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## Occurrence and Particle Character of Aminoacyl-tRNA Synthetases in the Post-Microsomal Fraction from Rat Liver

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A post-microsomal fraction (Fraction X) was isolated from cell sap from rat liver by centrifugation at  $105\,000\times g$  for 15 h. The activity of a number of aminoacyl-tRNA synthetases was considerably higher in the resulting pellet than in the supernatant. These enzymes (glutaminyl-, isoleucyl-, leucyl-, lysyl-, and methionyl-tRNA synthetase) were purified by gel filtration on Sephadex G-200, chromatography on DEAE-Sephadex A-50 and on hydroxyapatite. As compared with crude cell sap a 120- to 170-fold purification was achieved.

In all purification steps, including additional centrifugation on sucrose gradients or isoelectric focusing, the major peak of activity of the five enzymes coincided.

In the electron microscope circular and rectangular particles, 11–14 nm in size, could be visualized. From these findings we suggest that in rat liver cells, if not in all animal cells, part of the aminoacyl-tRNA synthetases occur in a particulate state.

A post-microsomal fraction, called Fraction X, was first described by Hoagland in 1961 [1]. Different functions have been ascribed to this fraction, for instance messenger RNA [2], interaction between ribosomes and messenger RNA [3,4] and transfer activity [5]. Recently we have reported that amino-acid-activating enzymes might play a major role in the observed stimulation of incorporation of amino acids [6].

In connection with this we investigated the distribution of aminoacyl-tRNA synthetases (amino acid-RNA ligases) in this fraction in more detail.

The synthetases have further been purified by chromatography on DEAE-Sephadex A-50 and hydroxyapatite. They have been characterized by electrophoresis on polyacrylamide gels, centrifugation in sucrose gradients, isoelectric focusing and in the electron microscope.

## MATERIALS AND METHODS

### *Materials*

$^{14}\text{C}$ -Labeled amino acids, [ $^{35}\text{S}$ ]methionine, and tetra-sodium [ $^{32}\text{P}$ ]pyrophosphate were purchased from the Radiochemical Centre (Amersham, England). GTP, pyruvate kinase and bovine serum al-

*Enzymes.* Aminoacyl-tRNA synthetases (EC 6.1.1); inorganic pyrophosphatase (EC 3.6.1.1).

bumin, were supplied by Boehringer Mannheim GmbH (Mannheim, Germany).

Phosphoenolpyruvate (sodium salt), ATP and L-amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Dithiothreitol (Cleland's reagent) was obtained from Calbiochem (Los Angeles, California). Inorganic pyrophosphatase (from bakers' yeast) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Tetrasodium pyrophosphate, ammonium sulphate and glycerol were purchased from Merck AG (Darmstadt, Germany). Polyethylene glycol (Carbowax 6000) was obtained from Fluka AG. Chemische Fabrik (Buchs, Switzerland). Sephadex G-25 (coarse), Sephadex G-200 and DEAE-Sephadex A-50 were products from Pharmacia (Uppsala, Sweden).

Bovine  $\alpha$  and  $\beta$ -crystallin were gifts of Dr G. van Kamp. Hydroxyapatite was prepared according to Levin [7].

### *Buffer Solutions*

Medium A consisted of 50 mM Tris-Cl pH 7.6, 25 mM KCl and 5 mM magnesium acetate. Phosphate buffers were, unless stated otherwise, composed of potassium phosphate and contained 10% (w/v) glycerol. To the buffer solutions either dithiothreitol (1 mM) or 2-mercaptoethanol (5 mM) were added immediately before use.

### Preparation of Subcellular Fractions from Rat Liver

Total cell sap, supernatant X and fraction X were prepared as described earlier [6]. When the isolation was performed on a large scale, the microsomes were sedimented by centrifugation at  $75000 \times g$  for 150 min in rotor 30 of the Spinco model-L preparative ultracentrifuge.

Total cell sap, supernatant X and fraction X were freed from endogenous amino acids and ATP by gel filtration on Sephadex G-25 (coarse), equilibrated in medium A. The protein-containing fractions were concentrated by addition of 1 mg dry Sephadex G-25 (coarse) per 5 ml protein solution; after 30 min at 2 °C the Sephadex was removed by centrifugation at 3000 rev./min for 5 min in a Martin Christ junior centrifuge. Medium A, containing 2 M sucrose was added to a final concentration of 0.35 M. The fractions were stored at -20 °C in small portions containing 10–17 mg protein/ml.

In a number of experiments supernatant X was freed from pH 5 enzymes. This fraction, devoid of glutaminyl-, isoleucyl, leucyl-, lysyl-, and methionyl-tRNA synthetase, was added to the incubation mixture when formation of aminoacyl-tRNA was assayed.

The solution was adjusted to pH 5.2 by gradual addition of 1 N acetic acid. The resulting suspension was stirred for 1 h in an ice bath and centrifuged at 4500 rev./min in the Martin Christ Jr. centrifuge. The supernatant was adjusted to pH 7.6 by addition of 1 N KOH. Passage through Sephadex G-25 (coarse) and storage of the protein was as described above.

Polyribosomes were isolated according to the method described by Bloemendal *et al.* [8].

### Isolation of RNA from Rat Liver

Transfer RNA was isolated from rat liver essentially according to the method of Brunngraber [9], with modifications as described by Konings [10]. The tRNA was stripped by incubation for 90 min at 37 °C in 1.8 M Tris-Cl pH 8.0.

Ribosomal RNA was isolated as described earlier [6].

### Assay for Amino-Acid-Dependent ATP—Pyrophosphate Exchange

The activation of amino acids was measured by the amino-acid-dependent ATP—pyrophosphate exchange assay [11]. The reaction mixture (0.25 ml) contained 100 mM Tris-Cl pH 7.6, 7 mM MgCl<sub>2</sub>, 5 mM ATP (neutralized with KOH), 2 mM L-amino acid, 5 mM KF, 2 mM tetrasodium pyrophosphate (20000–100000 counts/min per assay), 2 mM dithiothreitol, 250 µg bovine serum albumin and limit-

ing amounts of enzyme (dissolved in phosphate buffer). In blanks amino acid was omitted. After incubation at 37 °C for 10 min the reaction was stopped and ATP adsorbed to Norit A as described by Lemoine *et al.* [12], except that the volumes were twice as large. The suspension was filtered through glass-fibre paper (Whatman GF/A) and the filter was washed three times with 10-ml portions of distilled water. The paper was glued onto an aluminium planchet with the Norit surface down [13], dried and counted in a thin-window gas-flow counter.

One unit of enzyme activity is defined as that amount incorporating 1 µmol [<sup>32</sup>P]pyrophosphate into ATP in 10 min at 37 °C.

### Assay for Aminoacyl-tRNA Formation

The incubation mixture (0.2 ml) contained 50 mM Tris-Cl pH 7.6, 25 mM KCl, 5 mM magnesium acetate, 5 mM ATP (neutralized with KOH), 0.125 mM <sup>14</sup>C-labeled L-amino acid (or L-[<sup>35</sup>S]methionine) (25 µCi), 2 mM dithiothreitol, 200 µg rat liver tRNA, 100 µg bovine serum albumin and limiting amounts of enzyme. Enzymes were diluted in medium A, containing 0.35 M sucrose and 2 mg/ml (w/v) albumin. In blanks ATP was omitted. Incubation was at 37 °C for 10 min. The reaction was stopped by addition of 5 ml 5% trichloroacetic acid (w/v). The tubes were kept at 0 °C for 30 min. After centrifugation the precipitate was collected on a Whatman glass-fibre paper. Radioactivity was measured in a liquid scintillation spectrophotometer [6].

Incubation mixtures which contained [<sup>14</sup>C]cysteine, [<sup>14</sup>C]serine or [<sup>14</sup>C]tryptophan showed high blank values. In these mixtures the reaction was stopped essentially as described by Muench [14].

One unit of enzyme is equivalent to the formation of 1 nmol of aminoacyl-tRNA in 10 min at 37 °C.

### Assay for Incorporation of Amino Acids

Incorporation of [<sup>14</sup>C]leucine in the endogenous system was measured as described earlier [6].

### Assay of Inorganic Pyrophosphatase

Inorganic pyrophosphatase was assayed according to the method described by Heppel [15]. As control for activity inorganic pyrophosphatase from yeast was used.

### Protein Determination

Protein was measured by the method of Lowry *et al.* [16] using bovine serum albumin as standard. The standards and blanks contained the same concentration of 2-mercaptoethanol or dithiothreitol as did the protein samples. As 2-mercaptoethanol and dithiothreitol interfere strongly in this assay [17, 18], all samples were diluted ten-fold.

When the protein concentration in the column effluent was too small to enable a reliable determination, the protein content was estimated after dialysis by measuring the absorbance at 280 and 260 nm according to Warburg and Christian [19].

#### *Purification of Aminoacyl-tRNA Synthetase from Fraction X*

**Precipitation with Ammonium Sulphate.** Fraction X (400–450 mg protein) was concentrated by precipitation of protein between 30 and 60% saturation with ammonium sulphate [6]. After centrifugation the precipitate was dissolved in 3 ml of medium A and dialyzed during 3 h against this medium. A slight precipitate, which appeared during the course of the dialysis procedure, was removed by centrifugation.

**Gel Filtration on Sephadex G-200.** The sample (7–8 ml) was applied immediately to a column of Sephadex G-200, equilibrated in Medium A. In order to keep the filtration step short a column (column size 58 × 4 cm, LKB) was used with a peristaltic pump (velocity 15 ml/h) by which the sample and the buffer were introduced into the bottom of the column and pumped upward. This procedure improved the separation of the active fraction (which was the first protein peak [6]) from the inactive protein.

When no further purification was performed, the active fraction was concentrated by addition of Sephadex G-25 (coarse) and subsequent centrifugation. The solution was made 0.35 M in sucrose, frozen in small portions and stored at –20 °C.

When purification was continued, glycerol was added to a final concentration of 10% (w/v) and the protein was dialyzed during at least 3 h against 1 l of 20 mM phosphate buffer pH 7.5. The buffer was changed twice.

**Chromatography on DEAE-Sephadex A-50.** After dialysis, the sample was applied onto a column of DEAE-Sephadex A-50 (column size 12 × 1.2 cm), equilibrated in 20 mM phosphate buffer pH 7.5. The column was washed with 50 ml buffer and the enzymes were eluted with a linear salt gradient formed of 75 ml 0.02 M phosphate buffer pH 7.5 and 75 ml 0.5 M phosphate buffer pH 6.5. The flow rate was 15 ml/h and fractions of approximately 2 ml were collected. Fractions which were 0.09 to 0.2 M in phosphate were pooled, concentrated by dialysis against 1 l of 20 mM potassium phosphate buffer pH 7.0, containing 20% (w/v) polyethylene glycol, 10% (w/v) glycerol and 1 mM dithiothreitol, and subsequently dialyzed against the same buffer without polyethylene glycol.

**Chromatography on Hydroxyapatite.** The diffusate was loaded on a column of hydroxyapatite (column size 10 × 1.2 cm), equilibrated in 20 mM phosphate

buffer pH 7.0. The column was washed with 20 ml buffer. A linear gradient was applied ranging from 0.02 to 0.7 M phosphate pH 7.0. The total volume of the effluent was 150 ml. The flow rate was adjusted to 9 ml/h with the aid of a peristaltic pump and 2-ml fractions were collected.

The enzyme activities emerged at a phosphate concentration of about 0.35 M.

#### *Centrifugation on Sucrose Gradients*

Centrifugation on linear 10–35% sucrose gradients was performed as described earlier [6].

Exponential isokinetic sucrose gradients were prepared and analyzed essentially according to the method of McCarty *et al.* [20]. Isokinetic gradients were prepared in centrifuge tubes of the IEC SB-283 rotor. The sucrose solutions were in a buffer containing 20 mM potassium phosphate pH 7.5, 0.1 M KCl and 1 mM dithiothreitol. A particle density of 1.3 was assumed. The concentrations of sucrose solutions were, according to data of Noll [21], 15% (w/v) in the mixing vessel and 32.8% in the reservoir. Six identical gradients were generated simultaneously. The samples (0.5 ml) which had been dialyzed against the buffer in which the sucrose solutions were prepared, were carefully layered on top of the gradient. Centrifugation at 40000 rev./min and 2 °C was carried out in the IEC ultracentrifuge. After the run, fractions of approximately 0.27 ml were collected by top unloading of the gradient with the aid of a 2 M sucrose solution.

#### *Isoelectric Focusing*

Isoelectric focusing of purified synthetases was performed essentially as described by Surguchev *et al.* [22] with the following modifications. Ampholines pH 5–8 were used. Both dense and light solution contained 1 mM dithiothreitol. 1.5–2 mg of protein in 7–10 ml of 20 mM potassium phosphate buffer pH 7.5 were mixed with the dense solution. The cathode solution, containing 0.4 ml triethanolamine, 12 g sucrose and 14 ml distilled water, was poured into the lower part of the column. The anode solution, 0.1 ml phosphoric acid and 10 ml distilled water, was layered 1.5 cm above the upper electrode. The column was thermostatted at 0–2 °C. Maximally 1 W was applied onto the column. The duration of the run was 60–72 h at a final current of 1.2 mA and 800 V.

#### *Electrophoresis on Polyacrylamide Gels*

Separation of proteins on polyacrylamide gels was performed as described by Bloemendal [23] except that the composition of the gel was 4.5% acrylamide, 0.24% bisacrylamide 0.085 M Tris-borate pH 8.9, 0.4% 3-dimethylaminopropionitrile,

0.003%  $K_3Fe(CN)_6$  and 0.125% ammonium persulphate. All solutions were degassed before they were poured into the tubes.

Electrophoresis in sodium dodecylsulphate [24] was carried out as described by Weber and Osborn [25].

#### Electron Microscopy

Enzyme preparations were dialyzed overnight against 0.1 M ammonium acetate buffer pH 7.0 at 2 °C. Droplets of fluid were placed on a grid covered with a carbon film and almost completely removed with a piece of filter paper. A small drop of either 2% potassium phosphotungstate pH 6.7 or 0.5% uranyl oxalate pH 7.0 [26] or uranyl formate [27] was applied to the grid. Excess of negative-stain solution was removed with filter paper. Electron micrographs were taken with a Philips EM-300 microscope, operated at 60 kV, fitted with a cooling device.

### RESULTS AND DISCUSSION

#### Aminoacyl-tRNA Synthetase Activity

Fraction X stimulates the incorporation of amino acids in the presence of polyribosomes [1–6]. This fraction showed considerable aminoacyl-tRNA synthetase activity [6]. When fraction X was centrifuged in a linear sucrose gradient, amino-acid-activating enzymes appeared to be present in the same region in which activity of fraction X was located. In Fig. 1 is shown that leucyl-tRNA synthetase activity was concentrated in two different regions of the gradient. The corresponding sedimentation coefficients were 25 S and 18 S, respectively. Less than 20% of the activity of leucyl-tRNA synthetase remained in supernatant X. When the distribution of 20 aminoacyl-tRNA synthetases in total cell sap, supernatant X and fraction X was determined, it appeared that the amino-acid-activating enzymes were not uniformly distributed throughout these fractions (Table 1). In order to enable comparison of the distribution of the different synthetases, the total number of units in the total cell sap had been equalized to 100% for each enzyme. (Enzyme activity is expressed in units, defined as specific activity times mg protein.) The recovery of the individual enzyme activities was markedly different. In the case of lysyl-tRNA synthetase about 40% was lost. Presumably this reflects a different degree of stability of the individual enzymes, a phenomenon which has been reported for the synthetases from *Escherichia coli* [28]. Loss of activity however, was not paralleled with a loss of protein. The fractions derived from total cell sap contained together as much protein as the starting material.

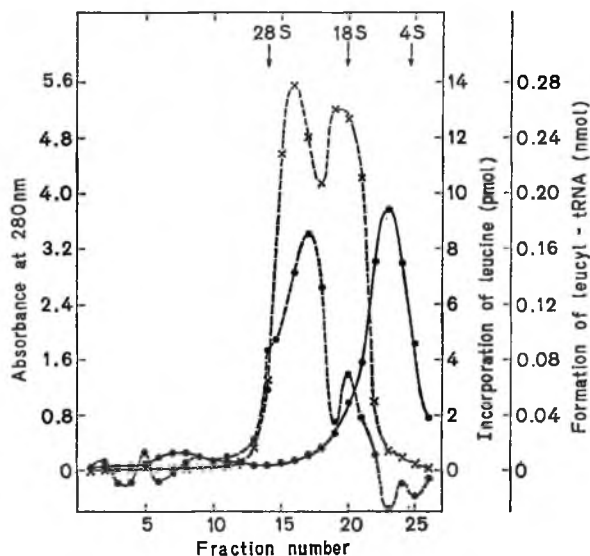


Fig. 1. Sedimentation of fraction X in a 10 to 35% linear sucrose gradient. An aliquot of 20 mg protein was layered on top of the gradient. Fractions of 13 drops were collected after 12 h of centrifugation at 25000 rev./min. For incorporation of amino acids, 50  $\mu$ l of each fraction were assayed. The incubation mixtures (0.25 ml) contained 0.5 mg of supernatant X protein and 100  $\mu$ g of polyribosomes. For formation of leucyl-tRNA 25  $\mu$ l of each fraction was assayed. The incubation mixtures (0.2 ml) were as described in Experimental Procedures. The arrows indicate the position of tRNA and of RNA isolated from rat liver polyribosomes after 12 h of centrifugation at 25000 rev./min in a 10 to 35% linear sucrose gradient containing 0.01 M sodium acetate (pH 5.1) and 0.1 M NaCl. ●—●, stimulation of incorporation of [ $^{14}$ C]leucine; ×—×, formation of [ $^{14}$ C]leucyl-tRNA; ●—●, absorbance at 280 nm

We also compared the specific activity of aminoacyl-tRNA synthetases in fraction X with their specific activity after purification of this fraction by gel filtration on Sephadex G-200. A number of synthetases accumulated in the first protein peak. This fraction was completely devoid of glycyl-, histidyl-, seryl-, and tryptophanyl-tRNA synthetase activity, whereas the specific activity of alanyl-, asparagyl-, cysteinyl-, threonyl- and tyrosyl-tRNA synthetases was lower than in fraction X before purification (Table 2). The behaviour of aspartyl-tRNA synthetase was quite unexpected. While the activity of this enzyme remained predominantly in supernatant X (Table 1), its activity was considerably enhanced in the purified fraction X (Table 2).

#### Purification of Five Aminoacyl-tRNA Synthetases

As glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase accumulated in fraction X, showing only a very low activity in supernatant X, we attempted to further purify these

Table 1. *Distribution of aminoacyl-tRNA synthetases in total cell sap, supernatant X and fraction X*

Enzyme activity was measured by means of formation of aminoacyl-tRNA as described in Experimental Procedures. The total number of units was calculated from the specific activity of the distinct enzyme (expressed in nmol amino acid attached to tRNA in 10 min at 37 °C per mg protein) times mg protein in each fraction. The values for supernatant X and fraction X are given proportional to the number of units present in total cell sap

Substrate	Total cell sap	Super-natant X	Fraction X	Recovery
	units	%	%	%
Alanine	9630	69	15	84
Arginine	4448	53	47	100
Asparagine	5786	52	27	79
Aspartic acid	5366	76	23	99
Cysteine	3280	74	20	94
Glutamic acid	2021	35	56	91
Glutamine	1010	10	66	76
Glycine	4146	60	20	80
Histidine	1942	66	19	85
Isoleucine	123	12	49	61
Leucine	682	15	61	76
Lysine	6324	5	55	60
Methionine	774	9	57	66
Phenylalanine	1260	60	37	97
Proline	3503	53	31	84
Serine	3503	68	30	98
Threonine	8567	52	27	79
Tyrosine	1010	79	22	101
Tryptophan	879	90	20	110
Valine	682	37	48	85

Table 2. *Specific activity of aminoacyl-tRNA synthetases in fraction X and in purified fraction X*  
Specific activity was measured as described in Table 1

Substrate	Specific activity	
	Fraction X	Fraction X after purification on Sephadex G-200
	units/mg protein	
Alanine	7.1	0.08
Arginine	10.3	32.9
Asparagine	7.8	1.0
Aspartic acid	6.2	40.9
Cysteine	3.3	3.0
Glutamic acid	5.6	13.8
Glutamine	3.3	10.5
Glycine	4.1	0
Histidine	1.8	0
Isoleucine	0.3	0.8
Leucine	2.1	8.3
Lysine	17.4	58.9
Methionine	2.2	8.7
Phenylalanine	2.3	4.0
Proline	5.4	5.9
Serine	5.2	0
Threonine	11.6	2.4
Tyrosine	1.1	0.1
Tryptophan	0.9	0
Valine	1.6	4.0

enzymes. We applied chromatography on DEAE-Sephadex A-50 and hydroxyapatite, techniques which had earlier successfully been used for the purification of bacterial synthetases [12–14, 29–35]. In addition, gel filtration on Sephadex G-200 was performed as a first step. The results of the different purification steps are summarized in Table 3. Enzyme activity has been measured by means of amino-acid-dependent ATP—pyrophosphate exchange.

Activity of glutamyl-tRNA synthetase could not be measured when no tRNA was present in the reaction mixture. Therefore, tRNA, from rat liver (200 µg) was added when glutamine-dependent ATP—pyrophosphate exchange had to be determined [36–38]. When Tris-maleate-KOH buffer was added to the incubation mixtures, we did not notice a shift in optimal pH to a lower value as reported for rat liver glutamyl-tRNA synthetase [38].

The active protein peak from the Sephadex G-200 step had an  $A_{280} : A_{260}$  absorbance ratio of 0.72–0.67 which, according to Warburg and Christian [19], indicates the presence of 10–12% nucleic acid.

The purification which was achieved in the subsequent DEAE-Sephadex step was rather low and was accompanied with a considerable loss of activity. However, as the RNA present in the Sephadex G-200 effluent was removed by this procedure, this step was included. The removal of RNA increased the lability of the enzymes. This phenomenon was also observed by Deutscher for rat liver glutamyl-tRNA synthetase [39]. Therefore, when the enzymes had to be stored after this step, the protein was dialyzed against potassium phosphate buffer pH 7.0 containing 50% (w/v) glycerol in order to stabilize the enzymes [34, 39, 40]. By this procedure the protein was concomitantly concentrated two-fold.

The purification of glutamyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase after the gel filtration step is illustrated in Fig. 2 and Fig. 3. Synthetase activity was localized by measuring the formation of aminoacyl-tRNA (Fig. 2).

In order to enhance formation of isoleucyl-tRNA, supernatant X freed from pH 5 enzymes, had to be added to the assay mixture (Bont, W. S., unpublished results). In the presence of this fraction the formation of glutamyl- and lysyl-tRNA was also stimulated.

The main peak of synthetase activities emerged from the column between 0.10 and 0.18 M phosphate. The capacity to attach amino acids to tRNA coincided with the amino acid activation as measured by ATP—pyrophosphate exchange.

Fig. 2 also shows the stimulation of incorporation of amino acids. Maximal stimulation coincided with maximal activity of aminoacyl-tRNA synthetases. These data and the results presented in Table 1 and Table 2 strongly suggest that, in contrast to conclusions of others [1–5], amino-acid-activating enzymes

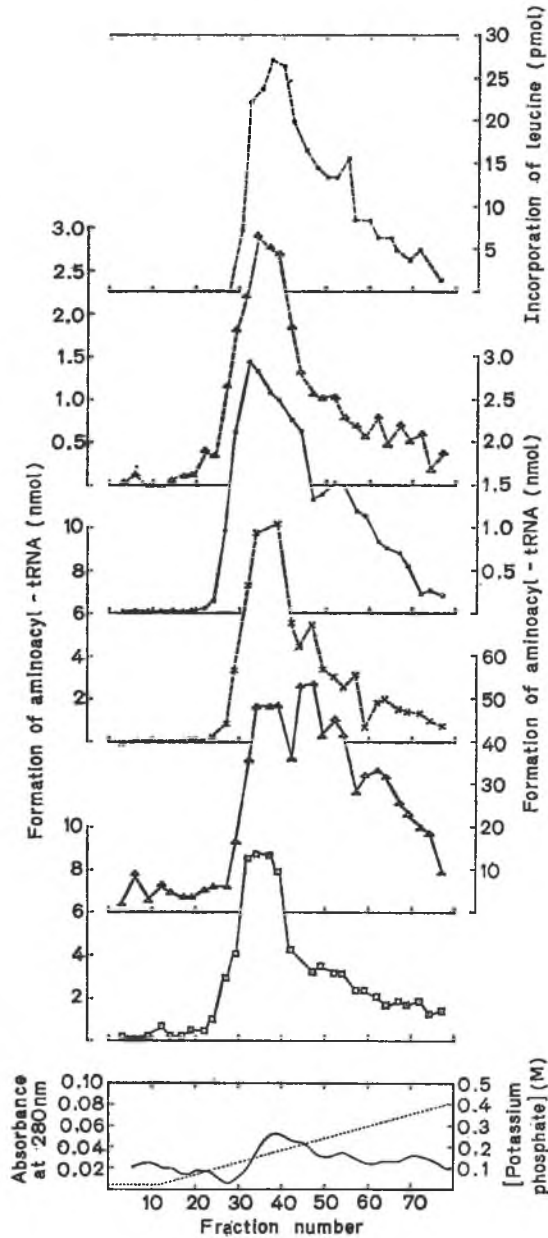


Fig.2. Elution from DEAE-Sephadex A-50 of proteins of fraction X. The first protein peak from the Sephadex G-200 step (9.5 mg protein in 19.5 ml) was dialyzed and applied onto a DEAE-Sephadex column, washed with 0.02 M potassium phosphate buffer pH 7.5 and eluted with a potassium phosphate-pH gradient (100 ml). Fractions were 1.22 ml. For incorporation of amino acids, 50  $\mu$ l of each fraction was assayed. The incubation mixtures (0.25 ml) contained 0.4 mg of supernatant X protein and 100  $\mu$ g of poly-ribosomes. For determination of synthetase activity each fraction was diluted, when necessary, in 100 mM Tris-Cl pH 7.6, 50 mM KCl, 10 mM magnesium acetate and 1 mM dithiothreitol in the proper proportion in order to

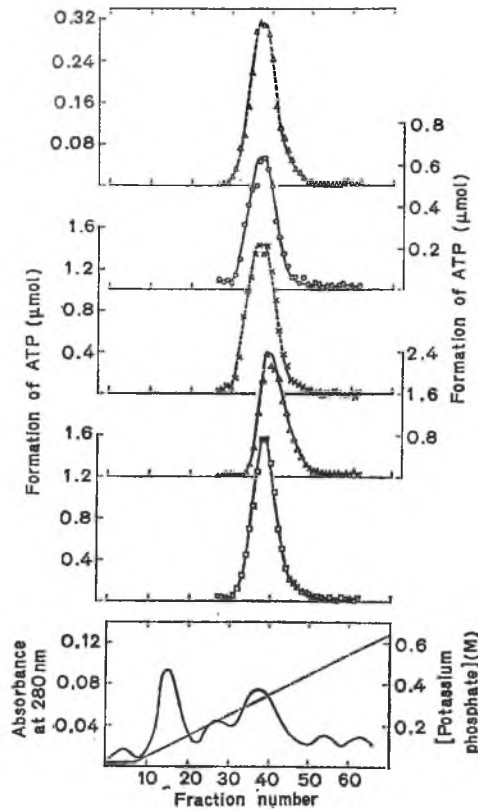


Fig.3. Elution from hydroxyapatite of proteins of fraction X. The active fraction from the DEAE-Sephadex step (5.1 mg protein in 46 ml) was dialyzed and applied onto a column of hydroxyapatite, washed with 20 mM potassium phosphate buffer pH 7.0 and eluted with a linear gradient of potassium phosphate (150 ml). Fractions were 2.0 ml. Synthetase activity was assayed by means of amino-acid-dependent ATP-pyrophosphate exchange. The enzyme activities have been expressed by formation of ATP in  $\mu$ mol per column fraction.  $\Delta$ ---- $\Delta$ , glutaminyl-tRNA synthetase;  $\circ$ --- $\circ$ , isoleucyl-tRNA synthetase;  $\times$ ---- $\times$ , leucyl-tRNA synthetase;  $\Delta$ --- $\Delta$ , lysyl-tRNA synthetase;  $\square$ --- $\square$ , methionyl-tRNA synthetase; —, absorbance at 280 nm;  $\cdots$ , concentration of potassium phosphate

achieve limiting enzyme concentration in the incubation mixtures. The incubation mixtures were as described in Experimental Procedures, except that the reaction mixture was reduced to 0.1 ml and 100  $\mu$ g of supernatant X freed from pH 5 enzymes was added instead of albumin. Synthetase activities have been expressed by formation of aminoacyl-tRNA in nmol per column fraction.  $\bullet$ --- $\bullet$ , stimulation of incorporation of [ $^{14}$ C]leucine;  $\Delta$ ---- $\Delta$ , glutaminyl-tRNA synthetase;  $\circ$ --- $\circ$ , isoleucyl-tRNA synthetase;  $\times$ ---- $\times$ , leucyl-tRNA synthetase;  $\Delta$ --- $\Delta$ , lysyl-tRNA synthetase;  $\square$ --- $\square$ , methionyl-tRNA synthetase; —, absorbance at 280 nm;  $\cdots$ , concentration of potassium phosphate



Table 3. Purification of glutamyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase

Enzyme activity was measured by means of amino-acid-dependent ATP-pyrophosphate exchange. The values for total protein have been calculated for the isolation of synthetases from 50 rat livers

Step	Specific activity of tRNA synthetase for							Recovery of tRNA synthetase activity for					Purification of tRNA synthetase for						
	Protein							units/mg protein					%						
	Gln	Ile	Leu	Lys	Met	Met	Met	Gln	Ile	Leu	Lys	Lys	Met	Met	Gln	Ile	Leu	Lys	Met
Total cell sep	0.026	0.106	0.197	0.036	0.081	0.081	100	100	100	100	100	100	100	1	1	1	1	1	1
Pellet (fraction X)	0.149	0.336	0.673	0.181	0.348	0.348	84	47	50	74	63	68	5.7	3.4	3.4	3.4	5.0	4.3	4.3
Sephadex G-200 eluate	1.10	3.90	6.89	2.92	4.60	4.60	32	28	27	55	44	44	42	37	35	35	72	57	57
DEAE-Sephadex eluate	1.72	5.22	11.8	3.88	6.77	6.77	8.0	6.0	7.3	9.7	10.2	10.2	66	49	60	60	80	84	84
Hydroxyapatite*	3.3	3.56	23.6	13.0	11.8	11.8	3.0	2.9	2.7	3.7	3.2	3.2	137	128	120	120	168	146	146

\* Four preparations were combined for chromatography on hydroxy-apatite.

are responsible for the stimulation of incorporation of amino acids by this fraction.

In Fig. 3 the elution pattern of the five synthetases from the column of hydroxyapatite is shown. An ultraviolet-absorbing peak was eluted at 10 mM potassium phosphate buffer pH 7.0 which did not contain synthetase activity.

Also in the preceding wash of the column of hydroxyapatite no synthetase activity could be measured. The most striking result in this purification step is that again the peaks of activity of glutamyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase coincided.

From the results summarized in Table 3, Fig. 2 and Fig. 3, one has to conclude that the five enzymes resist separation by the purification procedures described.

#### CHARACTERIZATION OF THE PURIFIED SYNTHETASES

##### Sedimentation Analysis

The results described above suggested that the five enzymes were aggregated into a rather stable complex. In order to verify this assumption, we also examined the sedimentation behaviour of the purified enzymes in a sucrose gradient. The result is depicted in Fig. 4. It has been reported that amino-acid-activating enzymes have molecular weights of about 100 000 to 200 000 and sedimentation coefficients of 5 to 8 S [30, 32–35, 40–47]. However, the five enzymes studied revealed a considerably higher sedimentation value. Assuming a particle density of 1.3, a value of 18.2 S can be derived for glutamyl-, lysyl- and methionyl-tRNA synthetase. Isoleucyl- and leucyl-tRNA synthetase showed each two peaks of activity which were localized in fractions in which proteins were expected with a sedimentation value of 12.5 and 18 S, respectively. Stimulation of incorporation of amino acids coincided with the synthetases which sedimented at 18 S. No synthetase activity or capacity to stimulate incorporation of amino acids could be detected in fractions 35 to 40, in which protein might be expected with a sedimentation coefficient up to 28 S.

When the eluate from the column of hydroxyapatite was centrifuged in an analytical ultracentrifuge, a rather broad peak was observed which was characterized by sedimentation coefficients ranging from  $s_{20,w} = 17.9$  to 21.2 S.

The sedimentation behaviour of the synthetases after purification by chromatography on DEAE-Sephadex and hydroxyapatite differed from the sedimentation behaviour of less purified enzymes. The less purified amino-acid-activating enzymes showed, in addition to 18-S components, components with a sedimentation value of approximately 25 S (compare Fig. 1). Stimulatory activity for incorpora-

tion of amino acids was also located in fractions corresponding to higher sedimentation coefficients. However, the purified synthetases sedimented only at 18 S. Presumably the amino-acid-activating enzymes had been converted to material which is characterized by the latter sedimentation coefficient.

#### Isoelectric Focusing

In a final attempt to separate the amino-acid-activating enzymes, we subjected the proteins present in the eluate from the column of hydroxyapatite to isoelectric focusing. Two peaks of ultraviolet-absorbing material were found, with *pI* 7.0 and 5.7, respectively. All five enzymes showed a peak of activity in fractions with *pI* 5.7. Smaller peaks of activity were observed at pH 6.1 and/or 6.5 (Fig. 5) *cf.* [48]. Activity was measured both by formation of aminoacyl-tRNA and by amino-acid-dependent ATP-pyrophosphate exchange. The curves of activity coincided in both methods. This is in contrast with the results of Surguchev *et al.* [22]. The capacity

to stimulate incorporation of amino acids was also examined in the fractions which were obtained after isoelectric focusing. Stimulation coincided with the fractions which had been focused at pH 5.7. In the other fractions no stimulation was detected.

#### Electrophoresis on Polyacrylamide Gels

In a 4.5% polyacrylamide gel six bands were visible from the eluate of the column of Sephadex G-200 (Fig. 6A). The protein isolated after chromatography on DEAE-Sephadex revealed a sharp and a diffuse band (Fig. 6B). Likewise the band pattern of proteins obtained after chromatography on hydroxyapatite contained one sharp and a diffuse band (Fig. 6C). However, the sharp protein band hardly penetrated into the gel. This band may represent protein which had been aggregated during the electrophoretic procedure. The diffuse band had a mobility comparable with a (diffuse) band which was also observed after electrophoresis of the protein from the Sephadex G-200 eluate. Probably this band represents the active material in fraction X.

Sodium dodecylsulphate dissociates the protein complex into eight components (Fig. 6D). Molecular weights of the proteins were estimated according to the procedure of Weber and Osborn [25] (*cf.* Fig. 6D). The same pattern was observed when protein from the peak which had been focused at pH 5.7 was sub-

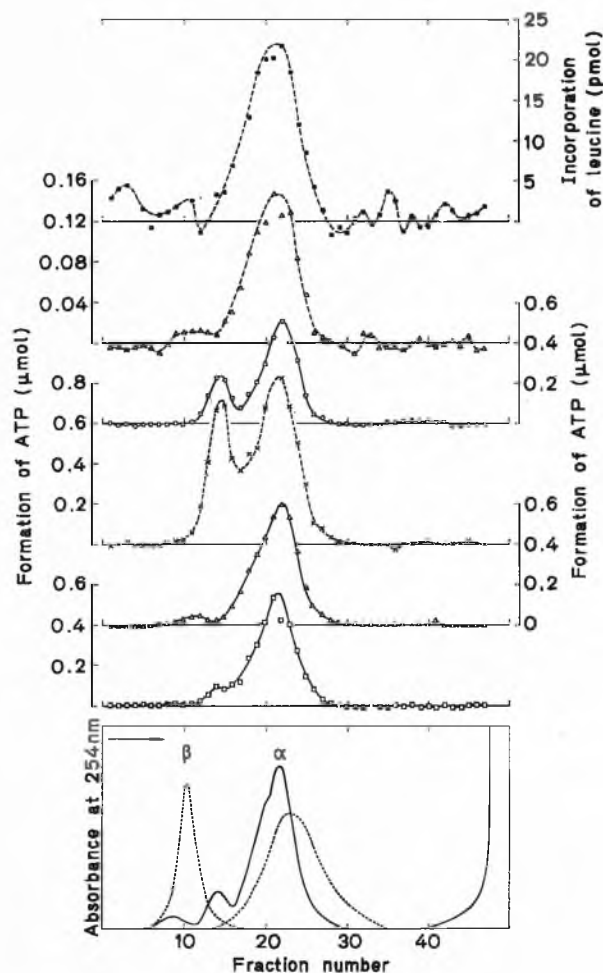


Fig. 4. Sedimentation in an isokinetic sucrose gradient. Fraction X was purified by gel filtration on Sephadex G-200 and by chromatography on DEAE-Sephadex A-50 and hydroxyapatite. The active fraction was concentrated by dialysis *in vacuo* against 20 mM potassium phosphate buffer pH 7.5, containing 0.1 M KCl and 1 mM dithiothreitol. 0.324 mg protein in 0.5 ml were layered on top of an isokinetic gradient.  $\alpha$ -Crystallin (sedimentation coefficient 19.8 S) and  $\beta$ -crystallin (sedimentation coefficient 7.9 S) were dissolved in the buffer as used for dialysis of purified fraction X. 0.406 mg  $\alpha$ -crystallin and 0.080 mg  $\beta$ -crystallin, each in 0.5 ml, were layered on separate, identical gradients. Fractions of 0.266 ml were collected, after 16 h of centrifugation at 40000 rev./min, by top unloading of the gradient with a 2 M sucrose solution. The absorbance was measured continuously at 254 nm. The bottom of the gradient is marked by the rapid increase in absorbance due to the 2 M sucrose solution. Each fraction was diluted fourfold in 1 mM potassium phosphate buffer pH 7.5, containing 1 mg/ml (w/v) albumin, 10% (w/v) glycerol and 1 mM dithiothreitol. For incorporation of amino acids 75  $\mu$ l of each diluted fraction was assayed. The incubation mixtures (0.25 ml) contained 0.5 mg supernatant X protein and 100  $\mu$ g polyribosomes. Synthetase activity was measured as described in Fig. 4. The enzyme activities have been expressed by formation of ATP in  $\mu$ mol per gradient fraction. The arrow indicates the direction of sedimentation.  $\odot$ — $\odot$ , stimulation of incorporation of [ $^{14}$ C]leucine;  $\Delta$ — $\Delta$ , glutamyl-tRNA synthetase;  $\circ$ — $\circ$ , isoleucyl-tRNA synthetase;  $\times$ — $\times$ , leucyl-tRNA synthetase;  $\triangle$ — $\triangle$ , lysyl-tRNA synthetase;  $\square$ — $\square$ , methionyl-tRNA synthetase; —, absorbance at 254 nm of fraction X in relative units;  $\cdots$ , absorbance at 254 nm of  $\alpha$ - and  $\beta$ -crystallin in relative units

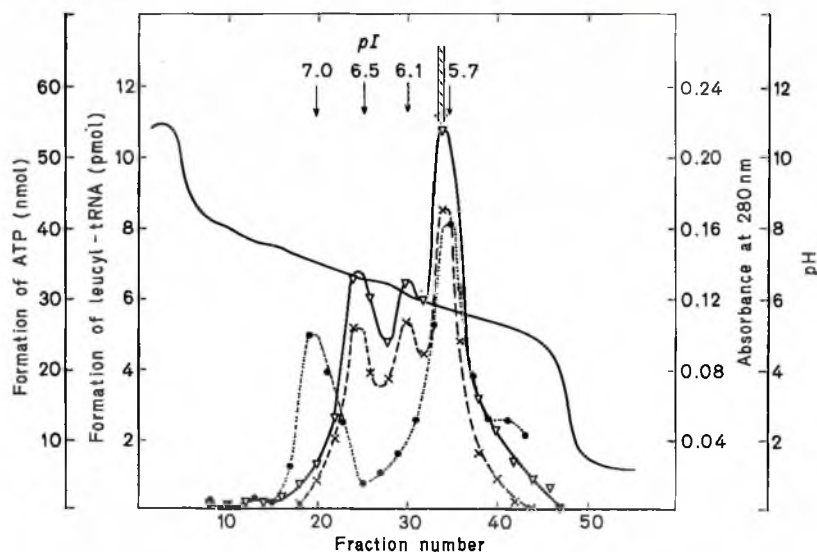


Fig. 5. Isoelectric focusing of leucyl-tRNA synthetase of fraction X. Fraction X was purified as described in Fig. 4; 1.6 mg protein in 10 ml was subjected to isoelectric focusing. Synthetase activity was measured both by leucine-dependent ATP-pyrophosphate exchange and by formation of leucyl-tRNA. For pyrophosphate exchange 100  $\mu$ l of each fraction was assayed. For formation of leucyl-tRNA 20  $\mu$ l of each fraction was tested as described in Fig. 3.

Stimulation of incorporation of amino acids was assayed after extensive dialysis of the fractions. The incubation mixtures (0.25 ml) contained 0.2 mg of supernatant X protein and 40  $\mu$ g of polyribosomes. Maximal stimulation of incorporation of [ $^{14}$ C]leucine is indicated by the hatched arrow.  $\nabla$ — $\nabla$ , formation of ATP;  $\times$ — $\times$ , formation of leucyl-tRNA;  $\bullet$ — $\bullet$ , absorbance at 280 nm; —, pH at 0  $^{\circ}$ C

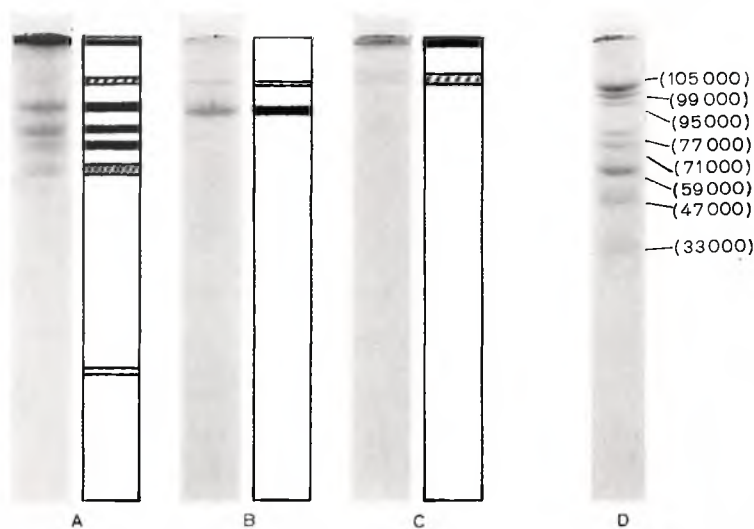


Fig. 6. Electrophoresis of fraction X on polyacrylamide gels. (A, B, C) Electrophoresis in 4.5% acrylamide gels at pH 8.9; (D) electrophoresis in a polyacrylamide gel containing sodium dodecylsulphate; (A) active fraction after gel filtration on Sephadex G-200; (B) active fraction after chromatography on DEAE-Sephadex A-50; (C, D) active fraction after chromatography on hydroxyapatite. Estimated molecular weights are given in parentheses

jected to electrophoresis in the presence of sodium dodecylsulphate.

#### Electron Microscopy

Electron microscopic studies of proteins which play a role in protein biosynthesis are scarce. Shelton

*et al.* [49] envisaged the possibility that transferase I exists as a particle which sediments at 19 S. When investigated in the electron microscope, our purified enzyme preparations revealed particle-like structures with a rather well-defined shape.

Fig. 7A shows the result after negative staining with potassium phosphotungstate. This particle

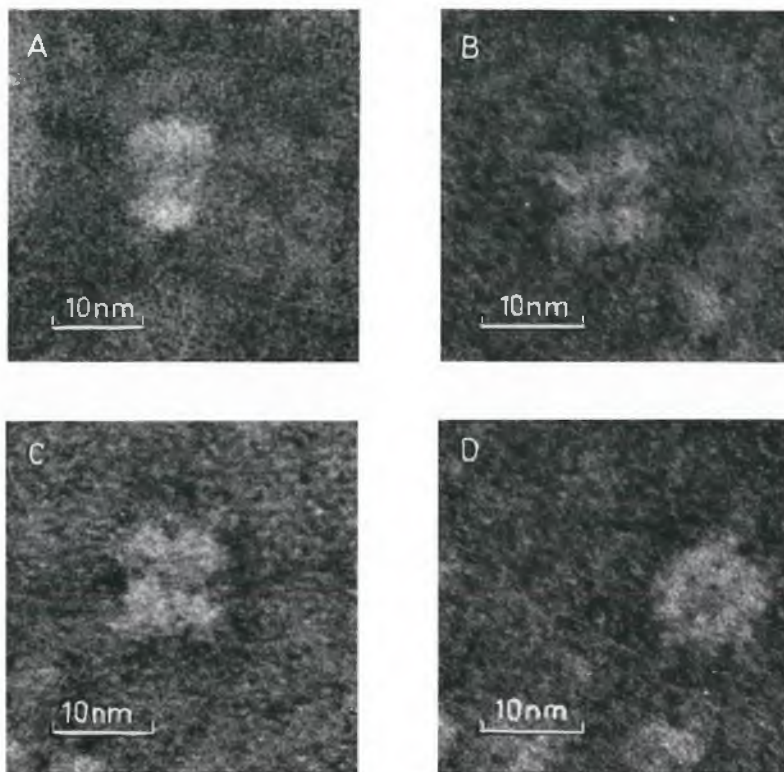


Fig. 7. *Electron micrographs of the active protein-peak after purification.* (A) Protein fraction after chromatography on DEAE-Sephadex A-50. Note the dumb-bell like feature. (B, C, D) Protein fraction after centrifugation in an isokinetic sucrose gradient. Note the distinct globular subunit structure in (B). Negative staining was performed with potassium phosphotungstate (A) or uranyl formate (B,C,D)

resembles those with transferase I activity [49], but is somewhat smaller, approximately  $9 \times 12.5$  nm. The particle frequently showed a dumb-bell-like feature. After centrifugation of the purified synthetases in a sucrose gradient particle-like structures could also be observed in the electron microscope (Fig. 7B–D). The particles could only be detected in fractions corresponding to number 18 to 25 in Fig. 4, where both stimulation of incorporation of amino acids and activity of all five aminoacyl-tRNA synthetases had been demonstrated. Two types of particles were observed. One type seemed clearly to be built up by distinct globular subunits (Fig. 7B). These structures occurred more frequently in the slower sedimenting part of the major protein peak (Fig. 4, fraction 18 to 21). The other type of particles revealed a more compact structure (Fig. 7C and D) and was mainly found in the faster sedimenting part of the protein peak (Fig. 4, fraction 21 to 25). The rectangular structure in Fig. 7C and the circular structure in Fig. 7D presumably represents the same particle viewed from axes perpendicular to each other. In fractions corresponding to numbers 5 to 15 (Fig. 4) no particle-like material was observed.

#### CONCLUSIONS

Our results enable a more direct interpretation of the action of fraction X. Under the assay conditions used, the activity of this fraction seems to be based mainly upon its content of certain amino-acid-activating enzymes, namely glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase. This can be deduced firstly from our finding that these enzymes were only present in minor quantities in supernatant X. Secondly from the capacity to stimulate incorporation of amino acids, which was always paralleled with the presence of the five synthetases in all purification steps. Thirdly from the observation that the effect of fraction X could only be measured when the incubation mixture contained free amino acids instead of aminoacyl-tRNA [6].

The stimulation achieved with the recombined supernatant X and purified fraction X was of the same order of magnitude as that obtained with supernatant X and crude fraction X. This indicates that the purified fraction still contained all active factors present before purification.

The sedimentation coefficient (18 S) of the purified enzymes was much higher than expected. As

particles with an organized structure were observed in the electron microscope, the results suggest a particle character of the five aminoacyl-tRNA synthetases.

Crude fraction X (Fig. 1) or partially purified fraction X (*cf.* [6]) showed a higher sedimentation value than more purified fraction X (Fig. 4). This probably results from aggregation of the distinct particles as was frequently observed by us. During further purification disaggregation seems to occur into separate particles.

In a recent paper evidence has been provided that in rat liver cells all aminoacyl-tRNA synthetases are integrated into a high molecular weight complex [50]. The authors state that this complex is not an artifact arising during the isolation procedure of the enzymes. The complex of synthetases is very labile and can even be disrupted by excess of homogenization or by freezing and thawing. Our results revealed that certain amino-acid-activating enzymes are lacking in the high molecular weight fraction after partial purification of fraction X (Table 2). This may suggest that some enzymes are more strongly associated than others.

It cannot definitely be excluded that transferase I is also a constituent of a more organized structure. Shelton *et al.* [49] suggest that transferase I is related with particles which resemble those obtained by us after chromatography on DEAE-Sephadex. However, transferase I seems to be bound less tightly to the complex as can be deduced from the results of Shelton *et al.* and from our earlier finding that supernatant X contains saturating quantities of this enzyme [6].

Possibly also initiation factors form an organized structure as the behaviour of factor  $M_2$  and  $M_3$  [51] and  $EF_m$  [52] during gel filtration on Sephadex G-200 suggests a high molecular weight. In our routine test system the concentration of magnesium ions was too high to allow the determination of activity of these factors. As at lower concentrations of magnesium ions (4 to 6 mM) the stimulatory effect was as high as when determined at higher magnesium concentrations (7 to 10 mM), it is not very likely that these factors are responsible for the effect of fraction X.

Taking all data available into consideration, we ascribe the action of fraction X mainly to glutaminyl-, isoleucyl-, leucyl-, lysyl- and methionyl-tRNA synthetase aggregated into a rather stable complex, which is preserved during gel filtration on Sephadex G-200, chromatography on DEAE-Sephadex A-50 and hydroxyapatite and during centrifugation in sucrose gradients.

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