coenzyme A becomes progressively less under these conditions. As previous authors have pointed out (e.g. Laties, 1953b; Lindberg & Ernster, 1954) such a result might be expected if a single dehydrogenase well supplied with substrate was sufficiently active to saturate an electron-transfer system normally shared by subsequent dehydrogenation steps.

In conclusion it may be mentioned that responses to coenzyme A and cocarboxylase may be expected when other preparations are investigated, although the high rates of oxygen uptake which have been recorded without benefit of such additions may indicate a degree of self sufficiency which would make these preparations less desirable for such demonstrations. However, we have already been able to show responses to these cofactors in the oxidation of oxaloacetate by preparations from cauliflower and potato.

SUMMARY

1. Particulate preparations have been prepared from the endosperm of germinating castor beans (Ricinus communis) which under appropriate conditions will oxidize the various intermediates of the Krebs cycle at high and sustained rates.

2. The activity of such preparations is related to the stage in germination, and a decline in activity which occurs after the fourth day has been shown to be due to the presence of a thermolabile factor.

3. A study has been made of the effects of added cofactors on the oxidation of the various acids by the Ricinus preparations.

4. Clear requirements have been established, not only for adenosine triphosphate and diphosphopyridine nucleotide but for the additional cofactors coenzyme A and cocarboxylase.

5. An explanation has been suggested for the apparent stimulation by cofactors of the oxidation of individual acids in which no primary role for these substances has yet been established.

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Some Requirements for Pyruvate Oxidation by Plant Mitochondrial Preparations

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Pyruvate oxidation by particulate preparations from plants and its stimulation by some of the acids of the Krebs cycle have already been demonstrated by a number of workers (e.g. Millerd, Bonner, Axelrod & Bandurski, 1951; Millerd, 1953; Laties, 1953b; Davies, 1953; Hackett & Simon,

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1954). Though it had seemed apparent that the addition of oxaloacetate or an acid that would readily give rise to oxaloacetate would facilitate the entry of pyruvate into the cycle, the actual mechanism of this key reaction remained obscure until Lipmann, Ochoa and others characterized coenzyme A (CoA) and established its role in pyruvate oxidation induced by enzymes isolated from bacteria and animal tissues (see Ochoa, 1954).

These workers have shown that in the presence of catalytic amounts of CoA the reaction

$$H_3C.CO.CO_2H + CoA \xrightarrow[\text{cocarboxylase}]{\text{DPN}} H_3C.CO.S.CoA + CO_2$$
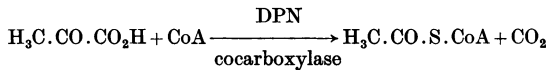
will not proceed unless there is also present an acetate- or acetyl-acceptor system which will regenerate CoA. In the presence of condensing enzyme, oxaloacetate will function as such an acceptor and CoA is released from its acetylated derivative, with the concomitant formation of citrate.

The separation from the endosperm of germinating castor bean (*Ricinus communis*) (Beevers & Walker, 1956) of a particulate fraction which is almost entirely free from substrate acids and cofactors has made possible a new and more detailed study of pyruvate oxidation in preparations from a higher plant. The present paper describes evidence, obtained with such preparations, which associates the stimulation of pyruvate oxidation by oxalo-

acetate with the function of the latter as an acetate acceptor in a CoA- and cocarboxylase-mediated reaction analogous to those recently described for enzyme systems isolated from animal and bacterial sources.

MATERIALS AND METHODS

These were as described in a previous paper (Beevers & Walker, 1956). Sodium pyruvate was supplied by H.M. Chemical Co. Ltd., Santa Monica, California, and oxaloacetate by California Foundation for Biochemical Research. The oxaloacetic acid was dissolved and neutralized immediately before it was added to the Warburg vessels. The total volume of liquid in the vessels was 2·0 ml. in all experiments.

RESULTS

Pyruvate oxidation; the requirement for a second acid of the Krebs cycle

Particulate preparations from the endosperm of 4-day castor beans which will oxidize acids of the Krebs cycle will not oxidize pyruvate even in the
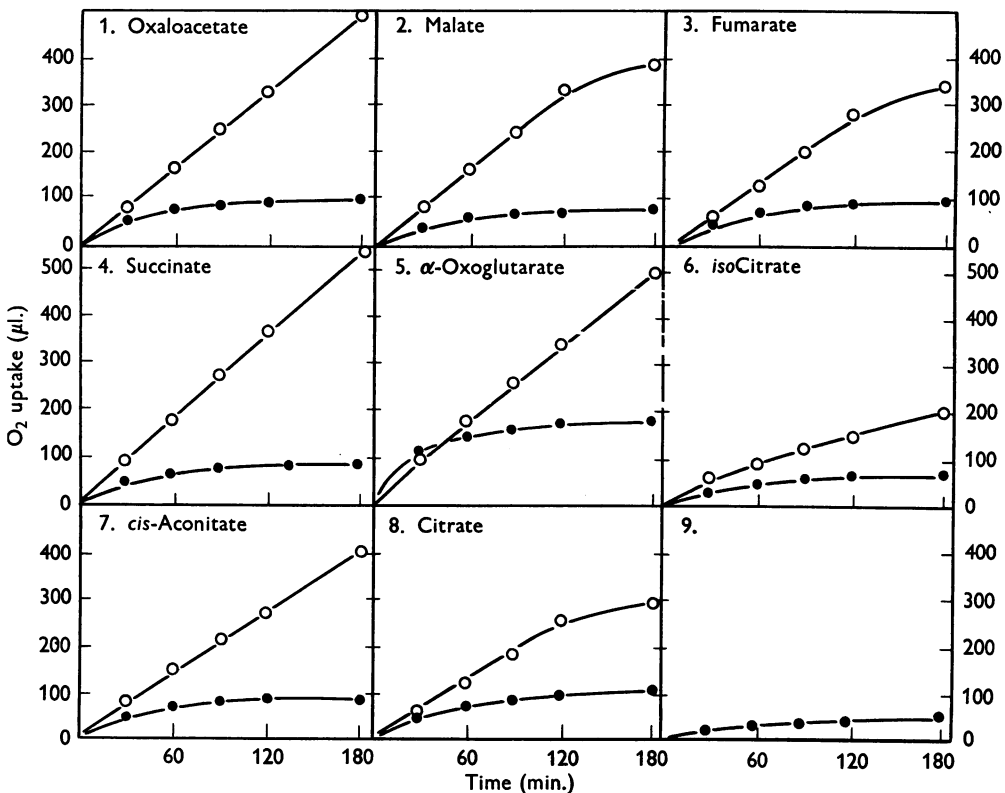

Fig. 1. Graphs 1–8: effectiveness of eight Krebs-cycle acids in inducing the oxidation of pyruvate by *Ricinus* mitochondria. The lower curve (●) in each case represents the O_2 uptake observed on adding the named acid (0·001 M) to the enzyme preparation in the presence of 1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA and 0·5 mg. cocarboxylase. The upper curve (○) represents the O_2 uptake observed when pyruvate (0·01 M) was also added. Graph 9 shows the O_2 uptake observed in the presence or absence of pyruvate, when no second acid was included.

presence of added diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP), CoA and co-carboxylase unless a small amount of one of the other acids is also added to the reaction mixture. Maximal rates of pyruvate oxidation are obtained only when all four cofactors are present in addition to the second acid. The stimulatory effects of the several acids on pyruvate oxidation are shown in Fig. 1, where the course of oxidation of 2 μmoles of the individual acids of the Krebs cycle is contrasted with that which resulted when 20 μmoles of pyruvate was also included in the reaction mixtures. The results were obtained in a single experiment and the individual rates can be directly compared. It will be seen that the individual acids were oxidized at more or less equal rates which declined as the substrates were consumed. In the presence of pyruvate, however, oxygen uptake proceeded in every case at much higher rates which in most cases were maintained for the 3 hr. period. At the end of this time the values for total oxygen uptake in the presence of pyruvate far exceeded those for the complete oxidation of the 2 μmoles of added 'cycle' acid, and it thus seems firmly established that the individual acids were acting catalytically in pyruvate oxidation. Bicarbonate does not appear to function in our system as a 'sparking' agent for the oxidation of pyruvate as it does in some mitochondrial preparations from liver (cf. Green, Loomis & Auerbach, 1948).

Oxidation of oxaloacetate

Although frequent references are made to the ability of plant mitochondria to oxidize 'all of the Krebs cycle acids' the oxidation of oxaloacetate as a single substrate seems to have been described only for cauliflower (Laties, 1953a, b). However, oxaloacetate has been shown to function as a 'sparker' of pyruvate oxidation by Laties (1953b) and by Brummond & Burris (1953) for lupin mitochondria, and such demonstrations would seem to be crucial and essential evidence for the operation of the cycle. When oxaloacetate was added to the *Ricinus* preparations in the absence of added cofactors no oxidation occurred. However, when it was added to a reaction mixture containing enzyme suspension together with ATP and DPN an unusual effect was observed (Fig. 2, curve *F*). There was an initial period greater than 1 hr. during which there was no detectable oxygen uptake; this induction period was eventually terminated by the inception of oxygen uptake, which then reached its maximal rate within 5 or 10 min.

When CoA was present in addition to ATP, DPN, oxaloacetate and enzyme suspension (Fig. 2, curve *C*) the period of induction was strikingly

shortened and a second marked diminution (Fig. 2, curve *B*) resulted when cocarboxylase was also included. In Fig. 2, it can be seen that the induction period in the presence of the full comple-ment of cofactors extends only for about 20 min., whereas the omission of individual cofactors (Fig. 2, curves *C–F*) causes the period to be lengthened by various time increments, which throughout a series of experiments remained remarkably constant.
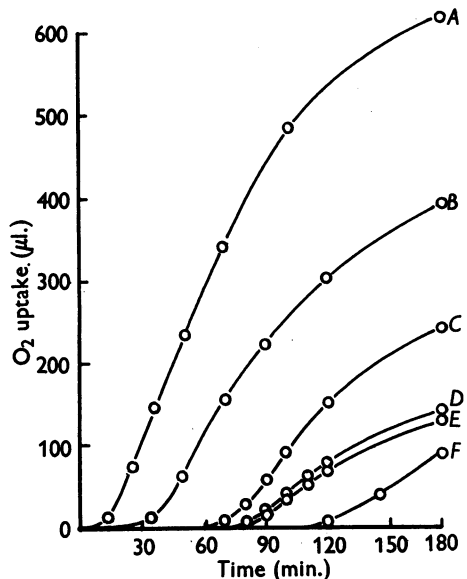

Fig. 2. Oxidation of pyruvate–oxaloacetate by *Ricinus* mitochondria. *A*, 0·01 M pyruvate, 0·01 M oxaloacetate, 1 ml. enzyme, 1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA, 0·5 mg. cocarboxylase; *B*, no pyruvate; *C*, no pyruvate, no cocarboxylase; *D*, no pyruvate, no ATP; *E*, no pyruvate, no CoA; *F*, no pyruvate, no cocarboxylase, no CoA.

Although the onset of oxygen uptake was de-layed, carbon dioxide was evolved from these reaction mixtures from the outset (at the rate of about 150 μl./hr. in a typical experiment). Since oxaloacetic acid undergoes ready autodecarboxyla-tion to yield pyruvate, and since this process is known to be catalysed by magnesium ions (Speck, 1949) which were present in the enzyme suspension, it was evident that the present system could be regarded as containing oxaloacetate as the sole substrate only at the outset. It seemed desirable therefore to investigate the effect of including pyruvate in the reaction mixture from the outset, previous experiments (Fig. 1) having shown that this acid was not oxidized alone, even in the presence of all four cofactors which were being used.

Table 1. *Effect of pyruvate concentration on*
the oxidation of 0·01 M *oxaloacetate*

Added pyruvate	Time to reach maximum rate (min.)	O_2 uptake (µl.) in subsequent 30 min.
0·02 M	2	224
0·01 M	5	231
0·005 M	6	234
0·001 M	7	213
Nil	20	251

Each flask contained 1 mg. DPN, 1 mg. ATP, 0·1 mg. CoA and 0·2 mg. cocarboxylase.
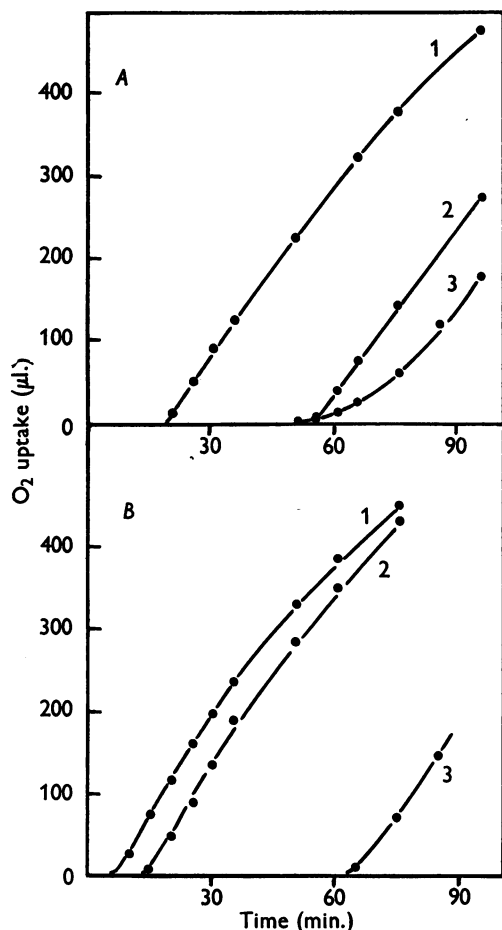

Fig. 3. Effects of cofactor omissions on the oxidation of: A, oxaloacetate 0·01 M; B, oxaloacetate (0·001 M) and pyruvate (0·01 M). Graph A: curve 1, oxaloacetate, 1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA, 0·5 mg. cocarboxylase; curve 2, as 1 minus cocarboxylase; curve 3, as 1 minus CoA. Graph B: curve 1, oxaloacetate plus pyruvate, 1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA, 0·5 mg. cocarboxylase; curve 2, as 1 minus CoA; curve 3, as 1 minus cocarboxylase.

Oxaloacetate–pyruvate oxidation

When pyruvate was added to a reaction mixture containing oxaloacetate, enzyme suspension and the full complement of cofactors, the period of induction was reduced to a minimum (Fig. 2, curve A). Table 1 shows that although the degree of diminution of this period was a function of the quantity of added pyruvate the rates of oxygen uptake after initiation were independent of the concentration.

Omission of cofactors. The effects of omitting cofactors from reaction mixtures containing both oxaloacetate and pyruvate were essentially the same as those observed when oxaloacetate was the only acid originally present, except that the induction period which resulted from the exclusion of CoA was considerably reduced (Fig. 3). Omission of all the cofactors again resulted in the complete absence of all oxygen uptake in excess of that from a blank containing no substrates.
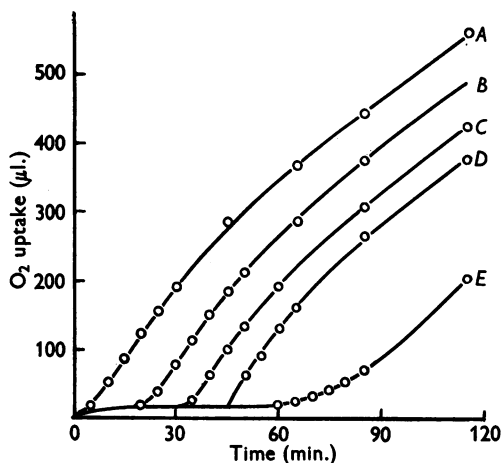

Fig. 4. Effect of cocarboxylase on pyruvate–oxaloacetate oxidation. 0·01 M pyruvate, 0·01 M oxaloacetate, 1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA, 1 ml. enzyme. A, 0·5 mg. cocarboxylase added at zero time; B, cocarboxylase added after 15 min.; C, cocarboxylase added after 30 min.; D, cocarboxylase added after 45 min.; E, no cocarboxylase added.

The effect of adding cocarboxylase during the course of the reaction is shown in Fig. 4, in which curve A shows the oxygen uptake in the complete system. As can be seen from curves B to D the induction period may be terminated at will by the addition of the cofactor from the side arm. Table 2 shows that the length of the induction period bears a definite relationship to the concentration of cocarboxylase, but that the rates after inception of oxygen uptake are largely independent of the amounts added.

Cofactor requirements for other preparations

Having established that cocarboxylase and CoA were required in addition to ATP and DPN, for the oxidation of pyruvate by *Ricinus* particles, we investigated two other preparations. Mitochondria were prepared from cauliflower tissues by methods similar to those employed by Laties (1953a). Such preparations were found to oxidize an oxalo-acetate–pyruvate mixture in the presence of the four cofactors, and it was of considerable interest to find that there were marked responses to CoA and cocarboxylase, although there was no sugges-

tion of any initial induction period (Fig. 5). With potato, however, there was observed to be a close correspondence between the behaviour of particles separated from the tuber [0·001M cysteine was included in the grinding medium in this case (Sharpensteen & Conn, 1954)] and those prepared from *Ricinus* endosperm. A similar lag period in the onset of O₂ uptake was observed by Brummond & Burris (1953) when lupin mitochondria were supplied with oxaloacetate–pyruvate (see Discussion).

Figs. 6 and 7 show the oxidation of an oxalo-acetate–pyruvate mixture by potato mitochondria

Table 2. *Effect of cocarboxylase on the oxidation of pyruvate (0·10M) plus oxaloacetate (0·001M), in the presence of ATP (1 mg.), DPN (1 mg.) and CoA (0·1 mg.)*

Cocarboxylase (mg.)	Time to reach maximum rate (min.)	Maximum rate of O₂ uptake/ min.	Rate after 90 min.
0·5	4	6·9	4·0
0·25	6	6·4	3·8
0·10	8	6·3	3·9
0·075	8½	6·9	3·8
0·050	9	6·8	3·9
0·035	11	6·7	3·7
0·001	15	6·9	3·9
0	90	4·6	4·6

Fig. 6. Oxidation of pyruvate–oxaloacetate by potato preparations. *A*, 0·01M pyruvate, 0·01M oxaloacetate, 1 mg. DPN, 1 mg. ATP, 0·1 mg. CoA, 0·5 mg. cocarboxylase, 1 ml. enzyme; *B*, no CoA; *C*, no CoA, no cocarboxylase.
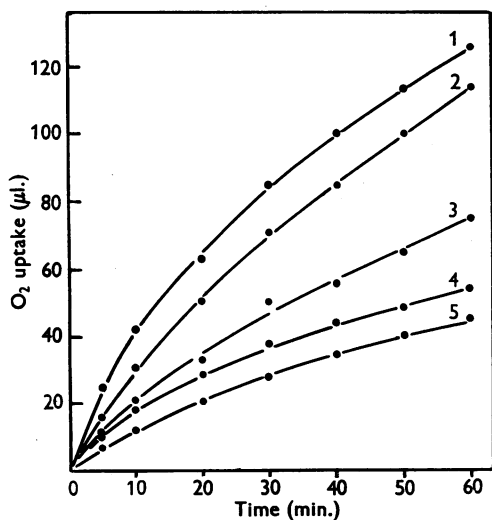

Fig. 5. Effects of omitting cofactors on the oxidation of pyruvate and oxaloacetate by cauliflower mitochondria. Curve 1, complete system; 1 ml. enzyme preparation, pyruvate (0·01M), oxaloacetate (0·001M), 1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA, 0·5 mg. cocarboxylase; curve 2, as 1 minus cocarboxylase; curve 3, as 1 minus CoA; curve 4, as 1 minus cocarboxylase and CoA; curve 5, as 1 minus pyruvate and oxaloacetate.
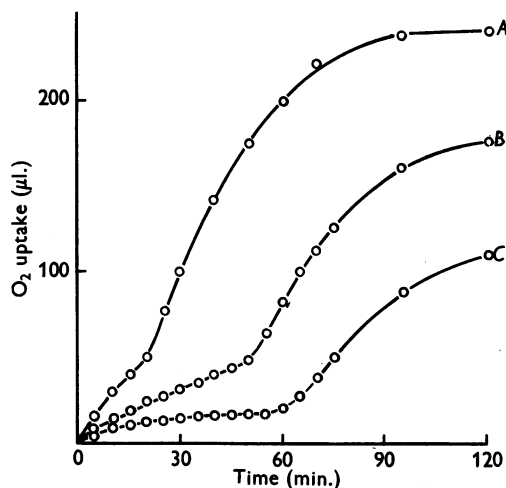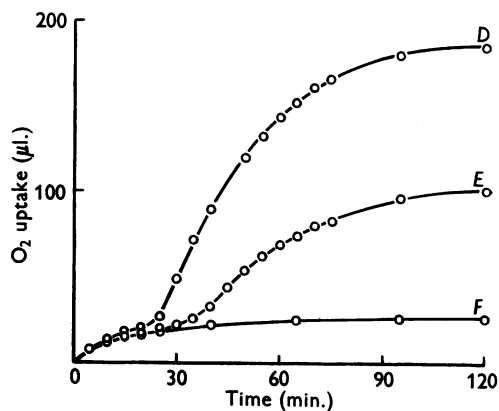

Fig. 7. Oxidation of pyruvate–oxaloacetate by potato preparations. Reaction mixture as for *A* in Fig. 6. *D*, no cocarboxylase; *E*, no ATP; *F*, no DPN.

and the effects of single omissions of the several cofactors. It will be seen that the initial induction period in the presence of the full complement of cofactors and substrates, which was only fleeting in *Ricinus*, is here extended. A second difference was that oxaloacetate was not oxidized at all in the absence of added pyruvate; a minimum concentration of 0·004 M pyruvate had to be provided if oxidation was to occur. Once this level had been reached, however, the reaction proceeded at maximal rates and was then independent of further increases in the pyruvate concentration.
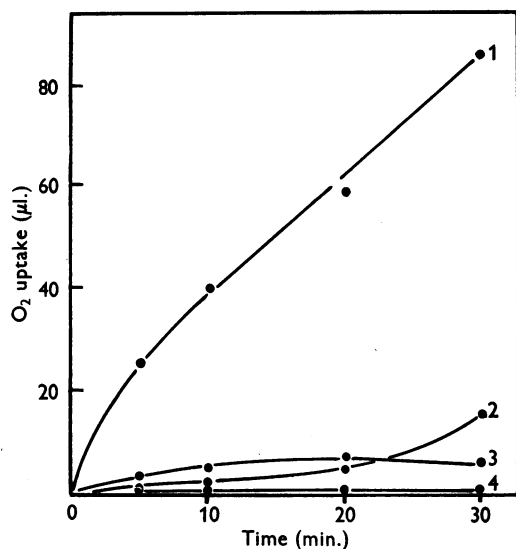

Fig. 8. Mitochondrial oxidation of pyruvate induced by transacetylase and phosphate as acetyl acceptor. Complete system (curve 1) contained mitochondria, 1 ml. of the standard preparation, cofactors (1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA, 0·5 mg. cocarboxylase) pyruvate (0·01 M) and 0·5 ml. of a preparation containing trans-acetylase from dried cells of *Clostridium kluyveri*; curve 2, mitochondria with cofactors and transacetylase; curve 3, cofactors, pyruvate and transacetylase; curve 4, transacetylase alone.
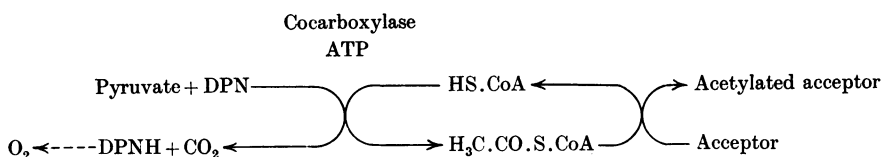
Necessity for an acetate or acetyl acceptor

In enzymes isolated from bacterial and animal preparations several workers (see Discussion) have demonstrated the requirement for an acetate or acetyl acceptor in reactions involving the CoA-mediated oxidation of pyruvate. The fact that pyruvate is not oxidized by *Ricinus* or potato preparations even in the presence of a full complement of cofactors (except in the presence of oxalo-acetate or its precursors) may be taken as evidence that this substance plays the role of an acetate acceptor in the present reaction.

This view is supported by two additional lines of evidence. First, it was shown chromatographically that citrate is formed in the reaction mixtures during the period of rapid oxygen uptake. Secondly, it was found possible to replace oxaloacetate by an entirely different acceptor. Phosphate has been shown to serve as an acetyl acceptor in the presence of a suitable donor and a transacetylating enzyme from *Clostridium kluyveri* (Stadtman, Novelli & Lipmann, 1951; Stadtman, 1952). Experiments were therefore performed in which an extract from dried cells of *C. kluyveri*, kindly supplied by Dr Stadtman and known to contain transacetylase, was tried as an acetyl acceptor. The extract was prepared by suspending 0·5 g. of the dried cells in 10 ml. of 0·01 M phosphate buffer pH 7·0 and allowing the suspension to stand at 25° for 4 hr. with occasional stirring, after which the cell particles were centrifuged off at 18 000 *g*. A portion (0·5 ml.) of the clear yellow supernatant was used, and as can be seen from Fig. 8 the preparation did not absorb oxygen (curve 4) and was incapable of oxidizing pyruvate even in the presence of all the cofactors (curve 3). When mitochondria were added with the cofactors to the transacetylase (curve 2) the oxygen uptake was again negligible, and the mitochondria themselves are incapable of oxidizing pyruvate. However, when pyruvate was added to a mixture of mitochondria, transacetylase and cofactors, a rapid oxygen uptake was evident from the outset (curve 1).

DISCUSSION

The results obtained in experiments designed to take advantage of the properties of a particulate fraction from castor-bean endosperm, which is largely devoid of endogenous substrates and co-factors, provide indications of the actual mech-anism of pyruvate oxidation in the higher plant. It is apparent that neither oxaloacetate nor pyruvate is oxidized alone and that the oxygen uptake in the presence of a combination of these substrates proceeds at its maximum rate from the outset only in the presence of a full complement of cofactors, including coenzyme A and cocarboxylase. The involvement of these two cofactors is clearly shown by their effects on the termination of the remark-able induction periods observed in deficient reaction mixtures. The demonstration in a number of preparations from plants of the beginning of pyruvate oxidation that follows the addition of an acid of the Krebs cycle, supports the accepted view that the oxidation of these acids proceeds from the citrate stage in a cyclic manner to give oxaloacetate which, as we have demonstrated, is involved in the oxidation of pyruvate. It appears that if oxalo-acetate is the sole added substrate no oxygen

uptake can be detected until some of the acid undergoes decarboxylation to yield pyruvate, which is then oxidized in a coenzyme A- and co-carboxylase-mediated reaction in which the remaining oxaloacetate plays the role of an acetate acceptor. This view is supported by the finding that pyruvate, which is also apparently incapable of oxidation as a single substrate, undergoes rapid oxidation when orthophosphate and transacetylase are substituted for oxaloacetate as the acceptor system which regenerates CoA. In the light of conclusions from spectrophotometric experiments with isolated enzymes from other sources (Ochoa, 1954) we may suppose with some justification that the oxidation of pyruvate by the *Ricinus* mitochondria proceeds as follows:

might easily be associated with the requirement for an initial decarboxylation to give a reaction mixture containing both acids; this view is supported by the experimental findings. The fact that induction periods brought about by the omission of single cofactors are also eventually terminated implies that although the level of the available cofactors originally present in the mitochondrial preparations is insufficient to meet the minimal requirement, this deficit is made good by some unknown mechanism during the lag period. This might conceivably occur either by synthesis from precursors or release from an unavailable condition. In the cauliflower preparations, which show no such lag effects, we may suppose that the minimal requirements for cofactors are already met.

$$
\begin{array}{c}
\text{Cocarboxylase} \\
\text{ATP} \\
\text{Pyruvate} + \text{DPN} \longrightarrow \quad \text{HS.CoA} \longleftarrow \quad \text{Acetylated acceptor} \\
\text{O}_2 \longleftarrow \text{-- -- --DPNH} + \text{CO}_2 \longleftarrow \quad \text{H}_3\text{C.CO.S.CoA} \longrightarrow \quad \text{Acceptor}
\end{array}
$$

where the acceptor may be oxaloacetate, which yields citrate in the presence of condensing enzyme, or orthophosphate, which yields acetyl phosphate in the presence of transacetylase.

In some of the mitochondrial preparations described, the assessment of the roles of the cofactors is complicated by the fact that, even when one or more of the cofactors is omitted, the reaction begins after a more or less prolonged lag period. In a polyphasic system of this type such a delay might be caused by barriers to the mutual access of enzymes and other reactants. However, induction periods are known in purely chemical reactions, and though the causal factors are varied it has been suggested (Friend, 1932) that, in a system of consecutive reactions which proceed at measurable rates, an induction period would be observed between the initiation of the first reaction and the appearance of the end product of the terminal reaction at its maximum rate. The duration of such an induction period would depend on the concentration of the various reactants. Such a thesis would be very attractive because the studies on partial reactions show that the oxidation of pyruvate in animal and bacterial preparations proceeds through a series of consecutive reactions involving coenzyme A, DPN, cocarboxylase and an acetate acceptor, and it is the presence and concentration of these various reactants that determine the duration of the induction periods in the plant preparations. The production of pyruvic acid from oxaloacetic acid occurs readily, and the oxidation of oxaloacetate after an initial induction period

A further possibility, which may well be a major cause of the lag effects which occur even when oxaloacetate, pyruvate and the four cofactors are present together, has been suggested to us by Dr C. A. Price. Because of the fact that the malic-oxaloacetic equilibrium under the influence of malic dehydrogenase and DPN is known to be very much in favour of malic acid, we might expect that when oxaloacetic acid is supplied to the system the malic–dehydrogenase reaction will compete with the enzymes transferring electrons to oxygen for the reduced DPN produced during the initial stages of pyruvate oxidation. The expected result of such an interaction would be that, even though pyruvate oxidation might be proceeding, its manifestation as oxygen uptake would be delayed to the extent of the time necessary for the oxaloacetic–malic equilibrium to be established. Clearly, if the initial stages of pyruvate oxidation were slowed down, e.g. by the omission of one or more co-factors, the lag period would be more extended; and since the oxaloacetate supplied would at the same time be subject to decarboxylation to pyruvate and condensation to citrate, its concentration would be a diminishing one, and hence the competition for reduced DPN would decrease during the experiments. Further experimental analysis of this possibility is contemplated.

There is at least one earlier reference to induction periods in a closely similar system. Lipmann & Tuttle (1945), measuring pyruvate decomposition with a preparation from *Escherichia coli*, reported that the final rate was reached only after prolonged

incubation, and suggested an autocatalytic activation of some part of the enzyme system as the cause. In the only previous case in which a time curve of oxaloacetate–pyruvate oxidation by plant mitochondria has been given, a lag phase is clearly apparent (Brummond & Burris, 1953); this was interpreted by the authors as an inhibition by the oxaloacetate, although the rate once established was as rapid as with malate, and it now seems to us that the alternative explanation outlined above is the more likely one.

Whatever may be the precise explanation of the spontaneous termination of the lag periods observed in the absence of one or more cofactors, the point that we wish to emphasize is that the marked stimulation of pyruvate oxidation by the full complement of cofactors, the initiation by oxaloacetate (or another system which would be expected to lead to the regeneration of coenzyme A) and the initial failure of the reaction to proceed at measurable rates in the absence of any one coenzyme provide strong grounds for believing that these substances are directly implicated in a reaction sequence analogous to that elucidated by recent work on isolated enzymes. This reaction sequence would appear to proceed both in the oxidation of an individual acid which was giving rise to an acetate acceptor as well as in the functioning of the cycle as an integrated unit, *in vivo*.

SUMMARY

1. The conditions under which pyruvate is oxidized by plant mitochondrial preparations, particularly those from the endosperm of germinating *Ricinus* seeds, have been investigated.

2. It has been established that maximal initial rates of oxygen uptake are not obtained unless coenzyme A, cocarboxylase and an acetyl-acceptor system, as well as adenosine triphosphate and diphosphopyridine nucleotide, are provided.

3. An induction period before the onset of oxygen uptake has been found to be a feature of pyruvate oxidation in certain circumstances. Possible explanations of this effect are discussed.

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Metabolic Effects of Azide on Electrically Stimulated Cerebral Cortex

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The metabolic rate of slices of cerebral tissue *in vitro* can be increased by the application of electrical pulses (McIlwain, 1951 *a*; McIlwain, Anguiano & Cheshire, 1951). Heald (1953) demonstrated that metabolic inhibitors were able to depress the response to applied electrical pulses at concentrations which had negligible effects on the normally respiring tissue. A greater sensitivity to inhibitors had previously been reported by Gerard & Doty (1950), who observed that azide could abolish the extra oxygen consumption of active nerve, while leaving the resting respiration virtually unaffected.

The present investigations were carried out to extend Gerard & Doty's (1950) observations and to determine the sensitivity of cerebral tissue to azide when metabolizing glucose as well as other substrates.