Hydrocortisone Requirement for the Induction of Glutamine Synthetase in Chick-Embryo Retinas

By LIANE REIF-LEHRER* AND HAROLD AMOS

Department of Bacteriology and Immunology, Harvard Medical School,
Boston, Mass. 02115, *U.S.A.*

Hydrocortisone has been found to induce glutamine synthetase activity in chick-embryo retinas in culture. Evidence is presented to show that the hydrocortisone is definitely required for transcription; its requirement for translation has not been ruled out. The possible identity of hydrocortisone with an active component of calf-serum diffusate reported earlier is discussed. The data also indicate that the glutamine synthetase messenger RNA is stable for at least several hours.

Corticosteroids have been shown to play an important role in the induction of enzymes in animal cells (Rosen & Nichol, 1963). Most of the evidence has been gained from the study of their action in the whole animal with enzymes whose formation was not initiated but was accelerated by the hormones. This 'secondary induction' has been found to be actinomycin D-sensitive, suggesting that the steroids in some way stimulate the synthesis of m-RNA† (Kenney & Albritton, 1965).

Recently the elevation of the activity of several enzymes in cultivated cells has also been demonstrated to require corticosteroids (Pitot, Peraino, Morse & Potter, 1964; Thompson, Tomkins & Curran, 1966; Moscona & Piddington, 1966; Melnykovych, 1966). In a study of the induction of tyrosine transaminase in a hepatic cell line, Thompson *et al.* (1966) have shown that the hormones were, in fact, required for both transcription and translation.

During attempts to identify a diffusible serum factor required for the induction of GS (Reif & Amos, 1966) in cultured chick retina, we found the presence of a corticosteroid to be obligatory for induction. This discovery was made independently before the recent report by Moscona & Piddington (1966).

It is the intent of this paper to present evidence that (1) hydrocortisone is active at the transcription stage of GS synthesis, (2) m-RNA for GS can be synthesized and can accumulate while protein synthesis is inhibited by puromycin or cycloheximide, (3) m-RNA for GS is stable for at least several hours and (4) translation of m-RNA for GS probably does not require the hormone.

METHODS AND MATERIALS

Retinas were removed from embryos at 10 days of development. Each retina was cultured in 5 ml. of Eagle's basal medium with 10% (v/v) of foetal calf serum in tightly stoppered 50 ml. Erlenmeyer flasks at 37°. The flasks were shaken on a gyrorotary shaker (70–72 rev./min.) for the culture period (usually 1–4 days). Hydrocortisone was used either as the free alcohol (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) or as the 21-sodium succinate derivative (obtained through the generosity of Upjohn Co., Kalamazoo, Mich., U.S.A.). It was kept frozen in aqueous solution. Puromycin and cycloheximide were obtained from Nutritional Biochemicals Corp. and were also stored frozen in aqueous solution. Actinomycin D was obtained through the courtesy of Merck and Co., Rahway, N.J., U.S.A., and was stored in aqueous solution in the dark at 4°. Flasks treated with actinomycin D were protected from light as far as possible.

Where indicated retinas were washed three times at room temperature with Hanks' balanced salt solution containing antibiotics. In some experiments the balanced salt solution used for washing also contained 10% (v/v) of foetal calf serum. After washing, the retinas were supplied with 5 ml. of fresh medium containing 10% (v/v) of foetal calf serum and additions as indicated in the text. Wash fluids were removed by decanting. In many experiments, the residues of the original medium and of the first wash were drawn off by suction through a sterile Pasteur pipette. L-[14C]Leucine (240 mc/m-mole), L-[14C]isoleucine (126 mc/m-mole) and L-[14C]phenylalanine (355 mc/m-mole) were obtained from Schwarz BioResearch Corp., Orangeburg, N.Y., U.S.A.

All analyses were done on the sample obtained from mild ultrasonic disruption of the saline-washed retina in 2 ml. of 0·06 M-sodium phosphate buffer, pH 7·4–7·6. This

---

* Present address: Howe Laboratory of Ophthalmology, Harvard University Medical School, Massachusetts Eye and Ear Infirmary, Boston, Mass. 02114, U.S.A.

† Abbreviations: m-RNA, messenger RNA; GS, glutamine synthetase.

involved a 5sec. treatment with a Branson model LS75 Sonifier whose probe tip (0·32cm. in diameter) was inserted just below the surface of the solution. The enzyme appears to be stable to ultrasonic treatment under the same conditions for at least 15sec. Enzyme activity was determined by the method of Waelsch (1955), with a 2hr. incubation; product formation in this assay is linear over this length of time at 37°. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Incorporation studies refer to radioactive amino acids incorporated into cold-trichloroacetic acid-precipitable protein. The ultrasonic preparation (0·5ml.) was precipitated with trichloro-

acetic acid and left at 4° overnight. The precipitate was collected, washed with 20–40 μg. of unlabelled amino acid in 5% (w/v) trichloroacetic acid/ml., drained and dissolved in 2·5ml. of 0·1 N-NH$_3$. A 0·5ml. sample from each preparation was used for the protein determination. Another 0·5ml. sample was used to determine the radioactivity incorporated on a Packard Tri-Carb scintillation counter with a scintillation fluid of composition: dioxan, 750ml.; anisole, 125ml.; diethyleneglycol dimethyl ether, 125ml.; 2,5-diphenyl-oxazole, 4g.; 1,4-bis-(5-phenyloxazol-2-yl)benzene, 100mg. All values of enzyme activity are reported as E_{540}/mg. of protein; all radioactivities are reported as specific radio-

Table 1. *Effect of hydrocortisone on the appearance of GS activity in foetal-calf-serum cultures*

Units of enzyme activity are E_{540} readings (Bausch and Lomb Spectronic 20) divided by the mg. of protein in the sample. Ten-day retinas assayed shortly after excision give values of about 0·023. This zero-point value has been subtracted from all values presented. L-Tyrosine hydroxamide (Nutritional Biochemicals Corp.) has on average E_{540} 0·22/mg. under these assay conditions. Each value presented in the Table is the average of two or three cultures. Retinas were treated with hydrocortisone after 2 days in culture and harvested 2 days after treatment except in Expt. 3, where they were treated at zero time and harvested after 2 days, Expt. 5, where they were treated after 1 day and harvested after 2 further days, and Expts. 8 and 9, where they were treated at zero time and harvested after 3 days. Hydrocortisone was used in the free alcohol form in aqueous solution except where stated otherwise. For comparison, a parallel set of cultures in Expt. 1, treated with calf-serum diffusate instead of hydrocortisone, had a specific activity of 0·29. We call attention to the fact that in Expts. 1, 8 and 9 (as in fact in about 30% of all experiments) the values for specific enzyme activity are lower than in the other experiments. These values should be compared only within an experiment and not from one experiment to another, since they reflect the state of the retinas and other factors.

| | Specific enzyme activity (E_{540}/mg. of protein) | | | |
| | Untreated cultures | | | |
Expt. no.	At time hydrocortisone is added to parallel cultures	At time of harvest of parallel cultures	Hydrocortisone-treated cultures	Final concn. of hydrocortisone in medium (μg./ml.)
1	0·016	0·016	0·22	4·0
		0·077*	0·16	2·0
			0·16	0·4
			0·23	0·1
			0·22	0·02
2	0·031	0·077	0·42	0·4
3	0·00	0·016	0·43	0·4
4	0·047	0·12	0·50	0·4
5	0·00	0·12	0·73†	0·4
			0·71	0·4
6	0·031	0·062	0·39†	0·4
			0·34†	2·0
7	0·016	0·062	0·48	0·4
8			0·27	0·4
			0·23	0·02
			0·27	0·01
			0·21	0·005
			0·18	0·0025
9			0·34	0·4
			0·28	0·02
			0·26	0·01
			0·24	0·005
			0·12	0·001

* This value is for a set of cultures that were changed to fresh foetal-calf-serum medium at the time when parallel cultures were treated with hydrocortisone.

† Free hydrocortisone replaced by the 21-sodium succinate derivative.

activities, i.e. per mg. of protein. [^{14}C]Uridine (25 mc/m-mole) was obtained from Schwarz BioResearch Corp., Orangeburg, N.Y., U.S.A.

RESULTS AND DISCUSSION

Chick-embryo retinal cells *in vivo* contain no detectable GS activity until the seventeenth day of development (Rudnick & Waelsch, 1955). If the retinas are removed from the embryo at an earlier stage (10 days in the experiments described below) and put into culture medium containing 10% (v/v) of calf serum, GS activity appears within 24 hr. and reaches a maximum after 3–4 days in culture (Moscona & Piddington, 1966; Moscona & Hubby, 1963; Kirk & Moscona, 1963; Moscona & Kirk, 1965). In contrast, retinas maintained in medium containing foetal calf serum in place of calf serum develop little or no GS activity (Moscona & Kirk, 1965; Moscona & Piddington, 1966), although general protein synthesis, as measured by incorporation of ^{14}C-labelled amino acids, is fairly comparable in the two kinds of media (Rief & Amos, 1966). If, however, hydrocortisone is added at any time to the foetal-calf-serum medium, a rather high specific enzyme activity develops during the subsequent 1–2 days in culture.

The hydrocortisone requirement is satisfied by any of a wide range of concentrations (Table 1). Although the response is lowered at very low concentrations, 0·001 μg./ml. induces a moderate amount of enzyme activity. The results obtained with this and lower concentrations of hormone are somewhat variable. Preliminary findings indicate that the variability is due, at least in part, to differences in the concentrations and binding affinities of transcortin and albumin in the sera (Daughaday, 1958; Slaunwhite & Sandberg, 1959).

It should be noted that retinas from different batches of eggs appear to vary considerably in their response to induction. This may reflect factors influencing their adapatability to the culture condition and is reasonably uniform within a given set of eggs. The experiments shown in Table 1 represent the limits of responsiveness found in cells from different lots of embryos treated with hydrocortisone for 48 or 72 hr. The results obtained on adding hydrocortisone to foetal-calf-serum medium closely resemble those obtained on addition of either calf-serum diffusate (Reif & Amos, 1966) in place of hydrocortisone or calf serum alone (Moscona & Piddington, 1966).

A feature common to both hydrocortisone and calf-serum diffusate is the requirement for some quantity of whole serum in the initial incubation medium. The maximum enzyme activity obtained with hydrocortisone or calf-serum diffusate in the absence of serum is at best small (Table 2). We have found with hydrocortisone that cultures first exposed to serum (48 hr. in our experiments) are subsequently capable of induction by hydrocortisone alone; this response is, however, only half as large as that observed in the continued presence of serum. A major caution concerns the extent to which serum carry-over influenced these results. This is especially important since preliminary experiments indicate that concentrations of foetal calf serum as low as 0·5% are sufficient to give at

Table 2. *Dependence on serum of the effect of hydrocortisone on GS activity*

Units of enzyme activity are E_{540} readings (Bausch and Lomb Spectronic 20) divided by the mg. of protein in the sample. Abbreviations: 10% FCS, medium containing foetal calf serum (10%, v/v); HC, medium containing hydrocortisone (0·4 μg./ml.); S (−), medium lacking serum.

Expt. no.	Medium	Specific enzyme activity (E_{540}/mg. of protein)	
		Day 2	Day 4
1	10% FCS	0·016	—
	10% FCS + HC	0·43	—
	S (−)	0·00	—
	S (−) + HC	0·077	—
2	S (−)	0·016	—
	S (−) + HC	0·077	—
3	10% FCS	0·016	0·062
	10% FCS for 2 days, then S (−) for 2 days	—	0·093
	10% FCS for 2 days, then S (−) + HC for 2 days	—	0·25
	10% FCS for 2 days, then 10% FCS + HC for 2 days	—	0·48
4	10% FCS	0·031	0·11
	10% FCS for 2 days, then S (−) for 2 days	—	0·11
	10% FCS for 2 days, then S (−) + HC for 2 days	—	0·40
	10% FCS for 2 days, then 10% FCS + HC for 2 days	—	0·60

least marginal stimulation with hydrocortisone. Although many other parallels have been observed between hydrocortisone and some component of the calf-serum diffusate, hydrocortisone has not yet been definitively established as an active compound in calf-serum diffusate.

Having clearly established that hydrocortisone was capable of initiating formation of GS, we undertook to determine at what stage of the inductive process it was active.

Actinomycin D added simultaneously with hydrocortisone to cultures receiving the latter for the first time completely prevents the appearance of GS activity. This indicates that m-RNA for GS is not present in the retinas before the addition of hydrocortisone. A reversible inhibitor of protein synthesis such as puromycin or cycloheximide also prevents the appearance of GS activity (Colombo, Fellicetti & Baglioni, 1965; Ennis & Lubin, 1964; Darken, 1964) when added with hydrocortisone. Subsequent removal of the inhibitor permits a rapid rise in the amount of enzyme (Table 3b). The washing procedure, which can be shown to remove the inhibitor, may or may not effectively remove hydrocortisone. This limitation has made it difficult to interpret certain experimental results that are discussed below.

Essential to the projected use of actinomycin D was the establishment of the fact that it would not affect the translation or stability of GS m-RNA once formed. It was, in fact, found that retinas permitted to form m-RNA in the presence of hydrocortisone continue to make enzyme for several hours subsequent to the interruption of RNA synthesis by actinomycin D (Table 3a). Knowing that actinomycin D did permit translation, we attempted to separate more clearly the transcription and translation phases of the induction. Experiments were designed to permit RNA synthesis under conditions that do not allow protein synthesis (stage I) followed by a second stage (II) permitting protein synthesis without further RNA synthesis (Table 3b). Hydrocortisone was provided during either or both stages of the experiment. In a representative experiment protein synthesis was inhibited for 13 hr., after which time the cells were washed three times with balanced salt solution containing 10% (v/v) of foetal calf serum and returned to foetal-calf-serum medium containing 0·2 μg. of actinomycin D/ml. As demonstrated in Expts. 1, 2 and 3b of Table 3(a), enzyme synthesis is not impaired by the presence of actinomycin D at a concentration that restricts RNA synthesis to 5% of the normal value. The same concentration of actinomycin D given along with hydrocortisone permits only 15% of uninhibited enzyme synthesis. It seems reasonable to conclude that the formation of enzyme must result from the translation during stage II of GS m-RNA accumulated during stage I. The extent of enzyme formation is not enhanced by the readdition of hydrocortisone with actinomycin D.

A comparison of enzyme formed in stage II in the presence of actinomycin D shows a large discrepancy between cultures that were and were not exposed to cycloheximide in stage I. This difference can be accounted for if (1) recovery from cycloheximide inhibition is slow and (2) much of the GS message has been destroyed by the time the system has fully recovered.

In support of this explanation is the finding (Table 3b) that virtually all the enzyme made in stage II from RNA synthesized in stage I is made during the first 7 hr. This interpretation is predicated on the assumption that intracellular GS is stable for 24 hr. Experiments in which protein synthesis was inhibited by cycloheximide show that the GS activity is in fact stable over the period of time considered here (Fig. 1). Another explanation for the low yield of enzyme from m-RNA made in the absence of protein synthesis could be the inhibition of RNA synthesis by the drug, in this case cycloheximide; however, we have found no difference in radioactive uridine incorporation between control cultures and cultures treated with cycloheximide during stage I (Table 4). It is also conceivable that GS m-RNA made in the absence of protein synthesis differs in stability from that made with concurrent protein synthesis. Sussman (1966) has so interpreted results obtained with UDP-galactose polysaccharide transferase induction in slime moulds.

The very low concentrations of hydrocortisone required in this system make it difficult to assess how effectively the hormone has been removed by the washing procedure; therefore although no additional hydrocortisone appears to be required in stage II, the possibility remains that traces are in fact required for translation.

We now comment briefly on the difficulties encountered in our attempts to make use of puromycin as an inhibitor of protein and enzyme synthesis. We found the effect of this antibiotic on the retinal cells to be highly variable. In experiments with puromycin obtained before the summer of 1966, concentrations ranging from 1 to 20 μg./ml. seemed to inhibit consistently the synthesis of GS, and this effect was in each case reversed on washing out the drug. Since that time we have usually been unable to reverse the inhibition of enzyme synthesis despite more careful washing procedures. Moreover, general protein synthesis appears to be recovering normally. We cannot say at this time whether this is a function of the eggs or some contaminating inhibitor in puromycin that specifically interferes with GS synthesis.

Table 3. *Translation of GS m-RNA after inhibition of RNA synthesis, and synthesis of GS m-RNA during inhibition of protein synthesis*

The experiments were done as follows. Retinas were preincubated in medium with 10% (v/v) foetal calf serum for 52 hr. (Expts. 1 and 2), 48 hr. (Expts. 3a and 3c) or 60 hr. (Expt. 3b). They were then treated as indicated under the heading 'Stage I' for 13 hr. (Expts. 1 and 2), 5 hr. (Expt. 3a), 12 hr. (Expt. 3b) or 24 hr. (Expt. 3c). At the end of this time, the retinas were washed, put into fresh medium and treated as described under the heading 'Stage II' for 24 hr. Units of enzyme activity are E_{540} readings (Bausch and Lomb Spectronic 20) divided by the mg. of protein in the sample. Experiment numbers in (a) correspond to experiment numbers in (b). Abbreviations: HC, hydrocortisone (0·4 μg./ml. except 0·02 μg./ml. in Expt. 3); Act-D, actinomycin D (1·5 μg./ml.); CY, cycloheximide (1 μg./ml.). The specific enzyme activity values given are the averages of at least two cultures each. In (b), mean deviations from the average are given; the average mean deviation was 0·01.

(a) Translation of GS m-RNA after inhibition of RNA synthesis

Expt. no.	Treatment		Specific enzyme activity (E_{540}/mg. of protein)
	Stage I	Stage II	
1	Harvested after preincubation	—	0·01
	HC	Harvested after stage I	0·20
	HC	Harvested after stage II	0·30
	HC	Fresh medium	0·42
	HC	Fresh medium + Act-D	0·40
	HC + Act-D	Harvested after stage I	0·03
2	Harvested after preincubation	—	0·01
	HC	Harvested after stage I	0·25
	HC	No change; harvested after stage II	0·41
	HC + Act-D	Harvested after stage I	0·04
	HC	Fresh medium	0·42
	HC	Fresh medium + Act-D	0·38
3a	No HC	Harvested after stage II	0·06
	HC	Harvested after stage I	0·03
	HC	Fresh medium + Act-D	0·14
3b	HC	Harvested after stage I	0·08
	HC	Fresh medium	0·20
	HC	Fresh medium + Act-D (harvested after 7 hr.)	0·28
	HC	Fresh medium + Act-D	0·28
	HC	Fresh medium + Act-D (harvested after 53 hr.)	0·19
3c	HC	Harvested after stage I	0·24
	HC	Fresh medium + Act-D	0·33

(b) Synthesis of GS m-RNA during inhibition of protein synthesis

| Expt. no.	Treatment		Specific enzyme activity (E_{540}/mg. of protein)
	Stage I	Stage II	
1	HC	Harvested after stage I	0·20 ± 0·02
	CY + HC	Harvested after stage I	0·04 ± 0·01
	CY + HC	Fresh medium	0·32 ± 0·01
	CY + HC	Fresh medium + Act-D	0·12 ± 0·03
	CY + HC	Fresh medium + Act-D (harvested after 7 hr.)	0·10 ± 0·004
2	HC	Harvested after stage I	0·25 ± 0·004
	CY only	Harvested after stage I	0·02 ± 0·006
	CY + HC	Fresh medium	0·22 ± 0·001
	CY + HC	Fresh medium + Act-D (harvested after 7 hr.)	0·10 ± 0·02
	CY + HC	Fresh medium + Act-D	0·13 ± 0·01
3b	HC	Harvested after stage I	0·19 ± 0·02
	CY + HC	Harvested after stage I	0·04 ± 0·002
	CY + HC	Fresh medium	0·07 ± 0·005
	CY + HC	Fresh medium + Act-D (harvested after 7 hr.)	0·13 ± 0·01
	CY + HC	Fresh medium + Act-D	0·11 ± 0·003
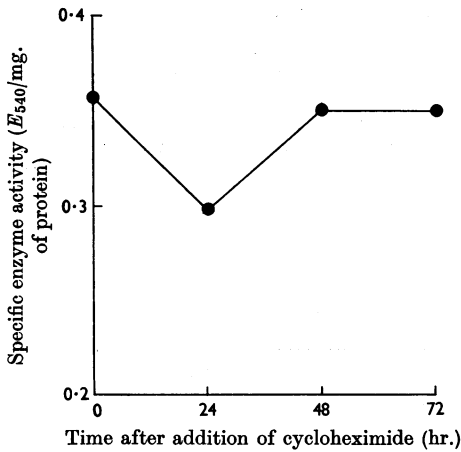
Fig. 1. Stability of GS. Retinas were exposed to 0·4 μg. of hydrocortisone/ml. after preincubation in 10% (v/v) foetal-calf-serum medium for 24 hr. During the subsequent 24 hr. a considerable amount of enzyme was produced. The retinas were then washed and returned to fresh medium with 10% (v/v) foetal calf serum and 0·5 μg. of cycloheximide/ml. At the times indicated duplicate cultures were harvested and assayed for enzyme activity. This concentration of cycloheximide reduced the incorporation of [^{14}C]phenylalanine over a 6 hr. period to 10% of the control.

Table 4. *Amino acid and uridine incorporation during cycloheximide treatment of chick-embryo retina cultures*

Retinas were preincubated in 10% (v/v) foetal-calf-serum medium for 48 hr. At this time hydrocortisone (0·4 μg./ml.) was added along with radioactive precursor (0·135 μc of phenylalanine or uridine/flask) and cycloheximide (1 μg./ml.) where used. Cultures were harvested after 18 hr. of treatment.

	Radioactivity incorporated (counts/min./mg. of protein in 24 hr.)	
	Controls (hydrocortisone only)	Hydrocortisone + cycloheximide
[^{14}C]Phenylalanine	5040	660
[^{14}C]Uridine	5700	6430

Clearly, the final resolution of the role of hydrocortisone in translation of GS m-RNA awaits a means of effective removal of traces of the corticosteroid whether they be inside the cell or on the cell surface. Meanwhile, it seems reasonable to propose that the hydrocortisone requirement for translation is at least an order of magnitude lower than that for transcription. We have clearly demonstrated that a specific m-RNA can be synthesized by retinal cells during the inhibition of protein synthesis by cycloheximide or puromycin. The synthesis of various classes of RNA in animal cells so inhibited has not been carefully studied to date. Although the GS m-RNA is synthesized, its stability or its rate of synthesis appears to be decreased in the absence of concomitant protein synthesis.

REFERENCES

Colombo, B., Fellicetti, L. & Baglioni, C. (1965). *Biochem. biophys. Res. Commun.* **18**, 389.

Daughaday, W. H. (1958). *J. clin. Invest.* **37**, 519.

Darken, M. A. (1964). *Pharmacol. Rev.* **16**, 223.

Ennis, H. L. & Lubin, M. (1964). *Science*, **146**, 1474.

Kenney, F. T. & Albritton, W. L. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 1693.

Kirk, D. L. & Moscona, A. A. (1963). *Developmental Biol.* **8**, 341.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall R. J. (1951). *J. biol. Chem.* **193**, 265.

Melnykovych, G. (1966). *Science*, **152**, 1086.

Moscona, A. A. & Hubby, J. L. (1963). *Developmental Biol.* **7**, 192.

Moscona, A. A. & Kirk, D. L. (1965). *Science*, **148**, 519.

Moscona, A. A. & Piddington, R. (1966). *Biochim. biophys. Acta*, **121**, 409.

Pitot, H. C., Peraino, C., Morse, P. A., jun. & Potter, V. R. (1964). *Monogr. nat. Cancer Inst.* no. 13, p. 229.

Reif, L. & Amos, H. (1966). *Biochem. biophys. Res. Commun.* **23**, 39.

Rosen, F. & Nichol, C. A. (1963). *Vitam. & Horm.* **21**, 135.

Rudnick, D. & Waelsch, H. (1955). *J. exp. Zool.* **129**, 309.

Slaunwhite, W. R., jun. & Sandberg, A. A. (1959). *J. clin. Invest.* **38**, 384.

Sussman, M. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 813.

Thompson, E. B., Tomkins, G. M. & Curran, J. F. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 296.

Waelsch, H. (1955). In *Methods in Enzymology*, vol. 2, p. 267. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.