Biochemical Pharmacology, Vol. 23, pp. 1955–1962. Pergamon Press, 1974. Printed in Great Britain.

EFFECTS OF ETHANOL ON TRYPTOPHAN HYDROXYLASE ACTIVITY FROM STRIATE SYNAPTOSOMES*

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(*Received* 22 *October* 1973; *accepted* 7 *December* 1973)

Abstract—Ethanol inhibits the conversion of tryptophan to serotonin (5-HT) by rat striate synaptosomes *in vitro* in concentrations as low as 0·1 M. This effect is apparently not mediated by a decrease in the uptake of radioactive tryptophan but through a noncompetitive inhibition of the activity of the enzyme tryptophan-5-hydroxylase (EC 1.99.1.4) itself. Experiments showing: (1) a correlation between the inhibitory potencies of a number of alcohols of increasing carbon chain length and their partition coefficients, and (2) a calculable increment of binding of 0·47 kcal/mole/methylene unit in the alcohol series (which should be between 0·36 and 0·95 kcal/mole if the methylene unit is the source of ΔF) suggest that the inhibition may operate via a hydrophobic site on the enzyme.

WE HAVE SHOWN that changes in the serotonin (5-HT) biosynthetic system induced by the acute administration of drugs lead to changes in tryptophan hydroxylase activity and that when the drugs are administered chronically the changes in enzyme activity tend to compensate for the acute effects of the drugs.[1–3] For example, lithium chloride *in vitro* and *in vivo* results in augmented tryptophan uptake into synaptosomes. Consequently, in the early phase (3–5 days) of lithium treatment, synaptosomal conversion of tryptophan to 5-HT is significantly increased. However, by 10 days after the initiation of lithium treatment conversion activity has returned to control levels and tryptophan hydroxylase activity is significantly below control levels.[2] Several workers have reported that chronic administration of ethanol to mice increased 5-HT turnover and tryptophan hydroxylase activity in the brain, in spite of the fact that acute administration of the drug appeared to have little or no effect.[4–6] It was our purpose in the work reported here to examine the effect of ethanol *in vitro* in light of our model of the adaptive regulation of the serotonergic system.[7] Using our measure of 5-HT biosynthetic capacity, i.e. regional synaptosomal conversion of tryptophan to 5-HT, we have found that ethanol inhibits that conversion *in vitro*. Our report explores this phenomenon in some detail.

MATERIALS AND METHODS

Materials. L-[1-^{14}C]-tryptophan (12 μCi/μmole) and L-[3-^{14}C]-tryptophan (29 μCi/μmole) were obtained from New England Nuclear Corp., Boston. DL-[1-^{14}C]-dihydroxyphenylalanine (Dopa; 53 μCi/μmole) was obtained from Amersham-

* Supported by National Institute of Mental Health Grant No. DA-00265-02 and Friends of Psychiatric Research of San Diego, Inc.

Searle, Arlington Heights, Ill. Impurities were removed from the isotopes by lyophilization from 0·1 M Tris-acetate buffer (pH 8·1). From Calbiochem, La Jolla, Calif., we obtained 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$), 6-methyl-5,6,7,8-tetrahydropterin (6-$MePH_4$) and pyridoxal-5′-phosphate. Triton X-100 (octylphenoxypolyethanol) was purchased from Sigma Chemical Co., St. Louis, Mo. All reagents used were of the highest purity obtainable.

Preparation of tissue. Adult male Sprague–Dawley rats (150–200 g each) from Hilltop Laboratories, Chicago, were decapitated and the brains were removed rapidly and rinsed in 0·32 M sucrose. For preparation of synaptosomes, the striate cortex (which is rich in serotonergic nerve endings) was immediately dissected free and homogenized with ten up-and-down strokes and a clearance of 0·10 cm in 25 vol. of ice-cold 0·32 M sucrose. The homogenates were initially centrifuged at 1000 *g* for 10 min, and the pellet, consisting of nuclei and cell debris, was discarded. The supernatant fraction was further centrifuged at 12,000 *g* for 20 min to obtain a synaptosomally enriched crude mitochondrial pellet (P_2) which was resuspended in the homogenization volume of 0·32 M sucrose.[8]

The midbrain, known to contain a high density of serotonergic cell bodies,[9] served as the source of soluble tryptophan hydroxylase.[10] The tissue, consisting primarily of the region bounded rostrally by the anterior border of the cerebellar peduncles and caudally by the pons, was homogenized in 25 vol. of 2 mM sodium phosphate buffer (pH 7·0), and the 45,000 *g* supernatant fraction was used in the assays.

Enzyme assays. Tryptophan-5-hydroxylase (EC 1.99.1.4) was assayed in the midbrain preparation according to the method of Ichiyama *et al.*[11,12] with modifications as described by Knapp and Mandell.[3,10] The reaction mixture contained 40 nmoles Tris-acetate buffer (pH 7·4), 760 nmoles $DMPH_4$ or 300 nmoles 6-$MePH_4$, 10 nmoles of β-mercaptoethanol, 5–10 units of aromatic l-amino-acid decarboxylase (EC 4.1.1.26; 3,4-dihydroxy-L-phenylalanine carboxy lyase) from rat kidney prepared by modification[3] of the method of Christenson *et al.*,[13] 0·2 ml of the tissue preparation (0·4 mg protein), and 4–6 nmoles L-[1-^{14}C]-tryptophan in a final volume of 0·7 ml. The following alcohols were added to the incubation mixture at various concentrations: ethanol, methanol, *n*-propanol and *n*-butanol. The enzymatic reaction was initiated by the addition of substrate and allowed to proceed at 37° for 45 min. At the end of the incubation period, the reaction was stopped by the addition of 0·5 ml of 2 N perchloric acid. The $^{14}CO_2$ evolved during the reaction was trapped in 0·1 ml NCS (Nuclear, Chicago) contained in plastic wells suspended from the stoppers which sealed the reaction tubes. The wells were placed directly in vials containing 10 ml of scintillation fluid consisting of a mixture of toluene phosphor [850 ml toluene containing 3·4 g 2,5-diphenyloxazole (PPO) and 0·41 g *p*-bis-[2-(5-phenyloxazolyl)]-benzene] (POPOP) and absolute ethanol in a 4-to-1 ratio. The radioactivity in each sample was determined with a Beckman LS 250 liquid scintillation spectrometer using external standard quench correction. All enzymatic activities were assayed in duplicate, and appropriate controls and reagent blanks were included in each assay.

Dopa decarboxylase (EC 4.1.1.26) from rat kidney was assayed according to a modification[3] of the method of Christenson *et al.*[13] The incubation mixture contained 20 nmoles Tris-acetate buffer (pH 8·1), 30 pmoles pyridoxal-5′-phosphate, 30 pmoles β-mercaptoethanol, 1·5 μmoles [1-^{14}C]-Dopa, 0·1 ml of the enzyme prep-

aration, and ethanol in an appropriate concentration in a total volume of 0·7 ml. The mixture was incubated at 37° for 30 min. The reaction was stopped, $^{14}CO_2$ was collected and radioactivity was determined as described above.

Conversion activity. The assay for the conversion of tryptophan to 5-HT in the striate synaptosomal fraction was similar to that used for the midbrain fraction except that $DMPH_4$, β-mercaptoethanol and kidney decarboxylase were excluded. The assay was buffered at its pH optimum of 8·1[10] and the total volume was 0·7 ml. This measure reflects the composite activity of intrasynaptosomal tryptophan hydroxylase, tryptophan transport and endogenous cofactor levels as well as decarboxylase activity.[3]

Synaptosomal tryptophan uptake. A 0·2-ml aliquot of the striate synaptosomal preparation (0·2 mg protein) was added to a mixture of 0·1 M Tris-acetate buffer (pH 7·4, containing 3·4 mM glucose), 12 μM L-[3-^{14}C]-tryptophan and an appropriate concentration of ethanol. The total assay volume was 0·6 ml. At the end of a 5-min incubation at 37°, uptake was stopped by dilution with 1 ml of ice-cold 0·32 M sucrose. The synaptosomes were collected on Millipore filters (25 mm diameter, 0·63 μm pores) with a Millipore multiple-sampling vacuum manifold. The filters were washed with an additional 1 ml of 0·32 M sucrose and were counted directly in vials containing 10 ml of scintillation fluid prepared as described above. When substrate concentration ranged from 10 to 100 μM, the transport of L-[3-^{14}C]-tryptophan was saturable at 100 μM. The 60 per cent inhibition observed in the presence of 2 mM ouabain supports the assertion that this tryptophan transport is an active uptake process. In addition, the uptake of L-[3-^{14}C]-tryptophan is reduced by 80 per cent of control values when the experiment is carried out at 4° rather than 37°. This uptake process is abolished when the synaptosomes are lysed.

RESULTS

Effect of ethanol on synaptosomal conversion of tryptophan to 5-HT. The effect of ethanol on the conversion of tryptophan to 5-HT in synaptosomes isolated from the rat striate cortex was studied with the alcohol in concentrations ranging from 0·05 to 0·5 M (0·23 to 2·3% w/v). Significant decreases in conversion activity were observed with ethanol in as low a concentration as 0·1 M (Fig. 1).

Effect of ethanol on synaptosomal uptake of tryptophan. Active uptake of L-[3-^{14}C]-tryptophan, as opposed to the conversion of the amino acid, was not affected by ethanol concentrations as high as 0·5 M in the incubation mixture (Fig. 1).

Effect of ethanol on midbrain soluble tryptophan hydroxylase activity. Ethanol inhibited the soluble hydroxylase from rat midbrain, although not quite to the same degree that it inhibited the striate synaptosomal preparation (Fig. 2). Ethanol in the concentrations used did not alter the activity of the exogenous rat kidney decarboxylase in the coupled assay. $DMPH_4$ was routinely used as a cofactor, but we determined that the effect was similar with the monomethyl cofactor 6-$MePH_4$ at a concentration of 0·65 mM. The kinetics of the inhibition are noncompetitive with respect to cofactor (Fig. 3) and substrate (Fig. 4) with ethanol at a concentration of 0·2 M. The degree of inhibition was identical to that seen when the supernatant from a 100,000 *g* centrifugation of a striate cortex fraction solubulized with Triton X-100 (0·5% w/v) was used as the enzyme source. This suggests that ethanol affects the

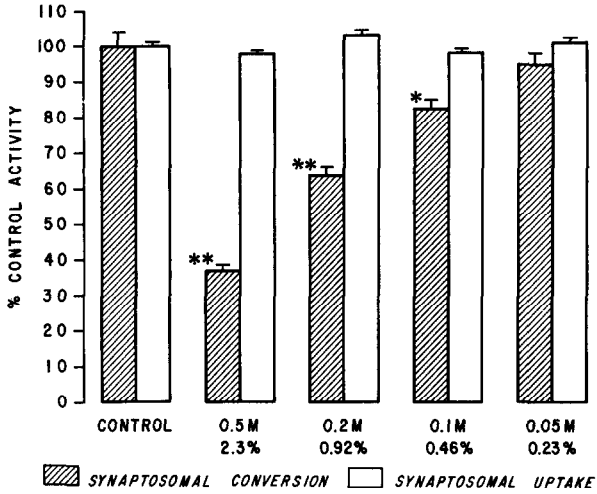

FIG. 1. Ethanol inhibition of the conversion of tryptophan to 5-HT by synaptosomes prepared from rat striate cortex. There is no effect on the active uptake of tryptophan by the same preparation. Each bar represents the mean of from three to six separate determinations. Data are expressed as per cents of mean control activities ± S.E. Mean net conversion of control samples: 1754 ± 79 dis./min/0·2 ml of synaptosomal fraction/45 min. Mean uptake of control samples: 13,511 ± 102 dis./min/0·2 ml of synaptosomal fraction/5 min. Concentrations of alcohol *in vitro* are shown in moles/liter and percentage (w/v). The single asterisk indicates $P < 0{\cdot}02$ and the double asterisk indicates $P < 0{\cdot}001$ by Student's *t*-test.[14]

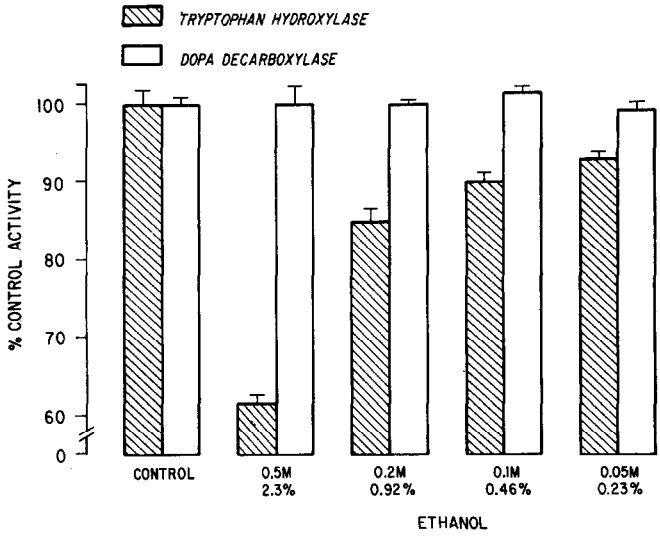

FIG. 2. Effects of ethanol on the activity of tryptophan hydroxylase from rat midbrain and Dopa decarboxylase from rat kidney. Ethanol concentrations are comparable to those in experiments represented in Fig. 1. The vertical axis in this figure is abbreviated. Data are expressed as means ± S.E. for at least six samples and two separate determinations respectively. Mean net activity of control samples: 1715 ± 145 dis./min/0·2 ml of enzyme source/45 min for tryptophan hydroxylase; 19,498 ± 545 dis./min/0·1 ml of enzyme source/30 min for Dopa decarboxylase.

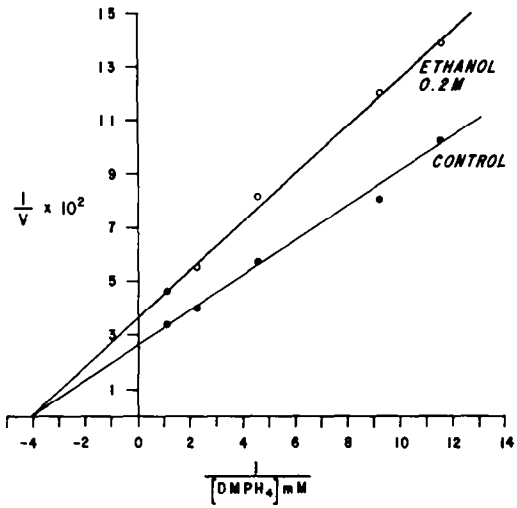

FIG. 3. Kinetic analysis of the inhibition of midbrain soluble tryptophan hydroxylase by ethanol according to the method of Lineweaver and Burk.[15] Units of $V(1/V \times 10^2)$ are pmoles 5-HT formed/0·2 ml of enzyme source/45 min. Substrate concentration was 10 μM; $DMPH_4$ concentration was 50 μM to 1 mM. V_{max} with regard to $DMPH_4$ was 1200 ± 150 dis./min/0·2 ml of enzyme preparation/45 min. Each point represents the mean of three determinations.

enzyme itself rather than an adherent membrane system. To determine if the alcohol-induced inhibition were reversible, we dialyzed samples. However, the lability of the enzyme resulted in such reduced activity in the control samples that valid data could not be obtained.

Effect of short-chain aliphatic alcohols on soluble tryptophan hydroxylase. A number of alcohols tested in addition to ethanol showed a concentration-related inhibition

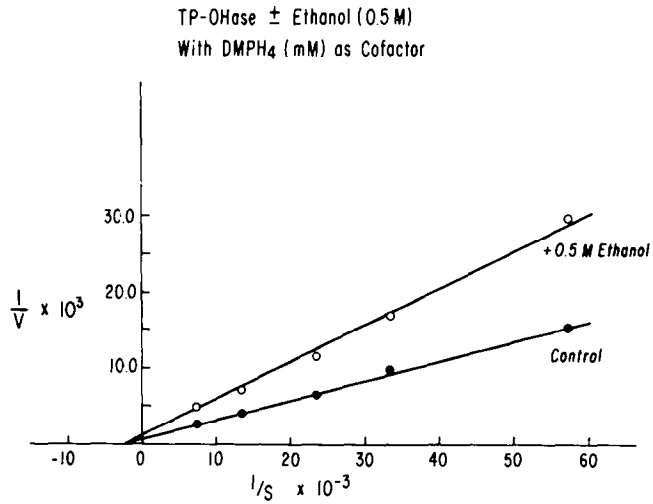

FIG. 4. Kinetic analysis of the inhibition of midbrain soluble enzyme. $DMPH_4$ concentration, 1 mM; substrate concentration, 10–100 μM. Units of $V(1/V \times 10^3)$ are pmoles 5-HT formed/0·2 ml of enzyme preparation/45 min. V_{max} with regard to tryptophan with $DMPH_4$ as cofactor: 900 pmoles/0·2 ml of enzyme preparation/45 min. Each point is the average value of two determinations.

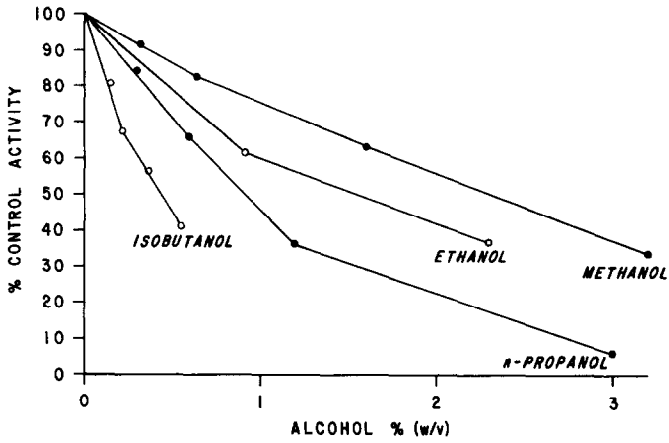

FIG. 5. Dose-related inhibition of tryptophan hydroxylase by various short-chain aliphatic alcohols at 1% (w/v) equals 0·122 M isobutanol, 0·15 M *n*-propanol, 0·21 M ethanol and 0·29 M methanol. Mean control activity from three experiments: 1400 ± 99 dis./min/0·2 ml of enzyme preparation/45 min.

of the soluble enzyme from rat midbrain. The more hydrophobic the alcohol (as measured by octanol–water partition coefficients) the greater its inhibitory effect (Fig. 5). The molar concentration of each alcohol that produced 50 per cent inhibition of enzyme activity (I_{50}) was taken as a measure of the inhibitory potency of the alcohol. We found excellent correlation between the logarithms of the I_{50} values of these alcohols and the logarithms of their partition coefficients (Fig. 6).

Using a model described by Hansch and Dunn[17] for the inhibition of enzymes by a series of organic compounds, we were able to relate the increment in methylene units linearly to the increment in degree of inhibition using the arbitrarily defined I_{50} (Fig. 7). It is possible to calculate the change in free energy ($\Delta\Delta F$) which can then

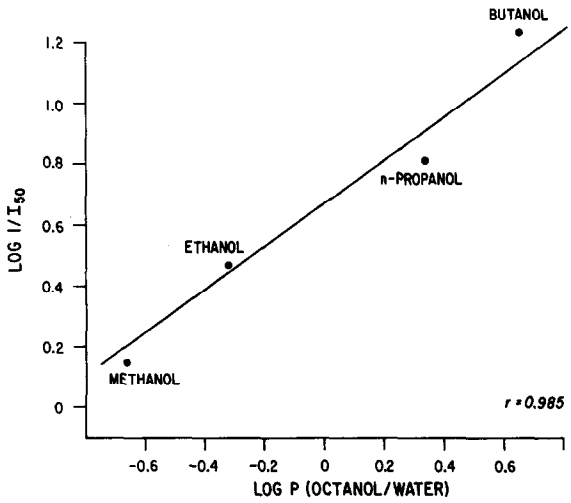

FIG. 6. Relationship of logarithm $1/I_{50}$ to log P for a number of alcohols, from the data presented in Fig. 5. The partition coefficients, P_1, are from the published data of Goldstein *et al.*[16]

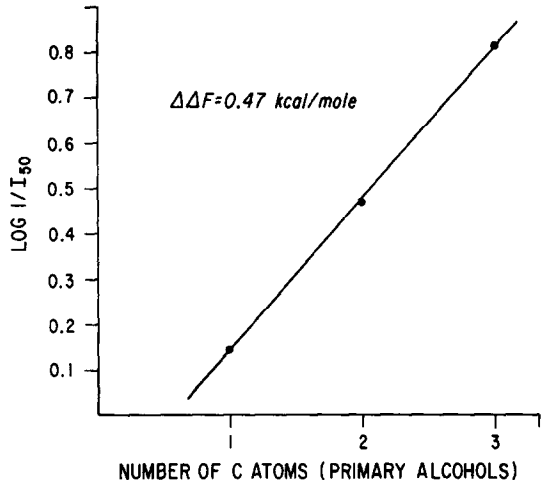

FIG. 7. Relationship of logarithm $1/I_{50}$ (Fig. 5) to the number of carbon atoms in three primary alcohols, demonstrating an increment in the free energy of binding of [0·47] kcal, mole^{-1} CH_2 group^{-1}.

be related to the type of physical interaction between the inhibitor and the enzyme by comparison with such values in a reference series of compounds. For example, Webb[18] and Hansch[19] have shown that, in simple organic series, if $\Delta\Delta F$ in methylene units is between 0·36 and 0·95 kcal/mole the interaction is probably hydrophobic. In this case the change in free energy per methylene group can be calculated from the equation

$$\Delta\Delta F = 2{\cdot}303\,RT\Delta pK_i \tag{1}$$

where R is the gas constant; T is the temperature in degrees Kelvin; and ΔpK_i is the change in the logarithm of the reciprocal of an index of inhibition (Fig. 7) as follows:

$$2{\cdot}3\,RT\,pK_i = \Delta\Delta F \tag{2}$$

$$2{\cdot}3\,(1{\cdot}98)\,(310) = 1411{\cdot}74 \tag{3}$$

$$\Delta F_1 - \Delta F_2 = 1411{\cdot}74 \log[(I_1)_{0{\cdot}5}/(I_2)_{0{\cdot}5}] \tag{4}$$

where 50 per cent inhibition is taken for both inhibitors. The reciprocals of the log of I_{50} for the series are calculated as follows (Fig. 7):

N (methylene units)	I_{50} (M)	$-\text{Log}\ I_{50}$	$1/I_{50}$
1	0·718	0·144	1·39
2	0·339	0·470	2·95
3	0·153	0·815	6·53

In the linear relationship log $1/I_{50}$ vs N, the slope is $-0{\cdot}336$, the intercept is 0·1946 and r is 0·999,[17] so that

$$\Delta\Delta F = (1411.74)\,(-0{\cdot}336) \tag{5}$$

$$[\Delta\Delta F] = 0{\cdot}47\ \text{kcal/mole} \tag{6}$$

This falls well within the range of free energy change established for a series of inhibitors whose mechanisms are related to hydrophobic interactions.[18]

DISCUSSION

Ethanol, in the concentrations we studied, appears to reduce the capacity of striatal synaptosomes to convert tryptophan to 5-HT. This effect was not the result of an alteration in the uptake of substrate into the synaptosomes, but a direct effect on enzyme activity. The kinetics of the inhibition as well as the relationships between degree of inhibition, length of carbon chain, and partition coefficients of the alcohols suggest the possible involvement of a hydrophobic site on the brain enzyme. Although we are not working with a purified enzyme preparation, it should be noted that systematic relationships have been established in the past with other impure systems. Hansch and Dunn[17] have reviewed such interactions involving a number of organic compounds and enzyme systems extensively. In the context of our model for the adaptive regulation of the neurotransmitter systems in the brain,[7] our finding a reduction of 5-HT biosynthesis in a nerve ending preparation *in vitro* is compatible with observations of increased 5-HT synthesis under the chronic administration of ethanol[4,5] on a feedback regulatory basis.[1–3] Rather high concentrations of ethanol were required to produce these effects *in vitro* and, as such, they may not be relevant to the clinical aspects of alcoholism.

Recent studies by Myers' group[20,21] and Fray *et al.*[22] have suggested that the serotonergic systems in the brain may be involved in the preference and/or the consumption of alcohol by rats, whether it is genetically based or augmented by stress. We are now examining the response of the serotonergic system to chronic administration of ethanol with some hope of relating the adaptive changes in brain tryptophan hydroxylase to alcohol-seeking behavior.

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