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

I. Accumulation of Glutamine Synthetase with Suppression of Macromolecular Synthesis

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ABSTRACT

The analogue of cytidine, cytosine arabinoside (Ara-C), elicited a significant increase in the level of glutamine synthetase (GS) in embryonic chick neural retina in the absence of the steroid inducer of the enzyme. The increase was due to *de novo* synthesis of GS and was mediated by RNA which accumulated in the presence of the effective concentration of Ara-C. Accumulation of GS did not result from the inhibition of DNA synthesis for which Ara-C is best known. This new effect of Ara-C involves differential suppression of macromolecular synthesis in this system: the concentration of Ara-C which caused maximum GS accumulation suppressed overall protein and RNA syntheses 65–75% without inhibiting the transcription and translation of templates essential for GS synthesis. Withdrawal of Ara-C resulted in restoration of RNA synthesis and cessation of GS accumulation, even though preformed templates for the enzyme were present; however, if all RNA synthesis was arrested with actinomycin D at the time of Ara-C withdrawal, GS continued to accumulate. The results are consistent with the hypothesis that Ara-C differentially affects the activity of structural and regulatory genes involved in the regulation of GS levels in the retina: Ara-C allows transcription of the enzyme-specific templates, but reversibly inhibits the expression of regulatory genes which limit the accumulation of GS.

INTRODUCTION

Clarification of the mechanisms controlling specific enzyme levels in eukaryotic cells is essential for understanding the processes of embryonic differentiation and enzyme induction. Experimental analysis of these problems requires embryonic systems responsive to chemically defined effectors which promote changes in the levels of tissue-specific enzymes by differentially modulating gene

expressions. The induction and regulation of glutamine synthetase (GS)[1] in the embryonic chick neural retina by hormonal and nonhormonal effectors meets these requirements.

On the 16th day of embryonic development, GS specific activity in the chick neural retina begins to rise sharply in correlation with the functional differentiation and maturation of this tissue (16, 23). However, for several days before this time, GS can be induced precociously by hydrocortisone (HC) and related 11β-hydroxycorticosteroids, both in vivo (17, 22, 24) and in organ cultures of isolated neural retina tissue (18, 23–25). This premature induction is accompanied by other phenotypic changes in the retina which normally appear later in development (22–24). Because it can be studied under rigorously controlled conditions in vitro, GS induction is a useful system for analyzing regulatory mechanisms in differentiation and in hormone action (19, 21). For a review see references 14 and 15.

Previous work on this system suggested that maintenance of low GS levels in the uninduced retina is associated with the continuous provision of regulatory gene products; treatment with HC counteracts these products, thereby resulting in increased synthesis and accumulation of GS (1, 15, 19). Accepting this concept, we reasoned that it should be possible to elicit an increase in the level of GS in the absence of the steroid inducer if a molecule could be found which would selectively inhibit the proposed regulatory gene products (20). To test this working hypothesis, various inhibitors of macromolecular synthesis were screened for one which might exert the differential action necessary to increase the level of GS in the embryonic retina.

We found that Ara-C (1-β-D-arabinofuranosylcytosine; cytosine arabinoside) elicited a prompt and significant increase of retinal GS activity in the absence of the steroid inducer and that this effect was not related to the conventional action of Ara-C as an inhibitor of DNA synthesis (3, 30). Furthermore, while the effective concentration of Ara-C inhibited a considerable proportion of RNA and protein syntheses in the retina, it did not prevent transcription and translation essential for the synthesis of GS. Our results demonstrate the feasibility of using Ara-C and possibly other nucleoside analogues as differential inhibitors of specific macromolecular synthesis in studies of regulation of specific gene expressions in embryonic cells.

[1] *Abbreviations used in this paper:* GS, glutamine synthetase (EC 6.3.1.2); HC, hydrocortisone; Ara-C, 1-β-D-arabinofuranosylcytosine, cytosine arabinoside, arabinosylcytosine; Act D, actinomycin D; Cyh, cycloheximide; GHA, glutamohydroxamic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Tissue Culture

Neural retina tissue isolated aseptically from 10-day chick embryos was cultured at 37°C in 25-ml Erlenmeyer flasks on a gyratory shaker at 70 rpm, as previously described (21). Each flask contained retina tissue from one eye in 3 ml of culture medium (20% fetal bovine serum in Tyrode's balanced salt solution with 50 U/ml each of penicillin and streptomycin). All cultures were gassed with 5% CO_2-95% air mixture. To elicit accumulation of GS in the absence of the steroid inducer, Ara-C (1-β-D-arabinofuranosylcytosine hydrochloride; Lot No. 88B-0470, Sigma Chemical Co., St. Louis, Mo.) was added at the beginning of incubation to appropriate culture flasks at a final concentration of 7.5 mM. In all cases, the presence of serum in the culture medium resulted in significantly higher levels of GS activity; however, serum was not obligatory for Ara-C to affect GS levels.

Hormonal induction of GS was accomplished by adding hydrocortisone (0.92 μM) to the culture medium at the beginning of incubation, as described before (19, 21). Cycloheximide (Sigma) and actinomycin D (Merck Chemical Div., Merck and Co., Inc., Rahway, N. J.) were used to inhibit protein synthesis and RNA synthesis, respectively. When retinas were transferred from one medium to another, they were rinsed in three changes of the final medium (37°C, 5 min each change) in flasks on a gyratory shaker set at 100 rpm. Retinas cultured in the presence or absence of radioactive precursors were collected after indicated periods of incubation and rinsed in ice-cold balanced salt solution; they were then prepared for scintillation counting and determination of GS specific activity as described below.

Chromatographic Analysis of Ara-C

Different commercial preparations of Ara-C were found to vary considerably with respect to their effect on GS levels. Preparations of the hydrochloride of Ara-C elicited significantly greater increases of GS activity than the free nucleoside. It should also be stressed that the magnitude of the biological response differed considerably, depending on the embryonic age of the retina. Thus, prescreening of different lots of Ara-C for one with maximum activity and careful staging of embryos were essential. The results reported here are purposely restricted to the effects of the lot of Ara-C given above and

to retinas from 10-day chick embryos; however, they are representative of all lots which elicited moderate to high increases of GS in retinas of this age.

We have monitored the purity of more than 24 different lots of Ara-C from Sigma, Upjohn Co. (Kalamazoo, Mich.), Calbiochem (San Diego, Calif.), Nutritional Biochemicals Corp. (Cleveland, Ohio), Pfanstiehl Chemical Corp. (Waukegan, Ill.), Terra-Marine Bioresearch (La Jolla, Calif.), Serva Feinbiochemica GmbH and Co. (Heidelberg, W. Germany), and Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England). All commercial preparations of Ara-C which increased GS levels in the retina were found to be free of steroidal and nonsteroidal contaminants according to the following criteria of purity. Thus, these findings strongly support the view that the reported biological effects of Ara-C in this system are attributable to the action of this arabinonucleoside, rather than to a minor contaminant.

All commercial lots of Ara-C yielded ultraviolet absorbance spectra at pH 2 and pH 12 and infrared absorbance spectra conforming to those reported for pure preparations of Ara-C (3). Each lot of Ara-C migrated as a single spot when examined for trace impurities by paper chromatography and thin-layer chromatography. Quantities of Ara-C up to 5 mg were applied to Whatman 3MM chromatographic paper; up to 250 μg Ara-C was spotted on MN-300-10 cellulose plates (Brinkmann Instruments, Inc., Westbury, N. Y.). Replicate chromatograms were developed with *n*-butanol-pyridine-water in each of the following combinations: (45:25:40), (66:25:19), and (22:25:63). Additional thin-layer chromatograms were obtained with isobutyric acid-water-concentrated ammonium hydroxide (66:33:1), pH 3.7–4.0 and ethanol-0.5 M ammonium acetate (5:2). Various steroids, purines, pyrimidines, nucleosides, and arabinonucleoside analogues were cochromatographed as markers. No trace of any of these classes of compounds was found in any preparation of Ara-C examined. In all cases, Ara-C eluted as a single peak when run on Sephadex G-10 columns (Pharmacia Fine Chemicals Inc., Piscataway, N.J.), thus eliminating the possibility of contaminants of molecular weight greater than 700. Extraction of Ara-C with ether, acetone, chloroform, or chloroform-methanol (1:1) did not diminish its subsequent effect on GS levels. Trimethylsilyl derivatives of various lots of Ara-C were prepared by reaction with pyridine-hexamethyldisilazane-trimethylchlorosilane (5:3:1.5). Gas chromatography of the supernates confirmed the absence of trace steroidal contaminants. Further data concerning the variability of different lots of Ara-C and the importance of embryonic age will be reported elsewhere[2].

[2] R. E. Jones, and A. A. Moscona. 1974. Manuscript in preparation.

Radioisotopic Procedures

Radioactive precursors (New England Nuclear, Boston, Mass.) were used to monitor DNA, RNA, and protein syntheses: [methyl-^{3}H]thymidine (> 20 Ci/mmol), [5-^{3}H]uridine (> 25 Ci/mmol), and [^{14}C]L-amino acid mixture (10 mCi/mmol each). They were prepared in sterile, neutral, balanced salt solution and were added to retina cultures for various periods as indicated below. After labeling, each retina was washed, suspended in 1.6 ml of 10-mM phosphate buffer (pH 7.1), and disrupted by ultrasound. Aliquots of the sonicates were used to determine isotope incorporation, total protein content, and GS specific activity.

Isotope incorporation was measured after precipitation of 50–100-μl aliquots of tissue sonicates with ice-cold 10% trichloroacetic acid (TCA). The precipitates were collected on Millipore filters (Millipore Corp., Bedford, Mass.), washed with excess 5% TCA, dried, and transferred to vials containing toluene-based fluors (PPO-POPOP, Spectrafluor; Amersham/Searle Corp., Arlington Heights, Ill.). In some cases, the washed TCA precipitates were solubilized with NCS (Amersham/Searle) before addition of fluors. To monitor [^{3}H]thymidine incorporation into DNA, additional aliquots of tissue sonicates were precipitated and heated in 5% TCA at 94°C for 30 min, as previously described (21).

Radioactivity was measured with a Nuclear-Chicago Mark II liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.); quench correction was by either the channels ratio method for filter disks or by ^{133}Ba external standardization for homogeneous samples. Total protein was determined by the method of Lowry et al. (11). The specific radioactivity of each sample was expressed as disintegrations per minute per milligram protein.

Enzyme Assay

GS specific activity in tissue sonicates was determined colorimetrically by the glutamyl-transferase reaction, as described before (8, 33). Units of enzyme specific activity were expressed as micromoles of γ-glutamylhydroxamate (GHA) formed per hour per milligram tissue protein. Addition of Ara-C directly to the enzyme assay mixture did not interfere with the assay or with GS activity.

Analysis of Cytoplasmic RNA

Neural retinas labeled with [^{3}H]uridine, as described below, were pooled, washed in balanced salt solution, suspended in buffer (0.01 M Tris-HCl, pH 7.4; 0.01 M NaCl; 0.0015 M $MgCl_2$; 0.25 M sucrose), and gently lysed in sterile Dounce homogenizers (Kontes Glass Co., Vineland, N. J.). Nuclear breakage was negligible as determined by phase-contrast microscopy. Lysates were centrifuged at 27,000 g for 15 min at 4°C. Sodium dodecyl sulfate (SDS) was added to the resultant post-

mitochondrial supernate to a final concentration of 0.5%. The mixture was incubated at 37°C for 5 min and layered over 10–30% linear sucrose density gradients prepared in SDS buffer (0.01 M Tris-HCl, pH 7.4; 0.01 M NaCl; 0.001 M EDTA; 0.5% SDS). The gradients were centrifuged at 52,200 *g* for 16 h at 18–22°C and were eluted as 1.2 ml fractions; the absorbance at 254 nm was continuously recorded. Each fraction was precipitated with cold TCA; the precipitates were collected on Millipore filters and processed for scintillation counting as described above.

Immunochemical Procedures

Immunochemical tests were performed to compare GS from Ara-C-treated retina, steroid-induced retina, and noninduced retina. For these tests partially purified GS was obtained from the following sources: (*a*) retinas from 10-day embryos cultured for 24 h with 7.5 mM Ara-C; (*b*) retinas from 12-day embryos induced in culture for 24 h with HC; (*c*) retinas from 15-day embryos. In each case the retinas (30, 25, and 85, respectively) were pooled, sonified in 20 ml of cold 0.15 M KCl-0.005 M 2-mercaptoethanol (KCl-ME) and centrifuged at 105,000 *g* for 30 min. GS was precipitated from the supernates at 0°C by titration to pH 4.2 with 0.1 N acetic acid. The precipitates were collected by centrifugation, washed, and resuspended in 2 ml cold KCl-ME; GS was solubilized by slowly raising the pH to 6.8 with 0.1 N NaOH. The mixture was stirred in the cold for 30 min and centrifuged for 15 min at 12,000 *g*; the supernates contained partially purified GS.

Immunotitration curves for each of these three preparations were obtained by reacting 20 μl of rabbit anti-GS serum (19) with a series of dilutions of the partially purified GS enzyme preparations (for further details see legend, Fig. 2). The precipitation efficiency in each of the three cases was identical.

Radioimmunoassay of GS

Amounts of newly synthesized GS in the retina were determined radioimmunochemically, as described before (1, 2). Isolated neural retina tissue from 10-day embryos was cultured in medium with 7.5 mM Ara-C; control cultures were without Ara-C. [^{14}C]amino acid mixture (0.5 μCi/ml) was added to all cultures at the beginning of incubation to label nascent GS. After 5, 12, or 24 h, the retinas were washed and sonicated in 10 mM PO_4-buffered saline, pH 7.1; 105,000 *g* supernates were obtained. GS was precipitated from aliquots of these supernates with 20 μl of anti-GS γ-globulin isolated (29) from rabbit antiserum against purified chick retinal GS (27); duplicate aliquots were precipitated with γ-globulin from normal rabbit serum to monitor nonspecific precipitation of radioactivity. Before the addition of γ-globulin, the enzyme activity units (GHA) in each aliquot were adjusted with unlabeled GS from adult chick retina to a uniform level optimal for maximal precipitation of the enzyme (for further details see legend, Fig. 4). The immunoprecipitates were collected by centrifugation, washed, dispersed by sonication, and treated with cold 10% TCA; the precipitates were collected on Millipore filters and processed for scintillation counting. In each case the specific precipitation of labeled GS was determined by subtracting the disintegrations per minute per milligram protein precipitated with normal γ-globulin from that precipitated with anti-GS γ-globulin.

RESULTS

Kinetics of Accumulation of GS Activity

When 10-day chick embryo neural retina was cultured in medium with 7.5 mM Ara-C, GS activity began to increase rapidly after a lag period of 2–3 h (Fig. 1). The increase of enzyme specific activity (GHA per milligram protein) represented accumulation of enzyme activity units (GHA) in the cells and was not due to a relative decrease in overall protein content. The kinetics of GS activity accumulation elicited by Ara-C differed only

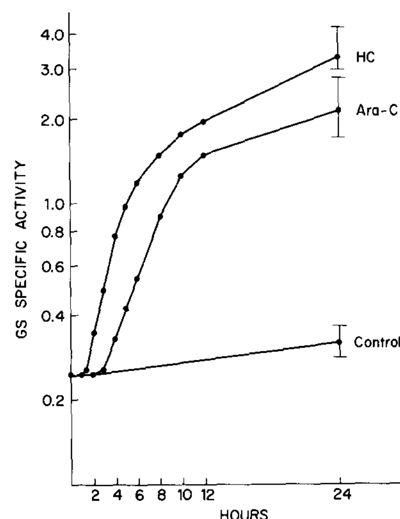

FIGURE 1 Comparison of accumulation of GS activity elicited by Ara-C and hydrocortisone (HC) in cultures of 10-day chick embryo neural retina. Ara-C (7.5 mM) or HC (0.92 μM) was added at 0 h; control cultures were without additions. The overall kinetics of accumulation of GS activity were similar for all lots of Ara-C tested; however, the absolute specific activity values varied with different commercial preparations (see Materials and Methods). Each point represents the average of at least five replicate cultures.

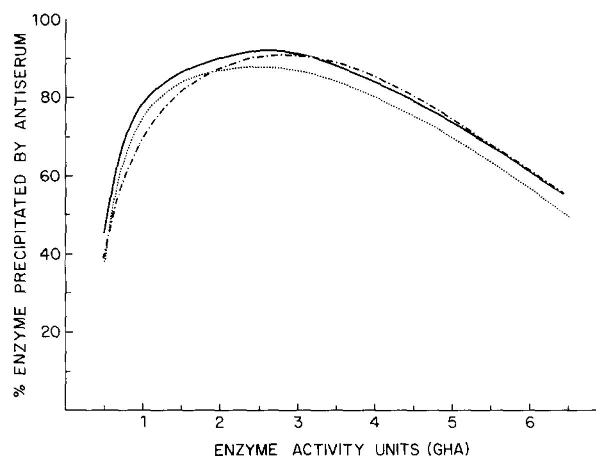

FIGURE 2 Immunotitration of GS activity from Ara-C-treated (——), HC-induced (— · —), and uninduced retina tissue (- - -). Aliquots of partially purified sonicates (see Materials and Methods), containing increasing amounts of enzyme activity units (GHA), were precipitated with 20 μl of anti-GS serum; in each case 500 μg bovine serum albumin was added and the final vol adjusted to 1.0 ml with 10 mM PO_4-0.85% NaCl buffer (pH 7.1). The mixture was incubated at 37°C for 45 min and allowed to precipitate overnight at 4°C. The immunoprecipitate and supernate were separated by centrifugation, assayed for GS activity, and the percent of the input GHA precipitated by the antiserum was determined.

slightly from those of GS induction by HC (Fig. 1): the lag period was longer, and the rate of increase during the initial hours was somewhat less than in hormonal induction.

Comparative Immunotitration of GS Activities

Previous immunological tests have established the antigenic similarity between GS from steroid-induced embryonic retina and GS from adult retina tissue (19). The following experiments immunochemically compared the GS activity in retina treated with Ara-C with the GS present in steroid-induced and noninduced retina tissue. The immunotitration results (Fig. 2) showed that the overall shape and equivalence zones (regions of maximal immunoprecipitation) of the three precipitin curves were practically identical. Therefore, the increased GS activity elicited in embryonic retina by Ara-C is in all likelihood due to the same enzyme as the GS activity in steroid-induced retina, in uninduced retina, and in the normal adult chick retina (19).

Requirement for Protein Synthesis

The increase of GS activity elicited by Ara-C was prevented by inhibiting protein synthesis in the retina. Addition of cycloheximide (2 μg/ml) to retina cultures together with Ara-C (7.5 mM), or at any time thereafter, inhibited incorporation of [^{14}C]amino acids into retina proteins 94–97% and completely prevented the rise in GS activity (Fig. 3). This inhibition could be partially reversed if the tissue was washed and transferred to fresh medium with Ara-C only (see *R*, Fig. 3). Therefore, as in the case of GS induction by hydrocortisone (21), the increase of GS activity by Ara-C requires continuous protein synthesis.

Evidence for Enzyme Synthesis

Radioimmunoassays established that the increased GS activity in Ara-C-treated retina was due to *de novo* enzyme synthesis (see Materials and Methods). The results (Fig. 4) showed that newly synthesized, [^{14}C]-labeled GS accumulated steadily in Ara-C-treated retina in close correspondence with the increase in GS activity. In controls (not treated with Ara-C), accumulation of labeled GS was significantly lower and paralleled the slow basal increase in GS activity characteristic of the uninduced retina. Therefore, we conclude that the increase of GS activity elicited by Ara-C results from *de novo* synthesis and accumulation of the enzyme, and is not due predominantly to activation of preexisting precursor molecules. Experiments are in progress to determine whether

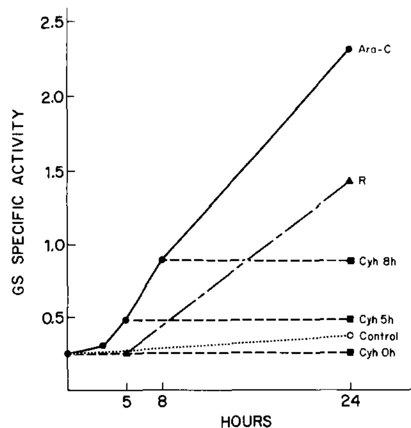

FIGURE 3 Effects of cycloheximide on GS accumulation elicited by Ara-C. Retinas were cultured in medium with 7.5 mM Ara-C; after 0, 5, or 8 h cycloheximide (Cyh; 2 μg/ml) was added to stop protein synthesis. Control cultures were without additions. *R:* retinas were incubated with both Ara-C and Cyh; after 5 h they were washed and transferred into fresh medium containing Ara-C without Cyh. Each point represents the mean of several replicate cultures.

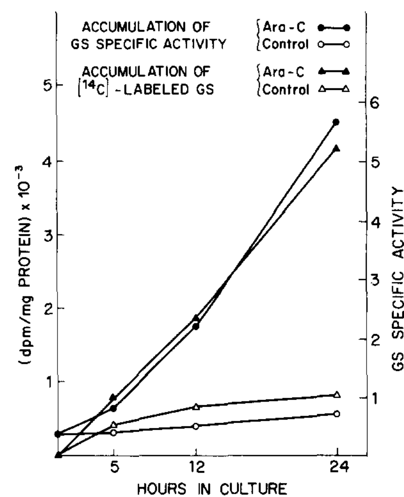

FIGURE 4 Accumulation of [^{14}C]-labeled GS and GS specific activity in embryonic retinas cultured in the presence and absence of 7.5 mM Ara-C. GS specific activities were determined in aliquots of 105,000 *g* supernates prepared from [^{14}C]-labeled retina tissue (see Materials and Methods). Replicate aliquots were precipitated immunochemically; the precipitation mixture contained labeled GS, unlabeled adult retina GS, 500 μg bovine serum albumin, 500 μg sodium deoxycholate, 20 μl 2.5% Triton X-100, 20 μl anti-GS (or normal) γ-globulin, and 10 mM PO_4-0.85% NaCl buffer (pH 7.1) to bring the final vol to 1.0 ml. The precipitation mixtures were incubated for 30 min at 37°C and overnight at 4°C. Newly synthesized GS was expressed as net disintegrations per minute per milligram protein (disintegrations per minute per milligram protein precipitated by anti-GS γ-globulin minus disintegrations per minute per milligram protein precipitated by normal γ-globulin). For further details, see text.

Ara-C elevates GS levels by accelerating the rate of enzyme synthesis or by decreasing its rate of degradation.

Inhibition of DNA Synthesis by Ara-C does not Stimulate GS Accumulation

Virtually all previous studies with Ara-C in both prokaryotic and eukaryotic systems have focused on its inhibitory effect on DNA synthesis which occurs at relatively low concentrations of the nucleoside (3, 30). Since significant increases of GS are elicited in the retina at relatively high concentrations of Ara-C, 7.5 mM being required for optimal response (Fig. 5), we examined if the effect of Ara-C on GS levels was related to inhibition of DNA synthesis.

Even low concentrations of Ara-C (0.01 mM) inhibited incorporation of [^{3}H]thymidine into retinal DNA > 91% within 15 min (Figs. 5 and 6); DNA synthesis remained inhibited 97-100% for the total 24-h culture period in the presence of Ara-C. However, only much higher doses elicited significant increases in GS levels. The fact that low doses of Ara-C which effectively inhibited DNA synthesis did not elicit GS accumulation (Fig. 5) demonstrates that the effect on GS does not result from this inhibition. Furthermore, other inhibitors of DNA synthesis (hydroxyurea, FUdR, phleomycin, mitomycin C, and methotrexate) elicited no significant increases of GS levels when added to cultures of embryonic neural retina over a wide range of concentrations.

The above findings preclude a direct causal relationship between inhibition of DNA synthesis by Ara-C and the increase of GS; however, the possibility remained that the effect on GS levels occurred only if the cells were engaged in DNA synthesis at the time of nucleoside addition. To test this, retinas were treated for 5 h with a low dose of Ara-C (0.01 mM) to inhibit ongoing DNA synthesis and were then transferred to medium with 7.5 mM Ara-C; GS activity was assayed at 24 h. The results (Fig. 7) showed that preinhibition of DNA

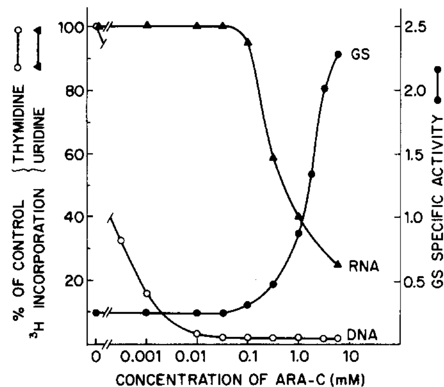

FIGURE 5 DNA synthesis, RNA synthesis, and GS accumulation in embryonic neural retina treated with Ara-C. Dose response curves. Retinas were cultured in medium containing different concentrations of Ara-C; in each case retina tissue from one eye was cultured in medium with Ara-C and the retina from the opposite eye was cultured in nucleoside-free medium (control). For determination of DNA synthesis, retina pairs were cultured for 1 h and then labeled for 1 h with 0.33 μCi/ml [^{3}H]thymidine; other retina pairs were cultured for 23 h and then labeled for 1 h with 0.33 μCi/ml [^{3}H]uridine to monitor total RNA synthesis. The cultures were harvested and processed for determination of specific radioactivity (disintegrations per minute per milligram protein); incorporation of label into DNA (O——O) and RNA (▲——▲) of each Ara-C-treated retina was expressed as % of its paired control. Each point represents the average of at least five pairs of retina. GS specific activities (●——●) were determined after 24 h of culture with Ara-C.

synthesis did not diminish the responsiveness of the retina to subsequent treatment with the effective dose of Ara-C. This agrees with earlier findings that GS induction by hydrocortisone is also independent of DNA synthesis (15). Taken as a whole, the above experiments show that neither DNA synthesis nor its inhibition are directly involved in the mechanism by which Ara-C increases GS levels in the retina.

Suppression of Protein and RNA Synthesis by Ara-C

To determine whether Ara-C affected the synthesis of other macromolecules coordinately with its effect on GS, we monitored the incorporation of labeled precursors into protein and RNA. Retinas were cultured for 23 h in the presence of 0.01 mM or 7.5 mM Ara-C, then labeled with 1 μCi/ml [^{14}C]amino acid mixture for 1 h and assayed for isotope incorporation into proteins and for GS activity. Table I shows that the low dose of Ara-C did not measurably affect total protein synthesis or GS levels; in contrast, 7.5 mM Ara-C suppressed protein synthesis 65%, yet it raised the level of GS nearly tenfold over that present in the freshly isolated retina. Since the rise of GS activity is due to *de novo* enzyme synthesis, these results clearly demonstrate that high concentrations of Ara-C differentially inhibit protein synthesis in the retina.

The following measurements showed that the inhibition of protein synthesis by Ara-C was associated with a massive reduction of overall RNA synthesis; the results revealed a dose-response relationship between the suppression of RNA synthesis and the increase of GS. Retinas were cultured for 23 h in the presence of different concentrations of Ara-C; they were then labeled for 1 h with 0.33 μCi/ml [^{3}H]uridine and assayed for isotope incorporation and GS activity. Fig. 5 shows that low concentrations of Ara-C (< 0.1 mM) neither inhibited the incorporation of [^{3}H]uridine nor increased GS levels. However,

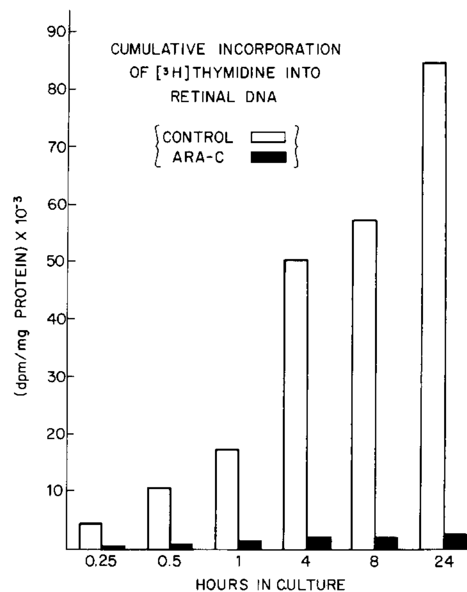

FIGURE 6 The effects of Ara-C on DNA synthesis. 10-day embryonic chick neural retina tissue was cultured in medium with Ara-C (0.01 mM); control cultures were without Ara-C. [^{3}H]thymidine (0.5 μCi/ml) was added to all cultures at the beginning of incubation. The cultures were harvested at the times shown above and the incorporation of label into TCA-precipitable material was determined. The values are averages of at least five replicate cultures.

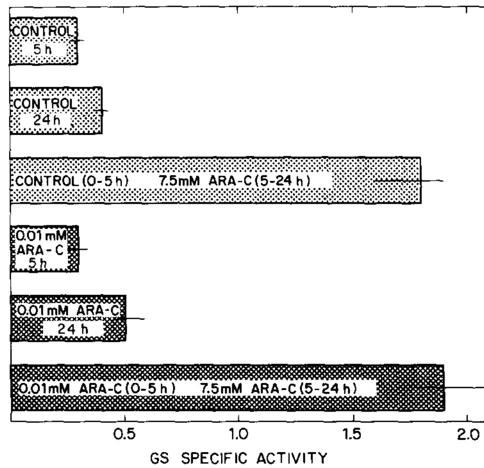

FIGURE 7 Stimulation of retinal GS accumulation by 7.5 mM Ara-C after inhibition of ongoing DNA synthesis with 0.01 mM Ara-C. The values shown above are averages of several replicate cultures. The horizontal lines indicate variability.

TABLE I

Effects of Low and High Concentrations of Ara-C on Protein Synthesis and GS Accumulation

Treatment	[^{14}C] amino acid incorporation (disintegrations per minute per milligram protein) $\times 10^{-3}$	Percent inhibition of protein synthesis	GS specific activity at 24 h*
Control	127.1	-	0.52
0.01 mM Ara-C	121.8	4.1	0.62
7.5 mM Ara-C	44.1	65.3	2.11

* GS specific activity of freshly isolated 10-day embryonic neural retina tissue was 0.22.

higher concentrations of Ara-C (0.1–7.5 mM) caused a dose-dependent reduction of RNA synthesis which closely correlated with increases of GS activity.

To determine the effect of 7.5 mM Ara-C on rapidly labeled RNA synthesis, retinas were cultured in the presence or absence of Ara-C for various lengths of time and then were labeled for 15 min with 10 μCi/ml [^{3}H]uridine. Table II shows that this concentration of Ara-C, which elicited maximal increases of GS, reduced the synthesis of rapidly labeled RNA 70–75%. This level of inhibition occurred within the first hour of exposure to Ara-C and persisted throughout the 24-h culture period.

As a first step toward a more detailed analysis of the effects of Ara-C on RNA synthesis, we examined its effect on the synthesis of cytoplasmic RNA after short term and long term labeling with [^{3}H]uridine. In the first series of experiments (Fig. 8) retinas were cultured for 5 h in the presence or absence of 7.5 mM Ara-C and were labeled for 15 min with 50 μCi/ml [^{3}H]uridine immediately before harvesting. The tissues were processed for analysis of cytoplasmic RNA synthesis by SDS-sucrose density gradients (see Materials and Methods). Ara-C inhibited the synthesis of tRNA (gradient fractions 1–7) by 62%; the radioactivity distributed throughout the rest of the gradient was reduced 79%. In the second series of experiments, the tissues were labeled with 2 μCi/ml [^{3}H]uridine for the final 3 h of culture to allow accumulation of the isotope in cytoplasmic ribosomal RNA. The results (Fig. 9) show that 7.5 mM Ara-C inhibited the synthesis of 18S and 28S ribosomal RNA 76%; under these conditions tRNA (gradient fractions 1–8) was inhibited 63%.

TABLE II

The Effects of Ara-C on Rapidly Labeled RNA Synthesis

Hours in culture	[^{3}H]uridine incorporation (disintegrations per minute per milligram protein) $\times 10^{-4}$		Percent inhibition
	Control	Ara-C-treated	
1	6.97	1.75	74.9
2	8.05	2.44	69.9
3	8.68	2.79	67.9
4	9.20	2.84	69.1
5	9.61	2.89	69.9
12	10.40	3.03	70.9
24	9.52	2.11	77.8

Neural retina tissue was explanted from 10-day chick embryos. Retina from one eye was cultured in medium containing 7.5 mM Ara-C; the other was cultured in nucleoside-free medium (control). Each pair was pulse-labeled with 10 μCi/ml [^{3}H]uridine for 15 min immediately before harvesting at the times shown above. The cultures were then processed for determination of TCA-precipitable ^{3}H incorporated into rapidly labeled RNA. The values above are averages of at least five cultures per case.

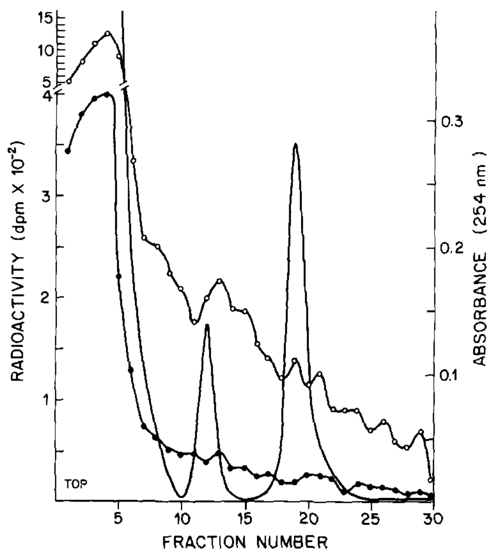

FIGURE 8 Sucrose density gradient analysis of cytoplasmic RNA in SDS-treated 27,000 *g* supernates prepared from retinas pulsed for 15 min with [^{3}H]uridine immediately before harvesting at 5 h. The absorbance profile at 254 nm (——) was identical for control and Ara-C-treated tissue. Radioactivity: control (O——O); 7.5 mM Ara-C (●——●).

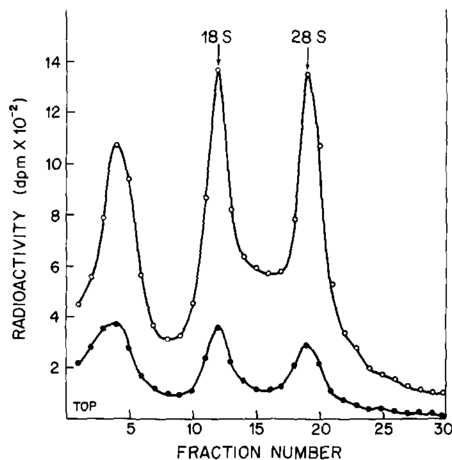

FIGURE 9 Sucrose density gradient analysis of cytoplasmic RNA in SDS-treated 27,000 *g* supernates prepared from retinas labeled for 3 h with [^{3}H]uridine immediately before harvesting at 5 h. Absorbance profiles were identical to that shown in Fig. 8. Radioactivity: control (O——O); 7.5 mM Ara-C (●——●).

These results show that Ara-C exerts no gross selectivity in inhibiting the major classes of cytoplasmic RNA resolvable by these techniques. However, since *de novo* synthesis of GS occurs while RNA synthesis is drastically curtailed, the possibility remained that these two effects were causally related at a more subtle level. Conceivably, Ara-C may differentially affect transcription of RNA species involved in the regulation of GS levels. To examine this question we first determined if transcription was required for the Ara-C-mediated increase of GS.

Requirement for RNA Synthesis

The effect of Ara-C on GS levels is dependent on RNA synthesis. Addition of 10 μg/ml actinomycin D (Act D) to retina cultures together with Ara-C (7.5 mM) inhibited incorporation of [^{3}H]uridine 98–99% and completely prevented the increase of GS (Fig. 10). Act D neither inactivates GS nor inhibits the translation required for GS accumulation (15, 19, 21). Since Ara-C itself rapidly inhibits 75% of RNA synthesis (Table II), it follows that the residual 25% of transcription which is blocked by Act D contains RNA essential for the increase of GS. Therefore, these results firmly indicate that (*a*) the increase of GS elicited by Ara-C depends

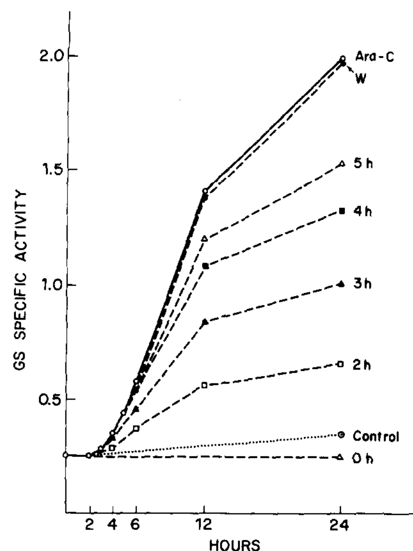

FIGURE 10 Effects of actinomycin D (Act D) on GS accumulation elicited by Ara-C. Act D (10 μg/ml) was added to retina cultures containing 7.5 mM Ara-C at the times shown above. Control cultures were without additions. *W:* retinas incubated for 5 h with 7.5 mM Ara-C, then washed and transferred into fresh medium containing 10 μg/ml Act D without Ara-C. Each point represents the mean of several replicate cultures from one set of experiments; the absolute values varied somewhat in different experiments.

on the provision of gene products for synthesis of the enzyme, and (*b*) Ara-C differentially inhibits RNA synthesis in the retina in that it does not stop the transcription essential for GS synthesis.

To determine whether the progressive rise of GS in Ara-C-treated retinas required continuous provision of new RNA, Act D (10 μg/ml) was added to retina cultures at various times after the addition of Ara-C. The results (Fig. 10) showed that the increase in the level of GS gradually became less dependent on ongoing RNA synthesis; thus, when RNA synthesis was completely halted with Act D after 5 h of exposure to Ara-C, GS continued to increase. This increase did not require the further presence of Ara-C: in retinas transferred after 5 h in Ara-C to medium with Act D only, GS continued to increase to levels similar to controls treated with Ara-C only (see *W*, Fig. 10). This transcription-independent increase of GS activity was inhibited by cycloheximide (condition 3, Fig. 11); therefore, it evidently represented enzyme synthesis mediated by preformed templates. These findings clearly indicate that (*a*) exposure to Ara-C results in accumulation of RNA templates for GS synthesis which can mediate GS formation even if transcription is later stopped; (*b*) Ara-C does not directly affect the translation of GS from preformed templates; (*c*) the massive inhibition of transcription by Ara-C does not include gene products for GS synthesis and therefore is distinctly selective.

The accumulation of RNA templates for GS synthesis in Ara-C-treated retina does not require concurrent protein synthesis. Retinas were cultured for 5 h in medium with both Ara-C (7.5 mM) and cycloheximide (2 μg/ml); they were then washed, transferred into medium containing only Act D (10 μg/ml), incubated for an additional 19 h, and assayed for GS activity. The results (condition 2, Fig. 11) showed that after transfer of the tissue to Act D, GS activity increased; therefore, templates for GS synthesis accumulated in the absence of protein synthesis and were expressed after translation was unblocked. A similar situation has been described and discussed for the induction of GS by hydrocortisone (14, 15, 21).

The Effects of Withdrawal of Ara-C

If after 5 h of treatment with Ara-C the retina was thoroughly washed and transferred to culture medium without Ara-C, GS activity increased at a progressively declining rate for the next 7 h and

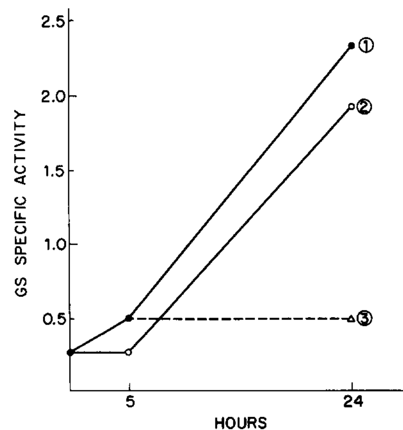

FIGURE 11 Effects of cycloheximide (Cyh) on transcription-independent increases in GS specific activity. The requirements for protein synthesis were examined under the following conditions: condition (1), retinas cultured for 5 h with Ara-C (7.5 mM), then washed and transferred into fresh medium containing Act D (10 μg/ml) without Ara-C (control); condition (2), retinas cultured for 5 h with both Ara-C and Cyh (2 μg/ml), then washed and transferred into fresh medium containing Act D only; condition (3), addition of both Cyh and Act D to retinas cultured for 5 h in Ara-C.

then leveled off (Fig. 12 A). The experiments with Act D, described above, showed that after 5 h of treatment with Ara-C the retina contains a pool of templates which will sustain continuous GS accumulation to high levels in the absence of further RNA synthesis (see Figs. 10 and 11); yet, if Ara-C is withdrawn after 5 h without simultaneous total inhibition of RNA synthesis, GS accumulation ceases. Thus, continued accumulation of GS under these conditions depends on processes which require either the constant presence of Ara-C or the halting of all RNA synthesis at the time of Ara-C withdrawal (*W*, Fig. 10; Fig. 11). The above results are consistent with the assumption that Ara-C indirectly controls GS accumulation by affecting transcriptional processes which regulate translational or posttranslational events. This possibility will be examined in the Discussion.

In all of the above experiments with Ara-C, accumulation of GS in the retina is associated with inhibition of RNA synthesis. If, as indicated earlier, these two phenomena are causally related, then the leveling off of GS after Ara-C withdrawal should involve restoration of RNA synthesis. To test this prediction retinas were cultured for 5 h in medium with 7.5 mM Ara-C, then the nucleoside was withdrawn as described above. At various

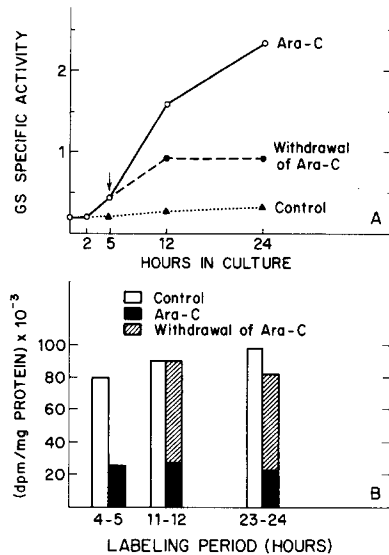

FIGURE 12 The effects of withdrawal of Ara-C. Retinas were cultured in medium containing 7.5 mM Ara-C; control cultures were without Ara-C. Arrow marks time of Ara-C withdrawal. In each case replicate cultures were labeled with 2 μCi/ml [^{3}H]uridine for 1 h before harvesting at 5, 12, and 24 h; these were assayed for (A) GS specific activity and (B) incorporation of label into total newly synthesized RNA.

times before and after withdrawal of the nucleoside, 2 μCi/ml [^{3}H]uridine was added simultaneously to experimental and control cultures; the retinas were collected 1 h later and processed for scintillation counting to monitor total RNA synthesis. The results (Fig. 12 B) show that the inhibitory effects of Ara-C on RNA synthesis are reversible. While inhibition of RNA synthesis was sustained in retinas cultured continuously in medium with Ara-C, complete recovery of RNA synthesis accompanied withdrawal of the nucleoside.[3]

DISCUSSION

The present work with Ara-C and the contrasting results obtained with proflavine (3,6-diaminoacridine) in this system (28, 34) demonstrate that relatively simple compounds can be effectively used as molecular probes for analysis of specific gene expressions in embryonic cells. The differential effects of these molecules offer unique and promising approaches to further analysis of the mechanisms controlling enzyme levels in differentiating cells. Proflavine reversibly and differentially inhibits the hormonal induction of GS in the retina by interfering with the provision of templates for synthesis of the enzyme (34). Dosages of proflavine which completely prevent the increase of GS only minimally inhibit RNA and protein syntheses. The effects of Ara-C in this system are just the opposite: under suitable conditions, GS specific activity increases to levels similar to those elicited by the steroid inducer, while RNA and protein syntheses are greatly reduced. The facts that changes in GS levels are characteristic of the developmental program of the retina and are correlated with other aspects of retinal differentiation (13, 22–24) add to the interest and potential usefulness of such molecular probes in the exploration of developmental processes.

Our results conclusively eliminate inhibition of DNA synthesis as the cause of the Ara-C effect on GS accumulation in the retina. More importantly, they indicate a causal relationship between the partial suppression of RNA and protein syntheses by Ara-C and the concurrent increase of retinal GS. These suppressions are clearly selective in that they allow transcription and translation of the RNA essential for the synthesis of GS. It should be stressed that induction of GS by steroids is not accompanied by measurable reductions of macromolecular synthesis (1, 14).

Our findings indicate that the effects of Ara-C on GS levels in the embryonic retina are achieved primarily through transcriptional processes. In the uninduced embryonic retina, templates mediating GS synthesis do not accumulate and GS levels remain almost unchanged; thus, the marked increase in enzyme content elicited by Ara-C involves more than simply permitting the basal-level transcription and expression of these templates. Our results strongly suggest that Ara-C elicits the accumulation of functional GS templates in the uninduced retina. The results of the actinomycin D experiments and the lag period observed to precede the initial rise of GS levels support this interpretation. While the duration of this lag period may reflect to some extent the sensitivity limits of the

[3] DNA synthesis remained inhibited after withdrawal of Ara-C. While our washing procedure was effective in reducing Ara-C to levels permitting renewal of RNA synthesis, a residual intracellular pool of the nucleoside may have continued to prevent synthesis of DNA (see Fig. 5). Alternatively, the effect of high dosages of Ara-C on DNA synthesis may be irreversible in these cells. In any event, this finding shows that the leveling of GS activity upon removal of Ara-C is not associated with restoration of DNA synthesis.

enzyme assay, the rapid inhibition of macromolecular synthesis by Ara-C demonstrates that the lag is not due simply to delayed uptake of the nucleoside.

The precise mechanisms by which two molecules as different as Ara-C and hydrocortisone independently elicit accumulation of active templates for GS synthesis are being investigated. Previous studies have suggested that the provision of GS templates in the uninduced retina is hindered by a labile, gene-controlled repressor(s) (15, 19); accordingly, Ara-C may selectively inhibit the formation or activity of this regulatory element, thereby allowing the accumulation of transcripts for GS synthesis. Alternatively, Ara-C may enhance the processing, transport, or stability of GS templates in the uninduced retina and thereby bring about their accumulation. In this connection, it is of interest that polyadenylate (poly[A]) synthesis is required in the induction of retinal GS by hydrocortisone (26), since poly(A) sequences associated with messenger RNA (4, 6, 9, 10) may play a role in processing, transport, or stability of messenger RNA (5, 7, 12).

The availability of templates for GS synthesis cannot be the only factor determining GS levels in the Ara-C-treated retina. This is evident from the fact that when Ara-C is withdrawn GS ceases to accumulate, even though the cells contain preformed templates for further synthesis of the enzyme. This strongly suggests that the nucleoside regulates the enzyme level by also affecting translational or posttranslational processes. Work in other systems supports the concept that enzyme regulation in eukaryotic cells can occur at several control levels (31, 32). In view of the evidence against a direct effect of Ara-C on GS translation, we suggest that Ara-C indirectly affects GS accumulation through action on regulatory genes which control template activity and/or enzyme degradation. Such regulatory genes have been previously postulated as essential components of the control mechanisms which maintain GS at a low level in the uninduced retina (15, 19). Accordingly, our working assumption is that the inhibition of RNA synthesis by Ara-C includes the activity of these regulatory genes but not the structural genes for GS; thus, GS accumulation is permitted. Withdrawal of Ara-C restores RNA synthesis and the activity of the regulatory genes, thereby preventing further GS accumulation. Tests of the above assumptions using Ara-C and other probes of gene action should provide further information concerning regulatory mechanisms controlling specific enzyme levels in embryonic cells.

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REFERENCES

1. Alescio, T., and A. A. Moscona. 1969. Immunochemical evidence for enzyme synthesis in the hormonal induction of glutamine synthetase in embryonic retina in culture. *Biochem. Biophys. Res. Commun.* **34**:176.
2. Alescio, T., M. Moscona, and A. A. Moscona. 1970. Induction of glutamine synthetase in embryonic retina. Effects of partial and complete inhibition of RNA synthesis on enzyme accumulation. *Exp. Cell Res.* **61**:342.
3. Cohen, S. S. 1966. Introduction to the biochemistry of D-arabinosyl nucleosides. *In* Progress in Nucleic Acid Research and Molecular Biology. J. N. Davidson and W. E. Cohn, editors. Academic Press, Inc., New York. **5**:1.
4. Darnell, J. E., R. Wall, and R. J. Tushinski. 1971. An adenylic acid-rich sequence in messenger RNA of HeLa cells and its possible relationship to reiterated sites in DNA. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1321.
5. Darnell, J. E., L. Philipson, R. Wall, and M. Adesnik. 1971. Polyadenylic acid sequences: role in conversion of nuclear RNA into messenger RNA. *Science (Wash. D.C.).* **174**:507.
6. Edmonds, M., M. H. Vaughan, Jr., and H. Nakazato. 1971. Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1336.
7. Johnston, R. E., and H. R. Bose. 1972. Correlation of messenger RNA function with adenylate-rich segments in the genomes of single-stranded RNA viruses. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1514.
8. Kirk, D. L., and A. A. Moscona. 1963. Synthesis of experimentally induced glutamine synthetase (glutamotransferase activity) in embryonic chick retina *in vitro*. *Dev. Biol.* **8**:341.
9. Lee, S. Y., J. Mendecki, and G. Brawerman. 1971. A polynucleotide segment rich in adenylic acid

in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1331.

10. LIM, L., and E. S. CANELLAKIS. 1970. Adenine-rich polymer associated with rabbit reticulocyte messenger RNA. *Nature* (*Lond.*). **227**:710.
11. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
12. MENDECKI, J., S. Y. LEE, and G. BRAWERMAN. 1972. Characteristics of the polyadenylic acid segment associated with messenger ribonucleic acid in mouse sarcoma 180 ascites cells. *Biochemistry.* **11**:792.
13. MORRIS, J. E., and A. A. MOSCONA. 1971. The induction of glutamine synthetase in aggregates of embryonic neural retina cells: correlations with differentiation and multicellular oganization. *Dev. Biol.* **25**:420.
14. MOSCONA, A. A. 1971. Control mechanisms in hormonal induction of glutamine synthetase in the embryonic retina. *In* Hormones in Development. M. Hamburgh and E. J. W. Barrington, editors. Appleton-Century-Crofts, New York. 169.
15. MOSCONA, A. A. 1972. Induction of glutamine synthetase in embryonic neural retina: a model for the regulation of specific gene expression in embryonic cells. *FEBS Fed. Eur. Biochem. Soc. Proc. Meet.* **24**:1.
16. MOSCONA, A. A., and J. L. HUBBY. 1963. Experimentally induced changes in glutamotransferase activity in embryonic tissue. *Dev. Biol.* **7**:192.
17. MOSCONA, A. A., and R. PIDDINGTON. 1966. Stimulation by hydrocortisone of premature changes in the developmental pattern of glutamine synthetase in embryonic retina. *Biochim. Biophys. Acta.* **121**:409.
18. MOSCONA, A. A., and R. PIDDINGTON. 1967. Enzyme induction by corticosteroids in embryonic cells: steroid structure and inductive effect. *Science* (*Wash. D. C.*). **158**:496.
19. MOSCONA, M., N. FRENKEL, and A. A. MOSCONA. 1972. Regulatory mechanisms in the induction of glutamine synthetase in the embryonic retina: immunochemical studies. *Dev. Biol.* **28**:229.
20. MOSCONA, A. A., M. MOSCONA, and R. E. JONES. 1970. Induction of glutamine synthetase in embryonic neural retina *in vitro* by inhibitors of macromolecular synthesis. *Biochem. Biophys. Res. Commun.* **39**:943.
21. MOSCONA, A. A., M. H., MOSCONA, and N. SAENZ. 1968. Enzyme induction in embryonic retina: the role of transcription and translation. *Proc. Natl. Acad. Sci. U. S. A.* **61**:160.
22. PIDDINGTON, R. 1967. Hormonal effects on the development of glutamine synthetase in the embryonic chick retina. *Dev. Biol.* **16**:168.
23. PIDDINGTON, R., and A. A. MOSCONA. 1965. Correspondence between glutamine synthetase activity and differentiation in the embryonic retina *in situ* and in culture. *J. Cell Biol.* **27**:247.
24. PIDDINGTON, R., and A. A. MOSCONA. 1967. Precocious induction of retinal glutamine synthetase by hydrocortisone in the embryo and in culture. Age-dependent differences in tissue response. *Biochim. Biophys. Acta.* **141**:429.
25. REIF-LEHRER, L. 1968. Induction of glutamine synthetase in chick embryo retina. Effects of serum and of steroid structure. *Biochim. Biophys. Acta.* **170**:263.
26. SARKAR, P. K., B. GOLDMAN, and A. A. MOSCONA. 1973. Involvement of poly-A in selective gene expression: suppression of enzyme induction in neural retina by inhibitors of poly-A synthesis. *Biochem. Biophys. Res. Commun.* **50**:308.
27. SARKAR, P. K., D. A. FISCHMAN, E. GOLDWASSER, and A. A. MOSCONA. 1972. Isolation and characterization of glutamine synthetase from chicken neural retina. *J. Biol. Chem.* **247**:7743.
28. SARKAR, P. K., A. W. WIENS, M. MOSCONA, and A. A. MOSCONA. 1972. Control aspects of glutamine synthetase induction in embryonic neural retina. *In* Molecular Genetics and Developmental Biology. M. Sussman, editor. Prentice-Hall, Inc., New Jersey. 303.
29. STELOS, P. 1967. Isolation of immunoglobulin. Salt fractionation. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications Ltd., Oxford. 4.
30. SUHADOLNIK, R. J. 1970. Nucleoside Antibiotics. John Wiley and Sons, Inc., New York. 156.
31. TOMKINS, G. M. 1969. Control of specific gene expression in mammalian cells. *In* Problems in Biology: RNA in Development. E. W. Hanly, editor. University of Utah Press, Salt Lake City, Utah. 145.
32. TOMKINS, G. M., E. B. THOMPSON, S. HAYASHI, T. GELEHRTER, D. GRANNER, and B. PETERKOFSKY. 1966. Tyrosine transaminase induction in mammalian cells in tissue culture. *Cold Spring Harbor Symp. Quant. Biol.* **31**:349.
33. WELLNER, V. P., and A. MEISTER. 1966. Binding of adenosine triphosphate and adenosine diphosphate by glutamine synthetase. *Biochemistry.* **5**:872.
34. WIENS, A. W., and A. A. MOSCONA. 1972. Preferential inhibition by proflavine of the hormonal induction of glutamine synthetase in embryonic neural retina. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1504.