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Effect of calcium on synthesis of dipicolinic acid in *Penicillium citreoviride* and its feedback resistant mutant

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Abstract. Dipicolinic acid synthesis in *Penicillium citreoviride* strain 3114 was inhibited by Ca^{2+} ions, but not by Ba^{2+}, Cu^{2+}or Fe^{2+}. Among the metals tested, only Zn^{2+} inhibited the synthesis of dipicolinic acid and promoted sporulation. None of these metals reversed the inhibition by Ca^{2+} or Zn^{2+} . A mutant 27133-*dpa-ca* selected for resistance to feedback inhibition by dipicolinic acid: Ca^{2+} complex showed cross-resistance to inhibition by dipicolinic acid: Zn^{2+}. Both 3114 and 27133-*dpa-ca* excreted a number of aliphatic and amino acids during secondary metabolism of dipicolinic acid. In the presence of 1000 ppm of Ca^{2+}, accumulation of citric acid and α-aminoadipic acid was completely inhibited under conditions of inhibition of dipicolinic acid in parent strain 3114 but not in the mutant. Citric acid with or without Ca^{2+} did not inhibit the *de novo* synthesis of dipicolinic acid in the strain 3114. In fact, citric acid in the presence of Ca^{2+} improved significantly rate of dipicolinic acid synthesis. Apart from resistance to feed back inhibition by dipicolinic acid: Ca^{2+} complex, mutant differed from the parent in three other aspects *viz.* (i) dipicolinic acid synthesis was not subject to catabolite repression by glucose, (ii) sporulation as well as dipicolinic acid synthesis was dependent on the presence of Ca^{2+} ions in the medium and (iii) Mg^{2+} requirement for the mutant increased three fold. Higher requirement of the Mg^{2+} could be partially relieved by Ca^{2+} during secondary metabolism. The results support the inference that *de novo* synthesis of dipicolinic acid is regulated through feedback inhibition by dipicolinic acid: Ca^{2+} complex.

Keywords. *Penicillium citreoviride;* dipicolinic acid; secondary metabolite; DPA regulation; feedback inhibition.

Introduction

Previous studies from this laboratory, have shown that (i) dipicolinic acid (pyridine, 2,6-dicarboxylic acid; DPA) was synthesised by the mold *Penicillium citreoviride* as a secondary metabolite, (ii) its *de novo* synthesis was inhibited by calcium, and (iii) though the mold showed increased sporulation in the presence of calcium, no detectable amount of DPA could be observed in the spores (Kalle 1978; Kalle and Khandekar 1983). This was in contrast to the observations in bacteria where DPA and calcium were incorporated directly into the spore cortex (Forman and Aronson, 1972; Tamir and Gilvarg, 1974). It was proposed that inhibition of DPA synthesis in the mold was as a result of feedback inhibition by DPA: Ca complex and that the increased sporulation in the presence of calcium was not related to the DPA metabolism (Kalle and Khandekar, 1983). Implicit in this mechanism, was an assumption that action of calcium in inhibiting DPA synthesis was specific

Abbreviations used: DPA, Dipicolinic acid —2,6-dicarboxylic acid; NTG, nitrosoguanidine.

and not related directly to sporulation. In this communication, we present evidence to show that among the metals which complex with DPA, only Ca^{2+} and Zn^{2+} exhibited the ability to inhibit DPA synthesis. Furthermore, mutants selected for resistance to feedback inhibition by DPA: Ca complex also lost their sensitivity to DPA: Zn complex. Such a mutant continued to show increased sporulation and produce DPA in the presence of calcium.

Materials and methods

Growth and studies

Media, maintenance of the mold *P. citreoviride* strain NRRL 3117, growth conditions and estimation procedures were described in our earlier communication (Kalle and Khandekar, 1983). Cultures were grown in 100 ml of synthetic medium (pH 7.0) in the presence and absence of Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgCl_2$), Zn^{2+} ($ZnCl_2$), Fe^{2+} ($FeCl_2$), Cu^{2+} ($CuSO_4$) and Ba^{2+} ($BaCl_2$). All experiments were carried out at 30°C on a rotary shaker in 500 ml flasks. Accumulated products were analysed by paper chromatography with a solvent system comprising of butanol: acetic acid: water in the ratio of 12:3:5 v/v. Amino acids were reacted with ninhydrin and compared visually with standards. Aliphatic acids were visualised by spraying with aniline-xylose mixture followed by heating at 105°C for 10 min (Schwartz, 1963). The presence of citric acid was also confirmed by standard pentabromoacetate method (McArdle, 1965). The effect of citrate on DPA synthesis was checked by adding pure citric acid to the medium in the presence and absence of added Ca^{2+} to the medium.

Isolation of mutants resistant to feedback inhibition by DPA: Cα complex

Strain 3114 was grown on Sabouraud's agar medium for 10–14 days till sporulation was completed. Spores were suspended to a concentration of 10^6 per ml in 0.05 M Tris-maleate NaOH buffer (pH 9.0) containing 3 mg/ml of nitrosoguanidine (NTG) and incubated for 30 min at 30° C. Action of NTG was terminated by dilution and plating directly on several Sabouraud's agar plates. The plates were incubated for 3–4 days at 30°C and colonies were transferred by replica plated to two agar plates containing synthetic medium supplemented with 1000 ppm Ca^{2+}, added as $CaCl_2$. On one set of these plates 3 ml of 0.1% ferrous sulphate solution was gently and uniformly poured without disturbing the colonies. Within 5 min, DPA producing colonies turned pink, because of the formation of a coloured DPA: Fe complex. Colonies from the other plate were marked, purified further and checked to determine if they produced DPA when grown in the presence of 1000 ppm Ca^{2+} in the synthetic medium. The DPA produced by the mutant was purified by ion-exchange chromatography (Kalle and Khandekar, 1983) and its identity confirmed spectrophotometrically by comparison with standard DPA and also that obtained, from the parent culture. The mutant strain 27133-*dpa-ca* was chosen for further studies. It was routinely maintained on Sabouraud's agar supplemented with 1000 ppm of Ca^{2+}.

Results and discussion

Effect of divalent metals on DPA synthesis in P. citreoviride strain 3114

Among the metal ions tested only Ba^{2+} showed a partial reversal of inhibitory effects by Ca^{2+} of DPA synthesis, whereas Cu^{2+}, Zn^{2+} and Fe^{2+} did not show similar effects (figure 1).Cu^{2+} when added alone (*i.e.* in absence of Ca^{2+}) showed

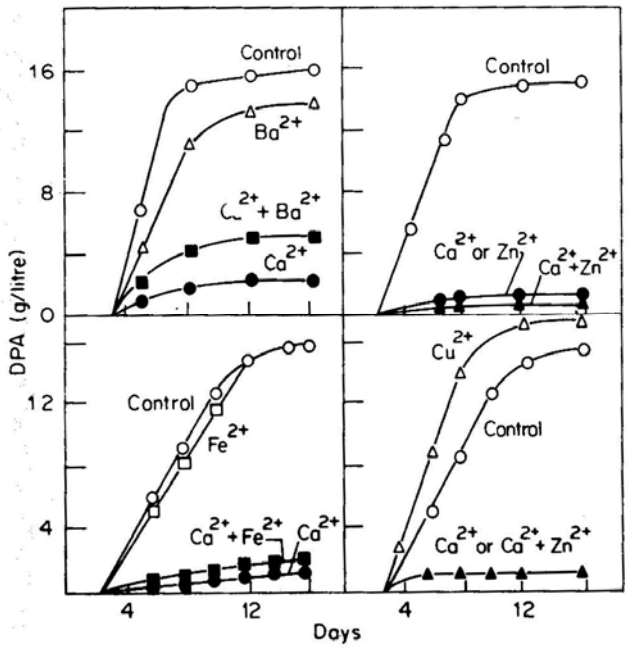

Figure 1. Influence of metal ions on DPA synthesis in *P. citreoviride* concentration of metal ions was 100 ppm each. All the flasks including the control contained 0.1 % of $MgSO_4 \cdot 7H_2O$.

stimulation of DPA synthesis and final levels reached in the medium were significantly higher than the control. The most interesting effect observed was inhibition of DPA synthesis by another metal ion Zn^{2+}, which emulated all the effects of Ca^{2+} including stimulation of sporulation in this mold. All the metal ions tested here (Ba^{2+}, Cu^{2+}, Zn^{2+}) have been shown to have a capacity to replace Ca^{2+} ions from DPA: Ca complex in bacterial spores (Gould and Dring 1974). Influence of metal ions on growth in relation to their effect on DPA synthesis is shown in the data presented in table 1. Generally, the addition of metal ions at 100 ppm did not affect adversely the growth pattern of the mold. Furthermore, on solid media, sporulation was extensive when Ca^{2+} or Zn^{2+} was present either alone or in combination with other metals. It is interesting to note that the final pH of the medium was alkaline whenever there was extensive sporulation. This has also been observed during sporulation in bacteria. In the presence ofCa^{2+} or Zn^{2+} there was a net inhibition of *de novo* DPA synthesis as indicated by low yields of this metabolite per unit increase in cell mass, and there was no net synergistic effect even under conditions when both Ca^{2+} and Zn^{2+} were present.

Table .1. Effect of metal ions on net synthesis of DPA in *Penicillium citreoviride*.

Metal ions	Final pH	DPA g/litre	DPA g/mg DW cells	Growth mg DW cells per 100 ml medium
Nil	3.8	14.0	0.27	5.4
Ca^{2+}	8.3	1.2	0.02	6.1
Zn^{2+}	8.0	0.9	0.02	5.9
Ba^{2+}	8.0	13.0	0.22	5.8
Cu^{2+}	5.2	17.1	0.36	4.9
Fe^{2+}	5.7	15.0	0.23	5.9
$Ca^{2+}+Zn^{2+}$	8.2	0.5	0.01	6.2
$Ca^{2+}+Ba^{2+}$	8.2	4.5	0.06	6.0
$Ca^{2+}+Cu^{2+}$	7.6	0.5	0.01	4.8
$Ca^{2+}+Fe^{2+}$	8.4	0.9	0.012	7.1

Concentration of all metal ions was maintained at 100 ppm. In flasks containing two metal ions, each was added at 100 ppm level. DPA and total mycellial concentrations was measured after 10 days of incubation at 30°C on rotary shaker (100 ml medium/ 500 ml flask).

Influence of calcium on accumulated products

Calcium has been shown to have an important role to play in the transport of aliphatic acids and amino acids in some molds (Ring *et al.*,1970; Hackette *et al.*, 1970; Cameron and Le John, 1972) and even in bacteria prior to events leading to sporulation (Murrell, 1967). It is possible to consider a role for calcium in which it aids the organism to retain the necessary pool of certain metabolites required for sporulation. In bacteria, there is evidence that some of the organic acids (tricarboxylic acid – cycle intermediates) accumulate prior to sporulation and these are utilised rapidly when sporulation is triggered, with the result that pH shifts rapidly to the alkaline side (Murrell, 1967). Somewhat similar conditions were observed in experiments where Ca^{2+} stimulated sporulation and inhibited DPA synthesis.

Examination of supernatant fluids from the culture in the presence and absence of Ca^{2+} showed that both qualitatively and quantitatively less number (and amount) of acids accumulated in the presence of calcium (figures 2 and 3). Only α-amino adipic acid and citric acid were identified using standards, since they have been reported to be key intermediates in biosynthesis of DPA (Tannenbaum and Kaneke1964); other amino acids were not identified. Accumulation of both these intermediates was completely inhibited in the presence of Ca^{2+} under conditions which inhibited DPA synthesis. This inhibition was not observed in the mutant 27133-*dpa-ca* which was selected for resistance to feedback inhibition by DPA:Ca complex. In order to determine if accumulation of intermediates, such as amino acids, aliphatic acids, etc. showed any correlation with pH changes and sporulation, they were monitored periodically during growth by chromatographic analysis. Relative intensities of these products were recorded (figure 3). In the absence of Ca^{2+}, both aliphatic acids and amino acids paralleled DPA synthesis and

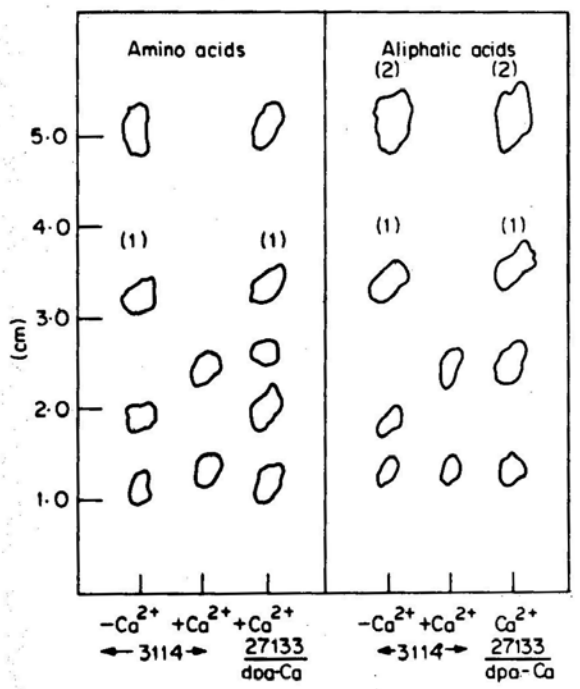

Figure 2. Chromatographic pattern of the accumulated products in growth medium of strain 3114 and 27133 *-dpa-ca*. Strains were grown in the presence and absence of 1000 ppm of Ca^{2+} for 10 days at 30°C on rotary shaker. The culture fluid after separation of mycellia was directly spotted on paper (1) α-aminoadipic acid; (2) citric acid.

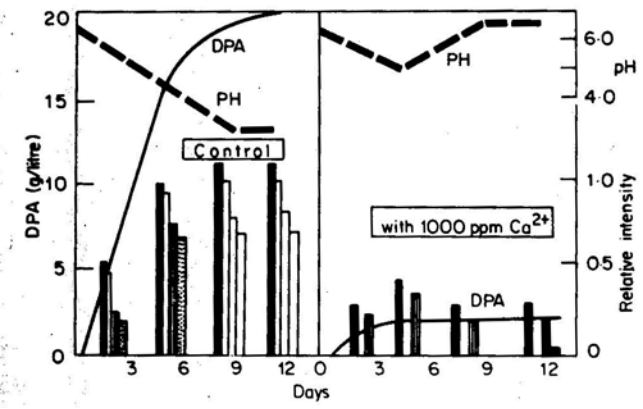

Figure 3. Accumulation of aliphatic acids and amino acids during various stages of DPA synthesis. Strain 3114 was grown in the presence of 1000 ppm Ca_{2+} and culture fluid was chromatogramed. The results are plotted as relative intensities on a 0-1 scale.

Alimphatic acids ; Citric acid
Amino acids α-Aminoadipic acid

decrease in pH. In the presence of Ca^{2+}, citric acid and α-aminoadipic acid were absent in the culture fluid throughout the growth of the organism. Other acids accumulated intially and then decreased as a function of time. The pH changes paralleled the accumulation pattern in that, it decreased initially and then increased till sporulation was complete. Zinc mimicked all the effects of calcium both in the parent strain 3114 and mutant 27133-*dpa-ca*,

Influence of citric acid on DPA synthesis

According to the pathway proposed by Tanenbaum and Kaneko (1964), citric acid is a precursor to DPA in *P. citreoviride* (see figure 8). Evidence available so far has indicated that Ca^{2+} tends to prevent accumulation of organic acids into the medium. Does it help in retention of these acids inside the cell, thus making them available for growth and sporulation ? Alternatively, does it have any role in transport of these acids into the cells ? We have attempted to answer these questions indirectly by studying the ability of the strain to use citrate in the presence of normal available carbon source-sucrose. Data are presented in figure 4. Addition of citrate

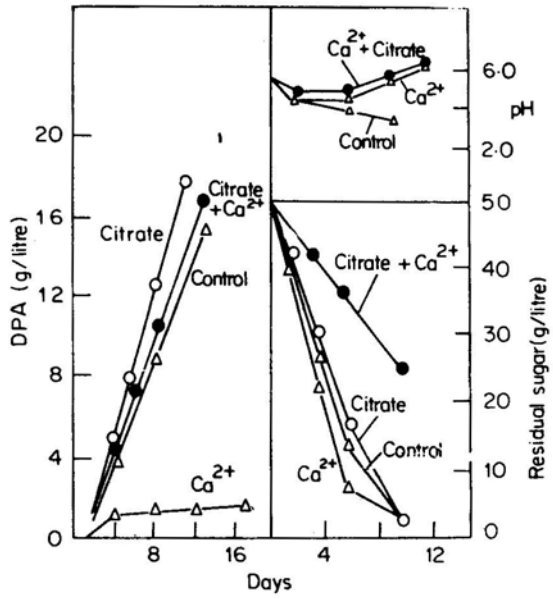

Figure 4. Influence of citric acid on DPA synthesis in the presence and absence of 1000 ppm Ca^{2+}. In the absence of Ca^{2+}, citric acid accumulation upto 3 g/litre. In the presence of Ca^{2+}, all of the citrate was used up from the medium. Concentration of citrate in the medium 10 g/litre.

or citrate with calcium had no inhibitory effect on *de novo* synthesis. In other words, citrate effectively reversed inhibitory effects of Ca^{2+} on DPA synthesis. In fact, there was a significant increase in the rate of DPA synthesis in the presence of citrate, an observation which may be explained if this metabolite was a precursor of DPA in this mold. The fact that citrate competes with sucrose in the presence of calcium is shown indirectly from the pattern observed in utilization of sucrose. The rate of sugar taken up during the DPA synthesis in the presence of calcium alone,

sucrose and sucrose with citrate was more or less the same. However, in the presence of calcium and citrate, sugar uptake was greatly reduced. Since growth rates in all the cases were the same, we feel that citrate competes with sucrose more efficiently in the presence of calcium as a carbon source, and also possibly, as a precursor to DPA synthesis.

Properties of mutant 27133-dpa-ca

The mutant 27133-*dpa-ca* differed from its parent 3114 in several properties, particularly in its response to calcium. The mutant produced DPA in the presence of 1000 ppm Ca^{2+}, but the rate of DPA synthesis and the sugar uptake were calcium dependent (figure 5). In the presence of calcium, the amount and the rates of DPA

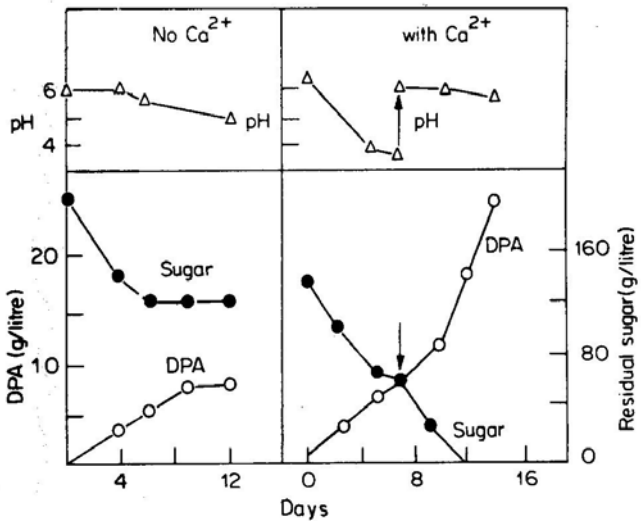

Figure 5. Effect of Ca^{2+} on DPA synthesis in the mutant. *P. citreoviride* 27133-ca-*r*-Medium contained 1000 ppm Ca^{2+} added as $CaCl_2$. In the presence of Ca^{2+}, the pH was adjusted to 6.0 with 1N NaOH on 8th day of fermentation.

synthesis were twice than that observed in the absence of calcium. In fact, there was a rapid drop in pH which had to be neutralised to enable further synthesis of DPA. Another distinguishing property of the mutant, was that unlike its parent (Kalle and Khandekar, 1983), the DPA synthesis was not subject to catabolite repression. In all these experiments, starting sugar was 15–20% at which DPA synthesis was repressed in the parent strain. Furthermore, the mutant unlike its parent sporulated only when Ca^{2+} was present in the medium. It did not sporulate in the absence of Ca^{2+}, a property not observed with the parent strain. The mutant showed resistance to inhibition by Zn^{2+} of *de novo* DPA synthesis, suggesting a common site of action for the two metal ions.

Requirement for Mg^{2+} for DPA synthesis

One of the possibilities for limitation in sugar uptake and DPA synthesis in the absence of calcium in the mutant 27133-*dpa-ca* could have been due to the increased requirement of Mg^{2+} for the mutant. A consequence of this assumption would be that calcium had a sparing action on the Mg^{2+} requirement of the strain

resulting in better rates of sugar uptake and higher DPA yields. In the case of the parent strain 3114, there is an obligate requirement of $MgSO_4$ for synthesis of DPA and at concentrations less than 0.05% of $MgSO_4$, the DPA levels in the medium were drastically reduced (figure 6). In the mutant strain, however in the presence of Mg^{2+} (0.1%$MgSO_4$), the sugar uptake and DPA synthesis came to a halt after 6 days. It resumed only when additional Mg^{2+} (0.05% $MgSO_4$) was added (figure 7). In the presence of 1000 ppm of Ca^{2+}, there was no cessation of

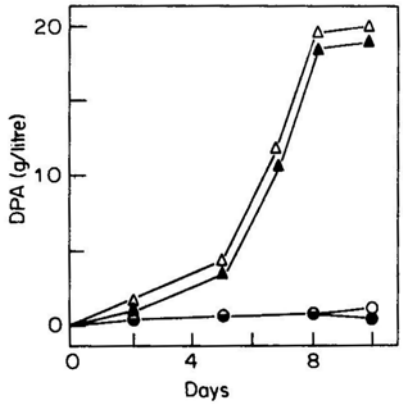

Figure 6. Effect of Mg^{2+} concentration on DPA synthesis in strain 3114. The mold was grown in synthetic medium with 0.1 % $MgSO_4$ $7H_2O$ for 6 days at 30° C. Mycellia centrifuged, washed and starved for one hour in medium without Mg^{2+}. It was then transferred to the medium containing at following concentrations of $MgSO_4$. (●),0%;(O),0.025%;(▲), 0.05% and (Δ),0.1%.

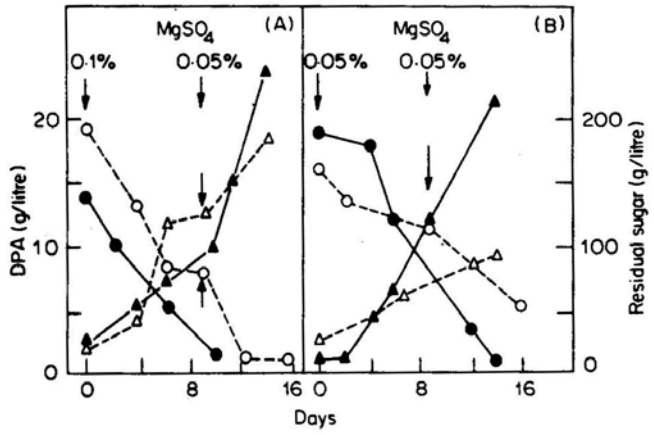

Figure 7 Effect of Ca^{2+} on magnesium requirement of the mutant 27133-*dpa-ca*. DPA(Δ), (No Ca^{2+});(▲), (Ca^{2+}: 1000 ppm); Sugar (●), (No Ca^{2+});(O), (Ca^{2+}: 1000 ppm).

DPA synthesis and in fact addition of 0.05% $MgSO_4$ showed significant improvement in DPA synthesis. When initial concentration of $MgSO_4$ was reduced to 0.05%, which was considered optimal for the parent strain, the rate of DPA synthesis was drastically reduced but improved after further supplementation of

Mg^{2+} ions. In the presence of calcium, this level of $MgSO_4$ was more than adequate for continued DPA synthesis (figure 7). This type of sparing action was not observed in the parent strain which was inhibited even at 10 ppm Ca^{2+} (Kalle and Khandekar, 1983).

Role of calcium in regulating biosynthesis of secondary metabolite DPA

Importance of trace metals in the regulation of secondary metabolite biosynthesis has been reviewed in great detail by Weinberg (1970). The metal ions may either inhibit or stimulate secondary metabolite synthesis and there is often a linear correlation between the yield of the product and the log of concentration of the metal ion. The mechanism of action of so called "key" metals has not been properly understood, and it is believed that they may influence the secondary metabolite biosynthesis by interferring at pre-transcription, transcription, as well as at post-translation level (Weinbergs 1970). We had proposed earlier that action of Ca^{2+} ions in inhibiting DPA synthesis was due to the feedback inhibition by DPA: Ca complex (Kalle and Khandekar, 1983). The data presented here substantiates this hypothesis on the basis of the following evidence.

Specificity of Ca^{2+}: All the metal ions tested here have capacity to chelate with DPA but their stability constants (log *K)* vary significantly anywhere from 10 for Cu^{2+} to 2.4 for Mg^{2+} (Murrel, 1967). Three of the metal ions Cu^{2+}, Zn^{2+} and Fe^{2+} had chelate stability for DPA greater than that of Ca^{2+}, and Ba^{2+} and Mg^{2+} had lower stability. And yet only Ca^{2+} and Zn^{2+} inhibited *de novo* DPA synthesis.

Resistance to feedback inhibition by DPA: Ca complex: The DPA synthesis in the mutant 27133-*dpa-ca* selected for resistance to feedback inhibition by DPA: Ca complex, was not inhibited by Zn^{2+} suggesting that the site of action for DPA synthesis for Ca^{2+} and Zn^{2+} were probably the same in this mold.

Inhibition of precursors of DPA by DPA: Cα complex: In the presence of Ca^{2+} and under conditions of DPA inhibition two of the metabolites, citric acid and α-aminoadipic acid were inhibited completely and selectively. Accumulation of other organic acids was inhibited only partially in the presence of Ca^{2+}. According to the pathway proposed by Tanenbaum and Kaneko (1964) both these metabolites are probably precursors of DPA biosynthesis (figure 8). Accumulation of α-aminoadipate can be explained if it is derived from α-ketoadipic acid which is not used for DPA synthesis.

Accumulation of citrate and its disappearance from the medium in the presence of calicum can be explained only if we consider citric acid as one of the precursors of DPA synthesis. Here calcium may be assisting in better citrate utilisation by the mold as evidenced from the fact that it successfully competes with sugar in supporting growth and DPA synthesis. It did not inhibit the *de novo* synthesis of DPA suggesting that the controlling site for DPA metabolism is probably between citrate and α-ketoadipic acid in the pathway. It is interesting to note that α-ketoadipic acid is a common precursor both to DPA and lysine biosynthesis and in

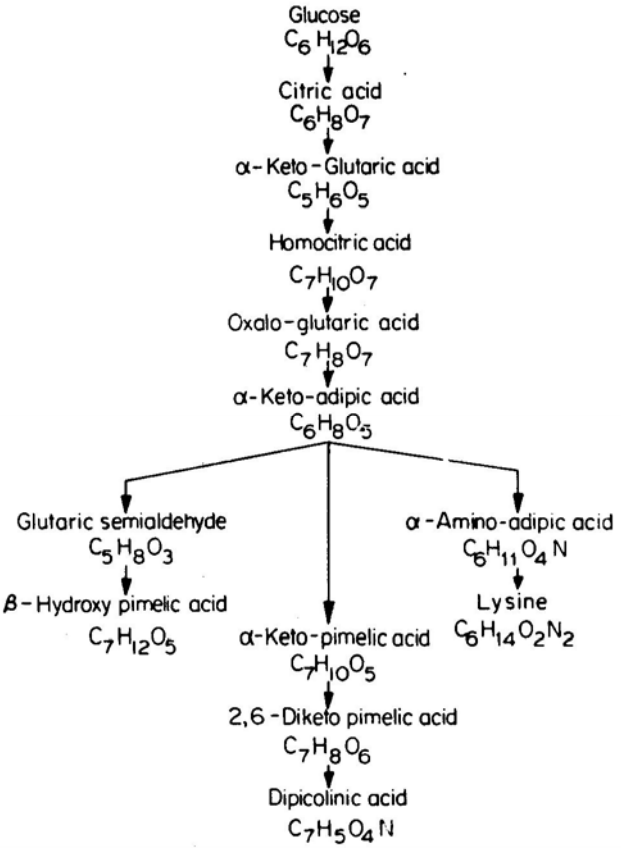

Figure 8. Proposed pathway for DPA synthesis in *P. citreoviride* adapted from Tanenbaum and Kaneko (1964).

P. chrysogenum, enzyme homocitrate dehydrogenase is the controlling enzyme specifically inhibited by lysine (Demain, 1966). It is likely that the same enzyme may also be inhibited by DPA: Ca complex.

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