INITIAL STEPS IN PYRIMIDINE SYNTHESIS IN EHRLICH ASCITES CARCINOMA[1]

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SUMMARY

The pyrimidine specific carbamyl phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase) of the Ehrlich ascites carcinoma have been found to exist as a complex with a molecular weight of 750,000-850,000 on sucrose gradients in the presence of 30% dimethyl sulfoxide (DMSO). In the presence of 10% DMSO, the activities separate and the remaining CPSase activity has a molecular weight of 150,000-200,000 while the ATCase now exists as two peaks of activity, with molecular weights of 300,00-400,000 and 525,000-700,000, depending on the exact conditions of the gradient. Dihydroorotase activity was found to co-sediment also with the two ATCase peaks under these conditions. It appears then that ATCase can be associated with CPSase and DHOase and that these first three enzymes of the pyrimidine biosynthetic pathway may exist as a complex.

INTRODUCTION

In E. coli carbamyl phosphate synthetase (CPSase) provides carbamyl phosphate (CAP) for both arginine and pyrimidine biosynthesis and is hence not the first enzyme unique to pyrimidine biosynthesis in that organism. This enzyme is feedback regulated by ornithine, a precursor of arginine, and a pyrimidine end-product. This activation and inhibition of E. coli CPSase apparently plays a significant role in the regulation of this enzyme. In Neurospora (Williams et al., 1970) and yeast (Lue and Kaplan, 1971) there

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are two CPSase's, one for arginine biosynthesis and one for pyrimidine biosynthesis. In these cells the pyrimidine CPSase has been shown to be associated in a single complex with the second enzyme in the biosynthetic pathway, aspartate transcarbamylase (ATCase), and a regulatory site. In yeast the molecular weight of the complex (in the presence of UTP, glutamine, Mg^{++}) is 800,000 with both enzymes fully sensitive to feedback inhibition by UTP. Omission of UTP from the gradient results in the two enzymes being complexed at 380,000 MW with CPSase fully inhibitable, but ATCase less susceptible to feedback inhibition. Omission of glutamine and Mg^{++} results in a CPSase of 250,000, fully insensitive to feedback inhibition, and an ATCase of 140,000 fully insensitive to feedback inhibition.

In the mammalian system the ATCase of mouse spleen (Inagaki and Tatibana, 1970) has been shown to exist in two forms on sucrose gradients. In rat, mouse, and chicken liver the ATCase was also found to exist in two macromolecular forms of approximately 600,000 and 900,000 (Koskimies et al., 1971). Jones (1971) has reported that in the Ehrlich ascites tumor cell CPSase and ATCase can exist as a complex with only one peak of ATCase activity. This communication reports the finding of a complex which contains the first, second, and third enzymes of the de novo pyrimidine biosynthetic pathway.

MATERIAL AND METHODS

Enzyme preparation: extracts of Ehrlich ascites tumor cells were prepared in 30% dimethyl sulfoxide (DMSO) and 5% glycerol as described earlier (Jones, 1971). The 30 min-100,000 x g supernatant is referred to as the crude extract. In order to study molecular weight characteristics, the enzymes were concentrated by centrifuging the crude extract for an additional four and one-half hours at 100,000 x g and 0°. This pelleted a large percentage (40-60%) of CPSase, ATCase, and dihydroorotase (DHOase). The supernatant was discarded and the pelleted material was redissolved in 1/24 its original volume of 5% glycerol, 0.05 M Tris, and 10% or 30% DMSO (pH of redissolving solution was 7.5). Appropriate molecular weight marker enzymes were added and 0.5 ml was layered on each sucrose gradient.

Sucrose gradients: The gradients were prepared by mixing equal volumes of 5 and 20% (w/v) sucrose dissolved in 5% (v/v) glycerol, 10 or 30% (v/v) DMSO, and 0.05 M Tris with a final pH of 7.5. The linear gradients had a total volume of 11 or 12 ml and were prepared at 24° or 4° and allowed to set at 4° for at least 4 additional hours. Gradients were centrifuged in an SW41 rotor at 40,000 rpm and 0° for 15-22 hours. Fractions were collected of 0.51-0.59 ml each, making a total of 20-22 fractions per gradient.

Enzyme assays: CPSase was assayed by the method of Hager and Jones (1967), ATCase as described by Bethell et al. (1968) using ^{14}C-carbamyl phosphate, and DHOase by measuring the carbamyl aspartate formed from dihydroorotate by the method of Prescott and Jones (1969). Lactate dehydrogenase (rabbit muscle, MW 136,000) was assayed by observing the rate of decrease in absorbancy at 340 nm as NADH is oxidized (Worthington Biochemical Corp., catalogue). Catalase (beef liver, MW 247,000) was measured by the disappearance of peroxide spectrophotometrically at 240 nm as described by Beers and Sizer (1952). Glutamine synthetase (E. coli, MW 592,000) was assayed by a modification of the method described by Elliott (1955).

RESULTS AND DISCUSSION

It was previously reported by Hager and Jones (1965) that when ^{14}C-bicarbonate and glutamine (both substrates for the pyrimidine CPSase) and glucose were incubated with intact Ehrlich ascites tumor cells, the only acid-soluble products accumulating are the uridine ribonucleotides. That is, none of the intermediates, carbamyl aspartic acid, dihydroorotic acid, orotic acid, or orotidine monophosphate accumulate unless orotic acid is added in addition to the substrates. This could indicate that the CPSase was the rate-limiting enzyme of the biosynthetic pathway or that the enzymes of de novo pyrimidine biosynthesis might be associated in some type of complex. The data reported here give preliminary evidence that some type of complex may exist for three of the six enzymes of this pathway.

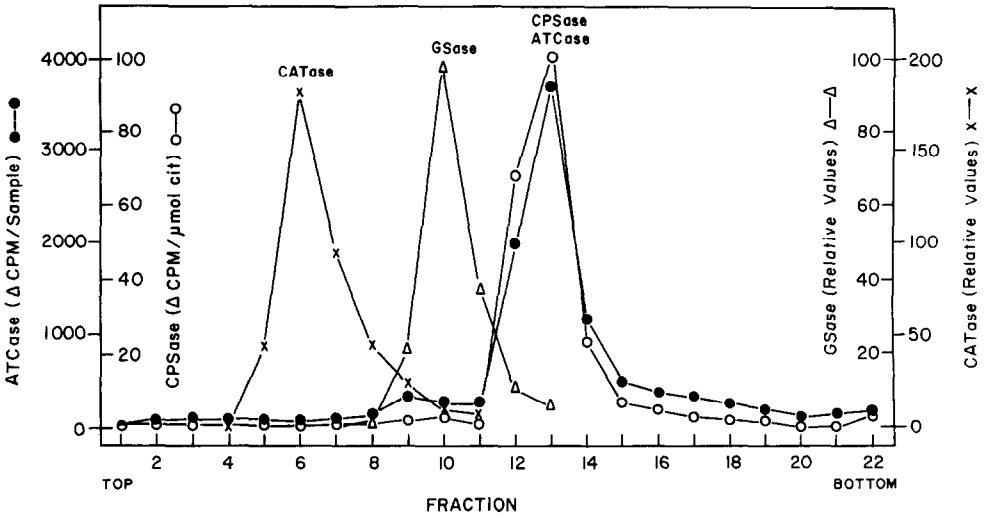

Figure 1. Sedimentation pattern of CPSase, ATCase, catalase (CATase) and glutamine synthetase (GSase) in 5-20% sucrose gradients in the presence of 5% (v/v) glycerol, 30% (v/v) DMSO, and 0.05 M Tris with a final pH of 7.5. The linear gradients (11.6 ml) were centrifuged at 0°, 17.5 hours, 40,000 rpm in a preparative ultracentrifuge using the SW41 rotor. Fractions were collected lightest first and were approximately 0.51-0.52 ml each.

Figure 1 shows a 5-20% sucrose gradient in 30% DMSO in which CPSase and ATCase co-sedimented with a molecular weight of approximately 800,000 as calculated from the marker enzymes catalase and glutamine synthetase by the method of Martin and Ames (1961). The 30% DMSO and 5% glycerol are necessary to give maximal stability to the CPSase which becomes more labile as the DMSO concentration is reduced. The aspartate transcarbamylase activity remains stable with changing DMSO concentration for the time period of these experiments.

As the DMSO concentration is reduced, the CPSase becomes markedly less stable, and dissociates from the ATCase as is seen in the sucrose gradient of Figure 2. This gradient is similar to the gradient of Figure 1 except that the DMSO concentration has been reduced to 10%. The remaining CPSase activity under these conditions has a molecular weight of 150,000-200,000. No CPSase activity is now seen associated with the ATCase. The ATCase in 10% DMSO or in 0.5% bovine serum albumin (with no DMSO) exists as two peaks

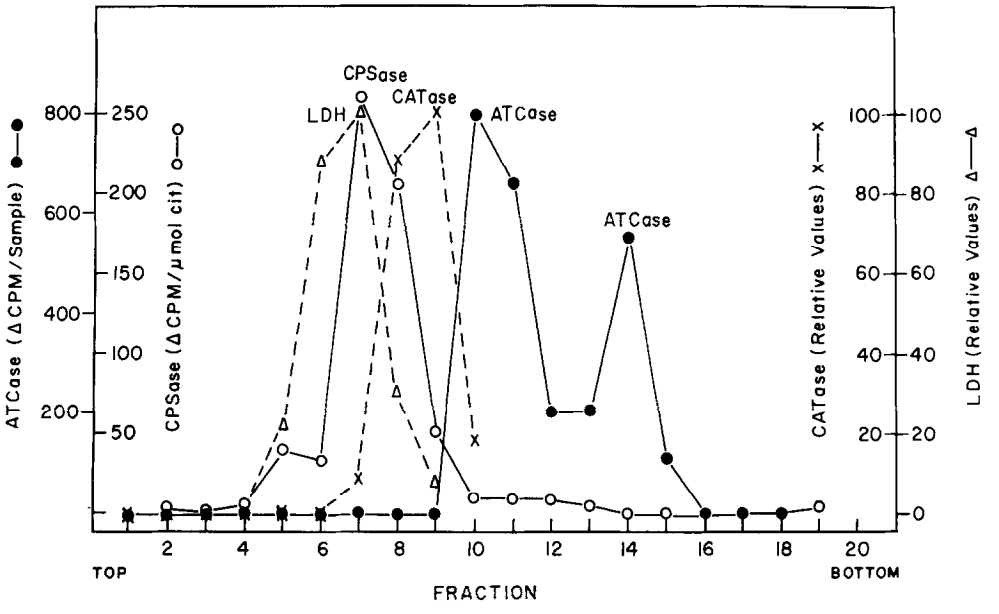

Figure 2. Sedimentation pattern of CPSase, ATCase, lactate dehydrogenase (LDH), and catalase (CATase) in 5-20% sucrose gradients in the presence of 5% (v/v) glycerol and 10% (v/v) DMSO. Urease, also used as a standard on this gradient, gave a peak around fractions 12-13. The linear gradients (10.7 ml) were centrifuged at 0°, 22 hours, 40,000 rpm in a preparative ultracentrifuge using the SW41 rotor. Fractions were collected lightest first and were approximately 0.50-0.51 ml each.

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of activity with molecular weights of 300,000-400,000 and 525,000-700,000 depending on the concentration and type of buffer used. The relative heights of the two ATCase peaks varies from preparation to preparation and the significance of this variation has not been investigated. Such variation of mammalian ATCase peaks has also been observed for rat, mouse, and chick tissues and this variation appears to be related to different physiological stages (Koskimies et al., 1971). We find both peaks of ATCase activity to be inhibited 30-40% by 10 mM cytidine triphosphate (CTP) when the CAP concentration is 10^{-4} M.

Figure 3 is essentially a repeat of Figure 2 (i.e., in 10% DMSO) except that this time ATCase and DHOase were assayed. As can be seen, the DHOase co-sediments in two peaks which are coincident with the two ATCase peaks. The two peaks are less separated in Figure 3 than in Figure 2 because of the

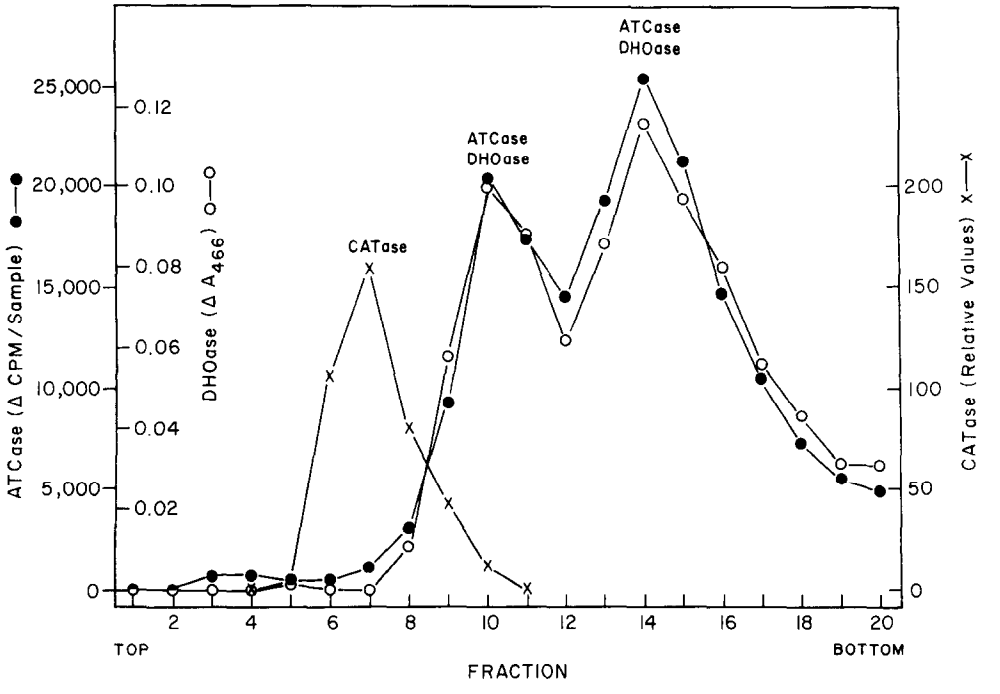

Figure 3. Sedimentation pattern of ATCase, DHOase, and catalase (CATase) in 5-20% sucrose gradients in the presence of 5% (v/v) glycerol, 10% (v/v) DMSO, and 0.05 M Tris with a final pH of 7.5. The linear gradients (12 ml) were centrifuged at 0°, 15 hours, 40,000 rpm in a preparative ultracentrifuge using the SW41 rotor. Fractions were collected lightest first and were approximately 0.58-0.59 ml each. The ATCase peaks were less well separated than in Figure 2 because of the shorter time interval used with this gradient.

shorter time of centrifugation. The estimated weight of each of the two peaks in Figure 3 (420,000; 700,000) is greater than those of Figure 2 (325,000; 525,000). This may be a result of a change in the buffer used for the two experiments as the two ATCase peaks seem to sediment differently with the type and concentration of buffer used. However, it may be that the ATCase peaks in Figure 3 contain some protein (enzyme or regulatory subunit) not present in the ATCase peaks of Figure 2.

In view of the data presented, it appears that some type of complex may exist for at least the first three enzymes of de novo pyrimidine biosynthesis in the Ehrlich ascites tumor cell. Experiments to verify this suggestion as well as whether other enzymes of pyrimidine biosynthesis might also be

a part of such a complex are in progress. We believe that these further studies will be fruitful and necessary for an understanding of the regulation of this pathway.

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