Relationship of 3α-Hydroxysteroid Dehydrogenase to Pyridine Nucleotide Transhydrogenases

Abstract. It appears that the α-hydroxysteroid dehydrogenases are not significant entities in promoting transhydrogenase reactions in animal tissues.

An enzyme catalyzing the oxidation of reduced triphosphopyridine nucleotide (TPNH) by diphosphopyridine nucleotide (DPN) was found originally in extracts of *Pseudomonas fluorescens* (*1*). This enzyme was purified and was shown to mediate a direct and reversible transfer of hydrogen between di- and triphosphopyridine nucleotides (*1–3*). The reaction is illustrated in Eq. 1.

$$\text{TPNH} + \text{DPN} \rightleftharpoons \text{TPN} + \text{DPNH} \quad (1)$$

The purified enzyme was shown to possess no dehydrogenase activity toward all substrates tested (*1*). Furthermore, attempts to duplicate transhydrogenase reactions with DPN- and TPN-linked dehydrogenases failed (*2*). A similar activity has been found in the mitochondrial fraction of many animal tissues (*4, 5*).

Recently, Talalay and his associates have reported that purified preparations of the soluble fractions of human placenta (*6, 7*) and rat liver (*8, 9*) which exhibit DPN- and TPN-linked dehydrogenase activity toward various 3α-hydroxysteroids can catalyze the above reaction (*6–9*). 3α-Hydroxysteroid dehydrogenase linked to both TPN and DPN has been purified from the latter fraction by Tomkins (*10*) and was employed by Hurlock and Talalay (*8, 9*) in the studies cited. We should like to clarify the relationship of the steroid dehydrogenases to the pyridine nucleotide transhydrogenase of mitochondria.

We have compared the activities of the TPNH-DPN transhydrogenase (Eq. 1) in the mitochondrial and soluble fractions of rat liver. Activities were determined by following the reduction of the 3-acetylpyridine analog of DPN by TPNH (*5*). The method is illustrated by Eq. 2.

$$\text{TPNH} + \text{acetylpyridine (DPN)} \rightarrow \text{TPN} + \text{acetylpyridine (DPNH)} \quad (2)$$

The data are given in Table 1. At *p*H 6.5, but not at *p*H 8.3 (*8*), a small, but perhaps significant, stimulation of analog reduction by the soluble fraction is obtained in the presence of TPNH. The net reaction is less than 1 percent of the reaction in mitochondria and does not appear to be influenced by the presence of androsterone. The relatively high rate of endogenous acetylpyridine (DPN) reduction in the unmodified rat liver soluble fraction appears to be due to the presence of DPN-linked dehydrogenase and endogenous substrates, particularly since the activities increase at higher *p*H values. The specific activity of the 20-fold purified enzyme of Hurlock and Talalay (*8*) may be calculated from their data to be 1.1 mμmole per minute per milligram of protein, and therefore the activity of their enzyme in the unmodified rat liver soluble fraction would be of the order of 0.05 mμmole per minute per milligram of protein, or about 0.05 percent of the specific activity of the reaction as it occurs in mitochondria. It is of interest that the calculated specific activity of the TPNH-DPN transhydrogenase linked to androsterone in the rat liver soluble fraction is quite insufficient to account for the small net rate of reaction 2 we have observed in this fraction at *p*H 6.5, even if the difference in the rate of reaction between DPN and its acetylpyridine analog, in the case of the placental enzyme (*7*), is taken into account.

We have pointed out elsewhere (*5*) that the purified beef heart transhydrogenase is not affected by estradiol-17β. Similarly, we have now found that the partially purified rat liver mitochondrial enzyme will not respond to additions of either androsterone or androstane-3,17-dione. Significant differences exist in the K_m and rates of reaction with the various pyridine nucleotides between the mitochondrial enzymes we have described (*5*) and the steroid-linked transhydrogenase reactions described by Talalay and his associates (*7*).

It is clear from the above that the enzyme from mitochondria is a different entity from the soluble steroid dehydrogenase, as Talalay and associates have stated (*9*). There is no evidence at the present time to suggest that steroids are involved in the transhydrogenase reactions of mitochcondria, although the possibility that the enzyme has a cofactor always exists. As Talalay has suggested (*6–9*), all steroid dehydrogenases might exhibit transhydrogenase activity, but it must be emphasized that this does not imply that all transhydrogenases are steroid dehydrogenases. In view of the extraordinarily great difference in transhydrogenase activity between the mitochondrial system and steroid-linked dehydrogenases, we would question their statement (*9*) that the latter enzymes function efficiently in the transhydrogenase reaction. Even if the low absolute activity of the transhydrogenase reaction catalyzed by hydroxysteroids is ignored, the fact that the activity of this reaction, as catalyzed by the placental enzyme, is about one-tenth the activity of the dehydrogenase reaction (*7*) casts doubt on the significance of these enzymes in the transhydrogenase reaction in the cell. It is difficult to see how such low activities could be of importance in regulating the flow of electrons between the two forms of the pyridine coenzymes, even in the soluble portion of the cell.

The fact that a certain enzyme can be manipulated to catalyze a given reaction does not imply that this is a physiological role for the catalyst. Triosephosphate dehydrogenase will catalyze the oxidation of acetaldehyde; this, however, does not impute a physiological role for this enzyme in the oxidation of acetaldehyde (*11*). Under conditions of high lactate concentration generated by TPNH, pyruvate, and concentrated lactic dehy-

Table 1. Reduction of the 3-acetylpyridine analog of DPN by TPNH in rat liver fractions. All cuvettes contained 3 μmole of KCN, 300 μmole of potassium phosphate buffer (*p*H 6.5) or 60 μmole of Tris · HCl (*p*H 8.3), and 0.6 μmole of 3-acetylpyridine (DPN) in a final volume of 3 ml. TPNH (0.4 μmole) and androsterone [2 μg in 0.01 ml of dioxane (*8*)] were added as indicated. The mitochondrial fraction was a suspension representing 250 mg of fresh liver per milliliter in 0.5 percent digitonin (*5*); 0.05 ml of suspension containing 0.4 mg of protein was added to the appropriate cuvettes. The soluble fraction was the supernatant of a rat liver homogenate centrifuged 2 hours at 105,000 *g*; 0.2 ml of this fraction, containing 2.4 mg of protein, was added to the appropriate cuvettes. The reactions were followed at 375 mμ (*5*), and the values were calculated assuming $E_{mM} = 5.1$ for all changes in optical density. All values for cuvettes containing TPNH have been corrected for small changes in optical density in control cuvettes containing TPNH but no analog. All values have been calculated from the slope of the initial rate of reaction.

Additions	Fraction	Acetylpyridine (DPNH) formed (mμmole/min per milligram of protein)	
		*p*H 6.5	*p*H 8.3
None	Mitochondria	2.2	1.5
TPNH	Mitochondria	114	66.5
None	Mitochondria	2.2	2.0
TPNH + androsterone	Mitochondria	121	67.9
None	Soluble	1.5	5.5
TPNH	Soluble	2.4	5.3
Androsterone	Soluble	1.5	5.3
TPNH + androsterone	Soluble	2.2	5.7

drogenase (0.8 mg/ml), Holzer and Schneider (*12*) were able to demonstrate a small reduction of DPN upon the addition of unusually large amounts of this pyridine nucleotide (12 mg/ml). Although a similar reaction proceeded with somewhat greater facility in the case of glutamic dehydrogenase (*12*), it is unnecessary to add that these observations do not establish physiological roles for either enzyme in pyridine nucleotide transhydrogenase reactions.

We suggest that the term *pyridine nucleotide transhydrogenase* be reserved for enzymes exhibiting these activities as quantitatively primary functions, and which, by virtue of concentration in unmodified tissue fractions or high purification, possess a reasonably high specific activity (*13*).

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Direct Experimental Observation of Cells in Phenomic Lag

Abstract. By employing cells mutating at a very high spontaneous rate and a grid plate containing a medium totally selective against mutant cells, direct microscopic observation was made of mutant cells of recent origin passing through a limited number of phenomic lag divisions.

The concept that cells immediately following mutation are phenotypically normal and require several generations for mutant phenotype expression has been invoked to interpret discrepancies in mutation rates estimated by various methods (*1*) and to interpret the effect of intermediate cultivation after exposure to mutagens on the number of mutants expressed (*2*). Ryan (*3*) reviewed the evidence for phenomic lag and concluded that it was not compelling.

The likelihood of direct observation of a newborn mutant in phenomic lag is unreasonably small when the mutation rate is low. When the rate is high (for example, 10 to 40 percent; as in the cytoplasmic mutation to respiration deficiency in some yeast strains) the direct observation of phenomic lag becomes possible on a medium totally selective against the mutant. Under these conditions one predicts that normal cells should form colonies (complete development), old mutant cells or dead cells should fail to divide (no development), but mutant cells of recent origin should exhibit only one or a few divisions (limited development), if phenomic lag does in fact occur.

Intermittent observation of the development of many individual cells on an agar surface may be made if each cell can be relocated on a grid. A convenient grid plate may be constructed as follows. Ten razor blades are clamped together with the edges aligned on a plane surface. The blade edges are used to cross stamp the dry agar surface of a 2-day-old plate lightly, yielding a grid of squares approximately 0.1 mm on edge. One drop of a cell suspension (about 1×10^4 cells/ml) is placed directly on the grid. If the agar is dry the drop is rapidly absorbed, and microscopic observation may be begun almost immediately. The mechanical stage is modified to accommodate a petri dish, the corner of the grid is located under low and then high power and, as the grid is scanned, each cell or configuration (singlet, doublet, triplet, and so on) is drawn on a paper facsimile of the grid. At timed intervals the grid is scanned and the number of cells in each configuration is again recorded. Intermittent microscopic observation may be made of the open plate for 48 hours without visible interference from airborne contaminants. The tendency of growth to become confluent interferes with longer periods of observation.

Table 1. Number of microcolonies of various terminal sizes on lactate grid plates. Cells of strain 14940 were plated from shaken cultures growing in lactate nutrient broth at 35°C.

No. of cells in initial configuration*	No. of cells in final configuration*														Total microcolonies	Total macrocolonies
	1	2	3	4	5	6	7	8	9	10	11–12	13–14	15–16	17–20		
1	55	91	87	65	45	14	19	10	3	1	9	1	3	5	408	160
2		68	22	26	32	22	20	15	16	13	16	11	7	9	277	699
3			4	2	3	4	3	0	5	3	5	2	2	1	34	229
4				0	0	0	1	1	0	0	1	1	0	0	4	76
5					0	0	0	0	0	1	0	0	0	0	1	25
6						0	0	0	0	0	0	1	0	0	1	16
7							0	0	0	0	0	0	0	0	0	2
Total															725	1207

* Buds scored as cells.

Two plating media have been employed. One, a glucose nutrient agar, allows both respiration-deficient and respiration-sufficient cells to form colonies. The other, a lactate, nutrient agar allows only respiration-sufficient cells to form colonies (*4*). When a respiration-deficient clonal isolate of strain 14940 (*5*) growing in exponential phase in glucose nutrient broth was transferred to glucose grid plates, essentially all cells or configurations developed to form colonies. A low frequency (less than 1 percent) failed to produce a single bud and were considered dead cells. When aliquots of the same culture were studied on lactate grid plates, not a single new bud was formed in more than 1000 configurations. To test whether the respiration-deficient cells had died on the lactate agar, a doughnut-shaped ring of filter paper impregnated with glucose was placed on the agar surface surrounding the grid. After some lag, essentially all configurations budded on the plates to which glucose had been added. These cells were therefore still viable. Although previous observations (*4*) had indicated that respiration-deficient cells were incapable of forming macro-colonies on lactate agar, the observation of the complete failure to form new buds on the lactate grid plate is more critical evidence for the totally selective character of the medium against such cells. It does not, however, yield any information concerning the ability of respiration-deficient cells recently arisen from respiration-sufficient cells to produce buds in the selective medium.

Strain 14940 exhibits a high spontaneous mutation rate to respiration deficiency when it is growing exponentially in lactate nutrient broth at 35°C (about 40 percent). When samples of this culture were transferred to glucose grid plates, essentially all configurations (except 1 percent inviable) developed to microcolonies within 24 hours and to macrocolonies by 48 hours. On lactate grids however, many configurations (30.9