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DRUG METABOLISM IN THE NOVIKOFF HEPATOMA

EVIDENCE FOR A MIXED FUNCTION OXIDASE SYSTEM AND PARTIAL PURIFICATION OF CYTOCHROME *P*-450 REDUCTASE

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Summary

Novikoff hepatoma microsomes catalyze the hydroxylation of benzphetamine and ethylmorphine at rates less than 1% of those of liver microsomes but catalyze the hydroxylation of *p*-nitroanisole and *p*-nitrophenetole at rates about 40% of those of liver microsomes. Benzo[*a*]pyrene hydroxylation is also catalyzed by Novikoff hepatoma microsomes at about 2% of the rate of liver microsomes. Like the hepatic microsomal system the rates of substrate hydroxylation by Novikoff hepatoma microsomes can be increased by pretreatment with phenobarbital/hydrocortisone or β-naphthoflavone and inhibited by carbon monoxide, SKF-525A, and 7,8-benzoflavone.

In addition, NADPH-cytochrome *P*-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) has been partially purified from Novikoff hepatoma ascites cells and some properties are described. The induction and inhibition characteristics of the Novikoff hepatoma microsomal hydroxylation activities and the isolation of a cytochrome *P*-450 reductase from the hepatoma are consistent with the presence of a functional mixed function oxidase system in the Novikoff hepatoma, analogous to that present in liver endoplasmic reticulum.

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Abbreviation: 'Minimal deviation' and 'multiple deviation' are terms originally introduced by Potter [9] and are used in this paper to denote the magnitude or number of enzymatic changes in a relative manner. Tumors with relatively few changes in enzyme patterns are classified as 'minimal deviation' tumors, whereas those with extensive changes such that the cell of origin cannot be identified with certainty are classified as 'multiple deviation' tumors.

Introduction

Cytochrome *P*-450-dependent drug metabolism systems have been described in the liver [1—3] and in other tissues [4—7]. Sugimura et al. [8] demonstrated aniline metabolism in microsomes prepared from 'minimal deviation' hepatomas 7316A, 7793, 7794A, and 7795. Other workers showed that azo dye *N*-demethylase [10] and benzo[*a*]pyrene hydroxylase [11] activities are induced in several 'minimal deviation' hepatomas by 3-methylcholanthrene, a known inducer of the cytochrome *P*-450-dependent polycyclic hydrocarbon hydroxylation system of liver [12,13]. Rogers et al. [14] obtained a 3-fold induction of hexobarbital metabolism in hepatoma 7800 by pretreating with daily injections of phenobarbital. In contrast, Miyake et al. [15] were unable to show induction of drug or polycyclic hydrocarbon metabolism by either phenobarbital or 3-methylcholanthrene pretreatment in 'minimal deviation' hepatoma 7777.

Fouts and coworkers [16,17] have assayed a 'multiple deviation' hepatoma, the Novikoff hepatoma, for studies on drug metabolism. These workers first reported that Novikoff hepatoma microsomes catalyzed only the reductive cleavage of the azo linkage of neoprontosil, the oxidative pathways being absent. Later, they reported the presence of hexobarbital metabolism in Novikoff hepatoma microsomes from phenobarbital-pretreated hosts [17]. Although these observations were the first indication that a rapidly growing, highly dedifferentiated tumor was able to support the metabolism of hexobarbital, the nature of the enzyme system which catalyzed this reaction was not identified and studied. This paper extends the range of substrates hydroxylated by Novikoff hepatoma microsomes and attempts to define the system involved in these hydroxylation reactions using specific inhibitors, kinetic analyses and partial purification of the reductase component of the Novikoff hepatoma microsomal drug metabolism system.

Materials and Methods

Novikoff hepatoma (ascites cells obtained from Dr. Walborg, M.D. Anderson Hospital and Tumor Institute, Houston) was transplanted by intraperitoneal injection of 1 ml ascites fluid (approx. $25 \cdot 10^6$ cells) into male Sprague-Dawley rats (80—100 g) obtained from Flow Laboratories, Dublin, Va. Tumor-bearing rats were treated with phenobarbital sodium (75 mg/kg body weight) in saline daily for 2 days followed by 2 injections/day phenobarbital sodium (75 mg/kg) and hydrocortisone-21-sodium succinate (50 mg/kg) for the 2 days immediately before use. Alternatively, the host rats were injected with a single daily dose of 80 mg/kg β-naphthoflavone (5,6-benzoflavone) in corn oil for 3 days before use. Control animals (which received corn oil injections) and treated rats were fasted for 16 h before use. The animals were killed by decapitation, the ascites fluid collected, diluted with 1 vol. 0.02 M Tris-HCl buffer, (pH 7.7) and centrifuged at $4200 \times g$ for 10 min. The pellet was resuspended in Tris buffer and recentrifuged until the packed hepatoma cells were free of erythrocytes (four wash cycles). The cells were suspended in 0.02 M Tris-HCl buffer/10 mM EDTA/0.25 mM phenylmethylsulfonyl fluoride and ruptured by passage through a French pressure cell; microsomes were

prepared by differential centrifugation. The microsomal pellet was resuspended in 0.25 M sucrose/10 mM EDTA and stored at −70°C.

Benzphetamine and ethylmorphine hydroxylation activities were determined spectrophotometrically at 30°C as benzphetamine-dependent oxidation of NADPH at 340 nm [18] or colorimetrically by formaldehyde liberation [18—21]. The extinction coefficient used was 6.2 $cm^{-1} \cdot mM^{-1}$ at 340 nm. NADPH-cytochrome *c* reductase activity was assayed at 30°C by a modification of the method of Phillips and Langdon [22]. Reaction mixtures (1 ml) contained 300 μmol potassium phosphate buffer (pH 7.7) and reduction of cytochrome *c* was followed at 550 nm (extinction coefficient 21 $cm^{-1} \cdot mM^{-1}$) [23]. In similar reaction mixtures, dichlorophenolindophenol reduction was measured at 600 nm (extinction coefficient 21 $cm^{-1} \cdot mM^{-1}$) [24]; ferricyanide reduction was measured at 420 nm (extinction coefficient 1.02 $cm^{-1} \cdot mM^{-1}$) [25]. Epinephrine cooxidation was measured at 480 nm (extinction coefficient, 4.02 $cm^{-1} \cdot mM^{-1}$) [26,27]. Benzo[*a*]pyrene hydroxylation was measured spectrofluorometrically in 1-ml reaction mixtures at 30°C (excitation wavelength, 386 nm, emission wavelength, 515 nm) [28].

The O-dealkylation activity of Novikoff hepatoma microsomes was estimated using *p*-nitroanisole and *p*-nitrophenetole. The rate of dealkylation was determined by formation of the product, *p*-nitrophenol [29].

Liver cytochrome *P*-450 was prepared by the method of Levin et al. [30] through the DEAE-cellulose step and determined by the method of Omura and Sato [31] (extinction coefficient, 91 $cm^{-1} \cdot mM^{-1}$ for $A_{450nm}-A_{490nm}$). Protein concentrations in microsomal preparations were determined by the method of Lowry et al. [32]; the method of Shaffner and Weissman [33] was used to determine protein in all other preparations (bovine serum albumin was used as standard). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [34].

NADPH-cytochrome *P*-450 reductase was purified from Novikoff hepatoma microsomes by a modification of the procedure of Dignam and Strobel [35]. 6 g Novikoff hepatoma phenobarbital-pretreated microsomes were suspended at 4°C in a 600 ml mixture containing 180 ml glycerol, 30 ml 2 M Tris-HCl (pH 7.7), 6 ml 0.01 M dithiothreitol and 12 ml of 0.5 M EDTA in water. 6 ml 25 mM phenylmethylsulfonyl fluoride was added as protease inhibitor. Microsomes were solubilized by the slow addition of 83 ml 10% (v/v) Renex 690 (polyoxyethylene (10) nonylphenyl ether). 13 ml 1.5% (w/v) protamine sulfate was then added slowly with stirring. The suspension was centrifuged at 100 000 × *g* for 1 h and the pellet was discarded. The supernatant solution was loaded onto a 1 l DEAE-Sephadex A-25 column (5 × 55 cm) equilibrated with 0.1 M Tris-HCl buffer, (pH 7.7)/20% glycerol/10 mM EDTA/0.1 mM dithiothreitol/0.15% (v/v) Renex 690. The column was washed with equilibration buffer to remove unbound material. Cytochrome *P*-450 reductase was eluted from the column with a linear 3 l 0—0.3 M KCl gradient in equilibration buffer. Fractions containing NADPH-cytochrome *c* reductase activity were pooled and concentrated by ultrafiltration with an Amicon XM-50 membrane. The concentrated fraction was loaded onto an NADP-Sepharose column (2 × 6 cm) equilibrated with 0.3 M potassium phosphate buffer, (pH 7.7)/0.1% (w/v) sodium deoxycholate/20% glycerol/0.1 mM dithiothreitol/1 mM EDTA [35].

The column was washed with equilibration buffer and the reductase eluted with equilibration buffer containing 1 mM $NADP^+$. The active fractions were concentrated by ultrafiltration and stored at −70°C until use. $NADP^+$ was removed from the enzyme by dialysis.

Materials. Horse heart cytochrome *c*, $NADP^+$, NADPH, and hydrocortisone-21-sodium succinate were purchased from Sigma Chemical Co. St. Louis, Mo. Renex 690 was obtained from ICI America, Philadelphia, Pa., dilauroyl phosphatidycholine, from Serdary Research Laboratories, London, Ont. Benzphetamine hydrochloride was a gift of Dr. J.W. Hinman of the Upjohn Company, Kalamazoo, Mich. Ethylmorphine and phenobarbital sodium were obtained from Merck, Rahway, N.J., DEAE-Sephadex A-25 from Pharmacia, Piscataway, N.J., and Agarose 0.5 m from Bio-Rad Laboratories, Richmond, Calif. Epinephrine hydrogen tartrate was purchased from British Drug House, Poole, Eng., benzo[*a*]pyrene, *p*-nitroanisole and *p*-nitrophenetole from Eastman, Rochester, N.Y., 5,6-benzoflavone (β-naphthoflavone) and 7,8-benzoflavone (α-naphthoflavone) from Aldrich Chemical Co., Milwaukee, Wisc. SKF-525-A (2 diethylaminoethyl-2,2-diphenylvalarate hydrochloride) was a gift of Smith, Kline and French Laboratories, Phildelphia, Pa. All other chemicals were reagent grade or better.

Results

Presence and inducibility of drug metabolism activities in Novikoff hepatoma microsomes

Novikoff hepatoma microsomes catalyze the hydroxylation of a variety of N- and O-alkyl drugs and the polycyclic hydrocarbon benzo[*a*]pyrene (Table I), though at rates much lower than those of liver microsomes. No hydroxylation of benzphetamine or ethylmorphine could be detected in control Novikoff

TABLE I

HYDROXYLATION ACTIVITIES OF CONTROL, PHENOBARBITAL/HYDROCORTISONE-PRETREATED AND β-NAPHTHOFLAVONE-PRETREATED NOVIKOFF HEPATOMA MICROSOMES

The hydroxylation of benzphetamine and ethylmorphine was determined by formaldehyde liberation at 30°C in 1.5-ml reaction mixtures. *p*-Nitrophenetole and *p*-nitroanisole hydroxylation were measured by formation of *p*-nitrophenol at 30°C in 1.5-ml reaction mixtures. Benzo[*a*]pyrene hydroxylation was determined spectrofluorometrically in 1-ml reaction mixtures at 30°C. Microsomal samples were obtained from control rats or rats pretreated with phenobarbital/hydrocortisone or β-naphthoflavone as described in Materials and Methods. All values are the mean of at least five determinations ± S.E.

Substrate	Microsomal prepration		
	Control	Phenobarbital/ hydrocortisone	β-Naphthoflavone
Benzphetamine	Undetectable	0.0565 ± 0.030 *	0.035 ± 0.007 *
Ethylmorphine	Undetectable	0.0376 ± 0.023 *	0.011 ± 0.002 *
p-Nitroanisole	0.144 ± 0.028 **	0.645 ± 0.163 **	0.302 ± 0.111 **
p-Nitrophenetole	0.35 ± 0.12 **	0.92 ± 0.19 **	0.33 ± 0.17 **
Benzo[*a*]pyrene	3.19 ± 1.78 ***	3.28 ± 1.49 ***	11.53 ± 2.25 ***

* nmol formaldehyde/mg per min.

** nmol *p*-nitrophenol/mg per min.

*** pmol 3-OH benzo[*a*]pyrene/mg per min.

hepatoma microsomes after incubation times of 30—40 min at high protein concentration (5—10 mg/assay). Slight activity was detected with both substrates when hepatoma microsomes from animals pretreated with known inducers of the drug metabolism system, such as phenobarbital [36] or β-naphthoflavone [37], were used. Even with pretreated microsomes, however, long incubation times and high protein concentrations had to be used to detect these activities which are 1% or less than those from pretreated liver microsomes (e.g. 20 nmol formaldehyde/min per mg produced from benzphetamine using phenobarbital/hydrocortisone-pretreated liver microsomes). Benzo[*a*]-pyrene hydroxylation activity could be detected in control microsomes and was induced 3-fold by pretreatment with β-naphthoflavone. Hydroxylation of *p*-nitrophenetole and *p*-nitroanisole could also be detected in control Novikoff hepatoma microsomes and was induced by phenobarbital/hydrocortisone pretreatment. The phenobarbital/hydrocortisone-induced activity is about 40% of that of induced liver (2.18 nmol *p*-nitrophenol/min per mg from *p*-nitrophenetole).

No cytochrome *P*-450 or *P*-448 could be detected, however, in any of our Novikoff hepatoma microsomal preparations by the method of Omura and Sato [31] but NADPH-cytochrome *c* reductase activity was detectable in all preparations. The mean reductase specific activity in phenobarbital/hydrocortisone- or β-naphthoflavone-pretreated rats, was 40.22 ± 11.47 and 39.84 ± 16.14 nmol cytochrome *c* reduced/min per mg, respectively, whereas the control activity was 28.56 ± 6.23 nmol/min per mg.

Characterization of the Novikoff hepatoma microsomal drug metabolism system

Two substrates, *p*-nitrophenetole and benzo[*a*]pyrene, were selected for study of response to protein concentration, length of assay period and substrate concentration. The data of Fig. 1A show that the hydroxylation of *p*-nitrophenetole is linear up to 4 mg microsomal protein, whereas hydroxylation by β-naphthoflavone-pretreated microsomes approximates to linearity up to 2 mg microsomal protein. The data in panel B indicate that hydroxylation of benzo[*a*]pyrene is linear up to 1 mg microsomal protein. In addition, the data in Fig. 1 show (as in Table I) that *p*-nitrophenetole hydroxylation was significantly induced by phenobarbital/hydrocortisone pretreatment, whereas benzo-[*a*]pyrene hydroxylation was induced significantly only by β-naphthoflavone pretreatment.

In order to determine the effect of incubation time on reaction velocity, 0.5 mg samples of control, phenobarbital/hydrocortisone-pretreated and β-naphthoflavone-pretreated hepatoma microsomes were assayed for *p*-nitrophenetole and benzo[*a*]pyrene hydroxylation after various incubation times. The hydroxylation rate was linear with time for at least 10 min (Figs. 2A and 2B). The K_m of Novikoff hepatoma microsomes for *p*-nitrophenetole is $1.22 \cdot 10^{-4}$ M and, for benzo[*a*]pyrene, the K_m is $2.94 \cdot 10^{-5}$ M.

Since the presence of cytochrome *P*-450 has not, thus far, been directly demonstrated in Novikoff hepatoma microsomes, the indirect means of showing the involvment of cytochrome *P*-448 or *P*-450 in hydroxylation reactions by use of known inhibitors was attempted. SKF-525-A (2-diethylamino-

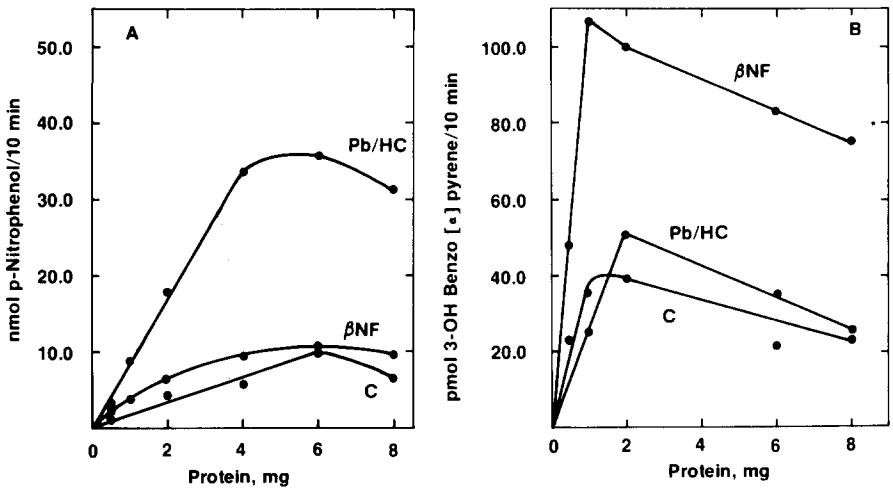

Fig. 1. Effect of Novikoff hepatoma microsomal protein concentration on *p*-nitrophenetole and benzo[*a*]pyrene hydroxylation rates. (A) The amount of *p*-nitrophenol formed from *p*-nitrophenetole in 10 min was determined at various concentrations of control (C), phenobarbital/hydrocortisone-pretreated (Pb/HC), β-naphthoflavone-pretreated (βNF) hepatoma microsomal protein. (B) The amount of 3-OH benzo[*a*]pyrene formed from benzo[*a*]pyrene was similarly determined.

ethyl-2,2-diphenylvalarate hydrochloride, an inhibitor of drug metabolism activity [38] shown to bind to cytochrome *P*-450 [39]), 7,8-benzoflavone (an inhibitor of cytochrome *P*-448-dependent polycyclic hydrocarbon hydroxylation [40,41]), and carbon monoxide, (which interacts with both cytochromes [42,43]), were used in this study. The effects of these substances on the hydroxylation system are shown in Table II. When reaction mixtures con-

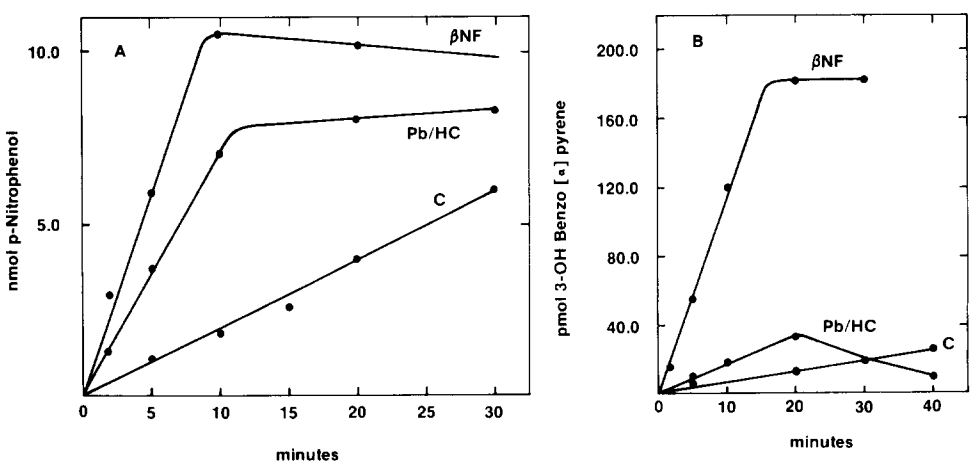

Fig. 2. Effect of incubation time on product formation from *p*-nitrophenetole and benzo[*a*]pyrene by Novikoff hepatoma microsomes. (A) *p*-nitrophenol production by 0.5 mg of control (C), phenobarbital/hydrocortisone-pretreated (Pb/HC) and β-naphthoflavone-pretreated (βNF) Novikoff hepatoma microsomes was determined after incubation at 30°C for various lengths of time. (B) 3-OH benzo[*a*]pyrene production by Novikoff hepatoma microsomes described for A was also determined after various incubation times. Microsomal protein concentrations were: control, 7.2 mg/assay; phenobarbital/hydrocortisone-pretreated, 3.6 mg/assay; β-naphthoflavone-pretreated, 2.8 mg/assay.

TABLE II

EFFECTS OF CARBON MONOXIDE, 7,8-BENZOFLAVONE AND SKF-525-A ON MIXED FUNCTION OXIDASE ACTIVITIES OF NOVIKOFF HEPATOMA MICROSOMES

The hydroxylation of *p*-nitrophenetole and benzo[*a*]pyrene was measured as described in Table I. Phenobarbital/hydrocortisone-pretreated hepatoma microsomes were used for *p*-nitrophenetole hydroxylation assays and β-naphthoflavone-pretreated hepatoma microsomes were used for benzo[*a*]pyrene hydroxylation assays. CO/O_2 mixtures were prepared in a gas burette. Reaction mixtures were equilibrated with carbon monoxide mixtures in reaction tubes with teflon caps. The tubes were flushed with the CO/O_2 mixture and then tightly capped. 7,8-Benzoflavone was dissolved in methanol, and added in 10-μl aliquots.

Addition	*p*-Nitrophenetole hydroxylation		Benzo[*a*]pyrene hydroxylation	
	Activity *	Inhibition (%)	Activity **	Inhibition (%)
None	4.03	0	4.53	0
SKF-525-A, 2.0 mM	2.27	44	4.03	27
CO/O_2				
10/1	2.53	37	2.15	61
100/1	1.15	71	1.60	71
7,8-Benzoflavone				
10^{-7} M	—	—	4.0	28
10^{-6} M	—	—	3.5	37
10^{-5} M	—	—	2.9	47
10^{-4} M	—	—	2.6	53
10^{-3} M	—	—	1.3	76

* nmol/10 min per 0.5 mg.
** pmol/10 min per mg.

taining Novikoff hepatoma microsomes were equilibrated with a 10 : 1 (v/v) mixture of CO and O_2 and incubated in sealed reaction tubes under the same gas mixture, *p*-nitrophenetole hydroxylation was reduced by 37% and benzo[*a*]pyrene hydroxylation was reduced by 61%. Increasing the ratio CO : O_2 increased the degree of inhibition. Exchanging N_2 for CO at these ratios resulted in no (or less than 10%) inhibition of hydroxylation activity. 2.0 mM SKF-525-A caused 44% inhibiton of *p*-nitrophenetole hydroxylation and 27% inhibition of benzo[*a*]pyrene hydroxylation. 7,8-Benzoflavone inhibited hepatoma microsomal benzo[*a*]pyrene hydroxylation with 50% inhibition occurring between 10 and 100 μM (Table II) whereas 50% inhibition of liver microsomal benzo[*a*]pyrene hydroxylation occurs at 10 μM. The effect of 7,8-benzoflavone on *p*-nitrophenetole hydroxylation could not be determined because of inhibitor interference with the assay for *p*-nitrophenol.

Resolution of cytochrome P-450 reductase from Novikoff hepatoma microsomes

When Novikoff hepatoma microsomes are solubilized with Renex 690, treated with protamine sulfate and then chromatographed on DEAE-Sephadex A-25 [35], cytochrome *P*-450 reductase bound tightly to the gel, while most other microsomal constituents passed through or were eluted in the wash step. In contrast to results with liver microsomes [44] (where cytochrome *P*-450 can be recovered in the non-adsorbed material) no cytochrome *P*-450 could be

detected in this fraction from solubilized Novikoff microsomes. However, the non-adsorbed fraction gave a peak at 420 nm in a reduced CO difference spectrum, suggesting the presence of cytochrome *P*-420.

Purification of Novikoff hepatoma NADPH-cytochrome P-450 reductase

The purification of cytochrome *P*-450 reductase from Novikoff hepatoma microsomes is summarized in Table III. The Novikoff hepatoma reductase was purified approx. 500-fold, with a 13% recovery. SDS-polyacrylamide gel electrophoresis of the Novikoff hepatoma reductase revealed some minor bands and a major band which accounts for 88% of the total stained material on the gel. The apparent minimum subunit molecular weight of the Novikoff hepatoma reductase is 77 000. A mixture of Novikoff hepatoma reductase and rat liver reductase, subjected to SDS gel electrophoresis, gave a single reductase band of molecular weight 77 000.

The visible spectrum of the reductase preparation is that of a flavoprotein with absorbance maxima at 380 and 450 nm and a shoulder at 480 nm. The presence of an absorbance peak at 418 nm suggests contamination of this preparation with an heme protein. Fluorometric analysis of the flavin content of this preparation by the method of Bessey et al. [45] indicate the presence of both FMN and FAD. The total flavin content, however, was 6.45 nmol/mg protein and there appeared to be twice as much FMN as FAD present in this preparation in contrast to the flavin content and distribution of liver reductase [35, 44]. Incubation of the hepatoma reductase with exogeneous FMN and FAD, however, did not stimulate the rate of cytochrome *c* reduction more than 10% above the rate with the untreated enzyme.

Reconstitution of drug metabolism with Novikoff hepatoma cytochrome P-450 reductase and hepatic cytochrome P-450

The reconstitution of drug metabolism activity from partially purified Novikoff hepatoma reductase and liver cytochrome *P*-450 is shown in Table IV. Novikoff hepatoma reductase is fully able to support cytochrome *P*-450-dependent benzphetamine hydroxylation. Hydroxylation activity is completely dependent upon the presence of added cytochrome *P*-450 and phosphatidylcholine. Benzphetamine-dependent NADPH oxidation in a reconstitued system, containing excess cytochrome *P*-450 and phosphatidylcholine, is proportional

TABLE III

PURIFICATION OF NADPH-CYTOCHROME *P*-450 REDUCTASE FROM NOVIKOFF HEPATOMA MICROSOMES

Preparation	Protein (mg)	Total activity *	Specific activity **	Yield (%)
Solubilized microsomes	6046	105.2	0.017	100
Treated with protamine sulfate	1618	129	0.080	123
DEAE-Sephadex A-25 eluate	81.8	36.3	0.445	35
NADP-Sepharose eluate; concentrated by ultrafiltration	1.5	13.3	8.89	12.6

* μmol cytochrome *c* reduced/min.

** μmol cytochrome *c* reduced/min per mg protein.

TABLE IV

BENZPHETAMINE HYDROXYLATION BY A SYSTEM RECONSTITUTED FROM NOVIKOFF HEPATOMA CYTOCHROME P-450 REDUCTASE AND LIVER CYTOCHROME *P*-450

Reaction mixtures contained 24 μg Novikoff hepatoma reductase, 0.24 nmol liver cytochrome P-450, 30 μg dilauroyl phosphatidylcholine and 100 μmol potassium phosphate buffer (pH 7.7) in 1-ml reaction mixtures. The reaction was initiated by the addition of 0.15 μmol NADPH.

System	nmol NADPH oxidized/min
Complete	6.42
No reductase	0
No cytochrome *P*-450	0
No lipid	0

to the amount of Novikoff hepatoma reductase added up to about 25 μg reductase protein (Fig. 3). Addition of larger quantities of reductase either caused either no increase in rate or a slight inhibition.

Kinetic properties of Novikoff hepatoma cytochrome P-450 reductase

The partially purified Novikoff hepatoma reductase also interacted with a number of exogenous electron acceptors and cooxidants. Kinetic analysis for the interaction of the Novikoff hepatoma reductase with cytochrome *c* is shown in Fig. 4. When the concentration of cytochrome *c* was varied at fixed concentrations of NADPH, double-reciprocal plots of the rate of cytochrome *c*

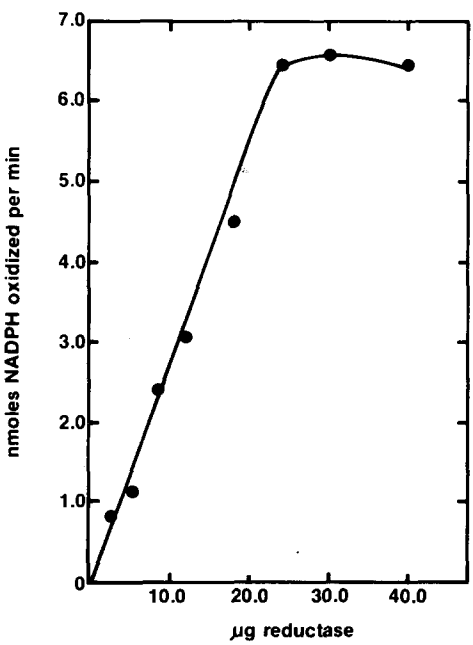

Fig. 3. Benzphetamine hydroxylation as a function of Novikoff hepatoma reductase added to a reconstituted system. Reaction mixtures contained 30 μg dilauroyl phosphatidylcholine, and 0.24 nmol liver cytochrome *P*-450 while the reductase was varied.

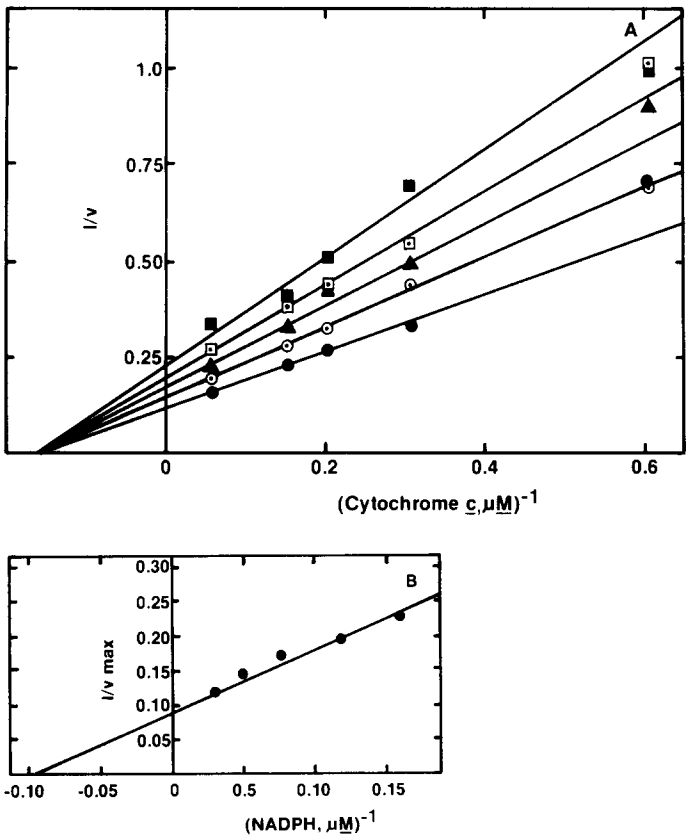

Fig. 4. Double-reciprocal plots of velocity for the reduction of cytochrome *c* by Novikoff hepatoma reductase. Reactions were carried out in 1 ml final volume containing 300 mM potassium phosphate buffer (pH 7.7), varying amounts of cytochrome *c* and 1.2 μg Novikoff hepatoma reductase. Velocity is expressed as $\mu mol^{-1} \times min^{-1} \times 10^3$. (A) ●, 3.2 mM NADPH; ○, 2.0 mM NADPH; ▲, 1.3 mM NADPH; □, 0.85 mM NADPH; ■, 0.63 mM NADPH. (B) Secondary double-reciprocal plot of $1/v$ intercept from A vs. concentration of NADPH.

reduction vs. concentration of cytochrome *c* at each concentration of NADPH extrapolated to a common point of intersection (Fig. 4A). This result is similar to that obtained with purified liver cytochrome *P*-450 reductase [35] and is consistent with a sequential reaction mechanism [46] for the interaction of NADPH and cytochrome *c* with Novikoff hepatoma reductase. The apparent K_m for cytochrome *c* determined from Fig. 4A is 6.2 μM. A secondary double-reciprocal plot of the $1/v$ intercept of each line from Fig. 4A vs. the fixed concentration of NADPH for each line is shown in Fig. 4B. The points lie on a straight line which, when extrapolated, gives an apparent K_m for NADPH in this reaction of 10.4 μM.

The activity of the purified Novikoff hepatoma reductase in electron transfer to artificial acceptors and cooxidants is summarized in Table V. Although the *V* values for reduction or cooxidation of these substrates by the purified Novikoff hepatoma reductase are considerably lower than the values for purified rat liver reductase, the apparent K_m values are all in good agreement with those of the normal liver reductase.

TABLE V

KINETIC PROPERTIES OF NOVIKOFF HEPATOMA NADPH-CYTOCHROME *P*-450 REDUCTASE IN ARTIFICIAL SYSTEMS

Reaction mixtures contained 300 μmol potassium phosphate buffer (pH 7.7) and the acceptor in a final volume of 1 ml. The reaction was initiated by addition of 0.15 μmol NADPH.

Substrate	K_m *	V **
Cytochrome *c*	6.2	9.5
Dichlorophenolindophenol	17.5	9.2
Potassium ferricyanide	24.1	35.5
Epinephrine	270.0	1.7
NADPH ***	10.4	—

* μM.

** μmol substrate reduced (or oxidized) per min per mg reductase.

*** Cytochrome *c* was used as electron acceptor.

Discussion

The results of this paper extend the preliminary observation [17] that the Novikoff hepatoma can catalyze the hydroxylation of drugs by showing that a variety of N-methyl and O-alkyl drugs and polycyclic hydrocarbons are hydroxylated by Novikoff hepatoma microsomes. The hydroxylation of these substrates can be induced by pretreatment of the rat hosts with known inducers of hepatic cytochrome *P*-450 (*P*-448)-dependent drug metabolism, phenobarbital [36] and β-naphthoflavone [37], as judged by an increase in specific activity after pretreatment. No cytochrome *P*-450 or *P*-448 was detectable in any tumor microsomal preparation by reduced CO difference spectroscopy, in agreement with the data of Sato and Hagihara [47] who reported the absence of cytochromes *P*-450 and b_5 in the Novikoff hepatoma. Cytochrome *c* reductase activity is detectable and the mean specific activity rises after pretreatment with inducers, though the rises are not statistically significant. This result is consistent with the observations of Stohs et al. [48] for cytochrome *c* reductase of small intestinal microsomes.

The linearity of benzo[*a*]pyrene and *p*-nitrophenetole hydroxylation activities with protein concentration and time is consistent with catalysis by an enzyme system, the nature of which was further studied using known inhibitors of cytochrome *P*-450/*P*-448-mediated reactions. CO and 7,8-benzoflavone inhibit benzo[*a*]pyrene hydroxylation by Novikoff hepatoma microsomes in a dose-dependent fashion. Similarly, carbon monoxide and SKF-525-A inhibit *p*-nitrophenetole hydroxylation. Since these inhibitors have been shown to exert their effects at the level of the cytochromes [39—43], their inhibition of hydroxylation activities catalyzed by Novikoff hepatoma microsomes provides indirect evidence for the existence and catalytic function of cytochrome *P*-450/*P*-448 in the Novikoff hepatoma even though the presence of these cytochromes cannot be demonstrated directly.

Additional support for the presence of a mixed function oxidase system is provided by the solubilization and resolution studies which led to the partial purification of Novikoff hepatoma cytochrome *P*-450 reductase. Although the presence of drug-metabolizing activity has been observed in the ascites form of

the Novikoff, this paper represents, to our knowledge, the first report of the purification of NADPH cytochrome *P*-450 reductase from the Novikoff hepatoma. The partially purified Novikoff hepatoma reductase has an apparent minimum molecular weight (77 000) and is indistuinguishable from liver reductase when a mixture of the reductases is electrophoresed on SDS gels.

The spectrum and the presence of both FMN and FAD determined in the sample of the partially purified preparation is in accord with the identification of the reductase as a flavoprotein. The two flavins are not present in equal amounts in the Novikoff hepatoma reductase as they are in liver reductase [44]; the apparent concentration of FMN per mg of protein is almost twice that of FAD. This appears in striking contrast to observations made in this laboratory and by Vermilion and Coon [49] that FMN is more readily removed from the liver reductase than is FAD. The relative affinities of FMN and FAD for the Novikoff hepatoma reductase must be determined before this observation can be clarified..

The total flavin content per 77 000 ng of Novikoff hepatoma reductase protein in this preparation is about 0.5 nmol whereas the total flavin content in liver reductase is 2 nmol per 80 000 ng of protein [33]. The turnover number of the Novikoff hepatoma reductase for cytochrome *c* reduction based on flavin content (1380 min^{-1}) is about 60% that for the liver reductase (2400 min^{-1}) calculated from previous data (35). Both the total flavin content and the turnover number of the Novikoff hepatoma reductase suggest that the preparations contains aporeductase. The presence aporeductase would give the appearance of high purity on gels but lower the turnover number.

Catalytically, the tumor reductase can substitute for liver reductase in in vitro benzphetamine hydroylation systems [18] reconstituted with partially purified hepatic cytochrome *P*-450. Benzphetamine hydroxylation in such a reconstituted system required the presence ofphosphatidylcholine, as has been shown for the resolved liver drug metabolism system [19,50]. The rate of hydroxylation is dependent on the amount of reductase added. Further, the tumor reductase will transfer electrons to a variety of artificial acceptors. Although in each case the V is lower than that of the liver reductase, the K_m values of the tumor reductase for electron acceptors and NADPH are similar to those [35] of normal liver reductase for the acceptors. This is, however, in contrast to the case of reductase purified from the 'minimal deviation' hepatoma 5123 t.c. (H), which exhibits a K_m for NADPH (75 μM) an order of magnitude above that of liver (5 μM) [51].

The ability of Novikoff hepatoma microsomes to catalyze the hydroxylation of a variety of substrates including N- and O-alkyl drugs and polycyclic hydrocarbons, the sensitivity of these reactions to specific inducers and inhibitors of cytochrome *P*-450-dependent reaction, and the presence of what appears to be a normal liver-like cytochrome *P*-450 reductase argue for the presence in the Novikoff hepatoma of an active mixed function oxidase system. Conclusive demonstration of the cytochrome component of the drug metabolism system would strengthen the argument; however, no such demonstration has been reported. A strong inducer such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which was used to induce the cytochrome *P*-448 in genetically non-responsive mice [52,53], may be useful in this regard.

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