

Changes in Macromolecular Synthesis Associated with the Induction of Glutamine Synthetase in Embryonic Retina

(hydrocortisone/differentiation/polysomes/cytosine arabinoside)

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ABSTRACT The hydrocortisone-mediated induction of glutamine synthetase in the neural retina of chicken embryo *in vitro* is correlated with enhanced incorporation into protein of [¹⁴C]aspartic acid, an amino acid abundant in this enzyme. In the induced retina labeled with [¹⁴C]-aspartic acid, a peak of radioactivity was detected in the region of the polysomal profile corresponding to polysomes comprising 12-14 ribosomes. In retinas labeled with [³H]uridine, an increased amount of radioactivity was also detected in the same polysomal region of the hydrocortisone-induced retina. If we assume a monocistronic messenger RNA for retinal glutamine synthetase, this region corresponds to the estimated size of the polysomes necessary for the translation of this enzyme. The evidence presented demonstrates a correlation between these changes in incorporation and the induction of glutamine synthetase.

The induction of glutamine synthetase in the embryonic neural retina by hydrocortisone lends itself well to studies on control mechanisms involved in differentiation (1, 2). The increase of the enzyme activity in the developing retina is correlated with its functional differentiation (3). The induction of the enzyme by hydrocortisone is specific for the neural retina and represents synthesis and accumulation of the enzyme (4, 5); it depends on gene action, since it is prevented by halting all RNA synthesis simultaneously with the initial addition of the inducer (1, 2); it involves an accumulation of stable RNA templates for glutamine synthetase, so that after 4-5 hr of induction, transcription can be completely stopped, yet the enzyme continues to be made for several hours on preformed templates (1, 2, 5, 6).

To further analyze specific changes elicited by hydrocortisone in the retina in connection with the induction of enzyme synthesis, we have examined the incorporation of [¹⁴C]Asp into nascent proteins in this system. The abundance of this amino acid in glutamine synthetase from other sources (7) prompted us to explore its usefulness for detecting induced synthesis of the retinal enzyme in tissue homogenates and at the polysomal level. We report here an enhancement by hydrocortisone of the incorporation of [¹⁴C]Asp into retina proteins, correlated with induction of the enzyme and with the synthesis of a new RNA.

MATERIALS AND METHODS

Tissue culture

Neural retinas from 12-, 14-, and 18-day chicken embryos were dissected aseptically and cultured at 37°C in 25-ml Erlenmeyer flasks containing 3 ml of medium, as described (2). Each flask contained retina tissue from a single eye. The culture medium consisted of: 79% Tyrode physiological salt

solution, 20% fetal-bovine serum, and 1% penicillin-streptomycin mixture (5000 units/ml). Cerebrum was obtained from 16-day chick embryos; each cerebral hemisphere was cultured in a separate flask containing 3 ml of medium. Sheets of skin from the backs of 8-day embryos were also cultured. Each flask contained skin from 4 embryos in 3 ml of medium.

Induction of glutamine synthetase was elicited by hydrocortisone (2) (3 µg/3 ml of culture medium); controls were cultivated without hydrocortisone.

Monolayer cultures of retina cells were prepared as described (8), from 10-day chick embryos; cells were plated in 30-mm Falcon culture dishes (about 4 × 10⁷ cells per dish) in 3 ml of medium. (79% Eagle's minimum essential medium, 20% fetal-bovine serum, and 1% penicillin-streptomycin.) After 40 hr of incubation at 37°C, the cells become noninducible for glutamine synthetase by hydrocortisone (8). The medium was then replaced by the Tyrode-serum-antibiotic medium described above and changed again to reduce the pool of free amino acids. The cultures were then exposed to hydrocortisone and radioactive precursors, as described (2), and harvested for further analysis.

Glutamine synthetase

The specific activity of glutamine synthetase was determined as described (2).

Homogenization and cell fractionation

Tissues and cells were washed in Tyrode solution and disintegrated by five gentle strokes in a Dounce homogenizer fitted with a loose pestle (cerebrum and skin required five additional strokes with a tight pestle); they were then lysed for 5-10 min in 0.5% Triton X-114 in buffer (0.05 M Tris·HCl (pH 7.4); 0.01 M magnesium acetate; and 0.1 M KCl) with 0.25 M sucrose. The lysates were centrifuged at 15,000 × *g* for 10 min to sediment nuclei, mitochondria, and debris. 1 ml of the supernatant was layered over 3-4 ml of 2 M sucrose in Tris·HCl-magnesium acetate-KCl buffer and centrifuged at 360,000 × *g* for 1 hr. The sediment was used as a source of polysomes.

Fractionation of polysomes

Polysomes were fractionated from 15,000 × *g*-supernatants in sucrose density gradients (15-40%) at 25,000 × *g* for 2 hr. 1-ml fractions were collected with an ISCO model D fractionator equipped with a UV-analyzer set at 254 nm.

Assays of radioactivity

For measurements of total incorporation of [¹⁴C]amino-acid mixture and [¹⁴C]Asp into proteins, labeled tissues or fractions

TABLE 1. Incorporation of [¹⁴C]Asp into proteins in retina and its correlation with the induction of glutamine synthetase

Expt.*	Tissue	[¹⁴ C]Asp incorporation (DPM/mg of protein)		Increase in incorporation (hydrocortisone/control)	Glutamine synthetase specific activity†		Increase in the specific activity of glutamine synthetase (hydrocortisone/control)
		Control	Hydrocortisone- treated		Control	Hydrocortisone- treated	
(i)	Retina—12 day	948	2,840	2.99 (2.14–3.39)	0.31	1.18	3.80
(ii)	Retina—12 day	13,741	27,200	1.97 (1.48–2.36)	0.35	1.23	3.50
(iii)	Retina—12 day	5,392	13,006	2.41	0.54	1.53	2.83
(iv)	Retina—14 day	5,656	14,856	2.62	0.72	1.81	2.51
(v)	Retina—18 day	7,664	10,364	1.35	4.23	4.60	1.09

* Standard labeling conditions (see *Results*) were used in experiments (i), (iii), (iv), and (v). In experiment (ii), proteins were labeled according to the standard conditions, except for omission of actinomycin D. In Expt. (i) and (ii), representative data are presented from a series of similar experiments; the range of increases in incorporation of [¹⁴C]Asp is shown in parenthesis. 80–90% of the acid-precipitable counts were from proteins and were insoluble in hot trichloroacetic acid.

† Specific activity of glutamine synthetase is expressed as μ mol of glutamyl hydroxamate formed per hr per mg of protein.

were sonicated in 1 ml of 0.01 M phosphate buffer (pH 7.1); aliquots from the sonicates were brought to 7.5% concentration of ice-cold trichloroacetic acid and left in the cold for 15–30 min. The precipitates were collected and washed on Millipore filters and the radioactivity was determined by liquid scintillation spectrometry (Nuclear Chicago Mark II Scintillation Counter). For measurement of radioactivity in polysomes labeled with [¹⁴C]Asp or [³H]uridine, fractions from sucrose gradients were precipitated with trichloroacetic acid, filtered, and counted as above.

Chemicals

[¹⁴C]L-amino-acid mixture, [¹⁴C]L-aspartic acid (> 150 Ci/mol) and [5-³H]uridine (>20 Ci/mmol) were obtained from New England Nuclear Corp.

RESULTS AND DISCUSSION

The following factors were important for demonstrating changes associated with the induction of glutamine synthetase by hydrocortisone as described here. (a) Preferential labeling with an amino acid abundant in the enzyme; for this [¹⁴C]Asp was chosen (see *Introduction*). (b) Conditions for optimal labeling of glutamine synthetase at which the enzyme synthesis proceeds at a fast rate, while that of other proteins is markedly reduced; these conditions, to be referred to as “standard labeling conditions,” consisted of culturing retina tissue for 4 hr in the presence of hydrocortisone to allow the accumulation of stable templates for the enzyme (controls were cultured without hydrocortisone); this was followed by a 2-hr inhibition of further RNA synthesis with actinomycin D (10 μ g/ml), to reduce the pool of short-lived mRNA; consequently, overall protein synthesis was decreased, but the glutamine synthetase formation continued, resulting in a relative “enrichment” of the enzyme synthesis. At the end of the second hour in actinomycin D, the cultures were pulsed for 15 min with radioactive amino acids, washed, harvested, and analyzed.

Incorporation of [¹⁴C]Asp into total proteins

The results of a 15-min pulse with [¹⁴C]Asp under standard labeling conditions (see above) showed that in the hydrocortisone-treated retina, in which glutamine synthetase was

induced, the incorporation of the label into total proteins was 2.1–3.4 times higher than in the noninduced controls [Table 1; Expt. (i)]. This difference was not observed if a mixture of [¹⁴C]amino acids was used, which indicates the preferential nature of labeling with [¹⁴C]Asp. Treatment with actinomycin D was not obligatory for the increased incorporation of [¹⁴C]Asp in the induced retina, but only served to amplify the difference; if it was omitted, incorporation of [¹⁴C]Asp was still higher in the hydrocortisone-treated retina than in the controls, albeit only by 1.4- to 2.5-fold [Table 1; Expt. (ii)], presumably because of continued synthesis of other proteins.

Correlation of [¹⁴C]Asp incorporation with the induction of glutamine synthetase

The increased incorporation of [¹⁴C]Asp into proteins in the hydrocortisone-treated retina is related to the magnitude of induction of glutamine synthetase and is not seen when hydrocortisone does not induce this enzyme. This was demonstrated by the following tests. (a) If glutamine synthetase induction was prevented by blocking RNA synthesis at zero hr with actinomycin D, there was no significant difference in [¹⁴C]Asp incorporation between the control and hydrocortisone-treated retina. (b) In normal ontogeny of the chicken embryo, glutamine synthetase activity in the retina is induced to rise sharply on the 16th day of development and, by day 18, the enzyme activity increases at a rapid rate that can only be slightly accelerated by the addition of hydrocortisone. If a direct correlation between increased glutamine synthetase accumulation and enhancement of [¹⁴C]Asp incorporation is assumed, one would expect this enhancement to be less in retinas from 18-day than in those from 12- or 14-day embryos since in the latter, hydrocortisone induces a much greater increase of the enzyme activity than in the former. The data show that in retinas from 18-day embryos that are treated with hydrocortisone, there was only about 30% increase in [¹⁴C]Asp incorporation over untreated controls, while in retinas from 12- and 14-day embryos, incorporation was 2.5–3 times higher than in untreated controls [Table 1; Expts. (iii)–(v)]; correspondingly, in the retinas of the younger embryos, hydrocortisone elicited in 6 hr a 3-fold increase of glutamine synthetase activity, as compared to only 10% increase in retinas from older embryos. (c) Disaggregated retina cells cul-

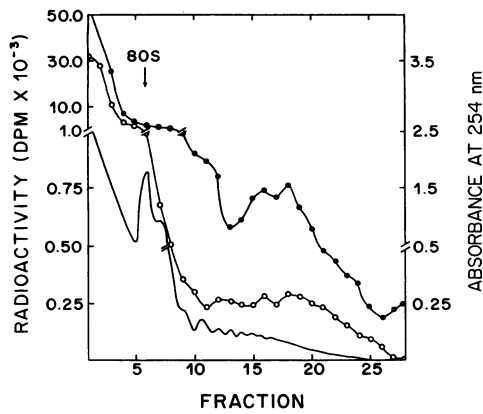


FIG. 1. Sucrose-gradient (15–40%) analysis of $15,000 \times g$ supernatants from control and hydrocortisone-induced retinas labeled in culture for 15 min with $[^{14}\text{C}]\text{Asp}$ ($3.3 \mu\text{Ci}/\text{ml}$), according to standard labeling conditions (see *Results*). Absorbance profiles (solid line) were identical in both cases. \circ — \circ , Radioactivity (control); \bullet — \bullet , Radioactivity (induced).

tured in a monolayer in the absence of hydrocortisone lose in 2 days 80–95% of their inducibility for the enzyme. When such monolayer cultures were pulsed with $[^{14}\text{C}]\text{Asp}$ in the presence or absence of hydrocortisone under standard labeling conditions, there was only a 15% increase in $[^{14}\text{C}]\text{Asp}$ incorporation in the hydrocortisone-treated cultures and, correspondingly, there was no significant increase of enzyme activity. (d) No significant difference in $[^{14}\text{C}]\text{Asp}$ incorporation was observed in control and hydrocortisone-treated tissues in which the enzyme is not inducible, e.g., skin or cerebrum of chick embryos, examined under standard labeling conditions.

Taken as a whole, these results demonstrate that the hydrocortisone-elicited increase in $[^{14}\text{C}]\text{Asp}$ incorporation into retina proteins is not due to a nonspecific effect of the hormone on embryonic cells. The increase in the incorporation of $[^{14}\text{C}]\text{Asp}$ is a characteristic of the neural retina; it occurs only when the retina cells are inducible for glutamine synthetase and is correlated with the magnitude of enzyme induction. While these data do not exclude the possibility that hydro-

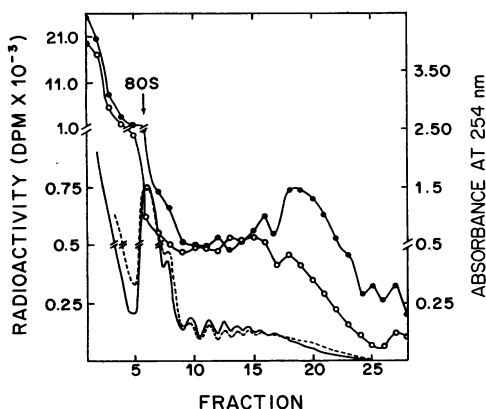


FIG. 2. Sucrose density gradient analysis of $15,000 \times g$ supernatants from control and retinas induced by cytosine arabinoside that were pulsed for 15 min with $[^{14}\text{C}]\text{Asp}$ at the end of the seventh hour in culture. — Absorbance (control); - - - Absorbance (induced); \circ — \circ Radioactivity (control); \bullet — \bullet Radioactivity (induced).

TABLE 2. Distribution of proteins labeled with $[^{14}\text{C}]\text{Asp}$ in different subcellular fractions from control and hydrocortisone-treated retinas of 12-day chick embryos

Fraction	Total incorporation of $[^{14}\text{C}]\text{Asp}$ (DPM/mg of protein)	
	Control	Hydrocortisone-treated
Pellet from $15,000 \times g$ (10 min) sediment (nuclei, mitochondria, and cell debris)	9,940	40,170
Pellet from $360,000 \times g$ (1 hr) sediment (ribosomal fraction)	8,060	16,810
Supernatant after $360,000 \times g$ (1 hr)	46,880	186,480

cortisone also enhances the incorporation of $[^{14}\text{C}]\text{Asp}$ into other proteins, they show that labeling with $[^{14}\text{C}]\text{Asp}$ under these experimental conditions is useful for further exploration of molecular events in the induction of glutamine synthetase.

Distribution of proteins labeled with $[^{14}\text{C}]\text{Asp}$ in different subcellular fractions

The distribution of $[^{14}\text{C}]\text{Asp}$ in different subcellular fractions of retinas from control and 12-day embryos that are treated with hydrocortisone was examined, under standard labeling conditions (Table 2). In both control and hormone-treated retinas, about 75% of the trichloroacetic acid-insoluble radioactivity was present in the soluble tissue fraction, representing the bulk of the newly made proteins. About 10% of the radioactivity was present in the polysomal fraction, presumably as nascent proteins, and about 15% was present in the fraction containing nuclei, mitochondria, and cell debris.

Analysis of polysome preparations

Analyses of polysomal profiles for the incorporation of $[^{14}\text{C}]\text{Asp}$ revealed significant differences between control and hydrocortisone-induced retinas (Fig. 1). (a) A generally higher amount of radioactivity throughout the entire profile from the induced retina; (b) a zone of increased radioactivity in the retina induced with hydrocortisone in the region of polysomes containing 8–15 ribosomes (gradient fractions 15–20), with an apparent peak in the region of 12–14 polyribosomes. Since the absorbance (254 nm) of the polysomal profiles from control and induced retina were essentially similar, the differences in $[^{14}\text{C}]\text{Asp}$ distribution are due to a greater incorporation of the label into nascent proteins in polysomes from the induced retina.

If we assume a monocistronic message, mRNA attached to 12–14 ribosomes corresponds to a protein chain of a molecular weight of 35,000–45,000; estimates of molecular weight of the subunit of glutamine synthetase from rat liver, pea, and sheep brain fall in the range of 40,000–50,000 (7). Preliminary data from sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggest that the subunit of retinal glutamine synthetase also has a molecular weight in the same range.* These considerations, together with the correspondence between $[^{14}\text{C}]\text{Asp}$ incorporation and the enzyme induction, suggest that the radioactivity peak in Fig. 1 is related to synthesis of the

* Sarkar, P. K., and A. A. Moscona, to be published.

enzyme in induced retinas. Clearly this suggestion remains to be tested by further experiments.

A correlation between glutamine synthetase induction by hydrocortisone and the presence of the [^{14}C]Asp-labeled peak in the polysomal profile was further supported by the absence of this peak in polysome preparations from cultures in which, as described earlier in this paper, hydrocortisone did not induce glutamine synthetase: cultures of retina-cell monolayers, cerebrum from 16-day embryos, and skin from 8-day embryos.

Further evidence that the peak of [^{14}C]Asp radioactivity in the polysomal profile is correlated with the induction of glutamine synthetase was provided by experiments in which the enzyme was induced by a preparation of cytosine arabinoside† (9). The distribution of [^{14}C]Asp radioactivity in polysomal profiles from retinas cultured in the presence and absence of this nucleoside (Fig. 2) showed a distinct peak in the region of 12- to 14- ribosome polysomes in the induced retina. The similarity, in this specific sense, between the effects of such different agents as hydrocortisone and cytosine arabinoside that share the property of inducing retinal glutamine synthetase, is consistent with the apparent correlation between the radioactivity peak and synthesis of the enzyme.

Distribution of rapidly labeled RNA in the polysomal profile

An increased amount of RNA labeled with [^3H]uridine was detected in the polysomal profile from hydrocortisone-induced retina in the region of the peak of [^{14}C]Asp incorporation (Figs. 1 and 2). For doing these experiments, advantage was taken of the rapid accumulation of stable templates for glutamine synthetase during the first 4 hr of induction, and of the fact that transcription can be halted thereafter without stopping the enzyme synthesis (2, 4, 5). Retinas cultured in medium with or without hydrocortisone were pulsed for 15 min with [^3H]uridine after 2.5 hr of cultivation, i.e., at the time of presumably rapid synthesis of templates for the enzyme. RNA synthesis was then stopped with actinomycin D (10 $\mu\text{g}/\text{ml}$) and, after 15 min, the retinas were washed, transferred into fresh culture medium with actinomycin D, and incubated for an additional 2 hr to allow the decay of short-lived messages. Since actinomycin D (10 $\mu\text{g}/\text{ml}$) requires 15 min to completely stop all RNA synthesis in these cells, the actual labeling time was between 15 and 30 min.

The absorbance profiles of polysomes from control and induced retinas were similar; however, in the profile from induced retina (Fig. 3), there was an increased amount of ^3H -radioactivity in the region of polysomes containing 8–15 ribosomes (gradient fractions 15–20), i.e., in the zone of high [^{14}C]Asp incorporation. These findings raise the possibility

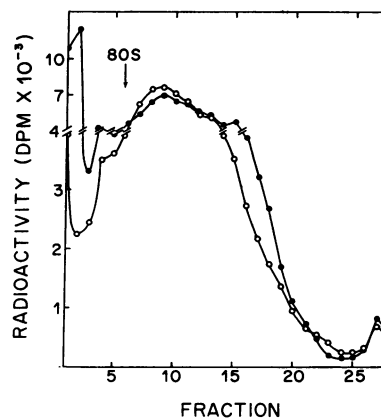


FIG. 3. Distribution of the rapidly labeled RNA in the polysomal profiles. Control and induced retinas were pulsed for 15–30 min with [^3H]uridine (33 $\mu\text{Ci}/\text{ml}$) after 2.5 hr in culture and incubated for 2 hr more in the presence of actinomycin D (10 $\mu\text{g}/\text{ml}$). 15,000 $\times g$ supernatant was prepared and fractionated on sucrose gradients. Absorbance profiles were identical in both cases and similar to that shown in Fig. 1. ○—○ Radioactivity (control); ●—● Radioactivity (induced).

that the hydrocortisone-induced RNA may represent mRNA for glutamine synthetase.

In summary, we have demonstrated changes at the polysomal level in the incorporation of [^{14}C]Asp into proteins and of [^3H]uridine into rapidly labeled RNA that are closely correlated with induction of the enzyme in the retina.

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† Cytosine arabinoside from Sigma (Lot no. 88B-0470) was used. It is not clear whether glutamine synthetase induction by this preparation is due to the nucleoside or to a contaminant; this is currently being examined. The mechanism of induction by this agent is also being investigated.