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The Inhibition of Alcohol and Aldehyde Dehydrogenases by Propranolol

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SUMMARY

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Propranolol inhibits horse liver alcohol dehydrogenase (EC 1.1.1.1), yeast alcohol dehydrogenase (EC 1.1.1.1), and pig brain aldehyde dehydrogenase (EC 1.2.1.3). In each case the inhibition is reversible. The form of the inhibitions is consistent with the formation of an enzyme-propranolol complex which in some cases may bind the coenzyme. Various kinetic constants for the inhibitions have been calculated; K_i values lie between 100 and 360 μM for these enzymes. Propranolol increases the dissociation constants of both 5'-AMP and phenanthroline from their complexes with liver alcohol dehydrogenase, but ternary complexes of enzyme with propranolol and 5'-AMP or phenanthroline are formed. Propranolol in concentrations up to 1 mM inhibits neither rat liver lactate dehydrogenase (EC 1.1.1.27) nor malate dehydrogenase (EC 1.1.1.37) from rat liver or from pig heart. Pronethalol inhibits liver alcohol dehydrogenase with a $K_{i\ slope}$ value of 84 μM. From these enzymatic results propranolol has the potential of slowing ethanol oxidation, and since aldehyde dehydrogenase is involved in the catabolism of the biogenic amines, propranolol may modify the metabolism of the deaminated biogenic amines.

INTRODUCTION

Propranolol is a widely used blocking agent of the *beta* adrenergic receptor site, and so may modulate the activities of a number of enzymes which are controlled via these receptors. The direct effect of propranolol on individual enzymes has not been extensively investigated, but the drug has been shown to inhibit serum cholinesterase (1), liver monoamine oxidase (2, 3) and the catecholamine-induced activation of adenylate cyclase (4). Isolated mitochondria and mitochondrial fragments are affected by propranolol (5, 6). The respiratory control coefficients for a number of substrates are decreased, and the oxidation of NADH and some NAD-linked substrates by submitochondrial particles is inhibited.

During the course of an investigation of the effects of drugs on the enzymes of catabolism of the biogenic amines, inhibition of aldehyde dehydrogenase (EC 1.2.1.3) by propranolol was noted. Aldehyde dehydrogenase is an enzyme involved in the biological oxidation of both acetaldehyde and the aldehyde products of monoamine oxi-

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dase. Factors influencing this enzyme may hence bring about changes in the metabolism of ethanol and the biogenic amines. The initial observation of inhibition of aldehyde dehydrogenase by propranolol led to the work reported here.

MATERIALS AND METHODS

DL-Propranolol [1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol] hydrochloride and DL-metanephrine were obtained from Sigma, Ltd.; coenzymes and commercial enzyme preparations, from Boehringer Mannheim Corporation; and pronethalol (α-[(isopropylamino)methyl]-2-naphthalene-methanol) hydrogen bromide, from Ayerst Laboratories, Montreal.

Propranolol HCl and pronethalol HBr were dissolved in water to give stock solutions of concentrations up to 40 mM. *o*-Phenanthroline and 5′-AMP were dissolved in warm water. New solutions were made up each day.

Enzymes were assayed at 25°.

3-Methoxy-4-hydroxyphenylacetaldehyde (the immediate precursor of homovanillic acid in brain) was synthesized from DL-metanephrine by the method of Robbins (7). The original synthesis (7) includes preparation of the bisulphite adduct of the aldehyde, but as the bisulphite anion inhibits aldehyde dehydrogenase (8), this step was not included. It was routinely verified that no inhibitor was present in the aldehyde solution before each set of kinetic determinations, because concentrated solutions of substituted acetaldehydes produce a potent inhibitor of alcohol and aldehyde dehydrogenases, probably via aldol condensation. Aldol products are known to inhibit aldehyde dehydrogenase.

Aldehyde dehydrogenase for kinetic studies was purified from acetone powders of pig brain as described previously (9). Maximum velocity, obtained from double-reciprocal plots, was about 15 nmoles/min/mg of protein with 3-methoxy-4-hydroxyphenylacetaldehyde as substrate. This is some 75% of maximum velocity with heptaldehyde (10). Aldehyde dehydrogenase was assayed fluorometrically at pH 7.2 in 100 mM phosphate buffer, by recording the rate of production of NADH.

Liver alcohol dehydrogenase was diluted to 3 mg/ml with a solution of 1 mM dithiothreitol in 50 mM phosphate buffer, pH 7.4, and then filtered through Sephadex G-25 into a fresh sample of the same buffer to remove ethanol added by the supplier. Liver and yeast alcohol dehydrogenases were assayed spectrophotometrically at pH 7.4 in 50 mM phosphate buffer with propionaldehyde and NADH, or at pH 10.0 in 100 mM glycine-NaOH buffer with NAD^+ and ethanol.

Malate dehydrogenase was assayed spectrophotometrically in 100 mM glycine-NaOH buffer, pH 10.0, using 500 μM L-malate (a nonsaturating concentration) and NAD^+.

Lactate dehydrogenase was assayed spectrophotometrically at pH 7.4 in 50 mM phosphate, using 5 μM pyruvate (which is nonsaturating) and NADH.

A homogenate of rat liver (1 part liver, 9 parts 0.25 M sucrose) was prepared and centrifuged at 25,000 × *g* for 40 min. The supernatant fraction was used as the source of rat liver malate and lactate dehydrogenases.

Coenzymes were standardized as described in ref. 11, and aldehydes were standardized using aldehyde dehydrogenase (10).

Spectra (absorption and fluorescence) were measured in 50 mM phosphate buffer, pH 7.4, at 25°.

When propranolol or pronethalol was found to inhibit an enzyme, the reversibility of the inhibition was verified by dilution. The enzyme was incubated with a known, high concentration of propranolol or pronethalol at room temperature for 10 min. Then a sample of the incubation mixture was taken and diluted into the assay system. Initial reaction rates obtained were compared with those of controls incubated in the absence of

inhibitor, with those of complete assay systems containing the initial high concentration of inhibitor, and with those of assay systems containing the inhibitor at the final dilution.

Lines were fitted to the experimental points in double-reciprocal and Dixon plots by eye, but it was first verified that such lines should theoretically meet at a single point. The criterion used for this was the linearity of replots of both slopes and vertical intercepts of the double-reciprocal plots (12). K_m and K_i values from secondary plots were obtained by linear regression analysis; K_i and K_{diss} values obtained from intersection points on double-reciprocal plots were obtained visually. Since most constants may be obtained from two or more different types of plots of the same data, correspondence between values of kinetic constants was ensured before the final values were derived.

RESULTS AND DISCUSSION

Pertinent Spectral Properties of Propranolol

Propranolol fluoresces at 345 nm with activation at either 240 nm or 290 nm (all wavelengths uncorrected). In anticipation of the requirements for controls and blanks in the experimental work, certain properties of propranolol were investigated. NADH (100 μM) quenches the fluorescence of 500 μM propranolol excited at either wavelength, but NAD^+ (100 μM) quenches only the fluorescence excited at the shorter wavelength. Zinc sulphate (100 μM) does not change the fluorescence spectrum of propranolol. The fluorescence of NADH is not quenched or augmented by propranolol, and NADH fluorescence is not induced by excitation at 240 or 290 nm in the presence of propranolol.

The absorption spectrum of a 100 μM solution of propranolol plus 130 μM NAD^+, when measured against a reference containing 130 μM NAD^+, is identical with that of 100 μM propranolol alone.

Inhibition of Dehydrogenases

Aldehyde dehydrogenase. Pig brain aldehyde dehydrogenase is reversibly inhibited by propranolol. The inhibition is of the mixed type (13) with respect to both aldehyde and NAD^+. The plot of reciprocal velocity against the reciprocal of the concentration of NAD^+ is shown in Fig. 1. Dixon plots (14) are linear and intersect at a single point. A K_i value of 105 μM may be obtained from the Dixon plot of inhibition at a series of NAD^+ concentrations. K_m for NAD^+ is 4.6 μM at an aldehyde concentration of 7 μM; K_m for 3-methoxy-4-hydroxyphenylacetaldehyde is 0.28 μM at an NAD^+ concentration of 18.6 μM.

Brain aldehyde dehydrogenase binds its substrates in a compulsory order: NAD^+ first, then aldehyde (10). The present results are consistent with the formation of an enzyme-propranolol-NAD^+ ternary complex. Enzyme-propranolol species cannot be kinetically active, or nonlinear Dixon plots would be obtained. The dissociation constant of NAD^+ from the ternary complex may be obtained from the point of intersection of the lines of Fig. 1 and has a numerical value of 200 μM, or some 40 times greater than K_m at the same aldehyde concentration.

The rate equation describing the form of inhibition proposed is

$$1/v = 1/V_{max}\left[1 + \frac{K_m}{NAD^+} \cdot\left(1 + \frac{i}{K_i} + \frac{i NAD^+}{K_i K_{diss}}\right)\right] \quad (1)$$

where i = concentration of propranolol in this case, K_{diss} is the dissociation constant of NAD^+ from the ternary complex, and K_m is a complex constant, containing terms in aldehyde concentration, as well as individual rate constants. By simple algebra it may be shown that $1/NAD^+ = -1/K_{diss}$ at the point of intersection of the lines plotted as in Fig. 1. The formation of quaternary enzyme-NAD^+-propranolol-aldehyde complexes can-

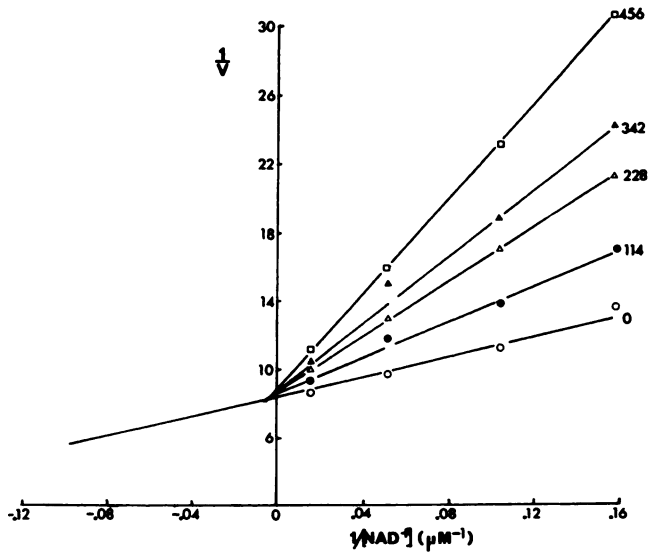


FIG. 1. *Aldehyde dehydrogenase inhibition by propranolol: double reciprocal plot*
The concentration of propranolol (micromolar) applicable to a line is inscribed beside that line. The aldehyde concentration was 7 μM. v is measured in arbitrary fluorescence units.

not be ruled out. In this case K_{diss} will also include terms in aldehyde concentration. If K_{diss} has a value of infinity, the form of the inhibition becomes competitive.

Yeast alcohol dehydrogenase. Propranolol reversibly inhibits yeast alcohol dehydrogenase. The inhibition is competitive with respect to NAD^+ and mixed with respect to ethanol. Dixon plots are linear and intersect at a single point. The reverse reaction is also inhibited, but the detailed kinetics has not been investigated. Yeast alcohol dehydrogenase binds its substrates in random order, and the various enzyme-substrate complexes are at equilibrium with each other and with free substrate (15). The inhibition described is consistent with binding of propranolol to the free enzyme form, preventing binding of NAD^+. An enzyme-ethanol-propranolol complex may also be formed. A K_{diss} for ethanol from this complex may be calculated from the point of intersection of the lines in Fig. 2, and has a value of 53 mM. K_m for ethanol from Fig. 2 is 15.7 mM. K_i for propranolol is 360 μM from Dixon plots of the inhibition at a series of NAD^+ concentrations.

Rat liver malate and lactate dehydrogenases. Neither of these enzymes is inhibited by propranolol. Malate dehydrogenase was assayed with NAD^+ concentrations from 64 to 640 μM (K_m = 175 μM under the assay conditions), and although malate was also nonsaturating, no inhibition could be detected with propranolol in concentrations up to 770 μM. Lactate dehydrogenase assayed at nonsaturating concentrations of both pyruvate and NADH is not inhibited by up to 1 mM propranolol. Commercial pig heart malate dehydrogenase assayed as detailed above is not inhibited by up to 1 mM propranolol, and commercial bovine heart lactate dehydrogenase assayed as described above is inhibited less than 5% by 1 mM propranolol. Yeast alcohol dehydrogenase is inhibited to exactly the same extent by 500 μM propranolol in the presence or absence of liver supernatant; therefore the lack of inhibition of rat liver malate and lactate dehydrogenases is due to the nature of those enzymes, not to an effect of the homogenate on propranolol.

Horse liver alcohol dehydrogenase. Propranolol reversibly inhibits this enzyme. Inhibition is of the mixed type with respect

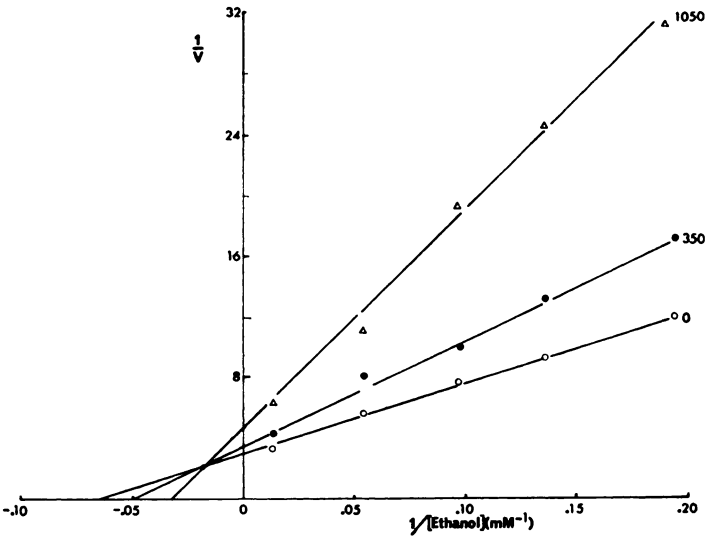


FIG. 2. *Yeast alcohol dehydrogenase inhibition by propranolol: double-reciprocal plot*
The concentration of propranol (micromolar) applicable to a line is inscribed beside that line. The NAD^+ concentration was 230 μM.

to both NAD^+ and NADH (Fig. 3). Dixon plots of these inhibitions are linear and give K_i values of 215 and 190 μM, respectively. The difference in these values may be ascribed to the fact that the assay is conducted at different pH values in the two directions. Inhibition is mixed with respect to ethanol.

The inhibition of the enzymes described above can best be explained by assuming that propranolol binds to the same form of the enzyme as does the coenzyme. It is of interest to study how propranolol may affect the coenzyme-binding site. Yonetani and Theorell (16) have described a simple graphical method for obtaining an interaction constant between two competitive inhibitors in an enzyme-inhibitor complex. The method provides a means of answering the kinetic question whether the inhibitors are mutually exclusive or whether an enzyme–inhibitor 1–inhibitor 2 complex is formed, and also gives information about the magnitude of various kinetic constants (12). The treatment of Yonetani and Theorell is applicable only to competitive inhibition, but may be extended to certain other forms of inhibition.

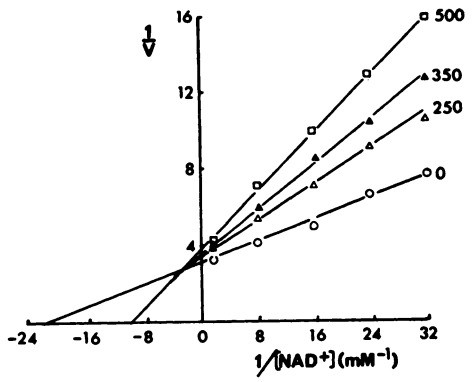
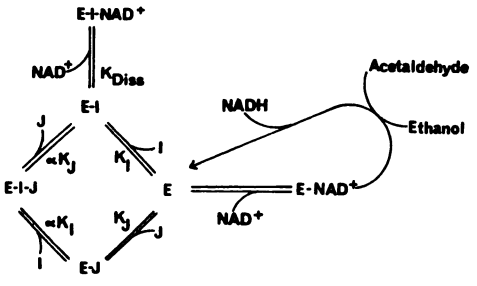


FIG. 3. *Liver alcohol dehydrogenase inhibition by propranolol: double-reciprocal plot*
The concentration of propranolol (micromolar) applicable to a line is inscribed beside that line. The ethanol concentration was 2.3 mM.

Liver alcohol dehydrogenase is inhibited by nucleotides and by phenanthroline. Both types of inhibitor compete with NAD but are not mutually exclusive. Binding of phenanthroline actually facilitates binding of 5′-AMP (16). Scheme 1 shows the kinetic pathway for liver alcohol dehydrogenase, which is consistent with the known reaction mechanism of the enzyme (15) and the inhibition results described above.



SCHEME 1. *Proposed inhibition pathways of liver alcohol dehydrogenase*

I represents propranolol, *J* represents either 5'-AMP or phenanthroline, *E* represents free enzyme, *E-I*, etc., represent enzyme-inhibitor, etc., complexes, K_I is the dissociation constant of *E-I*, αK_I is the dissociation constant of *I* from *E-I-J* (see the text), and K_{diss} is the dissociation constant of NAD+ from *E-I*-NAD+.

Application of the method of Cha (17) to Scheme 1 allows the following rate equation to be derived:

$$1/v = 1/V_{max}\left[1 + \frac{K_m}{NAD^+}\left(1 + \frac{I}{K_I} + \frac{J}{K_J} + \frac{IJ}{\alpha K_I K_J} + \frac{I\ NAD^+}{K_I K_{diss}}\right)\right] \quad (2)$$

The various terms are defined in the legend to Scheme 1.

The ratio of the dissociation constants of propranolol from the *E-I-J* complex (see Scheme 1) and from the *E-I* complex must, from the nature of the inhibition proposed, equal the ratio of the dissociation constants of the other inhibitor from the *E-I-J* and *E-J* complexes. This ratio of dissociation constants, α, gives a measure of how the presence of one inhibitor affects the interaction of the enzyme with the other inhibitor. When the binding of one inhibitor decreases the binding of the other, $\alpha > 1$; if binding of one inhibitor completely prevents the binding of the other, $\alpha = \infty$. When the binding of inhibitors is independent, $\alpha = 1$, and if binding of one inhibitor increases the binding of the other, $\alpha < 1$.

On a Yonetani-Theorell plot (of $1/v$ against concentration of *I* at a series of concentrations of *J*) it can be seen from the rate equation that the point of intersection of lines of constant *J* will have an *I* coordinate of $I = -\alpha K_I$. The converse plot, of $1/v$ against *J* at a series of constant *I*, intersects with a *J* coordinate of

$$J = -\alpha K_J[1 + (NAD^+/K_{diss})].$$

Yonetani-Theorell plots of the inhibition of liver alcohol dehydrogenase by phenanthroline and propranolol are shown in Fig. 4. It may be calculated that K_J for phenanthroline is 37 μM under these conditions, K_I for propranolol is 215 μM, α has a value of

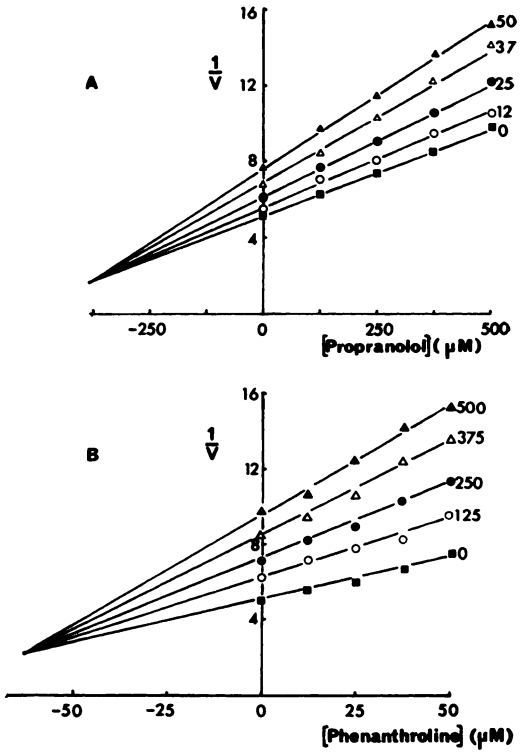


FIG. 4. *Liver alcohol dehydrogenase inhibition by propranolol and phenanthroline: Yonetani-Theorell plots*

A. $1/v$ against concentration of propranolol (micromolar). The concentration of phenanthroline (micromolar) applicable to a line is inscribed beside that line. B. $1/v$ against concentration of phenanthroline (micromolar). The concentration of propranolol (micromolar) applicable to a line is inscribed beside that line. For both A and B the NAD+ concentration was 60 μM and the ethanol concentration was 2.3 mM.

1.7, and K_{diss} is very large. The binding of one of these inhibitors decreases, but does not prevent, the binding of the other.

A Yonetani-Theorell plot of the inhibition of liver alcohol dehydrogenase by propranolol at a series of 5′-AMP concentrations gives an α value of 2.5. Thus 5′-AMP binding is decreased but not prevented by propranolol.

The over-all form of inhibition of liver alcohol dehydrogenase by propranolol is consistent with the formation of an enzyme-propranolol binary complex that alters the protein conformation in such a manner as to decrease binding at the coenzyme site.

From structural considerations, propranolol would be expected to be a metal-chelating agent. If this were the case, propranolol could perhaps inhibit dehydrogenases by chelation. It is unlikely, however, that propranolol inhibits the dehydrogenases by chelation of the zinc at the active center, because a much larger interaction between propranolol and phenanthroline [which does chelate the zinc (18)] would then be expected.

Certain of the constants may change in value if the ethanol forms a quaternary complex with the enzyme-coenzyme-inhibitor ternary complex. Breakdown of such a complex to give product is not consistent with the linear Dixon plots obtained. Moreover, the conclusions concerning the interaction of the pairs of inhibitors are valid even if a quaternary complex is formed. The present analysis was performed at a constant ethanol concentration of 2.3 mM.

Pronethalol is structurally related to propranolol and also blocks *beta* adrenergic receptors (19). Horse liver alcohol dehydrogenase is inhibited by pronethalol, as shown in Fig. 5, which illustrates the form of the inhibition with respect to NADH. The inhibition is reversible by dilution. Cleland (20) has defined inhibitor constants obtainable from the slopes (K_{is}) and vertical intercepts (K_{ii}) of such plots. For liver alcohol dehydrogenase, values of 84 μM for K_{is}, and 112 μM for K_{ii}, may be obtained from the

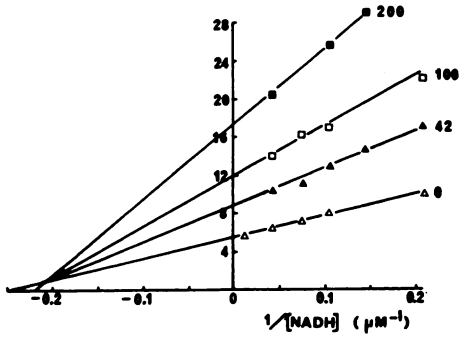


FIG. 5. *Liver alcohol dehydrogenase inhibition by pronethalol: double-reciprocal plot*

The concentration of pronethalol (micromolar) applicable to a line is inscribed beside that line. The aldehyde concentration was 240 μM.

results depicted in Fig. 5. Both slope and intercept replots are linear.

One of the problems encountered in working with competitive inhibitors is that of deciding whether the inhibitor binds to the enzyme or to the substrate. As propranolol is very nearly competitive with respect to NAD for a number of enzymes, it is necessary to consider that the inhibition may simply be due to binding of coenzyme. It has been shown that metronidazole inhibits liver alcohol dehydrogenase, perhaps via the formation of a charge transfer complex with NAD^+ (21). That neither malate nor lactate dehydrogenase is inhibited by propranolol, although yeast alcohol dehydrogenase in the same cuvette is strongly inhibited, is firm evidence that coenzyme binding is not involved. Further evidence is that the absorption spectra of NAD and propranolol are additive, and that the fluorescence spectrum of NADH is not changed by propranolol.

Propranolol is now known to interact with catecholamine metabolism at a number of sites. After administration of propranolol, the activity of tyrosine hydroxylase in rat striate cortex is increased (22). Liver monoamine oxidase is inhibited directly by propranolol (2, 3), as is brain aldehyde dehydrogenase. The amine receptor site is, of course, blocked by propranolol. The significance of these enzymological observations is

not clear, but changes in the catecholamine content of cat brain following intraventricular injection of propranolol have been reported (23).

Since both liver monoamine oxidase and brain aldehyde dehydrogenase are inhibited by propranolol, it is apparent that oxidative deamination of the biogenic amines is potentially slowed by the drug. Whether such a change actually occurs under physiological conditions is not known, but in view of the actions ascribed to the deaminated metabolites of biogenic amines in sleep (24–26) and other central functions (27–29), a change in the levels and proportions of the deaminated biogenic amines is of considerable interest.

Propranolol also interacts with the enzymes of ethanol oxidation; on the basis of this study both alcohol and aldehyde dehydrogenases are inhibited. The direct effect of propranolol on this metabolic pathway would be expected to be a decrease in the rate of ethanol oxidation, reinforced by the reported inhibition of NADH oxidation. Whether such a decrease in rate actually occurs is not known; the relatively high K_i values observed make a large effect unlikely. The K_i value for aldehyde dehydrogenase is less than that for alcohol dehydrogenase (105 μM as opposed to 215 μM), with the result that inhibition of aldehyde dehydrogenase is likely to be greater than inhibition of alcohol dehydrogenase. Under these circumstances an abnormal build-up of acetaldehyde may occur. Such a build-up would be of great interest in view of the known and postulated actions of acetaldehyde (30–32).

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