IMMUNOCHEMICAL EVIDENCE FOR ENZYME SYNTHESIS IN THE HORMONAL INDUCTION OF GLUTAMINE SYNTHETASE IN EMBRYONIC RETINA IN CULTURE*

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The hormonal induction of glutamine synthetase (GS) in the embryonic neural retina offers various advantages for detailed studies on the mechanisms of phenotypic changes in differentiation. In the normal ontogeny of the chick embryo, GS activity in the retina increases sharply on the 16th day of development in correlation with other aspects of differentiation; however, this enzyme can be induced to rise precociously in retinas from much younger embryos by treating this tissue in culture with certain 11β-hydroxycorticosteroids, such as hydrocortisone (Moscon and Piddington, 1966; Piddington, 1967). Previous studies have shown that RNA and protein synthesis are required for GS induction (Moscona et al, 1968), but left open the question whether the increase in GS activity was due to enzyme synthesis or to activation or assembly of pre-existing precursors. The relevance of such information to embryonic induction in general and the scarcity of embryonic system for studying enzyme induction prompted us to examine this problem. The immunochemical determinations summarized here offer evidence that the hormonal induction of GS in the embryonic retina is due primarily to enzyme synthesis and accumulation.

MATERIALS AND METHODS

Neural retina tissue from 12-day chick embryos was cultured for 24 hours, or less, in 25 ml Erlenmeyer flasks with 3 ml medium on a gyrator (70 rpm) at 37°C in Eagle's basal medium with 20% fetal bovine serum and 1% penicillin-streptomycin mixture (Moscona and Hubby, 1963; Moscona et al, 1968). C^{14}-labeled amino acids (New

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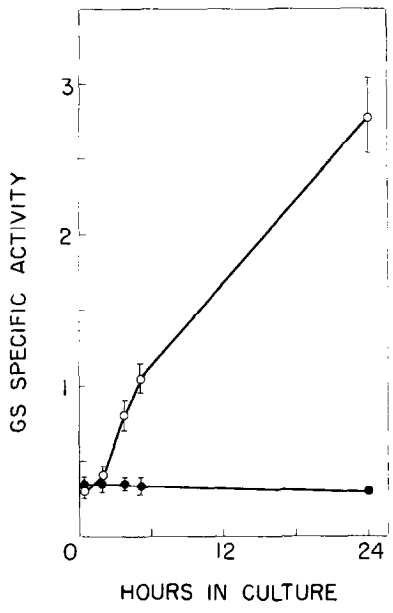
England Nuclear, NEC 445, S.A.~1mC/mg) were added as described below. GS was induced by hydrocortisone, 1μg/3ml, added to the culture medium at zero hour. Enzyme specific activity was determined as before (Kirk and Moscona, 1963). Proteins were determined according to Lowry et al (1951).

Antisera were prepared in rabbits against (1) GS from chick retina partially purified by adsorption to glass (Kirk, 1967) by a procedure modified from Kogut et al, (1956); (2) GS from sheep brain, partially purified according to Pamiljans et al, (1962). Both antisera were found to precipitate GS from sonicates of embryonic chick retina with very similar efficiencies. Immunological determinations were carried out on sonicates of whole retina in 0.01 M PO_4-buffered saline, pH 7.1, centrifuged at 40,000 rpm for 20 minutes ("40,000 rpm supernatant"). Antiserum was mixed with the supernatant at dilutions optimal for the highest efficiency of precipitation (see below); after 30 minutes at room temperature and overnight at 4°C, the precipitate was sedimented by centrifugation, washed, placed on filter discs and treated with cold TCA, preparatory to measurements of radioactivity. Since the antiserum did not destroy enzyme activity the residual GS activity in the supernatant was compared with the total amount present before the precipitation, as a measure of precipitation efficiency. C^{14}-incorporation into retina proteins in the 40,000 rpm supernatant was measured after TCA-precipitation on filter discs (Mans and Novelli, 1961). Radioactivity was measured with a Nuclear Chicago Mark 1 Liquid Scintillation Counter.

RESULTS

GS induction: Fig. 1 shows the 24-hour rise of GS activity in retinas induced by hydrocortisone in culture, and the unchanged enzyme level in the non-induced control cultures. Fig. 2 shows that there is no significant difference in the incorporation and accumulation of C^{14}-amino acids in total proteins of induced and control retinas.

Precipitation of GS by the antisera. The optimal proportion of GS to antiserum was determined from the precipitin curve illustrated in Fig. 3. Using this proportion the precipitation efficiency in the following experiments was at least



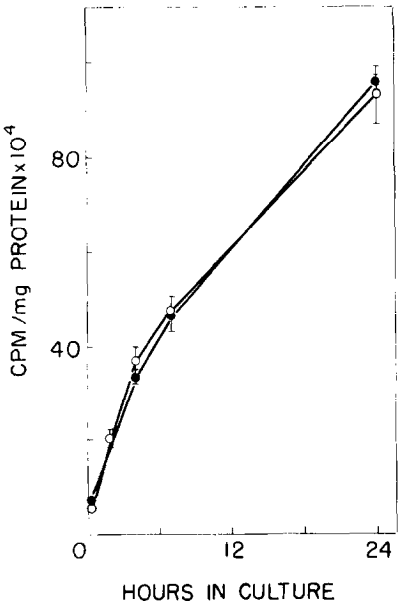


Fig. 1 Average GS specific activities in induced (open circles) and control (closed circles) retina, as a function of cultivation time. Standard errors ($s/\sqrt{n}$) in this and other figures are indicated when larger than the points. There is practically no cell proliferation in this tissue during this time.

Fig. 2 Average C^{14}-amino acids incorporation into TCA-insoluble protein in induced (open circles) and control (closed circles) retinas, in the continuous presence of 5μC/3 ml of the label.

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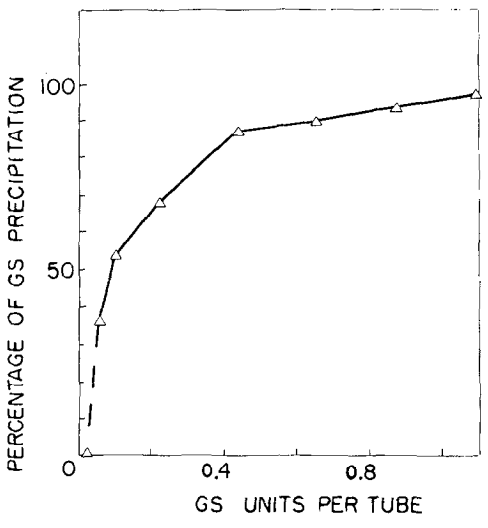
of the order of 80% for both antisera.

Accumulation of labeled GS was measured by adding C^{14}-amino acids at zero hour to cultures in inducing and in control media and determining the amount of radioactivity in material precipitated with anti-GS serum. Fig. 4 shows the stea accumulation of the labeled antigen in the induced retina which closely correspon to the increase in GS activity in this tissue. In the non-induced controls there was some accumulation of radioactivity in precipitable material between zero and 4 hours of culture, but practically none thereafter.

The above evidence for GS synthesis and accumulation in GS induction raises the question whether the low level of GS activity in the non-induced state is due to minimal synthesis and/or to a constant turnover of the enzyme. Changes in the



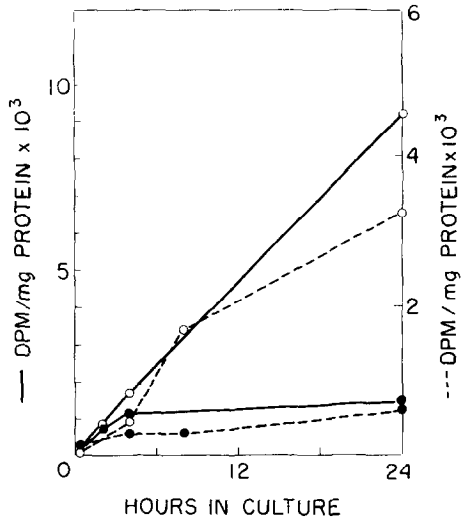


Fig. 3 Precipitation curve of GS: GS activity units precipitated by anti-GS serum (against sheep brain GS) from supernatant of sonicates of induced embryonic chick retinas; plotted as a function of GS concentration. Ten per cent anti-GS serum was mixed 1:1 with serial dilutions of the 40,000 rpm supernatant to give decreasing GS activity. The final volume of the mixture was always 1 ml. Enzyme activities in the precipitate and in the residual supernatant were found to be additive.

Fig. 4 Accumulation of C^{14}-labeled material precipitable with anti-GS serum in induced (open circles) and control (closed circles) retina. Solid lines left scale: results obtained with antiserum against chick retina GS (label: 5μC/3 ml). Broken lines - right scale: results obtained with antiserum against sheep brain GS (label: 2.5μC/3 ml).

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rate of degradation play an important role in enzyme regulation (Schimke et al, 1965; Kenney, 1967). In the embryonic chick retina measurements of protein turnover are complicated by the large pool of amino acids. In preliminary studies we determined the hourly rate of GS synthesis in induced and control retinas at different times after explantation, by pulsing for one hour with C^{14}-amino acids and measuring the amount of radioactivity precipitable with the antiserum. Two such experiments (summarized in Fig. 5) suggested that in induced retinas the rate of GS formation increased between 4 and 7 hours, then declined somewhat; in the non-induced controls there was a lower amount of precipitable radioactivity. The rate of GS formation was consistently higher in induced than in control retinas; however, the induced/control ratios differed sufficiently from one experiment to

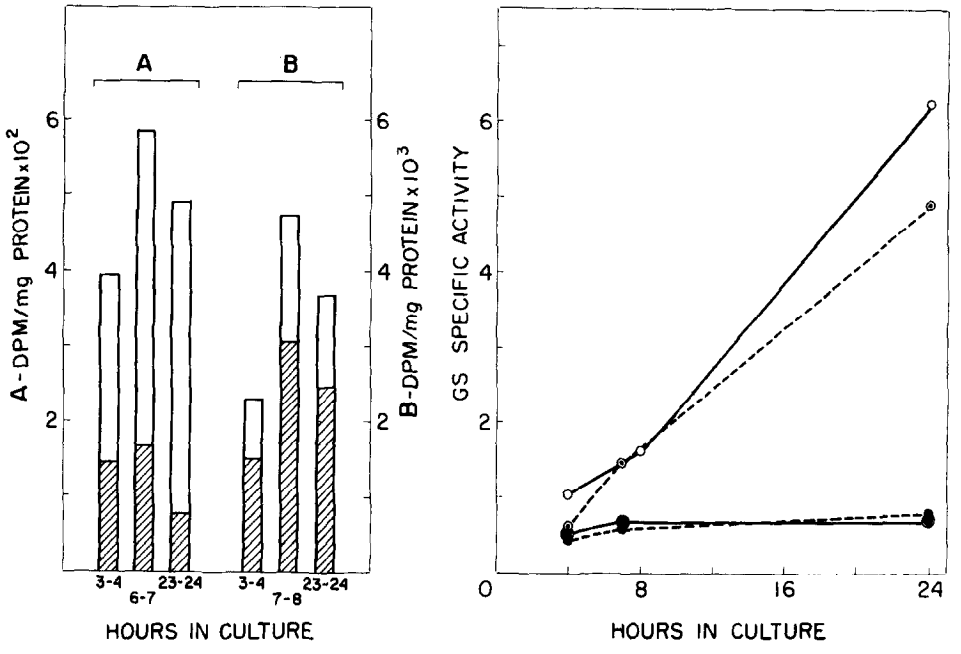

Fig. 5 Hourly rate of incorporation of C^{14}-amino acids into GS in induced (whole bar) and control (shaded part) retinas. A, left scale: radioactivity precipitated by anti-GS serum (against sheep brain GS) from retinas labeled for 1 hour with 2.5μC/ml of C^{14}-amino acids. B, right scale: radioactivity precipitated by anti-GS serum (against chick retina GS) from retinas pulsed for 1 hour with 5μC/ml of the label.

Fig. 6 GS specific activities in the experiments in Fig. 5. Solid lines: experiment in Fig. 5A; broken lines: experiment in Fig. 5B; induced-open circles; control-closed circles.

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another to preclude absolute measurements. Nevertheless, the data raise the possibility that there is synthesis of GS also in the non-induced retina; however, this point clearly requires further examination, particularly in view of the likelihood of non-specific and heterogeneous precipitation by the antiserum. If, in fact, the enzyme is being continuously made in the non-induced tissue it must evidently also be continuously degraded at a rate which results in an almost steady net level of GS activity in the non-induced retina (Fig. 6).

Assuming that turnover may be a factor in the regulation of GS accumulation, the question arose whether there is enzyme turnover also in the induced tissue when GS is made at a faster rate. From the data in Fig. 5 we calculated the

TABLE 1

	Experiment in Fig. 5A			Experiment in Fig. 5B		
	4 hours	24 hours	Increase Factor	4 hours	24 hours	Increase Factor
Calculated accumulation of radioactivity in GS; DPM/mg Protein	1.58	11.74	7.43	9.20	86.00	9.35
GS specific activity	1.05	6.22	5.92	0.63	4.90	7.78

theoretically expected accumulation of radioactive GS in the induced retina, given that there is no degradation of the enzyme, and compared this with the actual increase in GS specific activity in the same experiments (Fig. 6). For these calculations we assumed that the hourly rate of C^{14}-incorporation actually measured, applied in each case also to the preceding time interval. Accordingly, (Table 1) if there is no GS turnover in the induced retinas between 4 and 24 hours, there should be a sevenfold accumulation of radioactivity in the experiment in Fig. 5A, and a ninefold accumulation in the one in Fig. 5B. The data in Fig. 6 (and Table 1) show that this prediction corresponds closely to the actual increase in GS activity at that time, which is approximately sixfold and eightfold in the two respective experiments; since nearly 80% of the GS, immunochemically detectable as newly synthesized in that phase of induction can be accounted for as accumulation of enzyme activity, it follows that no more than 20% of the enzyme is turning over at that time. However, this value may be too high, possibly due to the error inherent in such calculations, or to non-specific precipitation by the antiserum (particularly with the anti-chick GS serum); therefore, during the induced accumulation of GS in the period covered by these tests there is little GS turnover, while in the non-induced state the low level of the enzyme is maintained by turnover.

COMMENTS

The steroid-induced rapid rise in GS activity in the embryonic retina has now been shown to involve primarily enzyme synthesis and accumulation. This

statement is supported by the good correlation between the increase in the amount of labeled antigen precipitated by the antiserum and the increase in specific enzyme activity. The available data also suggest, but do not prove, that the low level of GS in the non-induced retina is due to a slow rate of enzyme synthesis and to steady turnover. We, therefore, suggest, as a basis for further study, that GS induction in the embryonic retina represents an increase in the rate of enzyme synthesis and probably a reduction in the rate of its degradation, from the level in the non-induced state. Mechanisms that might be involved in the regulati of these processes were considered elsewhere in the light of the requirements for RNA synthesis in GS induction (Moscona et al, 1968). The present findings make possible the testing of these suggestions as a further step towards clarification of inductive processes in embryonic cells.

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