MALIC ENZYME INHIBITION BY PREGNENOLONE IN BOVINE CORPORA LUTEA

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L-Malate: $NADP^+$ Oxidoreductase (EC 1.1.1.82) obtained from cytoplasmic fraction of bovine corpora lutea has been found to be competetively inhibited by pregnenolone with respect to NADP. Progesterone and 17β-Estradiol were observed to inhibit the enzyme activity to one half of the inhibition produced by pregnenolone.

It has been reported by Taylor et al (1) that malate stimulates steroidogenesis in bovine corpora lutea. The malic enzyme in bovine adrenal mitochondria has been implicated (2) to be involved in the production of reduced pyridine nucleotides for steroid hydroxylations. NADP had been shown to stimulate steroid biosynthesis in luteal tissues (3). While pregnenolone has been shown to be inhibitory (4): Haksar and Ramonoff (5) reported that the pregnenolone inhibition in bovine corpus luteum is competetively reversed by NADP.

Control of reduced pyridine nucleotides generation by cytoplasmic NADP linked dehydrogenases has been known to be one of the mechanisms by which steroidogenesis is regulated in these tissues and steroids have been observed (6, 7) to affect these enzymes. The present study was made in order to test if steroids affect malic enzyme.

METHODS AND MATERIALS:

Non pregnant cows' ovaries obtained at slaughter house were immediately cooled in ice, brought to the laboratory, corpora lutea were separated and homogenized in 0.25M sucrose containing 10mM of $MgCl_2$ and 10mM tris-HCl buffer (pH 7.4), with eight volumes of tissue (w/v) and centrifuged at 500g for 10 minutes. The supernatant was centrifuged at 10,000g for 20 minutes, the resulting supernatant was further spun down at 105,000g for 90 minutes to yield the soluble fraction.

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The soluble fraction was dialysed for 10 hours against 0.005M tris HCl buffer containing reduced glutathione and fractionated with ammonium sulfate. The precipitate obtained between 50-80% saturation contained 70-80% activity of the soluble fraction. This fraction was used for inhibition studies and its specific activity was 4-5 fold higher than the crude 105,000g supernatant. Protein was determined by the method of Warburg and Christian (8).

L. Malate: $NADP^+$ Oxidoreductase (1.1.1.82) was estimated by the method of Ochoa (9) following the increase in absorbancy at 340mμ during 10 minutes of incubation at room temperature. The recordings at 340mμ were made in a Beckman recording Spectrophotometer. The incubation mixture containing 155μg of protein (12 units), 200 μ moles of tris HCl buffer pH 7.4, 5 μ moles $MnCl_2$, 100 mμ of NADP and 50 μl of propylene glycol in a final volume of 3.0 ml was preincubated for 5 minutes and the reaction started by adding 10 μ moles of L. Malate. The increase in optical density at 340 mμ was measured against a reference blank without malate. The steroids were dissolved in propylene glycol and added in a volume of 25-50 μl. Reference blanks were run simultaneously.

0.1M solution of L. Malate was made and neutralised with potassium hydroxide to a pH of 7.4. The steroids and NADP were purchased from Sigma Chemical Co.

The production of one mμ mole of NADPH/minute/mg protein was used as one unit of enzyme and the Molar extinction coefficient for NADPH 6.22×10^3 was used to calculate the concentration of NADPH formed.

RESULTS AND DISCUSSION:

Malate NADP: Oxidoreductase has been obtained in the cytoplasmic fraction of bovine corpus luteum. The present preparation precipitates at 50-80% saturation of ammonium sulfate which is in close agreement with mitochondrial malic enzyme preparation by Simpson and Estabrook (12). There was a linear increase in absorbancy at 340 mμ as a result of NADPH

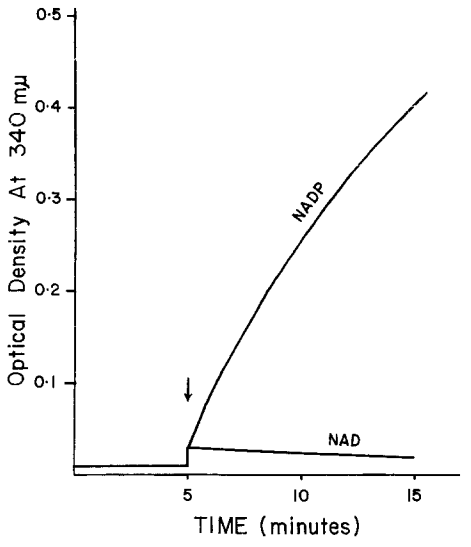

Fig. 1. EFFECT OF PYRIDINE NUCLEOTIDES ON MALIC DEHYDROGENASE incubation conditions are as described in text. L. Malate (10 μM) added and the optical density recorded at 340 mμ.

formation over a period of 15 minutes, while no such increase was found when NAD was used as electron acceptor for malate oxidation (Fig. 1). It has been shown that NAD linked malate dehydrogenase is present in rabbit, Cod testis (10) and rats (11). In addition malate could replace NADPH in adrenal mitochondria (14) for cholesterol side chain cleavage.

Pregnenolone inhibits the enzyme activity to about 60% at $1x10^{-6}$M concentration (Fig. 2). Lineweaver-Burk plot for NADP at various concentrations of Pregnenolone showed a competetive inhibition (Fig. 3). The high concentration of steroid required to inhibit the enzyme activity *in vitro* may be due to insolubility of the steroid.

There was no oxidation of NADPH under present experimental conditions indicating that the observed inhibition was not mere disappearance of NADPH due to steroid dehydrogenase. A noncompetitive type of inhibition of glucose-6-phosphate dehydrogenase (G6PD) has been observed by this steroid (6, 13), while McKerns (7) reported a competetive inhibition with bovine adrenal cortex G6PD. The competetive inhibition of malic enzyme by

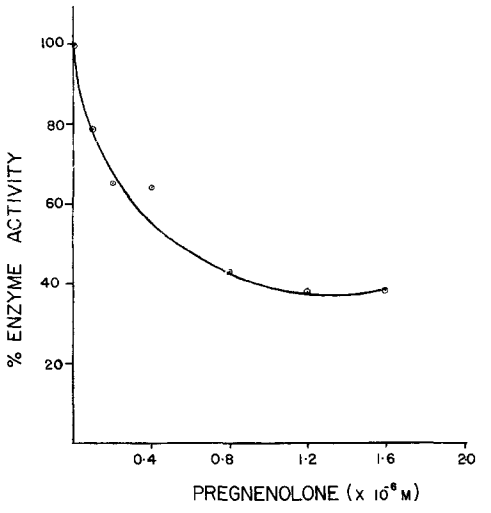

Fig. 2. EFFECT OF PREGNENOLONE ON MALE NADP: OXIDOREDUCTASE ACTIVITY incubation conditions were as described in the text and the enzyme activity presented as the rate of increase in absorbancy at 300 mμ per minute.

pregnenolone may suggest a regulatory mechanism for reduced pyridine nucleotides formation in this tissue. In support of this study Haksar and Romanoff (5) have shown that pregnenolone inhibits steroidogenesis from acetate, which is competitively reversed by NADP in bovine corpus luteum. Further malate has been shown to stimulate steroidogenesis in luteoinised rat ovary (1).

In addition to pregnenolone two other steroids, progesterone and 17β-Estradiol were found to be inhibitory. The latter steroids were one half as active as pregnenolone (Table 1). Studies are to be made to delineate the nature of inhibition by progesterone and estradiol. However it was reported (13) that the inhibitory activity of the steroids has a specificity for the dehydrogenase inhibited and the structure of inhibiting compound. The steroids with 17 or 20 keto group were potent inhibitors and the presence of ketone or β hydroxyl group at C_3 or a saturated or unsaturated bond at C_{4-5} or C_{5-6} have a little effect on the inhibitory action of G6PD from human erythrocytes. The most potent inhibitors of G6PD from bovine corpora

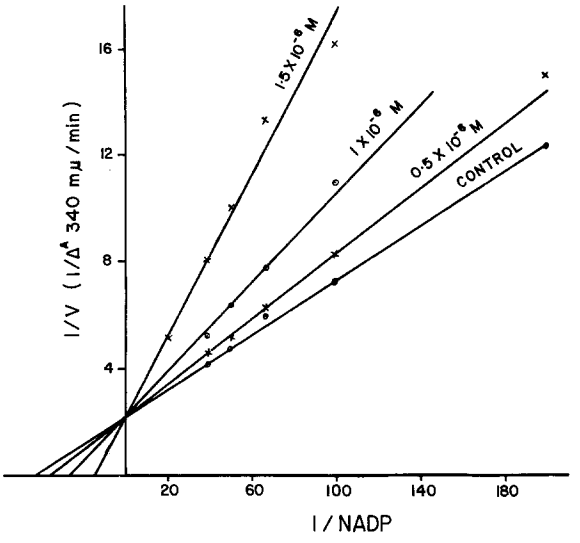

Fig. 3. LINEWEAVER-BURK PLOT DEMONSTRATING COMPETETIVE INHIBITOON OF BOVINE OVARIAN MALATE NADP. OXIDOREDUCTASE conditions were as described in text with variation in NADP concentrations: control, no steroid and concentration of pregnenolone as indicated on each curve.

lutea were shown to have Δ^5 3β hydroxy configuration where Δ^4 3 keto steroids had no or little activity. The mechanism of malate dehydrogenase inhibition is similar to the observation of Nelson and Warren (6) that pregnenolone was a superior inhibitor of G6PD than other steroids.

While it can not be ascertained the importance of malate dehydrogenase in producing reduced nucleotides from present studies, the inhibitory activity of pregnenolone on this enzyme is in support of the hypothesis that the intracellular steroid concentrations regulate the process of steroidogenesis. This may be operating in part by modulating the co-factors and the activity of preformed enzymes needed for steroidogenesis. It is not known if such mechanism is influenced by gonadotropins. It is conceivable that the intracellular concentration of pregnenolone is de-creased, due to further metabolism or secretion on treatment with gonado-tropins.

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TABLE 1

EFFECT OF STEROIDS ON THE ACTIVITY OF MALATE NADP: OXIDOREDUCTASE.

Steroids	percent activity	
	$5x10^{-8}$M, NADP	$1x10^{-7}$M, NADP
Control	100	100
Pregnenolone	51.5	73.3
Progesterone	77.3	89.3
17β-Estradiol	80.3	91.6

Steroids ($1x10^{-6}$M) were added in 50 µl of propelene glycol. Incubation conditions were as described in text.

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