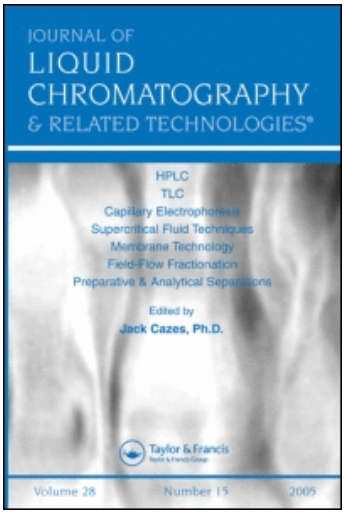

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Assay of the Enzymes of Pyrimidine Synthesis by Thin Layer Ion Exchange Chromatography and Video-Densitometry (CV-Technique)

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ASSAY OF THE ENZYMES OF PYRIMIDINE SYNTHESIS BY THIN LAYER ION EXCHANGE CHROMATOGRAPHY AND VIDEO-DENSITOMETRY (CV-TECHNIQUE)

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ABSTRACT

A simple and rapid method is described for the assay of the glutamine-dependent carbamoyl phosphate synthetase and aspartate carbamoyltransferase in minute amounts of liver tissue. From 60-120 µl assay mixtures 10-20 µl aliquots were chromatographed on thin layer ion exchange chromatoplates, and the amino acids were developed with ninhydrin. The intensity of the ninhydrin spots, i.e. the quantity of amino acids formed or consumed, was determined by video-densitometry and the specific activity of the enzymes was calculated.

INTRODUCTION

The activity of enzymes in tissue and cell extracts is usually determined by techniques requiring 1 ml or even a larger quantity of assay mixture and, obviously, a proportional amount of tissue extract. However, the available amount of the tissue sample to be assayed is often limited. In clinical diagnostics, for example, the tissue samples obtained from adult patients as well as that taken at early stages of fetal development often weigh only a few times 10 mg.

In previous investigations we have found (1, 2) that the activity of amino acid-transforming enzymes in rela-

tively small samples can be determined by the highly sensitive thin layer ion exchange chromatographic and videodensitometric procedure (CV-technique).

Since this technique has been found to be suitable for the kinetic investigation of ornithine carbamoyltransferase as well (2), we thought it would be worth examining other amino acid-transforming enzymes, too.

The present paper describes a method for the assay of aspartate carbamoyltransferase (EC 2.1.3.2) and carbamöyl phosphate synthetase (glutamine-dependent, EC 2.7.2.9.), enzymes participating in the de novo synthesis of the pyrimidines. They have an important role in the metabolism of fast growing, e.g. embryonic, regenerating, tumorous, hypertrophic, etc, tissues.

MATERIALS AND METHODS

Preparation of Cytosol Extracts from Liver Cells

CFY rats, weighing 200-250 g, were starved for a day and fed with water ad libitum, before the experiments. They were bled under ether anaesthesis. The livers (7-8 g) were rapidly removed and kept on ice until use.The samples were homogenized in a Potter-type tephlon homogenizer at $0^{o}C$ with 3 volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 500 μM EDTA and 5 mM dithiotreitol. The homogenate was centrifuged at 15 000 x g for 30 min to remove the debris and mitochondria. The resulting cytosol fraction was concentrated 5-fold on an Amicon Centriflo 50 CF membrane (Oosterhout, The Netherlands) by centrifu-

gation. The protein content of the concentrated samples was determined according to Lowry et al. (4).

Ornithine Carbamoyltransferase

Ornithine carbamoyltransferase was used for the determination of carbamoyl phosphate synthetase activity in a coupled reaction. The enzyme was isolated from bovine liver as described by Marshall and Cohen (5). The specific activity of the purified preparation was 420 mmol citrulline/hr/mg protein.

Determination of Enzyme Activity

For the determination of the activity of the enzymes the substrate and the product/s/ formed in the assay mixture were first separated by thin layer ion exchange chromatography on 20x20 cm chromatoplates (Fixion 50x8, Chromatronix, Palo Alto, CA, USA). The Dowex 50x8 type resin-coated chromatoplates were used either in Na^+ or in Li^+ form. Plates in Li form were prepared by prechromatography in 0.5 M LiCl solution (6). The assay mixtures were incubated at 37°C, then 10-20 µl samples were added to equal amounts of 10 % trichloroacetic acid drops,which had been applied onto the chromatoplate beforehand. The plates were dried by hot air, and were then chromatographed in the solutions descibed below.

For comparison the activity of aspartate carbamoyltransferase was determined also by the colorimetric procedure as described by Prescott and Jones (7), except that deacetylmonoxim-thiosemicarbazide was used instead

of diacetylmonoxim-antipyrine, and the absorption was measured at 520 nm (8).

Solutions Used for Chromatography

For the determination of aspartate and citrulline the chromatoplate was used in Na^+ form. Chromatography was carried out at 50°C in citrate buffer, pH 3.3, composed of 84 g of citric acid, 16 g of NaOH and 5.9 ml of concentrated HCl in 1000 ml final volume (9).

To measure the amount of glutamate chromatography was performed at 50°C on chromatoplates in Li^+ form in a lithium-citrate-formate system composed of 14.1 g of LiCl, 2.3 ml of 85 % formic acid and 8.0 ml of concentrated HCl in a final volume of 1000 ml at pH 2.8 (6).

After chromatography the plates were dried and the amino acids were developed with cadmium-ninhydrin reagent. The reagent was prepared freshly before use by mixing 100 ml of 1 % ninhydrin dissolved in acetone and 20 ml of 1 % cadmium-acetate dissolved in 10 % acetic acid (9).

Quantitative Determination of Amino Acids

A Telechrom reflexion video-densitometer (3) was used for the determination of the intensity of ninhydrin spots (10). The chromatoplates were placed into the equipment perpendicular to the direction of chromatography. The density of identical amino acid spots of samples, withdrawn from the assay mixture at different time intervals, together with that of the reference spot were taken as 100 % and the intensity of the individual spots was ex-

pressed in percentage values. The molar concentration of the individual amino acid spots was calculated from the percentual density of the reference amino acid (1, 2). The standard deviation of the video-densitometric evaluation of chromatograms was $\pm$ 3 %, as determined with lysine (10).

RESULTS AND DISCUSSION

Determination of the Glutamine-Dependent Carbamoyl Phosphate Synthetase Activity

This enzyme catalyzes the following reaction:

$$2\ ATP + glutamine + HCO_3^- \longrightarrow carbamoyl\ phosphate + glutamate + 2\ ADP + P_i.$$

If the assay mixture contained ornithine and ornithine carbamoyltransferase, carbamoyl phosphate was converted into citrulline: carbamoyl phosphate + ornithine $\longrightarrow$ citrulline + P_i.

The activity of carbamoyl phosphate synthetase can be determined by measuring the increase in glutamate or in citrulline concentrations. When the formation of glutamate was followed, the assay mixture of 120 µl final volume contained 25 mM ATP, 25 mM sodium-hydrogen-carbonate, 50 mM magnesium-sulphate, 50 mM glutamine and tissue extract equal to 12-15 mg of fresh liver, in 50 mM veronal-sodium buffer, pH 7.4. The mixture was incubated at 37°C, and at 30-min intervals 20 µl samples were withdrawn and applied onto a chromatoplate in Li^+ form then it was chromatographed in a lithium-formate

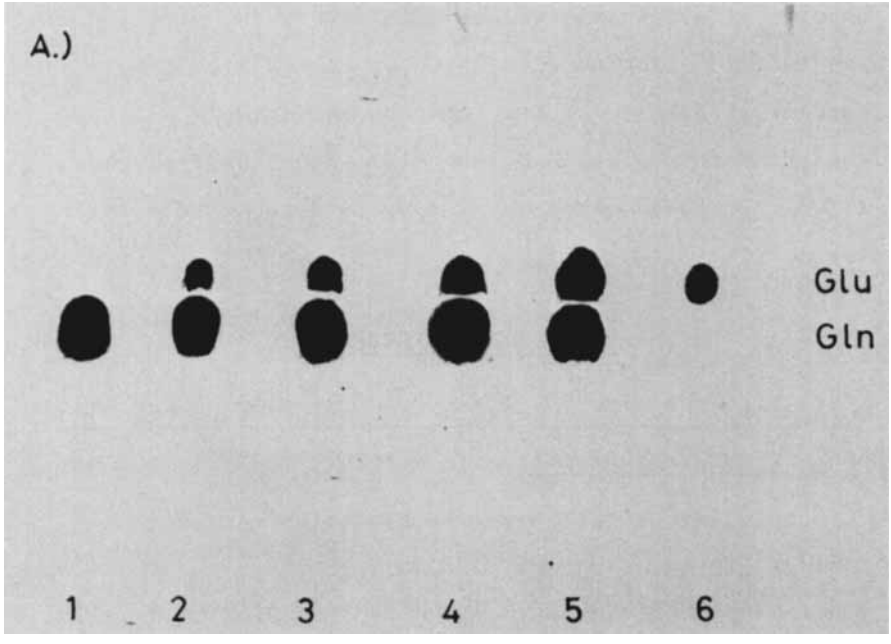

FIGURE 1 - *Carbamoyl phosphate synthetase assay by* CV-technique.
A./ Formation of glutamate from glutamine.

solvent system as described in Methods. As reference, 25 nmol of glutamate was also run (Fig. 1a). The specific activity of the enzyme measured in 15 liver samples was 430 $\pm$ 32 nmol glutamate/g wet tissue/h.

The glutamate-dependent carbamoyl phosphate synthetase can also be determined by measuring the formation of citrulline (2). In this case the above assay mixture was supplemented with 10 mM ornithine and 10 μl ornithine carbamoyltransferase. 20 μl samples were chromatographed on a chromatoplate in Na^+ form. The amount of citrulline formed in the reaction was measured (Fig. 1b).

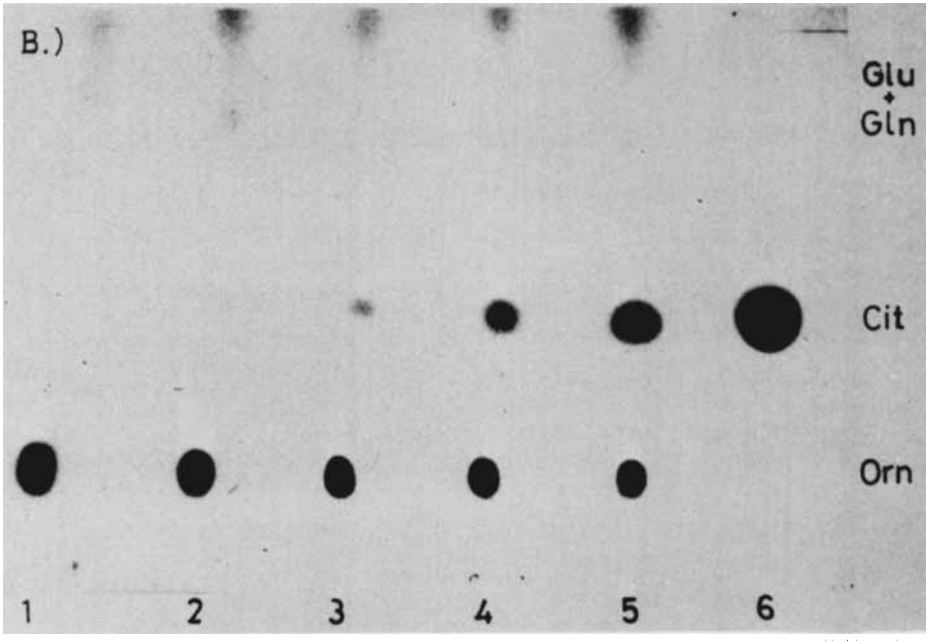

FIGURE 1 - (continued)

B./ Formation of citrulline from ornithine and carbamoyl phosphate. 1 - 5 samples taken in 30 min intervals, 6. reference amino acids. For details see Methods.

The specific activity of the same 15 liver samples as above was 520 $\pm$ 60 nmol citrulline/g wet tissue/h. This value is by 17 % greater than that obtained by measuring the glutamate formation. The difference may be explained by the fact that a portion of carbamoyl phosphate is further converted by ornithine carbamoyltransferase present in the liver extract. Hence, the reaction is pulled in the direction of carbamoyl phosphate synthesis. Thus, the activity of carbamoyl phosphate synthetase can be de-

termined more reliably by measuring the amount of glutamate.

Determination of Aspartate Carbamoyltransferase Activity

The enzyme catalyzes the following reaction in the pyrimidine synthesis:

aspartate + carbamoyl phosphate ⟶ carbamoylaspartate + P_i.

The activity of the enzyme was determined by measuring the changes in aspartate concentration. The assay mixture contained liver extract, corresponding to 1-2 mg of fresh liver, 10 mM aspartate and 15 mM carbamoyl phosphate in 20 mM triethanolamine-HCl buffer, pH 7.7, in a final volume of 60 µl. It was incubated at 37°C and at 30-min intervals 10 µl samples were withdrawn and applied onto a chromatoplate in Na^+ form. As reference, 50 nmol of aspartate was also run. A specific activity of 48 ± 3.3 µmol aspartate/g wet tissue/h was determined in the assay carried out with 25 samples of rat liver.

For comparison the activity of aspartate carbamoyltransferase was also determined by spectrophotometry. According to least squares regression analysis, $y = 0.96x + 1.02$, where y is the spectrophotometric activity and x is the activity determined by the CV-technique. The calculated determination coefficient, r^2, was 0.90, which indicates a 90 % correlation between the data obtained with the two procedures.

We may conclude that ion exchange chromatography combined with video-densitometry, i.e. the CV-technique, is

suitable for the assay of the activity of the above two enzymes, and of other enzymes as well (1,2). The sensitivity of the technique is reasonably high. The formation or consumption of even 1-2 nmol of amino acids can be detected by the CV-technique statisfactorily. Enzyme reaction can be assayed in 10-20 µl samples of a reaction mixture of even less than 100 µl final volume, and thus enzymes can be detected in extracts prepared from a few milligrams of tissues.

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