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EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE SUBSTRATE INTERACTION WITH CYTOCHROME P-450 IN THE OXIDATION OF DRUGS BY LIVER MICROSOMES*

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Abstract—Adrenalectomy or alloxan diabetes markedly decreased the magnitude of cytochrome P-450 spectral change induced by hexobarbital or aminopyrine in male rat liver microsomes. The decrease in the binding capacity of cytochrome P-450 for hexobarbital or aminopyrine was assumed to be responsible. On the other hand, the binding capacity of cytochrome P-450 for aniline or zoxazolamine was not significantly affected by adrenalectomy or alloxan diabetes. In contrast to male rats, the binding capacity by the liver of female rats to cytochrome P-450 was not affected by adrenalectomy or alloxan diabetes.

The K_m (Michaelis constant) value for hexobarbital hydroxylation and the K_s (spectral dissociation constant) value for the hexobarbital-induced spectral change were increased in liver microsomes from the adrenalectomized or diabetic male rats, whereas the K_m and K_s values for aniline were not affected.

These results suggest that the decrease in the binding capacity of cytochrome P-450 for hexobarbital or aminopyrine is a factor responsible for the decrease in the oxidation of drugs by liver microsomes from the adrenalectomized or diabetic male rats. The impairment of the androgen-dependent regulatory mechanism for the binding capacity of cytochrome P-450 was assumed to be a factor responsible for the decrease in the binding capacity in liver microsomes from the adrenalectomized or diabetic rats.

IN A PREVIOUS paper,[1] it was reported that the activities of drug-metabolizing enzymes of liver microsomes, such as hexobarbital hydroxylation and aminopyrine *N*-demethylation were markedly decreased by adrenalectomy or alloxan diabetes in male rats but not in female rats. However, the aniline hydroxylation and zoxazolamine hydroxylation in male and female rats were increased or not significantly altered by the same treatments.

Castration in male rats markedly decreased the hexobarbital hydroxylation and administration of methyltestosterone to such rats restored the activity to the level of the intact males.[1] Adrenalectomy or alloxan diabetes did not produce a further decrease in the hexobarbital hydroxylation in the castrated rats, but they did produce a marked decrease in the hexobarbital hydroxylation both in the methyltestosterone-treated castrated rats and the intact males.[1] These results indicate that adrenalectomy or alloxan diabetes decreases the hexobarbital hydroxylation and aminopyrine *N*-demethylation, which are increased by androgen, probably through an impairment of an androgen-dependent stimulating mechanism.[1–3]

* A preliminary account of the present studies was published briefly in *J. Biochem.* (*Tokyo*) **66**, 739 (1969).

Recent studies have established that a microsomal hemoprotein called P-450 is involved as the oxygen activating component in a number of NADPH-dependent monooxygenase reaction, such as hydroxylations of drugs and steroid hormone.[4-8] Imai and Sato,[9] and Schenkman *et al.*[10] have reported that a number of drugs which are substrates of hepatic microsomal hydroxylases react with the microsomal cytochrome P-450 to give two characteristic types of spectral change. These results have suggested that the spectral changes observed are indicative of substrate interaction with cytochrome P-450, presumably representing the primary binding of substrate for enzymic hydroxylation.[11-12]

On the other hand, Schenkman *et al.*[13] have demonstrated that the magnitude of the hexobarbital-induced spectral change is higher in liver microsomes from male rats than in those from female rats and castration in male rats abolishes this sex difference. These results have suggested that the sex difference in the hexobarbital hydroxylation may be due to the difference in the substrate interaction with cytochrome P-450.

The purpose of the present communication therefore is to investigate whether the decrease in the activities of hexobarbital hydroxylation and aminopyrine *N*-demethylation in liver microsomes from adrenalectomized or alloxan diabetic male rats is related to an impairment of the stimulation of the substrate interaction with cytochrome P-450 by androgen.

MATERIALS AND METHODS

Male and female rats (60 days old) of the Wistar strain, weighing about 180 and 150 g, respectively, were used. NADP, NADPH, glucose-6-phosphate, AMP and glucose-6-phosphate dehydrogenase were purchased from Böhringer and Mannheim GmbH. The other chemicals used were of reagent grade. The rats were adrenalectomized 5 days before sacrifice and maintained on 1% NaCl solution as drinking solution, or treated with alloxan (170 mg/kg, s.c.) 5 days before being killed; rats which had more than 0·5% glucose in urine at sacrifice were used. In preliminary experiments, it was observed that the sham operation (5 days before) did not significantly affect the magnitude of drug-induced spectral change and drug oxidation in the liver microsomes. The liver microsomes were prepared as described in a previous paper.[3] The microsomes were suspended with 1·15% KCl so that 1 ml was equivalent to 250 mg liver.

Determination of the activity of drug-metabolizing enzymes. The incubation mixture consisted of 1 ml of the microsomal suspension equivalent to 250 mg liver, 20 μmoles of glucose-6-phosphate, 10 μmoles of AMP, 1·0 μmole of NADP, 1·5 unit (one unit reduces 1 μmole of NADP per min) of glucose-6-phosphate dehydrogenase, 25 μmoles of nicotinamide, 12·5 μmoles of $MgCl_2$, 0·7 ml of 0·2 M sodium phosphate buffer (pH 7·4), various substrates and water to a final volume of 2·5 ml. The concentrations of the substrates were 2 mM, except for hexobarbital and zoxazolamine which were 1·6 mM and 0·4 mM, respectively. The mixture was incubated for 30 or 20 min (in kinetic studies) under air at 37° with constant shaking.

The hydroxylation of hexobarbital was determined by disappearance of the substrate according to the method of Cooper and Brodie.[14] The *N*-demethylation of aminopyrine was determined by the formation of formaldehyde according to the method of Nash.[15] The hydroxylation of aniline was measured by *p*-aminophenol formed according to the method described by Kato and Gillette.[1] The hydroxylation

of zoxazolamine was determined by the disappearance of the substrate according to Conney *et al.*[16]

Determination of microsomal protein and cytochrome P-450. Microsomal protein was measured according to the method of Lowry *et al.*[17] Cytochrome P-450 content was measured by CO-induced difference spectrum according to Omura and Sato.[4]

Determination of substrate-induced difference spectrum. The difference spectra of cytochrome P-450 produced on addition of substrates were measured according to Schenkman *et al.*[10] The microsomal suspension was diluted with 5 vol. of 0·1 M phosphate buffer (pH 7·4) and 2·8 ml of the sample solution (1·2 mg protein/ml) was placed in cuvettes of 1 cm optical path. The various amounts of drugs in 0·1 ml of distilled water were added to the sample cuvette and 0·1 ml of distilled water was added to the reference cuvette. Thirty to sixty sec later, the substrate-induced difference spectra were recorded at room temperature with a Hitachi EPS-3T recording spectrophotometer with an integral sphere attachment. The change of absorbance on addition of the substrate was expressed as ΔA per milligram microsomal protein or mμmole P-450 per ml according to Schenkman *et al.*[13]

Determination of the activity of microsomal NADPH-cytochrome c reductase. The activity of NADPH-cytochrome c reductase was measured by the method of Williams and Kamin.[18]

RESULTS

Effect of adrenalectomy or alloxan diabetes on the contents of microsomal protein and cytochrome P-450 and the activity of NADPH-cytochrome c reductase. Adrenalectomy or alloxan diabetes did not affect the content of hepatic microsomal protein in male and female rats. Adrenalectomy decreased the content of cytochrome P-450 in liver

TABLE 1. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE CONTENTS OF PROTEIN AND P-450 IN LIVER MICROSOMES OF MALE AND FEMALE RATS

	Control	Adrenalectomy	Alloxan diabetes
(A) Male			
Microsomal protein (mg/g wet wt.)	28·1 ± 0·3	26·9 ± 0·4 (−4%)	28·4 ± 0·3 (+1%)
P-450 (mμmole/g wet wt.)	28·9 ± 1·2	21·8 ± 1·7 (−25%)*	32·2 ± 2·3 (+11%)
P-450 (mμmole/mg protein)	1·03 ± 0·05	0·81 ± 0·05 (−21%)*	1·13 ± 0·06 (+10%)
(B) Female			
Microsomal protein (mg/g wet wt.)	28·4 ± 0·3	27·2 ± 0·4 (−5%)	28·4 ± 0·5 (0%)
P-450 (mμmole/g wet wt.)	21·8 ± 1·2	17·9 ± 1·4 (−18%)*	25·3 ± 1·9 (+16%)*
P-450 (mμmole/mg protein)	0·76 ± 0·04	0·65 ± 0·06 (−14%)	0·89 ± 0·0 (+17%)*

The rats were adrenalectomized 5 days before and maintained with 1% NaCl solution as drinking solution, or treated with alloxan (170 mg/kg, s.c.) 5 days before being killed.

The results represent averages ± S.E. obtained from 12–16 rats. The figures in the parentheses indicate the percentage difference from control values. The asterisks indicate significant difference ($P < 0·05$) from control values.

microsomes of male and female rats (Table 1). Alloxan diabetes did not significantly affect the content of P-450 in male rats, but increased it in the diabetic females. The activity of NADPH-cytochrome c reductase was not affected in liver microsomes from the adrenalectomized and alloxan diabetic rats.

Effect of adrenalectomy or alloxan diabetes on the hexobarbital-induced spectral change and hydroxylating activity in male rats. Adrenalectomy decreased the magnitude of spectral change induced by hexobarbital per unit of microsomal protein and of cytochrome P-450 in the males (Table 2). Similarly, the activity of hexobarbital hydroxylation by liver microsomes was decreased in the adrenalectomized males. Therefore, the ratio of the hydroxylating activity of hexobarbital to the spectral change was not significantly affected. The decrease in the magnitude of the spectral change per unit of cytochrome P-450 suggested a decrease in the binding capacity of cytochrome P-450 for hexobarbital in liver microsomes from the adrenalectomized rats.[3]

TABLE 2. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE HEXOBARBITAL (HB)-INDUCED SPECTRAL CHANGE AND HEXOBARBITAL HYDROXYLATION BY LIVER MICROSOMES OF MALE RATS

	Control	Adrenalectomy	Alloxan diabetes
HB Spectral change ($\Delta A \times 10^3$/mg protein)	17·5 ± 1·25	10·4 ± 1·02 (−41%)*	13·6 ± 1·22 (−22%)*
HB Spectral change ($\Delta A \times 10^3$/mμmole P-450)	17·0 ± 1·10	12·6 ± 0·90 (−26%)*	12·0 ± 0·90 (−29%)*
HB Hydroxylation (mμmole/mg protein)	129 ± 8·2	68·8 ± 5·6 (−47%)*	76·3 ± 5·9 (−41%)*
HB Hydroxylation (mμmole/mμmole P-450)	125 ± 6·3	84·9 ± 5·5 (−32%)*	67·5 ± 4·9 (−54%)*
HB Hydroxylation (mμmole/$\Delta A \times 10^3$)	7·34 ± 0·62	6·62 ± 0·72 (−10%)	5·59 ± 0·69 (−24%)*

The magnitude of the spectral change is determined by the decrement of the absorbance between 421 mμ and 500 mμ produced by addition of hexobarbital (1·6 mM) into microsomal suspension and expressed as the change of absorbance per mg protein or per mμmole P-450. The results represent averages ± S.E. obtained from 12–16 rats. The figures in the parentheses indicate the percentage difference from control values. The asterisks indicate the significant difference ($P < 0·05$) from control values. The hydroxylating activity is expressed as mμmole of the substrate oxidized per mg protein or mμmole P-450 for 30 min and per the magnitude of the spectral change ($\Delta A \times 10^3$) for 30 min.

On the other hand, alloxan diabetes decreased the magnitude of spectral change induced by hexobarbital in liver microsomes of the males and the hexobarbital hydroxylation by liver microsomes was more clearly decreased (Table 2). Thus the ratio of the hydroxylating activity to the magnitude of the spectral change was significantly decreased.

Effect of adrenalectomy or alloxan diabetes on the aniline-induced spectral change and hydroxylating activity in male rats. As shown in Table 3, the magnitude of spectral change induced by aniline was slightly decreased in liver microsomes from the adrenalectomized rats, but the magnitude of the spectral change per unit of cytochrome P-450 was unchanged. The aniline hydroxylation was also slightly decreased. Therefore, the ratio of hydroxylating activity to the spectral change was not significantly

TABLE 3. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE ANILINE(AN)-INDUCED SPECTRAL CHANGE AND ANILINE HYDROXYLATION BY LIVER MICROSOMES OF MALE RATS

	Control	Adrenalectomy	Alloxan diabetes
AN Spectral change ($\Delta A \times 10^3$/mg protein)	14·6 ± 0·90	11·5 ± 1·22 (−21%)*	17·3 ± 1·45 (+16%)
AN Spectral change ($\Delta A \times 10^3$/mμmole P-450)	14·2 ± 0·73	14·2 ± 1·10 (0%)	15·3 ± 1·13 (+8%)
AN Hydroxylation (mμmole/mg protein)	23·3 ± 1·9	20·0 ± 2·1 (−15%)	30·4 ± 1·9 (+30%)*
AN Hydroxylation (mμmole/mμmole P-450)	22·6 ± 1·4	24·8 ± 1·6 (+9%)	26·9 ± 2·0 (+19%)*
AN Hydroxylation (mμmole/$\Delta A \times 10^3$)	1·59 ± 0·15	1·73 ± 0·18 (+8%)	1·75 ± 0·16 (+10%)

The magnitude of the spectral change is determined by the increment of the absorbance between 431 mμ and 500 mμ produced by addition of aniline (2·0 mM) into microsomal suspension and expressed as the change of absorbance per mg protein or per mμmole P-450. * Significant difference ($P < 0·05$) from control values. See the legends for Table 2.

affected. Alloxan diabetes did not significantly increase the magnitude of spectral change induced by aniline but it increased the hydroxylating activity for aniline. However, the ratio of the hydroxylating activity to the magnitude of spectral change was not significantly altered.

These results suggested that the decrease in the hydroxylating activity in the adrenalectomized males may be related to the decrease in the binding capacity of cytochrome P-450 for hexobarbital. Hexobarbital induced type I spectral change, while aniline induced type II spectral change.[10] Therefore, it is conceivable that the difference between the alteration in the binding capacity of the cytochrome for hexobarbital and that for aniline in the adrenalectomized or diabetic males may be related to the difference in the type of spectral change.

On the other hand, the binding capacity of cytochrome P-450 for hexobarbital is androgen dependent, whereas that for aniline is not.[3,13] It is therefore probable that the above difference may be related to the difference in the degree of androgen dependence in the substrate interaction with cytochrome P-450. Thus both possibilities were investigated in the following experiments.

Effect of adrenalectomy or alloxan diabetes on the aminopyrine- or zoxazolamine-induced spectral change and oxidating activities in male rats. Both aminopyrine and zoxazolamine display type I spectral change and the binding capacity of cytochrome P-450 for aminopyrine is clearly dependent on the action of androgen, whereas the binding capacity of cytochrome P-450 for zoxazolamine is independent of androgen.[19,20]

Adrenalectomy decreased the magnitude of spectral change induced by aminopyrine per unit of microsomal protein and of cytochrome P-450 in liver microsomes of male rats (Table 4), whereas the same treatment did not significantly affect the magnitude of spectral change induced by zoxazolamine (Table 5). Similarly, the activity of aminopyrine *N*-demethylation was decreased in the adrenalectomized males, and the activity of zoxazolamine hydroxylation was not significantly affected.

TABLE 4. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE AMINOPYRINE (AP)-INDUCED SPECTRAL CHANGE AND AMINOPYRINE *N*-DEMETHYLATION BY LIVER MICROSOMES OF MALE RATS

	Control	Adrenalectomy	Alloxan diabetes
AP Spectral change ($\Delta A \times 10^3$/mg protein)	10·1 ± 0·67	5·25 ± 0·70 (−48%)*	7·28 ± 0·61 (−28%)*
AP Spectral change ($\Delta A \times 10^3$/mμmole P-450)	9·83 ± 0·52	7·71 ± 0·64 (−22%)*	6·50 ± 0·44 (−34%)*
AP N-Demethylation (mμmole/mg protein)	94·1 ± 5·2	55·3 ± 4·9 (−44%)*	59·3 ± 4·3 (−39%)*
AP N-Demethylation (mμmole/mμmole P-450)	91·4 ± 4·5	68·0 ± 4·2 (−26%)*	52·5 ± 4·5 (−43%)*
AP N-Demethylation (mμmole/$\Delta A \times 10^3$)	9·31 ± 0·69	10·48 ± 0·83 (+13%)	8·14 ± 0·59 (−13%)

The magnitude of the spectral change is expressed by the decrement of the absorbance between 420 mμ and 500 mμ produced by addition of aminopyrine (2·0 mM) into microsomal suspension and expressed as the change of absorbance per mg protein or per mμmole P-450. See the legends for Table 2.

* Significant difference ($P < 0·05$) from control value.

TABLE 5. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE ZOXAZOLAMINE (ZA)-INDUCED SPECTRAL CHANGE AND ZOXAZOLAMINE HYDROXYLATION BY LIVER MICROSOMES OF MALE RATS

	Control	Adrenalectomy	Alloxan diabetes
ZA Spectral change ($\Delta A \times 10^3$/mg protein)	7·37 ± 0·58	6·06 ± 0·67 (−18%)	8·99 ± 0·64 (+22%)*
ZA Spectral change ($\Delta A \times 10^3$/mμmole P-450)	7·19 ± 0·52	7·45 ± 0·58 (+4%)	7·89 ± 0·44 (+10%)
ZA Hydroxylation (mμmole/mg protein)	27·5 ± 1·7	23·1 ± 2·0 (−16%)	32·8 ± 2·2 (+19%)*
ZA Hydroxylation (mμmole/mμmole P-450)	26·7 ± 1·5	28·5 ± 2·0 (+7%)	29·0 ± 1·4 (+9%)
ZA Hydroxylation (mμmole/$\Delta A \times 10^3$)	3·72 ± 0·38	3·83 ± 0·34 (+3%)	3·66 ± 0·28 (−2%)

The magnitude of the spectral change is expressed by the decrement of the absorbance between 420 mμ and 500 mμ produced by addition of zoxazolamine (1·0 mM) into the microsomal suspension and expressed as the change of absorbance per mg protein or per mμmole P-450. See the legends for Table 2.

* Significant difference ($P < 0·05$) from control values.

Alloxan diabetes decreased the magnitude of spectral change induced by aminopyrine in the males, but increased the magnitude of spectral change induced by zoxazolamine. Similarly, the *N*-demethylating activity for aminopyrine was decreased in the diabetic males, whereas the hydroxylating activity for zoxazolamine was slightly increased.

These results indicated that the different effect of adrenalectomy or alloxan diabetes between the binding capacity of P-450 for hexobarbital and that for aniline may be related to the difference in the androgen dependence in the substrate interaction with cytochrome P-450.

Effect of adrenalectomy or alloxan diabetes on the hexobarbital- or aniline-induced spectral change and hydroxylating activities in female rats. As shown in Tables 6 and 7, the magnitude of spectral change induced by hexobarbital was markedly greater in liver microsomes from male rats than in those from the females, whereas that induced by aniline was similar in liver microsomes from both sexes.

TABLE 6. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE HEXOBARBITAL (HB)-INDUCED SPECTRAL CHANGE AND HEXOBARBITAL HYDROXYLATION BY LIVER MICROSOMES OF FEMALE RATS

	Control	Adrenalectomy	Alloxan diabetes
HB Spectral change ($\Delta A \times 10^3$/mg protein)	7·37 ± 0·52	6·82 ± 0·67 (−7%)	11·1 ± 0·90 (+51%)*
HB Spectral change ($\Delta A \times 10^3$/mμmole P-450)	9·69 ± 0·46	10·5 ± 0·73 (+8%)	12·5 ± 0·81 (+29%)*
HB Hydroxylation (mμmole/mg protein)	46·3 ± 3·1	41·9 ± 3·5 (−10%)	62·6 ± 5·0 (+35%)*
HB Hydroxylation (mμmole/mμmole P-450)	60·9 ± 5·3	64·5 ± 3·6 (+6%)	70·1 ± 4·1 (+15%)
HB Hydroxylation (mμmole/$\Delta A \times 10^3$)	6·28 ± 0·59	6·14 ± 0·72 (−2%)	5·62 ± 0·48 (−10%)

See the legends for Table 2.
* Significant difference ($P < 0·05$) from control values.

TABLE 7. EFFECT OF ADRENALECTOMY OR ALLOXAN DIABETES ON THE ANILINE (AN)-INDUCED SPECTRAL CHANGE AND ANILINE HYDROXYLATION BY LIVER MICROSOMES OF FEMALE RATS

	Control	Adrenalectomy	Alloxan diabetes
AN Spectral change ($\Delta A \times 10^3$/mg protein)	11·3 ± 0·73	9·86 ± 0·90 (−13%)	14·6 ± 1·16 (+29%)*
AN Spectral change ($\Delta A \times 10^3$/mμmole P-450)	14·9 ± 0·81	15·2 ± 1·02 (+2%)	16·4 ± 1·16 (+10%)
AN Hydroxylation (mμmole/mg protein)	18·4 ± 1·3	17·1 ± 1·8 (−7%)	26·6 ± 1·9 (+45%)*
AN Hydroxylation (mμmole/mμmole P-450)	24·2 ± 1·5	26·3 ± 1·8 (+9%)	29·9 ± 1·3 (+24%)*
AN Hydroxylation (mμmole/$\Delta A \times 10^3$)	1·62 ± 0·12	1·73 ± 0·16 (+7%)	1·82 ± 0·20 (+12%)

See the legends for Table 4.
* Significant difference ($P < 0·05$) from control values.

Adrenalectomy did not significantly affect the magnitude of spectral change induced by hexobarbital or the hydroxylating activity for hexobarbital in liver microsomes from the females (Table 6). On the other hand, the magnitude of spectral change and hexobarbital hydroxylation were increased in the microsomes from the diabetic females. Similarly, adrenalectomy did not significantly affect the magnitude of spectral change and the hydroxylating activity for aniline. Alloxan diabetes increased the magnitude of spectral change and the hydroxylating activity for aniline.

The results again support the postulation that the different effect of adrenalectomy or alloxan diabetes on the binding capacity of P-450 for hexobarbital and that for aniline in male rats may be related to the difference in the androgen dependence in the substrate interaction with cytochrome P-450.[21]

Kinetic studies on the hexobarbital and aniline hydroxylation in liver microsomes from adrenalectomized or diabetic male rats. As shown in Fig. 1 and Table 8, adrenalectomy or alloxan diabetes decreased the V_{max} (maximum velocity) value for the hexobarbital hydroxylation by liver microsomes of male rats, whereas it increased the K_m value.

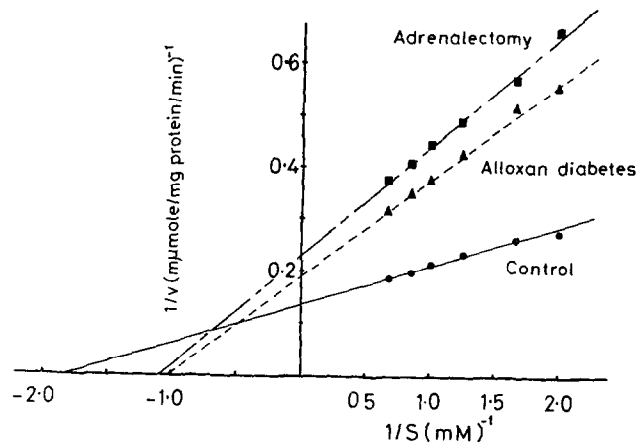

FIG. 1. Reciprocal plot of the activity of hexobarbital hydroxylation against hexobarbital concentration for liver microsomes from adrenalectomized or diabetic males. Hexobarbital hydroxylation was measured under conditions described in the methods except that hexobarbital concentration was varied as shown in the figure. The activity is expressed by mμmole hexobarbital metabolized per mg microsomal protein per min. The treatments of rats are the same as given in Table 1.

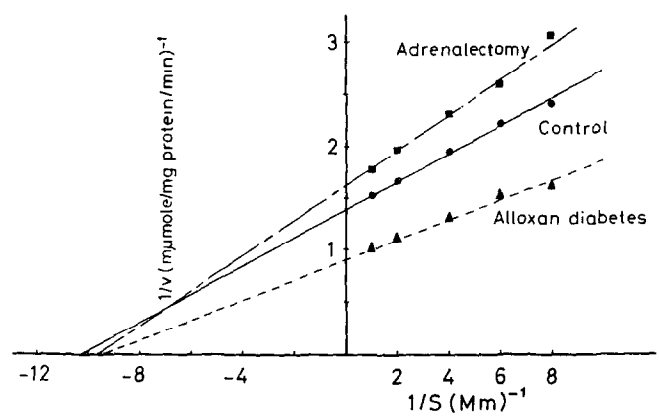

FIG. 2. Reciprocal plot of the activity of aniline hydroxylation against aniline concentration for liver microsomes from adrenalectomized or diabetic males. Aniline hydroxylation was measured under conditions described in the methods except that aniline concentration was varied as shown in the figure. The activity is expressed by mμmole *p*-aminophenol formed per mg microsomal protein per min.

On the other hand, the V_{max} and K_m values for the aniline hydroxylation by liver microsomes from adrenalectomized males were not significantly affected (Fig. 2). The V_{max} value for the aniline hydroxylation was significantly increased but the K_m value was not altered in liver microsomes from the diabetic males.

TABLE 8. KINETIC CONSTANTS FOR HEXOBARBITAL, AND ANILINE IN MICROSOMES FROM ADRENALECTOMIZED OR ALLOXAN DIABETIC MALE RATS

	Spectral change				Hydroxylation			
	K_s (mM)		ΔA_{max} ($\Delta A \times 10^3$/mg protein)		K_m (mM)		V_{max} (mμmole/mg protein/min)	
(A) Hexobarbital								
Control	0·065		18·1		0·55		7·46	
Adrenalectomy	0·110	(+75%)*	9·5	(−48%)*	0·94	(+70%)*	4·61	(−38%)*
Alloxan diabetes	0·106	(+63%)*	15·5	(−14%)	1·00	(+82%)*	5·06	(−32%)*
(B) Aniline								
Control	0·75		14·9		0·098		0·72	
Adrenalectomy	0·69	(−8%)	11·7	(−21%)*	0·10ɔ	(+8%)	0·64	(−11%)*
Alloxan diabetes	0·72	(−4%)	19·6	(+32%)*	0·106	(+8%)	1·10	(+52%)*

The K_m (Michaelis constant) and V_{max} (maximum velocity) values were calculated from experiments shown in Figs. 1 and 2. The K_s (spectral dissociation constant) and ΔA_{max} (maximum spectral change) were calculated from experiments shown in Figs. 3 and 4 according to Schenkman *et al.*[13] The results are expressed as averages from four experiments. Pooled livers from four to five rats were used for each experiment. The figures in the parentheses indicate the percentage differences. The asterisks indicate the significant difference ($P < 0·05$) from control values.

As reported in previous papers,[3,13] the K_m value for the hexobarbital hydroxylation is higher in female rats than in males and androgen is assumed to be a factor responsible for the sex difference. The present results, therefore, indicate that the ability of androgen to decrease the K_m value for the hexobarbital hydroxylation by liver microsomes is impaired by adrenalectomy or alloxan diabetes.[3,20] In further experiments, it was observed that the K_m value for the hexobarbital hydroxylation by liver microsomes from the adrenalectomized or diabetic females was not significantly altered.

Kinetic studies on the hexobarbital or aniline interaction with cytochrome P-450 in liver microsomes from adrenalectomized or diabetic male rats. As shown in Fig. 3 and Table 8, adrenalectomy decreased the ΔA_{max} (maximum spectral change) value for the hexobarbital-induced spectral change of cytochrome P-450 by liver microsomes in male rats, whereas it increased the K_s (spectral dissociation constant) value. Alloxan diabetes did not decrease the ΔA_{max} for hexobarbital-induced spectral change, but it increased the K_s value. On the other hand, the K_s values for the aniline-induced spectral change by liver microsomes from the adrenalectomized males were not significantly affected (Fig. 4). The ΔA_{max} value for the aniline-induced spectral change was significantly increased but the K_s value was not altered in the microsomes from the diabetic males.

As reported in previous papers,[3,13] the K_s value for the hexobarbital-induced spectral change is higher in liver microsomes from female rats than in those from

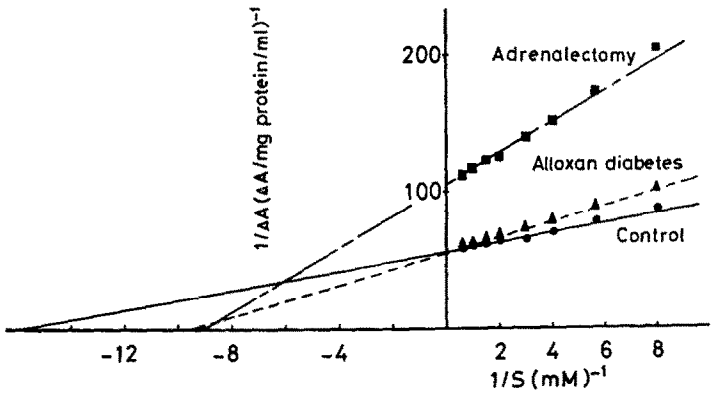

FIG. 3. Reciprocal plot of the spectral change induced by hexobarbital against hexobarbital concentration for liver microsomes from adrenalectomized or diabetic males. The decrement in absorbance between 421 and 500 mμ on addition of various concentrations of hexobarbital was measured from the hexobarbital-induced difference spectrum. The results are expressed by the change of absorbance per mg protein. From these results K_s (spectral dissociation constant) values were calculated according to Schenkman *et al.*[13]

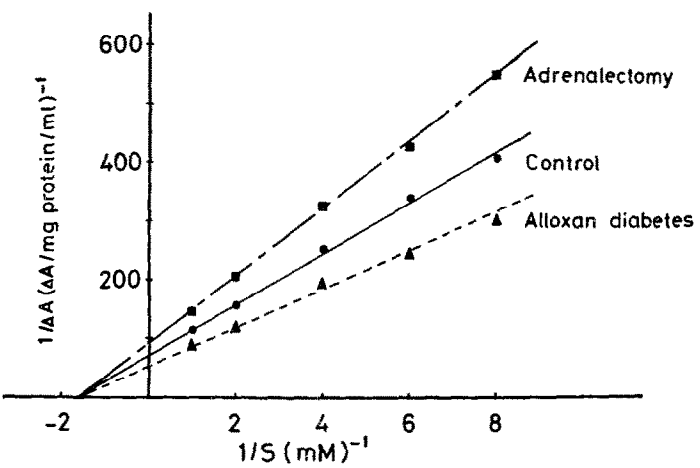

FIG. 4. Reciprocal plot of the spectral change induced by aniline against aniline concentration for liver microsomes from adrenalectomized or diabetic males. The increment of absorbance between 431 and 500 mμ on addition of various concentrations of aniline was measured from the aniline-induced difference spectrum. The results are expressed by the change of absorbance per mg protein. From these results the K_s (spectral dissociation constant) values were calculated according to Schenkman *et al.*[13]

males and androgen is assumed to be a factor responsible for the sex difference. The present results, therefore, indicate that the ability of androgen to decrease the K_s value for the hexobarbital-induced spectral change is impaired by adrenalectomy or alloxan diabetes.[3,20] In further experiments, it was observed that the K_s value for the hexobarbital-induced spectral change by liver microsomes from the adrenalectomized or diabetic females was not significantly altered.

DISCUSSION

The interaction of substrate with cytochrome P-450 causes the spectral change of liver microsomes, and the substrate interaction with P-450 is assumed to be an initial step for the oxidation of drugs.[9,10] The magnitude of spectral change induced by hexobarbital or aminopyrine and the hexobarbital hydroxylation or aminopyrine *N*-demethylation are regulated by androgen.[3,13] In the present studies, it has been shown that adrenalectomy or alloxan diabetes decreased the magnitude of hexobarbital- or aminopyrine-induced spectral change per unit of microsomal protein and of cytochrome P-450 in liver microsomes of male rats, whereas in the females the spectral change was not significantly decreased (Tables 2 and 6).

These results indicate that the binding capacity of P-450 for these substrates is impaired by adrenalectomy or alloxan diabetes.[3] Since the alteration in the hexobarbital hydroxylation or aminopyrine *N*-demethylation by adrenalectomy or alloxan diabetes ran parallel to that observed in the magnitude of the spectral change, the decreased capacity of cytochrome P-450 to interact with the substrate is assumed to be a factor responsible for the decrease in the oxidation of the drugs.[20,21] In addition, significant decrease in the ratio of the hydroxylating activity to the magnitude of the spectral change for hexobarbital was observed in liver microsomes from diabetic males. These results suggest that the efficiency of hydroxylation of bound substrate with cytochrome P-450 was decreased probably through a decrease in the rate of the substrate-bound P-450 by NADPH-linked electron transport system.[22,23]

On the other hand, the magnitude of aniline-induced spectral change per unit of microsomal protein was slightly decreased in the adrenalectomized rats, whereas that of P-450 was unaffected (Table 3). Alloxan diabetes did not significantly affect the magnitude of aniline-induced spectral change per unit of microsomal protein and of cytochrome P-450. These results, therefore, indicate that the binding capacity of P-450 for aniline is not impaired by adrenalectomy or alloxan diabetes. Similarly, the binding capacity of P-450 for zoxazolamine does not seem to be impaired.

The spectral change induced by aniline is type II spectral change, whereas the spectral change induced by hexobarbital or aminopyrine is type I.[10] Therefore, it is conceivable that the difference in the effect of adrenalectomy or alloxan diabetes may be related to the difference in the type of the spectral change. However, as shown in Table 5, the spectral change induced by zoxazolamine is type I,[19] but the magnitude of the spectral change is not dependent on the action of androgen as aniline-induced spectral change.[3,19] These results suggest that the failure of adrenalectomy or diabetes to decrease the magnitude of spectral change induced by aniline is not related to the type of spectral change, but may be related to the fact that the binding capacity of cytochrome P-450 for aniline is not dependent on the action of androgen.[3,13]

The results of the kinetic studies on the substrate-induced spectral changes and the hydroxylation of drugs showed that the K_s value for the spectral change induced by hexobarbital or aminopyrine is increased by adrenalectomy or alloxan diabetes in agreement with the increase in the K_m value for hexobarbital hydroxylation or aminopyrine *N*-demethylation (Table 8).[3] In contrast, the K_s and K_m values for the aniline-induced spectral change and aniline hydroxylation by liver microsomes from male rats were not significantly affected by adrenalectomy or alloxan diabetes. The administration of androgen increases both K_s and K_m values for hexobarbital and aminopyrine.[3] These results indicate that adrenalectomy or alloxan diabetes impairs the

action of androgen on the K_s and K_m values as well as on the ΔA_{max} and V_{max} values.

In further experiments, it was observed that adrenalectomy or alloxan diabetes did not cause a further decrease in the binding capacity of P-450 for hexobarbital in the castrated males, but it caused a significant decrease in the binding capacity in the testosterone-treated castrated males as in the intact males as observed in the case of morphine treatment.[20]

Therefore, the results obtained in the present investigation indicate that adrenalectomy or alloxan diabetes impaired the stimulatory action of androgen on the binding capacity of cytochrome P-450 to interact with hexobarbital or aminopyrine and then resulted in the decrease in oxidating activity for hexobarbital or aminopyrine. These results give further evidence to support the view that the substrate interaction with cytochrome P-450 is one of the rate-limiting steps for the overall oxidation of drugs by liver microsomes.[12, 23, 24]

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