EFFECTS OF CYTOSINE ARABINOSIDE ON DIFFERENTIAL GENE EXPRESSION IN EMBRYONIC NEURAL RETINA

II. Immunochemical Studies on the Accumulation of Glutamine Synthetase

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ABSTRACT

Cytosine arabinoside (Ara-C) elicits a significant increase in the level of the enzyme glutamine synthetase (GS) while it markedly reduces overall RNA and protein synthesis in cultures of embryonic chick neural retina. This increase was analyzed by radioimmunochemical procedures and compared with the induction of GS by hydrocortisone (HC). Accumulation of GS in Ara-C-treated retinas was found to be due to *de novo* synthesis of the enzyme; however, unlike the induction of GS by HC, Ara-C caused no measurable increase in the rate of GS synthesis. The results indicate that Ara-C facilitates GS accumulation largely by preventing degradation of the enzyme. Even though Ara-C inhibits the bulk of RNA synthesis in the retina, it does not stop the formation of GS-specific RNA templates. However, the progressive accumulation of these templates does not result in an increased rate of GS synthesis unless Ara-C is withdrawn from such cultures under suitable experimental conditions. Thus, it is suggested that the continuous presence of Ara-C imposes a reversible hindrance at the translational level which limits the rate of GS synthesis. The results demonstrate that the increase in retinal GS elicited by Ara-C is achieved through mechanisms which are quite different from those involved in the hydrocortisone-mediated induction of this enzyme.

During embryonic differentiation, genotypically identical cells undergo alterations in patterns of specific macromolecular synthesis and thus become phenotypically diverse. An experimental system which has proven useful for investigating mechanisms governing differential gene expression in embryonic cells is the retina-specific induction of the enzyme glutamine synthetase (GS) (EC 6.3.1.2) by hydrocortisone (HC) in cultures of embryonic chick neural retina tissue (for reviews, see references 25–27, 37). These studies have shown that GS induction involves: (*a*) complexing of the steroid with cytoplasmic receptors, (*b*) binding of these complexes to sites in the nucleus, (*c*) transcriptional and posttranscriptional modifications, and (*d*) an increased rate of synthesis and accumulation of the enzyme (20, 26, 27, 29, 30, 34).

Recently it has been found that relatively high concentrations of 1-β-D-arabinofuranosylcytosine

(cytosine arabinoside; Ara-C) which inhibit RNA synthesis in the retina by ~80% can elicit a several-fold increase in retinal GS activity in the absence of the steroid inducer (15, 28). This effect of Ara-C is dependent upon specific sites on both the sugar and pyrimidine moieties of the molecule.[1] Investigations of the mechanism of Ara-C action in the retina have demonstrated that this effect is not related to the conventional action of Ara-C as an inhibitor of DNA synthesis (7, 42). The evidence suggested that, under these conditions, the nucleoside acts primarily through transcriptional processes which indirectly lead to the provision and accumulation of template RNA essential for the subsequent synthesis of GS (14, 15). Moreover, in the preceding paper of this series (15), the possibility was raised that Ara-C may also act at translational or post-translational levels of control.

Previous measurements of GS specific activity in Ara-C-treated retinas (19, 29) did not resolve the question of whether the increases in GS levels were due to an accelerated rate of synthesis or a decreased rate of degradation of the enzyme. This and related questions bearing on the Ara-C-mediated changes in retinal GS levels were examined in the present study by radioimmunochemical measurements: rates of GS synthesis were monitored by precipitating radioactively labeled GS from retina sonicates, using a highly specific preparation of γ-globulin isolated from antiserum against purified retinal GS, the most sensitive method currently available for analysis of enzyme synthesis in this system. Comparison of the results obtained in this investigation with immunochemical data on GS induction by hydrocortisone demonstrates that the two effectors elicit increases in GS levels by different mechanisms.

MATERIALS AND METHODS

Tissue Culture

Neural retina tissue was aseptically isolated from white Leghorn chicks on the 10th day of embryonic development (14, 29). Each retina was cultured at 37°C in a 25-ml Erlenmeyer flask containing 3 ml of culture medium (Tyrode's balanced salt solution with 20% fetal bovine serum [FBS] and 50 U/ml each of penicillin and streptomycin). The medium was gassed with 5% CO_2-95% air mixture and the flasks were rotated 70 rpm, as previously described (15, 30). All culture media were supplemented with the same preparation of FBS (lot no. 2071382, tissue culture select grade, BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) which was prescreened to ensure the absence of effectors which increase retinal GS activity (14).

Increases in the level of GS were elicited by adding Ara-C hydrochloride (lot no. 3129W, Upjohn Co., Kalamazoo, Mich.) to the culture medium at the beginning of incubation to a final concentration of 7.5 mM. Steroidal induction of GS was achieved by adding hydrocortisone (free alcohol) to the culture medium to a final concentration of 0.92 μM, as previously described (29, 30). Actinomycin D (Act D) (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.) was added to retina cultures to a final concentration of 10 μg/ml, as indicated in the text. This dose of the antibiotic inhibits 98–99% of [^{3}H]uridine incorporation in the retina without inactivating GS or blocking the translation required for GS accumulation (26, 29, 30). Whenever retinas were transferred to fresh culture medium, they were washed in three changes of final medium in flasks rotated at 100 rpm, as described before (15). At the end of the incubation period, the retinas were rinsed in cold Tyrode's solution and prepared for enzyme assay, scintillation counting, or immunological determinations, as described below.

Physicochemical Analyses of Ara-C

The preparation of Ara-C used in this study and several other lots of the nucleoside obtained from different commercial suppliers have been extensively screened by the following methods: ultraviolet and infrared spectroscopy; recrystallizations from solution; organic solvent extractions; Sephadex column chromatography; paper, thin-layer, and gas-liquid chromatography. These tests[1] revealed no detectable steroidal contaminants in any preparation of Ara-C used in our studies.

Enzyme Assay

Retinas were suspended in 10 mM phosphate buffer, pH 7.1, and disrupted at 0°C with six 1-s bursts of ultrasound from a Biosonik III model B10-111 sonifier (Will Scientific, Inc., Rochester, N. Y.) tuned to maximum output. Aliquots of tissue sonicates were assayed for GS activity by the glutamyltransferase reaction (19); replicate aliquots were assayed for protein by the method of Lowry et al. (24), using bovine serum albumin as the standard. GS specific activity was expressed as micromoles of γ-glutamylhydroxamate (GHA) formed per hour per milligram of tissue protein. The presence of Ara-C, HC, or Act D in the enzyme assay mixture did not interfere with measurements of GS activity.

Radioisotopic Labeling

Retina proteins were labeled by adding ^{14}C-L-amino acid mixture (NEC-445, 10 mCi/mmol each; New England Nuclear, Boston, Mass.) or ^{3}H-L-amino acid mixture (NET-250; New England Nuclear) to the culture medium. The quantity of isotope and duration of expo-

[1] Jones, R. E. Manuscript in preparation.

sure to labeled precursors are indicated in the text and legends. Measurements of isotope incorporation into retina proteins, amino acid uptake, and procedures for liquid scintillation counting were as previously described (15, 29). The radioactivity of each sample was expressed as disintegrations per minute per milligram of protein.

Gamma Globulins

Normal (preimmune) rabbit serum and antiserum against highly purified chick retina glutamine synthetase were obtained as described before (33, 36). γ-Globulin was isolated by ammonium sulfate precipitation, according to the procedure of Stelos (41). The reactions of GS with normal and anti-GS γ-globulins were studied by quantitative immunoprecipitin tests (see legend, Fig. 1), polyacrylamide gel electrophoresis, and immunoelectrophoresis.

Immunoelectrophoresis

The immunological specificity of anti-GS γ-globulin was monitored by immunoelectrophoresis using glass slides coated with 1% agarose (Bio-Rad Laboratories, Richmond, Calif.) in 0.025 M barbital buffer, pH 8.2. Two antigen solutions were tested: (*a*) 105,000-*g* supernates of retina sonicates (25 mg of lyophilized adult retina tissue in 1 ml of 10 mM phosphate buffer, pH 7.1), and (*b*) highly purified chick retina glutamine synthetase prepared as described by Sarkar et al. (36). Approx. 15 μl of each antigen (≈250 μg of protein) were electrophoresed for 1 h in barbital buffer using an LKB model 3276-50 electrophoresis apparatus (LKB Instruments, Inc., Rockville, Md.); average voltage and current were 325 V and 6 mA, respectively. Antigens were then reacted with 175–200 μl of anti-GS γ-globulin (upper troughs), and with the same amount of normal γ-globulin (lower troughs). The slides were placed in a humidified chamber for 30 h at 4°C for development of precipitin arcs, then washed for 48 h at 4°C in several changes of 2% NaCl, rinsed with distilled water, and dried for 5 h at 37°C. The slides were stained for 5 min with 2% amido black 10 (Chroma-Gesellschaft, Schmid and Co., Stuttgart-untertürkheim, Germany) in glacial acetic acid-methanol-water (1:2:2), destained with 2% acetic acid, and dried. Replicate immunoelectrophoretograms were obtained using serially diluted preparations of antigen and γ-globulin.

Radioimmunoassay

The radioimmunoassay for measuring newly synthesized GS in the retina was modified from that previously described (1, 2, 30). Cultures of 10-day embryonic neural retina were supplied with ^{3}H- or ^{14}C-amino acid mixture; labeling conditions are noted in the text and legends. At the end of the labeling period, the retinas were thoroughly washed with cold Tyrode's solution and sonicated in 10 mM phosphate-0.85% NaCl buffer, pH 7.1 (PBS). 105,000-*g* supernates of retina sonicates were prepared and aliquoted for reaction with γ-globulins. Unlabeled carrier GS from adult chick retina was added to each aliquot to bring the total enzyme activity units to a uniform level (2.5 GHA) optimal for maximal precipitation of the enzyme. GS was precipitated from these samples with 20 μl of anti-GS γ-globulin; nonspecific precipitation of radioactivity was monitored by reacting replicate aliquots with γ-globulin isolated from normal rabbit serum (see below). The following additional components were included in the precipitation mixture: 500 μg of bovine serum albumin, 500 μg of sodium deoxycholate, 20 μl of 2.5% Triton X-100; PBS was added to bring the final volume to 1.0 ml. Immunoprecipitation reactions were performed in sterile plastic test tubes to avoid the adherence of labeled proteins to glass. The precipitation mixtures were incubated for 30 min at 37°C and 12–16 h at 4°C. The immunoprecipitates were collected by centrifugation, washed three times with PBS containing 0.05% sodium deoxycholate and 0.05% Triton X-100, and processed for liquid scintillation counting, as described before (15). The disintegrations per minute per milligram of protein precipitated with normal γ-globulin were subtracted from that precipitated with anti-GS γ-globulin to obtain the radioactivity due to specific precipitation of isotopically labeled GS.

Controls for Nonspecific Radioactivity in Immunoprecipitates

In addition to using normal γ-globulin as a control for nonspecific radioactivity in antigen-antibody complexes (see above), we have monitored nonspecific radioactivity by another widely used procedure (16, 17, 21, 32, 43, 44), originally described by Schimke et al. (39). For these tests, retinas were cultured either in medium containing 7.5 mM Ara-C or in control medium (without Ara-C). ^{3}H-L-amino acid mixture was added to all cultures at the beginning of incubation to a final concentration of 16.7 μCi/ml. After 24 h, the retinas were harvested and aliquots of 105,000-*g* supernates were reacted with anti-GS or normal γ-globulin, as described above. The supernates remaining after removal of materials precipitated by anti-GS γ-globulin were subjected to a second precipitation by again adding unlabeled carrier GS and anti-GS γ-globulin in antigen-antibody equivalence; the second precipitates were processed identically to the first. The radioactivity in the second precipitate was subtracted from the radioactivity in the first to calculate the quantity of labeled GS specifically precipitated by anti-GS γ-globulin.

Table I (column 6) shows that the average percent of total radioactivity precipitated by normal γ-globulin varied between 0.14 and 0.21% for control and Ara-C-treated retinas, respectively. Anti-GS γ-globulin precipitated only about 0.05% of the total radioactivity remaining after the first immunoprecipitation with anti-GS γ-globulin, regardless of the source of antigen. The facts that the values obtained with normal γ-globulin were significantly greater than the corresponding values ob-

TABLE I

*Comparison of Procedures for Determination of GS-Specific Radioactivity in Immunoprecipitates**

Treatment	Total acid-insoluble radioactivity	Retina protein reacted	Gamma globulin	Radioactivity precipitated by gamma globulin	Total radioactivity precipitated by gamma globulin	Net GS radioactivity	Net GS radioactivity expressed as % of total acid-insoluble radioactivity
	dpm/mg Protein $\times 10^{-6}$	*mg*		*dpm/mg Protein* $\times 10^{-4}$	*%*	*dpm/mg Protein* $\times 10^{-4}$	
First Immunoprecipitation							
Ara-C	6.08	0.64	Anti-GS	7.19	1.18	5.91‡	0.97
			Normal	1.28	0.21		
Control	7.03	0.75	Anti-GS	2.20	0.31	1.21‡	0.17
			Normal	0.99	0.14		
Second Immunoprecipitation							
Ara-C	6.01	0.63	Anti-GS	0.30	0.05	6.89§	1.15
Control	7.01	0.75	Anti-GS	0.40	0.06	1.80§	0.26

* Values shown are averages of triplicate determinations.

‡ Radioactivity precipitated by anti-GS γ-globulin minus radioactivity precipitated by normal γ-globulin.

§ Radioactivity precipitated by anti-GS γ-globulin during first immunoprecipitation minus radioactivity precipitated by anti-GS γ-globulin during second immunoprecipitation.

tained with the second immunoprecipitation procedure, and that they showed differences dependent upon whether or not the tissue was treated with Ara-C, suggest that the normal γ-globulin controls detect the entrapment, co-precipitation, or denaturation of labeled non-GS proteins; moreover, the generation of this nonspecific radioactivity is apparently restricted primarily to the incubation period of the first immunoprecipitation. Under such conditions, these labeled nonspecific proteins would be removed during the first immunoprecipitation with anti-GS γ-globulin and their contribution could not be detected by performing a second immunoprecipitation. Accordingly, we have corrected all immunoassay data in this report for nonspecific radioactivity by subtracting the radioactivity precipitated by normal γ-globulin from that precipitated by anti-GS γ-globulin, rather than employing the second immunoprecipitation procedure.

Polyacrylamide Gel Electrophoresis

Radioactively labeled immunoprecipitates (see legend, Fig. 2) were subjected to electrophoresis on gels containing 5% acrylamide, 0.19% *N,N'*-methylenebisacrylamide, and 0.2% sodium dodecyl sulfate (SDS), as described (8, 40). Immunoprecipitates were prepared for electrophoresis by incubating each sample at 37°C for 15 min with 200 μl of electrophoresis buffer and 50 μl of the following mixture: 5% SDS, 50 mM Tris·HCl (pH 8.0), 5 mM EDTA, 200 mM dithiothreitol, 50% sucrose, and 50 μg/ml of pyronin Y (8). Electrophoresis was performed at 8.5 mA/gel and terminated when the tracking dye had migrated 85 mm from the origin. The gels were cut into 1.5-mm slices and placed in scintillation counting vials (two slices per vial). Radioactive materials were eluted by incubating the slices for 4 h at 50°C with 1 ml of NCS (Amersham/Searle Corp., Arlington Heights, Ill.); toluene-based fluors and ^{133}Ba external standardization were utilized for liquid scintillation counting, as described before (15).

GS from chicken retina was obtained as previously described (36); 10 μg of enzyme was treated with SDS, as described above, electrophoresed as marker, and localized by staining with Coomassie Blue R-250.

RESULTS

Specificity of Anti-GS γ-Globulin

A typical quantitative immunoprecipitin reaction between anti-GS γ-globulin and GS is shown in Fig. 1. In this test, anti-GS γ-globulin precipitated up to three enzyme activity units at an efficiency >92%; γ-globulin from normal serum precipitated <1% of the input GS activity units. When a crude extract of total retina proteins was subjected to immunoelectrophoresis, only a single precipitin arc was obtained upon reaction with anti-GS γ-globulin (Fig. 1, inset *A*). An identical arc was obtained when highly purified GS was used as the antigen (Fig. 1, inset *B*). Serially diluted preparations of antigen or γ-globulin yielded identical immunoelectrophoretograms. There were no detectable arcs when the electrophoresed antigens were reacted with normal γ-globulin.

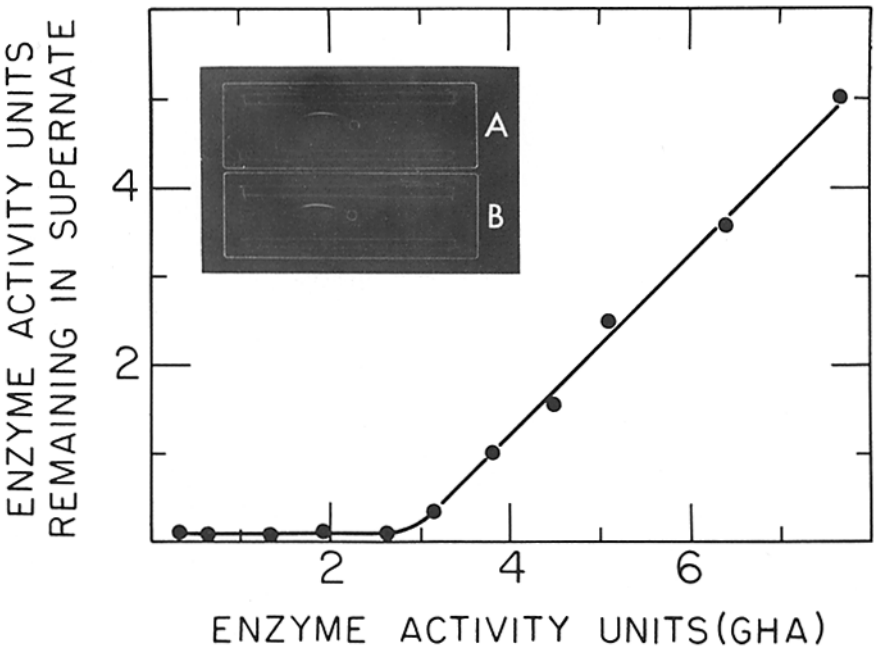

FIGURE 1 Reactions between GS and anti-GS γ-globulin. Lyophilized adult retina tissue was sonified in 10 mM PO_4-0.85% NaCl buffer, pH 7.1 (PBS), and 105,000-*g* supernates were diluted with PBS to a final concentration of 10 GS enzyme activity units (GHA) per ml. Aliquots of this mixture containing increasing quantities of GHA were precipitated with 20 μl of anti-GS γ-globulin (see Materials and Methods). Immunoprecipitates were collected by centrifugation and the supernates were assayed for GS activity. A quantitative immunoprecipitin curve was obtained by comparing the GHA added to the reaction mixture with the GHA remaining in the supernate after immunoprecipitation. Inset: Immunoelectrophoretograms (for details, see Materials and Methods). Upper troughs, anti-GS γ-globulin; lower troughs, normal γ-globulin. Antigen(s) in center well: (*A*) total retina proteins (105,000-*g* supernates); (*B*) highly purified GS from chicken retina.

Fig. 2 shows the results of polyacrylamide gel electrophoresis of immunoprecipitates formed by the reaction of anti-GS or normal γ-globulin with radioactively labeled proteins from control and Ara-C-treated retinas. The immunoprecipitates obtained with anti-GS γ-globulin contained only one predominant peak of radioactivity which electrophoresed precisely to the location of the GS subunit marker. Conversely, there was no significant radioactivity detectable in the GS region when precipitates formed with normal γ-globulin were subjected to electrophoresis. Background radioactivity across the gel (presumably due to nonspecific entrapment of labeled materials) was virtually identical in immunoprecipitates formed by either anti-GS or normal γ-globulin. Thus, the above tests indicate that (*a*) anti-GS γ-globulin selectively precipitates this enzyme from a mixture of total retina proteins, and (*b*) the low levels of nonspecific radioactivity in immunoprecipitates obtained with anti-GS γ-globulin can be monitored reliably by determining the radioactivity precipitated by normal γ-globulin.

Accumulation of GS in Embryonic Neural Retinas Treated with Ara-C

When neural retina tissue from 10-day chick embryos is cultured for 24 h in medium containing 7.5 mM Ara-C, GS specific activity increases several-fold (Fig. 3). Previous work has shown that the kinetics of GS accumulation closely resemble those obtained with the steroid inducer hydrocortisone and that RNA and protein synthesis are required for the increase in GS activity mediated by the nucleoside (15). As shown in Fig. 3 [+(Act $D)_{0\ h}$], addition to the cultures of 10 μg/ml Act D together with Ara-C prevented the increase of GS. However, if Act D was added after 5-h exposure

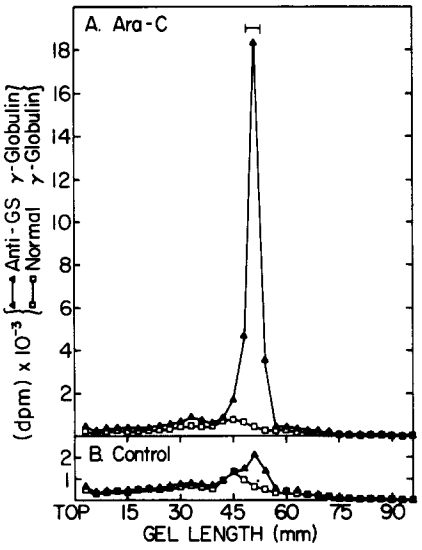

FIGURE 2 Polyacrylamide gel electrophoresis of immunoprecipitates. Retinas were cultured either in medium containing 7.5 mM Ara-C or in medium lacking Ara-C (controls). ^{3}H-L-amino acid mixture was added to all cultures at the beginning of incubation to a final concentration of 16.7 μCi/ml. After 24 h of labeling, the retinas were harvested, sonicated in PBS, and centrifuged at 105,000-*g* for 30 min. Aliquots of the supernates (each containing 1.91 mg protein) were precipitated with either anti-GS γ-globulin or normal γ-globulin, using the radioimmunoassay protocol given in Materials and Methods. The immunoprecipitates were washed three times with PBS containing 0.05% sodium deoxycholate and 0.05% Triton X-100, treated with SDS, and electrophoresed on 5% polyacrylamide gels (see Materials and Methods). Data above show the distribution of radioactivity obtained when 760 μg of each solubilized immunoprecipitate was subjected to electrophoresis. Horizontal bar indicates position of GS subunits run in parallel gels as marker (molecular mass ~42,000 daltons).

to Ara-C, GS activity continued to accumulate throughout the 24-h culture period to levels close to those obtained with Ara-C alone [+(Act D)$_{5\,h}$]. Furthermore, when retinas were cultured for 5 h in medium with Ara-C, then thoroughly washed and transferred into medium containing only Act D, GS activity increased to levels that often exceeded those reached in the continuous presence of the nucleoside [W(Act D)$_{5\,h}$]. No measurable increase in GS activity was elicited when Act D was added to retinas cultured in the absence of Ara-C, regardless of the time of administration of the antibotic. Previous findings have shown that cycloheximide prevents the increase in GS activity which occurs during Act D treatment (15), indicating that this transcription-independent increase in enzyme activity requires protein synthesis. The following immunochemical experiment demonstrated that the increase in GS activity during Act D treatment represents accumulation of newly synthesized GS.

Retinas were cultured in either the presence or absence of 7.5 mM Ara-C and were labeled with 1.0 μCi/ml ^{14}C-amino acid mixture from the beginning of incubation. After 5 h, Act D (10 μg/ml) was added to half of the Ara-C-treated cultures; the remainder was incubated continuously with the nucleoside. After varying periods of incubation, GS specific activities were determined and the quantity of ^{14}C-labeled GS was measured by immunoassay. The results (Table II) showed that increases of enzymatic activity coincided with the accumulation of ^{14}C-labeled GS, whether or not Act D was added after the 5th h of culture with Ara-C. There was only slight accumulation of labeled GS in control cultures, which corresponded to the basal increase in GS specific activity typical of retinas cultured in medium without added effectors (see also Table I and Fig. 2).

The above results have indicated that (*a*) RNA synthesis is required during the first 5 h of treatment with Ara-C in order to initiate the increase

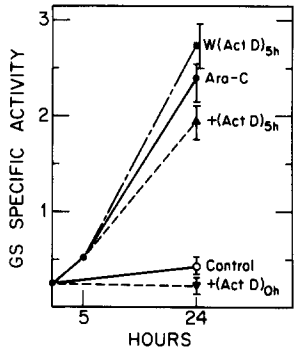

FIGURE 3 Effects of Act D on GS accumulation in Ara-C-treated retinas. Retinas were cultured in medium containing either Ara-C (7.5 mM) or no additions (controls). The tissues were treated with Act D (10 μg/ml) and GS specific activities were compared after 24 h of incubation under the following conditions: +(Act D)$_{0h}$, Act D added at 0 h with or without Ara-C; +(Act D)$_{5h}$, Act D added after the 5th h of incubation to cultures with Ara-C; W(Act D)$_{5h}$, retinas cultured for 5 h in medium with Ara-C, then washed and transferred into medium containing only Act D. Points shown are averages of four determinations; vertical lines indicate range of values obtained in five different experiments.

TABLE II

Accumulation of ^{14}C-Labeled GS in Embryonic Retinas Treated with Act D after Exposure to Ara-C

Treatment	Incorporation of ^{14}C-amino acids into total retina proteins	Retina protein reacted	Immunoprecipitated ^{14}C-labeled GS	GS sp act
	dpm/mg Protein $\times 10^{-5}$	*mg*	*Net dpm/mg protein*‡ $\times 10^{-3}$	*GHA/mg Protein*
Control, 5 h	2.56	2.10	0.41	0.40*
Ara-C, 5 h	1.92	2.23	0.76	0.74
Control, 8 h	4.00	1.85	0.52	0.45
Ara-C, 8 h	2.96	1.78	1.58	1.40
Ara-C, 0–8 h; Act D, 5–8 h	2.42	1.92	1.25	1.30
Control, 24 h	5.23	0.72	0.70	0.61
Ara-C, 24 h	3.92	0.66	3.53	3.80
Ara-C, 0–24 h; Act D, 5–24 h	2.67	0.79	3.42	3.18

Retinas were cultured in medium containing 7.5 mM Ara-C; controls were cultured in nucleoside-free medium. 1.0 μCi/ml ^{14}C-amino acid mixture was added to all cultures at the beginning of incubation. Act D (10 μg/ml) was added after 5-h exposure to Ara-C, as indicated above. Retinas were collected after 5, 8, and 24 h and 105,000-*g* supernates were prepared; duplicate aliquots were assayed for incorporation of ^{14}C-amino acids into total retina proteins, GS specific activity, and for ^{14}C-labeled GS, as described in Materials and Methods.

* GS specific activity of freshly isolated neural retina was 0.30.

of GS activity, and to allow the subsequent accumulation of GS to become independent of further transcription; (*b*) the RNA templates which accrue during the 0–5-h exposure to Ara-C can mediate GS accumulation when further transcription is arrested with Act D; (*c*) the increase in GS levels in retinas treated with Ara-C alone, or with Act D after 5-h exposure to Ara-C, is due to accumulation of GS molecules which are synthesized *de novo*.

Previous work on the induction of GS by HC has shown that the steroid elicits accumulation of active GS-specific RNA templates in the embryonic retina (29) which results in a several-fold increase in the rate of GS synthesis (30). Thus, it was imperative to determine whether the accumulation of GS elicited by Ara-C also resulted from increases in the rate of GS synthesis. This was examined by comparing directly the rates of GS synthesis in retinas cutured with Ara-C or HC.

Comparison of the Rates of GS Synthesis in Retinas Treated with Ara-C or HC

Retinas were cultured in medium containing either 7.5 mM Ara-C or 0.92 μM HC; controls did not have these additions. After various periods of incubation, the cultures were pulse-labeled for 15 min with 3.3 μCi/ml ^{14}C-amino acid mixture. The brief exposure to radioisotope excluded enzyme turnover as a major factor in interpretation of these results (17, 30). The retinas were sonicated and 105,000-*g* supernates were prepared (see Materials and Methods). GS was selectively precipitated from the mixture of radioactively labeled proteins by addition of anti-GS γ-globulin; the specificity and precipitation efficiency of this reaction were identical to those shown in Figs. 1 and 2. Nonspecific immunoprecipitation was monitored by treating duplicate samples with normal γ-globulin; though slight, the radioactivity precipitated by normal γ-globulin was always subtracted from that precipitated by anti-GS γ-globulin. The corrected immunoprecipitated radioactivity provided a direct measurement of the rate of GS synthesis.

The results (Table III) showed that Ara-C did not elicit an increase in the rate of GS synthesis over the basal level. Increases in enzyme accumulation resulting from *de novo* enzyme synthesis can only be due to either an increase in the rate of synthesis or a decrease in the rate of degradation of the enzyme. In the case of Ara-C-treated retinas, GS accumulates even though the rate of enzyme synthesis remains identical to that of untreated controls; this strongly suggests that Ara-C mediates GS accumulation by preventing the turnover of GS which normally limits enzyme accumulation in the uninduced retina.

TABLE III

Rates of GS Synthesis in Ara-C- and HC-Treated Retinas

Hours in culture	Treatment	Retina protein reacted	Rate of synthesis: Total protein	Rate of synthesis: GS	GS sp act
		mg	*dpm/mg Protein* $\times 10^{-4}$	*Net dpm/mg protein*	*GHA/mg protein*
2	Control	2.07	7.50	73	0.36
	Ara-C	2.24	2.90	69	0.39
	HC	2.11	7.61	110	0.59
5	Control	1.84	8.16	80	0.42
	Ara-C	1.79	2.26	75	0.80
	HC	1.65	8.32	450	1.38
8	Control	1.88	8.68	72	0.44
	Ara-C	1.91	2.46	79*	1.26
	HC	1.55	8.60	590	1.77

Retinas were cultured in medium containing either 7.5 mM Ara-C or 0.92 μM HC; control cultures were without these additions. The cultures were pulse-labeled for 15 min with 3.3 μCi/ml ^{14}C-amino acid mixture immediately before collection at the times shown above. The retinas were washed, sonicated, and 105,000-*g* supernates were prepared. Rates of total protein synthesis were monitored by determining the total quantity of TCA-insoluble radioactivity in the retina; rates of GS synthesis were determined by radioimmunoassay and corrected for nonspecific radioactivity, as described in Materials and Methods. Replicate aliquots of each 105,000-*g* supernate were assayed for GS specific activity.

* The rate of GS synthesis in Ara-C-treated retinas remained in the basal range between the 8th and 24th hours of culture.

In accord with previous findings (30), the increase of GS activity in retinas induced by hydrocortisone was due to acceleration of the rate of enzyme synthesis (Table III). Thus, considering the effects on rates of GS synthesis, the mechanism by which Ara-C increases GS levels in the embryonic retina is distinctly different from that of HC.

The rate of total protein synthesis in HC-induced retinas never deviated more than 2% from controls (Table III); thus, the increase in the rate of GS synthesis represents a selective response to the steroid inducer, as noted previously (30). Neither Ara-C nor HC measurably altered the uptake of ^{14}C-amino acids into the acid-soluble fraction of the retina. In contrast to HC, the effective concentration of Ara-C (7.5 mM) inhibited the incorporation of ^{14}C-amino acids into total retina proteins 60–70% (Table III). Yet, this massive inhibition neither prevented *de novo* synthesis of GS nor measurably reduced the rate of GS synthesis below control levels; this indicates that the suppression of retinal protein synthesis by Ara-C is highly differential.

The above findings have led to two seemingly incongruent conclusions: first, the evidence suggests that active templates for GS synthesis accumulate in Ara-C-treated retinas but not in untreated controls (Fig. 3); second, while the rate of GS synthesis in Ara-C-treated retina is not higher than in controls, GS accumulates to levels considerably above those of controls (Table III). The following experiments were undertaken as a first step toward resolution of this apparent paradox.

Effects of Ara-C on the Expression of Preformed Templates for GS Synthesis

Retinas were cultured in the presence of 7.5 mM Ara-C. After 5 h, the cultures were divided into two groups: the first group was washed to remove Ara-C; the second group was not. Act D (10 μg/ml) was then added to both sets of cultures in order to stop further transcription; therefore, the subsequent synthesis of GS was restricted to that mediated by templates which had accumulated during the initial 0–5-h culture period. After varying periods of treatment with Act D, the retinas were labeled for 15 min with 3.3 μCi/ml ^{14}C-amino acid mixture and then immediately harvested. Rates of GS synthesis were determined by

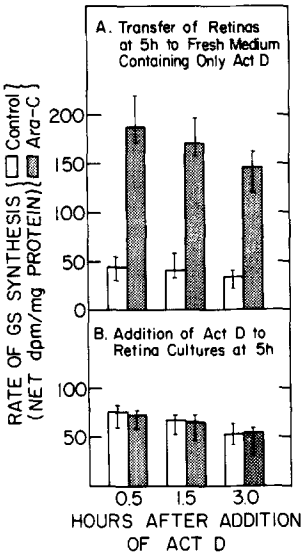

FIGURE 4 Rates of GS synthesis in embryonic retinas treated with Act D after exposure to Ara-C. Neural retina tissue from 10-day chick embryos was cultured in medium containing 7.5 mM Ara-C (shaded bars); control cultures were without Ara-C (white bars). After the 5th h of incubation, experimental and control tissues were simultaneously treated with Act D (10 μg/ml) according to one of two procedures: (A) retinas thoroughly washed to remove Ara-C, then transferred to fresh medium containing only Act D; (B) Act D added directly to retina cultures without removal of Ara-C. For each case, the retinas were labeled for 15 min with 3.3 μCi/ml ^{14}C-amino acid mixture immediately before collection after 0.5, 1.5, and 3.0 h of exposure to Act D. The tissues were subjected to radioimmunoassay for determination of the rate of GS synthesis, as described in Materials and Methods. Values shown are the average of four independent determinations; vertical lines indicate range of values obtained in two separate experiments.

immunoprecipitation of the radioactively labeled enzyme, as described above. The following conditions were compared: (*a*) absence of Ara-C during treatment with Act D, and (*b*) presence of Ara-C during Act D treatment.[2] Controls consisted of retinas that were not exposed to Ara-C; either they were washed after 5 h and transferred to fresh medium with Act D or Act D was added to the culture medium at 5 h.

As shown in Fig. 4 A, when Ara-C was withdrawn with simultaneous addition of Act D at 5 h, there was a prompt 4.2-fold increase in the rate of GS synthesis relative to controls; this increase persisted for at least 3 h, though its absolute level declined somewhat with time. When Ara-C was not withdrawn at the time of Act D addition, the rate of GS synthesis did not increase above control levels (Fig. 4 B). Thus, marked differences can exist between the rates of GS synthesis in Ara-C-treated retinas, depending upon whether the nucleoside is present or absent during Act D treatment.

The results of these immunochemical measurements (Fig. 4 A) further support the suggestion that exposure of the retina to 7.5 mM Ara-C causes an accumulation of templates for GS synthesis; they show that these templates can mediate a sustained increase in the rate of GS synthesis after the nucleoside is removed and further transcription is halted with Act D. Acceleration of the rate of GS synthesis was not an indiscriminate cellular response evoked by Act D: Act D did not elicit an increase in the rate of GS synthesis either in control cultures (Fig. 4) or in the continued presence of Ara-C (Fig. 4 B).

By comparing the results shown in Table II, Table III, and Fig. 4, it can be deduced that the effects of Ara-C on retinal GS levels are not attributable to major alterations in the specific activity of amino acid pools or of amino-acyl tRNA. For example, if the accumulation of ^{14}C-labeled GS in Ara-C-treated retinas (Table II) was due to increases in the specific activity of either the amino acid pools or the amino-acyl tRNA, one would expect to find a concomitant increase in the incorporation of ^{14}C-amino acids into total retina proteins, rather than the reduction observed under these conditions (Tables II and III). Conversely, the possibility that the low rates of synthesis of GS and total protein in Ara-C-treated retinas (Table III) may be associated with either an inhibition in the charging of tRNA or a reduction in the specific activity of amino acid pools is inconsistent with the finding that accumulation of ^{14}C-labeled GS increases ~400% during the 24-h culture period (Table II). Measurements of total uptake and incorporation of ^{14}C-amino acids by the retina also provided indirect evidence that the fourfold increase in the rate of GS synthesis occurring with

[2] Previous findings suggested that increases in GS activity elicited by Ara-C may be associated with the inhibition of regulatory gene transcription; withdrawal of Ara-C restores RNA synthesis and presumably the activity of the regulatory genes, resulting in cessation of GS accumulation (15). Addition of Act D at the time of Ara-C withdrawal prevents restoration of transcription while permitting continued synthesis of GS [see Fig. 3, W(Act D)$_{5\,h}$]. Accordingly, to most easily evaluate the effects of Ara-C on translation of preformed GS templates, Act D was used in these experiments to prevent possible reinitiation of regulatory gene activity upon withdrawal of Ara-C.

addition of Act D after Ara-C withdrawal did not simply reflect an equivalent increase in the specific activity of the amino acid pool. First, uptake of labeled amino acids into the acid-soluble fraction of the retina did not increase upon withdrawal of Ara-C and transfer to fresh medium containing Act D. Second, this treatment caused only a 20% increase in ^{14}C-amino acid incorporation into total retina proteins; the increase was detectable during only the first 30 min of exposure to Act D and occurred in both control and Ara-C-treated retinas. This increase in total protein synthesis is not sufficient to account for the >400% increase in the rate of GS synthesis which occurs in Ara-C-treated retinas under these conditions (Fig. 4 A).

Presently, the most plausible interpretation of the above results is that Ara-C limits or hinders some translational process(es) necessary for maximal expression of messenger RNA for GS. Thus, in retinas cultured continuously with Ara-C, the rate of GS synthesis does not increase over the basal level (Table III), despite the accumulation of potentially translatable GS templates (Fig. 3); withdrawal of Ara-C removes this translational hindrance and permits expression of the accumulated templates, resulting in a greater than basal rate of GS synthesis (Fig. 4 A).

DISCUSSION

Comparison of the Mechanism of Action of Ara-C and HC

HC, an 11-β-hydroxycorticosteroid, and Ara-C, a synthetic nucleoside, independently cause significant increases in GS levels in the embryonic neural retina. The increase of GS elicited by Ara-C is not causally related to inhibition of DNA synthesis by the nucleoside; however, there is a direct correlation between the selective inhibition of RNA synthesis by Ara-C and the concomitant increase in retinal GS (15). Both Ara-C and HC bring about accumulation of RNA templates which mediate *de novo* synthesis of the enzyme (1, 15, 29); however, the rate of expression of these templates is different with each effector. The present study demonstrates that the increases in the level of GS elicited by Ara-C and HC are achieved through different mechanisms; these differences apply to control processes governing the accumulation of RNA templates for GS synthesis, the rate of GS synthesis, and the rate of GS turnover.

PROVISION AND EXPRESSION OF GS-SPECIFIC TEMPLATE RNA: The initial events in the induction of GS by HC are similar to those known from other cases of steroid-cell interactions (4, 9, 12, 13, 31). HC binds to specific cytoplasmic receptors in the retina (6, 20), and the steroid-receptor complexes ultimately bind to sites within the nucleus (34, 35). Shortly thereafter, GS-specific template RNA begins to accumulate and the rate of enzyme synthesis in the retina increases (25, 29), but there are no gross changes in total RNA or protein synthesis (25, 26). According to a hypothesis advanced earlier (26, 30), HC may elicit accumulation of active templates for GS synthesis by causing a reduction of repressor molecules which prevent the accumulation of these templates in the uninduced retina.

It is unlikely that the effects of Ara-C involve complexing of the nucleoside with HC-specific cytoplasmic receptors; furthermore, reactions of Ara-C with the genome must be assumed to differ from those of HC. This is emphasized by the fact that the concentrations of Ara-C which increase GS levels also cause a very marked inhibition of macromolecular synthesis in the retina (15); it is possible that this results in a reduction of the postulated repressor which, in turn, results in accumulation of templates for GS synthesis. This assumption presupposes a differential inhibition of RNA synthesis in the retina by the effective concentrations of Ara-C and, in fact, it has been shown that Ara-C elicits such a differential inhibition which directly correlates with increased provision of the RNA templates which mediate GS synthesis (15).

Since both Ara-C and hydrocortisone elicit an accumulation of GS-specific RNA templates (15, 26), one might have expected to find a marked increase in the rate of GS synthesis in retinas treated with either effector; however, this expectation proved to be incorrect. In the steroid-induced retinas, immunochemical determinations confirmed previous findings (30) that, as the pool of GS-specific templates increased, the rate of GS synthesis was driven upward (Table III). In contrast, it was found that the rate of GS synthesis did not increase over the basal level in the Ara-C-treated retinas (Table III), despite the progressive accumulation of templates for GS synthesis (Fig. 3 and reference 15). These results suggest that translation of GS templates is hindered in the presence of Ara-C; this possibility is supported by the finding that the hindrance can apparently be reversed by withdrawing the nucleoside (Fig. 4). Thus, in the embryonic retina, the rate of synthesis of a specific protein (GS) may not always be directly related to the quantity of available tem-

plate RNA; furthermore, in this case, the translational hindrance caused by Ara-C neither prevents the accumulation of GS-specific templates (Fig. 3) nor stops the continuous *de novo* synthesis of the enzyme at a basal rate (Tables II and III). That the low rate of GS synthesis in Ara-C-treated retinas is not due to a defective messenger RNA for GS is shown by the fact that, under appropriate experimental conditions, the rate of GS synthesis can increase promptly after withdrawal of Ara-C (Fig. 4 A).

The nature of the reversible translational hindrance elicited by Ara-C is presently unknown; however, the following possibilities suggest themselves: (*a*) Ara-C may directly inactivate some component(s) required for optimal translation of mRNA for GS; (*b*) Ara-C may indirectly limit the provision of an essential translation factor(s) as a consequence of its inhibition of macromolecular synthesis; (*c*) the nucleoside may cause a general metabolic reduction of the ATP or GTP pools in the retina, thus limiting the rate of translation of all mRNA.

TURNOVER OF GS: Regulation of the turnover rate of specific proteins is an important control mechanism in eukaryotic cells (5, 38, 39). Accumulation of newly synthesized enzyme molecules reflects the balance between the rates of synthesis and degradation of the enzyme (38). Our radioimmunoassays have shown that the rate of GS synthesis was virtually identical in retinas cultured in either the presence or absence of 7.5 mM Ara-C (Table III); yet, in the Ara-C-treated retinas, there was a several-fold greater accumulation of newly synthesized GS than in controls (Table II). The most plausible interpretation of the above results is that treatment of the retina with Ara-C causes cessation of GS turnover, and this possibility should eventually lend itself to direct examination.[3]

In contrast to Ara-C, HC appears not to halt the turnover of GS in the retina (30); thus, in hormonal induction, accumulation of GS is due largely to the increased rate of GS synthesis (Table III and reference 30). These differences with respect to GS turnover provide a further example of disparities between the mechanisms by which Ara-C and HC elicit increases in the level of GS in the retina.

Studies on the hormonal induction of retinal GS have suggested that GS degradation may be mediated by a degrader molecule which has a relatively short half-life and which requires continuous RNA synthesis for its production or activity (30). The turnover of inducible enzymes in other tissues as well may be dependent upon the continuous synthesis and provision of specific degradative enzymes of short half-life (10, 17, 18, 21–23). In the embryonic neural retina, provision of the postulated degrader of GS can apparently be stopped by completely inhibiting RNA synthesis with Act D (26, 30), or by arresting protein synthesis with cycloheximide or puromycin (29). We suggested above that Ara-C elicits accumulation of GS by stopping the turnover of this enzyme; since this occurs only at concentrations of Ara-C which severely inhibit RNA and protein syntheses in the retina (14, 15), it is quite possible that the nucleoside inhibits the formation of the labile degrader of GS, thereby causing cessation of GS degradation. However, an alternative possibility is suggested by the finding that degradation of tyrosine aminotransferase in cultured hepatoma cells is reduced by inhibitors of ATP production (11). As noted earlier, Ara-C may reduce ATP levels in the retina, and this reduction could account for both the cessation of GS turnover and the low rate of GS synthesis. Future studies will attempt to resolve these alternatives.

Usefulness of Ara-C as a Molecular Probe of Gene Expression

The finding that Ara-C may be employed to elicit increases in retinal GS in the absence of the typical steroid inducer of this enzyme can be of significant benefit to further studies of enzyme regulation in embryonic cells. The potential usefulness of Ara-C in this context is evident from the facts that Ara-C is chemically different from the inducing corticosteroids and causes increases in the level of GS by a mechanism different from that of steroid inducers. An essential aspect of the mechanism of action of Ara-C is that it inhibits both RNA and protein syntheses in the retina in a remarkably discriminatory manner: Unlike some

[3] Conventional "pulse-chase" experiments for measurement of protein degradation in the retina are not presented in this report since (*a*) we were unable to determine unequivocably the extent of reutilization of ^{14}C-amino acids derived from the breakdown of proteins labeled during the initial pulse period; (*b*) we did not wish to use additional drugs to block reutilization of the labeled amino acids; and (*c*) conclusions concerning rates of GS degradation in Ara-C-treated retinas could be derived implicitly by comparing rates of GS synthesis with rates of GS accumulation.

of the conventional inhibitors of macromolecular synthesis, Ara-C does not stop the formation of RNA templates for GS, yet it blocks the turnover of the enzyme. Similarly, cessation of GS turnover in Ara-C-treated retinas must be achieved through a mechanism more selective than that of other inhibitors of protein synthesis which stop enzyme degradation (3, 17, 21-23): Ara-C blocks GS turnover without preventing *de novo* synthesis of the enzyme; as a result, GS accumulates continuously. In contrast, cycloheximide and puromycin inhibit both GS degradation and GS synthesis in the retina, causing enzyme levels to plateau (29, 30). Finally, an additional experimental advantage of Ara-C is that its effects on accumulation and translation of GS template RNA, and on GS degradation, are reversed if the nucleoside is withdrawn (Fig. 4 and reference 15).

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