The Purification and Properties of the Alanyl-Transfer Ribonucleic Acid Synthetase of Tomato Roots

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1. The alanyl-s-RNA synthetase of tomato roots has been purified by ammonium sulphate precipitation, adsorption on calcium phosphate gel and DEAE-cellulose chromatography and its properties have been investigated. 2. Enzyme activity was measured by using the hydroxamate assay, the [^{32}P]pyrophosphate-ATP-exchange assay and the [^{14}C]alanyl-s-RNA assay. The purified enzyme was specific for L-alanine and was activated by Mg^{2+} ions and to a smaller extent by Co^{2+} and Mn^{2+} ions. It was free from adenosine triphosphatase, pyrophosphatase and ribonuclease, and possessed a specific activity comparable with that of the most highly purified aminoacyl-s-RNA synthetases from animal and microbial systems. 3. The properties of the purified enzyme were similar in many respects to most other highly purified aminoacyl-s-RNA synthetases. It differed, however, in that the pH optimum of the hydroxamate assay was almost the same as that of the pyrophosphate-ATP-exchange assay and in requiring a high concentration of L-alanine for maximum activity ($100\text{ }\mu\text{moles/ml.}$). 4. The purified enzyme was not absolutely specific for tomato-root s-RNA; slight activity was also observed with yeast s-RNA. 5. The properties of this enzyme are fully consistent with the suggestion that the enzymic formation of alanyl-s-RNA proceeds via the intermediate formation of alanyl acyl-adenylate with the elimination of pyrophosphate from ATP. It remains to be shown the extent to which alanyl-s-RNA participates further in subsequent stages of protein synthesis in plants.

The first step in protein synthesis is apparently the carboxyl activation of amino acids in which amino acids react with ATP to form enzyme-bound aminoacyl-adenylates in the presence of specific enzymes (Hoagland, 1955; Kingdom, Webster & Davie, 1958). This step is represented by reaction (1):

These enzyme-bound aminoacyl-adenylates are then transferred to specific soluble s-RNA† (Holley, 1957; Hoagland, Zamecnik & Stephenson, 1957; Ogata & Nohara, 1957), to which they become bound by an ester linkage through the ribose 2'- or 3'-hydroxyl groups of the terminal adenosine residue (Zachau, Archs & Lipmann, 1958; Wong & Moldave, 1960).

The enzymes catalysing these reactions have been detected in animal preparations (Cole, Coote

& Work, 1957; Campbell, 1960), microbial preparations (Nismann, 1959) and plant preparations (Davis & Novelli, 1958; Clark, 1958; Marcus, 1959; Webster, 1959). Until the work of Moustafa & Lyttleton (1963), however, these enzymes have been purified and characterized only from animal systems (Webster & Davie, 1961; Webster, 1961) and microbial systems (Berg, 1956). In plants, moreover, the actual role of free amino acids in the synthesis of proteins has been somewhat uncertain (Steward & Bidwell, 1962).

To examine more fully protein synthesis in plants an investigation was carried out to detect, purify and characterize an enzyme isolated from plant material bringing about these initial steps in protein synthesis. The earlier investigation of protein synthesis in barley seedlings (Cocking & Yemm, 1961), which demonstrated the ready incorporation of ^{15}N -labelled ammonium phosphate into the alanine of the root proteins, suggested the suitability of the study of alanyl-s-RNA synthetase. This enzyme [L-alanine-s-RNA ligase (AMP), EC 6.1.1.7] has now been highly purified from seedling tomato roots and its properties have been investigated and

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† Abbreviation: s-RNA, transfer or 'soluble' RNA.

correlated with other aminoacyl-s-RNA synthetases purified from different sources.

EXPERIMENTAL

Materials. All amino acids used were purchased from F. Hoffmann-La Roche and Co. (Basle, Switzerland), and their purity was checked by partition chromatography. L- α -Amino-n-butyric acid, D- α -amino-n-butyric acid and DL-alanyl-DL-alanine were obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.). ATP (dipotassium salt) was purchased from Pabst Research Biochemical Laboratories (Milwaukee, Wis., U.S.A.), and CTP, calcium phosphate gel and L- α -alanine hydroxamate from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Hyamine 10X [2-methyl-4-(1,1,3,3-tetramethylbutyl)phenoxyethoxyethyl-dimethylbenzylammonium chloride monohydrate] and liquid scintillator NE213 were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh. [32 P]Pyrophosphate and L-[14 C]alanine were purchased from The Radiochemical Centre (Amersham, Bucks.).

Analytical procedures. The protein concentration of enzyme preparations was determined by using the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951), with egg albumin as a standard. Orthophosphate was determined by the method of Fiske & Subbarow (1925), and RNA by the method of Slater (1958).

Adenosine-triphosphatase activity was measured by the method of Webster (1961) and ribonuclease activity determined spectrophotometrically by the method of Kunitz (1946). Pyrophosphatase activity was measured as described by Heppel (1955). Radioactivity measurements were made with a liquid-scintillation counter (Panax type SCLP). Portions (0.1 ml., aqueous) were blended with the NE213 scintillator by using a molar solution of Hyamine 10X in methanol (A.R.) (Brown & Badman, 1961). Samples were placed in the dark for 15 min. before counting; the normal background was 36 counts/min. and the counting efficiency was approximately 30%. The s-RNA was isolated by the modification of Kirby's phenol-extraction method (Monier, Stephenson & Zamecnik, 1960).

Hydroxamate assay. The complete reaction mixture contained 10 μ moles of $MgCl_2$, 100 μ moles of tris-HCl buffer, pH 8.3, 100 μ moles of L-alanine, 9 μ moles of dipotassium ATP neutralized to pH 7.0 with KOH, m-hydroxylamine hydrochloride neutralized to pH 7.0 with KOH and enzyme preparation to a final volume of 1.0 ml. Reaction mixtures were incubated at 37° for 1 hr. unless otherwise stated since experiments had shown that the rate of hydroxamate formation was linear for at least 1 hr. The reaction was terminated by the addition of 1.0 ml. of m-trichloroacetic acid and the precipitated protein removed by centrifugation. Portions (1.0 ml.) of the supernatant were taken for the estimation of hydroxamate (Schweet, 1955). The intensity of the colour produced when 1M-ferric chloride-2N-HCl (1:1, v/v) was added to the supernatant was determined after 5 min. to ensure maximum colour development. L- α -Alanine hydroxamate was used as standard. The activity of the enzyme is reported as the amount of hydroxamate formed with the complete assay mixture minus that formed with the control assay mixture without added amino acid. In this way the influence of all chromogenic impurities in the assay mixture are eliminated. In the crude enzyme preparation assays amino acid hydroxamates will

be readily formed in the control assay owing to the presence of endogenous amino acids. The addition of an amino acid under these conditions will often fail to increase the quantity of hydroxamate formed and in certain instances may apparently reduce the quantity of hydroxamate formed, probably because different amino acid hydroxamates yield ferric-amino acid hydroxamates with different extinction coefficients. If the quantity of hydroxamate formed in the control mixture is greater than that formed in the complete assay mixture a negative activity is reported. A unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 μ mole of hydroxamate/hr. The specific activity is expressed as the number of units/mg. of protein.

Exchange assay. The complete reaction mixture contained 10 μ moles of $MgCl_2$, 50 μ moles of tris-HCl buffer, pH 8.3, 9 μ moles of ATP (dipotassium salt) neutralized to pH 7.0 with KOH, 9 μ moles of [32 P]pyrophosphate (specific activity 70 000 counts/min./ μ mole), 100 μ moles of L-alanine and enzyme preparation to a final volume of 1.0 ml. The reaction mixture was incubated at 37° for 30 min. and the reaction stopped by the addition of 1.0 ml. of 10% trichloroacetic acid. The ATP and pyrophosphate were separated by the method of Crane & Lipmann (1953) as modified by DeMoss & Novelli (1956) by using activated acid-washed charcoal (Ultrasorb Z.X., British Carbo-Norit Union Ltd.) (Pennington, 1960). The ATP was then hydrolysed with acid and the radioactivity and orthophosphate were determined in suitable portions. With every exchange assay control experiments were carried out without the addition of L-alanine to eliminate the effects of any endogenous amino acids or of any exchange of pyrophosphate into ATP independent of L-alanine. The specific activity of the crude enzyme preparation and of the purified enzyme is reported as μ moles of [32 P]pyrophosphate exchanged/hr./mg. of protein (DeMoss & Novelli, 1956). The activity of the enzyme at various stages of its purification is, however, more conveniently expressed as the counts in ATP/min./mg. of protein due to the addition of L-alanine to the above reaction mixture containing [32 P]-pyrophosphate.

L-Alanyl-s-RNA assay. The complete reaction mixture contained 10 μ moles of $MgCl_2$, 50 μ moles of tris-HCl buffer, pH 7.5, 9 μ moles of ATP (dipotassium salt) neutralized to pH 7.0 with KOH, 1.0 μ mole of L-[14 C]-alanine (activity 360 000 counts/min./ μ mole), 10 μ g. of pyrophosphatase (kindly provided by Dr M. Kunitz), 10 mg. of s-RNA and enzyme preparation to a final volume of 1.0 ml. The tubes were incubated at 37° for 30 min. The reaction was stopped and the RNA, together with any L-alanyl-s-RNA produced, precipitated by the addition of 1.0 ml. of 1M-trichloroacetic acid. The precipitate was washed twice with cold 5% trichloroacetic acid, twice with ethanol and once with ether. When only small amounts of s-RNA were available, necessitating the use of only a few milligrams of s-RNA in the incubation mixture, 10 mg. of carrier RNA was added before precipitation with trichloroacetic acid. Finally the washed precipitate was dissolved in Hyamine hydrochloride, by the method of Brown & Badman (1961), and counted by the liquid scintillation technique. Control experiments were carried out, both with no enzyme and with boiled enzyme to eliminate errors due to the adsorption of the L-[14 C]alanine on to protein or RNA. The specific activity of the enzyme is reported as

the number of μ moles of L-[^{14}C]alanyl-s-RNA formed/hr./mg. of protein.

Growth of tomato plants and preparation of crude enzyme extract. Tomato seeds (var. Sutton's 'Best of All') were soaked in distilled water for 16 hr. They were then sterilized in a 1% bromine solution for 4 min. and washed in six changes of sterile distilled water. The seeds were then sown in coarse sterile sand and placed in a slightly humid constant-temperature room at 27°. They were kept initially in the dark for 3 days to encourage germination. Later they were placed under white fluorescent tubes giving 250 ft.-candles at the surface of the seed boxes. Growth was continued for a further 14 days (shoot height 1–2 in.). The young seedlings were then carefully removed from the sand and well washed in distilled water to remove sand particles. The roots of the seedlings were excised and placed immediately in distilled water. They were gently dried between filter paper and their fresh weight was recorded. All further manipulations were carried out in a cold room at 0–4°. The root material (0.5 g. fresh wt.) was macerated in 0.02 M-potassium phosphate buffer, pH 6.8 (0.1 g. fresh wt./1.0 ml. final vol.) with an all-glass macerator. The extract was then centrifuged at 1150g for 30 min. and the supernatant collected. The residue was washed by resuspending it as a suitable volume of the buffer; after maceration and centrifugation the supernatant from this washing was added to the initial supernatant to give the crude enzyme preparation.

Sephadex gel filtration. Endogenous amino acids were removed from the crude enzyme preparation by adding 1.0 ml. of this extract (1.1 mg. of protein) to a column containing 6.0 g. of Sephadex G-25 (medium grade) equilibrated with 0.02 M-potassium phosphate buffer, pH 6.8, and eluting with the same buffer.

Purification of L-alanyl-s-RNA synthetase present in the crude enzyme preparation. The crude enzyme extract was brought to 45% saturation with ammonium sulphate solution at pH 5.5 and allowed to stand for 30 min. The precipitate was collected by centrifugation at 1,150g for 30 min. and discarded. Ammonium sulphate solution at pH 5.5 was added to the supernatant to 65% saturation and the solution was again allowed to stand for 30 min. The precipitate was collected by centrifugation at 1,150g for 30 min. and the supernatant discarded.

The precipitate obtained by the above procedure was resuspended in 0.02 M-phosphate (pH 5.0) buffer and added

to 1 ml. of a suspension of calcium phosphate gel (0.21 g. of calcium phosphate gel/ml. of distilled water adjusted to pH 5.0). The mixture was shaken continually for 10 min. to prevent sedimentation, centrifuged at 1150g for 10 min. and the supernatant discarded. The gel to which the enzyme had become adsorbed was resuspended in 1.0 ml. of 0.1 M-potassium phosphate buffer, pH 6.8, and the above procedure of mixing and shaking repeated. The mixture was then centrifuged as before and the supernatant collected.

The partially purified enzyme (the supernatant from the calcium phosphate gel) was applied to the top of a DEAE-cellulose column and eluted step-wise with 0.02 M-, 0.05 M-, 0.1 M- and 0.2 M-potassium phosphate buffer, pH 6.8. Fractions (3.0 ml.) were collected and analysed for protein and nucleic acid by measurement of the extinction of light at 280 m μ and 260 m μ respectively. Purification of the L-alanyl-s-RNA synthetase was followed with both the hydroxamate assay and the pyrophosphate-ATP-exchange assay. At intermediate stages in the purification only the hydroxamate assay was employed and assays were carried out in the presence of 45 μ moles of ATP and at pH 8.3, since these conditions had been shown to be optimum for this assay when the enzyme was only slightly purified. The hydroxamate assay was used to assay all the peaks separated by DEAE-cellulose chromatography. The peaks showing L-alanyl-s-RNA-synthetase activity as deduced from the hydroxamate assay were then assayed by the more sensitive [^{32}P]pyrophosphate-ATP-exchange method. The hydroxamate assay apparently detected activity in two peaks: peak A eluted in 0.05 M-buffer and peak B eluted in 0.2 M-buffer, pH 6.8. The pyrophosphate-ATP-exchange assay, however, detected activity only in peak A (see the Discussion section). A summary of the purification procedure is shown in Table 1. The purified enzyme was found to be unstable to storage at 37°, 4° or –10°. The best stability was obtained at 37°. At 37° after 72 hr. there was only 35% loss of activity; at 4° there was complete loss of activity after 24 hr. At –10° there was 19% loss of activity after 24 hr., 27% loss after 33 hr. and complete loss of activity after 72 hr. This instability at –10° is rather unusual; the stability could not be maintained satisfactorily by the addition of Mg^{2+} ions, s-RNA or glutathione. All further experiments were therefore carried out with freshly purified enzyme.

Table 1. *Purification of the alanyl-s-ribonucleic acid synthetase of tomato roots*

Fraction	Stage	Yield* (%)	Total protein (mg.)	Sp. activity (μ moles of alanine hydroxamate formed/hr./ mg. of protein)	Sp. activity (μ moles of [^{32}P]pyro- phosphate exchanged/ hr./mg. of protein)
Crude enzyme preparation	1	100	3.60	0.5	0.019
(NH_4) $_2\text{SO}_4$ precipitation	2	97	0.222	7.5	†
Calcium phosphate-gel ad- sorption	3	75	0.111	12	†
DEAE-cellulose chromato- graphy	4				
Peak A		60	0.022	48	66.8
Peak B		45	0.039	24	0

* Determined from hydroxamate assay.

† No determinations.

RESULTS

Amino acid-activating enzymes in the crude enzyme preparation. The ability of the crude enzyme extract to form hydroxamates of all the amino acids and amides normally found in proteins was demonstrated. The reaction mixture was similar to that for the hydroxamate assay as used in most subsequent stages of purification and contained 100 μ -moles of each amino acid or amide together with 45 μ -moles of ATP rather than only 10 μ -moles of ATP. Published data were used to ascertain the probable pH optimum for enzyme activity. Only after endogenous amino acids had been removed from the crude enzyme preparation by Sephadex

(G-25) filtration was it possible to demonstrate the presence of certain of the enzymes, and, moreover, in most instances the specific activity was also increased by this treatment (Table 2).

Properties of the purified alanyl-s-RNA synthetase.

(a) Hydroxamate formation and pyrophosphate-ATP-exchange activity. The requirements for the hydroxamate and pyrophosphate-ATP-exchange assays are shown in Table 3. Both systems showed a definite requirement for ATP, Mg^{2+} ions and L-alanine. The enzyme is specific for L-alanine; no significant activity was demonstrated with D-alanine or with either the D- or L-configurations of the closely related α -amino-*n*-butyric acid, nor was any significant activity demonstrated with β -alanine, DL-phenylalanine, DL-serine or DL-alanyl-DL-alanine. DL-Alanine showed only one-fifth of the activity of L-alanine. Owing to the relative insensitivity of the hydroxamate assay the more sensitive pyrophosphate-ATP-exchange assay was employed; each assay mixture contained 15 mg. of the purified enzyme (peak A, stage 4, Table 1) and 100 μ -moles of each amino acid.

As illustrated in Fig. 1 for both the hydroxamate assay and the pyrophosphate-ATP-exchange assay sharp pH optima at pH 8.30 were observed.

Fig. 2 demonstrates the effect of L-alanine on the rate of hydroxamate formation and of [^{32}P]pyrophosphate exchange into ATP. From this graph

Table 2. *Amino acid-activating enzymes present in the crude enzyme preparation from tomato seedling roots*

The hydroxamate assay was used without the addition of inorganic pyrophosphatase. The complete reaction mixture contained 10 μ -moles of $MgCl_2$, 100 μ -moles of tris-HCl buffer (at the appropriate pH), 100 μ -moles of the amino acid or amide, 45 μ -moles of ATP (dipotassium salt) neutralized to pH 7.0 with KOH, 1M-hydroxylamine hydrochloride neutralized to pH 7.0 with KOH and enzyme preparation (300 μ g. of protein) in a final volume of 1.0 ml. L-Alanine hydroxamate was used as the standard in the estimation of the μ -moles of amino acid hydroxamate formed.

Amino acid or amide	pH of assay	Sp. activity (μ -moles of amino acid hydroxamate formed/hr./mg. of protein)	
		Before Sephadex treatment	After Sephadex treatment
L-Alanine	8.3	0.62	6.00
L-Arginine	8.3	(-0.36)	1.88
DL-Aspartic acid	6.8	0.32	4.81
DL-Asparagine	6.8	0.32	20.38
L-Cysteine	6.8	1.09	6.73
L-Cystine	6.8	0.0	6.73
DL-Glutamic acid	7.5	3.41	15.38
DL-Glutamine	6.8	0.78	4.81
Glycine	8.3	1.05	0.00
DL-Histidine	7.5	1.40	9.42
L-Hydroxyproline	8.3	0	3.85
DL-Isoleucine	7.5	0	2.89
DL-Leucine	7.5	0.32	0.77
DL-Lysine	8.3	0.30	0.39
DL-Methionine	8.3	0.62	0.39
DL-Phenylalanine	8.3	1.55	0.39
DL-Proline	8.3	0	0.39
DL-Serine	7.5	0.30	0.39
DL-Threonine	8.3	(-1.55)	0.78
DL-Tyrosine	8.3	1.24	1.88
DL-Tryptophan	8.3	3.41	3.77
DL-Valine	8.3	0.19	0.39

Table 3. *Requirements for the action of the L-alanine-activating enzyme*

Both hydroxamate assay and the pyrophosphate-ATP-exchange assay were employed. The hydroxamate assay was carried out without the addition of inorganic pyrophosphatase. Each assay mixture contained 15 μ g. of protein of the purified enzyme preparation (peak A, stage 4, Table 1).

Modification to assay system	Hydroxamate formed (μ -moles/hr./mg. of protein)	[^{32}P]Pyrophos- phate-ATP- exchange assay ($10^4 \times$ counts in ATP/min./mg. of protein)
None (complete)	51	22.0
Minus ATP	0	0
Minus Mg^{2+}	0	0.16
Minus L-alanine	0	0
Complete (boiled)	0	0.59
Minus enzyme	0	0
None (10 μ -moles of $MgCl_2$ present)	40	5.2
10 μ -moles of $MnCl_2$ in place of 10 μ -moles of $MgCl_2$	20	1.0
10 μ -moles of $CoCl_2$ in place of 10 μ -moles of $MgCl_2$	20	4.5

Fig. 1. Effect of pH on the rate of alanine activation. The hydroxamate assay (●) mixtures contained 10 μ moles of $MgCl_2$, 100 μ moles of tris-HCl buffer and 1M-hydroxylamine adjusted to the required pH, 9 μ moles of neutralized ATP (dipotassium salt), 100 μ moles of L-alanine and purified enzyme preparation (15 μ g. of protein) to a final volume of 1ml. The exchange-assay (○) mixtures contained 10 μ moles of $MgCl_2$, 50 μ moles of tris-HCl buffer adjusted to the required pH, 9 μ moles of neutralized ATP (dipotassium salt), 9 μ moles of tetrasodium [^{32}P]pyrophosphate and purified enzyme preparation (15 μ g. of protein) to a final volume of 1ml. Enzyme incubations were carried out in duplicate and three portions taken from each for the determination of activity.

the Michaelis constant of the enzyme for L-alanine was calculated by using the method of Hoftee (1952) and shown to be $7.1 \times 10^{-2}M$ with respect to the hydroxamate assay and $2.8 \times 10^{-2}M$ with respect to the exchange assay.

Lower concentrations of ATP and Mg^{2+} ions were required for maximum activity. In both the hydroxamate assay and the exchange assay 10 μ moles of ATP supported maximum activity (Fig. 3). Both assay reactions required the presence of Mg^{2+} ions although the amount required for maximum activity in the hydroxamate assay was 10 μ moles and that required in the exchange assay was only 5 μ moles (Fig. 4). Accordingly in the hydroxamate assay the maximum activity was obtained when the concentrations of ATP and Mg^{2+} ions were approximately equimolar. In both assays (Table 3) Mn^{2+} and Co^{2+} ions were able to replace Mg^{2+} ions, but were never able to support as much activity as the Mg^{2+} ions. Maximum activity was measured with the hydroxamate assay when

Fig. 2. Derivation of the Michaelis constant for alanine activation. The hydroxamate assay (●) and the [^{32}P]pyrophosphate-ATP-exchange reaction (○) were both employed. Both assays were carried out as described in the Experimental section and each contained 15 μ g. of protein of the purified enzyme preparation (peak A, stage 4, Table 1).

Fig. 3. Effect of ATP concentration on the [^{32}P]pyrophosphate-ATP-exchange reaction and on the hydroxamate assay. The hydroxamate assay (●) and exchange-assay (○) mixtures both contained 15 μ g. of protein (purified enzyme). Composition of the assay mixtures was as described in the Experimental section, apart from the variation indicated in the concentration of ATP.

600 μ moles of hydroxylamine were added to the reaction mixture (Fig. 5).

The addition of both pyrophosphatase and s-RNA increased the rate of hydroxamate formation

Fig. 4. Effect of Mg^{2+} ion concentration on the $[^{32}P]$ -pyrophosphate-ATP-exchange reaction and on the hydroxamate assay. The hydroxamate-assay (●) and exchange-assay (○) mixtures both contained $15\mu g.$ of protein (purified enzyme). Composition of the assay mixtures was as described in the Experimental section apart from the variation indicated in the concentration of $MgCl_2$.

Fig. 5. Effect of hydroxylamine concentration on the formation of alanine hydroxamate. Mixtures were incubated at 37° with the concentration of hydroxylamine shown. Assays also contained $10\mu moles$ of $MgCl_2$, $100\mu moles$ of tris-HCl buffer, pH 8.3, $100\mu moles$ of L-alanine, $9\mu moles$ of neutralized ATP (dipotassium salt) and purified enzyme ($15mg.$ of protein) to a final volume of $1.0ml.$

whereas addition of pyrophosphate decreased this rate (Table 4). Added s-RNA decreased the rate of exchange of $[^{32}P]$ pyrophosphate into ATP (Table 4).

An assay mixture was prepared with $9\mu moles$ of $[^{32}P]$ orthophosphate instead of $[^{32}P]$ pyrophosphate. The ATP was separated from the orthophosphate or, in the control experiment, from the pyrophos-

Table 4. Effect of added pyrophosphatase, pyrophosphate and s-ribonucleic acid on the activity of the alanine-activating enzyme

In the investigation of the action of pyrophosphatase and pyrophosphate only the hydroxamate assay was employed, whereas in the study of the effect of added s-RNA both the hydroxamate and the pyrophosphate-ATP-exchange assays were used. Each assay mixture contained $15\mu g.$ of protein of the purified enzyme preparation (peak A, stage 4, Table 1). Pyrophosphate was added in the form of the tetrasodium salt, and since Na^+ ions are sometimes inhibitory solutions of NaCl were prepared to give concentrations of Na^+ ions equivalent to those present in the samples of pyrophosphate added.

Modification to the assay mixture	Hydroxamate formed ($\mu moles/hr./mg.$ of protein)	$10^{-4} \times$ Counts in ATP/min./mg. of protein
(1) Effect of added crystalline pyrophosphatase		
None	40	
Plus $40\mu g.$ of pyrophosphatase	93	
(2) Effect of added pyrophosphate		
None	80	
Plus $1.0\mu mole$ of pyrophosphate	14	
Plus $10\mu moles$ of pyrophosphate	0	
Plus Na^+ ($\equiv 1.0\mu mole$ of pyrophosphate)	80	
Plus Na^+ ($\equiv 10\mu moles$ of pyrophosphate)	80	
(3) Effect of added soluble yeast RNA		
None	50	15.2
Plus $0.1mg.$ of s-RNA	150	*
Plus $1.0mg.$ of s-RNA	190	*
Plus $5.0mg.$ of s-RNA	*	0.51
Plus $10mg.$ of s-RNA	*	0

* No determinations.

phate, and the radioactivity in the ATP determined. When $[^{32}P]$ orthophosphate was used in the assay mixture no radioactivity was detected in the ATP; however, when $[^{32}P]$ pyrophosphate was used the expected amount of radioactivity, resulting from the pyrophosphate-ATP exchange, was detected in the ATP.

(b) The enzymic formation of L-alanyl-s-RNA. In the presence of ATP, Mg^{2+} ions, s-RNA, pyrophosphatase and L- $[^{14}C]$ alanine the purified enzyme was capable of catalysing the formation of L-alanyl-s-RNA. The s-RNA was extracted from baker's yeast and *Candida utilis* by the modified phenol method of Monier *et al.* (1960). For the extraction of s-RNA from tomato roots the roots

were prepared as for the crude enzyme preparation but were homogenized with acid-washed sand. After centrifugation to remove the sand an equal volume of 90% (w/v) phenol was added to the supernatant and the s-RNA extracted as for yeast s-RNA. s-RNA isolated not only from tomato roots but also from baker's yeast and *Candida utilis* was active in this reaction. However, the amount of [^{14}C]alanyl-s-RNA formed was greatest with tomato-root s-RNA. With tomato-root s-RNA (10mg.) the specific activity of the purified enzyme was $0.97 \mu\text{mole}$ of [^{14}C]alanyl-s-RNA formed/hr./mg. of protein and with *Candida utilis* s-RNA (10mg.) and baker's yeast s-RNA (10mg.) the specific activities were 0.06 and 0.05 respectively.

When the enzyme's activity was assayed at various pH values activity was detected over the range pH 6.5–8.2 (Fig. 6). The addition of increasing amounts of baker's yeast s-RNA caused a steady increase in the quantity of [^{14}C]alanyl-s-RNA formed in a given time. The quantity of [^{14}C]alanyl-s-RNA formed was almost doubled by increasing the amount of baker's yeast s-RNA from 4 to 17mg. Similarly the addition of increasing amounts of the purified enzyme caused an increase in the quantity of [^{14}C]alanyl-s-RNA formed. However, after 0.5ml. of the purified enzyme had

been added no further increase was observed, since after this the concentration of L-[^{14}C]alanine became limiting as the quantity of L-[^{14}C]alanine present was only $1 \mu\text{mole}$ (Fig. 7). The apparent activity of the boiled enzyme in Fig. 7, which increases with increase in the concentration of boiled enzyme, is probably due to absorption of L-[^{14}C]alanine.

Effect of potassium fluoride on the activity of crude enzyme preparation and on the activity of purified enzyme. Potassium fluoride was added to both enzyme preparations and the activity measured by using the hydroxamate assay. The addition of at least $20 \mu\text{moles}$ of potassium fluoride was necessary for the detection of any increased rate of hydroxamate formation in the crude enzyme preparation. No effect was observed on the purified enzyme (Table 5).

Assay of crude enzyme preparation and purified enzyme for ribonuclease, adenosine triphosphatase and inorganic pyrophosphatase activities. Both the purified enzyme and the crude enzyme preparation were assayed for the presence of these three enzymes. All three enzymes were present in the crude extract but the purified enzyme had no detectable ribonuclease, pyrophosphatase or adenosine-triphosphatase activities.

Effect of added CTP and ATP on the enzymic formation of L-alanyl-s-RNA. The effect of pre-treating baker's yeast s-RNA with ATP and CTP

Fig. 6. Effect of pH on the enzymic synthesis of alanyl-s-RNA. The complete assay mixture (●) contained $10 \mu\text{moles}$ of MgCl_2 , $50 \mu\text{moles}$ of tris-HCl buffer adjusted to the required pH with N-HCl , $9 \mu\text{moles}$ of neutralized ATP (dipotassium salt), $1.0 \mu\text{mole}$ of L-[^{14}C]alanine, $10 \mu\text{g.}$ of pyrophosphatase, 10mg. of s-RNA and purified enzyme preparation ($15 \mu\text{g.}$ of protein) to a final volume of 1.0ml. Tubes were incubated at 37° for 30 min. and the reaction was stopped, and the [^{14}C]alanyl-s-RNA formed precipitated by the addition of 1.0ml. of 1M -trichloroacetic acid. Control assays were used both without added enzyme (■) and with boiled enzyme (▲) to correct for adsorption of [^{14}C]alanine on to enzyme protein or on to s-RNA.

Fig. 7. Effect of enzyme concentration on alanyl-s-RNA synthesis. The complete assay mixture (●) contained varying amounts of purified enzyme ($10 \mu\text{g.}$ of protein/ml.) but was in other respects identical with that described in the Experimental section. Control assays were used both with no added enzyme (■) and with boiled enzyme (▲) to correct for adsorption of [^{14}C]alanine on to enzyme protein or on to s-RNA. The volume of the reaction mixture was 1.0ml. until 0.5ml. of purified enzyme had been added. Thereafter it was 1.5ml.

Table 5. *Effect of potassium fluoride on the activity of the alanine-activating enzyme*

The hydroxamate assay was employed for the determination of activity in both the crude and purified enzyme preparations. Crude enzyme preparations (300 μ g. of protein/ml. of assay mixture) were assayed in the presence of 45 μ moles of ATP and purified enzyme preparations (15 μ g. of protein/ml. of assay mixture) were assayed with 10 μ moles of added ATP. No pyrophosphatase was added.

KF added (μ moles)	Hydroxamate formed (μ moles/hr./mg. of protein)	
	Crude enzyme preparation (stage 1, Table 2)	Purified enzyme (peak A, stage 4, Table 1)
0	1.5	50
10	1.5	52
20	2.5	50
50	6.0	50
100	6.4	50

was investigated. s-RNA (10 mg.) was mixed with 10 μ moles of CTP and 10 μ moles of ATP and allowed to stand at 37° for 15 min. Then ATP, Mg^{2+} ions, pyrophosphatase, L-[^{14}C]alanine and purified enzyme were added as in the standard procedure. There was a 65% increase in the amount of L-[^{14}C]alanyl-s-RNA formed when the s-RNA was pre-treated with ATP and CTP.

DISCUSSION

One of the chief difficulties in assessing the purification of aminoacyl-s-RNA synthetases rests in the difficulty of adequate assay of enzyme activity at the various stages of purification. This is particularly the case when the crude enzyme preparations are assayed for enzyme activity. The pyrophosphate-ATP exchange is a valid assay only if the concentrations of the reactants remain constant during the course of the reaction. The presence of adenosine triphosphatase or inorganic pyrophosphatase in the enzyme preparation will almost certainly ensure that the concentrations of ATP and pyrophosphate do not remain constant. The hydroxamate assay is likewise beset with difficulties, particularly if pyrophosphatase is not added to assay mixtures and if s-RNA is present in excess (Stulberg & Novelli, 1963; Webster, 1961). The best assay is one which measures the overall reaction, namely the incorporation of the amino acid into s-RNA; this is not normally, however, readily applicable to crude enzyme preparations owing to the presence of high activity of ribonuclease, particularly in plant extracts.

The alanyl-s-RNA synthetase, which was purified over 3000-fold from extracts of tomato roots (Table 1), was highly specific for L-alanine and possessed a specific activity, based on pyrophosphate-ATP exchange, comparable with that described for other highly purified aminoacyl-s-RNA synthetases (Norton, Ravel, Lee & Shive, 1963). Further, its properties were similar in many instances to the properties of aminoacyl-s-RNA synthetases purified from other sources, particularly the L-alanyl-s-RNA synthetase isolated by Webster (1961) from pig liver and the D-alanine-activating enzyme isolated by Baddiley & Neuhaus (1960). The apparent inhibition of L-alanine-stimulated pyrophosphate exchange by D-alanine is, however, a noteworthy difference, since D-amino acids have not usually been observed to be inhibitory; DL-amino acids are usually found to be one-half as active in promoting exchange as L-amino acids (DeMoss & Novelli, 1956). Moreover, the enzyme differed from most other aminoacyl-s-RNA synthetases in requiring a relatively high concentration of amino acid for maximum activity (100 μ moles of L-alanine/ml. of reaction mixture). The Michaelis constant based on measurements from the exchange assay was $2.8 \times 10^{-2} M$ for L-alanine. This unusually high value is in accordance with the data published by Baddiley & Neuhaus (1960) for their D-alanine-activating enzyme, but contrary to reports that amino acid-activating enzymes have high substrate affinities with Michaelis constants of the order of $6 \times 10^{-5} M$ (e.g. for tyrosine) (Schweet & Allen, 1958), and that amino acid molarities of $10^{-4} M$ are sufficient to saturate the enzymes. As has been suggested by Baddiley & Neuhaus (1960), high K_m values may account for the failure to detect the presence of these enzymes, particularly if a fixed and too low a concentration of the amino acid is employed. It is not surprising therefore that Davis & Novelli (1958) were unable to detect the presence of alanyl-s-RNA synthetase by using the pyrophosphate-ATP-exchange assay with 1 μ mole of alanine/ml. of reaction mixture. It has come to be recognized that differences in the pH optima may also account for inability to detect the presence of these enzymes (Webster, 1959). However, even for the same enzyme the pH optima may be different for the pyrophosphate-ATP exchange and the hydroxamate assay (Webster, 1961) and not nearly the same as in the present study, and may be altered by the type of buffer employed in the assay system (Bergman, Berg & Dieckmann, 1961).

The postulated mechanism for the enzymic formation of aminoacyl-s-RNA compounds, which involves the intermediate formation of aminoacyl-adenylates and of pyrophosphate, is fully

substantiated by the present results. The overall reaction may be represented as:

The fact that pyrophosphate inhibits and pyrophosphatase stimulates the rate of enzymic formation of amino acid hydroxamates (Table 4) is therefore to be expected, since the hydroxamate assay depends on the reaction of hydroxylamine with the intermediate enzyme-bound amino acyl-adenylate to form the amino acid hydroxamate. As would also be expected from the above reaction pathway s-RNA decreases the rate of exchange of pyrophosphate into ATP (Table 4), but increasing amounts of s-RNA stimulate the rate of formation of alanyl-s-RNA. The stimulation of the rate of alanine hydroxamate formation by added s-RNA (Table 4) is probably more indirect, involving a reaction between alanyl-s-RNA and hydroxylamine, as suggested by Webster (1961):

The inhibition of the pyrophosphate-ATP-exchange reaction by s-RNA probably accounts for the failure to detect enzyme activity in peak B (Table 1) of the DEAE-cellulose chromatogram even though activity is readily demonstrable in this peak by using the hydroxamate assay. Peak B was shown to contain a greater amount of ribonucleic acid than peak A. The failure to obtain any enzymic phosphate-ATP exchange with the purified enzyme is further evidence for the suggested pathway for the synthesis of the alanyl-s-RNA, and serves to demarcate this reaction from the reactions involved in the enzymic synthesis of the amides glutamine and asparagine which in plants involve a phosphate-ATP exchange (Webster & Varner, 1955), although it should be noted that the synthesis of amides in bacteria often involves a pyrophosphate-ATP exchange (Ravel, Norton, Humphreys & Shive, 1962). When the overall enzymic reaction involving the formation of alanyl-s-RNA is carried out the purified enzyme is, as would be expected from reaction (2), active in the presence of L-alanine, ATP and Mg^{2+} ions, and the rate of the reaction is increased by increasing amounts of s-RNA.

The specific activity of the purified alanyl-s-RNA synthetase as measured by alanyl-s-RNA formation ($0.97 \mu\text{mole}$ of alanyl-s-RNA formed/hr./mg. of protein) compares favourably with that of aspartyl-

s-RNA synthetase ($0.058 \mu\text{mole}$ of aspartyl-s-RNA formed/hr./mg. of protein) purified by Norton *et al.* (1963). It is lower, however, than the specific activity of the alanyl-s-RNA synthetase purified by Webster (1961) from pig liver cytoplasm and also lower than the specific activity determined either by pyrophosphatase-ATP exchange or by the hydroxamate reaction (Table 2). This is not unexpected, since there is evidence in the present investigation for the concentration of s-RNA being rate-limiting.

The specific activity of the crude enzyme preparation as measured by the hydroxamate assay is far greater than that measured by the pyrophosphate-ATP-exchange assay, whereas the specific activity of the purified enzyme as measured by the hydroxamate assay is less than that measured by the pyrophosphate-ATP-exchange assay (Table 1). In the hydroxamate assay the amount of amino acid hydroxamate present is estimated colorimetrically by adding Fe^{3+} ions in acid solution. The colour is formed by formation of a complex of Fe^{3+} ions and hydroxyl groups. However, aminoacyl-s-RNA complexes also produce intensely coloured complexes with Fe^{3+} ions (de Kloet, Shuurs, Koningsberger & Overbeek, 1960). Evidence has accumulated in the present work that these aminoacyl-s-RNA complexes are readily formed in crude enzyme preparations from endogenous soluble RNA on the addition of amino acids but not in purified enzyme preparations from which soluble RNA has been removed. This will result in purified enzyme preparations, when assayed by the hydroxamate assay and compared with crude enzyme extracts assayed by the same procedure, being apparently less highly purified than the same preparations compared and assayed by the pyrophosphate-ATP-exchange method (Table 1). Moreover it is also evident that the presence of adenosine triphosphatase in the crude enzyme preparation but not in the purified enzyme preparation will greatly increase the apparent purification of the enzyme as deduced from the pyrophosphate-ATP-exchange assay.

High endogenous amounts of amino acids coupled with the presence of adenosine triphosphatase in plant extracts often make difficult the detection of these activating enzymes. Sephadex treatment is particularly useful in removing endogenous amino acids and potassium fluoride can be used to inhibit adenosine-triphosphatase activity (Table 5), although it should be noted that with certain plant material potassium fluoride also apparently causes an inhibition of aminoacyl-s-RNA-synthetase activity (Peterson & Fowden, 1963). It remains to be shown the extent to which these enzymes are associated with structural compounds of the plant cell and the extent to which the products of their action, the amino-

acyl-s-RNA compounds, participate further in subsequent stages of protein synthesis in plants.

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