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Hepatic Organelle Interaction

I. Spectral Investigation During Drug Biotransformation

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SUMMARY

A technique which permits the measurement of levels of mitochondrial and endoplasmic reticulum pigments in the hepatocyte, the examination of changes in the oxidation-reduction states of these pigments, and the study of interactions between the mitochondrial and endoplasmic reticulum electron transport chains is described. The content of cytochrome P-450 was about 30 nmoles/g of liver, wet weight. Based on the microsomal concentration of cytochrome P-450, the microsomal protein content ranged from 50 to 70 mg/g of liver. The total mitochondrial pigment concentration was 83 nmoles/g of liver, wet weight, and 1.16 nmoles/mg of mitochondrial protein. Based on these figures there were about 75 mg of mitochondrial protein per gram of liver. The rate of reduction of cytochrome P-450 in liver slices was also measured; the endogenous rate of reduction was 1.5–3.0 nmoles/g of liver per minute, and it increased to 4.5 nmoles/g/min in the presence of NADPH. The addition of 8 mM aminopyrine doubled the rate of reduction of cytochrome P-450 to 5.0–6.0 nmoles. When a Krebs cycle intermediate such as succinate was added in the presence of aminopyrine, a further doubling of the rate of reduction of cytochrome P-450 occurred (9.0–10.0 nmoles/g of liver per minute). The presence of succinate in the medium containing both aminopyrine and NADPH increased the P-450 reductase activity synergistically to 23 nmoles/g of liver per minute. These data indicate that the mitochondria may be a site of cellular control of drug biotransformation in the endoplasmic reticulum.

INTRODUCTION

The oxidative metabolic pathway of the cellular organelle, the mitochondrion, has been substantially elucidated by the efforts of a multitude of investigators during the past two or three decades. The transfer of electrons along the cytochrome chain in the mitochondrion to the final acceptor, molecular oxygen, has been well established (1). Less well understood, however, are the electron transport chains in another cellular organelle, the endoplasmic reticulum. One of these chains contains a flavoprotein reductase, NADH–cytochrome b_5 reductase, and the hemoprotein acceptor cytochrome b_5; the function of this pathway is not yet known, and the terminal electron acceptor has yet to be described. The other endoplasmic reticulum electron transport chain also consists of a flavoprotein, NADPH–cytochrome P-450 reductase, and a hemoprotein, cytochrome P-450. Like the mitochondrial cytochrome oxidase, cytochrome P-450 is a terminal oxidase, transferring electrons to

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molecular oxygen; the involvement of this terminal oxidase in drug metabolism was shown conclusively by Cooper *et al.* (2).

The purpose of this paper and the following one (3) is to focus attention on the interaction between cellular organelles, and to indicate that the mitochondria may be a site of cellular control of drug biotransformation in the endoplasmic reticulum. In this paper a technique is described whereby the levels of cellular pigments may be measured in surviving hepatocytes of liver slices. Levels of hepatocellular pigments, as well as those in the mitochondria and endoplasmic reticulum, are determined and compared. Interactions between mitochondrial and endoplasmic reticular electron transport chains are described.

MATERIALS AND METHODS

Young, untreated, male Sprague-Dawley rats (150 g) were obtained commercially and fed a standard laboratory diet. Livers were removed immediately following decapitation, chilled to about 0° with cold 0.9% NaCl, and then perfused gently with 0.9% NaCl through the hepatic veins and the portal venous system by means of a syringe, to remove blood and hemoglobin. The livers were beige or tan in color following perfusion. Care was taken not to apply too much pressure during perfusion, because the capillary bed is fragile and easily ruptures, resulting in poor liver slices.

The livers were then transferred to a cold room, and slices were prepared using a Stadie-Riggs microtome. Each slice was immediately placed in a Petri dish filled with the oxygen-saturated medium described below, cut to the appropriate length and width, and placed into the slot of a special cuvette, designed for spectral analysis of liver slices. A schematic design of the type of cuvette employed is shown in Fig. 1. The cuvettes were made from 1.25-cm² stock Rohm and Haas Plexiglas (UVT) cut into 4.4-cm-long segments. These cuvettes permit continuous circulation of fluid bathing the tissue slices during spectral measurements. The liver slice is spread evenly and carefully on one inner wall, as shown in Fig. 1, in order to avoid stretching it; black plastic tape is then used to seal the chamber tightly. A series of reservoir bottles containing bathing medium and substrates under a variety of gaseous atmospheres can be connected to the cuvettes; fluid flow occurs by gravity at room temperature (23°). The circulating fluid enters the cuvette chamber near its base, filling the chamber and exiting from the top of the cuvette. The liver slice is kept in place (a) by movement of the fluid filling the chamber from the base, (b) by capillary attraction between fluid, wall, and slice, and

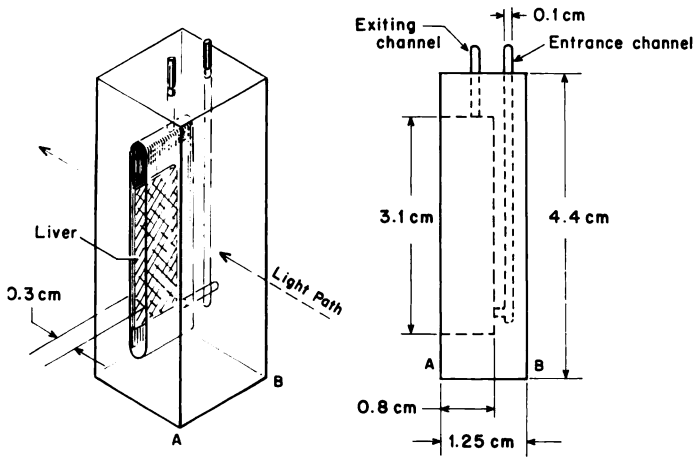

FIG. 1. *Cuvette designed for spectral analysis of liver slices*
The bathing medium flows through the 0.1-cm entrance channel, filling the chamber from the bottom, and leaves by way of the exiting channel. The width of the chamber is 0.3 cm, the depth is 0.8 cm, and the height is 3.1 cm. The cuvette is cut from Plexiglas UVT.

(c) by the narrow width of the chamber. Both sides of the liver slice are in contact with the fluid medium. In our studies the liver slices were bathed with oxygen-saturated medium for 10–15 min before beginning the experiment, and a baseline of equal light absorption, measured as a function of wavelength, was obtained. The difference spectra were recorded with an Aminco-Chance dual-wavelength split beam spectrophotometer. All studies were performed at 24° unless otherwise stated.

After spectral analysis, the slices were removed from the cuvette, blotted to remove excess fluid, and weighed. The volume of each slice was determined by dividing its weight by the density of liver (1.1 g/ml), and the thickness was determined from its area and volume. Slices used for spectral studies were 0.4–0.6 mm thick and weighed between 75 and 125 mg. Slices of approximately equal spectral density were chosen, as judged from their spectral absorbance at about 420 nm. With practice it was possible to match slices closely by eye.

The iso-osmotic medium which bathed the slices consisted of 125 mM NaCl, 6 mM KCl, 12 mM Na_2HPO_4, 3 mM NaH_2PO_4, and 1 mM $MgSO_4$; the pH was adjusted to 7.4 with 1 M HCl.

Liver mitochondria were generally prepared in 0.25 M sucrose and 1.0 mM EDTA as described by Simpson *et al.* (4). Microsomal fractions were prepared at 0° from 0.25 M sucrose homogenates of rat liver as described elsewhere (5), and were resuspended in 0.15 M KCl containing 0.05 M Tris-Cl buffer at pH 7.5 to a final protein concentration of 6 mg/ml.

Preparation of tissue for electron microscopy. At the conclusion of a typical experiment, liver slices were removed from the cuvettes, rinsed in cold buffer medium, and placed for 60 min in a solution of 3% glutaraldehyde in 0.025 M cacodylate buffer, pH 7.0, containing 4.5% dextrose. Then the slices were removed and rinsed in cacodylate buffer, followed by refixation for 60 min in 1% osmium tetroxide in 0.025 M cacodylate buffer, pH 7.0, containing 4.5% dextrose. The slices were then dehydrated through a graded series of ethyl alcohol concentrations (50–100%) and embedded in Epon. Sections of embedded material were made on an LKB Ultramicrotome, and were stained for 30 sec in lead citrate to enhance contrast. The sections were viewed in a Hitachi 11B electron microscope.

All chemicals and biochemicals were of the highest purity available commercially and, with the exception of aniline, CO, and N_2, were not purified further. Aniline was redistilled under vacuum, and the two gases were bubbled through sintered glass gassing tubes into a vanadyl chloride deoxygenating medium (6).

RESULTS

Cytochrome content of mitochondria in rat liver. The amount of the mitochondrial hemoproteins in the rat liver slices was determined by difference spectrum. Two slices of about equal thickness were bathed for 15 min in oxygen-saturated medium, and a baseline of equal light absorbance was recorded. The fluid in the sample cuvette was then switched to an anaerobic, N_2-saturated medium; the reference slice remained bathed by the oxygenated medium. A spectrum of the respiratory pigments similar to that seen with fresh isolated mitochondria appeared within 2 min (Fig. 2, spectrum *A*). In the 420–430 nm region, mitochondrial spectra exhibit a shoulder which is generally attributed to cytochromes *b*, *c*, and c_1. However, in liver slices a peak often develops here and increases slightly with time, indicating the reduction of an extra-respiratory chain pigment (Fig. 2). When the 420 nm shoulder increases, subtraction of the fully reduced mitochondrial spectrum (see Fig. 2, inset, spectrum *D* minus spectrum *A*) yields a typical difference spectrum of cytochrome b_5. The magnitude of this hemoprotein spectrum never reaches more than 15–20% of the total liver slice cytochrome b_5, calculated from the content of endoplasmic reticulum.

Although reduction of the respiratory pigments occurred in the anaerobic medium using endogenous reducing equivalents, maximal reduction was observed only after the addition of a Krebs cycle intermediate, such as succinate; the shoulder in the 420–430 nm region increased more rapidly in the presence of succinate than in its absence

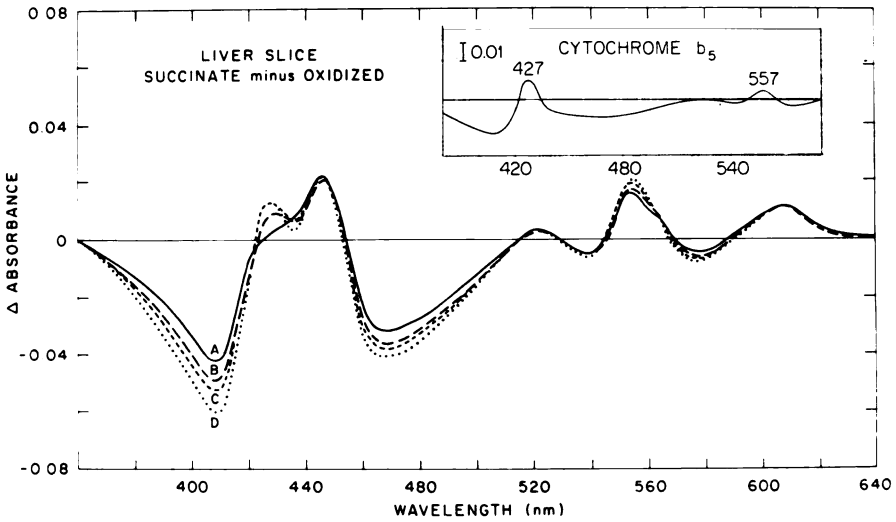

FIG. 2. *Difference spectra of intracellular hemoproteins in rat liver slices, using sodium succinate*
The liver slices were bathed in O_2-saturated medium for 15 min, after which a baseline of equal light absorption was recorded. The fluid bathing the liver slice in the experimental cuvette was changed to an N_2-gassed, deoxygenated medium containing 5 mM succinate. Difference spectra were recorded after 2, 4, 6, and 10 min, represented by spectra *A*, *B*, *C*, and *D*, respectively. Spectrum *A* is qualitatively similar to the spectrum obtained under N_2 alone, but in the latter case reduction of the mitochondrial pigments was not maximal (75–80%, even after 15 min). The inset represents the spectrum obtained by subtracting spectrum *A* from spectrum *D*. Liver slices were 0.47 mm thick.

(Fig. 2, spectra *B–D*). It was also observed that the supply of available endogenous substrate(s) capable of providing reducing equivalents could not be depleted by bathing the liver slices with the oxygen-saturated medium, even for 1 hr.

Dithionite reduction (Fig. 3) of the experimental liver slice gave a spectrum similar to *D* in Fig. 2, with the exception of a slightly higher absorption peak in the 420–430 nm region; the trough in the 410 nm region, observed with dithionite, was also similar to that of spectrum *D*, Fig. 2. Since only a slight increase in the cytochrome b_5 absorption band occurred on dithionite addition, cytochrome b_5 might already have been present in a reduced state. In order to test this possibility, both liver slices were bathed for 10 min in oxygenated medium containing 1 mM potassium ferricyanide. The medium was changed to an oxygenated medium without ferricyanide for 5 min, followed by substitution of a dithionite-containing, N_2-saturated medium. This treatment did not cause further appearance of the cytochrome b_5 spectrum.

Table 1 shows the concentration of the mitochondrial respiratory pigments in liver slices. The pigment content of rat liver mitochondrial preparations were also determined (Table 1) and compared with the values reported by Chance and Hess (7) and Estabrook and Holowinsky (8). The total mitochondrial pigment content of the liver slice was 83.3 nmoles/g. The data obtained with the isolated mitochondria compare favorably with the values reported in the literature (7, 8). The total mitochondrial pigment content on the basis of mitochondrial protein is 1.16 nmoles/mg; hence the amount of mitochondrial protein per gram of liver, wet weight, is approximately 72 mg, agreeing with the data of Schollmeyer and Klingenberg (9).

Cytochrome P-450 content of liver slices. The content of cytochrome P-450 in liver slices was determined by bathing an experimental slice with CO-saturated medium containing dithionite, while dithionite plus N_2-saturated medium bathed the reference slice. The resultant P-450 absorption spectrum was very similar to that obtained with microsomal

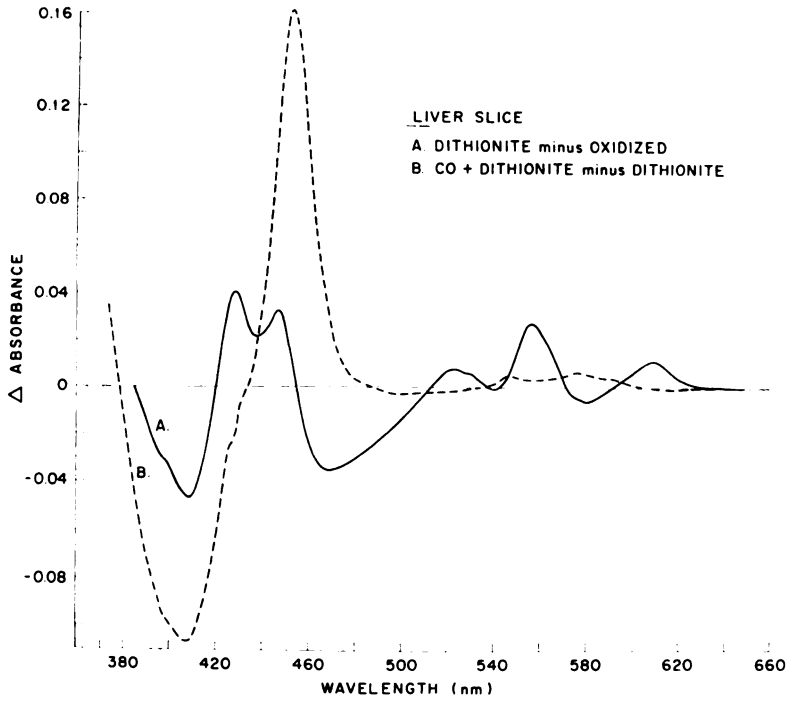

FIG. 3. *Mitochondrial and microsomal hemoprotein spectra from rat liver slices*

A baseline was recorded as described in Fig. 2. N_2-saturated medium containing sodium dithionite was used to bathe the liver slice in the experimental cuvette, and after 3 min the difference spectrum was recorded (——). The medium bathing the liver slice in the reference cuvette was then changed to the same medium as in the experimental cuvette, and a baseline was recorded. The fluid bathing the experimental slice was then changed to one saturated with CO and containing sodium dithionite, and a difference spectrum was recorded (- - -). Liver slices were 0.52 mm thick.

preparations: the absorption peak was maximal at 450 nm, with a trough at about 410 nm.

Table 2 shows the concentration of cytochrome P-450 in normal male rat livers. There was considerable variation in the P-450 content of different rat livers (17–41 nmoles/g). Different sets of slices from the same liver, however, did not vary appreciably. The content of cytochrome P-450 per milligram of microsomal protein ranged from 0.44 to 0.81 nmole. The amount of microsomal protein per gram of liver, wet weight, was determined by dividing the amount of cytochrome P-450 per gram of liver by the concentration of P-450 in the microsomes; the results indicated 52 ± 6 mg of endoplasmic reticulum protein per gram of liver. This value is slightly higher than that previously reported (10). The range of microsomal protein calculated for different livers was 50–70 mg, although the yield of microsomes obtained by standard fractionation procedures in this laboratory (11) is only 15–20 mg/g of liver. This indicates that approximately 50–70 % of the microsomal protein is lost during the preparative procedure (12).

Figure 3 shows a comparison of the liver slice mitochondrial pigment absorption spectrum with that of cytochrome P-450. It is immediately obvious that P-450 represents the major cytochrome in the hepatocyte, being 2–3 times greater than any individual mitochondrial cytochrome, i.e., approximately 25 nmoles/g vs. approximately 11 nmoles/g. As indicated above, the absorption peak at 420–430 nm is partly due to cytochrome b_5. Even the chemical reducing agent $Na_2S_2O_4$ does not reveal the complete absorption spectrum of b_5 in the liver slice.

Type I and II spectral changes in liver slices. Liver slices were bathed in medium

TABLE 1

Concentration of mitochondrial respiratory pigments in rat liver

The reduced minus oxidized difference spectra of respiratory pigments were obtained as follows. For mitochondria, ADP was added, at a final concentration of 2.0 mM, to 6 ml of mitochondrial suspension (2.0 mg of protein per milliliter) diluted in 50 mM Tris buffer, pH 7.4. The solution was divided between two cuvettes, and a baseline of equal absorbance was recorded. To the sample cuvette was added 5 mM sodium succinate, and the reduced minus oxidized difference spectrum was recorded after utilization of oxygen in the cuvette was complete. For liver slices, after the oxygenated medium had bathed the slices for 15 min and a baseline recorded, the sample cuvette was switched to an anaerobic medium containing succinate and the difference spectrum was recorded. Values obtained in this study are the means and standard errors of 10 livers. The numbers in parentheses represent ratios of the pigment concentrations relative to cytochrome *a* (taken as 1.0).

Pigment	Wavelength[a]	ϵ	This study		Mitochondria	
			Mitochondria	Liver slices	Chance and Hess (7)	Estabrook and Holowinsky (8)
	nm	$mM^{-1}\ cm^{-1}$	*nmoles/mg protein*	*nmoles/g*	*nmoles/mg protein*	
Cytochrome *a*	605–625	16	0.175 ± 0.006 (1.0)	11.16 ± 0.87 (1.0)	0.20 (1.0)	0.21 (1.0)
Cytochrome *b*	562–575	20	0.096 ± 0.005 (0.5)	12.52 ± 1.22 (1.1)	0.18 (0.9)	0.10 (0.5)
Cytochrome *c*	551–540	19.1	0.158 ± 0.009 (0.9)	12.62 ± 1.40 (1.1)	0.23 (1.2)	0.20 (1.0)
Cytochrome c_1	551–540	19.1	0.079 ± 0.006 (0.5)	6.35 ± 0.71 (0.6)	0.11 (0.6)	0.11 (0.5)
Cytochrome a_3	444–455	60	0.183 ± 0.006 (1.0)	11.36 ± 1.06 (1.0)	0.22 (1.1)	0.20 (1.0)
Flavoprotein	460–500	11.5	0.473 ± 0.041 (2.7)	29.24 ± 4.99 (2.6)	0.72 (3.6)	0.69 (3.3)

[a] The first wavelength represents the absorption maximum, while the second wavelength indicates an absorption minimum or an isosbestic point.

TABLE 2

Concentration of cytochrome P-450 in rat liver

Cytochrome P-450 was determined by the CO plus dithionite minus dithionite difference spectrum in both liver slices and microsomes. The P-450 values and the amount of microsomal protein per gram of liver, wet weight, are means ± standard errors; the extinction coefficient for 450–490 nm is 91 $mM^{-1}\ cm^{-1}$.

No. of rats	Sets of slices per liver	Cytochrome P-450		Microsomal protein *mg/g liver*
		nmoles/g tissue	*nmoles/mg microsomal protein*	
18	2	29.1 ± 2.8	0.56 ± 0.02	52 ± 6

containing different substrates of the mixed-function oxidase system. In confirmation of previous studies of substrate interaction with cytochrome P-450 in liver microsomes (11), 5 mM aminopyrine, ethylmorphine, Amytal, or hexobarbital in the medium bathing the sample slice caused the appearance of a type I spectral change. The difference spectrum obtained had the same 420–422 nm trough and 390 nm absorption peak as seen with liver microsomes. Aniline caused the typical amine type II spectral change. The magnitude of the spectral changes was dependent upon both the concentration of the substrates added and the thickness of the slices.

Rate of reduction of cytochrome P-450 in liver slices. As noted above, there are sufficient quantities of endogenous reducing equivalents present in hepatocytes to reduce not only the mitochondrial pigments, but also cytochrome P-450 in the liver slice. Attempts were made to deplete these reducing equivalents, but even after 24, 48, 72, and 96 hr of fasting the rats the liver slices were still capable of reducing these pigments without addition of exogenous compounds. Therefore the endogenous rate of reduction

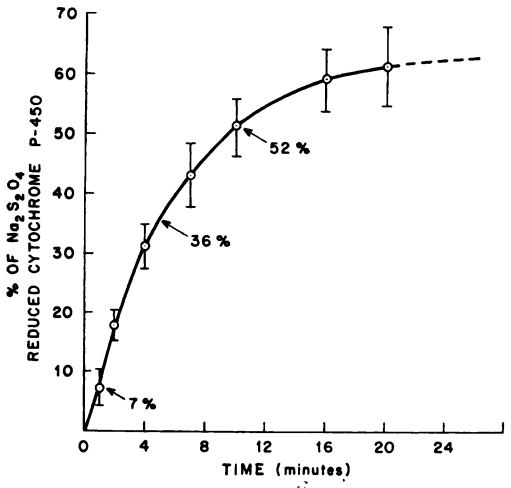

FIG. 4. *Endogenous reduction rate of cytochrome P-450*

The baseline was obtained as described in Fig. 2. The experimental cuvette was then switched to a CO-saturated medium, and the reference cuvette, to an N_2-saturated medium (CO minus N_2), and the enzymatic reduction of cytochrome P450 was determined at different time intervals. At the end of the experiment, dithionite was added to both cuvettes to attain maximal reduction of cytochrome P-450. The percentage reduction after 1, 5, and 10 min is indicated in the figure. Slices ranged from 0.4 to 0.58 mm thick. Values are expressed as a percentage of dithionite-reduced cytochrome P-450.

of cytochrome P-450 had to be determined for each liver (Fig. 4). Continuous spectra were recorded for a period of up to 20 min. The rate of reduction was determined from the change in absorbance at 450 nm relative to that at 490 nm with time. The initial endogenous rate of reduction of cytochrome P-450 was found to be quite slow; only 1.5–3.0 nmoles were reduced per minute per gram of liver. This value is approximately equal to the NADPH-supported rate in 1 mg of microsomal protein at the same temperature (13); assuming a microsomal content of about 50 mg/g of liver, the hepatocytes could attain a maximal rate of reduction of P-450 of 75 nmoles/min/g. In the liver slices, after 1 min the endogenous reduction of cytochrome P-450 had reached 7%, while 36% reduction occurred by 5 min; 52% reduction was reached by 10 min. Only about 60–70% of cytochrome P-450 was reduced by endogenous reducing equivalents at 24°. Raising the temperature to 37° increased the extent of reduction of cytochrome P-450 to over 80% by 10 min, and to over 92% by 20 min. In agreement with our above observations, deprivation of food for up to 4 days did not change the endogenous rate or extent of reduction of P-450.

In contrast to a previous report (12), the addition of mitochondrial tricarboxylic acid cycle intermediates, such as succinate and isocitrate, to the medium did not cause an increase in the rate of reduction of cytochrome P-450. Furthermore, after reduction of the hemoprotein by endogenous reducing equivalents had reached 50%, the addition of Krebs cycle intermediates caused no further reduction. The reason for this discrepancy is unclear, but it may be accounted for by endogenous reduction.

The effect of pyridine nucleotides on the reduction of cytochrome P-450 was also studied, since it has been shown in microsomes that these substances transfer reducing equivalents to P-450 (14). Slices were oxygenated for 15 min as described above and then switched to a system containing

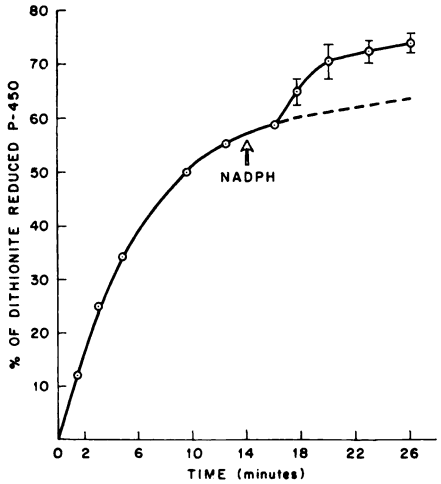

FIG. 5. *Effect of NADPH on Reduction of cytochrome P-450*

The procedure was the same as described in MATERIALS AND METHODS and Fig. 4, except that 1 mM NADPH was added to the experimental cuvette medium after 10 min of recording (CO minus N_2). Liver slices were 0.45–0.55 mm thick. Values are expressed as a percentage of dithionite-reduced cytochrome P-450.

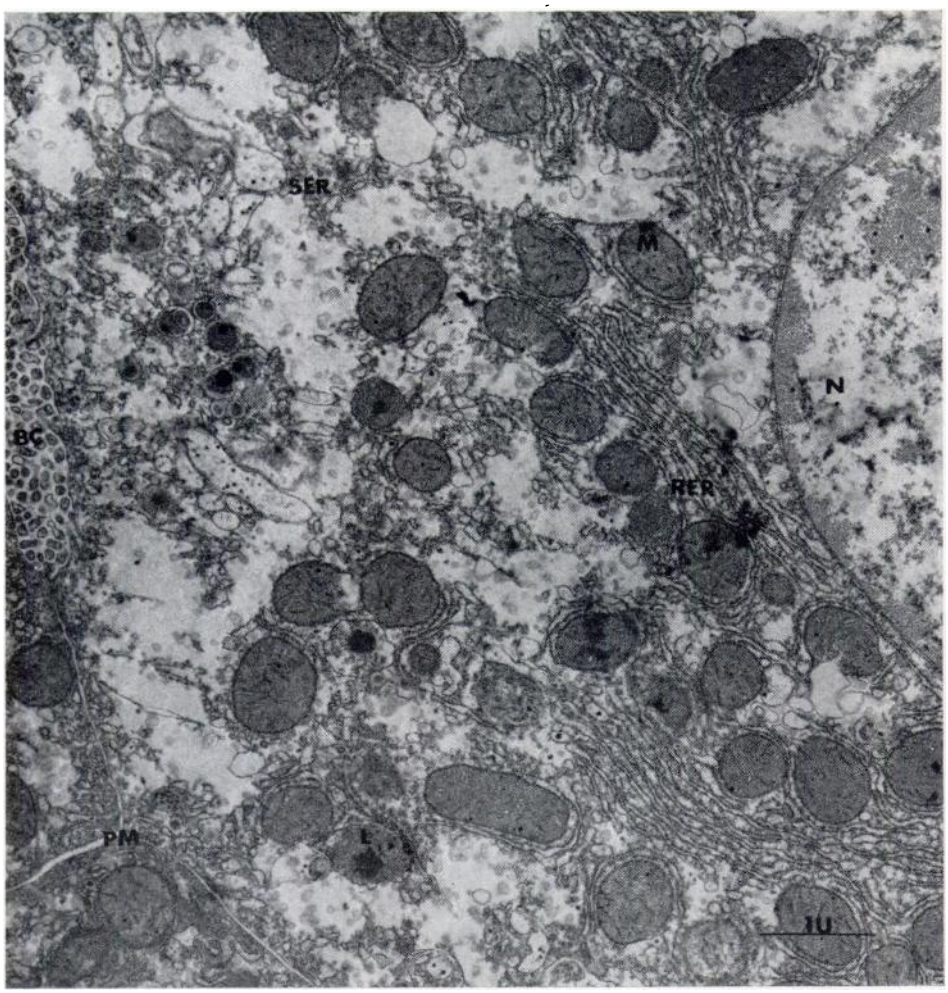

FIG. 6. *Electron micrograph of experimental liver slice*

N, nucleus; M, mitochondria; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; BC, bile canaliculus; PM, plasma membrane; L, lysosomes (Electron microscopy courtesy of Dr. Joan Higgins.)

CO but not N_2. The rate of endogenous reduction was followed for 10–13 min, after which time NADH or NADPH was added. As shown in Fig. 5, NADPH caused an increase in the rate as well as the magnitude of reduction of cytochrome P-450. The same effect was obtained with NADH, NAD, and NADP. Thus, under these conditions, the pyridine nucleotides penetrate into the liver cells. However, unlike succinate (see above), the addition of NADPH to the CO-containing medium at zero time increased the rate of reduction of cytochrome P-450 relative to the endogenous rate; about 4.5 nmoles/g of liver per minute were reduced compared with about 2.5–3.0 nmoles/g/min in the absence of NADPH.

In order to determine whether pyridine nucleotide penetration was due to a gross loss of the cell membrane integrity, we examined the membrane morphology by electron microscopy. At the end of a typical experiment liver slices were removed from the cuvette, rinsed, and fixed as described under MATERIALS AND METHODS. The experimental tissues were compared with fresh, unincubated liver slices fixed in the same way. Figure 6 is an electron micrograph of a

section of an experimental liver slice. Morphologically the hepatocytes did not differ from those of the control slice. All borders of the plasma membranes appeared normal, as did the bile canaliculi. The mitochondria and smooth and rough endoplasmic reticulum also appeared structurally well preserved, with slight dilation of the endoplasmic reticular cisternae; the latter did not occur in all sections. Although morphologically the hepatocytes appeared well preserved, this does not preclude alterations in the biochemical integrity[2] of the plasma membrane.

Rate of reduction of cytochrome P-450 in liver slices in the presence of the mixed-function oxidase substrate, aminopyrine. When drugs were added to the medium bathing the liver slice, the reduction of cytochrome P-450 was greatly increased (Fig. 7). In the presence of aminopyrine the initial rate of P-450 reduction was 5–6 nmoles/g of liver per minute, as compared with the initial rate of 1.5–3.0 nmoles/g/min in the absence of the drug. When the slices were bathed in medium containing aminopyrine and a Krebs cycle intermediate such as succinate, an even greater rate of P-450 reduction was observed (Fig. 7), i.e., 8–10 nmoles/g/min, but there was no difference in the extent of reduction of the hemoprotein. Malate, another Krebs cycle intermediate, did not affect the initial rate of aminopyrine-stimulated cytochrome P-450 reduction, but diminished the extent of reduction of the hemoprotein by about 15–20%.

In order to ascertain whether the stimulation provided by succinate was due to a limitation of reducing equivalents, the experiment was repeated in the presence of NADPH (Fig. 8). The initial endogenous rate of reductase activity was 3 nmoles/min/g of liver. NADPH stimulated the initial rate 1.6-fold, and aminopyrine plus NADPH provided a 4-fold increase in the initial rate. When succinate plus NADPH and aminopyrine were present, the initial rate was stimulated 8-fold; however, the extent of reduction was unchanged from that obtained with NADPH plus aminopyrine. Similar effects are described in the following paper (3), in which drug biotransformation was measured.

[2] Tests performed to determine biochemical integrity of liver slices include inulin "space," isocitrate dehydrogenase output, and vital dye uptake. The vital dye data were ambiguous because of technical difficulties, but inulin space was 18.4% ± 2.7 (SE; $n = 5$), suggesting no loss of cell membrane control over inulin exclusion. Isocitrate dehydrogenase (NADP enzyme) levels increased in the medium bathing the liver slices for at least 2 hr. Livers used in our experiments were generally discarded by 30 min.

DISCUSSION

In the past, most biochemical studies concerned with cellular metabolism have been

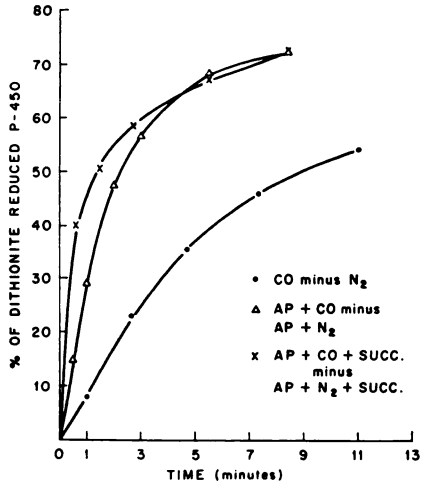

FIG. 7. *Effect of aminopyrine (AP) on rate of reduction of cytochrome P-450*

A baseline was obtained when the oxygenated medium containing aminopyrine bathed both slices for 10 min. The experimental slice was switched to a medium containing aminopyrine and CO, and the reference slice to a medium containing aminopyrine and N_2, and spectra were recorded. The aminopyrine-containing medium bathed a new set of slices from the same liver, and a similar baseline was recorded. The experimental slice was then switched to a medium saturated with CO and containing aminopyrine and succinate, while the reference cuvette contained the same medium, except that N_2 replaced CO; continuous spectra were recorded. The endogenous rate of reduction was determined from a third set of slices from the same liver. Slices were 0.44–0.55 mm thick. Values are expressed as a percentage of dithionite-reduced cytochrome P-450. The aminopyrine concentration was 8 mM, and the succinate concentration was 10 mM.

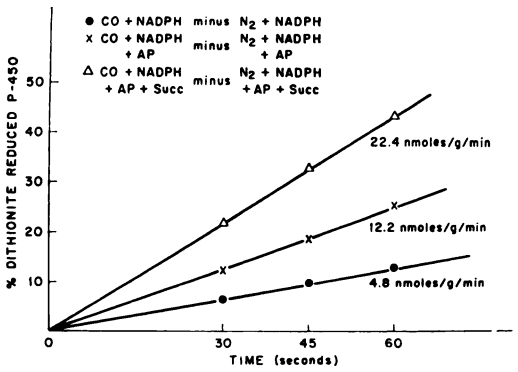

FIG. 8. *Effect of aminopyrine (AP) on rate of reduction of cytochrome P-450 in the presence of 1 mM NADPH*

The procedure was the same as described in Fig. 7, except that 1 mM NADPH was added to the medium bathing the three sets of slices. Slices were 0.4–0.6 mm thick. Values are expressed as a percentage of dithionite-reduced cytochrome P-450. The aminopyrine and succinate concentrations were 8 mM and 10 mM, respectively.

performed on isolated and/or purified enzymes, or on cell organelles like nuclei, microsomes, lysosomes, and mitochondria. The technique described in this paper permits the spectrophotometric measurement of oxidation-reduction states of different hemoproteins in the surviving hepatocyte during changes in the metabolism of the cell. By employing an iso-osmotic buffer system which has been equilibrated with various gasses, cellular substrates, and drugs, it is possible to distinguish spectrally between the pigments of the mitochondria and endoplasmic reticulum, and their effects during drug biotransformation.

The feasibility of this technique was shown in Table 1, where the mitochondrial pigment ratios, relative to cytochrome *a*, in liver slices were compared with the ratios obtained in isolated mitochondria; the data are in excellent agreement. The technique permits the first direct determination of the amount of mitochondrial and endoplasmic reticulum protein in the liver. The amount of mitochondrial protein per gram of liver obtained with our technique is 72 mg, which agrees well with the estimated value reported by Schollmeyer and Klingenberg (9), based on cytochrome *c* determination. The amount of cytochrome P-450 present in normal rat liver is about 29 nmoles/g. Comparing the content of cellular organelle cytochromes indicates that cytochrome P-450 is the major hemoprotein in the rat hepatocyte; it is normally present at 2–3 times greater concentrations than any of the other cytochromes. Since this is an inducible hemoprotein, with a capacity to increase 5-fold (15) in the endoplasmic reticular membrane, it is of interest to speculate on the extent to which this extramitochondrial respiratory chain contributes to total hepatocellular respiration. Estabrook *et al.* (12) suggested that this contribution may be 10–25%, but the relatively slow rates of drug oxidation (3) indicate that this range is probably a bit high in the noninduced hepatocyte.

Spectral investigation of cellular pigments revealed an anomaly; the ratio of cytochrome b_5 to cytochrome P-450 in the isolated microsomes was about 0.6–0.7, while a relatively minor Soret band for cytochrome b_5 was observed in the liver slices. Based upon the level of cytochrome P-450, one should be able to detect about 15–20 nmoles of cytochrome b_5 per gram of liver. This should be manifested by a Soret peak of about 0.1 absorbance unit in Fig. 3, at about 425 nm. The lack of appearance of this Soret band was considered to be due to one of the following three possibilities. First, it is possible that cytochrome b_5 exists in the cell mainly in its reduced form; since this cytochrome is not a terminal oxidase, it seemed the best possibility, especially since Strittmatter (16) reported cytochrome b_5 to be only slowly autoxidizable. However, bathing the liver slices in potassium ferricyanide-containing, oxygenated medium did not cause the appearance of the Soret absorption band of this hemoprotein. Also, when liver slices were homogenized and difference spectra recorded, cytochrome b_5 was present in the oxidized form reducible by NADH and dithionite. A second possibility is that cytochrome b_5 is in some way buried in a pocket of the endoplasmic reticulum and thus is inaccessible to agents like ferricyanide and dithionite. From the ease with which cytochrome b_5 can be removed from the microsomes, and from the fact that this hemoprotein can be readily reduced in isolated

microsomes, this possibility is unattractive. The third possibility is that the cytochrome b_5 spectrum, as seen in isolated microsomes, is an artifact of cellular disruption. The possibility was considered that in the hepatocyte this hemoprotein may be linked to some non-membrane-bound acceptor for its reducing equivalents. Interaction with this hypothetical acceptor moiety may be the reason why the hemoprotein could not be detected in the intact hepatocyte. However, addition of a concentrated cell sap fraction to a microsomal suspension, or resuspending a microsomal pellet in undiluted, organelle-free cell sap, did not prevent the $Na_2S_2O_4$-induced appearance of the usual cytochrome b_5 reduced minus oxidized spectrum.

The interaction between cytochrome P-450 and substrates of the microsomal mixed-function oxidase system, which has been demonstrated in liver microsomes (11), was also observed in the liver slices. Aminopyrine, ethylmorphine, Amytal, and hexobarbital all caused the typical type I spectral change, and aniline caused the typical type II spectral change.

The results indicate that the hepatic endoplasmic reticulum mixed-function oxidase, in the absence of oxidizable substrates, is reducible by an as yet unknown endogenous supply of reducing equivalents. Under these conditions, intermediates of the mitochondrial Krebs cycle, although reducing mitochondrial pigments, were without effect on the flow of electrons to cytochrome P-450. In agreement with the report by Chance (17), we were also unable to see a synchronous oxidation-reduction change in cytochrome b_5 and the mitochondrial pigments; however, this may be because, as Chance also observed, we did not detect any appreciable change in the cytochrome b_5 oxidation-reduction state. The responses of the mitochondrial electron transport chain are more rapid than those of the endoplasmic reticulum transport chain; hepatocyte mitochondrial cytochromes are completely reduced by 5 mM succinate within 2 min in an anaerobic medium, while cytochrome P-450 is reduced only about 50% by 10 min.

The question of a cellular permeability barrier to pyridine nucleotides was considered in light of the increase in extent of cytochrome P-450 reduction when the liver slices were bathed in medium containing pyridine nucleotides. In order to exclude the possibility that the slices incurred damage during the experimental period, either by cell autolysis or by membrane distortion and disruption, cell morphology was examined. The electron micrographs of various liver slice sections under different experimental conditions showed structurally well preserved plasma membranes; the morphology of the hepatocyte in the experimental slice appeared the same as that seen in the control. This evidence and the biochemical evidence described in the following paper (3) and by Everse (18) indicate that the cell membranes are permeable to pyridine nucleotides.

Finally, in agreement with the data reported by Gigon *et al.* (19) and Schenkman (20), the initial rate of reduction of cytochrome P-450 was increased by the presence of drugs like aminopyrine. Furthermore, under the stress of an increased demand for reducing equivalents, or by virtue of some allosteric mechanism triggered by substrate interaction, certain mitochondrial Krebs cycle intermediates caused a marked potentiation of NADPH–cytochrome P-450 reductase activity. This synergistic effect could also be demonstrated in liver homogenates,[3] but was not obtained in microsomal suspensions or in the absence of drug substrates of the microsomal mixed-function oxidase.

In the absence of drug substrates, the cytochrome P-450 reductase activity of liver slices was about 2.5 nmoles of P-450 reduced per gram of liver per minute. In the presence of aminopyrine this rate was about doubled (5 nmoles/min/g), and when succinate and aminopyrine were both present the rate doubled again (10 nmoles/min/g). These effects were magnified in the presence of exogenous NADPH, when the synergistic action of succinate was even more pronounced (the activity was increased 8-fold to about 23 nmoles of cytochrome P-450 reduced per minute per gram of liver).

This indicates that a direct interaction exists between the two cellular organelles,

[3] D. L. Cinti and J. B. Schenkman, unpublished observations.

the mitochondrion and the endoplasmic reticulum, and suggests that the former exerts a control over the function of the latter in drug biotransformation. The exact nature of this interaction is at present unknown, but it may occur via a carrier system or shuttle. The results are in agreement with the following paper (3), which describes mitochondrial control of drug oxidation in the hepatocyte endoplasmic reticulum.

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