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The Incomplete Conversion of Hepatic Cytochrome P-450 to P-420 by Mercurials

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SUMMARY

The conversion of hepatic microsomal cytochrome P-450 to P-420 by the mercurial compound mersalyl (sodium *o*[(3-hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetate) was incomplete. In contrast, high concentrations of urea caused complete conversion of cytochrome P-450 to P-420. During the conversion the total heme concentration of microsomes did not change, but the sum of the cytochrome concentration measurable as P-450 and P-420 showed a net decrease. The lack of complete conversion of cytochrome P-450 to P-420 was independent of the nature of the mercurial sulfhydryl reagent employed and was largely unaffected by changes in the type of buffer, ionic strength, and the presence of type I or type II substrates. The loss of the ability to demonstrate a type I but not a type II binding spectrum paralleled the loss of cytochrome P-450. The conversion of cytochrome P-450 to P-420 could not be completely reversed by the addition of reduced glutathione. The transition of the iron of the cytochrome P-450 from a low-spin to a high-spin state, observed in the electron paramagnetic resonance spectrum, which occurs upon the mercurial conversion of cytochrome P-450 to P-420, was investigated and compared with both the spin state changes obtained by lowering the pH and the optically determined conversion of cytochrome P-450 to P-420 by mercurials.

INTRODUCTION

From the original investigations on the characterization of cytochrome P-450 by Omura and Sato (1), it became evident that attempts to isolate the purified cytochrome were hampered by its ability to convert readily to a pigment, termed P-420. Much of the study on the conversion of cytochrome P-450 to P-420 has thus centered around procedures such as detergent treatment, organic solvent treatment, urea dissociation, and enzymatic digestion (1-3), which are commonly employed for purification purposes. Other reported treatments which result in the conversion of cytochrome P-450 to P-420 are alteration of the pH far from neutrality and treatment with sulfhydryl reagents, such as mercurials (4).

Preliminary observations (5), demonstrating an inability to achieve complete conversion of rat liver microsomal cytochrome P-450 to P-420 by mercurials, prompted a more intensive examination of the conditions influencing the action of mercurials on the cytochrome. The majority of experiments were performed using the

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organic mercurial mersalyl (sodium *o*-[(3-hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetate), but cross-correlation with other mercurial compounds suggested that the conversion of cytochrome P-450 to P-420 was independent of the mercurial sulfhydryl reagent employed.

Throughout this paper, what has hitherto been termed cytochrome P-420 will be referred to as P-420, since the term cytochrome should be applied to hemoproteins functional in oxidation-reduction reactions (6).

MATERIALS AND METHODS

Animals were treated with phenobarbital, and hepatic microsomes were prepared as described previously (5). All difference spectra were determined with an Aminco-Chance dual-wavelength split-beam recording spectrophotometer. For the cytochrome determinations, microsomes (2 mg of protein per milliliter) were suspended at room temperature (22°) in a buffer generally containing 50 mM Tris-chloride (pH 7.4)–150 mM KCl–10 mM $MgCl_2$, with the appropriate mercurial. After 1 min a few crystals of sodium dithionite were added, and the mixture was divided between two cuvettes. After establishment of a baseline of equal light absorbance, the contents of the sample cuvette were gassed for 3 min with CO. The difference spectrum was then recorded. Cytochrome P-450 and P-420 concentrations were determined using extinction coefficients of 91 and 110 mM^{-1} cm^{-1}, respectively, with a negative extinction coefficient of −41 mM^{-1} cm^{-1} for the contribution of cytochrome P-450 to the P-420 absorption maximum (7). Concentrations of total microsomal heme were determined by the pyridine-hemochromogen method, in which microsomes were suspended at a protein concentration of 2 mg/ml in 20% pyridine in 0.1 N NaOH (1). The absorbance change at 557 nm relative to 575 nm upon the addition of a few grains of sodium dithionite to the contents of the sample cuvette was determined using a buffer solution in the reference cuvette. An extinction coefficient of 32.4 mM^{-1} cm^{-1} for these wavelengths was employed.

Electron paramagnetic resonance spectral determinations were performed with a Varian E4 EPR spectrometer with samples cooled to a temperature of −172°. The microsomal samples (12–20 mg of protein per milliliter) were incubated in appropriate buffers, with or without mersalyl, for 6 min and then frozen in liquid nitrogen. Instrument settings were 12.5-gauss modulation amplitude and a microwave power setting of 50 mW. Signal heights are quoted as the peak to trough distance of the first derivative spectrum in each case. Although this method of expressing electron paramagnetic signals has its limitations, it was used as the most practical method of expressing our determinations, since no broadening of the signals was detectable. The low-spin g value quoted (2.25) is the position of maximal absorption (i.e., the midpoint of the peak to trough distance in the derivative spectrum), and the high-spin g value (6.1) is the position of the maximum signal in the derivative spectrum.

Nicotinamide, $NADP^+$, *N*-ethylmaleimide, mersalyl acid, and *p*-chloromercuriphenylsulfonic acid were purchased from Sigma Chemical Company. *p*-Chloromercuribenzoate sodium was obtained from Calbiochem; ethylmorphine hydrochloride, from Merck & Company; and hexobarbital, from Winthrop Laboratories. Trypsin was purchased from Worthington Biochemicals Corporation, and ovomucoid trypsin inhibitor, from Sigma.

RESULTS

Derivation of standard method. The addition of mercuric chloride or mersalyl to rat liver microsomes produced a spectral change in the difference spectrum of the oxidized pigments with an absorption maximum at 402 nm and an absorption minimum at 423 nm. The magnitude of this spectral difference was largest approximately 2 min after the mercurial addition and, at the lower mercurial concentrations investigated, remained nearly maximal for a further 8 min (Fig. 1A). At higher mercurial concentrations the magnitude of the absorbance change decreased soon after reaching a maximum, possibly because of the lability of P-420 under aerobic conditions (8). The maximal spectral change obtained depended upon the mersalyl concentration added (Fig. 1B), up to a concentration of about 80 nmoles/mg of microsomal

protein. Adding more mercurial to a concentration of 200 nmoles/mg produced very little additional spectral change.

It was necessary to determine whether the spectral change observed upon the addition of a mercurial to microsomes was due to light scattering changes or to a conversion of cytochrome P-450 to P-420. The hemoprotein concentrations were determined using CO as a ligand for the reduced pigments, since the

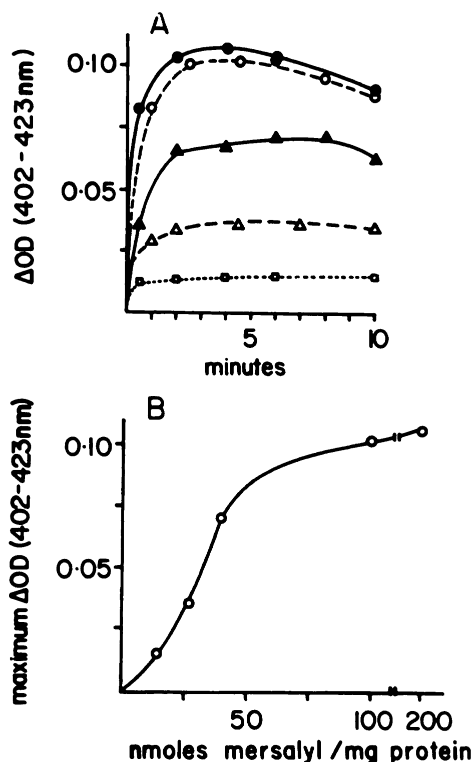

FIG. 1. *Mercurial-induced difference spectrum in aerobic rat liver microsomes*

Hepatic microsomes (2 mg of protein per milliliter) from phenobarbital-treated rats were suspended in the standard buffer (50 mM Tris-chloride, pH 7.4, containing 150 mM KCl and 10 mM $MgCl_2$), and the sample was divided between two cuvettes. The difference spectra were recorded at various intervals for up to 10 min after the addition of various concentrations of mersalyl to the contents of the sample cuvette (A): □, 14; △, 27; ▲, 40; ○, 100; ●, 200 nmoles of mersalyl per milligram of protein. The absorbance changes between the absorption maximum (402 nm) and minimum (423 nm) were then plotted against the mersalyl concentration (B).

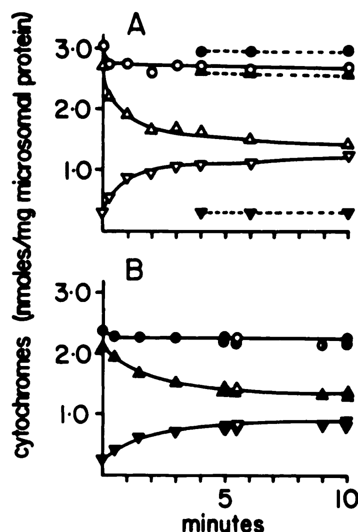

FIG. 2. *Conversion of cytochrome P-450 to P-420 after addition of mercurial*

Liver microsomes (2 mg of protein per milliliter) from phenobarbital-induced rats were suspended in the standard buffer; a few crystals of sodium dithionite were added, the mixture was divided between two cuvettes, and a baseline of equal light absorbance was determined. After the contents of the sample cuvette had been gassed with CO for 3 min, the mercurial (mersalyl at a concentration of 80 nmoles/mg of protein in A; CMPS at a concentration of 48 nmoles/mg of protein in B) was added and the difference spectrum was recorded at the times indicated. B. In addition to the above, experiments were performed in which sodium dithionite was added 10 sec (symbols, half-filled horizontally), or 1 min (open symbols) after the CMPS, and also an experiment in which CMPS was added 1 min after the dithionite (symbols, half-filled vertically). A. controls are shown (filled symbols) in which no mercurial was added to the sample cuvette (○, total of P-450 and P-420; △, cytochrome P-450; ▽, P-420).

extinction coefficients for these complexes had previously been reported. For these experiments liver microsomal pigments were reduced by adding dithionite, the sample was gassed with CO, and the spectral changes were determined after the addition of mersalyl (Fig. 2A) or CMPS[2] (Fig. 2B) to the sample. This showed that the conversion of

[2] The abbreviations used are: CMPS, *p*-chloromercuriphenylsulfonic acid; CMB, *p*-chloromercuribenzoate.

cytochrome P-450 to P-420 which would be obtained at the mercurial concentration employed was essentially complete within 5 min, or slightly longer than the spectral changes observed for the pigments in the oxidized state (see Fig. 1A). This is in contrast to the results reported for the conversion of cytochrome P-450 to P-420 by neutral salts (7), which was accelerated by dithionite. After the period of extensive conversion there was a further slow conversion of cytochrome P-450 to P-420, amounting to about 1–2% of the initial cytochrome concentration per minute. Using the standard assay procedure described under MATERIALS AND METHODS, in which the mercurial was added to the microsomes in the oxidized state, followed 1 min later by dithionite reduction, this slow conversion rate was also approximately 1–2% irrespective of the type or concentration of mercurial used (CMB, CMPS, or mersalyl). In the absence of mercurial no conversion of cytochrome P-450 to P-420 was detectable during the time periods employed. Whether dithionite was added to the microsomal sample before or after the mercurial made little difference in the extent of conversion of cytochrome P-450 to P-420 observed after 5 min or more (Fig. 2B). The duration of CO gassing and the timing of the initiation of gassing after dithionite addition also had no effect on the conversion of cytochrome P-450 to P-420.

Conversion of cytochrome P-450 to P-420. When hepatic microsomes were treated with mersalyl as described under MATERIALS AND METHODS the conversion of cytochrome P-450 to P-420 did not proceed to completion: about 50% of the cytochrome P-450 appeared to be refractory. Concentrations of mersalyl from 0 to 75 nmoles/mg of protein caused conversion of cytochrome P-450 to P-420, but increasing the mersalyl concentration to more than 150 nmoles/mg had little further effect (Fig. 3). This compares favorably with spectral changes seen when mersalyl interacts with the microsomal pigments in the oxidized state (Fig. 1B). Accompanying the formation of P-420 was a loss of spectrophotometrically measurable cytochrome, which depended on the amount of conversion of cytochrome P-450 to P-420.

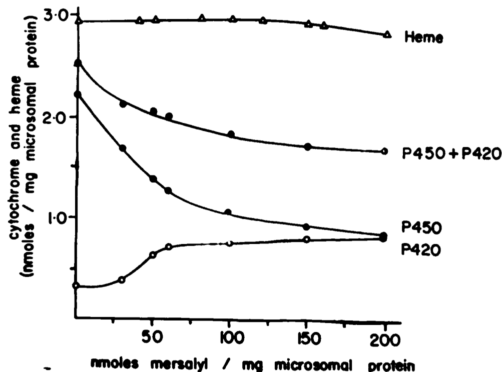

FIG. 3. *Effect of mersalyl conversion of microsomal cytochrome P-450 to P-420 on heme content of Microsomes*

Hepatic microsomes (2 mg of protein per milliliter) from phenobarbital-treated rats were treated with mersalyl by the procedure described under MATERIALS AND METHODS. The cytochrome concentrations were calculated from the difference spectrum obtained 10 min after the addition of mersalyl to the microsomes. Total heme (using the pyridine-hemochromogan assay described in the text) was determined using a separate sample of microsomes which had been treated with mersalyl for the same period of time.

Extensive gassing of the sample with argon prior to the addition of mersalyl did not prevent the loss of detectable cytochrome. It must be emphasized, however, that the loss of CO-combining cytochrome was only a loss of measurable cytochrome by our method, since the total heme content of the microsomes was unchanged after mercurial treatment.

A similar pattern of conversion of cytochrome P-450 to P-420 was observed using two other mercurials, CMPS and CMB (Fig. 4), but not with another sulfhydryl reagent, *N*-ethylmaleimide. Mercuric chloride at low concentrations caused a loss of cytochrome P-450 similar to that observed for other mercurials but at concentrations above 30 nmoles/mg of protein the recovery of P-420 was much less, suggesting that the P-420 formed was susceptible to degradation in the presence of mercuric chloride. In contrast to the incomplete conversion of cytochrome P-450 to P-420 by mercurials, urea (Table 1) caused almost complete conversion of cytochrome P-450 to P-420. However, a loss of

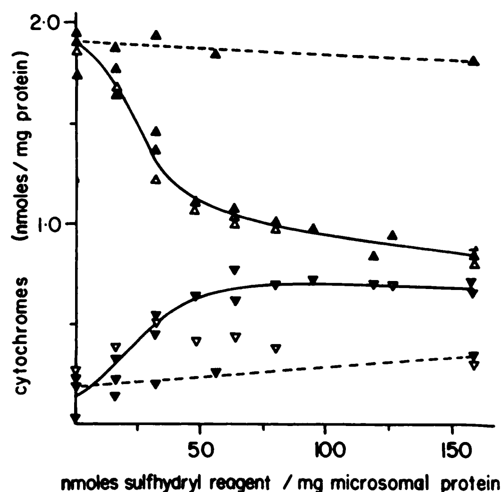

FIG. 4. *Conversion of microsomal cytochrome P-450 to P-420 by various sulfhydryl reagents*

Liver microsomes (2 mg of protein per milliliter) from phenobarbital-treated rats were treated with sulfhydryl reagents (filled symbols, CMPS; open symbols, $HgCl_2$; symbols half-filled vertically, CMB; symbols half-filled horizontally, *N*-ethylmaleimide) by the procedure described under MATERIALS AND METHODS, and the difference spectra were determined 6 min after the addition of the sulfhydryl reagents to the microsomes (△, cytochrome P-450; ▽, P-420).

CO-combining cytochrome also occurred with this urea treatment.

The general pattern of conversion of cytochrome P-450 to P-420 by increasing mercurial concentrations described above was evident under a variety of conditions *in vitro*. It was dependent on the concentration of mersalyl per milligram of microsomal protein. The addition of compounds which produce type II substrate binding spectra (nicotinamide) and type I substrate binding spectra (ethylmorphine, hexobarbital) (Table 2) produced only slight changes in conversion. $NADP^+$ (which is known to protect the microsomal oxidative metabolism from inhibition by mersalyl) added in either the presence or absence of nictinamide [a substance known to prevent $NADP^+$ destruction by pyridine nucleotidases (9)] did not protect the cytochrome P-450 from attack by mersalyl. The conversion was essentially independent of the buffer used and was influenced very little by up to 10-fold changes in hydrogen ion concentration above of below pH 7.4 (Table 3).

The failure to observe complete conversion of hepatic cytochrome P-450 to P-420 was at variance with the reported results of Murakami and Mason (4), although Ichikawa and Yamano (3) failed to show 100% conversion of cytochrome P-450 upon CMB treatment of microsomes at concentrations up to 500 nmoles/mg of protein. However, Murakami and Mason employed Lubrol-solubilized microsomes in their studies, which, in our hands, caused some conversion of cytochrome P-450 to P-420 in the absence of mercurials, and in the presence of mercurials permitted the conversion of more cytochrome P-450. Also, we were able to confirm that Lubrol has a disruptive effect on intact microsomes (10), as least as manifested by inhibition of ethylmorphine demethylation activity.

However, disruption of the microsomes does not necessarily mean a greater ease of conversion of cytocrome P-450 to P-420 by mercurials. Treatment of the microsomes with trypsin (which completely releases NADPH–cytochrome *c* reductase activity from the microsomes in 16 min) for up to 30

TABLE 1

Conversion of microsomal cytochrome P-450 to P-420 by urea

Hepatic microsomes (2 mg of protein per milliliter) from phenobarbital-treated rats were treated with urea at various concentrations. The CO difference spectra were recorded 10 min after the addition of urea to the microsomes, using the procedure described under MATERIALS AND METHODS.

Urea	Cytochromes		
	Total P-450 + P-420	P-450	P-420
M	*nmoles/mg protein*		
0	4.00	3.30	0.70
1	3.82	2.98	0.84
2	3.21	2.24	0.97
3	2.39	1.01	1.38
4	2.16	0.55	1.61
5	1.87	0.12	1.75
6	1.80	0.04	1.76

TABLE 2

Effects of ethylmorphine, hexobarbital, and nicotinamide on conversion of microsomal cytochrome P-450 to P-420 by mersalyl

Liver microsomes (2 mg of protein per milliliter) from phenobarbital-treated rats were suspended in standard buffer containing 8 mM ethylmorphine, 10 mM nicotinamide, both nicotinamide and ethylmorphine, or 3 mM hexobarbital, and treated with mersalyl as described under MATERIALS AND METHODS. The CO difference spectra were recorded 6 min after the addition of mersalyl to the microsomes.

Mersalyl	% of Original cytochrome (P-450 + P-420) concentration present after mersalyl treatment									
	No addition		Ethylmorphine		Nicotinamide		Ethylmorphine + nicotinamide		Hexobarbital	
	P-450	P-420	P-450	P-420	P-450	P-420	P-450	P-420	P-450	P-420
nmoles/mg protein	*% total*		*% total*		*% total*		*% total*		*% total*	
0	87	13	90	10	88	12	89	11	89	11
13			79	14	78	14				
27	73	19	61	19	68	18				
32	66	26					81	13	78	14
40	57	33	57	21	60	21				
53	50	25	55	19	56	22				
67	38	28	52	22	56	27				
80	46	30					62	16	60	20
133	36	29	45	22	44	24				
160	39	34					53	17	53	25

TABLE 3

Effect of different buffers and changes in pH on conversion of microsomal cytochrome P-450 to P-420 by mersalyl

Hepatic microsomes (2 mg of protein per milliliter) from phenobarbital-treated rats were suspended in the buffers shown and treated with mersalyl as described under MATERIALS AND METHODS. The CO difference spectra were recorded 10 min after the addition of mercurial to the microsomes.

Mersalyl	50 mM Tris–150 mM KCl–10 mM $MgCl_2$				100 mM potassium phosphate			
	pH 7.4		pH 8.4		pH 7.4		pH 6.8	
	P-450	P-420	P-450	P-420	P-450	P-420	P-450	P-420
nmoles/mg protein	*nmoles/mg protein*		*nmoles/mg protein*		*nmoles/mg protein*		*nmoles/mg protein*	
0	2.01	0.50	1.49	0.56	2.09	0.40	2.14	0.36
26	1.19	0.75	0.89	0.72	1.59	0.69	1.59	0.68
48	0.84	0.76			0.95	0.83		
70	0.73	0.88	0.62	0.77	0.79	0.78	0.81	1.02
96	0.69	0.89			0.71	0.81		
160	0.52	0.87	0.48	0.83	0.62	0.80	0.62	0.91

min did not greatly increase the conversion of cytochrome P-450 to P-420 by mercurials (Table 4).

Effect of mersalyl on substrate binding spectra. As there have been reports (11, 12) of a differential destruction of type I and type II substrate-binding sites of microsomal cytochrome P-450, the effect of mersalyl on these was determined using hexobarbital and aniline as the respective ligands (Table 5). It was evident that the loss of the ability to demonstrate a hexobarbital substrate binding spectrum occurred to approximately the same extent as the loss of cytochrome P-450.

TABLE 4

Effect of trypsin digestion of microsomes on conversion of cytochrome P-450 to P-420 by mersalyl

Liver microsomes from phenobarbital-treated rats were incubated at 25° with trypsin (4 μg/mg of microsomal protein) for 0, 16, or 30 min. The digestion was halted with trypsin inhibitor (7 μg/mg of microsomal protein), and the effect of mersalyl on the cytochrome P-450 of these microsomes was determined using the standard method, recording the CO difference spectra 10 min after the addition of mersalyl to the microsomes.

Mersalyl	Cytochrome concentration after trypsin digestion								
	0 min			16 min			30 min		
	P-450	P-420	P-450 + P-420	P-450	P-420	P-450 + P-420	P-450	P-420	P-450 + P-420
nmoles/mg protein	*nmoles/mg protein*			*nmoles/mg protein*			*nmoles/mg protein*		
0	3.38	0.75	4.13	2.60	0.86	3.46	2.26	0.91	3.17
32	2.38	1.07	3.45	1.96	1.05	3.01	1.78	1.03	2.81
80	1.52	1.25	2.77	1.24	1.27	2.51	1.18	1.22	2.40
160	1.21	1.28	2.49	0.97	1.42	2.39	0.89	1.26	2.15

TABLE 5

Effect of mersalyl on microsomal substrate binding spectra

Liver microsomes (2 mg of protein per milliliter) in the standard buffer were incubated with various concentrations of mersalyl at room temperature for 10 min. After a baseline of equal absorbance had been established, the mixture was divided between two cuvettes and the difference spectra were recorded 1 min after the addition of 3 mM hexobarbital or saturating concentrations of aniline to the sample cuvette.

Mersalyl	P-450	Peak to trough signal of binding spectrum		
		Hexobarbital	Aniline	Aniline[a]
nmoles/mg protein	%	%	%	%
0	100	100	100	100
32	69	77	84	88
64	53	48	65	67
96	47	44	63	65
160	34	45	60	62

[a] With 3 mM hexobarbital in both reference and sample cuvettes.

The aniline binding spectrum, however, was less affected. Since aniline has been suggested to produce some type I as well as type II substrate binding spectra (13), and since mersalyl also produced shifts in the wavelengths of the absorption maximum and minimum of the aniline substrate binding spectrum similar to those caused by hexobarbital, it may be that mersalyl, like hexobarbital, increases the peak to trough signal heights and therefore gives the smaller percentage loss observed in the aniline binding spectrum. When the aniline binding studies were conducted in the presence of hexobarbital, however, the same percentage loss upon mersalyl treatment was observed as in the absence of hexobarbital, thus confirming that the type II substrate-binding site is less affected by mersalyl than the type I substrate-binding site.

Reconversion of microsomal P-420 to cytochrome P-450. It has been claimed that some reconversion (65%) of P-420 to cytochrome P-450 was obtained with at least a 20-fold excess of glutathione over mercurial (3). Under the present conditions only about 20% reconversion of P-420 to cytochrome P-450 could be achieved with reduced glutathione in concentrations up to 5 times that of the mercurial (Fig. 5). The small reconversion obtained was independent of the time period (longer than 3 min) during which the reduced glutathione was in contact with the mercurial-treated microsomes. Mixing the reduced glutathione and mersalyl together before their addition to microsomes demonstrated that reduced glutathione, under conditions in which it cannot reconvert P-420 to cyto-

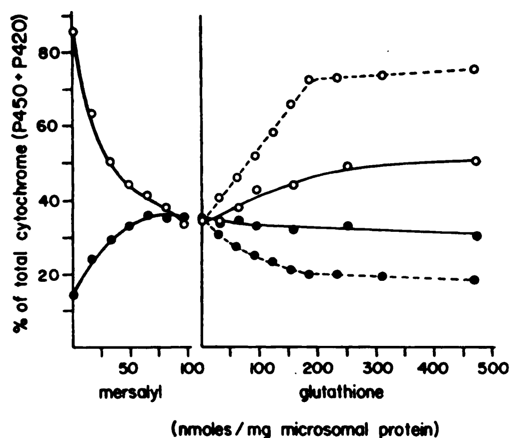

FIG. 5. *Reconversion of mercurial-produced microsomal P-420 to cytochrome P-450 by reduced glutathione.*

On the left, the conversion of cytochrome P-450 to P-420 was determined as described under MATERIALS AND METHODS, except that sodium dithionite was added 5 min after the mersalyl had been added to the microsomes. The CO difference spectra were recorded 10 min after the mersalyl addition. In the experiments shown on the right, 96 nmoles of mersalyl per milligram of protein were added to the microsomes, followed by reduced glutathione after 2 min and sodium dithionite after a further 3 min. The CO difference spectrum was then recorded at 10 min, and the cytochrome concentrations were determined (——). These were compared with similar assays in which the reduced glutathione and mersalyl were added together before their addition to microsomes (- - -). ○, cytochrome P-450; ●, P-420.

chrome P-450, can bind the mercurial and prevent the initial conversion of cytochrome P-450 to P-420. This suggests that mercurial conversion of cytochrome P-450 to P-420 is largely irreversible.

Changes in microsomal electron paramagnetic resonance with mersalyl treatment. Since some of the hepatic cytochrome P-450 in phenobarbital-treated animals is refractory toward mercurials, it was of interest to investigate changes in the spin state of the heme iron associated with the optically measurable conversion of cytochrome P-450 to P-420. Earlier workers have shown that cytochrome P-450 in the low-spin form is quantitatively converted to P-420 in the high-spin form (4) by pH changes. In our experiments a peak to trough low-spin signal (g = 2.25) of unity gave a peak to trough high-spin signal (g = 6.1) of 0.4. Although lowering the pH caused quantitative changes in hepatic microsomes from phenobarbital-induced rabbits (Fig. 6A), conversion of cytochrome P-450 to P-420 by mersalyl caused a change in the low-spin EPR signal without the concomitant appearance of an equivalent amount of high-spin signal, possibly because of the inability to detect P-420 by ESR under certain conditions (4). In addition, the amount of conversion of cytochrome P-450 with mersalyl, as shown by the decrease in

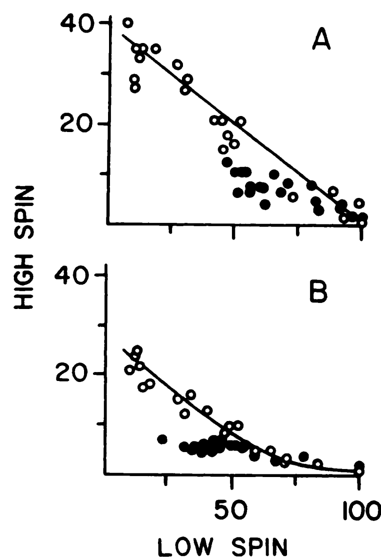

FIG. 6. *Effect of pH changes and mersalyl on electron paramagnetic resonance spectra of rat and rabbit liver microsomes*

A. Hepatic microsomes from phenobarbital-treated rabbits were used at concentrations of 12–15 mg of protein per milliliter. Changes in the high-spin (g = 6.1) and low-spin (g = 2.25) signals were obtained with changes in pH from 7.4 to 4.0 (○——○) and with mersalyl concentrations (●) from 0 to 200 nmoles/mg of protein. The low-spin signal obtained at pH 7.4 in each preparation was normalized to 100, and the high-spin signal was adjusted to the same extent.

B. Liver microsomes from phenobarbital-treated rats were used at concentrations of 15–19 mg of protein per milliliter. Signal heights were again normalized as with rabbit liver microsomes. pH changes were from 7.4 to 4.0 (○——○), and mersalyl concentrations (●) of 0–240 nmoles/mg of protein were used.

low-spin signal, never approached that obtainable with pH changes. Mersalyl binding as a substrate, with conversion of the low-spin cytochrome P-450 to a high-spin form not detectable under our conditions, seems unlikely, because of the similarity in the losses of cytochrome P-450 and the hexobarbital substrate binding spectrum already discussed.

With hepatic microsomes from phenobarbital-treated rats, lowering the pH did not cause the same conversion of the low-spin (g = 2.25) to the high-spin (g = 6.1) signal (Fig. 6B) seen in rabbit microsomes. When the low-spin loss was less than about 40% there was little increase in the magnitude of the observable high-spin signal. Nevertheless, as in the experiments using rabbit liver microsomes, mersalyl always produced less high-spin signal for a given loss of the low-spin signal than did lowering the pH.

A correlative study of the conversion of cytochrome P-450 to P-420 by mersalyl (Fig. 7), observed both optically and by electron spin resonance spectroscopy, showed that the loss of the low-spin signal and cytochrome P-450 and the gain in the high-spin signal and formation of P-420 (above that already present in the native microsomes), caused by mersalyl, were correlated very closely. This indicates that the discrepancy observed in the spin resonance studies (assuming the relationship between the high- and low-spin signals observed in rabbit microsomes) may correspond with measurable hemoprotein "lost" in the spectral studies. The apparent lack of high-spin signal for P-420 present in native microsomes (i.e., those not treated with mersalyl) may also reflect an error in the determination of cytochrome P-420 using accepted extinction coefficients. Thus mersalyl, as well as converting cytochrome P-450 to P-420, also appears to destroy a portion of the hemoprotein present in microsomes. Although the difference between P-420 and the high-spin signal has been explained as an inability to detect all the optically measurable P-420 as the high-spin signal (4), the correlation demonstrated here between cytochrome P-450 and the low-spin signal, on the one hand, and P-420 and the high-spin signal, on the other, strongly suggests that mersalyl also converts some of the cytochrome to a form that cannot be measured either as an EPR signal or optically as a carbon monoxide derivtive.

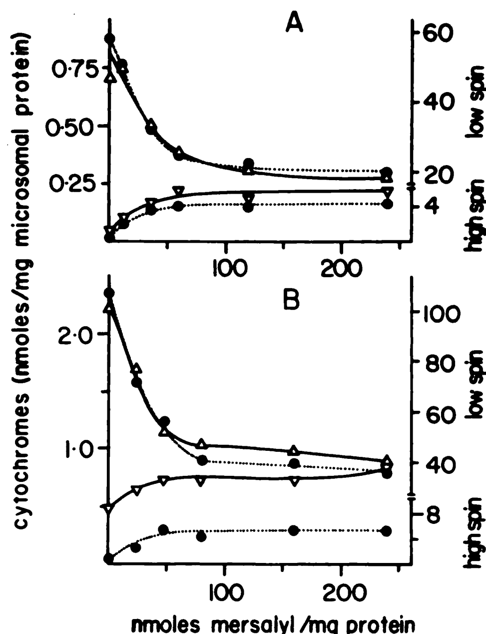

FIG. 7. *Correlation of effects of mersalyl on optical and electron paramagnetic resonance spectra of rat microsomal cytochrome P-450*

The optical CO difference spectra were obtained 6 min after the addition of mersalyl to the microsomes, using the standard procedure described under MATERIALS AND METHODS. Microsomes were used at a concentration of 2 mg of protein per milliliter. Electron paramagnetic resonance samples, consisting of aerobic microsomes treated with mersalyl, were frozen in liquid nitrogen at the time the optical spectra were determined (6 min after the addition of mersalyl), using microsomal concentrations of 20 and 15 mg of protein per milliliter in control (A) and phenobarbital-treated (B) animals, respectively. The high-spin signal is plotted on the same scale as the low-spin, using the ratio derived from the pH conversion of rabbit liver microsomes (i.e., 1 arbitrary unit of low-spin gives 0.4 unit of high-spin signal). △ and ▽, optical determinations; ●, electron paramagnetic resonance determinations.

DISCUSSION

The results presented here demonstrate that mercurials convert hepatic microsomal cytochrome P-450 to P-420, but that in mi-

crosomes from phenobarbital-treated animals this does not proceed to completion. The cytochrome P-450 is not partially refractory to all methods used for conversion, since high urea concentrations result in complete disappearance of cytochrome P-450.

The concentration range of mercurial (about 80 nmoles/mg of protein) at which conversion of cytothrome P-450 to P-420 occurs is much greater than the concentration of cytochrome P-450 (2–3 nmoles/mg of protein) present in the microsomes. Although recent evidence (14) implicates a sulfhydryl group as a lignad to the heme in cytochrome P-450, the original assumption, based on the mercurial conversion of cytochrome P-450, would seem to be rather fortuitous. Our evidence indicates either that there exists a heterogeneous population of cytochrome P-450 molecules, as regards the susceptibility of the ligand —SH group to mercurials, or that the ligand —SH group is not titrated at all and the conversion of cytochrome P-450 to P-420 is caused by an attack on other —SH groups, either on the cytochrome P-450 itself or in the membrane proteins concerned with the structural organization of the electron transport chain.

Earlier work (15) has shown that the —SH groups on the NADPH–cytochrome *c* reductase necessary for enzyme activity are protected from mercurial attack by $NADP^+$ The lack of protection by $NADP^+$ toward the conversion of cytochrome P-450 to P-420 by mersalyl suggests that the mode of attack of mersalyl is not through disruption of this key component—key in that quantitative determination of the components of the microsomal electron transport chain (16) requires that many cytochrome P-450 molecules be linked in some way to this flavoprotein. Also, after trypsin digestion, which releases the flavoprotein from the microsomal membrane, there appears to be no remarkable change in the mercurial conversion of cytochrome P-450. That the conversion of cytochrome P-450 to P-420 is affected only slightly by compounds which bind to the cytochrome in the oxidized state (giving type I and type II binding spectra), in a region of the molecule close enough to the heme to cause perturbation of the heme group (17), again suggests that the mercurial attack occurs at some point more distant from the "active" heme area. The lack of reconversion of P-420 to cytochrome P-450 by reduced glutathione suggests either that the thiol groups in the microsomes have a very high affinity for the mercurial or that the mercurial interaction causes or permits irreversible disruption of the protein.

The loss of total cytochrome upon mersalyl treatment, observed with optical as well as electron paramagnetic resonance studies, the asymmetry of the loss of cytochrome P-450 with the increase in P-420, and the low amounts of P-420 obtained when cytochrome P-450 is converted by mercuric chloride may all indicate that nascent P-420 is labile toward mercurials. Conversion by lowering the pH, however, as measured by spin state changes, would indicate that with pH changes, at least in rabbit liver microsomes, the conditions are less drastic as regards the destruction of P-420. Alternatively, the loss of measurable hemoprotein may be due to separation of the heme from its apoprotein or to preferential destruction of a pigment with a high extinction coefficient (18). However, since the cytochrome with a high extinction coefficient is presumed to be a high-spin hemoprotein which is undetectable under our conditions, one would expect a smaller loss of low-spin signal for a given loss of optically measurable cytochrome P-450. This matter has been investigated further in the following paper (19), using 3-methylcholanthrene-treated animals, in which the presumed high extinction coefficient of cytochrome P-450 represents a larger percentage of the total cytochrome P-450 in the microsomes (14).

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