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Enzymes and Ribonucleic Acid Associated with the Incorporation of Amino Acids into Proteins of Wheat Endosperm

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When intracellular components of wheat endosperm are incubated with ^{14}C -labelled amino acids under suitable conditions, radioactivity is incorporated into the major protein components (Morton & Raison, 1964). The characteristics of this enzymic process and the properties of the intracellular components concerned with the incorporation reactions have been described (Morton & Raison, 1964; Morton, Palk & Raison, 1964). The protein-body preparation [containing proteoplasts (protein-forming plastids)] and the supernatant preparation (containing elements of the endoplasmic reticulum) catalyse incorporation into the storage proteins and the supernatant proteins respectively.

Each of the two systems concerned with amino acid incorporation has now been fractionated, and transfer RNA and amino acid-activating enzymes have been identified. The present paper describes the procedure for extraction and assay of the enzymes and nucleic acid components associated with the formation of amino acyl-RNA in the two systems. It also describes the incorporation of radioactivity from ^{14}C -labelled amino acids into soluble protein and nucleic acid components in a supernatant fraction obtained from disrupted protein-forming plastids.

MATERIALS

Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Uniformly labelled

^{14}C]proline, ^{14}C]glutamic acid, ^{14}C]leucine and ^{14}C]glycine were of the same specific activities as described by Morton & Raison (1964). Uniformly-labelled mixed amino acids (Hallowes & Winteringham, 1958) were obtained from the protein of *Chlorella* grown on ^{14}C]bicarbonate as the sole carbon source; the mixed amino acids had a specific activity of $145\text{ }\mu\text{C}/\text{mg}$. Carrier-free $\text{K}_2\text{H}^{32}\text{PO}_4$ was obtained from N. V. Philips-Duphar, Amsterdam, Holland, and was converted into ^{32}P]pyrophosphate by pyrolysis at 450° (Lee Peng, 1956). The disodium salt of ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.) was converted into the acid salt by using Dowex 50 resin (H^+ form) and neutralized with 0.1N -KOH and 0.1M -potassium phosphate buffer, pH 7.2. 'Low-salt' hydroxylamine was prepared by the following modification of the method of Beinert *et al.* (1953). The hydroxylamine hydrochloride and KOH were mixed in anhydrous methanol, ground in a glass mortar for 2 min. and then shaken for 16 hr. with a Microid flask shaker (Griffin and George Ltd.). Norit SX-2 charcoal was obtained from Harrington Brothers Ltd., London, and washed by repeated boiling in 6N -HCl and then in water. DEAE-cellulose and Sephadex G-75 were obtained from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. Phosphoenolpyruvate, Nonidet P40 detergent, pyruvate kinase (EC 2.7.1.40) and amino acids were obtained from the sources indicated by Morton & Raison (1964).

METHODS

Preparation of enzyme fractions

Endosperm was obtained from immature wheat grain (Morton & Raison, 1964) stored at -15° for about 3 months and homogenized in 0.1M -tris-HCl buffer, pH 7.4, containing 1% (v/v) of detergent (Nonidet P40) and magnesium chloride (6 mM), by the methods described by Graham, Morton & Raison (1963). The protein-body and supernatant preparations were obtained by differential centrifugation of the homogenate as described by Morton & Raison (1964).

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Enzyme fractions from the supernatant preparation. The supernatant preparation was further separated by centrifuging to yield a 105000g supernatant fraction (see Morton & Raison, 1964). Part of the 105000g supernatant (referred to as the 'supernatant fraction') was dialysed against 0.1M-tris-HCl buffer, pH 7.4, containing magnesium chloride (6 mM), at 2° for about 16 hr. The dialysed preparation is referred to as the 'dialysed supernatant fraction'. Part of the supernatant fraction was adjusted to pH 5.0 by the addition of 0.5N-acetic acid and the precipitate was collected and washed as described by Hoagland, Keller & Zamecnik (1956). The precipitate was finally dissolved in 0.1M-tris-HCl buffer, pH 7.4, containing magnesium chloride (6 mM), and is referred to as the 'pH 5 enzyme' fraction. Part of the pH 5 enzyme fraction was separated into protein and nucleic acid fractions by chromatography with DEAE-cellulose (see Fig. 1).

Enzyme fractions from the protein-body preparation

Preparation and extraction of an acetone-dried powder. A protein-body preparation, obtained from 100 g. wet wt. of isolated endosperm as described by Morton & Raison (1964), was suspended in 10 ml. of 0.1M-tris-HCl buffer, pH 7.4, and poured slowly into 500 ml. of redistilled acetone, precooled to -15°. During the addition of the protein-body preparation and for 90 sec. afterwards, the acetone was vigorously agitated with a Vibromix (Shandon Scientific Co. Ltd.). The precipitate was rapidly filtered off on Whatman no. 1 paper by using vacuum filtration. The precipitate was washed with cold (-15°) acetone and ether and dried in a vacuum desiccator over phosphorus pentoxide and solid paraffin. The dried powder was extracted with 10 ml. of 0.1M-tris-HCl buffer, pH 7.4, by using a Vibromix. The mixture was centrifuged at 5000g for 15 min. and the clear supernatant, containing about 20% of the total nitrogen in the protein-body preparation, was removed; this supernatant is referred to as the 'acetone extract' of the protein-body preparation.

Disruption and extraction of protein-body preparation. Suspensions of protein bodies (usually 8 ml. containing approx. 20 mg. of N) were stored at -15° for various periods up to 1 week. The preparations were thawed slowly for several hours and treated by ultrasonic vibration with an ultrasonic drill, type E 7680/3 (Mullard Equipment Ltd., London) operating at 20 kcy./sec. and 60 w for 5 min. The temperature was maintained at 2-4°. The mixture was centrifuged at 20000g for 30 min. and part of the supernatant was further centrifuged at 105000g for 90 min. Both the 20000g and the 105000g supernatants were used.

Separation and purification of transfer ribonucleic acid. The RNA component of the enzyme fractions from both the protein-body and supernatant preparations was separated from the protein components by chromatography (see Fig. 1).

The RNA fraction eluted with 1.0M-KCl in 0.1M-tris-HCl buffer, pH 7.4, appeared as a single component on rechromatography (see Fig. 1b). To prevent breakdown during storage at 2°, the RNA fraction was shaken with 0.5 vol. of water-saturated phenol. After centrifuging at 1000g for 15 min. the RNA was recovered in the aqueous phase, extracted several times with diethyl ether, and dialysed against water to remove residual phenol. Preparations purified in this way were used for enzyme studies and for sedimentation-velocity measurements.

Determination of enzymic activity

Hydroxamic acid formation. The basic reaction mixtures (8 ml.) contained 500 μmoles of glycylglycine buffer, pH 7.2, 6.0 m-moles of 'low-salt' hydroxylamine, 50 μmoles of magnesium chloride and 80 μmoles of a mixture of 15 amino acids (leucine, isoleucine, histidine, tryptophan, methionine, glutamic acid, proline, valine, serine, threonine, phenylalanine, glycine, alanine, lysine and arginine). ATP and an ATP-generating system, consisting of phosphoenolpyruvate, pyruvate kinase and ADP, were added to the basic reaction mixture where indicated. The reaction mixtures were incubated at 30° for 20 min. Portions (3 ml.) were removed and added to 1.4 ml. of 100% (w/v) trichloroacetic acid adjusted to pH 0.9 (Clark, 1958) and 0.6 ml. of 2M-ferric chloride. The mixture was centrifuged at 10000g for 15 min. The extinction of the clear supernatant was determined at 540 mμ in a 1 cm. cuvette (Clark, 1958).

Fig. 1. (a) Separation of protein and s-RNA by chromatography on DEAE-cellulose essentially as described by Holley *et al.* (1961). A DEAE-cellulose column (1 cm. diam. $\times$ 8 cm. high) was equilibrated to pH 7.4 with 0.1M-tris-HCl buffer. The sample of pH 5 enzyme fraction (containing 120 mg. of protein) was applied to the surface of the column and washed with 200 ml. of 0.1M-tris-HCl buffer, pH 7.4. The eluate was collected in 10 ml. fractions, and the concentration of protein was estimated from the $E_{260\text{ m}\mu}/E_{280\text{ m}\mu}$ ratio (Warburg & Christian, 1942). Fractions 4 and 5 were combined and are referred to as the 'RNA-free pH 5 enzyme' fraction. (b) Material eluted in 1.0M-KCl in 0.1M-tris-HCl buffer, pH 7.4, was dialysed against 0.1M-tris-HCl buffer, pH 7.4, and re-treated by column chromatography as described above. The eluents were: A, 0.1M-tris-HCl buffer, pH 7.4; B, 1.0M-KCl in 0.1M-tris-HCl buffer, pH 7.4; C, 0.1N-NaOH.

Pyrophosphate exchange reaction. The reaction mixture (total vol. 1 ml.) contained 100 μ moles of tris-HCl buffer, pH 7.4, 4.8 μ moles of [32 P]pyrophosphate, 4.8 μ moles of ATP, 6 μ moles of magnesium chloride, 10 μ moles of KF and the enzyme fraction (containing 0.5–1.0 mg. of protein). The mixture was incubated at 37° for 20 min. and the reaction was stopped by the addition of 0.25 ml. of 20% (v/v) perchloric acid. Protein was sedimented by centrifuging and 1 ml. of the clear supernatant was mixed with 0.6 ml. of a suspension of charcoal (equivalent to 45 mg. of dry charcoal). The charcoal was collected by vacuum filtration on to disks of Whatman no. 41 filter paper cut to a diameter of 25 mm. to fit the planchet holder of a thin-mica-end-window Geiger-Müller counter (Ekco Electronics Ltd.). The charcoal was washed on the filter paper with 20 ml. of 0.1 M-sodium pyrophosphate buffer, pH 7.0, and with 5 ml. of diethyl ether.

Amino acyl-ribonucleic acid formation. The formation of amino acyl-RNA was determined from the amount of 14 C-labelled amino acid linked to transfer RNA (s-RNA) by an alkali-labile ester bond. The reaction mixture (final vol. 3 ml.) contained 300 μ moles of tris-HCl buffer, pH 7.4, 2 μ moles of GSH, 5 μ moles of KCl, 1 μ mole of magnesium chloride, 0.1 μ mole of s-RNA, 2.5–7.5 μ g. of a mixture of 14 C-labelled amino acids (approx. 0.02–0.06 μ mole) and 1.0 ml. of the enzyme fraction, containing 0.4–2.2 mg. of protein. The mixture was incubated at 30° for 20 min. and applied to the surface of a DEAE-cellulose column (1 cm. diam. $\times$ 4 cm. high) equilibrated to pH 7.4. After the removal of amino acids and protein from the column, amino acyl-RNA was eluted with 1.0 M-KCl in 0.1 M-tris-HCl buffer, pH 7.4, as described above (see also Fig. 1). The amino acyl-RNA fraction was dialysed against 100 vol. of water for 3 hr. and the radioactivity was determined with a liquid-scintillation counter. The dialysed amino acyl-RNA fraction was adjusted to pH 9.8 with 0.1 N-KOH-0.1 M-Na₂CO₃ buffer, pH 9.8, incubated for 1 hr. at 37° and again dialysed for 16 hr. against 500 vol. of water. Amino acids linked to s-RNA by ester bonding were determined by the loss of radioactivity after incubation at pH 9.8 and dialysis. At least 95% of the s-RNA present in the original reaction mixture was recovered in the RNA fraction eluted from the DEAE-cellulose column.

Determination of radioactivity

Measurements were made at room temperature with a type N664A scintillation counter and a thin-mica-end-window Geiger-Müller counter, type N620 (Ekco Electronics Ltd.). The 14 C radioactivity in 0.2 ml. samples was determined with the liquid-scintillation counter in 5 ml. of Ditol (Herberg, 1960). Under these conditions 0.25 μ C of [14 C]glutamic acid gave 6760 counts/sec. The 32 P radioactivity in the charcoal on paper planchets was determined with the Geiger-Müller counter; the paper planchets were positioned on the metal planchet holder by a metal collar. Under these conditions 0.012 μ C of K₂H³²PO₄ (0.01 ml.), evenly distributed over an equivalent amount of charcoal on a paper planchet, gave 102 counts/sec.

Other determinations

Sedimentation measurements were carried out with a Spinco model E ultracentrifuge. Nitrogen was determined by a micro-Kjeldahl method. Protein was determined from the $E_{260\text{ m}\mu}/E_{280\text{ m}\mu}$ ratio (Warburg & Christian, 1942).

RESULTS

Extraction and purification of transfer ribonucleic acid. The nucleic acid components of the pH 5 enzyme fraction from the supernatant preparation, and of the enzyme fraction from the protein-body preparation, were separated from the protein components by chromatography on DEAE-cellulose (Fig. 1). The nucleic acid fraction appeared as a single component on rechromatography (Fig. 1b). The absorption spectrum of the rechromatographed fraction had E_{max} 259 m μ , and the $E_{260\text{ m}\mu}/E_{280\text{ m}\mu}$ ratio was 1.96. These values are similar to those reported for s-RNA by Holley & Goldstein (1959) and by Ofengand, Dieckmann & Berg (1961). The s-RNA prepared by chromatography was further purified by treatment with water-saturated phenol as described in the Methods section. This treatment did not alter the absorption spectrum but provided a preparation of s-RNA that was stable on storage at 2°. In the ultracentrifuge the purified s-RNA fraction appeared as a single component; the sedimentation coefficient ($S_{s,w}$) was 3.4.

Fractionation of the protein-body preparation. By treatment of the protein-body preparation with acetone and extraction of the acetone-dried powder with 0.1 M-tris-hydrochloric acid buffer, pH 7.4, as described in the Methods section, 20% of the total nitrogen was extracted (Table 1). After disruption of the protein-body preparation by ultrasonic vibration, approx. 19% of the nitrogen remained in the 20000g supernatant fraction (Table 1).

When a suspension of protein bodies isolated by density-gradient centrifugation as described by Graham *et al.* (1963) was extracted by treatment in 0.01 M-sodium pyrophosphate buffer, pH 7.4, with a Vibromix, 15% of the total nitrogen remained in the supernatant obtained by centrifuging the homogenate at 10000g for 15 min. (Table 1).

The supernatant fractions obtained from the protein-body preparation treated with either acetone or ultrasonic vibration contained amino acid-activating enzymes and catalysed the formation of amino acyl-RNA.

Amino acid-activating enzymes. Fractions obtained from both the supernatant and protein-body preparations catalysed an ATP-[32 P]pyrophosphate exchange.

(a) From the supernatant preparation. Without dialysis the supernatant fraction catalysed a pyrophosphate exchange in the absence of added ATP or of added amino acid. The rate of exchange was linear with protein concentration between 2 and 5 mg. of protein (Fig. 2), and linear with time for at least 20 min. After dialysis, pyrophosphate exchange catalysed by the dialysed supernatant fraction was dependent on the addition of both ATP (Table 2) and amino acids (Table 3). The

Table 1. *Fractionation of the protein components of the protein-body preparation*

Portions of separate protein-body preparations, and of protein bodies obtained by density-gradient centrifugation (Graham *et al.* 1963), were treated as follows. In Expt. 1 the protein-body preparation was treated with acetone and the dried powder was extracted with 0.1 M-tris-HCl buffer, pH 7.4, and centrifuged at 5000g for 15 min. (see the Methods section). In Expt. 2 the protein-body preparation was treated by ultrasonic vibration for 5 min. and centrifuged at 20000g for 30 min. (see the Methods section). In Expt. 3 the isolated protein bodies (Graham *et al.* 1963) were suspended in 0.01 M-sodium pyrophosphate buffer, pH 7.0, homogenized with the aid of a Vibromix and centrifuged at 10000g for 15 min.

Expt. no.	Original preparation (mg. of N)	Fractions		N extracted in the supernatant fraction (%)
		Supernatant (mg. of N)	Precipitate (mg. of N)	
1	4.50	0.89	3.61	19.6
2	6.50	1.20	4.60	18.5
3	0.290	0.045	0.245	15.0

Fig. 2. Effect of protein concentration on the rate of ATP-[^{32}P]pyrophosphate exchange. The reactions were carried out as described (see the Methods section) with the supernatant fraction from the supernatant preparation.

Table 2. *Effect of adenosine triphosphate on the adenosine triphosphate-[^{32}P]pyrophosphate-exchange activity catalysed by the dialysed supernatant fraction*

[^{32}P]Pyrophosphate exchange was estimated as described in the Methods section; 0.25 ml. of the dialysed supernatant fraction from the supernatant preparation (containing 5 mg. of protein) was used in the assay.

ATP added (μmoles)	[^{32}P]Pyrophosphate exchanged/20 min. (μm-moles)
—	15
2.0	865
4.0	1130
4.8	1465
8.0	795

Table 3. *Effect of added amino acids on the adenosine triphosphate-[^{32}P]pyrophosphate-exchange activity of the dialysed supernatant fraction*

[^{32}P]Pyrophosphate exchange was estimated as described in the Methods section; 0.25 ml. of the dialysed supernatant fraction from the supernatant preparation (containing 5 mg. of protein) was used in the assay.

Amino acid mixture added (μmoles)	[^{32}P]Pyrophosphate exchanged/20 min. (μm-moles)
—	150
2	735
4	945
8	1465

Table 4. *Effect of adenosine triphosphate and of an adenosine triphosphate-generating system on the formation of hydroxamate catalysed by the supernatant fraction*

ATP and an ATP-generating system (50 μmoles of ADP, 0.1 ml. of a 5% solution of pyruvate kinase, and phosphoenolpyruvate) were incubated with the basic reaction mixture (see the Methods section) and the supernatant fraction from the supernatant preparation. Hydroxamate formation was estimated from $E_{540\text{ m}\mu}$.

ATP added (μmoles)	Phosphoenolpyruvate added (μmoles)	$E_{540\text{ m}\mu}$
—	—	0.593
10	10	0.624
10	50	1.11
10	100	1.18

addition of ATP to the reaction mixture at a concentration exceeding the concentration of pyrophosphate decreased the rate of pyrophosphate exchange. The addition of amino acids at a concentration greatly exceeding the concentration of ATP had no inhibitory effect.

The dialysed supernatant fraction also catalysed the formation of amino acyl-hydroxamates (Table 4). As found by other workers (Elliott & Coleman,

1961; Clark, 1958) the formation of hydroxamate depended on the addition of a large excess of an ATP-generating system.

(b) From the protein-body preparation. Fractions obtained by ultrasonic vibration of a protein-body preparation gave high values in the pyrophosphate-exchange assay in the absence of added ATP, even after dialysis for 20 hr. at 2° against 0.1M-tris-hydrochloric acid buffer, pH 7.4. A sample of the enzyme fraction was further purified by passing through Sephadex G-75 with 0.05M-tris-hydrochloric acid buffer, pH 7.4. This preparation also gave high values in the pyrophosphate-exchange assay in the absence of added ATP, and the rate of pyrophosphate exchange decreased when ATP was added to the system (Table 5). The addition of amino acids to another portion of the same preparation caused an increase in the rate of pyrophosphate exchange, but caused a decrease when the amount of amino acid added greatly exceeded the concentration of pyrophosphate (Table 6).

Amino acyl-ribonucleic acid formation. Enzyme fractions obtained from the supernatant and protein-body preparations catalysed the formation of amino acyl-RNA (Table 7). With the dialysed supernatant fraction the addition of ATP greatly stimulated amino acyl-RNA formation. With the dialysed enzyme fraction from the protein-body

preparation, the addition of ATP stimulated amino acyl-RNA formation but not to the same extent as the stimulation found with the enzyme fraction from the supernatant preparation.

Incorporation of amino acids into the enzyme fraction from the protein-body preparation. The incorporation of ¹⁴C-labelled amino acids into non-

Table 7. *Formation of amino acyl-ribonucleic acid by enzyme fractions from the supernatant and protein-body preparations*

The reactions were carried out as described in the Methods section by using the dialysed 105000g supernatant fraction from the protein-body preparation (containing 0.4 mg. of protein) and the supernatant preparation (containing 22 mg. of protein). The amino acyl-RNA was isolated as described in Fig. 1 and amino acids linked to RNA by ester bonds were determined as described in the Methods section.

Source of the enzyme fraction	ATP added (μmoles)	Radioactivity of the alkali-labile amino acids (counts/sec.)
Supernatant preparation	0	20
	10	41
Protein-body preparation	0	68
	10	83

Table 5. *Effect of adenosine triphosphate on the adenosine triphosphate-³²P pyrophosphate-exchange activity of an enzyme fraction from the protein-body preparation*

The enzyme fraction was obtained from the protein-body preparation after disruption by ultrasonic vibration and was purified by gel-filtration through Sephadex G-75. [³²P]Pyrophosphate exchange was measured as described in the Methods section in a reaction mixture containing 0.64 mg. of protein.

ATP added (μmoles)	[³² P]Pyrophosphate exchanged/20 min. (μ-moles)
0	13.7
2	6.0
4	4.6

Table 6. *Effect of added amino acids on the adenosine triphosphate-³²P pyrophosphate-exchange activity of an enzyme fraction from the protein-body preparation*

The preparation of the enzyme and the conditions of the reaction were as described in Table 5.

Amino acid mixture added (μmoles)	[³² P]Pyrophosphate exchanged/20 min. (μ-moles)
0	2.0
4	5.6
8	4.6

Fig. 3. Incorporation of ¹⁴C-labelled amino acids into protein and RNA components of an enzyme fraction from disrupted protein bodies. Incorporation was carried out in a reaction mixture (final vol. 3 ml.) containing 1.25 μC each of [¹⁴C]proline, [¹⁴C]glutamic acid, [¹⁴C]leucine and [¹⁴C]glycine, 500 μmoles of tris-HCl buffer, pH 7.5, and the enzyme fraction (0.5 mg. of protein). The mixture was incubated for 30 min. at 30° and the reaction was stopped by the addition of 1 ml. of 8M-urea. After dialysis against several changes of 100 vol. of 0.01M-tris-HCl buffer, pH 7.5, over a period of 20 hr., the protein and RNA components were separated on DEAE-cellulose (see the Methods section). A total of 2120 counts/sec. was applied to the column. The eluents were: A, 0.1M-tris-HCl buffer, pH 7.5; B, 1.0M-KCl in 0.1M-tris-HCl buffer, pH 7.5. Fractions (10 ml.) were collected. ○, Radioactivity (counts/sec.); ●, $E_{260\mu\mu}$.

Table 8. *Stability of radioactivity incorporated into the protein and nucleic acid components of the enzyme fraction from disrupted protein bodies*

The protein fractions (fractions 2, 3, 4 and 5 in Fig. 3) were combined and taken up in 10 ml. of 0.1M-sodium carbonate, and adjusted to pH 9.8 with 1.0N-NaOH. The mixture was incubated at 30° for 60 min. and dialysed against water for 18 hr. at 2°. The RNA fractions (fractions 12, 13 and 14 in Fig. 3) were combined, dialysed against water for 6 hr., adjusted to pH 9.8, and incubated at 30° for 60 min. The incubation mixture was again dialysed against water and the non-diffusible material was applied to a DEAE-cellulose column equilibrated with 0.1M-tris-HCl buffer, pH 7.4. The column was washed and fractions were collected; the radioactivity and $E_{260\text{ m}\mu}$ of each fraction were determined. All of the radioactivity was found in the first three fractions eluted with 1.0M-KCl in 0.1M-tris-HCl buffer, pH 7.4. These fractions were combined to give the s-RNA. The recovery of material after incubation and dialysis, determined from the $E_{260\text{ m}\mu}/E_{280\text{ m}\mu}$ ratio, was 100% of the nucleic acid fraction and 93% of the protein fraction.

Fractions	Radioactivity (counts/sec.)		Loss of radioactivity (%)
	Before incubation	After incubation	
Protein	937	831	11
RNA	230	66	71

diffusible protein and RNA components of the enzyme fraction from the protein-body preparation is shown in Fig. 3. Of the total of 5300 counts/sec. of radioactivity incorporated into non-diffusible components 51% was in the protein components and 18% in the s-RNA fraction separated by chromatography on DEAE-cellulose. Most of the radioactivity incorporated into the protein components remained associated with the protein after incubation at pH 9.8 and further dialysis. Most of the radioactivity associated with the nucleic acid fraction was lost when this fraction was treated in a similar manner (Table 8).

DISCUSSION

The protein-body and supernatant preparations used as the source of enzyme fractions were similar to the preparations that catalyse the separate incorporation of amino acids into protein (Morton & Raison, 1964). With both preparations, the incorporation of amino acids was inhibited by specific reagents known to inhibit protein synthesis, and the reactions involved in the incorporation showed many of the characteristics of the enzyme reactions linked with protein synthesis (Morton & Raison, 1964). By further fractionation, the enzymes and RNA components associated with the initial

activation of amino acids and the formation of amino acyl-RNA have been identified in isolated fractions from both the protein-body and the supernatant preparations.

The chemical and morphological composition of the protein-body preparation has been described (Morton & Raison, 1963; Morton *et al.* 1964). After treatment with acetone or with ultrasonic vibration approx. 20% of the total nitrogen of the protein-body preparation remained in the supernatant fraction after centrifuging at 20000g for 30 min. Since the protein-body preparation contains the protein-forming plastids (proteoplasts), the release of nitrogenous material from preparations that had been previously washed with buffer solutions appears to depend on the disruption of the plastid membrane. Although a similar proportion of nitrogenous material was extracted from the protein-body preparation by the two methods used (Table 1), the enzymic activity of fractions prepared by ultrasonic disruption was higher than that of fractions prepared by using acetone. A similar proportion of the total nitrogen of a protein-body fraction prepared by density-gradient centrifugation (Graham *et al.* 1963) was extracted after vigorous homogenizing with a Vibromix. With protein-body fractions obtained from endosperm that had incorporated [^{35}S]sulphate into the storage and cytoplasmic proteins (Graham *et al.* 1963), the fraction not sedimented by centrifugation after homogenizing had a specific activity (counts/sec./mg. of N) lower than that of the original protein-body fraction and considerably lower than that of the cytoplasmic proteins (Graham, Morton & Raison, 1964). These findings suggest that a portion of the proteins in the proteoplast are functionally distinguishable from the storage and cytoplasmic proteins.

The activation of amino acids was measured by the exchange of [^{32}P]pyrophosphate with ATP in reaction mixtures containing equimolar concentrations of pyrophosphate and ATP. With the dialysed supernatant fraction obtained from the supernatant preparation, pyrophosphate exchange was almost completely dependent on the addition of ATP and amino acids (Tables 2 and 3), and the rate of exchange increased with an increasing amount of enzyme fraction (Fig. 2). This fraction also catalysed the formation of amino acyl-RNA with s-RNA isolated from the supernatant preparation (Table 7).

A fraction that catalysed a pyrophosphate-exchange reaction and the incorporation of amino acids into protein was also obtained from the protein-body preparation after treating the preparation with ultrasonic vibration. No enzymic activity could be detected in the buffer solutions used to wash the protein-body preparation. The

release of enzymes thus appears to depend on disruption of the membranes enclosing the protein-forming plastids (Morton & Raison, 1963) or on disruption of the vesicular elements associated with the protein bodies in the protein-body preparation (Morton *et al.* 1964). After dialysis and purification by gel-filtration, the pyrophosphate-exchange reaction catalysed by the enzyme fraction from a disrupted protein-body preparation was only partially dependent on the addition of amino acids (Table 6) and was considerably inhibited by the addition of ATP (Table 5). The formation of amino acyl-RNA catalysed by this enzyme fraction was not dependent on the addition of ATP but was stimulated when ATP was included in the reaction mixture (Table 7).

It has been shown that the protein-body preparation contains a system for the generation of ATP from ADP and inositol hexaphosphate (phytate) and that this system is extracted from the protein-body preparation after treatment with ultrasonic vibration (Morton & Raison, 1963). The lack of dependence of the formation of amino acyl-RNA on the addition of ATP, and the inhibition of the pyrophosphate-exchange reaction by the addition of ATP, can thus be explained by the presence of ATP and the ATP-generating system in the enzyme fraction from the protein-body preparation. Since the pyrophosphate-exchange reaction is influenced greatly by the relative concentration of ATP and pyrophosphate in the reaction mixture, the apparent inhibition of the pyrophosphate-exchange reaction by added ATP is probably due to the high concentration of ATP present in the enzyme fraction. Added ATP did not inhibit the formation of amino acyl-RNA catalysed by the enzyme fraction from the protein-body preparation, and this indicates that ATP did not inhibit the amino acyl-RNA synthetases. The difference in the pyrophosphate-exchange reactions observed with enzymes from the protein-body and supernatant preparations can thus be attributed to the generation of ATP in the enzyme fraction from the protein-body preparation and the influence of the concentration of ATP on the pyrophosphate-exchange reaction. There is no evidence to suggest that the amino acyl-RNA synthetases from the protein-body and supernatant preparations are different enzymes. Since the amino acyl-RNA synthetases from the protein-body preparation catalysed the formation of amino acyl-RNA with s-RNA from the supernatant preparation, it is probable that the enzymes and s-RNA in the two preparations catalyse essentially the same reactions.

More definite evidence of the presence of amino acyl-RNA synthetases in the enzyme fraction from disrupted protein-body preparations was obtained

from the direct incorporation of a number of labelled amino acids into non-diffusible components that were separated, by chromatography, into protein and RNA (Fig. 3). The loss of the major proportion of the radioactivity associated with the RNA component after incubation in alkaline conditions and subsequent dialysis indicates that amino acids were attached to the RNA by acyl linkage, and further demonstrates the formation of amino acyl-RNA (without added ATP) by enzymes and components in the enzyme fraction from the protein-body preparation.

A part of the radioactivity (29%; cf. Table 8) not lost from the RNA component after alkali treatment was probably due to incorporation of the labelled amino acids into enzyme proteins which are known to be associated with s-RNA and are not separated from the RNA fraction by chromatography on DEAE-cellulose (see Holley *et al.* 1961).

The incorporation of labelled amino acids into alkali-stable bonds associated with the protein components separated from the amino acyl-RNA by chromatography indicates that the enzyme fraction from the protein-body preparation contained all of the components essential for the incorporation of amino acids into protein. Previous studies have shown that the enzyme fraction from the protein-body preparation contains ribosomes (Morton *et al.* 1964), and it is evident from the present studies that s-RNA and amino acyl-RNA synthetases are also present in this fraction.

The evidence presented above indicates that the amino acyl-RNA synthetases and s-RNA associated with the incorporation of amino acids into the storage protein of wheat endosperm are localized in the protein-forming plastids in the protein-body preparation. Similarly, the synthetases and s-RNA associated with the incorporation of amino acids into cytoplasmic proteins are localized in the supernatant preparation. The localization of enzymes and s-RNA associated with the initial stages of protein synthesis in both the protein-body and supernatant preparations is in accordance with the previous biochemical and morphological evidence (Morton *et al.* 1964; Morton & Raison, 1964) for the existence of two independent systems in the wheat endosperm for the synthesis of storage and cytoplasmic proteins.

SUMMARY

1. Methods are presented for the separation and purification of amino acyl-RNA synthetases and s-RNA from the protein-body and supernatant preparations of wheat endosperm.

2. The activity of the synthetases was demonstrated by ATP-[³²P]pyrophosphate exchange and

by the formation of amino acyl-RNA. A method is described for the separation of amino acyl-RNA from enzyme reaction mixtures by chromatography on DEAE-cellulose.

3. The pyrophosphate-exchange reaction catalysed by the synthetases from the protein-body preparation showed an apparent inhibition with added ATP. The pyrophosphate exchange catalysed by the enzymes from the supernatant preparation was almost completely dependent on ATP and stimulated by the addition of ATP. The formation of amino acyl-RNA by enzyme preparations from both the protein-body and supernatant preparations was stimulated by the addition of ATP.

4. An enzyme fraction obtained from the protein-body preparation after treatment with ultrasonic vibration catalysed the incorporation of amino acids into amino acyl-RNA and soluble protein.

5. The localization of enzymes and RNA associated with the initial stages of protein synthesis in the two preparations from wheat endosperm, and the significance of these findings in relation to the independent synthesis of storage and cytoplasmic proteins of wheat endosperm, is discussed.

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Photoassimilation of Acetate and the Biosynthesis of Amino Acids by *Chlorobium thiosulphatophilum*

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The green sulphur bacteria *Chlorobium limicola* and *Chlorobium thiosulphatophilum*, unlike other photosynthetic bacteria, are both strict anaerobes and strict autotrophs. With the exception of the green organism isolated from the symbiotic aggregate *Chlorochromatium* by Mechsner (1957) and *Chloropseudomonas ethylicum* isolated by Shaposhnikov, Kondratieva & Fedorov (1960), growth of the green sulphur bacteria has not been obtained in media containing organic compounds in place of

reduced compounds of sulphur. However, Larsen (1953) found that washed suspensions of *C. thiosulphatophilum* carboxylated propionate to succinate, and Sadler & Stanier (1960) found that *Chlorobium limicola* grew in the presence of low concentrations of some simple organic compounds, added to the standard basal medium for autotrophic growth, and that this resulted in a greater final cell yield. Acetate was the most effective of the organic compounds tested in promoting increased cell yield and chlorophyll formation. It was shown that acetate was assimilated by growing cultures only if both bicarbonate and a source of reducing power such as sulphide were available.

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