ELECTRON TRANSPORT AND PROGESTERONE BIOSYNTHESIS IN THE BOVINE CORPUS LUTEUM HOMOGENATE.

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SUMMARY

The role of electron transport in progesterone biosynthesis was investigated in bovine corpus luteum homogenate. Although amytal and malonate inhibited progesterone synthesis, rotenone under several different incubation conditions was without effect. On the basis of differences in the effects of amytal and rotenone it is suggested that for the side-chain cleavage of cholesterol, although formation of some high energy intermediate of oxidative phosphorylation is obligatory, the electron shuttle between NAD and flavin is not.

In an earlier report we described the stimulatory effect of ATP on progesterone biosynthesis in the bovine corpus luteum homogenates (1). It occurred to us that the stimulation by ATP may be due to the energy-linked reversal of electron transport and transhydrogenation of NADP which would result in the generation of NADPH - a cofactor essential for the conversion of cholesterol to pregnenolone. There is some evidence for the participation of reversed electron transport in the side-chain cleavage of cholesterol in the adrenal cortex (2,3). Therefore, it seemed of interest to see if such a mechanism is involved in progesterone biosynthesis in the corpus luteum as well.

MATERIALS AND METHODS.

ATP, NADP, G-6-P and rotenone were purchased from Sigma Chemical Company. Amytal was a gift of Eli Lilly & Company (Courtesy Dr. J.M. McGuire). Progesterone 7α-^{3}H was purchased from New England Nuclear Corporation.

Bovine ovaries judged by appearance to be in the early or mid-luteal phase of the cycle were obtained at the slaughter house and transported to the laboratory in ice-cold 0.9% sodium chloride solution. The corpora lutea were removed from the stromal tissue and homogenized in a buffer (PH 7.4) containing 0.30 M Na_2HPO_4, 0.0088 M KH_2PO_4, 0.007 M $MgCl_2$ and 0.126 M sucrose. The tissue concentration in these experiments was 200 - 250 mg/ml of the medium. All incubations were carried

out in duplicate in a Dubnoff Metabolic Shaker at 37°C in air.

The incubations were terminated by pouring ethanol:acetone (1:1,v/v) into the beakers at the end of 15 minutes and the contents were transferred to 50 ml centrifuge tubes. To each tube was added a tracer amount of progesterone-7α-^{3}H and extraction was completed with more ethanol:acetone by repeated shaking, centrifuging and removing the supernatant layer.

Progesterone was isolated and purified from the ethanol:acetone extract by the method described previously (4,5). Recovery of progesterone in each sample was calculated on the basis of tritium found in the final product.

RESULTS AND DISCUSSION

Table 1 shows the stimulatory effect of 1 mM ATP on progesterone synthesis. The optimum concentration of ATP probably lies in a small range around 1 mM. Higher concentrations (5-10 mM) were inhibitory (1). Increase in progesterone synthesis by NADP (with or without G-6-P) shows that the NADP - dependent dehydrogenases were active in the homogenate and did contribute NADPH towards progesterone synthesis. A small increase in progesterone synthesis was also observed with 2 and 5 mM succinate (25-40%) (Table 2).

Amytal caused a substantial inhibition of progesterone synthesis, ranging from 60% to almost 100% in the various preparations from different corpora

Additions	*De novo* Synthesis of Progesterone µg/g tissue	
	Expt. HAc-34	Expt. HAc-35
None (control)	31.6	55.1
ATP 1 mM	52.0	-
ATP 5 mM	26.1	-
Amytal 5 mM	5.6	23.4
Amytal 10 mM	3.6	-
Amytal 10 mM + ATP 1 mM	6.6	-
Amytal 10 mM + ATP 5 mM	10.6	-
NADP 0.5 mM	-	66.2
NADP 0.5 mM + G-6-P 1 mM	-	65.4
NADP 0.5 mM + Amytal 5 mM	-	35.4
NADP 0.5 mM + G-6-P 1 mM + Amytal 5 mM	-	31.9

Table 1 Effect of ATP, NADP and Amytal on the *de novo* synthesis of progesterone. The incubation conditions are described in the "Methods" section.

Additions	De novo synthesis of progesterone, µg/g tissue	
	Expt. HAc-34	Expt. HAc-43
None (control)	31.6	34.0
Succinate 2 mM	40.8	43.3
Succinate 5 mM	-	41.2
Succinate 10 mM	40.1	-
Amytal 5 mM	5.6	2.0
Amytal 10 mM	3.6	0.3
Malonate 10 mM	-	0.5
Malonate 20 mM	-	0.0
Succinate 2 mM + Amytal 10 mM	3.5	0.5
Succinate 5 mM + Amytal 10 mM	-	0.0
Succinate 10 mM + Amytal 10 mM	2.0	-
Succinate 2 mM + Malonate 10 mM	-	3.2
Succinate 5 mM + Malonate 10 mM	-	7.7

Table 2. Effect of Succinate, Amytal and Malonate on the de novo synthesis of Progesterone. The incubation conditions are described in the "Methods" section.

lutea (Tables 1 and 2). Amytal also prevented the increase in progesterone synthesis due to ATP (Table 1) or succinate (Table 2). However, it had no effect on the stimulation caused by NADP or NADP + G-6-P. In the experiment reported in Table 1, NADP caused an increase of 11 µg over the control (55.1 and 66.2 µg). In the presence of amytal, NADP still produced an increase of about 12 µg (23.4 and 35.4 µg). Similarly NADP and G-6-P produced an increase of 10 µg over the control in the absence of amytal. In the presence of amytal the increase was about 9 µg. Therefore it would appear that amytal did not interfere with the NADP dependent dehydrogenases.

The results with amytal suggested that reversal of electron transport may be involved in the production of NADPH required in the conversion of cholesterol to progesterone. Malonate, an inhibitor of succinate dehydrogenase was, therefore, used to block the flow of electrons from succinate to the respiratory chain. In several experiments the inhibition with 5 or 10 mM malonate ranged from 50-100%. In the experiment reported in Table 2, complete inhibition of progesterone synthesis was observed with 10 and 20 mM malonate. In the same experiment 10 mM malonate partially prevented the increase in progesterone synthesis due to

2 mM succinate. The increase due to 5 mM succinate was not affected by malonate. These data indicate that in the homogenate there is apparently substantial amount of succinate and 10 mM malonate is not enough to inhibit succinate oxidation completely when more succinate has been added exogenously.

Rotenone is a more specific inhibitor of electron transport than amytal (6). It is, therefore, surprizing that in the work on adrenal, the investigators have not made use of this inhibitor (3,4). In our initial experiments we observed that rotenone did not have any appreciable effect on progesterone synthesis under the usual conditions of incubation. Therefore, we utilized several different methods of adding rotenone to our tissue preparation (Table 3). Under these conditions rotenone is known to inhibit electron transport in other tissues (7). However, in our tissue homogenate rotenone did not have any appreciable effect on progesterone synthesis even when added in a relatively high concentration of 2.5 mM.

It is improbable that the ineffectiveness of rotenone was due to its low solubility because we had sufficient amounts of ethanol and bovine serum albumin in addition to the relatively high concentrations of the inhibitor itself. Bovine serum albumin is also known to reduce the non-specific binding of rotenone (7). To overcome the lag period of rotenone (8) we tried preincubation at $4^{o}C$ for varying times. This procedure did not affect the results. Incubation at lower temperatures (20^{o} and $30^{o}C$) usually employed by others (7,8) also did not give any different results.

Koritz (3), on the basis of results obtained with amytal and Krebs' cycle intermediates, had suggested that in the adrenal mitochondria NADPH required for the conversion of cholesterol to pregnenolone may be produced by the reversal of electron transport from succinate to NAD followed by transhydrogenation of NADP. Hall (4) provided further evidence for the participation of this pathway by showing that hyperbaric oxygen was detrimental to steroid synthesis from labelled cholesterol. Our results with amytal and malonate are similar to those of Koritz (3) and Hall (4). But we have extended the work further by studying the effect of rotenone.

Rotenone and amytal inhibit electron transport at the same site in the respiratory chain (7). Amytal, however exerts an effect on some other reactions while rotenone does not. Thus unlike amytal, rotenone does not effect the Pi - ATP exchange (9), the ATPase reactions in the presence or absence of 2,4-dinitrophenol (10,11) and does not lower the P:O ratio with succinate as the substrate (6). Rotenone, therefore, blocks the NAD - flavin linked electron transport without interfering with the energy conservation reactions which are affected by amytal. Ernster et al. (6) have further suggested that the electron shuttle between NAD and flavin is not obligatory for the formation of a high energy intermediate(which may not involve phosphate) in oxidative phosphorylation.

Table 3. Effect of Rotenone on the de novo synthesis of progesterone.

Exp.	Mode of Addition and Incubation Conditions				De novo synthesis of progesterone, ug/g tissue			
	Preincubation	Incubation	Ethanol v/v	BSA, W/V	CONTROL	Rotenone 10^{-4}M	Rotenone 10^{-3}M	Rotenone 2.5×10^{-3}M
1	None	15mts;37°C	None	None	34.1	34.7	30.8	-
	"	"	5%	"	40.5	40.2	39.9	-
	"	"	"	1%	50.0	48.3	41.5	-
2	"	"	"	2%	59.9	-	53.9	53.0
	20mts;4°C	"	"	"	60.7	-	58.0	57.6
	40mts;4°C	"	"	"	55.0	-	51.4	53.2
3	15mts;4°C	15mts;20°C	10%	"	33.4	-	38.0	33.3
	"	15mts;30°C	"	"	44.6	-	46.4	50.2

In our experiments although amytal and malonate produced inhibition of progesterone synthesis, rotenone was without any effect. It would thus appear that mere transport of electrons is not obligatory for progesterone synthesis although the formation of some high energy intermediate is. The precise role of the high energy bond in the conversion of cholesterol to progesterone can not be defined yet.

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