The Mechanism of Protein Synthesis in the Developing Chick Embryo

THE INCORPORATION OF FREE AMINO ACIDS

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The mechanisms for the synthesis of protein that have been suggested in recent years have usually visualized free amino acids as being the starting material of the process. The suggestion has been made, however, that in embryonic tissues existing or storage proteins are converted into new cell protein through intermediates that do not include free amino acids. Walter & Mahler (1958) reached this conclusion from results obtained after the injection of various precursors into the developing hen's egg, and Francis & Winnick (1953) were similarly persuaded by results obtained with embryonic cells in culture. It has also been suggested that a direct incorporation of peptide-bonded amino acids into protein can occur in adult tissues in special circumstances, e.g. in the synthesis of haemoglobin from peptides derived from that protein (Brown & Brown, 1960) and in tumours (Babson & Winnick, 1954).

Confirmation of these results has not been forthcoming. Askonas, Campbell, Godin & Work (1955) and Godin & Work (1956) were unable to implicate blood proteins or peptides derived from casein as precursors of milk proteins in the goat except by routes involving free amino acids. Campbell & Stone (1957) concluded that tissue and tumour proteins, and serum globulin, were not derived from serum albumin in the rat, and Walter & Zipper (1962) have shown that free amino acids are the starting material in haemoglobin synthesis.

The chick embryo is an ideal organism for the study of the mechanisms that control and initiate protein synthesis during development, and more direct evidence is therefore being sought on the route by which the egg proteins are transformed into new cell proteins. The present paper, the first part of this study, is concerned with the incorporation of free amino acids by tissues and tissue fractions of the embryo.

MATERIALS AND METHODS

Materials. All solutions for the tissue incubations were made up in glass-distilled water in acid-washed glassware.

ATP was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.; GTP was from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and

phosphoenolpyruvate and pyruvate kinase were from Sigma Chemical Co., St Louis, Mo., U.S.A.; tris was from L. Light and Co., Colnbrook, Bucks., and was recrystallized from ethanol.

L-[¹⁴C]lysine hydrochloride was obtained from The Radiochemical Centre, Amersham, Bucks., and used at specific activities of 1.44–5.80 $\mu\text{C}/\mu\text{mole}$.

Hydroxide of hyamine 10X, 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from the Packard Instrument Co., La Grange, Ill., U.S.A.

Preparation of tissues and tissue fractions. Eggs of the cross Rhode Island $\times$ Light Sussex (Withers, Appleby Farm, Egerton, Ashford, Kent) were incubated in a Glevum incubator at 38°. Embryos were removed from the egg during fifth and ninth days of incubation, approximately at stages 25 and 33 (Hamilton, 1952), and rinsed in 0.155M-KCl to remove any yolk and the extra-embryonic membranes. The head was cut off and the embryo transferred to ice-cold 0.44M-sucrose, or the liver was dissected out and this transferred to sucrose for storage until all the eggs had been treated.

For the incubation of minced tissues, one embryo was chopped finely with scissors and transferred to a flask containing the incubation medium described below.

The tissue was fractionated into subcellular components after being homogenized in 0.44M-sucrose in the all-glass device of Dounce, Witter, Monte, Pate & Cottone (1955) and being filtered through gauze. The particulate fractions were separated by centrifuging as follows: nuclei, 600g for 10 min. in the MSE Major centrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex) fitted with rotor no. 6876; mitochondria, 10000g for 10 min., and microsomes, 105000g for 60 min., both in the MSE Super-Speed 40 centrifuge fitted with rotor no. 29390. The fractions were washed by resuspension in 0.44M-sucrose and centrifuging again, and appeared to be essentially similar to those defined in previous work (Carey & Greville, 1959).

Tissue incubation conditions. Minced tissues were added to 3 ml. of medium containing phosphate buffer, pH 7.5 (0.01M), or tris buffer, pH 7.5 (0.01M), together with glucose (0.05M), KCl (0.12M) and [¹⁴C]lysine as indicated in the Results section. The suspension was shaken at 37° with air in the gas phase, and the reaction was arrested by the addition of 3 ml. of 10% (w/v) trichloroacetic acid or by cooling to 0° if the tissue was to be separated into cell fractions after the incorporation of radioactivity.

The washed microsomal fraction was incubated in 1.0 ml. of medium similar to that described by Korner (1962), consisting of tris buffer, pH 7.5 (0.01M), MgCl₂ (5 mM), NaCl (50 mM), KCl (80 mM), phosphoenolpyruvate (10 mM), pyruvate kinase (20 μg .), ATP (0.8 mM), GTP (0.5 mM),

[^{14}C]lysine or [^{14}C]leucine (1.0 μC), microsomal suspension in 0.44M-sucrose (0.3 ml.; 0.4–3.8 mg. of protein/ml.) and cell sap in 0.44M-sucrose (0.2 ml.; 1.3–4.1 mg. of protein/ml.). The reaction was stopped with 1.0 ml. of 10% trichloroacetic acid containing 1 mg. of [^{14}C]lysine/ml. Zero-time blanks were prepared by the addition of the trichloroacetic acid before the enzymic fractions, fractionated and determined as for the experimental incubations, and their radioactivities subtracted from the experimental values.

Chemical fractionation of tissues and tissue fractions. The minced tissues precipitated with trichloroacetic acid were washed twice with 5% (w/v) trichloroacetic acid at room temperature, extracted twice with the same solution at 90° for 10 min. and subsequently cooled in ice before being centrifuged. The precipitate was then washed successively in 70% (v/v) ethanol, ethanol, ethanol-ether (3:1, v/v) and ether, and then dried.

The isolated cell fractions were extracted with cold and hot trichloroacetic acid as described above, except that the solution contained 1 mg. of [^{14}C]lysine/ml. Unless this was done, high and variable values were found in the zero-time blank. The cell fractions were not extracted with fat solvents since it was found that the hot-trichloroacetic acid-insoluble material could sometimes be dissolved in 70% ethanol (cf. Delbro, Tarver & Korner, 1957). The protein precipitate was dissolved in 0.1N-NaOH for the measurement of radioactivity.

Determination of radioactivity. Samples (15–20 mg.) of the protein powders from the minced tissues were washed with ether on to weighed aluminium planchets (diam. 2 cm.). Then 2–3 drops of 0.1% paraffin wax in ether were added, and the material was dried and weighed. It was counted in a windowless gas-flow counter, to a standard error of less than 1% (at least 10000 counts collected), and the observed activity corrected for self-absorption.

Protein solutions and other extracts from the isolated cell fractions were counted in a Packard Tri-Carb liquid-scintillation spectrometer, also to a standard error of less than 1% and with an efficiency of about 50%. A 0.1 ml. sample was mixed either with 1.0 ml. of M-hyamine and 15 ml. of the scintillator solution or with 0.5 ml. of M-hyamine and 8 ml. of scintillator. The scintillator solution was that developed by Dr K. Smith and described by Carey & Goldstein (1962), except that 2 ml. of acetic acid was added for each 250 ml. of dioxan, since acidification eliminates the photoluminescence of protein in this mixture (Herberg, 1958).

Protein determination. The dried protein weighed on the aluminium planchets was considered to be mainly the protein of the minced tissue. The other protein fractions were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine or porcine serum albumin as reference material.

RESULTS

Incorporation of [^{14}C]lysine by minced tissues of the 8-day embryo. Incubation of minced tissues of the 8-day embryo in a buffered glucose medium led to the rapid incorporation of radioactivity from added [^{14}C]lysine into the protein fraction. The rate of incorporation was dependent on the concentration of lysine in the incubation medium (Fig. 1).

At 1.0 mM about 1.0 μg . of lysine was incorporated/mg. of protein/hr., if it is assumed that no dilution by non-radioactive lysine occurred within the cell. At 2.0 mM 1.5 μg . of lysine was incorporated/mg. of protein/hr., which was the highest observed.

Distribution of radioactivity between cell fractions after the incorporation of [^{14}C]lysine by minced tissues. The distribution of radioactivity varied with the duration of incubation (Fig. 2). Although the proportion of activity in the nuclei and mitochondria was low and varied little at different incubation periods, the microsomes, at short time-

Fig. 1. Effect of the concentration of [^{14}C]lysine on its incorporation into protein by minced tissues of the 8-day chick embryo. Experimental details are given in the text. The incubation time was 30 min. The specific activity of the [^{14}C]lysine was 1.44 $\mu\text{C}/\mu\text{mole}$.

Fig. 2. Ratio of the specific activities of the cell-fraction proteins to that of the homogenate, after incubation of minced tissues of the 8-day chick embryo with [^{14}C]lysine. Experimental details are given in the text. The specific activities of the homogenate proteins were (counts/min./mg. of protein): after incubation for 3 min., 506; after incubation for 6 min., 1460; after incubation for 9 min., 3240. The concentration of lysine was 0.066 μM , and the specific activity 5 $\mu\text{C}/\mu\text{mole}$.

intervals, were twice as radioactive as the homogenate (and also had the highest percentage of the total activity), but this ratio fell with more prolonged incubation. The cell sap, on the other hand, had less radioactivity to start with, but its proportion rose during incubation.

In an attempt to trace the movement of radioactivity between fractions of the cell, the following experiment was carried out. Five incubations of minced tissues with [^{14}C]lysine were made, one for each of the points on the curves in Fig. 3. Two were for 3 and 9 min. and three for 4, 6 and 9 min., 0.1 ml. of [^{14}C]lysine (50 mg./ml.) being added at 3 min., causing a 140-fold dilution of the radioactivity. The incubations were stopped by cooling in ice, and the tissue was washed once in 0.25 M-sucrose before being homogenized in 0.44 M-sucrose. The cell fractions were separated and treated to obtain the material soluble in cold trichloroacetic acid and the hot-trichloroacetic acid-insoluble residue.

The cold-trichloroacetic acid-soluble radioactive material of the cell sap comprised about 80 % of the total of this material present in the tissues, and changes in this fraction were paralleled by similar changes in cold-trichloroacetic acid-soluble material from the particulate fractions. It is clear from Fig. 3 (a) that the lysine of this fraction (cold-

Fig. 3. Incorporation of [^{14}C]lysine into cell fractions by minced tissues of the 8-day chick embryo. Five embryos were incubated separately with [^{14}C]lysine. Each incubation was stopped by cooling in ice, the tissue was washed and homogenized in sucrose, cell fractions were prepared and their protein and radioactivity were determined. Two incubations were for 3 and 9 min. with 0.066 μmole (0.33 μC) of [^{14}C]lysine/ml. (O); three were for 4, 6 and 9 min. with the above lysine concentration for 3 min., 9.1 μmoles of [^{14}C]lysine/ml. being added at this point (●). (a) Cold-trichloroacetic acid-soluble fraction of cell sap; (b) nuclear protein; (c) mitochondrial protein; (d) microsomal protein; (e) cell-sap protein; (f) homogenate protein.

trichloroacetic acid-soluble fraction of cell sap) exchanged rapidly with that in the medium, and steady levels of radioactivity/mg. of cell-sap protein were reached in a few minutes. On the addition of [^{14}C]lysine to the medium, the radioactivity fell to a new constant level, but not to zero. Results indicated that contamination with radioactivity from outside the cell could not have exceeded 3 %.

The incorporation of radioactivity into the protein of the nuclei and the mitochondria (Figs. 3b and 3c) was brought to a halt by the dilution of the [^{14}C]lysine with [^{12}C]lysine, suggesting that the proteins that become radioactive in these fractions continue to reside in them after the incorporation.

The microsomal protein fraction was the only one to show a decrease in radioactivity after the addition of the diluting [^{12}C]lysine (Fig. 3d), whereas incorporation continued at a high rate into the cell-sap protein in the intervals immediately after this addition (Fig. 3e).

Incorporation into the protein of the unfractionated homogenate (Fig. 3f), which represents the total of the above fractions, was rapidly arrested by the dilution of the [^{14}C]lysine.

The above results are obviously subject to some variation, so the reproducibility was checked in a similar experiment in which three duplicate incubations were carried out, one for 3 min., one for 4 min. and the third for 4 min., the excess of [^{12}C]lysine being added after 3 min. The results obtained were closely similar to those depicted in Fig. 3, and some of them were pooled with the comparable results in Fig. 3 for a statistical analysis. The mean values are shown in Table 1. Since the error showed a significant tendency to increase as the radioactivity value increased, the mean is a geometric mean and the standard error is expressed as a proportion. The changes observed between the two time-intervals in the five fractions given in Table 1 were significant in all cases except that of the mitochondria. In the same interval, where [^{12}C]lysine was not added the specific activities rose by 150–170 % in all fractions.

The small rise that occurs (Table 1) in the specific activity of the homogenate must be due to incorporation occurring before complete mixing of the added [^{12}C]lysine, and this could conceivably explain the increase in the specific activity of the nuclear fraction.

The increase in the specific activity of the cell-sap fraction, however, is much higher, being close to that in the flasks where the [^{14}C]lysine was not diluted, as depicted in Fig. 3.

Incorporation of radioactive amino acids by microsomal fractions in vitro. The microsomal fraction from tissues of embryos at various ages incorporated amino acids *in vitro* (Table 2). The

Table 1. *Specific activity of cell fractions of the 8-day chick embryo after the incorporation of [^{14}C]lysine by minced tissues*

Experimental details are given in the text. The results are means of 3 values; the standard error is 1.063–0.944 times the mean in each case.

Incubation conditions ...	Sp. activity (counts/min./mg. of protein)		Percentage change at 4 min.	P
	3 min.	4 min.; [^{14}C]lysine added at 3 min.		
Cell fraction				
Homogenate	1056	1214	+15	< 0.01
Nuclei	592	739	+25	< 0.01
Mitochondria	765	805	+5	> 0.7
Microsomes	2059	1704	-17	< 0.01
Cell sap	707	1052	+49	< 0.01

Table 2. *Incorporation of [^{14}C]lysine by microsomes from tissues (whole embryo minus head) and liver of the chick embryo*

The complete incubation mixture is given in the Materials and Methods section. The radioactivity of the zero-time control (which varied between 1 and 37 counts/min.), where trichloroacetic acid was added before the enzymes, has been subtracted. One-half of the material in the incubation was used for the determination of radioactivity.

Omissions from incubation mixture	Radioactivity (counts/min.)			
	5-day-embryo tissues	8-day-embryo tissues	8-day-embryo liver	17-day-embryo liver
None (complete mixture)	320	49	106	125
ATP	—	27	52	38
GTP	—	4	10	-6
ATP-generating system	—	7	-2	9
ATP, GTP and ATP-generating system	10	0	24	-6
Microsomes	—	1	1	-15
Cell sap	-1	5	-2	-4
Amount ($\mu\text{g.}$) of microsomal protein in sample counted	159	114	81	258

Fig. 4. Progress curve of the incorporation of [^{14}C]lysine into protein by microsomes isolated from 8-day chick embryo tissues. Experimental details are given in the text. The lysine specific activity was $5.8 \mu\text{C}/\mu\text{mole}$.

omission of ATP, GTP or an ATP-generating system decreased or abolished the incorporation, and both the microsomal and the cell-sap fractions were required. If either the microsomal fraction or

the cell sap was preincubated at 80° for 5 min., incorporation did not occur above control levels.

Fig. 4 shows the progress curve of the incorporation, which in other experiments was shown to be proportional to the microsomal concentration below 0.4 mg. of protein/incubation flask.

DISCUSSION

The incorporation of free amino acids into the proteins of embryonic tissues has been demonstrated by a number of workers. For instance Hultin & Bergstrand (1960) have shown this to occur in the sea urchin, and Feldman & Waddington (1955), Waddington & Perry (1958) and Prockop, Peterkofsky & Udenfriend (1962) have demonstrated it in the chick embryo. Walter & Mahler (1958) showed that free amino acids were incorporated into the chick-embryo proteins after injections into the egg, although they concluded that peptide-bonded amino acids were a preferred source of new cell protein.

The results reported in this paper confirm that the chick embryo incorporates free amino acids into protein, and demonstrate further that the microsomal fraction operates in a manner

similar to that described for other tissues. When tissues were incubated with ^{14}C -labelled amino acid *in vitro* for short time-intervals, this fraction had the highest proportion of the radioactivity, decreasing as the incubation proceeded, whereas the proportion in cell sap rose. In addition, the dilution of the specific activity of the incorporated amino acid resulted in a lower specific activity of the microsomal protein, whereas that of the cell sap continued to increase, thus suggesting that some at least of the amino acid incorporated into cell-sap protein passes through a hot-trichloroacetic acid-insoluble fraction in the microsomes.

Radioactivity was incorporated into the proteins of the nuclear and mitochondrial fractions also, but here the dilution resulted only in the arresting of any further increase in specific activity. In other organisms these latter fractions have been shown to incorporate amino acids when isolated from other fractions of the cell (Truman & Korner, 1962; Roodyn, Reis & Work, 1961; Allfrey, Mirsky & Osawa, 1957), so that the complete system for the incorporation appears to be present within these particles. Walter & Mahler (1958) found, with labelled protein precursors injected into the egg, that either the nuclear or the mitochondrial proteins of the embryo had the highest specific activity, and they suggested that these are the main sites of protein synthesis at some stages of development. In the results described above no support is given to this suggestion.

The radioactivity in the cold-trichloroacetic acid-soluble fraction fell rapidly by only about one-half when the specific activity of the amino acid in the medium was diluted 140-fold. This cold-trichloroacetic acid-soluble radioactive material was not further incorporated into protein, so that it did not appear to be part of an 'internal' or 'conversion' amino acid pool such as that described by Cowie & Walton (1956).

The incorporation of amino acids by the isolated microsomal fractions observed is very similar to that found in the adult tissues of other animals. The nature of the factors in the cell sap required has not yet been established beyond the fact that they are heat-labile. Deuchar (1961) has shown, however, that amino acid-activating enzymes are present in the chick embryo, and so it is reasonable to assume that these are involved.

Minced tissues of the embryo were found to incorporate free lysine at a rate of about $1.5\ \mu\text{g.}/\text{mg.}$ of protein/hr. The estimate of Singer, Hochstrasser & Cerecedo (1956) showed that the embryonic protein contains 7.5% of lysine, so that this incorporation is equivalent to a synthesis of $20\ \mu\text{g.}$ of new protein/mg. of cell protein/hr. If the observed incorporation can be taken as indicative of the rate of synthesis by the embryo, it may then

be predicted that the protein of the stage 33 embryo (about 40 mg.), increasing logarithmically as it does, would result in there being 7.3 g. of protein at hatching. In actual fact the unincubated egg contains 6.5 g. of protein, some of which is deaminated and catabolized during development. A similar calculation can be made from results given by Prockop *et al.* (1962). They observed that, 1 hr. after the injection of ^{14}C proline into the 9-day embryo, the specific activity of this amino acid in protein was 10 disintegrations/min./ μmole , and the average specific activity of free proline during this period was 450 disintegrations/min./ μmole . Thus each mole of ^{14}C proline incorporated was diluted with 45 moles already present in the protein or 2.2 moles were incorporated/100 moles present/hr., which is equivalent to a synthetic rate of $22\ \mu\text{g.}$ of new protein/mg. of cell protein/hr. This rate would give about 13.9 g. of protein at hatching if the embryos are presumed to have been at stage 35. Such rough calculations are of course open to objection, but they do indicate that the rate of incorporation of free amino acids observed is of the right order to account for the amount of protein synthesis taking place.

The results obtained in the present study so far do not contradict the suggestion that there is an alternative route for protein synthesis from peptide-bonded reserves, other than that through free amino acids. They do demonstrate, however, that, if such a route exists in the chick embryo, it does so in conjunction with one from free amino acids which resembles those found elsewhere, and which alone could probably account for all the protein synthesis occurring in the embryo.

SUMMARY

1. Free ^{14}C lysine is incorporated into protein by minced tissues of the 8-day chick embryo *in vitro*. The maximal incorporation rate is about $1.5\ \mu\text{g.}$ of lysine/mg. of protein/hr.

2. The protein of all fractions of the cell was radioactive. At shorter time-intervals, the microsomes had the highest specific activity and percentage of the total activity.

3. Dilution of the ^{14}C lysine with a large amount of ^{12}C lysine led to a rapid cessation of incorporation into the homogenate protein. The nuclear and mitochondrial protein also ceased to gain radioactivity. The radioactivity in the microsomal protein diminished, whereas the cell-sap protein continued to increase in specific activity at a high rate.

4. Microsomes isolated from embryonic tissues at various ages incorporated free ^{14}C lysine and ^{14}C leucine into protein with cofactor requirements similar to those of microsomes from other sources.

5. The results indicate that free amino acids are utilized by embryonic tissues by routes similar to those found in adult tissues, and at rates that could probably account for the requirements of the organism for protein synthesis.

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The Utilization of *p*-Fluorophenylalanine for Protein Synthesis by the Phenylalanine-Incorporation System from Rabbit Reticulocytes

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The amino acid analogue *p*-fluorophenylalanine is incorporated into the proteins of both bacteria (Richmond, 1962) and animals, including specific proteins such as haemoglobin (Kruh & Rosa, 1959), aldolase and glyceraldehyde 3-phosphate dehydrogenase (Westhead & Boyer, 1961). The replacement of phenylalanine by *p*-fluorophenylalanine is only partial, however, and it therefore seemed possible that different amino acyl-transfer ribonucleic acids could act on the two amino acids and be responsible for the less efficient utilization of the analogue. Alternatively, the existence of degeneracy in the code for phenylalanine, i.e. the participation of more than one transfer RNA in the incorporation of phenylalanine, could result in discrimination against *p*-fluorophenylalanine, if one of the codes happened to favour phenylalanine

more than the other. Recent work showing that polyuridylic acid is capable of stimulating the incorporation of phenylalanine by cell-free preparations from *Escherichia coli* (Nirenberg & Matthaei, 1961; Lengyel, Speyer & Ochoa, 1961) or mammalian cells (Arnstein, Cox & Hunt, 1962; Griffin & O'Neal, 1962; Maxwell, 1962; Weinstein & Schechter, 1962) made possible a study of the incorporation of phenylalanine and *p*-fluorophenylalanine into protein in relation to the code for phenylalanine.

The cell-free system used in the present work consisted of ribosomes, amino acid-activating enzymes and transfer RNA from rabbit reticulocytes, and is capable of catalysing the whole process of protein synthesis, i.e. 'activation' of the amino acids followed by attachment to the appro-