Purification, Properties and Comparative Specificities of the Enzyme Prolyl-Transfer Ribonucleic Acid Synthetase from *Phaseolus aureus* and *Polygonatum multiflorum*

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1. A prolyl-s-RNA synthetase (prolyl-transfer RNA synthetase) has been purified about 250-fold from seed of *Phaseolus aureus* (mung bean), a species not producing azetidine-2-carboxylic acid, and more than 10-fold from rhizome apices of *Polygonatum multiflorum*, a liliaceous species containing azetidine-2-carboxylic acid. The latter enzyme was unstable during ammonium sulphate fractionation. 2. The enzymes exhibited different substrate specificities towards the analogue. That from *Phaseolus*, when assayed by the ATP-PP_i exchange, showed azetidine-2-carboxylic acid activation at about one-third the rate with proline. Both labelled imino acids gave rise to a labelled aminoacyl-s-RNA. The enzyme from *Polygonatum*, however, activated only proline. 3. The enzyme from *Polygonatum* also formed a labelled prolyl-s-RNA with *Phaseolus* s-RNA but at a lower rate than when the *Phaseolus* enzyme was used. No reaction occurred when the *Phaseolus* enzyme was coupled with *Polygonatum* s-RNA, and only a very slight one was observed when both enzyme and s-RNA came from *Polygonatum*. 4. Protein preparations from seeds of *Pisum sativum*, another species not producing azetidine-2-carboxylic acid, also activated the analogue in addition to proline, whereas those from rhizome and seeds of *Convallaria*, the species from which the analogue was originally isolated, failed to activate it. However, a liliaceous species not producing the analogue, *Asparagus officinalis*, activated it. 5. Of the other proline analogues investigated, only 3,4-dehydro-DL-proline and L-thiazolidine-4-carboxylic acid were active with the enzyme preparation from *Phaseolus*. 6. pH optima of 7.9 and 8.4 were established for the enzymes from *Phaseolus* and *Polygonatum* respectively. 7. The *Phaseolus* enzyme was specific for ATP and PP_i. Mn²⁺ partially replaced the requirement for Mg²⁺ as cofactor. Preincubation with *p*-chloromercuribenzoate at a concentration of 0.5 mM or higher produced over 99% inhibition of the *Phaseolus* enzyme. One-half the enzymic activity was destroyed by preheating for 5 min. at 62° in tris-hydrochloric acid buffer, pH 7.9. 8. All experimental evidence supports the hypothesis that azetidine-2-carboxylic acid and proline are activated by the same enzyme in *Phaseolus* preparations, whereas the analogue was inactive in all *Polygonatum* preparations. The possible nature of this different substrate behaviour is discussed.

L-Azetidine-2-carboxylic acid, the lower homologue of proline, was isolated from leaves of *Convallaria majalis* (Fowden, 1955, 1956) and later shown to have a restricted distribution within the Liliaceae and related Agavaceae (Fowden & Steward, 1957). This imino acid may occur generally within the plant or be restricted to a particular organ, but it is always present in the 'soluble amino acid pool' and excluded from proteins

(Fowden & Bryant, 1958). When azetidine-2-carboxylic acid was supplied to germinating seeds of four species in which it does not normally occur, it produced growth inhibitions and was lethal at high concentration (Fowden, 1963). The degree of inhibition was dependent on the species studied and on the concentration of imino acid supplied, and was correlated with a stoichiometric replacement of proline residues of protein by azetidine-2-carboxylic acid in newly developed plant tissues. This inhibitory action was reversed when proline was supplied simultaneously, and the different

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degrees of growth inhibition observed in the various plant species could be explained by differences in their endogenous proline concentration. Azetidine-2-carboxylic acid therefore forms a potent proline analogue and similar growth inhibitions have been reported in carrot-tissue cultures, *Escherichia coli* and chick embryos (Steward, Pollard, Patchett & Witkop, 1958; Fowden & Richmond, 1963; Fowden, 1963).

In liliaceous species in which it occurs, azetidine-2-carboxylic acid is often accumulated to high concentrations, e.g. it may represent as much as 70% of the aqueous-ethanol-soluble nitrogen, or 50% of the total nitrogen present in the rhizome of *Polygonatum multiflorum* (Fowden & Bryant, 1959). Even in the presence of this extraordinary accumulation, the imino acid is completely excluded from the protein of *Polygonatum* tissues, although one-fiftieth of the concentration is lethal to *Phaseolus aureus* seedlings (Fowden, 1963). Such results suggest that some difference must exist in the specific selection mechanisms for amino acids in relation to protein synthesis operating in these two groups of plants. This difference must be fairly widespread and of considerable importance, since, of 88 species examined, azetidine-2-carboxylic acid was recorded in 23, representing 17 genera of the Liliaceae (Fowden & Steward, 1957).

Various mechanisms for excluding azetidine-2-carboxylic acid from liliaceous protein were considered, and the possibility that members of the Liliaceae containing this imino acid could have evolved a more selective enzymic mechanism operative at one (or more) point(s) during the elaboration of the protein molecule was examined. The first system investigated was the L-proline-s-RNA* ligase (AMP) (trivial name, prolyl-s-RNA synthetase or proline-activating enzyme) isolated from *Phaseolus aureus* (mung bean) and *Polygonatum multiflorum* (a Solomon's seal). The activity of the enzyme was measured by the ATP-PP_i exchange reaction and by the formation of aminoacyl-s-RNA with labelled proline and azetidine-2-carboxylic acid. The investigations established that the imino acid substrate specificities of these two enzyme preparations differed sufficiently to account for the biological phenomena outlined above; preliminary notes on aspects of the work have been published (Peterson & Fowden, 1963, 1964).

EXPERIMENTAL

Materials

Plant materials. Seeds of *Phaseolus aureus*, *Pisum sativum* (var. Little Marvel) and *Convallaria majalis* were obtained respectively from the Bombay Emporium Ltd. (London),

Carters Tested Seeds Ltd. (London) and Thompson and Morgan Ltd. (Ipswich). The first two species were ground before use in a laboratory mill with 0.5 mm. mesh size; the last-named species was ground manually with a pestle and mortar. Rhizomes of *Polygonatum multiflorum* and *Convallaria majalis* were collected from the Nuffield Gardens, Regent's Park, London, in mid-March, washed free of soil and stored at 1°. Only the shoot apices (length 1 in.) developing on the rhizome were used for enzyme and s-RNA preparation.

Chemicals. ATP (disodium salt), GTP (disodium salt) and ITP (trisodium salt) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.); CTP (disodium salt) and UTP (disodium salt) were obtained from California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.).

L-Azetidine-2-carboxylic acid, *trans*-4-hydroxy-L-pipecolic acid and 4-methylene-DL-proline were isolated from plant sources (cf. Fowden, 1963). DL-Pipecolic acid was prepared by catalytic hydrogenation of pyridine-2-carboxylic acid (Stevens & Ellman, 1950). Imino acids obtained commercially were: L-proline and 2-carboxypyrrole (L. Light and Co., Colnbrook, Bucks.); D-azetidine-2-carboxylic acid (California Corp. for Biochemical Research); L-thiazolidine-4-carboxylic acid (Mann Research Laboratories Ltd., New York, N.Y., U.S.A.); *trans*-4-hydroxy-L-proline (Roche Products Ltd., Welwyn Garden City, Herts.). Gifts received were: *cis*-4-hydroxy-L-proline, *trans*- and *cis*-3-hydroxy-L-proline, and D-proline (Dr F. Irreverre, Bethesda, Md., U.S.A.); *trans*-4-methyl-L-proline (Dr R. C. Sheppard, Liverpool); 4,5-dehydro-DL-pipecolic acid (baikiain) (Professor T. J. King, London); 3-hydroxy-DL-pipecolic acid (Dr H. Plieninger, Heidelberg, Germany); *trans*-5-hydroxy-L-pipecolic acid (Professor A. I. Virtanen, Helsinki, Finland).

Protein amino acids, herring-sperm DNA and yeast RNA were obtained from L. Light and Co. Ltd., Roche Products Ltd., or British Drug Houses Ltd. (Poole, Dorset).

All reagents used were of A.R. quality.

Calcium phosphate gel was prepared by the method of Keilin & Hartree (1938) and stored for 1 month at 1° before use.

Darco G.60 charcoal (obtained from Honeywell-Atlas Ltd., London) was used after acid-washing according to the procedure of Thomson (1960). Complete adsorption of ATP or other triphosphate was checked by measuring the extinction of the supernatant at 259 mμ in a Hilger Uvispek H 700 spectrophotometer (Hilger and Watts Ltd., London) or by assay of inorganic phosphorus after hydrolysis of the charcoal-adsorbed ATP.

Radioactive chemicals. [³²P]PP_i was prepared from carrier-free sodium [³²P]P_i, pH 2-3, obtained from The Radiochemical Centre (Amersham, Bucks.) and its purity checked by paper chromatography as described by Peterson & Fowden (1963). Samples were stored at -45° for up to 2 weeks and were stable during this period.

L-[U-¹⁴C]Proline (specific activity 103 μc/mg.) and L-[U-¹⁴C]protein hydrolysate from *Chlorella vulgaris* (specific activity 200 μc/mg.) were obtained from The Radiochemical Centre.

DL-[4-³H]Azetidine-2-carboxylic acid (specific activity 14.4 mc/mg.) was obtained from California Corp. for Biochemical Research and further purified by chromatography on Whatman no. 3MM chromatographic-grade paper with as solvent the upper phase of ethyl acetate-pyridine-water (2:1:2, by vol.).

* Abbreviations: s-RNA, transfer or soluble RNA; ATPase, adenosine triphosphatase.

Enzyme. Ribonuclease (EC 2.7.7.16), protease-free from bovine pancreas, was obtained from L. Light and Co. Ltd.

Methods

Ionophoresis. To remove a possible proline contamination ionophoresis of L-thiazolidine-4-carboxylic acid was conducted on Whatman no. 3 paper in a pH 2 buffer [1.5N-formic acid-2N-acetic acid (1:1, v/v)] for 2 hr. at 60 v/cm. in the apparatus of Locarte Ltd. (London).

Isolation of prolyl-s-RNA synthetases. (a) Homogenates. All manipulations were carried out in a cold room at 1° and plant material was precooled before use.

Mung-bean and pea-seed flour (45g.) were ground manually by pestle and mortar with acid-washed sand (5g.) for 5 min. with 0.1M-tris-HCl buffer, pH 7.5 (135 ml.), containing sucrose (0.4M). *Convallaria* seed material was similarly treated except that only 25g. of material was used with an appropriate decrease in solution volume and manual grinding was increased to 1 hr.

Apical rhizomous tissue from *Polygonatum* (60g.) and *Convallaria* (30g.) were homogenized at 12000 rev./min. in an Ato-Mix blender (Measuring and Scientific Equipment Ltd., London) for ten 1 sec. intervals with the above tris-sucrose solution (150 and 75 ml. respectively). In each case the slurry of plant tissue was pressed through four layers of muslin at 2000 lb./in.² in a Hafco hydraulic press (Fisher and Co., Düsseldorf, Germany) and the liquid was collected and centrifuged at 20000g for 15 min. in an 8 × 50 ml. head of a Super-Speed 27500 centrifuge (Measuring and Scientific Equipment Ltd.) at 1°.

In a preliminary experiment the subcellular distribution of the prolyl-s-RNA synthetase was investigated by centrifuging the mung-bean extract in progressively higher gravitational fields to yield the fractions described in Table 1. Each fraction was washed once by resuspending in tris-sucrose solution and resedimented by centrifuging under the original conditions.

(b) pH fractionation. The layer of fat present in seed homogenates was removed from the top of centrifuged solutions and the supernatant was poured off and diluted with water (50 ml.). The pH was slowly decreased with 0.1N-acetic acid to pH 4.2 by using a Dynacap pH-meter (Pye and Co., Cambridge) and the solution was centrifuged at 12000g for 5 min. The supernatant was discarded, the inside of the centrifuge tubes dried with strips of filter paper and the protein redissolved in 0.1M-tris-HCl buffer, pH 7.6, (100–200 ml.), with the aid of a Potter glass homogenizer. Protein solutions were stirred (15 min.) and centrifuged as above to remove undissolved protein.

The dissolved protein was then fractionated by stepwise lowering of the pH with acetic acid as above, and the series of precipitates were collected by centrifugation. The protein fractions redissolved in 0.1M-tris-HCl buffer, pH 7.6 (10 ml., or, if to be further purified by (NH₄)₂SO₄ fractionation, 15 ml.). After pH-activity curves had been determined, the final pH for dissolving protein was 7.9 for mung bean and pea seed and 8.4 for *Polygonatum* and *Convallaria* rhizome.

(c) (NH₄)₂SO₄ fractionation. The solid salt was added to give the required percentage saturation values calculated for 1° and then allowed to stand for 15 min. before centrifuging as above. The protein fractions were redissolved in tris-HCl buffer as above for enzyme assay or in distilled

water if further purification was to be attempted. Only mung-bean preparations were purified further, since the *Polygonatum* enzyme was relatively unstable.

(d) Calcium phosphate-gel fractionation. One-quarter volume of gel was added to the enzyme solution (10 ml.), which was stirred for 15 min. and centrifuged. The supernatant was discarded and the gel suspended in 0.1M-tris-HCl buffer, pH 7.9 (5 ml.), homogenized for 10 min. and centrifuged. The supernatant was again discarded and the residual gel extracted twice with successive portions of 0.1M-potassium phosphate buffer, pH 7.9 (7.5 ml.), 10% saturated with (NH₄)₂SO₄, and centrifuged after each extraction. The enzyme was finally precipitated from the combined supernatants by saturating with (NH₄)₂SO₄, centrifuging and redissolving in 0.1M-tris-HCl buffer, pH 7.9 (usually 7.5 ml.).

(e) Polyacrylamide-gel ionophoresis. The purified prolyl-s-RNA synthetase was subjected to ionophoresis on polyacrylamide gel at room temperature for 1.5 hr. at 3 ma./tube, by using the method and apparatus of Davis (1962). Proteins were fixed and stained with Amido Schwarz in 7% (v/v) acetic acid and electrophoretically destained.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was followed, with crystalline serum albumin as standard. Corrections were made for interference of the tris-HCl buffer in dilute protein solutions.

Isolation of s-RNA. The phenol method was used where mung-bean flour (75g.) or *Polygonatum* apices (150g.) were ground and homogenized with 0.05M-tris-HCl buffer, pH 7.5 (200 and 300 ml. respectively). Water-saturated phenol (150 and 250 ml. respectively) was added and the mixture blended (500 rev./min. for 1 hr. at 1°). The procedure of Moustafa & Lyttleton (1963) was then followed, except that the s-RNA finally precipitated was dissolved in 0.05M-tris-HCl buffer, pH 7.9 (15 ml.), and dialysed for 15 hr. against two changes of 2l. of buffer. The non-diffusible material (s-RNA) was stored at -45° for up to 2 months and thawed when required: it lost no detectable activity during this period.

RNA determination. RNA was determined by the orcinol method of Schneider (1957), with yeast RNA as a standard with appropriate correction based on phosphate determinations by the method of Chen, Toribara & Warner (1956). The assay as applied to s-RNA isolated from plant tissue gave high values due to the presence of contaminating polysaccharide giving a ribose reaction. True s-RNA values were obtained on samples precipitated with HClO₄. However, the contaminating polysaccharide did not interfere with the action of the proline-s-RNA synthetase system.

DNA determination. DNA was determined by the method of Schneider (1957), with herring-sperm DNA as a standard.

Lipid determination. Lipids were extracted with an ethanol-ether mixture (19:1, v/v) and dried, and the residue was weighed on a micro-balance.

Incubation procedures. The enzyme incubation mixtures were duplicated in tapered glass centrifuge tubes, which each contained the reactants added in the order listed.

(a) ATP-PP_i system. The incubation medium consisted of: tris-HCl buffer, pH 7.6 (100 μmoles), in early experiments, but, after pH-activity curves had been determined, pH 7.9 was used for mung-bean enzyme and pH 8.4 for *Polygonatum* enzyme; MgSO₄ (10 μmoles); single amino acids (5 μmoles), or 2.5 μmoles of each amino acid when a 'complete mixture'

of the 20 protein amino acids was used; ATP (disodium salt) (4 μ moles); [32 P]PP_i (4 μ moles; 10⁵ counts/min.); enzyme solution (0.6–0.7 ml., to give final volume of 1 ml.). The mixtures were incubated at 37° for 15 min. with periodic agitation.

Amino acid hydrochlorides were neutralized before use with KOH.

The endogenous exchange was measured by using the above incubation solution minus amino acids. There was no reaction in the absence of enzyme, or with inactive protein (e.g. egg albumin) or with enzyme boiled for 10 min.

In some experiments pH values, time of incubation, enzyme concentration, MgSO₄ concentration and amino acid concentration were altered (see the text). In certain mixtures ATP was replaced by other triphosphate, or Mg²⁺ by other metal ions, and in some experiments the enzyme preparations were preincubated either at various elevated temperatures, or with *p*-chloromercuribenzoate or ribonuclease before assay.

(b) Prolyl-s-RNA system. The reactants were as follows: tris-HCl buffer, pH 7.9 (100 μ moles); MgSO₄ (10 μ moles); ATP (disodium salt) (10 μ moles); [14 C]proline (0.08 μ mole; 1 μ c) or [3 H]azetidine-2-carboxylic acid (0.003 μ mole; 4.4 μ c) or 14 C-labelled protein-hydrolysate amino acids (0.05 μ mole; 1 μ c); enzyme solution (0.2 ml.); s-RNA solution (0.6 ml.); the final volume was 1 ml. The mixtures were incubated at 37° for 30 min. with continuous agitation.

The addition of the above reactants to 1 ml. of 0.8N-HClO₄ was a zero-time control. Controls also were included in which either enzyme or s-RNA was omitted.

Mixtures were appropriately modified to study the dependence of the reaction on Mg²⁺ and ATP, and to determine the relationship between the rate of reaction and other factors such as the time of incubation, and the concentration of s-RNA and of enzyme.

Determination of enzymic reaction. (a) ATP-PP_i reaction. The enzymic reaction was stopped by placing the incubation tubes into ice-cold water and adding ice-cold 7.5% (w/v) trichloroacetic acid (2 ml.). After stirring, the mixture was kept at 0° for 10 min.; the precipitated protein was sedimented by centrifuging at 1500g for 10 min. in a Super-Speed Medium Centrifuge (Measuring and Scientific Equipment Ltd.). ATP was adsorbed on charcoal (added at 100 mg./ml.), according to the method of Crane & Lipmann (1953) as modified by DeMoss & Novelli (1956), and finally the two terminal phosphate groups were cleaved from the ATP by hydrolysis with N-HCl for 15 min. at 100°. Samples (1 ml.) of the hydrolysate were assayed for radioactivity in glass planchets under a thin end-window Geiger-Müller tube (Mullard MX 123) connected to a 1700 scaler (Isotope Developments Ltd., Reading, Berks.) with an efficiency of 4%. Corrections for background, radioactive decay and paralysis time were made and samples counted to give a standard error of 1.4% in all practical cases and a 1% error when possible.

The results of ATP-PP_i exchange are given in terms of 'percent exchange' and 'rate of exchange' (Davie, Koningsberger & Lipmann, 1956).

Chromatographic methods were used to establish that [32 P]PP_i was incorporated solely into ATP. For this purpose the reaction was stopped by the addition of 2 vol. of ethanol to the incubation mixture, and, after removal of precipitated protein, samples of the supernatant were applied to paper chromatograms. The nucleotides were also separated from

the PP_i by adsorption on to charcoal as above but were recovered by suspending the charcoal in 10% (v/v) pyridine and incubating the suspension in a stoppered tube at 37° for 3 hr. The supernatant was evaporated at laboratory temperature and the residue was redissolved in water and chromatographed on Whatman no. 4 paper with the 2-methylpropionic acid solvent system of Thomson (1960). Nucleotides were located under a u.v. Chromatolite lamp (Hanovia Ltd., Slough, Bucks.) at 254 m μ ; phosphates were detected by spraying with the ammonium molybdate reagent of Burrows, Grylls & Harrison (1952) and exposure to u.v. light for not less than 2 hr. Radioactive compounds were located by using Kodirex double-coated X-ray film (Kodak Ltd.).

When crude enzyme preparations were used, some 32 P label was found in ADP, indicating the presence of an ATPase, but, after enzyme purification by (NH₄)₂SO₄ fractionation, radioactivity was detected only in ATP. The presence of an ATPase in the crude enzyme preparations was also indicated by the failure to account completely for the added ATP in the form of the two terminal phosphate groups released as P_i after acid hydrolysis of the charcoal-adsorbed ATP.

The presence of a weak pyrophosphatase activity was also noted in the crude extracts only, from the recovery of PP_i, determined by the method of Flynn, Jones & Lipmann (1954).

Proline was determined by the method of Chinard (1952) and was completely recovered at the end of the reaction.

(b) Aminoacyl-s-RNA formation. The formation of labelled aminoacyl-s-RNA was assayed by the method of Moustafa & Lyttleton (1963); 14 C-labelled compounds were dried on aluminium planchets at infinite thinness before assay of radioactivity, and 3 H-labelled samples were assayed by a scintillation technique (see below).

A phenol isolation of s-RNA was carried out at the end of the reaction period from a system containing s-RNA and purified prolyl-s-RNA synthetase from mung bean to show that the radioactivity detected in [14 C]prolyl-s-RNA was truly associated with s-RNA and not with enzyme protein. The isolated material contained 97.5% of the radioactivity present in the original [14 C]prolyl-s-RNA.

Similarly, radioactivity associated with the aminoacyl-s-RNA was confirmed as [14 C]proline by subjecting the postulated [14 C]prolyl-s-RNA, precipitated as in the assay above, to mild alkaline hydrolysis at pH 10–11 for 2 hr. at 37°. This treatment hydrolyses the aminoacyl-s-RNA link but not the internucleotide bonds (Hoagland, 1958). After neutralization, 2 vol. of ethanol was added and the mixture was stirred for 30 min. at –10°. The s-RNA was centrifuged down and the supernatant containing [14 C]proline was concentrated and chromatographed on Whatman no. 4 paper with butan-1-ol-acetic acid-water (90:10:29, by vol.) as solvent. Radioautography proved that all the radioactivity was associated with proline.

The 3 H-labelled aminoacyl-s-RNA samples, when precipitated, contained approx. 0.05 ml. of 0.2N-HClO₄, and were dissolved in N-NH₃ (0.3 ml.) and added to 0.25 ml. of water in liquid-scintillation vessels. Liquid scintillator (8.5 ml.) (NE 220; Nuclear Enterprises Ltd. Edinburgh) was added rapidly and the solutions were stirred for a few seconds until a single phase was produced. Samples were kept in the dark (10 min.), then counted immediately in a tritium scintillation counter type 6012 (Isotope Developments Ltd.). A

value of 18.2% efficiency for the counting of a standard ^3H sample was obtained, but an internal standard of similar overall composition to that of the aminoacyl-s-RNA sample was not available to check the exact counting efficiency applicable to the experimental determinations.

RESULTS

Enzyme purification

Mung-bean preparations. The ability of different subcellular fractions of seed homogenate to give measurable ATP-PP_i exchange in the presence of 20 protein L-amino acids is shown in Table 1 on both a percent-exchange and rate-of-exchange basis. Fraction 2 containing wall debris, plastids, nuclei etc. (characterized microscopically), although yielding a low percent exchange, catalysed the highest rate of exchange, owing partly to the absence of an active ATPase and partly to the low proportion of seed storage protein in this fraction. The strongest ATPase activity was associated with the fraction

sedimented at 20000g (presumed to be largely composed of mitochondria), the removal of which increased the exchange of the soluble fraction.

Most of the activity in the homogenate was encountered in the 'soluble' enzyme fraction when the total protein was considered, and so this fraction was most suitable for use in later experiments. In practice, however, the 100000g fraction (presumably mainly ribosomes) was not normally sedimented because it had only a slight effect on the exchange.

The addition of L-proline alone to these crude preparations did not significantly stimulate the exchange above the high endogenous rate observed on the omission of added amino acid. To investigate the prolyl-s-RNA synthetase, a marked lowering of the endogenous rate was essential; and the purification steps used were as follows.

(a) pH fractionation. A direct acetic acid fractionation of the 'soluble' fraction decreased the endogenous rate slightly, and showed that the

Table 1. *Comparative rates of ATP-PP_i exchange in subcellular fractions of mung bean*

The incubation system and assay procedures are described in the Experimental section; 2.5 μ moles of each of the 20 protein L-amino acids as listed in Table 3 were added to the incubation system. The particulate fractions listed were washed once with the initial extraction medium. Protein recovery was 98.7% of the initial homogenate. The rate of exchange is given in terms of μ moles of PP_i exchanged/hr./mg. of protein, and corrected for ATPase activity.

Fraction	Percent exchange	Rate of exchange	Protein in fraction (mg.)	ATP recovery* (μ moles)
1. Whole homogenate	39.85	0.044	4860	2.7
2. Walls and large particles (sedimented at 3000g for 15 min.)	3.57	0.349	33	3.9
3. Homogenate minus fraction 2	40.20	0.045	4830	—
4. 'Mitochondrial' fraction (sedimented at 20000g for 15 min.)	26.82	0.228	187	2.2
5. Homogenate minus fractions 2 and 4	54.68	0.089	4660	3.5
6. 'Ribosomal' fraction (sedimented at 100000g for 60 min.)	19.15	0.193	56	3.6
7. Soluble enzymes	55.00	0.093	4520	3.6

* 4 μ moles of ATP added initially.

Table 2. *Typical purification of prolyl-s-RNA synthetase from mung bean and Polygonatum (Solomon's seal)*

The activity of the enzyme was assayed by the ATP-PP_i exchange reaction. The incubation system, assay procedure and details of the protein purification scheme are described in the Experimental section. The activity of the enzyme is expressed as μ moles of PP_i exchanged/hr./mg. of protein. Owing to the high endogenous rate in fraction 1, an approximate rate only can be given.

Fraction	Species	Protein (mg.)	Synthetase activity	Relative purification	Yield (%)
1. Centrifuged homogenate	Mung bean	3400	(0.060)	(1)	(100)
Centrifuged homogenate	Solomon's seal	1500	(0.065)	(1)	(100)
2. pH 4.2-4.6 fraction	Mung bean	60	1.770	29.5	52.1
pH 4.6-5.3 fraction	Solomon's seal	122.5	0.474	7.3	59.6
3. 60-70% sat. (NH ₄) ₂ SO ₄ fraction	Mung bean	8.55	8.960	149	37.5
60-70% sat. (NH ₄) ₂ SO ₄ fraction	Solomon's seal	17.2	0.626	9.6	11.0
4. Calcium phosphate-gel eluate	Mung bean	2.26	14.82	247	16.2

fraction sedimented below pH 5 contained most of the proline-stimulated ATP-PP_i-exchange activity. When protein was precipitated first by adjusting to pH 4.2, then rapidly redissolved at pH 7.6 and fractionated, a considerable purification was achieved (Table 2), and the fraction showing maximum activity contained over half the recovered activity of the soluble fraction. The prolyl-s-RNA synthetase was used at this stage of purification for investigating the pH optimum, and the relationship existing between the rate of reaction and other factors (e.g. time, enzyme concentration, heat denaturation of the enzyme before incubation, inhibition by *p*-chloromercuribenzoate, nucleotide specificity, and dependence on PP_i and metal ions).

(b) Ammonium sulphate fractionation. The protein precipitated between pH 4.2 and 4.6 with acetic acid was further fractionated with solid ammonium sulphate. The fraction collected between 60 and 70% saturation had the highest specific activity and contained the major proportion of the prolyl-s-RNA synthetase activity. This enzyme preparation was used to study the behaviour of imino acid analogues and D-isomers as substrates, as well as the polynucleotide content of the enzyme.

(c) Calcium phosphate-gel fractionation. The adsorption of the enzyme and subsequent elution further purified the enzyme (Table 2). An initial elution with tris-hydrochloric acid buffer alone failed to remove the enzyme from the gel, although some inactive protein was eluted. The enzyme was displaced only when a phosphate buffer 10% saturated with ammonium sulphate was used.

(d) Polyacrylamide-gel ionophoresis. The purity of the enzyme preparation was investigated by ionophoresis on polyacrylamide-gel columns. There was a striking contrast between the protein pattern of the initial seed homogenate, with its one predominant band of storage protein, and that of the enzyme preparation after step (c), which showed two major and three minor bands scarcely visible in

the initial homogenate (see Fig. 1). It is not surprising that more than one protein band was detected because the presence or absence of other synthetase enzymes was the main criteria for enzyme purity.

Solomon's-seal preparations. A method of fractionation comparable with that described in the mung-bean study was used to obtain a synthetase enzyme from rhizome apices (Table 2). However, fractionation was not as definite, because the homogenates were mucilaginous. The maximum prolyl-s-RNA-synthetase activity was found in the acetic acid fraction collected between pH 4.6 and 5.3. Only three properties of the enzyme were examined at this stage of purification, namely variation of exchange rate with pH, with the time of reaction and with protein concentration.

A considerable loss of activity occurred on ammonium sulphate fractionation, although most of the recovered enzyme activity was collected between 60 and 70% saturation, as with mung-bean enzyme. Even though much inactive protein was discarded, the relative purification was only slightly increased. Insufficient rhizome tissue was available to examine systematically the reasons for this loss of activity. The enzyme was not further purified and only its imino acid substrate specificity was examined.

Aminoacyl-s-RNA-synthetase activities

The activities of the other aminoacyl-s-RNA synthetases present in the protein fractions were also examined (Table 3). In the mung-bean experiments, the highest enzymic activity was due to the prolyl-s-RNA synthetase, and repeated purification steps removed most of the other contaminating aminoacyl-s-RNA synthetases. A 65–75% saturated ammonium sulphate fraction showed only prolyl-s-RNA-synthetase activity, although insufficient enzyme was present for this fraction to be useful throughout.

A shortage of rhizome tissue limited the survey of other aminoacyl-s-RNA synthetases present in the Solomon's seal fractions. The valyl-s-RNA synthetase showed the highest activity in the acetic acid-precipitated fraction (pH 4.6–5.3); the prolyl-s-RNA synthetase had only moderate activity. A closely similar group of amino acids stimulated ATP-PP_i exchange both in this fraction and in the one studied from mung bean (Table 3), although their relative activities as substrates differed.

Substrate specificity

Substrates involved in the overall formation of aminoacyl-s-RNA are amino acid, ATP, PP_i and s-RNA. The specificity of prolyl-s-RNA synthetase towards these substrates was examined.

Fig. 1. Diagrammatic representation of the protein bands separated by electrophoresis on polyacrylamide gel and stained with Amido Schwarz. (a) Extract obtained from initial homogenate of mung-bean seeds; (b) enzyme preparation after step (c) purification. Heavily stained bands are shown solid, and less intense bands are hatched.

Table 3. *Stimulation of ATP-PP_i exchange by individual amino acids*

Mung-bean homogenates were initially precipitated to pH 4.2 and redissolved in tris-HCl buffer, pH 7.6, before fractionation. Details of the fractionation procedure and assay system are given in the Experimental section. Rate of exchange values have been calculated in terms of μ moles of PP_i exchanged/hr./mg. of protein and converted into a percentage of the rate with proline. The rate-of-exchange values have been corrected for an endogenous exchange, i.e. without addition of amino acid. The 'complete mixture' contained the 20 protein L-amino acids. The L-amino acids threonine, isoleucine, alanine and phenylalanine showed activities less than 10% of the proline rate after step (a) and were inactive with enzyme after step (b). Glutamic acid, glycine, arginine, aspartic acid, cyst(e)ine, methionine and glutamine were all inactive with enzyme at step (a).

Amino acid	Stimulation of ATP-PP _i exchange		
	Enzyme after step (a) purification (pH 4.2-4.6 fraction)	Enzyme after step (b) purification [60-70% sat. (NH ₄) ₂ SO ₄ fraction]	Enzyme after step (c) purification (calcium phos- phate-gel eluent)
L-Azetidine-2-carboxylic acid	38	35	37
Complete mixture	193	171	—
L-Proline	100	100	100
L-Valine	97	45	47
L-Serine	80	0	—
L-Lysine	31	0	—
L-Asparagine	18	0	—
L-Tyrosine	18	4.5	5.4
L-Histidine	15	0.6	0
L-Tryptophan	13	0.3	0
L-Leucine	10	2.1	4

Proline and azetidine-2-carboxylic acid. (a) Mung-bean preparations. In all experiments involving the prolyl-s-RNA synthetase isolated from mung-bean seeds, a stimulation of ATP-PP_i exchange by the addition of proline was always accompanied by a stimulation by azetidine-2-carboxylic acid at about one-third the rate with proline under the standard assay conditions (i.e. at 5 μ moles of imino acid; see the Experimental section). When the substrate concentration was altered, the ratio of rates varied slightly. Proline at 2.5 μ moles saturated the enzyme but maximal rates were not obtained with azetidine-2-carboxylic acid even at 50 μ moles, when the azetidine-2-carboxylic acid/proline activity ratio had increased to 43%.

The relative rates for the two substrates remained the same at all stages of enzyme purification (see Table 3), and in all other acetic acid and ammonium sulphate fractions exhibiting lower activity with proline. This suggests that either one enzyme or two enzymes with similar physical properties are involved. In the ammonium sulphate fraction, activation rates were additive when different aminoacyl-s-RNA synthetase activities were assayed. However, when proline and azetidine-2-carboxylic acid were present together in either 5 or 10 μ mole amounts, the ATP-PP_i exchange rate observed was slightly less than that with proline alone. This finding supports the one-enzyme hypo-

thesis, and additional evidence for this view is discussed below.

The prolyl-s-RNA synthetase was specific for the L-isomers of proline and azetidine-2-carboxylic acid, D-isomers being completely inactive. There was no competitive inhibition by the D-isomers.

Acetic acid fractions from *Pisum sativum*-seed homogenates containing prolyl-s-RNA synthetase likewise activated azetidine-2-carboxylic acid, but at a lower rate than proline.

(b) Solomon's-seal preparations. In all experiments involving rhizome tissue, stimulation by azetidine-2-carboxylic acid was never observed, although stimulation by proline of the ATP-PP_i exchange was at a high level.

These conclusions were substantiated with rhizome tissues from another liliaceous species, *Convallaria majalis*, where azetidine-2-carboxylic acid again did not stimulate the ATP-PP_i exchange. Similarly, when seed of either of these liliaceous species was the source of enzyme, no stimulation by azetidine-2-carboxylic acid occurred.

Other imino acid analogues. (a) Mung-bean preparations. Of the proline analogues investigated, 3,4-dehydro-DL-proline was shown to be activated at 57% of the rate with proline, whereas 2-carboxypyrrole was inactive. 4-Methylene-DL-proline, an apparently naturally racemic compound, showed a slight reaction (3% of that with proline), but paper

chromatography revealed slight proline contamination sufficient to account for the observed stimulation of ATP-PP_i exchange. L-Thiazolidine-4-carboxylic acid was activated to a small extent (2% of that with proline) and contamination with proline was first suspected. However, the rate of stimulation of the ATP-PP_i exchange by material, either twice recrystallized or purified by ionophoresis, remained the same as with the original sample, thus indicating a true activation of this compound.

The following ten compounds were tested and found to be inactive: *trans*-4-methyl-L-proline; *trans*- and *cis*-4-hydroxy-L-proline; *trans*- and *cis*-3-hydroxy-L-proline; DL-pipecolic acid; 3-hydroxy-DL-pipecolic acid; *trans*-4-hydroxy-L-pipecolic acid; *trans*-5-hydroxy-L-pipecolic acid; 4,5-dehydro-DL-pipecolic acid (baikiain).

(b) Solomon's-seal preparations. Apart from the marked difference of selectivity for azetidine-2-carboxylic acid between the prolyl-s-RNA synthetase from this source and that from mung bean, the only other difference detected was that 3,4-dehydro-DL-proline stimulated exchange at only 28% of the rate with proline. L-Thiazolidine-4-carboxylic acid and other possible analogues of proline were not tested owing to lack of initial plant material.

Nucleotide specificity. Replacement of ATP by comparable concentrations of GTP, UTP, CTP or

ITP yielded rates of 0.6, 0.3, 0.1 and 0.1% respectively of that with ATP with the prolyl-s-RNA synthetase from mung bean.

PP_i specificity. The ability of [³²P]P_i to participate in an exchange reaction with ATP was examined with mung-bean enzyme only. No activity (less than 0.1% of that with PP_i) was measured when equimolar amounts of P_i replaced PP_i.

s-RNA specificity. An initial investigation into the factors affecting the formation of prolyl-s-RNA with [¹⁴C]proline and purified prolyl-s-RNA synthetase and s-RNA isolated from mung bean showed the following. (i) The formation increased with time until it reached a maximum value after 20 min. with the reaction mixture described (see the Experimental section), indicating the complete filling of all the proline sites on the s-RNA. When maximum transfer was sought, a 30 min. incubation of the reactants was therefore adopted. (ii) The amount of prolyl-s-RNA formed was linearly related to prolyl-s-RNA synthetase concentration when a 10 min. incubation time was used with excess of s-RNA. (iii) The formation of prolyl-s-RNA was linearly related to s-RNA concentration (30 min. incubation) when excess of prolyl-s-RNA synthetase was present.

Aminoacyl-s-RNA formation also was measured in the presence of a ¹⁴C-labelled protein hydrolysate and the results are compared with those obtained with [¹⁴C]proline in Table 4. An acetic acid fraction

Table 4. *Activation of amino acids with enzymes and s-RNA from both mung bean and Solomon's seal*

Details of the assay system are given in the Experimental section. The enzymes from mung bean and Solomon's seal used for the activation of the amino acids present in the protein hydrolysate were pH 4.2-5.3 fractions collected after purification step (a) (see the Experimental section) and containing 3.6 and 3.7 mg. of protein respectively; enzyme obtained after the (NH₄)₂SO₄ precipitation step (b) (see the Experimental section) was used for the activation of proline and contained 0.36 and 1.2 mg. of protein respectively. Mung-bean and Solomon's-seal s-RNA preparations contained 1.5 and 1.1 mg. of RNA respectively. The same preparations of enzyme and s-RNA were used in the different combination experiments. The activation of amino acids is expressed in units of counts/min. of ¹⁴C-labelled mixed amino acids or proline transferred to s-RNA, corrected for a zero-time blank in which 1 ml. of 0.8 N-HClO₄ was added before the addition of enzyme.

Combination	Species		Labelled amino acid System	Activation of amino acids	
	Enzyme	s-RNA		... Protein hydrolysate	Proline
1	Mung bean	Mung bean	Complete	4315	181.7
			Minus enzyme	0	0
			Minus s-RNA	226	0.8
2	Mung bean	Solomon's seal	Complete	2175	-1.7
			Minus enzyme	0	0
			Minus s-RNA	504	0.5
3	Solomon's seal	Solomon's seal	Complete	1861	6.5
			Minus enzyme	0	0
			Minus s-RNA	653	-1.8
4	Solomon's seal	Mung bean	Complete	3843	43.4
			Minus enzyme	0	0
			Minus s-RNA	441	1.9

(after step *a*) was used as the enzyme source for transfer of the mixed ^{14}C -labelled amino acids and further-purified enzyme (step *b*) with ^{14}C proline. In each case the graphs relating the formation of the mixed aminoacyl-s-RNA derivatives (expressed as ordinates) with other factors under conditions (i), (ii) and (iii) above were hyperbolic.

The formation of prolyl-s-RNA and the mixture of aminoacyl-s-RNA derivatives when both the prolyl-s-RNA synthetase and s-RNA originated from mung beans (designated combination 1) is illustrated in Table 4. There was no reaction in the absence of enzyme, but significant reaction was observed with the crude enzyme in the absence of s-RNA. The formation of prolyl-s-RNA was confirmed by hydrolysis and subsequent chromatography of the products (see the Experimental section).

The addition of ^3H azetidine-2-carboxylic acid instead of ^{14}C proline gave rise to labelling of the s-RNA but at only 3% of the rate with proline.

The replacement of mung-bean s-RNA by *Polygonatum* s-RNA (combination 2, Table 4) eliminated prolyl-s-RNA formation, but a transfer of label from the mixed protein ^{14}C -labelled amino acids to the s-RNA still proceeded. The absence of labelling with proline alone is not surprising because only slight formation of prolyl-s-RNA was observed when both the prolyl-s-RNA synthetase and the s-RNA were from *Polygonatum* (combination 3, Table 4).

An appreciable formation of prolyl-s-RNA was obtained when mung-bean s-RNA was used together with *Polygonatum* enzyme (combination 4, Table 4).

Other properties of prolyl-s-RNA synthetase

The enzyme from both *Polygonatum* and mung bean was used to investigate the dependence of the rate of ATP-PP_i exchange on pH, on enzyme concentration and on the time of incubation, but only the latter enzyme was used when examining the other properties reported below.

pH optima. The pH-activity curves showed definite maxima at pH 7.9 and 8.4 for mung-bean enzyme and *Polygonatum* enzyme respectively.

With mung-bean enzyme, the stimulation of exchange by azetidine-2-carboxylic acid also was maximal at pH 7.9. No ATP-PP_i exchange attributable to the presence of azetidine-2-carboxylic acid was observed at any of the pH values tested when the prolyl-s-RNA synthetase was obtained from Solomon's seal.

Effect of enzyme concentration. The rate of ATP-PP_i exchange observed in the presence of proline or azetidine-2-carboxylic acid as imino acid substrate was related linearly to the concentration of mung-bean enzyme (up to 7 mg. of protein in the standard assay mixture), whereas the proline-dependent

exchange catalysed by the *Polygonatum* enzyme was linear with up to 16 mg. of protein per assay. The analogue was inactive with the latter enzyme at all enzyme concentrations studied.

Effect of time of incubation. The rate of exchange was constant during a 15 min. incubation period for the enzyme preparations from both tissues.

Metal ion dependence. The addition of Mg^{2+} was essential for enzymic reaction; its omission gave an insignificant exchange (less than 0.05% of that with standard assay). An optimum concentration of 10 $\mu\text{moles/ml.}$ was established.

The possible replacement of Mg^{2+} was also examined and the activities measured with other bivalent cations are listed as percentages of the rates determined for Mg^{2+} ; in decreasing order of activity they were Mn^{2+} , Co^{2+} , Cd^{2+} , Zn^{2+} and Ni^{2+} , at 24.2, 18.0, 9.9, 6.6 and 3.6% respectively; Ca^{2+} , Ba^{2+} and Sr^{2+} were inactive.

Inhibition by *p*-chloromercuribenzoate. The proline stimulated ATP-PP_i exchange was inhibited by preincubation for 5 min. with *p*-chloromercuribenzoate. A concentration of 0.5 mM or higher produced over 99% inhibition. The addition of an equimolar amount of GSH partially diminished the inhibition to 81%. Only 16% inhibition was produced with 0.1 mM *p*-chloromercuribenzoate.

Effect of heat incubation. In 0.1 M-tris-hydrochloric acid buffer, pH 7.9, the enzyme was stable during a 5 min. preincubation at 40°, and it retained complete activity during the 15 min. incubation at 37°. Preincubation for 5 min. at 80° completely destroyed activity, but only half the activity was lost at about 62°.

When the above solution of enzyme in 0.1 M-tris-hydrochloric acid buffer was adjusted to pH 4.2 with acetic acid, no activity was lost on standing for 15 min. at 1°, but 41% of the activity was lost at 30°.

Heat-denatured enzyme was not reactivated after cooling and standing for 18 hr. at 1°.

Partial heat inactivation of the enzyme did not alter the ratio of ATP-PP_i exchange rates observed with proline and azetidine-2-carboxylic acid.

Effect of ribonuclease. The crude mung-bean enzyme obtained after step (a) fractionation contained 3% of polynucleotide material, whereas the purest preparation of prolyl-s-RNA synthetase contained 1.8%. To test whether this material was an integral part of the enzyme, preincubation for 15 min. at 30° was carried out with the ribonuclease at 1, 10, 100 $\mu\text{g./ml.}$ before assay by the ATP-PP_i exchange technique. Treatment with ribonuclease decreased the nucleotide content of the enzyme preparation to less than 0.3% without affecting the proline- or azetidine-2-carboxylic acid-stimulated exchange reaction.

DISCUSSION

The results obtained on the species specificity of the prolyl-s-RNA synthetase with regard to azetidine-2-carboxylic acid and, to a smaller extent to 3,4-dehydro-DL-proline, as substrate are of great interest since this question does not appear to have been considered previously. Species specificity of some aminoacyl-s-RNA synthetases in relation to s-RNA, is however, well documented but incompletely understood (see Stulberg & Novelli, 1962). Although only one liliaceous species containing azetidine-2-carboxylic acid, i.e. *Polygonatum multiflorum*, and one dicotyledonous species, i.e. mung bean (*Phaseolus aureus*), were studied in detail, the results have been confirmed by the use of an additional species in each group, namely *Convallaria majalis* and *Pisum sativum* respectively. In experiments not reported here, enzyme preparations from a liliaceous species lacking azetidine-2-carboxylic acid, namely *Asparagus officinalis*, were shown to activate this imino acid in a manner similar to mung-bean enzyme. This evidence suggests that species producing azetidine-2-carboxylic acid do not normally activate it, whereas those incapable of synthesizing it generally activate it. This hypothesis has received support from Atherly & Bell (1964), who concluded that a prolyl-s-RNA-synthetase preparation from rat liver also activated azetidine-2-carboxylic acid. They also observed that the enzyme had a low affinity for the latter imino acid, which is in agreement with the present findings.

The toxicity of azetidine-2-carboxylic acid to mung-bean seedlings and to other systems can now be reasonably attributed to a failure of their prolyl-s-RNA-synthetases to discriminate between the normal substrate and molecules of the imino acid analogue. Expressed in different terms the more exacting substrate selection shown by the prolyl-s-RNA synthetase of *Polygonatum* protects these plants against their own lethal product. An alternative protective mechanism apparently exists in cells of an *Agrobacterium* species, which may utilize azetidine-2-carboxylic acid as a sole nitrogen source and possess an extremely active enzyme system degrading this imino acid by a hydrolytic ring-fission mechanism (Dunnill & Fowden, 1965). Although other experiments have shown that the prolyl-s-RNA synthetase from this *Agrobacterium* sp. activates azetidine-2-carboxylic acid, no incorporation of the imino acid analogue into the microbial protein could be demonstrated. Presumably in this instance the high activity of the hydrolytic enzyme ensures that little or no azetidine-2-carboxylic acid reaches the site of the prolyl-s-RNA synthetase.

The concept of different substrate specificities has also been considered by Lewis (1963) with a meth-

ionyl-s-RNA synthetase. The methionyl-s-RNA synthetase from the wild-type strain of a basidiomycete fungus, *Coprinus lagopus*, activated both methionine and ethionine, but a comparable enzyme from a mutant form activated only methionine. Similarly, Neidhardt & Fangman (1963) and Fangman & Neidhardt (1964) have reported a phenylalanyl-s-RNA synthetase with an altered specificity towards *p*-fluorophenylalanine isolated from an analogue-resistant mutant of *Escherichia coli*. Whereas the prolyl-s-RNA and methionyl-s-RNA synthetases mentioned above failed completely to activate their respective analogues, the phenylalanyl-s-RNA synthetase from the *E. coli* mutant had 5% of the ability of the wild-type strain to utilize the analogue for protein synthesis.

No information is available about the amino acid sequences in aminoacyl-s-RNA synthetases. However, the single gene mutation observed to be responsible for the altered specificity of methionyl-s-RNA synthetase (Lewis, 1963) may be associated with a single amino acid substitution at or near to the active site of the enzyme. Similarly, the differences of specificity reported in the present paper between various higher plants could be the result of a mutation of long evolutionary standing. Possibly within such a time more than one amino acid substitution has occurred; indeed, the amino acids in the active site may be totally different in the two sets of plants, for more than one conformation can effect a reaction at the catalytic site of a hydrolytic enzyme (see Sanger, 1963; Boyer, 1963).

The structure and mode of action of aminoacyl-s-RNA synthetases is not well known, but their active centres probably contain a thiol group because *p*-chloromercuribenzoate or oxygen causes inhibition that can be reversed by GSH. The substrate also partially protects against inhibition by *p*-chloromercuribenzoate (Allen, Glassman & Schweet, 1960; Stulberg & Novelli, 1962).

There are differences between individual aminoacyl-s-RNA synthetases in their sensitivity towards *p*-chloromercuribenzoate. Those enzymes that are the most stable to storage are least sensitive to *p*-chloromercuribenzoate (Stulberg & Novelli, 1962). The leucyl-s-RNA and isoleucyl-s-RNA synthetases are strongly inhibited by 0.1 mM *p*-chloromercuribenzoate (Ogata, Nohara, Ishikawa, Morita & Asaoka, 1961), whereas a concentration of 1 mM was required to inhibit strongly the tryptophanyl-s-RNA and tyrosyl-s-RNA synthetases (Davie *et al.* 1956; Schweet & Allen, 1958). The prolyl-s-RNA synthetase used in the present study was not unduly labile and was inhibited only slightly by 0.1 mM concentration.

Aminoacyl-s-RNA synthetases are large protein molecules (mol. wt. of isoleucyl-s-RNA synthetase is 112000; Norris, 1964) with some associated poly-

nucleotide material. Generally a higher enzyme purity is associated with a smaller polynucleotide content. The prolyl-s-RNA synthetase from mung bean contained 1.8% of polynucleotide material, whereas an isoleucyl-s-RNA synthetase from pig liver contained 1.46% at a similar stage of purification (Ogata *et al.* 1961). There are discrepancies between results of different workers on the effect of the treatment of these enzymes with ribonuclease. In the present study, preincubation with ribonuclease had no effect on the rate of ATP-PP_i exchange stimulated by proline. However, such treatment slightly increased the rate of exchange catalysed by a purified alanyl-s-RNA synthetase (Goldstein & Holley, 1960), and was used as a method for purifying the methionyl-s-RNA synthetase (Berg, 1956). In contrast, treatment with ribonuclease severely decreased the exchange stimulated by purified isoleucyl-s-RNA synthetase (Ogata *et al.* 1961).

The prolyl-s-RNA synthetase was absolutely specific for ATP of the trinucleotides tested, in agreement with the specificity observed for an alanyl-s-RNA synthetase (Webster, 1961). ITP has, however, been shown to replace ATP at about 20% efficiency with a general aminoacyl-s-RNA-synthetase preparation from *E. coli* (DeMoss & Novelli, 1956).

Of the nine bivalent cations tested, the most active after Mg²⁺ in the prolyl-s-RNA-synthetase system was Mn²⁺. Atherly & Bell (1964) have commented on the activity of Mn²⁺ for this enzyme isolated from rat liver.

The observed specificity of the prolyl-s-RNA synthetases from the plant tissues studied towards proline and 3,4-dehydropoline, and from mung-bean seeds towards azetidine-2-carboxylic acid and thiazolidine-4-carboxylic acid, indicate that the heterocyclic ring must be either smaller than or similar in size to that of proline if activation is to occur. *cis* or *trans*-Substitution of hydroxyl or methyl groups at ring positions 3 or 4 of proline yielded inactive compounds. Such substitution can produce an alteration in the properties of the ring: for example, the planarity of ring atoms associated with proline is lost in *trans*-4-hydroxy-L-proline, where C-4 is displaced about 0.4 Å from a plane, defined by the other four ring atoms, on the opposite side of which lies the carboxyl group (Donohue & Trueblood, 1952). Therefore it is impossible to decide whether this compound is inactive as a result of the increased size of the hydroxyl group replacing the hydrogen, or as a result of the loss of planarity of the ring. The former possibility seems the most likely because some alteration of the relative positions of C-3 and C-4 is permissible, as seen by the activation of 3,4-dehydropoline.

The absence of activation of the substituted

prolines is of interest when compared with the results for aromatic aminoacyl-s-RNA synthetases. Davie *et al.* (1956) and Sharon & Lipmann (1957) have shown with a tryptophanyl-s-RNA synthetase from pancreas that substitution of an aromatic hydrogen by any markedly bulkier group, e.g. methyl or hydroxyl, gave rise to inactive compounds. They obtained active compounds only with fluoro analogues, i.e. 6-fluoro and 5-fluoro-tryptophan. Similarly, Schweet & Allen (1958) showed that a pancreatic tyrosyl-s-RNA synthetase failed to activate 11 substituted tyrosines, although 3-fluorotyrosine could be used as a substrate. Likewise, with phenylalanine, the *p*-fluoro derivative is activated (Nisman & Hirsch, 1958).

In contrast, replacement of C-4 of proline by the larger S of thiazolidine-4-carboxylic acid (4-thio-proline), although slightly enlarging the ring, still gave an active compound. This compound was slightly active in promoting ATP-PP_i exchange when catalysed by a rat-liver prolyl-s-RNA synthetase (Fraser & Klass, 1963), but the purity of the test sample was not examined. Similarly, the replacement of a ring C of tryptophan by N (as in 7-azatryptophan or tryptazan) also gave active compounds (Davie *et al.* 1956; Sharon & Lipmann, 1957).

The activity of prolyl-s-RNA synthetase from mung bean was measured by both the exchange reaction and by the formation of prolyl-s-RNA. This enzyme has not been isolated previously from plant tissue, and few other aminoacyl-s-RNA synthetases have been studied in detail in plant extracts let alone comparisons of substrate specificities between plant groups. The enzyme catalysed the coupling not only of proline but also of azetidine-2-carboxylic acid with s-RNA from mung bean. The rate of transfer measured for the analogue was only 3% of the rate with proline, but this value may be low owing to uncertainty about the purity of ³H-labelled material used. Arnstein & Richmond (1964) found strong competition between phenylalanine and *p*-fluorophenylalanine for the phenylalanyl-s-RNA synthetase; the presence of phenylalanine at only 20% of the concentration of the analogue completely inhibited analogue-s-RNA formation. By analogy, since azetidine-2-carboxylic acid was added at high specific activity, the presence of a residual trace of proline might have markedly decreased the rate of the azetidine-2-carboxylic acid transfer reaction, but the addition of high carrier concentrations of unlabelled analogue would have made radioactivity assay difficult.

The virtual absence of prolyl-s-RNA formation with materials from *Polygonatum* was probably due to the s-RNA having few sites available for proline. The enzyme was active when measured either by the ATP-PP_i reaction or by the formation of

prolyl-s-RNA from mung-bean s-RNA. Therefore enzyme activities measured by the ATP-PP_i reaction were unrelated to those measured by aminoacyl-s-RNA formation. Similar observations have been noted by many workers; e.g., Moustafa & Lyttleton (1963) noted that an alanyl-s-RNA synthetase showed 40% of the activity of a valyl-s-RNA synthetase when measured by the exchange reaction, yet only 3% if compared by aminoacyl-s-RNA formation.

When the *Polygonatum* enzyme was coupled with mung-bean s-RNA, less labelled proline was transferred to RNA than when mung-bean enzyme was used. This difference could be due to differences in the endogenous proline content of the two enzyme preparations or to some species specificity inherent in the s-RNA. At present there are many conflicting reports on the subject of species-specific s-RNA. Some of the results obtained have shown that particular aminoacyl-s-RNA synthetases exhibited absolute specificity towards particular s-RNA molecules, whereas other reports indicate some activity between heterologous mixtures of enzyme and s-RNA (see Stulberg & Novelli, 1962).

In one of the few studies on higher plants, Moustafa & Lyttleton (1963) showed that s-RNA from pea would accept certain amino acids activated by enzymes from wheat germ. Experiments by Berg, Bergmann, Ofengand & Dieckmann (1961) suggested that the s-RNA specific for methionine in *E. coli* was heterogeneous, 40% being capable of reacting with the methionyl-s-RNA synthetase from either *E. coli* or yeast, and the remainder only with enzyme from *E. coli*. Weisblum, Benzer & Holley (1962) have isolated two different molecular species of s-RNA specific for leucine, and Sueoka & Yamana (1962) have isolated two specific for serine. Recently five s-RNA molecules specific for serine and phosphoserine have been proposed by Carlsen, Trelle & Schjeide (1964). Since three trinucleotide codes have been proposed for serine (Wahka *et al.* 1963), this leads to the speculation that more than one s-RNA may be required for each amino acid because the code is degenerate. In the present experiments, the difference of labelling of s-RNA from mung bean with prolyl-s-RNA synthetase from either mung bean or Solomon's seal has been interpreted as being possibly due to differences in traces of proline present in the preparations. However, four triplet codes have been assigned to proline (Jones & Nirenberg, 1962), and so the results could equally well be explained by assuming that different molecular species of s-RNA, specific for proline, exist in mung bean, but that only one of these accepted proline activated by *Polygonatum*.

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