Comparison of Transhydrogenase and Pyridine Nucleotide-Cytochrome c Reductase Activities in Rat Liver and Novikoff Hepatoma*

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In a recent discussion of the biochemical aspects of tumor uniformity and heterogeneity, it was suggested that TPNH[1]-cytochrome c reductase was a strategically important oxidative component that needed further study in tumors (25). It is the object of this report to examine that enzyme, together with the related enzymes, DPN-cytochrome c reductase and transhydrogenase, in rat liver and in the Novikoff hepatoma. The latter tumor was chosen because of its relation to rat liver, which has been studied extensively in this laboratory in young rats and in adult rats following partial hepatectomy, and because of the extensive data available on the Novikoff hepatoma (16, 21, 32–34).[2]

It would be impossible to detail the many developments that facilitated the present study, but three are particularly noteworthy: first, the development of the centrifugal technics for cell fractionation, which in turn led to the studies by Hogeboom and Schneider (10) and by DeDuve *et al.* (4), showing that TPNH-cytochrome c reductase activity occurs both in the mitochondria and in the "microsome fraction"; second, the discovery of the enzyme called pyridine nucleotide transhydrogenase in *Pseudomonas fluorescens* by Colowick *et al.* (3) and its demonstration in animal tissues by Kaplan *et al.* (14); and, third, the demonstration of the mitochondrial membrane (29, 31) and the use of "depleted" mitochondria (15, 17, 28).

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[1] Abbreviations used: DPN and TPN = oxidized diphospho- and triphosphopyridine nucelotides, respectively; DPNH and TPNH = reduced diphospho- and triphosphopyridine nucleotides, respectively; Mt = mitochondria; Mc = microsomes.

[2] We are indebted to Dr. Claude Allard for making available valuable unpublished data.

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MATERIALS AND METHODS

Rats bearing Novikoff hepatoma were kindly supplied by Dr. A. Novikoff, and his method of transplanting the tumor intraperitoneally was used (21). Normal male rats, 160–200 gm., were obtained from the Holtzman Rat Company, Madison, Wisconsin. After the rats were killed by decapitation, the required tissues were removed and placed in cold isotonic sucrose. All subsequent manipulations were carried out at 0° C.

Preparation of homogenates and cell fractions.—Homogenates, in water or isotonic sucrose, were prepared in a glass-to-glass Potter-Elvehjem homogenizer. Mitochondria were prepared in a model PR-1 International refrigerated centrifuge by fractionation of a 10 per cent homogenate in 0.25 M sucrose by the method of Schneider and Hogeboom (27). The fluffy layer that appears after washing the mitochondria was always decanted from the well packed pellet and was never used in a regular experiment except in a single one, in which it had characteristics intermediate between those of mitochondria and microsomes as far as the enzymes studied are concerned.

The "microsome fraction" was prepared from the mitochondrial supernatant by centrifugation at 105,000 × *g* for 50 minutes in a Spinco preparative ultracentrifuge.

Preparation of depleted mitochondria and microsomal vesicles.—Mitochondria and microsomes equivalent to 2.5 gm. of tissue were resuspended in 5 ml. of glass-distilled water and incubated at room temperature for 2 hours with constant and gentle shaking in an Erlenmeyer flask. The cell fractions were then sedimented by centrifugation

at 0° C. at the same speed and time as were used for their preparation. The pellets were resuspended in cold isotonic sucrose and used for the enzyme assays. The supernatant from the depleted mitochondria was further centrifuged in the Spinco at 105,000 × *g* for 50 minutes to remove any possible microsomal contamination. This supernatant (washing water), as well as that from depleted microsomes, was then checked for enzyme activity and protein content.

Protein determinations were carried out by the biuret reaction (8).

Reaction mixtures.—The standard reaction mixture contained the following final concentrations in a total volume of 3.0 ml.: 0.04 M nicotinamide; 0.033 M phosphate buffer, pH 7.2; 3.0 mg. oxidized cytochrome c; 3.3×10^{-4} M potassium cyanide; and 350 μg. of TPNH, DPN, DPNH, or combinations thereof. In many cases isotonicity was achieved by addition of the right amount of solid sucrose to the medium, but it was shown that, with hypotonic reaction mixtures, the results were essentially the same. The cell fractions were kept in cold isotonic sucrose prior to use. The amount of tissue used was at a level of 1–1.5 mg. of protein/reaction mixture. In the case of liver mitochondria this was roughly equal to about 25 mg. of fresh whole liver. However, the amount varied above and below this figure, depending on the rate of the reaction; but the fractions were usually added in a volume of 0.1 ml. of isotonic sucrose. Nicotinamide was used in the reaction mixture to prevent enzymatic inactivation of pyridine nucleotides by the specific nucleosidases (19). The cyanide was added to inhibit cytochrome oxidase, which is present in the mitochondrial fraction and which evidently is present to a small extent in the microsome fraction. No reduction of cytochrome c occurs in the absence of cyanide in mitochondria, but a fairly rapid reduction occurs when microsomes are used in the absence of cyanide (Chart 1). The results at the highest level of cyanide are believed to depend upon the time of exposure of the cytochrome c to cyanide, as shown earlier by Potter (25).

Assay procedure.—Assays were developed according to technics based on those used by Potter (25) and Lockhart and Potter in 1941 (18) for the study of the DPNH-cytochrome c reductase, except that TPNH was also used and the existence of transhydrogenase was recognized. In general, all the experiments were done at room temperature, and the reduction of cytochrome c was measured at a wave-length of 550 mμ. A model B Beckman spectrophotometer was used, and the change in absorbancy was read every 15 seconds for the fast reactions and every minute for the slower ones. A conversion factor for the μmoles of cytochrome c reduced for a given change in the E value was found by standardization against a Model DU Beckman spectrophotometer. With the use of a 13 × 100-mm. calibrated colorimeter tube as the reacting cell for the Beckman B, the change in the E value, when oxidized cytochrome c was converted into the reduced form, was lower than the corresponding value obtained in a Beckman DU with the proper cell by a factor equal to 1.5, which was used to correct our figures. Usually reactions were started by adding one of the pyridine nucleotides.

Reduction of cytochrome c upon the addition of DPNH was used as a measure of the DPNH-cytochrome c reductase and its rate as a measure of the activity. Similarly, the reduction upon the addition of TPNH was interpreted as due to the TPNH-cytochrome c reductase. The increase in the rate of cytochrome c reduction by TPNH, when DPN was already present, was an expression of the transhydrogenase.

Chemical materials.—DPN, DPNH, TPNH, and heart cytochrome c were products of the Sigma Chemical Company. Other materials were commercial products of reagent grade.

RESULTS

Comparison of normal liver with Novikoff hepatoma.—Chart 2 represents a typical experiment comparing the activity of normal liver mitochondria and Novikoff hepatoma mitochondria in promoting the reduction of cytochrome c upon the addition of DPNH, TPNH, or the combination of DPN and TPNH. In general, the amount of tissue was equivalent to 25 mg. wet weight of original tissue. However, in the case of liver mitochondria with DPNH, the amount used was

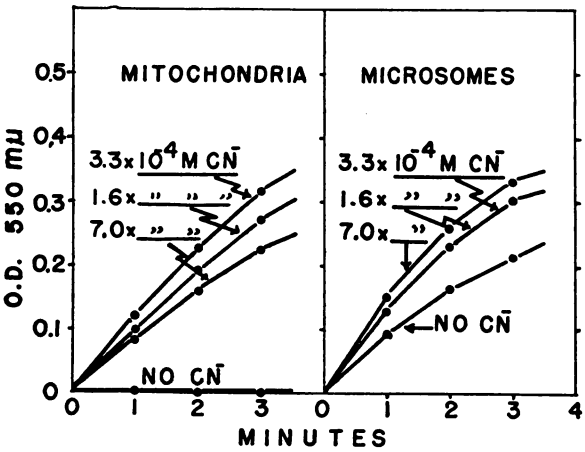

CHART 1.—Cytochrome c reduction by TPNH in liver mitochondria and microsome fractions at different levels of cyanide. Reaction mixtures contained the standard additions with cyanide used at the indicated levels. Reactions were started by addition of TPNH.

one-fifth as much because of the rapid rate; and in the case of hepatoma with TPNH ± DPN, the amount was increased up to as much 150 mg. equivalent. It can be seen (Chart 2) that DPNH-cytochrome c reductase in liver mitochondria was more active than that present in hepatoma mitochondria, which nevertheless were sufficiently active to give a vigorous reaction. In normal liver mitochondria there was a slow rate with TPNH and a somewhat faster reaction when DPN was added to bring transhydrogenase into play. In the hepatoma mitochondria neither TPNH-cytochrome c reductase nor transhydrogenase activity was in evidence, as shown by results with TPNH ± DPN. Chart 3 shows the results with the microsome fraction. In this case the DPNH system was active in both liver and hepatoma. In liver there was activity with TPNH and no stimulation when DPN was added, which fact

suggested either a lack of transhydrogenase or saturation with DPN. The former explanation appears to be the correct one. With the microsome fraction from hepatoma, there was no activity with TPNH ± DPN, which observation indicated

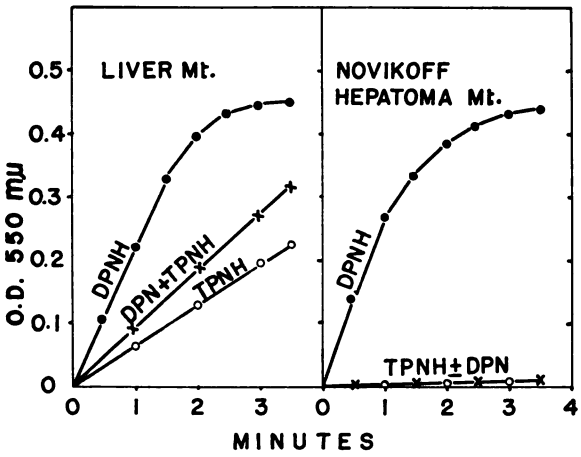

CHART 2.—Pyrimidine nucleotide-cytochrome c reductases and transhydrogenase activity of rat liver and hepatoma mitochondria. All reaction mixtures contained the standard additions plus 25 mg. equivalent of tissue, except for liver DPNH and tumor TPNH±DPN, in which 5 and 150 mg. equivalents of mitochondria were used, respectively.

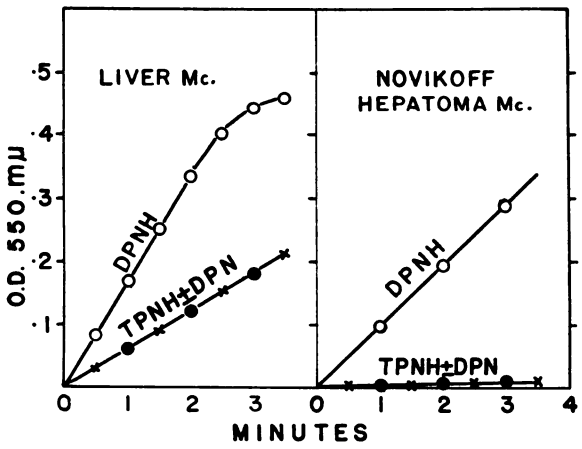

CHART 3.—Pyridine nucleotide-cytochrome c reductases and transhydrogenase activity of rat liver and hepatoma "microsome fraction." All reaction mixtures contained the standard additions plus 25 mg. equivalent of tissue, except for liver DPNH and tumor TPNH±DPN, in which 5 and 150 mg. equivalents of microsome fraction were used, respectively.

absence or negligible amounts of transhydrogenase and TPNH-cytochrome c reductase.

Results in Chart 4 were obtained from experiments with whole homogenates of liver and of hepatoma. This experiment was done because it seemed possible that activity missing from one cell fraction might have passed into another fraction or might have been in a discarded fraction. However, the results with whole homogenate support the results with mitochondria and with the microsome fraction: DPNH-cytochrome c reductase was present in both liver and hepatoma, but TPNH-cytochrome c reductase and transhydrogenase were too weak to be demonstrated in the hepatoma. It may be stated here that neither reductase nor transhydrogenase was demonstrable in the supernatant from the microsomal fraction from liver or hepatoma in isotonic sucrose.

Table 1 summarizes a series of experiments in which TPN and DPN-cytochrome c reductase were determined in whole homogenate and cell fractions of normal liver and Novikoff hepatoma.

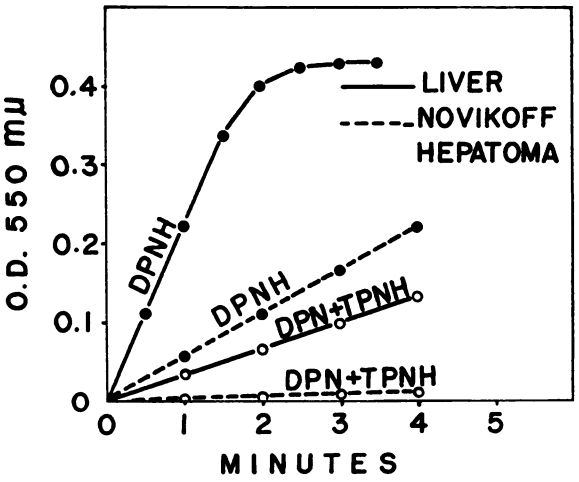

CHART 4.—Cytochrome c reduction by DPNH, TPNH, or DPN+TPNH in whole homogenates from liver and Novikoff hepatoma. Reaction mixtures contain standard additions plus one-fourth (about 0.25 mg. of protein) the regular amount of tissue, except for tumor DPN+TPNH in which 25 mg. of tissue was used.

It is evident that DPNH-cytochrome c reductase activity in hepatoma was less than in liver, but the more striking result was that in hepatoma the TPN-cytochrome c reductase activity was negligible, both in comparison with the TPN enzyme of liver and with the DPN enzyme of the hepatoma itself.

To learn whether the lack of activity with Novikoff hepatoma on TPNH ± DPN was simply an artefact or the result of some sort of inhibitor present in hepatoma, whole tumor and liver homogenates were incubated together for 20 minutes at room temperature in a reaction mixture containing all ingredients but cytochrome c. The reactions were started by adding cytochrome c. Data

in Chart 5 show the results on TPNH-cytochrome c reductase, which is almost absent in Novikoff hepatoma. The activity in liver appeared intact after a period of 20 minutes of incubation with tumor homogenate, as compared with that of liver alone incubated for the same period of time. Fluoride, ATP (5), and CoA (1) did not show any effect on the TPN reductase and transhydrogenase activity of mitochondria from Novikoff hepatoma.

Experiments with "depleted" mitochondria and microsomes.—Since the enzyme transhydrogenase promotes the following reversible reaction (13):

$$TPNH + DPN^+ \rightleftharpoons DPNH + TPN^+,$$

its demonstration, by the present methods, depends upon the availability of DPN in the cell fraction studied, when TPNH is the hydrogen donor. The liberation or "depletion" of endogenous nucleotides including DPN from mitochondria may be accomplished by different procedures (15, 17, 28). The one we describe under "Methods" was the most satisfactory for our purposes.

In Chart 2 it was shown that liver mitochondria showed TPNH-cytochrome c reductase activity in the absence of added DPN, and it was thought that if this result were due to intramitochondrial DPN the activity would disappear if the mitochondria were depleted. Chart 6*a* shows that this result was obtained: there was essentially no TPNH-cytochrome c reductase activity in the absence of added DPN; but, when DPN was added with TPNH, cytochrome c reduction was restored (Chart 6*a*). This does not necessarily mean that mitochondria lack TPNH-cytochrome c reductase and that the TPNH activity seen in fresh mitochondria (Chart 2) is exclusively due to intramitochondrial DPN, because Chart 6*b* shows that the TPNH activity lost when mitochondria were depleted could be recovered in the supernatant fluid or wash water. It may also be seen (Chart 6*b*) that the activity of the supernatant enzyme with TPNH alone was not stimulated when DPN was added, and no activity was observed with DPNH. Thus, this activity may be considered due to TPNH cytochrome c reduction per se. A similar experiment was carried out with microsomes, because Palade (22, 23) has shown that the microsome fraction yields vesicles that behave as osmometers and swell in distilled water. However, Chart 6*c* shows that microsomes thus treated not only retained DPNH activity but also TPNH activity; and no indication of transhydrogenase was found, since the activity on TPNH would appear not to require DPN (Chart

TABLE 1

TOTAL AND SPECIFIC ACTIVITY OF DPN AND TPN-CYTOCHROME C REDUCTASES IN WHOLE HOMOGENATE AND CELL FRACTIONS FROM NORMAL RAT LIVER AND NOVIKOFF HEPATOMA

The activity is expressed in terms of μmoles cytochrome c reduced/min/gm of fresh tissue or fraction therefrom or per gram of protein. Figures are averages of two to four experiments.

	Rat liver				Novikoff hepatoma			
	DPN-cyt. c reductase activity		TPN-cyt. c. reductase activity		DPN-cyt. c reductase activity		TPN-cyt. c. reductase activity	
Fresh cell fractions	Per gm. protein	Per gm. tissue	Per gm. protein	Per gm. tissue	Per gm. protein	Per gm. tissue	Per gm. protein	Per gm. tissue
Homogenate	*	59.6	*	5.5	*	9.4	*	0.1†
Mitochondria	278.3	32.3	16.4	2.2	94.2	5.7	0.24†	0.02†
Microsomes	208.5	21.3	21.7	2.6	41.4	3.2	0.42†	0.03†

* The biuret method for protein gave questionable results with whole homogenates.

† Values for hepatoma TPN-cytochrome c reduction are at the margin of precision for the present method.

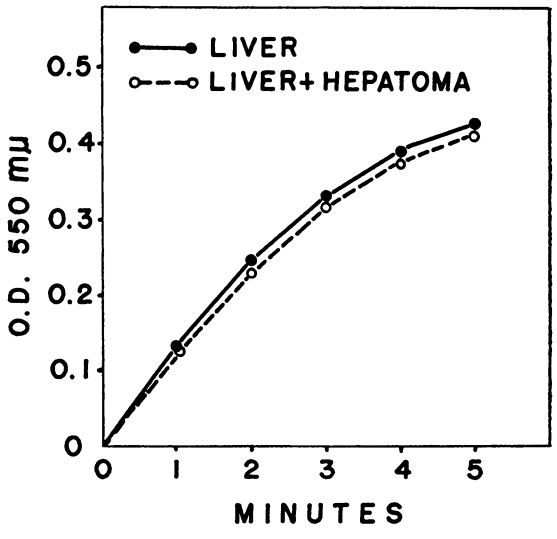

CHART 5.—TPNH-cytochrome c reductase activity in whole liver homogenate alone, and in a mixture of whole liver and tumor homogenates; 10 mg. of liver or 20 mg. of an equa mixture of liver and tumor homogenates were incubated for 20 minutes at room temperature in 2.8 ml. of reaction mixture containing all ingredients but cytochrome c. Reactions were started by adding 0.2 ml. of a solution containing 3 mg. of oxidized cytochrome c.

6c). Chart 6d indicates that the microsomal fluid or wash water did not have any considerable activity.

From experiments of the type reported in Chart 6, specific activity for each one of the enzymes was calculated and summarized in Table 2.

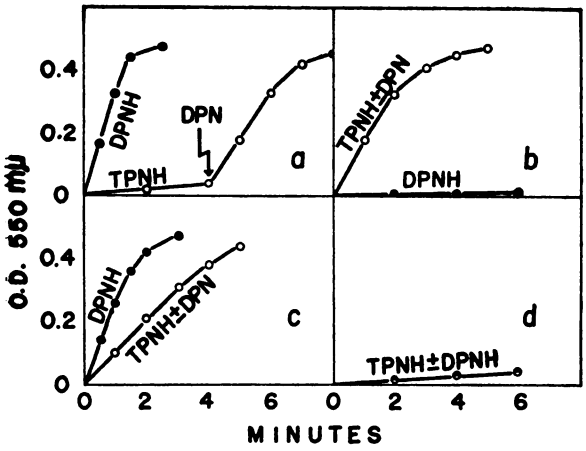

CHART 6.—Pyridine nucleotide-cytochrome c reductases and transhydrogenase activity in depleted liver mitochondria (*a*) and microsome (*c*) fractions and their wash waters (*b* and *d*, respectively). Reaction mixtures contain the standard additions plus the following amount of tissue protein: mitochondria DPNH system = 0.28 mg.; mitochondria TPNH ± DPN system = 1.4 mg.; mitochondrial wash water = 0.75 mg.; microsome DPNH system = 0.3 mg.; microsome TPNH ± DPN system = 1.3 mg.; microsomal wash water = 1.0 mg. protein.

In those experiments carried out with fresh liver mitochondria, the increase in the rate of cytochrome c reduction by TPNH in the presence of added DPN when endogenous DPN was also present was used merely as an evidence of transhydrogenase activity. However, it was not possible to calculate its specific activity from that kind of data, since, under the conditions employed, transhydrogenase and TPNH-cytochrome c reductase are performing cytochrome c reduction simultaneously, and we do not know to what extent each enzyme contributes to the rate of such a reduction. Therefore, it would be erroneous to calculate specific activity of transhydrogenase by taking either the over-all reduction (DPN + TPNH), or the difference between this over-all reduction and that due to TPNH-cytochrome c reductase (TPNH alone). The use of depleted mitochondria obviates this difficulty, because during depletion TPNH-cytochrome c reductase is solubilized into the wash water (Chart 6*b* and Table 2), leaving transhydrogenase attached to the nonsoluble part of the mitochondria (Chart 6*a* and Table 2), thus making it possible to calculate its specific activity without interference.

In microsomes, where the process of depletion failed to separate TPNH-cytochrome c reductase, and in soluble fractions (wash water in Table 2) no evidence of transhydrogenase activity was obtained, because it was demonstrated that the rate of cytochrome c reduction with TPNH was not

TABLE 2

SPECIFIC ACTIVITY OF PYRIDINE NUCLEOTIDE-CYTOCHROME C REDUCTASES AND TRANSHYDROGENASE IN DEPLETED CELL FRACTIONS AND THEIR WASH WATERS FROM NORMAL LIVER AND NOVIKOFF HEPATOMA

The data are expressed in terms of μmoles cytochrome c reduced per minute per gram of protein. Figures are averages of two to four experiments.

	Depleted cell fractions	Liver activity/gm protein	Novikoff hepatoma activity/gm protein
Transhydrogenase	Mitochondria	35.1	0.3*
	Mit. wash water	0.0	0.0
	Microsomes	0.0	0.0
	Mc. wash water	0.0	0.0
TPN -cyt. c reductase	Mitochondria	1.5	0.4*
	Mit. wash water	45.6†	0.1
	Microsomes	20.5	0.4
	Mc. wash water	2.1	0.3
DPN -cyt. c reductase	Mitochondria	351.0†	112.2
	Mit. wash water	2.5	3.4
	Microsomes	200.0	39.5
	Mc. wash water	1.8	0.9

* Values for hepatoma transhydrogenase and TPNH -cytochrome c reductase are at the margin of precision for the present method.

† In an experiment designed to test the relation between the activities in fresh and depleted mitochondria, 95 per cent of the original DPNH-cytochrome c reductase was recovered in the depleted mitochondria, and 67 per cent of the original apparent TPNH-cytochrome c reductase was recovered in the mitochondrial wash water. Cf. Chart 6*a* and 6*b*.

affected by DPN (Chart 6*b* and 6*c*). Measurements on endogenous DPN in the "depleted" microsome fraction have not been made, but it is assumed that none is present on the basis of the results with depleted mitochondria (Chart 6*a*).

Experiments with irradiated, regenerating, and newborn rat livers.—Since it is believed that the enzymes here studied might play an important role in many physiological as well as pathological processes, exploratory experiments were carried out to see if these enzymes show any change under different conditions. Results with a pool of livers from a litter of 7-day-old rats, as well as with 12-, 18- or 24-hour regenerating livers, did not show any gross variation from that described for adult normal rat liver. Also, one experiment with a 200-gm. rat, irradiated with 1500 r (20 roentgens/min) and killed 6 hours later, showed results not significantly different from the normal control.

Experiments with other tissues.—Preliminary experiments with certain other normal tissues, such as rat heart and brain and guinea pig spleen and pancreas, have been carried out. All three enzymes studied were found in these tissues in different amounts and with characteristic patterns of intracellular distribution for each one. No data comparable to the hepatoma data were obtained thus far, although negligible or negative values have been reported (12) for transhydrogenase in certain normal tissues. On the other hand, HeLa cells, Yoshida ascites cells, primary rat hepatoma, and mouse 129 hepatoma showed results markedly similar to the hepatoma results, which might be in agreement with the fact that TPN in tumors is present at very low levels and almost exclusively in the reduced form (7). A pooled sample of cultured human liver cells (Chang line), maintained by Dr. D. L. Walker, was provided to us. The assay results with whole homogenate resembled the data with hepatoma and not the data with rat liver, thus showing a tumorlike behavior in this respect (24).

DISCUSSION

From the results presented in this paper it appears that normal rat liver contains all three of the enzymes studied, to an extent easily demonstrable by the present method. DPNH-cytochrome c reductase occurs in both mitochondria and the microsome fraction, although the pattern of intracellular distribution of this enzyme appears different from that reported by Hogeboom and Schneider (9, 11) and DeDuve *et al.* (4) in that mitochondria are the site of the major activity of DPNH-cytochrome c reductase. Our values for this enzyme, expressed per gram of tissue, are somewhat higher than values obtained by McIlwain and Tresize (20) and by Palade and Siekevitz (22), but lower than those reported by Hogeboom (9). Transhydrogenase appears to be limited to the mitochondria, being absent from the microsome and soluble fractions of the cell. TPN-cytochrome c reductase, like DPNH-cytochrome c reductase, is present in both mitochondria and microsome fractions, with a pattern again somewhat different from that described by other workers (4, 10).

The studies with depleted mitochondria suggest that DPNH-cytochrome c reductase and transhydrogenase are firmly attached to the nonsoluble part of the mitochondria, which might well be the "mitochondrial membrane" studied by Siekevitz and Watson (29) and by Ball and Cooper (2), whereas the mitochondrial TPNH system is readily dissociated with water and passes into a form not sedimented at speeds that ordinarily bring down microsomes. Furthermore, it appears not to be microsomal in origin, because it has a negligible DPNH-cytochrome c reductase activity. The absence of the latter enzyme requires that the TPNH activity observed must be a true TPNH-cytochrome c reductase and not an artefact due to transhydrogenase, and must also be different from that of the microsome fraction, since the enzyme from this fraction is not dissociated with distilled water by the present method. However, much additional work will have to be done to learn the distribution of the enzymes in the various cell fractions and their role in oxidative phosphorylation (15). The apparent lack of either the transhydrogenase or the true TPNH-cytochrome c reductase in the Novikoff hepatoma must of course be considered as a possible artefact, and further work needs to be done. Similarly, the question of an absolute lack must be raised with the physiological implication pointed out by Potter in 1956 (26). Our studies with a few other normal and tumor tissues should not be taken to indicate that the combined deficiencies of TPNH-cytochrome c reductase and transhydrogenase activity are unique characteristics of tumors thus fulfilling the requirements for the fundamental alteration that distinguishes cancer tissue from normal tissue (26). Results obtained by Glock and McLean (7) showing that hepatoma and other tumors examined contain low levels of TPN might favor this conclusion. However, it appears that the deficiencies of TPNH-cytochrome c reductase and transhydrogenase are not exclusive characteristics of neoplastic tissues, since Humphrey (12) reported negligible and negative values

of transhydrogenase for certain normal tissues not yet studied in this laboratory. Also, a possible indication that it may occur in some tumors might be inferred by the report of Emmelot and Brombacher (6), who found that thyroxine uncoupled oxidative phosphorylation in some tumor mitochondria and not in others, and it appears by the work of Ball and Cooper (2) that the thyroxine acted by inhibiting the transhydrogenase in a preparation believed to be mitochondrial membranes; thus, a tissue lacking the enzyme should accordingly not be affected by thyroxine. Data presented by Waravdekar *et al.* (30) showed, incidentally, a significant TPNH-cytochrome c reductase activity in whole homogenate of Sarcoma 37, and Emmelot (personal communication) has found the same enzyme in the microsome fraction from certain tumors although at low levels.

It would be premature to discuss extensively the implications of these findings until additional work is carried out in a variety of tumors and normal tissues. Nevertheless, it would appear to be worth while to explore the physiological consequences of the enzymatic defect described in hepatoma and to relate it to the excellent work being done in other laboratories on the same tumor.

SUMMARY

1. Pyridine nucleotide-cytochrome c reductases and transhydrogenase have been examined in whole homogenate and in cell fractions of normal rat liver and Novikoff hepatoma.

2. DPNH-cytochrome c reductase was present in mitochondria and microsome fractions from both liver and hepatoma.

3. In liver the enzyme transhydrogenase was limited to the mitochondria and was firmly attached to the nonsoluble part of this cell fraction. It was absent from the microsome and soluble fractions.

4. Transhydrogenase was absent or present only to a negligible extent in whole homogenate and cell fractions from Novikoff hepatoma, under the conditions employed thus far.

5. There was a true TPNH-cytochrome c reductase in mitochondria and microsomes from normal liver.

6. TPNH-cytochrome c reductase from liver was firmly associated with microsomes but was readily dissociated from mitochondria, passing into a form that was not sedimented at a speed of 105,000 × *g*. This soluble fraction did not contain DPNH-cytochrome c reductase.

7. TPNH-cytochrome c reductase activity was absent or present in negligible amounts in whole homogenate and cell fractions of Novikoff hepatoma, as determined by the present method.

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