

granules resembling mitochondria and areas of lipoprotein membranes were present, protein bodies were the predominant cellular components of the protein-body preparations. Lipoprotein fragments and ribosomes were present in the supernatant preparation.

3. The fine structure of protein bodies in intact tissue and of bodies obtained by ultrasonic disruption of endosperm shows that the bodies and associated ribosomes are contained within a distinctive lipoprotein membrane. Evidence is presented to show that protein bodies are formed within proteoplasts.

4. Ribosomes isolated from the protein-body preparation have lower sedimentation coefficients than ribosomes obtained from the supernatant preparation under similar conditions. The significance of these observations is discussed.

5. The protein-body preparations contain protein, lipid, nucleic acids and phytic acid.

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## The Separate Incorporation of Amino Acids into Storage and Soluble Proteins Catalysed by Two Independent Systems Isolated from Developing Wheat Endosperm

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With intact heads of immature wheat grain it has been shown that [<sup>35</sup>S]sulphate and <sup>14</sup>C-labelled amino acids are taken up and accumulate in proteins associated with distinctive cellular components of the endosperm tissue (Graham & Morton, 1963). Each of the protein components separated by starch-gel electrophoresis was found

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to contain radioactivity (Graham & Morton, 1963; Graham, Morton & Raison, 1964). The cellular components of developing endosperm tissue include protein bodies and the associated protein-forming plastids (Morton & Raison, 1963; Morton, Palk & Raison, 1964a). These may be separated from the supernatant-protein fraction by differential centrifugation of suitable homogenates of endosperm tissue (Graham, Morton & Raison, 1963b). With intact heads, the incorporation of radioactivity

into each of these protein fractions occurs simultaneously but incorporation into the protein-body fraction is relatively independent of incorporation into the supernatant proteins (Graham *et al.* 1964). These findings suggest that proteins may be synthesized by the protein-forming plastids (with their associated protein bodies) and by other cellular components by independent processes.

The present paper describes studies of the incorporation of radioactivity from [<sup>35</sup>S]sulphate and from <sup>14</sup>C-labelled amino acids into the proteins of intact endosperm, endosperm homogenates and isolated cellular components. Incorporation of radioactivity into the protein-body fraction and into the supernatant-protein fraction proceeds on the addition of the radioactive component only and is substantially inhibited by chloramphenicol and by puromycin and other reagents. Incorporation into the supernatant fraction is distinguished from the incorporation into the protein-body fraction by a differential response to specific reagents.

The implication of these findings in relation to the synthesis of storage proteins in wheat endosperm is discussed.

## MATERIALS

*Wheat endosperm.* Heads from wheat plants (*Triticum durum* cultivar. Dural) grown under field conditions were harvested at about 19 days after flowering. Endosperm was obtained as described below from grain harvested in November 1962 and stored at -15° for up to 7 months, and also from grain harvested in February 1963 and used within 24 hr.

*Radioactive compounds.* These were obtained from The Radiochemical Centre, Amersham, Bucks. The specific activities of labelled amino acids were: L-[<sup>14</sup>C]proline, 33 c/mole; L-[<sup>14</sup>C]leucine, 6 c/mole; [<sup>14</sup>C]glycine, 5.7 c/mole; L-[<sup>14</sup>C]glutamic acid, 27 c/mole; L-[<sup>35</sup>S]cysteine, 42 c/mole. The sodium [<sup>35</sup>S]sulphate was carrier-free.

*Phosphoenolpyruvate.* This was prepared from bromopyruvic acid and trimethyl phosphite according to the method of M. V. Clarke (personal communication) and was used as the cyclohexylammonium salt.

*Nonidet P40.* This commercial non-ionic detergent was obtained from Shell Chemical Co. (Aust.) Pty. Ltd.

*Lactate dehydrogenase.* Commercial lactate dehydrogenase (British Drug Houses Ltd.) was used as a source of p; ruvate kinase (EC 2.7.1.40).

*Other reagents.* Chloramphenicol (micronized powder; Parke Davis and Co. Ltd.), puromycin hydrochloride (Nutritional Biochemicals Corp.) and actinomycin D [Merk Sharp and Dohme (A/asia) Ltd.] were from the sources indicated. L-Amino acids were used except for DL-methionine, DL-valine and DL-isoleucine.

## METHODS

*Preparation of isolated endosperm and cell-free homogenates.* Endosperm was obtained from fresh and frozen grain by dissection by hand. For the homogenates, cut pieces of

endosperm (2-3 g. wet wt.) were mixed with 5 ml. of 0.1M-potassium phosphate buffer, pH 7.2, containing magnesium chloride (6 mM), and disintegrated by ultrasonic vibration with a Disintegrator (Ultrasonic Industries Inc., New York) operating at 80 kcyc./sec. and 60 w for 5 min. The temperature was maintained at 2-4°. Intact cells and large starch granules were removed by filtration through no. 10 nylon mesh and by subsequent centrifuging at 100g for 10 min.

*Isolation of the protein-body and supernatant preparations.* All procedures were carried out at 2-4°. Endosperm was obtained from frozen grain by the rolling method described by Graham *et al.* (1963b) and washed with medium A [0.1M-potassium phosphate buffer, pH 7.2, containing magnesium chloride (6 mM)]. The homogenate was then fractionated as shown in Scheme 1. Centrifuging was carried out with a Super Minor centrifuge, with head no. 6451 (Measuring and Scientific Equipment Ltd., London), and a Vacufuge centrifuge, with rotor 9 RA-V (Lourdes Instrument Co. Ltd.), and suspensions were agitated with a Vibromix (Shandon Scientific Co. Ltd., London).

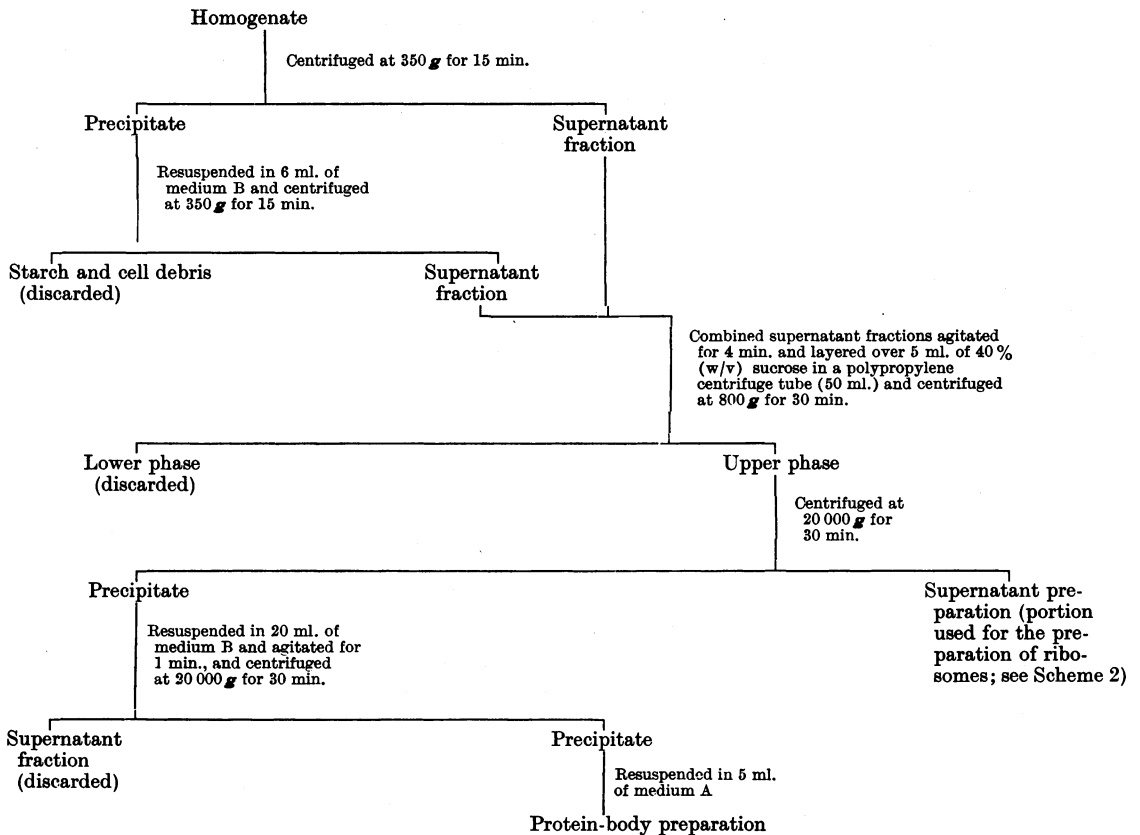
*Preparation of ribosomes and of high-speed supernatant fraction.* These fractions were obtained from the supernatant preparation (Scheme 1) as shown in Scheme 2. The 8000g and 50000g precipitates (Scheme 2) were obtained by using the 16 RA-V rotor of a Vacufuge centrifuge and the 105000g precipitates (Scheme 2) by using the no. 40 rotor of a Spinco model L centrifuge.

*Other determinations.* Nitrogen was determined by a micro-Kjeldahl procedure. Protein was determined from the  $E_{280\text{ m}\mu}/E_{280\text{ m}\mu}$  ratio (Warburg & Christian, 1942).

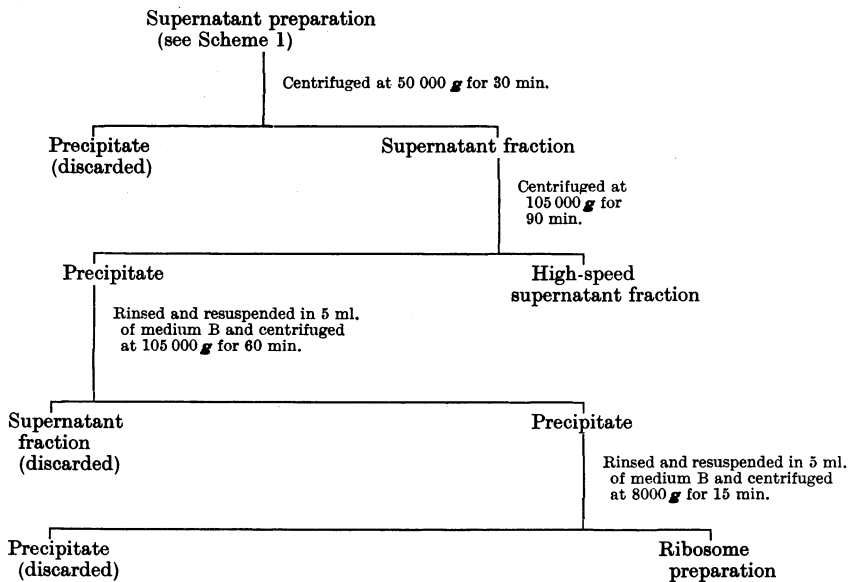
*Starch-gel electrophoresis.* Protein components were separated by starch-gel electrophoresis as described by Graham (1963), and the separated components were eluted from the gel by a continuous electrophoresis method and collected in a fraction collector (J. K. Raison, unpublished work).

*Determination of radioactivity.* Radioactivity was determined at room temperature with a type N 664 A scintillation counter and a type N 530 F scaler (Ekco Electronics Ltd., Essex). Preparations were counted in 5 ml. of Ditol (Herberg, 1960). Under the conditions used, 0.25  $\mu\text{C}$  of [<sup>14</sup>C]glutamic acid gave 6760 counts/sec.

The radioactivity associated with the N-terminal amino acids was determined by treating approx. 2 mg. of protein with phenyl isothiocyanate at pH 9 as described by Niall & Edman (1962). The phenylthiocarbonyl-protein derivatives were hydrolysed in N-HCl at 100° for 1 hr. The 3-phenyl-2-thiohydantoin-amino acids were extracted from the hydrolysate with ethyl acetate and concentrated in an air stream. The radioactivity of the 3-phenyl-2-thiohydantoin-amino acids and the residual protein hydrolysate was then determined. The radioactivity associated with the C-terminal amino acids was determined by decarboxylation of carboxyl groups with ninhydrin. Protein samples (0.5-1.0 mg.) were heated with 0.1% (w/v) ninhydrin in acetone for 15 min. in a boiling-water bath; acetone was evaporated from the heated mixture *in vacuo*. The radioactivity of the treated sample was then determined. Samples of protein were treated similarly but without ninhydrin. The radioactivity of the carboxyl groups which were decarboxylated was calculated from the change in radioactivity due to ninhydrin.



Scheme 1. Procedure for obtaining the protein-body preparation and the supernatant preparation used in the incorporation studies.



Scheme 2. Procedure for obtaining the ribosome preparation and the high-speed supernatant fraction.

Table 1. *Incorporation of  $^{35}\text{S}$  and  $^{14}\text{C}$  radioactivity into intact endosperm*

The reaction mixtures (final vol. 35 ml.) contained 5 g. wet wt. of intact endosperm, 200  $\mu\text{moles}$  of potassium phosphate buffer, pH 7.2, and each of the following components: Expt. 1, 67.5  $\mu\text{C}$  of [ $^{35}\text{S}$ ]sulphate, and 5  $\mu\text{moles}$  each of glycine, valine, leucine, isoleucine, serine, threonine, alanine, arginine, lysine, histidine, tyrosine, phenylalanine, tryptophan, proline, glutamic acid and aspartic acid; Expt. 2, 160  $\mu\text{C}$  of [ $^{14}\text{C}$ ]proline and 5  $\mu\text{moles}$  of each of the amino acids used in Expt. 1 but with the omission of proline. The mixtures were gently shaken at room temperature (22°) for 3 hr. under oxygen at 1.06 atm. The reaction was stopped by rapidly cooling to -15°. The frozen endosperm was homogenized in 35 ml. of 0.2 M-potassium phosphate buffer, pH 7.2, containing magnesium chloride (6 mM) and 2% (v/v) of detergent. Protein bodies were obtained by centrifuging at 12000g for 30 min., and the supernatant was centrifuged at 105000g for 90 min.; the final supernatant so obtained was dialysed for 16 hr. against 200 vol. of water.

Expt. no.	Radioactive compound	Sp. activity of fractions (counts/sec./mg. of N)		Ratio of sp. activity of supernatant to sp. activity of protein bodies
		Final supernatant	Protein bodies	
1	[ $^{35}\text{S}$ ]Sulphate	2270	100	22.7
2	[ $^{14}\text{C}$ ]Proline	2060	370	5.6

Table 2. *Incorporation of [ $^{14}\text{C}$ ]proline into homogenates of endosperm*

The homogenates (10 ml., containing 1 mg. of N) were incubated with 0.5  $\mu\text{C}$  of [ $^{14}\text{C}$ ]proline for 3 hr. at 30° under oxygen at 1.06 atm. The reaction was stopped by the addition of 10 ml. of 0.1 M-potassium phosphate buffer, pH 7.2, containing magnesium chloride (6 mM) and 2% (v/v) of detergent, precooled to 2°. After homogenization with a Vibromix for 4 min., the protein bodies were isolated by centrifuging at 12000g for 30 min., and the supernatant was centrifuged at 105000g for 90 min. The final supernatant was dialysed for 16 hr. against 200 vol. of water at 2°.

Endosperm	Fraction	Sp. activity of fractions (counts/sec./mg. of N)
Fresh	Protein bodies	1130
	Supernatant	2450
Stored	Protein bodies	2320
	Supernatant	2260

## EXPERIMENTAL AND RESULTS

*Incorporation of [ $^{35}\text{S}$ ]sulphate and of [ $^{14}\text{C}$ ]proline into isolated endosperm.* When isolated endosperm was incubated in oxygen, [ $^{35}\text{S}$ ]sulphate and [ $^{14}\text{C}$ ]proline were incorporated into the non-diffusible components of the protein-body and supernatant fractions (Table 1). There was preferential incorporation of [ $^{14}\text{C}$ ]proline as compared with [ $^{35}\text{S}$ ]sulphate into the protein bodies (Table 1). Similar trends had been found in the relative incorporation of the radioactivity of these compounds into the protein-body and supernatant fractions of intact wheat heads (Graham *et al.* 1963b, 1964).

*Incorporation of [ $^{14}\text{C}$ ]proline into the protein-body and supernatant fractions of endosperm homogenates.* [ $^{14}\text{C}$ ]Proline was incorporated into the protein-body and supernatant fractions of homogenates

prepared from both fresh and stored grain (Table 2). The total radioactivity recovered in the proteins of the two fractions from both homogenates represents approx. 1% of the radioactivity added to the reaction mixtures. Since the homogenates were prepared from endosperm of grain at different stages of development and grown under different climatic conditions (see the Materials section), no significance is attributed to the differences between the fresh and stored grain in the relative distribution of radioactivity into the protein-body and supernatant fractions.

The amount of [ $^{14}\text{C}$ ]proline incorporated into the protein-body and supernatant fractions of an homogenate of fresh endosperm increased with time up to 2 hr. (compare Expts. 1 and 2 in Table 3). There was no decrease in the amount of radioactivity incorporated when both fractions were dialysed against [ $^{14}\text{C}$ ]proline at 2° for 16 hr. (compare Expts. 3 and 1 in Table 3). Moreover, when the protein-body fraction containing [ $^{14}\text{C}$ ]proline was isolated from the homogenate and further incubated at 30° for 1 hr. with [ $^{14}\text{C}$ ]proline or with [ $^{14}\text{C}$ ]glutamate, there was no significant loss of radioactivity (compare Expts. 6 and 7 with Expts. 2 and 5 in Table 3). Dilution of [ $^{14}\text{C}$ ]proline with a tenfold excess of [ $^{12}\text{C}$ ]proline during the incubation greatly diminished the incorporation of radioactivity into the protein-body and supernatant fractions (compare Expts. 4 and 2 in Table 3), but dilution with [ $^{12}\text{C}$ ]glutamate, [ $^{12}\text{C}$ ]leucine and [ $^{12}\text{C}$ ]lysine had little effect on the incorporation of radioactivity from [ $^{14}\text{C}$ ]proline (compare Expts. 5 and 2 in Table 3).

*Incorporation of radioactivity from  $^{14}\text{C}$ -labelled amino acids into the protein components of the protein-body preparation from endosperm tissue.* When the protein-body preparation (cf. Scheme 1) was incubated with buffered [ $^{14}\text{C}$ ]proline, radioactivity was incorporated into the protein bodies (Table 4).

The incorporation was slightly stimulated by the addition of ATP and an ATP-generating system, but the further addition of ribosomes and high-speed supernatant fractions (cf. Scheme 2) caused no further increase (Table 4). When the protein-body preparation was incubated with [ $^{14}\text{C}$ ]proline, high-speed supernatant fraction, ATP and an ATP-generating system, the addition of an amino acid mixture caused a stimulation of incorporation of radioactivity into the protein bodies (Table 5). However, the further addition of ribosomes caused a decreased incorporation into the bodies (Table 5).

The incorporation of radioactivity from [ $^{14}\text{C}$ ]proline into the protein-body preparation was prevented by 1 mM-mercuric chloride (Expt. 5 in Table 6), by 2M-urea (Expt. 6 in Table 6) and by heating the protein-body preparation at 100° for 1 min. (Table 7). With the protein-body preparation and [ $^{14}\text{C}$ ]proline, the rate of incorporation of radioactivity into protein bodies decreased after about 1 hr. (Expts. 1–4 in Table 6). The incorporation in 90 min. was increased slightly by addition of ATP and an ATP-generating system added after incubation for 60 min. (see Expts. 4 and 7 in Table 6) but there was no similar effect on the addition of the high-speed supernatant fraction (see Expts. 4 and 8 in Table 6).

With protein-body preparations obtained from different samples of endosperm there was some variation in the rate of incorporation of radioactivity from buffered [ $^{14}\text{C}$ ]proline and in the

ability to maintain incorporation for more than 1 hr. As shown in Table 7, when a protein-body preparation was incubated for 3 hr. at 30° before addition of [ $^{14}\text{C}$ ]proline, the subsequent rate of incorporation was considerably increased as compared with the appropriate control. After dialysis of a protein-body preparation for 18 hr. at 2°, the rate of incorporation from [ $^{14}\text{C}$ ]proline was almost linear between 1 and 3 hr., but there was no further incorporation after 3 hr. (Fig. 1).

The ability of protein-body preparations from frozen endosperm to incorporate radioactivity from buffered [ $^{14}\text{C}$ ]proline into protein bodies declined considerably after about 2 months of storage of grain at -15° and was almost negligible after storage for 7 months (Table 8). None of several treatments investigated, including the addition of ATP, of GTP, of an ATP-generating system and of high-speed supernatant fraction, restored activity.

As shown in Table 9, radioactivity from several  $^{14}\text{C}$ -labelled amino acids was incorporated into protein bodies by the protein-body preparation.

The inhibition of amino acid incorporation was also investigated. Table 10 shows the effect of several reagents on the incorporation of radioactivity from  $^{14}\text{C}$ -labelled amino acids into the protein bodies as observed in a number of experiments carried out during a period of about 4 months. There was considerable inhibition by chloramphenicol and by puromycin.

Table 3. *Effects of amino acids on the incorporation of [ $^{14}\text{C}$ ]proline into the protein-body and the supernatant fractions of endosperm homogenates*

The reaction mixtures (final vol. 4 ml.) contained 2 ml. of homogenate of fresh endosperm, 750  $\mu\text{moles}$  of potassium phosphate buffer, pH 7.2, and 5  $\mu\text{C}$  of [ $^{14}\text{C}$ ]proline. After incubation at 30° with [ $^{14}\text{C}$ ]proline as shown, or after subsequent incubation for 1 hr. under similar conditions with other amino acids (unlabelled), 4 ml. of 0.1M-potassium phosphate buffer, pH 7.2, containing 2% (v/v) of detergent, and precooled to 2°, was added and the mixture was homogenized in a Potter-Elvehjem-type homogenizer for about 30 sec. The protein bodies were collected by centrifuging at 20000g for 30 min., resuspended twice in 4 ml. of 0.1M-potassium phosphate buffer, pH 7.2, and then precipitated by centrifuging at 20000g for 30 min. In Expts. 6 and 7, the protein bodies were then further incubated for 1 hr. with 0.5  $\mu\text{mole}$  of either proline (Expt. 6) or glutamic acid (Expt. 7) in 4 ml. of potassium phosphate buffer, pH 7.2, before precipitation as described above. The supernatants remaining after precipitation of the protein bodies were centrifuged at 105000g for 90 min. The protein-body and the supernatant fractions were separately dialysed for 16 hr. at 2° under the conditions shown. For determination of radioactivity, the protein-body fraction was hydrolysed in 6N-HCl at 110° for 18 hr., the HCl was removed by evaporation *in vacuo* and the residue was suspended in potassium phosphate buffer, pH 7.2. The volume of the dialysed supernatant fraction was decreased by freeze-drying before counting.

Expt. no.	Incubation time with [ $^{14}\text{C}$ ]proline (hr.)	Amino acids in subsequent incubation mixture	Dialysis solution	Total radioactivity (counts/sec.)	
				Protein-body fraction	Supernatant fraction
1	1	—	Water	44	17
2	2	—	Water	135	25
3	1	—	0.25M-Proline	53	19
4	1	Proline (2.5 $\mu\text{moles}$ )	Water	76	15
5	1	Glutamic acid (2.5 $\mu\text{moles}$ ), leucine (2.5 $\mu\text{moles}$ ) and lysine (2.5 $\mu\text{moles}$ )	Water	110	22
6	2	Proline (0.5 $\mu\text{mole}$ )	Water	105	—
7	2	Glutamic acid (0.5 $\mu\text{mole}$ )	Water	136	—

Table 4. *Effects of the addition of adenosine triphosphate, high-speed supernatant fraction and ribosomes on the incorporation of radioactivity from [<sup>14</sup>C]proline into the protein-body preparation*

In each experiment, the reaction mixtures (final vol. 4 ml.) were incubated with 5  $\mu$ C of [<sup>14</sup>C]proline for 1 hr. at 30°, after which the reaction mixture was diluted by the addition of 4 ml. of 10 mM-proline in 0.1M-potassium phosphate buffer, pH 7.2, precooled to 2°. The protein bodies were collected by centrifuging at 20000g for 30 min. and dialysed against 1 l. of water for 16 hr. at 2°. Each of the reaction mixtures comprised 1.5 ml. of the protein-body preparation, containing 0.4 mg. of N. In Expt. 1, there were no further additions. In Expt. 2, the additions were 10  $\mu$ moles of ATP, 50  $\mu$ moles of phosphoenolpyruvate and 0.1 ml. of the pyruvate-kinase preparation. In Expt. 3, the additions were as in Expt. 2 together with 0.2 ml. of ribosome preparation (containing 0.1 mg. of protein) and 1 ml. of high-speed supernatant fraction (containing 0.12 mg. of N).

Expt. no.	Components	Total radioactivity of protein bodies (counts/sec.)
1	Control	6400
2	ATP and ATP-generating system added	8030
3	ATP, ATP-generating system, ribosomes and high-speed supernatant added	7420

Table 5. *Effect of amino acids and of ribosomes on the incorporation of radioactivity from [<sup>14</sup>C]proline into the protein-body preparation*

In each experiment, the reaction mixtures (final vol. 5 ml.) were incubated with 5  $\mu$ C of [<sup>14</sup>C]proline for 1 hr. at 30°, after which the reaction mixtures were diluted by the addition of 5 ml. of 10 mM-proline in 0.1M-potassium phosphate buffer, pH 7.2, precooled to 2°. The protein bodies were collected by centrifuging at 20000g for 30 min. and dialysed against 1 l. of water for 16 hr. at 2°. Each of the reaction mixtures comprised 2 ml. of the protein-body preparation (containing 0.6 mg. of N), 1 ml. of the high-speed supernatant fraction (cf. Scheme 2) (containing 0.12 mg. of N), 10  $\mu$ moles of ATP, 50  $\mu$ moles of phosphoenolpyruvate, 0.1 ml. of the pyruvate-kinase preparation and 20  $\mu$ moles of KF. In Expt. 1, there were no further additions. In Expt. 2, 0.16  $\mu$ mole of each of the amino acids (except proline) as listed in Expt. 1 in Table 1 was added. In Expt. 3, 0.2 ml. of the ribosome preparation (containing 0.1 mg. of protein) was added to the components shown for Expt. 2.

Expt. no.	Components	Total radioactivity of protein bodies (counts/sec.)
1	Control	2470
2	Amino acids added	3100
3	Amino acids and ribosomes added	1520

*Incorporation of radioactivity from [<sup>14</sup>C]-labelled amino acids into the soluble protein fraction catalysed by the supernatant preparation.* As shown in Table 9, when the supernatant preparation (cf. Scheme 1) containing both ribosomes and soluble proteins (high-speed supernatant fraction; cf. Scheme 2) was incubated with [<sup>14</sup>C]-labelled amino

Table 6. *Effect of the supernatant fraction and of adenosine triphosphate on the rate of incorporation of [<sup>14</sup>C]proline into the protein-body preparation*

The complete reaction mixtures (final vol. 4 ml.) contained 3 ml. of a protein-body suspension (containing 0.36 mg. of N), 200  $\mu$ moles of potassium phosphate buffer, pH 7.2, and 5  $\mu$ C of [<sup>14</sup>C]proline. Samples were separately incubated at 30° and the reactions were stopped by addition of 1 ml. of 8M-urea. In Expt. 5, 4  $\mu$ moles of HgCl<sub>2</sub> were added before addition of [<sup>14</sup>C]proline. In Expt. 6, 8  $\mu$ moles of urea were added before the addition of [<sup>14</sup>C]proline. In Expt. 7, 2 ml. of high-speed supernatant was added after incubation for 60 min. and the sample was incubated for a further 30 min. In Expt. 8, 10  $\mu$ moles of ATP, 50  $\mu$ moles of phosphoenolpyruvate and 0.1 ml. of the pyruvate-kinase preparation were added after incubation for 60 min. and the sample was incubated for a further 30 min. In Expts. 1-4, 7 and 8, the reactions were stopped by the addition of 8M-urea (final concn. 2M) and the protein bodies were collected as described in Table 4.

Expt. no.	Additional components	Total incubation time (min.)	Total radioactivity of protein bodies (counts/sec.)
1	None	0	1350
2	None	30	6270
3	None	60	17590
4	None	90	19730
5	HgCl <sub>2</sub>	90	1720
6	Urea	60	1560
7	Supernatant	90	16380
8	ATP and phosphoenolpyruvate	90	22390

Table 7. *Effect of treatments on the incorporation of [<sup>14</sup>C]proline into the protein-body preparation*

The reaction mixtures (total vol. 5 ml.) contained 3 ml. of a suspension of protein bodies (containing 0.82 mg. of N), 400  $\mu$ moles of potassium phosphate buffer, pH 7.2, and 5  $\mu$ C of [<sup>14</sup>C]proline. They were incubated at 30° for the periods shown. In Expt. 3, the suspension of protein bodies was incubated under the conditions shown before the addition of [<sup>14</sup>C]proline. The reactions were stopped and the protein bodies were treated as described for Expts. 1-4, 7 and 8 in Table 6.

Expt. no.	Further treatment	Total radioactivity (counts/sec.)	
		After 1 hr.	After 2 hr.
1	None	7640	22850
2	Heated at 100° for 1 min.	1260	—
3	Incubated at 30° for 3 hr.	12230	27980

acids, radioactivity was incorporated into the soluble proteins.

The ability of the supernatant preparations to catalyse this incorporation decreased markedly after storage of the grain at  $-15^{\circ}$  for about 2 months and was negligible after storage for 3 months (Table 8).

The inhibition of amino acid incorporation was also investigated. Table 11 shows the effects of several reagents on incorporation of radioactivity from [ $^{14}\text{C}$ ]proline. There was considerable inhibition by chloramphenicol, hydroxylamine and fluoroacetate.

*Starch-gel electrophoresis of proteins after the incorporation of  $^{14}\text{C}$ -labelled amino acids.* The radioactivity incorporated into the high-speed supernatant fraction of the supernatant preparation was associated with each of the protein components separated by starch-gel electrophoresis (Graham *et al.* 1964). Most of the radioactivity was associated with the fractions that migrated from the gel between 6 and 10 hr. (Fig. 2). The mobilities of these components correspond to those of the fast-moving protein components of the supernatant fraction of wheat endosperm (Graham *et al.* 1963*b*).

Most of the radioactivity incorporated into the protein-body preparation was associated with two major groups of components. These migrated from the gel between 4 and 11 hr., and between 22 and

30 hr., and thus correspond to the fast- and slow-moving components of the protein-body preparations (Graham *et al.* 1963*b*, 1964).

*Determination of radioactivity in the N- and C-terminal amino acids.* A sample of the protein-

Table 8. *Effect of storage of grain on the incorporation of [ $^{14}\text{C}$ ]proline into the protein-body preparation and the supernatant preparation*

The protein-body preparations and the supernatant preparations were obtained as shown in Scheme 1. With the protein-body preparations, the reaction conditions were essentially those described for Expt. 1 in Table 4 except that the reactions were stopped by the addition of 8*M*-urea and then treated as described in Table 10. With the supernatant preparations, the reactions were carried out as described in Table 11. The results are given for various preparations obtained from wheat grain stored at  $-15^{\circ}$  for the periods shown.

Storage period (weeks)	Sp. activity (counts/sec./mg. of N)	
	With protein-body preparations	With supernatant preparations
9	13850	—
10	11190	2250
20	9340	290
21	9940	140
24	2620	—
25	890	—
27	630	—

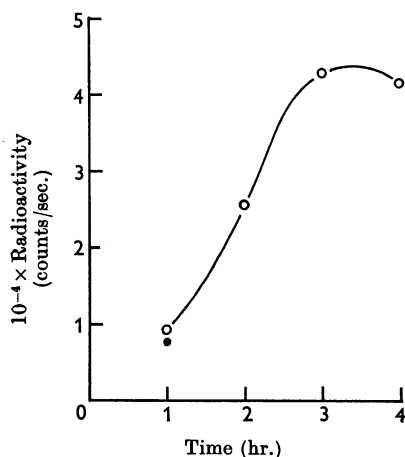


Fig. 1. Variation of the incorporation of [ $^{14}\text{C}$ ]proline into the protein-body preparation during incubation. The reaction mixtures were similar to those for Expt. 1 in Table 7. The suspension of protein bodies was dialysed against 0.1*M*-potassium phosphate buffer, pH 7.2, for 18 hr. at  $2^{\circ}$  before addition to the reaction mixture. For point A (●) 2.5  $\mu\text{moles}$  of ATP, 25  $\mu\text{moles}$  of phosphoenolpyruvate and 0.2 ml. of the pyruvate-kinase preparation were included in the reaction mixture. The reactions were stopped and the protein bodies were treated as described for Expts. 1-4, 7 and 8 in Table 6.

Table 9. *Separate incorporation of  $^{14}\text{C}$ -labelled amino acids into the protein-body preparation and the supernatant preparation*

The reaction mixtures A (final vol. 4 ml.) consisted of 3 ml. of the protein-body preparation (containing 1.5 mg. of N), 300  $\mu\text{moles}$  of potassium phosphate buffer, pH 7.2, and 5  $\mu\text{C}$  of the  $^{14}\text{C}$ -labelled amino acid. They were incubated for 1 hr. at  $30^{\circ}$ . The reactions were stopped by adding 1.0 ml. of 8*M*-urea and the mixtures were dialysed against 200 vol. of water for 16 hr. at  $2^{\circ}$ . The reaction mixtures B (final vol. 10 ml.) consisted of 8 ml. of the supernatant preparation (containing 3.5 mg. of N), 1 m-mole of potassium phosphate buffer, pH 7.2, and 5  $\mu\text{C}$  of the  $^{14}\text{C}$ -labelled amino acid. They were incubated for 1 hr. at  $30^{\circ}$ . The reactions were stopped by the addition of 5 ml. of 8*M*-urea and the mixtures were centrifuged at 105000*g* for 90 min. The supernatants were dialysed against 100 vol. of water for 16 hr. at  $2^{\circ}$ .

$^{14}\text{C}$ -labelled amino acid used	$^{14}\text{C}$ -labelled amino acid incorporated ( $\mu\text{g}/\text{mg. of N}$ )	
	Reaction mixture A (with protein bodies)	Reaction mixture B (with supernatant)
Proline	0.95	0.21
Glutamic acid	1.47	0.14
Leucine	0.73	0.24
Glycine	0.56	0.21

body preparation that had been incubated with [ $^{14}\text{C}$ ]proline as described in Table 6 contained 15340 counts/100 sec. It was treated with phenyl isothiocyanate, and the *N*-terminal amino acids were removed as described in the Methods section. The remaining protein contained 14600 counts/100 sec., corresponding to a loss of 4.8% of the total radioactivity resulting from the removal of the *N*-terminal amino acids. By similar procedures a sample of protein bodies isolated from intact heads that had been exposed to [ $^{14}\text{C}$ ]proline (Graham *et al.* 1964) lost 3.4% of the radioactivity.

A sample of the protein-body preparation that had been incubated with  $2\mu\text{C}$  of uniformly-labelled mixed amino acids (see Morton, Raison & Smeaton, 1964*b*) as described in Table 6 contained 1405 counts/sec. The sample was treated with ninhydrin as described in the Methods section. After this treatment the sample contained 903 counts/sec. compared with a similar sample treated with acetone alone that contained 1026 counts/sec. The loss of radioactivity after decarboxylation of the free carboxyl groups was 8.6% of the total radioactivity of the protein sample.

Table 10. *Effect of various reagents on the incorporation of  $^{14}\text{C}$ -labelled amino acids into the protein-body preparation*

The reaction mixtures contained 300  $\mu\text{moles}$  of potassium phosphate buffer, pH 7.2, the protein-body preparation and, in Expt. 1,  $5\mu\text{C}$  of [ $^{14}\text{C}$ ]proline, and, in Expts. 2-4, a mixture of  $1.25\mu\text{C}$  of each of [ $^{14}\text{C}$ ]proline, [ $^{14}\text{C}$ ]glutamic acid, [ $^{14}\text{C}$ ]leucine and [ $^{14}\text{C}$ ]glycine (total radioactivity  $5\mu\text{C}$ ). The mixtures were incubated at  $30^\circ$  for 1 hr. The reactions were stopped by adjusting to a final concentration of 2*M*-urea with 8*M*-urea. The bodies were then dialysed against 200 vol. of water for 16 hr. at  $2^\circ$ . Between Expt. 1 and Expt. 4 there was an interval of 4 months (see also Table 8).

Expt. no.	Amount of protein-body preparation (mg. of N)	Reagent added	Concn. of reagent (mM)	Sp. activity (counts/sec./mg. of N)	Inhibition (%)
1	1.16	None	—	9950	—
	1.26	Chloramphenicol	0.33	2140	78
	1.32	Hydroxylamine	10	8560	14
	1.22	Sodium fluoroacetate	10	10230	0
2	1.54	None	—	4220	—
	1.54	Chloramphenicol	0.33	1790	57
	1.89	Chloramphenicol	0.66	40	99
	2.24	Puromycin	5	2470	42
	2.66	KCN	10	1940	54
	2.94	Sodium arsenate	100	1930	54
3	10.5	None	—	2590	—
	12.4	Chloramphenicol	0.16	1420	43
	11.5	KCN	10	1670	35
4	0.32	None	—	3930	—
	0.39	Chloramphenicol	0.66	1120	71
	0.45	Puromycin	1	1980	47
	0.49	Puromycin	5	1190	68
	0.45	Actinomycin D	0.26	4140	0
	0.52	Actinomycin D	0.50	3990	0

Table 11. *Effect of various reagents on the incorporation of radioactivity from  $^{14}\text{C}$ -labelled amino acids into the supernatant preparation*

The reaction mixtures (final vol. 8 ml.) contained about 800  $\mu\text{moles}$  of potassium phosphate buffer, pH 7.2, supernatant preparation (see Scheme 1) and  $5\mu\text{C}$  of [ $^{14}\text{C}$ ]proline. The mixtures were incubated at  $30^\circ$  for 1 hr. The reactions were stopped by adjusting to a final concentration of 2*M*-urea with 8*M*-urea and the mixtures were dialysed against 200 vol. of water for 16 hr. at  $2^\circ$ . The mixtures were then centrifuged at 105000*g* for 90 min. and radioactivity in the supernatant fraction was determined.

Amount of supernatant preparation (mg. of N)	Reagent added	Concn. of reagent (mM)	Sp. activity (counts/sec./mg. of N)	Inhibition (%)
3.64	None	—	2250	—
4.37	Chloramphenicol	0.33	490	78
2.63	Hydroxylamine	10	630	72
3.70	Sodium fluoroacetate	10	360	84



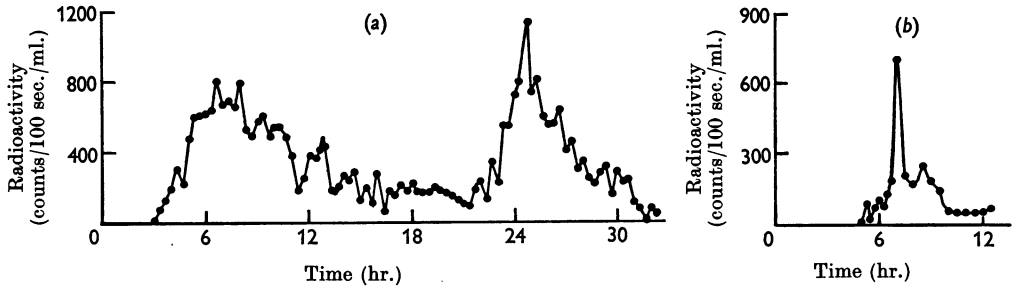


Fig. 2. Distribution of radioactivity in protein components of the protein-body preparation (a) and supernatant preparation (b) separated by continuous starch-gel electrophoresis. [ $^{14}\text{C}$ ]Proline, [ $^{14}\text{C}$ ]glutamic acid, [ $^{14}\text{C}$ ]leucine and [ $^{14}\text{C}$ ]glycine were incorporated into protein bodies as described for Expt. 2 in Table 10, and into the soluble proteins of the supernatant preparation as described in Table 11. The protein bodies and soluble proteins of the supernatant preparation were dialysed as described in Tables 10 and 11, freeze-dried and suspended in 2M-urea to give a final concentration of 3 mg. of protein nitrogen/ml. The protein components of each fraction were separated by starch-gel electrophoresis, as described by Graham (1963), in an apparatus so modified that the separate protein components were continuously eluted from the gel into 17 mM-aluminium lactate buffer, pH 3.2, containing urea (2M), and collected in a fraction collector (J. K. Raison, unpublished work). Samples (5 ml.) were collected at 20 min. intervals, and the radioactivity of each fraction was determined as described in the Methods section.

## DISCUSSION

*System for the incorporation of amino acids into proteins of the protein-body preparation.* The protein-body preparation used in the present studies contains protein bodies and the protein-forming plastids (Morton & Raison, 1963) that were described by Morton *et al.* (1964a). As shown by Table 4, this preparation incorporates amino acid into the storage protein. The incorporated amino acid is in a stable linkage in the protein. In the relative ability to incorporate a number of radioactive amino acids, the protein-body preparation has characteristics resembling intact heads (Graham *et al.* 1963b, 1964), isolated endosperm (Table 1) and tissue homogenates (Tables 2 and 3). Because of the high proportion of proline in the protein-body preparation (Jennings & Morton, 1963a), [ $^{14}\text{C}$ ]proline has been used for most of the present studies. The relative amount of amino acid incorporated into the storage protein of the protein-body preparation is closely related to the proportion of the amino acid in the storage protein (see Table 9, and also Table 5 of Jennings & Morton, 1963a). Moreover, as shown by Table 3, the incorporation of proline is independent of the presence or absence of other amino acids. The incorporation process is thus relatively specific for each amino acid. As with intact endosperm and with endosperm homogenates, the incorporation of proline by the protein-body preparation did not depend completely on the addition of other amino acids or on the addition of a system for generating and maintaining ATP. Developing wheat endo-

sperm contains a relatively high proportion of non-protein nitrogenous compounds, consisting predominantly of free amino acids (Jennings & Morton, 1963a, b; Graham, Morton & Simmonds, 1963a). The isolated protein-body preparation retains acid-soluble ninhydrin-positive compounds (probably free amino acids) even after dialysis of the preparation for about 16 hr. at 2° (see Table 1, Morton *et al.* 1964a).

The activity of a protein-body preparation was clearly enhanced by dialysis or preincubation before the addition of the labelled amino acid (Table 7 and Fig. 1). Although not dependent on the addition of ATP, the incorporation of proline was slightly stimulated by the addition of ATP and of an ATP-generating system, particularly if the addition was made after a period of incubation (Table 6). The protein-body preparation thus appears to have partly retained a reactive phosphoryl donor that may be utilized for ATP formation and thus for amino acid incorporation.

Analyses of protein-body preparations indicate the presence of acid-soluble acid-stable organic phosphate which is almost certainly phytate (Morton *et al.* 1964a), and it has now been shown that ATP may be formed from added phytate and ADP, the reaction being catalysed by ATP-phosphoinositol phosphotransferase present in the protein-body preparation (Morton & Raison, 1963). The generation of ATP by this system accounts for the lack of dependence on ATP of amino acid activation (Morton *et al.* 1964b) and of amino acid incorporation catalysed by the protein-body preparation.

The protein-body preparation has associated ribosomes (Morton *et al.* 1964*a*) and amino acid-activating enzymes and RNA (Morton *et al.* 1964*b*) which are probably derived from the protein-forming plastid (see Morton *et al.* 1964*a*). Thus the protein-body preparation appears to have at least partly retained some of each of the components known to be associated with protein synthesis in other cellular systems.

As shown by Table 3, the radioactive amino acid incorporated into the protein components of the protein-body preparation was not displaced by further incubation with unlabelled amino acid or by prolonged dialysis. The incorporated amino acid was associated with a number of the protein components separated by starch-gel electrophoresis (Fig. 2), and neither *N*-terminal nor *C*-terminal degradation of the labelled proteins removed any substantial proportion of the radioisotope. It therefore seems likely that the labelled amino acid is incorporated into a peptide linkage not confined to the *N*- or *C*-terminal positions of the protein.

The inhibition of incorporation of amino acid into the protein-body preparation by chloramphenicol and puromycin is similar to the inhibition of protein syntheses in bacterial and animal systems caused by these compounds (Brock, 1961; Yarmolinsky & de la Haba, 1959). The failure of actinomycin D to inhibit incorporation suggests that a DNA-dependent synthesis of RNA (Goldberg & Rabinowitz, 1962) is not obligatory for the incorporation of amino acid.

The incorporation of  $^{14}\text{C}$ -labelled amino acids into protein by systems *in vitro* does not necessarily establish that the system is involved in net protein syntheses. However, the characteristics of the incorporation reactions described above satisfy a number of the criteria that Hoagland (1960) has indicated must be met before amino acid incorporation can be equated with protein synthesis.

*System for the incorporation of amino acids into proteins of the supernatant preparation.* The supernatant preparation used in the present incorporation studies is relatively free of protein bodies but contains amino acid-activating enzymes and transfer RNA (Morton *et al.* 1964*b*), together with membrane fragments and ribosomes (Morton *et al.* 1964*a*). The results presented above were obtained with preparations that were not further dialysed before use. Hence the lack of dependence of the incorporation of  $^{14}\text{C}$ -labelled amino acid on any further additions to the system is probably due to the presence of free amino acids and of an ATP-generating system in the supernatant preparation. Although clearly concerned with incorporation of amino acids into protein components that, by starch-gel electrophoresis, are distinct from protein

components of the protein-body preparation, many of the characteristics of the incorporation catalysed by the supernatant preparation resemble those of the protein-body preparation. Thus the amounts of several amino acids incorporated are closely related to the amino acid composition of the proteins of the supernatant fraction (see Table 9, and also Table 5 of Jennings & Morton, 1963*a*); incorporated amino acid is not displaced by further incubation with additional amino acids (Table 3) and incorporation is inhibited by chloramphenicol (Table 11).

As found also in studies with intact wheat heads, the incorporation of labelled amino acid into the proteins of the supernatant fraction is inhibited by 10 mM-fluoroacetate (Graham *et al.* 1964), which, however, has a negligible effect on the incorporation of amino acids catalysed by the protein-body preparation. The mechanism of the inhibition by fluoroacetate has not been established; it was used on the assumption that fluoroacetate would preferentially interfere with a respiratory-dependent system for ATP generation possibly required for incorporation into the supernatant preparation.

*Biochemical and structural aspects of formation of endosperm proteins.* The two systems described in the present paper are associated with distinctive cellular components (Morton *et al.* 1964*a*). Each system catalyses incorporation of amino acids into characteristic protein components which differ in the two systems. The results obtained with the isolated preparations show much in common with results obtained with intact wheat heads (Graham *et al.* 1964) in which the relative amounts of radioactivity incorporated from  $^{35}\text{S}$  sulphate,  $^{14}\text{C}$ -leucine and  $^{14}\text{C}$  proline had indicated that the formation of storage protein and that of supernatant protein are relatively independent processes. With intact heads also there was inhibition by fluoroacetate of the incorporation of radioactivity from  $^{35}\text{S}$  sulphate into the supernatant protein but no inhibition of incorporation into storage protein.

The close similarity between the results obtained with the isolated preparations from wheat endosperm and with intact wheat heads suggests that the results reflect aspects of the biosynthesis of the proteins of wheat endosperm. It is considered that the occurrence of the protein-forming plastids in endosperm cells accounts for the synthesis of storage protein independently of the synthesis of supernatant protein. Based on the present understanding of the control of protein synthesis, the independent formation of two groups of proteins separately localized within the one cell requires that the messenger RNA and ribosomes, which code for the synthesis of one group of protein, be morphologically separated from the other group. Thus the ribosomes that are observed within the

protein-forming plastid (Morton *et al.* 1964*a*) appear to carry messenger RNA which is distinct from that carried by the ribosomes of the general endoplasmic reticulum (Morton *et al.* 1964*a*) which are found in the supernatant preparation.

The systems described above and in related papers (Morton *et al.* 1964*a, b*) are unusual in the considerable stability of the various enzymic processes involved in amino acid incorporation. With isolated ribosomes from maize kernels, Rabson & Novelli (1960) observed that the incorporation of amino acid into ribosomal protein was maintained in preparations stored for up to 48 hr. at 2° and that preparations frozen in liquid nitrogen retained about 50 % of their activity after about 2 months. It would therefore appear that there is a relatively high stability of the ribosome-bound messenger RNA involved in protein synthesis in plant seeds. Since the seed represents the end product in the life of the annual plant, it is unlikely that the synthesis of storage protein would be dependent on an unstable messenger RNA, the re-formation of which requires intervention of nuclear DNA. The lack of inhibition of amino acid incorporation by actinomycin D in the protein-body preparation supports this view.

The ribosomes from the supernatant preparation contained some radioactivity when the supernatant preparation was incubated with [<sup>14</sup>C]-proline. However, most of the radioactivity was associated with protein components that remained in solution after centrifuging at 105000*g*. Similarly, with the protein-body preparation, radioactivity was incorporated into the protein components of storage protein.

Other workers (Webster, 1959; Raacke, 1959) have described systems from pea epicotyl which appear to be involved in net protein synthesis, but Ts'o, Bonner & Vinograd (1956) and Lett & Takahashi (1962) were able to establish only incorporation into ribosomal particles from pea epicotyl.

If indeed it is established that the amino acid incorporation as described in the present paper can be regarded as a measure of protein synthesis, then these systems would have considerable merit for elucidating terminal stages of protein formation.

#### SUMMARY

1. The characteristics of the incorporation of <sup>14</sup>C-labelled amino acids into the storage and soluble proteins of wheat endosperm have been investigated by using isolated endosperm, cell-free homogenates, and isolated cellular components.

2. With homogenates of endosperm tissue the <sup>14</sup>C-labelled amino acids incorporated into both the storage and soluble proteins were retained after

dialysis against water and against solutions of amino acids, and after further incubation with non-labelled amino acids. The incorporation of <sup>14</sup>C-labelled amino acids was relatively specific for each amino acid.

3. The incorporation of <sup>14</sup>C-labelled amino acids into the storage proteins catalysed by the protein-body preparation did not require the addition of ATP or of amino acids. Incorporation was inhibited by heating, and by the addition of urea, mercuric chloride, chloramphenicol or puromycin. There was no inhibition with actinomycin D or fluoroacetate.

4. The incorporation of <sup>14</sup>C-labelled amino acids into the soluble proteins catalysed by the supernatant preparation did not depend on the addition of ATP or of other amino acids, but was inhibited by heating and by the addition of urea, mercuric chloride or chloramphenicol.

5. The radioactivity incorporated into the storage and soluble proteins by the isolated preparations was not confined to the *N*- or *C*-terminal amino acid residues, and remained associated with the protein components that were separated by starch-gel electrophoresis.

6. The implication of these findings in relation to the independent synthesis of the storage and soluble proteins of wheat endosperm is discussed.

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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 <sup>14</sup>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 <sup>14</sup>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

[<sup>14</sup>C]proline, [<sup>14</sup>C]glutamic acid, [<sup>14</sup>C]leucine and [<sup>14</sup>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 [<sup>14</sup>C]bicarbonate as the sole carbon source; the mixed amino acids had a specific activity of 145 μC/mg. Carrier-free K<sub>2</sub>H<sup>32</sup>PO<sub>4</sub> was obtained from N. V. Philips-Duphar, Amsterdam, Holland, and was converted into [<sup>32</sup>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<sup>+</sup> 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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