapid conversion of the glycol to the carbinol occurred.

Pyruvate Substrates

In contrast, during the dissimilation of the first two thirds of the substrate, the carbinol was the major product, its rate of formation being over four times that of the glycol. During the dissimilation of the remainder of the substrate the rate of carbinol formation fell off more rapidly than did that of the glycol. (In some cases (Fig. 6a) this effect was so marked that the glycol level finally exceeded that of the carbinol). When the carbinol + glycol level had become maximal, rapid conversion of the glycol to the carbinol again occurred, the latter phase being identical with both glucose and pyruvate substrates.

The Equilibrium between Acetylmethylcarbinol and 2:3 Butylene Glycol

A complex equilibrium seemed to exist between the carbinol and the glycol during the time these compounds were actually being formed. The washed cells of *A. aerogenes* were capable of catalysing the oxidation of 2:3 butylene glycol in synthetic mixtures of the carbinol and the glycol (Fig. 8). In such experiments the 2:3-butylene glycol disappearing was quantitatively oxidised to acetylmethylcarbinol. In the absence of the concurrent fermentation of glucose or pyruvate no evidence of the reverse reaction could be obtained. Unless the glycol is the first four-carbon compound formed from pyruvate, which seems unlikely in view of the results with cell free enzymes (SILVERMAN AND WERKMAN[14]), it seems probable that the reduction of the carbinol to the glycol must be dependent upon the presence of some suitable hydrogen donor or linked hydrogen donating reaction. The cells were not, however, capable of catalysing the reduction of the carbinol during the fermentation of formate which, therefore, does not presumably act in this capacity during normal fermentation.

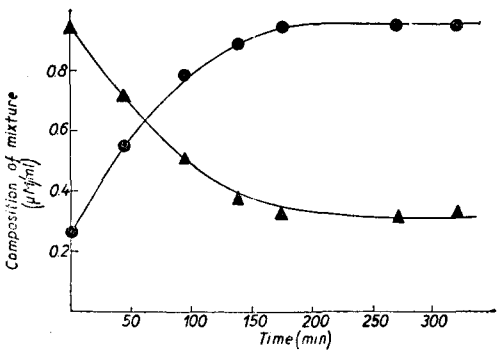

Fig. 8. Effect of washed cell suspensions of *A. aerogenes* on synthetic mixture of AMC (—●——●—) and 2:3 BG (—▲——▲—)

The addition of ascorbic acid to pyruvate dissimilations caused a marked increase in the relative proportion of the glycol formed (Fig. 9), and its effect extended to cause a slight temporary reversal of the normal oxidation of glycol to carbinol. The slight increase in the rate and amount of glycol production was reflected by similar increases in the total production of both four-carbon compounds.

The addition of thioglycollic acid did not exhibit this duality of effect (Fig. 10); there was an increase in the rate and amount of acetylmethylcarbinol production only.

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The thioglycollic acid was without effect on the conversion of the carbinol to the glycol, and the production of the latter compound therefore showed no difference from the controls.

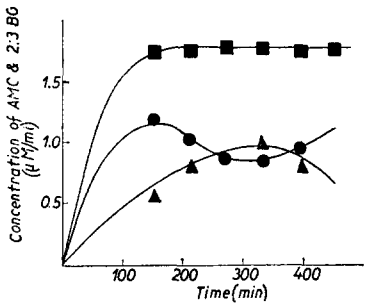

Fig. 9. Effect of 10 μg/ml ascorbic acid on progress curve of AMC and 2:3 BG production from pyruvate by washed cell suspensions of *A. aerogenes*. —●——●— AMC. —▲——▲— 2:3 BG. —■——■— AMC + 2:3 BG. (Compare with Fig. 7b)

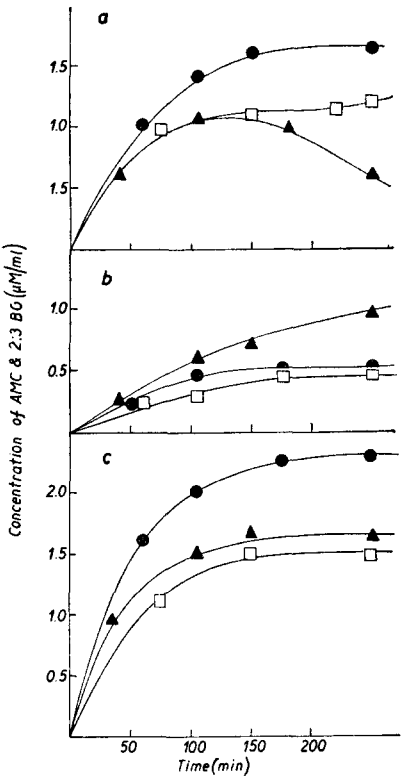

Fig. 10. Effect of hydrogen donors on (a) AMC production (b) 2:3 BG production and (c) AMC + 2:3 BG production from pyruvate by washed cell suspensions of *A. aerogenes*. —□——□— Control (No addition). —▲——▲— 100 μg/ml ascorbic acid added. —●——●— 100 μg/ml thioglycollic acid added

DISCUSSION OF RESULTS

The cells of *A. aerogenes* are capable of forming acetylmethylcarbinol and 2:3-butylene glycol from pyruvic acid, which has been considered as the simple precursor of these compounds but if this was so, it would not be easy to see why significantly more than twice the amounts of the four-carbon compounds should be formed from an equimolar amount of glucose (Fig. 3). Indeed it would seem that less than two molecules of pyruvic acid will result from each molecule of glucose entering the glycolytic cycle and the magnitude of the effect may be more than is apparent. A simple decarboxylation of pyruvate to acetaldehyde followed by condensation to acetylmethylcarbinol seems improbable. The effects of the concurrent fermentation of small amounts of glucose on the production of the four carbon-compounds from pyruvate suggest a connection with the glycolytic cycle (Fig. 5). The results are explicable either in terms of the intervention of an intermediate or a linked reaction of the glycolytic cycle in the formation of acetylmethylcarbinol and 2:3-butylene glycol from pyruvic acid.

Observations of the relative production from glucose and pyruvic acid of acetylmethylcarbinol and 2:3-butylene glycol and of the equilibrium between these two show

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that a most complicated relationship exists (Fig. 7). In something over 200 minutes the combined concentration of acetylmethylcarbinol and 2:3-butylene glycol reaches a maximum and then remains constant. Conversion of the glycol to the carbinol then occurs, the final equilibrium being markedly in favour of the latter compound. Depending upon whether glucose or pyruvate is the substrate, the final equilibrium is achieved in slightly differing fashion since the initial amounts of the two compounds formed differ with each substrate. If the carbinol is the first four-carbon compound formed then differences in the production of acetylmethylcarbinol and 2:3-butylene glycol may be due to the rate of the reducing reaction being increased, in glucose substrates, by the presence of a suitable hydrogen donating system. If such a system is present in greater amounts during the fermentation of glucose, the rate of the reducing reaction may be sufficiently stimulated to allow only slight accumulation of the carbinol. (Some evidence in favour of this view may be seen in the phenomenon of the decrease in rate of formation of the carbinol before that of the glycol. This may be due to the reducing reaction starting to remove the carbinol as fast as it is formed, whereas in the initial stages of the reaction when the rate of formation of the four-carbon compounds is greater, it is unable to do this.) The identity of the hydrogen donating system is of interest. Reduced Coenzyme I (produced by the glycolytic cycle) is a possibility especially in view of the decrease in lactic acid production normally associated with the formation of the four-carbon compounds. The reduction of the carbinol to the glycol only occurs when the four-carbon compounds are actually being formed, and it remains possible that the normal hydrogen donating system is directly involved in the breakdown of pyruvic acid. The reduction may be linked with the oxidative decarboxylation of pyruvic acid, the effect of an oxidation reaction of the glycolytic cycle thus being supplementary but not essential to the formation of the glycol from the carbinol. The conversion of the glycol to the carbinol after formation of the four-carbon compounds has ceased may, on these views, occur when a suitable hydrogen donating system is no longer available to poise the equilibrium in favour of the glycol.

The effects of the additions of hydrogen donors to pyruvate dissimilations are in accordance with these views (Figs 9 and 10). Ascorbic acid is able to act in place of the natural hydrogen donating system and enable conversion of the carbinol to the glycol to occur when pyruvate is no longer being metabolised. In addition ascorbic acid has a dual effect since it influences the rate of formation of the four-carbon compounds. Thioglycollic acid, on the otherhand, only affects the formation of these compounds and not the equilibrium between them. Hydrogen acceptors have long been known to increase (or in the case of yeast, initiate) the enzymic formation of acetylmethylcarbinol, and this would be expected if an oxidative decarboxylation of pyruvic acid occurs. It is not easy to see why hydrogen donors should have a similar effect upon the formation of the four-carbon compounds unless another reductive process is involved apart from the reduction of the carbinol to the glycol. The occurrence of diacetyl as the immediate precursor of acetylmethylcarbinol might account for the observed similarity in the effects of hydrogen donors and acceptors.

Acetaldehyde and acetic acid have both been shown to be condensed to acetylmethylcarbinol and 2:3-butylene glycol during the fermentation of glucose (MICHELSON AND WERKMAN[4]; SLADE AND WERKMAN[5]; STAHYL AND WERKMAN[22]). The observation that suitable hydrogen donors affect the actual formation of acetylmethylcarbinol and 2:3-butylene glycol suggests that acetaldehyde may have a dual action. It was formerly

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considered that acetic acid was reduced to the aldehyde before condensation to the four-carbon compounds. The possibility now arises that added acetaldehyde is first oxidised (acting as a hydrogen donor) to acetic acid and that the acetic acid, or a compound closely related to it, is the immediate precursor of acetylmethylcarbinol and 2:3-butylene glycol.

The cells of the test organism can oxidise the glycol to the carbinol in the absence of interfering reducing factors (Fig. 8). Complete oxidation of biologically produced 2:3-butylene glycol occurs (Fig. 7), whereas the cells can only oxidise about two-thirds of the synthetic material. It is probable that this anomaly is due to the specificity of the enzyme system involved for optical isomers of the glycol. This is in accordance with the results of STANIER AND FRATKIN[23] who showed that *A. aerogenes* only oxidised the *laevo* and *meso* isomers of the glycol. Their studies were solely manometric and showed an uptake of oxygen far in excess of the requirements for conversion to the carbinol. This was interpreted as indicating further oxidation of the carbinol. No evidence was found during the present studies that such an oxidation occurs, but it remains possible that a strain difference may account for the conflicting evidence. The transient VOGES-PROSKAUER reaction observed with several strains of *A. aerogenes* may be due to further oxidation of the carbinol, but reduction to the glycol may also be responsible for the failure of some cultures to give a positive result.

SUMMARY

The formation of acetylmethylcarbinol and 2:3-butylene glycol has been studied using washed cell suspensions of *Aerobacter aerogenes*. The results show marked differences in the production of the two four-carbon compounds from glucose and pyruvic acid and make it improbable that a simple decarboxylation of pyruvic acid to acetaldehyde followed by condensation occurs. The results with mixed glucose-pyruvic acid substrates suggest that the formation of acetylmethylcarbinol from pyruvic acid may be associated with a reaction of the glycolytic cycle. The effects of hydrogen donors on pyruvate dissimilations support the hypothesis that this association may take the form of a linked oxidation-reduction reaction. The implication of these findings on the problem of the mechanism of acetylmethylcarbinol and 2:3-butylene glycol formation is discussed.

RÉSUMÉ

En employant des suspensions cellulaires lavées de *Aerobacter aerogenes* la formation d'acetylmethylcarbinol et de 2:3-butylène glycol est étudiée. On peut constater des différences distinctes entre la production de ces deux corps en expérimentant soit avec du glucose soit avec de l'acide pyruvique; ainsi la supposition qu'il y aurait une simple decarboxylation de l'acide pyruvique jusqu'au acetaldehyde suivie d'une condensation est rendue improbable. Les expériences faites avec des mélanges de glucose et d'acide pyruvique donnent à penser que la formation d'acetylmethylcarbinol à partir de l'acide pyruvique peut s'associer à une réaction du cycle glycolytique. Les effets produits par les donateurs d'hydrogène sur la décomposition de l'acide pyruvique fournissent un support à l'hypothèse que cette association pourrait avoir la forme d'une réaction conjointe d'oxydation et de réduction.

Les déductions tirées de ces observations et concernant les problèmes du mécanisme de la formation d'acide pyruvique et de 2:3-butylène glycol sont discutées.

ZUSAMMENFASSUNG

Es wurde die Bildung des Acetoins und des 2:3-Butylen-glykols bei Verwendung einer ausgewaschenen Zellsuspension von *Aerobacter aerogenes* untersucht. Die Resultate zeigten eine deutliche Verschiedenheit in der Produktion von diesen beiden Verbindungen je nachdem die Experimente mit der Glukose oder mit der Brenztraubensäure angestellt wurden; dadurch hat es sich als unwahrscheinlich erwiesen, dass eine einfache Decarboxylation der Brenztraubensäure zum Acetoin mit

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nachfolgender Kondensation vor sich gehen sollte. Die Resultate, welche mit den Mischungen von Glukose und Brenztraubensäure erhalten wurden, deuten darauf hin, dass die Bildung von Acetoin aus der Brenztraubensäure in einer Vereinigung mit einer Reaktion aus dem glykolytischen Cyclus verlaufen könne. Die Wirkung von Wasserstoffschenkern auf die Zersetzungen von Brenztraubensäure liefert eine Unterstützung für die Hypothese dass diese Vereinigung die Form einer oxydativ-reduktiven gekoppelten Reaktion annehmen kann. Die Folgerungen dieser Befunde in Bezug auf das Problem des Enstehungsprocesses von Acetoin und 2:3-Butylenglykols werden erörtert.

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