Biochemical Pharmacology, Vol. 30, No. 19, pp. 2657–2662, 1981.
Printed in Great Britain.

0006-2952/81/192657-06 $02.00/0

COMPENSATORY INCREASE IN SYNAPTOSOMAL ALDEHYDE REDUCTASE ACTIVITY IN RAT BRAIN AFTER CHRONIC BARBITAL TREATMENT

AKEMI MINEGISHI, RYUJI FUKUMORI, TETSUO SATOH* and HARUO KITAGAWA
Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 260, Japan

(*Received* 28 *July* 1980; *accepted* 25 *February* 1981)

Abstract—The changes in brain aldehyde reductase (AlR) activities during long-term treatment of rats with barbital were studied. NADH-linked AlR activity in the synaptosomal fraction increased rapidly, and supernatant AlR activity rose later during barbital treatment. With respect to NADPH-linked AlR, two distinct K_m values were observed for *p*-nitrobenzaldehyde. The low-K_m enzyme had a higher K_i value than the high-K_m enzyme. Although the elevation of NADPH-linked AlR activity under the routine assay conditions was less remarkable, V_{max} values of the low-K_m enzyme were greatly increased in both the synaptosomal and the supernatant fractions by chronic barbital treatment. In addition, the K_i value of low-K_m AlR in synaptosomes was greater in barbital-treated animals than in control animals. K_m values were unchanged in either fraction by chronic barbital treatment. These data suggest that chronic barbital treatment resulted in a compensatory increase in the activity of AlR with low K_m, which is less sensitive to barbital and utilizes either NADH or NADPH, in synaptosomes.

In recent years, considerable interest has been focused on the aldehyde derived from biogenic amines, in view of their biological activities. These aldehydes were postulated to be related to the sleep mechanism [1, 2], maintenance of seizure susceptibility [3, 4], and regulation of body temperature [5, 6]. The concentrations of these aldehydes are controlled by the activities of aldehyde-metabolizing enzymes [7]. In the brain, the conversions of these aldehydes to the corresponding alcohols and acids are catalyzed by aldehyde reductase (AlR) (alcohol: NADP oxidoreductase, EC 1.1.1.2) and aldehyde dehydrogenase (AlDH) (aldehyde: NAD oxidoreductase, EC 1.2.1.3) respectively.

Barbiturates are known to be potent inhibitors of NADPH-linked AlR [8]. In addition, it has been suggested that the inhibition of aldehyde metabolism by barbiturates may be involved in their pharmacological actions on the central nervous system [9, 10]. Recently, we found that the activity of NADH-linked AlR in brain cytosol, which is less sensitive to barbiturates, was compensatorily increased by chronic treatment of rats with barbital or phenobarbital [11]. It has generally been thought that most of the aldehyde-reducing activity in rat brain appears in the soluble supernatant fraction, with either NADH [12] or NADPH [8, 13]. It may be more important, however, to examine the change in activity located in the nerve-ending synaptosomal fraction that may actually participate in the regulation of the levels of biogenic aldehydes. Therefore, the present investigation was designed to study the time courses of changes in the amounts and kinetic properties of the AlR activities in the supernatant and synaptosomal fractions during long-term barbital treatment.

* Author to whom all correspondence should be addressed: Dr. Tetsuo Satoh, Department of Biochemical Pharmacology, Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1–33, Chiba 260, Japan.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing nearly 140 g at the beginning of experiments were used. Body weight and water intake were checked periodically during the experimental period (Table 1). Barbital was dissolved in drinking water containing sodium saccharin (10 mg/0.5 g of barbital) to disguise the bitter taste of barbital; the water was given *ad lib*. The dose regimen was based on the method of Morgan *et al.* [14] with minor modifications (Table 1). The saccharin control rats received water containing the same concentration of sodium saccharin as that given to the barbital-treated group. Unless otherwise noted, "control" refers to this saccharin control group. The water control group received water without additional drugs.

Chemicals

NADPH, NADH and NAD were purchased from Boehringer Mannheim, GmbH, West Germany. Barbital was obtained from the Wako Pure Chemical Co., Tokyo, Japan; *p*-nitrobenzaldehyde from the Tokyo Kasei Co., Tokyo; and L-glutamic acid monopotassium salt from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Enzyme preparations

Rats were killed after 6, 13, 21, 28 and 49 days of drug treatment. The brain was immediately removed and homogenized in 9 vol. of 0.32 M

Table 1. Daily barbital intake levels during barbital treatment*

Concn of barbital (g/l)	Days of administration	Barbital consumption ($mg \cdot rat^{-1} \cdot day^{-1}$)	($mg \cdot kg^{-1} \cdot day^{-1}$)
1.0	3	22.8 ± 0.7 (12)	150.0 ± 4.5 (12)
1.5	3	35.1 ± 1.7 (12)	200.3 ± 8.5 (12)
2.0	3	48.7 ± 3.3 (12)	251.1 ± 14.2 (12)
2.5	4	59.7 ± 4.3 (10)	274.3 ± 14.5 (10)
3.0	5	70.9 ± 3.1 (9)	304.0 ± 12.2 (9)
3.5	5	90.7 ± 2.5 (8)	358.9 ± 13.7 (8)
4.0	12	116.8 ± 3.6 (7)	378.8 ± 15.6 (7)
5.0	12	129.8 ± 6.1 (6)	398.4 ± 13.8 (6)

* Each value is the mean ± S.E.M. Figures in parentheses indicate the number of determinations. For details, see Materials and Methods.

sucrose. After discarding the pellet from 750 *g* centrifugation, the supernatant fraction was centrifuged at 10,000 *g* for 10 min. The resulting supernatant fraction and pellet were used as the enzyme sources for the supernatant fraction and crude synaptosomal AlR respectively.

Supernatant fraction. The supernatant fraction from 10,000 *g* centrifugation was recentrifuged at 40,000 *g* for 1 hr and the resulting supernatant fraction was precipitated with 80% ammonium sulfate. After centrifugation at 12,000 *g* for 15 min, this pellet was dissolved in sucrose solution consisting of 0.25 M sucrose, 2 mM mercaptoethanol, and 10 mM sodium phosphate (pH 7.4), and used for the supernatant AlR assay.

Crude synaptosomal fraction. The pellet from 10,000 *g* centrifugation was washed with 0.32 M sucrose and suspended in 0.001 M Tris–acetate buffer (pH 7.4) to solubilize the enzyme in the synaptosomes hypotonically according to the method of Knapp and Mandell [15]. This suspension was centrifuged at 35,000 *g* for 45 min, and the resulting supernatant fraction was treated with ammonium sulfate as described above, and then used for the assay of crude synaptosomal AlR.

Enzyme assays

AlR activity was determined spectrophotometrically by measuring the change in NAD(P)H absorbance at 340 nm according to the method of Tabakoff and Erwin [8]. Assay mixture consisting of the enzyme solution, 1.6×10^{-4} M NAD(P)H, 6.5×10^{-4} M *p*-nitrobenzaldehyde and 0.1 M sodium phosphate (pH 7.0), in a total volume of 3.0 ml, was routinely used. The reaction was initiated after addition of the aldehyde. For kinetic studies, enzyme solutions prepared from eight animals in each 49-day treatment group were pooled and used under the standard assay conditions. Glutamate dehydrogenase [L-glutamate:NAD(P) oxidoreductase, deaminating, EC 1.4.1.3] activity was measured spectrophotometrically at 340 nm by the method of Strecker [16]. The protein content of the enzyme solution was determined by the method of Lowry *et al.* [17].

RESULTS

Drug intakes

During the drug treatment period, the animals consumed 20–30 ml of water per day, and the volume consumed was not affected by the concentration of barbital or saccharin. The mean volumes of water ingested by the barbital-treated group, the saccharin control group, and the water control group were 25.0 ± 0.8, 25.8 ± 1.1, and 27.5 ± 2.1 ml respectively. In accordance with the observation of Morgan *et al.* [14], the daily barbital intake (Table 1) increased as the period of drug intake was prolonged. Neither body weight nor other variables were altered in the barbital-treated animals compared with the control animals.

AlR activities in the synaptosomal and supernatant fractions in control rats

The total activity of NADPH-linked AlR in the crude synaptosomal fraction was relatively high compared with that in the supernatant fraction (approximately two-fifths of the activity in the supernatant fraction) (Table 2). In contrast, the activity of

Table 2. Aldehyde reductase activities in the rat brain*

Enzyme	Enzyme activity [$nmoles \cdot (g\ brain)^{-1} \cdot min^{-1}$]	[$nmoles \cdot (mg\ protein)^{-1} \cdot min^{-1}$]
Crude synaptosomal AlR		
NADPH-linked	125.7 ± 4.6 (10)	14.21 ± 0.48 (15)
NADH-linked	7.1 ± 0.2 (10)	0.87 ± 0.03 (16)
Supernatant AlR		
NADPH-linked	291.6 ± 8.6 (10)	10.95 ± 0.27 (14)
NADH-linked	31.6 ± 1.9 (10)	1.18 ± 0.04 (13)

* Procedures for obtaining the enzyme preparations and for determinations of enzyme activities are described in Materials and Methods. Figures in parentheses indicate the number of animals used.

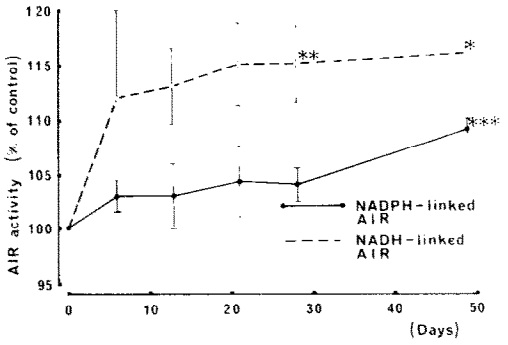

Fig. 1. Time course of changes in crude synaptosomal AlR activity during chronic treatment with barbital. Each value is the mean ± S.E.M. of five to seven determinations, expressed as a percentage of the specific activity of tissue from control animals. Statistical significance is expressed as follows: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$.

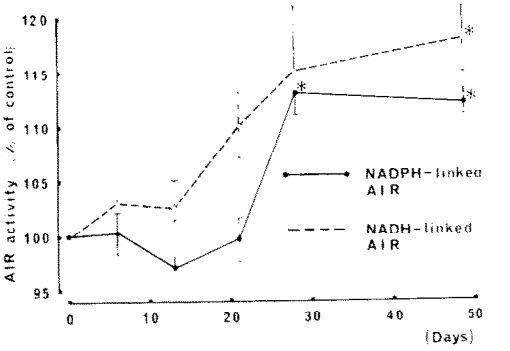

Fig. 2. Time course of changes in supernatant AlR activity during chronic treatment with barbital. For details, see the legend to Fig. 1. Asterisks indicate statistical significance ($P < 0.01$).

NADH-linked AlR was largely contained in the supernatant fraction. Thus, the relative activity of NADH-linked AlR with respect to NADPH-linked AlR in the crude synaptosomal fraction was much smaller than in the supernatant fraction. The data on the specific activities of tissues further confirmed that activities compatible with those of the supernatant enzymes were localized in synaptosomes.

In addition, we ascertained that the activity of glutamate dehydrogenase, a marker enzyme of the mitochondrial matrix, was not detectable in the crude synaptosomal fraction, but remained in the hyposmotically treated mitochondrial pellet [98.0 nmoles of NADH produced·min^{-1}·(g brain)$^{-1}$]. It is known that membrane-bound enzymes in mitochondria, such as monoamine oxidase (MAO) and succinate dehydrogenase, are also not released by hyposmotic shock [18]. Therefore, the activity in the crude synaptosomal fraction is essentially not contaminated by the mitochondrial activity. In harmony with our observation, Turner and Tipton [13], using density gradient centrifugation, demonstrated that the activity found in the crude mitochondrial fraction was synaptosomal rather than truly mitochondrial.

Changes in the AlR activities during chronic barbital treatment

Chronic administration of barbital resulted in an increase in both synaptosomal and supernatant AlR activities (Figs. 1 and 2). In synaptosomes, the activity of NADPH-linked AlR increased gradually and became slightly but significantly higher than that of the control after 49 days of drug treatment. In contrast, the activity of NADH-linked AlR increased rapidly and reached over 115 per cent of the control value. The NADH-linked AlR activity in the supernatant fraction began to increase rather later and reached a plateau (more than 115 per cent of control) after 30 days. In this fraction, the overall change in the NADPH-linked activity, although less marked, resembled that in the NADH-linked activity.

Kinetic characteristics of AlR in control and barbital-treated rats

Double-reciprocal plots for the substrate *p*-nitrobenzaldehyde indicated the presence of two NADPH-linked AlRs with two different K_m values (Table 3). The lower value was in the micromolar

Table 3. Apparent Michaelis constants of AlR from control and barbital-treated animals*

	Apparent K_m (10^{-6} M)			
	Crude synaptosomal fraction		Supernatant fraction	
Substrate	Control	Barbital-treated	Control	Barbital-treated
p-Nitrobenzaldehyde (NADPH, 0.16 mM)	44.1 & 7.2	44.1 & 8.3	93.5 & 17.5	96.2 & 19.6
p-Nitrobenzaldehyde (NADH, 0.16 mM)	64.0	67.6	90.9	96.2
NADPH (*p*-nitrobenzaldehyde)	5.6	6.4	11.9	11.9

* After 49 days of treatment with barbital or the vehicle, each activity was determined with the pooled enzyme solution (from eight animals) under the standard assay conditions. Apparent K_m values were calculated from the usual double-reciprocal plots. Values are averages of the values obtained from two separate experiments. K_m values for NADH were not measurable owing to the low activity under our experimental conditions.

Table 4. Maximum velocities of AlR from control and barbital-treated animals*

Enzyme	V_{max} [nmoles·(mg protein)$^{-1}$·min^{-1}]					
	Crude synaptosomal fraction			Supernatant fraction		
	Control	Barbital-treated	% Increase	Control	Barbital-treated	% Increase
NADPH-linked AlR						
High-K_m	13.81	14.57	5.5	11.28	12.30	9.0
Low-K_m	3.16	4.33	37.0	2.99	3.71	24.1
NADH-linked AlR	0.79	1.01	27.8	1.00	1.10	10.0

* V_{max} values were determined with various concentrations of *p*-nitrobenzaldehyde, 0.16 mM NAD(P)H, and the pooled enzyme solution, and were calculated from Lineweaver–Burk plots. For details, see the legend to Table 3.

range and the higher was in the range of 10 μM. We could, however, detect only one K_m value for the cofactor, NADPH, by the present techniques. The value for *p*-nitrobenzaldehyde with NADH was in the range of 10 μM being similar to the higher K_m value with NADPH. The K_m values of crude synaptosomal AlR were generally lower than the corresponding values of supernatant AlR. Despite the treatment of animals with barbital for 49 days, the K_m values were nearly unchanged.

The values of maximum velocity (V_{max}) of NADPH-linked AlR with high K_m (Table 4) were compatible with the activities under our routine assay conditions (Table 2), as were the V_{max} values of NADH-linked AlR. This indicates that the substrate and cofactors were essentially at saturating levels under our routine assay conditions (Table 2). On the other hand, the V_{max} values of low-K_m NADPH-linked AlR were increased 37 per cent in synaptosomes by barbital treatment and 24.1 per cent in the cytosol (Table 4).

Effect of chronic barbital treatment on the in vitro *inhibition of NADPH-linked AlR by barbital*

When 1 mM barbital was added to the assay mixture, synaptosomal AlR activities from control and barbital-treated animals were inhibited by 84.8 and 82.4 per cent respectively (Table 5). Under the same conditions, supernatant AlR from control and barbital-treated animals was inhibited by 72.0 and 71.9 per cent respectively. Thus, the crude synaptosomal AlR was more strongly inhibited by 1 mM barbital than the supernatant enzyme. The apparent increase in the synaptosomal enzyme activity by barbital treatment was greater (25.8 per cent) when assayed in the presence of barbital (1 mM) than in the absence of barbital (9.0 per cent). Such a change was not seen in the supernatant fraction.

A Lineweaver–Burk plot of the kinetic data indicated that the inhibition by barbital was noncompetitive with respect to *p*-nitrobenzaldehyde, in agreement with the finding of Erwin *et al.* [19]. K_i values were higher in the low-K_m enzyme than in the high-K_m enzyme, and higher in the synaptosomal fraction than in the supernatant fraction (Table 6). The K_i values were unchanged by barbital treatment except for the value of the low-K_m enzyme in the synaptosomal fraction, which was approximately two times higher in barbital-treated animals than in control animals.

DISCUSSION

Our previous report [11] demonstrated that prolonged administration of barbiturates to rats resulted in an increase in cytosol NADH-linked AlR [12]. In the present study, it was confirmed that NADH-linked AlR activity was elevated more markedly than the NADPH-linked activity in both the synaptosomal and the supernatant fractions (Figs. 1 and 2). In

Table 5. Effect of chronic barbital treatment on the *in vitro* inhibition of NADPH-linked AlR by barbital in the rat brain*

Enzyme	Barbital (1 mM)	Enzyme activity [nmoles·(mg protein)$^{-1}$·min^{-1}]		Apparent increase (%)
		Control	Barbital-treated	
Crude synaptosomal AlR	(−)	13.70 ± 0.17	14.93 ± 0.14	9.0
	(+)	2.09 ± 0.20	2.63 ± 0.15	25.8
% Inhibition		84.8	82.4	
Supernatant AlR	(−)	10.58 ± 0.15	11.70 ± 0.13	10.6
	(+)	2.97 ± 0.13	3.28 ± 0.07	10.7
% Inhibition		72.0	71.9	

* After 49 days of treatment with barbital or the vehicle, brain NADPH-linked AlR activity was determined in the presence and absence of barbital (1 mM) under routine assay conditions. Each value is the mean ± S.E.M. obtained from four to five animals.

Table 6. Effect of barbital treatment on the inhibitor constants for barbital with NADPH-linked AlR*

Enzyme	K_i (10^{-5} M)	
	Control	Barbital-treated
Crude synaptosomal AlR		
High-K_m	8.7	9.7
Low-K_m	37.2	69.0
Supernatant AlR		
High-K_m	6.5	6.6
Low-K_m	30.0	30.4

* After 49 days of treatment with barbital or the vehicle, K_i values were determined from Lineweaver–Burk plots. Values are averages of the values obtained with two different concentrations of inhibitors. NADPH concentration was 0.16 mM with various concentrations of *p*-nitrobenzaldehyde (from 10 to 500 μM) and pooled enzyme solution (from eight animals in each case) in 0.1 M sodium phosphate (pH 7.0).

particular, very rapid elevation of the activity was observed in the synaptosomal fraction. In contrast, the activity of NADPH-linked AlR in the synaptosomal fraction was increased much later, and only slightly, by chronic barbital treatment.

Kinetic studies indicated that the increases in the activities of AlR upon chronic barbital treatment were not due to changes in K_m values (Table 3). We obtained two distinct K_m values for *p*-nitrobenzaldehyde with NADPH-linked AlR in either fraction. This is in line with the report of Reyes and Erwin [20] on synaptosomal NADPH-linked AlR. It is thought that low-K_m AlR is probably responsible for the reduction of biogenic aldehydes, since the levels of these aldehydes in the brain are very low. Therefore, it is of interest that the V_{max} values of low-K_m NADPH-linked AlR in synaptosomes and cytosol were greatly increased (by 37.0 and 24.1 per cent respectively) by barbital treatment (Table 4), despite the increases of only about 10 per cent in the activities under routine assay conditions (Figs. 1 and 2), which correspond to the V_{max} values of high-K_m NADPH-linked AlR. This suggests that the increase in the NADPH-linked activity observed under the routine assay conditions (Figs. 1 and 2) may be mainly due to the increase in this low-K_m AlR activity induced by barbital treatment.

Inhibition by 1 mM barbital was more potent in the case of synaptosomal NADPH-linked AlR than in that of the supernatant enzyme under the routine assay conditions (Table 5). However, the K_i values were rather higher in the synaptosomal fraction (Table 6). This discrepancy may be explained by the difference in the composition ratio of low-K_m enzyme to high-K_m enzyme between synaptosomes and cytosol (Table 4). Since the relative activity of low-K_m enzyme, which has a higher K_i value than the high-K_m enzyme, with respect to the high-K_m enzyme was lower in the synaptosomal fraction, the entire activity in the synaptosomal fraction may become apparently more sensitive to high concentrations of barbital than the supernatant fraction activity. In harmony with the present data, Ris *et al.* [21, 22] reported that barbiturates caused potent inhibition of the major NADPH-linked AlR with high-K_m in rat brain, whereas these drugs were less inhibitory to the minor component with low-K_m. Further, the low-K_m enzyme was reported to utilize NADH as well as NADPH as a cofactor [22]. This view is also compatible with our finding that the relative activity of NADH-linked AlR with respect to NADPH-linked AlR in the synaptosomal fraction was lower than that in the supernatant fraction. In the barbital-treated animals, the K_i value of the low-K_m NADPH-linked AlR in synaptosomes was greater than in control animals, although there were no changes in the K_i values of other enzymes. Thus, the apparent increase in the synaptosomal activity by 25.8 per cent in the presence of 1 mM barbital (Table 5) may have resulted from a combination of the effects of barbital treatment on the K_i and on the amount of the enzyme activity.

The present results indicate that the synaptosomal AlR activities with either NADH or NADPH were increased during barbital exposure. In particular, it is likely that the activity of low-K_m NADPH-linked AlR, which is less sensitive to barbital and also utilizes NADH as a cofactor, was selectively increased by barbital treatment. The low-K_m AlR may be mainly responsible for the metabolism of the aldehydes that are physiologically generated and present in low concentrations in the brain. Thus, the elevation of the activity (37 per cent) and the K_i value (85 per cent) of the low-K_m enzyme may compensate for the decreased biogenic aldehyde-metabolizing capacity in the brain. When calculated from the obtained V_{max} and K_i values, the low-K_m AlR activity in the barbital-treated animals is 56 per cent of the normal value even in the presence of 1 mM barbital, while that in the control animals is only 27 per cent. In the presence of 0.1 mM barbital, the activity in the control animals is estimated to be 79 per cent of the normal value, but in the barbital-treated animals the activity seems to be rather higher than the normal value. These barbital concentrations correspond with brain levels of barbiturates achieved with doses of the barbiturates that produce pharmacological effects in rats [23, 24].

It has been assumed that the inhibitory effects of barbiturates on NADPH-linked AlR are involved in their anticonvulsant action [9]. Further, we suggested [10, 23] that the inhibition of biogenic alde-

hyde metabolism may be responsible for the increased hypnotic response to barbiturates caused by disulfiram, a brain AlDH inhibitor [25–27]. Chronic administration of barbiturates leads to an attenuation of hypnotic response to a challenging dose of barbiturates, i.e. tolerance, and a reduction of convulsive threshold, i.e. physical dependence, which appear to be functional adaptations of the CNS. Our present findings may be one of the biochemical adaptive mechanisms during barbiturate treatment. Whether these biochemical effects may be involved in the pharmacological actions during chronic barbiturate treatment is under investigation.

REFERENCES

1. M. Jouvet, *Science* **163**, 32 (1969).
2. H. C. Sabelli, W. J. Giardina, S. G. A. Alivisatos, P. K. Seth and F. Ungar, *Nature, Lond.* **223**, 73 (1969).
3. R. Fukumori, A. Minegishi, T. Satoh, H. Kitagawa and S. Yanaura, *Psychopharmacology* **69**, 243 (1980).
4. T. Satoh, R. Fukumori, I. Nakagawa, A. Minegishi, H. Kitagawa and S. Yanaura, *Life Sci.* **24**, 2031 (1979).
5. I. Barofsky and A. Feldstein, *Experientia* **26**, 990 (1970).
6. P. K. Gessner and A. G. Soble, *J. Pharmac. exp. Ther.* **186**, 276 (1973).
7. R. J. S. Duncan and T. L. Sourkes, *J. Neurochem.* **22**, 663 (1974).
8. B. Tabakoff and V. G. Erwin, *J. biol. Chem.* **245**, 3263 (1970).
9. V. G. Erwin and R. A. Deitrich, *Biochem. Pharmac.* **22**, 2615 (1973).
10. A. Minegishi, R. Fukumori, T. Satoh, H. Kitagawa and S. Yanaura, *Res. Commun. Chem. Path. Pharmac.* **24**, 273 (1979).
11. T. Satoh, R. Fukumori, A. Minegishi, H. Kitagawa and S. Yanaura, *Res. Commun. Chem. Path. Pharmac.* **23**, 297 (1979).
12. V. G. Erwin, W. D. W. Heston and B. Tabakoff, *J. Neurochem.* **19**, 2269 (1972).
13. A. J. Turner and K. F. Tipton, *Eur. J. Biochem.* **30**, 361 (1972).
14. W. W. Morgan, K. A. Pfeil and E. G. Gonzales, *Life Sci.* **20**, 493 (1977).
15. S. Knapp and A. J. Mandell, *J. Pharmac. exp. Ther.* **193**, 812 (1975).
16. H. J. Strecker, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 2, p. 220. Academic Press, New York (1955).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. V. G. Erwin and R. A. Deitrich, *J. biol. Chem.* **241**, 3533 (1966).
19. V. G. Erwin, B. Tabakoff and R. L. Bronaugh, *Molec. Pharmac.* **7**, 169 (1971).
20. E. Reyes and V. G. Erwin, *Neurochem. Res.* **2**, 87 (1977).
21. M. M. Ris, R. A. Deitrich and J. P. von Wartburg, *Biochem. Pharmac.* **24**, 1865 (1975).
22. M. M. Ris and J. P. von Wartburg, *Eur. J. Biochem.* **37**, 69 (1973).
23. R. Fukumori, A. Minegishi, T. Satoh, H. Kitagawa and S. Yanaura, *Brain Res.* **181**, 241 (1980).
24. S-J. Liu, C. L. Huang and I. W. Waters, *J. Pharmac. exp. Ther.* **194**, 285 (1975).
25. A. Minegishi, T. Satoh, R. Fukumori, H. Kitagawa and S. Yanaura, *Life Sci.* **24**, 1131 (1979).
26. R. Fukumori, A. Minegishi, T. Satoh, H. Kitagawa and S. Yanaura, *Life Sci.* **25**, 123 (1979).
27. R. Fukumori, A. Minegishi, T. Satoh, H. Kitagawa and S. Yanaura, *Eur. J. Pharmac.* **61**, 199 (1980).