THE ENZYMIC FORMATION OF ACETYLMETHYLCARBINOL AND RELATED COMPOUNDS

by

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INTRODUCTION

Acetylmethylcarbinol, 2:3 butylene glycol and diacetyl are of widespread occurrence as products of carbohydrate metabolism. The problem of the mechanism of their formation has attracted numerous workers, the first investigations being made by NEUBERG and his associates working with yeast[1,2,3,4,5,6,7]. They suggested that pyruvic acid was first decarboxylated (by carboxylase) to acetaldehyde which then condensed to form acetylmethylcarbinol. They postulated an enzyme "carboligase" to catalyse this reaction. Although acetylmethylcarbinol is not formed during the normal fermentation of glucose by yeast, additions of acetaldehyde (or other hydrogen acceptors) cause its formation. The anomaly that the carbinol is not formed from glucose alone was later suggested by KLUYVER AND DONKER[8] to be due to the rapid removal of acetaldehyde by reduction. No satisfactory biosynthesis of acetylmethylcarbinol using acetaldehyde alone was achieved although subsequently several reports of such a reaction have appeared (TOMIYASU[9,10] and KUZIN[11]). DIRSCHERL[12,13] has suggested that the carbinol is formed by the non-enzymic condensation of active (*i.e.* biologically produced) acetaldehyde, a hypothesis that gained support by the failure of several workers to demonstrate a separate carboxylase and carboligase (TANKO AND MUNK[14]). Isotopic studies have since confirmed that acetaldehyde added to yeast fermentations is converted to acetylmethylcarbinol but they did little to elucidate the actual mechanism of its conversion (GROSS AND WERKMAN[15,16]).

The data obtained from enzyme studies with mammalian tissue extracts are in many ways analogous to those obtained during studies with yeast. The addition of acetaldehyde to the decarboxylation of pyruvic acid by an enzyme system isolated from pigs heart causes a marked increase in the rate of both carbon dioxide and acetylmethylcarbinol production (GREEN, WESTERFIELD, VENNESLAND, AND KNOX[17]), and both systems are able to form acetylmethylcarbinol from acetaldehyde alone.

Somewhat conflicting data have been obtained in studies with bacteria. Pyruvic acid acts as a precursor of both carbinol and glycol (BARRITT[18]) and the addition of acetaldehyde (MICHELSON AND WERKMAN[19]) or acetic acid (REYNOLDS AND WERKMAN[20]) to fermentations of glucose by *Aerobacter indologenes* and *Bacillus polymyxa* (STAHYL AND WERKMAN[21]) cause an increased formation of the four-carbon compounds. The position was further complicated by the preparation of a cell free enzyme system from *A. aerogenes* which catalyses the anaerobic decarboxylation of pyruvic acid with concurrent formation of acetylmethylcarbinol (SILVERMAN AND WERKMAN[22]), but does not

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utilize acetaldehyde or acetyl phosphate during the dissimilation of pyruvate. Later isotopic studies (GROSS AND WERKMAN[15]) confirmed these results and indicated that at least two mechanisms existed. It was suggested that whilst the condensation of acetaldehyde can be accomplished by whole cells this system is absent from cell-free extracts which produce acetylmethylcarbinol by another mechanism.

The studies reported in this paper are primarily concerned with the mechanism of formation of acetylmethylcarbinol by bacteria and we have endeavoured to relate the results obtained with those from studies with other systems.

EXPERIMENTAL METHODS

Materials

The sample of co-carboxylase used was synthesised by the method of TAUBER[23]; sodium pyruvate was prepared as previously described (HAPPOLD AND SPENCER[24]).

Methods of Estimation

Pyruvic acid was determined colorimetrically as the 2:4 dinitrophenylhydrazone following a specific extraction procedure (FRIEDEMANN AND HAUGEN[25]). Protein nitrogen was estimated by a micro-Kjeldahl method (MARKHAM[26]). Acetylmethylcarbinol was determined by the method described in a previous paper[24]. No 2:3 butylene glycol was formed by the system. Carbon dioxide was measured by conventional WARBURG technique using air as the gas phase in the flasks. Preliminary experiments showed that carbon dioxide evolution was the only gas exchange catalysed by the crude enzyme, oxygen uptake being zero or negligible. Carbon dioxide evolution was measured at 37° C, pyruvate being added from the side arm at zero time. The main chamber of the flasks normally contained the enzyme solution and other addenda in *M*/15 phosphate buffer at pH 5.9. In the experiments when parallel estimations of carbon dioxide and acetylmethylcarbinol were made, the carbon dioxide production was first estimated manometrically and immediately afterwards samples were withdrawn from the flasks, pipetted into a protein precipitant (normally 10% trichloracetic acid) and the acetylmethylcarbinol determined. Parallel control experiments using a boiled sample of the enzyme and without addition of the substrate were always performed.

Growth of Cells

A. aerogenes (laboratory strain) was maintained by fortnightly sub-culturing on ordinary agar slopes. The organism was grown in bulk in 10 l quantities of the following medium: 1.0% glucose, 0.3% peptone, 0.8% dipotassium hydrogen phosphate, 10% tap water (metals). The tap water and phosphate were autoclaved separately and mixed asceptically after sterilization. The bulk medium was inoculated with the whole of the growth from a 24 hour slope culture of the organism and was incubated for 20 to 24 hours at 37° C. The cells were harvested in the form of a paste by means of an Alfa Laval Centrifugal Oil Separator.

Preparation of Cell-Free Enzyme Extracts

SILVERMAN AND WERKMAN[22] ground their cells with powdered glass in preparing their extracts whilst GALE[27] shook cell suspensions with glass beads to liberate the acetylmethylcarbinol forming system from the bacteria. Both methods have been used in the present work.

Active extracts were also obtained from acetone dried bacteria prepared by the following method which overcomes the difficulties of drying cells producing mucilagenous polysaccharide in their growth.

The cells were grown in the usual glucose-peptone media adjusted to pH 6.0 before inoculation. The bulk medium was inoculated by transferring a loop of growth from a 24 hour agar slope culture of the organism to 9 ml of sterile water and agitating until the suspension became just observably turbid. The bulk medium was inoculated with the whole of this suspension and was incubated at 30° C for a maximum of 20 hours. These details resulted in somewhat impaired yields but limited the amount of polysaccharide formed by the cells and so minimised their resistance to the action of the acetone. Failure to observe these details always resulted in a yield of cells unsuitable for acetone drying. The cells from 10 l of medium were harvested, washed, resuspended in 200 ml of distilled water and cooled to 0° C. The chilled suspension was then slowly added to 5 volumes of ice cold acetone, the addition being made slowly and with constant agitation; the suspension was stored at −15° C for 15 to 24 hours. The cells were finally separated by filtration, washed first with 750 ml of 50% acetone-ether and finally with 250 ml of ether, care being taken to see that the powder did not become dry until after the final washing with ether. Final drying was then accomplished by suction of air through the filter.

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Acetone-dried powders of *A. aerogenes* prepared in this way were quite stable if stored in a desiccator at room temperature. The enzyme system was extracted with *M*/15 phosphate buffer, pH 6.0, the optimum time of extraction being 12 hours at 0° C. The cell debris was sedimented by centrifugation for 30 to 45 minutes at 7000 r.p.m. The average yield of cell free extract from 10 l of medium was about 15 ml.

General

Enzyme extracts obtained by all three methods quickly lost activity, five hours standing at room temperature being sufficient to cause inactivation. Irreversible inactivation occurred after about 3½ hours at 37° C even in the presence of the substrate, and at 0° C complete inactivation occurred after 30 hours. Additions of inert proteins, protective colloids and reducing agents did not stabilize the system. The cell free extracts were, therefore, stored in a frozen condition at −15° C. SILVERMAN AND WERKMAN reported that if frozen solid, extracts could be stored for a considerable period with little loss of decarboxylating activity. Our experience has confirmed this but has also shown that freezing and thawing are not without effect upon the acetylmethylcarbinol activity. The practice of storing extracts before use was, therefore, avoided.

EXPERIMENTAL RESULTS

Determination of the optimum pH *of the enzyme system*

A sample of the cell free extract was obtained by the glass grinding technique. The production of carbon dioxide and acetylmethylcarbinol from pyruvate by the enzyme preparation was measured in citrate-phosphate buffers at various pH's determined by a Glass Electrode. There was no appreciable change in the pH of any of the reactants during incubation. The results shown in Fig. 1 are typical of those obtained.

The determinations of the optimum pH of carbon dioxide and acetylmethylcarbinol were made on different samples of the extract and the relative amounts of the two compounds formed are not comparable. The pH optimum of the system is about 5.9 for both acetylmethylcarbinol and carbon dioxide production. All extracts were inactive below a pH of 4 when protein precipitation occurred.

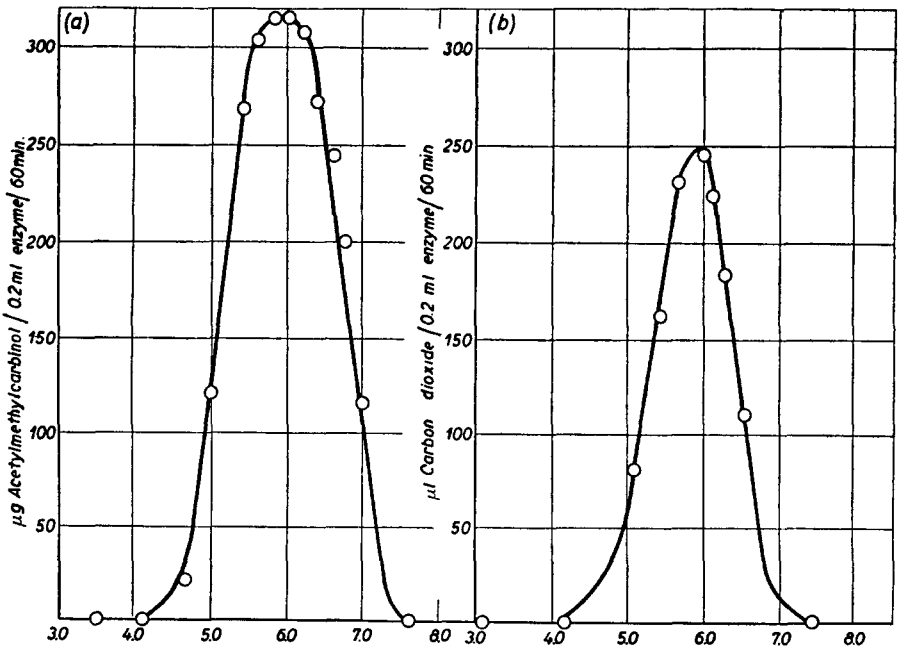

Fig. 1. pH activity curves for acetylmethylcarbinol enzyme of *A. aerogenes*. (a) acetylmethylcarbinol production. (b) carbon dioxide production.

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Comparison of the cell-free extracts obtained by the three methods of preparation

The extracts prepared by the three methods of preparation were compared by means of an arbitrary unit of activity. This was defined as: that amount of acetylmethylcarbinol (in μg) or carbon dioxide (in μl) produced by an amount of enzyme containing 1 mg protein nitrogen after 30 minutes incubation at 37° C in $M/15$ phosphate buffer at pH 5.9 in the presence of excess pyruvate. The results place the shaking method as the most efficient but the extracts obtained from the acetone dried powders are the most convenient to prepare, and such extracts have been used in all further studies unless it is stated otherwise.

In the course of these studies, whilst comparing KCl with the $M/15$ phosphate as an extraction agent for the enzyme system, it was observed that variations in the strength of the KCl employed (from $^1/_8$ to $^1/_2$ saturation) caused CO_2 evolution to drop from 110 to 32 μl/30 min/mg N_2, whereas acetylmethylcarbinol production fell only from 290 to 260 μg.

The effect of co-carboxylase, manganese and magnesium ions on the enzyme system

SILVERMAN AND WERKMAN reported that co-carboxylase, manganese and, to a lesser extent, magnesium ions, increase the rate of decarboxylation of pyruvate. These effects were reinvestigated by measuring the effects of these ions on both carbon dioxide and acetylmethylcarbinol production.

Saturation of the system with either co-carboxylase or manganese ions stimulates the initial rate of carbon dioxide and of acetylmethylcarbinol production. The observation that magnesium ions acted similarly could not be confirmed. Co-carboxylase alone causes a disproportionate stimulation of the initial rate of carbon dioxide production. In fresh samples of the extract equality in the magnitude of the effect on the two activities is restored by concurrent saturation of the system with manganese ions.

It proved impossible to resolve the system by dialysis (with a view to its subsequent reconstruction from known dialysable factors) since, owing to its instability at temperatures above its freezing point, irreversible inactivation of the protein moiety occurs. Dialysable factors are, however, involved since a dialysate (obtained by dialysis of a cell free extract against distilled water) after concentration in a vacuum at 25° C shows a stimulating action on the initial rates of both carbon dioxide and acetylmethylcarbinol production.

Adsorption of the enzyme complex

When an active phosphate buffer extract of acetone dried powder is shaken with kieselguhr, the supernatant removed and the kieselguhr washed with phosphate buffer at pH 5.9 an interesting phenomenon occurs. The carbon dioxide activity of the control is 430 μl/30 min/0.5 ml, the equivalent of the supernatant and the washings 322 and 121 μl respectively. Apparently no adsorption takes place and the agreement between the control and the summated fractions is excellent. When aliquots of the same preparations are tested for acetylmethylcarbinol, however, the summated activity of the fractions are apparently greater than the starting material alone, moreover, the acetylmethylcarbinol/carbon dioxide ratio (μM/ml) changes from 1.29 with the control material to 1.63 with the supernatant and 2.1 with the washings. Clearly some fraction is removed by the kieselguhr which has no effect on carbon dioxide formation but results in a greater total acetylmethylcarbinol formation.

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Fractionation of the enzyme system by precipitation

The enzyme system was precipitated without loss of activity by the following method for which acetone dried powders were suitable only if freshly prepared. The powder was extracted with $M/15$ phosphate buffer (pH 5.9) at 0° C for 10–12 hours and the cell debris removed by centrifugation at low temperature (−2° C). The cell free extract was held in a freezing mixture at 0° C and acetone (previously cooled to −15° C) added drop by drop with constant stirring to a series of samples of the extract. As soon as sufficient acetone had been added to prevent the extract freezing, the temperature was lowered to −8 to −10° C. The samples of precipitated protein corresponding to known additions of acetone, 0–45%, 0–50%, 0–55% . . . 0–80%, were sedimented by centrifugation and redissolved in equal volumes of $M/15$ phosphate buffer (pH 5.9). The acetylmethylcarbinol and carbon dioxide forming activity of each sample was determined, and a determination of Kjeldahl nitrogen made as a measure of protein precipitation. The results shown in Fig. 2 are typical of those obtained.

It will be observed that an increase in the concentration of acetone above 60% to 65% (v/v), etc., caused a small additional precipitation of protein and this coincided with an inhibition of both the carbon dioxide and acetylmethylcarbinol producing activity of the precipitated protein. This could be due to a separate and distinct enzyme substrate competition or to the precipitation of some other inhibitor or, less likely, to enzyme denaturation.

When fractionation was achieved by stepwise addition of acetone to a single extract, 0–40%, 40–45%, 45–50% etc., the precipitates corresponding to each addition were

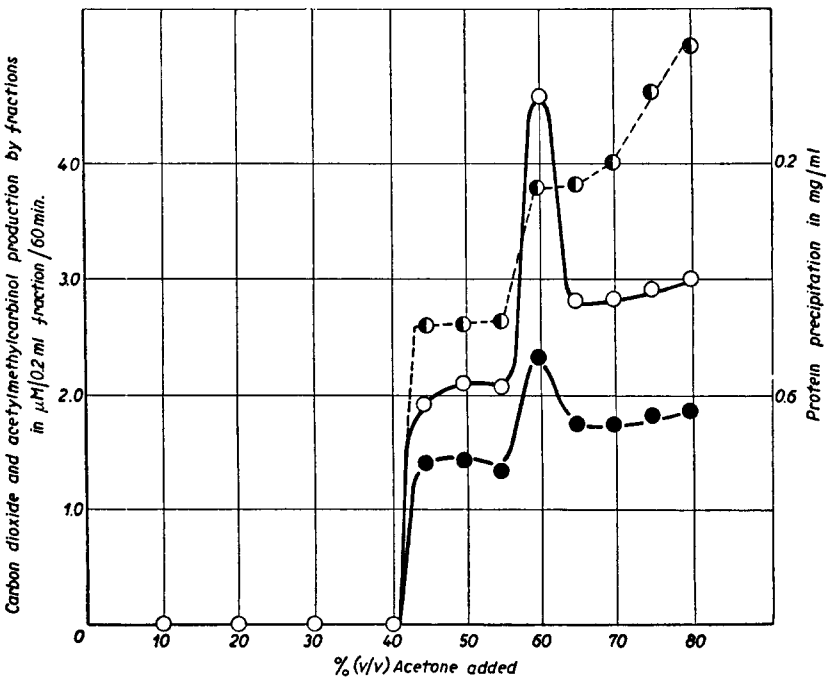

Fig. 2. Fraction curve for precipitation of acetylmethylcarbinol enzyme system of *A. aerogenes* with acetone. -O—O—O- carbon dioxide activity, -●—●—●- acetylmethylcarbinol activity and -◐—◐—◐- protein precipitation.

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separated by centrifugation and redissolved in equal volumes of phosphate buffer.

TABLE I

CARBON DIOXIDE AND ACETYLMETHYLCARBINOL ACTIVITIES OF SEPARATED ACETONE PRECIPITATED FRACTIONS OF ENZYME COMPLEX

Composition of Reaction Mixtures: 0.5 ml (10 mg/ml) sodium pyruvate
2.0 ml $M/15$ phosphate buffer, pH 5.8
0.2 ml enzyme fraction

Fraction (*vol. acetone added*)	*Carbon dioxide* ($\mu M/60$ *min/0.2 ml*)	*Acetylmethylcarbinol* ($\mu M/60$ *min/0.2 ml*)
0–35%	nil	nil
35–40%	1.4	0.9
40–45%	7.4	4.9
45–50%	nil	nil
50–55%	0.8	0.4
55–60%	nil	nil
60–70%	nil	nil

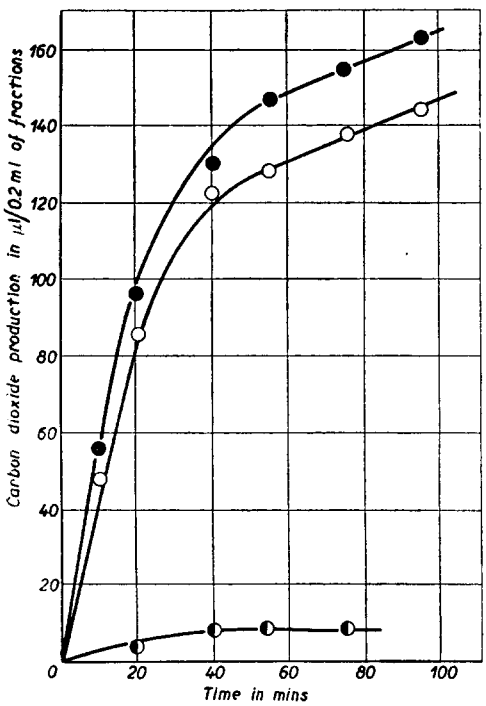

Fig. 3. Progress curves for carbon dioxide production by the acetone precipitated fractions of the acetylmethylcarbinol enzyme system of *A. aerogenes*.
-O—O—O- 0.2 ml 0–45% fraction,
-◑—◑—◑- 0.2 ml 50–55% fraction
and -●—●—●- 0.2 ml 45% fraction plus 0.2 ml 55% fraction

Activity tests conducted with these fractions gave results shown in Table I. There are two very unequal zones of activity, one precipitated between 35 and 45% (v/v) acetone and a second zone of lesser activity between 50–55%.

A partial reconstruction of the whole extract was achieved by additions of equal quantities of the 45–50%, 50–55%, 55–60% and 60–70% fractions to the 0–45% fraction. The results of such combinations (Table II) confirm that the 50–55% fraction (almost without activity) considerably enhances the activity of the 45% fraction when added to it. These findings are readily reproducible and have furthermore been obtained with enzyme extracts from pigs heart muscle (HAPPOLD, HULLIN, NOBLE, AND SPENCER[28]). The progress curves for carbon dioxide production by the whole extract indicate the presence of two carbon dioxide producing steps in the reaction. The usual course of the reaction is an initial rapid linear production of carbon dioxide for about 30 minutes followed by a sharp break and a subsequent linear period in which the carbon dioxide production rate is markedly de creased. The progress curves for carbon dioxide production by the 45% and 50–55% fractions alone and when in combination (Fig. 3) indicate that some separation of these two hypothetical steps is occurring. The stimulating effect of the 55% fraction on the carbon dioxide production of the 45% fraction is limited to increasing the initial rate of carbon dioxide production only.

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TABLE II

CARBON DIOXIDE AND ACETYLMETHYLCARBINOL ACTIVITIES OF COMBINATIONS OF THE ACETONE PRECIPITATED FRACTIONS

Composition of Reaction Mixtures: 0.5 ml (10 mg/ml) sodium pyruvate
1.8 ml *M*/15 phosphate buffer, pH 5.8

0.2 ml 45% fraction with additions of 0.2 ml of fraction as stated	*Carbon dioxide (μM/60 min/0.2 ml)*	*Acetylmethylcarbinol (μM/60 min/0.2 ml)*
0–45% alone	4.5	2.9
0–45% + 45–50%	4.9	3.2
0–45% + 50–55%	6.9	3.4
0–45% + 55–60%	7.2	3.6
0–45% + 60–65%	6.4	3.2
0–45% + 65–70%	6.5	3.2

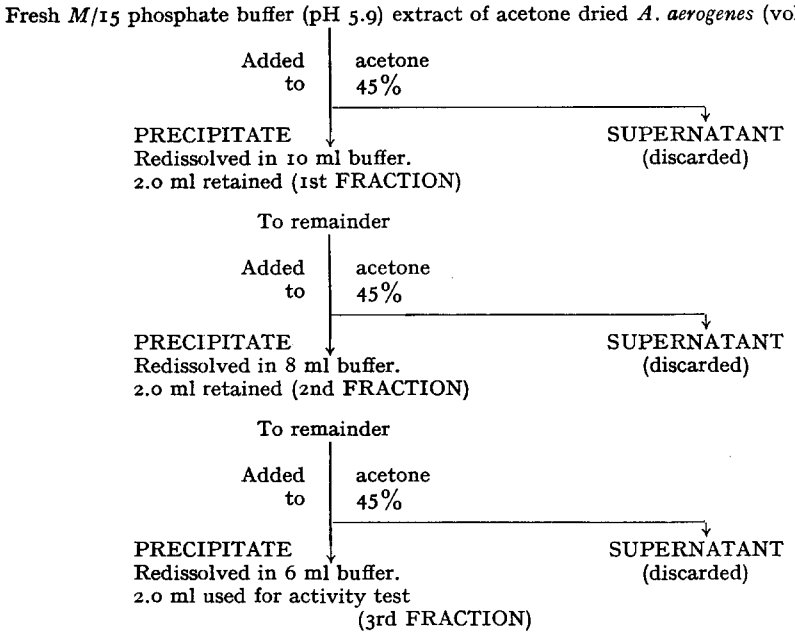

Fig. 4. Scheme for fractionation and further separation of the 45% acetone precipitated fraction of the acetylmethylcarbinol enzyme system of *A. aerogenes*.

The 45% fraction was further separated by submitting it to the fractionation scheme shown in Fig. 4. The activity figures for the fractions together with the relative amounts of pyruvate utilized (Table III) were obtained at the end of the reactions as indicated by a cessation of carbon dioxide evolution (140 minutes). Repeated precipitation of the fraction leads to a marked decrease in pyruvate utilization, acetylmethylcarbinol and carbon dioxide formation; it was found impossible to reprecipitate the fraction more than twice and still retain appreciable activity. The molar ratio between the carbon dioxide and acetylmethylcarbinol produced remained constant but pyruvate utilization does not decrease at the same rate, with the result that the molar ratio pyruvate utilized: carbon dioxide produced, increases as follows: whole enzyme 1.5;

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1st fraction, 1.8, 2nd fraction, 3.2. Reprecipitation of the 45% fraction results in the concentration of a pyruvate utilizing enzyme which does not form acetylmethylcarbinol or carbon dioxide.

TABLE III

ACTIVITIES OF THE FRACTIONS OBTAINED ON FURTHER SEPARATION OF THE 45% ACETONE PRECIPITATED FRACTION

Fraction	*Pyruvate utilization* (μM/140 min/1.0 ml)	*Carbon dioxide production* (μM/140 min/1.0 ml)	*Acetylmethylcarbinol production* (μM/140 min/1.0 ml)
Whole enzyme	17.2	11.5	5.6
1st. fraction	14.0	7.6	3.9
2nd. fraction	5.8	1.8	1.1
3rd. fraction	0.1	nil	nil

Carbon balance studies with the enzyme system

The results obtained in the previous sections had frequently shown a variation in the theoretical molar ratio of 2:1 for the carbon dioxide and acetylmethylcarbinol formed at various times during the course of the reaction. In addition the fractionation studies had shown the possibility of disproportionate pyruvate utilization. SILVERMAN AND WERKMAN[22] using large scale reaction mixtures had been able to demonstrate a molar ratio of 2:2:1 for pyruvate utilized, carbon dioxide and acetylmethylcarbinol produced at the end of the reaction.

A serial analysis of carbon dioxide and acetylmethylcarbinol production during the course of the enzymic reactions with the unfractionated extracts gave the results shown in Table IV.

The theoretical ratio of 2:1 for carbon dioxide and acetylmethylcarbinol is only reached after about three hours incubation when the reaction was in fact complete; *i.e.* the acetylmethylcarbinol formation lags behind that of the carbon dioxide during the course of the reaction. The reverse effect is apparent with the 0–45% fraction (Fig. 2 and Table II); this fraction when isolated gives carbon dioxide acetylmethylcarbinol ratios of 1.5:1, 1.5:1 and 1.4:1 after 60 minutes incubation, when the 50–55% fraction is present in addition to the 45% fraction, the observed ratio then becomes 2.0:1 and 1.9:1 and thus approximates to the theoretical.

TABLE IV

SERIAL ANALYSIS OF CARBON DIOXIDE AND ACETYLMETHYLCARBINOL PRODUCTION DURING THE COURSE OF THE ENZYMIC REACTION

Composition of Reaction Mixtures: 0.5 ml (5.0 mg/ml) sodium pyruvate
1.7 ml M/15 phosphate buffer, pH 5.9
0.5 ml enzyme extract

Time (in minutes)	*Carbon dioxide produced* (μM)	*Acetylmethylcarbinol produced* (μM)	*Molar ratio* CO_2:*AMC*
30	3.8	1.5	2.54
60	5.6	2.1	2.67
100	6.5	2.9	2.24
140	6.7	3.2	2.10
180	6.7	3.3	2.03

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Possible intermediates in the enzymic reaction

The three compounds most likely to occur as intermediates seemed to be acetaldehyde, lactic and acetic acids. The work of WERKMAN[22] and his associates showed that acetaldehyde as such was not involved in the reaction. Lactic acid can not be detected in samples from large scale reaction mixtures at any time during the reaction by either the thiophene reaction of HOPKINS or UFFELMANN's reagent. Similarly, after removing interfering ions by the procedure of HUTCHENS AND KASS[29] negative results are obtained in the lanthanum nitrate test for acetate. No volatile acids can be detected in the distillate after removal of pyruvic acid by redistillation from an acid magnesium-mercuric sulphate mixture.

The effect of both acetaldehyde acetic and lactic acids on the enzymic reaction was investigated. The results confirm the findings of SILVERMAN AND WERKMAN that acetaldehyde does not enter into the reaction. Lactate is without effect either upon the rates of formation or final amounts of carbon dioxide and acetylmethylcarbinol formed or upon the amount of pyruvate utilized, in addition the enzyme system is without effect upon lactate alone. Acetate, on the other hand, stimulates both the rate and amount of carbon dioxide and acetylmethylcarbinol production (Fig. 5 and Table V), its effect on the former being limited to increasing the initial rate of carbon dioxide formation.

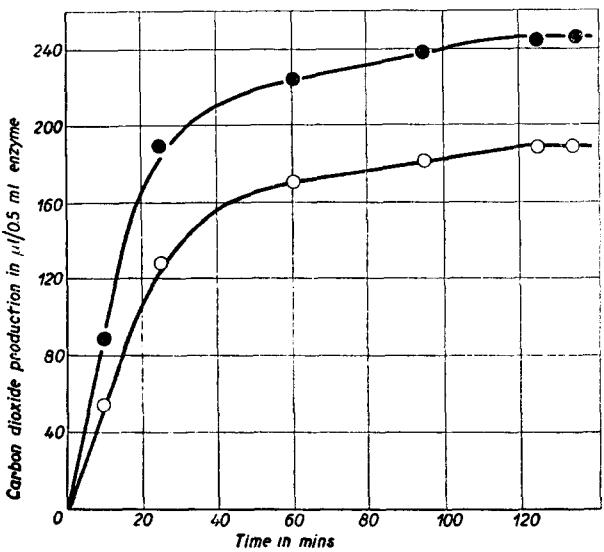

Fig. 5. The effect on carbon dioxide production of the addition of acetate to pyruvate dissimilations by the acetylmethylcarbinol enzyme system of *A. aerogenes*.
—○—○—○— 23 μM pyruvate,
—●—●—●— 23 μM pyruvate plus 20 μM acetate.

TABLE V

EFFECT OF ADDITIONS OF ACETATE TO PYRUVATE DISSIMILATIONS ON THE TOTAL AMOUNTS OF CARBON DIOXIDE AND ACETYLMETHYLCARBINOL FORMED

Composition of Reaction Mixtures: 0.5 ml (5.0 mg/ml) sodium pyruvate
0.5 ml enzyme extract
1.2 ml $M/15$ phosphate buffer, pH 5.9

Addendum	*Carbon dioxide produced* (μM)	*Acetylmethylcarbinol produced* (μM)
0.5 ml phosphate buffer	7.6	3.7
0.5 ml phosphate buffer	7.2	3.6
0.5 ml phosphate buffer	7.3	3.7
0.5 ml phosphate buffer + 0.5 ml (20 μM/ml) acetate	8.9	4.2
0.5 ml phosphate buffer + 0.5 ml (20 μM/ml) acetate	9.0	4.2
0.5 ml phosphate buffer + 0.5 ml (20 μM/ml) acetate	9.2	4.5

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No carbon dioxide or acetylmethylcarbinol are formed by the system from acetate alone.

The "catalytic" effect of acetate on the enzymic reaction was further demonstrated by an examination of the effect of acetate concentration on the magnitude of the stimulation produced. The results shown in Fig. 6 are typical of those obtained. With the sample of the extract used, as little as 2 μM of acetate produces a maximum effect. Above this concentration the magnitude of the stimulation is largely independent of the acetate concentration though an additional stimulation is invariably noticed when the molar acetate concentration approaches that of the pyruvate.

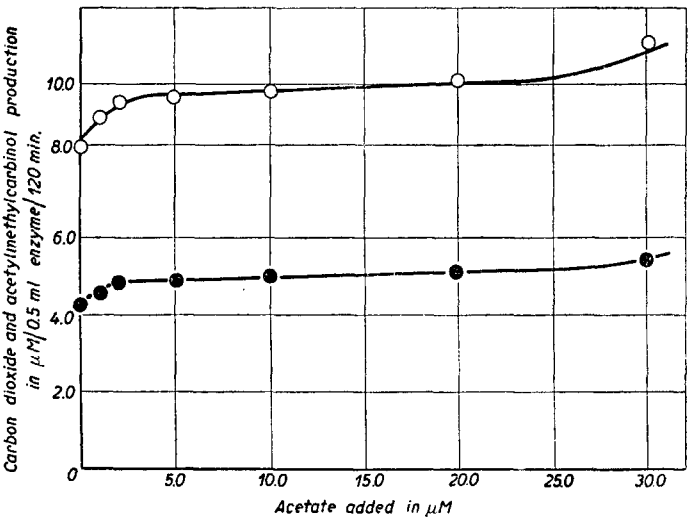

Fig. 6. The effect of concentration of acetate on carbon dioxide –O——O——O– and acetylmethylcarbinol production –●——●——●– by enzyme system from *A. aerogenes* during dissimilation of 23 μM of pyruvate.

DISCUSSION

Since these studies were completed JUNI[30] briefly reports a more complete fractionation of the enzyme from *A. aerogenes*. Of the two fractions he obtained, one acts on pyruvic acid to form α-acetolactic acid and carbon dioxide and the other decarboxylates this to yield acetylmethylcarbinol and carbon dioxide, the latter is without effect upon pyruvic acid. In addition, a variety of Vosges Proskauer positive organisms and the acetylmethylcarbinol enzyme system from pigs heart have been shown to be able to decarboxylate α-acetolactic acid (WATT AND KRAMPITZ[31]; KRAMPITZ AND EVERETT[32]) whilst organisms which do not ordinarily produce acetylmethylcarbinol are capable of catalysing this reaction. The first "pyruvate decarboxylase" fraction has been shown to be cocarboxylase dependent whilst the α-acetolactic acid decarboxylase system is dependent upon manganese or magnesium ions but not cocarboxylase.

This work leaves little doubt that the synthesis of acetylmethylcarbinol can occur through α-acetolactic acid as an intermediate. OCHOA[33] is of the opinion that the mechanism of acetylmethylcarbinol synthesis is best explained by the following reactions:

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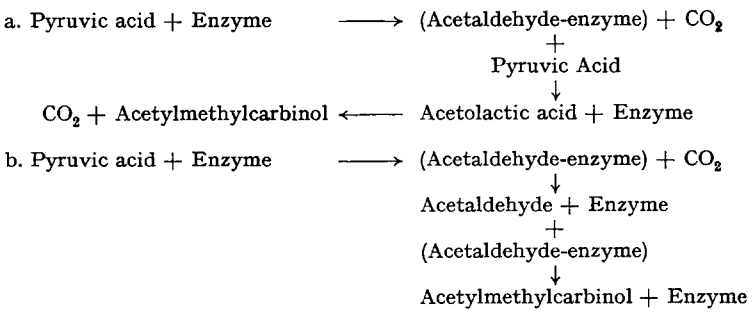

There is evidence against the occurrence of acetaldehyde as an intermediate in the bacterial enzymic synthesis of acetylmethylcarbinol and the formation of an enzyme complex is postulated to explain this. The second mechanism is postulated to account for the condensation of added acetaldehyde by the system from yeast and animal tissues, α-acetolactic acid not occurring as an intermediate in this scheme.

The results reported in this paper confirm much of the findings reviewed above. We have shown that the whole enzyme system has a resultant sharp optimum pH at about 5.9 for both acetylmethylcarbinol and carbon dioxide formation. This is in agreement with the original results of SILVERMAN AND WERKMAN[22] who stated that the pH optimum for the system was in the region of 5.6 to 6.0. The results obtained by these workers were irregular and some of their preparations were apparently as active at pH 4.5 as at 5.6. Our preparations were all comparatively inactive at a pH of 4.5, considerable protein precipitation occurring below this pH. Use of half saturated potassium chloride as an extracting agent for the system and the kieselguhr effect both indicated the presence of two enzymic fractions. Similarly the relative effects of coenzyme factors and metallic ions on acetylmethylcarbinol and carbon dioxide production are in accord with the presence of two distinct systems, one cocarboxylase dependent and one manganese dependent. The partial fractionation achieved indicated the presence of a pyruvate utilizing fraction and of a second fraction that was inactive towards pyruvate in the absence of the first fraction. It seems most probable that our second fraction is identical with the α-acetolactic decarboxylase of JUNI[30]. The kinetics of carbon dioxide production by the whole system are in agreement with the conception of two distinct decarboxylating fractions, the first being associated with the initial attack on pyruvate and the second with the α-acetolactic decarboxylase. Furthermore it appears that the decarboxylating activity of the second fraction is conditional upon prior decarboxylation of the pyruvate by a system contained in the first fraction.

In contrast, however, further attempted purification of our first fraction indicates that a "pyruvate utilizing non-carbon dioxide forming" system may be present which suggests that JUNI's first fraction may not be a single enzyme. Serial analysis of the products during the course of the reaction indicates that the theoretical molar ratio of 2:1 for carbon dioxide and acetylmethylcarbinol produced is only obtained at the end of the reaction. In addition it has been shown that acetate (but not acetaldehyde) is condensed by the bacterial enzyme system during the concurrent decarboxylation of pyruvate; and causes an increase in both the rate and amount of formation of carbon dioxide and acetylmethylcarbinol. These results are not entirely compatible with the reaction paths suggested by OCHOA[33].

Hydrogen acceptors have long been known to act on the enzymic formation of

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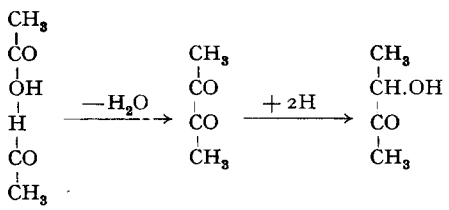
acetylmethylcarbinol by increasing (or in the case of yeast, initiating) its formation, and it has been supposed that they exerted an "acetaldehyde sparing" action thus providing more of the two-carbon compound for condensation to acetylmethylcarbinol. Studies with washed cell suspensions of *A. aerogenes* (HAPPOLD AND SPENCER[26]) have indicated that an oxidative reaction may be involved in the conversion of pyruvic acid to acetylmethylcarbinol and that such an oxidative reaction may be linked with a reductive reaction (other than the reduction of the carbinol to the glycol) which also occurs in the reaction chain. An added hydrogen donor or a hydrogen donating reaction of the glycolytic cycle is able to act in place of the normal hydrogen donating system. MARTIUS[34] has proposed a hypothesis to explain pyruvate metabolism in which he postulates that the first reaction of pyruvate in the presence of co-carboxylase is dehydrogenation, and some evidence has been presented by SOUMALINER AND JÄNNES[35] that dehydrogenation is the primary phase of pyruvate breakdown prior to acetylmethylcarbinol formation by yeast. It therefore seems possible that an oxidative decarboxylation of pyruvate occurs and that acetate (or a compound closely related to it other than acetaldehyde) is the two-carbon compound involved in acetylmethylcarbinol formation. In this case, the absence of an acetaldehyde effect with the bacterial enzyme extract may be due to the absence of an aldehyde oxidase. In contrast, with extracts from mammalian tissue acetaldehyde may have a dual role (*i.e.* as a source of supply of the two-carbon compound and as a hydrogen donor). This would account for the fact that although the acetate effect can be observed with mammalian enzyme extracts, the analogous acetaldehyde effect is markedly greater.

If acetate is involved two possibilities arise. α-acetolactic acid could arise by interaction of acetate and lactic acid. Such a scheme would involve an oxidative decarboxylation and a reduction of pyruvate and might account for the observed effects of hydrogen donors and acceptors on the system. The fact that lactate is not condensed by the bacterial system may be due to its not appearing as such in the reaction (enzyme complex?). Alternatively, a condensation between acetate and acetaldehyde (as an enzyme complex) is possible:



A reaction of this nature might pass through diacetyl as an intermediate which would provide the symmetrical intermediate that the isotopic studies indicate. There is no direct evidence for the occurrence of diacetyl in this role but SOUMALINER AND JÄNNES[35] claim to have detected diacetyl in yeast dissimilations of pyruvic acid under conditions that make its formation by fortuitous oxidation of the carbinol unlikely. In addition, an enzyme system has been isolated from mammalian tissue which catalyses the reduction of diacetyl to acetylmethylcarbinol (STUMPF, GREEN AND ZARUDNAYA[36]). The isolated system uses a second molecule of diacetyl as a hydrogen donor but it is possible that, if this system is involved in the enzymic synthesis of acetylmethylcarbinol, it might be linked to the oxidative decarboxylation of pyruvate. Previous work with

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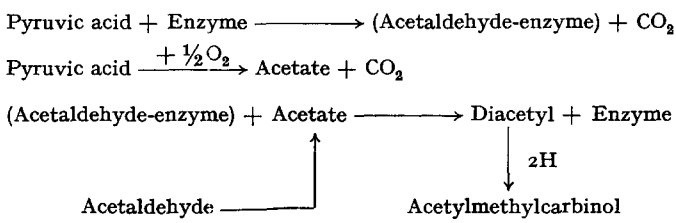
substituted aldehydes (NEUBERG AND HIRSCH[1]; BERG AND WESTERFIELD[37]) makes it appear probable that the condensation of acetaldehyde involves the coupling of the aldehyde (or its derivative) with a two-carbon compound and not with pyruvate or lactate, in which case α-acetolactic acid may not be involved in this pathway. The bulk of the evidence at the present time seems to indicate that this is the case, the kinetic data for carbon dioxide production on additions of acetate indicating that the increased rate involves the decarboxylation associated with the first fraction and not the α-acetolactic decarboxylase fraction. To explain the increased rate of carbon dioxide formation on addition of acetate it is necessary to assume that the condensation between pyruvate and the acetaldehyde-enzyme complex is the rate limiting step, added acetate thus being able to compete with the excess pyruvate.

OCHOA's scheme for the intervention of acetaldehyde (reaction scheme b) may be redrafted as below:



Such a modification provides an explanation of the effects of acetate and of hydrogen donors on the reaction. It does not appear that this mechanism operates through α-acetolactic acid as an intermediate and hence it must be supposed to function as an alternative and in addition to that reaction path.

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SUMMARY

A cell-free enzyme extract which catalyses the anaerobic decarboxylation of pyruvic acid with the formation of acetylmethylcarbinol has been isolated from *Aerobacter aerogenes* by three methods (*viz.* by grinding with powdered glass, by shaking with glass beads and by extraction of an acetone dried preparation). Adsorption of the complex, fractionation studies and an examination of the effects of coenzyme factors indicate that at least two systems are involved. The whole system is able to condense acetate but not acetaldehyde during the concurrent decarboxylation of pyruvate. It is suggested that acetate may be involved in one type of enzymic synthesis of acetylmethylcarbinol and that acetaldehyde is condensed only after oxidation to acetate. The possibility of the occurrence of diacetyl as an intermediate in the reaction involving acetate is discussed.

RÉSUMÉ

Un suc enzymatique dépourvu de cellules agissant comme catalisateur de la décarboxylation anaérobique de l'acide pyruvique avec la production d'acétylmethylcarbinol a été obtenu de l'*Aerobacter aerogenes* au moyen de trois méthodes: en broyant les bactéries avec du verre pulverisé, en les agitant avec des billes de verre, ou enfin par l'extraction d'une préparation sechée avec de l'acétone. Les données fournies: par l'adsorption de ce suc enzymique, par les résultats du fractionnement et l'effet des agents activateurs indiquent qu'au moins deux systèmes enzymatiques y sont présent. L'ensemble est capable de condenser l'acétate mais non pas l'acétaldehyde lors de la décarboxylation simultanée du pyruvate. On peur supposer que l'acétate intervient dans un genre de synthèse enzymatique d'acétylmethylcarbinol et que l'acétaldehyde est condensé seulement après avoir été transformé en acétate par oxydation. La possibilité de la presénce du diacetyl comme intermédiaire dans cette réaction est discutée.

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ZUSAMMENFASSUNG

Ein zellenfreier Enzymsaft, welcher die anaerobe Decarboxylation der Brenztraubensäure unter Bildung von Acetoin katalisiert, wurde aus dem *Aerobacter aerogenes* auf dreifachem Wege, nämlich durch Zerreiben mit pulverisiertem Glass, durch Schütteln mit Glassperlen und durch Extrahieren eines mit Aceton getrockneten Präparates erhalten. Die Adsorption des Enzymsaftes, die Ergebnisse der Fraktionierung, sowie die Beobachtungen über die Wirkung der Aktivatoren weisen darauf hin, dass wenigstens zwei Enzymsysteme dabei beteiligt sind. Das System als Ganzes ist imstande Acetat, nicht aber Acetaldehyd während der gleichzeitigen Decarboxylation der Brenztraubensäure zu kondensieren. Es wird angenommen, dass das Acetat in einer der enzymatischen Synthesen des Acetoins beteiligt sein könnte und dass Acetaldehyd erst nach Oxydation zu Acetat kondensiert wird. Die Möglichkeit des Auftretens von Diacetyl als einer Zwischenstufe in dieser Reaktion wird erörtert.

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