Amino Acid Incorporation by Cell Fractions from the Oviduct of the Laying Hen and the Synthesis of Egg-White Proteins

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(Received 29 November 1965)

1. Homogenates of the magnum of the hen oviduct have been fractionated by differential centrifuging. 2. Centrifuging at 600g for 10 min. gave a pellet containing most of the particulate material of the cell, but on washing this fraction some particles were removed from it. The washed 600g pellet contained most of the DNA of the homogenate. 3. Centrifuging the 600g supernatants at 10000g for 10 min. gave particulate fractions (*I* particles) richer in RNA than other fractions which were active in incorporating amino acids into protein in isolation. When minced tissue was incubated with radioactive amino acids before homogenizing these particles were the most radioactive of the cell fractions. 4. The pellet obtained by centrifuging the 10000g supernatant at 105000g for 60 min. was very small; its RNA/protein ratio was raised compared with that of the homogenate. It only incorporated amino acids in isolation to a small extent or not at all. 5. The 105000g supernatant contained a large proportion of the protein of the homogenate. 6. The *I* particles could be subfractionated by layering over denser sucrose to give a pellet with lower RNA content and incorporating activity, and also suspended material richer in both these properties. 7. Treatment of the *I* particles with deoxycholate gave rise to particles sedimenting at 105000g for 60 min. containing most of the RNA of the original particles, but only about 34% of the protein, and with a high activity in incorporating amino acids. 8. The *I* particles, or particles derived from them by deoxycholate treatment, required GTP and phosphoenolpyruvate for the incorporation of free amino acids. The omission of ATP reduced the incorporation to varying extents. Chicken-liver cell sap stimulated the activity. 9. Radioactive amino acids linked to transfer RNA were transferred to protein by *I* particles. GTP and phosphoenolpyruvate were required for this transfer. The phosphoenolpyruvate requirement could not be replaced by increasing the GTP concentration. ATP partially inhibited the transfer. 10. After incorporation by the cell-free system, the hot-trichloroacetic acid extract, but not the lipid extract, was radioactive. Ribonuclease and puromycin inhibited at low concentrations. Lecithinase-C was much less inhibitory. Transfer of amino acid, from a radioactive lipid-amino acid complex prepared from hen oviduct, was not detected. 11. After short periods of incubation of minced tissue with [¹⁴C]lysine some of the radioactive protein of the isolated *I* particles behaved as ovalbumin. The distribution of radioactivity in the protein resembled that in ovalbumin in soluble extracts of the tissue obtained after longer periods of incubation.

The oviduct of the laying hen seems likely to be a very useful tissue in which to study protein synthesis, not merely because it is capable of producing a large quantity of egg protein in a short time, but also because these proteins are produced by the tissue in part as a response to stimulation by hormones. A close examination of the mechanisms involved might well lead to information on the

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means by which hormones evoke the production of specific proteins in target organs.

As a result of his studies on this tissue, Hendler has concluded that the mechanisms of protein synthesis proposed for other tissues, involving nucleic acid-linked intermediates, do not operate or are less important in the magnum of the oviduct. He suggests that lipid-amino acid complexes may be more important (e.g. Hendler, 1961). He has also shown that differential centrifuging of a homogenate

of the tissue does not yield fractions which correspond morphologically to those obtained with for example liver, but that almost all of the particles are sedimented by 600g applied for 10 min. (Hendler, 1957, 1959).

The work described here, while confirming the latter result, is derived from the observation that washing the 600g pellet removes from it some of the particulate material. It is shown below that this material has many of the properties of the liver microsome fraction, in spite of the different behaviour in the centrifuge, and that it may well be involved in the synthesis of egg-white proteins. Some of this work has been reported briefly elsewhere (Carey, 1964a, 1965).

MATERIALS AND METHODS

Materials. The following materials were obtained from the sources indicated: ATP, GTP, PEP*, RNA, DNA, pyruvate kinase, sodium deoxycholate, pancreatic ribonuclease, lecitinase-C (*Clostridium welchii*) and ovalbumin (five-times recrystallized) were from Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP was also obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.; puromycin was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; L-[U-¹⁴C]lysine hydrochloride (specific activities 33–328 μ C/mg.), L-[U-¹⁴C]leucine (82 μ C/mg.) and ¹⁴C-labelled algal-protein hydrolysate (100 μ C/mg.) were from The Radiochemical Centre, Amersham, Bucks.; hydroxide of Hyamine 10-X [p-(diisobutylresorxyethoxyethyl)dimethylbenzylammonium hydroxide], diphenylloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Packard Instruments Ltd., La Grange, Ill., U.S.A.

Birds. Young White Leghorn hens were grown from fertile eggs obtained from Withers, Appleby Farm, Egerton, Kent.

Preparation of cell fractions. Hens were killed by cervical dislocation and bled. A section of the magnum of the oviduct was removed into ice-cold 0.44M-sucrose. As much as possible of the peritoneum was removed and a 5g. portion was minced finely with scissors and homogenized in 25 ml. of 0.44M-sucrose in a Dounce homogenizer (Dounce, Witter, Monte, Pate & Cottone, 1955) with a loosely fitting pestle. The homogenate was filtered by squeezing it through several layers of surgical gauze, the filtrate rehomogenized with a tighter pestle, and then centrifuged at 600g for 10 min. in an MSE Major centrifuge, rotor no. 6876 (Measuring and Scientific Equipment Ltd., Crawley, Sussex). Subsequent fractionation was as shown in Schemes 1 and 2. The pellet was washed three times by suspending it in successive 6 ml. volumes of 0.44M-sucrose with a Pasteur pipette and re sedimenting. The washed pellet was called *P*₁. In early experiments (Scheme 1) the particles washed out of the pellet were sedimented by centrifuging at 10000g for 10 min. in the MSE Superspeed 40, rotor no. 29390. The resulting pellet constituted fraction *P*₁W. In these experiments the

supernatant from the first 600g centrifuging was centrifuged at 10000g for 10 min. giving a pellet which was washed at 10000g to give fraction *P*₂. For later studies (Scheme 2) the four supernatants from the 600g centrifuging were combined before centrifuging at 10000g for 10 min. giving a pellet, fraction *I*. All the 10000g supernatants were combined and centrifuged at 105000g for 60 min. (MSE Superspeed 40 centrifuge, rotor no. 29390), giving a pellet which was washed to give a particulate fraction *P*₃ and the cell-sap fraction *S*₃.

The particles of fraction *I* were subfractioned by two procedures. In the first (Scheme 2i) they were treated with 0.4% deoxycholate in 0.44M-sucrose and centrifuged at 105000g for 60 min., the particles so obtained being washed by centrifuging again from 0.44M-sucrose. The particles and supernatant have been called *I*_p and *I*_s. In the second procedure (Scheme 2ii) they were suspended in 0.44M-sucrose and layered over denser sucrose, usually 0.88M, in a sector tube and centrifuged at 2000g for 30 min. in the MSE Major, rotor no. 6876. The pellet obtained in this case has been called *I*_m. The particulate material in the top layer in 0.44M-sucrose, collected by centrifuging at 20000g for 15 min. was fraction *I*_v, whilst the fine particulate material distributed through the denser sucrose and similarly collected by centrifuging was called *I*_c.

Chicken-liver cell fractions were prepared by centrifuging a 20% (w/v) homogenate in 0.44M-sucrose at 10000g for 15 min. The pellet was discarded and the supernatant centrifuged at 105000g for 60 min. The pellet was washed and comprised the microsome fraction. The supernatant was the cell sap.

Incubation conditions. To test the incorporation of amino acids by whole cells minced oviduct was incubated in an equal volume of a bicarbonate-buffered salt solution (Hendler, 1956) with [¹⁴C]lysine, penicillin (500 units/ml.) and streptomycin (3.3 mg./ml.) added. After the incubation at 37° for the times stated, the mixture was cooled in ice, centrifuged and the tissue washed in 0.25M-sucrose. Cell fractions were separated as above.

Incorporation by separated cell fractions was tested by incubation in the following mixture: tris buffer, pH 7.5 (0.01M), NaCl (50 mM), KCl (80 mM), MgCl₂ (5 mM), ATP (0.8 mM), GTP (0.5 mM), PEP (10 mM), pyruvate kinase (8 μ g.), [¹⁴C]lysine or [¹⁴C]leucine (1.0 μ C), liver cell sap (0.2 ml. of the preparation described above); total volume 1.0 ml. A portion (0.2 or 0.3 ml.) of the cell fraction was added to start the incubation, which was for 30 min. at 37°. The reaction was stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid (containing 1 mg. of [¹²C]lysine or of [¹²C]leucine/ml. where appropriate). Zero-time controls, in which the trichloroacetic acid was added before the cell sap and particles, were subtracted. The protein content of the sample of cell fraction added to the mixture is given with each experiment in the Results section.

Chemical fractionations. The precipitates in trichloroacetic acid from the incubation mixtures referred to above, or unincubated cell fractions precipitated with 5% (w/v) trichloroacetic acid, were fractionated chemically, first by washing with 5% (w/v) trichloroacetic acid at room temperature. Where lipids were to be extracted the precipitate was then suspended in water and 3 vol. of ethanol added. The precipitate was washed in ethanol, ethanol-ether (3:1, v/v) and finally ether and the lipid extracts were combined. The residue, with or without lipid extraction, was extracted with 5% (w/v) trichloroacetic acid at 90° for

* Abbreviations: PEP, phosphoenolpyruvate; t-RNA, transfer RNA.

Scheme 1. Flow-sheet of the procedures for separating cell fractions from a homogenate of the magnum of the laying hen.

10 min. and centrifuged. The extraction was repeated twice; the combined extracts were considered to contain the nucleic acids and the residue to contain the protein.

Separation of egg-white proteins from oviduct preparations. Two procedures have been used. In the first the minced tissue was incubated with [¹⁴C]lysine as described above for 24 hr. It was then homogenized in the incubation medium and ovalbumin precipitated with (NH₄)₂SO₄ (Hendler, 1957). The crude ovalbumin was dissolved in water and dialysed first against water, then 0.1M-ammonium acetate, pH 4.4, before chromatography on a CM-cellulose column (Rhodes, Azari & Feeney, 1958). Protein in the column

eluate was measured by extinction at 280 mμ ($E_{280} 1.00 \equiv 1.41$ mg. of protein/ml.).

In the second procedure the minced oviduct (10 g.) was incubated for 15 min. with [¹⁴C]lysine, at which time 70% or more of the radioactivity in protein is to be found in fraction I (Table 2), and then cooled in ice and washed with 0.25M-sucrose and the I particles were separated as described above. A sample of the particles was taken for protein and radioactivity determinations and the remainder incubated at 37° for 30 min. in 10mM-tris-(pH 7.5)-5 mM-MgCl₂-80 mM-KCl-50 mM-NaCl, which solubilizes 50% of the protein. The mixture was cooled, centrifuged at

Scheme 2. Subfractionation (i) by deoxycholate treatment, (ii) by centrifuging through 0.88 M-sucrose, of a combined fraction I containing the particles present in fractions $P_1 W$ and P_2 .

10000g for 10min. and the protein and radioactivity of samples of the supernatants and pellet were measured. Non-radioactive ovalbumin was added to the remaining supernatants to triple the protein concentration, the mixture dialysed against 0.1M-ammonium acetate, pH4.4, and fractionated on a CM-cellulose column.

Preparation of [^{14}C]leucine-lipid. Oviduct (20g.) was minced and added to 40ml. of buffered salt solution

(Hendler, 1956) containing 50 mg. of streptomycin sulphate, 7500 units of penicillin, and $40\mu\text{C}$ (0.49 mg.) of [^{14}C]leucine and incubated for 3 hr. at 37° with $\text{O}_2 + \text{CO}_2$ (95:5) in the gas phase. The mixture was then cooled in ice, homogenized and precipitated with 60ml. of 10% trichloroacetic acid containing 1 mg. of [^{12}C]leucine/ml. The precipitate was washed three times with 5% trichloroacetic acid and then extracted successively three times with ethanol, twice with

ethanol-ether (3:1, v/v) and once with ether. The combined lipid extracts were evaporated with a stream of air to an oily residue, which was dissolved in 25 ml. of chloroform and shaken with water. The layers were separated by centrifuging and the chloroform was washed three times more. Finally the chloroform was evaporated to about 2.5 ml. and centrifuged to clarify. At each washing the 'fluffy layer' at the interface was discarded.

To test the transfer of the [^{14}C]leucine from the lipid extract to protein, 0.5 ml. of the chloroform extract was evaporated, and the oily residue shaken with 1.0 ml. of dilute detergent (0.1 ml. of Shandon RBS concentrate in 50 ml. of water), until fully dispersed. The emulsion produced was stable for several days at least and contained 25770 counts/min./ml.

Preparation of ^{14}C -labelled aminoacyl-t-RNA. Chicken liver (160 g.) was homogenized in 2.5 vol. of medium containing 0.15 M-sucrose, 25 mM-KCl, 10 mM-MgCl₂ and 35 mM-tris, pH 7.8 (Rendi & Hultin, 1960), and centrifuged at 80000g for 60 min. The supernatant (cell sap) was taken to pH 5.2 and the precipitate produced centrifuged and dissolved in 1 ml. of 0.4 M-tris, pH 7.8, for each 10 ml. of cell sap, and diluted with 4 vol. of water (pH 5 enzyme preparation) (Decken & Campbell, 1962). The solution was shaken with 1 vol. of water-saturated phenol, the phenol layer washed with 0.5 vol. of water and the RNA precipitated from the water layers by adding 0.1 vol. of 20% (w/v) potassium acetate and 2 vol. of ethanol and allowing to stand several hours at -10° to -20° . The precipitate was dissolved in water, dialysed against glass-distilled water and freeze-dried. t-RNA (30 mg.) was charged with ^{14}C -labelled amino acids by incubation (at 37° for 15 min.) in a total vol. of 22 ml. with 88 μC (0.88 mg.) of ^{14}C -labelled algal-protein hydrolysate, MgCl₂ (7 mM), ATP (15 mM) and 2 ml. of some fresh pH 5 enzyme preparation and the RNA re-extracted by treatment with phenol as above. The pH 5 enzyme solution was freshly prepared, since on freezing a fibrous precipitate formed and activity was lost.

The preparation was dialysed extensively against water, and the radioactivity of samples was determined (i) without treatment, (ii) after washing with cold 5% (w/v) trichloroacetic acid, (iii) after washing with hot (90° , 10 min.) 5% (w/v) trichloroacetic acid, (iv) after incubation at pH 9.0 and 37° for 60 min., followed by washing with cold 5% (w/v) trichloroacetic acid. A small quantity of bovine serum albumin was added in each case. Treatment with cold

trichloroacetic acid solubilized about 38% of the radioactivity, indicating that this portion was not covalently bound to the t-RNA. Treatments (iii) and (iv) reduced the radioactivity of the precipitate to zero, which would be expected with covalently bound amino acid. The involvement of the bound amino acid only in the transfer of radioactivity to protein during incubations with cell-free particles is suggested by the fact that the addition of a mixture of unlabelled amino acids did not reduce the transfer, and also that a much higher proportion of the added radioactivity was recovered in protein with the aminoacyl-t-RNA than when free amino acids were used. Up to 2500 counts/min. were transferred from aminoacyl-t-RNA of 18300 counts/min. added, whereas up to 1000 counts/min. were incorporated from free amino acid of about 1000000 counts/min. added to the incubation mixture.

Chemical determinations. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. DNA extracted with the hot trichloroacetic acid was determined by the indole method (Dische, 1955), with calf-thymus DNA as standard. The RNA of the hot trichloroacetic acid extracts, after destruction of hexoses by heating for 15 min. at 100° with 0.1 N-NaOH (Solomon, 1957), was measured by the orcinol method (Dische, 1955), with yeast RNA as standard. Hexoses from egg-white protein are extracted by hot trichloroacetic acid and unless removed will interfere with the determination of ribose by orcinol.

Determination of radioactivity. Samples (0.1 ml.) of soluble extracts or 0.1 ml. of N-NaOH solution of the proteins were mixed with 0.5 ml. of Hyamine and 8 ml. of scintillator solution and counted in a Packard Tri-Carb liquid-scintillation spectrometer (Carey, 1964b).

RESULTS

Distribution of RNA, DNA and protein between oviduct cell fractions. The distribution of nucleic acids and protein is shown in Table 1. It is clear that most of the DNA remained in fraction P_1 , resulting in a high DNA/protein ratio. The large amount of the RNA which must have sedimented in the first centrifuging (600g, 10 min.) was washed out subsequently and was found in fraction P_1W , giving this fraction a high RNA/protein ratio. Very

Table 1. *Distribution of protein and nucleic acids between hen-oviduct cell fractions*

The results are averages of two (RNA) and three (DNA) experiments. Percentages are the amounts of material recovered in each fraction compared with that of the unfractionated homogenate. For nomenclature of fractions see Scheme 1.

Cell fraction	Protein (%)	RNA (%)	RNA/protein (g./g.)	Protein (%)	DNA (%)	DNA/protein (g./g.)
Homogenate	(100)	(100)	0.036	(100)	(100)	0.0083
P_1	8.2	13.2	0.057	23.1	91.5	0.0370
P_1W	13.2	32.5	0.088	8.1	1.9	0.0022
P_2	1.9	5.6	0.103	1.4	0.4	0.0043
P_3	1.6	2.8	0.058	2.3	0.3	0.0014
S_3	67.2	30.1	0.017	66.0	7.6	0.0013
Recovery	92	84	—	101	102	—

Table 2. *Distribution of radioactivity between the protein of hen-oviduct cell fractions*

Minced tissue (1 g.) was incubated for the times specified with 1 μ C (30 μ g.) of [14 C]lysine in 2 ml. of medium, then cooled in ice, washed with sucrose, homogenized and fractionated. The specific activity of the protein is given in counts/min./mg., and the total activity of the fraction is given as a percentage of that of the unfractionated homogenate. For nomenclature of fractions see Scheme 1.

Incubation period (min.)	Incorporation							
	5		15		30		60	
	%	Sp. activity	%	Sp. activity	%	Sp. activity	%	Sp. activity
Homogenate	(100)	62	(100)	383	(100)	562	(100)	700
P_1	33	60	30	374	19	455	31	824
$P_1 W$	60	132	53	845	44	1270	40	1590
P_2	5	112	16	1630	15	1910	23	2820
P_3	0	0	5	561	3	850	3	1040
S_3	0	0	2	9	2	21	3	38
Recovery	98	—	106	—	83	—	100	—

small amounts of material were recovered in fractions P_2 and P_3 but the ratio of RNA to protein was high in the former fraction. Fraction S_3 contained a large but variable proportion of the protein of the homogenate, but it also appeared to contain quite a large amount of the RNA and some of the DNA.

Distribution of amino acid-incorporating activity. When minced oviduct was incubated with [14 C]-lysine, it was found that the large particulate fractions became radioactive first (Table 2). In particular fractions $P_1 W$ and P_2 had high specific activities, and a large proportion of the activity incorporated into protein was localized in them. Fraction P_3 , although its specific activity was high after longer incubation periods, contained only a small proportion of the incorporated amino acid, and activity in fraction S_3 was low, rising slowly at later time-intervals. Fraction P_1 (from which all of the $P_1 W$ material is not removed even by three washings) was also radioactive, but its specific activity was lower than either fractions $P_1 W$ or P_2 . When isolated cell fractions were incubated with [14 C]lysine in the mixture of salts and cofactors described in the Materials and Methods section, amino acid was incorporated into protein (Table 3). The incorporation was observed to be proportional to the particle concentration within the ranges used in this experiment. Fractions $P_1 W$ and P_2 were the most active in incorporating amino acid in isolation. Fraction P_3 did not incorporate amino acid in this experiment. In some experiments this fraction did show a slight activity, but since it represents quite a small proportion of the particulate material of the cell its incorporating activity is a correspondingly small proportion of the total activity of the cell particles.

Subfractionation of particles having incorporating activity. Because the particles of fractions $P_1 W$ and

Table 3. *Incorporation of [14 C]lysine by hen-oviduct cell fractions*

Incubation was for 30 min., the conditions being as described in the Materials and Methods section. [14 C]Lysine (1 μ C, 488 μ g.) and the following amounts of particle protein were added to the incubation mixture: P_1 , 1.23 mg.; $P_1 W$, 0.73 mg.; P_2 , 0.116 mg.; P_3 , 0.133 mg. For nomenclature of fractions see Scheme 1.

Cell fraction	Incorporation (counts/min./mg. of protein)
P_1	84
$P_1 W$	195
P_2	224
P_3	0

P_2 have similar sedimentation and amino acid-incorporating properties they were combined for the purposes of further fractionation and designated I . Electron-microscopic examination showed that this fraction contains a mixture of particles (Carey, 1965). It was also noticed that upon spinning fraction I at 10000g the pellet had a layered appearance. Subfractionation was achieved by centrifuging through denser sucrose solutions as described in the Materials and Methods section. The distribution of protein, RNA and amino acid-incorporating activity in the fractions obtained is shown in Table 4. The top layer (I_+) was richer in both RNA and incorporating activity than the pellet (I_m). The middle layer (I_c) resembled I_+ more than I_m . Electron-microscopic observations showed that the mitochondria present in the original fraction were largely recovered in the pellet I_m .

The addition of deoxycholate to a suspension of I particles in 0.44M-sucrose produced an opalescent suspension, which upon centrifuging at 105000g

Table 4. *Distribution of protein, RNA and amino acid-incorporating activity between subfractions of hen-oviduct I particles*

For amino acid incorporation, $1\text{ }\mu\text{C}$ ($4.2\text{ }\mu\text{g.}$) of [^{14}C]lysine and the following amounts of particle protein were added to the reaction mixture: I , 0.97 mg. ; I_m , 0.38 mg. ; I_c , 0.54 mg. ; I_v , 0.58 mg. For nomenclature of fractions see Scheme 2.

Cell fraction	Expt. 1			Expt. 2	
	Protein (mg.)	RNA (mg.)	RNA/protein (g./g.)	Protein (mg.)	Incorporation of [^{14}C]lysine (counts/min./mg. of protein)
I	28.0	1.63	0.058	19.4	579
I_m	10.8	0.35	0.033	3.8	121
I_c	4.9	0.48	0.098	5.4	270
I_v	8.7	0.89	0.101	5.8	725
Total of fractions I_m , I_c and I_v	24.4	1.72	—	15.0	—

Table 5. *Distribution of protein, RNA and amino acid-incorporating activity between deoxycholate subfractions of hen-oviduct I particles*

[^{14}C]Lysine ($1\text{ }\mu\text{C}$, $4.2\text{ }\mu\text{g.}$) was added to the incubation mixture for amino acid incorporation, and also 0.96 mg. of I particle protein or 0.33 mg. of I_p protein. For nomenclature of fractions see Scheme 2.

Cell fraction	Protein (mg.)	RNA (mg.)	RNA/protein (g./g.)	Incorporation of [^{14}C]lysine (counts/min./mg. of protein)	
					RNA (counts/min./mg.)
I	6.4	0.78	0.122	289	2360
I_p	2.2	0.55	0.249	510	2050
I_s	3.5	0.09	0.026	—	—
Total of I_p and I_s	5.7	0.64	—	—	—

for 60 min. gave a pellet which was almost clear, compared with the opaque pellet of the original I particles centrifuged at $1000g$. The particles of the pellet (I_p) contained only 34% of the protein but 70% of the RNA of the original particles (Table 5). The incorporation of [^{14}C]lysine into protein was also higher in fraction I_p than in the original particles when referred to their protein content, but was the same when referred to the RNA content.

Incorporation of free amino acid by cell-free particles. I particles from the oviduct, or the small particles derived from them by deoxycholate treatment (I_p), incorporated [^{14}C]lysine *in vitro* in the presence of a mixture of cofactors (Table 6). The omission of the catalytic quantity of ATP from the mixture did not always cause a fall in incorporation. This was sometimes observed, however (cf. Table 7), and so ATP was always included in the mixture. ATP was required by the I_p particles for maximum incorporation. Catalytic quantities of GTP were

Table 6. *Incorporation of [^{14}C]lysine by hen-oviduct cell fractions in vitro*

The particles were prepared on separate occasions. The complete mixture contained $1\text{ }\mu\text{C}$ ($4.2\text{ }\mu\text{g.}$) of [^{14}C]lysine, 1.86 mg. of I particle protein and 1.59 mg. of I_p protein and other constituents in concentrations given in the Materials and Methods section. The incorporation is given per mg. of I particle protein except when particles were omitted, when it is given per mg. of liver cell-sap protein.

Incubation mixture	Incorporation (counts/min./mg. of protein)	
	By I particles	By I_p particles
Complete mixture	1080	1160
Minus ATP	1120	728
Minus GTP	222	194
Minus PEP	10	3
Minus ATP, GTP, PEP	16	10
Minus liver cell sap	434	10
Minus particles	0	4
Plus 0.1 mM -puromycin	44	—

Table 7. *Incorporation of radioactive amino acids by hen-oviduct I particles and chicken-liver microsomes in vitro*

The complete mixture contained 0.92 mg. of *I* particle protein or 0.74 mg. of chicken-liver microsomal protein, 1 μ C (12.2 μ g.) of [14 C]leucine or 18300 counts/min. (0.3 mg.) of 14 C-labelled aminoacyl-t-RNA in addition to other constituents at concentrations given in the Materials and Methods section.

Radioactive substrate	Incubation mixture	Incorporation (counts/min./mg. of protein)	
		By hen-oviduct <i>I</i> particles	By chicken-liver microsomes
14 C-labelled aminoacyl-t-RNA	Complete	1860	990
	Minus ATP	2200	1370
	Minus GTP	835	345
	Minus PEP	275	225
	Minus ATP, GTP	975	305
	Minus ATP, PEP	340	265
	Minus GTP, PEP	235	225
[14 C]Leucine	Complete	960	445
	Minus ATP	780	540
	Minus GTP	510	131
	Minus PEP	96	60

necessary for incorporation in both types of particle, but the greatest diminution resulted from the omission of PEP. The omission of the liver cell sap from the mixture merely lowered the incorporation by *I* particles but the *I_p* particles were inactive in the absence of the soluble enzymes. Puromycin in low concentration virtually abolished the incorporation by *I* particles.

Incorporation of amino acids bound to t-RNA. Amino acids bound to t-RNA were transferred to protein by *I* particles, but, as shown in Table 7, there were some unusual features of the transfer. The parallel experiment with chicken-liver microsomes is included for comparison. The presence of ATP was found to decrease the transfer. In Table 7 three paired comparisons can be made between incubations with and without ATP, both with oviduct *I* particles and chicken-liver microsomes (Table 7, lines 1 and 2, 3 and 5, 4 and 6), and in all except one case the presence of ATP resulted in a lower transfer of activity to protein. The incorporation of free [14 C]leucine on the other hand was slightly reduced by the omission of ATP with this preparation. The requirement for GTP for the transfer of activity from t-RNA was still apparent as expected, but as with the incorporation of free amino acid the greatest diminution resulted from the omission of PEP. The results with the chicken-liver microsomes were very similar in all cases.

These effects obtained with the *I* particles were explored further, as shown in Table 8. The two commercial preparations of ATP had very little effect on the incorporation of [14 C]leucine, but a

marked difference was found with the 14 C-labelled aminoacyl-t-RNA. A diminished transfer was observed in the presence of ATP and one preparation produced a greater diminution than the other. This effect has not been studied further.

An attempt to see whether the requirement for PEP in the incorporation from aminoacyl-t-RNA was direct, or just to replenish GTP, was inconclusive (Table 8). A higher concentration of GTP did not increase the incorporation in the absence of PEP, but it also caused an inhibition of the activity found in the presence of PEP. This latter effect of high GTP concentration was obtained with [14 C]leucine or 14 C-labelled aminoacyl-t-RNA as substrate.

Possible involvement of lipid intermediates in incorporation in vitro. Hendler (1959) has shown that, when minced oviduct is incubated with radioactive amino acid, the lipid fraction becomes radioactive, and appears to have the properties of an intermediate in the incorporation into protein. To test this possibility in isolated particles a radioactive lipid extract was prepared as described in the Materials and Methods section, and incubated with isolated *I* particles in the presence of ATP, GTP, PEP, liver cell sap, salts and buffer. [14 C]-Leucine bound to oviduct lipids was not transferred to protein under these conditions (Table 9). The dilute detergent used to emulsify the lipid may have caused a slight inhibition of the incorporation of free lysine. The radioactive lipid emulsion itself, however, reduced the incorporation of free lysine even further.

Table 8. Incorporation of ^{14}C -labelled amino acids by hen-oviduct I particles

The complete mixture contained 0.78 mg. of I particle protein, $1\text{ }\mu\text{C}$ ($12.2\text{ }\mu\text{g.}$) of [^{14}C]leucine or 18300 counts/min. (0.3 mg.) of ^{14}C -labelled aminoacyl-t-RNA in addition to other constituents at concentrations given in the Materials and Methods section. Except where stated the concentration of GTP was 0.5 mM.

Incubation mixture	Incorporation (counts/min./mg. of protein)	
	[^{14}C]Leucine	^{14}C -labelled aminoacyl-t-RNA
Complete, Pabst ATP	1060	1960
Complete, Sigma ATP	1120	2580
Minus ATP	1070	3400
Minus PEP	87	351
Minus ATP plus 5.0 mM-GTP	305	1110
Minus PEP plus 5.0 mM-GTP	26	180
Minus ATP, PEP plus 5.0 mM-GTP	64	328
Plus 5.0 mM-GTP	78	465

Table 9. Incorporation of ^{14}C -labelled amino acids by hen-oviduct I particles *in vitro*

The incubation mixture contained 2.8 mg. of I particle protein, and $1\text{ }\mu\text{C}$ ($30\text{ }\mu\text{g.}$) of [^{14}C]lysine or the lipid emulsion containing 5150 counts/min. were added as indicated. The lipid was that extracted from approx. 0.8 g. of oviduct. Other constituents were present in concentrations given in the Materials and Methods section.

Additions to the incubation mixture	Incorporation (counts/min./mg. of protein)
[^{14}C]Lysine	150
[^{14}C]Lysine plus detergent	133
[^{14}C]Leucine-lipid in detergent	0
[^{14}C]Leucine-lipid in detergent plus [^{14}C]lysine	64

During the incorporation of free lysine by isolated I particles it was found (Fig. 1) that the hot-trichloroacetic acid extract contained radioactivity, but the lipids were not radioactive; it should be noted that the extraction of lipids preceded the extraction with hot trichloroacetic acid in this experiment. The lipid extract here was the combined ethanol and ether extracts, evaporated to small volume, dissolved in chloroform and washed with water and included the activity in the 'fluffy layer' which is obtained at the interface between the chloroform solution of the lipids and the water. It is also noticeable that the incorporation into protein reaches a peak and then activity is reduced. This phenomenon is at present unexplained.

A further indication of the degree of involvement of lipid-bound or nucleic acid-bound intermediates in the cell-free preparation is given in Fig. 2. Ribonuclease added to the incubation mixture inhibited the incorporation of [^{14}C]lysine very greatly at low concentration; by contrast lecithinase-C

Fig. 1. Incorporation of [^{14}C]lysine by hen-oviduct I particles *in vitro*. Each point is the result of the incubation of 1.78 mg. of I particle protein. ●, Lipid-soluble radioactivity; ▲, radioactivity soluble in hot trichloroacetic acid; ○, radioactivity in the residual protein precipitate.

Fig. 2. Effect of ribonuclease and lecithinase-C on the incorporation of amino acids by I particles *in vitro*. The curves are composed of the results of several experiments and show the effect of lecithinase-C on [^{14}C]lysine (○) or [^{14}C]leucine (●) incorporation, and the effect of ribonuclease on [^{14}C]lysine incorporation (▲).

Fig. 3. Chromatography on a CM-cellulose column (1 cm. $\times$ 5 cm.) of a soluble extract of *I* particles from oviduct after incubation with [^{14}C]lysine for 15 min. The soluble extract contained 9.5 mg. of protein; 20 mg. of unlabelled ovalbumin was added as carrier. The eluate was divided into 3 ml. fractions. Radioactivity was determined in 0.1 ml. samples. $\circ$, E_{280} ; $\bullet$, radioactivity (counts/min.). The starting buffer was 0.1 M-ammonium acetate, pH 4.4. Peak 1, material that did not attach to the column; peak 2, ovalbumin A_1 ; peak 3, ovalbumin A_2 ; peak 4, ovalbumin A_3 .

was much less inhibitory either with [^{14}C]lysine or [^{14}C]leucine as substrate.

Incorporation of radioactivity into egg-white proteins. The incubation of minced oviduct with ^{14}C -labelled amino acids leads to the incorporation of radioactivity into protein. After such an incubation, egg-white proteins isolated from the oviduct are radioactive (Anfinson & Steinberg, 1951; Hendler, 1956). In this study, ovalbumin has been isolated from two types of oviduct preparation (see the Materials and Methods section).

Ovalbumin isolated from the oviduct after incubation for 24 hr. was abnormal in that ovalbumin A_1 was absent (or on one occasion present in low concentration), whereas it normally constitutes the bulk of this protein isolated from egg white. Precipitation of the ovalbumin once only, or three times, did not alter the distribution pattern of the three forms of ovalbumin on the CM-cellulose column.

In the second procedure, radioactive ovalbumin has been sought in the *I* particles after short incubations. During the early periods of incubation by minced tissue, most of the radioactivity in protein is localized in the *I* particles and the presence of radioactive ovalbumin in them would strongly suggest that they are derived from the site of egg-protein synthesis in the cell.

Chromatography of a soluble extract of the radioactive particles obtained as described in the Materials and Methods section gave rise to two peaks of radioactivity, which coincided with those of ovalbumin A_2 and A_3 (Fig. 3). The ovalbumin A_1 peak of the carrier ovalbumin was not radioactive, and thus the distribution of radioactivity resembled the pattern shown by ovalbumin isolated from

Table 10. *Distribution of radioactivity among proteins derived from I particles pre-labelled by incubation of oviduct in vitro*

Pre-labelling was for 15 min. with [^{14}C]lysine.		
Preparation	Radioactivity (counts/min.)	Protein (mg.)
<i>I</i> particles	74300	23.5
<i>I</i> particles incubated at 37°, 10000g residue	48300	10.3
Soluble protein from <i>I</i> particles incubated at 37°	23800	9.5
Ovalbumin A_2 and A_3 peaks from the soluble fraction separated by chromatography	6200	—

soluble extracts of the whole tissue. As shown in Table 10, the combined activity of the ovalbumin A_2 and A_3 peaks amounted to 26% of that found in the soluble extract from the *I* particles or 8% of the activity of the original *I* particles themselves.

DISCUSSION

The results presented here confirm the observations of Hendler (1957, 1959) that in the fractionation of a homogenate of the hen-oviduct magnum most of the particles are sedimented by low centrifugal fields and the small amount of material sedimented by 100000g possesses little protein-synthetic activity. In other tissues, the microsome fraction has been shown to be derived from the

endoplasmic reticulum, which is believed to be a major site of protein synthesis in the cell. In the oviduct, however, the particles which sediment at 100000g have only a small ability to incorporate amino acids. Hendler, Dalton & Glenner (1957) have shown that material derived from the endoplasmic reticulum is found, with most of the other particles of the homogenate, in the 600g pellet. The results in this paper suggest that this derivative can subsequently be removed from the 600g pellet. The residual 600g pellet (P_1) contained most of the DNA of the homogenate and must therefore have included most of the nuclei. The particles washed out of the first 600g pellet, which could be sedimented at 10000g, had no DNA, a higher RNA/protein ratio than the homogenate and a high activity in incorporating amino acid *in vitro* either in isolation or in whole cells.

The reason why these intermediate-size particles sedimented at first with the nuclear fraction is not clear. Hendler, in his study, washed the 600g pellet, but apparently did not observe a similar phenomenon. The methods here differ only in the use of 0.44M instead of 0.25M sucrose as homogenizing medium and that a Dounce homogenizer, instead of a Potter-Elvehjem homogenizer, was used. It is possible that the denser sucrose prevented the particles from resedimenting, and, since Anderson (1956) suggested that the Dounce homogenizer is less likely to damage cell particles, this may have had some effect. These factors do not seem to provide a complete explanation, however, since the small particles were regularly found to change their characteristics during the fractionation, becoming increasingly difficult to sediment as the fractionation proceeded. Nevertheless a fraction can be isolated (I particles) which contains the most important agents in amino acid incorporation into protein *in vitro*.

This incorporation by isolated I particles showed many similarities in its cofactor requirements with those exhibited by the microsome preparations of other tissues. The omission of catalytic quantities of GTP or of the ATP generator reduced or abolished the incorporation. The catalytic quantity of ATP itself was not always required for incorporation, but this may have been due to the presence of small amounts of ATP in the particle preparation, a suggestion borne out by the partial requirement for it seen with the I_p particles produced by treatment of I particles with deoxycholate.

The I particles also showed only a partial requirement for cell-sap enzymes from liver for incorporation. It was not practicable to use oviduct cell sap because this contains large quantities of protein, which have been found to interfere with subsequent preparative procedures; however, with the amounts used, incorporation is higher with liver cell sap. In

view of the partial requirement for cell sap it is possible that the particles themselves contain some t-RNA and activating enzymes. These, however, appear to be solubilized by deoxycholate treatment, since the I_p particles required the presence of cell sap for activity.

The I particles were also able to transfer radioactive amino acid from t-RNA to protein. The labelled t-RNA used in these experiments was prepared with crude algal-protein hydrolysate as the source of radioactive amino acids. About 38% of the activity of this labelled t-RNA was soluble in cold trichloroacetic acid, a result which has since been obtained by using purified single amino acids as the label. However, details given in the Materials and Methods section indicate that this free radioactivity did not contribute to the transfer of activity to protein by cell-free preparations in the experiments described in this paper.

GTP and PEP were required for transfer of the activity from t-RNA to protein by I particles and the requirement for PEP in the system was not replaced by a tenfold increase in GTP concentration, in agreement with Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958). On the other hand this concentration of GTP was inhibitory even in the presence of PEP. ATP had an inhibitory effect on the transfer of amino acids from t-RNA, which was found to different extents with two commercial preparations of ATP and was also seen when chicken-liver microsomes were incubated under the same conditions.

Hendler (1959) has suggested that lipid-bound amino acids are intermediates in protein synthesis in the tissue. There is no support for this suggestion from results with cell-free preparations. The incorporation of free amino acids by isolated I particles was inhibited by low concentrations of puromycin and ribonuclease, but less so by lecithinase-C. During the incorporation in cell-free preparations, the hot-trichloroacetic acid extract, but not the lipid extract, became radioactive. The radioactivity of a lipid extract prepared by incubating chopped tissue with [^{14}C]leucine was not transferred to protein by isolated I particles under the conditions used. Treatment with deoxycholate, which probably destroys the lipid membranes, did not abolish amino acid incorporation, which was still a property of the RNA-rich particles produced. Although these results do not support the hypothesis that lipid-bound amino acids are involved in protein synthesis they cannot be said completely to exclude the hypothesis, since Hendler (1962) has suggested that two routes of incorporation may exist in whole cells, and it is possible that the route involving lipids does not survive when the cell is broken.

The results obtained here are in agreement with

those obtained with many other tissues, indicating the presence of a route of incorporation involving nucleic acid-bound intermediates in the cell-free system. In view of the high activity of the particles isolated in incorporating amino acids when compared with other cell fractions, it seems very likely that they are involved in the synthesis of egg-white protein.

In an attempt to test this possibility, ovalbumin was isolated from the oviduct and its cell fractions and separated into its three components, ovalbumin A₁, A₂ and A₃. When the oviduct was incubated for 24 hr. with radioactive amino acid, ovalbumin precipitated from a soluble extract of the tissue was radioactive, but usually ovalbumin A₁ was absent, the bulk of the preparation comprising ovalbumins A₂ and A₃. After short periods of incubation (15 min.) most of the radioactivity incorporated into protein was found in the I particles. Some of the radioactivity of a soluble extract of these labelled particles, to which carrier ovalbumin was added, separated with ovalbumins A₂ and A₃. It is possible that some or all the activity in these peaks was due to contaminating proteins other than ovalbumin. However, the ovalbumin A₁ peak, which was eluted before the A₂ and A₃ peaks, contained no radioactivity, and the elution pattern of the radioactivity resembled that found for the ovalbumin of the whole-tissue extract after prolonged incubation. These results suggest the presence of an ovalbumin-like protein within the I particles which becomes labelled during short incubations. It seems probable that this is a precursor of the radioactive ovalbumin extracted from the whole tissue after longer incubation.

The finding that ovalbumin A₁ was not observed in soluble extracts by the techniques described here, whereas both ovalbumins A₂ and A₃ were, is curious in view of the results of Sanger & Hocquard (1962), who showed that in the isolated tissue ovalbumin A₃ (no phosphate present) is synthesized first, with little or no ovalbumin A₂ (one phosphate) as intermediate in the transformation into ovalbumin A₁ (two phosphates). However, Oades & Brown (1965) have found that immature hens need to be treated with oestrogen and progesterone or oestrogen and testosterone to maintain the normal pattern of water-soluble proteins in the magnum found in laying birds, and in particular the dominant peak of ovalbumin A₁ seems to be absent in birds treated with oestrogen alone. It is possible therefore that the absence of the normal hormone balance experienced by the isolated mature tissue could result in an abnormal proportion of incorporation into the end-product proteins. This effect may be variable, depending perhaps on the stage of the egg-laying cycle, and in that case could account for the disagreement with Sanger & Hocquard (1962).

The results in this paper indicate that protein synthesis in the oviduct occurs in a portion of the cell, probably similar to the endoplasmic reticulum of other tissues, which in homogenates gives rise to large particles. There is at the moment little information available to suggest that the synthetic apparatus is arranged in special structures in this tissue. However, results in some other tissues and organisms have suggested that this is possible (for references see Morton & Raison, 1963; Carey, 1965), and the features in common seem to be the rapid synthesis of large quantities of protein, which may sometimes be secreted from the cell. It is possible therefore that the particles isolated are derived from special structures within the tissue and that they represent an adaptation to these features of the synthesis.

It is a pleasure to thank Miss Janet Sterlini and Miss Ruth Coxon for expert technical assistance. The author is also very grateful to the Endowment Fund of St Thomas's Hospital and the British Egg Marketing Board for grants, and to Professor F. T. G. Prunty for his advice and encouragement.

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