MECHANISM OF ACETOIN SYNTHESIS BY α-CARBOXYLASE*

by

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Since the discovery of acetoin (acetylmethylcarbinol, AMC**) production by fermenting yeast[3], the formation of this substance has been demonstrated in a variety of bacteria, invertebrates, vertebrates, and higher plants[4–8]. The intermediate steps in the bacterial synthesis of AMC from pyruvate have been recently elucidated. The mechanism of AMC formation in plants and animal tissues, where pyruvate plus acetaldehyde or acetaldehyde alone are the reactants, has not been completely understood[9]. Available evidence indicates that α-acetolactate, an intermediate in AMC synthesis by bacteria, is not involved in AMC formation in yeast, pig heart[10] or filariae[8], and the same is true of wheat germ.

Although differences exist in the conditions required for AMC synthesis in yeast, pig heart, and in higher plants, the fundamental mechanism of the reaction might be the same in all of these cases, and this mechanism has been the subject of a long-standing controversy[9,11,12]. Until recently, most of the experimental work on the mechanism of AMC formation has been performed with yeast. It has been suggested that a special enzyme, carboligase, exists in yeast, which accomplishes the condensation of "nascent acetaldehyde" with acetaldehyde, or other aldehydes, to acyloins[3,13]. The "nascent acetaldehyde" was thought to arise from the decarboxylation of pyruvate by α-carboxylase. The opponents of this view have attempted to prove that AMC synthesis is a by-product of the decarboxylation of pyruvate by α-carboxylase and that the condensation occurs *in situ* on α-carboxylase[14,15]. DPT is known to be a requisite for the formation of AMC by animal and plant tissues, but there is little agreement on the exact function of DPT in this reaction. The resolution of these problems appears to be in the use of highly purified enzyme preparations for the study of AMC synthesis.

Partially purified preparations of α-carboxylase from yeast have been reported[16,17] but AMC formation was not followed in the course of purification. The availability of the α-carboxylase of wheat germ in a highly purified state[18] has opened the way for a re-examination of these questions. The results are reported in this paper.

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* This research was supported by grants from The National Vitamin Foundation, Inc., the Division of Research Grants and Fellowships, National Institutes of Health, U.S. Public Health Service, and the Williams-Waterman Fund of the Research Corporation. Preliminary reports of this work have been published[1,2].

** The following abbreviations are used: AMC, acetylmethylcarbinol; DPT, diphosphothiamin; DPN, diphosphopyridine nucleotide; $DPNH_2$, reduced DPN; TPN, triphosphopyridine nucleotide; A.R., activity ratio.

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MATERIALS AND METHODS

The crystalline dimer of AMC was prepared from commercial AMC (Fairmount) by a modification[8] of the method of KLING[19]. It was thoroughly washed with ether before use. Preparations of (—) AMC, isolated from *A. aerogenes* and from filariae[8], were kindly supplied by Dr E. BUEDING. Diacetyl (Eastman) was redistilled immediately before use. DPN was a commercial product (Schwartz Laboratories, Inc.); TPN was isolated by the method of LE PAGE AND MUELLER[20], and $DPNH_2$ was prepared by OHLMEYER'S method[21]. Alcohol dehydrogenase was purified from yeast[22]. Acetaldehyde and AMC were determined colorimetrically, after deproteinization with $Zn(OH)_2$[23], by the methods of STOTZ[24] and WESTERFELD[25], respectively. In order to correct for the slight color produced by high concentrations of acetaldehyde and pyruvate in the AMC determination, aliquots from each tube were removed and deproteinized at zero time and the color given by the "initial" samples was substracted from that of the corresponding incubated samples. Unless otherwise stated, in experiments on AMC synthesis the amount of enzyme present in 3 ml incubation mixture was chosen so as to give 400 μl CO_2 evolution in 5 minutes, in the standard manometric α-carboxylase assay[18]. The purified enzyme preparations mentioned in the experimental section are those described in the preceding paper[18]. Other methods and materials were as previously described[18].

RESULTS

AMC production in wheat germ preparations. Extracts of wheat germ and preparations of α-carboxylase isolated therefrom[18] at all stages of purity catalyze the formation of AMC from pyruvate plus acetaldehyde or from acetaldehyde alone. The formation of AMC progresses linearly with time and it is proportional to the enzyme concentration, even in the crudest extracts (Fig. 1). Deviation from a linear rate occurs only when the concentration of acetaldehyde or of pyruvate falls below the saturation level. It may be noted that the synthesis of AMC from acetaldehyde plus pyruvate is considerably more rapid than from acetaldehyde alone. Addition of pyruvate always increases the rate of AMC formation over that obtained from acetaldehyde alone, the increase being 2- to 4-fold, depending upon the relative concentrations of acetaldehyde, pyruvate, and enzyme.

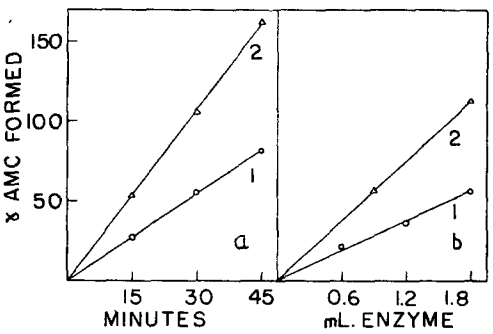

Fig. 1. Proportionality of AMC formation with time and enzyme concentration. Enzyme, water extract of wheat germ, centrifuged 1 hour at 10,000 r.p.m., 106 mg dry weight per ml. Each tube contained in 3 ml total volume 0.75 ml. 0.2 *M* succinate buffer, pH 6.0, 0.1 ml 0.03 *M* $MgSO_4$, 0.15 ml $3.9 \cdot 10^{-4}$ *M* DPT, and the appropriate substrates. (a) 1.8 ml enzyme; time as shown. (b) Enzyme as shown; 30 minutes incubation. Curve 1, 0.1 *M* acetaldehyde alone. Curve 2, 0.05 *M* acetaldehyde plus 0.005 *M* pyruvate.

Like decarboxylase activity[18], the formation of AMC requires the addition of DPT and of a metallic ion for full activity. It is of interest in connection with the question of the identity of α-carboxylase with the AMC synthesizing enzyme that in the course of the purification of α-carboxylase the requirements for both cofactors appear at the same stage, and to the same degree, whether the decarboxylase or the AMC-synthesizing activities are measured. The optimal concentrations of DPT ($1.9 \cdot 10^{-5}$ *M*) and of Mg^{++} ($1 \cdot 10^{-3}$ *M*) are identical for both types of activity. The same bivalent metals activate AMC synthesis from acetaldehyde and pyruvate, or from acetaldehyde alone, as were found to be active in the decarboxylase assay. The affinity constant, K_M, of DPT for the enzyme was $1.37 \cdot 10^{-6}$ *M* when AMC syn-

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thesis from acetaldehyde alone was studied, and $1.44 \cdot 10^{-6} M$ when acetaldehyde plus pyruvate were the substrates. These values agree excellently with the K_M established in decarboxylase measurements, $1.35 \cdot 10^{-6} M$. Similar agreement was found when the K_M of Mg^{++} in the AMC-synthesizing system was compared with the value found in decarboxylase assays. In all of these experiments the enzyme preparations used were at stages 4 and 5 of the purification procedure.

The pH activity curve for AMC formation from acetaldehyde plus pyruvate coincides with the pH-activity curve for the decarboxylation of pyruvate (Fig. 5a in [18]). The same is true of the pH curve for AMC synthesis from acetaldehyde alone, at least in the region of the optimal range and on the acid side of the curve. On the alkaline side of the optimum the decline is more moderate than in the presence of pyruvate. As pointed out in the DISCUSSION, this may indicate the presence of an electropositive group in the active center, which is necessary for the binding of pyruvate, but not of acetaldehyde, to the enzyme.

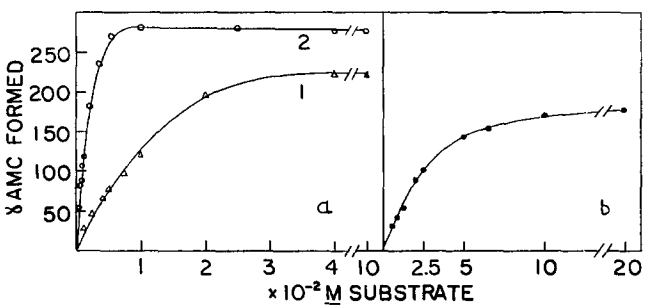

Fig. 2. Influence of substrate concentration on AMC synthesis. (a) Acetaldehyde plus pyruvate; (b) acetaldehyde alone. In (a) Curve 1 represents acetaldehyde concentration in the presence of $5 \cdot 10^{-3} M$ pyruvate, Curve 2 represents pyruvate concentration in the presence of $5 \cdot 10^{-2} M$ acetaldehyde. Experimental conditions as in Fig. 1, except that incubation time was 1 hour. Enzyme in (a) was at stage 5 of the purification procedure, A.R. = 4,000, 0.21 mg protein per 3 ml in Curve 1, 0.24 mg protein in Curve 2. In (b) the enzyme preparation was similar, A.R. = 4,150, 0.32 mg protein per 3 ml.

The influence of substrate concentration on the rate of AMC synthesis is shown in Fig. 2. When both acetaldehyde and pyruvate are present, maximal velocity is reached at approximately $5 \cdot 10^{-3} M$ pyruvate and $5 \cdot 10^{-2} M$ acetaldehyde. Half-saturation is reached at $1.3 \cdot 10^{-3} M$ pyruvate and $8.6 \cdot 10^{-3} M$ acetaldehyde (Fig. 2a). With acetaldehyde alone as substrate, saturation is reached at a concentration of about $2 \cdot 10^{-1} M$ and half-saturation at $2 \cdot 10^{-2} M$ (Fig. 2b).

When pyruvate ($5 \cdot 10^{-3} M$) is the sole substrate, the amount of AMC synthesized is less than 5% of the amount formed in the presence of excess acetaldehyde (Fig. 2a, curve 1). This may first seem like a paradoxical situation, since the enzyme preparation is known to decarboxylate pyruvate and thus form its own acetaldehyde. This observation may be readily explained, however, on the basis of two properties of the enzyme. First, a relatively high concentration of acetaldehyde is required for rapid AMC synthesis. Second, the accumulation of acetaldehyde strongly inhibits further decarboxylation of pyruvate. Thus acetaldehyde is not produced in sufficient amounts to meet the saturation requirements for AMC synthesis (*cf.* DISCUSSION). However, when the decarboxylation is accelerated by raising the pyruvate concentration to $5 \cdot 10^{-2} M$, a

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significant amount of AMC accumulates without added acetaldehyde, although never as much as with acetaldehyde added in excess.

Identification of product as AMC. Since the measurements of AMC in the foregoing experiments were based solely on a colorimetric method, it was felt desirable to identify further the product. Definite proof that the substance measured was AMC came from its conversion to diacetyl, from periodate degradation, and from the isolation of a derivative.

In a typical experiment designed to accumulate a relatively large amount of ketol, 12.6 mg of an alcohol-precipitated enzyme preparation, A.R. = 3,700, were incubated with 0.15 M succinate buffer, pH 6.0, $1 \cdot 10^{-3}$ M $MgSO_4$, $3.8 \cdot 10^{-5}$ M DPT, $2.5 \cdot 10^{-1}$ M pyruvate, and $2.5 \cdot 10^{-1}$ M acetaldehyde in a total volume of 5 ml at 30°. During the incubation (190 minutes) the pH was maintained between 6.5 and 7.0 by intermittent addition of 1 N acetic acid. After deproteinization with $Zn(OH)_2$ at 0°, 0.5 ml 0.5 M KH_2PO_4 was added and the solution was distilled *in vacuo* at a bath temperature of 25° to 30°. The first ml of the distillate, containing most of the residual acetaldehyde, was discarded; the rest was collected in a flask surrounded by dry ice. The concentration of the AMC in the distillate (4.2 ml), determined colorimetrically[25], was 10.0 mg per ml. This was the stock solution used in the succeeding experiments.

One-tenth ml of this solution was oxidized with $FeCl_3$ in H_2SO_4 to diacetyl[25], and the latter was determined by the method of WHITE *et al.*[26] The result indicated that 0.1 ml of the stock solution of the enzymically produced AMC (1.0 mg AMC) gave rise to exactly the same amount of diacetyl as 1.0 mg authentic AMC.

An aliquot of the stock solution of the enzymically synthesized AMC was degraded with periodic acid[27]. Determinations of the periodate consumption and of the acetaldehyde and acetic acid formation were in good agreement with the concentration of AMC calculated from colorimetric measurements (Table I). In another experiment the periodate degradation of an AMC sample, which had been formed from acetaldehyde alone as substrate, gave the same results.

TABLE I

PERIODATE OXIDATION OF ENZYMATICALLY PRODUCED AMC

AMC used	*Periodate consumed*	*Acetaldehyde produced*	*Acetic acid produced*
μM*	μM	μM	μM
67.7	65.2	62	67.1

* Concentration of stock solution estimated colorimetrically. The reaction was carried out in 10 ml 0.05 M phosphate, pH 7.1, containing 67.7 μM (5.96 mg) AMC and 300 μM KIO_4 at 25° for 30 minutes. Acetaldehyde was removed by brief aeration at 100° and collected in a bisulfite tower; its concentration was estimated iodometrically[28]. Aliquots of the remaining solution were used for determination of the unused periodate[27] and for steam distillation and titration of volatile acids.

For further identification 2.2 ml of the stock solution used in the foregoing experiments (22 mg AMC) were treated with semicarbazide hydrochloride. The semicarbazone, isolated in good yield, melted* at 180.5–181°; an authentic sample of acetoin semi-

* All melting points are corrected.

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carbazone melted at 180.5–181°; "mixed m.p.", 181°. The melting point remained unchanged on recrystallization from 25% ethanol.

*Analysis**. $C_5N_3O_2H_{11}$

Calculated	C 41.37, H 7.64, N 28.95
Found, derivative of enzymically produced compound	C 41.45, H 7.75, N 28.73

Added proof of the identity of the enzymically accumulated compound with AMC came from studies of the stoichiometry of its formation.

Stoichiometry of AMC synthesis. The stoichiometry of AMC formation from acetaldehyde as the sole substrate is shown in Table II. The results are in good agreement with the formulation: 2 $CH_3CHO \longrightarrow CH_3COCHOHCH_3$. In another experiment, performed under similar conditions but with both acetaldehyde and pyruvate added at equal concentrations, at the completion of the reaction 1 mole of AMC accumulated for each mole of pyruvate used, in accord with the equation:

$$CH_3COCOOH + CH_3CHO \longrightarrow CH_3COCHOHCH_3 + CO_2.$$

TABLE II

STOICHIOMETRY OF AMC FORMATION FROM ACETALDEHYDE

Time	*AMC produced*	*Acetaldehyde utilized*	
		Found	*Calculated*
hrs	*μM per ml**	*μM per ml**	*μM per ml**
1	8.81	17.6	17.6
2	15.9	31.6	31.8
3	19.3	39.6	38.6

* All concentrations are expressed as μM per ml reaction mixture. The latter contained 3 mg enzyme (Step 5, A.R. = 4,500), $7.5 \cdot 10^{-2}$ *M* imidazole buffer, pH 6.35, $1 \cdot 10^{-3}$ *M* $MgSO_4$, $1.9 \cdot 10^{-5}$ *M* DPT, and $5 \cdot 10^{-2}$ *M* acetaldehyde in a total volume of 1.52 ml. The solution was incubated at 30°, and aliquots were removed at zero time and at the intervals indicated. After deproteinization with $Zn(OH)_2$, the concentration of acetaldehyde was determined colorimetrically on distillates[24] and AMC was estimated directly in the filtrates[25].

Irreversibility of AMC formation. AMC utilization was tested with preparations varying from crude aqueous extracts of wheat germ to the most highly purified fractions of α-carboxylase, using racemic and (—) AMC, as well as a preparation containing predominantly the (+) form of AMC. In no case could a net utilization be detected, under aerobic or anaerobic conditions. This is to be expected from the fact that in the presence of a sufficient amount of enzyme the formation of AMC from either acetaldehyde or acetaldehyde plus pyruvate is essentially quantitative. Further evidence of the irreversibility of AMC formation is presented in Fig. 3. In this experiment alcohol dehydrogenase and $DPNH_2$ were used to trap any acetaldehyde that might be formed and also to drive the reaction in the direction AMC $\longrightarrow$ 2 CH_3CHO. As seen in Fig. 3, no oxidation of $DPNH_2$ and, therefore, no acetaldehyde formation could be detected, although subsequent addition of 2.5 μ*M* acetaldehyde caused immediate oxidation of the $DPNH_2$, and the system could have detected the presence of as little as 0.1 μ*M*

* The microanalysis was carried out by Dr E. W. D. HUFFMAN, Denver, Colorado.

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of acetaldehyde. Additional evidence for the irreversibility of AMC formation by this enzyme will be presented in the section on *Optical Activity*.

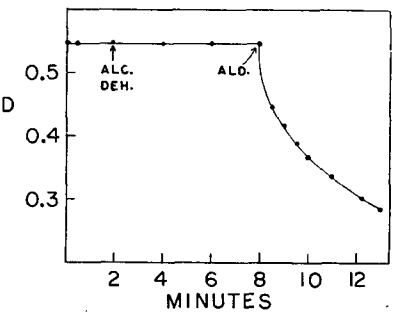

Fig. 3. Demonstration of the irreversibility of AMC formation. The values on the ordinate are optical densities at 340 mμ in 1 cm cuvettes, determined in the Beckman spectrophotometer. Each cuvette contained, in a total volume of 3 ml, 0.5 ml $1 \cdot 10^{-1}$ *M* imidazole buffer, pH 6.5, 1.4 mg enzyme protein (Step 5, A.R. = 4,600), 0.3 ml $1 \cdot 10^{-2}$ *M* $MgSO_4$, 0.15 ml $3.9 \cdot 10^{-4}$ *M* DPT, and 0.3 ml $5 \cdot 10^{-2}$ *M* racemic AMC. The solution was incubated for 15 minutes and 0.15 ml $1.8 \cdot 10^{-3}$ *M* $DPNH_2$ was added at zero time. Alcohol dehydrogenase (0.2 mg purified protein) and 0.15 ml $1 \cdot 10^{-2}$ *M* acetaldehyde were added at the times indicated by the arrows. All values are calculated for 3 ml volume and are corrected for the slight absorption given by alcohol dehydrogenase. In control experiments the amount of α-carboxylase protein used here, in the presence of the same components and excess acetaldehyde, synthesized 5 μ*M* AMC in 15 minutes.

Identity of α-carboxylase and AMC-synthesizing enzyme. The close agreement in the apparent pH optima and in the requirements for DPT and a metallic cofactor between α-carboxylase and the enzyme responsible for AMC synthesis suggested that the two may be identical. More conclusive evidence for this view came from purification and inactivation studies.

Table III demonstrates that the ratio of decarboxylase activity to AMC-synthesizing activity (from acetaldehyde and pyruvate) is essentially constant throughout the 2,700-fold purification. The same constancy holds towards AMC synthesis from acetaldehyde alone. As a matter of fact, the addition of pyruvate brings about a constant increase (about 2.6-fold) in the rate of AMC formation, over that given by acetaldehyde alone as substrate, in all fractions tested, provided that the experimental conditions are kept constant*.

TABLE III

DECARBOXYLATION AND AMC SYNTHESIS AT VARIOUS STAGES OF PURIFICATION

*Purification step**	Q_{CO_2}	*Ratio*** *of decarboxylation/ AMC synthesis*
Crude water extract	25	66.0
After precipitation at pH 4.9	1,190	67.9
After ethanol fractionation	18,000	61.2
After $(NH_4)_2SO_4$ precipitation and dialysis	67,200	65.7

* For details *cf.*[18]

** μ*M* Co_2 per mg per hour/μ*M* AMC produced per mg per hour from acetaldehyde and pyruvate. CO_2 evolution from pyruvate was measured in the standard α-carboxylase assay and the conditions for AMC synthesis were those given in METHODS and in the legend of Fig. 1.

* The experimental conditions referred to are the same as in Fig. 1, except that the enzyme concentration selected is such as to give 400 μl CO_2 evolution in 5 minutes in the standard α-carboxylase assay. At constant concentration of pyruvate and acetaldehyde, the amount of enzyme protein present determines the increase in AMC formation afforded by pyruvate. When the amount of α-carboxylase present is greater than stated, the increased AMC formation in the presence of pyruvate is also greater, up to a limiting value of about 4 times the rate on acetaldehyde alone.

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It was pointed out in the last paper[18] that the most purified fractions of α-carboxylase (Q_{CO_2} about 67,500), while apparently homogeneous in electrophoretic measurements, are contaminated with some 7 to 10% impurity, which was detected and separated in the ultracentrifuge. No AMC-synthesizing activity was found in either of the two minor ultracentrifugal components present in the purified enzyme.

As an independent line of evidence for the identity of α-carboxylase with the AMC-synthesizing enzyme, systematic inactivation studies were carried out. Table IV demonstrates that the loss of AMC-synthesizing activity parallels the degree of inactivation in the decarboxylase test, attendant upon denaturation by heat, aging or inhibition by *p*-chloromercuribenzoate.

TABLE IV

EFFECT OF PARTIAL INACTIVATION

Treatment	*Inactivation*	
	Decarboxylation	*AMC synthesis**
	per cent	*per cent*
Heated, 54° for 5 mins	64	66
Aged 8 days at 6°	80	79
p-Chloromercuribenzoate, $2 \cdot 10^{-6}$ *M*	82	84

* From acetaldehyde plus pyruvate or from acetaldehyde alone. The various activities were measured as in the experiments in Table III. Heat inactivation experiment: enzyme solution at Step 4 of purification, A.R. = 1,400, dissolved in $2 \cdot 10^{-1}$ *M* succinate buffer, pH 6.0, to give 2.95 mg protein per ml and heated 5 minutes at 54°. Ageing experiment: enzyme preparation as above, dissolved in $1 \cdot 10^{-3}$ *M* NaCl to give 0.33 mg protein per ml, pH 5.5, and kept 8 days at 6°. *p*-Chloromercuribenzoate experiment: enzyme, same as in heat inactivation experiment, incubated with the inhibitor at pH 6, 30°, for 10 minutes at a protein concentration of 0.021 mg per ml prior to addition of the substrate.

In view of the evidence cited, it is concluded that in wheat germ preparations the AMC synthesizing and α-carboxylase activities are associated with the same enzyme.

Optical activity of the enzymically produced AMC. We have reported in a preliminary communication[29] that the AMC produced by the purified α-carboxylase consists of about 72% of the (+) and 28% of the (—) form. This figure is based on the following facts. Concentrated solutions of enzymically synthesized AMC, prepared as described in the section on *Identification of product*, consistently gave optical rotations of $[\alpha]_D^{27} = 36° (\pm 1°)$. The optical rotation of (—) AMC, isolated from preparations of *A. aerogenes*, filarial homogenates, and animal tissues is $[\alpha]_D = -84° (\pm 2°)$[6,8,10], a value which we have been able to confirm. Incubation of pure (—) AMC with our enzyme preparation, under the same conditions as used for enzymic synthesis of AMC, resulted in quantitative recovery of the (—) AMC without any change in optical rotation, demonstrating that neither the enzyme preparation nor the isolation procedure causes racemization of added AMC. The lack of racemization of (—) AMC further supports the findings on the essential irreversibility of AMC synthesis.

The proportion of (+) and (—) AMC produced by the enzyme from wheat germ, given above, is calculated on the assumption that the AMC produced by animal tissues and bacteria is the pure (—) isomer. However, the fact that the AMC produced by wheat

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germ is partly racemic involves no such assumption. It should be added that essentially the same specific rotation was observed when α-carboxylase preparations of varying purity were used to accumulate the AMC from either acetaldehyde alone as substrate or from acetaldehyde and pyruvate.

The possible mechanism of this partially asymmetric enzymic synthesis will be dealt with in the DISCUSSION.

DISCUSSION

The evidence presented confirms the hypothesis of DIRSCHERL AND LANGENBECK[11,12] that in cells containing an active α-carboxylase, AMC formation is the consequence of the action of this enzyme on pyruvate, rather than the function of a separate carboligase. Although definitive evidence for this view has been presented only for wheat germ thus far, there is good reason to believe that the same is true of other higher plants[6] and

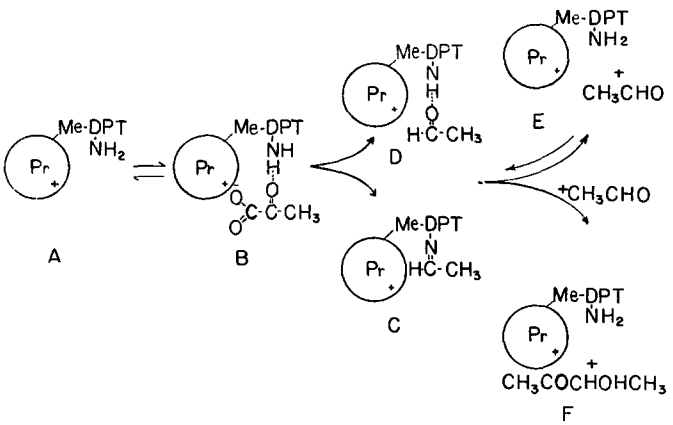

Fig. 4. Intermediate steps in the action of α-carboxylase.

of yeast[10,15]. The objections which have been leveled against this view will be more conveniently discussed after the presentation of the proposed mechanism of action of α-carboxylase, which follows.

Fig. 4 is a schematic representation of the postulated course of AMC synthesis. The reactants are pyruvate and the holoenzyme; the latter consists of a protein-metal-DPT complex (A). Pyruvate may be initially oriented toward the active center of the enzyme by electrostatic forces, and, upon closer approach, a two-point combination may occur between the enzyme and pyruvate (B). The carbonyl group of the latter is visualized as approaching the free amino group of DPT, whereas the carboxyl is attracted to an electropositive site on the enzyme. The activation step would involve the formation of an intermediate complex of finite stability, which may be either a Schiff's base (C) or a hydrogen-bonded structure (D) between the carbonyl and amino groups, and CO_2 would be split off. The resulting intermediate enzyme-acetaldehyde complex is known to be in reversible equilibrium with free acetaldehyde and free enzyme (E); its dissociation is the cause of acetaldehyde formation in the typical α-carboxylase reaction. The intermediate enzyme-acetaldehyde complex (C or D) can arise either directly from acetaldehyde or, indirectly, by decarboxylation of pyruvate *in situ*. While

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acetaldehyde is still attached to the enzyme, in the presence of a sufficient amount of free acetaldehyde, a second molecule of the latter could react with the complex, forming an unstable enzyme-AMC complex, which would rapidly and irreversibly break down to free AMC and free enzyme (F). According to this scheme it is the fate of the intermediate enzyme-acetaldehyde complex (C or D) which determines whether acetaldehyde formation ("carboxylase action") or AMC formation ("carboligase action") takes place, and its accumulation in the presence of free acetaldehyde inhibits further decarboxylation of pyruvate.

The production of partially asymmetric AMC by the wheat germ enzyme has been ascribed to incomplete steric hindrance offered by the side-chains of the protein in the condensation step[29]. In terms of this hypothesis the proportion of (+) and (—) AMC produced would represent the probability of the reaction of acetaldehyde with complex C or D from either one of two opposite directions.

The existence of an electropositive site on the enzyme protein is postulated for the following reasons. Pyruvate is completely ionized in the pH range where the enzyme acts, and acetaldehyde is, of course, non-ionizable. As mentioned under *Results*, the pH-activity curves of the decarboxylation of pyruvate and of AMC formation from pyruvate and acetaldehyde essentially coincide, but AMC formation from acetaldehyde alone declines considerably less sharply on the alkaline side of the pH optimum (pH 6.3). The reason for this may be the presence of a dissociable group in the enzyme, the ionization of which is repressed above pH 6.3, and to which the carboxyl group of pyruvate is attracted. The inactivity of pyruvamide as a substrate is in harmony with this explanation.

If it is admitted that in the purified preparations used here AMC synthesis is catalyzed by a single enzyme, the fact that the AMC produced is optically active must mean that the condensation of the two acetaldehyde molecules occurs on the surface of the enzyme. The formation of a relatively stable enzyme-acetaldehyde complex[9,15], which reacts *in situ* with a second molecule of acetaldehyde, is the key to the postulated reaction scheme.

Acetaldehyde is undoubtedly tightly bound by the α-carboxylase of wheat germ; the strong inhibition of decarboxylation by internally produced acetaldehyde is evidence thereof. The high affinity of the enzyme for acetaldehyde explains why the latter is a sufficient substrate for AMC synthesis. The relatively large concentration of acetaldehyde needed for AMC synthesis (Fig. 2) probably reflects the requirements for the second molecule of acetaldehyde, which reacts with this complex.

Acetaldehyde is less inhibitory toward yeast α-carboxylase, and is probably, therefore, less tightly bound by that enzyme. Accordingly, acetaldehyde alone is insufficient for AMC formation by the partially purified yeast enzyme. In contrast to this, the enzymically produced acetaldehyde may be so tightly associated with the active center of pig heart α-carboxylase that no free acetaldehyde is liberated into solution, and the formation of AMC is practically quantitative[7]. The fact that the enzyme from pig heart also forms AMC from acetaldehyde alone[7,30], and the demonstration that with this enzyme ketol formation from pyruvate and an added aldehyde *follows* decarboxylation, may well indicate that the mechanism of AMC formation presented in this paper also applies to animal carboxylases. Thus the differences in the products and substrate requirements of the various α-carboxylases may be explained in terms of their respective dissociation constants for acetaldehyde and other aldehydes.

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The main objections[13] to the hypothesis that α-carboxylase may be responsible for ketol formation in various cells have been that AMC synthesis can proceed in the absence of α-keto acids and HOFMANN's finding[31] that yeast decarboxylates α-keto-*n*-valerate without forming from it a ketol. The answer to the first of these objections is apparent from the foregoing. The lack of *n*-butyroin synthesis from α-keto-*n*-valerate probably reflects the low affinity of *n*-butyraldehyde for the yeast enzyme, since the affinity of aldehydes for α-carboxylase decreases with increasing chain-length[18], and thus the activated enzyme-aldehyde complex may never form from this homologue.

Space does not permit a detailed consideration of the reaction rates in the postulated scheme, but it is important to mention that the rate of formation of the activated complex (C or D) from pyruvate must exceed its rate of formation from acetaldehyde, in order to account for the more rapid synthesis of AMC from pyruvate plus acetaldehyde than from the latter alone. It should be kept in mind that the inhibition of the decarboxylation of pyruvate by acetaldehyde is never complete; a sufficiently large fraction of the enzyme molecules is always free for combination with pyruvate to account for the increased rate of AMC synthesis which the addition of pyruvate effects.

It remains to consider the function of DPT in the activity of α-carboxylases. LANGENBECK's postulate[32] that the amino group of DPT reacts with the carbonyl groups of α-keto acids was subsequently incorporated into various modifications of his reaction scheme (*cf.* [9]). The reaction between the amino group of DPT and the carbonyl groups of pyruvate and acetaldehyde has been retained in the scheme presently proposed, but subsequent intermolecular reactions[15,32] or intramolecular oxido-reductions[33] of the intermediate are not deemed necessary to explain the action of simple α-carboxylases. The condensation of free acetaldehyde with the enzyme-acetaldehyde complex to yield AMC might occur by addition to the double bond in structures C or D or by a substitution reaction of acetaldehyde on complex C, with retention of the C = N linkage.

The same type of protein-DPT-acetaldehyde complex, which has been discussed in connection with α-carboxylases, may conceivably be the first step in the action of enzymes which oxidize pyruvate. There is a striking similarity between the α-carboxylase of wheat germ and the pyruvic oxidase recently isolated by SCHWEET *et al.*[34] in certain respects (AMC formation, requirements for cofactors, high molecular weight, etc.). It seems possible that the enzyme-acetaldehyde complex, under the influence of a second prosthetic group attached to the protein, may be oxidized to an enzyme-acetyl compound, which could in turn be hydrolyzed to acetate or react with coenzyme A. If the dissociation constant of the enzyme-acetaldehyde intermediate were small enough, no free acetaldehyde would be liberated*. This line of reasoning leads to the concept that α-carboxylase is the prototype of enzymes for the activation of α-keto acids and that enzymes capable of oxidizing the latter would have to possess, besides DPT and a metal ion, a receptor site for the attachment of an additional prosthetic group involved in electron transfer**. The verification of this concept would establish NEUBERG's "nascent acetaldehyde"[3] as a key intermediate in carbohydrate metabolism.

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* The unreactivity of acetaldehyde in many pyruvate oxidases might be ascribed to the need for the presence of a carboxyl group in the substrate for initial attraction to the enzyme proteins of these systems.

** We have attempted to investigate whether the purified α-carboxylase from wheat germ would react with oxidizing agents. Using acetaldehyde or pyruvate as a substrate no reduction of DPN or TPN could be observed spectrophotometrically, nor could ferricyanide or methylene blue act as an electron acceptor.

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It is a pleasure to acknowledge the invaluable help of Dr EDNA B. KEARNEY in the development of the hypothesis presented.

SUMMARY

1. Highly purified preparations of α-carboxylase from wheat germ catalyze the synthesis of acetylmethylcarbinol (AMC) from pyruvate and acetaldehyde and from acetaldehyde alone.

2. The AMC thus synthesized has been isolated and its identity established by conversion to diacetyl, periodate degradation, and by analysis of the semicarbazone.

3. It has been shown that the decarboxylase and AMC-synthesizing activities are associated with the same enzyme, α-carboxylase. The conclusion is based on the constant ratio of decarboxylation to AMC synthesis throughout a 2,700-fold purification, on the identical effects of partial inactivation and of pH, and on the identical requirements for diphosphothiamin and a metallic ion for the various activities.

4. The AMC synthesized by α-carboxylase consists of 72% of the (+) and 28% of the (—) optical isomer.

5. A scheme has been proposed for the enzymic transformations catalyzed by α-carboxylase. Known facts about the α-carboxylases of yeast, higher plants, and of animal tissues may be explained in terms of the formation of a relatively stable enzyme-acetaldehyde complex, wherein the aldehyde is linked to the diphosphothiamin moiety of the enzyme. It has been suggested that the formation of a similar intermediate may be the first step in the enzymic oxidation of pyruvate.

RÉSUMÉ

1. Des préparations hautement purifiées de α-carboxylase de germe de blé catalysent la synthèse de l'acétylméthylcarbinol (AMC) à partir du pyruvate et de l'aldéhyde acétique ainsi qu'à partir de l'aldéhyde acétique seule.

2. L'AMC ainsi synthétisé a été isolé et son identité établie par transformation en diacétyl, par dégradation au perjodate et par analyse de la semicarbazone.

3. Nous avons montré que les activités de décarboxylase et de synthèse d'AMC sont associées au même enzyme, l'α-décarboxylase. Cette conclusion est basée sur la constance du rapport décarboxylation/synthèse d'AMC au cours d'une purification poussée (2700 fois), sur l'identité des effets produits par une inactivation partielle et par le pH et sur les besoins identiques de diphosphothiamine et d'un ion métallique pour les diverses activités.

4. L'AMC synthétisé par l'α-carboxylase contient 72% d'isomère optique (+) et 28% d'isomère (—).

5. Nous avons proposé un schéma des transformations enzymatiques catalysées par l'α-carboxylase. Des faits connus concernants l'α-carboxylase de la levure, de plantes supérieures et de tissus animaux peuvent s'expliquer par la formation d'un complexe relativement stable enzyme-aldéhyde acétique, où l'aldehyde est liée à la diphosphothiamine de l'enzyme. Nous avons suggéré l'idée que la formation d'un intermédiaire semblable serait le première étappe de l'oxidation enzymatique du pyruvate.

ZUSAMMENFASSUNG

1. Weitgehend gereinigte α-Carboxylase-Präparate aus Weizenkeimen katalysieren die Synthese von Acetylmethylcarbinol (AMC) aus Pyruvat und Acetaldehyd oder aus Acetaldehyd allein.

2. Das auf diese Weise synthetisierte AMC wurde isoliert und seine Identität durch Umwandlung in Diacetyl, durch Abbau mit Perjodat und durch Analyse des Semicarbazons festgestellt.

3. Es wurde gezeigt, dass die Decarboxylase- und die AMC-Synthese-Aktivität zu demselben Enzym, der α-Carboxylase gehören. Dieser Schluss gründet sich auf das konstante Verhältnis Decarboxylase/AMC-Synthese bei 2700 facher Verdünnung, auf die Gleichheit der Wirkungen von partieller Inaktivierung und pH und auf den gleichen Bedarf an Diphosphothiamin und einem Metallion für die verschiedenen Aktivitäten.

4. Das AMC, welches durch die α-Carboxylase synthetisiert wird, enthält 72% des optischen (+)-Isomeren und 28% des (—)-Isomeren.

5. Ein Schema der enzymatischen, durch α-Carboxylase katalysierten Umwandlungen wurde vorgeschlagen. Bekannte Tatsachen über α-Carboxylasen von Hefe, höheren Pflanzen und tierischen Geweben können durch die Bildung eines relativ stabilen Komplexes Enzym-Acetaldehyd, wo das Acetaldehyd an den Diphosphothiaminteil des Enzyms gebunden ist, erklärt werden. Die Bildung eines ähnlichen Zwischenproduktes könnte die erste Stufe der enzymatischen Pyruvatoxydation sein.

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Received December 28th, 1951