Organ-specific properties of cytochromes P-450$_{s21}$ (steroid 21-hydroxylases) of liver and adrenocortical microsomes

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Organ specificities of cytochrome P-450$_{s21}$ (steroid 21-hydroxylase) were investigated. The substrate specificity of the liver cytochrome P-450$_{s21}$ was found to be different from that of adrenocortical cytochrome P-450$_{s21}$. The steroid 21-hydroxylase activity of liver microsomes decreased with treatment by sodium phenobarbital or β-naphthoflavone and was inhibited by anti-cytochrome b_5 immunoglobulin, although that of adrenocortical microsomes was not. The liver cytochrome P-450$_{s21}$ was immunochemically similar to adrenocortical cytochrome P-450$_{s21}$. The bovine liver and adrenocortical cytochromes P-450$_{s21}$ showed immunoprecipitin lines against antibody to bovine adrenocortical cytochrome P-450$_{s21}$. The molecular masses of the bovine liver and adrenocortical cytochromes P-450$_{s21}$ were 60 ± 1 and 50 ± 1 kDa, respectively. The content of cytochrome P-450$_{s21}$ and its percentage for the total microsomal cytochrome P-450 were immunochemically determined using the antibody to the bovine adrenocortical cytochrome P-450$_{s21}$.

Cytochrome P-450 *Steroid 21-hydroxylase* *Microsomal monooxygenase* *Microsomal mixed function oxidase* *Cytochrome P-450$_{s21}$*

1. INTRODUCTION

Cytochrome P-450$_{s21}$ (steroid 21-hydroxylase) exists not only in adrenocortical microsomes, but also in extra-adrenal tissue microsomes such as those of liver, kidneys, lungs, duodenum, brain, spleen and aorta [1–4]. Recently, cytochrome P-450$_{s21}$ was purified as a single protein from bovine adrenocortical microsomes [2–5]. Here, the immunochemical properties of the cytochrome P-450$_{s21}$ isozymes were investigated with respect to those of the liver and adrenocortical microsomes of various animals by using antibodies to the bovine adrenocortical cytochrome P-450$_{s21}$.

Abbreviations: SDS, sodium dodecyl sulfate; cytochrome P-450$_{s21}$, cytochrome P-450 catalyzing steroid 21-hydroxylation

2. MATERIALS AND METHODS

2.1. *Materials*

Fresh adrenal glands and livers of Holstein-Friesian oxen and Yorkshire swine were obtained from a local slaughterhouse. The fresh tissues were kept on ice in a jar and transported to this laboratory within 1 h. Adrenal glands and livers of male Wistar albino rats (150 g) and male New Zealand White rabbits (2.5 kg) were obtained from the Animal Research Center of this university.

2.2. *Microsomal preparation*

The livers and adrenal glands were washed and perfused with ice-cold saline to remove as much blood as possible. Fat and connective tissues and main blood vessels were removed carefully from the tissues. Bovine and swine adrenocortices were separated carefully from their medullae. The livers or adrenocortices were homogenized in 5 vols of

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0.25 M sucrose (adjusted to pH 7.4). Microsomes of livers or adrenocortices were prepared as in [6].

2.3. *Purifications of enzymes*

Cytochrome P-450$_{s21}$ of adrenocortices was purified from the microsomes as in [3]. Cytochrome b_5 was purified from bovine liver microsomes as in [7].

2.4. *Induction of cytochrome P-450*

Cytochrome P-450 of rabbit liver microsomes was induced by sodium phenobarbital (50 mg/kg body wt per day) or β-naphthoflavone (30 mg/kg body wt per day) for 7 days as described in [8]. The control rabbits were injected with the reagent solvents: saline or corn oil only.

2.5. *Enzymic activities*

Steroid 21-hydroxylase activity was determined as in [3]. Cytochrome P-450 content was determined as in [9].

2.6. *Protein content*

Protein content was measured by the biuret reaction method in the presence of 0.3% (w/v) deoxycholate to remove the turbidity of the microsomes [10], or as in [11].

2.7. *Electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed as in [12] with some modifications, using 10% (w/v) acrylamide.

2.8. *Serology*

Anti-cytochrome b_5 immunoglobulin was obtained by inoculation of bovine liver cytochrome b_5 into rabbits and anti-cytochrome P-450$_{s21}$ immunoglobulin was obtained by inoculation of bovine adrenocortical cytochrome P-450$_{s21}$ into rabbits as in [3].

2.9. *Contents and* M_r *values of cytochromes P-450$_{s21}$ in the liver and adrenocortical microsomes*

Cytochrome P-450$_{s21}$ contents were estimated immunochemically as in [13] with modifications [14]. The liver or adrenocortical microsomes were subjected to SDS-polyacrylamide gel electrophoresis. The protein bands of these microsomes were electrophoretically transferred to a cellulose nitrate sheet (Toyo membrane filter, Type TM-2). The sheet was treated with bovine serum albumin solution and then incubated with anti-cytochrome P-450$_{s21}$ immunoglobulin for 1 h at 25°C and washed with saline in 0.05 M Tris–HCl buffer at pH 7.4. The treated sheet was incubated with anti-rabbit immunoglobulin–immunoglobulin complex with peroxidase for 1 h at 25°C. After the sheet was carefully washed with the same saline solution, a solution of the substrate of the peroxidase, 30 mg 4-chloro-1-naphthol, 10 ml methanol, and 0.01% hydrogen peroxide per 60 ml saline containing 0.05 M Tris–HCl buffer at pH 7.4, was added to the sheet and incubated for 5 min at 25°C. The peroxidase reaction was stopped by washing with saline. The contents of cytochrome P-450$_{s21}$ were estimated from the immunostaining intensity at 590 nm of the electrophoretograms using purified adrenocortical cytochrome P-450$_{s21}$ at various concentrations as standards.

The M_r values of the liver and adrenocortical cytochromes P-450$_{s21}$ were estimated from the immunochemical staining band on the cellulose nitrate sheet using standard marker proteins stained immunochemically.

2.10. *Densitometry*

A Shimadzu dual-wavelength TLC scanner, Model CS-910, equipped with a Shimadzu recorder U-225MCS, was used to measure densitometric absorption of the SDS-polyacrylamide gels and cellulose nitrate sheets.

2.11. *Chemicals*

[1,2-^{3}H]Progesterone, 17α-[1,2-^{3}H]hydroxyprogesterone, 11-[1,2-^{3}H]deoxycortisol, and 11-[1,2-^{3}H]deoxycorticosterone were obtained from New England Nuclear.

3. RESULTS

Table 1 shows the steroid 21-hydroxylase activities of liver and adrenocortical microsomes of oxen, swine, rats, and rabbits. The substrates used were both progesterone and 17α-hydroxyprogesterone. The substrate specificity of the 21-hydroxylase activity with respect to liver and adrenocortical microsomes is summarized in this table. The ratio of the 21-hydroxylase activities for the two substrates in bovine liver microsomes was 0.34

Table 1

Steroid 21-hydroxylase activities of microsomes of livers and adrenocortices of various animals

Animal	Tissues	Specific activities of steroid 21-hydroxylases		Ratio (A/B)
		A, 17α-Hydroxyprogesterone	B, Progesterone	
Oxen	Adrenocortex	3.02 ± 0.40	1.07 ± 0.04	2.82
	Liver	0.11 ± 0.03	0.32 ± 0.06	0.34
Rabbits	Adrenal gland	1.28 ± 0.18	2.40 ± 0.47	0.53
	Liver	0.08 ± 0.02	0.27 ± 0.03	0.30
Swine	Adrenocortex	2.42 ± 0.51	1.51 ± 0.33	1.60
	Liver	0.10 ± 0.00	0.26 ± 0.04	0.38
Rats	Adrenal gland	–	–	–
	Liver	0.15 ± 0.06	0.29 ± 0.02	0.52

The steroid 21-hydroxylase activity is expressed as nmol·min^{-1}·mg protein^{-1} of microsomes. 3–5 experiments were done and the values given are the means and standard deviations

while that in bovine adrenocortical microsomes was 2.82. This indicates a dramatic difference in the substrate specificities for the 21-hydroxylases of the livers and adrenocortices. This phenomenon was observed regarding the other animal livers and adrenocortices.

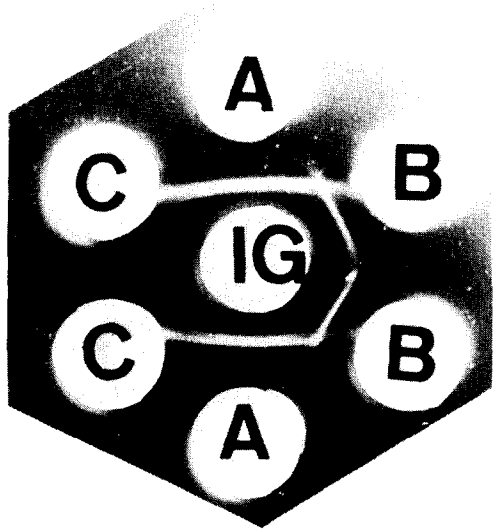

Fig.1. Immunochemical reactions in the Ouchterlony double diffusion agar test. The center well contained anti-cytochrome P-450$_{s21}$ immunoglobulin (15 μg) of bovine adrenocortical microsomes and the surrounding wells contained the following bovine liver and adrenocortical cytochrome P-450$_{s21}$. (A) Bovine adrenocortical microsomes; (B) bovine liver microsomes; (C) saline.

Cytochrome P-450 has multiple molecular forms and some cytochromes P-450 are induced by sodium phenobarbital, 3-methylcholanthrene, or β-naphthoflavone. However, the steroid 21-hydroxylase activity of the liver microsomes was not induced but decreased by the treatment (not shown).

The immunochemical properties of the liver and adrenocortical cytochromes P-450$_{s21}$ were examined. Fig.1 shows an example of the immunochemical reaction in an Ouchterlony double diffusion agar test of bovine liver and the adrenocortical cytochromes P-450$_{s21}$ against the antibody to bovine adrenocortical cytochrome P-450$_{s21}$. Crossed spur precipitin lines are observed between the liver and the adrenocortical cytochromes P-450$_{s21}$. This demonstrated that the bovine liver and adrenocortical cytochromes P-450$_{s21}$ have some common antigenic determinants. The liver and the adrenocortical cytochromes P-450$_{s21}$ of swine and rats also showed immunochemical precipitin reactions against the antibody to bovine adrenocortical cytochrome P-450$_{s21}$.

When the SDS-polyacrylamide gel electrophoretograms of purified adrenocortical cytochrome P-450$_{s21}$, solubilized adrenocortical microsomes, and liver microsomes after being transferred to a cellulose nitrate sheet, were stained with the anti-cytochrome P-450$_{s21}$ im-

munoglobulin, a sharp staining band appeared in the lane containing the solubilized adrenocortical microsomes at the same position as purified adrenocortical cytochrome P-450$_{s21}$ (fig.2). The M_r of bovine adrenocortical cytochrome P-450$_{s21}$ was estimated to be 50000 ± 1000 from standard marker proteins. There was no staining band at M_r 50000 in the lane containing solubilized liver microsomes. Instead, there was a relatively sharp band which appeared at M_r 60000 ± 1000. This protein must correspond to the one which formed a precipitin line with anti-adrenocortical cytochrome P-450$_{s21}$ in the Ouchterlony double diffusion agar plate. Thus, this protein was tentatively designated as liver microsomal cytochrome P-450$_{s21}$ because of its immunochemical relatedness to the adrenocortical cytochrome P-450$_{s21}$. The cytochrome P-450$_{s21}$ was 13% of the total cytochrome P-450 content of the bovine liver microsomes. The cytochrome P-450$_{s21}$ content was estimated to be about 75% of the total cytochrome P-450 of bovine adrenocortical microsomes. This high percentage may be overestimated because of the following factors: (a) the total microsomal cytochrome P-450 values do not include cytochrome P-420; (b) spectrally determined cytochrome P-450 does not include any apocytochrome P-450, while immunochemically determined cytochrome P-450$_{s21}$ contains both

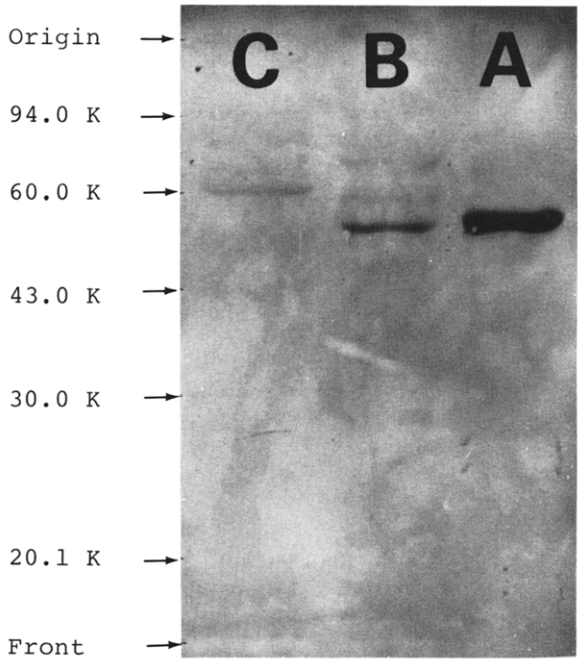

Fig.2. Immunochemical staining of SDS-polyacrylamide gel electrophoretogram after transfer to a nitrocellulose sheet. Cytochrome P-450$_{s21}$ purified from bovine adrenocortical microsomes, bovine liver, and the adrenocortical microsomes were electrophoresed. The resolved proteins were electrophoretically transferred to nitrocellulose sheet which was stained as described here. (A) Purified cytochrome P-450$_{s21}$; (B) adrenocortical microsomes; (C) liver microsomes.

Table 2

M_r values and contents of liver and adrenocortical cytochromes P-450$_{s21}$ and total cytochrome P-450 of various animal microsomes

Animal	Tissue microsomes	Cytochrome P-450$_{s21}$[a]		Total cytochrome P-450[b]
		M_r[c]	Content	
Oxen	Liver	60000 ± 1000	0.12 (13%)	0.94 ± 0.11
	Adrenocortex	50000 ± 1000	0.80 (75%)	1.07 ± 0.10
Rabbits	Liver	–	–	1.55 ± 0.20
	Adrenal gland	–	–	1.75 ± 0.12
Swine	Liver	–	–	0.52 ± 0.10
	Adrenocortex	49000 ± 1000	0.88 (78%)	1.14 ± 0.20
Rats	Liver	–	–	0.75 ± 0.10
	Adrenal gland	–	–	0.85 ± 0.10

[a] Cytochrome P-450$_{s21}$ content was determined immunochemically

[b] Total cytochrome P-450 content was determined by the heme content of cytochrome P-450 and the values given are the means and standard deviations of 5–10 experiments

[c] The values are the means and ranges

The values in parentheses show the percentage of cytochrome P-450$_{s21}$ in the total content of microsomal cytochrome P-450. Values of cytochrome P-450$_{s21}$ and total cytochrome P-450 are expressed as nmol/mg microsomal protein

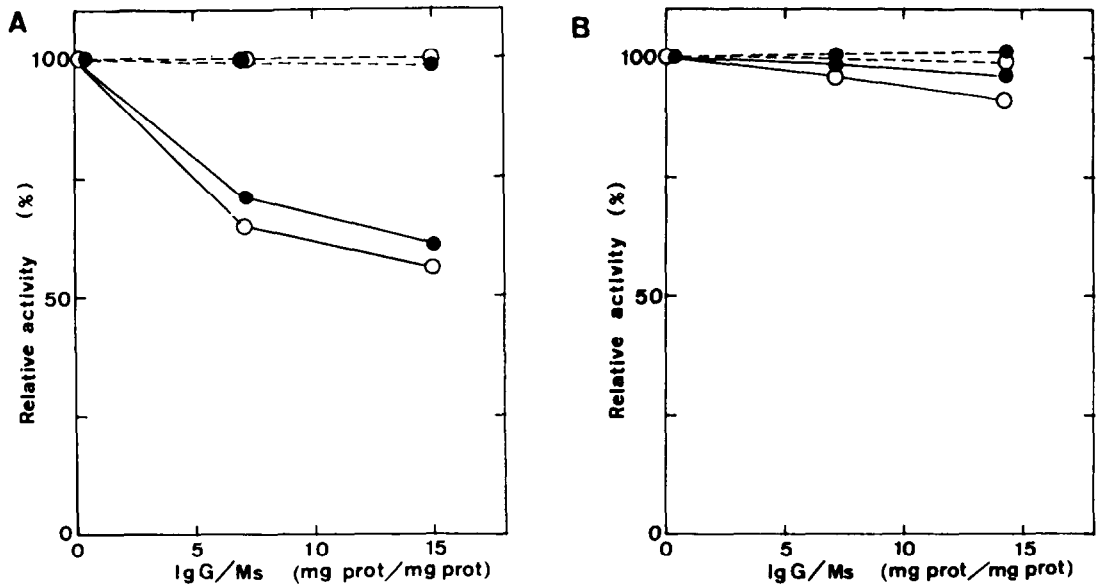

Fig.3. Effects of anti-cytochrome b_5 immunoglobulin on the steroid 21-hydroxylase activities of the liver and adrenocortical microsomes of oxen. The activities shown are the percentages of the steroid 21-hydroxylase activities in the absence of anti-cytochrome b_5 immunoglobulin of bovine liver microsomes. The abscissa is expressed as the concentrations of anti-cytochrome b_5 immunoglobulin/mg microsomal protein. (A) Bovine liver microsomes; (B) bovine adrenocortical microsomes; (○) 17α-hydroxyprogesterone; (●) progesterone; (——) in the presence of anti-cytochrome b_5 immunoglobulin; (– – –) in the absence of anti-cytochrome b_5 immunoglobulin.

apo- and holocytochromes P-450. Nevertheless, the dominance of cytochrome P-450$_{s21}$ in bovine adrenocortical microsomes is obvious. The cytochrome P-450$_{s21}$ contents in bovine liver microsomes and swine adrenocortical microsomes were estimated in the same manner, assuming that both cytochromes have the same immunochemical character with the cytochrome P-450$_{s21}$ in bovine adrenocortical microsomes. The M_r values and contents of the liver and adrenocortical cytochromes P-450$_{s21}$ are summarized in table 2.

Fig.3 shows the effects of anti-cytochrome b_5 immunoglobulin on the steroid 21-hydroxylase activity of liver and adrenocortical microsomes. A 15-fold protein amount of antibody to bovine liver cytochrome b_5 was used for the steroid 21-hydroxylase activity on the basis of the microsomal protein content. The steroid

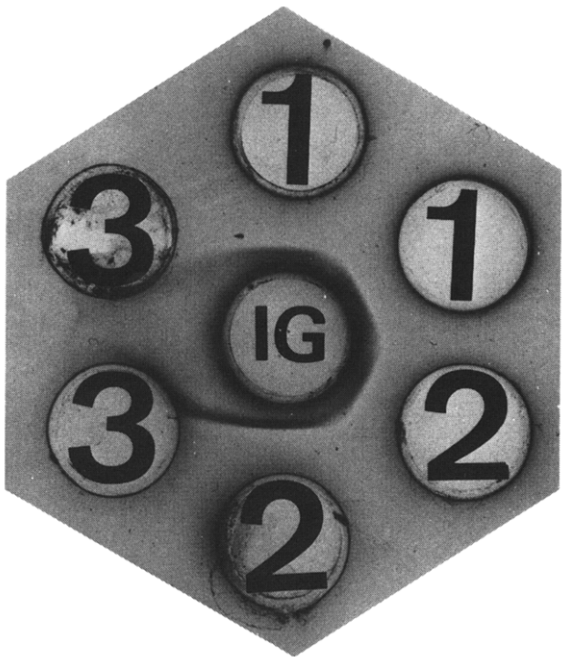

Fig.4. Immunochemical reactions in the Ouchterlony double diffusion agar test. The center well contained anti-cytochrome b_5 immunoglobulin (15 μg) of bovine liver microsomes and the surrounding wells contained bovine liver and the adrenocortical cytochrome b_5. 1, cytochrome b_5 purified from bovine adrenocortical microsomes; 2, cytochrome b_5 purified from bovine liver microsomes; 3, saline.

21-hydroxylase activity of the liver microsomes was inhibited by the anti-cytochrome b_5 immunoglobulin, but not that of the adrenocortical microsomes. Inhibition of the liver 21-hydroxylase activity depended on the amount of anti-cytochrome b_5 immunoglobulin. The absence of the effect of the anti-cytochrome b_5 immunoglobulin on the adrenocortical steroid 21-hydroxylase activity was not due to the liver specificity of cytochrome b_5. Both the bovine liver and adrenocortical cytochromes b_5 formed immunochemical precipitin lines against the antibody to the liver cytochrome b_5 and these two lines fused (fig.4). This suggests that the bovine liver and adrenocortical cytochromes b_5 are immunochemically identical.

4. DISCUSSION

The substrate specificity and M_r of cytochrome P-450$_{s21}$ of the liver differed from that of adrenocortical microsomes. The cytochrome P-450$_{s21}$-linked monooxygenase system of liver microsomes was inhibited by anti-cytochrome b_5 immunoglobulin, while the activity of adrenocortical microsomes was not. The adrenocortical cytochrome P-450$_{s21}$-linked monooxygenase system was reconstituted with components purified from the adrenocortical microsomes and the effect of cytochrome b_5 on the reconstituted system was examined [3]. The result corroborated the finding that the bovine adrenocortical steroid 21-hydroxylase activity was not affected by the addition of either the bovine adrenocortical or the liver cytochrome b_5. Accordingly, the different effects of the anti-liver cytochrome b_5 immunoglobulin on the liver and adrenocortical steroid 21-hydroxylase activities were considered not to be due to differences between the immunochemical properties of the liver and the adrenocortical cytochrome b_5. The synergistic effect of cytochrome b_5 suggests that the cytochrome P-450$_{s21}$-linked monooxygenase system of the liver microsomes is a conjugated electron transport system, but that of adrenocortical microsomes was a single electron transport system. That is, the synergistic effect of the cytochrome b_5 was liver-specific.

The M_r of bovine adrenocortical cytochrome P-450$_{s21}$ reported previously [3] differed slightly from the value estimated by the immunological staining of cellulose nitrate sheet. The different values between methods may be affected by the concentration of acrylamide of the electrophoretic gel, because the liver and adrenocortical cytochromes P-450$_{s21}$ have heme prosthetic groups and probably carbohydrate. Accordingly, the exact M_r of the cytochromes P-450$_{s21}$ should be determined by Ferguson plots [14].

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