PROBLEMS INTERPRETING THE SIGNIFICANCE OF MULTIPLE FORM PATTERNS OF RAT LIVER TYROSINE AMINOTRANSFERASE

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Summary. Cortisol induced tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5) of rat liver cytosol can be resolved into several peaks of activity by CM-Sephadex chromatography. These separable activities have been considered distinct molecular species and are referred to as multiple forms of this enzyme. The present study demonstrates that a number of in vitro manipulations considerably alter the pattern of multiple forms of tyrosine aminotransferase. These include homogenization of the liver in buffers of varying ionic strength and pH, freezing and thawing, and sonication. Based on these findings we conclude that one must proceed cautiously when attempting to relate multiple form patterns observed in vitro to the form(s) of the enzyme in vivo.

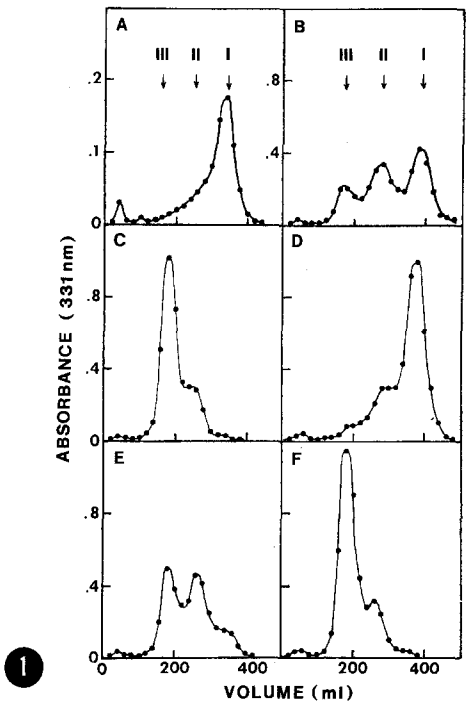
Several reports have indicated that tyrosine aminotransferase exists in multiple forms and that the proportion of each form can be affected by various hormones and changes in diet (1-8); also, that these forms are interconvertible and that one may be the substrate for the inactivating system (8). Although this is an attractive hypothesis, the observations presented here indicate that the distribution of these multiple forms can be altered by several in vitro procedures. It is the purpose of this communication to stress that caution should be observed in interpreting the significance of multiple forms of rat liver tyrosine aminotransferase, and, perhaps, of multiple forms of other enzymes as well.

Materials and Methods. Animals--Adrenalectomized male rats of the Sprague-Dawley strain, weighing about 200 g, were obtained from the Charles River Laboratories (Wilmington, Massachusetts). Animals were maintained on laboratory chow and 0.9% NaCl drinking water for several days. Three to four hours prior to use each animal received 5 mg/100 g body weight of hydrocortone phosphate (Merck, and Co., West Point, Pa.) intraperitoneally. After sacrifice, the livers were perfused with ice cold saline, removed, and blotted to remove excess fluid. Preparation of liver for column chromatography--Approximately 1 g of liver was treated as indicated in the legends of each figure and the extract was centrifuged at 4° for 35 min at 105,000 x g. A 1 ml aliquot of the supernatant was diluted appropriately with ice cold distilled water to an ionic strength of 0.05. Depending on the initial buffer concentration, from 1 to 25 ml was used to charge a CM-Sephadex (C-50) column.

Preparation of CM-Sephadex columns--Usually, 6 samples were analyzed simultaneously. The morning or night before a run was to be made, 12 g of dry CM-Sephadex (C-50) were hydrated with 700 ml of buffer A that contained 50 mM potassium phosphate (pH 6.5); 2.5 mM α-ketoglutarate; 1 mM EDTA; and 1 mM β-mercaptoethanol. The slurry was mixed slowly with a magnetic stirrer for 10-15 min at room temperature and allowed to settle for about 1 hr; after which about 60 to 70 ml of the CM-Sephadex layer was poured into each of 6 columns (2 x 40 cm) having coarse fritted-glass filters covered with about 2 cm height of acid-washed sand. The columns were allowed to settle for about 30 min and then drained. About 125 ml of buffer A were added and the columns equilibrated for several hours at 4°. Each column, containing about a 20 cm height of CM-Sephadex, was drained again and charged with the diluted extract. Unbound protein (about 50 % of the total protein) was washed through the column with about 50 ml of cold (4°) buffer A. A linear gradient of 200 ml buffer A and 200 ml of buffer A + 0.4 M KCl was used for eluting tyrosine aminotransferase from each column. Approximately 22 fractions containing about 20 ml each were collected over a 6-7 hour period from each column. Our experience with hundreds of such columns has been that 85% or more of tyrosine aminotransferase charged is recovered. Similarly treated samples from the same animal yielded elution patterns that were reproducible within $\pm$ 2%. That is, the areas under each peak of activity in duplicate columns showed very little variation.

Tyrosine aminotransferase activity--Aliquots of original liver extracts (0.015 ml, 5 min) and fractions from columns (0.5 ml, 20 min) were assayed according to the procedure of Diamondstone (9). Enzyme activity is expressed on a relative basis as absorbance (331 nm) or as p-hydroxyphenylpyruvate formed per hour per unit of sample.

Results. Effects of pH and ionic strength of the homogenizing buffer on distribution of multiple forms of tyrosine aminotransferase--Homogenates of rat liver prepared 3-4 hours after administration of hydrocortisone phosphate (5 mg IP/100 g body weight) can be shown to contain at least three reproducibly separable forms of tyrosine aminotransferase following CM-Sephadex column chromatography. The distribution of enzyme activity in each peak, however, can be altered readily simply by varying the composition of the homogenizing medium. The data in panels A, B, and C of Fig. 1 demonstrate a shift from enzyme form I to form III, when rat liver samples are broken with an electrically driven glass-teflon tissue homogenizer in increasingly concentrated potassium phosphate buffers having the same pH (6.5). It should be noted that the ordinate scale of panel A is one-fourth that of the other panels. In this experiment, there was approximately a 4-fold difference in total soluble tyrosine aminotransferase activity between liver samples homogenized in 0.01 and 0.05 M buffer (compare panels A and B, Fig. 1). Following homogenization in buffers of low ionic strength, about 80% of the enzyme remains



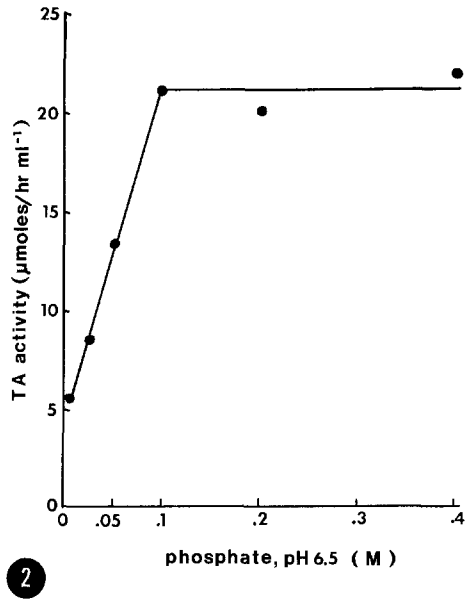


Fig. 1. The effects of homogenizing liver samples in various buffers on tyrosine aminotransferase multiple form patterns as analyzed by CM-Sephadex chromatography. Six 1 g samples of liver were obtained from a hydrocortone phosphate treated animal as indicated in Materials and Methods. Homogenization of each sample was carried out at 0° in glass-teflon tissue grinders in the following media: panel A, 4 ml 0.01 M potassium phosphate, pH 6.5; panel B, 4 ml 0.05 M potassium phosphate, pH 6.5; panel C, 4 ml 0.25 M potassium phosphate, pH 6.5, panel D, after homogenizing in 0.01 M potassium phosphate, pH 6.5, as in panel A, 1.22 ml of 1 M potassium phosphate (pH 6.5) was added to make the final potassium phosphate concentration 0.2 M; panel E, 4 ml of 0.03 M potassium phosphate, pH 7.4; panel F, 0.05 M potassium phosphate, pH 7.4. Following homogenization, each was centrifuged for 35 min at 105,000 x g at 4° . One ml aliquots were brought to an ionic strength of 0.05 with ice cold distilled water and chromatographed on CM-Sephadex columns. Total enzyme activity charged (μmoles/hr/ml) : A, 11.8, B, 55.1; C, 74.3;, D, 69.4; E, 64.3; F, 88.1.

Fig. 2. Ionic strength-dependent solubilization of rat liver tyrosine aminotransferase. Six g of liver from an animal treated with hydrocortone phosphate (as indicated in Materials and Methods) were homogenized in a glass-teflon tissue grinder with 24 ml ice cold 0.01 M potassium phosphate buffer, pH 6.5. To 1.2 ml aliquots of this homogenate was added varying amounts of ice cold 1 M potassium phosphate (pH 6.5) to give molar concentrations of 0.01, 0.025, 0.05, 0.1, 0.2 and 0.4 when each was brought to a final volume of 2.0 ml. Each sample was centrifuged at 4° for 35 min at 105,000 x g and an aliquot of the supernatant fluid assayed for tyrosine aminotransferase activity.

bound to particulate structures, but is readily released as the solute concentration is increased (Fig. 2). The multiple form pattern of released enzyme is dependent on the ionic strength of the medium in which the liver cells are initially broken. When liver homogenates were prepared initially in 0.01 M phosphate buffer, pH 6.5,

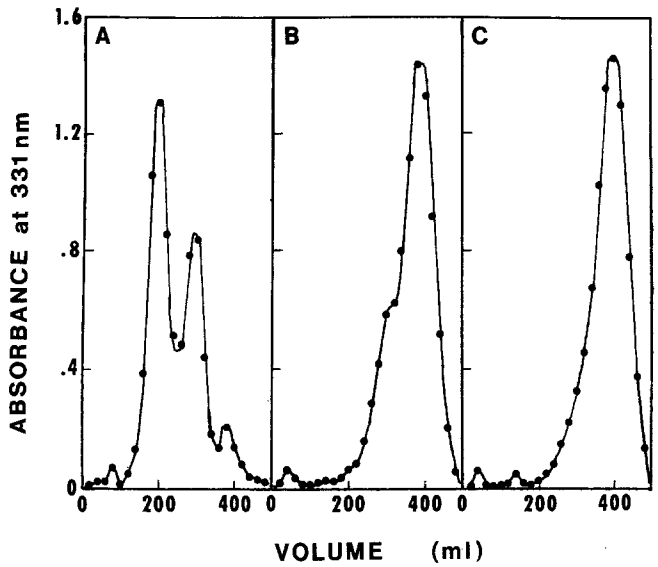


Fig. 3. Effect of freezing and thawing on tyrosine aminotransferase multiple forms as analyzed by CM-Sephadex chromatography. Two grams of liver from a hydrocortone phosphate induced rat was homogenized in 8 ml of 0.05 M potassium phosphate buffer (pH 7.6) containing 0.07 M potassium chloride (final pH 7.4; final ionic strength, about 0.15). Half the homogenate was frozen in liquid nitrogen and then thawed at 20°. Both samples were centrifuged for 35 min at 105,000 x g at 4° and 1 ml of each supernatant chromatographed on CM-Sephadex columns. Panel A, control (187 μmoles/hr/ml); panel B, frozen-thawed sample (176 μmoles/hr/ml): Another 1 g sample of the same liver was frozen directly in liquid nitrogen, thawed at room temperature; and then macerated with a cold porcelain mortar and pestle. The resultant paste was extracted with 4 ml of the above buffer, centrifuged, and 1 ml of the supernatant (167 μmoles/hr/ml) chromatographed (panel C). In all cases the ionic strength was adjusted to 0.05 prior to charging the sample on the column.

(as in panel A. Fig. 1) and then brought to 0.2 M, pH 6.5, an increase in total tyrosine aminotransferase activity was observed as described above, but the enzyme was predominantly in form I (panel D, Fig. 1). Homogenates prepared directly in 0.25 M potassium phosphate, pH 6.5, yielded enzyme mainly in form III (panel C, Fig. 1). Similar changes were observed in homogenates prepared in 0.2 M sucrose or KCl (pH ranged between 6.5-6.7) indicating that increased phosphate was not essential for the changes observed. Form III predominated not only when homogenates were prepared in solutions of high solute content, but also when pH was increased (and ionic strength kept within narrow limits, compare panels B, E, and F, Fig. 1). Effect of freezing and thawing, and sonication--Liver homogenates prepared in 0.05 M phosphate buffer (pH 7.4) containing potassium chloride (0.07 M) were shown to contain tyrosine aminotransferase predominantly as form III (panel A, Fig. 3) Quick freezing of the liver, either before or after homogenizing in the same buffer, caused almost all the enzyme to shift to form I (panels B and C, Fig. 3).

Liver slices disrupted by short bursts of sonic oscillation in buffers of

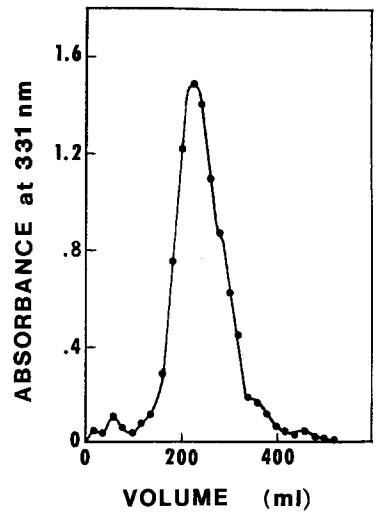


Fig. 4. CM-Sephadex chromatography of rat liver exudate. Slices of rat liver obtained from a hydrocortone phosphate induced animal were crushed between glass slides. As much of the broken tissue mass as possible was transferred by Pasteur pipet to a centrifuge tube and spun for 35 min at 105,000 x g. The small supernatant volume was carefully removed and added to 4 ml ice cold distilled water. This entire volume was charged onto a CM-Sephadex column and analyzed as indicated in Materials and Methods.

varying ionic strengths produce essentially the same multiple form patterns as those observed after homogenization in glass-teflon tissue grinders (see panels A, B, and C of Fig. 1). However, if the initial sonicate, which was prepared in 0.05 M phosphate buffer (pH 6.5), was centrifuged and the supernatant fluid exposed again to three 15 sec sonic bursts, a shift in the multiple form pattern of tyrosine aminotransferase was observed. No such shift was seen when these procedures were carried out at pH 7.4 instead of 6.5.

Enzyme pattern in intact cell--By the very nature of the information sought, it is difficult to say with certainty what forms of the enzyme, as presently described, actually occur in the hepatocyte. To gain some insight into this question liver slices were crushed between glass slides and the contents transferred to centrifuge tubes. After centrifuging for 35 min at 105,000 x g the small supernatant volume was chromatographed in the usual way. The data in Fig. 4 indicate that the enzyme obtained by this procedure was almost entirely form III.

In other experiments, biopsy samples of the liver were taken from pentobarbital anesthetized rats prior to, and 3 and 9 hours after glucocorticoid administration and treated in a manner similar to that described above. At each time the enzyme pattern resembled the one shown in Fig. 4 indicating predominantly one form of the enzyme (form III).

Discussion. Separable zones of tyrosine aminotransferase activity have been

observed following disc gel electrophoresis (3), and hydroxyapatite (4,5) and CM-Sephadex column chromatography. Although it can be interpreted that these activities arise from separate genes, Johnson et al. (8) presented evidence that these enzyme activities were not isozymes, but post-translational modifications of a single gene product. Their data indicated that: 1) forms I, II, and III of tyrosine aminotransferase had about the same molecular weight; b) all reacted equally with antityrosine aminotransferase antibodies; c) all had similar inactivation temperatures; d) all had similar specific activities; e) forms I and II were quantitatively converted to form III in liver homogenates prepared in 0.05 M potassium phosphate (pH 6.5) containing 10 mM potassium cyanate; and f) forms II and III were quantitatively converted to form I when the supernatant fraction from a homogenate prepared in 0.05 M potassium phosphate was incubated at 25° for 30 min. Based on such data, and additional observations that indicated multiple form patterns of tyrosine aminotransferase were changed following hormone induction, these investigators indicated that turnover of this enzyme may be related to the proposed post-translational modification process (8).

The present data demonstrate that multiple form patterns can be easily altered by varying the medium in which the liver is homogenized, and by procedures commonly used to disrupt cells such as sonication and freeze-thawing. As clearly shown in Fig. 1, elevating ionic strength or pH of the homogenizing medium yields predominantly form III of tyrosine aminotransferase, while lowering these parameters favors form I. Of the buffer concentrations tested, 0.25 M potassium phosphate is probably closest in ionic strength to that found within the cell. This would suggest, then, that form III should predominate *in vivo*. Breaking liver cells between glass slides and analyzing the exudate indicated that most of the enzyme was in this form (Fig. 4). Therefore, there is no substantial reason to believe, as has been indicated elsewhere (8), that form I of tyrosine aminotransferase is the immediate gene product. Indeed, there is reason to question whether multiple forms of this enzyme exist, as presently envisioned, in significant amounts *in vivo*. This uncertainty is strengthened by our observations that multiple form patterns of tyrosine aminotransferase are not distinctly apparent after hormone administration when cell exudates of biopsy samples (prepared by crushing pieces of liver between glass slices) are analyzed by CM-Sephadex column chromatography.

Why, then, are distinctly different multiple form patterns of tyrosine aminotransferase observed under certain conditions, and how can one explain previously reported alterations of these patterns following changes in diet or hormone administration? Unfortunately, we cannot resolve these questions at this time. We wish to stress, however, that *detailed* conditions of tissue preparation are necessary to allow evaluation and comparison of data from different laboratories. It is apparent that problems exist in interpreting multiple form patterns of tyrosine aminotransferase.

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