

EFFECT OF WASHOUT OF INDUCING STEROID ON γ -GLUTAMYL TRANSFERASE ACTIVITY IN THE CULTURED CHICK EMBRYO RETINA

LIANE REIF-LEHRER. From the Department of Ophthalmology, Harvard Medical School, and the Department of Connective Tissue Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114

Glutamine synthetase (GS), as measured by its γ -glutamyl transferase (GT) activity¹, can be precociously induced in chick embryo retinas in tissue culture at least 7 days before its normal rapid elevation *in ovo* (Reif-Lehrer, 1968). The enzyme can be induced by a number of glucocorticoids (Chader and Reif-Lehrer, 1972), cortisol being the one most commonly employed. In such experiments, the question arises whether the continual presence of inducer is necessary to maintain the elevated enzyme level initially achieved. In some systems such as in liver-derived cells, simple washing to remove inducer leads to a fairly rapid drop in induced tyrosine transaminase activity (Tomkins et al., 1969; Auricchio et al., 1969; Baxter and Tomkins, 1970; Gerschenson et al., 1970). In some cultured hepatoma cell lines in which GT activity is inducible by dexamethasone, removal of inducer steroid results in a 50% drop in GT activity in 24 h (Kulka and Cohen, 1973). This does not appear to be the case for GS in the chick retina. In this system, retinas treated with steroid for 60 min or less develop very little additional GT activity in 24 h after washout of steroid. In contrast, retinas treated for 5 h or more, and then washed, develop enzyme levels approaching those of retinas left in contact with steroid during the whole of the 24-h period. The enzyme activity attained by retinas after 24 h in cortisol remains unchanged or, more often, increases by 72 h after the hormone has been washed out of the culture medium.

METHODS AND MATERIALS

Retinas from 12-day chick embryos were cultured and assayed for GT activity and protein as previously described (Reif-Lehrer, 1971), except that Eagle's basal medium without glutamine was used throughout, and the fetal calf serum used was treated with Norit (Fisher Scientific Co., Medford, Mass.) before use to eliminate

¹ In recent years, assays for GS and GT activity have been used interchangeably to measure GS enzyme. Evidence is now accumulating that this may not, in fact, be valid (Reif-Lehrer and Coghlin, 1973; Herzfeld, 1973; Herzfeld and Estes, 1973).

endogenous cortisol (Reif-Lehrer and Chader, 1969).

Retinas were incubated overnight (except as otherwise indicated) in medium containing 0.01 $\mu\text{g/ml}$ (2.6×10^{-8} M) cortisol or [³H]cortisol (2.2 $\mu\text{Ci/ml}$, i.e., 11 $\mu\text{Ci/flask}$ containing one retina.) Several control samples received no cortisol. At the end of this preincubation, the retinas were washed three or four times with 5 ml of prewarmed medium, which was removed as completely as possible by suction each time. The second and/or third wash included either a 20- or 60-min incubation on the gyrotory shaker. Some retinas were harvested before and some after the washing, while the rest were put into fresh medium without cortisol (after washing) for varying periods of time up to 72 h. In some experiments, additional cultures were included in which the final medium contained cycloheximide (2 $\mu\text{g/ml}$), actinomycin-D (2 $\mu\text{g/ml}$), cortisol (0.01 $\mu\text{g/ml}$), or combinations of these.

The extent to which the washing procedure eliminated steroid from the system was determined from cultures containing radioactive cortisol. For these cultures radioactivity of all media (triplicates, 10 μl each) and all washes (500 μl each) was determined after filtration to eliminate any cellular debris. Aliquots (500 μl) of the 2-ml retinal lysate were also counted. Aquasol (5 ml) in minivials (Rochester Scientific Co., Rochester, N. Y.) was used in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). All samples (except for the fourth wash) had at least 200 cpm (20 times background).

The volume of a 12-day retina was measured by determining the displacement of fluid resulting from addition of freshly dissected retinas to HBSS contained in a 10 ml graduated burette.

Medium was obtained from Microbiological Associates (Bethesda, Md.); dialyzed fetal calf serum and Hanks' Balanced Salt Solution were from Grand Island Biological Co. (Grand Island, N. Y.); cortisol was from Nutritional Biochemical Corp. (Cleveland, Ohio) or Sigma Chemical Co. (St. Louis, Mo.); tritiated cortisol and Aquasol were from New England Nuclear (Boston, Mass.); cycloheximide was from Nutritional Biochemical Corp.; actinomycin-D and glutamohydroxamic acid (used as a standard in the enzyme assay) were both from Sigma Chemical Co.

RESULTS

When retinas were incubated with 0.01 $\mu\text{g/ml}$ cortisol, the glutamotransferase activity increased

at least threefold overnight compared to controls incubated without steroid. If the tissue, after extensive washing, was further incubated in medium devoid of hormone, no drop in enzyme activity occurred in the next 56–72 h (Fig. 1). In fact, the specific activity of GT at the end of this period in noninducing medium was often higher than the activity just after washing. This change was variable but ranged all the way from no increase to as much as a 50% increase. In the presence of 2 $\mu\text{g}/\text{ml}$ cycloheximide (Fig. 1), enzyme activity neither increased nor decreased during the time in culture in the absence of cortisol (Kenney, 1967). An approximately two-fold increase in enzyme activity was observed when actinomycin-D was present during the terminal phase of incubation (Fig. 1). Similar results with this antibiotic were obtained in earlier experiments with a different objective (Reif-Lehrer, 1971). As expected, cycloheximide abolished the

enhancing effect of actinomycin-D on enzyme activity (Fig. 1).

The extent of cortisol washout was measured by using tritium-labeled cortisol at 0.01 $\mu\text{g}/\text{ml}$ (2.2 $\mu\text{Ci}/\text{ml}$, $1.1\text{--}1.4 \times 10^6$ cpm/ml); the results are summarized in Table I. The retinal volume used to calculate some of the values in the table was an average of independent measurements on 10 retinas and was 0.025 ± 0.007 ml.

When retinas were treated with steroid for periods of less than 24 h, the intraretinal concentrations of cortisol were somewhat higher than expected (compared to those left in steroid for 24 h) both immediately after washing, and after washing followed by 24 h of incubation in cortisol-free medium (Table II). However, steroid treatment for 1 h or less gave rise to only little additional enzyme activity in the subsequent 24 h in noninducing medium (Fig. 2). In contrast, although there was, in fact, a gradual change,

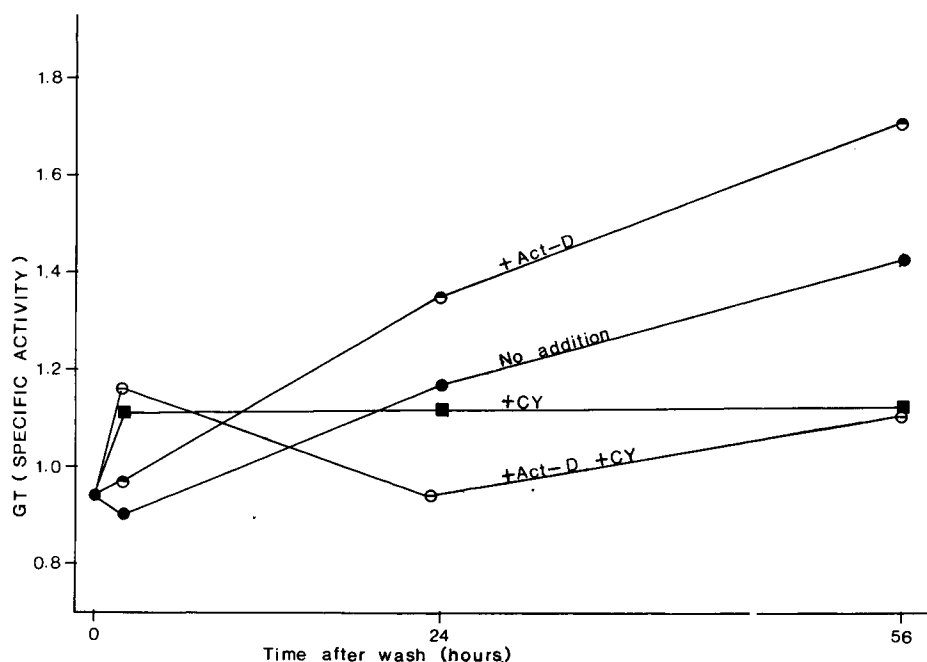


FIGURE 1 Effect of cycloheximide and actinomycin-D on additional accumulation of GT activity after inducer is washed out. The data in the figure are for a representative experiment. Similar results were obtained in experiments carried out to 72 h. The basic experiment in which there were no additions after washout of cortisol was repeated seven times; other conditions were repeated two to three times. Each time point in each experiment was the average of measurements obtained with three to six retinas independently incubated and assayed. The ordinate represents the specific activity of GT in micrograms of glutamohydroxamic acid formed per hour per microgram protein. After overnight incubation with 0.01 $\mu\text{g}/\text{ml}$ cortisol, the retinas were washed as described in the text (20-min incubation during wash). The abscissa represents the number of hours the retinas were subsequently incubated in noninducing medium. The concentration of actinomycin-D (Act-D) or cycloheximide (CY) in the postwash medium was 2.0 $\mu\text{g}/\text{ml}$.

TABLE I
Removal of [³H]Cortisol from Retinas by Washing*

Washing procedure	% of cortisol* remaining in retinal lysate		
	Immediately after washing	After 24 h in medium without cortisol‡	After 72 h in medium without cortisol‡
Three washes (1-h incubation during wash no. 2)	10-20	8 [2.0 × 10 ⁻³]	—
Four washes (1-h incubation during both washes no. 2 & 3)	10	1.4 (9.0 × 10 ⁻⁶)§ [3.6 × 10 ⁻⁴]	1.2 (7.7 × 10 ⁻⁶)§ [3.1 × 10 ⁻⁴]

* This table is a composite of the data obtained in four experiments. Retinas were incubated for 24 h in medium containing [³H]cortisol. At the end of this time about 1% of the cortisol in the medium had been taken up by the retinas (average of 4-6 replicates in each of two experiments). It is the percent of the amount of cortisol in the retina at the end of 24 h (before washing) that is tabulated above. The percent of [³H]cortisol in the original medium, which is found in medium in which washed retinas have been further maintained for 24 and 72 h is <0.02 and <0.015, respectively, when retinas were washed four times with two 1-h incubations. The 24-h value is 0.1% for retinas which were washed four times with only one 1-h incubation during wash no. 3.

‡ These have also been washed as described.

§ Value in () = micrograms of cortisol per total retina.

|| Value in [] = micrograms of cortisol per milliliter retinal lysate. This value is calculated using our measured value of 0.025 ml as the volume of an average 12-day retina.

TABLE II
Uptake of Cortisol by and Removal of Cortisol from the Retina

Time in [³ H]cortisol*	Percent of total cpm in original medium taken up by retina in that time	µg cortisol per ml retinal lysate (× 10 ³)‡		
		Prewash	Immediately post wash	24 h post wash
1 min	0.26	5.1	0.7	0.034
10 min	0.49	9.6	2.3	0.15
1 h	0.69	13.7	3.2	0.34
5 h	1.1	22.2	5.2	0.92
24 h	1.2	24.0	6.8	2.0

The values in the table are a composite from two experiments. Each value is an average derived from three to five retinas.

* 0.01 µg/ml [³H]cortisol

‡ Calculated values based on our measured volume of 0.025 ml for an average 12-day retina.

retinas induced for 4 h or more usually showed appreciable increase in enzyme activity during this period. Such retinas developed a level of GT activity approaching or equal to that obtained in retinas maintained for 24 h in the continual presence of the steroid (Fig. 2).

DISCUSSION

Our findings in the present study indicate that thorough washout of inducing steroid from 12-day retinas in culture leads to a decreased rate of accumulation of GT activity but does not, in most cases, lead to a leveling off of this activity. The experiments also indicate that even the most thorough washing procedure appears to leave a measureable cortisol level in the retina; the latter is, however, probably not responsible for additional increase in enzyme activity.

In our experiments, retinas washed after 5 h in steroid develop GT activity equal to that of retinas left in cortisol for 24 h. When retinas, after 24 h in steroid, were washed four times (with two 1-h incubations at 37°C), the GT activity increased about 30% in the 72 h after removal of cortisol. Moscona et al. (1970) have reported that the washout of steroid, after 4-5 h, from cultured retinas caused a leveling off of glutamine synthetase (GS) activity², and they concluded that GS activity continued to increase only if all further RNA synthesis was stopped. Data given in a recent report by Schwartz (1973) indicate, in agreement with our results, that the washing procedure causes no significant repression of GS² increase.

² As measured by GT activity.

It is difficult to assess the significance of incomplete washout of cortisol from the retinas with respect to induction. Induction of GT in retinas, after 24 h, was found by Chader and Reif-Lehrer (1972) to be half maximal when the medium concentration of steroid was 1×10^{-3} $\mu\text{g/ml}$, whereas 1×10^{-4} $\mu\text{g/ml}$ resulted in no induction (96% of the cortisol remains as the unmetabolized steroid under these conditions, Reif-Lehrer and Chader, 1969). Intermediate steroid concentrations (3×10^{-4} and 5×10^{-4} $\mu\text{g/ml}$) led to sporadic induction (Reif-Lehrer, unpublished results), probably reflecting the threshold responsiveness of the particular batch of eggs. In the present study, in which 0.01 $\mu\text{g/ml}$ cortisol (11 $\mu\text{Ci/flask}$ containing one retina) was used in the original medium, an inducing concentration of cortisol within the retinal lysate was not unusual immediately after washing and by the end of 24 h

(Table II). (The level of cortisol in the medium at the latter time is always well below an inducing concentration.) Schwartz, using 1 $\mu\text{g/ml}$ cortisol (3 $\mu\text{Ci/flask}$ containing one retina), reported 0.19 pmol cortisol/retina at the end of 19 h postwash. Using our measured value of 0.025 ml as the volume of the retina, this gives a value of 3×10^{-3} μg cortisol/ml retinal volume. However, the additional accumulation of enzyme activity in these retinas probably does not result from the residual intraretinal steroid. This conclusion is supported by the finding that actinomycin-D enhances this additional accumulation of enzyme (Fig. 1). Actinomycin-D is known to prevent formation of GT messenger RNA (mRNA) in this system, but to enhance translation of preexisting message (Reif-Lehrer, 1971).

The rate of total uptake (free steroid, nonspecifically bound and bound to specific receptor sites) of

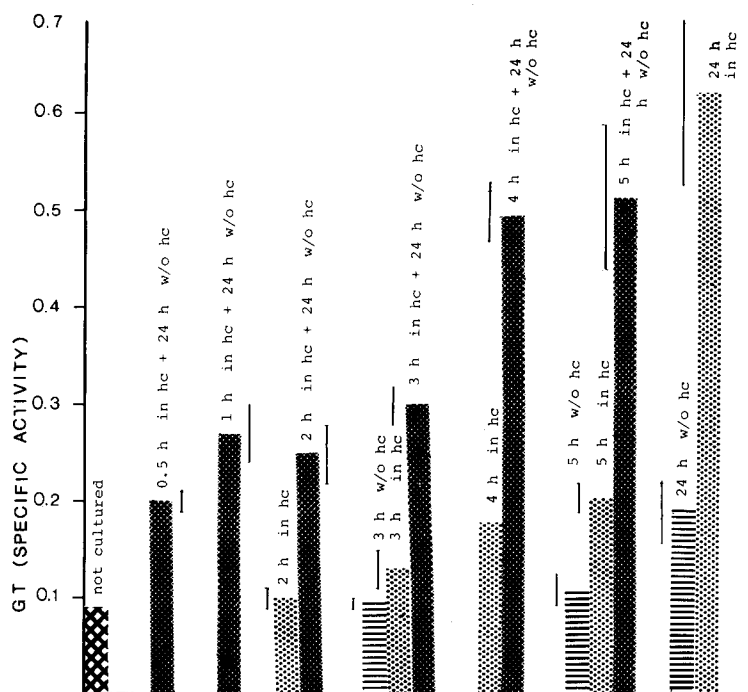


FIGURE 2 ■ Retinas excised and immediately frozen for assay (i.e. not put in culture). ▨ Incubated for number of hours (h) shown, in medium without (w/o) cortisol (hc). ▩ Incubated for number of hours shown, in medium containing 0.01 $\mu\text{g/ml}$ cortisol. ■ Incubated for number of hours shown, in cortisol; washed four times with 1-h incubation during both wash no. 3 and wash no. 4; then incubated in medium without steroid for 24 h. In this experiment the medium contained 5% commercially dialyzed fetal calf serum treated for 1 h at 45°C with twice the usual concentration of Norit (i.e. 100 mg Norit/1 ml serum). Each bar derives from the average of five retinas cultured independently. The ordinate represents the specific activity of GT in micrograms of glutamohydroxamic acid formed per hour per microgram of protein.

cortisol by retinas is rather high (Chader and Reif-Lehrer, 1972) and is maximal by 30–45 min (Reif-Lehrer, unpublished results) but, as can be seen from Fig. 2, 1 h in cortisol is not enough time in inducer to subsequently give rise to appreciable GT activity. Thus, the continued presence in the medium of some minimal concentration of cortisol is necessary until there is a sufficient accumulation of mRNA which can later be translated into enzyme. The rate of efflux of cortisol from retinas transferred to fresh medium lacking cortisol is also high (at least 50% as high as uptake; Reif-Lehrer, unpublished results). This would imply that the residual intraretinal steroid found in the present study might be that which is tightly bound to specific receptors. It would seem *a priori* reasonable that this bound species might be the active one with respect to induction. However, despite a greater inducibility in retinas from older compared to younger retinas (Reif-Lehrer, 1968), Lippman, Wiggert, Chader, and Thompson (1974) have found no increase in concentration of retinal cytoplasmic cortisol receptor in retinas between day 6 and 15 of development.³

Retinas left in noninducing medium after 5 h in cortisol develop a level of GT activity which is comparable to that attained by retinas in contact with steroid throughout the whole 24-h period. In a previous report concerning an enhancing effect of actinomycin-D on the translation phase of GT induction, the effect was also found to be maximal when transcription was allowed to proceed for 4–5 h (Reif-Lehrer, 1971). Retinas left in cortisol for 5–12 h have a relatively greater increase in GT activity in a subsequent 19–12 h in noninducing medium than do retinas left in steroid for 24 h, in an ensuing 24-, 48-, or 72-h period (Reif-Lehrer, unpublished results). Data given herein and elsewhere (Reif-Lehrer and Amos, 1968) indicate that the GT activity itself (but possibly not the GS activity, T. Reid, private communication) is quite stable under these circumstances; therefore, decay of GT activity would not be expected to contribute to the observed results. The above observations probably reflect the rate of accumulation of mRNA for GT as well as an appreciable half-life of this messenger.

The present study indicates that GT activity can

³ Koehler and Moscona (in press) have recently reported a greater concentration of cytoplasmic cortisol receptors in younger chick embryo retinas as compared to older.

continue to increase after washout of inducer if a sufficient concentration of mRNA for the enzyme has accumulated in the retina. The increased activity may be independent of residual steroid present in the retina.

SUMMARY

Cortisol-treated retinas can be washed free of steroid, although traces do remain within the tissue. Retinas which are left in an inducing concentration of steroid for a sufficient length of time and are then thoroughly washed can continue to accumulate GT activity in the virtual absence of steroid. Treatment with cortisol for less than 1 h results in little increase in enzyme activity when retinas are subsequently placed in inducer-free medium. 5-h treatment leads to an increased activity comparable to that attained by retinas maintained in the continued presence of the hormone. The postinducer increase in GT activity is doubled by actinomycin-D and is completely blocked by cycloheximide.

The author would like to thank Ms. Dianne Lindewall and Ms. Joy Bergenthal for their excellent technical assistance.

This work was supported by a grant from the National Eye Institute no. EY00374.

Received for publication 21 January 1974, and in revised form 13 May 1974.

REFERENCES

- AURICCHIO, F., D. M. JUN, and G. TOMKINS. 1969. Control of degradation and synthesis of induced tyrosine aminotransferase studies in hepatoma cells in culture. *Nature (Lond.)* **224**:806.
- BAXTER, J. D., and G. TOMKINS. 1970. The relationship between glucocorticoid binding and tyrosine aminotransferase induction in hepatoma tissue culture cells. *Proc. Natl. Acad. Sci. U. S. A.* **65**:709.
- CHADER, G. J., and L. REIF-LEHRER. 1972. Hormonal effects on the neural retina: corticoid uptake, specific binding and structural requirements for the induction of glutamine synthetase. *Biochim. Biophys. Acta.* **264**:186.
- GERSCHENSON, L. E., M. ANDERSSON, J. MOLSON, and T. OKIGAKI. 1970. Tyrosine transaminase induction by dexamethasone in a new rat liver cell line. *Science (Wash. D. C.)* **170**:859.
- HERZFELD, A. 1973. The distinction between γ -glutamyl-hydroxamate synthetase and L-glutamine-hydroxylamine glutamyltransferase activities in rat tissue: studies *in vitro*. *Biochem. J.* **133**:49.

- HERZFELD, A., and N. A. ESTES III. 1973. The distinction between γ -glutamylhydroxamate synthetase and L-glutamine-hydroxylamine glutamyltransferase activities in rat tissues: studies *in vivo*. *Biochem. J.* **133**:59.
- KENNEY, F. T. 1967. Turnover of rat liver tyrosine transaminase: stabilization after inhibition of protein synthesis. *Science (Wash. D. C.)* **156**:525.
- KULKA, R. G., and H. COHEN. 1973. Regulation of glutamine synthetase activity of hepatoma tissue culture cells by glutamine and dexamethasone. *J. Biol. Chem.* **248**:6738.
- LIPPMAN, M., B. O. WIGGERT, G. J. CHADER, and E. B. THOMPSON. 1974. Glucocorticoid receptors: characteristics and ontogenesis in the embryonic chick neural retina. *J. Biol. Chem.* In press.
- 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.
- REIF-LEHRER, L. 1971. Actinomycin-D enhancement of glutamine synthetase activity in chick embryo retinas cultured in the presence of cortisol. *J. Cell Biol.* **51**:303.
- REIF-LEHRER, L. 1968. Development of glutamine synthetase activity in chick embryo retina cultures in the absence of added hydrocortisone. *Biochem. Biophys. Res. Commun.* **33**:984.
- REIF-LEHRER, L., and H. AMOS. 1968. Hydrocortisone requirement for the induction of glutamine synthetase in chick-embryo retinas. *Biochem. J.* **106**:425.
- REIF-LEHRER, L., and G. J. CHADER. 1969. The steroid induction of glutamine synthetase in chick embryo retinas in culture. *Biochim. Biophys. Acta.* **192**:310.
- REIF-LEHRER, L., and J. COGLIN. 1973. Conversion of glutamic acid to glutamine by retinal glutamine synthetase. *Exp. Eye Res.* **17**:321.
- SCHWARTZ, R. J. 1973. Control of glutamine synthetase in the embryonic chick neural retina. *J. Biol. Chem.* **248**:6426.
- TOMKINS, G. M., T. D. GELEHRTER, D. GRANNER, D. MARTIN, H. H. SAMUELS, and E. B. THOMPSON. 1969. Control of specific gene expression in higher organisms. *Science (Wash. D. C.)* **166**:1474.