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Dehydrogenation of Reduced Pyridine Nucleotides by Leydig Cell Tumors of the Rat Testis (39224)

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The growth (1), biologic properties (1), and ultrastructural characteristics (2) of tumors derived from interstitial cells of the rat testis have been described. The H.540 tumor secretes androgens and estrogens, is more highly differentiated, and has a slow rate of growth. The H.372 is more undifferentiated, grows rapidly, and elaborates no significant hormonal products. These tumors have been transferred through several generations in inbred Fisher rats.

Some biochemical properties of these tumors have been studied in our laboratory. This work has centered on the reduction and dehydrogenation of a nicotinamide adenine dinucleotide diphosphate (NADP), because it is known that NADPH is an essential cofactor for steroid hydroxylation in testis microsomes (3) and mitochondria (4). In addition the dehydrogenation-reduced nicotinamide adenine nucleotide (NADH) was also studied because of the function of NADH in the generation of reducing equivalents for respiration in the mitochondria.

In this communication the activity of the cytochrome *c* reductases and of the D-T diaphorase and their roles in the dehydrogenation of NADPH and of NADH by the Leydig cell tumors will be reported.

Materials and methods. The enzymatic activity of the tumors was compared with the activity of the interstitium of normal Fisher rats. The interstitium of the testis which is composed predominantly of Leydig cells was dissected following the method of Christensen and Mason (5) and then homogenized by hand in 0.1 *M* phosphate buffer with 0.154 *M* KCL. The tumors were homogenized in the same media. To further localize the activity of the enzymes the tumor homogenates were subjected to differential centrifugation in a Spinco L preparative ultracentrifugue. Due to the small amount of tissue obtained by dissection of the testicular interstitium the control interstitium was not subjected to differential centrifugation.

The mitochondria were sedimented at 12,000*g*, washed twice, and resedimented again each time at 18,000*g*. The microsomes were sedimented at 105,000*g* for 1 hr, washed once, and resuspended. The same buffer used for the homogenization was used for the washing of the different fractions. When our homogenization medium was substituted with sucrose 0.25 *M* and Tris buffer 0.25 *M*, pH 7.5, the same results were obtained. The cytochrome *c* reductases were assayed following the method of Omura (6) and the D-T diaphorase with the method described by Ernster (7) using dichlorophenolindophenol (DCPIP) and menadione as acceptors. Protein was estimated by the method of Nayyar and Glick (8).

Results. The measurements of cytochrome *c* reductase activities on the total homogenates are presented in Table I. The NADH cytochrome *c* reductase activity in the functional tumor (H.540) is about nine times greater than control values as represented by determinations on the total homogenate of normal interstitial tissue. Table II shows the results obtained with the D-T diaphorase in total homogenates. The nonfunctional tumor (H.372) shows much greater activity than the normal interstitium.

The results obtained with the subcellular fractions obtained from the tumors are shown in Tables III and IV. Table III shows the activities of cytochrome *c* reductase. It can be seen that the increased activities observed in the H.540 tumors are localized exclusively in the particulate fractions. Likewise, the H.372 tumor shows the same lo-

calization, although the activity of the enzyme is much smaller than observed in the H.540 tumor.

In Table IV the results obtained for the subcellular distribution of the D-T diaphorase are presented. The activity is localized largely in the supernatant fraction. In the H.540 tumor, when NADH is used as a substrate, DCPIP is reduced in mitochondria and microsomes. The observed activity is high; however, it is not presented in the tables because it is known that DCPIP can be reduced by the action of the cytochrome *c* reductases (9). Furthermore, some investigators used DCPIP reduction instead of cytochrome *c* for the measurement of the enzyme activity (9, 10). When menadione was used as a substrate and the decrease in optical density of reduced pyridine nucleotides at 340 mm was followed, the same changes were observed in the H.372 tumor as when DCPIP was used as a substrate for the diaphorase.

Discussion. When compared with the normal interstitium the two tumors studied show significant changes; however, these changes are of different magnitude and probably of different significance.

The increase in the activity of NADH cytochrome *c* reductase in the H.540 tumor lends itself to two different interpretations. It could be either that this tumor utilized NADH for the steroid hydroxylation or that the observed increase of the enzymatic action could be simply a manifestation of cellular hyperactivity. This tumor is also reported to show a fivefold increase in the activity of the transhydrogenase (11). In a

TABLE I. CYTOCHROME *C* REDUCTASE ACTIVITY TOTAL HOMOGENATE.[a]

	Substrate	
	NADPH	NADH
Control interstitium	20 ± 3:11 (4)	120 ± 18 (4)
H.540	48 ± 14 (8)	1016 ± 54 (8)
H.372	11 ± 3 (7)	37 ± 5 (7)

[a] Activity expressed as micromicromoles of reduced cytochrome *c* per microgram of protein present in incubation mixture, ±1 SEM. In parenthesis the number of animals used for each determination.

TABLE II. D-T DIAPHORASE ACTIVITY TOTAL HOMOGENATES.[a]

	Substrate	
	NADPH	NADH
Control interstitium	49.8 ± 1.4 (4)	34.7 ± 3.3 (5)
H.540	124 ± 20 (9)	450 ± 28 (10)
H.372	384 ± 48 (8)	478 ± 45 (8)

[a] Activity expressed as micromicromoles of reduced DCPIP present in incubation mixture per minute per microgram of protein, ±1 SEM. In parenthesis the number of animals used for each determination.

TABLE III. CYTOCHROME *C* REDUCTASE LEYDIG CELL TUMORS SUBCELLULAR FRACTIONS.[a]

	H.540		H.372	
Fraction	NADPH	NADH	NADPH	NADH
Homogenate	48 ± 14 (7)	1016 ± 54 (8)	11 ± 3 (7)	37 ± 5 (7)
Mitochondria	48 ± 8 (9)	1709 ± 114 (9)	13 ± 4 (6)	103 ± 23 (6)
Microsomes	280 ± 52 (8)	4874 ± 280 (9)	24 ± 6 (7)	99 ± 32 (6)
Supernatant	Nil	Nil	Nil	Nil

[a] Activity expressed as micromicromoles of reduced cytochrome *c* per minute per microgram of protein present in the incubation mixture; ±1 SEM. In parenthesis, the number of animals analyzed.

TABLE IV. D-T DIAPHORASE ACTIVITY LEYDIG CELL TUMORS SUBCELLULAR FRACTIONS.[a]

	H.540		H.372	
Fraction	NADH	NADPH	NADH	NADPH
Homogenate	450 ± 28 (10)	124 ± 20 (9)	384 ± 48 (6)	478 ± 45 (6)
Mitochondria	High	Low	Low	Low
Microsomes	High	Low	Low	Low
Supernatant	120 ± 37 (9)	148 ± 23 (9)	651 ± 73 (6)	861 ± 52 (6)

[a] Activity expressed as micromicromoles of reduced DCPIP per minute per microgram of protein present in the incubation media; ±1 SEM. In parenthesis, the number of animals analyzed.

study on the metabolism of reduced pyridine nucleotides in tumor tissues, Foster and Taylor (12) have shown the electrons derived from NADH are more readily incorporated into cholesterol than the ones derived from NADPH. Therefore, it may be that in neoplastic cells, NADH is used preferentially in reductive synthesis instead of NADPH. This hypothesis will be tested in our laboratory. It should be emphasized that in the functional tumor (H.540), the activity of cytochrome *c* reductases is localized in the particulate fractions of the cells (Table III). The study of these enzymatic functions in one mouse Leydig cell functional cell tumor has shown similar increase in the activity of the cytochrome *c* reductases.

The H.372 tumor shows striking changes in the activity of the D-T diaphorase. The D-T diaphorase was described by Ernster in liver supernatant. This enzyme is a flavoprotein (7) that accepts electrons either from NADH or NADPH and reduces several artificial electron acceptors, among them DCPIP and menadione. Ernster has postulated that the enzyme physiologically constitutes a bypass of the normal phosphorylating electron-transfer pathway between NADH and cytochrome *c*, provided that an artificial electron mediator, such as Vitamin K_3, is present. In our laboratory it has been demonstrated that in the normal testis, NADPH and NADH can be oxidized by mitochondria only if menadione is present in the incubation medium.

The increase in the activity of the soluble D-T diaphorase in the H.372 tumor shown in Table IV suggests a type of anaplerotic reaction. It is known that the activity of the shuttles for the oxidation of extramitochondrial-reduced pyridine nucleotides are decreased or absent in neoplastic cells (13). In ascitic tumor cells (14), it has been shown that glucose can contribute reducing equivalents for respiration only if Vitamin K is present. Although a diaphorase type activity was suggested to explain those findings, the specific activity of the enzyme was not measured.

It should be emphasized that in our work on two tumors of similar origin, we have observed that the more poorly differentiated nonfunctional tumor, H.372, showed the greater increase in the D-T diaphorase activity; meanwhile, the well differentiated one shows a striking increase in the cytochrome *c* reductase activity. Furthermore, these enzymatic functions are being studied in tissue culture cell lines derived from the tumors. The functional H.540 tumor shows a higher activity of the cytochrome *c* reductase activity and the H.372 line a high activity of the D-T diaphorase. These results will be reported separately.

There have been numerous reports on the biochemical properties of experimental tumors especially of experimental hepatomas (15, 16). It has been reported that the more differentiated hepatomas show a decrease in the activity of NADPH dehydrogenating enzymes. Although those reports seem to be at variance with our results, determinations were performed either on mitochondria or microsomes (17, 18). However, when the dehydrogenation of NADPH and NADH was studied on the supernatant, elevated enzyme activities similar to the ones observed in the testicular tumor were observed in both slowly and rapidly growing hepatomas (19).

Summary. The activities of the cytochrome *c* reductases and of the D-T diaphorase in rat Leydig cell tumors have been described. The increase in enzymatic activity of the NADH cytochrome *c* reductase activity in functional tumors derived from interstitial cells of the rat testis is interpreted as being possibly related to hydroxylation of steroids by the neoplastic cells. Meanwhile, the increase in the activity of the D-T diaphorase in the other tumor is interpreted as being an anaplerotic reaction to substitute for the deficient shuttles for the transfer of reducing equivalents from the cytoplasm to the mitochondria observed in tumors.

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