The Effect of Cortisol, Administered *in Vivo*, on the *in Vitro* Incorporation of DNA and RNA Precursors by Rat Thymus Cells

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SUMMARY

Cortisol, administered *in vivo*, decreases the rate at which radioactive labeled deoxycytidine and uridine are incorporated into macromolecular material by rat thymus cells incubated *in vitro*. The degree of inhibition of uridine conversion to acid-insoluble material, observed after 3 hr of cortisol treatment, remains constant at both high and low levels of precursor specific activity, whereas the inhibition of deoxycytidine-^{3}H incorporation observed using precursor of high specific activity is obliterated when the labeled precursor is diluted with large amounts of the nonradioactive compound. This observation is consonant with the hypothesis that one action of cortisol on rat thymocytes is to increase the intracellular pool of DNA precursors and not, as has been supposed, to inhibit the rate of DNA synthesis. Inhibition of RNA synthesis does seem to occur within a few hours after cortisol administration.

INTRODUCTION

In 1945, Dougherty and White (1) demonstrated that glucocorticoid treatment results in a marked inhibition of mitosis in lymphoid structures. Since that time, other investigations (2–6) more chemical in nature have supported the contention that glucocorticoids effect a decrease in DNA synthesis in lymphatic tissues. Makman, Dvorkin, and White (7) showed that thymocytes from rats treated with cortisol *in vivo*, when incubated *in vitro*, incorporated less radioactive deoxycytidine into DNA than did cells from control animals. Addition of the steroids *in vitro* yielded similar inhibitions of both thymidine and deoxycytidine incorporation. The investigations of Pratt and Aronow (8) demonstrate that those glucocorticoids which depress the rate of growth of mouse fibroblasts growing *in vitro* decrease the rate of thymidine incorporation into macromolecular material. Several of these same studies, as well as others, have also shown that the rate of RNA and protein precursor incorporation is depressed in antianabolic systems such as the thymus (9–12), lymph nodes (13, 14), lymphoma cells in culture (12), and fibroblasts growing *in vitro* (8).

METHODS AND MATERIALS

Male Sprague-Dawley rats weighing about 100 g each were obtained from Simonsen Laboratories Inc., Gilroy, California. The rats were bilaterally adrenalectomized by the dorsal route and were maintained on Purina Lab Chow and 0.9% saline ad libitum for 7 days before experimental use. Groups of five animals received intraperitoneal injections of either cortisol, 5 mg/100 g body weight dissolved in 1 ml of 50% aqueous dimethylsulfoxide,[2]

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[2] There was no reaction to the intraperitoneal administration of 1.0 ml of 50% aqueous dimethylsulfoxide save for very transitory signs of local irritation.

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or 1 ml of vehicle alone. At various time intervals after injection, the animals were sacrificed by decapitation, and the thymus glands were immediately excised and placed in an iced petri dish containing Joklik medium (obtained in powdered form from General Biochemicals) supplemented with 6% calf serum, serine (10^{-4} M), and pyruvate (10^{-3} M) in a constant atmosphere of 5% CO_2 in air. The glands were then minced with scissors and suspended in approximately 8 ml of the above medium; the cells were suspended by 3 strokes with a loose-fitting pestle in a Dounce-type homogenizer. Tissue fragments were eliminated by straining through 2 layers of cheese cloth, and additional chilled medium was added until the final concentration of cells was approximately 3 to 4×10^7 cells/ml as determined by hemacytometer count. The resulting cell suspension was preincubated for 15 min at 37° (95% air, 5% CO_2 atmosphere) and strained again through 2 layers of cheese cloth to remove clotted material which formed during preincubation; 1.0 ml aliquots were distributed into glass centrifuge tubes containing 0.1 ml of radioactive precursor solution. The tubes were immediately stoppered and incubated for 1 hr at 37° with shaking. Care was taken to maintain the atmosphere at 5% CO_2 in air.

The incubation was terminated by placing the tubes in ice and adding 4 ml of cold 5% trichloroacetic acid containing per milliliter 10 μg of nonradioactive deoxycytidine and uridine. After mixing, the tubes were centrifuged at 9000 rpm and the acid-insoluble material was washed twice by resuspension in cold trichloroacetic acid and centrifugation. The washed acid-insoluble material was suspended in either 3 or 4 ml of cold 2.5% trichloroacetic acid, duplicate 1.0-ml samples were collected by filtration with mild suction onto 2.4-cm Whatman glass-paper disks and washed once with cold water. The glass-paper disks were then dried and placed in a liquid scintillation counting vial. Ten ml of scintillator solution [4 g of 2,5-diphenyloxazole and 0.1 g of 2,2-*p*-phenylenebis(5-phenyloxazole) per liter of toluene] was added. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Protein was determined by the method of Oyama and Eagle (15) on triplicate 1-ml cell suspensions which had been pipetted into 4 ml of Earle's salt solution, centrifuged and washed twice with the salt solution to remove traces of serum protein, and dissolved in 2.0 ml of 1 N NaOH.

RESULTS AND DISCUSSION

Freshly isolated rat thymus cells suspended in a growth medium at 37° readily incorporate labeled deoxycytidine and uridine into acid-insoluble material. The incorporation of each of the precursors increases with the duration of the incubation and is temperature dependent. Incubations carried out in an ice bath for 1 hr or precipitated with trichloroacetic acid immediately after the addition of radioactive precursor yielded 2% of the acid-insoluble radioactivity observed for deoxycytidine, and 8% of that observed for uridine, after a 1-hr incubtaion at 37°. The specificity of the system with respect to the incorporation of each precursor into a particular type of macromolecule was examined, with the following results: (a) 93% of the acid-insoluble deoxycytidine-^{3}H radioactivity was recovered in the DNA fraction (0.3 N NaOH-insoluble material, solubilized by 10% trichloroacetic acid at 90° for 20 min), 7% was found to be incorporated into the RNA fraction (0.3 N NaOH-soluble material); (b) 85% of the uridine-^{14}C incorporated into macromolecular material was solubilized by extraction with 0.3 N NaOH for 18 hours at 37°. There was, therefore, no more than 15% incorporation of radioactivity from uridine into DNA.

The incorporation of radioactive deoxycytidine and uridine was studied in isolated thymocytes taken from rats which had received cortisol 0.5, 1, and 3 hr previously. There is little or no depression of any precursor incorporation if the animals are sacrificed 30 min after steroid administration. At 1 hr there is a variable response; after 3 hr of steroid action *in vivo*, depressed incorporation is always observed. Table 1

TABLE 1

Influence of cortisol on the incorporation of radioactive deoxycytidine and uridine by rat thymus cells

Five 150-g adrenalectomized rats were injected intraperitoneally with 7.5 mg of cortisol dissolved in 1 ml of aqueous dimethylsulfoxide (1:1). Five control rats received 1 ml of the vehicle. Three hours after injection the animals were sacrified, and thymus cell suspensions were prepared as described in the text. Of the cell suspension, 1.0 ml was added to each test tube containing one of the following precursors in an atmosphere of 5% CO_2: 1.25 mμmoles deoxycytidine-U-^{3}H (2.4 C/mmole), or 16.5 mμmoles uridine-2-^{14}C (30 mC/mmole). After 1 hr of incubation with shaking at 37°, the acid-insoluble material was collected as described in the text. The figures in the table represent the means and standard errors of three replicate incubations.

	Deoxycytidine			Uridine		
Expt. No.	Control (cpm/10 μg protein)	+Steroid (cpm/10 μg protein)	Inhibition[a] (%)	Control (cpm/10 μg protein)	+Steroid (cpm/10 μg protein)	Inhibition[a] (%)
1[d]	15.5 ± 1.1	6.6 ± 0.1	57	18.6 ± 0.3	8.5 ± 0.5	54
2[d]	13.7 ± 2.0	6.6 ± 0.1	52[b]	35.9 ± 0.2	19.9 ± 0.3	45
3	98.6 ± 2.8	35.2 ± 3.5	64	100.1 ± 1.6	36.8 ± 0.8	63
4	50.3 ± 2.2	31.7 ± 5.6	37	35.0 ± 3.5	28.4 ± 0.5	19[c]
5	61.8 ± 2.4	29.0 ± 1.7	53	37.8 ± 0.7	29.5 ± 0.3	22
6	52.0 ± 1.7	37.5 ± 1.7	28	43.2 ± 0.3	34.8 ± 1.6	19
Average			49 ± 5			37 ± 8

[a] All values in the column are significant at $p < 0.01$ except as noted.

[b] Significant at $p < 0.05$.

[c] Not significant.

[d] In experiments 1 and 2, a 15-min equilibration was not carried out.

presents the results of 6 separate experiments, each a 1-hr incubation with labeled precursors, carried out on thymus cells from rats which had received cortisol 3 hr prior to sacrifice. The degree of inhibition of deoxycytidine-^{3}H incorporation resulting from cortisol treatment ranged from 28% to 64%, with a mean depression of 49%. Uridine incorporation is inhibited an average of 37%. The low standard errors within each experiment attest to the internal reliability of the incorporation system; however, in these experiments, as in those of Makman, Dvorkin, and White (7), there is a considerable variability of response from one experiment to another in terms of the degree of cortisol-mediated inhibition of both precursor incorporations. The reason for the variation in percent inhibition observed from experiment to experiment is not known, but it may well have to do with the speed of absorption and distribution of the steroid from the peritoneal cavity to the thymus gland.

Both the studies from White's laboratory and our experiments (Table 1) were carried out using tritiated deoxycytidine of high specific activity as a monitor of DNA synthesis.[3] In addition, Makman, Dvorkin, and White found that the incorporation of ^{3}H-labeled uridine (1.8 C/mmole) into the DNA fraction and the incorporation of deoxyuridine-^{14}C (9.3 mC/mmole) into acid-insoluble material were depressed by cortisol treatment. But depressions observed under these conditions might be obtained if the effect of the steroid were merely to increase the size of the precursor pools. If this were the case, the depression of precursor incorporation observed in the cortisol-treated cells would reflect a steroid-mediated dilution of the specific activity of the precursor and not necessarily a decrease in the rate of macromolecular synthesis.

In order to investigate the possibility that the steroid is effecting a dilution in deoxycytidine or uridine specific activity

[3] Makman and White employed deoxycytidine-^{3}H at 2.4 C/mmole.

This One
14LH-BDC-0JH0

TABLE 2

Cortisol effect on incorporation into macromolecular material under conditions of high and low precursor specific radioactivity

Six 150-g adrenalectomized rats were injected intraperitoneally with 7.5 mg of cortisol dissolved in 1 ml of aqueous dimethylsulfoxide (1:1). Six control rats received vehicle injections. After 3 hr, both groups of rats were sacrified and thymus cell suspensions were prepared as described in the text. Of the cell suspension 1.0 ml was added to each test tube containing one of the following precursors in an atmosphere of 5% CO_2: in experiment A, 1.25 mμmoles deoxycytidine-U-^{3}H (2400 mC/mmole, high specific activity), 62.5 mμmoles deoxycytidine-U-^{3}H (48 mC/mmole, low specific activity); in experiments B and C, 0.6 mμmole deoxycytidine-U-^{3}H (5000 mC/mmole, high specific activity), 30.0 mμmoles deoxycytidine-U-^{3}H (100 mC/mmole, low specific activity); in experiments A, B, and D, 33 mμmoles uridine-2-^{14}C (30 mC/mmole, high specific activity), or 3.3 μmoles uridine-2-^{14}C (0.3 mC/mmole; low specific activity). After 1 hr of incubation at 37°, the acid-insoluble material was collected and assayed for radioactivity as described in the text. The values presented in the table represent the means and standard errors of three replicate incubations.

Experiment	Specific radioactivity of precursor[e]	Deoxycytidine			Uridine		
		Control (cpm/10 μg protein)	+Steroid (cpm/10 μg protein)	Ratio of steroid to control	Control (cpm/10 μg protein)	+Steroid (cpm/10 μg protein)	Ratio of steroid to control
A	High	9.3 ± 0.2	5.8 ± 0.3	0.62[a]	30.0 ± 0.1	26.3 ± 0.4	0.87[a]
	Low	2.3 ± 0.1	2.5 ± 0.3	1.06[d]	7.5 ± 0.1	6.4 ± 0.3	0.85[b]
B	High	20.4 ± 1.2	16.2 ± 0.7	0.80[c]	100.0 ± 1.9	79.5 ± 0.6	0.80[a]
	Low	9.3 ± 0.6	9.2 ± 0.6	0.99[d]	79.0 ± 4.0	70.3 ± 0.5	0.89[d]
C	High	26.0 ± 1.8	17.9 ± 0.8	0.69[b]	—	—	—
	Low	10.5 ± 0.6	11.3 ± 1.4	1.08[d]	—	—	—
D	High	—	—	—	25.9 ± 0.7	20.4 ± 0.3	0.79[a]
	Low	—	—	—	8.6 ± 0.2	6.8 ± 0.1	0.80[a]

[a] Significantly different from control at $p < 0.01$.
[b] Significantly different from control at $p < 0.02$.
[c] Significantly different from control at $p < 0.05$.
[d] Not significantly different from control.
[e] Note that amount of radioactivity is kept constant. Under conditions of low specific activity there is a higher concentration of precursor as specified in the legend.

by increasing the amount of nonradioactive deoxycytidine or uridine (or their phosphorylated derivatives) in the intracellular pool, replicate thymus cell suspensions from 3-hr cortisol-treated and control rats were incubated in triplicate for 1 hr with both high and low specific activity deoxycytidine or uridine. The amount of radioactive label was kept constant, but the total concentration of precursor was increased 50-fold in the case of deoxycytidine and 100-fold in the case of uridine, in order to achieve low specific activity conditions.

The data shown in Table 2 reveal two facts. First, in control cultures, the incorporation of radioactivity was reduced much less than the specific radioactivity of the precursor in the external medium. Under conditions of low specific radioactivity, for example, the incorporation was only about ¼ that found under conditions of high specific radioactivity rather than 1/50 or 1/100. Second, in steroid-treated cultures, the depression observed for uridine-^{14}C incorporation under conditions of high specific activity was maintained under conditions of low specific activity, but the depression of deoxycytidine-^{3}H incorporation observed using small amounts of the precursor was obliterated under conditions when the precursor was present in much greater amounts.

With respect to the first finding, it could not be expected that there be a direct rela-

tionship, since the size of the internal, nonradioactive precursor pool will change the specific activity ratios between the high and low conditions to some ratio other than that of the added precursor. Considering the control culture only, if there is an intracellular pool of deoxycytidine triphosphate (dCTP) of the order of 20 μM, and we add (approximately) 1 μM deoxycytidine at 2400 μC/μmole, then the specific activity of the intracellular dCTP would approximate $1/21 \times 2400 = 115$ μC/μmole. If we add instead 57 μM deoxycytidine at 48 μC/μmole, then the specific activity of the intracellular dCTP would approximate $(57/77) \times 48 = 36$ μC/μmole. Then the ratio of specific activities intracellularly under these two conditions is about 3 to 1, instead of the 50 to 1 of the external, added solutions. Consequently, one would expect to see the incorporation under conditions of high specific activity reduced to about one-third this value under conditions of low specific activity, or about the amount actually observed (Table 2).

Although these concentrations of nucleotides are about what one might suppose to exist intracellularly (16), it would be very unwise, in view of the uncertainties, to make any more extensive calculations. We wish merely to show here that the extent of decreased incorporation observed in the control cells in the presence of precursor material of low specific activity is in the expected range. Another possible explanation, that the higher concentrations of deoxycytidine or uridine stimulated nucleic acid synthesis either by directly increasing the intracellular concentration of precursors, or in some other unspecified way, cannot be ruled out but there is no reason to believe this actually occurs.

With respect to the second finding, the observation that the steroid-mediated depression of deoxycytidine-^{3}H incorporation is obliterated in the presence of high concentrations of deoxycytidine at low specific activity can be interpreted in two possible ways. First, the steroid could have caused the intracellular pool of deoxycytidine triphosphate to expand, so that the apparent decrease in incorporation seen when using precursor in very small amounts at high specific activity is actually a pool-dilution effect. The other possibility is that deoxycytidine itself, at high concentration, has an effect on DNA synthesis, and is blocking or hiding the steroid effect in some unspecified way. However, even at our higher concentration the concentration of deoxycytidine is only 57 μM, and there is no reason to believe this would have any effect on DNA synthesis.

Cortisol could effect an increase in the size of the DNA precursor pool in two ways: (a) by increasing the rate of precursor synthesis; (b) by increasing the rate of DNA breakdown. With respect to the first hypothesis, it is known that certain steroids can affect the rate of synthesis of DNA precursors. Weiss, Zagerman, and Kokolis (17) found that the levels of both thymidylate synthetase and the thymidine kinases in the seminal vesicle of the mouse were increased markedly in response to testosterone treatment. With respect to the second hypothesis, Haynes and Sutherland (18) have demonstrated that rat thymus slices taken 1 hr after cortisol injection release over twice as much DNA into the incubation medium as do control slices. These authors also noted that thymus slices made from adrenalectomized rats treated for 2 hr with cortisol *in vivo* incorporated thymidine-^{3}H into DNA at about 50% the rate of control slices when the incubations were carried out with 0.01 mM thymidine-^{3}H. With 1 mM thymidine-^{3}H, however, slices from treated rats incorporated the same amount or slightly more than the controls.

Experiments such as presented here, and those of Haynes and Sutherland approach the question of a pool dilution phenomenon indirectly. Direct measurements of pool sizes and effects of glucocorticoids upon them must be carried out.

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