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The Reduction of Vinylacetate by *Clostridium kluyveri* and its Dependence on Catalytic Amounts of High-energy Acetate

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Growing cultures of *Clostridium kluyveri* ferment ethanol plus acetate, yielding mainly butyrate, hexanoate and hydrogen (Bornstein & Barker, 1948). Stadtman & Barker (1949*a-e*) and Kennedy & Barker (1951*a*) have investigated the mechanism of butyrate synthesis by this organism, using dried cells and cell-free extracts which catalyse reactions 1 and 2:

During an examination of possible intermediates, the reduction of vinylacetate (3:4-butenate) according to reaction 3 was observed (Stadtman & Barker, 1949*e*).

Several other C_4 compounds at the same oxidation level as vinylacetate were examined, including crotonate (*trans*-2:3-butenate), isocrotonate (*cis*-2:3-butenate) and 3-hydroxybutyrate; all were excluded as intermediates in reactions 1 and 2. Crotonate and isocrotonate were neither oxidized nor reduced by the preparations. No direct test was carried out with 3-hydroxybutyrate, but the preparations reduced acetoacetate, giving a quantitative yield of 3-hydroxybutyrate, indicating that the latter is not reduced. Vinylacetate was the only compound of those tested which was oxidized and reduced at rates compatible with the role of intermediate. In the presence of a nitrogen gas phase, vinylacetate was shown to undergo two other reactions—an anaerobic oxidation with the evolu-

tion of H_2 (reaction 4) and a dismutation (reaction 5).

However, isotope experiments indicated that free vinylacetate was not a true intermediate in reactions 1 or 2. In the presence of unlabelled vinylacetate, [*carboxy*- ^{14}C]butyrate gave rise to acetate with a higher specific activity than the vinylacetate recovered at the end of the experiment. Again, in the presence of unlabelled vinylacetate, [*carboxy*- ^{14}C]acetate yielded labelled butyrate, but no isotope appeared in the residual vinylacetate.

Kennedy & Barker (1951*a*) later observed that dried cells could oxidize butyrate and vinylacetate in the absence of added phosphate, though at a reduced rate, and under these conditions acetoacetate was the main product. These observations led to the hypothesis that the C_4 compounds are oxidized and reduced in the form of complexes with a coenzyme, possibly coenzyme A (CoA) (cf. Fig. 2). This concept is supported by the observations reported in the present paper, which indicate that vinylacetate must be activated before it is reduced.

MATERIALS AND METHODS

Organism and suspensions of dried cells. Strain K1 of *Cl. kluyveri*, which was isolated by Barker & Taha (1942) and used in the studies on fatty acid synthesis by Stadtman & Barker, was used throughout.

The dried cell preparation used (Lot YE9) was made by the method of Stadtman & Barker (1949*a*), with the following modifications: (1) a slight change was made in the

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medium for large-scale cultivation described by Stadtman & Barker (1949*a*) which contains ethanol, acetate, salts and growth factors—0.05% Difco Yeast Extract replaced the biotin and *p*-aminobenzoic acid; (2) the cells were washed three times in 0.03% $\text{Na}_2\text{S}_2\text{O}_3$ instead of twice; (3) the washed cells were freeze-dried instead of being dried *in vacuo* over CaCl_2 at room temperature.

Aeration inactivates the butyrate oxidizing system of *Cl. kluyveri*, and the preparations are reactivated by 1:3-dimercaptopropanol (BAL) (Kennedy & Barker, 1951*a*). The dried cells were therefore suspended in water containing 0.002*M* BAL; this operation was carried out anaerobically in an evacuated Thunberg tube. Samples of cell suspensions were added to manometer cups immediately before gassing with H_2 .

Extract of boiled yeast (EBY). This was prepared by heating a 20% (w/v) suspension of '20-40' dried yeast (Fleischman, New York) at 100° for 10 min. The solid material was removed by centrifuging and the supernatant was brought to pH 8 and stored at -18° until used.

Acetyl phosphate. This was used as the dilithium salt prepared by the method of Stadtman & Lipmann (1950).

Acetyl coenzyme A. This was prepared as described by Stadtman (1952*a*) from a sample of CoA assaying 69 units/mg. (units defined by Kaplan & Lipmann, 1948). In the final chromatographic separation, the solvent used contained acetate buffer, the removal of which was essential since it inhibits the reduction of vinylacetate (see below). This was accomplished by adjusting the pH of the eluate from the chromatogram to 4-5 and passing it through a 1 x 1 cm. diam. column of charcoal. The charcoal was Nuchar C190 (West Virginia Pulp and Paper Co., Covington, Va.) and before use was treated by soaking overnight in 5*N*-HCl and then washing with distilled water until the pH of the eluate rose to 4-5. After application of the acetyl CoA concentrate, the column was washed with 10 ml. of water to remove the acetate, after which the absorbed acetyl CoA was eluted with 40% aqueous acetone containing 0.0165*N*- NH_3 . The first 6 ml. of eluate was collected and evaporated to dryness under reduced pressure at room temperature. The product was used in aqueous solution, the concentration being determined as described by Stadtman (1952*a*).

Diphosphopyridine nucleotide (DPN). This was obtained from Dr A. Kornberg and was approximately 90% pure.

Triphosphopyridine nucleotide (TPN). The preparation used was made from hog liver by the method of LePage & Mueller (1949) and contained 8.7% of TPN.

Vinylacetate. The anhydrous sodium and potassium salts of vinylacetic acid were prepared from the free acid (Rietz, 1944) by neutralizing to pH 7 with the appropriate hydroxide, followed by freeze-drying (R. G. Bartsch, private communication).

When assayed by the catalytic-hydrogenation method of Harrison (1939), the hydrogen uptakes observed were 98% (Na salt) and 101% (K salt) of the theoretical values.

Reduction of vinylacetate. This was followed by using molecular H_2 as hydrogen donor and measuring the gas uptake in Warburg manometers at 26° (Stadtman & Barker, 1949*e*). The reaction was carried out at pH 8.0 in presence of KH_2PO_4 - K_2HPO_4 or aminotrihydroxymethylmethane (tris) buffer. [Tris was recrystallized from ethanol: H_2SO_4 or KH_2PO_4 was added to an aqueous solution to give the requisite pH and a final tris concentration of 1.0*M*.]

Stadtman & Barker (1949*e*) showed that the reduction of vinylacetate by their preparation gave rise to an amount of butyrate equivalent to the H_2 uptake. An equivalence between the H_2 uptake (or butyrate production) and the amount of vinylacetate added was not shown. The formation of butyrate during the reduction of vinylacetate by the present preparation (YE9) was checked qualitatively by separating the volatile fatty acids by distillation from acid solution in the apparatus of Markham (1942). The Na salts of the acids, obtained by titration and evaporation of the distillates, were examined by paper chromatography with an ethanol-ammonia solvent as described by Kennedy & Barker (1951*b*). The results showed that the vinylacetate disappeared and butyrate appeared. No major changes in any other volatile acid were observed, although in the case of acetic acid any change would be obscured since the enzyme preparations yielded appreciable amounts of acetic acid on distillation.

The total H_2 uptakes observed varied somewhat in the range 55-70% of the theoretical quantity required for complete reduction of the added vinylacetate to butyrate. The fate of the vinylacetate not reduced to butyrate has not been established. Apart from reduction to butyrate, vinylacetate might also be removed by the anaerobic oxidation or the dismutation mentioned above (reactions 4 and 5), though these reactions were demonstrated by Stadtman & Barker (1949*c*) only in the presence of N_2 . In either case, the products other than butyrate would be acetyl phosphate and acetate, and under H_2 these would be reduced to butyrate. The net result would still be an uptake of 1 mole of H_2 for each mole of vinylacetate reduced to butyrate. It therefore seems reasonable to assume that the H_2 uptake observed in the present experiments may be used as a measure of the reduction of vinylacetate to butyrate.

After a short lag period of doubtful significance, H_2 uptake followed a linear course, until it was complete (see, for example, curve *A*, Fig. 1*a*). The rates of H_2 uptake quoted were measured over the linear phase.

RESULTS

Effect of boiled-yeast extract (EBY) on the reduction of vinylacetate by dried cells of *Clostridium kluyveri*. When the present work was started, difficulty was experienced in obtaining preparations of dried cells which would catalyse the reduction of vinylacetate by H_2 under the conditions of Stadtman & Barker (1949*e*), although some older preparations which had been stored at -18° for up to 2 years were still highly active. Certain modifications in the method of preparation of the dried cells have been mentioned above and may account for the observed differences in behaviour.

It was found possible to activate several of the batches of dried cells prepared according to the modified method, by the addition of EBY to the test system. The results of a typical experiment are given in Table 1. This indicated that the preparation was deficient in some cofactor necessary for the reduction of vinylacetate, and attempts were therefore made to replace the EBY by various known substances.

Replacement of EBY by acetyl phosphate. The results obtained when various supplements were added to the system are given in Table 2. DPN, TPN and CoA—singly or all three together—were without effect. Neither was any activity observed when a solution of metal salts was added so as to give the concentrations obtaining in the medium used for growing the organism, nor when a small amount of ethanol was added. Difco Yeast Extract could not replace the EBY.

Table 1. *Requirement for extract of boiled yeast (EBY) for the reduction of vinylacetate by dried cells of Clostridium kluyveri*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001 M BAL; 0.05 M-KH₂PO₄-K₂HPO₄, pH 8.0; 0.4 ml. of EBY. Na vinylacetate (17.0 μ moles) was added from the side arm after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°.

System	Rate of H ₂ uptake (μ l./hr.)
EBY absent, no substrate	2
EBY absent + vinylacetate	16
EBY present, no substrate	2
EBY present + vinylacetate	392

Table 2. *Tests on various substances for their ability to replace EBY in the reduction of vinylacetate*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001 M BAL; 0.05 M-KH₂PO₄-K₂HPO₄, pH 8.0. Sodium vinylacetate (17.0 μ moles) was added from the side arm after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°. All supplements were initially present in the main compartment, except for the ethanol and acetyl phosphate, which were added from the side arm with the substrate. Metal ions were added where indicated as 0.2 ml. of a solution giving the following concentrations (μ g./ml.) in the reaction mixture: MgSO₄·7H₂O, 200; CaCl₂, 10; Na₂MoO₄·2H₂O, 2; MnSO₄·4H₂O, 2; FeSO₄·7H₂O, 2.

Supplements	Rate of H ₂ uptake (μ l./hr.)	
	Expt. 1	Expt. 2
None	6	7
EBY (0.4 ml.)	150	155
DPN (770 μ g.)	7	—
TPN (170 μ g.)	5	—
CoA (30 units)	12	—
DPN + TPN + CoA (amounts as above)	—	8
Difco Yeast Extract (4 mg.)	—	11
Metal ions	—	6
Ethanol (0.1 μ mole)	—	8
Lithium acetyl phosphate (0.2 μ mole)	—	157

However, when a small amount (0.2 μ mole) of acetyl phosphate was added, hydrogen was taken up at the same rate as in the presence of EBY, suggesting that acetyl phosphate can replace the active factor in the EBY. A more detailed analysis

of the effect of acetyl phosphate is shown in Table 3. Acetyl phosphate, added in the absence of vinylacetate, had no effect on the H₂ uptake, showing that the stimulation observed in presence of vinylacetate was not due to an increase in the rate of endogenous H₂ uptake.

Table 3. *Stimulation of the reduction of vinylacetate by a catalytic amount of acetyl phosphate*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001 M BAL; 0.05 M-KH₂PO₄-K₂HPO₄, pH 8.0. Sodium vinylacetate (17.0 μ moles) and/or acetyl phosphate (0.2 μ mole) were added where indicated from the side arm after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°.

System	Rate of H ₂ uptake (μ l./hr.)
Acetyl phosphate absent, no substrate	18
Acetyl phosphate absent + vinylacetate	22
Acetyl phosphate present, no substrate	16
Acetyl phosphate present + vinylacetate	126

Table 4. *Further observations on the effect of acetyl phosphate and EBY on the reduction of vinylacetate*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001 M BAL; 0.05 M-KH₂PO₄-K₂HPO₄, pH 8.0. EBY (0.4 ml.) was added from the side arm or was initially present in the main compartment as indicated. Acetyl phosphate (0.1 μ mole) and Na vinylacetate (10 μ moles) were added from the side arm, the former where indicated and the latter in all cases except that indicated, after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°.

System	H ₂ uptake	
	Total volume (μ l.)	Rate (μ l./hr.)
No supplements	13	12
+ EBY (side arm)	105	114
+ acetyl phosphate	128	168
+ EBY (side arm) + acetyl phosphate	113	122
+ EBY (side arm), vinylacetate absent	22	10
+ hydrolysed acetyl phosphate	15	10
+ EBY (main compartment)	19	18

The stimulating action of acetyl phosphate appears to be similar to that of EBY, since when both were added to the system the total H₂ uptake was not significantly greater than with either singly (Table 4). Neither was the rate of H₂ uptake; indeed in this particular experiment the rate was significantly lower in presence of both supplements than with acetyl phosphate alone. Previously, the EBY had been placed in the main compartment of the manometer along with the *Cl. kluyveri* preparation, but in this experiment it was added from the

side arm for reasons given below. A further control (Table 4) showed that even in these changed circumstances there was no marked H_2 uptake in the absence of vinylacetate. Hydrolysis of acetyl phosphate destroyed its activating properties, indicating that the intact molecule is necessary.

The measurement of vinylacetate reduction involves a preliminary incubation before tipping in the substrate, during which hydrogen is taken up by the *Cl. kluveri* preparation (Stadtman & Barker, 1949e). Stimulation of vinylacetate reduction by EBY was not obtained consistently when this supplement was preincubated with the enzyme system in the main compartment of the manometer, although in the same experiment it was active when added from the side arm along with the substrate (cf. Tables 1 and 4). This suggested that the active principle in the EBY might be unstable, and this point was examined in more detail.

Need for the simultaneous presence of vinylacetate and acetyl phosphate for reduction. In order to investigate further the stability of the activating factor, an experiment was carried out in which acetyl phosphate and vinylacetate were added separately to the system in different sequences. The results are shown in Fig. 1.

When the acetyl phosphate and vinylacetate were added simultaneously, the H_2 uptake observed was linear with time until it ceased (curve A, Fig. 1a). The total H_2 uptake observed was less than that required for the quantitative reduction of vinylacetate to butyrate but was proportional to it, since when only half the amount of vinylacetate was added the H_2 uptake was approximately halved (curve B, Fig. 1a). The significance of the blank H_2 uptake in the absence of both vinylacetate and acetyl phosphate (curve C, Fig. 1a) is difficult to assess. A slow but prolonged H_2 uptake was observed, whereas, in the manometers where vinylacetate was reduced, no such slow uptake was found after the rapid uptake due to the substrate had ceased. When the acetyl phosphate was added at zero time and the vinylacetate added 90 min. later, no H_2 was taken up (curve D, Fig. 1a), but when the times of addition of these two compounds were interchanged, H_2 was taken up at the same rate and the total uptake was approximately the same as when both components were added at zero time (curve E, Fig. 1a). It follows that whereas vinylacetate added alone is relatively stable in this system, the acetyl phosphate (or activating compound derived from it) is not.

Once the H_2 uptake due to the addition of substrate plus activator had ceased, addition of either acetyl phosphate or vinylacetate had no effect (compare curves B, F, G, Fig. 1b). On the other hand, addition of both led to a further H_2 uptake

Fig. 1. Dependence of hydrogen uptake on the simultaneous presence of vinylacetate and acetyl phosphate. Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluveri*; 0.001M BAL; 0.05M KH_2PO_4 - K_2HPO_4 ; pH 8.0. Acetyl phosphate (0.2 μ -mole) and sodium vinylacetate (10 or 20 μ moles) were added from the side arm as indicated below, after saturation of the enzyme system with H_2 . Figs. 1a and 1b are drawn from data obtained in a single experiment. Curve A: acetyl phosphate and vinylacetate (20 μ moles) added at zero time. Curve B: acetyl phosphate and vinylacetate (10 μ moles) added at zero time. Curve C: no additions. Curve D: acetyl phosphate added at zero time and vinylacetate (20 μ moles) at 90 min. Curve E: vinylacetate (20 μ moles) added at zero time and acetyl phosphate at 90 min. Curve F: acetyl phosphate and vinylacetate (10 μ moles) added at zero time and additional acetyl phosphate at 90 min. Curve G: acetyl phosphate and vinylacetate (10 μ moles) added at zero time and additional vinylacetate (10 μ moles) at 90 min. Curve H: acetyl phosphate and vinylacetate (10 μ moles) added at zero time and again at 90 min.

proportional to the amount of vinylacetate added (curve *H*, Fig. 1*b*). The fact that the second addition of a small amount of acetyl phosphate was without effect suggests that the uptake of H_2 ceases when the vinylacetate is exhausted. It will be noticed, however, that the total H_2 uptake was equivalent to only 55 % of the vinylacetate added.

Since the second addition of vinylacetate was not reduced in the absence of extra acetyl phosphate, it follows that the activating principle must have been destroyed during the interval between

but is stabilized during the metabolism of vinylacetate.

Influence of the amount of acetyl phosphate on the reduction. It seemed possible that there might be a threshold concentration of acetyl phosphate below which the rate of reduction would be dependent on the concentration of this factor. However, experiment showed that, if reduction occurred at all, both the rates and total H_2 uptake in each experiment were approximately equal, whatever the amount of acetyl phosphate added (Table 5). No

Table 5. *Effect of the amount of added acetyl phosphate on the reduction of vinylacetate*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001M BAL; 0.05M-KH₂PO₄-K₂HPO₄, pH 8.0. Sodium vinylacetate (14 μ moles in Expt. 1; 10 μ moles in Expts. 2 and 3) and acetyl phosphate in the amounts indicated were added from the side arm after saturation of the enzyme system with H_2 . Gas phase H_2 ; temp. 26°.

Acetyl phosphate added (μ moles)	H_2 uptakes					
	Expt. 1		Expt. 2		Expt. 3	
	Total (μ l.)	Rate (μ l./hr.)	Total (μ l.)	Rate (μ l./hr.)	Total (μ l.)	Rate (μ l./hr.)
0.2	190	121	—	—	—	—
0.1	211	133	110	122	124	128
0.05	214	134	—	—	100	128
0.025	—	—	—	—	27	14
0.02	—	—	115	134	—	—
0.013	—	—	—	—	120	124
0.006	—	—	—	—	109	116
0.004	—	—	11	8	—	—
0.003	—	—	—	—	117	124
0.0008	—	—	7	8	—	—
0.00016	—	—	10	8	—	—
0.00003	—	—	10	8	12	6
0	38	29	10	8	—	—

the cessation of the H_2 uptake and the addition of the extra vinylacetate. In a separate experiment, an attempt was made to determine the time interval required for destruction of the activating principle by making the second addition of vinylacetate at various times relative to the point at which the H_2 uptake due to the first addition of vinylacetate ceased. This interval proved too short to measure; extra vinylacetate was reduced only if it was added during the linear phase of the reaction.

The fact that H_2 uptake occurs at a linear rate until it is complete shows that the activating principle is quite stable in presence of vinylacetate. Other evidence supports this view. With the manometer containing the larger amount of vinylacetate, H_2 uptake was still proceeding at the initial rate after 90 min. (curve *A*, Fig. 1*a*) whereas, after the same time, the system with half that amount of vinylacetate required more acetyl phosphate in order to reduce extra vinylacetate (curve *H*, Fig. 1*b*). It may be concluded that acetyl phosphate, or some activating compound derived from it, is rapidly destroyed by the dried-cell suspension

intermediate rate was observed, except possibly in the vessel in which 0.025 μ mole was added in Expt. 3, where the observed rate was only slightly higher than with the lowest amount of acetyl phosphate. This particular observation appears anomalous, since in the same experiment three lower amounts of acetyl phosphate were effective, but no explanation can be offered. The amount of acetyl phosphate required to stimulate reduction is variable; thus in Expt. 3 0.003 μ mole was effective, whereas in Expt. 2 0.004 μ mole did not stimulate reduction. The amount of acetyl phosphate usually added in subsequent experiments was 0.1 μ mole; this amount never failed to stimulate reduction.

Activation by the products of the reaction between acetyl phosphate and dried cells. It appeared possible that the reducing system might be activated, not by acetyl phosphate as such, but rather by some product derived from it, especially since EBY, which also stimulates, is unlikely to contain any acetyl phosphate. An experiment was therefore carried out to determine whether the interaction of dried cells with acetyl phosphate would yield a product capable of activating the

reducing system. For this purpose a relatively large amount of acetyl phosphate was used, since this compound was known to be unstable in presence of dried cells, and the time of incubation was kept short. After incubation, the enzymes and excess of acetyl phosphate were destroyed by heating at pH 3.5, and the soluble products remaining after centrifuging tested for activating properties. Corresponding products from control incubations in which the acetyl phosphate or the dried cells were omitted were also tested.

Table 6. *Stimulation of vinylacetate reduction by the products obtained by incubating acetyl phosphate with dried cells of Clostridium kluyveri*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001M BAL; 0.05M tris-KH₂PO₄, pH 8.0. Sodium vinylacetate (10 μ moles) and incubation products as indicated were added from the side arm after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°. The figures have been corrected by subtracting the values from corresponding flasks without vinylacetate. These latter values were all within the range 2–19 μ l.

The incubation products were prepared as follows. Dried cells of *Cl. kluyveri* (100 mg.) and 10 μ moles of acetyl phosphate were incubated for 15 min. at 26° in a total vol. of 2.0 ml. containing 0.04M tris-KH₂PO₄, pH 8.0, and 0.03% Na₂S₂O₅. Blank mixtures from which dried cells and acetyl phosphate were omitted respectively were treated likewise. After incubation the mixtures were cooled, the pH was adjusted to 3.5 with HCl, the mixture heated at 100° for 15 min. and cooled, the pH adjusted to 8 with NaOH, the mixture centrifuged and 0.5 ml. of the resulting supernatant used for the manometric tests.

	H ₂ uptake	
	Total vol. (μ l.)	Rate (μ l./hr.)
1. Product tested from incubation of		
Dried cells + acetyl phosphate	130	130
Dried cells alone	18	8
Acetyl phosphate alone	6	5
2. Acetyl phosphate tested directly	109	96

The results (Table 6) show that in the presence of dried cells acetyl phosphate is converted into a product which is heat-stable and capable of activating the reduction of vinylacetate. The rate of reduction is in fact rather higher than in the presence of acetyl phosphate.

Activation by acetyl-coenzyme A. Preparations of *Cl. kluyveri* are known to contain phosphotrans-acetylase, which catalyses the transfer of the acetyl group from acetyl phosphate to CoA (reaction 6), and the results of the experiments described above are compatible with the activation of the reducing

system by acetyl-CoA. This possibility was tested more directly by using a purified sample of acetyl-CoA.

The compound was found to activate the system (Table 7), and the rate of reduction was again slightly higher than that observed with acetyl phosphate. The stimulation observed with EBY may be due to the presence of acetyl-CoA, since this compound has been isolated from yeast by Lynen & Reichert (1951).

Table 7. *Stimulation of vinylacetate reduction by acetyl-CoA*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001M BAL; 0.05M tris-KH₂PO₄, pH 8.0. Sodium vinylacetate (5 μ moles), acetyl phosphate (0.1 μ mole) and acetyl-CoA (0.08 μ mole; preparation described under Methods) were added, as indicated, from the side arm, after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°.

System	H ₂ uptake	
	Total vol. (μ l.)	Rate (μ l./hr.)
Vinylacetate present, no supplements	6	2
Vinylacetate present + acetyl phosphate	68	82
Vinylacetate present + acetyl-CoA	77	98
Vinylacetate absent + acetyl-CoA	8	2

Table 8. *Inhibition of vinylacetate reduction by acetate*

Manometers contained in a total vol. of 2.0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0.001M BAL; 0.05M tris-KH₂PO₄, pH 8.0. Sodium vinylacetate (5 μ moles), acetyl phosphate (0.1 μ mole) and sodium acetate, potassium acetate or NaCl as indicated were added from the side arm after saturation of the enzyme system with H₂. Gas phase H₂; temp. 26°. The figures given have been corrected by subtracting the values obtained in the absence of vinylacetate which lay within the range 6–11 μ l./hr.

Additions	Rate of H ₂ uptake (μ l./hr.)
None	85
Sodium acetate (0.005M)	1
Potassium acetate (0.005M)	0
NaCl (0.005M)	87

Inhibition of the reduction of vinylacetate by acetate. It is apparent from the earlier experiments that, in the absence of vinylacetate, acetyl phosphate is removed from the system by some reaction in which the energy-rich acyl bond is presumably destroyed. One such reaction already known to be catalysed by preparations of *Cl. kluyveri* is the reduction of acetyl phosphate plus acetate to butyrate. Consequently it was expected that the addition of acetate to the system might accelerate

the disappearance of acetyl phosphate and in this way prevent the activation of the system reducing vinylacetate. The effect of acetate was therefore tested.

When 0.005M sodium acetate was added to the usual test system, the hydrogen uptake was completely inhibited (Table 8; the H_2 uptake required to remove the small amount of acetyl phosphate present is negligible). This appears to be a true effect of the acetate ion, since complete inhibition was also found with potassium acetate, whereas there was no inhibition with NaCl.

Inhibition of the reduction of vinylacetate by arsenate. Previous work (Stadtman & Barker, 1949c, e) has shown that the oxidation of vinylacetate to acetyl phosphate plus acetate and the reduction of acetyl phosphate plus acetate to butyrate are both inhibited by arsenate, and it was therefore of interest to investigate the effect of this inhibitor on vinylacetate reduction.

Table 9. *Inhibition of vinylacetate reduction by arsenate*

Manometers contained in a total vol. of 2.0 ml.: 100 mg. of dried cells of *Cl. kluyveri*; 0.001M BAL; 0.05M tris- KH_2PO_4 , pH 8.0; 0.5 ml. of extract of boiled *Cl. kluyveri* (1 g. of dried cells of *Cl. kluyveri*, lot A0, were suspended in 5 ml. of water and heated at 100° for 20 min. The mixture was then centrifuged and the clear supernatant used). Potassium vinylacetate (10 μ moles), acetyl phosphate (0.1 μ mole) and potassium arsenate were added as indicated from the side arm after saturation of the enzyme system with H_2 . Gas phase H_2 ; temp. 26°.

System	Rate of H_2 uptake (μ l./hr.)
Vinylacetate absent, no arsenate	21
Vinylacetate present, no arsenate	378
Vinylacetate absent + arsenate (0.025M)	15
Vinylacetate present + arsenate (0.025M)	21

The experiment was carried out after the dried cell preparation used in the previous experiments (Lot YE9) had been stored at -18° for 12 months. The preparation had lost most of its activity during this period, in contrast to the earlier batches of cells prepared exactly according to Stadtman & Barker (1949a) and referred to in the Materials and Methods section above. However, by increasing the concentration of dried cells and adding a boiled extract of a different dried cell preparation (Lot AO, prepared according to Stadtman & Barker, 1949a) a rapid reduction of vinylacetate was obtained. With these modified conditions, it was found that 0.025M arsenate completely inhibited the uptake of H_2 in the presence of vinylacetate (Table 9).

DISCUSSION

Kennedy & Barker (1951a) suggested that in *Cl. kluyveri* vinylacetate was reduced in the form of a complex with a coenzyme, possibly CoA, as indicated in Fig. 2. The results presented in this paper show quite clearly that acetyl phosphate or acetyl-CoA is necessary for the reduction of vinylacetate, indicating that the vinylacetate molecule must be activated in some way before reduction can occur. This evidence therefore supports the scheme proposed by Kennedy & Barker and suggests that the conversion of vinylacetate into the active complex requires a high-energy acyl bond.

It had previously been observed that acetyl phosphate was required for the oxidation of butyrate by dialysed extracts of *Cl. kluyveri* (Stadtman & Barker, 1949b), but in these experiments the amounts of acetyl phosphate used were large (10 μ moles). From the present experiments it is quite evident that the role of acetyl phosphate in the reduction of vinylacetate is essentially catalytic, since only a fraction of a micromole is necessary to permit the reduction of several micromoles of vinylacetate. It follows that the energy of the acyl link used to activate vinylacetate must be conserved in some way.

It is now known that in animal tissues the fatty acids are oxidized in the form of their thioester derivatives of CoA (Mahler, 1953; Lynen, 1953), and extracts of *Cl. kluyveri* have been shown to oxidize butyryl-CoA (Stadtman, 1953a). The current views on the synthesis and oxidation of butyrate by bacterial systems are summarized in Fig. 2 (cf. Stadtman, 1953a).

The activation and reduction of vinylacetate can therefore be readily explained by the following series of reactions, in which coenzyme A is represented by CoASH for convenience.

Activation process

Cyclic reduction process

Reaction 6 is the phosphotransacetylase reaction and reaction 7 is catalysed by the CoA transphorase of *Cl. kluyveri*. Both these enzymes have been

partially purified and examined in some detail by Stadtman (1952*b*, 1953*b*). Thus the initial activation of vinylacetate by conversion into vinylacetyl-CoA is explicable in terms of enzyme systems known to occur in *Cl. kluyveri*.

There has been no direct demonstration of reaction 8 in this organism, though Stadtman & Korkes (quoted by Stadtman, 1953*a*) have shown that butyryl-CoA is oxidized to acetoacetyl-CoA, presumably via a butenoyl derivative of CoA. From animal tissues, Mahler and co-workers (Green, Mii, Mahler & Bock, 1954; Mahler, 1954) have obtained, in a highly purified state, a cupro-

Reaction 10 is known to be catalysed by the CoA transphorase of *Cl. kluyveri* (Barker, Stadtman & Kornberg, 1955).

Reactions 6-9 could thus be accomplished by known enzyme systems, and such a sequence would explain the dependence of vinylacetate reduction on activation by acetyl phosphate or acetyl-CoA. The requirement for only catalytic amounts of the activator is also explicable in terms of these reactions, since the thioester linkage is preserved in reactions 8 and 9, and the system becomes self-sustaining once a catalytic amount of vinylacetyl-CoA has been formed.

Fig. 2

flavoprotein which catalyses the oxidation of butyryl-CoA to a butenoyl-CoA. The position of the double bond in the butenoyl derivative has not been finally settled, but it is known that crotonyl-CoA can act as substrate for the reverse reaction [Mahler, 1954; also cf. the work of Seubert & Lynen (1953) with the *N*-acetylthioethanolamine analogues of the thioesters of CoA]. It is possible that in presence of the appropriate hydrogen donor this enzyme might catalyse the reduction of vinylacetyl-CoA to butyryl-CoA directly, or alternatively reduction may be preceded by conversion of vinylacetyl-CoA into crotonyl-CoA. This latter conversion would appear possible by way of 3-hydroxybutyryl-CoA as intermediate, since the unsaturated fatty acyl-CoA hydrazes of animal tissues reversibly hydrates both these unsaturated derivatives (Wakil & Mahler, 1954).

Reaction 9 may be catalysed directly by CoA transphorase, though this reaction has not so far been reported. Alternatively, this transfer of CoA might be effected in two stages in the presence of catalytic amounts of acetate by reaction 10 followed by reaction 7.

If the scheme postulated above is correct, then the reduction of vinylacetate should be CoA-dependent. Experiments designed to test this have so far proved inconclusive. Preparations from which CoA was removed by treatment with Dowex 1 resin (Dow Chemical Co., Midland, Mich.), as described by Chantrenne & Lipmann (1950), were not reactivated by CoA alone, although boiled extracts of *Cl. kluyveri* would restore activity (Peel & Barker, 1953). Cofactors other than CoA, which may well be required for the hydrogen-activating or electron-transporting systems, would be removed by the resin treatment.

The exact structure of the butenoyl-CoA intermediate in butyrate synthesis and oxidation remains undecided (see Anfinsen & Kielley, 1954, for a full discussion), though arguments based on specificity and energy relationships indicate that crotonyl-CoA is more likely to be the true intermediate, at least in animal tissues (Green *et al.* 1954). Vinylacetyl-CoA was formerly considered the more likely intermediate in the *Cl. kluyveri* system, since preparations of the organism will metabolize vinylacetate but not crotonate. Stadtman (1953*a*), however, has pointed out that this may simply be due to the inability of the system to

activate crotonate, since the CoA transphorase of *Cl. kluyveri* catalyses the transfer of CoA from acetyl-CoA to vinylacetate but not to crotonate. The ultimate solution of this problem must await further studies on the isolated enzyme systems.

The instability of acetyl phosphate in the absence of vinylacetate has been explained in terms of its conversion into butyrate. The inhibitory action of acetate now requires further consideration in terms of the mechanism proposed for the reduction of vinylacetate. Acetate is a CoA acceptor and would tend to remove vinylacetyl-CoA and butyryl-CoA by reversing the vinylacetate-activating reaction 7 and promoting reaction 10: thus in the presence of acetate there would be a leakage of the thioesters concerned in the cyclic reduction process (reactions 8 and 9). The resulting acetyl-CoA would be converted into acetoacetyl-CoA by β -ketothiolase and then reduced by hydrogen. The condensation involves a net loss of acyl thioester bonds, and would result in the complete removal of such bonds from the system, provided that sufficient free acetate were present.

The inhibition by arsenate can be explained along similar lines. In the presence of arsenate, phosphotransacetylase would rapidly break down acetyl-CoA to CoA and free acetate. This acetate could then react with other CoA derivatives in the presence of CoA transphorase, yielding more acetyl-CoA, which in turn would undergo arsenolysis. The overall result would again be the removal of the acyl-CoA derivatives on which the reduction of vinylacetate depends.

The explanations offered above for the phenomena connected with the reduction of vinylacetate are over-simplified. Only one reaction bringing about a removal of acetyl phosphate has been taken into account, namely the reduction of acetyl phosphate plus acetate to butyrate (reaction 1), whereas preparations of *Cl. kluyveri* also catalyse a slow hydrolysis of acetyl phosphate (Stadtman & Barker, 1950; Lieberman, 1954). It is also possible that, after the initial activation of vinylacetate, acetyl phosphate may be generated by dismutation according to reaction 5. Both these reactions would affect the number of high-energy acyl bonds present in the system and thus might exert a controlling effect on the activation and reduction of vinylacetate. The fact that all the added vinylacetate is not reduced to butyrate would be understandable if, in the later stages of the reaction, the production of acyl bonds by the dismutation of vinylacetate could not keep pace with the hydrolysis of acetyl phosphate. Such considerations, however, involve modifications to the proposed scheme for the reduction of vinylacetate rather than any change of the basic principles involved.

So far, no mention has been made of the hydro-

gen-activating system of *Cl. kluyveri* which is involved in the test system used for the experiments described in this paper. Arsenate has no effect on this system, since it does not inhibit the reduction of acetoacetate to 3-hydroxybutyrate by molecular hydrogen (Stadtman & Barker, 1949*d*). The possibility that the stimulation of vinylacetate reduction by acetyl phosphate might be due to an effect on the hydrogen-activating system would appear to be ruled out by the following observations. Preparations of *Cl. kluyveri* possess a strong yellow colour which is largely due to the presence of flavin compounds, principally flavin-adenine dinucleotide (Peel, 1955). In the presence of H_2 this yellow colour disappears, presumably owing to the reduction of these flavins, and, with the preparation YE9 used in the above experiments, this occurs without the addition of acetyl phosphate. It is known that the butyryl-CoA dehydrogenase of animal tissues is a cuproflavoprotein (Mahler, 1954), and a survey of the flavin content of a variety of micro-organisms has revealed a correlation between a high flavin content and the metabolism of fatty acids (Peel, 1955). Consequently, it appears probable that a similar enzyme operates in *Cl. kluyveri*. If so, acetyl phosphate is not required for its reduction by molecular hydrogen.

SUMMARY

1. In presence of molecular hydrogen, dried cells of *Cl. kluyveri* reduce vinylacetate (3:4-butenate) to butyrate. With some dried cell preparations extract of boiled yeast is required for this reaction.
2. The extract of boiled yeast can be replaced by catalytic amounts of acetyl phosphate or acetyl-CoA, but not by various known cofactors, including CoA.
3. In the absence of vinylacetate and in the presence of hydrogen, acetyl phosphate is destroyed by the preparations and stimulates reduction only if added at the same time as, or after addition of, the vinylacetate.
4. Whenever reduction occurred, the rates of hydrogen uptake and the total hydrogen uptakes were the same, whatever the amount of acetyl phosphate added. No intermediate rate was observed. The minimum amount of acetyl phosphate required to stimulate reduction could not be established with certainty. The lowest effective amount (in 2 ml.) was 0.003 μ mole.
5. The reduction of vinylacetate is completely inhibited by 0.005M acetate or 0.025M arsenate.
6. These observations can be explained on the assumption that vinylacetate is reduced, not in the free state, but in the form of vinylacetyl-CoA, which is produced by a transfer of CoA from acetyl- or butyryl-CoA.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

3. INVESTIGATION OF THE PRODUCTS OF METABOLISM AFTER INCUBATION OF 5 β -PREGNANE-3 α :20 α -DIOL WITH RABBIT-LIVER HOMOGENATE*

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It is well established that when progesterone (pregn-4-ene-3:17-dione) is administered to human subjects a considerable proportion remains unaccounted for by the known progesterone metabolites, mainly 5 β -pregnane-3 α :20 α -diol ('pregnanediol') subsequently recoverable from the urine. Moreover, Sommerville & Marrian (1950) found that when 'pregnanediol' itself was administered orally to human subjects the proportion of administered steroid subsequently excreted in the urine as 'pregnanediol' was no higher than when progesterone was given to the same subjects by the

same route. It was suggested by these authors that these low recoveries of urinary 'pregnanediol' might be due to excretion of that steroid by other routes or to conversion into further metabolites as yet unidentified, or to a combination of both processes.

To explore the possibility that further metabolism of 'pregnanediol' occurs in the body, Grant & Marrian (1950) and Grant (1952) investigated the metabolism of 'pregnanediol' dihemisuccinate by rabbit- and rat-liver preparations. Liver slices, acetone powders and homogenates were able to metabolize 'pregnanediol' under aerobic conditions, and addition of diphosphopyridine nucleotide

* Part 2: Taylor (1955).