SEPARATION OF TWO LEUCYL-RIBONUCLEIC ACID SYNTHETASES FROM RAT LIVER[1]

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The presence of two different aminoacyl-RNA synthetases for phenylalanine and aspartic acid has been demonstrated by Imamoto et al.(1965) and by Barnett and Epler (1966) in Neurospora Crassa;Yu and Rappaport (1966) have separated two leucyl-RNA synthetase activities in E.Coli by acrylamide-gel electrophoresis.Very recently (1967) Strehler et al.have shown chromatographycally different leucyl-synthetase activities in rabbit heart.

In the present communication the separation of two leucyl-RNA synthetase activities from rat liver is reported;moreover,kinetic experiments and a different behaviour to heat lead to the conclusion that the two activities are bound to two different enzymes.

METHODS

Enzyme preparation.About 60 gm of rat liver were treated as described by Keller and Zamecnik (1956) with the exception that medium A contained 0.01 M $KHCO_3$ instead of 0.035 M. The "pH 5" precipitate was dissolved in 0.04 M phosphate buffer pH 7.2 to give a solution containing about 10 mg of protein per ml. Solid ammonium sulphate was then added to the enzyme solution and the precipitate obtained between 33 and 65 % saturation was dissolved in 0.04 M phosphate buffer pH 7.2 containing 5 mM β-mercaptoethanol.To remove excess ammonium sulphate a total of 4-5 ml of this solution was passed through a 1x25-cm column of Sephadex G 50 (bead form) pre-equilibrated with the phosphate buffer,and elution

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was continued with the same buffer.Following Sephadex filtration the enzyme was immediately loaded on a hydroxylapatite (Levin,1962)column,1.8x17 cm,pre-equilibrated with phosphate buffer pH 7.2, 0.04 M containing 5 mM β-mercapto-ethanol.The column was washed at a rate flow of about 20 ml/hour with a linear gradient of the same buffer from 0.04 to 0.2 M;each fraction contained 10 ml. Two peaks of protein were eluted with this gradient without any leucyl-synthetase activity.It must be noted that,in some experiments,this linear gradient of phosphate buffer was replaced by the buffer solution 0.15 M;however in this case some enzyme activity was not retained in the column.

After the concentration of phosphate had reached a value of 0.15 M this first elution was stopped and a new gradient of the same buffer from 0.2 to 0.5 M was started and the flow rate was reduced to 4-5 ml/hour;each fraction was collected every hour.

Two protein peaks were eluted from the column with this second gradient and leucyl-synthetase activity (Enz I) was observed in the second peak.When no more enzymatic activity was detected in the effluent solution,sodium phosphate buffer pH 7.5, 0.5 M was run through the column and a single peak of protein with a second enzymatic activity (Enz II) was eluted from the column,(See fig.1).

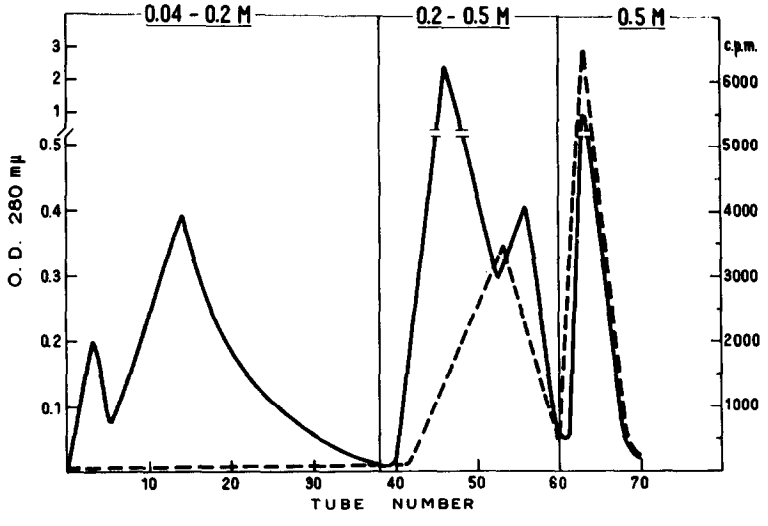


Fig. 1. Chromatography of rat liver leucyl-RNA synthetase activities on hydroxylapatite column.Activity was assayed as described in the text using 10 μl of protein solution.Continuous line represents Optical Density at 280 μm;dotted line represents enzyme activity.

5 mM β-mercaptoethanol was always present in the solutions of phosphate buffer used for the chromatography.

Assay of leucyl-synthetase activity. Leucyl-synthetase was assayed measuring the incorporation of C^{14}-L-leucine (spec.act. 3 mC/mM; The Radiochemical Center Amersham, England) into t-RNA prepared according to Brunngraber (1962).

The standard reaction mixture contained (in umoles): cacodilate buffer pH 7.5: 100; ATP: 0.75; $MgCl_2$: 0.5; GSH: 0.5; C^{14}-leucine: 0.05 and t-RNA: 250 μg to a final volume of 250 μl. The reaction was started by the addition of the enzyme (from 4 to 15 μgm of protein) and after 10 minutes of incubation at 37°, 50-μl aliquots were pipetted on Wathman 3-MM filter disks and analysis for C^{14}-leucyl-RNA was performed as described by Kelmers et al. (1965). Radioactivity was measured with a gas flow counter made by SELO Comp., Milano, Italy.

RESULTS AND DISCUSSION

Kinetic experiments. The effect of different concentrations of L-leucine on the formation of Leucyil-RNA by both the enzymatic activities is shown in fig.2. The values of Km calculated from the plots of the reciprocal of velocity vs. the reciprocal of leucine concentration (Lineweaver and Burk, 1934) were found to be 0.45×10^{-7} M for Enz I and $1,05 \times 10^{-8}$ M for Enz II. As one can ob

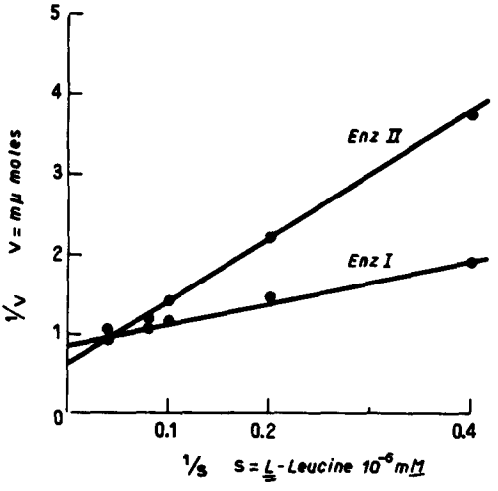
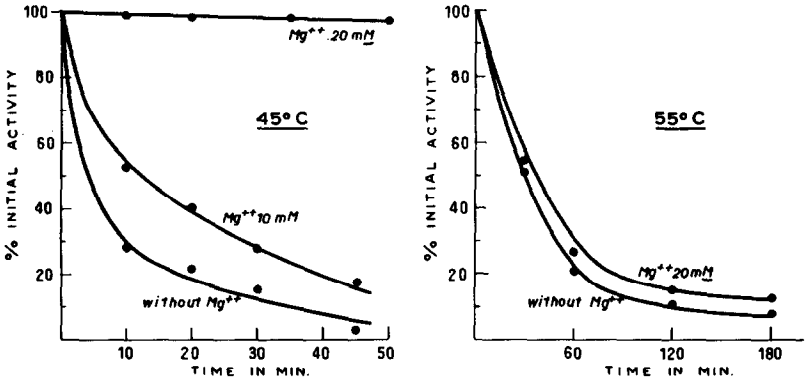


Fig.2 Lineweaver-Burk plots for two leucyl-synthetase activities of rat liver. Velocity is expressed as mumoles of leucyl-RNA formed after 10 min. of incubation at 37°. The enzyme assay was performed as described in the text, except that the final concentration of RNA was of 500 μg.

serve from the plots reported in figure 2,also the values of Vmax are different for the two activities.

The different values for Km and Vmax for the two preparations of leucyl-synthetase suggest that the two activities may be enzymatically distinct. A support to this hypothesis is given by the study on the heat stability of both the activities.

Heat Inactivation. The experiments on the effect of heat on the leucyl-synthetase activities are reported in figs. 3 and 4; they demonstrate clearly that the second enzyme loses almost completely its activity when incubated at 45° for 20 minutes; at this same temperature the first enzyme remains stable for many hours. Inactivation of the latter enzyme occurs only after 3 hours at 55°.



Figs. 3-4. Effect of heating and of $MgCl_2$ on leucylsynthetase activities. The enzyme II (fig.3)was preincubated at the temperature and times indicated at a concentration of 0.7 mg per ml in 0.5 M phosphate buffer pH 7.2. Enzyme I (fig.4) was preincubated at a concentration of 0.85 mg protein per ml in 0.03 M phosphate buffer pH 7.2.At given times,aliquots (10 µl) of preincubated enzymes were assayed for activity as described in the text.In the experiments with $MgCl_2$,this was added to the enzyme solution soon before preincubation

Effect of $MgCl_2$ on heat inactivation. The difference in the stability to heat between the activities of Enz I and Enz II would suggest that the enzymatic activities are bound to two structurally different proteins.A further support to the hypothesis of the existence of two different leucyl-synthetases is given by experiments in which Mg^{++} ions were added to Enz I and Enz II exposed to heating.

At a concentration of 20 mM, $MgCl_2$ exertes a protective effect on the heat inactivation of Enz II,while at the same concentration it does not have any effect at all on the activity of Enz I (Fig.3 and 4).

The biological meaning of the presence into bacterial cells of more than one aminoacyl-synthetase as well as more than one aminoacyl-tRNA specific for a single aminoacid is not yet well established.Some investigators suggest that the multiplicity of both synthetases and acceptor RNA's for one aminoacid may play a role in the regulation of the traslation process of the genetic message (Ames and Hartman 1963;Stent 1964;Taylor et al.1967).Recently (Strehler et al.1967) this multiplicity has been observed also in mammalian tissues.These findings might be used to support the view,so long ipothesized,of multisite cytoplasmatic regulation in mammals.

Moreover very recently we have found (unpublished results),in rat liver,at least three chromatographycally distinct specific leucyl acceptor RNA's.

Work is in progress to relate the two leucyl-synthetases studied and the different leucyl acceptor RNA's in rat liver.

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