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Effect of Environmental Temperature on Cytochrome P-450 and the Associated Electron Transfer System: Proposal for the Appropriate Dosage Regimen Corresponding to Seasonal Change

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The effect of environmental temperature on cytochrome P-450 and the associated electron transfer system was studied in male rats. Rats kept at 15 °C for two weeks (group A) showed a significant increase in the activity of nicotinamide adenine dinucleotide phosphate reduced form (NADPH)–cytochrome c (P-450) reductase, whereas they showed a slight reduction in the content of cytochrome P-450 when compared with rats kept at 30 °C for two weeks (group B). Further, aniline hydroxylase activity in group A increased significantly, while aminopyrine N-demethylase activity was unchanged. The activity of NADH–cytochrome b_5 reductase and the content of cytochrome b_5 showed no significant difference between the two groups. From the endocrinological viewpoint, the serum level of 3,5,3′-triiodothyronine (T_3) in group A was higher than that in group B. The differences in enzyme activity and content were observed from 4 d after the start of exposure of rats to each temperature.

Keywords——cytochrome P-450; NADPH–cytochrome c (P-450) reductase; cytochrome b_5; NADH–cytochrome b_5 reductase; temperature change; thyroid hormone

It has been demonstrated that variations of environmental temperature influence the pharmacological effects of drugs[1–3] and exposure to a high environmental temperature increases the toxicity of many drugs.[4,5] The reason for this may be related to change of drug metabolism. Lockwood and Houston[6] reported that the chronic or acute exposure of rats to a low environmental temperature (4 °C for 4 d) caused an increase in hepatic N-demethylation of aminopyrine and its monomethyl metabolite. Furthermore, Kaplanski and Ben-Zvi[7] demonstrated that the chronic exposure of rats to high temperature (35 °C for 1 month) resulted in a reduction in hepatic N-demethylation of *p*-chloro-*N*-methylaniline and aniline hydroxylation.

Research has spread from model compounds to clinically inportant drugs. According to Albin *et al.*,[8] the elimination half-lives for theophylline, digoxin and propranolol in male rats were significantly prolonged by chronic hyperthermia (35 °C for 1 month). They suggested this was due to a reduction in cytochrome P-450 content. However, the exact mechanisms of these phenomena remain to be unsettled.

In order to clarify the relationship between temperature and liver microsomal mixed function oxidases, we examined microsomes prepared from male Wister rats kept at 15 °C (group A) and 30 °C (group B) for 2 weeks. Acclimation processes of mixed function oxidases corresponding to the change of temperature were also examined.

Experimental

Materials and Reagents——Cytochrome c was obtained from Sigma Chemical Company, St. Louis, U.S.A. Nicotinamide adenine dinucleotide (reduced form; NADH), nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. Both aminopyrine and aniline were obtained from Aldrich Chemical Company, Inc., Milwaukee, U.S.A. Other common chemicals were purchased from commercial sources and used without further purification.

Treatment of Animals——Male Wister rats weighing 230 to 250 g were divided into two groups; one group (group A) was kept at a constant temperature of 15 ± 1 °C under 60% relative humidity, and the other group (group B) was kept at a constant temperature of 30 ± 1 °C under the same humidity. The light (2000 lux) was on from 6 a.m. to 6 p.m. in both cases. The rats, which had been kept on a commercial diet (Oriental Yeast Co., Ltd. Tokyo, Japan), were fasted for 24 h prior to the experiment. Water was given *ad libitum*. On the day of the experiment, all animals were weighed and killed by decapitation between 9:00 to 10:00 a.m.

Preparation of Microsomes——The liver was excised quickly, washed, weighed, and homogenized in 3 vol. of 0.25 M sucrose solution containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris–HCl buffer (pH 7.4) in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Homogenates were centrifuged at $9000 \times g$ for 20 min and aliquots of the supernatant fraction were further centrifuged in Hitachi model 55P-7 preparative ultracentrifuge at $105000 \times g$ for 60 min to obtain microsomes. Microsomal pellets were resuspended in 0.15 M KCl containing 50 mM Tris–HCl buffer (pH 7.4) at a concentration of 5 to 6 mg protein per milliliter. All steps for the preparation of microsomes were performed at 4 °C.

Assay of Enzyme Activities——The contents of cytochrome P-450 and cytochrome b_5 in isolated microsomes were determined according to the methods of Matsubara *et al.*[9] and Oshino and Sato,[10] respectively. The activities of NADPH–cytochrome c (P-450) reductase and NADH–cytochrome b_5 reductase were measured by the methods of Omura and Takesue[11] and Takesue and Omura,[12] respectively. Microsomal aminopyrine N-demethylase activity was measured by the method of Nash.[13] Microsomal aniline hydroxylase activity was measured by the method of Imai *et al.*[14] Protein determination was performed by the method of Lowry *et al*,[15] using bovine serum albumin (Fraction V) as a standard. All assays described above were done by using Shimadzu MPS-2000 spectrometer.

Determination of Serum Thyroxin (T_4) and 3,5,3′-Triiodothyronine (T_3) in Rats——Blood samples were collected *via* Jugular puncture and samples were kept on ice until the serum was separated by centrifugation ($900 \times g$ for 20 min at 4 °C). The serum was then kept at −15 °C till the assay for T_4 and T_3. Serum levels for T_4 and T_3 were determined by using a Clinical Assays Gammacoat T_4 radioimmunoassay kit and a Gammacoat T_3 radioimmunoassay kit (Japan Travenol Laboratories Inc. Tokyo, Japan), respectively.

Results

The effects of environmental temperature on body weights, liver weights and liver microsomal mixed function oxidase are summarized in Table I. Exposure of rats to a temperature of 15 °C for two weeks gave rise to a significant ($p < 0.05$) increase in liver weight and liver weight per body weight ($p < 0.01$) compared with rats exposed to 30 °C for two weeks. As for the effect of temperature on the microsomal electron transfer system, there were

Table I. Effects of Environmental Temperature on Body Weight, Liver Weight, Liver Weight per Body Weight and Hepatic Microsomal Mixed Function Oxidase Activity

	Group A (15 °C)	Group B (30 °C)
Body weight (g)	296±7	286±6
Liver weight (g)	12.49±0.39[c]	10.90±0.32
Liver weight/body weight (×100)	4.22±0.02[d]	3.81±0.04
Cytochrome P-450[a]	0.560±0.018[c]	0.657±0.035
NADPH–cytochrome c (P-450) reductase[b]	0.072±0.004[d]	0.049±0.005
Cytochrome b_5[a]	0.450±0.016	0.491±0.045
NADH–cytochrome b_5 reductase[b]	3.29±0.04	3.77±0.36

Rats were kept at 15 °C (A group) or 30 °C (B group) for 14 d. Each value represents the mean ± S.E. of 6 rats. *a*) nmol mg protein^{-1}. *b*) μmol min^{-1} mg protein^{-1}. Significantly different from the corresponding mean of group B, *c*) $p < 0.05$ or *d*) $p < 0.01$ by F-test.

TABLE II. Effects of Environmental Temperature on Aniline Hydroxylase and Aminopyrine N-Demethylase Activities

	Group A (15 °C)	Group B (30 °C)
Aniline hydroxylase[a)]	0.713±0.040[b)]	0.504±0.052
Aminopyrine N-demethylase[a)]	12.25±0.77	11.56±0.59

Rats were kept at 15 °C (A group) or 30 °C (B group) for 14 d. Each value represents the mean ± S.E. of 6 rats. *a*) nmol min^{-1} mg $protein^{-1}$. Significantly different from the corresponding mean of group B, *b*) $p<0.05$ by F-test.

TABLE III. Effects of Environmental Temperature on Thyroxin (T_4) and 3,5,3′-Triiodothyronine (T_3) Levels in Rats

	Group A (15 °C)	Group B (30 °C)
Thyroxin ($\mu g\,dl^{-1}$)	5.35±0.41	4.80±0.31
3,5,3′-Triiodothyronine ($ng\,dl^{-1}$)	82.0±5.8[a)]	67.0±3.6

Rats were kept at 15 °C (A group) or 30 °C (B group) for 14 d. Each value represents the mean ± S.E. of 6 rats. Significantly different from the corresponding mean of group B, *a*) $p<0.05$ by F-test.

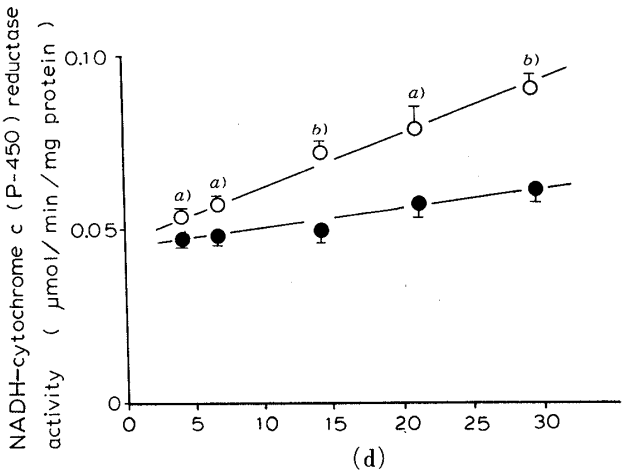

Fig. 1. Acclimation Process of NADPH–Cytochrome c (P-450) Reductase Activity in Microsomes Prepared from Rats Kept at 15 °C and 30 °C

—○—, 15 °C (group A); —●—, 30 °C (group B). Significantly different from the corresponding mean of group B; *a*) $p<0.05$; *b*) $p<0.01$.

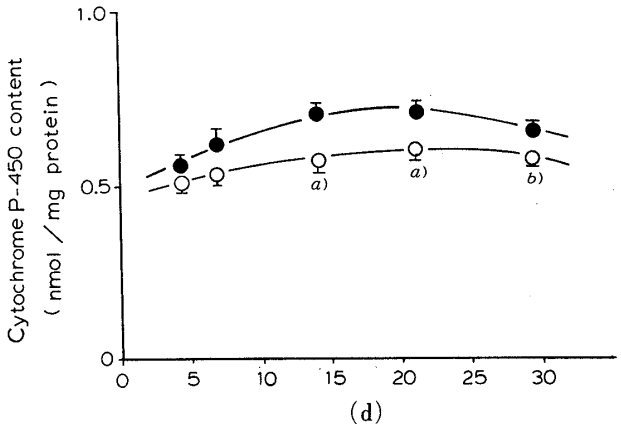

Fig. 2. Acclimation Process of Cytochrome P-450 Content in Microsomes Prepared from Rats Kept at 15 °C and 30 °C

—○—, 15 °C (group A); —●—, 30 °C (group B). Significantly different from the corresponding mean of group B; *a*) $p<0.05$; *b*) $p<0.01$.

significant differences in the activity of NADPH–cytochrome c (p-450) reductase ($p<0.01$) and the content of cytochrome P-450 ($p<0.05$). The activity of NADPH–cytochrome c (P-450) reductase in group A was approximately 1.5 times higher than that in group B. However, the content of cytochrome P-450 in group A was slightly lower than that in group B; this difference was significant in terms of the F-test ($p<0.05$). On the other hand, there was no significant difference in the activity of NADH–cytochrome b_5 reductase or the content of cytochrome b_5 between the two groups.

Table II shows the effect of environmental temperature on the *in vitro* hepatic aminopyrine N-demethylase and aniline hydroxylase activities. There was a significant

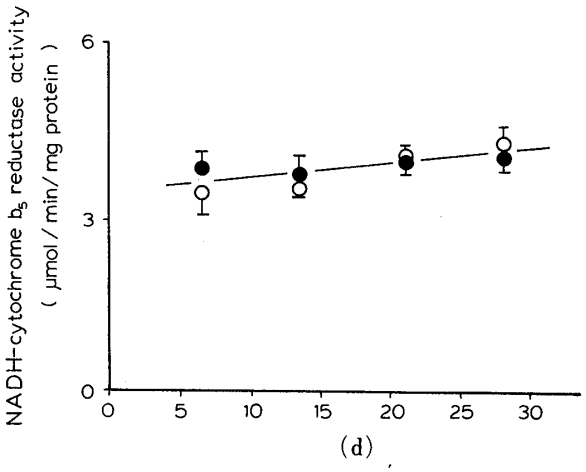

Fig. 3. Acclimation Process of NADH–Cytochrome b_5 Reductase Activity in Microsomes Prepared from Rats Kept at 15 °C and 30 °C
—○—, 15 °C (group A); —●—, 30 °C (group B).

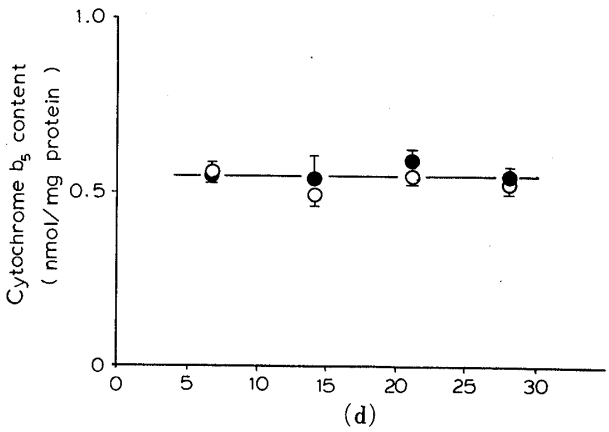

Fig. 4. Acclimation Process of Cytochrome b_5 Content in Microsomes Prepared from Rats Kept at 15 °C and 30 °C
—○—, 15 °C (group A); —●—, 30 °C (group B).

increase in the aniline hydroxylase activity in group A even though the aminopyrine N-demethylase activity was unchanged. The increase of aniline hydroxylase activity was 40% compared with group B. The effects of environmental temperature on thyroid hormones (T_4 and T_3) are shown in Table III. The serum level of T_3 was significantly higher in rats kept at 15 °C for two weeks than in those kept at 30 °C for two weeks. The same tendency was observed in the serum level of T_4, though the difference was not statistically significant.

The acclimation process of mixed function oxidase in liver microsomes prepared from each group was also examined. As presented in Fig. 1, a significant difference in the activity of NADPH–cytochrome c (P-450) reductase between the two groups was first observed at 4 d after the start of feeding rats at each temperature. The difference became larger as time passed. The content of cytochrome P-450 in group A was always lower than that in group B. The difference in cytochrome P-450 content between the groups increased gradually and reached the maximum after two or three weeks, followed by a decrease (Fig. 2). The acclimation process of NADH–cytochrome b_5 reductase activity and cytochrome b_5 content are presented in Figs. 3 and 4, respectively. There was no significant difference in the activity of NADH–cytochrome b_5 reductase or the content of cytochrome b_5 between the two groups at any time. However, the content of cytochrome b_5 in group B tended to be higher than that in group A after two weeks.

Discussion

The results of the present studies show that environmental temperature affects cytochrome P-450 and the associated electron transfer system of liver microsomes.

As described in the preface, Albin *et al.*[8] observed a significant reduction in the metabolism of theophylline, propranolol, and digoxin in chronically heat-exposed rats (35 °C for 1 month) when compared with the control (20—22 °C for 1 month). They speculated that the reduction in the activity of drug metabolism was due to a decrease in the content of cytochrome P-450. In the present work, we obtained the opposite result, *i. e.*, that the content of cytochrome P-450 in group B (30 °C for 2 weeks) was significantly higher ($p < 0.05$) than in group A (15 °C for 2 weeks). However, the activity of NADPH–cytochrome c (P-450) reductase in group B was far lower than that in group A. We suggest that the reduction in the metabolism of drugs after heat exposure of rats was not due to the reduction in the content of

cytochrome P-450 but was due to a significant reduction in the activity of NADPH–cytochrome c (P-450) reductase.

It has been suggested that plasma corticosterone increases in stress situations, followed by changes of drug metabolism.[16] However, Kaplanski and Ben-Zvi[7] demonstrated that there was no difference in plasma corticosterone between a heat-exposed group (35 °C for 1 month) and the control (22 °C for 1 month). Corticosterone seems to be independent of the reduction in drug metabolism in heat-exposed rats. Thyroid hormones may hold the key to the solution of this problem. As is generally known, the secretion of thyroid hormones is controlled by environmental temperature in order to maintain basal metabolism. According to Quimby *et al.*[17] and Osiba,[18] the function of the thyroid gland is accelerated in winter and reduced in summer. In this study, environmental temperature did affect thyroid hormone; the serum level of T_3 was significantly different in the two groups.

Smith *et al.*[19] first pointed out the correlation of thyroid hormone with the content of cytochrome P-450. They reported that thyroid hormone regulated heme oxidation in the liver. Triiodothyronine (T_3), when administered for 5 d at a dose of 6 μg/100 g of body weight/d, stimulated basal heme oxygenase activity 2-fold compared to diluent-treated animals, followed by a decrease of cytochrome p-450 content. On the other hand, Rumbaugh *et al.*[20] reported a relation between thyroid hormone and NADPH–cytochrome c (p-450) reductase. According to their report, thyroxin (T_4) administration at a dose of 5 μg/100 g/d for 7 d to hypophysectomized rats increased the activity of NADPH–cytochrome c (P-450) reductase whereas it reduced the content of cytochrome P-450. Corresponding with the change of enzyme activity, ethylmorphine demethylase, aniline hydroxylase and benzo[a]pyrene hydroxylase were all maximally stimulated following the administration of T_4. When we compare the thyroid hormone level of group A and group B, the serum level of T_3 in group A was significantly higher than that in group B. The T_4 level was also higher in group A than group B, though the difference was not significant. The higher level of thyroid hormone in group A is thus considered to be responsible for the higher level of NADPH–cytochrome c (P-450) reductase activity and slightly lower level of cytochrome P-450.

An interesting observation in the present *in vitro* study was that aniline hydroxylase activity in group A was significantly higher than that in group B even though aminopyrine N-demethylase activity was unchanged, suggesting that NADPH–cytochrome c (P-450) reductase may be the rate-limiting enzyme for aniline hydroxylation but not for aminopyrine N-demethylation. The report from Kitada *et al.*[21] also supports this hypothesis. They examined the effect of addition of purified NADPH–cytochrome c (P-450) reductase in a reconstituted cytochrome P-450 system on drug oxidative activities. Rather than aminopyrine N-demethylation, aniline hydroxylation was enhanced by the addition of NADPH–cytochrome c (P-450) reductase. The hypothesis is consistent with the report by Albin *et al.*[8] that chronic hyperthemia (35 °C for 1 month) prolonged the elimination half-lives of theophylline, digoxin and propranolol, but not that of phenytoin. It appears that phenytoin metabolism is independent of the change of NADPH–cytochrome c (P-450) reductase activity caused by hyperthermia. In other words, NADPH–cytochrome c (P-450) reductase seems to be the rate-limiting enzyme for theophylline, digoxin and propranolol metabolism, but not for phenytoin metabolism.

On the basis of the present observations and the reports of Smith *et al.*[19] and Rumbaugh *et al.*,[20] it is possible to summarize the relationship between environmental temperature and drug metabolism as shown in Fig. 5. A change of environmental temperature affects thyroids hormones first, and then NADPH–cytochrome c (P-450) reductase and heme oxygenase activities are changed as a consequence. However, NADPH–cytochrome c (P-450) reductase is more susceptible to the change of thyroid hormones than cytochrome P-450. Thus, the overall oxidative metabolism is influenced primarily through NADPH–cytochrome c (P-450)

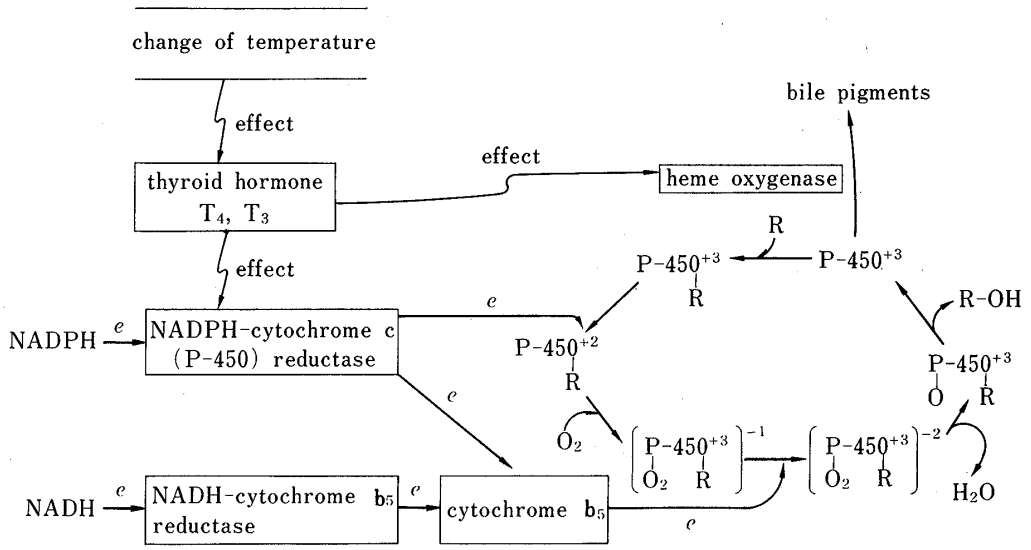

Fig. 5. Effects of Environmental Temperature on the Catalytic Cycle of Cytochrome P-450 and the Associated Electron Transfer System of Liver Microsomes

P-450, cytochrome P-450; R, substrate.

reductase activity when environmental temperature is changed. In particular, drug oxidation for which NADPH–cytochrome c (P-450) reductase is the rate-limiting enzyme, *i. e.*, aniline hydroxylation, is most strongly influenced by the change of temperature.

It is noteworthy that the acclimation of rats to a change of environmental temperature was very fast as judged from the experimental finding that the activity of NADPH–cytochrome c (P-450) reductase in microsomes prepared from rats kept at 15 °C (group A) is significantly higher than in the case of rats kept at 30 °C (group B) after only 4 d. Two weeks is clearly adequate for the acclimation of animals to an environmental temperature.

In the previous work,[22] the effect of environmental temperature on the metabolism of theophylline in rabbits was examined under the same conditions as used in the present experiment. The cross-over experiment between 15 and 30 °C also confirmed the present observations. Rabbits kept at 15 °C for 2 weeks showed a significant increase in the metabolism of theophylline in comparison with those kept at 30 °C. It is very interesting that the effect of environmental temperature on the metabolism of drugs is observed not only in rats but also in rabbits, and even in humans.[23]

Prior to phase I testing of new drugs, pharmacokinetic studies are often performed using volunteers. It seems clear that the effect of seasonal changes of environmental temperature on drug metabolism should be taken into account in evaluating the results.

The range of drug concentration in plasma required to achieve a satisfactory pharmacological response without significant toxicity is known as the therapeutic range. The upper limit of the therapeutic range is defined by the onset of side effects. The therapeutic ranges of digoxin, theophylline and propranolol are very narrow, and if the metabolism of these drugs in man is influenced by changes of temperature, as is the case in rats and rabbits, therapeutic equivalence of formulations throughout all seasons can not be assured. The present work suggests the necessity for establishing appropriate drug administration schedules corresponding to the change of season.

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