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Preferential Inhibition by Proflavine of the Hormonal Induction of Glutamine Synthetase in Embryonic Neural Retina

(acridines/enzyme induction/gene regulation/differentiation)

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ABSTRACT **The hormonal induction of glutamine synthetase (EC 6.3.1.2) in embryonic neural retina tissue *in vitro* is blocked preferentially and reversibly by proflavine (3,6-diaminoacridine) in the absence of cell or DNA replication; cell viability is not affected, and the synthesis of total cellular proteins and RNA is only slightly reduced. In the induction of this enzyme, there is a rapid increase in the synthesis and accumulation of the enzyme selectively elicited by the steroid inducer; for this effect, transcription is essential. Radioimmunochemical measurements have shown that proflavine inhibits induction by prevention of *de novo* synthesis of catalytically active and immunologically reactive glutamine synthetase protein. It does not measurably affect the uptake of the steroid inducer by the retina cells. Since the translation of this enzyme by preformed RNA templates is not stopped by proflavine, the inhibitory effect of proflavine on induction is apparently due to interference with transcriptional or pretranslational processes required for the provision of active transcripts for enzyme synthesis. The finding that proflavine inhibits preferentially a tissue-specific, inducible differentiation in postmitotic embryonic neural cells offers new approaches to the study of regulation of gene expression in eukaryotes.**

The feasibility of experimentally controlling specific gene expression in embryonic cells under defined conditions would be of considerable usefulness in the analysis of mechanisms of differentiation. In the course of studies on the hormonal induction of glutamine synthetase (EC 6.3.1.2) in embryonic neural retina (1), we have explored the possibility of selectively blocking this differential gene expression by chemical agents. We found that proflavine (3,6-diaminoacridine) inhibited this induction preferentially and reversibly. Since a selective inhibition by an acridine of an experimentally inducible, gene-controlled differentiation in embryonic cells is of general interest, we report here our findings.

The induction of glutamine synthetase in primary cultures of neural retina tissue from chick embryos by 11β-hydroxycorticosteroids, and its relation to retina differentiation have been described (2); this induction involves synthesis and accumulation of the enzyme. In the embryonic retina glutamine synthetase activity begins to increase rapidly on day 16 in response to a rise in systemic corticosteroids (3). However, glutamine synthetase can be induced precociously in retinas isolated from younger embryos by treatment with hydrocortisone (1, 4); the hormone elicits promptly a rapid increase of enzyme synthesis, without affecting overall protein synthesis in the retina (5–7). Transcription is essential for this induction, and results in accumulation of stable, active RNA templates for the enzyme synthesis (1, 7). Cell proliferation or DNA replication is not involved, and glutamine synthetase can be induced in the isolated retina under conditions of completely arrested DNA synthesis (2); accordingly, 5-bromodeoxyuridine, which interferes with the expression of differentiated functions in some other tissues (8), does not inhibit the induction of glutamine synthetase (2).

The inhibition of glutamine synthetase induction by proflavine is not a general property of acridines, and proflavine is by far the most effective among several acridine derivatives tested thus far; their degree of inhibition of the induction is related to number and position of amino and methyl groups in the analogue (manuscript in preparation).

MATERIALS AND METHODS

Flask Cultures of Neural Retina tissue isolated aseptically from 12-day chick embryos were prepared and incubated at 38° on a gyratory shaker (1). The freshly obtained retinas were placed in 25-ml Erlenmeyer flasks (1 retina per flask) with 3 ml of medium [Tyrode solution, 20% fetal-bovine serum, and 1% penicillin-streptomycin mixture (5000 units/ml); culture flasks with proflavine (Nutritional Biochemicals) were wrapped with foil. To induce glutamine synthetase, hydrocortisone was added at a concentration of 0.91 μM. The specific activity of the enzyme was assayed in tissue sonicates by the glutamyl-transferase reaction (1). Addition of proflavine or of sonicates of proflavine-treated retinas directly to the enzyme assay mixture did not interfere with the assay and did not modify the enzyme activity.

Incorporation of Radioactive Precursors into retina DNA, RNA, and proteins was measured after trichloroacetic acid precipitation of tissue sonicates on Millipore filter disks (1, 7). For *radioimmunochemical measurements* of the enzyme, antiserum prepared against purified retinal glutamine synthetase was used (5, 7). For *electrophoretic separation* of labeled glutamine synthetase, polyacrylamide–sodium dodecyl sulfate gels were used (9); these were run for 3 hr at 10 mA/gel and sliced; the slices were placed in 1-ml tissue solubilizer (NCS, Amersham/Searle), and processed for counting in a scintillation counter (1).

RESULTS

Inhibition of glutamine synthetase induction by proflavine

The induction of retinal glutamine synthetase was completely prevented by 8 μM proflavine (2.5 μg/ml) added to the retina cultures simultaneously with the steroid inducer (Fig. 1). The effect of proflavine was concentration dependent; the dose–

response relationship was linear between 1 μM, which did not inhibit glutamine synthetase induction, and 8 μM, which inhibited it completely.

Reversibility of inhibition

The inhibitory effect of 8 μM proflavine was reversible (Fig. 2); after removal of the tissue from proflavine-containing medium, repeated washing, and transfer to culture medium with inducer, the retina regained at least 60% of its inducibility during 18 further hours of cultivation. The degree of reversibility was related to the efficacy of the washing procedure. Reversibility did not require DNA synthesis; it took place in the presence of 10 μM cytosine arabinoside, which blocks DNA replication in retina (2, 10). It is, therefore, unlikely that, under these experimental conditions, the inhibition by proflavine of glutamine synthetase induction is due to mutagenic effects of the kind that may be produced by proflavine in other systems (11, 12). This conclusion is in accord with the fact that the inhibition of glutamine synthetase induction by proflavine can occur without ongoing DNA replication, i.e., in the presence of 10 μM cytosine arabinoside (10).

Cell viability and steroid uptake

The inhibition of glutamine synthetase induction by 8 μM proflavine is not due to general cytotoxicity; monolayer cultures of retina cells maintained for 48 hr in this concentration of proflavine remained alive after transfer to proflavine-free medium. The blocking of the enzyme induction is also not due to simple interference by proflavine with the uptake of the steroid inducer by the retina cells; measurements of [^{14}C]-hydrocortisone accumulation revealed no significant difference between retina cultured in the presence and absence of proflavine.

Proflavine (8 μM) suppressed about 40% of DNA synthesis in retina, as determined by measurements of [^{3}H]thymidine incorporation; however, the block to glutamine synthetase induction is not due to this suppression, since even complete inhibition of DNA synthesis in 12-day embryonic retina by cytosine arabinoside does not prevent the induction by the steroid inducer (2, 10).

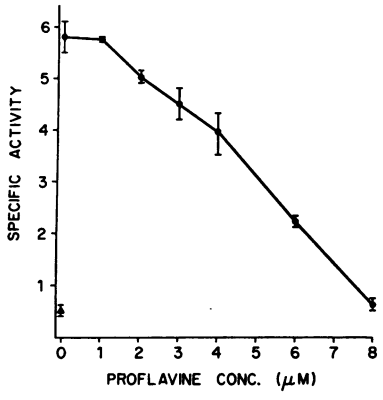

FIG. 1. Inhibition of the induction of glutamine synthetase by proflavine. Neural retinas from 12-day chick embryos were cultured in medium with the steroid inducer (hydrocortisone) and with different concentrations of proflavine; after 24 hr, the specific activity of glutamine synthetase was determined. The inhibitory effect of proflavine was concentration dependent; in 8 μM proflavine the level of synthetase activity was as in uninduced controls (▲). *Points* represent averages obtained in different experiments; *vertical lines* indicate the ranges.

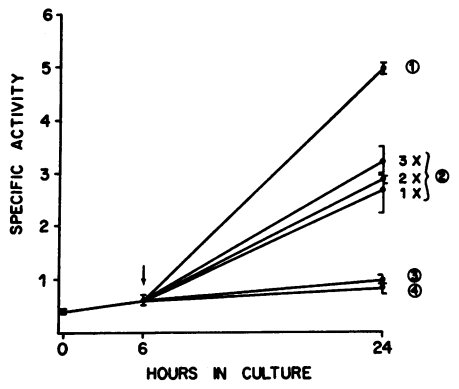

FIG. 2. Reversibility of the proflavine block of glutamine synthetase induction in neural retina. The recovery of inducibility after removal of proflavine was examined in retinas cultured for 24 hr as follows: (*1*) no proflavine; inducer (hydrocortisone) added at 6 hr (*arrow*); (*2*) 8 μM proflavine for the first 6 hr, then washed in proflavine-free medium 1, 2, or 3 times and transferred to medium with hydrocortisone; (*3*) control cultures without proflavine or hydrocortisone; (*4*) control cultures with 8 μM proflavine, to which hydrocortisone was added at 6 hr without removal of proflavine.

Differential effect of proflavine on protein synthesis in retina

Overall protein synthesis in retina tissue was only slightly reduced by 8 μM proflavine; in retinas cultured with and without proflavine for 4 or 24 hr and pulsed with a mixture of [^{14}C]aminoacids for the last 15 min of either period, there was only a 5-10% reduction by proflavine of ^{14}C incorporation into total proteins. To determine if gross differences existed between the proteins made in proflavine-treated retina and in controls, retinas were cultured for 24 hr in the presence of [^{14}C]aminoacids and inducer, with and without proflavine; from these, supernatant fractions of material soluble at 100,000 × *g* were prepared (for methods, see Table 1) and fractionated by polyacrylamide-sodium dodecyl sulfate gel electrophoresis. No major differences were found in the ^{14}C distribution profiles between proflavine-treated and untreated retinas; the absence of such differences indicates that proflavine did not massively alter the character of the majority of proteins made in these cells during the labeling period, and points to a preferential nature of proflavine action on glutamine synthetase induction in retina.

Since the induction of glutamine synthetase involves synthesis of the enzyme protein, we examined if the block by proflavine was due to inhibition of enzyme synthesis, and thus to a differential effect on protein synthesis in these cells. Using radioimmunoprecipitation procedures to measure directly the synthesis of glutamine synthetase, we found that in the presence of 8 μM proflavine the enzyme synthesis was completely suppressed.

Table 1 shows that in induced retina the amount of immunoprecipitated radioactivity was several times greater than that in the uninduced controls; addition of proflavine together with the inducer totally prevented this increase, and the amount of immunoprecipitable counts was similar to that of uninduced controls.

That the labeled immunoprecipitate from proflavine-treated retina contained no newly made glutamine synthetase was

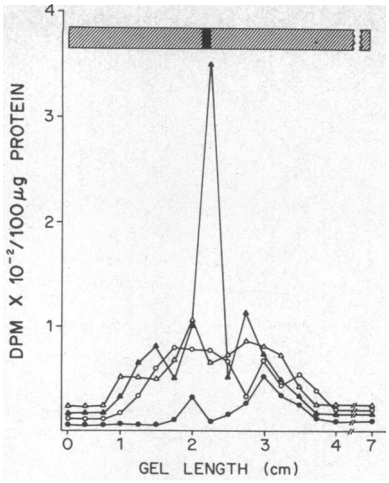

FIG. 3. Gel electrophoresis of immunoprecipitates, showing the inhibition by proflavine of glutamine synthetase synthesis in neural retina. Immunoprecipitates were obtained as described in Table 1. After washing, the precipitates were dissolved and dissociated in 2% sodium dodecyl sulfate (SDS), and fractionated with polyacrylamide–SDS gel electrophoresis (10% gels, 70 × 6 mm, run 3 hr at 10 mA/gel). Profiles of radioactivity obtained by fractionation of preparations obtained from retina tissue cultured with the steroid inducer (▲——▲) show a peak of radioactivity corresponding in position to the band of glutamine synthetase subunits (*inset on top*); the peak represents glutamine synthetase made during the labeling period and precipitated by the anti-glutamine synthetase serum. This peak is absent if proflavine (8 μM) was added to the cultures together with the inducer (O——O), or if neither inducer nor proflavine were present (controls) (Δ——Δ). The specificity of glutamine synthetase precipitation by the anti-glutamine synthetase serum was tested by the use of normal serum (●——●).

confirmed by fractionation of the precipitates by polyacrylamide–sodium dodecyl sulfate gel electrophoresis (Fig. 3): ^{14}C distribution profiles on the gels revealed that while the immunoprecipitates from induced retinas contained a radioactivity peak in the position corresponding to that of the glutamine synthetase band, this peak was absent in material

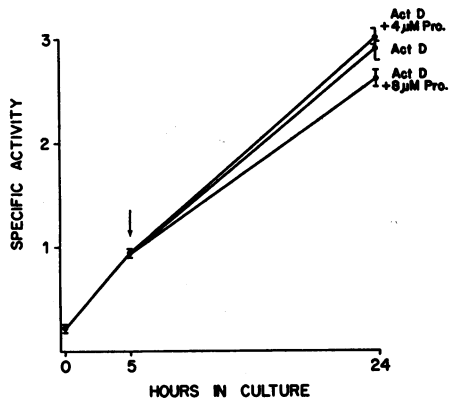

FIG. 4. Evidence that proflavine does not block the translation of preformed templates for glutamine synthetase. Retinas were cultured for 5 hr in medium containing the steroid inducer; transcription was then stopped (*arrow*) with actinomycin D (10 μg/ml), and the cultures were continued for 19 hr, either in the absence of proflavine or with proflavine (*Pro*) added simultaneously with actinomycin D (*Act D*).

from retinas treated with proflavine together with inducer, as well as from uninduced retinas. Since mixing experiments of sonicates from induced and proflavine-treated retina failed to demonstrate in the latter the presence of glutamine synthetase destroying activity, we conclude that the blocking of this induction by proflavine results in the absence of synthesis of the enzyme.

Translation of templates for glutamine synthetase is not stopped by proflavine

That the inhibition of glutamine synthetase induction is not due to a direct action of proflavine on the translation of the messenger RNA for the enzyme was demonstrated in the following experiments. Retinas were induced for 5 hr to elicit the synthesis and accumulation of stable, active RNA templates for the enzyme; transcription was then stopped with actinomycin D (10 μg/ml), which does not prevent continued enzyme synthesis by the preformed templates (1, 6, 7) (Fig. 4). Addition of 8 μM proflavine together with actinomycin D did not stop the enzyme increase, a result showing that the inhibition by proflavine of the synthesis of this enzyme is not exerted at the level of translational processes.

Transcription of rapidly labeled RNA is essential for glutamine synthetase induction, and complete inhibition of RNA synthesis with actinomycin D at the time of addition of the inducer totally prevents induction (1, 7). It is, therefore, of interest that 8 μM proflavine, which blocks induction, inhibits only 12% of RNA synthesis in the retina of 12-day chick embryos; the possibility arises that the induction-blocking effect of proflavine might be due to preferential interference with the provision of functional transcripts essential for the synthesis of glutamine synthetase. Since 8 μM proflavine does not prevent the translation of the preformed enzyme templates,

TABLE 1. *Inhibition of the synthesis of glutamine synthetase by proflavine*

Tissue	Counts precipitated by anti-glutamine synthetase serum (dpm/mg of protein)
(*1*) Uninduced control	7,760
(*2*) Hydrocortisone-induced	17,062
(*3*) Hydrocortisone and 8 μM proflavine	5,960

Neural retinas from 12-day embryos were cultured for 24 hr as follows: (*1*) without inducer (control); (*2*) with inducer (+hydrocortisone); (*3*) with inducer and proflavine (8 μM). After 1 hr of incubation, 0.5 μCi/ml of [^{14}C]aminoacid mixture was added to all cultures for the remaining 23 hr of incubation. The tissues were then washed, sonicated in 0.01 M phosphate buffer (pH 7.2), and centrifuged at 100,000 × *g* for 30 min; the supernatant was obtained and immunoprecipitations were performed with anti-glutamine synthetase serum, with normal serum as a control for non-specific precipitation. The precipitated material was washed, taken up in 0.1 M NaOH, reprecipitated with 15% trichloroacetic acid, collected on Millipore filters, washed, dried, and counted. The data represent the difference between the counts precipitated by antiserum and normal serum. The amount of radioactivity precipitated specifically by the anti-glutamine synthetase serum provided a measure of accumulation of newly-made synthetase during the labeling period.

the block to induction is evidently due to inhibition of either the transcription essential to this induction or of post-transcriptional processing, transfer, or stability of the relevant transcripts.

It should be pointed out that the inhibition of glutamine synthetase induction is unlikely to be the only differential effect of proflavine on the synthesis of proteins in these cells; the susceptibility of other specific cell functions to proflavine is being investigated.

DISCUSSION

The finding that proflavine blocks preferentially and reversibly the hormonal induction of glutamine synthetase in embryonic neural retina offers a new means for study of control mechanisms in the differentiation of this tissue. The immediate issue raised by this finding concerns the basis of this apparently selective effect of proflavine on a specific, inducible gene expression. Information about proflavine effects in other inducible embryonic tissues could provide further insight into this problem.

Our results show that this preferential blocking of glutamine synthetase induction does not involve DNA replication, that proflavine does not hinder the uptake of the inducer, and that it does not directly prevent the translation of glutamine synthetase message. Concerning the mechanism and site of this blocking effect, the evidence directs attention to transcriptional and pretranslational processes involved in the control and regulation of glutamine synthetase induction. Among the most readily envisioned levels of proflavine action in this tissue are: reaction of the inducer (or inducer-receptor product) with the genome; transcription of the structural and regulatory genes involved in the enzyme synthesis and accumulation; or processing, stability, and intracellular transport of these gene products. A heuristic model has been proposed for the mechanism of glutamine synthetase induction (2, 7) that indicates feasible approaches towards analysis of the site and mode of proflavine action in this system.

Concerning the preferential character of the proflavine effect on this system, it is of interest that the selective mutagenicity of proflavine in T4 bacteriophage differs uniquely from that of other mutagens (11), and may be related to base sequences at the sensitive site, especially to the presence of monotonous regions (12). Intercalation of acridines into DNA (13), the apparent cause of frameshift mutations in T4 bacteriophage (14), may conceivably produce the kind of reversible nonmutagenic effects that could block glutamine synthetase induction; interference by proflavine with DNA-dependent RNA polymerase (15) at particular sites could block transcription of the affected cistrons; alternatively, frameshift misreading of a specific region (14) intercalated by proflavine could result in transcription of nonsense sequences. Conceivably, proflavine might associate with RNA and hinder its normal activity; or it might interfere with the formation or properties of polyadenylate sequences involved in the provision of functional messenger RNA.

Since there is no present evidence that the selectivity of proflavine action in our system reflects affinity for particular base sequences, other possibilities should not be excluded. Thus, at physiological pH, proflavine is positively charged and might, therefore, interact with anionic groups on DNA (16) or with acidic chromosomal proteins; the latter have been implicated in the control of hormonal induction of protein synthesis (17). Finally, it should be of interest to determine the range of the inhibitory selectivity of proflavine in retina and in other eukaryote cells—whether this range is limited to certain tissue-specific inducible gene expressions, or includes other products for which continuous transcription is required.

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