Merthiolate, when incubated with the enzyme, resulted in losses of enzymatic activity (Fig 3) The reaction of Merthiolate with the enzyme reaches equilibrium after 10 min Thus, it would appear that Merthiolate dissociates the enzyme into subunits, and that the subunits are not active

1 mole of thiosalicylate (RSH) is formed when 1 mole of Merthiolate (R–S–Hg–Et) reacts with a protein sulfhydryl group The reaction follows the course shown in Eqn 1

$$R\text{–}S\text{–}Hg\text{–}Et + \text{enzyme–SH} \rightarrow RSH + (\text{subunit(s)})\text{–}S\text{–}Hg\text{–}Et \quad (1)$$

Reversal of this reaction by sulfhydryl groups would be predicted To test this possibility, excess dithiothreitol was added after preincubation of the enzyme with Merthiolate

The data in Table I and Fig 4 indicate that the reaction of succinyl-CoA synthetase with Merthiolate was reversible The subunits reassociated to an active form (Table I) that was serologically similar to the native enzyme (Fig 4) The fact that full activity was not restored may simply indicate that the rate of reaggregation was very slow under the conditions employed

The effect of Merthiolate on succinyl-CoA synthetase may be similar to that of *p*-mercuribenzoate It has been observed that *p*-mercuribenzoate dissociates the phosphorylated form of the enzyme into phosphorylated and nonphosphorylated subunits[5], although details describing this finding are not yet at hand It is important now to establish whether the Merthiolate subunit of mol wt 70 000 is unique or is a dimer of subunits of mol wt 35 000, and it will be of interest to determine which of these subunits is (are) phosphorylated

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Preparation of aminoacyl synthetases from higher plants*

Although the preparations of mixed aminoacyl synthetases (amino acid tRNA ligases (AMP)) from bacteria[1] and individual synthetases from yeast and from animal

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tissues[2] have been well documented, the corresponding enzymes from plants have been much less widely studied The literature contains references to the demonstration of synthetase activity in tomato seedling roots[3], pea epicotyls[4], maize[5] and wheat germ tissues[6] Detailed studies of plant synthetases are, however, rare and optimal procedures for their isolation have yet to be established

In our hands, the well-established procedures for the isolation of bacterial enzymes failed when applied without modification to plant tissue This paper describes the modifications necessary to prepare active synthetases from pea roots Some properties of the enzymes are described

Enzyme extraction Alaska peas (*Pisum sativum* var Alaska) were sterilized and germinated at 25° and 100% relative humidity for 48 h The roots were removed by a procedure developed in this laboratory (R O MORRIS, personal communication) for the bulk isolation of seedling roots and were frozen in liquid nitrogen The frozen roots were powdered under liquid nitrogen and were suspended in a slurry of polyvinylpolypyrrolidone (Polyclar AT, General Aniline and Film Corp) in a buffer containing 10 mM Tris chloride (pH 7 4), 5 mM $MgCl_2$, 1 mM reduced glutathione and glycerol (10%, v/v) Optimum conditions for preparation resulted when ratios were observed equivalent to 1 g of tissue suspended in 1 5 ml of buffer containing 0 1 g polyvinylpolypyrrolidone, which had been previously equilibrated overnight with buffer

All subsequent operations were carried out at 2° 10 min after mixing, the slurry was filtered through bolting silk and was clarified by centrifugation at 20 000 × *g* for 10 min The absorbance of the supernatant was measured at 260 nm, and protamine sulfate (Eli Lilly and Co) was added to a level of 5 $\mu g/A_{260\ nm}$ unit After 10 min the precipitate was removed by centrifugation at 27 000 × *g* for 10 min The solution at this point was designated the crude supernatant

Solid $(NH_4)_2SO_4$ was added to 80% satn , and the precipitate was resuspended in the minimum volume of homogenization buffer from which Mg^{2+} had been omitted It was then dialyzed against an excess of Mg^{2+}-free buffer for 30 min Solid KCl was added to a concentration of 0 15 M

DEAE-cellulose chromatography The solution was applied to a column of DEAE-cellulose (2 5 cm × 10 cm) which had been equilibrated with Mg^{2+}-free buffer containing 0 15 M KCl Upon elution with the same buffer, synthetase activity appeared in the void volume together with the bulk of the protein applied to the column Prior to this point synthetase activity was highly variable It was only after elution from DEAE-cellulose that stable high levels were seen

Exclusion chromatography The column eluate was brought to 80% satn with satd $(NH_4)_2SO_4$ solution (pH 7 0), and the precipitate was dissolved in a small volume of extraction buffer It was then subjected to chromatography on a column of polyacrylamide (Bio-Gel P-150, 2 5 cm × 35 cm) Synthetase activity eluted in the void volume and was concentrated by precipitation with $(NH_4)_2SO_4$ to 80% satn as before.

tRNA was prepared from the root tissue by a modification of the procedure of VON EHRENSTEIN[7] It was stored frozen at −70°

Amino acid attachment was measured by an assay containing the following components in a final volume of 0 2 ml Tris chloride, pH 7 4 (20 μmoles), $MgCl_2$ (2 μmoles), ATP (0 2 μmole), GSH (0 8 μmole), [^{14}C]amino acid (1 0 μC, specific

activity 100–500 mC/mmole), tRNA (1 $A_{260\ nm}$ unit) and enzyme (50 μg) The mixture was incubated at 30° for 15 min, and the reaction was terminated with an equal volume of ice-cold trichloroacetic acid solution (40%, w/v) After standing at 0° for 10 min, the precipitate was collected by filtration on a Schleicher and Schuell B6 membrane filter, was washed with cold trichloroacetic acid (5%, w/v) and then was dried at 80° Radioactivity was determined by the use of a Triton X-100–toluene system[8] and a Packard Tri-Carb scintillation counter

Where necessary, protein was measured by applying the procedure of LOWRY *et al* to protein which had been precipitated with trichloroacetic acid

TABLE I

PURIFICATION OF AMINOACYL SYNTHETASES

Determined by measurement of leucine acceptor activity, enzyme limiting A unit of enzyme activity in the assay results in the production of 1 pmole leucyl-tRNA in 15 min at 30° All figures were corrected by control assays (*minus* added tRNA)

Fraction	*Vol (ml)*	$\frac{A_{280\ nm}}{A_{260\ nm}}$	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*
Crude homogenate	52	0 60	81	1670	20 6
After protamine	45	0 57	53	1070	20 1
After $(NH_4)_2SO_4$	9 5	1 04	17 6	1620	92 1
Eluate from DEAE-cellulose	3	1 27	15 3	1800	118
Eluate from P-150	1	1 60	5 1	480	94 3

The ability of various stages of the enzyme preparation to attach leucine to pea root tRNA is outlined in Table I In the early stages of purification, individual preparations differed widely in their total activity and in their dependence upon added tRNA In some cases, activity was insignificant prior to DEAE-cellulose chromatography Subsequent to chromatography, however, there was a marked increase in the $A_{280\ nm}/A_{260\ nm}$ ratio, and individual preparations showed uniform high activity together with dependence upon added tRNA

Chromatography on P-150 gave a further increase in the $A_{280\ nm}/A_{260\ nm}$ ratio and usually in the specific activity of the preparation The major consequence of P-150 chromatography was, however, to impart dependence upon ATP Prior to this point, preparations were capable of catalyzing significant amounts of acylation in the absence of added ATP Presumably endogenous ATP was being carried through the previous stages Treatment with P-150 not only eliminated the endogenous activity but also materially reduced the ribonuclease activity present Assuming small loss between the original homogenate and the crude supernatant, the total purification was of the order of 4-fold in terms of specific activity

The best preparations catalyzed the attachment of about 96 pmoles of leucine per $A_{260\ nm}$ unit of tRNA during 15-min incubation at 30° This may be compared with a value of 160 pmoles for the corresponding *Escherichia coli* system[7], 14 pmoles for the myeloblast enzyme (J W CARNEGIE AND G S BEAUDREAU, personal communication) and 40 pmoles for the maize enzyme[5]

It was found that the presence of glycerol and the use of liquid nitrogen during the initial steps of tissue homogenization were both mandatory for success No activity was observed when glycerol was omitted[1] Disruption of the tissue with a

homogenizer at 0° gave only a fraction of the activity obtained by the use of liquid nitrogen Polyvinylpolypyrrolidone was not essential in the preparation, but it improved the $A_{280\ nm}/A_{260\ nm}$ ratio of the final product and gave a slight enhancement of activity, presumably by the adsorption of polyphenols[9] The enzyme was stable after elution from P-150 (but not before) and could be maintained at −70° for 3 weeks without appreciable loss of activity

TABLE II

SPECIFICITY OF ACYLATION BY THE PEA ROOT ENZYMES

Assays were performed as described in the text Acylation is expressed as pmoles amino acid attached to 1 nmole tRNA in 15 min at 30°

Amino acid	*Acylation (pmoles)*	*Amino acid*	*Acylation (pmoles)*
Alanine	1 96	Lysine	5 84
Arginine	7 62	Methionine	4 31
Aspartic acid	1 08	Phenylalanine	0 837
Glutamic acid	0 604	Proline	0 099
Glycine	1 85	Serine	2 61
Histidine	1 03	Threonine	1 48
Isoleucine	4 51	Tyrosine	1 52
Leucine	7 92	Valine	2 03

Chromatography of the deproteinized, dialyzed reaction mixtures upon benzoylated DEAE-cellulose[10] and Freon reverse phase columns[11] revealed that at least 70% of the acid-precipitable radioactivity was attached to specific tRNA fractions. As shown in Table II, the preparation was able to attach all amino acids tested with the exception of proline

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