SPECIES DIFFERENCE IN DRUG METABOLISM
BY LIVER MICROSOMES IN ALLOXAN
DIABETIC OR FASTED ANIMALS
(II) THE SUBSTRATE INTERACTION WITH
CYTOCHROME P-450 IN DRUG OXIDATION

RYUICHI KATO, KIN-ICHI ONODA AND AKIRA TAKANAKA

Department of Pharmacology, National Institute of Hygienic Sciences, Setagaya-ku, Tokyo

Received for publication June 10, 1970

Imai and Sato (1), and Schenkman et al. (2) showed that a number of drugs which are substrates of hepatic microsomal monooxygenase react with a microsomal cytochrome called cytochrome P-450 to give characteristic types of spectral change. These results have suggested that the spectral changes observed are indicative of substrate interaction for enzymic hydroxylation (2) and that the magnitude of the substrate binding with cytochrome P-450 is one of important factors controlling the rate of the over-all hydroxylation of drugs by liver microsomes (3-6).

In the preceding paper (7), it was demonstrated that, in contrast to the results obtained with male rats, the hexobarbital hydroxylation by liver microsomes was not decreased in alloxan diabetic or fasted male mice and rabbits. These results supported the hypothesis that the decrease in the hexobarbital hydroxylation in alloxan diabetic or fasted rats is due to an impairment of the androgen-dependent stimulating mechanism for drug-metabolizing enzymes (8, 9). It has been reported that the magnitude of hexobarbital-induced spectral change was greater in male rats than in the females, and that androgen administration increased the magnitude of the spectral change (3, 6). On the other hand, there was no clear sex difference in the magnitude of hexobarbital-induced spectral change in liver microsomes from mice and rabbits and the administration of androgen did not stimulate the magnitude of the spectral change (6, 10).

In the present studies, therefore, the effect of alloxan diabetes or starvation on the binding of cytochrome P-450 with substrate in liver microsomes of both sexes of rats, mice and rabbits was investigated in relation to the effect on the hydroxylating activity for hexobarbital and aniline.

MATERIALS AND METHODS

Male and female rats of the Wistar strain, weighing about 180 and 160 g, respectively, male and female mice of the dd strain, weighing about 26 and 22 g, respectively, and male and female rabbits, weighing about 2.3 and 2.0 kg, respectively, were used.
rats and mice were treated subcutaneously with alloxan, 170 and 410 mg/kg, respectively, 5 days before the experiment and the rats, mice and rabbits were fasted for 48, 36 and 72 hours, respectively, before killing.

Liver microsomes were prepared as described in a previous paper (4). The spectral change induced by hexobarbital or aniline was determined as described in the previous paper (4). The difference spectrum was recorded at room temperature in a Hitachi EPS-3T recording spectrophotometer with an integral sphere attachment. The concentrations of hexobarbital and aniline were 1.6 and 2 mM respectively, and microsomal protein concentration was about 1.2 mg/ml.

The content of microsomal protein was measured according to the method of Lowry et al. (11). The cytochrome P-450 content was determined by the difference spectrum of the carbon monoxide complex as described in the previous paper (4), and the results were expressed as \( \mu \text{mole} \) per mg microsomal protein according to Omura and Sato (12). The determination of hexobarbital and aniline hydroxylation by liver microsomes was carried out as reported in the preceding paper (7).

**RESULTS**

1. **Effect of alloxan diabetes or starvation on the spectral change induced by hexobarbital in liver microsomes**

As shown in Tables 1 and 2, the hexobarbital-induced spectral change was greater in male rats than in the females when expressed as per unit of microsomal protein as well as per unit of cytochrome P-450, but there was no clear sex difference in mice and rabbits (7). These results indicated that the amount of hexobarbital binding with cytochrome

| Table 1. Effect of alloxan diabetes on the spectral change induced by hexobarbital in liver microsomes. |
|---------------------------------------------------------------|
| Species | Sex | Hexobarbital-induced spectral change |
|         |     | Control | Alloxan diabetes | Difference (%) |
|         |     | (\( \mu \text{OD} \times 10^3 / \text{mg protein/mI} \)) | (\( \mu \text{OD} \times 10^3 / \text{\mu m mole P-450/mI} \)) |
| Rats    | M   | 17.9±1.1 | 13.6±1.0          | -24*           |
|         | F   | 6.0±0.6  | 8.3±1.0           | +36*           |
| Mice    | M   | 7.3±0.5  | 7.8±0.7           | +6             |
|         | F   | 7.4±0.5  | 8.2±0.6           | +11            |
| Rats    | M   | 18.8±0.9 | 12.5±0.9          | -34*           |
|         | F   | 8.5±0.6  | 9.6±0.8           | +13            |
| Mice    | M   | 6.8±0.3  | 6.6±0.5           | -3             |
|         | F   | 7.2±0.5  | 7.3±0.6           | -1             |

The rats and mice were treated subcutaneously with alloxan, 170 and 410 mg/kg, respectively, 5 days before the experiment. The results are expressed by the difference in the optical density between 421 \( \mu \) and 500 \( \mu \) on addition of hexobarbital and given as means±S.E. from 8-12 determinations. Pooled livers from 3 mice were used for one determination. The asterisks indicate significant difference (\( p<0.05 \)) from control values.
TABLE 2. Effect of starvation on the spectral change induced by hexobarbital in liver microsomes.

| Species | Sex | Hexobarbital-induced spectral change | Control | Starvation | Difference (%) |
|---------|-----|--------------------------------------|---------|------------|----------------|
|         |     | (\OD \times 10^4/mg protein/ml)      |         |            |                |
| Rats    | M   | 18.8±0.9                             | 16.6±1.2| -12        |
|         | F   | 6.0±0.4                              | 9.1±0.5 | +52*       |
| Mice    | M   | 7.1±0.5                              | 8.3±0.6 | +17        |
|         | F   | 7.6±0.4                              | 8.6±0.9 | +13        |
| Rabbits | M   | 15.2±1.1                             | 17.0±1.5| +12        |
|         | F   | 16.3±1.0                             | 17.3±1.6| +6         |
|         |     | (\OD \times 10^4/m\mu mole P-450/ml)|         |            |                |
| Rats    | M   | 19.0±1.0                             | 14.1±0.6| -26*       |
|         | F   | 8.2±0.4                              | 8.8±0.7 | +8         |
| Mice    | M   | 6.3±0.4                              | 5.8±0.6 | -8         |
|         | F   | 7.3±0.5                              | 6.4±0.8 | -12        |
| Rabbits | M   | 8.3±0.6                              | 9.5±0.9 | +14        |
|         | F   | 7.9±0.5                              | 9.9±0.8 | +13        |

The rats, mice and rabbits were fasted for 48, 36 and 72 hours, respectively before the experiment. The results are given as means ± S.E. from 8-12 determinations. Pooled livers from 3 mice were used for one determination. The asterisks indicate significant difference (p<0.05) from control values.

P-450 and the binding capacity of cytochrome P-450 in liver microsomes are higher in male rats than in the females.

The magnitude of spectral change induced by hexobarbital was significantly decreased in liver microsomes from the diabetic male rats, but it was increased in the microsomes from the diabetic female rats and not significantly affected in those from the diabetic male and female mice (Table 1). On the other hand, the starvation resulted in an increase in the magnitude of the spectral change per unit of microsomal protein in female rats, but it did not result in any significant change in male rats and both sexes of mice and rabbits. However, the magnitude of the spectral change per unit of cytochrome P-450 was decreased only in liver microsomes from the diabetic or fasted male rats and it was not significantly affected in the other species of animals.

The increase in the magnitude of spectral change per unit of microsomal protein induced by hexobarbital in the microsomes from the diabetic or fasted female rats was indicative of an increase in the amount of cytochrome P-450-hexobarbital complex. However, since the magnitude of the spectral change per unit of cytochrome P-450 in liver microsomes from these animals was not significantly affected, the increased spectral change per mg microsomal protein may be due to an increase in the content of cytochrome P-450. On the other hand, the decrease in the magnitude of the spectral change per unit of cytochrome P-450 in liver microsomes from the diabetic or fasted male rats was indicative of a decrease in the binding capacity of cytochrome P-450 with hexobarbital.
2. Effect of alloxan diabetes or starvation on the spectral change induced by aniline in liver microsomes

In contrast to the case of hexobarbital, there was no clear sex difference in liver microsomes of rats as well as in those of mice and rabbits concerning the magnitude of the spectral change induced by aniline per unit of microsomal protein and of cytochrome P-450 (7).

The magnitude of spectral change induced by aniline was increased in liver microsomes from the diabetic rats but not in those from the diabetic mice (Table 3). However, the magnitude of the spectral change per unit of cytochrome P-450 was not significantly affected in liver microsomes from these animals.

| Species | Sex | Aniline-induced spectral change |
|---------|-----|--------------------------------|
|         |     | Control | Alloxan diabetes | Difference (%) |
|         |     | (ΔOD × 10⁶/mg protein/ml) |
| Rats    | M   | 13.1±0.7 | 15.4±0.9 | +18* |
|         | F   | 9.9±0.4  | 12.6±0.7 | +27* |
| Mice    | M   | 15.1±0.3 | 16.5±0.5 | +9  |
|         | F   | 14.0±0.5 | 15.2±0.6 | +9  |

See the legends for Table 1. The results are expressed by the difference in the optical density between 430 mμ and 500 mμ on addition of aniline.

| Species | Sex | Aniline-induced spectral change |
|---------|-----|--------------------------------|
|         |     | Control | Starvation | Difference (%) |
|         |     | (ΔOD × 10⁶/mg protein/ml) |
| Rats    | M   | 12.4±0.8 | 15.9±1.2 | +28* |
|         | F   | 9.1±0.5  | 14.0±0.9 | +53* |
| Mice    | M   | 14.3±0.3 | 18.3±0.6 | +27* |
|         | F   | 13.8±0.5 | 17.9±0.8 | +29* |
| Rabbits | M   | 21.0±1.4 | 21.4±1.9 | +2  |
|         | F   | 22.3±1.2 | 21.5±2.0 | -4  |

See the legends for Tables 2 and 3.
On the other hand, the magnitude of spectral change induced by aniline was increased in the microsomes from the fasted rats and mice, but not in those from the fasted rabbits. However, the magnitude of the spectral change per unit of cytochrome P-450 was not significantly affected in liver microsomes from these animals (Table 4). These results indicated that the increase in the magnitude of the spectral change per unit of microsomal protein is due to the increased amount of cytochrome P-450 in microsomes from the diabetic or fasted animals.

3. Effect of alloxan diabetes or starvation on the ratio of hexobarbital hydroxylation to the spectral change in liver microsomes

The hexobarbital hydroxylation per unit of the magnitude of spectral change induced by hexobarbital was decreased in liver microsomes from the diabetic or fasted male rats (Table 5). These results indicated that the ratio of hexobarbital hydroxylation to the amount of cytochrome P-450-hexobarbital complex was decreased in the microsomes from the diabetic or fasted male rats in addition to the decrease in the binding capacity of cytochrome P-450 (13–15). On the other hand, the hexobarbital hydroxylation per unit of the magnitude of the spectral change was not significantly affected in the microsomes from the diabetic or fasted female rats, and mice and rabbits of both sexes.

4. Effect of alloxan diabetes or starvation on the ratio of aniline hydroxylation to the spectral change in liver microsomes

The aniline hydroxylation per unit of the magnitude of spectral change induced by aniline was increased in liver microsomes from the diabetic male and female rats, but it was not significantly affected in those from the diabetic mice (Table 6). These results indicated that the ratio of aniline hydroxylation to the amount of cytochrome P-450-

---

**Table 5. Effect of alloxan diabetes or starvation on the ratio of hexobarbital hydroxylation to the spectral change by liver microsomes.**

| Species | Sex | Hexobarbital hydroxylation (μmole/μOD x 10³/30 min) | Difference (%) |
|---------|-----|-----------------------------------------------------|----------------|
|         |     | Control                                             | Alloxan diabetes |                  |
| Rats    | M   | 6.12 ± 0.54                                         | 4.42 ± 0.43      | -28*            |
|         | F   | 6.88 ± 0.68                                         | 6.50 ± 0.59      | -5              |
| Mice    | M   | 5.54 ± 0.49                                         | 6.26 ± 0.57      | +15             |
|         | F   | 5.51 ± 0.51                                         | 6.20 ± 0.48      | +15             |
| Rats    | M   | 5.87 ± 0.54                                         | 4.37 ± 0.51      | -25*            |
|         | F   | 6.82 ± 0.60                                         | 5.77 ± 0.60      | -15             |
| Mice    | M   | 5.66 ± 0.32                                         | 5.34 ± 0.40      | -6              |
|         | F   | 5.44 ± 0.30                                         | 5.20 ± 0.52      | -4              |
| Rabbits | M   | 5.45 ± 0.42                                         | 5.25 ± 0.59      | -4              |
|         | F   | 4.80 ± 0.41                                         | 5.08 ± 0.51      | +6              |

See the legends for Tables 1 and 2. The hydroxylating activity (μmole/ mg microsomal protein/30 min) is expressed as the ratio to the magnitude of spectral change (μOD x 10³/mg microsomal protein).
TABLE 6. Effect of alloxan diabetes or starvation on the ratio of aniline hydroxylation to the spectral change by liver microsomes.

| Species | Sex | Aniline hydroxylation |
|---------|-----|-----------------------|
|         |     | (mumole/mOD x 10³/30 min) | Control | Alloxan diabetes | Difference (%) |
| Rats    | M   | 1.55±0.13              | 1.95±0.17  | +25*             |
|         | F   | 1.57±0.16              | 2.01±0.23  | +28*             |
| Mice    | M   | 2.56±0.12              | 2.90±0.17  | +13              |
|         | F   | 2.64±0.13              | 3.11±0.20  | +12              |
| Rats    | M   | 1.70±0.14              | 1.86±0.17  | +10              |
|         | F   | 1.75±0.16              | 2.00±0.21  | +14              |
| Mice    | M   | 2.72±0.12              | 2.93±0.15  | +8               |
|         | F   | 2.68±0.11              | 2.76±0.17  | +3               |
| Rabbits| M   | 1.29±0.10              | 1.59±0.16  | +32*             |
|         | F   | 1.68±0.08              | 1.41±0.17  | +31*             |

See the legends for Tables 1, 2 and 5.

Moreover, the aniline hydroxylation per unit of the magnitude of the spectral change in liver microsomes was not affected in the fasted rats and mice, but it was increased in the fasted rabbits. These results indicated that the ratio of aniline hydroxylation to the amount of cytochrome P-450-aniline complex was increased in liver microsomes from the fasted rabbits (13–15).

DISCUSSION

In the present studies, it has been demonstrated that the binding capacity of cytochrome P-450 with hexobarbital in liver microsomes was decreased in the diabetic and fasted male rats, but it was not affected in all other diabetic or fasted animals. Moreover, the binding capacity of cytochrome P-450 with aniline in liver microsomes was not affected in rats, mice and rabbits. On the other hand, these results indicated that the increase in spectral change induced by hexobarbital in liver microsomes from the diabetic or fasted female rats and the increase in spectral change induced by aniline in the microsomes from the diabetic rats and the fasted rats and mice were due to the increases in the amount of cytochrome P-450 in these animals as reported in the preceding paper (7).

It has been shown that the binding capacity of cytochrome P-450 for hexobarbital in rat liver microsomes was dependent upon the androgen-dependent stimulating mechanism, while the binding capacity for aniline was independent, and that this androgen-dependent stimulating mechanism is assumed to be absent in mice and rabbits (3, 6). These results therefore are in accordance with the postulation in the previous paper that decrease in the binding capacity of cytochrome P-450 for hexobarbital in liver microsomes from the diabetic or fasted male rats may be due to an impairment of the ability of androgen to stimulate the binding capacity (6).
The ratio of hexobarbital hydroxylation to the magnitude of the spectral change was decreased in the liver microsomes from diabetic or fasted male rats, but it was not affected in those from other animals. On the other hand, the ratio of aniline hydroxylation to the magnitude of the spectral change was increased in the microsomes from the diabetic rats and fasted rabbits. These results indicated that the ratio of hexobarbital hydroxylation to the amount of cytochrome P-450-hexobarbital complex was decreased in the microsomes from the diabetic or fasted male rats. The decrease in this ratio may be related to either a decrease in the rate of cytochrome P-450-hexobarbital complex by NADPH-cytochrome P-450 reductase or a decrease in the transfer of activated oxygen from cytochrome P-450 into hexobarbital molecule (the efficiency of hydroxylation). On the other hand, the ratio of aniline hydroxylation to the amount of cytochrome P-450-aniline complex was increased in liver microsomes from the diabetic rats and fasted rabbits and these results may be related to either an increase in the rate of cytochrome P-450-aniline complex by NADPH-cytochrome P-450 reductase or an increase in the efficiency of the hydroxylation.

It is recently suggested that the binding of the substrate with cytochrome P-450 modifies the rate of the reduction of cytochrome P-450, and that the binding of hexobarbital which induces type I spectral change increases the rate of the cytochrome P-450 reduction, whereas the binding of aniline which induces type II spectral change decreases the rate of the cytochrome P-450 reduction (13, 14). The binding site of hexobarbital and aniline has been assumed to be different. Therefore it is possible that the alloxan diabetes and starvation may modify the rate of reduction of substrate-binding cytochrome P-450 according to the binding nature of the substrate. However, the mechanism by which the species differences were produced in the effects of alloxan diabetes and starvation on the ratio of hydroxylations of hexobarbital and aniline to the amount of cytochrome P-450-hexobarbital complex is not yet known.

In addition, it is clearly demonstrated that, in contrast to the results obtained with rats, there is no clear sex difference concerning the effect of alloxan diabetes and starvation on the magnitude of spectral change and the binding capacity of cytochrome P-450 with hexobarbital in liver microsomes from mice and rabbits. These species differences appear to be related to the absence of sex difference in the intact mice and rabbits (7).

**SUMMARY**

The magnitude of spectral change per unit of protein induced by hexobarbital was significantly decreased in liver microsomes from alloxan diabetic male rats, but it was increased in those from the diabetic female rats. On the other hand, the magnitude of the spectral change was not significantly affected in liver microsomes from the diabetic male and female mice. Starvation resulted in the increase in the magnitude of the spectral change in the microsomes of female rats, but it did not result in any significant change in the microsomes of male rats and both sexes of mice and rabbits.
However, the magnitude of the spectral change per unit of cytochrome P-450 was decreased only in the microsomes from the diabetic or fasted male rats and it was not significantly affected in the other species of animals. These results indicated that the alloxan diabetes or starvation decreased the binding capacity of cytochrome P-450 with hexobarbital in liver microsomes of male rats, but not in those of male mice and rabbits.

On the other hand, the magnitude of spectral change of cytochrome P-450 induced by aniline was increased in the microsomes from the diabetic rats and fasted rats and mice, but it was not significantly altered in those from the diabetic mice and fasted rabbits. However, the magnitude of the spectral change per unit of cytochrome P-450 was not affected in liver microsomes from the diabetic or fasted animals.

These results are in accordance with our postulation that the decrease in the binding capacity of cytochrome P-450 for hexobarbital in liver microsomes from the diabetic or fasted male rats is caused through an impairment of the ability of androgen to stimulate the binding capacity of cytochrome P-450 with hexobarbital.

REFERENCES

1) Imai, Y. and Sato, R.: J. Biochem. 62, 239 (1967)
2) Schenkman, J.B., Remmer, H. and Estabrook, R.W.: Mol. Pharmac. 3, 113 (1967)
3) Schenkman, J.B., Frey, I., Remmer, H. and Estabrook, R.W.: Mol. Pharmac. 3, 516 (1967)
4) Kato, R., Takanaka, A. and Takayanaghi, M.: J. Biochem. 68, 395 (1970)
5) Kato, R., Takanaka, A. and Onoda, K.: J. Biochem. 66, 739 (1969)
6) Kato, R. and Onoda, K.: Biochem. Pharmac. 19, 1649 (1970)
7) Kato, R., Onoda, K. and Takanaka, A.: Jap. J. Pharmac. 20, 546 (1970)
8) Kato, R. and Gillette, J.R.: J. Pharmac. exp. Ther. 150, 279 (1965)
9) Kato, R. and Gillette, J.R.: J. Pharmac. exp. Ther. 150, 285 (1965)
10) Kato, R., Onoda, K. and Takayanaghi, M.: Jap. J. Pharmac. 20, 157 (1970)
11) Lowry, O.H., Rosebrough, H.J., Farr, A.L. and Randall, R.J.: J. biol. Chem. 193, 265 (1951)
12) Omura, T. and Sato, R.: J. biol. Chem. 239, 2370 (1964)
13) Holtzman, J.L., Gram, T.E., Gigon, P.L. and Gillette, J.R.: Biochem. J. 110, 407 (1968)
14) Gigon, P.L., Gram, T.E. and Gillette, J.R.: Mol. Pharmac. 5, 109 (1969)
15) Kato, R., Takanaka, A. and Takahashi, A.: J. Biochem. 68, 613 (1960)