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THE REGULATION OF ADRENAL FUNCTION BY ESTROGENS AND OTHER HORMONES

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SUMMARY

1. A number of cytoplasmic enzymes of the adrenal cortex are active in the reduction of NADP. However, the rate of corticoid synthesis from endogenous cholesterol would seem to be regulated primarily by the rate of reduction of NADP by glucose-6-*P* dehydrogenase. Estrogens and several other hormones are competitive inhibitors of NADP-binding to NADP-specific dehydrogenases. The competitive inhibition by estrogens of the rate of reduction of NADP by glucose-6-*P* dehydrogenase was correlated with the competitive inhibition to the rate of corticoid synthesis. Examples of the molecular structure and mode of action of hormone inhibitors are given. Inhibition is non-competitive for substrate such as glucose-6-*P*.

2. A second possible biochemical site of action of estrogen involves a non-competitive activation of the NAD-specific lactic dehydrogenase of the cytoplasm. The K_m for the activation of lactic dehydrogenase by estradiol in the presence of excess lactate is $2.7 \cdot 10^{-9}$ M.

3. The inhibitions and activations by estrogens were also found to occur with the dehydrogenase enzymes of the anterior pituitary. By an interplay of inhibition or activation of metabolic pathways, estrogen may act as a regulatory hormone in tissues that are target organs of estrogen.

INTRODUCTION

Estrogens have been found previously to inhibit the formation of corticoids by rat adrenals *in vitro*[1] and after administration[2] *in vivo* of high doses. Inhibition caused by low doses of estrogen can be compensated for by an increase in corticotrophin secretion. A number of dehydrogenase enzymes of the cytoplasm were also found to be inhibited by estrogens, androgens[3] and thyronine compounds[4]. It seemed reasonable that the inhibition of NADP-specific dehydrogenase by estrogens and other hormones could explain the inhibition to corticoid synthesis. This is now proven to be the case. It was also suggested that these hormones regulate the dynamic balance between the adrenal and the pituitary hormones, and in the case of estrogens, lead to a stimulus from the pituitary to pentose shunt metabolism in a number of tissues[5] such as adipose[6] and liver[7]. The mechanisms of the estrogen inhibition have been extended and clarified in this paper. In addition, the peculiar importance of the

pentose shunt dehydrogenases in the rate of reduction of NADP for corticoid synthesis is demonstrated. The competitive relationship between estrogens and NADP for binding sites on glucose-6-*P* dehydrogenase (EC 1.1.1.49) and the relationship of this inhibition to corticoid synthesis is shown. It has also been found that estrogens can activate the oxidation of lactate by NAD-specific lactic dehydrogenase (EC 1.1.1.37) of the cytoplasm at concentrations (10^{-9} M) which have no effect on NADP dehydrogenases. This seems to be an additional regulatory mechanism of estrogen in both adrenal cortex, anterior pituitary, and possibly other hormone-sensitive tissues. Preliminary reports of some of these results have appeared[8,9].

EXPERIMENTAL PROCEDURE

The methods for assay of extramitochondrial enzymes, purification of glucose-6-*P* dehydrogenase, and the determination of Michaelis constants have been described elsewhere[4]. Batches of slices of fresh cow adrenal cortex were made with a Stadie–Riggs slicer from adrenals of 8 or 10 cows. Slices consisting mostly of fasciculata cells were homogenized in 0.154 M KCl without preincubation, immediately frozen, and used within one week. Rat adrenals were handled and preincubated as described[10,11]. Estrogen in freshly distilled dioxane or ethanol was added to homogenates with rapid mixing before reactions were started by the addition of substrate or pyridine nucleotide. After incubation of adrenal tissue the corticoids were extracted with methylene dichloride and measured by the blue tetrazolium method[12].

RESULTS

Relative activities of extramitochondrial dehydrogenases and effects of estrogens

All the NADP dehydrogenases shown in Table I were inhibited by estrogens at 10^{-7} M. The enzymic activities of the controls vary somewhat in different batches of cow adrenal. The NAD-specific lactic dehydrogenase was stimulated by estrogens. At 10^{-9} M there was no effect of estrogen on the NADP dehydrogenases (at least at the fairly high NADP level of one μmole), but the reaction

$$\text{lactate} + \text{NAD} \rightarrow \text{pyruvate} + \text{NADH}_2$$

was stimulated 33% by estradiol, while the reverse reaction

$$\text{pyruvate} + \text{NADH}_2 \rightarrow \text{lactate} + \text{NAD}$$

was stimulated only 9%. Estrone and estradiol were slightly less effective. There was no reduction of NADP or NAD when substrates or nucleotides were omitted from the reaction mixture. When the reduction of added NADP or NAD was allowed to go to completion there was no re-oxidation or effect of estrogen. No transhydrogenase was detected in the cytoplasm.

The relation of dehydrogenase activity to corticoid synthesis

The importance of the dehydrogenase enzymes of the pentose shunt for corticoid synthesis is demonstrated by the data of Table II. The basic principles of enzyme kinetics were applied to these metabolism studies. NADP was added at a level ($9 \cdot 10^{-5}$ M) to give a good, but less than maximal, rate of corticoid synthesis with

TABLE I

RELATIVE ACTIVITIES OF EXTRAMITOCHONDRIAL DEHYDROGENASES IN COW ADRENAL CORTEX AND EFFECT OF ESTROGENS AT 10^{-7} M

Each enzyme system consisted of an appropriate buffer, 3 μmoles substrate indicated (lactate 150 μmoles), 100 μl of high-speed supernatant fraction from a 150 mg/ml adrenal-cortex homogenate in a total volume of 3 ml. Each cuvette had either 1 μmole NADP, 2 μmoles NAD for the lactate, or 0.2 μmole of $NADH_2$ for the pyruvate substrate The reaction was started by injecting 100 μl of substrate from a micro-syringe into the cuvette. A Beckman Model-DU Spectrophotometer coupled to a Honeywell Electronik High Speed Recorder was used to measure the initial velocity at 340 mμ. Temperature 25°. Values are the means of 5 determinations. Velocity expressed as μmoles $NADH_2$, NAD or $NADPH_2$ per min per 15 mg cortex. I, inhibition; S, stimulation.

	NADP-specific dehydrogenases				*NAD-specific dehydrogenases*	
	Glu-6-P	*6-P-gluconic*	*Isocitric*	*Malic*	*Lactic*	*Pyruvic*
Velocity control	0.76	0.70	0.25	0.65	0.57	0.63
Estrone (% change)	10 I	4 I	13 I	10 I	24 S	19 S
Estradiol (% change)	37 I	31 I	24 I	38 I	25 S	14 S
Estriol (% change)	25 I	0	11 I	3 I	32 S	18 S

TABLE II

THE RELATION OF DEHYDROGENASE ACTIVITY TO CORTICOID SYNTHESIS

The incubation medium for corticoid synthesis contained 7.8 μmoles of a substrate with 0.187 μmole ($9 \cdot 10^{-5}$ M) of NADP or NAD with lactate, or just 0.187 μmole of $NADH_2$, 0.4 ml of homogenate (100 mg), $2.2 \cdot 10^{-4}$ M $CaCl_2$ and Krebs–Ringer bicarbonate buffer to 2 ml. Incubation was for 30 min at 37° under O_2–CO_2 (95:5). The corticoids formed were extracted with methylene dichloride and estimated by the blue tetrazolium method. Enzyme activities at 25° were measured as in Table I using 50 μl of the high-speed supernatant fraction from the same batch of homogenate used for corticoid synthesis. Values are the means of 4 or more closely agreeing values.

	Glu-6-P	*6-P-gluconic*	*Isocitric*	*Malic*	*Lactic*	*$NADPH_2$*
	per 100 mg adrenal cortex					
Corticoids (μg/30 min)	26.2	12.0	2.8	4.2	0.6	4.6
$NADPH_2$ or $NADH_2$ (μmoles/min)	7.7	1.8	4.0	0.8	2.0	

glucose-6-*P* as substrate. The 30-min incubation time was selected from time studies to ensure that the rate of synthesis of corticoids was linear. Substrates were in excess so as not to be rate-limiting. Only under these conditions could the relative efficiencies of the various $NADPH_2$-generating systems be evaluated. Most of the corticoid synthesis occurred when added NADP was reduced by the enzyme systems glucose-6-*P* and 6-*P*-gluconate dehydrogenase. Isocitrate dehydrogenase produced considerably less corticoids than its capacity to reduced NADP would indicate. The malic dehydrogenase system was of low activity both in the rate of reduction of NADP and in corticoid synthesis. The hydroxylation and other systems of the mitochondria generating corticoids from preformed cholesterol are specific for $NADPH_2$ as shown by the fact that the generation of $NADH_2$ by the lactic dehydrogenase system produced no significant amounts of corticoids in the system *in vitro*. The stimulation

of lactic dehydrogenase by estrogens may be important *in vivo* due to a possible increase in the rate of generation of $NADH_2$ with subsequent electron transport to ATP. This could increase protein synthesis.

Rather surprisingly, $NADPH_2$ itself, when added to the homogenate, generated corticoids at a slow rate. $NADPH_2$ was purchased from Pabst Laboratories, Milwaukee, Wisc., and was found by analysis to be the completely reduced nucleotide. One would have expected that preformed $NADPH_2$ would be as good as a $NADPH_2$-generating system unless (as seems to be the case) there is some unique association of the pentose shunt dehydrogenases in the cytoplasm with the hydroxylation enzymes of the mitochondria or microsomes.

TABLE III

RELATIVE ACTIVITIES OF EXTRAMITOCHONDRIAL DEHYDROGENASES IN WHOLE RAT-ADRENAL HOMOGENATES

Enzyme assays were carried out as described in Table II.

	Glu-6-P	*6-P-Gluconic*	*Isocitric*	*Malic*	*Lactic*	*Pyruvic*
			per 100 mg whole adrenal			
$NADPH_2$ or $NADH_2$ (μmoles/min)	1.68	0.86	2.68	0.59	3.51	—

The incubation of homogenates without substrate or NADP produced no measurable corticoids. However, considerable precursor steroid—probably cholesterol—was present in the adrenals. The rate of corticoid synthesis seems thus to be regulated by the rate of reduction of NADP, primarily by hydrogen transfer from glucose-6-*P*.

Similar inhibitory effects of estrogens on NADP-specific dehydrogenases and a similar unique association of glucose-6-*P* and 6-*P*-gluconate dehydrogenases with corticoid synthesis were found for rat adrenals. The relative enzyme activities for whole rat adrenals are given in Table III. Isocitrate dehydrogenase showed more $NADPH_2$-generating capacity than glucose-6-*P* dehydrogenase but stimulated the rate of corticoid synthesis at only 10% the rate. The corticoid values are not given since no attempt was made to separate the medulla or slice the cortex and the relationship between enzyme activity and corticoid synthesis is therefore less precise than in the case of cow adrenal cortex (primarily fasciculata cells).

Competitive inhibition of NADP-binding by estrone

The competitive relationship between NADP and estrogens for binding sites on purified glucose-6-*P* dehydrogenase is shown by the example in Fig. 1. The 17-ketoandrogens, Δ^4-androstendione and dehydroepiandrosterone[3], as well as stilbestrol[5], hexestrol, estradiol, estriol and the thyronine hormones[4], were also found to be competitive inhibitors of NADP-binding to NADP-specific dehydrogenases. The corticoid series such as 17α-hydroxycorticosterone and the progesterone series are very weak inhibitors[3]. Thus, all these hormones may participate in varying degrees in the regulation of adrenal metabolism depending upon their inhibitory activity and the amount of non-protein-bound hormone in the circulation. These hormones,

at least in the case of the estrogens and thyronine compounds, are non-competitive with respect to the substrate glucose-6-*P*.

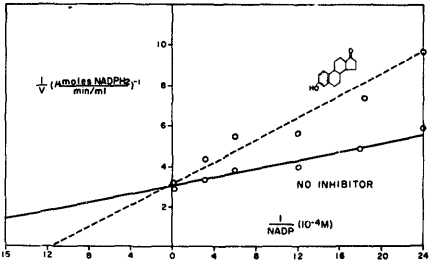

Fig. 1. Competitive inhibition of the binding of NADP to glucose-6-*P* dehydrogenase by estrone as demonstrated by a Lineweaver–Burk plot. Each point represents the average of 5 or more determinations. Each reaction mixture in Tris buffer (pH 8.0) contained enzyme, 10^{-3} M glucose-6-*P* and varying amounts of NADP. Estrone was added to the inhibitor series at $1 \cdot 10^{-5}$ M. K_m (NADP) $= 3.5 \cdot 10^{-6}$ M, $K_1 = 3.8 \cdot 10^{-5}$ M.

TABLE IV

EFFECT OF INCREASING NADP CONCENTRATION ON ESTRADIOL INHIBITION OF CORTICOID SYNTHESIS IN HOMOGENATES OF RAT ADRENALS

Quartered adrenal glands from 40 rats were preincubated for 1 h in Krebs–Ringer bicarbonate buffer with added glucose. The incubation medium was discarded and the glands were homogenized in 10.5 ml 0.1 M KCl solution. The final incubation with homogenate was for 30 min at which time the rate of corticoid synthesis was still linear. Each beaker of the series of 32 contained in 2 ml final volume of buffer 23 mg of homogenate, $4 \cdot 10^{-3}$ M Glu-6-*P*, $2.2 \cdot 10^{-2}$ M $CaCl_2$, and NADP as shown. Estradiol was added to the inhibitor series at $7.5 \cdot 10^{-6}$ M. There were four controls without added NADP, Glu-6-*P* or estradiol. Incubation was carried out at 37° under O_2–CO_2 (95:5). The corticoids formed were extracted and estimated as in Table II.

NADP concentration ($M \times 10^{-4}$)	*Corticoid production* (μg/100 mg adrenal)*		*Inhibition* (%)
	NADP control	*NADP + estradiol*	
1.5	6.3	3.1	51
3.0	13.8	10.5	24
9.0	37.3	29.4	21
27.0	40.8	37.5	8

* Each value is the mean of 4 determinations.

Competitive relationship between estradiol and NADP in the synthesis of corticoids

The rate of reduction of NADP by the glucose-6-*P* dehydrogenase system would seem to be the major rate-limiting step in the synthesis of corticoids. If estrogen is competitive with the binding of NADP this should manifest itself in a competitive fashion with the rate of corticoid synthesis. That this is so can be appreciated from the date of Table IV. (As the amount of added NADP increased the percentage inhibition decreased.) The data from a similar experiment are expressed in Fig. 2

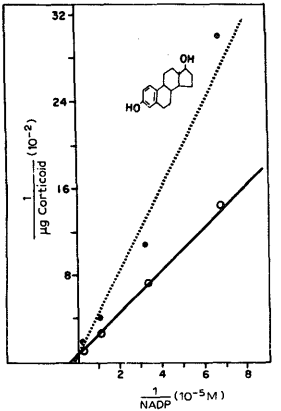

Fig. 2. A Lineweaver–Burk plot of the competitive relationship between 17β-estradiol and NADP for binding sites on glucose-6-*P* dehydrogenase, expressed in terms of corticoid synthesis from rat-adrenal homogenates. The incubation procedure with glucose-6-*P*, NADP and estradiol and the measurement of corticoids were the same as in Table IV.

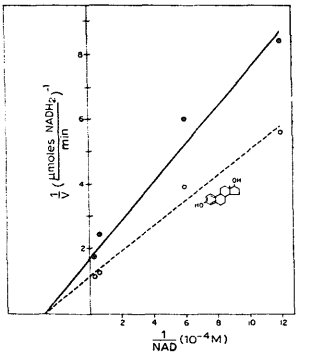

Fig. 3. The estrogen activation of lactic dehydrogenase in the high-speed supernatant fraction of cow adrenal-cortex tissue as demonstrated by a Lineweaver–Burk plot. Each point represents the average of 5 or more determinations. Each reaction mixture in glycine buffer (pH 7.0) contained excess sodium lactate and varying amounts of NAD. Estradiol was added to the steroids series at 10^{-9} M. The K_m for activation of lactic dehydrogenase by estradiol was determined to be $2.7 \cdot 10^{-9}$ M. Estradiol was non-competitive for NAD.

as a Lineweaver–Burk plot and the competitive relationship is clearly seen. Even the lowest level of added NADP is probably much higher than that found *in vivo*, so that it is not unlikely that concentrations of estrogen much lower than $7.5 \cdot 10^{-6}$ M are effective inhibitors.

Activation of lactic dehydrogenase by estrogen

A kinetic study was made of the stimulatory effect of estrogen on the very active lactic dehydrogenase system of adrenal cortex. As shown in Fig. 3, estradiol was non-competitive for NAD. The K_m for activation is extremely low. The mechanism of this effect is unknown and is being studied further. The lactic dehydrogenase in the cytoplasm of anterior pituitary tissue was also stimulated by estrogen and this activation may be a general enzymic site of action of the stimulatory effects of low levels of estrogens observed in many tissues.

DISCUSSION

The present paper relates the competitive inhibition of glucose-6-*P* dehydrogenase by estrogen to the inhibition of corticoid synthesis.

There seems to be a special requirement for the reduction of NADP by the

dehydrogenase enzymes of the pentose shunt pathway in the synthesis of corticoids. Glucose-6-*P* dehydrogenase in the cytoplasm is by far the most important system for the generation of $NADPH_2$ required by the hydroxylating enzymes of the particulate fraction. The regulation of the rate of synthesis of corticoids seems to be primarily dependent upon the rate of reduction of NADP. Adequate stores of preformed cholesterol are normally present, unless excessive stimulation to the adrenal has occurred. The regulation of the rate of corticoid synthesis can be influenced by estrogens and certain other hormones. Examples of the classes of hormones that are competitive with NADP for binding sites on NADP dehydrogenases and are thus regulators of the metabolic activity of the adrenal cortex are shown in Fig. 4. The

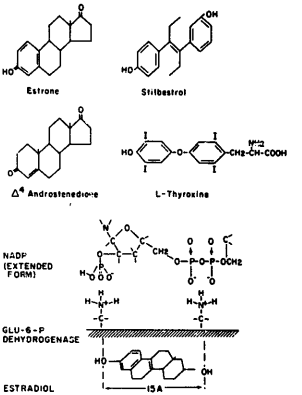

Fig. 4. Structural relationship of the competitive inhibitors of NADP-specific glucose-6-*P* dehydrogenase to NADP. The spatial separation of the ketone of phenolic hydroxyl groups of the inhibitors is in the range 12–15 Å. Thus the hydrogen-bonding groups for NADP on the dehydrogenase must also be in this range.

possible significance of some of these hormone effects on the adrenal–pituitary axis and the metabolism of other tissues has been discussed previously[4,5]. Since the inhibitors are competitive with NADP-binding, the amount of inhibition depends on the tissue level of NADP but was found to be independent of the level of substrate. Of the various potent inhibitors of NADP dehydrogenases the estrogens would seem, in the normal human female, to be of special physiological significance in the regulation of metabolism. The levels of circulating estrogen synthesized by the ovary vary cyclicly with the phases of the menstrual cycle and large amounts of estrogens are produced by the placenta during pregnancy (as much as 100 mg per day during late pregnancy). The inhibitory effects of the lower levels of estrogen on the adrenal cortex can clearly be compensated for by an increase in the rate of corticotrophin synthesis and an enlargement of the adrenal as discussed previously[5,6]. The higher levels of estrogen found in late pregnancy may have an effect on the adrenal beyond the capacity of the pituitary to compensate for. It is difficult to assess the physiological

effectiveness of the adrenal secretions in pregnancy since there are several other changing parameters. The cortisol-binding protein increases[13] and most of the cortisol circulates in the protein-bound and presumably inactive form, with a slower inactivation time than for free cortisol. The metabolic activity of the liver[7] and the liver-inactivation rate for cortisol increases and the turnover time for free cortisol is increased[14]. These changing parameters of cortisol metabolism are at least partially, if not wholly, compensated for by an enlarged adrenal and an increased rate of synthesis of the adrenal hormones. Whether the physiologically effective amount of corticoids is changed or not during late pregnancy, it is entirely possible that a large rebound phenomenum occurs after the separation of the placenta and the removal of the estrogen block to the adrenal. The rate of corticotrophin secretion is high and the enlarged adrenal could suddenly increase its rate of synthesis of corticoids. This may be a useful mechanism since it has been shown that single large doses of cortisol and related compounds are the trigger to the synthesis of milk in the mammary gland[15]. It seems certain that the exogenous administration of high levels of estrogen can be inhibitory to adrenal function. The daily administration of estrogen in the form of contraceptive pills has been shown to be inhibitory to cortisol synthesis in the human[16]. High doses of administered estrogen are inhibitory to adrenal function in the intact rat[2] while even low doses are inhibitory in the hypophysectomized rat.

It is interesting that further evidence of a unique role for glucose-6-*P* dehydrogenase has recently been demonstrated in the human[17]. This enzyme showed a marked increase in activity after corticotrophin administration. It had been shown previously that glucose-6-*P* dehydrogenase is linked with the production of reduced NADP required for 11β-hydroxylation[18, 19] and other steps in corticoid biosynthesis[20].

A second biochemical site of estrogen action involves the activation of lactic dehydrogenase. The level required for activation is extremely low and is independent of both NAD and lactate level. The stimulation to the rate of oxidation of lactate to pyruvate with an increase in the rate of production of $NADH_2$ and thus ATP, could increase protein synthesis in those tissues high in lactic dehydrogenase. Thus, for example, it would be possible to have a direct stimulus for the increase of adrenal size because of the increase in protein synthesis as well as the indirect stimulus to increased size from the pituitary which has been discussed previously[5]. As the level of estrogen rises, the inhibitory effect on the rate of reduction of NADP would be expected to predominate and inhibit metabolic function. Whether the effects of stimulation to the rate of $NADH_2$ or inhibition to the rate of $NADPH_2$ production predominate may also depend on the relative proportion of these enzymes in the target tissue and their ability to bind estrogen. For example, both enzyme systems were found to be active in the adrenal, pituitary and placenta; on the other hand, the myometrium of the human cervix of the uterus was relatively more active in NAD lactic dehydrogenase than in NADP dehydrogenases. The inhibitory effects of estrogen on NADP dehydrogenases and stimulatory effects on NAD-specific lactic dehydrogenase at low levels were also found with the enzymes of the anterior pituitary. Thus, these effects of estrogen may be a general regulatory mechanism in tissues responsive to estrogen. The significance of the effects of estrogen on dehydrogenase enzymes and on gonadotrophin synthesis in the anterior pituitary during the menstrual cycle, in pregnancy and with exogenous administration of estrogen will be discussed in a subsequent communication.

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