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REVERSIBLE INHIBITION OF AROMATIC HYDROXYLATION OF METHAMPHETAMINE IN RAT LIVER MICROSOMAL PREPARATIONS PRETREATED WITH METHAMPHETAMINE

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Abstract—The effects of a single and repeated administration of methamphetamine (MP) *in vivo* in rats on its own metabolism *in vitro* were investigated. In both cases, the *p*-hydroxylation of MP to *p*-hydroxymethamphetamine by a microsomal fraction from rat liver was inhibited for a period of 16 hr after the last injection of MP. This inhibition was diminished by dialysis of the microsomal preparations. In contrast, the reduced level of cytochrome P-450 in hepatic microsomes from rats pretreated with the SKF 525-A did not revert to the control value after dialysis. When microsomes were preincubated with *N*-hydroxymethamphetamine, which is the metabolite of MP and a potent substrate for the formation of a metabolic intermediate (MI) complex with cytochrome P-450, the content of the MI was increased and the MP-hydroxylation activity decreased in direct proportion to the length of the preincubation. These results suggest that the inhibition of MP-hydroxylation may be due to reduction of the level of cytochrome P-450 that accompanies the formation of the MI complex. Furthermore, it appears that the complex can be dissociated by dialysis.

Amphetamine (AP)† is known to inhibit the cytochrome P-450-dependent metabolism of AP [1], *p*-nitroanisole and benzo[*a*]pyrene [2] *in vitro*. This inhibitory action appears to be related to the formation of metabolic intermediate (MI) complexes which absorb visible light maximally at a wavelength around 455 nm [3]. Methamphetamine (MP) also forms an MI-complex when MP is incubated with a microsomal fraction from rat liver [4], but there are no reports concerning the inhibitory effects of MP on cytochrome P-450-mediated reactions.

Our previous report [5] has shown that MP and AP are hydroxylated by cytochrome P-450-dependent monooxygenases. These monooxygenases are not induced by phenobarbital(PB), 3-methylcholanthrene(3-MC) or pregnenolone-16alpha-carbonitrile and their activities are depressed by pretreatment with $CoCl_2$, SKF 525-A or iprindole. Demethylation of MP is catalyzed, in part, by cytochrome P-450-mediated monooxygenases having activities which are depressed by pretreatment with 3-MC, $CoCl_2$ or SKF 525-A and which are induced by treatment with PB. Demethylation of MP is also catalyzed, in part, by the flavin-containing monooxygenases which are inhibited by methimazole in microsomal fractions of rat liver. Substrate inhibition of hydroxylation of AP occurs at a concentration of AP greater than 0.5 mM [6].

Following the chronic administration of AP to rats, a marked tolerance develops to symptoms such as hyperthermia and anorexia, while the effects of AP on locomotor stimulation and stereotyped behavior are potentiated [7, 8]. In the case of MP, however, there are few reports of behavioral changes; in addition, the biochemical mechanisms of these changes are not well understood [9, 10].

In the present study, we investigated the effects of single and repeated administration of MP on its own metabolism *in vitro*. We also studied restoration by dialysis of the inhibited activity of MP-hydroxylation in microsomal preparations.

MATERIALS AND METHODS

Materials. Methamphetamine(MP) hydrochloride was purchased from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan), SKF 525-A was a gift from Smith, Kline & French Laboratories (Philadelphia, PA). *p*-Hydroxymethamphetamine sulfate and *p*-hydroxyamphetamine hydrobromide were prepared from *p*-methoxyphenyl acetone by the method of Buzas and DuFour [11]. AP sulfate and *N*-hydroxymethamphetamine oxalate were prepared by the method of Magidson and Garkusha [12] and Beckett *et al.* [13], respectively. The purity of the products was checked by gas chromatography (GLC) and GLC–mass spectrometry. All other chemicals were of analytical reagent grade.

Animals and treatment. Adult, male Wistar rats

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† Abbreviations used: MP, methamphetamine; AP, amphetamine; PB, phenobarbital; 3-MC, 3-methylcholanthrene; MI, metabolic intermediate.

Table 1. Acute toxicity of methamphetamine (25 mg/kg, i.p.) in rats pretreated with methamphetamine and iprindole

Pretreatment	Mortality			
	Hours after dosage:			
	0–4	4–8	8–24	%
Saline	8/20	8/20	0/20	80
Methamphetamine[a]	0/10	0/10	0/10	0
Iprindole[b]	3/10	4/10	0/10	70

[a] In the methamphetamine-pretreatment group (twice daily; 5, 10 mg/kg and 20 mg/kg for 5 days), methamphetamine HCl (25 mg/kg, i.p.) was administered 16 hr after the last injection.

[b] Iprindole HCl (50 mg/kg, i.p.) was administered 1 hr before the treatment with methamphetamine HCl (25 mg/kg, i.p.).

(body wt 160–200 g) were used. In the group for chronic administration of MP, rats were injected twice daily (10:00 a.m. and 5:00 p.m.) with MP HCl (i.p.) for 7 days. The doses of the drug were increased step wise to allow development of tolerance (Table 1), as follows: 1st day, 5 mg/kg; 2nd day, 10 mg/kg; 3rd to 7th days, 20 mg/kg. The rats were sacrificed 16 or 40 hr after the last injections. Control animals received equal volumes of saline over the same periods.

Enzyme preparation. Each rat was decapitated and the liver was removed, cleaned and weighed. Portions of the liver (5 g) were homogenized in 45 ml of 0.25 M sucrose–10 mM phosphate buffer (pH 7.4)–1 mM EDTA, with a Potter-Elvehjem homogenizer and a Teflon pestle. Microsomal fractions were prepared by ultra-centrifugation [5]. The resulting pellets were suspended in 0.15 M KCl–0.05 M phosphate buffer (pH 7.4, 20 ml) and used as enzyme preparations. These procedures were performed in an ice-bath. The protein concentration was determined by the modified method of Lowry *et al.* [14], as described by Albro [15], with bovine serum albumin as the standard.

Hepatic metabolism of methamphetamine in vitro. The assay mix (1.5 ml) contained 50 μmol phosphate buffer, pH 7.4, 60 μmol nicotinamide, 2 μmol NADP, 10 μmol glucose-6-phosphate, 25 μmol $MgCl_2$, glucose-6-phosphate dehydrogenase (1 unit). To the assay mix were added 0.5 ml containing 300 nmol MP and 1 ml of the hepatic microsomal fraction (2 mg of protein) to give a final volume of 3 ml. The enzymatic reaction was started by the addition of substrate and incubated for 15 min at 37°. The reactions were stopped by the addition of 1 ml 15% (w/v) $ZnSO_4$ and 1 ml saturated $Ba(OH)_2$. Enzymatic activities for *N*-demethylation and aromatic hydroxylation of MP are expressed as the amounts of AP and *p*-hydroxymethamphetamine formed in the incubation mixtures.

Extraction and determination of the metabolites were carried out as described previously [5]. The quantitative analysis was performed using a GLC–mass spectrometer (JEOL, D-300) equipped with a computer (JMA-2000). The operational conditions were as follows: column, 2% OV-17 on Uniport HP (80–100 mesh), glass column (2 mm i.d., 2 m length); temperature, of column oven 150°, of sample injector port, 200°, of separator 250°, of ionizing chamber, 150°; carrier gas, helium (inlet pressure 0.8 kg/cm^2); ionizing voltage, 70 eV; ionizing current, 300 μA.

Determination of MI complex. Complexes of MP or *N*-hydroxymethamphetamine with hepatic cytochrome P-450 were assayed by a slightly modified version of the method described earlier [4]. The incubation mixture (final volume, 3 ml) contained 50 μmol phosphate buffer, pH 7.4, 60 μmol nicotinamide, 2 μmol NADP, 10 μmol glucose-6-phosphate, 25 μmol $MgCl_2$, glucose-6-phosphate dehydrogenase (1 unit) and MP (15 μmol, finally

Table 2. Effect of single and repeated administration of methamphetamine on its own metabolism, levels of cytochrome P-450 and rate of formation of MI complex in rat liver microsomes

Treatment	Hours after the last injection	N[a]	Activity (pmol/min/mg[b]) methamphetamine to amphetamine	Activity (pmol/min/mg[b]) methamphetamine to p-OH methamphetamine	Cytochrome P-450 (nmol/mg)	MI complex[c] (ΔA × 10^4/min/mg)
Single[d]	control	8	31.1 ± 2.2	7.00 ± 0.25	0.81 ± 0.02	1.77 ± 0.09
	16	8	22.8 ± 1.5**	5.72 ± 0.37*	0.69 ± 0.03**	1.40 ± 0.07**
	40	8	32.3 ± 3.7	7.29 ± 0.61	0.75 ± 0.03	1.71 ± 0.14
Repeated[e] Injection	control	15	30.4 ± 1.2	6.55 ± 0.37	0.79 ± 0.03	2.04 ± 0.08
	16	15	28.8 ± 1.7	3.55 ± 0.28**	0.74 ± 0.02	1.77 ± 0.08
	40	5	28.3 ± 1.9	5.54 ± 0.37	0.76 ± 0.03	—[f]

Each value represents the mean ± SE.
Statistically different from the control groups (*$P < 0.05$, **$P < 0.01$).
[a] Number of rats.
[b] Incubation was carried out at 37° for 15 min with methamphetamine (0.1 mM) as a substrate.
[c] Incubation was performed at 37° for 30 min with methamphetamine (5 mM) as a substrate.
[d] Rats were injected with methamphetamine HCl (5 mg/kg, i.p.).
[e] Rats were repeatedly injected twice daily with methamphetamine HCl (5, 10 mg/kg and 20 mg/kg for 5 days, i.p.).
[f] Not determined.

Table 3. Effect of dialysis of hepatic microsomes, obtained from control rats or from rats that received repeated doses of MP, on the metabolism of methamphetamine *in vitro*

Dialysis[a]	Group	Activity (pmol/min/mg of protein) (%)[b] methamphetamine to amphetamine	methamphetamine to p-OH methamphetamine
–	control	33.8 ± 1.6 (100)	6.83 ± 0.48 (100)
	treated	26.3 ± 3.3 (78)	4.10 ± 0.35** (60)
+	control	51.9 ± 9.0 (100)	6.63 ± 1.08 (100)
	treated	53.2 ± 3.9 (102)	10.39 ± 0.60* (157)

Each value represents the mean ± SE of three animals.
Statistically different from the control groups (*$P < 0.05$, **$P < 0.01$).
[a] Microsomes (6 mg of protein, 3 ml) were dialyzed overnight against one liter of 10 mM phosphate buffer (pH 7.4) at 4°.
[b] Percent control activity is expressed in parentheses.

5 mM) or *N*-hydroxy methamphetamine (300 nmol, finally 0.1 mM) and the hepatic microsomal fraction (6 mg of protein). Each reaction mixture, in a 20 ml Erlenmeyer flask, was incubated for 30 min at 37°. The amount of MI complex formed was measured in a Hitachi model 557 spectrophotometer, with cuvettes of 1 cm optical path. The incubation mixture without substrate and the complete mixture were placed in the reference and sample cells, respectively. After recording the spectra (500–400 nm), the difference in absorbance (ΔA) was recorded between values at 455 nm and 490 nm. When MP was used as a substrate, the rate of formation of the MI-complex was linear during incubation for up to 90 min (6 mg of protein), and for protein concentrations of up to 9 mg/tube (data not shown).

Determination of cytochrome P-450 content. Cytochrome P-450 content was determined by the method of Omura and Sato [16].

Dialysis of microsomal suspensions. The microsomal suspensions (6 mg of protein, 3 ml) were dialyzed overnight against one liter of 10 mM phosphate buffer, pH 7.4 at 4°.

RESULTS

Effect of methamphetamine-treatment on its own metabolism in vitro.

Sixteen hours after a single injection of MP (5 mg/kg, i.p.), both the *N*-demethylation of MP (AP formation) and the *p*-hydroxylation of MP, formation of *p*-hydroxymethamphetamine, by hepatic microsomes were inhibited. The level of cytochrome P-450 and the rate of formation of the MI complex were also depressed. However, these inhibitory effects were not observed in animals sacrificed 40 hr after a single injection of MP (Table 2). After repeated administration of MP, aromatic hydroxylation was significantly inhibited in the liver microsomes of rats 16 hr after the last administration, but inhibitory effects on other parameters were not observed (Table 2). Forty hours after the last injection, there were no changes in the metabolism of MP nor the levels of cytochrome P-450.

Restoration of aromatic hydroxylating activity after dialysis

As shown in Table 3, the activity of MP-hydroxylation was depressed in microsomes prepared from rats 16 hr after the last pretreatment with MP. However, dialysis of the tested microsomes increased the activity to 157% of the control value. However, dialysis of the control microsomes had no effect on the activity. In contrast, MP *N*-demethylase activity was not significantly decreased in microsomes prepared from rats pretreated with MP; furthermore, dialysis of microsomes from both untreated and treated rats increased *N*-demethylase activity to the same extent.

Effect of dialysis on the amounts of MI complex induced by SKF 525-A or MP and on the amount of cytochrome P-450 in the microsomal fractions

Microsomes, fortified with a NADPH-generating system, were incubated either with SKF 525-A (0.2 mM), which exerts an inhibitory effect on cytochrome P-450-mediated monooxygenase by the formation of an oxygenated complex having a maximum absorbance at 455 nm [17], or they were incubated with MP (5 mM) at 37° for 30 min. In both cases, MI complexes were formed and levels of cytochrome P-450 decreased (Table 4). After dialysis of microsomes preincubated with MP, the amount of the MI complex was reduced markedly (49%) and the level of cytochrome P-450 was restored to the control value. In the case of SKF 525-A, however, the levels of cytochrome P-450 and MI complex were not changed by dialysis.

Relationships between formation of MI complex and inhibition of methamphetamine hydroxylation induced by N-hydroxymethamphetamine

Hepatic microsomes were preincubated with *N*-hydroxymethamphetamine, a potent substrate for cytochrome P-450 complex formation [18], for different periods of time in the presence of the NADPH-generating system. The amount of MI complex was measured, and then the ability of these mixtures to hydroxylate MP was measured with 2 mg

Table 4. Effect of dialysis on the MI complex induced by SKF 525-A or methamphetamine and on the levels of cytochrome P-450

Dialysis[b]	Incubation[a] SKF 525-A (0.2 mM)	Incubation[a] methamphetamine (5 mM)	MI complex ($\Delta A \times 10^3$/mg)	Cytochrome P-450 (nmol/mg)
−	−	−	−	0.71 (100)
	+		3.83 (100)	0.54 (76)
+	−	−	−	0.71 (100)
	+		3.94 (103)	0.48 (68)
−		−	−	0.69 (100)
		+	5.59 (100)	0.38 (55)
+		−	−	0.60 (100)
		+	2.76 (49)	0.56 (93)

Each value represents the mean of triplicate assays.
[a] Incubation was carried out at 37° for 30 min.
[b] The microsomes (6 mg of protein, 3 ml), treated with SKF 525-A or methamphetamine, were dialyzed overnight against one liter of 10 mM phosphate buffer (pH 7.4) at 4°.

of protein used each time as a source of enzyme. As shown in Fig. 1, the amount of MI complex increased and the aromatic hydroxylation of MP decreased in direct proportion to the duration of preincubation.

Residual MP and AP in microsomes from the pretreated rat

The trace amount of MP and AP in microsomal fractions prepared from rats singly and repeatedly pretreated with MP was measured by GC/MS. The detection limits of these compounds were approximately 50 ng in tubes. Under these conditions, we could not detect any amounts of these compounds in microsomal fractions (data not shown). In the case of MP, 50 ng in a 3 ml tube is comparable to 0.1 μM.

Acute toxicity in rats treated with iprindole

The acute toxicity observed with a sublethal dose of MP (50 mg/kg, MP-tolerant animals did not die at this dose. Table 1) in rats pretreated with iprindole (a specific inhibitor for MP hydroxylation [19]) was similar to that observed for a control group (Table 1).

DISCUSSION

The *p*-hydroxylation of AP and MP in hepatic microsomes has been shown to be catalyzed by cytochrome P-450-mediated monooxygenase(s) [5], and the AP-hydroxylation is markedly depressed by chronic administration of AP [3]. In this study, we

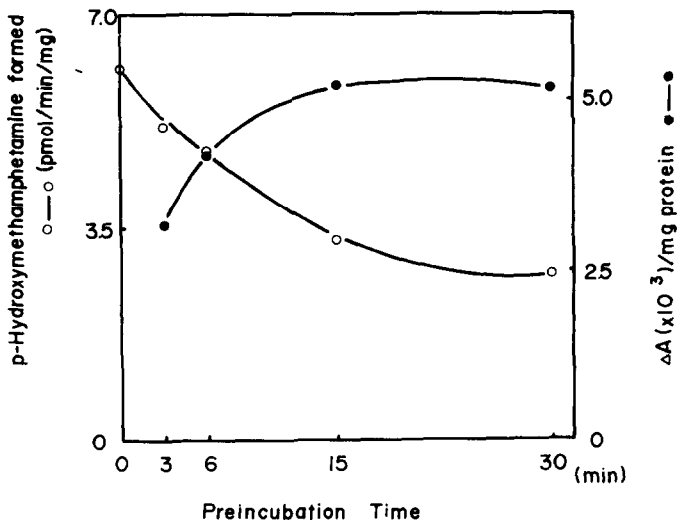

Fig. 1. Relationships between formation of MI complex and inhibition of methamphetamine hydroxylation induced by *N*-hydroxymethamphetamine. After preincubation with *N*-hydroxymethamphetamine (0.1 mM) and hepatic microsomes (6 mg of protein) for various times at 37°, the difference spectra ($\Delta A_{455-490}$) were measured and then the methamphetamine-hydroxylating activity was assayed using these mixtures (2 mg of protein) as enzymes. Reaction was started by addition of methamphetamine (0.1 mM) and incubated at 37° for 15 min.

observed that either a single or repeated dose of MP also inhibits the *p*-hydroxylation of MP in microsomes prepared from rats 16 hr after the last injection.

Apart from the inhibition of MP-hydroxylation, significant inhibition of MP-demethylation and depression of cytochrome P-450 content and MI-complex formation were observed only in the microsomal preparation obtained 16 hr after a single injection of MP; repeated administration inhibited only the *p*-hydroxylation but not demethylation. Although *p*-hydroxylation and in part demethylation of MP were catalyzed by cytochrome P-450(s), the enzymatic characterizations were different [5]. All of these results are consistent with the interpretation that these reactions are catalyzed by different isozymes of cytochrome P-450 and that each isozyme exhibits a different rate of MI-complex formation with MP. The absence of effects at 40 hr after the last administration of MP could be due to the dissociation of the MI-complex with cytochrome P-450 and/or to the elimination of MP prior to or during the preparation of microsomes. Moreover, MP-hydroxylation was not inhibited *in vitro* by adding MP (ranging from 0.02 mM to 12.5 mM) as a substrate [5], nor were the activities of *p*-nitroanisole *O*-demethylation and benzo[*a*]pyrene hydroxylation inhibited by less than 1 mM of MP [2]. These results and the absence of residual MP and AP in our pretreated preparations suggest that the levels of the MI complex in microsomal preparations, obtained from rats 40 hr after the last injection, are less than the levels necessary to inhibit MP hydroxylation.

Ellison *et al.* [20] found that prolonged administration of AP to cats led to the development of tolerance not accompanied by changes in the metabolism of AP. Sever *et al.* [21] reported that women who have developed tolerance to administered AP excreted more unchanged AP, norephedrine and 4-hydroxynorephedrine in 24 hr than control subjects. In our studies, MP hydroxylation was inhibited after repeated administration of MP, but the animals which showed depressed MP-hydroxylation by iprindole were not tolerant to acute toxic effects. *N*-hydroxymethamphetamine is a potent substrate for the formation of a complex with cytochrome P-450 [18] and in addition, *N*-hydroxymethamphetamine formed from MP by the action of the 10,000 *g* supernatant of homogenized rat liver is the ultimate metabolite of MP to form an MI-complex [22–24]; therefore, the inhibition of MP metabolism observed in this report could be due to the formation of an MI-complex with *N*-hydroxymethamphetamine.

The increase in MP-hydroxylation in dialyzed microsomes prepared from rats repeatedly treated with MP suggests that MP could induce its own metabolism, but the activity of MP-hydroxylation is masked by endogenous component(s). However, it is not clear why these effects occurred.

Schenkman *et al.* [25] demonstrated that simultaneous addition of SFK 525-A, a substrate which is metabolized by cytochrome P-450-mediated monooxygenases, competitive inhibition of drug metabolism was observed; however, after microsomes were aerobically preincubated with SKF 525-A and NADPH, a non-competitive inhibition of drug metabolism occurred. The SKF 525-A spectral complex was even retained during the isolation of microsomes from livers of rats treated with up to 50 mg/kg SKF 525-A for one to three weeks [25]. Studies with less than 12.5 mM of MP did not show inhibition of MP-hydroxylation, but SKF 525-A (100 μM) showed significant inhibition [5]. Our results suggest that the mechanisms of the inhibitory effects caused by MP and SKF 525-A on MP hydroxylation may be different since dialysis had opposite effects on hydroxylation of MP from MP and SKF 525-A treated microsomes. We would suggest that the metabolite of MP, *N*-hydroxymethamphetamine could be less tightly bound to cytochrome P-450(s) than SKF 525-A.

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REFERENCES

1. J. Jonsson, *Acta Pharmac. Toxicol.* **40**, 517 (1977).
2. M. R. Franklin, in *Microsomes and Drug Oxidations* (Eds. V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney), p. 284. Pergamon Press, Oxford (1977).
3. J. Jonsson and B. Lindeke, *Acta Pharm. Succina* **13**, 313 (1976).
4. M. R. Franklin, *Xenobiotica* **4**, 133 (1974).
5. T. Yamamoto, R. Takano, T. Egashira and Y. Yamanaka, *Xenobiotica* **14**, 867 (1984).
6. J. A. Jonsson, *Biochem. Pharmac.* **23**, 3191 (1974).
7. E. Ellinwood, A. Sudilovsky and L. Nelson, *Biol. Psychiatry* **4**, 215 (1972).
8. D. S. Segal, in *Neurobiological Mechanisms of Adaptation and Behavior* (Ed. A. J. Mandel), p. 242. Raven Press, New York (1975).
9. J. E. Peachey, B. Rogers, J. F. Brien, A. Maclean and D. Rogers, *Psycopharmac.* **48**, 271 (1976).
10. Y. Yamanaka, T. Yamamoto and T. Egashira, *Jap. J. Pharmac.* **33**, 33 (1983).
11. A. Buzas and C. DuFour, *Bull. Soc. Chim. France* 139 (1950).
12. O. Y. Magidson and G. A. Garkusha, *J. gen. Chem.* **11**, 339 (1941).
13. A. H. Beckett, R. T. Coutts and F. A. Ogunbona, *Tetrahedron* **29**, 4189 (1973).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. D. W. Albro, *Analyt. Chem.* **64**, 485 (1975).
16. T. Omura and J. Sato, *J. biol. Chem.* **239**, 2370 (1964).
17. K. J. Netter in *Hepatic Cytochrome P-450 Monooxygenase System* (Eds. J. B. Schenkman and D. Kupfer), p. 741. Pergamon Press, Oxford (1982).
18. B. Lindeke, U. Paulsen and E. Anderson, *Biochem. Pharmac.* **28**, 3629 (1979).
19. R. E. Billings, P. J. Murphy, R. E. McMahon and J. Ashmore, *Biochem. Pharmac.* **27**, 2525 (1978).
20. T. Ellison, R. Okun, A. Silverman and M. Siegel, *Archs Int. Pharmacodyn.* **190**, 135 (1971).
21. P. S. Sever, J. Caldwell, L. G. Dring and R. T. Williams, *Eur. J. Clin. Pharmac.* **6**, 177 (1973).
22. R. T. Coutts and S. H. Kovach, *Biochem. Pharmac.* **26**, 1043 (1977).
23. R. T. Coutts, G. R. Jones and S.-F. Liu, *Biomed. Mass Spectr.* **5**, 418 (1978).
24. R. T. Coutts and G. R. Jones, in *Biological Oxidation of Nitrogen* (Ed. J. W. Grorrod), p. 31. Elsevier/North Holland Biomedical Press, New York (1978).
25. J. B. Schenkman, B. J. Wilson and D. L. Cinti, *Biochem. Pharmac.* **21**, 2373 (1972).