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The Diversion of Dimethylallylpyrophosphate from Polyisoprenoid to Cyclopiazonic Acid Biosynthesis in *Penicillium cyclopium* Westling

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During the production of α-cyclopiazonic acid (αCA) by *Penicillium cyclopium*, dimethylallyltransferase (EC. 2.5.1.1) *T*, isopentenyl pyrophosphate isomerase (EC. 5.3.3.2) *I*, and a prenyl–aryl transferase, *S*, which produces β-cyclopiazonic acid (βCA) are all induced at the same time. This last enzyme appears maximally before the highest rate of α- or βCA production. Both transferases are not utilized to their maximum capacity, and the production of their end products seems to bear no relationship to their concentrations. Other controls therefore must play an important role in the utilization of their common substrate dimethylallylpyrophosphate (DMAPP). There are two possible control systems: (a), a direct competition by *S* and *T* for DMAPP; and (b), control by compartmentation. The first possibility is the more likely, in view of some of the controls that could apply to the deflection. The three enzymes were separated so that possible controls on the deflection of DMAPP from polyisoprenoids could be studied. They all possessed a subunit structure and exhibited maximum molecular weights (in the absence of divalent cations and presence of a thiol reductant) of 96 000 (**S**) and 64 000 (*I* and *T*) daltons. Mg^{2+} caused a diminution in size to 75 000 (*S*) and 50 000 (*I* and *T*) daltons. Mn^{2+} had the same effect on *I* and *T* but caused major disruptive changes to **S**. These effects were reversible by addition of EDTA. **S** was quite specific for DMAPP and *cyclo*aceto-acetyl-L-tryptophan (cAATrp) and exhibited Michaelis constants as follows; K_m^{cAATrp}, 6.0 μM and K_m^{DMAPP} 2.0 μM. It had no obvious requirement for a divalent cation and had an isoelectric point of 5.3. I had a K_m of 6.7 μM and an isoelectric point of 4.5, and either Mg^{2+} or Mn^{2+} was essential. The Michaelis constants for *T* could not be given but its isoelectric point was 5.1. The enzyme carried out the two reactions normally associated with it (i.e., two additions of IPP to produce farnesyl pyrophosphate) and required Mg^{2+} to do so. The pH optima for *S*, *I*, and *T* were 6.5–7.5, 6.0, and 8.0 respectively. The early and major controlling factor was the appearance of the cosubstrate of *S*, cAATrp. Other factors were: (a), the appearance of αCA which inhibited *T* more effectively than *S*; (b), the removal of free Mn^{2+} and Mg^{2+}, both essential for *I* and *T* but not for *S*, possibly brought about by chelation with cAATrp, α- and βCA; (c), the observed low pH of 6.0 when the activity of *S* was unaltered, *I* was at its highest, and *T* exhibited 50% of its maximum; and (d), an activation of *I* by low physiological levels of βCA and cAATrp which would enhance the rate of appearance of DMAPP to react with an existing pool of cAATrp.

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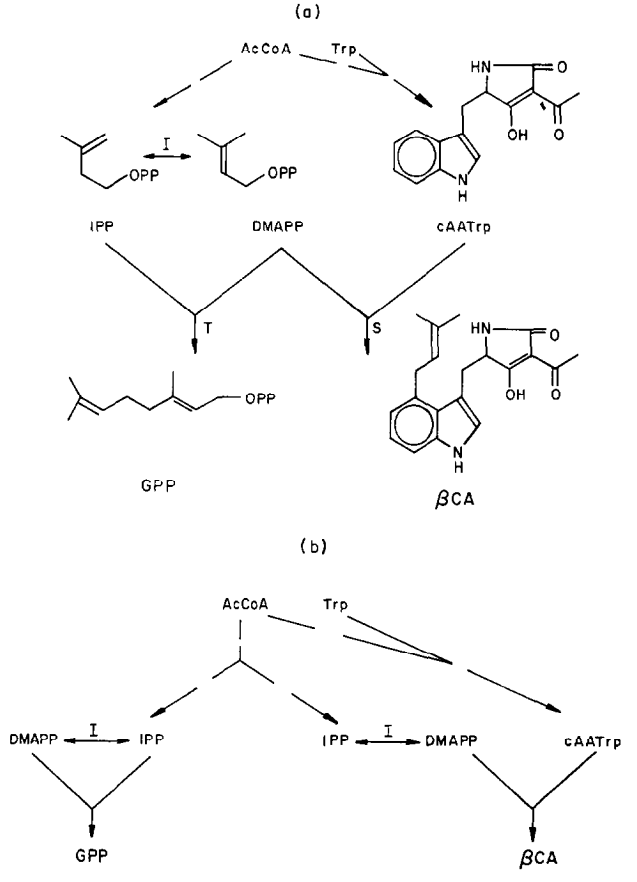
INTRODUCTION

In *Penicillium cyclopium* Westling strain 1082, the single chemical species of greatest concentration is α-cyclopiazonic acid (αCA)[2], where it can rise to more than 1 g/liter of well-defined culture medium, i.e., about 10% (w/w) of the total dry weight. It is produced via the isoprenylation of *cyclo*acetoacetyl-L-tryptophanyl (cAATrp) by dimethylallylpyrophosphate (DMAPP) brought about by dimethylallylpyrophosphate: *cyclo*acetoacetyltryptophanyl dimethylallyltransferase (β-cyclopiazonic acid synthetase) (*1*). The β-cyclopiazonic acid (βCA) so produced is oxidized and cyclized by β-cyclopiazonate oxidocyclase to yield αCA (*2*).

The metabolite DMAPP is also important in polyisoprenoid biosynthesis (*3*), being utilized by dimethylallyltransferase to yield farnesylpyrophosphate (E.C. 2.5.1.1) (*4*).



SCHEME 1. Isoprenoid and β-cyclopiazonic acid branchpoints: (a), *S* and *T* competing for one pool of DMAPP; (b), compartmentation of DMAPP into two pools.

[2] Abbreviations used: α- and βCA, α- and β-cyclopiazonic acid; Trp, tryptophan; cAATrp, cycloacetoacetyl-L-tryptophanyl; DMAPP, dimethylallylpyrophosphate; IPP, isopentenylpyrophosphate; GPP, geranylpyrophosphate; CFE, cell-free extract; S, secondary dimethylallyltransferase (β-cyclopiazonate synthetase); I, prenyl isomerase; T, primary dimethylallyltransferase; μ, ionic strength; mU, milliunits enzyme activity; and DTE, dithioerythritol.

This enzyme, important in primary metabolism, is abbreviated as *T*, while β-cyclopiazonic acid synthetase appears as *S*. These two enzymes will be in competition with each other for DMAPP presumably directly as in Scheme 1(a), but the possibility of indirect competition via compartmentation does exist, Scheme 1(b). Nevertheless, it is to be expected that not only will the ratio of the concentrations of these enzymes help control the branch, but also the concentrations of inhibitors and activators, and of the co-substrates isopentenylpyrophosphate (IPP) and cAATrp.

This paper describes the separation of *S* and *T* so that a study of the control of the branchpoint could be made. The enzyme prenylisomerase (E.C. 5.3.3.2) is also included because it is responsible for the production of DMAPP from IPP. It was hoped that a study of these three enzymes would also lead to a distinction between compartmentation and direct competition. Though no definite answer to this latter point can be given, on balance direct competition is favored. Irrespective of this, some interesting results were obtained concerning the effect of divalent cations, pH, and the cyclopiazonic acids on the enzymes.

MATERIALS

[1-^{14}C]IPP was purchased from Amersham, U.K. The syntheses of GPP, IPP, DMAPP, [1-^{14}C]DMAPP, *N*-acetoacetyl-L-tryptophan, and cAATrp (both generally ^{3}H-labeled in Trp) have been described (*1*).

METHODS

Protein was determined by the method of McGrath modified to contain half of the reported concentration of cyanide (*5*).

Penicillium cyclopium Westling, strain 1082, was grown at 25°C in shake culture (180 rpm) in a synthetic medium (*6*). A more complete discussion of growth and metabolic changes will appear later (*24*).

Cell-free extracts (CFE) were prepared from 60-hr-old mycelium, as described (*1*), at which time all three enzymes were at, or near, maximum concentration (Fig. 7). It should be noted that EDTA cannot be used in the preparation of CFE because *S* is inactivated. α- and βCA were isolated by tlc and estimated spectrophotometrically (*1*).

Gel columns were always recalibrated between fractionations using Blue Dextran 2000, albumin, myoglobin (whale), and [1-^{14}C]IPP.

Enzyme Assays

The *S* assay was modified from that described (*1*) to an incubation at 30°C for 5 min consisting of: [1-^{14}C]DMAPP, 50 nmol, 35 000 dpm; cAATrp, 50 nmol; Tris–acid maleate, pH 7.2 $\mu = 0.05$; 1–5 milliunits (mU) of enzyme; and H_2O to 1 ml.

The reaction was stopped by the addition of 2 drops of concentrated HCl, and the mixture was extracted with 1.0 ml of benzene containing 10 μg βCA. After centrifuging, 0.5 ml of the benzene layer was added to a glass scintillation vial and steamed in a pressure cooker for 15 min at atmospheric pressure. The ^{14}C remaining in each vial was due to βCA, no other radioactive material being found. This assay correlated well with that described, using silica gel partition tlc to isolate the βCA (*1*).

The assays for **I** and **T** were modified from those described by Popják (*4*).

For enzyme *T*, 1-ml reaction mixtures were incubated for 5 min at 30°C and contained: [1-^{14}C]IPP, 50 nmol, 240 000 dpm; DMAPP, 50 nmoles; $MgSO_4 \cdot 7H_2O$, 10 μmol; Tris–acid–maleate, pH 7.9, $\mu = 0.05$; and approximately 0.5 mU of enzyme. The reaction was stopped by the addition of 2 drops of concentrated HCl, saturated with $MgSO_4 \cdot 7H_2O$, made alkaline with NH_4OH, and the mixture was extracted with 1 ml of toluene which contained geraniol, nerolidol, and farnesol each at a concentration of 1% (v/v) toluene. After centrifuging, 0.5 ml of the toluene layer was taken for radioactivity determination (*1*).

For *I* the assay assumed that the transferase, if present, increased the radioactive acid-labile prenylpyrophosphates threefold; therefore in solutions where *T* was known to be present the observed activity was always divided by 3.

One-milliliter reaction mixtures were incubated for 5 min at 30°C and contained: [1-^{14}C]IPP, 50 nmol, 240 000 dpm; $MnSO_4 \cdot 7H_2O$, 5 μmol; Tris–acid maleate, pH 6.0, $\mu = 0.05$; and approximately 0.5 mU of the enzyme. The procedure thereafter was identical to that described for *T*.

Enzyme Units

One unit of *S*, *I*, or *T* will produce 1 μmol of product in 1 min at 30°C under the conditions described in the assays.

Separation of the Enzymes

All the following steps were carried out at 0–5°C.

CFE was centrifuged at 110 000*g* for 1 hr and the clear straw colored supernatant carefully separated from floating lipid to give fraction 110_k. This was adjusted to contain Tris–chloride buffer (pH 7.9, $\mu = 0.02$), 5 m*M* dithioerythritol (DTE), and 5 m*M* EDTA, before addition of ammonium sulphate. If this were not done, the enzymes would lose up to 70% activity. The enzyme *S* could be separated from *I* and *T* at this stage: the bulk of *S* precipitated between 45 and 60% saturation with ammonium sulfate (AS_{45}), while *I* and *T* precipitated between 60 and 75% saturation (AS_{60}) (Table 1).

TABLE 1

AMMONIUM SULPHATE FRACTIONATION OF 110_k[a]

Ammonium sulfate fraction (%)	Total activity (mU)		
	S	**I**	**T**
0–30	0	0	0
30–45	20 (0.1)[b]	0	0
45–60	1570 (6.0)	60 (0.3)	170 (0.6)
60–75	450 (2.0)	1250 (5.6)	1080 (4.0)
75–90	130 (1.6)	170 (2.0)	140 (1.6)
Total	2170	1480	1390
110_k[a]	2600 (3.4)	1950 (2.5)	1700 (2.2)

[a] Supernatant after centrifuging CFE at 110 000*g*.

[b] Specific activities in parentheses.

Fraction AS_{45} was dissolved in the minimum amount of Tris–chloride, pH 7.9, $\mu = 0.01$ containing 10 m*M* DTE and 1 m*M* EDTA, and fractionated on a Bio-gel A 0.5-m column (100–200 mesh) which had been equilibrated with the same buffer. The *S* enzyme appeared to have a molecular weight of 95 000 daltons; it chromatographed as shown in Fig. 5 though, naturally, *I* and *T* would be absent from the AS_{45} load. (In Fig. 5 an ammonium sulfate concentrated protein load was used which contained all three enzymes.) The dilute enzyme was concentrated by dialysis against a 50% (w/v) glycerol–water solution which contained 10 m*M* DTE. In this form it was stable for at least 6 months at –20°C. Table 2 summarizes the procedure and indicates a 16-fold purification from the CFE. The enzyme is free from both *I* and *T* which usually cochromatograph with molecular weights of about 64 000 daltons.

TABLE 2

SUMMARY OF ISOLATION STEPS FOR SECONDARY DIMETHYLALLYLTRANSFERASE

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity	Total activity (mU)
CFE[a]	30	6.0	3.3	600
110_k[b]	30	5.6	3.6	600
AS_{45}[c]	3	30.0	5.3	480
Biogel A (0.5 m, concentrate)	11	0.5	50.0	275

[a] CFE, cell-free extract.
[b] 110_k, supernatant after centrifuging CFE, at 110 000*g*.
[c] AS_{45}, 45–60% saturated ammonium sulphate fraction.

Unfortunately *I* and *T* could only be separated using the LKB isoelectric focusing apparatus and the CFE. Any other load, whether the ammonium sulfate or gel permeating fraction, lost all activity during isoelectric focusing, while no other technique separated *I* and *T*. The 110-ml apparatus was used as described by Vesterberg (*7*), except that the density zones were stabilized by glycerol ranging in concentration from 65 to 10% (w/v). The anode was always at the bottom and consisted of a 60% (w/v) sucrose solution containing H_2SO_4. Ampholytes were a 1% (w/v) solution with pH 4.0–6.0. Strong reductant caused the isoelectric points of I and T to become the same (Fig. 1a). Therefore, the experiments were carried out using no additions to a 5-ml CFE load. Tubes 11 and 16 (Fig. 1b) had Tris–chloride (pH 7.9) and DTE added to bring them to 0.05μ and 0.01 *M*, respectively. The enzymes could then be stored for at least 6 months at –20°C.

S, not shown in Fig. 1, was well separated from *T* having a pI of 5.3. However, because of large losses on isoelectric focusing, the method was not suitable for large-scale isolation.

It is impossible to give purification factors for *I* and *T* obtained above because of the direct assay used, i.e., the presence of *T* would make *I* spuriously high. However, inspection of Fig. 1b shows that most of the protein is found in Tubes 12 to 15 (68 of the 75 mg loaded), which suggests that the purification factor must be reasonably high. For the purposes of this work it was sufficient only to separate the activities.

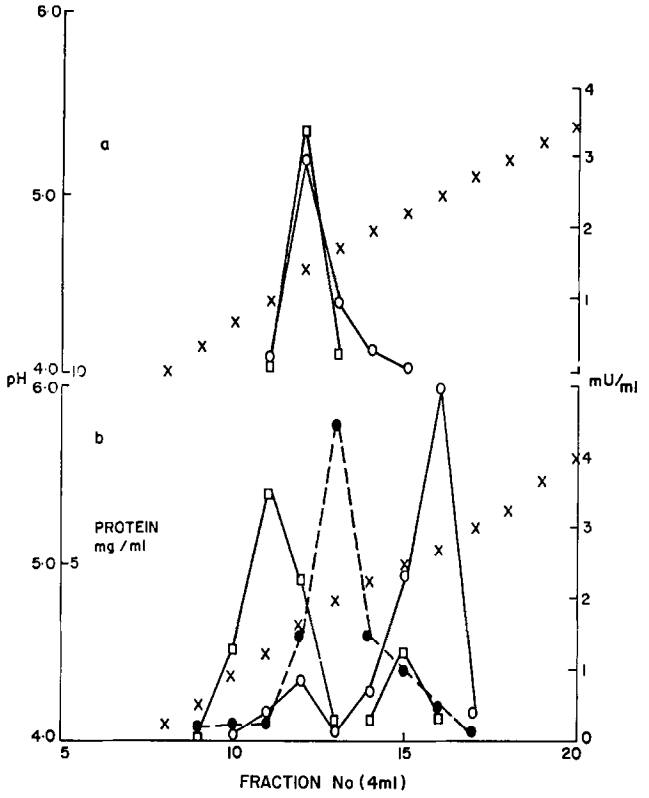


FIG. 1. (a) Five milliliters of CFE were isoelectric focused for 48 hr in glycerol containing 10 m*M* DTE. *I* (□), *T* (○), and pH (×) are shown. (b) This is identical to (*2*) except that DTE was omitted. In addition protein (●) is shown.

RESULTS AND DISCUSSION

Homologous Substrates (*Table 3*)

T used GPP or DMAPP with equal facility as cosubstrates with IPP. These two reactions paralleled each other during ammonium sulfate fractionation, gel permeation, and isoelectric focusing, as well as showing identical stabilities and labilities under varying conditions. Holloway and Popjak concluded on similar evidence that the one protein from pig liver carried out both reactions (*8*), and the same is probably true for the *T* in *P. cyclopium*. There is a considerable accumulation of evidence for the existence of both activities on the one protein, and the recent isolation of homogenous proteins from yeast and avian liver (*9*, *10*), which also carry out both reactions, confirms the one enzyme–two activities concept.

The requirement of *S* for DMAPP and cAATrp was quite specific, neither IPP, GPP, Trp, nor *N*-acetoacetyltryptophan being used. This specificity of *S* for a cyclized Trp substrate is paralleled by the work of Allen on *cyclo*-L-alanyl-2(1,1-dimethylallyl)-L-tryptophanyl synthetase (*11*). The substrate, in this case a diketopiperazine, *cyclo*-L-alanyl-L-tryptophanyl, could be replaced by *cyclo*-L-prolyl-L-tryptophanyl (*12*).

TABLE 3

HOMOLOGOUS SUBSTRATES[a] FOR THE ENZYMES *S* AND *T*

Enzyme	Substrates A	Substrates B	dpm in product
S	[1-^{14}C]DMAPP	cAATrp	5800
	[1-^{14}C]IPP	cAATrp	0
	[1-^{14}C]DMAPP	cAATrp	5700
	[1-^{14}C]DMAPP + IPP, 0.10 μM	cAATrp	5500
	[1-^{14}C]DMAPP + IPP, 0.80 μM	cAATrp	5000
	DMAPP	cAATrp, ^{3}H(G)	2800
	GPP	cAATrp, ^{3}H(G)	0
	[1-^{14}C]DMAPP	*N*-acetoacetyl Trp ^{3}H(G)	0
	[1-^{14}C]DMAPP	Trp	0
T	[1-^{14}C]IPP	DMAPP	10 200
	[1-^{14}C]IPP	GPP	9500

[a] The substrates were all at 0.05 μM concentration unless a value is given to the contrary.

It is of interest that a secondary transferase ,4-dimethylallyl-L-tryptophan synthetase, utilized Trp as a cosubstrate with DMAPP en route to the clavine alkaloids (*13*), while in this case Trp is not used at all, not even binding to the enzyme (Table 4).

TABLE 4

INHIBITION (%) CAUSED BY Trp, cAATrp, βCA, AND αCA

Substrate	Concentration (mM)	Inhibition (%) S	I	T
αCA	1	16	25	33
	2	24	40	50
βCA	1	31	63	28
	2	48	75	52
cAATrp	0.1	—	−10	25
	0.2	—	−20	50
TRP	0.1	0	0	0
	0.2	0	0	0

K_m Values

These were obtained graphically from replots of the slope and intercepts obtained from double reciprocal plots and are, for **I**, $K_m^{IPP} = 6.7\ \mu M$, and for *S*, $K_m^{cATTrp} = 6.0\ \mu M$ and $K_m^{DMAPP} = 2.0\ \mu M$. K_m values for **T** utilizing GPP and IPP are not given because the data showed that the enzyme gave a nonlinear double reciprocal plot, i.e., the mechanism did not conform to a regular substrate reaction. Difficulty was also met in an attempt to interpret the results when DMAPP and IPP were the cosubstrates of **T**.

pH Optima

The two primary enzymes show optima remarkably like those from pig liver (*8*, *14*) with an optimum of 6.0 for *I* and 8.0 for *T*. The secondary enzyme *S* has a much broader optimum ranging from pH 6 to 8 (Fig. 2).

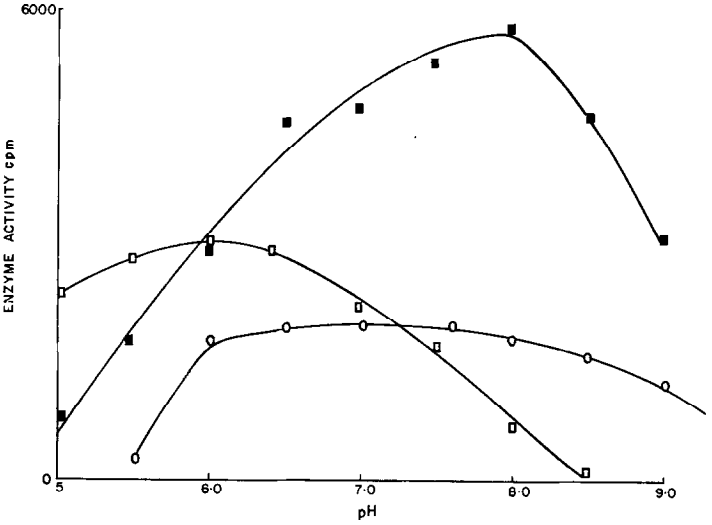


FIG. 2. pH dependence of the enzymes, *S* (○), *I* (□), and *T* (■). Tris–acid maleate buffers at constant ionic strength were used throughout the assays.

TABLE 5

CYCLOPIAZONIC ACID PRODUCTION AND pH

Incubation (hr)	36	48	60	72	84	96	108	120
αCA (m*M*)	0.05	0.25	0.40	0.81	0.81	1.06	1.74	2.7
βCA (m*M*)	0	0.05	0.20	0.40	0.56	0.84	0.55	0.26
pH	7.4	7.5	7.6	7.2	6.9	6.0	6.0	6.0

In the control of the branchpoint, pH effects cannot be ignored: at pH 8.0 there would be less DMAPP for the competing enzymes, while at pH 6.0 the DMAPP concentration would increase through activation of **I**; the activity of T would decrease by about 50%, and that of *S* would be relatively unaffected. As predicted the production of secondary metabolites increased considerably when the pH of the medium dropped from 7.2 to 6.0 between Days 3 and 4 (Table 5).

This argument would hold true whether or not the control was by direct competition or by compartmentation.

Divalent Cation Requirement (Fig. 3)

Enzymes *I* and *T* have a requirement for a cation, *I* preferring Mn^{2+} marginally more than Mg^{2+}, while *T* has a pronounced preference for Mg^{2+} against Mn^{2+}. On the other hand *S* is not inhibited by EDTA (both *I* and *T* are completely inactivated), which could mean either that the enzyme does not require a divalent cation, or that, if it does, the cation is not removed from the enzyme by EDTA.

The prominent feature is the inhibition of *S* by Mn^{2+}, which might be alleviated by the removal of *free* Mn^{2+} by, for example, chelation with α- or βCA or cAATrp. One has to postulate that only free Mn^{2+} is affected, otherwise enzyme *I* would be inhibited,

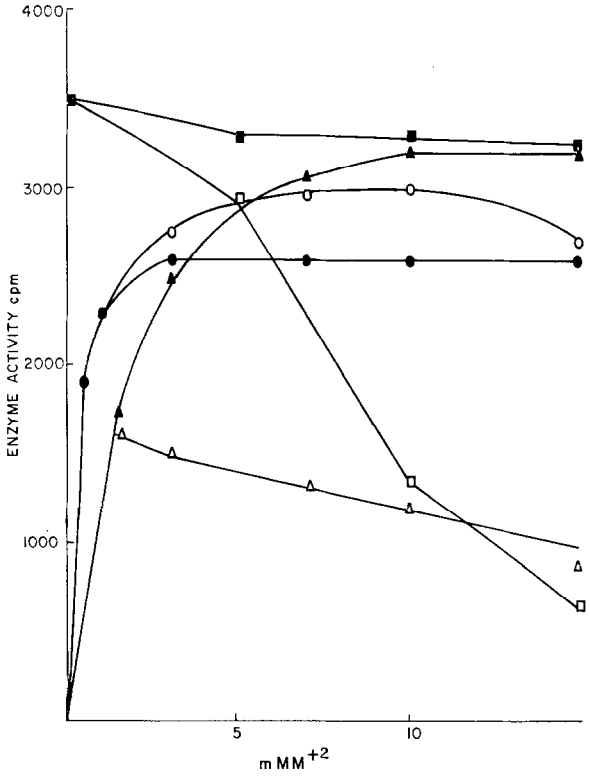


FIG. 3. The effect of Mg^{2+} and Mn^{2+} respectively, on *S* (■, □), (*I*●,○), and *T* (▲,△).

stopping the production of DMAPP. Potentially the competition of the ligands, protein and tetramic acids, for essential trace elements could be an excellent means of control. Later puzzling results (Table 6) as to activation and inhibition of enzyme *I* may owe their

TABLE 6

THE EFFECT OF cAATrp AND βCA ON ISOPRENYL ISOMERASE

Concentration of βCA (m*M*)	Concentration of cAATrp (m*M*)				
	0	0.5	1.0	2.0	3.0
	Observed *v* (dpm)				
0	10 000	14 000	17 000	16 800	15 000
0.5	20 400	23 200	23 000	16 000	12 000
1.0	2000	2000	2200	2400	2660
2.0	1600	2000	2000	2400	2500
3.0	1600	2000	1800	2000	2100

final explanation to chelation. That such chelation exists with Cu^{2+} is already known (*15*), and another tetramic acid, tenuazonic acid, has recently been found to exist in nature as a chloroform soluble Mg^{2+} salt (*16*). The stoichiometry for αCA and Cu^{2+}, and for tenuazonic acid and Mg^{2+}, is 2:1 (see Fig. 4).

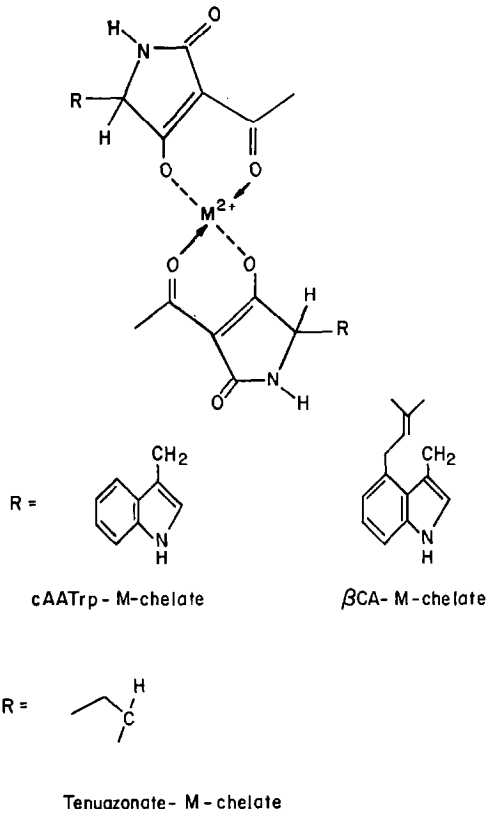


FIG. 4. Suggested chelates of the indole tetramic acids and tenuazonic acid (*14*).

The Effect of Mg^{2+} and Mn^{2+} on the Enzymes

Mn^{2+} has a profound effect on *S*, destroying it as a distinct entity (Fig. 5d), though total activity (area under the peak) was unchanged. This recovery of activity may well be due to the reversibility of the destruction, which is shown in Fig. 5b, when the enzymes stored in 2 m*M* Mn^{2+} reverted to "normal" size when the Mn^{2+} was removed by gel filtration. Mg^{2+} did not exhibit a similar destructive change on *S* though it did reduce the molecular weight (Fig. 5c). The effect of the two cations on *I* and *T* was the same, causing equivalent changes in molecular size.

The inhibition brought about by Mn^{2+} in the assay of *S* is approximately linear with the 50% inhibition occurring at a Mn^{2+} concentration of 8 m*M* (Fig. 3). In the assays performed in Fig. 5 the concentration of Mn^{2+} after dilution was only 100 μ*M*, which is not enough to cause noticeable inhibition. Because the process of molecular weight reduction is reversible there is no reason to assume, as one might from Fig. 5d, that the

reduction of enzyme size is not related to inhibition. Indeed, it is likely to be so related, though not certainly.

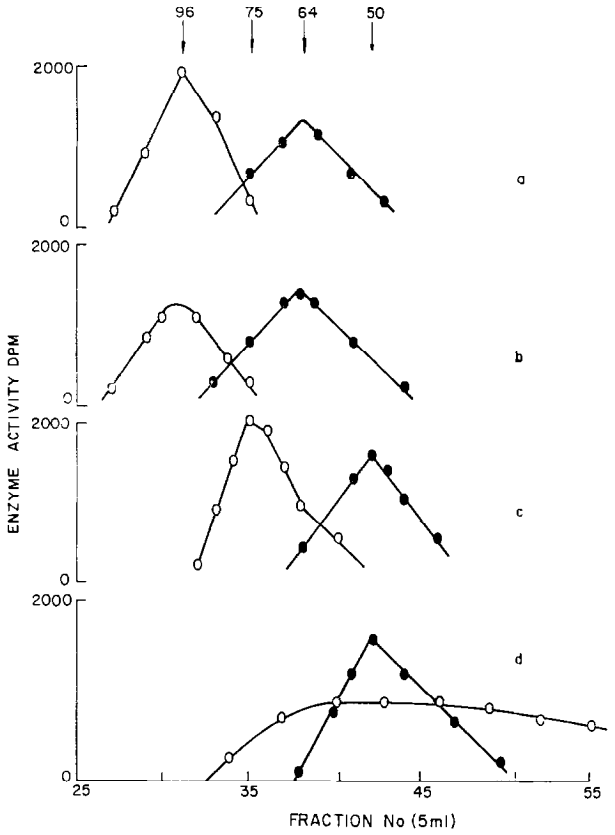


FIG. 5. (a) Ammonium sulfate-concentrated enzymes (40–80% saturated) in Tris-chloride buffer (pH 7.9, $\mu = 0.01$; DTE, 10 m*M*; EDTA, 1 m*M*) and loaded onto a Bio-Gel 0.5 m, 100–200 mesh column (79 × 2.5 cm) equilibrated in the above buffer. (b) This was loaded from a concentrate which had been stored in 2 m*M* Mn^{2+}. (c) This was loaded as (a), but the column eluant contained 2 m*M* Mg^{2+}. (d) As (c), but contained 2 m*M* Mn^{2+} in place of Mg^{2+}. Five milliliter fractions were collected of which 100 μl were assayed for *S* (o), *I*, and *T* (●) activity. The arrowed numerals are approximate molecular weights in thousands; the column was calibrated using BSA, myoglobin dextran blue 2000, and [1-^{14}C]IPP.

Enzyme Size Variations in Cell-Free Extracts

It was found that mycelium grown from inocula made at different times often gave enzymes with different molecular weights. Techniques, media, and culture were the same in each case.

Amicon concentrated CFEs were fractionated by gel filtration on Sephadex G-100; three results are shown in Fig. 6. Only in Fig. 6a were the three enzymes separable, and unfortunately we never were capable of reproducing this pattern; Fig. 6c was more normal. Enzymes of the Fig. 6a type were stable in frozen mycelium (–20°C) for at least a year while the others had a halflife of about 6 weeks. However the isoelectric points

were always the same: *S*, 5.3; *I*, 4.5; and *T* 5.1, though recoveries were very different, *S* sometimes disappearing completely from the Fig. 6b or c type.

These data are not juxtapositioned with that concerning the role of Mg^{2+} and Mn^{2+} to suggest a relationship between these metals and the observed molecular weights in

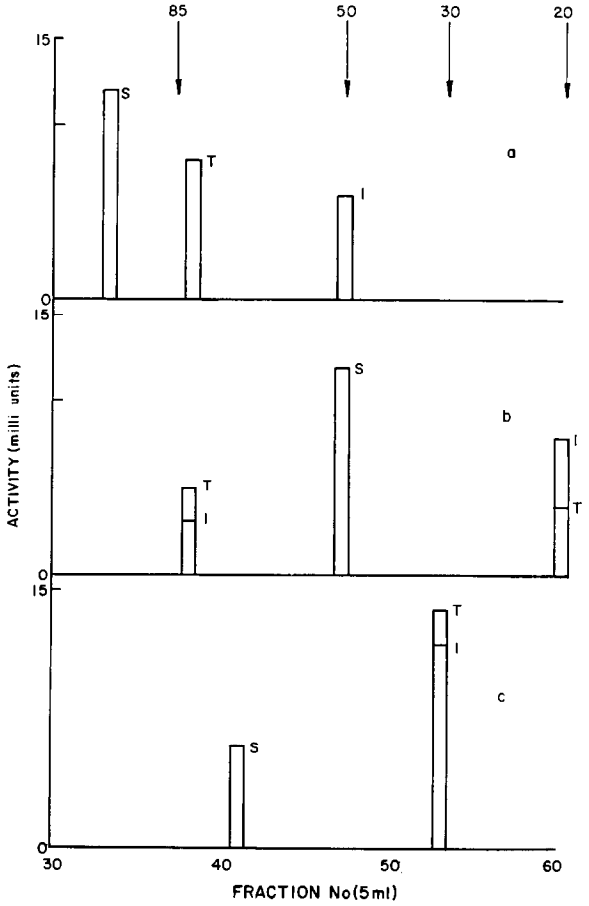


FIG. 6. Sephadex G-100 filtration of CFE concentrated 10-fold using an Amicon Model 52 apparatus with a P.M. 10 membrane. The CFEs (a–c) were prepared from three different batches of mycelium grown under identical conditions. The gel column (91 × 2.5 cm) was eluted with Tris-chloride, pH 7.5, $\mu = 0.01$, containing 2 m*M* DTE. The histograms represent peak height only. Arrowed numerals are approximate molecular weights in thousands calibrated using BSA, myoglobin, Dextran Blue 2000, and [1-^{14}C]IPP.

the CFE, even though trace metals do play an important role in secondary metabolism (*17*), but rather to underline the fact that different subunits exist in the cell itself.

The Effect of αCA, βCA, cAATrp and Trp

The early, and possibly the major, control of the diversion of DMAPP to secondary metabolites away from polyisoprenoids, was the appearance of cAATrp (the co-substrate with DMAPP for *S*) which is essential for this deflection. Furthermore, this

metabolite inhibited *T*, the competitor with *S* for DMAPP, while at the same time it activated *I* (Table 5). However, the product βCA inhibited *I* very strongly which led to an examination of the mixed effect of these two metabolites on the enzyme (Table 6). βCA was found to activate *I* in concentrations up to 0.5 m*M* after which it became strongly inhibitory; it was about this concentration (Table 5, Fig. 7) that β-cyclopiazonate oxidocyclase (*2*) was induced or utilized to convert β- to αCA. The effect of this activation of *I* may be to favor secondary metabolism because cAATrp exists in the cell as a pool, and the production of cyclopiazonic acids depends on the DMAPP concentration (*1*), while farnesylpyrophosphate production will depend not only on DMAPP, but more importantly on IPP (requiring 2 molecules). Thus it is likely that a faster conversion of IPP to DMAPP would promote *S* at the expense of *T*.

In compartmentation this argument would only apply if isomerization were the rate limiting step. In view of current knowledge of regulation of the isoprene pathway (*18*), it is not likely that isomerization would be the rate limiting step though this is by no means certain (Cholesterol does inhibit the isomerase from Mycoplasma (*19*).) How-

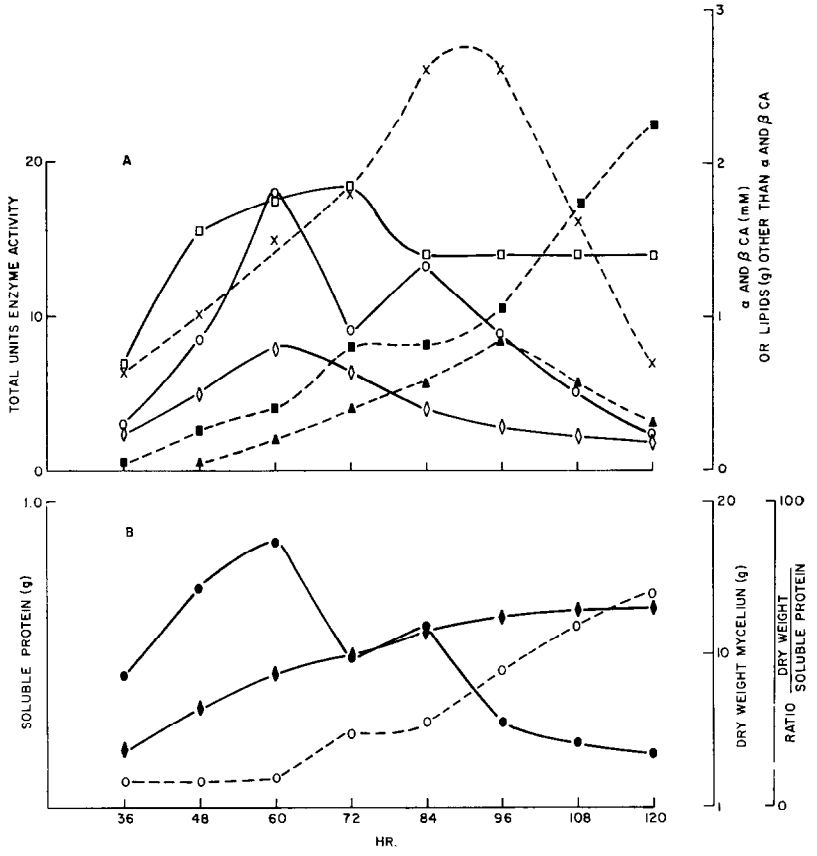


FIG. 7. Parameters of *P. cyclopium* during shake culture growth in one liter of chemically defined media: (a), αCA (■), βCA (▲), and chloroform soluble lipids other than α- and βCA (×). The total enzyme level is shown for *S* (⊙), *I* (◇) and *T* (□). (b) In the same experiment total soluble protein (●) and mycelial dry weight (♦), plus the ratio, mycelial dry weight/soluble protein (○), are shown.

ever, assuming that the rate limiting step lies between the compartmented branch and *I*, then activation of *I* by cAATrp or βCA would not favor deflection. Therefore, providing that isomerization is not rate limiting, the observed activation of *I* would tend to support Scheme 1(a), direct competition, where activation of *I* would be an important control favoring αCA production.

In spite of the activation by physiological levels of βCA, αCA is an inhibitor of all three enzymes though preferentially of *T*. The fact that it does not stop its own production implies either compartmentation, or that inhibition is never complete.

We have suggested previously that the tetramic acid ligands could further their own production by chelating free Mn^{2+}, an inhibitor of *S*. The effects noted here may well be due to competition by the tetramic acids and the enzymes for the essential divalent cations.

Enzyme Activity during Secondary Metabolite Production

The relative activities of *S* and *T* must naturally be of prime importance in considering the branch point. During a study of the concentrations of these enzymes a number of events were discernable (Fig. 7).

The first and highest level of *S* at 60 hr did not correspond to the greatest production of secondary matabolites. It would thus appear that the production of the cyclopiazonates is not simply related to *S*. In fact the relationship of *S* to soluble protein concentration is better than that to αCA production. The latter association is weak because maximum cyclopiazonate production occurs while the concentration of *S* is falling.

The enzyme *T*, with a very minor exception, is always at a greater concentration than *S* which is especially true at 120 hr when, in spite of this, α- and βCA production is still increasing at the cost of all other lipid material. It is apparent, therefore, that simple enzyme concentrations alone do not control the branch point. Nor are they working at maximum capacity; a simple calculation, based on the average enzyme units during the 36 to 96 hr period, indicated that S should have produced 41 mmol of βCA and **T**, 57.6 mmol of GPP. For the secondary metabolites at least, this represents 23 times more than that produced. This assumes that the cyclopiazonates are not further catabolized, and no evidence of such catabolism has been discovered after many radioactive feeding experiments (*1*). If the polyisoprenoids are not catabolized at a fast rate in *P. cyclopium*, then *T*, like *S*, will also not have been maximally utilized. Enzymes generally are present in excess of their normal requirements (*20*), and those in *P. cyclopium* are no exception.

As already noted, the two peak activities of *S* seem to be more associated with soluble protein production than with αCA production; i.e., they are produced during primary biosynthesis. As the first peak of *S* declines αCA begins to rise, then at 72 hr the second peak of *S* appears and αCA production falls. During this same period, and in step with the above parameters, mycelial dry weight, which had begun to fall, regained its original rate of appearance. To accentuate this process the parameter "dry weight/soluble protein" is shown in Fig. 7(b). This ratio represents the result of biological activity (biomass) divided by a producer of biomass, the proteins. A shallow or no slope has been found by us to correlate with *P. cyclopium* growing in the trophophase ("log" phase), while an inflexion indicates the onset of the idiophase (stationary phase) and secondary metabolism. Thus, in the experiment shown, the organism seemed to "hunt" or balance between primary and secondary metabolism at 60 to 84 hr.

The early production of nonutilized secondary enzyme has been noted before by Weinberg and Tonnis (*21*). *Bacillus subtilis* grown from an inoculum which contained Mn^{2+} produced secondary metabolites even when protein synthesis inhibitors were added just before metabolite production. On the other hand, if Mn^{2+} were omitted but added with the inhibitors, then secondary metabolism did not occur; thus in addition they noted a metal dependence of this early enzyme production.

Another notable feature in Fig. 7 is the disappearance of both lipids and βCA as αCA begins its rapid rate of production at 96 hr. This suggests an increased input to the DMAPP pathway, rather than utilization as an energy source, because the organism is going into a phase of less energy requirement (the idiophase). Presumably the organism has adapted to dispose of 5 molecules of AcCoA via αCA rather than allow it to spill into polyisoprenoid production.

CONCLUSION

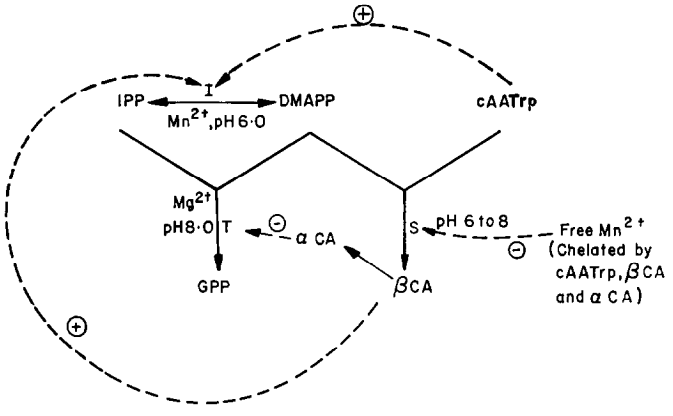
We have previously demonstrated that cAATrp exists as a pool in cultures of *P. cyclopium* and that DMAPP additions cause an increase in secondary metabolite production (*1*). Here we have shown that the concentrations of the enzymes, or their ratios, do not play a major role in deflecting DMAPP either to polyisoprenoid or αCA production. Therefore other enzyme regulatory mechanisms must be more influential.

The first important step will be the appearance of cAATrp, otherwise no αCA production would be possible. As cAATrp pools and DMAPP becomes rate limiting, the effect of trace element chelation may become important by removing free Mg^{2+} and Mn^{2+}, the former essential for the activity of **T** and the latter an inhibitor of **S**. However, it is not possible to make simple statements concerning metal chelation because the enzymes themselves are excellent chelators. Thus a competitive system for available divalent cations could be set up. A further complication would be the turnover of the enzymes involved in chelation.

No results in this work can unambiguously confirm either one of the postulated controls; i.e., either direct competition by **S** and **T** for DMAPP, or indirect competition via compartmentation. However, because of the existence of βCA and cAATrp activation of *I* and its apparent uselessness in a compartmented system (providing isomerization is not rate limiting), we tend to favor the more simple concept of direct competition. The presence of only one form of *I* may also support the simpler mechanism. Scheme 2 summarizes the possible controls on the direct competition of *S* and *T* for DMAPP.

Irrespective of how the branchpoint is controlled, the early presence of a major secondary metabolizing enzyme raises a question as to the correctness of Bu'lock and Powells' hypothesis of induced enzyme mechanisms (*22*). It would rather appear in this case that the enzyme exists before the need for it is manifest. Perhaps it belongs to an operon, but nevertheless the operon is activated 48 hr before the enzymes are maximally utilized. It would seem here at any rate, and perhaps in *B. subtilis* (*21*). that the system for secondary metabolism exists before the primary pools become unbalanced—a built-in overflow mechanism. This assumes Woodruffs' hypothesis (*23*), that secondary metabolism serves to keep primary pools balanced, is correct. A major control of metabolite production would then be availability of substrates.

An interesting point arises from our earlier observation that no pool of Trp was detectable in the organism (*1*); a branch must occur between cAATrp and Trp–tRNA production. Whether this branch serves as a shunt which depends on Trp that is not required for protein synthesis, or whether it competes for Trp is not known. We feel the former is more likely, in view of the early appearance and nonutilization of the



SCHEME 2. Possible control of the branchpoint caused by competition of secondary transferase *S* with primary dimethylallyltransferase *T*.

penultimate enzyme *S*, and that as primary growth ends Trp is utilized along with AcCoA to yield cAATrp. AcCoA is likely to be in excess at this time, the end of primary growth, because its main utilizer, the Krebs' cycle, will be slowing down. This metabolite in turn deflects excess DMAPP away from isoprenoid biosynthesis. We have suggested here that a large amount of normal lipid material is deflected into αCA, which would have noticeable effects on the cell structure, and in fact electromicrographs show that the main organelle affected is the mitochondria (*24*). Thus one might see secondary metabolism as an interlock mechanism, linking in this case protein biosynthesis and energy metabolism, and perhaps polyisoprenoid biosynthesis, which is called into play when a restraint is placed on a specific area of primary metabolism.

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