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Isolation of Seryl and Phenylalanyl Ribonucleic Acid Synthetases from Baker's Yeast*

Maynard H. Makman† and Giulio L. Cantoni

ABSTRACT: As part of a study of the interaction of proteins and nucleic acid polymers in purified systems a seryl s-RNA synthetase has been isolated from yeast in crystalline form; the enzyme was found to be homogeneous as determined by equilibrium ultracentrifugation. The purification procedure used also yielded a preparation of phenylalanyl s-RNA synthetase of at least 90% homogeneity. The seryl and phenylalanyl s-RNA synthetases have molecular weights (determined by equilibrium ultracentrifugation) of 89,000 and 180,000 respectively; $s_{20,w}$ values (determined by sucrose density gradient centrifugation) of 6.7 and 8.2, respectively; and turnover numbers for catalysis of aminoacyl s-RNA formation of about 50 moles of seryl s-RNA and 100 moles of phenylalanyl s-RNA per minute per mole of enzyme, respectively. The partial purification of the s-RNA synthetases for arginine and leucine from yeast is also described.

The nature and specificity of interaction of proteins and nucleic acid polymers in biological systems are as yet poorly understood. A potentially useful and interesting system for studying this interaction is that involving an amino acid–specific soluble (transfer) RNA (s-RNA) and the corresponding aminoacyl s-RNA synthetase. The synthetases catalyze the following two-step reaction:

$$\text{enzyme} + \text{ATP} + \text{amino acid} \rightleftharpoons \text{enzyme–aminoacyl adenylate} + \text{PP} \quad (1)$$

$$\text{enzyme–aminoacyl adenylate} + \text{s-RNA} \rightleftharpoons \text{aminoacyl s-RNA} + \text{enzyme} + \text{AMP} \quad (2)$$

Purified preparations of certain synthetases (Davie *et al.*, 1956; Van de Ven *et al.*, 1958; Schweet and Allen, 1958; Webster, 1961; Clark and Eyzaguire, 1962; Norris and Berg, 1964) and also of certain amino acid–specific s-RNA species (Holley and Merrill, 1959; Zachau *et al.*, 1961; Tanaka *et al.*, 1962; Ingram and Sjoquist, 1963; Von Ehrenstein and Dais, 1963; Goldstein *et al.*, 1964) have been reported by a number of investigators. Also some data on species specificity and kinetics of aminoacyl s-RNA formation have been obtained with the use of systems containing one of the macromolecular components in purified or partially purified form (Berg *et al.*, 1961; Clark and Eyzaguire, 1962; Bennett *et al.*, 1963; Lagerkvist and Waldenstrom, 1964). However, no study of the interaction of an s-RNA substrate with its specific enzyme has been reported in a system in which both macromolecular components were present in highly purified form.

In recent work in this laboratory, serine-specific s-RNA from yeast has been isolated in essentially pure form (Cantoni *et al.*, 1963). It was decided therefore to attempt to isolate the corresponding aminoacyl s-RNA synthetase in order to study protein–nucleic acid interaction with this system. The present paper describes the isolation of seryl s-RNA synthetase (serine-activating enzyme) from baker's yeast in pure and crystalline form, preparation of a nearly homogeneous phenylalanyl s-RNA synthetase, and also partial purification of the synthetases for arginine and leucine. A later paper will describe studies concerning the interaction of the seryl s-RNA synthetase with its s-RNA substrate.

Experimental

Measurement of Aminoacyl s-RNA Synthetase Activity. For routine assay, reaction mixtures contained

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0.02 M potassium phosphate buffer (*p*H 7.5), 0.005 M ATP[1] (sodium salt, neutralized), 0.013 M $MgCl_2$, 0.004 M reduced gluthathione, 0.2 mg/ml bovine serum albumin (twice crystallized), 0.025 μmole/ml [^{14}C]amino acid (specific activity, 20 μc/μmole), and 40 absorbancy units (260 mμ)/ml yeast s-RNA (about 3 mμmoles/ml in terms of acceptor capacity for the [^{14}C]amino acid present) in a final volume of 0.19 ml. Assay tubes were incubated for 30 minutes at 37° with suitable dilutions of enzyme. For dilution of the enzyme, a solution containing 0.05 M potassium phosphate buffer (*p*H 7.5) and 0.25% gelatin was used. Purified seryl s-RNA synthetase was not stable when diluted in buffer alone. Assay incubations were terminated by addition of 0.025 ml of 0.02 M [^{12}C]amino acid to each tube and precipitation of aminoacyl s-RNA with cold 10% trichloroacetic acid. Precipitation on paper disks, washing, and counting with a Packard Tri-Carb liquid scintillation spectrometer were carried out as described by Mans and Novelli (1961) with the following modification of the wash procedure: After the 10% trichloroacetic acid wash, the paper disks were washed successively in 3% perchloric acid (four times), ethanol (four times), and ether (two times) (3 ml per disk per wash), and then dried. Under these conditions the reaction rate is directly proportional to enzyme concentration over a tenfold range (i.e., proportional to aminoacyl s-RNA formation up to about 0.3 mμmole aminoacyl s-RNA/ml). A unit of enzyme catalyzes the formation of 1 μmole [^{14}C]aminoacyl s-RNA during the standard assay incubation.

Measurement of Total Acceptor Capacity of s-RNA for an Amino Acid. Assay of s-RNA acceptor capacity was carried out as described for enzyme assay with the following modifications: the concentration of [^{14}C]-amino acid was 0.065 μmole/ml, the concentration of s-RNA was 0.25–1.5 mμmoles/ml in terms of acceptor capacity for the [^{14}C]amino acid present (about 0.13–0.80 absorbancy unit [260 mμ]/ml for assay of pure adenosine-terminal yeast serine-specific s-RNA), and an excess of enzyme was used.

Materials. [^{14}C]Amino acids were obtained from New England Nuclear Corp. Yeast s-RNA was prepared according to the procedure of Monier *et al.* (1960) as modified by Apgar *et al.* (1962). Glass beads (No. 150, 75 μ) were obtained from Minnesota Mining and Manufacturing Co. The beads were treated with nitric acid (70% nitric acid diluted 1:1 with water) in a steam bath for 60 minutes, washed with distilled water, and dried prior to use.

The XE-64 (Amberlite) resin was 100–400 mesh and was obtained from Rohm and Hass Co. Prior to use the resin was washed by suspension and (following settling) decantation of the supernatant fluid repeated several times in, successively, water, 1 N NaOH, water, 1 N HCl, and water. The complete cycle was repeated three times. The resin was then suspended in 0.14 M potassium phosphate buffer and with suitable *p*H adjustments equilibrated with the buffer over a period of several days at *p*H 6.8 and 2–4°. DEAE-cellulose was purchased from Eastman Organic Chemicals and washed with water, 0.1 N NaOH, water, and finally 0.03 M potassium phosphate buffer, *p*H 7.2, prior to use.

[1] Abbreviation used in this work: ATP, adenosine triphosphate.

Results

Enzyme Purification. All operations were carried out in the cold room unless otherwise indicated. All centrifugations were carried out in refrigerated centrifuges.

STEP 1. INITIAL EXTRACTION AND CENTRIFUGATION. Each 100-g portion of pressed baker's yeast (Fleishman's) was homogenized with 300 g of glass beads, 160 mg of reduced gluthathione, 0.4 ml of *n*-octyl alcohol, and 100 ml of buffer containing 0.016 M K_2HPO_4, 0.004 M KH_2PO_4, and 0.002 M $MgSO_4$ (Monier *et al.*, 1960). Homogenization was carried out in a Waring Blendor, cooled with ice, at the speed obtained by connection to a powerstat set at 90 v. In order to keep the temperature below 15° during the procedure, homogenization was carried out for five intervals of 3 minutes each separated by periods of additional cooling on ice; the whole procedure usually required 45 minutes. After homogenization an additional 200 ml of buffer was added, and the suspension was mixed, allowed to settle, and decanted. The glass beads were washed twice with 100 ml of buffer each time. For routine preparation, 400 g of yeast (in four Waring Blendors) was processed in this manner. The extract and washings were combined and centrifuged for 30 minutes at 9000 × *g*. The supernatant fluid was then poured off and centrifuged for 60 minutes at 78,000 × *g* in a Spinco Model L ultracentrifuge. About 1700 ml of supernatant fluid containing 30 g of protein was recovered and subjected to further purification. This supernatant fraction contained aminoacyl s-RNA synthetase activity for all amino acids which were examined (serine, threonine, arginine, lysine, phenylalanine, leucine, isoleucine, valine, tyrosine, histidine, glutamate, aspartate, methionine, proline, alanine, glycine, and tryptophan).

Use of certain other procedures for disruption of yeast cells, such as treatment with toluene, incubation with bicarbonate, and freezing in a dry ice–ethanol bath, were found to be unsatisfactory for preparation of the serine enzyme. Sonic disintegration, or freezing in liquid nitrogen followed by homogenization with glass beads (Bennett *et al.*, 1963), resulted in crude extracts with seryl s-RNA synthetase activity comparable to that obtained by the standard procedure. Further fractionation of extracts prepared by these other procedures was not attempted.

STEP 2. AMMONIUM SULFATE FRACTIONATION AND DIALYSIS. To the supernatant fraction from step 1, 351 g of solid ammonium sulfate per liter of supernatant fluid was added slowly with stirring. The mixture was allowed to stand for 20 minutes with stirring and then centrifuged in a refrigerated Lourdes centrifuge. The precipitate was removed and 81 g of solid ammonium sulfate was added per liter of supernatant fluid. After

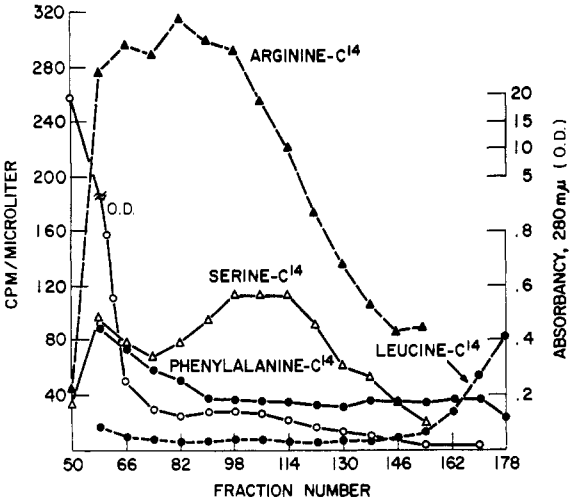

FIGURE 1: Elution pattern from XE-64 (step 3). A total of 4.1 g of protein was placed on a 6.6-cm × 67-cm column of XE-64 equilibrated with 0.14 M potassium phosphate buffer, *p*H 6.8. Elution was carried out with the same buffer. Fractions of 21 ml each were collected. Other conditions were as described in the text. Activity is expressed as cpm/μl of column fraction assayed in 0.19 ml of reaction mixture (standard assay system). Counting efficiency was about 70%. ▲---▲ [^{14}C]-arginine incorporation; (△—△) [^{14}C]serine incorporation; (●—●) [^{14}C]phenylalanine incorporation; (●---●) [^{14}C]leucine incorporation; (○—○) absorbancy at 280 mμ.

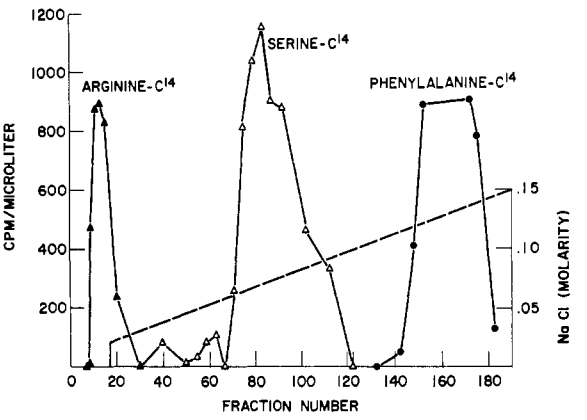

FIGURE 2: Elution pattern from DEAE-cellulose under a gradient of increasing sodium chloride concentration (step 4). For this experiment the step 3 fractions from four preparations (total starting material was 1600 g of yeast) were pooled and placed on a column of 80 ml bed volume. Fractions of 14 ml each were collected. Other conditions were as described in the text. Activity is expressed as cpm/μl of column fraction assayed in 0.19 ml reaction mixture (standard assay system). Counting efficiency was about 70%. (▲—▲) [^{14}C]-arginine incorporation; (△—△) [^{14}C]serine incorporation; (●—●) [^{14}C]phenylalanine incorporation; (---------) molarity of NaCl for elution.

stirring for 20 minutes the mixture was centrifuged, the supernatant fluid discarded, and the precipitate (55–67% saturation ammonium sulfate fraction) was resuspended in a small volume of buffer (0.016 M K_2HPO_4, 0.004 M KH_2PO_4, 0.0001 M EDTA, 0.0002 M reduced glutathione) and dialyzed against the same buffer overnight or until free of ammonium sulfate. The final protein concentration after dialysis was 80–100 mg/ml. If desired the dialyzed ammonium sulfate fraction could be stored at −80° for several weeks without appreciable loss of seryl s-RNA synthetase activity.

STEP 3. XE-64 COLUMN FRACTIONATION. To each ml of dialyzed ammonium sulfate fraction was added 0.075 ml of 1 M K_2HPO_4 and 0.065 ml of 1 M KH_2PO_4 (final phosphate concentration of 0.14 M). A total of 45–50 ml of this solution containing about 4 g of protein was passed through a 6.6 × 67-cm column of XE-64 resin pre-equilibrated with 0.14 M potassium phosphate buffer (*p*H 6.8) and 0.001 M EDTA, and elution was continued with the same buffer. Flow rate was 160 ml per hour and 21-ml fractions were collected. Under these conditions the synthetase activity for serine was retarded to a greater extent than most of the protein put on the column (Figure 1). In addition to serine activity the serine peak contained synthetase activity only for arginine and phenylalanine.

The serine peak fractions were pooled (with the later fractions containing leucyl s-RNA synthetase carefully excluded) and enzyme was concentrated by the addition of 561 g of solid ammonium sulfate per liter and centrifugation at 56,000 × *g* for 30 minutes. The precipitate was transferred to a single tube by resuspension in a small portion of the supernatant fluid and recentrifugation. Finally, the precipitate was taken up in 3 ml of buffer containing 0.02 M K_2HPO_4, 0.01 M KH_2PO_4, 0.001 M reduced glutathione, and 0.0001 M EDTA. Insoluble residue was removed by centrifugation. In order to remove ammonium sulfate the slightly hazy supernatant solution was then passed through a Sephadex G-25 (coarse) column equilibrated with 0.05 M potassium phosphate buffer at *p*H 7.5, 0.001 M reduced glutathione, and 0.0001 M EDTA. The extract could be stored at this stage at −80° for 14 days, or, prior to the Sephadex step, as an 80% ammonium sulfate suspension, in the cold room for 10 hours with only slight losses of seryl s-RNA synthetase activity.

The early, small peak of seryl s-RNA synthetase activity shown in Figure 1 (fraction 58) may represent enzyme bound to RNA; it is absent if immediately prior to step 2 most of the RNA in the extract is removed by precipitation with 0.02 M $MnCl_2$ at *p*H 6.4 (this precipitation step was not incorporated into the standard procedures).

STEP 4. DEAE-CELLULOSE COLUMN FRACTIONATION. Following Sephadex filtration the enzyme was diluted with 0.6 volume of H_2O and immediately placed on a 1.2- × 21-cm column containing DEAE-cellulose in

0.03 M potassium phosphate buffer, *p*H 7.2. (For this step, step 3 fractions from several preparations may be pooled after storage as specified above and fractionated on a correspondingly larger DEAE-cellulose column.) The column was washed with 30 ml of the phosphate buffer and then eluted with 600 ml of a linear gradient of 0.02 M NaCl plus 0.03 M phosphate buffer to 0.15 M NaCl plus 0.03 M phosphate buffer. Fractions of 10 ml each were collected in tubes each of which already contained 5.5 g of solid ammonium sulfate, 1 mg of reduced glutathione, and 0.005 ml of 0.2 M EDTA. The contents of the tubes were mixed in order to dissolve the ammonium sulfate within 30 minutes after each fraction was collected. Separate peaks were obtained for aminoacyl s-RNA synthetase activities for arginine, serine, and phenylalanine (Figure 2). Each of these peaks was found to be free of all other synthetase activities. The two small peaks of seryl s-RNA synthetase activity which appear before the major serine peak in Figure 2 were usually not found during this step and their significance is not known.

STEP 5. FINAL AMMONIUM SULFATE FRACTIONATION. The fractions from each DEAE-cellulose enzyme peak were pooled separately and the precipitates (containing the enzymes) were collected by centrifugation at 56,000 × *g* for 20 minutes. Successive extractions of the precipitates with ammonium sulfate solutions, such as has been described by Pontremoli *et al.* (1961) for preparation of crystalline transaldolase, were then carried out. For this step, ammonium sulfate was recrystallized in the presence of EDTA (Sutherland and Wosilait, 1956) and the solutions used for extraction contained 0.0005 M EDTA. The precipitates containing the serine and phenylalanine enzymes were each suspended in 1 ml and the arginine enzyme precipitate in 2 ml of 65% saturated (430 g/l) ammonium sulfate. The suspensions were centrifuged at 41,000 × *g* for 15 minutes. Identical extractions were also carried out with 55% (251 g/l) and with 45% (277 g/l) saturated ammonium sulfate solutions. The distribution of enzyme activity in the three fractions obtained for each of the synthetases is summarized in Table I. The 55% fraction in each case contained most of the enzymatic activity.

STEP 6. CRYSTALLIZATION OF SERYL S-RNA SYNTHETASE. The serine enzyme, stored in 55% saturated ammonium sulfate in the presence of EDTA in the refrigerator (3°), was stable for a period of at least 6 months. A small amorphous precipitate generally formed during the first 3 days of storage and was discarded. Under these conditions, with the enzyme solution in a small loosely covered test tube, small needle-shaped crystals developed slowly over a period of several weeks. Crystal formation appeared to be facilitated by occasionally bringing the enzyme solution to room temperature and immediately recooling in the refrigerator. Crystal formation was sometimes hastened by addition of several portions of extremely small amounts of saturated ammonium sulfate solution or solid ammonium sulfate. After the first trace of ammonium sulfate was added a small amorphous precipitate formed and was discarded. Further additions of ammonium sulfate were then carried out with caution in order to avoid formation of more amorphous precipitate instead of crystals. With either procedure significant crystal formation usually occurred at a final ammonium sulfate concentration of about 57–58% saturation. At this point the test tube containing the enzyme was tightly stoppered in order to avoid further increase in ammonium sulfate concentration and concomitant conversion of crystalline to amorphous precipitate.

TABLE I: Distribution of Enzymes in the Final Ammonium Sulfate Extracts.[a]

Aminoacyl s-RNA Synthetase	65% Extracts (% total)	55% Extracts (% total)	45% Extracts (% total)
Seryl	18	76	6
Phenylalanyl	6	77	17
Arginyl	6	87	7

[a] Relative distribution of enzymatic activity in the series of extracts obtained at step 5 upon fractionation of each of the step 4 enzyme preparations.

Seryl s-RNA Synthetase. The purification of seryl s-RNA synthetase at each step is summarized in Table II. The final crystalline preparation was found to be homogeneous as determined by equilibrium ultracentrifugation (see below).

Phenylalanyl s-RNA Synthetase. Small amounts (0.1–0.2 mg) of highly purified phenylalanyl s-RNA synthetase were also obtained (Table III). The final fraction was found to be better than 90% homogeneous as determined by equilibrium ultracentrifugation (see below).

Arginyl s-RNA Synthetase. This preparation was not homogeneous, although no other synthetase activities could be detected. Additional purification was obtained by fractional precipitation with the addition of small amounts of a saturated ammonium sulfate solution to the step 5, 55% ammonium sulfate fraction (Table III, last line).

Leucyl s-RNA Synthetase. The late XE-64 column fractions (step 3) contained a leucine enzyme with a specific activity of 0.63 unit/ml. The enzyme as eluted was quite labile but could be stabilized when concentrated by ammonium sulfate precipitation.

Removal of CCA-Pyrophosphorylase during Enzyme Purification. Maximum incorporation of amino acid nto s-RNA was appreciably greater using crude yeast extract than with the purified synthetases. The decreased capacity of the purified enzymes could be restored by the addition of a partially purified protein fraction from yeast. This fraction by itself was without synthetase activity for these amino acids. The effect of this fraction could be reproduced by a preincubation with s-RNA in the presence of ATP and was shown to be due to the terminal addition of adenosine to those s-RNA molecules present which lacked this adenosine. This

TABLE II: Purification of Seryl s-RNA Synthetase.[a]

Fraction (Step Number)	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)	Purification
1. Supernatant (78,000 × *g*)[b]	1700	29,900	38.9	0.0013	100	
2. $(NH_4)_2SO_4$ fraction	42	3,900	27.3	0.0070	70	5.4
3. XE-64 column fraction	1200	150	15.3	0.102	40	78
4. DEAE-cellulose eluate	80	4	4.4	1.10	11	846
5. $(NH_4)_2SO_4$ extract (55%)	1	0.75	1.9	2.53	5	1946

[a] Starting material was 400 g pressed baker's yeast. [b] Dialyzed prior to assay.

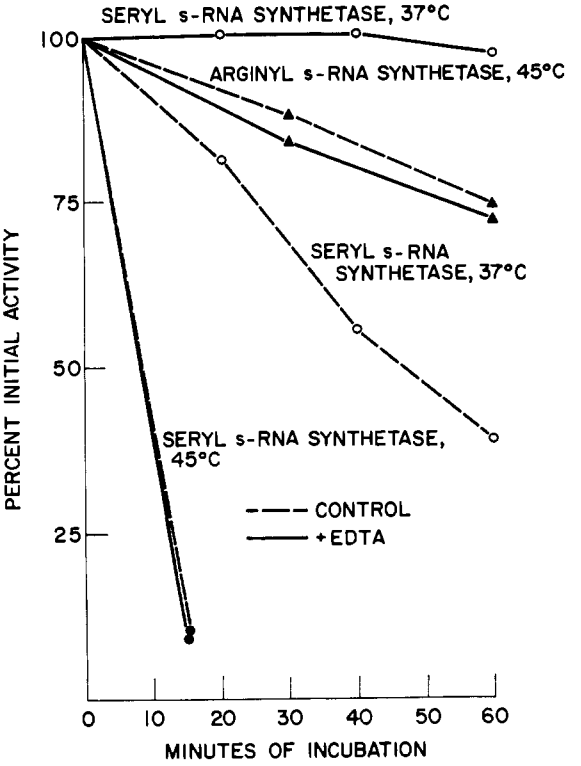

FIGURE 3: Effect of temperature and EDTA on stability of seryl- and arginyl s-RNA synthetases. The step 5 enzyme preparations were diluted to a final enzyme protein concentration of 10 μg/ml in 0.25% gelatin, 0.05 M potassium phosphate buffer, *p*H 7.5, and incubated as indicated above prior to assay in the standard assay system. The concentration of EDTA used in this experiment was 1×10^{-3} M.

fraction has subsequently been further purified and the enzyme provisionally identified as a CCA pyrophosphorylase.[2] The removal of this enzyme during purification of the synthetases results in specific activities and yields that are apparently lower in the purified fraction than are actually obtained when the assay system is fortified with CCA pyrophosphorylase. This was particularly true for the phenylalanine enzyme, since the phenylalanine s-RNA chains present in unfractionated yeast s-RNA often had a much lower proportion of chains terminating in adenosine than did the serine and arginine s-RNA species (e.g., s-RNA preparation I in Table IV).

TABLE III: Summary of Purification of Enzymes.

Aminoacyl s-RNA Synthetase	Specific Activity Step 1[a] (units/mg)	Specific Activity Step 5 (units/mg)	Purification
Seryl	0.0013	2.53[b]	1946
Phenylalanyl	0.0340	6.13[b]	180
Arginyl	0.0078	0.89[b]	116
		2.49[c]	427

[a] Dialyzed prior to assay. [b] 55% saturated ammonium sulfate extracts. [c] Extract obtained by further ammonium sulfate fractionation of the 55% extract.

Stability of Seryl s-RNA Synthetase. In the presence of small amounts of EDTA ($1\text{–}5 \times 10^{-4}$ M), the purified serine enzyme was stable for at least 6 months when stored at 2–4° in ammonium sulfate solution. In the absence of EDTA a variable, but sometimes large loss in activity occurred over a period of several weeks. During the incubation of the enzyme at 37° the protective effect of EDTA was clearly evident (Figure 3). At 45° the enzyme is rapidly inactivated and EDTA is no longer effective. The arginyl s-RNA synthetase, shown for comparison (Figure 3), is much more stable at 45° than is the serine enzyme. No effect of EDTA on the stability of the arginine enzyme has been noted at any temperature studied. Further studies of the heat stability of the serine enzyme in the presence of various substrates will be presented in a subsequent paper.

Substrate Affinity; Turnover Number. The K_m values

[2] P. L. Ipata, M. H. Makman, and G. L. Cantoni, to be published.

TABLE IV: Maximal Amino Acid Incorporation into Yeast s-RNA.[a]

s-RNA Preparation	Purified Synthetase[c]	μMole [^{14}C] Amino Acid Incorporated/ OD_{260} Unit s-RNA[d] Without Crude Enzyme	With Crude Enzyme
		× 10^6	× 10^6
I[b]	Seryl	15.3	36.8
	Arginyl	14.2	40.2
	Phenylalanyl	2.2	14.4
II[b]	Seryl	2.2	29.0
	Arginyl	0.9	26.9
	Phenylalanyl	0.9	28.0

[a] Assay for total acceptor activity for each of the amino acids was carried out as described under methods, with the appropriate purified synthetase and without or with a crude yeast enzyme fraction. [b] I and II represent two different batches of unfractionated yeast s-RNA. [c] Step 5, 55% saturated ammonium sulfate extracts (see text). [d] Absorbancy unit at 260 mμ.

TABLE V: Sedimentation and Molecular Weight of Purified Yeast Synthetases.

s-RNA Synthetase	Sucrose Gradient Method[a] $s_{20,w}$	Molecular Weight	Equilibrium Ultracentrifugation Method[b] Molecular Weight
Seryl	6.7	114,000	89,000
Phenylalanyl	8.2	155,000	180,000

[a] Method of Martin and Ames (1961). Centrifugation of the purified enzymes was carried out with a linear gradient of 5 to 20% sucrose at 3°, in the presence of 0.05 M potassium phosphate buffer, *p*H 7.5, and 0.001 M reduced glutathione. Centrifugation was carried out for 10 hours for the phenylalanine enzyme and for 14 hours for the serine enzyme. Beef liver catalase was used as a marker. [b] Method of Yphantis (1964). Determinations carried out as described in the text and in Figures 4 and 5.

for ATP in the formation of seryl, arginyl, and phenylalanyl s-RNA were all in the range 0.0005–0.001 M. The optimal ratio of Mg/ATP was investigated for the seryl s-RNA synthetase: Rates were very much decreased with ratios below 1. The greatest reaction rates were obtained with ratios of 1.5 to 2.5. The K_m for serine in the formation of seryl s-RNA was found to be 1×10^{-5} M.

The K_m value of the seryl s-RNA synthetase for purified yeast serine s-RNA was found to be 2.4×10^{-7} M; the turnover number was 50 moles seryl s-RNA/min/mole of enzyme. More detailed kinetic data for the seryl s-RNA synthetase will be given in a subsequent paper.[3] The turnover number for the phenylalanyl s-RNA synthetase was estimated as about twice that of the serine enzyme.

Molecular Weight and Sedimentation Constants. The purified seryl and phenylalanyl s-RNA synthetases were subjected to both sucrose density gradient centrifugation (Martin and Ames, 1961) and to equilibrium ultracentrifugation (Yphantis, 1964). The latter method is the more accurate and permits a direct calculation of molecular weight. These results are summarized in Table V. Experimental details for the equilibrium ultracentrifugation studies, carried out in collaboration with Marc Lewis, are presented below.

Equilibrium ultracentrifugation was carried out essentially as described by Yphantis (1964) with a Spinco Model E ultracentrifuge equipped with the rotor temperature indicator and control unit and with the Rayleigh interference optical system. Determinations were carried out at 3.0°. A cell with a 12 mm double sector centerpiece and sapphire windows was used. Plates were measured on a Nikon Model VI optical comparator.

Ultracentrifugation studies of the purified seryl s-RNA synthetase were carried out with equilibrium speeds of 13,410 rpm and 19,160 rpm used. Two buffer systems were used: (1) a buffer containing 220 g of solid ammonium sulfate per liter, 8×10^{-4} M EDTA, 1.6×10^{-3} M $MgCl_2$, and 3.7×10^{-5} M ATP; (2) a buffer containing 0.087 M K_2HPO_4, 0.013 M KH_2PO_4, 0.002 M $MgCl_2$, and 0.001 M EDTA. The serine enzyme preparation appeared to be homogeneous under these conditions and the molecular weight was calculated at 89,000. The partial specific volume assumed for the calculations both for this enzyme and for the phenylalanyl s-RNA synthetase was $\bar{V} = 0.72$. Measurements on one pattern obtained for the serine enzyme are presented in Figure 4.

The purified phenylalanyl s-RNA synthetase was studied in phosphate buffer (identical with the phosphate buffer system for the serine enzyme) and with an equilibrium speed of 19,160. Measurements on one pattern are presented in Figure 5. By appropriate extrapolations and calculations (Yphantis, 1964) values of M_w = 199,300 and M_z = 240,000 were obtained, with the molecular weight for the major component of 180,000. Assuming a two-component system, the enzyme preparation appeared to contain a major component which comprised 95% of the total protein and a contaminant of much higher molecular weight. Sucrose gradient centrifugation of the phenylalanine enzyme showed a single peak of enzymatic activity corresponding to an $s_{20,w}$ value of 8.2 (Table V). Within the limits of accuracy of the gradient technique this peak fits quite well with

[3] M. H. Makman and G. L. Cantoni, to be published.

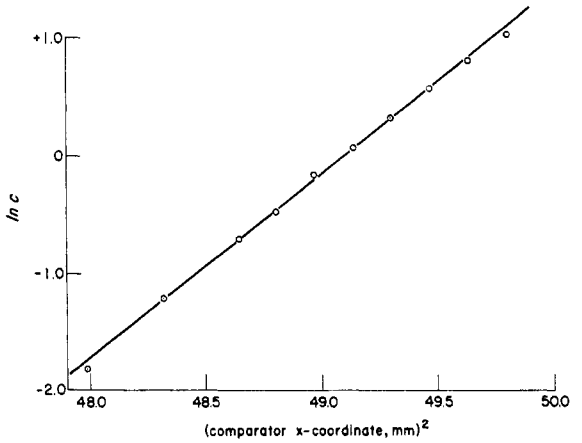

FIGURE 4: Equilibrium centrifugation of purified yeast seryl s-RNA synthetase. The data were obtained after 47 hours in an ammonium sulfate solution (see text) at 19,160 rpm and at 3.0°. The ordinate gives the natural logarithm of the concentration (in fringes) and the abscissa the square of the distance from the axis of rotation (in mm²). The experiment was carried out in collaboration with Marc S. Lewis.

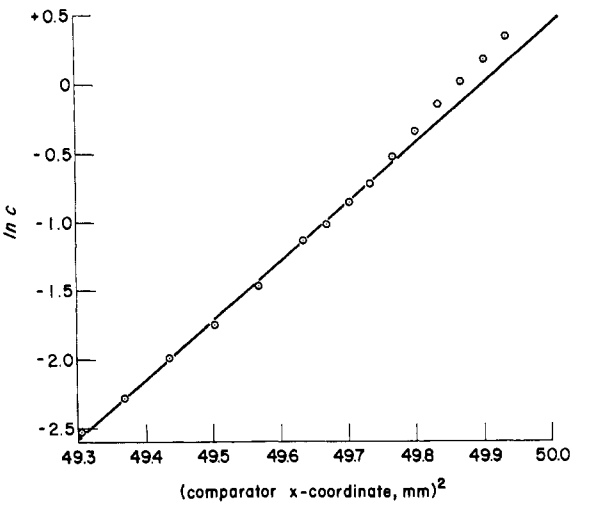

FIGURE 5: Equilibrium centrifugation of purified yeast phenylalanyl s-RNA synthetase. The data were obtained after 23 hours in a phosphate buffer (see text) at 19,160 rpm and at 3.0°. The ordinate gives the natural logarithm of the concentration (in fringes) and the abscissa the square of the distance from the axis of rotation (in mm²). The experiment was carried out in collaboration with Marc S. Lewis.

the major component found in the equilibrium centrifugation run, and also the possibility of an enzymatically active higher molecular weight material is ruled out.

The molecular weight of the phenylalanine enzyme (mw 180,000) is about twice that of the serine enzyme (mw 89,000). The $s_{20,w}$ (Table V) values for the two enzymes suggest that not only the molecular weight but also the hydrodynamic shape of the molecules are different.

Nucleic Acid, Tyrosine, and Tryptophan Content of Seryl s-RNA Synthetase. The purified serine enzyme has a ratio of absorbancy at 280 mμ to that at 260 mμ of 1.48. Estimation of tryptophan and tyrosine content of the enzyme (Goodwin and Morton, 1946) indicates the presence of 23 moles of tyrosine and 8 or 9 moles of tryptophan per mole of enzyme (mw 89,000). A protein which contains this amount of tyrosine and tryptophan (*e.g.*, yeast enolase (Malmstrom *et al.*, 1959) and no nucleic acid would have an expected absorbancy ratio of about 1.75 (Warburg and Christian, 1941). The observed ratio of 1.48 indicates the presence of about one nucleotide per mole of seryl s-RNA synthetase (Warburg and Christian, 1941). It is possible that the nucleotide is present as ATP or as seryl adenylate bound to enzyme (e.g., see Wong and Moldave (1960); Webster and Davie (1961); Norris and Berg (1964)). However the direct determination and identification of this nucleotide remain to be performed.

Discussion

The purification procedure described in this paper utilizes for the first time column chromatography on XE-64 resin for purification of aminoacyl s-RNA synthetases. Satisfactory chromatographic results with this resin generally have been obtained with relatively stable proteins (Paleus and Neilands, 1950; Hirs *et al.*, 1953; Tallan and Stein, 1953; Prins and Huisman, 1955; Morrison *et al.*, 1957) most often of low molecular weight (Hirs *et al.*, 1953). For successful chromatography of the rather labile seryl s-RNA synthetase and also of other synthetases on XE-64 as described here the ionic strength of the buffer used for elution had to be carefully calibrated. Use of 0.135 or 0.145 M buffer in place of the 0.14 M phosphate buffer resulted in appreciable change in the chromatographic behavior of the enzymes.

A number of investigators have described previously the purification of aminoacyl s-RNA synthetases from mammalian tissues (Davie *et al.*, 1956; Webster and Davie, 1961; Webster, 1961; Clark and Eyzaguire, 1962; Holley *et al.*, 1961; Allende and Allende, 1964), *E. coli* (Bergmann *et al.*, 1961; Conway *et al.*, 1962; Norris and Berg, 1964), and yeast (Van de Ven *et al.*, 1958; Lagerkvist and Waldenstrom, 1964). Included in these studies are the partial purification of a serine enzyme from beef pancreas (Webster and Davie, 1961) and the purification of a phenylalanine enzyme from *E. coli* (Conway *et al.*, 1962). In certain of these reports ultracentrifugal and electrophoretic studies were carried out in order to estimate the purity of the enzyme preparations: a tryptophan enzyme from pancreas (Davie *et al.*, 1956) and a tyrosine enzyme from yeast (Van de Ven *et al.*, 1958) were found to be associated with peaks representing 70–80% of the total proteins; preparations

of an alanine enzyme from liver (Webster, 1961), a tyrosine enzyme from pancreas (Schweet and Allen, 1958; Clark and Eyzaguire, 1962), and an isoleucine enzyme from *E. coli* (Norris and Berg, 1964) were found to be homogeneous. In most of these studies molecular weight determinations have not been carried out. However, Clark and Eyzaguire (1962) with use of a sucrose gradient technique obtained a molecular weight of 118,000 for the tyrosine enzyme of hog pancreas. Norris and Berg (1964) obtained a value of 112,000 for the isoleucine enzyme for *E. coli* using the equilibrium sedimentation method of Baldwin.

The homogeneous preparation of seryl s-RNA synthetase and the nearly homogeneous preparation of the phenylalanine enzyme, reported here, have molecular weights of 89,000 and 180,000 respectively. This finding is of particular interest and it might reflect a basic difference in the protein of phenylalanine enzyme, or it might represent merely a greater tendency for subunits or molecules of this enzyme to exist in an aggregated state during the molecular weight determination. The amino acid s-RNA synthetases represent a unique example of a family of enzymes obtained from the same cell and capable of catalyzing the same reaction, but with different specificity for the amino acid and polynucleotide substrates. One might have expected that nature would have solved the problem of specificity with relatively minor alterations of the molecular parameters. The existence of large differences in molecular weight between s-RNA synthetases derived from the same organism suggests that this expectation is too naive.

A K_m value of 3.2×10^{-8} M has previously been reported for tryosine s-RNA with the tyrosine enzyme from hog pancreas (Clark and Eyzaguire, 1962). These authors used unfractionated yeast s-RNA for their rate studies. Also from the data of these authors a turnover number of 14 moles of tyrosyl s-RNA per minute per mole of enzyme may be obtained. We have obtained somewhat higher turnover numbers for the serine and phenylalanine enzymes and a somewhat lower s-RNA substrate affinity for the serine enzyme, as presented in this paper. Other values are not available from the literature for comparison. A kinetic analysis of seryl s-RNA formation catalyzed by the yeast enzyme for both the homologous and heterologous serine-specific s-RNA substrate will be presented in a subsequent paper.[3]

The ratio of light absorbed by the purified yeast seryl s-RNA synthetase preparation at 280 and 260 mμ indicates the presence of about one nucleotide per enzyme molecule. This may be compared to the much lower ratios (0.9–1.0) which have been reported for the alanine enzyme of liver (Webster, 1961) and the tryptophan enzyme of pancreas (Davie *et al.*, 1956). In the latter study the main component of nucleotide present was identified as guanosine monophosphate. In contrast the tyrosine enzyme of pancreas (Schweet and Allen, 1958) has been reported to contain little or no nucleotide.

The existence of seryl s-RNA synthetase in an homogeneous crystalline form should make this enzyme useful for a variety of physical studies. Attempts to obtain large amounts of crystalline enzyme have not been successful to date, due to the small amount of enzyme obtained per preparation and the relatively low yield of crystals from the mother liquor. Apart from the observation of Davie *et al.* (1956) of the formation of large crystal plates on freezing of salt-free solutions of tryptophanyl s-RNA synthetase, the seryl s-RNA synthetase of yeast is the first of the aminoacyl synthetases to be prepared in crystalline form.

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Chemical Studies on Amino Acid Acceptor Ribonucleic Acids. IV. Position of the Amino Acid Residue in Aminoacyl s-RNA: Chemical Approach*

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ABSTRACT: A chemical method for determining the position of the aminoacyl group in the aminoacyladenosines isolated from s-RNA after pancreatic RNAase treatment was developed, based on the use of 2-cyanoethyl phosphate to phosphorylate the nonacylated hydroxyl groups. It was found that the 2-cyanoethyl group is removed by mild alkaline hydrolysis without formation of a cyclic phosphate intermediate even when a *cis*-hydroxyl group is present. The use of the reagent revealed that 65% of the aminoacyl groups in aminoacyladenosine isolated from aminoacyl s-RNA were 3′ while 35% were 2′.

The observation that the activation of amino acids for protein synthesis involved a ribonuclease-sensitive step (Holley, 1957) and that the amino acids were in fact bound to s-RNA in an intermediate step (Hoagland *et al.*, 1957, 1958; Ogata and Nohara, 1957; Ogata *et al.*, 1957) quickly led to the recognition that the amino acids were attached to either the 2′- or the 3′-hydroxyl of the terminal adenosine residue of the s-RNA molecule (Zachau *et al.*, 1958; Preiss *et al.*, 1959; Hecht *et al.*, 1959). The determination of the exact site of amino acid attachment, i.e., whether 2′ or 3′, is an important problem in the elucidation of the detailed mechanism of protein synthesis. The site of amino acid attachment is also of interest in the study of the action of inhibition of protein synthesis by puromycin which is thought to act as an analog of aminoacyl s-RNA (Yarmolinsky and De la Haba, 1959). The present paper and number V in this series describe experiments which were designed to define the site of amino acid attachment; some of the results obtained have already been briefly reported (McLaughlin and Ingram, 1963).

The *initial* site of attachment of an amino acid has been the subject of some speculation. It was predicted on the basis of the reactivity of the two hydroxyl groups of the terminal adenosine residue that the attachment would be to the 2′-hydroxyl group (Zamecnik, 1962), while on the basis of stereochemical considerations it was thought that the initial site would be 3′ (Urry and Eyring, 1962). An experimental approach to the problem has been the development of chemical methods to distinguish between the 2′- and 3′-aminoacyl esters of the adenosine end group of the s-RNA molecule (Frank and Zachau, 1963; McLaughlin and Ingram, 1963; Feldman and Zachau, 1964; Wolfenden *et al.*, 1964).

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[1] Abbreviations used in this work: ATP, adenosine triphosphate; CTP, cytidine triphosphate; pAp-3′, 3′,5′-adenosine diphosphate; and pAp-2′, 2′,5′-adenosine diphosphate.