INDUCTION OF GLUTAMINE SYNTHETASE IN EMBRYONIC NEURAL RETINA IN VITRO BY INHIBITORS OF MACROMOLECULAR SYNTHESIS

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Summary - In the embryonic neural retina glutamine synthetase (GS) is induced by hydrocortisone and related 11β-hydroxycorticosteroids which cause the accumulation of stable, active templates for the synthesis of this enzyme. It has now been shown that, under suitable experimental conditions GS can be induced by exposure of the isolated retina to cytosine arabinoside, or by treatment with cycloheximide followed by inhibition of transcription. It is suggested that these agents induce GS in the retina by blocking the production of inhibitor(s) which in the uninduced cells prevent the accumulation of stable, active templates for this enzyme.

The induction of glutamine synthetase (GS) in the embryonic neural retina of the chick is associated with the functional differentiation of this tissue and lends itself well to studies on control mechanisms in embryonic development (1,2). Retinal GS can be induced specifically by hydrocortisone and related 11β-hydroxycorticosteroids *in vivo* and in isolated retina in organ culture (3,4,5). The rapid increase of GS activity induced by the steroid is due to an increase in the rate of enzyme synthesis (6,7). The induction requires RNA synthesis (1,8,9) but continued exposure to the inducer results in the accumulation of stable and active templates for GS, so that after 4-5 hours of induction transcription can be completely halted (with Actinomycin D) yet GS continues to be made for a considerable time (1). Previous studies have suggested that the activity of these stable templates is subject to post-transcriptional control by a labile inhibitor (suppressor) which is counteracted by the inducer. This short-lived inhibitor can be stopped in the induced retina by blocking all RNA synthesis, whereupon GS continues to be made by the preformed, stable templates (1).

The present experiments were directed towards the question whether de-inhibition was involved also in the initial phases of GS induction; more specifically, whether in the uninduced retina, the accumulation of stable templates for GS is prevented by short-lived products and whether the removal of these products would confer upon the retina an induced state with respect to GS. Assuming such products to be protein or RNA, we attempted to stop their production in uninduced retina by treatment with inhibitors of macromolecular synthesis and to find out if this would lead to a subsequent increase of GS without addition of the steroid inducer. We report here that cycloheximide and cytosine arabinoside, applied to the isolated neural retina under conditions suitable for testing the above assumption, elicited an induction of GS in the absence of the steroid inducer. Studies are in progress to further determine if these results actually substantiate the underlying assumptions; however, since the available information may be of immediate interest to others concerned with enzyme induction in eukaryotic cells it is briefly described here.

Retina tissue freshly obtained from 12-day chick embryos was maintained in flask cultures at 37°C under 5%CO_2-air mixture in Tyrode's physiological salt solution with 20% fetal bovine serum (non-inducing medium) (1). Cycloheximide (a translational inhibitor of protein synthesis) was added at concentrations of 200-500 µg/ml. Controls contained no cycloheximide. After 5 hours of incubation, cycloheximide was thoroughly washed out to allow resumption of protein synthesis; some of the cultures were transferred into fresh, non-inducing medium, others into medium with Actinomycin D (Act D; 10 µg/ml) to halt all further RNA synthesis. The purpose of halting transcription at this time was twofold: (a) to determine if stable and potentially active templates for GS had accumulated during the treatment with cycloheximide (1); from previous experience such templates should mediate GS synthesis in the total absence of further transcription (1); (b) to stop RNA for the post-transcriptional inhibitor (1). After additional 19 hours of incubation the retinas were collected and the specific activity of

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(1 to 5 hours). Thus, the transient treatment of the uninduced retina with this dose of cycloheximide simulates the effect of the steroid inducer in the sense of allowing the accumulation of stable, potentially active templates for GS.

The fact that the subsequent expression of these templates (i.e., the increase of GS after removal of cycloheximide) occurs only if further RNA synthesis is inhibited (or if the steroid inducer is added, Fig. 1) is consistent with the notion of the post-transcriptional labile inhibitor, as explained above, and parallels the situation in steroid induction: if the steroid inducer is removed by thorough washing after 4 - 5 hours of induction (by which time stable templates are present) GS levels off (Fig. 2); but if, at the same time all further RNA synthesis is stopped, GS continues to increase (Fig. 2). Thus, the requirements for the steroid inducer in the initial and in the

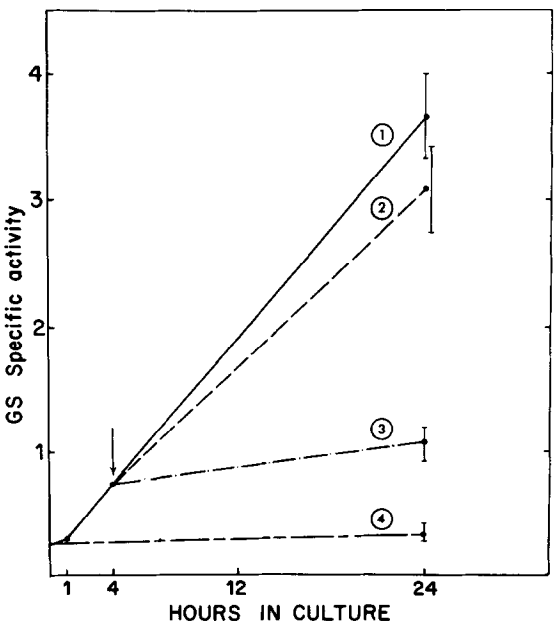

FIGURE 2. Induction of GS by hydrocortisone. ① Retinas cultured for 24 hours in the presence of hydrocortisone (1 µg/3ml medium). ② Hydrocortisone was washed out at 4 hours (arrow) and the retinas were transferred to non-inducing medium containing 10 µg/ml of Act D; GS activity continued to increase. ③ Hydrocortisone was washed out at 4 hours and the retinas were transferred to non-inducing medium; GS activity leveled off. ④ Retinas cultured in non-inducing medium for 24 hours.

later phases of GS induction can be replaced by the consecutive applications of the inhibitors of macromolecular synthesis, cycloheximide and Act D.

The concentrations of cycloheximide required to obtain the above results are higher than necessary for the complete blocking of protein synthesis and cause also inhibition of approximately 40% of total RNA synthesis (over a period of 4 hours in culture). Therefore, it is possible that this partial inhibition of transcription, rather than the block to translation, is primarily responsible for the effect on GS. To examine this possibility we determined if other agents which partially inhibit transcription but, unlike cycloheximide, do not completely stop protein synthesis, would cause GS induction. Of those tested so far, cytosine arabinoside was found to be highly effective.

Cytosine arabinoside was added at a concentration of 10^{-3}M to cultures of retina from 12-day chick embryos in non-inducing medium. After 24 hours of incubation in the continued presence of cytosine arabinoside, GS specific activity was found to have risen to levels similar to those elicited by the steroid inducer. Cytidine (10^{-3}M) or arabinose (10^{-3}M) had no such effect. If RNA synthesis was completely blocked with Act D at the time of the addition of cytosine arabinoside there was no induction of GS. However, as in the case of induction by the steroid, after 4 - 5 hours of exposure to cytosine arabinoside, further RNA synthesis could be completely halted, without stopping the increase of GS. Evidently, cytosine arabinoside, like the steroid inducer and like cycloheximide allows the accumulation of stable, active templates for GS.

At the concentration required for GS induction cytosine arabinoside inhibits approximately 40% of total RNA synthesis in the retina (11). It is of interest that a quantitatively similar inhibition of RNA synthesis with a low dose of Act D (0.05 - 0.1 μg/ml) does not result in GS induction in the retina. Experiments are in progress to determine if the induction of GS by cycloheximide or by cytosine arabinoside is in fact due to their partially inhibitory effect on RNA synthesis; if so, their differential mode of action with respect to that of Act D should prove useful for further analysis of the con-

trol mechanisms of GS. One possibility is that the RNA stopped by cycloheximide and cytosine arabinoside (as used in these experiments) includes the postulated labile inhibitor of template accumulation and that the blocking of its formation accounts for the induction of GS by these agents.

Since enzyme levels in eukaryotic cells are subject to multiple types of regulation (12,13,14), it remains to be determined if the similarities in the final outcome on GS between the effects of the steroid inducer and of the other two inducing agents reflect common levels and modes of regulation, or are due to quite different reasons.

The present information is consistent with the working hypothesis that the induction of GS in the embryonic neural retina involves the accumulation of stable, active templates for GS and that in the uninduced retina this is prevented by a labile inhibitor(s); treatment with steroid inducer, cycloheximide or cytosine arabinoside counteracts this inhibition. The available evidence further supports earlier suggestions that also the expression of the stable templates is subject to post-transcriptional inhibition by a labile product which requires the synthesis of RNA. It remains to be determined whether the same regulatory mechanisms are involved in inhibiting the accumulation and the expression of the templates for GS in the embryonic retina.

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