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Effects of 8-Azaguanine on the Induction of Uterine Glucose-6-Phosphate Dehydrogenase Activity by Estradiol or $NADP^+$ (38184)

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Glucose-6-*P* dehydrogenase activity (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49) in the uterus of the ovariectomized rat is increased by intravenous administration of estradiol or by intrauterine application of the cofactor, $NADP^+$ (1). Both the estrogen and $NADP^+$-mediated increases in uterine glucose-6-*P* dehydrogenase (G6PD) activity are prevented by cycloheximide (1). However, doses of actinomycin D which are sufficient to inhibit the estrogen-induced increase in this enzyme do not inhibit the cofactor-induced response (1). While actinomycin D inhibits RNA synthesis, it also accelerates the breakdown of nuclear RNA without interfering with the activity of pre-existing pools of cytoplasmic RNA (2). The insensitivity of the $NADP^+$-induced increase in enzyme activity to actinomycin D suggested that the cofactor acted at a posttranscriptional step perhaps by stimulating the expression of a cytoplasmic pool of messenger RNA (mRNA). The nucleic acid base analogue, 8-azaguanine, either inhibits protein synthesis or causes tissues to synthesize defective proteins by its incorporation into RNA where it causes formation of fradulent or inactive messengers (3). These observations suggest that 8-azaguanine could be used to determine the timing and importance of increased RNA synthesis in the response of uterine G6PD to estradiol without the side effects of actinomycin. The incorporation of 8-azaguanine into cytoplasmic pools of mRNA would indicate the importance of the integrity of these pools to the response of the enzyme to $NADP^+$.

Materials and methods. Female albino rats (Small Animal Supply Company, Omaha) were ovariectomized 3–4 wk previous to use and maintained on Wayne Lab-Blox and water *ad lib* throughout the course of these studies. Animals weighing 240–260 g received either 5.0 μg of estradiol, 10.0 μmoles of $NADP^+$, or the appropriate saline carrier. Estradiol-17β was administered by tail vein injection in 0.5 ml of 0.15 *M* NaCl containing 5% ethanol. A nonsurgical procedure (1) was used for the intrauterine (iu) application of $NADP^+$ in 0.05 ml of 0.3 *M* NaCl divided equally between the 2 uterine horns. 8-Azaguanine and 4-amino-5-imidazolecarboxamide were prepared for injection and administered intraperitoneally as previously described (3). Animals were killed by cervical dislocation and their uteri were quickly excised, trimmed of extraneous tissue, and immediately frozen between 2 blocks of dry ice. Tissues were stored at −20° without loss of glucose-6-*P* dehydrogenase activ-

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ity before assay as previously described (4). Enzyme activities are expressed as units/uterus with a unit equal to that amount of enzyme capable of reducing 1.0 μmole of $NADP^+$/min under the conditions of the assay. The *t* test was used to determine the statistical significance of differences between means.

Results. Dose of 8-azaguanine required for maximum inhibition of the uterine response to estradiol. Three doses of 8-azaguanine were tested for their inhibitory effect on the response of uterine G6PD activity to estradiol. Table I indicates that 200 mg/kg of 8-azaguanine gave maximum inhibition of the response. 8-Azaguanine (200 mg/kg) is not an effective inhibitor without the simultaneous administration of 4-amino-5-imidazolecarboxamide which inhibits the deamination of 8-azaguanine (5). 4-Amino-5-imidazolecarboxamide treatment alone had no effect on the base level of enzyme activity or the response of uterine G6PD to estradiol. A dose of 200 mg/kg of 8-azaguanine with 50 mg/kg of 4-amino-5-imidazolecarboxamide given at 0, 1 and 12 hr (depending on the length of the response being measured) was used in all further experiments.

Effect of 8-azaguanine on enzyme induction by estradiol or $NADP^+$. The effects of 8-azaguanine on the induction of uterine G6PD by either estradiol or $NADP^+$ are given in Table II. 8-Azaguanine inhibited the increases in this enzyme induced by either estradiol or $NADP^+$ at 12 hr and had no effect on the basal enzyme level.

In addition to their direct effects on RNA and protein synthesis, many inhibitors of RNA and protein synthesis are thought to suppress some responses of the uterus to estradiol by increasing the secretion of adrenocortical steroids (6). To determine the importance of adrenocortical hyperactivity to the inhibition by 8-azaguanine of enzyme induction by estradiol, ovariectomized rats were adrenalectomized 24 hr before injection with estradiol and the inhibitor. The results given in Table III indicate that adrenalectomy did not abolish the inhibitory effect of 8-azaguanine on uterine G6PD induction by estradiol. Thus, adrenocortical hyperactivity does not appear to be the mechanism of the suppressive effects of 8-azaguanine.

Effect of delayed 8-azaguanine treatment on the uterine response to estradiol. Several studies have demonstrated the importance

TABLE I. Effect of 8-Azaguanine and 4-Amino-5-Imidazolecarboxamide on the Induction of Uterine Glucose-6-*P* Dehydrogenase Activity by Estradiol.

Treatment[a]	Enzyme activity (units/uterus)	Percent inhibition of estrogen response
Saline	0.137 ± 0.012	—
Saline + AIC	0.146 ± 0.010	—
Saline + AIC + Az (400 mg)	0.152 ± 0.028	—
Estradiol	0.309 ± 0.023	—
Estradiol + Az (200 mg)	0.252 ± 0.057	33[b]
Estradiol + AIC	0.312 ± 0.026	—
Estradiol + AIC + Az (100 mg)	0.237 ± 0.029	44[c]
Estradiol + AIC + Az (200 mg)	0.174 ± 0.013	83[c]
Estradiol + AIC + Az (400 mg)	0.175 ± 0.020	83[c]

[a] Ovariectomized mature rats received either 0.5 ml of saline (0.15 *M* NaCl + 5% ethanol) or estradiol (5 μg in 0.5 ml saline) intravenously at 0 time. 4-Amino-5-imidazolecarboxamide, AIC, (50 mg/kg body weight) and 8-azaguanine, Az, (amounts per kg body weight as indicated in parentheses) were given at 0, 1 and 12 hr and all animals were killed at the 24th hr after the initial treatments. The data represent the mean ± SEM of individual determinations on 5 rats per treatment group.

[b] Compared to the saline and estradiol treatment groups which did not receive AIC.

[c] Compared to the saline and estradiol treatment groups which also received AIC.

TABLE II. Effect of 8-Azaguanine on the Induction of Uterine Glucose-6-*P* Dehydrogenase Activity by Estradiol or NADP+.

Treatment[a]	Enzyme activity (units/uterus)	Percent inhibition of response
Saline	0.145 ± 0.004	—
8-Azaguanine	0.142 ± 0.004	—
Estradiol	0.228 ± 0.017	0
Estradiol + 8-azaguanine	0.154 ± 0.004	89[b]
NADP+	0.210 ± 0.013	0
NADP+ + 8-azaguanine	0.168 ± 0.012	65[c]

[a] Ovariectomized mature rats received estradiol (5 μg per animal given intravenously at 0 time), NADP+ (10 μmoles per animal given by the intrauterine route in 0.05 ml of 0.3 *M* NaCl at 0 time) and 8-azaguanine (200 mg/kg body weight given intraperitoneally at 0 time and at 1 hr) as indicated. All animals received 50 mg/kg body weight of 4-amino-5-imidazolecarboxamide at 0 time and at 1 hr after the initial treatment. The data represent the mean ± SEM of individual determinations on 6 rats per treatment group.

[b] Compared to the estrogen treatment group.

[c] Compared to the NADP treatment group.

of early RNA synthesis in various estradiol-induced responses in the immature or ovariectomized mature rat uterus (7–9). The RNA necessary for G6PD induction could be synthesized during either a short initial period or continuously throughout the response after stimulation by estradiol. Since 8-azaguanine inhibited protein synthesis through its incorporation into and formation of fradulent messenger RNA (3), it is likely that administration of this inhibitor at various times after estradiol might permit timing of the synthesis of the RNA necessary for the increased rate of G6PD synthesis. Groups of rats were given estradiol and treatment with 8-azaguanine was begun at 4 hr intervals throughout the 0–24 hr uterine response to the hormone. As indicated in Fig. 1, administration of 8-azaguanine at any time after the injection of estradiol inhibited additional increases in uterine G6PD levels in response to the hormone.

Effect of 8-azaguanine on preinduced

TABLE III. Effect of Adrenalectomy on the Inhibition by 8-Azaguanine of the Induction of Uterine Glucose-6-Phosphate Dehydrogenase by Estradiol.

Treatment[a]	Enzyme activity (units/uterus)	
	Control	Adrenalectomized[b]
Saline	0.157 ± 0.015	0.167 ± 0.011
Estradiol	0.277 ± 0.025	0.269 ± 0.016
Estradiol + 8-azaguanine	0.190 ± 0.017	0.181 ± 0.011

[a] Ovariectomized mature rats received estradiol (5 μg per animal given intravenously at 0 time) and 8-azaguanine (200 mg/kg body weight given intraperitoneally at 0 time and at 1 hr) as indicated. All animals received 50 mg/kg body weight of 4-amino-5-imidazolecarboxamide at 0 time and at 1 hr after the initial treatment. The data represent the mean ± SEM of individual determinations on 6 or 7 rats per treatment group.

[b] Adrenalectomies performed 24 hr prior to administration of the above treatments.

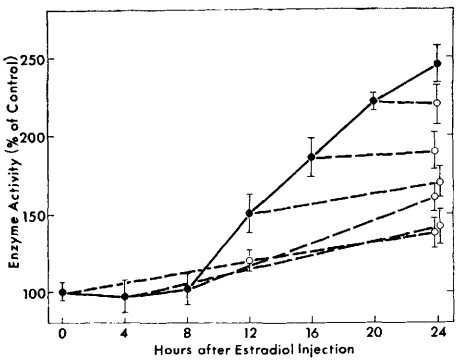

FIG. 1. Effect of administration of 8-azaguanine at various times after estradiol on the response of uterine glucose-6-*P* dehydrogenase activity to estradiol. Control animals were injected with estradiol alone (●) at 0 time and killed at 4 hr intervals. Other groups were injected with estradiol at 0 time and with 8-azaguanine and 4-amino-5-imidazolecarboxamide (○) beginning at 4 hr intervals after the estradiol injection and killed at 24 hr. 8-Azaguanine was injected at an interval after the estradiol injection and at 1 and 12 hrs later. The dashed line indicates the period during which each group was exposed to the inhibitor. Each point represents the mean percent of the 0 time control ± SEM of individual determinations on 5 rats per treatment group.

levels of G6PD activity. Inactivation or degradation of liver tyrosine transaminase can be inhibited by cycloheximide (10), 8-azaguanine, or 5-azacytidine (11). To determine whether or not 8-azaguanine might affect the rate of uterine G6PD inactivation, the inhibitor was administered to rats in which the levels of G6PD activity were previously elevated by daily injections with estradiol for 2 and 3 days, respectively. Individual groups at each time after estrogen treatment were given either another injection of estradiol or saline, with and without 8-azaguanine. As shown in Fig. 2, 8-azaguanine treatment inhibited the continuation of the response to estradiol between days 2 and 3 and decreased the level of G6PD activity normally maintained by estradiol between days 3 and 4. Following the withdrawal of estradiol treatment at days 2 or 3, enzyme levels declined and administration of 8-azaguanine did not alter the rate of decline. These results indicate

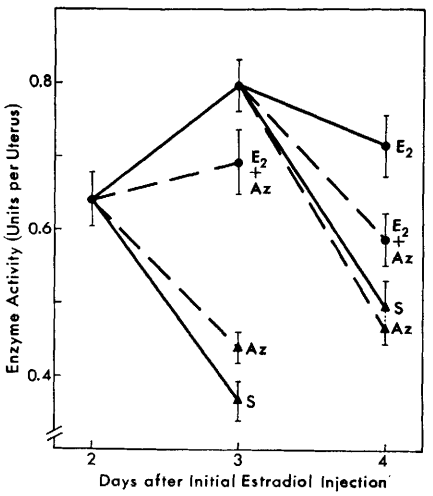

FIG. 2. Effect of 8-azaguanine on preinduced levels of glucose-6-*P* dehydrogenase activity. Ovariectomized rats received estradiol (5 μg, iv) for either 2 or 3 days before receiving estradiol (E_2, ●) or saline (S, ▲), with (- - -) or without (——) 8-azaguanine (Az). 8-Azaguanine (200 mg/kg body weight) and 4-amino-5-imidazolecarboxamide (50 mg/kg body weight) were given at 0, 1, and 12 hr. Each point represents the mean ± SEM of individual determinations on 6 rats per treatment group.

that 8-azaguanine does not cause a "superinduction-like" response as is seen when actinomycin is given in a similar experiment (12) and it does not alter the rate of enzyme inactivation or degradation which normally occurs after estrogen withdrawal.

Discussion. 8-Azaguanine inhibits protein synthesis in bacterial and mammalian systems (13) and causes a partial conversion of rat liver polyribosomes to inactive monomers and dimers (3), apparently through the formation of fraudulent or defective messenger RNA. Since the residual polyribosomes from 8-azaguanine-treated rats incorporated amino acids at rates comparable to those of controls, 8-azaguanine probably does not inhibit protein synthesis by affecting polyribosomes through competition with normal cofactors (GTP) or the production of fraudulent transfer RNA (3). Studies of the incorporation of precursors into uterine RNA (6–8) and the inhibition by actinomycin D of the estradiol-induced increase in uterine G6PD activity (1) indicate the importance of RNA synthesis to the uterine response to estradiol. Since 8-azaguanine inhibited the continuation of the uterine G6PD response to estradiol when injected at any time after estradiol (Fig. 1), the mRNA necessary for the synthesis of uterine G6PD must be synthesized continuously after initiation of the uterine response to estradiol.

Levels of enzyme activity in tissues are regulated by the control of rates of enzyme synthesis and degradation. The biochemical mechanisms of physiological protein degradation are not understood but appear to be controlled by hormonal or nutritional variables (14). Several studies have demonstrated the importance of continued protein synthesis to enzyme degradation in the liver. Cycloheximide, 5-azacytidine, or 8-azaguanine inhibited the degradation of hepatic tyrosine transaminase (9, 10), and a similar effect of cycloheximide on G6PD inactivation in the uterus has been observed (12). However, the results presented in Fig. 2 suggest that 8-azaguanine has no effect on the rate of enzyme inactivation in the uterus. Immunochemical determination of the decrease in prelabeled G6PD after

8-azaguanine treatment would provide more decisive proof of this negative result.

Some increase in G6PD activity was consistently observed after estradiol or $NADP^+$ treatment even at the dose of 8-azaguanine which was maximally effective. $NADP^+$ or estradiol may stimulate the expression of a small pre-existing pool of mRNA without further RNA synthesis. Such post-transcriptional effects have been observed with the induction of hepatic tyrosine transaminase by hydrocortisone after 8-azaguanine treatment (15). The G6PD enzyme protein of animals treated with estradiol, estradiol + 8-azaguanine, or saline were immunologically identical as determined by the endpoint of immunotitration (unpublished observations, E. R. Smith, B. C. Moulton, and K. L. Barker), indicating that the inhibitor probably caused a sufficient alteration in the G6PD messenger to render the messenger ineffective rather than inducing minor changes in the primary structure of the enzyme.

Intrauterine administration of $NADP^+$ appears to increase the level of G6PD in the ovariectomized rat uterus as a result of stimulation of the rate of enzyme synthesis rather than by activation of apoenzyme or by stabilization of existing enzyme (1, 4). The response to $NADP^+$ is sensitive to cycloheximide, and no activation or stabilization effects have been observed during the course of these studies. Since the induction of G6PD by $NADP^+$ was not inhibited by actinomycin D, a posttranscriptional mechanism for this induction was implicated. Enhanced translation after $NADP^+$ treatment would require the integrity of a pool of cytoplasmic mRNA for the enzyme. Inhibition of the response of G6PD to $NADP^+$ by 8-azaguanine could result from the synthesis of fraudulent mRNA which could replace or compete with the cytoplasmic pool of authentic G6PD messenger preventing the synthesis of active enzyme.

Summary. The purine analogue, 8-azaguanine, inhibits the induction of uterine glucose-6-*P* dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49) by estradiol or the enzyme's cofactor, $NADP^+$. Inactivation of the enzyme after estradiol withdrawal was not affected by 8-azaguanine treatment. Since 8-azaguanine limited the uterine response to estradiol when injected at any time after the steroid, the RNA necessary for the synthesis of uterine glucose-6-*P* dehydrogenase must be synthesized continuously after the initiation of the uterine response to estradiol.

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