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The Oxidative Activity of Particulate Fractions from Germinating Castor Beans

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During an investigation of the catabolic changes in germinating castor beans (*Ricinus communis*) highly active preparations were separated from the endosperm by methods similar to those employed by Laties (1953*a*, *b*) and by other workers with a variety of plant materials. The general properties of these particulate fractions are similar to those which have been described earlier, but some interesting distinguishing features were noted. Preparations from other tissues frequently show appreciable endogenous oxygen uptake and may be capable of rapid oxidation of Krebs-cycle acids without added cofactors or upon the single addition of adenosine triphosphate (ATP) or diphosphopyridine nucleotide (DPN) (e.g. Millerd, Bonner, Axelrod & Bandurski, 1951; Davies, 1953). The *Ricinus* preparations, on the other hand, are characterized by an extremely low endogenous uptake, and, with the sole exception of succinate, the unassisted oxidation of acids is slow. However, high rates of oxidation are obtained in all cases after the addition of a full complement of cofactors, and these features make the preparations especially suitable for the study of substrate–cofactor interrelationships. Such an investigation has led to the demonstration of distinct requirements, not only for adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN) but also for coenzyme A (CoA) and cocarboxylase.

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MATERIALS AND METHODS

Substrates. All the substrate acids were obtained commercially; *cis*-aconitic acid and oxaloacetic acid of 99±1% purity were obtained from the California Foundation for Biochemical Research, Los Angeles, California. Sodium DL-*iso*citrate, sodium pyruvate and α-oxoglutaric acid were supplied by the H.M. Chemical Co., Santa Monica, California. L-Malic acid was obtained from Eastman Kodak Co. Ltd., Rochester, New York. For use, all the substrates were dissolved in water and carefully adjusted to pH 7, using a Beckman Model H2 pH meter.

Cofactors. DPN '90' and disodium ATP were products of the Sigma Chemical Co., St Louis, Missouri, and crystal-

line cocarboxylase was purchased from the Nutritional Biochemical Corporation, Cleveland, Ohio. CoA of at least 70–75% purity was obtained from the Nutritional Biochemical Corporation or from Pabst Laboratories, Milwaukee, Wisconsin.

Castor beans. Variety U.S. 74 was purchased from the Baker Seed Co., Vernon, Texas.

Measurement of activity. Oxygen uptake at 25° was measured by the Warburg manometric method with air as the gas phase and potassium hydroxide in the centre well. In almost all instances reactants were added directly to the main chamber of the flasks and made up to a final volume of 2 ml. The reactions were started by adding the enzyme to the flasks as soon as it was prepared, and the flasks were then immediately connected to the manometers. A 5 min. equilibration period was allowed.

Separation of a particulate fraction from Ricinus *seedlings.* Washed castor beans were planted in vermiculite (heat-expanded mica) and germinated under controlled conditions in the dark at 30° and 76% humidity. The seedlings were harvested after 4 days, when the primary root had a length of 7–8 cm. At this stage secondary roots of 2–3 cm. are present and the hypocotyl does not exceed 1 cm. The testa, hypocotyl and roots were discarded, but the cotyledons were not separated from the endosperm, which was shown to provide the bulk of the activity.

The endosperm tissues were then washed in distilled water, dried by pressing gently between sheets of absorbent paper, and weighed. In a typical preparation 90 beans yielded 45 g. of endosperm tissue, and this was ground rapidly in a chilled mortar in cold 0·5 M sucrose, 0·02 M magnesium chloride and 0·1 M phosphate (NaH_2PO_4) adjusted to pH 7 by the addition of potassium hydroxide. For each gram of tissue 1·5 ml. of grinding medium were used. (Equally active preparations have recently been obtained when blending for 15 sec. in a chilled Waring Blendor was substituted for manual grinding.) The homogenate was then squeezed through a cloth and the filtrate centrifuged for 5 min. at low speed (500 *g*) to remove cell debris. The creamy homogenate was then centrifuged at high speed (18 000 *g*) for 30 min. in an 'International' refrigerated centrifuge set at −10°. The supernatant and the fat pellicle were discarded and the pale-brown particulate fraction washed by resuspending in cold-grinding medium with a motor-driven pestle (Potter–Elvehjem homogenizer). The particles were resedimented by high-speed centrifuging and suspended in 18 ml. of cold-grinding medium for use. A portion (1 ml.) of such a preparation (equivalent to 2·5 g. of tissue and containing 10–12 mg. of protein) was used in each flask and new preparations were made daily and used immediately. Though the cytological identity of the particles has not been proved they were found to be spherical bodies up to 1–2 μ. in diameter and they correspond in their preparation to fractions that have been termed mitochondria by various authors (see Stafford, 1951). In the presence of succinate the *Ricinus* preparations stain with Janus Green B, and, if air is subsequently excluded, the dye is reduced to the pink form.

RESULTS

Changes in activity during germination

At the end of 7 days under the described germination conditions the endosperm had been completely utilized, and the cotyledons remained covered only by a thin membrane. The endosperm originally has a high fat content, and it was shown that the level of ether-soluble substances remained high until the fourth day and then declined sharply. The water-soluble fraction (mostly sugars) increased in the fourth and fifth days and then this too decreased as the endosperm became absorbed by the cotyledons. The respiratory activity of the endosperm increases during the first 6 days of germination. Fig. 1 shows that there are considerable differences in the relative activities of particulate preparations obtained from seedlings of different ages and that the maximal activity was associated with the critical 4-day stage, at which the major metabolic

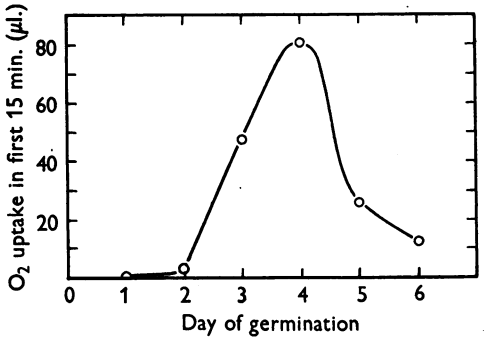

Fig. 1. Variation in activity of particulate preparations with stage in germination. Substrate, 0·01 M succinate. In each case the sample of enzyme preparation added to the flasks represented five beans of the age indicated.

Table 1. *Inactivation of* Ricinus *preparations by a thermolabile factor in 6-day extracts. Manipulations were performed at 0° and the supernatants maintained at this temperature before the treatment*

Treatment	µl. O_2 uptake in 20 min. with substrate		
	Citrate	α-Oxoglutarate	Succinate
A. Four-day particles resuspended in and resedimented from 4-day supernatant	47	25	90
B. Four-day particles resuspended in and resedimented from 6-day supernatant	20	4	57
C. Four-day particles resuspended in and resedimented from boiled 6-day supernatant	39	27	105

changes outlined above were initiated. The results in Table 1 show that the supernatant fraction from 6-day beans contains a thermolabile factor which is capable of inactivating the particulate fraction from 4-day beans, and it seems that the development of this (enzymic?) factor after the fourth day of germination may be responsible for the lowered activities recorded for the older beans.

Table 2. *Oxidation of Krebs-cycle acids by* Ricinus *particles*

	O_2 uptake (μl.) in first 60 min.	
	No additions	Added co-factors*
0·01 M Citrate	54	293
0·01 M *cis*-Aconitate	56	262
0·02 M DL-*iso*Citrate	41	263
0·01 M α-Oxoglutarate	33	262
0·01 M Succinate	174	465
0·01 M L-Malate	39	358
0·01 M Fumarate	30	407
No substrate	0	36

* 1 mg. DPN, 1 mg. ATP, 0·1 mg. CoA, 0·5 mg. co-carboxylase.

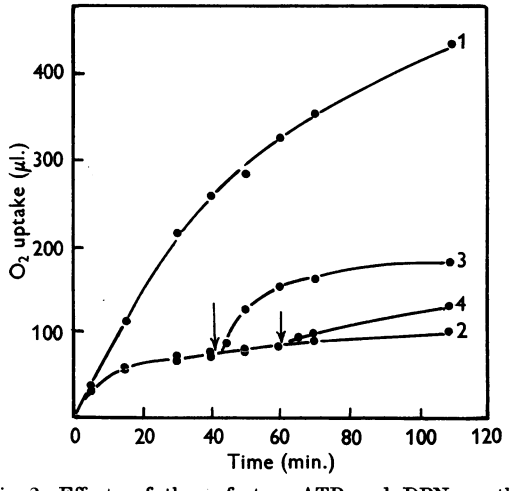

Fig. 2. Effects of the cofactors ATP and DPN on the oxidation of succinate 0·01 M. Curve 1, 1 mg. ATP and 1 mg. DPN present from the outset; curve 2, no co-factors added; curves 3 and 4, cofactors added at arrows, after 40 and 60 min. respectively.

Oxidation of acids of the Krebs cycle by 4-day Ricinus *particles*

When washed particulate preparations from the endosperm of 4-day castor beans are shaken in Warburg flasks they appear inert, and throughout a considerable number of experiments the recorded endogenous uptake of 1 ml. of enzyme preparation was not in excess of 5 μl./hr. When Krebs-cycle acids were added to the preparations this rate was augmented, the degree of stimulation depending on the acid added (Table 2). Only succinate was oxidized at a high rate under these conditions, and, as can be seen from Fig. 2, the rate declines sharply after the first 15–20 min.

Cofactor requirements

The addition of cofactors such as the phosphopyridine nucleotides, either alone or in any combination, brings about only a slight increment in the endogenous oxygen uptake of the washed particles (see the foot of Table 2). The effects of these co-factors on oxygen uptake when the preparations were also provided with Krebs-cycle acids are described in the following sections.

Effects of DPN and ATP. Added singly, both DPN and ATP cause slight stimulation of acid oxidation by the particles. Reference to Tables 4 and 5 indicates that ATP tends to be more effective in stimulating the oxidation of malate, fumarate and succinate, whereas DPN has the larger effect on

Table 3. *Specific requirement for DPN*

	O_2 uptake (μl./hr.)		
Additions	0·01 M Succinate	0·01 M α-Oxo-glutarate	0·01 M Citrate
None	97	12	69
2 mg. ATP	175	87	80
2·1 mg. ATP	195	82	103
2 mg. ATP + 0·1 mg. DPN	434	243	264

Table 4. *DPN, ATP, and CoA stimulation of the oxidation of Krebs-cycle acids by* Ricinus *preparations*

	O_2 uptake (μl./hr.)					
Additions*	0·01 M Malate	0·01 M Fumarate	0·01 M Succinate	0·01 M Citrate	0·01 M *cis*-Aconitate	0·02 M DL-*iso*Citrate
None	14	22	128	64	68	60
ATP	44	26	264	104	84	66
DPN	32	18	98	125	92	100
ATP and DPN	84	66	448	270	202	180
ATP and DPN and CoA	376	370	600	376	294	290

* ATP 1 mg., DPN 1 mg., CoA 0·1 mg.

Table 5. *Effect of co-factors on the oxidation of* 0·01 M *α-oxoglutarate*

Additions	O_2 uptake (μl./hr.)
None	40
DPN	102
ATP	148
DPN + ATP	256
DPN + ATP + CoA	304
DPN + ATP + CoA + cocarboxylase	386

Quantities: 1 mg. DPN, 1 mg. ATP, 0·1 mg. CoA, and 0·5 mg. cocarboxylase.

α-oxoglutarate and the citric acid group. The effect of DPN on succinate is small, and on several occasions it has been observed to bring about a slight depression in the rate of oxidation of this substrate. None of the stimulations due to single cofactors is large, but a considerable further acceleration results if they are both included in the same reaction mixture, though their combined stimulation of the endogenous rate remains small. Reference to Tables 3–5 indicates that the combined stimulation of the two factors in the presence of substrate acids is considerably greater than the sum of the individual stimulations. The high initial rate of succinate oxidation in the absence of DPN and ATP is only slightly augmented by the addition of these factors. At the end of the first 15–20 min., however, the DPN–ATP assisted rate is maintained, whereas the unassisted rate declines sharply (see Fig. 2). Addition of DPN and ATP after the inception of the decline results only in much smaller stimulations (Fig. 2, curves 3 and 4).

In experiments designed to determine the quantitative requirements for ATP and DPN it was found that saturating levels were not reached until amounts of 1 mg. were supplied in systems containing 1 ml. of enzyme suspension and 0·1 M substrate. Therefore, in all subsequent experiments in which ATP or DPN was used, 1 mg. was the quantity added. In the few instances in which it has been tried triphosphopyridine nucleotide appears to be able to bring about all the effects associated with DPN, and adenosine diphosphate can be substituted with equal effect for ATP.

Effect of coenzyme A. Like DPN and ATP, CoA added alone causes only low stimulation of the endogenous rate and only small increments in the rate of acid oxidation (Fig. 3). The stimulation of the endogenous rate by DPN, ATP and CoA together is slight, but the addition of CoA to a reaction mixture containing certain of the substrate acids and saturating concentrations of DPN and ATP results in striking stimulations. CoA may stimulate the oxygen uptake when the rate is already limited by the omission of either DPN or ATP, and in the case of succinate, for example, such an addition may result in the doubling of the rate of DPN–ATP assisted oxidation. But, for every acid considered, DPN, ATP and CoA together give rates considerably in excess of those achieved by any other combination of these three factors (see Fig. 3).

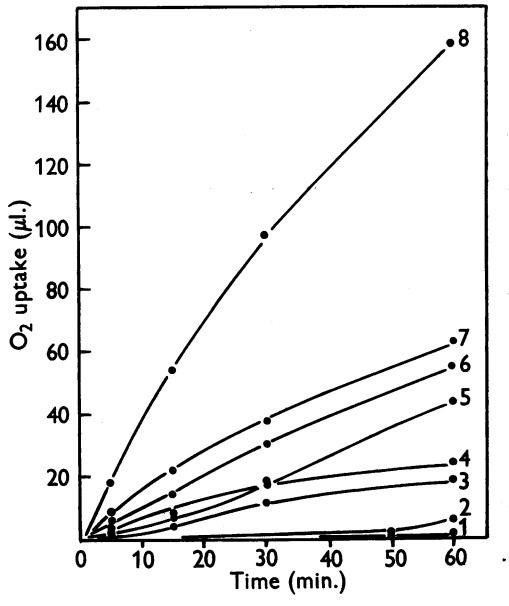

Fig. 3. Effects of three cofactors (1 mg. ATP, 1 mg. DPN, 0·1 mg. CoA) in various combinations on the oxidation of malic acid (0·01 M) by the particulate preparation. Additions to the mitochondria + malate: Curve 1, none; curve 2, DPN; curve 3, CoA; curve 4, ATP; curve 5, DPN and CoA; curve 6, ATP and CoA; curve 7, ATP and DPN; curve 8, DPN, ATP and CoA.

Tables 4 and 5 show the extent of the various responses. The oxidation of fumarate and malate, which is relatively slow in the presence of ATP and DPN, is particularly stimulated by addition of CoA, and in its presence the rates of oxidation of the various acids (with the exception of succinate) are quite similar. [There are several reports that malate and fumarate oxidation is much slower than that of the other acids (e.g. Millerd *et al.* 1951; Brummond & Burris, 1953) and it seems possible that CoA was limiting in the preparations described.] Over a large number of experiments involving different samples of CoA and different enzyme preparations, stimulations of 100–700 % of the DPN–ATP assisted rates of oxidation of these acids have been observed. The CoA stimulation of the citric acid group is about 50 % for each of the three acids (citric, *cis*-aconitic and *iso*citric), and it has been further observed that in the presence of CoA the decline in the rate of oxygen uptake which occurs in the later stages of DL-*iso*citrate oxidation

is diminished. It has been shown subsequently that much more striking effects of CoA on succinate oxidation are observed at lower substrate levels. Thus in one experiment the stimulation due to CoA was 15% when the succinate concentration was 0·02 M, and 179% when the succinate level was reduced to 0·002 M.

In the presence of saturating quantities of DPN and ATP the degree of stimulation is at first related to the concentration of the added CoA in a linear manner but at higher concentrations the increment per unit addition becomes progressively less. Accelerations follow the addition of as little as 0·01 mg., and 0·1 mg. was found to be saturating.

Effect of cocarboxylase. The highest prolonged rates of oxidation of the Krebs acids by *Ricinus* particles were obtained only when cocarboxylase was added in addition to the other cofactors. The degree of stimulation by cocarboxylase is never so pronounced as that induced by CoA, but definite increments follow the addition of cocarboxylase in systems containing substrate acids and saturating amounts of DPN, ATP and CoA. The greatest immediate stimulation by cocarboxylase is observed with α-oxoglutarate, and its effect and that of the other cofactors is shown in Table 5. With malate oxidation the effect of cocarboxylase became more noticeable with time. Thus in one experiment the increase due to the addition of cocarboxylase was 13·5% during the first hour, whereas in the second hour this had become 34%.

Relative rates of oxidation in the presence of all four added cofactors. The relative rates of oxidation of the various acids by a single enzyme preparation in the presence of ATP, DPN, CoA and cocarboxylase are shown in Table 2, where they are contrasted with the unassisted rates. It will be seen that in all cases the degree of stimulation is considerable. Succinate is still oxidized at the fastest rate, but the addition of the full complement of cofactors has also resulted in acceleration and prolongation of the oxidation of the other acids, so that high and maintained rates are now observed with every substrate.

It was of interest to find that the oxidative ability of the preparations was not lost when they were incubated with cofactors in the absence of substrates for 1 hr. at 25°. The subsequent addition of the acids resulted with one exception in initial rates of oxygen uptake which were not less than 80% of those observed in controls in which the substrates were added at the outset. However, with succinate, fumarate and malate these rates declined rather rapidly, and the fact that the rates of oxidation in the controls were maintained at high levels over the same period indicates a degree of substrate protection in these cases. It seems likely that this protection can be referred to that of a complex enzyme system controlling a single stage in the cycle, namely that concerned with the early stages of oxaloacetate oxidation, since when oxaloacetate was the substrate added after 1 hr. under conditions otherwise favourable for its oxidation (Walker & Beevers, 1956) it was shown that the oxidative ability had been virtually lost, whereas in a control system oxaloacetate oxidation proceeded at a high rate for at least 2 hr.

Comparison with mitochondria from other sources

Although the list of *plants* which have yielded particulate preparations capable of oxidizing Krebs-cycle acids is still small, it is of interest to observe (Table 6) that a variety of different *tissues* have yielded active preparations. Further work will no doubt increase the list and so lend support to the now generally accepted concept that the seat of integrated respiratory activity in the cell is the particulate mitochondrion. It is noteworthy, however, that, up to the present, attempts to isolate active mitochondria from leaves have been

Table 6. *Relative activities of mitochondrial preparations from various plants. Except for no. 5 the substrate in each case was succinate*

Authors	Material	Temp. of measurement (°)	Concn. of substrate (M)	μl. O_2 uptake/mg. N/hr. (q_{O_2})	μl. O_2 uptake/g. original fresh wt.
1. Millerd (1953)	Mungbean hypocotyl	30	0·03	260	45
2. Davies (1953)	Pea hypocotyl	30	0·025	—	25
3. Laties (1953*a*, *b*)	Cauliflower bud	30	0·02	566	88–176
4. Hackett & Simon (1954)	Arum spadix	30	0·02	818	500
*5. Brummond & Burris (1953)	Lupin cotyledons	30	0·02	60–120	34–43
6. Millerd, Bonner & Biale (1953)	Avocado fruit	25	0·02	338	34–48
7. Beaudreau & Remmert (1955)	Black Valentine bean hypocotyl	30	0·016	565	18
8. Price & Thimann (1954)	Pea internodes	25	0·02	800	126
9. Present	Potato tuber	25	0·01	—	30
10. Present	Castor-bean endosperm	25	0·01	280–375	180–240
11. Present	Castor-bean endosperm A	25	0·01	940–1250	—

* Substrate was 0·01 M malate + 0·01 M pyruvate.

less successful, and the recent work of Brummond & Burris (1954) indicates that, in lupin leaves at least, a different spatial distribution of enzymes is found. When the activities of the various preparations are compared on a unit nitrogen basis (see Table 6), it is seen that the majority lie in the range of q_{O_2} 300–600. It is quite probable that these figures are in general subject to improvement, since, as we have shown for the castor-bean preparations, separation of fractions with different levels of activity can be made during centrifuging. Thus in one experiment 67% of the activity (succinate oxidation) was recovered in a fraction (A in Table 6) containing 21% of the protein of the original suspension, giving a preparation (A) with a much higher q_{O_2}. When the fact that the measurements on castor-bean preparations were made at 25° is also taken into account it is clear that such preparations may be considered as having a relatively high activity.

The values for activity per unit fresh weight in Table 6 are calculated from the maximum rates recorded by the various authors; they are only approximate, as it is not clear in all cases exactly how much of the original tissue was represented by the mitochondria added to the flasks. It is evident, however, that considerable differences exist, reflecting as they do the amount of mitochondria originally present in the tissue, the efficiency of their extraction, and their intrinsic activity. Judged on this basis as a source of mitochondria the endosperm of the 4-day castor bean is inferior to only one reported and somewhat inaccessible tissue, the spadix of the English arum. As a convenient and easily handled material capable of providing preparations with equally high activity from day to day the germinating castor bean has clear advantages over some of the other sources which have been used.

DISCUSSION

In a system which is capable of oxidizing all the Krebs-cycle intermediates it is clearly difficult to associate cofactor requirements with any single step (Laties, 1953*b*), since the end product of the immediate reaction will serve as a new substrate for the next, and the contribution of subsequent reactions with different cofactor requirements will become progressively greater with time. For the results with ATP and DPN, it is clear that the *Ricinus* preparations do not need merely the adenylic residue, which Laties (1953*a*) has demonstrated to be the requirement in cauliflower preparations. There is in fact marked stimulation of oxygen uptake by traces of each cofactor in the presence of saturating quantities of the other (Table 3). Striking increases in rate are observed on the further addition of coenzyme A to systems saturated with DPN and ATP, and maximal rates are not obtained until cocarboxylase is also included. There is thus a clear requirement for a full complement of cofactors, and the results show that such additions are effective, not only in the initiation but also in the maintenance of high oxidation rates.

The coenzyme A and cocarboxylase stimulations seem to be most marked with α-oxoglutarate and malate. The marked response of malate oxidation to coenzyme A does not necessarily implicate this coenzyme in the first oxidative reaction of this substrate, since the stimulative effects of the coenzyme may be more appropriately ascribed to the facilitation of subsequent steps of oxidation and condensation. Similarly, the ability of ATP and DPN to prevent the decline in the initially high rate of succinate oxidation might be attributed to the effects of these cofactors on oxidation steps subsequent to the primary oxidation of succinate. Again, comparable effects are observed in the coenzyme A stimulations of citrate, *iso*citrate and *cis*-aconitate oxidation. On the other hand, the effects of ATP and DPN on the oxidation of these acids appear to be primary and are manifest from the outset. In α-oxoglutarate oxidation the response to coenzyme A and cocarboxylase is also immediately apparent, and in this case it seems that the primary reaction is the one stimulated. These results are in accord with the findings concerning the cofactor requirements for oxidations by purified enzymes (Ochoa, 1954), and it now appears that the responses to coenzyme A and cocarboxylase in this reaction and in the important initial steps of pyruvate oxidation will go far towards explaining all the stimulative effects we have observed with these cofactors on the oxidation of the various substrate acids.

Declining rates of oxygen uptake similar to those observed when a cofactor essential for a secondary reaction is omitted might be expected if the primary reaction was isolated in other ways, e.g. by selective inhibition or inactivation of the enzyme controlling the secondary reaction. In this connexion we have been able to show that the oxaloacetate–pyruvate oxidizing system becomes selectively inactivated if preparations are incubated in the absence of substrate for an hour at 25°; when attempts were made to oxidize succinate in preparations which had been so treated it was found that the initially high rate of oxygen uptake declined rapidly even in the presence of the four cofactors. In untreated preparations fortified with cofactors, it appears that the primary reaction during succinate oxidation becomes increasingly isolated from subsequent oxidative steps as the substrate level is increased, since the response to

coenzyme A becomes progressively less under these conditions. As previous authors have pointed out (e.g. Laties, 1953*b*; 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, 1953*b*; Davies, 1953; Hackett & Simon, 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).

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