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EVIDENCE FOR THREE ISOZYMES OF CHORISMATE MUTASE IN ALFALFA

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SUMMARY

Three forms of chorismate mutase (CM_1, CM_2, CM_3) have been found in alfalfa plants. The isozymes are separable by DEAE-cellulose fractionation and gel electrophoresis. They are distinguishable by different molecular weights (46 000, 58 000 and 69 000, respectively), different activation energies (11 300, 20 800 and 13 500 cal/mole, respectively) and differing sensitivity to metabolic inhibitors. CM_1 and CM_3 are inhibited by phenylalanine and tyrosine and activated by tryptophan. CM_1 and CM_2 are inhibited by caffeic and chlorogenic acid. CM_3 is unaffected by these dihydroxycinnamic compounds, but is activated by 3,4-dimethoxycinnamic acid and inhibited by ferulic acid, two compounds which have no effect on CM_1 and CM_2. All isozymes are inhibited by *p*-coumaric acid. All three forms are present in all plant parts of the mature alfalfa and in green as well as etiolated seedlings.

INTRODUCTION

In both microorganisms and plants the shikimic acid pathway (Fig. 1) has been established as the main pathway for the biosynthesis of the aromatic amino acids, phenylalanine, tyrosine and tryptophan[1,2]. These amino acids, in turn, are the

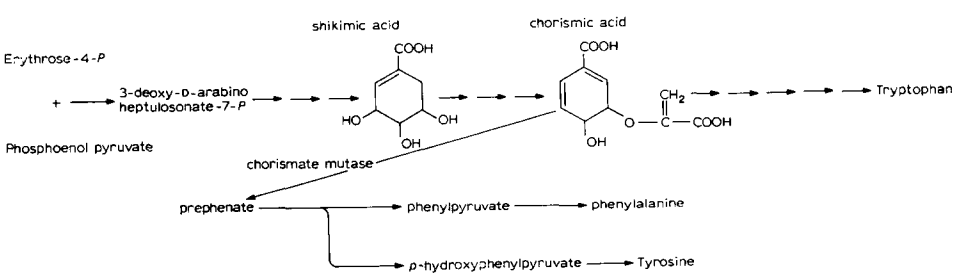

Fig. 1. The shikimic acid pathway.

Abbreviations: CM_1, CM_2 and CM_3, chorismate mutase isozymes 1, 2 and 3, respectively.

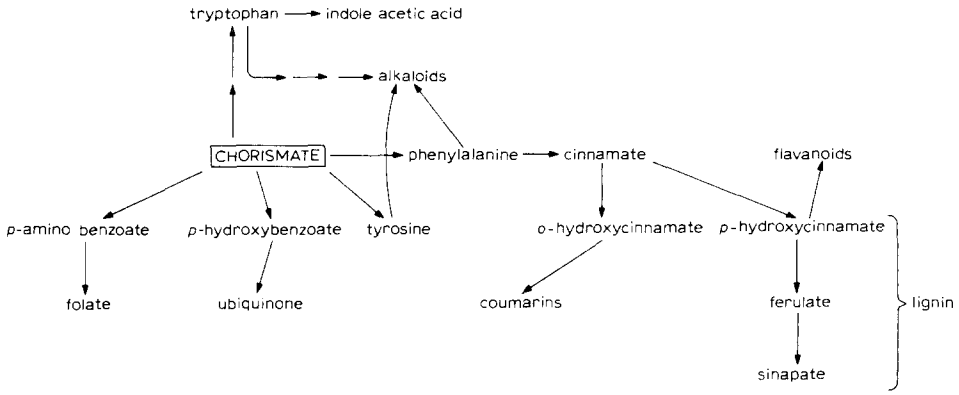

Fig. 2. Some of the possible metabolites of chorismate.

precursors of a wide variety of secondary metabolites including (1) the alkaloids, (2) the coumarins and their metabolites such as the flavanoids and some of the phytoallexins and (3) the lignin precursors, caffeic, cinnamic, *p*-coumaric, ferulic and sinapic acid. It is the disposition of chorismate which is one of the factors determining whether the products of the shikimic acid pathway will be diverted to the production

TABLE I

SUMMARY OF THE NUMBER AND PROPERTIES OF CHORISMATE MUTASE ISOZYMES IN A VARIETY OF ORGANISMS

Those places where? occurs indicate that no evidence for isozymes occurs, since tests such as sucrose density centrifugation followed by analysis and location of each enzyme in the shikimate pathway have either not been reported or performed.

Organism	*Number of chorismate mutases*	*Occurrence of aggregates including chorismate mutase*	*Inhibitors*	*Activators*	*Ref. No.*
Bacillus lichenformis	2	No	Prephenate	None	3
Bacillus subtilis	2	Yes	Prephenate	None	3
Escherichia coli	2	Yes	CM_P; Phe	None	4
Salmonella thyphimurium	2		CM_T; Tyr	None	5
Aerobacter aerogenes	2				4
Claviceps SD58*	1	No	Phe	Trp	6
Neurospora crassa	1		Tyr		7
*Saccharomyces cerevisiae***	1				8
Mung Bean	2	?	CM_1; Phe, Tyr CM_2; none	CM_1; Trp CM_2; none	9
Alfalfa	3	?	CM_1,CM_3; Phe, Tyr CM_2; none	CM_1,CM_3; Trp CM_2; none	This laboratory
Penicillium chrysogenum	2	?	As in alfalfa	CM_1 and CM_3	This laboratory

* *Claviceps* Pb156 contains two chorismate mutases, one as above, and one insensitive to phenylalanine and tyrosine but activated by tryptophan.

** *S. cerevisiae* chorismate mutase is not sensitive to phenylalanine.

of phenylalanine and tyrosine or to tryptophan, or, at least in microorganisms (this pathway has not yet been demonstrated in plants), to the vitamin precursors *p*-hydroxybenzoic and *p*-aminobenzoic acid (Fig. 2). Chorismate mutase, the enzyme which commits the shikimate pathway to production of phenylalanine and tyrosine, has been investigated in a wide variety of microorganisms[3–8] and more recently in higher plants[9]. Comparison of these studies illustrates the existence of varied isozyme and control patterns for this enzyme as well as for the pathway as a whole, Table I.

The present paper describes the isozyme and control pattern of chorismate mutase in the higher plant *Medicago sativa*.

METHODS AND MATERIALS

Plant material

For purification studies mature alfalfa at the prebloom stage was gathered either in the field or greenhouse. The main stem was stripped of all leaves and side branches and used as a starting material. Young seedlings were grown on cheesecloth wicks above distilled water for six to seven days. Green seedlings were germinated one to two days in darkness before being transferred to the greenhouse.

Preparation of extracts

Crude extract. Plant tissue was frozen with liquid nitrogen and ground to a fine powder either by hand or in a Waring blender set at high speed. The powder was then extracted twice with cold (4 °C) 0.1 M potassium phosphate buffer, pH 6.5, at a ratio of 2 g powder to 1 ml buffer. The first extract was used for subsequent purification. The material from the second extract was assayed in order to determine total mutase activity per g wet wt of plant material and then discarded. The buffer slurries were filtered through cheesecloth and then centrifuged for 15 min at 40 000 × *g* in a Sorvall refrigerated centrifuge. The resulting supernatant is designated as crude extract. The second extract usually contained about 1/10 the number of units in the first. Acetone powders were made by adding cut up fresh stems to a Waring blender and grinding for 1 min with acetone precooled to −10 °C. The resultant slurry was put in a Buchner funnel and washed with cold acetone until the filtrate was colorless. The acetone powder was air-dried and then extracted with 0.1 M potassium phosphate, pH 6.5, at a ratio of 1 g powder to 5 ml buffer.

Protamine sulfate precipitate. A 1% solution of cold salmon sperm protamine sulfate (Sigma Grade I) was added to the crude extract dropwise with stirring until no more precipitate formed. This was usually at a ratio of 1 g protamine sulfate to 4 g biuret protein. The suspension was centrifuged for 10 min at 27 000 × *g* and the resulting supernatant used for further studies. Much of the green color was precipitated at this step. Recovery of enzyme in the supernatant ranged from 95 to 120%, the wide range possibly reflecting the difficulty of analyzing by a spectral assay the highly colored crude extract and possible inhibition by low-mol.-wt substances in the extract. Less than 0.5% of the mutase activity could be recovered in the precipitate.

$(NH_4)_2SO_4$ concentration. In order to concentrate the mutase activity present in the dilute supernatant, enough solid $(NH_4)_2SO_4$ was added to give 90–100% saturation. The pH was adjusted to 6.5 and the solution stirred for about 1 h. The

resultant precipitate was collected by centrifuging at 27 000 × *g* for 15 min, dissolved in a minimal amount of 0.01 M potassium phosphate buffer, pH 6.5, and dialyzed against three changes of the same buffer.

DEAE chromatography. The $(NH_4)_2SO_4$ fraction (200 mg) was applied to a 2.5 cm × 12.0 cm column of DEAE-cellulose that had previously been equilibrated with 0.01 M potassium phosphate buffer, pH 6.5. The column was washed with buffer of the same concentration until $A_{280\ nm}$ of the eluate was less than 0.05. The column was then eluted either with a linear gradient (250/250 ml) of 0.01–0.05 M potassium phosphate buffer, pH 6.5, and/or directly with 0.05 M buffer until $A_{280\ nm}$ was less than 0.05. The column was then eluted with a linear gradient (250/250 ml) of 0.05–0.3 M potassium phosphate buffer, pH 6.5. Protein was determined by absorbance at 280 nm. A flow rate of 120 ml/h was maintained and the eluate collected in 5-ml fractions. Phosphate concentration in peak tubes of activity was determined by the Fiske–SubbaRow method[10]. Fractions from each elution step exhibiting high activity were pooled, concentrated on a Zeineh microconcentrator (Biomed Instruments, Inc.), dialyzed against appropriate buffer, reapplied to the column and refractionated. Fractions with high activity were again pooled and concentrated. Unless otherwise noted, these fractions were used for all subsequent work.

Fractionation of cell organelles

Alfalfa leaves were ground gently in isotonic sucrose solution[11] and separated by differential centrifugation[12] with appropriate washing techniques.

Sephadex gel filtration

1–5 ml of the concentrated fraction from DEAE chromatography were applied to a 2.5 cm × 30 cm Sephadex G-100 column previously equilibrated with 0.1 M potassium phosphate buffer, pH 6.5, and eluted with the same buffer. 1–5-ml fractions were collected. The flow rate was maintained at 20 ml/h. For determination of molecular weight 1-ml fractions were applied and collected. The following marker proteins were used: hexokinase, malate dehydrogenase, ovalbumin, α-chymotrypsin, lysozyme and cytochrome *c* and detected either by appropriate absorbance or activity measurements[13]. The elution volume (62 ml) of Dextran Blue was taken as the void volume (V_0).

Gel electrophoresis

Electrophoresis was performed on 6.0% polyacrylamide gels prepared according to Ornstein and Davis[14] with the exception that no spacer or sample gels were employed. Bromophenol blue was used as the tracking dye. 50 μl of sample made 10% in sucrose was applied gently under buffer to each of six gels. Each run lasted about 1.5 h with two sets of six gels, each approximately 9.0 cm long, subjected to a current of 3–4 mA/tube. Immediately at the end of each run 5 gels of each set were frozen in liquid nitrogen and stored overnight at −20 °C. One gel of each set was stained for protein by immersion for 12 h in 2.5% Amido Schwarz. These were destained by repeated flushing with 7% acetic acid.

Gel extraction and assay

The frozen gels were lined up with dye fronts matching and cut into sections

3 mm long. Each section was put in a 1.2 cm × 7.5 cm test tube to which was added 1.0 ml of 0.1 M potassium phosphate buffer, pH 6.5. Gels and buffer were incubated for 4–6 h with occasional hand shaking. When the dye band was evenly distributed between gel and buffer, we assumed protein elution was complete. Appropriate aliquots were used for assays of chorismate mutase, using a slight variation of a method developed by Nishioka and Woodin[15]. Assay mixtures consisting of 0.6 ml of 0.1 M potassium phosphate buffer, pH 6.5, containing 1 μmole chorismic acid and an appropriate aliquot of gel eluate were incubated at 37 °C for 30 min. The reaction was stopped by adding 0.2 ml of 20% trichloroacetic acid. After 15 min incubation at room temperature, 2.5 ml of 1.25 M borate–2.8 M phosphate, pH 6.5, was added. Absorbance at 300 nm was read after 30 min, using a Coleman-Hitachi Model III single beam spectrophotometer.

Enzyme assays

All other assays were performed at 37 °C as outlined by Nishioka and Woodin[15] with the exception that when assaying for sensitivity to effectors, only 0.25 μmole chorismate was used (approximate K_m concentration). One unit of enzyme activity represents 1 nmole of prephenate produced in 1 min. Specific activity represents the number of units produced by 1 mg of extract protein.

Prephenate dehydratase and prephenate dehydrogenase were determined according to Cotton and Gibson[4]. Protein concentration in early fractions was determined by the biuret method[16]. In fractions containing less than 1.0 mg/ml protein, absorbance at 280 nm was used to determine protein content. Proteins in active fractions of the gel were determined by the method of Lowry *et al.*[17].

Chemicals

Chorismic acid was prepared according to Gibson[18]. All other chemicals were purchased as the purest grade available and used without further purification. Chorismic, caffeic, ferulic, and 3,4-dimethoxycinnamic acid were chromatographed on Brinkmann thin-layer plates (Cellulose NM300 UV_{254}) in methanol–butanol–benzene–water (2:1:1:1, by vol.). Each exhibited only one ultraviolet absorbing spot.

RESULTS

$(NH_4)_2SO_4$ fractionation

Initial attempts to purify mutase isozymes from crude extracts of nitrogen or acetone powders of mature alfalfa stems by means similar to those successful with mung bean extracts[9] failed. Mutase activity was recoverable in all fractions resulting from fractional precipitation with $(NH_4)_2SO_4$ or stepwise elution from DEAE columns, and no fraction exhibited sensitivity to inhibition by phenylalanine or tyrosine or activation by tryptophan. Analysis of an $(NH_4)_2SO_4$ fractionation of an extract purified through the protamine sulfate and $(NH_4)_2SO_4$ concentration steps outlined above revealed activity present in each fraction but differential response of each fraction to the aromatic amino acids, indicating that more than one mutase isozyme might be present in alfalfa extracts. Apparently, phenolics or other substances which interfere with separation of plant enzymes by conventional means[19] were removed

by prior treatment with protamine sulfate enabling more successful fractionation with protamine sulfate-treated extracts compared to extracts not so treated.

DEAE fractionation

Our first attempts at DEAE fractionation using extracts purified through the $(NH_4)_2SO_4$ concentration step were similar to methods employed previously with mung bean[9]; namely, elution with 0.01 M potassium phosphate, pH 6.5, until $A_{280\ nm}$ was less than 0.05, followed by a linear gradient of potassium phosphate buffer, pH 6.5, from 0.05 to 0.3 M. We were successful in obtaining two distinct mutases with elution pattern and effector sensitivities similar to chorismate mutase isozymes 1 and 2 (CM_1 and CM_2) of mung bean; however, the form isolated on the linear gradient (CM_1) exhibited anomalies in the plot of its activity *versus* substrate concentration. It was for this reason we began to suspect the presence of another mutase isozyme and decided to subject the CM_1 fraction to a double gradient elution as detailed in Methods and Materials. (The velocity *versus* substrate concentration curve of the mutase form eluting with 0.01 M potassium phosphate was hyperbolic for the same range of substrate concentrations.)

The three forms of mutase were detected on DEAE chromatography using the double gradient elution technique, or the two-step one gradient elution technique detailed in Methods and Materials. Results were essentially the same whether extracts made from nitrogen or acetone powder were used. The three peaks of activity are designated CM_2, CM_3 and CM_1 respectively, ranging from the least to the most

TABLE II

ELUTION PATTERNS FROM DEAE CELLULOSE OF PARTIALLY PURIFIED STEM CHORISMATE MUTASE ISOZYMES

Methods of procedure are detailed in Methods and Materials. The mutase isozymes added to each column were purified through the first fractionation on DEAE-cellulose. The recovery of activity from reapplication of CM_3 was highly variable. Most reapplications resulted in appearance of only one peak of activity (in the 0.05-M phosphate eluate) but only 50–80% recovery of applied units.

Number of units of each isozyme added	*Recovered (units)*		
Eluate:	*0.01 M phosphate (CM_2)*	*0.05 M phosphate (CM_3)*	*0.05–0.3 M phosphate (CM_1)*
1600 units CM_2 + 790 units CM_3	1800	620	0
4400 units CM_1	0	0	4400
2700 units CM_2	2700	0	0
1500 units CM_3	0	1200	0
600 units CM_2 + 1360 units CM_1	600	0	1360

anionic species. CM_3 eluted at 0.04 M and CM_1 at 0.13 M phosphate. In comparison CM_1 and CM_2 of mung bean elute at 0.01 M and 0.13 M phosphate (pH 6.5) respectively. We felt there was a possibility CM_3 represented a hybrid of CM_1 and CM_2 which arose as an artifact due to our means of extraction or fractionation. Therefore, each fraction was reapplied to the column either alone or in a known combination with another fraction. Each isozyme subsequently eluted as a single peak of activity in the position and with the activity expected (Table II).

TABLE III

EFFECTS OF ADDITIVES ON CHORISMATE MUTASE ACTIVITY

Details of assay procedure are given in Methods and Materials. Figures in parenthesis represent the final concentration of each additive in the assay mixture. All additives were combined with enzyme directly before addition of chorismate. Controls included tubes containing enzyme and additive as well as the usual buffer *plus* chorismate and buffer *plus* enzyme. Mutases used were purified both by double fractionation on DEAE-cellulose and passage through Sephadex. (Results were the same for each.) At least four assays using varied time and enzyme concentration were made with each additive affecting chorismate mutase activity. The effects of each additive were also confirmed by testing them using the chorismate mutase assay described by Gibson[4].

Additive	*% original activity*		
	CM_1	CM_2	CM_3
Aromatic amino acids ($1.7 \cdot 10^{-4}$ M)			
Tyrosine	45	100	50
Phenylalanine	45	100	50
Tryptophan	175	100	150
Lignin precursors and analogues ($1.7 \cdot 10^{-5}$ M)			
Cinnamic acid	70	100	100
p-Hydroxycinnamic (*p*-coumaric) acid	40	90	40
3,4-Dihydroxycinnamic (caffeic) acid	35	50	100–110
Chlorogenic (caffeyl quinic) acid	40	65	100
3-Methoxy-4-hydroxycinnamic (ferulic) acid	95	100	60
3,4-Dimethoxycinnamic acid	100	100	190
3,5-Dimethoxy-4-hydroxycinnamic (spinapic) acid	70	65	110
3,4-Dihydroxybenzoic acid	100	97	80
Coumarins ($1.7 \cdot 10^{-5}$ M)			
o-Hydroxycinnamic (*o*-coumaric) acid	55	60	80
Coumarin	70	85	85

Occasionally, when doing scaled up preparations, CM_1 and CM_3 would elute together. In such cases they separated well when reapplied to the column and, once separated, (1) behaved as unique entities when applied to the column once more and (2) exhibited characteristic metabolite sensitivities.

Metabolite sensitivity

In order to establish the identity of the alfalfa mutase isozymes in relation to those already characterized from mung bean[9], we investigated their sensitivity to metabolite effectors (Table III). CM_1 of mung bean is sensitive to inhibition by caffeic acid but not by the three amino acids. CM_2 is sensitive to all four metabolites. Neither mutase is sensitive to 3,4-dimethoxycinnamic acid[9] (Woodin, T. and Nishioka, L., unpublished). CM_1 and CM_2 of alfalfa are similar to CM_1 and CM_2 of mung bean both in their metabolite sensitivity and their behavior on DEAE-cellulose chromatography. Although similar to CM_1 in its sensitivity to the phenolic amino acids and to *p*-coumaric acid, CM_3 is different in several respects including its activation by 3,4-dimethoxycinnamic acid and its inhibition by ferulic acid. None of the isozymes were sensitive to the following: oxidized or reduced pyridine nucleotides (either NAD or NADP); pyridoxal phosphate; the fully or partially phosphorylated forms of adenosine; 3′,5′-cyclic AMP; phosphoenolpyruvate; the precursors of chorismate, shikimate and quinate; or the aromatic compounds, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid and cinnamyl alcohol. When assaying for sensitivity to high-energy

compounds, assays were run both in the usual phosphate buffer and in 0.1 M maleate buffer, pH 6.5, using enzyme dialyzed against the same buffer. None of the following amino acids inhibited or activated either CM_1 or CM_3 (CM_2 was not tested); alanine, glutamate, glutamine, histidine, aspartate, asparagine, leucine or serine. So neither mutase form exhibits the tight control by amino acids (such as histidine, leucine and methionine) external to the shikimate pathway demonstrated for prephenate dehydrogenase or anthranilate synthetase in *Bacillus subtilis*[20]. As evidenced by a lack of sensitivity to AMP, ATP, phosphoenolpyruvate, NADH and NADPH, none of the mutase isozymes is sensitive to energy charge[21]. In all subsequent work, sensitivity to 3,4-dimethoxycinnamic acid, caffeic acid, tryptophan and/or phenylalanine were used to establish the isozyme identity and integrity of any active fraction.

Gel electrophoresis

In order to establish that the appearance of three mutase isozymes was not a result of exposure to DEAE-cellulose chromatography or protamine sulfate, we subjected to electrophoresis crude extracts from acetone and nitrogen powders as well as extracts which had been purified through the $(NH_4)_2SO_4$ concentration step. Results were essentially the same in all cases, three forms of mutase in the indicated ratio and moving as expected, CM_1, CM_3, CM_2, with CM_1 being the most anionic and therefore the faster moving species (Fig. 3). Each isozyme also displayed the expected

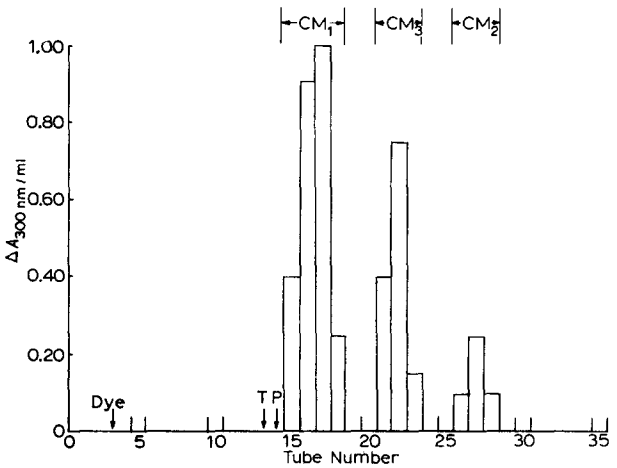

Fig. 3. Gel electrophoresis distribution pattern of alfalfa stem chorismate mutase. Details of gel composition, enzyme elution and assay are given in Methods and Materials. P and T indicate the elution peaks of prephenate dehydratase and dehydrogenase respectively.

sensitivity to the tested effectors. Assays for prephenate dehydrogenase and prephenate dehydratase indicated that neither enzyme was associated with any mutase isozyme and that each migrated slightly ahead of CM_1. As further proof of the integrity of the three mutase isozymes, each isozyme isolated by prior fractionation on DEAE-cellulose was subjected to gel electrophoresis using as a reference an extract which had been purified through the $(NH_4)_2SO_4$ step (Table IV). The identity of all isozymes was confirmed by challenging them with the appropriate effectors. Note

TABLE IV

COMPARATIVE ELECTROPHORESIS PATTERNS OF CHORISMATE MUTASE IN CRUDE EXTRACT AND OF PARTIALLY PURIFIED MUTASE ISOZYMES

Details of procedure are given in Methods and Materials. The identity of each isozyme was confirmed by testing for inhibitor sensitivity. Specific activity is nmoles prephenate produced per mg protein.

Added			*Recovered*								
Species	*Units*	*Spec. act.*	CM_1			CM_2			CM_3		
			Units	R_F	*Spec. act.*	*Units*	R_F	*Spec. act.*	*Units*	R_F	*Spec. act.*
Crude	17.2	10.0	9.0	0.47	100.0	1.6	0.11	50.0	4.4	0.25	88.0
CM_1	9.6	15.0	9.2	0.47	475.0	0	—	—	0	—	—
CM_3	2.86	45.0	0	—	—	0	—	—	2.76	0.25	100.0
CM_2	11.7	1.0	0	—	—	8.5	0.11	100.0	0	—	—

that in each case we recovered from 70 to 100% of original activity and that each isozyme migrated as only one peak with a predictable R_F and effector sensitivity pattern. The specific activity of the eluted fractions indicated a 10-fold purification on the gel. In all gels containing crude extracts, a dark brown band, presumably of phenolics, moved ahead of and a light green band moved directly behind the dye band.

At this point we were convinced that alfalfa stem had three forms of the mutase, but the question arose was the difference in chorismate mutase isozyme content between alfalfa and mung bean unique to the species, or did it reflect the fact we were comparing etiolated mung bean seedlings with mature alfalfa stems. So we examined the mutase isozyme pattern in extracts from alfalfa stem, leaves

TABLE V

PATTERNS OF CHORISMATE MUTASE ISOZYMES CONCENTRATION IN DIFFERENT PLANT PARTS OF ALFALFA

Details of procedure are given in Methods and Materials. (a) Nitrogen powders separated by gel electrophoresis. (b) Acetone powders separated by gel electrophoresis. (c) Nitrogen powders separated by DEAE chromatography (d) Acetone powders separated by DEAE chromatography.

Part		*Units (g wet wt)*	*Units (g dry wt)*	*Spec. act.*	*% of total chorismate mutase*			*% recovery*
					CM_1	CM_2	CM_3	
Seedlings (6 day)								
Green	(a)	36	350	4.0	63	10	27	92
Etiolated	(a)	14	140	2.0	61	10	28	88
Mature plant								
Leaves	(a)	37	145	2.0	63 ± 1.9*	12 ± 1.4*	25 ± 2.6*	75 ± 7.7*
Stem	(a)	50	252	2.6	59	13	28	100
	(b)				53	17	30	83
	(c)				62	14	24	100
	(d)				60	19	20	100
Flowers	(a)	24	185	1.2	60	12	28	84

* ± figures represent S.D. In cases where these are given, at least six separate experiments using either the same or different extracts were analyzed.

TABLE VI

SUBCELLULAR DISTRIBUTION OF CHORISMATE MUTASE ISOZYMES

Details of procedure are given in Methods and Materials.

Fraction	*Total units*	*Spec. act.*
Crude	21 000	4.0
Cell debris + nuclei	0	0
Chloroplasts	40	0.8
Mitochondria	10	0.7
Microsomes	30	0.4
Cell Sap	23 070	38.0

and flowers as well as from 6-day-old green and etiolated seedlings. In all cases three forms of chorismate mutase were present (Table V).

Distribution of chorismate mutase in cell organelles

Since it is known that the mitochondria and chloroplasts of higher plants contain phenolics such as ubiquinone and plastoquinone which are unique to these organelles, and, since recent evidence suggests mitochondria and chloroplasts possess a high degree of autonomy, we decided to determine if any or all of the mutases could be found in or were confined to any cell organelle. As shown in Table VI, chorismate mutase activity is confined to the cell sap and no appreciable mutase activity is discernable in any organelle.

Activation energy

In order to further distinguish the three isozymes, the activation energy of each

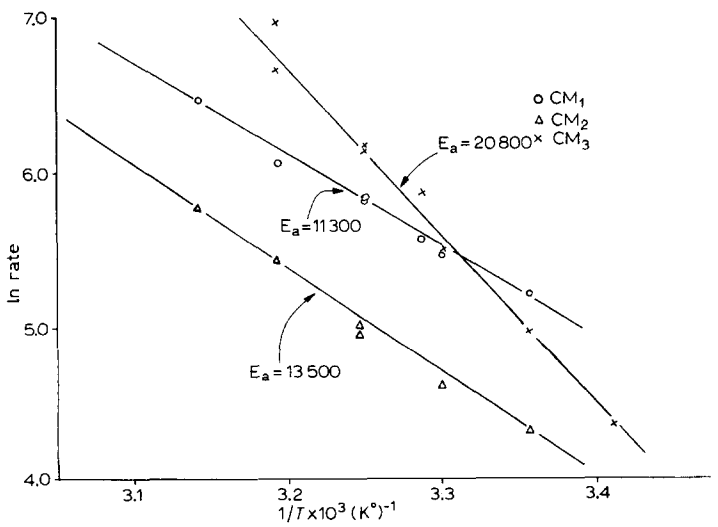

Fig. 4. Arrhenius activation energy of alfalfa chorismate mutase isozyme. Details of assay procedure are given in Methods and Materials. At each temperature assays were run for 15 and 30 min and at two different enzyme concentrations to ensure linearity with time. Temperature was maintained in a water bath and monitored with a calibrated thermometer.

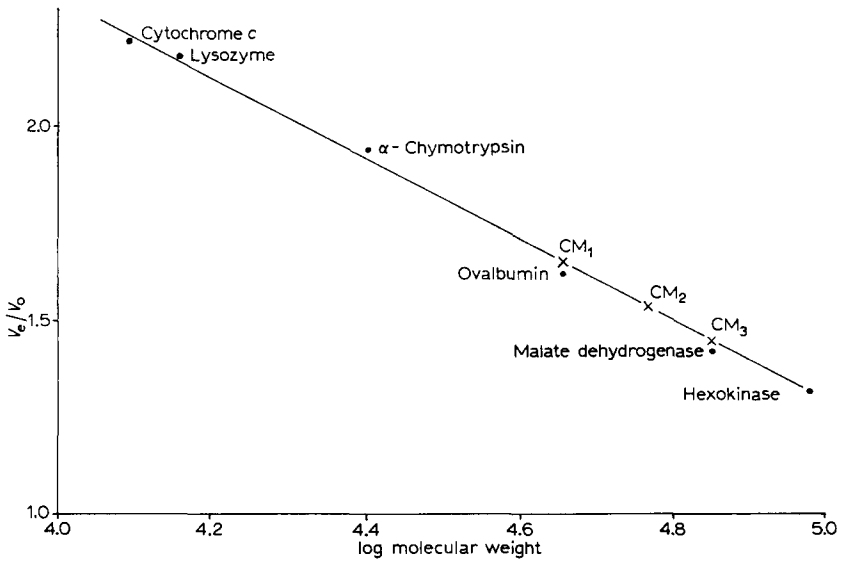

Fig. 5. Molecular weight of alfalfa chorismate mutase isozymes. Details of procedure are given in Methods and Materials. Each isozyme eluted as one symmetrical peak of activity. The insert indicates the percent recovery of total units and change in specific activity for each isozyme.

Mutase	*Specific activity*		*Total units*	
	Added	*Recovered*	*Added*	*Recovered*
CM_1	51	73	42	45
CM_2	17	43	32	32
CM_3	18	58	37	37

was determined (Fig. 4). Each exhibits a different activation energy with that isozyme (CM_1) sensitive to the most effectors having the lowest activation energy.

DISCUSSION

Based on their separability by a wide variety of methods (DEAE chromatography, gel electrophoresis, Sephadex fractionation) and the fact, that once separated from its fellows, each isozyme behaves uniquely and as an entity towards either further fractionation procedures or in the presence of such effectors as the aromatic amino acids or the lignin precursors and their analogues, we feel we have demonstrated the existence of three distinct mutase isozymes in alfalfa. Furthermore, the fact that although CM_3 is intermediate to the other two in both activation energy and anionic properties (as judged by its behavior on DEAE-cellulose and gel electrophoresis), it is not intermediate either in molecular weight or behavior toward lignin precursors or analogues, strongly supports CM_3 as a true isozyme and not as a hybrid of the other two mutases or an artifact arising during purification.

It is also apparent that our initial attempts at separating alfalfa mutase isozymes were discouraging because we failed to recognize the fact that the smear of

mutase activity during early fractionation attempts reflected not only the known recalcitrance of various plant extracts to clean fractionation, probably due to their high content of phenolics and polysaccharides[19], but also to the existence of a third isozyme, CM_3, probably intermediate to the other two in charge.

Extracts from mature mung bean as well as green and etiolated seedlings exhibit only two isozymes upon electrophoresis. (Hsu, A., Chen, S. and Woodin, T. S., unpublished.) Studies presently under way in our laboratory indicate that most plants, including a wide variety of monocots and dicots, possess three mutase isozymes. Only *Pisum sativum* and *Phaseolus vulgaris* of the dicots and a few more primitive plants such as fern and pine are limited to two mutase forms. (Nishioka, L., Hsu, A. and Woodin, T.S., unpublished.)

We are as yet unsure of the significance (but not the fact) of the presence of two mutases in one set of plants such as *Phaseolus* spp. and *Pisum* spp. and three in most others. Clarification of this point may reveal interesting taxonomic relationships. Or it may be the third form is so unstable in the apparent two isozyme species that it is destroyed during extraction. This latter point is unlikely in view of the stability of mutase activity in crude extracts from nitrogen powders.

As can be seen from Fig. 2, the shikimic acid pathway once committed to production of phenylalanine and tyrosine *via* the action of chorismate mutase has at least one more branch point, the production of either *p*-hydroxycinnamic acid leading to the production of the lignin precursors or the production of the *o*-hydroxycinnamic acids leading to the production of the coumarins. Upon discovering the three forms of mutase present in alfalfa, it was our original thought that each might be concerned with the production of a different endproduct; namely, the aromatic amino acids, the coumarins or the lignin precursors and that such differences would be reflected in their sensitivities to the various intermediates in each pathway. This does not appear to be the case. Instead it appears that one form, CM_1, which constitutes the bulk of the mutase activity, is sensitive to metabolites representative of each pathway. As to the other forms, one is sensitive to the lignin precursors and the other is sensitive to both aromatic amino acids and a few lignin precursors.

In alfalfa CM_2 and CM_3 are relatively minor components of the total mutase activity. On the basis of inhibitor effects, it appears that CM_2 can provide a steady low level of prephenate despite high levels of the aromatic amino acids or the coumarins; however, it can only operate optimally when lignin precursors are at low concentrations; CM_3 on the other hand can provide a steady low level of prephenate despite high levels of the lignin or coumarin precursors (although it is somewhat sensitive to *p*-coumaric and ferulic acid) but can only operate at maximal efficiency when levels of the aromatic amino acids, tyrosine and phenylalanine are low and tryptophan is high. If one takes as 100 units the total mutase activity when aromatic amino acids, lignin and coumarin precursors are low, then one could predict (1) in the presence of high levels of tyrosine and/or phenylalanine the level of mutase activity drops to 54 units due to the combined effects of these metabolites on CM_1 and CM_3; (2) in the presence of caffeic acid the level of mutase activity drops to 56 units due to the combined effect of this metabolite on CM_1 and CM_2; and (3) in the presence of tryptophan the total mutase activity increases to 165 due to the combined effect on CM_1 and CM_3. The fact that somewhat purified crude extracts do not react predictably to these effectors may reflect the fact these extracts still contain

interfering low-mol. wt metabolites or that the effector sensitivity of each mutase is somewhat altered in the presence of the other isozymes. The result of combining various lignin precursors, the aromatic amino acids and coumarin, as well as what levels of these metabolites are necessary for them to affect mutase activity, will be discussed in a succeeding paper (Nishioka, L. and Woodin, T.S., unpublished).

Another intriguing problem is the physical relation among the three mutases. On the basis of Sephadex molecular weight, it appears that no one mutase represents a moneric form of the other, although it is conceivable that they share common monomers combined in different ratios. This may be the case, but we have not observed any dissociation and/or rearrangement of the monomers. More definite information on this point must await purification to homogeneity of each isozyme.

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