# MACROMOLECULAR FORMS OF ASPARTATE TRANSCARBAMYLASE IN RAT LIVER[1]

I.T.Oliver,* O.Koskimies,** R.Hurwitz, and N.Kretchmer

Department of Pediatrics, Division of Developmental Biology

Stanford University School of Medicine

Stanford, California

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Summary. Zonal centrifugation of crude extracts of rat liver shows the presence of two major components of aspartate transcarbamylase, M.W. 900,000 and 600,000. The sedimentation behavior is unaffected by treatment with RNase and detergents. The activity of the ATCase is stabilized in the presence of bovine serum albumin. Experiments in the presence of dithiothreitol or EDTA show that the heavy components do not result from complex formation with albumin.

Aspartate transcarbamylase (ATCase), an enzyme characteristic of de novo pyrimidine biosynthesis, is distributed ubiquitously in nature. During mammalian and avian development ATCase and other enzymes of the de novo pyrimidine biosynthetic pathway progressively decrease in activity as the animal matures (1,2). A striking example is encountered with rat heart, where activity of ATCase falls from an elevated value in fetal heart to a negligible level in the neonatal organ (1). A similar phenomen is encountered with rat liver, except that there is low activity in adult liver, which is stimulated considerably in response to partial hepatectomy (3).

Studies of the regulatory aspects of ATCase in mammalian tissue have been difficult to interpret (4,5,6) since the enzyme has not been sufficiently purified. ATCase has been purified from bacteria (7), neurospora (8), and

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*Present address, Department of Biochemistry, University of Western Australia, Nedlands, Western Australia.

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yeast (9). From each of these organisms ATCase has been isolated as a macromolecule which can be dissociated into enzymatically active smaller units.

ATCase and carbamyl phosphate synthetase were co-purified from yeast in macromolecular aggregates with an average molecular weight (M.W.) of 600,000 (9). The complex dissociated into units of M.W. 300,000 when UTP was removed. Finally, after passing through a column of Sephadex the M.W. of the unit was reduced to 140,000, in which only ATCase activity was detected.

On the basis of these previous investigations, we subjected extracts of rat liver (LE) to zone sedimentation analysis. ATCase was found in association with macromolecular components, the major peaks having M.W. of 900,000 and 600,000.

## METHODS

Homogenates were prepared from freshly excised rat liver in a Teflon-glass homogenizer with 4 ml per gm of liver of 0.25 M sucrose-TKM (0.05M Tris-HCl, pH 7.5; 0.025 M KCl; and 0.005 M $MgCl_2$). The homogenates were centrifuged at 75,000 x g for 1 hr. at $4^{\circ}$ and the supernatant fluid was used as the liver extract (LE).

Zone centrifugation was carried out in 2" x ½" polyallomer tubes in the SW65 Ti rotor of the Spinco L2-65 ultracentrifuge. Linear sucrose density gradients (20%-30% sucrose) of total volume 4.8 ml were prepared using the Beckman density gradient former. Where specified, TKM buffer and additives were maintained at a constant concentration throughout the sucrose gradient.

For sedimentation analysis, 200 µl of LE were layered on top of the gradient and the tubes centrifuged at 65,000 rpm (300,000 x $g_{av}$) for 3-5 hours (exact times are noted for particular experiments). The rotor was accelerated on the automatic mode, the timer set when full speed was attained, and the centrifuge decelerated without braking. The contents of each tube were fractionated by puncturing the bottom of the tube with a needle and fractions were collected containing 9 drops (0.15 ml).

The assay for ATCase was a modification of the method described by

Porter et al (10). Each fraction (0.15 ml) received 75 µl glycylglycine buffer (0.5 M, pH 9.2); 30 µl $^{14}$C-aspartic acid, 300,000 cpm in 0.1 M potassium aspartate (a dilution of uniformly labeled $^{14}$C-aspartic acid, New England Nuclear or Armersham/Searle, 8.7mC/mMole); and was preincubated for 5 min. at 37°. The reaction was initiated by addition of 75 µl of freshly dissolved dilithium carbamyl phosphate, 56 mMoles, and incubated for 15 min. Carbamyl aspartate was formed at a linear rate for at least 30 minutes. The reaction was stopped by pipetting 0.2 ml of reaction mixture into a 2 ml pool of 0.5M acetic acid placed on top of a 1.2 x 1.5 cm column of Dowex 50 W-X8 ($H^+$form). The column was washed with 3 x 1 ml of water and all the effluent was collected in a centrifuge tube. To each tube was added 0.5 ml of 50% trichloracetic acid and the volume adjusted to 6.0 ml with water. After mixing, the tubes were centrifuged and 1.0 ml aliquots of supernatant fluid were transferred to glass scintillation vials to which 10 ml of Bray's solution (11) were added. The radioactivity was determined in a Packard Tri-carb liquid scintillation spectrometer Model 2003. An aliquot of the effluent was evaporated to a small volume and subjected to thin layer chromatography (12). Carbamyl aspartate was the predominant product with, possibly, a small amount of dihydroorotate.

Pyruvate kinase was assayed using a modification (13) of the method of Bücher and Pfleiderer (14). 0.1 M Tris-HCl buffer, pH 7.5, was used instead of phosphate buffer. Protein was estimated at 290 mµ in the fractions adjusted to 0.1 M KOH.

## RESULTS

Negligible activity of ATCase was detected in the nuclear or mitochondrial fractions of liver. Centrifugation of the post-mitochondrial supernatant fluid at 100,000 x g for one hour showed that less than 5% of the total activity of ATCase was concentrated in the pellet.

Activity of ATCase in the supernatant fluid was unchanged after storage at 4° for as long as 36 hours. In the first experiments, preparations sub-

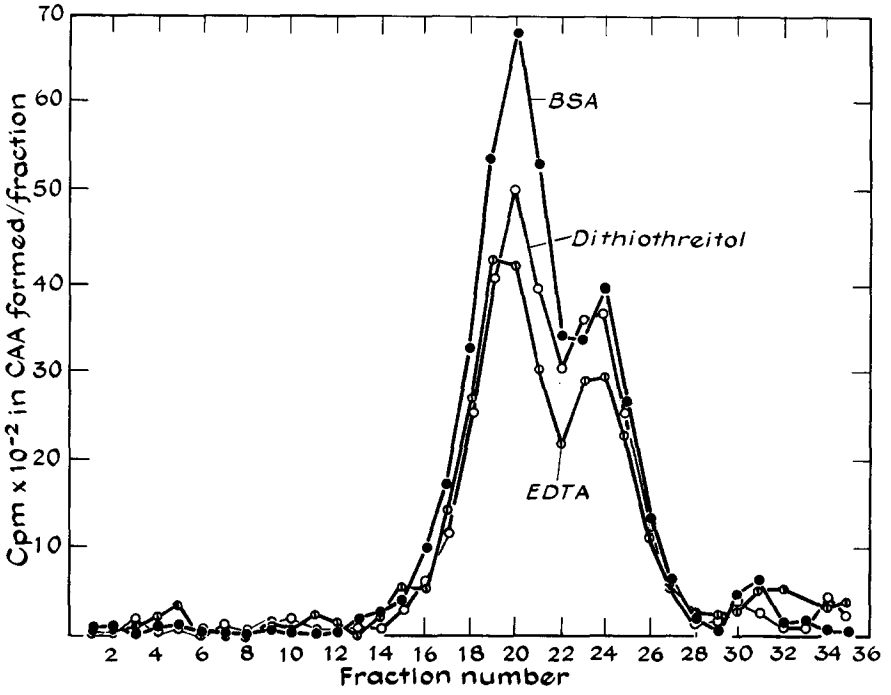


Figure 1. Sedimentation pattern of rat liver ATCase in the presence of protective agents: The fractions were obtained from 20-30% (Density: 1.074-1.113) sucrose density gradients of newborn-rat-liver extract centrifuged at 300,000 x $g_{av}$ for 3-1/3 hours. The highest density, 1.113, is fraction 1. The protective agents throughout each gradient were 0.5% BSA, EDTA ($10^{-3}$M) or dithiothreitol ($10^{-3}$M) as indicated. For assay, the enzyme was incubated for 15 minutes.

jected to zonal centrifugation showed no activity in any of the fractions. Addition of bovine serum albumin (BSA) to a concentration of 0.5% throughout the gradients resulted in 70-90% recovery of the initial activity. Dithiothreitol ($10^{-3}$M) and EDTA ($10^{-3}$M) when added throughout the gradient were partially effective in reducing loss of activity (55% and 45% recovery respectively). A combination of protective agents did not improve on the results obtained with the addition of BSA alone. Consequently, all other experiments were conducted in the presence of BSA.

The results shown in Fig. 1 indicate the presence of two major components which were obtained with LE from rats two days of age, regardless of the protective agent in each of the three preparations. The major portion of the

protein (91%) was located in the last five fractions (30 to 35).

Zonal centrifugation of the LE at 300,000 x $g_{av}$ for 5 hours indicates that the sedimentation velocities of liver ATCase are considerably higher than that of E. Coli ATCase (M.W. 310,000) run simultaneously (Fig. 2). Comparison of bacterial ATCase with purified rabbit muscle pyruvate kinase (M.W. 240,000), centrifuged under the same conditions, showed that the bacterial ATCase was not dissociated into sub-units. From these experiments the molecular weights of the two rat LE ATCase peaks were calculated at 900,000 and 600,000 (15,16). The sedimentation pattern for ATCase of rat liver was similar in all age groups studied, from the 17 day-old fetus through normal adult. The pattern is the same with freshly prepared LE and with LE stored 12 hr at 4° prior to zonal centrifugation.

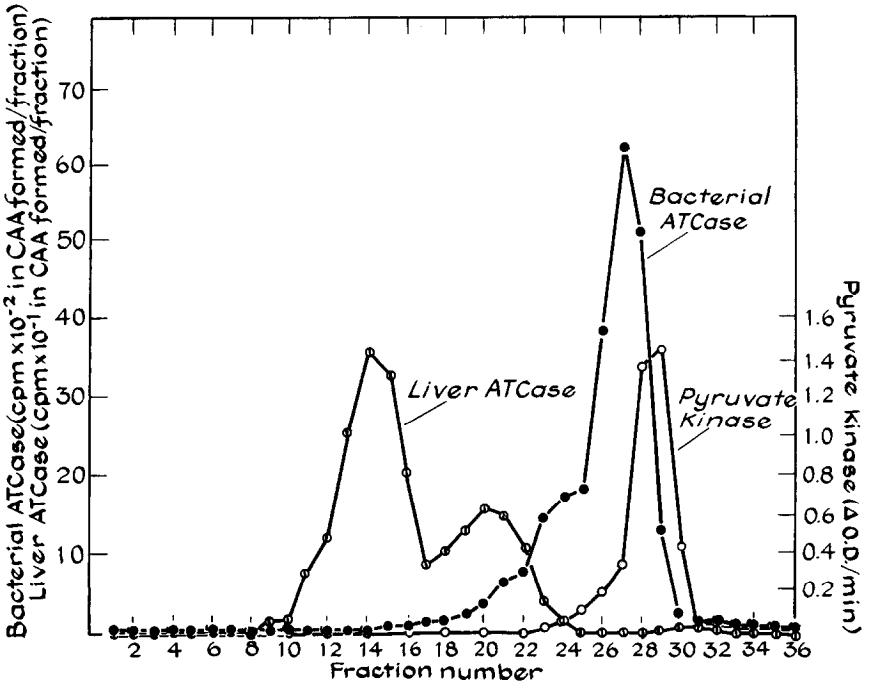


Figure 2. The relative distribution of rat-liver-ATCase, purified bacterial ATCase, and pyruvate kinase in sucrose density gradients: The fractions are derived from a 20-30% sucrose density gradient containing 0.5% BSA, centrifuged at 300,000 x $g_{av}$ for 5 hours. ATCase in fractions from the gradient with neonatal rat liver was incubated for 15 minutes at pH 9.2 and from the gradient of E. Coli ATCase for 5 minutes at pH 7.0. Pyruvate kinase was assayed as described using 50 µl aliquots from each fraction.

Preincubation of LE with RNase (500 μg/ml) for 2 hr at 4° did not alter the sedimentation properties. Preparation of liver homogenates in 0.25M sucrose containing hexadecyltrimethylammonium bromide (0.08%), TRITON X-100 (0.1%), NON-IDET P-40 (0.5%), or potassium phosphate (0.05M, pH 7.5) had no effect on the subsequent sedimentation profile. Addition of sodium deoxycholate and sodium dodecyl sulphate (0.5%) resulted in complete loss of activity.

When a solution of the enzyme precipitated from the extract with 30% saturated ammonium sulphate was subjected to zonal sedimentation, a pattern typical of a polydisperse system was observed.

## DISCUSSION

The results of zone sedimentation analysis of liver ATCase indicate that the enzyme exists in at least 2 macromolecular forms, both of which are considerably larger than bacterial ATCase. The heavier and predominant form has an approximate M.W. of 900,000, while the lighter form is about 600,000. Since the sedimentation behavior is similar in the presence of BSA, dithiothreitol, or EDTA, it is unlikely that artifacts have arisen due to the formation of a complex with BSA as described by Porter et al (10) for the catalytic subunit of the ATCase of E. Coli.

With an extended time of centrifugation both peaks of ATCase shifted and there was no evidence of banding to density equilibrium characteristic of lipid-containing-membrane particles. Treatment with 3 different detergents did not alter the sedimentation characteristics of the enzyme. Incubation with RNase was also without effect and thus the enzyme is probably not associated with RNA.

Both components of ATCase can be demonstrated in extracts of several other mammalian and avian tissue preparations (17).

The data presented in this paper indicate that rat liver ATCase occurs in at least two forms of high molecular weight. These macromolecules may possess the organizational complexity necessary for regulatory function.

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