PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 355-358 (1976)

Experimental Hepatomas: Dehydrogenation of Reduced Pyridine Nucleotides by the D-T Diaphorase[1] (39544)

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In this communication, the enzymatic activity of the soluble D-T diaphorase (reduced NAD(P): (acceptor) oxidoreductase (EC 1.6.99.2)) (1) in experimental hepatomas is reported. These studies were prompted by the fact that Leydig cell tumors of the rat testis show a D-T diaphorase activity several times that of non-neoplastic interstitial tissue (2). This finding has led to the hypothesis that the increased enzyme activity represents an anaplerotic mechanism compensating for the disappearance from neoplastic tissues of the shuttles normally used in the oxidation of extramitochondrial NADH and NADPH (3).

The dehydrogenation of NADH and NADPH by mitochondrial and microsomal enzymes has been studied in experimental hepatomas. It has been found that the NADH- and NADPH-cytochrome *c* reductases are decreased in the mitochondria and microsomes of the more undifferentiated hepatomas (4–6). Furthermore, the activity of the microsomal mixed oxidases are either reduced or absent in microsomes obtained from hepatomas (6, 7).

The slowly growing 7787 hepatoma and the rapidly growing 5123tc hepatoma were used in this study.

Materials and methods. The animals were killed by sudden decapitation. The livers of the control animals, the livers of the host rats, and the hepatomas were immediately dissected and homogenized. A 10% homogenate in 0.25 *M* sucrose buffered with Tris, pH 7.4, 0.1 *M* (the same results were obtained when potassium chloride 0.154 *M* with phosphate buffer, pH 7.4, 0.1 *M*, was used in the centrifugation medium), was prepared from the different specimens and then subjected to differential centrifugation in a Spinco L preparative ultracentrifuge. The supernatant was obtained after the centrifugation of the postmitochondrial fraction for 2 hr at 105,000*g*.

The enzymes were assayed in a Unicam recording spectrophotometer using dichloroindophenol (DCPIP) as an acceptor and NADH and NADPH as substrates. The composition of the incubation medium was phosphate buffer, 3.3×10^{-2} *M*; DCPIP, 3.33×10^{-5} *M*; and NADPH and NADH, 7.6×10^{-5} *M*. The final volume was 1.3 ml. In each determination approximately 70 μg of protein were used. The reaction was started with either NADH or NADPH, and the reduction of DCPIP was followed for 1 min at 600 nm. The extinction coefficient of 22.1 mM^{-1} cm^{-1} was used for the determination of the enzyme activity. Protein was determined by the method of Nayyar and Glick (8).

Results. The results obtained with the 7787 tumor are shown in Table I. Experiments with the 5123tc tumor are shown in Table II. The enzymatic activity in the experimental hepatomas is more than four times that of normal liver and three times that of the host liver. Also, from these results it can be seen that the increased activity of the D-T diaphorase is maintained throughout the different generations of the 5123tc tumor. It can also be observed that the host livers exhibit a higher activity than the livers of the control animals. These findings will be discussed elsewhere.

The kinetics of DCPIP reduction by NADH and NADPH in the tumors has been studied. The apparent Michaelis constant appears, with small variations, to be the same in the liver of the control and of the host animals and the hepatomas. Although

[1] Supported by the E. G. Schlieder Educational Foundation (N.A.S.) and in part by USPHS Grant CA10729 (H.P.M.).

the concentration of NADH and NADPH used in these experiments was near the K_m concentration, similar differences in activities were observed when the substrate concentration was either lowered fivefold or increased five- and tenfold, respectively. These results will be communicated in full in a forthcoming paper.

A critique often made of this type of work is that the enzymatic activity observed in the supernatant may constitute an artifact produced by a leakage of particulate enzymes into the cytoplasm. When the activities of the cytochrome *c* reductases of the particulate fractions of livers and hepatomas were compared with those of the supernatants, the degree of reduction of cytochrome *c* was tenfold higher using NADH as a substrate (the same results are obtained if DCPIP is used as an acceptor instead of cytochrome *c*); whereas in the supernatant the degree of reduction of DCPIP was almost equal using either NADPH or NADH as the substrate. Furthermore, the soluble enzymes will reduce cytochrome *c* only if a mediator such as menadione is present in the incubation mixture; meanwhile, the particulate enzymes reduce DCPIP without an electron mediator. These determinations tend to indicate different properties of the particulate and soluble enzymes which dehydrogenate reduced pyridine nucleotides in normal livers; these differences are retained in hepatomas. Thus, it appears that a real increase of activity of the D-T diaphorase is observed in experimental hepatomas and that this increase is not an artifactual result of the procedures used. The enzyme was inhibited by Dicumarol, hydroxycoumarin, and 7,8-benzoflavone. When other hydrogen acceptors such as cytochrome *c* and nitroblue tetrazolium plus vitamin K_3 were used, the same differences between normal livers and hepatomas were observed.

TABLE I. D-T DIAPHORASE ACTIVITY HEPATOMA 7787.[a]

Substrate	NADH	NADPH
Control liver	387.7 ± 31.6 (6)	468.1 ± 36.2 (6)
Hepatoma (*t*:23, *d*:65)[b]	1462.7 ± 151.2 (6)	2021.4 ± 265.2 (6)
Host liver	434.4 ± 86.5 (6)	454.7 (6) ± 122.2

[a] Activities expressed as micromicromoles of reduced DCPIP per microgram of protein present in the incubation mixture per minute in the soluble fraction obtained after centrifugation at 105,000*g*/2 hr.

[b] *t*, Number of generations of the tumor; and *d*, days of inoculation of the tumor before sacrifice.

Discussion. The interpretation of these findings at this moment is speculative. A possible interpretation is that this increase in activity is an anaplerotic mechanism to substitute for the decreased activity of the

TABLE II. D-T DIAPHORASE ACTIVITY HEPATOMA 5123tc.[a]

Substrate	NADH	NADPH
Experiment 1		
Control liver	210.7 ± 20.5 (5)	284.2 ± 44.1 (5)
Hepatoma (*t*:128, *d*:25)[b]	1153 ± 162.2 (6)	1374.1 ± 246.1 (6)
Host liver	320.8 ± 50.64 (6)	367.50 ± 50.1 (6)
Experiment 2		
Control liver	149.70 ± 20.3 (8)	219.2 ± 28.5
Hepatoma (*t*:135, *d*:26)[b]	878.3 ± 99 (7)	1270.4 ± 195.1 (7)
Host liver	201 ± 29.2 (8)	306 ± 30.1 (8)
Experiment 3		
Control liver	254.3 ± 48.8 (6)	276.6 ± 54.9 (6)
Heptatoma (*t*:138, *d*:45)[b]	1186.42 ± 127.7 (6)	1474 ± 257.7 (6)
Host liver	419.14 ± 77.4 (6)	514.1 ± 57.8 (6)

[a] Activities expressed as micromicromoles of reduced DCPIP per microgram of protein present in the incubation mixture per minute in the soluble fraction obtained after centrifugation at 105,000*g*/2 hr. In parentheses, the number of animals used.

[b] *t*, Number of generations of the tumor; and *d*, days of inoculation of the tumor before sacrifice.

shuttles used in the oxidation of NADH and NADPH by neoplastic mitochondria (3). It is known that the endogenous O_2 consumption of hepatoma mitochondria is enhanced (9); therefore, the D-T diaphorase could be the enzyme responsible for the transfer of reducing equivalents to the mitochondria. This explanation agrees with observations made by Conover and Ernster on the function of this enzyme (10, 11). These workers have postulated that the enzyme can transfer reducing equivalents to the mitochondrial chain using vitamin K_3 as an intermediate. In ascites tumor cells, it has been shown that glucose can contribute reducing equivalents for respiration only if vitamin K_3 is present (12). Although a diaphorase-type activity was suggested to explain these findings, the specific activity of the enzyme was not measured. A recent report by Lanoue *et al.* (13) on the defects of anion and electron transport in Morris hepatoma mitochondria has shown that the iron-sulfur protein which transfers electrons from NADH to ubiquinone in mitochondria is decreased in neoplasia. These authors have also confirmed and enlarged previous observations of Boxer and Devlin (3) on the activity of the shuttles utilized in the transfer between reduced pyridine nucleotides and mitochondria.

The extent of the defect in mitochondrial respiration according to Lanoue and her collaborators (13) does not appear to be a function of growth rate. Likewise, the results reported in this communication regarding the activity of the D-T diaphorase in the rapidly growing 5123tc and slowly growing 7787 hepatomas does not correlate with the growth rate of these hepatomas.

A second alternative may be deduced from a recent report which has shown that the D-T diaphorase may be related to the aryl hydrocarbon hydroxylase (AHH) system of liver microsomes (14). Microsomal mixed oxidases are greatly reduced or absent in microsomes obtained from hepatomas (6, 7). Although the relation of the D-T diaphorase to the hydroxylative system is not yet clear, it has been shown that the activity of the enzyme can be induced *in vivo* by methyl cholanthrene which induces the AHH system of liver microsomes (13). Likewise, 7,8-benzoflavone (12), an *in vitro* inhibitor of the AHH system, suppresses the activity of the D-T diaphorase extracted from either liver or hepatomas. Therefore, it may be postulated that the increase of the activity of the enzyme may be related to this process. Experiments conducted in our laboratory have shown that the activity of the enzyme is increased three- and twofold when rats are fed dimethylbenzanthracene and acetylaminofluorene. These changes are observed 24 hr after the administration of the carcinogen (15). The reducing equivalents transferred by the enzyme may also be utilized for the conversion of ribonucleotides to deoxyribonucleotides. This biochemical function is increased in hepatomas and is localized in the postmicrosomal fraction (16).

Summary. Major differences were observed in the dehydrogenation of reduced pyridine nucleotides when experimental transplantable hepatomas were compared with livers of control animals and livers of tumor-bearing animals. The enzymatic activity of the D-T diaphorase was increased almost fivefold in the hepatomas. The same increase in enzymatic activity was observed in one slowly growing and one rapidly growing Morris hepatoma. The possible functional significance of these findings is discussed.

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Received April 12, 1976. P.S.E.B.M. 1976, Vol. 153.