DISTRIBUTION AND PROPERTIES OF ISOCITRITASE IN PLANTS [1]

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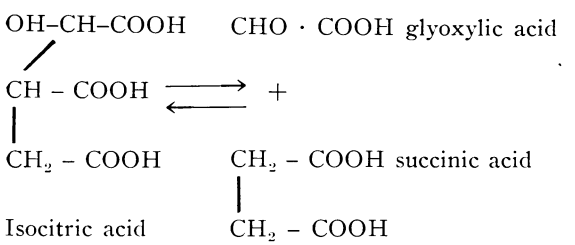
Isocitritase or isocitric lyase is the enzyme which catalyzes the reversible cleavage of isocitric acid to succinic and glyoxylic acids.



It was described originally in extracts from species of Pseudomonas and has since been shown to be present in a variety of bacteria (2, 9, 11, 12, 13) and fungi (8, 11, 13).

The metabolic significance of isocitritase appears to be that it catalyzes one of the steps of the glyoxylate cycle (8), which provides a mechanism by which acetate units can be quantitatively converted to malate. Since malate may be regarded as a precursor of hexose, the reactions of this cycle appeared to offer a solution to the long standing difficulty of explaining the formation of carbohydrate from acetate units in fatty seedlings (6, 7). The germinating castor bean, a prime example of such a seedling contains the enzymes of the glyoxylate cycle (6, 7). In particular, isocitritase was shown present in amounts adequate to account for the rates of reaction observed in vivo.

Since this was the first report of the occurrence of isocitritase in cells other than those of fungi and bacteria, it was of interest to find whether the enzyme was of general distribution in higher plants and to inquire into its properties more closely. One intriguing possibility, denied in the outcome, was that the activity of this enzyme and an ancillary glyoxylic reductase might account for the widespread occurrence of glycolate in plant cells. In fact, isocitritase appears to be confined, rather strictly, to those tissues in which fat is being converted to carbohydrate. A preliminary report of this work has appeared previously (3).

MATERIALS AND METHODS

PLANT MATERIALS: Castor beans, variety Cimarron, supplied by the Baker Seed Corporation, Vernon, Texas, were the principle source of isocitritase preparations. The castor beans were soaked for 24 hours in water with a sprinkling of Arasan (disinfectant). The seeds were then transferred into vermiculite in wooden flats, thoroughly soaked with water and allowed to germinate at 30° C in an incubator. No water was added subsequently. Five-day-old seedlings, i.e., 5 days from placing the seeds in vermiculite, were used for the most part in the preparation of the extracts. The seedling proper and testa were removed, and the "beans," consisting of the endosperm and the enclosed cotyledons, were then washed and a weighed sample taken.

Other seeds examined for isocitritase activity were grown in a similar manner, except that the initial soaking was for a period of from 3 to 6 hours. The other plant materials mentioned were collected from the greenhouse or from commercial sources.

PREPARATION OF EXTRACTS: The plant material was ground in a blendor for 25 to 30 seconds with about 1.4 times its weight of 0.05 M potassium phosphate buffer, pH 7.6. The suspension so obtained was squeezed through muslin and centrifuged for 15 minutes at 12,000 × G in an International refrigerated centrifuge set at −7° C. All glassware and the buffer were previously cooled to 0° C. The top fatty layer which separated during centrifuging was discarded, and the supernatant solution, which usually contained from 15 to 22 mg protein/ml was used as the crude enzyme preparation.

PREPARATION OF SUBSTRATES AND OTHER CHEMICALS: *Isocitrate*: The monopotassium salt of D-isocitric acid lactone was a gift from Dr. H. B. Vickery and prepared for use according to his directions. Twenty-one and two tenths milligrams (100 μM) of the acid were dissolved in 1.8 ml of 0.1 *N* KOH and 1 ml of H_2O and the solution warmed on a steam bath for 15 minutes. This was then adjusted to the desired pH (usually 6.0) with concentrated HCl and made up to 5 ml to give a concentration of 20 μM/ml. DL-Isocitric acid lactone, *allo*-free, from Sigma Chemical Company, St. Louis, Missouri, was prepared in a similar manner. Three ml of 0.1 *N* KOH was used to dissolve 17.6 mg (100 μM) of the acid lactone and the same procedure followed as with the D-isocitric acid. A fresh solution of isocitrate was prepared daily.

Glyoxylate: The monohydrate of sodium glyoxylate was obtained from Mann Chemical Laboratories, New York, N. Y. The dinitrophenyl hydrazone derivative was prepared and used as a standard for spectrophotometric and chromatographic identification of products of the isocitritase reaction.

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METHODS OF ASSAY FOR ISOCITRITASE ACTIVITY: Two methods were employed; each involved the estimation of the glyoxylate produced in the reaction mixture. In the 1st of these, aliquots were removed and analyzed by the Friedemann and Haugen method (4).

In a typical experiment, 200 μM (micromoles) of potassium phosphate buffer, pH 7.6, 15 μM of $MgSO_4$, 6 μM of cysteine HCl or glutathione adjusted to pH 7.6, 24 μM of isocitrate and water were added to a test tube to a final volume of 2.8 ml. At time zero, 0.2 ml of the enzyme preparation was added. One milliliter aliquots were taken at appropriate intervals and placed in a centrifuge tube containing 0.2 ml of 100 % trichloroacetic acid. After spinning down the precipitated protein, the supernatant was decanted into a test tube, and 0.33 ml of 0.1 % dinitrophenyl hydrazine in 2 *N* HCl added. After incubation for 15 minutes on a 30° C water bath, 1.67 ml of 2.5 *N* NaOH was added slowly. The solution (3 ml) was placed in a Corex cell and readings made at 445 mμ on a Beckman B spectrophotometer and compared with a glyoxylate standard.

The 2nd assay method was that used by Olson (personal communication), in which the reaction was followed continuously. Glyoxylate reacts with semicarbazide in the assay mixture and the production of semicarbazone is measured at 252 mμ on the spectrophotometer. The formation of the glyoxylate semicarbazone is catalyzed by acid and therefore the reaction must be carried out at pH 6.0 or less. (As will be shown later, this is not the optimum pH for the enzyme activity.) The reaction mixture consisted of 200 μM of potassium phosphate buffer, pH 6.0, 15 μM of $MgSO_4$, 6 μM of cysteine HCl or glutathione, and 60 μM of semicarbazide HCl adjusted to pH 6.0 with 0.1 KOH. Freshly prepared reaction mixture was placed in a cuvette with H_2O, and 0.05 ml of the enzyme preparation. At time zero, 0.3 ml of isocitrate (6 μM) was added to give a final volume of 3 ml. Generally in the enzyme experiments a linear rate of glyoxylate formation was attained only after a lag of 2 to 4 minutes. Enzyme activity was expressed as increase in optical density per minute over the linear portion of the curve, usually for a period of 4 to 6 minutes (fig 1). (O.D. at 252 mμ/3.6 = μM glyoxylate). The progress of glyoxylate formation at 2 levels of isocitritase is shown in figure 1.

PROTEIN AND NUCLEIC ACID: Protein estimation and the determination of "percent nucleic acid," in the enzyme preparations were carried out by methods described by Layne (10).

RESULTS AND DISCUSSION

ISOCITRITASE ACTIVITY OF PLANT EXTRACTS: In the earlier reports (6, 7) it was shown that, since the crude preparations may contain isocitric dehydrogenase, glyoxylic reductase and a common pyridine nucleotide, some of the added isocitrate might be converted to α-ketoglutarate rather than to glyoxylate. No such interference was evident after ammonium sulfate precipitation, and completely unequivocal demonstration of isocitritase activity and the stoichiometry of the reaction were possible with such preparations (7). In the present survey, interference from isocitric dehydrogenase was prevented by first treating the crude preparations with about 5 mg Darco charcoal per ml. This treatment was shown to remove added triphosphopyridine nucleotide from crude extracts while the isocitritase was unaffected.

Table I shows the results of isocitritase assays on extracts of the 25 different plant materials which were tested. Those extracts in which only a trace or no activity was present are listed first (1 through 16) and the rest in order of increasing isocitritase content (table I).

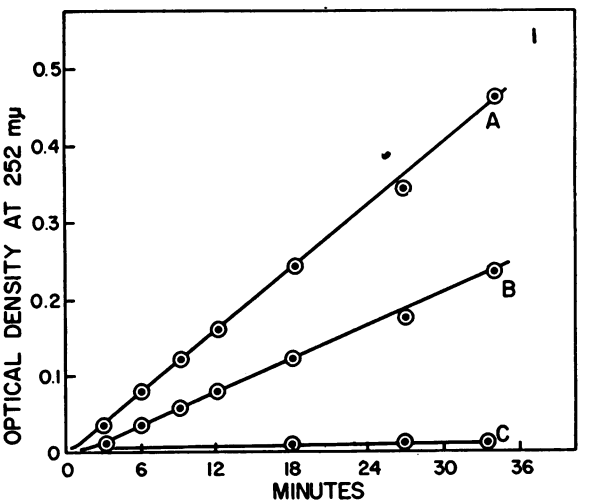


FIG. 1. Progress of the isocitritase reaction as measured by the semicarbazone assay. Cell A contained 0.10 ml isocitritase; cell B, 0.05 ml; and cell C, 0.10 ml boiled enzyme under the standard assay conditions.

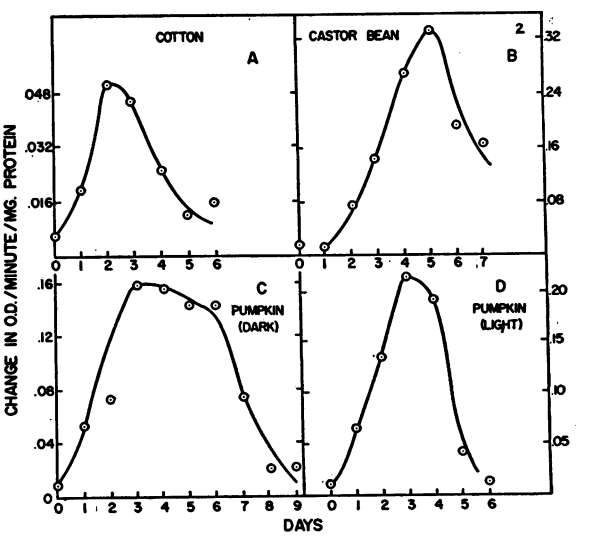


FIG. 2. Changes in isocitritase activity during germination of various seeds.

The results show at once that isocitritase is not of universal distribution in plants. On the contrary, it is clear that it is confined to those tissues, principally fatty seedlings, in which an active fat metabolism may be anticipated. Isocitritase was not detectable in roots and leaves and stems in which the predominant catabolic event is the breakdown of carbohydrate. Moreover it is noteworthy that in the castor bean seedling itself, which is the best source we have found, the enzyme is localized in the endosperm tissue, to which the fat is also confined, and it is not found in the adjacent parts of the seedling proper. Again, the enzyme was not found in castor bean seeds maturing on the parent plant.

DEVELOPMENTAL STUDIES: (*a*) *In the germinating castor bean*: The endosperm tissue of the germinating castor bean contains virtually all of the food reserve of the seed. On the 3rd or 4th day of germination a rapid breakdown of the fat begins and by the 10th day the endosperm has been completely absorbed by the seedling proper. The disappearance of the fat is accompanied by the appearance of an almost equivalent amount of carbohydrate in the endosperm and in the young seedling (15).

It will be recalled that in the endosperm of the castor bean maturing on the parent plant, isocitritase activity could not be detected, while the 5-day-old seedling gave an extract with the highest specific activity of any tissue tested. The questions of the time sequence of development of the activity of the isocitritase, and of what happens to the activity as the endosperm is absorbed and disappears, thus became of considerable interest (figs 1 & 2).

TABLE I

ISOCITRITASE ACTIVITY IN VARIOUS PLANT TISSUES *

	SOURCE OF PREPARATION	ACTIVITY *
1.	Maturing castor bean endosperm	None
2.	Castor bean root, 5-day-old seedling	"
3.	Castor bean hypocotyl, 5-day-old seedling	"
4.	Castor bean leaves	Trace
5.	Apple fruit	None
6.	Apple leaves	"
7.	Barley seedlings, 3-day-old	"
8.	Bean seedlings, 4-day-old	"
9.	Carrot root	"
10.	Coconut	"
11.	Corn seedlings, 3-day-old	Trace
12.	Pea seedlings, 4-day-old	Trace
13.	Pepper fruits	None
14.	Tobacco leaves	None
15.	Tomato fruits	None
16.	Oat seedling, 3-day-old	Trace
17.	Sunflower cotyledons, 4-day-old seedlings	0.004
18.	Flax seedlings	0.022
19.	Soybean cotyledons, 3-day-old seedlings	0.035
20.	Peanut cotyledons, 7-day-old seedlings	0.047
21.	Avocado fruit	0.048
22.	Watermelon cotyledons, 3-day-old seedlings	0.074
23.	Cotton cotyledons, 3-day-old seedlings	0.086
24.	Pumpkin cotyledons, 4-day-old seedlings	0.216
25.	Castor bean endosperm, 5-day-old seedlings	0.380

* Expressed as change in optical density at 252 mμ per minute per mg protein in the semicarbazone assay. (Charcoal treated preparations.) No activity is defined as a change in optical density of less than 0.001 per minute in the reaction mixture. Trace activity is defined as a change in optical density between 0.001 and 0.002 per minute.

Enzyme preparations were made at daily intervals and assayed for isocitritase by the semicarbazone method. Figure 2 B shows the isocitritase activity in germinating castor beans over a 7-day period. The activity after 24 hours of soaking was very low and it did not increase during the next 24 hours. Beginning on the 2nd day, there was a striking increase in activity up to a peak at the 5th day. After this there was an equally striking decline. It is therefore clear that during the 1st few days of germination isocitritase is produced or becomes active in the endosperm. What is more significant perhaps is that the enzyme activity reaches its peak at the time of most active fat breakdown in the seed. The later decline coincides with the softening and absorption of the endosperm material into the seedling proper. (*b*) *In germinating pumpkin and cotton seeds*: In the germination of the castor bean, the endosperm is, by the 5th day, in a state of decreasing metabolic activity and it is, perhaps, not unexpected that isocitritase activity should also decline. On the other hand, in seeds such as those of pumpkin and cotton, the fat is stored in the cotyledons, and the tissue remains metabolically active long after the fat has disappeared. It was of interest, therefore, to compare the isocitritase activity of the 2 different types of fatty seeds during germination.

Isocitritase activity in the germinating cotton seed is shown in figure 2 A. Here again there is an initial striking increase in activity, and a rapid decline occurs thereafter. By the 6th day, activity was only 20 % of that of the peak on the 2nd day, in spite of the fact that the cotyledons were still actively expanding.

Heydeman (5) recently reported that in marrow, a variety of *Cucurbita pepo*, isocitritase activity increased up to at least 6 days. In a study with pumpkin, another variety of *Cucurbita pepo* (fig 2 C), it was found that, in the dark, isocitritase activity increased until the 3rd day, then leveled off to about the 6th day, after which there was a rapid decline. When pumpkin seedlings were grown in the greenhouse exposed to sunlight, isocitritase activity once again reached a peak at the 3rd day (fig 2 D) and isocitritase activity declined even more rapidly than in seedlings grown in the dark.

From the 4 developmental studies carried out, it appears that isocitritase activity in the germinating seedling is ephemeral whether the fat is stored in the endosperm, which is completely absorbed during germination, or whether it is contained in cotyledons which persist as functional leaves. Enzyme activity, which was very low in the ungerminated seeds, rose to a peak when fat breakdown was at its height, and then declined again.

The picture which emerges from the results in this section is that the isocitritase enzyme is confined in the castor bean plant to the germinating seedling

and still further localized in the endosperm tissue. The likelihood that isocitritase is intimately concerned with the fat to carbohydrate conversion in the various seedlings is still further enhanced by the striking increases which occur in its activity as the seedlings progress from the dormant condition to the time of rapid fat mobilization, and by the disappearance of the enzyme as the seedling becomes independent of its fatty reserves. Such changes in enzyme activity are not, of course confined to isocitritase; our previous work has shown that mitochondrial activities show somewhat similar changes.

The appearance and disappearance of the isocitritase during germination can be compared with the behavior of the enzyme in microorganisms. When the cells are grown on carbohydrate as a carbon source, the enzyme is absent, but it appears, apparently "adaptively" when the cells are made to grow on acetate as the sole carbon source (11, 13).

PURIFICATION OF ISOCITRITASE: As shown in the preceding section extracts of 5-day-old castor bean seedlings had a higher specific isocitritase activity than any other source examined. In addition to this qualification as a source of isocitritase activity, the castor beans germinated well, were resistant to infection, and were uniform in growth.

The following purification procedure was adopted after many empirical trials, the details of which will not be reported here. In most of the experiments, from 150 to 200 g of endosperm were used, in 50 to 55 g lots, and the volume of the crude enzyme preparation (see Materials and Methods) was approximately 1 ml per 1 g of endosperm tissue used. This preparation usually contained from 15 to 22 mg of protein per ml. Solid ammonium sulfate was added to the crude preparation to a concentration of 22 %. This was done while the preparation was immersed in an ice bath and stirred by a magnetic stirrer. The preparation was allowed to stand at 5° C for 1 hour, then centrifuged for 15 minutes at 12,000 × G using the refrigerated centrifuge. The supernatant was then made to an ammonium sulfate concentration of 45 % as described above, and again kept at 5° C for 1 hour. The precipitated protein which contained from 70 to 80 % of the total enzyme activity was centrifuged and the supernatant discarded. Three-fourths of the original volume of 0.05 M potassium phosphate buffer, pH 7.6, was added and the protein was allowed to go into solution gradually with occasional swirling while at 0 to 5° C. $Ca_3(PO_4)_2$ gel was added in the ratio of 15 mg of gel to 100 mg of protein, and allowed to stand for 10 to 15 minutes before centrifuging. The precipitate was discarded. The $Ca_3(PO_4)_2$ gel step was repeated on the supernatant using the same ratio of gel to protein. (The use of larger ratios of $Ca_3(PO_4)_2$ resulted in loss of isocitritase activity.) The solution, which was now straw-colored and clear, was made up to 20 % with respect to $(NH_4)_2SO_4$, and then to 32 % in the manner described earlier; and the final precipitate was taken up in 0.05 M buffer. Two further $Ca_3(PO_4)_2$ gel treatments as described above usually resulted in a 9- to 14-fold purification. A final ammonium sulfate fractionation of 25 to 31 % provided a clear solution containing only minute amounts of nucleic acid, and 30 to 45 % of the total original isocitritase activity, with a specific activity of 18 to 30 times that of the original extract (table II). Such enzyme preparations were inactivated by dialyzing against either buffer, or buffer with cysteine and $MgCl_2$, and were used in the investigation of properties without further treatment.

TABLE II

ISOCITRITASE PURIFICATION PROCEDURE

STEPS IN PURIFICATION	ACTIVITY *	PROTEIN MG/ML	NUCLEIC ACID	ACTIVITY / MG PROTEIN
Crude preparation	.100	.327	12 %	0.306
22 to 45 % $(NH_4)_2SO_4$	.115	.193	10 %	0.600
$Ca_3(PO_4)_2$ Gel 15:100 to protein **	.113	.118	7 %	1.000
$Ca_3(PO_4)_2$ Gel 15:100 to protein	.113	.093	9 %	1.200
20 to 32 % $(NH_4)_2SO_4$	.131	.063	4 %	1.570
$Ca_3(PO_4)_2$ Gel 15:100 to protein	.130	.044	2 %	2.900
$Ca_3(PO_4)_2$ Gel 15:100 to protein	.131	.036	2 %	3.640
25 to 31 % $(NH_4)_2SO_4$	.150	.016	1 %	9.400

* Semicarbazone assay.

** After each treatment with $Ca_3(PO_4)_2$, the material precipitating with the gel was discarded; the supernatant solution was used for the succeeding step.

PROPERTIES OF ISOCITRITASE: (*a*) *Sulfhydryl requirement*: Sulfhydryl and divalent cation requirements for isocitritase activity have been reported by several workers (11, 12, 14).

As shown in figure 3, cysteine and gluthathione were equally effective as activators of the castor bean enzyme over the concentration range tested. Increase in enzyme activity was linear with increase in sulfhydryl concentration from 0 to 1 μM/ml, with no further increases in activity at higher levels. By extrapolating the curve to the abscissa, it can be deduced that the residual sulfhydryl concentration in the enzyme preparation was about 0.1 μM/ml.

The minimum concentration necessary to give maximum activity of the enzyme (1 μM/ml) agrees closely with that reported by Smith and Gunsalus (14) in a recent description of the properties of a bacterial isocitritase. However, whereas these authors found that cysteine was more effective than gluthathione, the 2 were equally effective as activators of the castor bean enzyme.

(*b*) *Divalent ion requirement*: Seven divalent ions were tested; Mg^{++}, Mn^{++}, Co^{++}, Fe^{++}, Cu^{++}, Ca^{++}, and Ni^{++}. Only the 1st 4 caused an increase in isocitritase activity over the residual

TABLE III

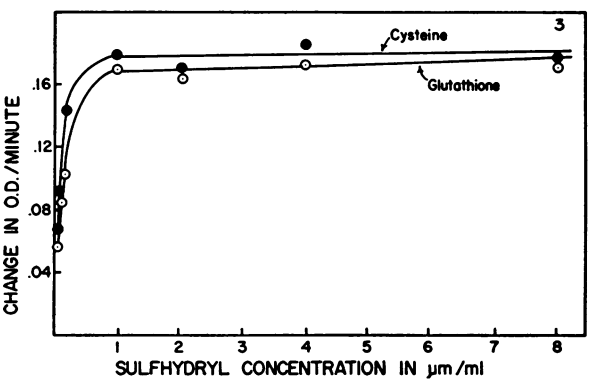
EFFECT OF pH ON ISOCITRITASE ACTIVITY

pH	5.5	6.0	6.5	7.0	7.5	8.0	8.5
Activity as % of that at pH 7.5	31	50	58	52	100	87	66

value. Salts of these ions were then added at various concentrations to reaction mixtures. (The Fe^{++} experiments were carried out by the Friedemann Haugen method because precipitation occurred when the carbazone mixture was used.) The results are shown in figure 4.

Mg^{++} was superior to all other ions tested; a plateau was reached at a concentration of 1.5 μM/ml. Mn^{++} and Co^{++} were approximately 30 % as effective as Mg^{++} at its optimum concentration in restoring isocitritase activity, and Fe^{++} was somewhat more effective.

(*c*) *Influence of pH on the reaction*: Measurements of isocitritase activity by the Friedemann and Haugen assay at several pH levels between pH 5.5 and 7.5 are shown in table III. They show that the optimum for activity was pH 7.5. At pH 6.0, that used in the carbazone assay method, activity is only one half of that at pH 7.5 (table III).



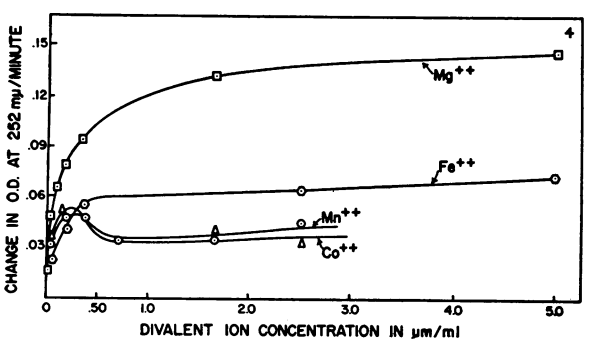
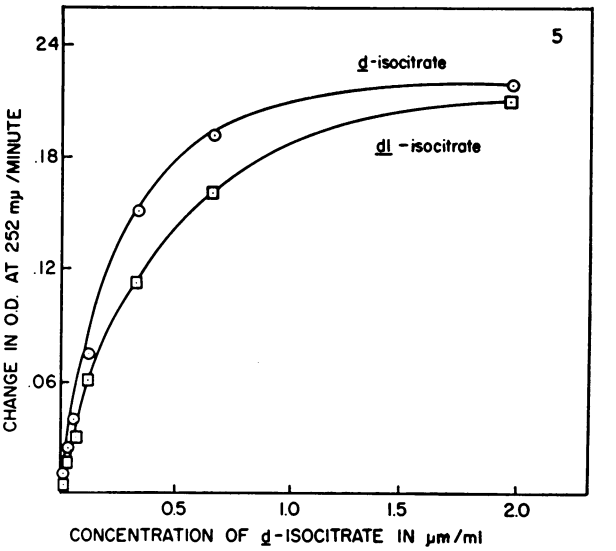


FIG. 3. The sulfhydryl requirement for isocitritase activity. The standard semicarbazone assay mixture was used, with sulfhydryl additions as shown.

FIG. 4. The divalent cation requirement for isocitritase activity. The standard semicarbazone assay mixture was used with the cations added as shown.

(*d*) *Sensitivity to heat*: The enzyme was rapidly inactivated at slightly elevated temperatures. Thus more than 50 % of the original activity was lost during 1 minute of exposure at 50° C and a 5 minute treatment at 40° C was sufficient to reduce activity by a similar amount.

(*e*) *Effect of substrate concentration*: The influence of isocitrate concentration on the rate of reaction is shown in figure 5. Rates obtained with



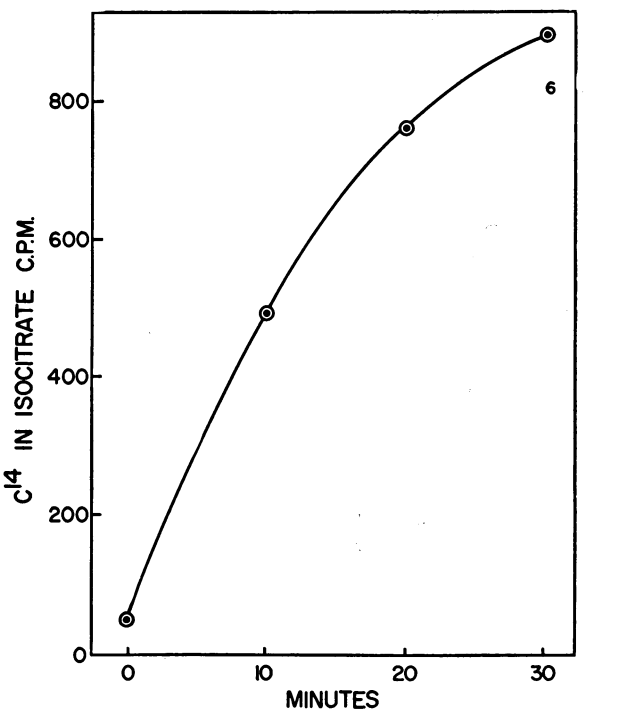


FIG. 5. Isocitritase activity as a function of substrate concentration.

FIG. 6. Progress of incorporation of succinate 2,3-C^{14} into isocitrate. (See text.)

both the natural form and the DL mixture are shown, and these are plotted as a function of the D component only. It will be seen that at the lower levels, the rates of reaction in the presence of the L form are definitely lower than in its absence, while at 2 μM/ml the rates are virtually the same (figs 5 & 6).

The maximum rate of reaction is reached at a substrate concentration somewhat above 1.0 μM/ml. When the reciprocals of reaction rate and substrate concentrations were plotted against each other, a straight line was obtained and the Km was calculated to be 3.2×10^{-4} M. The enzyme from *Pseudomonas aeruginosa* which was described recently had a Km of 4.5×10^{-4} M (14).

(*f*) *Reversal of the reaction*: Although the function of isocitritase would appear to be the splitting of isocitrate, the reverse reaction can be shown to occur. For example Smith and Gunsalus (14) measured isocitrate synthesis by the enzyme from *P. aeruginosa*, although they emphasized that cleavage is strongly favored in dilute solutions.

The synthesis of isocitrate from succinate and glyoxylate by the castor bean enzyme was demonstrated in the following manner.

Twenty micromoles of succinate 2,3-C^{14}, 20 μM of glyoxylate, 10 μM $MgSO_4$ and 200 μM potassium phosphate, pH 7.5 were made up to a volume of 2 ml. At zero time 1 ml of an isocitritase preparation (18-fold purification) was added. Aliquots of 0.5 ml were removed at 0, 10, 20 and 30 minutes and placed immediately in a boiling water bath. They were then chromatographed on Whatman no. 1 paper using mesityl oxide:formic acid:water (200:41:200, v/v/v) as solvent. Radioautographs were subsequently prepared.

The only radioactive component other than residual succinate was one which moved very slowly indeed (R_f 0.05) and which corresponded in position to known isocitrate. Figure 6 shows the progress of incorporation of radioactivity into this area during the experiment; after 30 minutes it was calculated that at least 8 % of the succinate had been so incorporated. The identity of this material with isocitrate was confirmed by the following observations.

1) Radioactive material eluted from the paper was co-chromatographed in another solvent (equal parts of propionic acid:water (62:79 v/v) and *n*-butanol:water (30:20 v/v) with known isocitrate, and the 2 were found to coincide at R_f 0.25.

2) When the eluted material was incubated with fresh isocitritase under the conditions of the usual enzyme assay there was a rapid production of glyoxylate semicarbazone as judged spectrophotometrically.

3) After incubation with isocitritase the products were separated in the propionic acid:butanol solvent. A radioautograph revealed that the radioactive material at R_f 0.25 had virtually disappeared and that 94 % of the radioactivity originally in the presumed isocitrate had now appeared in succinate.

The synthesis of isocitrate from succinate and glyoxylate under the conditions described above was found to be inhibited by malonate. Thus when malonate was included at a concentration equal to that of succinate, the incorporation of C^{14} into isocitrate was reduced by 10 %; when the malonate:succinate ratio was increased to 5:1 the inhibition was 94 %.

Summary and Conclusions

On the basis of the evidence from 25 different tissues it is concluded that, far from being widespread in higher plants, isocitritase is limited to certain species and confined, in these, to those parts in which active fat breakdown is occurring.

The developmental studies on germinating fatty seedlings showed that the early increases in enzyme activity and the later declines coincided with changes in the pace of fat breakdown. These findings furnish further presumptive evidence for the thesis advanced earlier (6, 7) that isocitritase activity is intimately concerned with the conversion of fats to carbohydrates in these tissues.

The isocitritase from castor beans has been somewhat purified. The best preparations had an activity some 30 times greater than that of the original extracts. The specific activity of such preparations is at least equal to that of the purified enzyme described recently from *P. aeruginosa*. The castor bean enzyme has several features in common with its counterpart from microorganisms. Thus a sulfhydryl compound and magnesium are mandatory for optimal activity which is shown at pH 7.5. The Km was found to be 3.2×10^{-4} M. The enzyme was very sensitive to heat. The enzyme was shown to bring about the synthesis of isocitrate from glyoxylate and succinate, and malonate inhibited this reaction.

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CHANGES IN ENDOGENOUS GROWTH SUBSTANCES DURING FLOWER DEVELOPMENT [1]

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The theories advanced to explain the flowering phenomenon generally involve the assumption that specific substances are manufactured by plants at the time of flower initiation. Such are, for example, the views of Cajlachjan (1) who suggested the existence of a "florigen," of Melchers (15) who thought that a different substance, "vernalin," was involved in the flowering of biennials, of Hamner and Bonner (8), of Resende (25) who speculated on the possible role of native auxins and antiauxins, of Lang et al (14) who actually caused flowering in Hyoscyamus and Samolus with extracts of Echinocystis endosperm, etc.

Nevertheless, investigations on the natural regulators actually present in plants during the onset of flowering are rare and, in general deceiving. Thus, Cooke (3) found that the auxin level in Xanthium was related to day-length rather than to flowering, flowering plants placed under long days having higher auxin contents than similar plants placed under short days. On the contrary, Vlitos and Meudt (26) detected more auxin under short than under long days in 2 other short-day plants, soybean and the Maryland Mammoth tobacco. In long-day plants, Konishi (13) did not find drastic differences between the auxin levels of rosetted and bolting plants, despite the dramatic morphological difference between these 2 states. Finally, Gilson (6) concluded from his auxin determinations in induced and non-induced Hyoscyamus that the observed auxin fluctuations were a consequence rather than a cause of the flowering process.

The reason why the results of the cited experiments are often unconvincing is probably 2-fold: 1) the techniques used were crude and unspecific, and 2) no convenient and quantitative test for flower-forming substances has yet been devised.

In the light of this situation, it was attempted to take advantage of the more accurate methods known today for the determination of auxins and gibberellins in order to get a clearer picture of the fluctuations of these substances during flower development, even though these substances may have only an accessory role in the mechanism of flowering. Such a study is reported below. It has been presented as a Master's thesis (9) and short summaries of it have been published (11, 18).

MATERIALS AND METHODS

PLANT MATERIAL: Three different physiological types of plants were used in the present study, namely: a long-day plant, *Rudbeckia speciosa* Wenderoth; a cold-requiring plant, *Chrysanthemum morifolium* Ram. variety Shuokan (cuttings were kindly supplied by Dr. H. M. Cathey, U.S.D.A., Beltsville, Maryland); and a short-day plant, *Chrysanthemum morifolium* variety Shasta. Rudbeckia appeared to be a favorable material because of its clear-cut response to photoperiodic treatments and also because its photoperiodic behavior has been studied in detail (5, 7, 13, 16). The Shuokan chrysanthemum is a Japanese variety (large pompon type, yellow) which bolts and flowers rapidly after a low-temperature treatment (23, 24). Unlike other chrysanthemums, it initiates and develops flowers regardless of the day-length. If the plant has not been exposed to cold, it remains vegetative and in a rosetted state for a long time[3]. The chrysanthemum Shasta is a short-day, commercial variety (anemone type, white) which usually requires 10 weeks to full bloom from the beginning of the short-day treatment.

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[3] At a minimum temperature of 16° C, bolting and flowering finally occur without cold treatment, but only after some 30 weeks, as was observed during the course of these experiments.