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# Ethanol, Catecholamines and Alkaloids: Interface of Neurochemistry and Alcoholism

Joel Allen Rubenstein  
*Loyola University Chicago*

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ETHANOL, CATECHOLAMINES AND ALKALOIDS:  
INTERFACE OF NEUROCHEMISTRY AND ALCOHOLISM

by

Joel Allen Rubenstein

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

June

1974

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Joel Allen Rubenstein  
Loyola University of Chicago

ETHANOL, CATECHOLAMINES AND ALKALOIDS:  
INTERFACE OF NEUROCHEMISTRY AND ALCOHOLISM

The theory has been advanced that tetrahydroisoquinoline (TIQ) alkaloids may form in neuronal and chromaffin cells during alcohol metabolism via condensation of catecholamines (CAs) and alcohol-derived aldehydes. The cyclic CA-type alkaloids could assume a physiological role in the development of alcohol dependence (G.Cohen and M. Collins, Science 167: 1749-1751).

As groundwork for in vivo studies on TIQs during alcoholism, the following questions required study.

(1) What are suitable chronic alcoholic animal models for the investigation of TIQ formation and CA changes?

(2) What are the possible products of catabolism of TIQs? Can the catabolism of TIQs be blocked through methods similar to those used to inhibit catabolism of endogenous amines?

(3) What pharmacological methods can be employed to maximize TIQ formation in vitro and in vivo? What are the effects of these methods on the enzymes of ethanol and acetaldehyde metabolism?

In the studies with chronic alcoholic animal models, mice and rabbits were provided with liquid Metrecal/ethanol (EtOH) diets as the sole sources of food. This protocol produced intoxication and slight alcohol withdrawal symptoms in the mice, agreeing somewhat with the results of G. Freund (Arch. Neurol. 21: 315, 1969). Rabbits fed Metrecal/

ethanol for 80 days displayed negligible signs of intoxication, despite developing substantial blood EtOH and occasionally high blood acetaldehyde (AcD) concentrations. Fatty liver changes were demonstrated. Malnutritional aspects of this diet were possible factors contributing to the pathologies seen.

4-Hydroxylated TIQ alkaloids derived from the neurotransmitter NE and AcD or formaldehyde were found to be readily O-methylated in vitro in rat brain and liver homogenates. Since it was significantly inhibited by pyrogallol (PG; 1,2,3-trihydroxybenzene), the catechol alkaloid O-methylation was due in part to the same enzyme that catabolizes the endogenous CAs, catechol-O-methyltransferase (COMT). Brain COMT O-methylated the TIQ alkaloids and NE to similar degrees, but liver COMT O-methylated the TIQs to a significantly greater extent than NE in these experiments. Furthermore, there was no evidence for monoamine oxidase action on the cyclic alkaloids, although the open-chain NE precursor did produce oxidation products.

PG was found to have a novel effect on AcD and its metabolism. Rats treated with PG prior to EtOH intoxication demonstrated significantly higher blood AcD levels than did non-PG-treated, intoxicated controls. It was determined in in vitro experiments that this effect was due in part to PG inhibition of liver aldehyde dehydrogenase (ALDH). Several other compounds structurally related to PG, notably the clinically-important decarboxylase inhibitor R044602 (for Parkinsonism therapy), were found to inhibit ALDH in vitro; this property may lead these or similar trihydroxy-benzene-containing drugs to clinical application as disulfiram-like deterrents in human alcoholics.

Thus PG was found to be potentially useful in enhancing TIQ

alkaloid levels in vivo through three biochemical mechanisms, two of which were demonstrated in these studies: (1) Oxidation of AcD via ALDH is inhibited and the concentration of one (AcD) of the two TIQ precursor molecules is maximized. (2) O-Methylation (and possibly inactivation) of TIQs is decreased, favoring the isolation of catechol TIQs by alumina procedures currently in use. (3) Combined with the well-established inhibition of COMT by PG, which serves to maximize NE, epinephrine and dopamine (precursors of TIQs), effects (1) and (2) should lead to detectable TIQ levels during intoxication. In support of this, recent studies by Bigdeli in this laboratory (M. Bigdeli, Ph.D. Dissertation, Loyola University of Chicago, 1974) have shown that adrenal and brain CA-derived TIQs are detectable by gas chromatography when EtOH-intoxicated rats are pretreated with PG.

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Above all, the author acknowledges the inspiration and love that has been his good fortune to gain from Marsha Patinkin Rubenstein, his wife.



## LIFE

Joel Allen Rubenstein was born in Chicago, Illinois, on September 15, 1947. After graduating from Bowen High School, Chicago, in 1965, he received the Bachelor of Science degree in Zoology from the University of Michigan in 1969. At that time he returned to the Chicago area to enroll in the Department of Biochemistry and Biophysics of Loyola University of Chicago.

At Loyola, he began his doctoral research under the tutelage of Dr. Michael A. Collins, studying aspects of the neurochemistry of alcoholism. The National Defense Education Act Title IV Fellowship was awarded to Mr. Rubenstein for the period 1970-73. He presently holds membership in several scientific societies. In December, 1974, he will receive the degree of Doctor of Medicine from Loyola University of Chicago Stritch School of Medicine.

Mr. Rubenstein was married to Marsha Patinkin on December 23, 1972.

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## PART ONE

### INTRODUCTION AND REVIEW OF THE LITERATURE

#### A. BACKGROUND

##### I. THEORIES OF ALCOHOL DEPENDENCE

At present there is relatively poor consensus concerning an adequate definition of alcoholism. As with most diseases that are expressed primarily as behavioral disorders, many social and environmental factors affect the definition of the problem. Perhaps the most widely accepted definition of alcoholism is that it is a disorder characterized by excessive drinking that results in injury to a person's health or adequate social functioning or both (Mendelson, 1970). A number of investigators have employed specific pharmacologic criteria of addiction for selection and description of their research subjects. These pharmacologic criteria for alcohol addiction are the same as those employed for a variety of other addictive disorders--tolerance and physical dependence.

Tolerance is demonstrated when, after repeated ingestion of a drug, increasing dosage must be used to give effects obtained in the original dose. Although there is tolerance observed in alcoholics, there is little evidence that altered EtOH metabolic processes can adequately account for the degree of tolerance that occurs. Cellular adaptation to EtOH has been advanced as a more parsimonious hypothesis to explain the phenomenon of tolerance (Mendelson, 1970).

Axelrod (1968) has reviewed data relevant to the several mechanisms that may underlie cellular adaptation in the development of tolerance to drugs. Although the models he has described were based upon

the possible action of narcotic analgesics, they have relevance for other centrally-acting drugs such as EtOH. Three possible factors are discussed: (1) reduced activity of the drug receptor, (2) depletion of endogenous substances that indirectly cause or mediate the drug action (transmitters), (3) enhanced metabolism or inactivation of the drug. There is much evidence against the last of these factors with regard to EtOH.

Axelrod (1956), studying morphine tolerance, has suggested that the first factor, drug-induced reduction of receptor-site activity, may cause the development of tolerance to that drug. These receptor sites may be enzymes, since liver N-demethylases, morphine catabolic enzymes, decrease in activity after continuous exposure to morphine. He postulated that a similar phenomenon might occur in the brain since the molecular structure of drug receptors in the central nervous system closely resemble the N-demethylating enzymes in the liver microsomes. However, challengers to this theory have argued that development of tolerance to narcotics can occur in rats without reduction of N-demethylating activity (Axelrod, 1956). In summary, Axelrod stresses the fact that isolation and characterization of analgesic receptors in the central nervous system are necessary precursors to testing hypotheses concerning reduction of receptor site activity by centrally acting analgesic drugs.

The tolerance to EtOH may be mediated by endogenous putative transmitters, the second factor mentioned by Axelrod. There is some indirect evidence that this tolerance may be associated with a decreased release of acetylcholine. However, the findings of Gage (1965) indicate that EtOH acts to increase rather than to decrease the rate of transmitter release. This is consistent with neurophysiologic findings that alcohol depressed spinal cord reflexes without affecting synaptic transmitters.

In line with these results are reports by Israel (1970), that EtOH suppresses active transport of ions in nervous tissue by inhibiting  $\text{Na}^+$ - $\text{K}^+$ - $\text{Mg}^{++}$ - ATPases through competitive inhibition with potassium.

One of the major difficulties in exploring cellular adaptation in laboratory animals has been the surprising lack of effect of EtOH on a number of measurable indices when EtOH is administered in relatively physiologic concentrations. Suppression of several types of neurochemical and neurophysiologic activities has been reported with extraordinarily large doses of alcohol in in vitro systems. However, it has been difficult to ascertain an appreciable inhibition of cellular function when concentrations of EtOH are employed that produce inebriation rather than death (Israel, 1970).

Goldstein and Goldstein (1968) have developed an enzyme repression theory of drug tolerance and physical dependence. This theory, which conceives of tolerance and physical dependence as a unitary mechanism, is based on the processes of repression and derepression of enzyme synthesis. In terms of an excitatory neuro-compound, for example NE, it is known that the synthesis of the NE synthetic enzymes (NSE) is decreased by increased levels of NE, i.e. end-product repression. These authors postulate that inhibition of NSE activity by a drug such as EtOH would produce an immediate effect by decreasing the concentration of NE. This, in turn, would produce a depression of the NE excitatory function. Simultaneously, a decrease in end-product repression of NSE would result, i.e. derepression. As the concentrations of enzyme NSE increased, more NE would be synthesized. In order to produce continued inhibition of NSE, more drug (EtOH) would be needed. This could explain the phenomenon of tolerance. Interestingly, chronic EtOH was seen to depress tyrosine

hydroxylase activity in rats, leading to depressed NE levels (Friedhoff, 1972).

In terms of physical dependence, it is thought that abrupt withdrawal of a drug EtOH would result in a very large concentration of active enzyme NSE. This large concentration of enzyme, in turn, would effect an enhanced synthesis of excitatory NE, and this great overproduction would have marked excitatory action (withdrawal symptoms). Thus, physical dependence results (Goldstein and Goldstein, 1968).

Of course, the origin of the alcohol withdrawal syndrome is quite unclear, as are the critical determinants of the onset of withdrawal symptoms, since either a relative decrease in blood EtOH levels or an abrupt alcohol abstinence may precipitate this syndrome. Abstinence signs have been observed in subjects with blood alcohol levels as high as 100 mg/100 ml (Isbell et al, 1955). Withdrawal onset does not appear to be related to dosage or duration, since Mello and Mendelson (1970) have also observed partial withdrawal signs of tremors in subjects who have been drinking small amounts of alcohol for as little as four days. In chronically intoxicated alcoholic humans, many of whom have other associated pathologies, withdrawal symptoms can involve tremor, hallucinations, and grand mal seizures, with a peak incidence 24 hours after the cessation of drinking. The syndrome of delirium tremors (D.T.'s), a state characterized by gross tremor and agitation, disorders of sense perception, and increased psychomotor and autonomic nervous system activity, has its onset between 72 and 96 hours following the cessation of drinking (Victor and Wolfe, 1973). Thus, two "subsyndromes" may follow EtOH withdrawal.

EtOH toxicity and withdrawal symptoms may be due to (1) direct

actions of EtOH; (2) psychoendocrine factors, e.g. increased CA secretion and enhanced release of adrenocorticotrophic hormones can be induced by EtOH; or (3) through neurophysiologic "denervation supersensitivity," wherein EtOH may be causing a depression which is followed by a rebound super-excitation upon removal of the EtOH. This latter concept was noted by Jaffe and Sharpless (1968), since many withdrawal phenomena involve an exaggeration of behavior that is ordinarily suppressed by the agent that induces dependence. Finally, (4) EtOH may cause the formation of some new products, possibly alkaloids, that may play a part in the neurological aspects of withdrawal syndrome and physical dependence.



## II. ETHANOL:METABOLISM

"The sway of alcohol over mankind," according to William James, "is unquestionably due to its power to stimulate the mystical facilities of human nature" (James, 1902). But mystical sway has been variously interpreted as having psychological, social, or biochemical bases, or combinations of these. The last, biochemical factors in alcoholism, has created intense interest in biomedical science of late. The study of alcohol and its related pathologies is vital to the betterment of over nine million Americans who have been classified as alcoholics. The origin of their affliction may be psychological or physical, if such a distinction can be made, and as such these heterogeneous persons are now labeled as psychologically dependent and/or physically dependent, rather than by the older term of "addicted " (Mendelson, 1970).

While it is generally recognized that ethyl alcohol is the chief intoxicant that immediately affects one after he drinks commercial liquor, there are probably other chemicals involved. Chemicals used for coloring and taste purposes, called congeners, are known to have toxicities of their own, for example, in bourbon. Hangovers in the "morning after" have been suggested as stemming from alcohol-induced brain meningeal edema, or possibly from toxic glycol products of catecholamines (CAs) created by a metabolic shift caused by EtOH (Smith and Gitlow, 1967). Others believe that increased diuresis, from suppression by EtOH of antidiuretic hormone, is responsible for some of alcohol's effects. More specific evidence has recently pointed at acetaldehyde (AcD), the proximal metabolite of ethanol, as being at the root of some temporally later effects of EtOH as well as of some neurological aspects.

The importance of AcD in the actions of EtOH as well as being

a factor in the etiology of alcoholism has been minimized in the past largely because of the relatively small amounts produced in the normal metabolism of alcohol. This appraisal has been based largely upon a limited amount of blood level data obtained by chemical methods which have been criticized recently for lack of specificity and sensitivity (Truitt and Duritz, 1967). Furthermore, AcD has been recognized as being directly responsible for a large number of actions in the acute and chronic intoxication syndromes produced by alcohol. AcD is a potent hypnotic in its own right, or in its tricyclic form of sustained release which is paraldehyde. In addition, it can induce nausea, vomiting, and sweating. It causes release of CAs and depression of oxidative phosphorylation in isolated brain tissue. Because the pharmacologic effects of AcD are generally subjectively unpleasant, it has been suggested that "while EtOH actions may be the reason that people drink alcohol, the actions of AcD may be more related to why they stop." (Truitt and Duritz, 1967).

EtOH can be regarded as a drug as well as an oxidizable substrate. It acts as a general depressant, despite the behavioral stimulation that is reflected in giddiness and increased activity. This pseudo-stimulation is most probably due to depression of inhibitory pathways in the brain. There seems to be fairly good agreement that most of the effects of EtOH as a depressant are not due to its metabolites but rather to the cellular effects of the alcohol molecule itself (Israel, 1970).

As a group, general depressants have a high oil/water partition coefficient, and the relative potencies of depressants have a good correlation to their lipid solubility. This concept is correlated with the higher permeability of the blood-brain barrier for uncharged, lipophilic molecules. Pauling showed that the general depressants share the property of forming hydrated micro-crystals of the clathrate type (Pauling, 1961). These crystals would be formed by the depressant surrounded by water and the charged side group of proteins, and could conceivably prevent the catalytic activity of enzymes and other processes by increasing the rigidity of the molecule in the biological system. EtOH, as a depressant, should not be considered as a specific molecule, but rather should be classified in a larger group. It is, therefore, important to study the effects of other general depressants to determine whether they produce effects similar to those of EtOH by virtue of their depressant ability alone.

EtOH is metabolized chiefly in the liver by alcohol dehydrogenase (ADH) to form acetaldehyde (AcD). It is generally accepted that EtOH is not metabolized to any great extent in brain and that activity

of brain ADH is very low (Raskin and Sokoloff 1972). Until the recent interest in the microsomal ethanol-oxidizing system (MEOS), (Orme-Johnson and Ziegler, 1965), it was believed that ADH was the major, if not the exclusive, enzyme responsible for catalyzing 90-98% of the initial oxidation of EtOH to AcD in liver. Hepatic ADH is a zinc-containing enzyme found mainly in the soluble fraction of the liver cell. It has a pH optimum of 10.4 and is NAD<sup>+</sup>dependent.

Recently, another hepatic enzyme system has been implicated as having a potent, important role in the oxidation of EtOH to AcD and methanol to formaldehyde. In 1965, Orme-Johnson and Ziegler described a hepatic MEOS that appeared similar to a variety of drug-metabolizing enzymes found in the liver. This microsomal oxidase system is dependent on NADPH and has a pH optimum of 7.2. Interestingly, chronic administration of EtOH to rats resulted in as much as a 200-fold increase in MEOS activity but with no significant increase in ADH activity (Lieber and De Carli, 1968). These authors also reported that pyrazole, an inhibitor of ADH, did not block the induced increase in rate of blood EtOH clearance seen in EtOH-treated animals. As stated before, alcohol tolerance does not appear to stem from increased ADH synthesis. However, Von Wartburg (1971) has described an atypical ADH in human liver. Atypical ADH has a different substrate specificities, different metal-binding characteristics, and different pH activity curves compared to typical ADH isoenzymes. However, the fall in plasma alcohol concentration in individuals with atypical ADH is only slightly increased after acute EtOH drinking.

The degree of importance of the MEOS, if it truly is a discrete enzyme, in the metabolism of EtOH and for the development of metabolic tolerance and cross-tolerance in alcoholics, remains to be determined.

Khanna et al (1970) have recently reported that contamination of in vitro systems by a "catalase-type " enzyme produced by cellular disruption, was responsible for the oxidation of EtOH by hepatic microsomal preparations. It is known that EtOH is a substrate for catalase, although methanol has four times the affinity for the enzyme. Most investigators still believe that ADH is the major enzyme responsible for the initial degradation of EtOH to AcD.

However, the old idea that the ADH activity was the slowest and thus rate-limiting step in the degradation of EtOH has recently been challenged by Goldstein (1969). He points out that the Michaelis constant (Km) for the interaction of EtOH with ADH is about  $2 \times 10^{-2}$ M. However, high blood alcohol concentration of 400 mg/100 ml. would be equivalent to  $8 \times 10^{-2}$ M., and moderate intoxication with blood EtOH levels of 100 mg/100 ml. would yield values equivalent to  $2 \times 10^{-2}$ M. It is therefore apparent that ADH is half-saturated at moderate blood levels. Goldstein suggests that the availability of hepatic NAD is a major factor in regulating the zero-order kinetics of EtOH oxidation (that is, metabolism proceeding at a constant rate independent of the concentration of EtOH). This hypothesis is consistent with a number of studies that have clearly shown that in vivo metabolism of EtOH is associated with a significant decrease in hepatic NAD/NADH ratios.

EtOH metabolism can be appreciably enhanced by administration of fructose (Carpenter and Leete, 1967). This knowledge has not been applied extensively clinically, although it has been postulated that intermediates of fructose metabolism affect the dissociation of the ADH-NAD complex.

The second major step in the degradation of EtOH involves the

conversion of AcD to acetate or acetyl coenzyme A. This reaction is catalyzed by ALDH and requires NAD as a cofactor (Racker, 1949). An NADPH-dependent aldehyde reductase, different from ADH, also exists in liver and brain. It can reduce AcD and aldehydes of biogenic amines. Low concentrations of barbiturates can inhibit aldehyde reductase, both in vivo and in vitro (Tabakoff and Erwin, 1970).

It is well-known that the rate of oxidation of AcD is very rapid and exceeds the rate of oxidation of EtOH. Despite the short half-life of AcD, it has been shown that a number of unique effects of AcD cannot be produced by EtOH alone. For example, AcD has been shown to inhibit coenzyme A activity (Ammon et al, 1969) and pyruvate-stimulated oxidative phosphorylation in brain mitochondria (Truitt and Duritz, 1967). AcD appeared to compete with or bind to pyruvate in the later experiments. AcD may also affect the biotransformation and metabolism of catechol amines and biogenic amines by stimulating the release of NE from neural storage depots (Truitt and Duritz, 1966) and shifting the catabolism of NE and 5HT from oxidative to reductive pathways (Walsh and Truitt, 1969).

The mechanisms of action of ALDH are complicated to analyze since the enzyme seems to exist in multiple forms and in multiple areas of the cell. The enzyme was discovered by Racker (1949) in beef liver acetone powder extract. Dietrich (1966) found it to be highest in liver, and existing in most mammalian tissues including the brain, which is highest in ALDH in the caudate nucleus. Both mitochondria and cytosol contain the enzyme, with recent work pointing to the mitochondria as the more important during in vivo AcD (Marjanen, 1972) metabolism.

Several forms of ALDH seem to exist, Dietrich (1973) having found by gel columns and isoelectric focusing two forms in mitochondria and the

following three forms in supernatant-- (1) phenobarbital-induced, (2) not phenobarbital-induced, disulfiram-resistant, oxidizing p-carboxybenzaldehyde but not propionaldehyde, and (3) having glycolaldehyde as a substrate. The enzyme or enzymes require NAD, optimally perform at pH 9.6-10, denaturing irreversibly above pH 10, and oxidize benzaldehydes, aliphatic aldehydes, succinic semialdehyde, and aldehydes of endogenous amines (Erwin and Dietrich, 1966). AcD was found by the same workers to have two  $K_m$ 's, indicating either the existence of two enzymes or substrate activation, the latter mechanism having been reported by Duncan and Tipton (1971) for the substrates AcD and propionaldehyde. There may be NADP-dependent ALDH in mitochondria and microsomes, according to recent reports by Tottmar and Kiessling (1973).

ALDH assays are usually done on tissue from male animals, largely because steroids, which vary greatly in levels in females, can inhibit or stimulate the enzyme (Maxwell and Topper, 1961). Progesterone, testosterone and others inhibit ALDH independent of aldehyde concentration, while estrone, diethylstilbesterol and others stimulate or inhibit the enzyme depending on substrate concentration.

Induction of rat liver ALDH after injection of mice with phenobarbital for four days has been reported (Cohen and Redmond, 1971), probably due to the induction of the supernatant fraction of the enzyme. These workers postulated that cross-tolerance between EtOH and barbiturates might exist if such an increase in AcD turnover occurred in animals or man.

Several inhibitors of ALDH have been found, most of which have little structural similarity to each other. The pre-treatment of rabbits with 2-chloroacetophenone blocks ALDH in brain slices, as evidenced by

decreased formation of acids from labelled NE (Rutledge and Dietrich, 1971). Sulfhydryl groups within the enzyme appear to be crucial, since administration of glutathione or various sulfhydryl reagents blocks the inhibition. A compound that inhibits ADH effectively, pyrazole, was found to have no effect on ALDH activity in rat liver or brain (Dietrich et al, 1971). However, aldehyde reductase was inhibited by pyrazole in the liver (but not in brain), the half-life of inhibition being 76 hours.

Both disulfiram (tetraethylthiuramdisulfide, Antabuse) and its monomer, diethyldithiocarbamate (DDC), irreversibly decrease liver ALDH activity after their injection into the live animal (Dietrich and Erwin, 1971). This inhibition seems to involve sulfhydryl groups of the enzyme. The return of activity appears to be dependent on protein synthesis, being blockable by cycloheximide.

The severity of and unexplained human deaths from the disulfiram-EtOH reaction have prompted a search for other drugs with a disulfiram-(DS) like action that is sufficiently powerful to discourage drinking but does not endanger life. Ferguson (1956) has suggested citrated calcium carbimide, also called Temposil, as another drug in the treatment of alcoholism. Its effects and mode of action seem to be similar to those of DS, but its time relations are different. A sensitizing action to alcohol (flushing, palpitations, sweating, hypotension) is apparent within 30 minutes (instead of 5-10 minutes with DS), and it lasts for about 8-12 hours (rather than one-half to several hours after DS). However, it has been argued that lowered dosage of DS is all that is needed in most cases to remove the severe effects that are intolerable to or life-threatening for certain individuals (Fox, 1973).

The hypoglycemic sulfonylureas may produce a reaction similar to



the DS-EtOH reaction and cause the AcD concentration of the blood to rise (Truitt and Duritz, 1972).

At least two pharmacologically important compounds are known to inhibit both ALDH and ADH: the sedative chloral hydrate and the beta-adrenergic blocker, propranolol. Chloral hydrate is reduced to trichloroethanol, a substrate and competitive inhibitor of ADH and thus a potentiator of EtOH intoxication, as in "Mickey Finn" knockout drops (Cooper and Friedman, 1958). The mechanism of ALDH inhibition by chloral hydrate remains to be elucidated.

Propranolol is the widely used beta-blocker whose structure is 1-(Isopropylamino)-3-(1-naphthyloxy)-2-propanol. According to Duncan (1973), the drug has been shown to inhibit several enzymes, including cholinesterase, monoamine oxidase, and the  $Ca^{++}$ -induced activation of adenylate cyclase while it is able to increase the activity of tyrosine hydroxylase in rat striate cortex. Duncan (1973) has recently shown that the inhibition of ADH by propranolol is competitive with respect to  $NAD^+$  but mixed with respect to EtOH, despite the hydroxyl group possessed by propranolol. Propranolol inhibition of pig brain ALDH is reversible and of the mixed type with respect to both  $NAD^+$  and aldehyde (3-methoxy-4-hydroxy-phenylacetaldehyde).

The kinetics of ALDH in pig brain have been described by Duncan and Tipton (1971). The enzyme binds its substrate and releases its products in a compulsory order:  $NAD^+$  is bound first, followed by aldehyde, and NADH is released after the acid product. The reaction is irreversible and is not inhibited by the acid products. High aldehyde concentrations are inhibitory. Substrate activation by AcD or propionaldehyde, as seen with other mammalian ALDH enzymes (Erwin and Dietrich, 1966), was

seen by the authors, who postulated the existence of sub-unit inter-action or of different enzymes with different  $K_m$  and  $V$  values.

In the present studies of ALDH, several compounds structurally similar to PG were tested for in vitro inhibition of ALDH. Of the various pyrogallol-related drugs tested for inhibition of ALDH, only a limited amount is known about the various actions and uses of these compounds.

Pyrogallol inhibits catechol O-methyl transferase (COMT), dehydroshikmate reductase, D-amino acid oxidase, and histidone decarboxylase (Webb, 1966). It can be prepared from gallic acid, and its antioxidant properties make it useful as a photographic developer and as an external antimicrobial and skin irritant (Merck). Propyl gallate is a very popular antioxidant for foods, fats, and oils (Merck). Hydroquinone is also a useful antioxidant and photographic developer (Merck). It is used occasionally as a depigmentation agent in the treatment of hypermelanosis. This latter action may be due to inhibition of tyrosinase (Merck). D-amino acid oxidase is also inhibited by hydroquinone (Webb, 1966). The two methoxylated compounds, 3-methoxycatechol and 2,3-dimethoxyphenol, are the products of O-methylation of pyrogallol by COMT in vitro (Archer et al, 1960). Shikimic acid (3,4,5-trihydroxy-cyclohexenoic acid), a non-aromatic compound with three vicinal hydroxyl groups, is an inhibitor of plan dehydroshikmate reductase (Webb). In plants, it is an important precursor in aromatic biosynthesis (Merck). RO 4-4602, an inhibitor of DOPA decarboxylase, has been used for the latter purpose to enhance L-DOPA levels in patients treated for Parkinsonism. Having the structure N - (DL-seryl)-N<sup>2</sup> - (2,3,4-trihydroxybenzyl) hydrazide, RO 4-4602 is basically a derivative of pyrogallol and seems to possess some of the same actions. Finally, 1,2,4-trihydroxybenzene, 3,4-dihydroxy-tolvene, and tetrahydroxyquinone,

all have two or more hydroxyl groups on a benzene nucleus, and are thus structurally related to pyrogallol.

III. ETHANOL:NEUROCHEMISTRY

More scientific light has been shed recently on the interrelation of alcohol, aldehydes, and biogenic amines. In vitro work by Majchrowicz (1972) points to enzyme inhibition by AcD as being the major factor in the effects of EtOH on amine metabolism. He found that (1) the reductive shift was not necessarily caused by an increased NADH/NAD ratio, since addition of NAD did not affect the increase in formation of indole glycols; (2) monoamine oxidase (MAO) and catechol O-methyltransferase (COMT) activity and NE uptake into synaptic vesicles are unaffected by EtOH 200 mM or AcD 25 mM, and finally (3) ALDH is inhibited by EtOH-derived AcD, and its decrease corresponds to decreased oxidation products of indole- and catecholamines. In support of point 3, the aldehyde-amine metabolism data, is the work of Collins and Dietrich (1970). When EtOH, 100-400 mg/100 ml, was added to mouse tissue in vitro, a pyrazole-reversible 60% inhibition of liver ALDH was seen. The pyrazole effect suggests that AcD is responsible for the ALDH inhibition. Another newly-discovered enzyme, aldehyde reductase, which reconverts AcD to EtOH and is important in brain tissue, was increased 41% in mouse brain tissue following thirty days of EtOH-metrecal drinking.

EtOH administration has produced contradictory results on neuroamine levels and on amine-biosynthetic enzyme activity as well as on protein synthesis in general. Acute EtOH administration depressed brain protein synthesis in one study, while increasing synthesis when given for ten days (Von Korff, 1970). Tewari and Noble (1971), saw the reverse: decreased synthesis of brain protein and RNA following chronic ingestion of 10% EtOH. NE synthesis is also variably affected by EtOH administration in rats. Hoping that an increased NE synthesis would be seen in

order to explain alcohol tolerance, the opposite was seen by Friedhoff (1972). The activity of tyrosine hydroxylase, supposedly the rate-limiting enzyme that converts tyrosine to dihydroxyphenylalanine (DOPA) en route to NE, was depressed in caudate nucleus after repeated alcohol injection. Acute administration caused no change, stated Friedhoff.

EtOH is also known to be an inhibitor of ion transport in nervous tissue (Israel, 1972), probably contributing to its role as an inhibitor of NE reuptake. EtOH at 0.1-0.25% depresses  $\text{Na}^{23}$  efflux (active transport) without changing the action potential in rat brain cortex slices. However, both active transport and action potentials were inhibited in squid giant axons, although the active transport was more affected. EtOH appears to compete with potassium ( $\text{K}^+$ ) for a site on the  $\text{Na}^+-\text{K}^+-\text{ATPase}$  enzyme; thus, the  $\text{K}^+$  concentration should be known in all experiments involving EtOH and ATPase. In the absence of added  $\text{K}^+$ , the concentration of EtOH necessary to inhibit the ATPase by 50% is of the order of 0.2-0.3%. This inhibition could be partly counteracted by increasing the concentration of  $\text{K}^+$  in the medium. EtOH (1%) significantly (25%) inhibited the active accumulation of radioactive NE by rat brain slices incubated with 6 mM  $\text{K}^+$ . EtOH (1%), however, did not affect the passive efflux of NE from preloaded brain slices nor its release when elicited by electrical stimulation, thus indicating that the action potentials are not inhibited.

The theory of EtOH as a homeostatic regulator of amine levels has been advanced by Bacopoulous and coworkers (1972). They have found an existing correlation between alcohol preference and brain neurotransmitter level changes in CF1 mice. Animals that "pursued" 2% alcohol solution in preference to water, had neuroamine levels resembling mice

that had been stressed by electroshock. Thus, increased 5HT and decreased NE levels were seen in stressed mice and in EtOH pursuers. EtOH apparently served to restore these "stress" levels to that of controls. That is, "the reinforcing properties of alcohol are attributed to its intrinsic stabilization of the labile neurotransmitter levels." As such, one may partly explain "addictive personalities" of animals and possibly humans in terms of inherited amine stability.

#### IV. CATECHOLAMINES: BIOSYNTHESIS AND METABOLISM

The CAs, NE, E, and DA, are 3,4-dihydroxyl derivatives of phenylethylamine. They are widely distributed throughout the animal kingdom, most often in nerve cells. CAs are found in the peripheral sympathetic nervous system and in the adrenal medulla, and are unequally distributed in different areas of the central nervous system (CNS). Small amounts of CAs are also found in chromaffin cells scattered throughout the body. The subject of their pharmacology and biochemistry has been reviewed by various authors (Axelrod, 1971; Kopin, 1969; Molinoff and Axelrod, 1971).

NE is highly localized in peripheral postganglionic sympathetic nerves. Its concentration in some sympathetic ganglia may be as high as 100 microgram/gm, while in sympathetically innervated tissue its concentration ranges from 0.1 to 0.2 microgram/gm. The nerve endings make up only a small part of the total mass of the tissue, and thus the actual concentration of NE in the endings is probably between 100 and 500 microgram/gm. Postganglionic stimulation leads to almost complete disappearance of NE from tissues, which suggests that it is wholly contained within the sympathetic nerves. In the CNS, the highest concentration of NE is found in the hypothalamus and brain stem. (Axelrod, 1971)

NE is released from sympathetic nerve terminals as a neurotransmitter and exerts most of its effect locally on post-synaptic cells. Epinephrine (E) functions mainly as a hormone, being released into the general circulation primarily from the adrenal medulla. Small amounts of E have been found in mammalian brain and heart. A third CA, DA, serves as a precursor of NE and E in both the peripheral and central nervous systems. Its most important function may be in the brain, where high concentrations are found in the striatum. DA is thought to be a synaptic

transmitter functioning to mediate inhibition in a motor-coordinating nigro-striatal pathway. Deficiency of DA in this area appears to be a biochemical correlate of Parkinson's disease.

It has recently been possible to visualize CAs *in situ*. A highly specific and sensitive fluorescent histochemical method has permitted the direct visualization at a cellular level of the biogenic amines DA, NE, E, and 5HT. The use of this technique has confirmed the conclusion that the NE present in peripheral mammalian tissue is localized almost exclusively in sympathetic nerves. More specifically, the use of gradient centrifugation techniques, the autoradiographic localization of tritium-labelled NE, and electron microscopy have combined to show that NE is localized not only within the sympathetic nerve endings, but also largely within specific vesicles contained in these nerve endings. (Axelrod, 1971)

CAs influence the actions of a wide variety of tissues such as vascular smooth muscle, adipose tissue, liver, heart, and brain. In many cases they act by causing an increase in the activity of a specific enzyme or enzymes. These effects are frequently mediated by adenosine 3', 5'- monophosphate (cyclic AMP).

The biosynthesis of CAs begins with tyrosine. The four enzymes involved in CA biosynthesis -- tyrosine hydroxylase (TH), DOPA decarboxylase (DOPA-D), dopamine B-hydroxylase (DBH), and phenylethanolamine N-methyltransferase (PNMT) -- do not have the same subcellular distribution. Thus, intracellular migration of substrates for these enzymes takes place as tyrosine is converted to NE in the sympathetic nerves and to E in the adrenal medulla.

Although the pathway of tyrosine to DOPA to DA to NE to E is the major pathway of CA biosynthesis, lack of specificity of several enzymes



involved permits several alternate pathways. One such pathway from tyrosine to NE and E may exist via tyrosine, tyramine, octopamine and synephrine. There is also a pathway from tyrosine to E which bypasses NE. In this reaction sequence, DA is converted to epinine by a non-specific phenylalkaline N-methyltransferase which has been found in rabbit lung. Epinine can then be beta-hydroxylated by DBH to form E.

Although CAs are rapidly inactivated when they enter the bloodstream, they are stored in high concentration in the adrenal medulla, sympathetic neurons, and certain areas of the brain. In the adrenal medulla they are held in granules that contain high concentrations of ATP in a CA/ATP ratio of 4:1; DBH; a group of nine specific proteins called chromogranins, having a molecular weight of about 77,000; and an ATPase dependent on  $Mg^{++}$  and  $Ca^{++}$ . These granules can take up radioactive E from solution by an energy-dependent process inhibited by drugs such as reserpine. On the basis of these observations, it is believed that the CAs in the granules are bound in a protein-ATP complex that may involve a divalent anion.

In the sympathetic nerve endings, NE is similarly found in the particulate fraction that contains dense-core vesicles. Only those amines that are bound in vesicles are released by nerve stimulation; and if binding in vesicles is prevented, even NE present in the soluble fraction is not released. Drugs such as reserpine, which deplete NE, cause disappearance of the dense-core vesicles. NE and E appear to be stored in two different granule types in the adrenal medulla.

The chemical structures relevant to binding have also been studied. NE has a catechol group, a beta-hydroxyl group, and an amine group. To determine which chemical structures may be involved in the

binding mechanism, related compounds that lack one or more of these groups have been studied. The amine group is necessary for binding. Phenylethylamine, which has only the amine group but none of the hydroxyl groups, is not bound; but phenylethanolamine can be bound by the vesicles to a slight extent. The beta-hydroxyl group, therefore, appears to contribute to binding. DA, which lacks only the beta-hydroxyl group of NE, is relatively strongly bound to the vesicles; thus, the catechol group appears to be involved in the binding mechanism. When either of the catechol hydroxyl groups of NE is lacking, as in m-octopamine or p-octopamine, the avidity for binding sites appears to be diminished in vivo as well as in vitro. This incomplete specificity of binding allows replacement of NE by other amines, so-called "false transmitters." Accumulation of false transmitters in vesicles diminishes the amount of NE that is released, thereby causing a partial adrenergic blockade when less active NE reaches the receptor site. (Molinoff and Axelrod, 1971).

Endogenous amines apparently are released by nerve stimulation only if they are bound in vesicles. However, mechanisms for releasing a compound from nerve endings that do not involve vesicle binding may also be present. Bretylium is a quaternary amine that interferes with the release of NE from the sympathetic nerves. Although not bound in vesicles, bretylium accumulates in sympathetic nervous tissue and is released during nerve stimulation. It is thus apparent that a substance need not be bound in vesicles in order to be released.

NE that is released at the adrenergic nerve ending reaches an effector cell where it reacts with a specific site, the receptor, to initiate a sequence of events that leads to a response. Adrenergic receptors have been separated into two main types, alpha and beta, based

on the structure-activity relation of various compounds (agonists) that interact with the receptor. The order of potency at the alpha receptor (e.g. vasoconstriction) is  $NE > E > \text{isoproterenol}$ . At beta receptors (e.g. vasodilatation or myocardial chronotropicity) this order is reversed (Kopin, 1969).

Further, the notion that there are two main types has been greatly strengthened by the discovery of drugs that specifically block one but not the other type of receptor (Kopin, 1969).

In addition to responses that involve muscular contraction or glandular secretion, CAs have several metabolic effects (e.g. hyperglycemia). The mechanism of a number of these effects has been shown to be increased formation of cyclic AMP from ATP as a consequence of activation of adenylyl cyclase. There is some evidence that the beta receptor is pharmacologically similar to the site of interaction that results in activation of adenylyl cyclase (Kopin, 1969).

After release, rapid inactivation of CAs occurs, primarily (80%) due to uptake into the presynaptic nerve endings. The neuronal uptake mechanism obeys saturation kinetics of the Michaelis-Menten type, requires energy derived from glycolysis or oxidation, is stereospecific for the L-isomer of NE, and requires the sodium ion. Many other amines can be taken up and stored in sympathetic nerves by a neuronal uptake process. These amines include tyramine, alpha-methyl NE, metaraminol, E, and DA. Cocaine and antidepressant drugs such as imipramine and sympathomimetic amines (amphetamines) block the uptake of NE in the nerve. Compounds that block uptake also prevent the inactivation of NE and thus prolong its physiologic actions (Molinoff and Axelrod, 1971).

An extraneuronal uptake process for NE, called uptake 2, has been

found which is blocked by normetanephrine (a metabolite of NE) and by adrenergic blocking agents like phenoxybenzamine. Uptake 2 operates at all concentrations of CAs to transport the amines into nonneuronal tissues where they are subsequently metabolized. Compounds such as E and isoproterenol which have a relatively low affinity for intraneuronal uptake and a high affinity for Uptake 2 may be inactivated mainly by the latter process (Axelrod, 1971).

According to Molinoff and Axelrod (1971), the main CA catabolic enzymes are catechol O-methyl-transferase (COMT) and monoamine oxidase (MAO). These enzymes act on CAs to produce physiologically inactive metabolic products, but neither of these enzymes plays an enormous role in terminating the physiological action of the neurotransmitter NE or of the hormone E. The rapid inactivation of CAs is due to reuptake into presynaptic endings. It has been estimated that 80% of NE released in sympathetic nerve endings is inactivated by reuptake, with 20% being catabolized by enzymes. COMT appears to be more important than MAO in metabolizing E and NE in the rat. In addition to these two major enzymes of CA metabolism, two other enzymes -- aldehyde dehydrogenase and aldehyde reductase -- are present and act on the products of MAO and COMT actions. In humans, vanillylmandelic acid (VMA), the product of COMT, MAO and aldehyde dehydrogenase action on NE and E, makes up about 40% of the total urinary CA metabolites. The primary metabolite of NE from brain is 3-methoxy-4-hydroxy-phenylglycol, formed via COMT, MAO, and aldehyde reductase. About one-third of the total CA metabolites in human urine comes from DA (Molinoff and Axelrod, 1971).

The search for CA O-methylating enzyme was prompted by the observation that NE and E are metabolized to the corresponding 3-O-methylamines, normetanephrine and metanephrine, respectively. COMT, first described in rat liver, requires S-adenosylmethionine (SAM) as a methyl donor. SAM is synthesized throughout the body from methionine and ATP. COMT requires divalent cations such as  $Mg^{++}$  for activity, but other ions can substitute, including manganese, cobalt, zinc, iron, cadmium, and nickel.

Rock et al (1970) compared the properties of rat brain COMT with that of liver. The two enzymes have similar pH optima, heat stabilities, molecular weights, and responses to inhibitors. However, brain COMT was found to have one less activity band (by electrophoresis) and one-tenth the specific activity of the liver enzyme. The optimum activity of COMT from either organ was obtained at pH8, using 0.01 M  $Mg^{++}$ .

The substrates for COMT include NE, E, DA, DOPA, 3, 4-dihydroxy-mandelic acid, 3, 4-dihydroxyphenylacetic acid, 3-hydroxyestradiol, and ascorbic acid. The latter can be O-methylated on the alpha-carbon after forming a cyclic ether. The enzyme can also O-methylate exogenously administered catechols such as 3,4-dihydroxyephedrine, 3,4-dihydroxy-amphetamine, and a variety of substituted catechols and polyphenols but not monophenols (Axelrod, 1968).

In vivo, O-methylation occurs mainly on the meta position. Polyphenols with non-polar side-chains such as acetophenones, however, may

have up to 50% para O-methylation. A novel type of interconversion of 4-methoxy-3-hydroxy-acetophenone to 3-methoxy-4-hydroxy-acetophenone occurs in vivo. This results from the O-demethylation of the 4-methoxyl compound to form a catechol which is subsequently O-methylated on the 3-position by COMT, according to Creveling et al (1970), finding that COMT has a molecular weight of approximately 24,000. At least two separate forms have been identified on starch-block electrophoresis.

The enzyme can be inhibited by dichloromercuribenzoate, which suggests that the enzyme has a sulfhydryl group in the region of its active site. Chelating agents such as tropolone can inhibit COMT. A normally occurring catechol compound that blocks O-methylation in vitro and in vivo is 3-hydroxyestradiol. Other competitive inhibitors are dihydroxyphenylacetamide, pyridoxal phosphate, and polyphenols such as pyrogallol (1,2,3-trihydroxybenzene). Another group of COMT inhibitors are substituted 3,5-dihydroxy-4-methoxybenzoic acids, which act non-competitively and are effective in vivo. (Nikodejèvic et al, 1970)

The inhibition of COMT by pyrogallol (PG) is of interest because of the bearing it has on the metabolism of E and NE. Bacq (1936) observed that PG increased the responses of tissues to sympathetic nerve stimulation and to E. However, he then attributed this action to the anti-oxidant properties of PG. The inhibition of COMT was reported by Bacq who believed that this could explain the sensitivity of smooth muscle to the CAs by PG and other phenolic compounds. The half-life of NE in mice is increased from 22 to 42 minutes by 10 mg PG, (Webb, 1966), while O-methylation is inhibited 99%, indicating other pathways for NE metabolism. Probably the MAO pathway is also important. The administration of PG to rats does not by itself increase brain NE levels, but it does

in conjunction with iproniazid (MAO inhibitor), since the two major NE degradation pathways are blocked (Jaattela and Paasonen, 1961).

Repeated administration of PG causes a rise in blood pressure, but this is soon followed by a loss of response or tachyphylaxis. The rate of urinary excretion of O-methylated derivatives of the CAs is briefly decreased by PG, but if the administration is continued, the rate returns to normal. Long-term treatment with PG leads to an increase in COMT and MAO activity in the liver of rats, so it may well be that these enzymes are adaptively altered. Excretion of O-methylated products of CAs reach a minimum at five days, returning to normal in twenty-five days. Urinary CAs, on the other hand, increase for twenty days, then slowly return to their pre-PG levels, despite continued PG injections (Wylie *et al*, 1960)

The kinetics of *in vivo* inhibition of COMT have been studied by Crout (1960). Inhibition occurs very rapidly in liver, heart and brain even when the PG is injected i.p., and by 30 minutes has developed appreciably. PG inhibition lasts 30-60 minutes with a single dose of 200 mg/kg, but lasts for several hours after injections of 50 mg/kg every 30 minutes for several hours before *in vitro* assay, measuring NE conversion to normetanephrine. The inhibition by PG is only partly competitive (actually the curves appear to indicate noncompetitive inhibition) despite the fact that PG is a substrate for the enzyme (Archer, 1960). The  $K_i$  of 0.008 mM for PG indicates the high potency of the inhibition ( $K_m$  for NE is 0.3 mM). The only other animal enzymes reported to be inhibited by PG are D-amino acid oxidase, dehydroshikimate reductase, and histidine decarboxylase (Webb, 1966).

COMT is localized in highest concentrations in the liver and

kidney in most animals. There is an unequal distribution of COMT in the brain, activity being highest in the area postrema and lowest in the cerebral cortex. Although most of the enzyme is found in the soluble fraction of the mammalian cell, there are also small amounts present in fat-cell membranes, erythrocyte membranes, and in microsomes. In sympathetic tissue, COMT is present mainly outside the neuron, in contrast to MAO which is present intraneuronally.

Recently the so-called peripheral beta-adrenergic receptor has been suggested to be a membrane catechol-binding protein which may be related to COMT (Cuatrecasas *et al*, 1974). Some well-known COMT inhibitors (tropolone, pyrogallol, and 3,4-dimethoxy-5-hydroxy-benzoic acid) which do not have beta-adrenergic activity could inhibit the binding of NE to liver microsomes, the significance of which is controversial.

Physiologically, COMT is involved in the metabolism of CAs released into the circulation and in the inactivation of NE in tissues lacking an abundant adrenergic innervation. COMT may also be associated with an extraneuronal uptake mechanism (Uptake 2).

Thus, the current concept of the adrenergic nerve has been described. The cell body manufactures vesicles and various synthetic enzymes which are transported by axoplasmic flow to the nerve terminal. Fast flow along the neurotubules carries the enzyme DBH, which converts DA to NE. The DA is derived from tyrosine which passes into the axon and is acted upon by synthetic enzymes. When stimulated by calcium, the vesicle passes along a contractile neurofilament to fuse with the cell membrane and extrude the contents of the vesicle, including proteins, DBH, ATP and NE. After the "exocytosis," the vesicle reaggregates as a smaller granule. Release can be increased by reserpine, tyramine, and



amphetamine. The released NE can enter into any of four processes: (1) NE can activate the receptor on the effector organ, this activation being diminished when structurally similar "false transmitters" replace some of the NE in the vesicles; (2) NE can pass into the circulation, where other tissues will probably inactivate it; (3) NE can be taken up by the neuron (Uptake 1) into presynaptic vesicles, and may be oxidized by MAO before vesiculization, thus having to pass out of the cell into the circulation; (4) NE can be taken up by other cells (Uptake 2), where COMT and possibly MAO catabolize NE, the products of which will then be excreted, probably as a glucuronide conjugation product of the liver.

V. ETHANOL AND AMINE-DERIVED ALKALOIDS

Scientific interest in aldehyde-plus-amine-derived isoquinolines in alcoholism was stimulated in the 1960's by a fluorescence method for the visualization of CAs in tissues which is based on formation of isoquinolines. When freeze-dried tissue slices are exposed to moist formaldehyde vapor at 80°C., the aldehyde and the endogenous CAs undergo a Pictet-Spengler (1911) type of condensation via a Schiff base (imine) intermediate to yield TIQ alkaloids. Subsequently, these alkaloids are oxidized to 3,4-dihydroisoquinolines which tautomerize to fluorescent quinone imines (Corrodi and Hillarp, 1964).

Collins and Cohen (1968), Robbins (1968), and others recognized the structural similarities between these products and plant isoquinoline alkaloids. Robbins stated the possibility that trace amounts of TIQs may form from AcD in vivo and have a relationship to alcohol addiction. He also added that "pharmacologically significant formation of these alkaloids might occur enzymatically, intracellularly or at sites of localized, high concentrations of the reactants." Possibly, they would act as sympathomimetics during alcohol withdrawal, creating a need for a depressant such as alcohol to restore physiological normalcy. Collins and Cohen reported the preliminary results of bovine adrenal perfusions with AcD, in which simple TIQs derived from NE and E were detected.

Following this, several reports emerged of CA-derived TIQ formation in animals. In vitro, TIQs were formed in cow adrenals after perfusion with AcD. (100 µg/ml) also created TIQs through adrenal perfusion (Cohen, 1971). Similar results in vivo were seen in rat adrenals after intraperitoneal injections of methanol, using detection by fluorescence microscopy (Cohen and Barrett, 1969), or by thin-layer chromato-

graphy (Collins and Cohen, 1970).

These studies were involved primarily with the CAs, norepinephrine (NE) and epinephrine (E), while Davis and co-workers (1970) probed the condensation reactions involving the CA, dopamine (DA), the immediate NE precursor localized chiefly in the caudate nucleus of the mammalian brain.

Rather than examine only the AcD-DA condensation product, "sal-solinol," Davis et al studied the formation of a possible morphine precursor, tetrahydropapaveroline (THP), from the condensation of DA with the aldehyde metabolite of DA. DA is known to be enzymatically oxidized in vivo by monoamine oxidase to 3,4-dihydroxyphenylacetaldehyde (DA-Ald). This unstable product is quickly oxidized by aldehyde dehydrogenase (ALDH), and a competitive inhibition of ALDH would occur, thus raising the levels of DA-Ald. This increased DA-Ald could then condense with the parent amine, DA, creating the larger molecule THP. EtOH-evoked diversion of DA biotransformation to this benzyltetrahydroisoquinoline alkaloid could be related to the dependence-producing properties of alcohol, argued Davis. That THP provides the starting point for the elaboration of the complete morphine skeleton by the opium poppy has been supported by tracer experiments (Leete, 1959). The possibility was suggested that animal systems could also mediate this specific reaction sequence. Davis makes the controversial case that many opium addicts revert to alcohol and vice versa, because the biochemical mechanisms of alcohol and opiate addiction are both opiate-based. It is known, however, that rats susceptible to morphine addiction can also be addicted to alcohol with greater ease than those which do not prefer morphine-containing water (Davis et al, 1970).

Davis et al (1970) did find that the formation of the alkaloid

THP in rat brain homogenate occurred to a greater degree following the addition of EtOH or AcD. This effect was apparently due to inhibition of ALDH, leading to decreased formation of 3,4-dihydroxyphenylacetic acid from DA-Ald. Further, the effect on THP formation of the cofactor nicotinamide adenine dinucleotide (NAD) was seen. Enhanced THP formation was seen, apparently as a result of greater acetaldehyde inhibition of the oxidation of DA-Ald. Another reason cited was the decreased formation of salsolinol, due to rapid AcD oxidation, thus allowing a significant increase in the amount of DA available for reaction with its intermediate aldehyde to form THP.

In the category of putative neurotransmitters in animals, the CAs are joined by histamine, acetylcholine, serotonin (5-HT), and others. It is likely that many of these are affected by EtOH or AcD. Interestingly, the idea of AcD condensation products from 5HT-like amines has been advanced by McIsaacs since 1961. He reported the presence of a 1,2,3,4-tetrahydro-beta-carboline alkaloid in the urine of rats treated with EtOH, 5-methoxytryptamine, iproniazid (a monoamine oxidase inhibitor), and disulfiram (an ALDH inhibitor). Various beta-carbolines can be synthesized, e.g. from 5HT plus AcD, analogous to TIQ formation from CAs. 6-methoxy-1,2,3,4-tetrahydro-beta carboline is a brain serotonin elevator in mice, possibly acting as an inhibitor of 5HT synthesis (McIsaacs et al, 1972). Thus, isoquinolines and beta-carbolines may form after alcohol ingestion. They may act by altering neuroamine levels or by direct effect upon receptor sites.

VI. METABOLISM AND PHARMACOLOGY OF BIOGENIC AMINE-DERIVED ALKALOIDS

The various alkaloid theories of alcoholism have been mentioned:

(1) CAs combining with simple alkaloids to form TIQs; (2) DA combining with its aldehyde metabolite to create THP; and (3) serotonin derivatives combining with simple aldehydes to yield beta-carbolines. All of the concepts revolve around the idea that plant-like alkaloids may form in animals after alcohol ingestion. The properties of these alkaloids possibly create some of the physiology of alcohol toxicity and withdrawal symptoms. Therefore, interest in isolating these compounds and their metabolites in animal tissues has increased in recent years.

Metabolites of alkaloids in plants are reviewed in great detail by Reti (1954). O- and N-methylation are the principal processes involved in the interconversion of TIQ alkaloids, for example. Many of the products of these reactions are more toxic in certain ways than are their precursors. Demethylation also occurs, as in the conversion of salsoline to salsolinol in desert cacti.

Many metabolic pathways followed by plant alkaloids are active in animal tissue as well. Numerous O- and N-methylating enzymes, as previously described, methylate numerous endogenous amines. Catechol O-methyltransferase is widespread, inactivating CAs; guaiacol O-methyltransferase is reported to add a second methoxy group to O-methylated DA products (Friedhoff et al, 1971); phenylethanolamine N-methyltransferase will N-methylate NE and other compounds (Axelrod, 1962); a nonspecific N-methyltransferase in rabbit lung can act on 5HT, tryptamine, tyramine and the CAs (Axelrod, 1961); histamine N-methyltransferase, hydroxyindole O-methyltransferase, demethylases, and water methylases also exist (Axelrod 1962).

Mixed-function liver oxidases are also known to form N-oxides of hydrazines and of various arylamines such as morphine, nicotine, chlorpromazine, and amphetamine. The nitroso products can be reconverted to their precursor using cytochrome reductase. The regulatory site can be activated by amphetamine or various phenylethylamines. Modifiers of the oxidases include barbiturates, which increase oxidative demethylase activity, and amphetamines, activators of N-oxidase in human and pig liver. N-oxides of heterocyclic compounds such as TIQs are seen in plants (Ziegler, 1972).

The mechanisms of actions of TIQ alkaloids in animals have been investigated. They easily form in vitro in adrenal tissue, as stated. Other areas where these alkaloids might form are in the adrenergic fibers of the sympathetic nervous system and brain. These alkaloids retain the original CA structure, but ring closure prevents rotation of the ethanolamine side chain. If the resultant conformational site were to correspond to that required for binding to smooth muscle or brain receptor sites, these substances could be unique neurotransmitter or blocking agents. They appear to be bound to adrenal, brain and nerve tissue, possibly at the same loci as CAs. Cohen and Collins (1970) suggested that TIQs alkaloids, either actively secreted or leaked from nerve terminals, could contribute to the behavioral changes caused by alcohol. A portion of the characteristic neurologic disturbances, such as hyperexcitability, tremulousness, hallucinosis, and seizures, which occur when blood concentrations of alcohol are falling or absent, could be due to the persistent physiologic action of the TIQ alkaloids.

The TIQ alkaloids derived from the CAs are structurally similar to a number of naturally occurring alkaloids, such as anhalamine, salso-

line, and carnegine, which are found in desert cacti, (Reti, 1954) and which exhibit pharmacologic actions such as excitation, narcosis, blood pressure changes and convulsions. Five to ten milligrams of pelletine, an anhalamine derivative, can cause convulsions in a frog. The most toxic of this group, lophophorine, tetanizes a frog at levels of 250 micrograms. But dose-dependent qualities exist, especially so at the 12.5 milligram level, above which blood pressure drops in intravenously injected rabbits, while higher pressure occurs below 12.5 milligrams.

According to Schultes, (1969) the TIQ alkaloids found in peyote are hallucinogenic and are responsible for the differences between the intoxication state produced by peyote versus that produced by pure mescaline, the major active ingredient. The reported pharmacological properties of TIQ derivatives of CAs include inhibition of uptake and storage of CAs in nerve and brain. Cohen (1973) demonstrated that 6,7-dihydroxy-TIQ (DA-derived) is accumulated by rat iris, sympathetic nerve endings, and brain synaptosomes. This TIQ was shown to block NE and DA uptake in brain synaptosomes. Intraventricular injection of this TIQ caused a dose-related decrease in the body temperature of rats, shown to be related to the release of CAs. Thus a DA-derived TIQ, as well as other related alkaloids (Alpers et al, 1974), possess properties that would serve to prolong or enhance the physiological effects of the CAs. Biosynthesized TIQs may then function as false adrenergic transmitters.

Direct pharmacological effects of CA-related TIQs have been shown. The complex benzylisoquinoline derivative of DA, tetrahydro-papaveroline (THP), is reported to act as a direct beta-sympathomimetic agent (Santi et al, 1972). The EEG stimulant effect of condensation products of AcD with mescaline, spermidine, tryptamine, glutamine, and

histidine, is greater for the condensation product than for the parent amine (Beck et al, 1968). Similarly, it has been seen that the reversal of pentobarbital sedation occurs to a larger extent with the TIQs than with NE (Goldstein, 1970).

Of possible significance is the report by Heath (1965) that schizophrenics under aldehyde-elevating disulfiram therapy are noticed to undergo a worsening of their mental condition. This response might in part be due to the stimulant effect of condensation products formed from endogenous amines with the increased aldehydes. Of course, other toxic effects of disulfiram may be active in this case as well. In a survey of pharmacologic actions of synthetic, phenolic TIQs, all lacking the 4-hydroxyl group of NE-derived TIQs, Hjort et al (1938) observed effects on blood pressure, respiration, and smooth muscle, as well as anti-anesthetic properties, blockade of pressor responses to epinephrine, tremor, and convulsions. Recently, L. Goldstein (1970) found a mescaline-like reversal of pentobarbital sedation following injection of a 6,7-dimethoxy-TIQ in rabbits. The pharmacology of the 4-hydroxy-TIQ alkaloids used in the experiments in this dissertation research has not yet been studied.

A. Collins et al (1973) recently reported the O-methylation in rat brain and liver of both salsolinol, a DA-AcD condensation product, and of THP, a DA-derived benzylisoquinoline. Salsolinol was a better substrate in vitro for COMT than was its parent amine, DA. Salsolinol also served as a moderate competitive inhibitor of both COMT and MAO, although no specific metabolite of these alkaloids was detected after incubation with MAO. It is not known whether MAO can break the heterocyclic ring of the TIQs in order to achieve possible deamination. THP



was found by these workers to form two O-methylated products, while salsolinol yielded only one. O-methylation in vivo and in vitro via COMT also was reported for trimetoquinol (Meshi, 1970), another benzylisoquinoline related to THP. Similarly, N-methylation has been reported for harmaline, a compound that has a beta-carboline structure and MAO inhibitory activity (Ho et al, 1971). However, the metabolism of beta-hydroxy-TIQs, such as those derived from NE, has not been studied prior to this work.

Thus, much of alkaloid metabolism in animals is similar to that in plants. And further, CA-derived TIQ alkaloids seem in the limited studies done thus far, to be handled through the same metabolic pathways as their parent CAs.

## VII. ANIMAL MODELS FOR ALCOHOLISM

Since the demonstration by Isbell et al (1955) that the alcohol withdrawal syndrome can be induced in man under controlled laboratory conditions, progress in the research of EtOH dependence has been slowed by the lack of suitable animal models. Richter (1956) has never observed overt alcohol intoxication with withdrawal symptoms in laboratory rats. He found intoxication in only three of thirty wild Norway rats drinking aqueous EtOH solutions as the only liquid. Rats have a tendency to avoid such solutions entirely, or drink only at night, becoming sober by sun-up. More recently, convulsions and "hallucinatory behavior" were induced in five dogs following withdrawal of alcohol administration through surgically implanted gastric cannulae (Essig and Lam, 1968). The evaluation of this animal model is difficult because the dogs developed gastrointestinal ulcerations and possible hypoglycemia, due to absent or markedly diminished food intake during the withdrawal period.

Ellis and Pick (1971) have also developed an alcohol intubation technique that can produce human-like withdrawal symptoms in monkeys and dogs, after three and six weeks of EtOH ingestion, respectively. The mean daily dose of EtOH is increased from 2 g/kg to 5-6 g/kg, an amount that is approximately equal to two pints of 100-proof distilled beverage consumed daily by a human subject weighing 150 pounds. The blood EtOH generally fluctuates between 100 and 500 mg/100 ml. Withdrawal symptoms were classified into the following stages of progressive severity: (1) tremulous, (2) spastic, and (3) convulsive. Death may or may not follow stage 3 in untreated animals. Administration of EtOH or barbiturates in these animals will entirely interrupt the withdrawal reactions at any stage.

For reasons of economy, however, most investigators prefer to use smaller animals, specifically rodents, in their search for an animal model for alcoholism. In the past, the two reasons cited for the inability to induce alcohol withdrawal reactions in laboratory rodents by the forced drinking of aqueous EtOH solutions were: (1) The total amount of EtOH which can be administered orally in aqueous solution is too small, and (2) the EtOH is consumed predominately during the night, leaving the daytime for recovery. Preliminary experiments by Freund (1969) with 10-25% aqueous EtOH solutions as the only drinking fluid demonstrated that mice consumed only up to 22% of calories in the form of EtOH and drank only during the night. Liquid diets of an EtOH-Metrecal mixture as the sole food were lethal within two weeks in Freund's lab when 45% or more of calories were supplied in the form of EtOH. Feeding of liquid diets containing 35% of EtOH-derived calories resulted in nearly even distribution of fluid intake between the day and night hours and were well tolerated. Freund has described an animal model that used initial 30% weight reduction of mice followed by forced choice presentation of liquid diets containing EtOH 6% of volume, or 35% of total calories. Apparently, weight reduction decreased the rate of EtOH degradation in these animals.

Freund claims to have induced seizure disorders, and later learning deficits, after abrupt replacement of alcohol by isocaloric sucrose in the basic liquid diet. Withdrawal was begun after the mice had been moderately to severely intoxicated in at least two-thirds of the daily ratings for four to five days. Light intoxication was defined as ataxic but rapid gait; severe as coma without righting reflexes; and moderate as in between light and severe. After substituting isocaloric amounts of

sucrose for EtOH, four stages of withdrawal were noted: (1) overactivity, squealing, generalized tremors 1-5 seconds in duration; (2) stage 1 plus rapid tail-beating; forward arching of the tail over the back to the nose (so-called Straub tail); slow-broad-based gait and retropulsion; repetitive movements; (3) generalized tonic-clonic convulsions of 6-20 seconds' duration; (4) death during convulsion, or the animal may recover gradually, exhibiting the signs of Stage 2 for 8-24 hours while crouched against the cage wall with eyes open. Freund reported that 7 of 10 of these ICR-DUB mice reached the convulsive stage. Freund ruled out hypoglycemia, starvation ketosis, and Metrecal diet as sources of convulsions. In later experiments, he replaced previous weight reduction with cold-room environments, (Freund, 1973 ), thus stimulating greater drinking by the mice.

One critic of Freund's animal model, Mendelson (1971), believes that malnutrition may in part be responsible for the withdrawal symptoms seen. He states that the Freund diet did not make it clear if the mice, while on 1-12 weeks of alcohol diet, had regained their basal pre-alcohol administration weight.

The frequency of malnutrition in human alcoholics, is, in part, associated with social and environmental conditions, states Mendelson. It is possible that vitamin deficiencies, which are believed to be of great import in alcohol-related neuropathologies, have been reduced because of greater availability of vitamin-enriched foods.

The poor dietary intake by alcoholics when they are drinking cannot be totally attributable to the fact that alcohol, which contains 7 calories/gm, suppresses appetite or replaces a need for other caloric intake. Recent studies by Mendelson (1970) and co-workers indicate that

alcoholics may consciously refrain from eating because they wish to obtain a better or greater response from a given dose of alcohol. The mechanism underlying this phenomenon is related to a decreased rate of EtOH metabolism that occurs as a consequence of food deprivation or starvation. Thus, alcoholics learn that enhanced inebriation can be obtained through decreased food intake during a drinking episode. To a lesser extent, the suppression of appetite due to EtOH also plays a role in food abstinence. Mendelson postulated that brain damage in alcoholics results from a combination of factors -- neuropathophysiological effects of direct EtOH toxicity, food deprivation, and alcohol withdrawal states -- rather than from a single cause (Mendelson, 1970).

One recent alcohol dependence experiment in the rat involves gastric intubation. Wallgren *et al* (1973) used tert-butanol, primarily because it is not oxidized, thus avoiding the complication of its actions by aldehydes. The tert-butanol was given to rats twice daily for 21 days along with a liquid diet, the latter to avoid constipation. A state resembling a severe withdrawal illness was observed when the tert-butanol was stopped. Thus, EtOH-like withdrawal symptoms can result after administration of alcohols other than EtOH.

Mello and Mendelson (1973) maintained monkeys in a reward-association paradigm in which a drinking response yielded a dry food pellet. The delivery of the pellets is accompanied by consumption of large quantities of fluid. However, neither marked intoxication nor evidence of withdrawal signs were seen during six-months of daily 3-hour polydipsia sessions.

PART TWO

## EXPERIMENTAL

A. MATERIALS

Acetaldehyde-Eastman, redistilled into small vials when needed and stored at - 20°C.

3'5' - adenosine triphosphate - disodium salt, equine muscle - Sigma.

Adsorbosil-1 TLC powder - Applied Science Labs.

Aluminum oxide - Grade I Neutral - Woelm. activated by HCl

Ascorbic acid - Lilly

BBS-3 scintillation solubilizer - Beckman

Bovine serum albumin - Sigma.

Carbosieve-B G.C. column support - 60/80 mesh - Supelco Co.

Cellulose MN 300 TLC plates - Brinkman

Diethyldithiocarbamate - Eastman

DL-3,4-Dihydroxymandelic acid - Grade II Sigma

3,4-dihydroxy phenylglycol - Sigma

3,4-dihydroxytoluene - Pfaltz and Bauer

2,3-dimethoxyphenol - Aldrich

Disulfiram (antabuse) - Tetraethylthiuramdisulfide - Ayerst

Bis - 2,5-diphenyloxazole (PPO) - Scintillation Fluor - Beckman

Dowex AG-50 Ion-Exchange Resin - Bio-Rad Labs-

Ellagic Acid Dihydrate - Aldrich

Epinephrine HCl - Sigma

Ethanol, 95% - Mallinckrodt

Ethanol, Absolute-Commercial Solvents Corp.

Folin reagent - Sigma

Gallic acid monohydrate - (3,4,5-trihydroxy benzoic acid) Aldrich

Gas chromatographs - Barber Coleman Model 5320, Varian Model 2100

Gas chromatography glass columns - Lance Glass Works Inc.

Beta-Glucoronidase Type II - Sigma

Heparin Sodium Salt, Grade I - Sigma

1,4-Hydroquinone - Eastman

Ink (recorder) - Applied Science

Ion-exchange glass columns - Ace glassware

Kreb's Ringer's - phosphate buffer - NaCl, KCl, MgSO<sub>4</sub>, 0.154 M each; Ca Cl<sub>2</sub> 0.11 M, Na<sub>2</sub>HPO<sub>4</sub> buffer pH 7.4 0.1 M. - Mallinckrodt

Liquid scintillation spectrometer, Model LS-250 - Beckman

DL-Methionine - Nutritional Biochemicals Co.

3-Methoxycatechol - Aldrich

Metrecal Shape liquid-Gazzolo Drug Co.

Microlance, Blood Lancet - Becton, Dickinson and Co.

Micropipet, 20 microliters - DADE

Beta-Nicotinamide Adenine Dinucleotide (NAD)-Grade II - Sigma

Norepinephrine (Arterenol) HCl - Sigma

7-<sup>3</sup>H-(DL)-Norepinephrine HCl - International Chemical and Nuclear Co.

DL-Normetanephrine HCl - Sigma

pH Meter - Model 12B-Corning

Planimeter - Gelman Instrument Co.

Porapak Q-S GC Column Support - 80/100 mesh - Waters Associates

Propionaldehyde - Eastman

Propylgallate - Aldrich

Pyrogallol (1,2,3-trihydroxybenzene) - Sigma

RO 4-4602 - (N' - (DL-seryl)- N<sup>2</sup>-(2,3,4-trihydroxybenzyl)  
hydrazide - Hoffmann - LaRoche (gift)

S-Adenosyl-L-Methionine chloride (70-80%)- Sigma

Salsolinol HBr 98% - K and K Labs

Scintillation bottles and polyethylene-lined screw caps-  
Scientific Products.

Septum, G.C. - Applied Science

Serum Bottle 30 cc and Stopper - Scientific Products

Shikimic acid - Aldrich

Snoop Leak detector soap - Applied Science

Spectrophotometer - Model Acta CIII-Beckman

Syringe, Gas-tight - Pressure-Lok A-2 - Pierce

Tetrahydroisoquinolines (4,6,7-trihydroxy)- synthesized by M. Collins,  
F. Kernozek. (1972).

Tetrahydroxy - 1,4-quinone dihydrate - Aldrich

Toluene, Scintillation Grade - Beckman

1,2,4-trihydroxybenzene - Aldrich

Vanillylmandelic acid - (DL-4-hydroxy-3-methoxy-mandelic acid)-  
Sigma

Zero Gases - Argon, Air, Helium, Hydrogen, Nitrogen - Size 1A-  
Liquid Carbonic



## B. METHODS

### I. IN VITRO CATABOLISM OF TETRAHYDROISOQUINOLINES

Male Holtzman Sprague-Dawley rats, weighing 300-400 gm each, were injected with pyrogallol (PG) 250 mg/kg i.p. in 1 ml 0.9% saline or with 0.9% saline only. One-half hour following pyrogallol or saline injection, rats were decapitated and livers and brains were removed and washed in cold Krebs-Ringer's phosphate buffer pH 7.4. Homogenization was done with a chilled Teflon homogenizer in 2 volumes of ice-cold buffer which contained L-ascorbic acid, 0.5 mg/ml. To each homogenate containing 500 mg of tissue was added 100  $\mu$ moles  $MgCl_2$  (0.1 ml), 2 nanomoles (1  $\mu$ C) of the catechol substrate to be metabolized (1  $\mu$ l), and a self-regenerating COMT cofactor system (Schweitzer and Friedhoff, 1969) consisting of 5  $\mu$ moles each of DL-methionine (0.25 ml), adenosine triphosphate (ATP) (0.1 ml) and S-adenosyl-L-methionine chloride (SAM) (0.1 ml). The solutions of ATP and SAM were made immediately before addition to the incubate because of their lability in solution. The total volume of each incubation mixture was 2.05 ml.

Incubations were carried out in open 25 ml Ehrlenmeyer beakers in a 37°C shaking water bath. Control incubations were either heat-denatured homogenates (90°C, 5 min) or those without the added COMT cofactors system. After 2 hours the reaction was stopped by deproteination with 1 ml 2N HCl and centrifuged at 10,000 g for 15 min at 0°C. The precipitate was washed once with 1 ml 2N HCl. Catechol compounds were separated from the non-catechol products using an aluminum hydroxide (AH) slurry method (Cohen, 1971). The supernatant and wash were combined in 50 ml beakers to which 3 ml freshly-prepared AH was added. (The AH was composed of  $Al_2(SO_4)_3 \cdot 18H_2O$ , 10% w/v adjusted to pH 8.3). The AH

suspension was shaken, centrifuged for 15 minutes at 10,000 g, washed twice in 1 volume 95% EtOH by resuspension and recentrifugation, and the non-catechol-containing supernatant was saved. The precipitate was then dissolved in 2 ml 4N HCl for 30 minutes, and the aluminum ions reprecipitated as aluminum phosphate by adding 1 ml  $\text{KH}_2\text{PO}_4$  and adjusting to pH 3.5 with 1N NaOH. This step frees the catechol compounds into the supernatant. After addition of 4 volumes of acetone: absolute EtOH (1:1 v/v), centrifugation is done for 20 minutes, the precipitate being resuspended and recentrifuged. The final supernatant had contained the free catechols, as confirmed by thin layer chromatography (vide infra).

The radioactivity of a 1 ml aliquot of each fraction was measured in a solution of toluene: 2,5-diphenyloxazole: BBS-3 (Beckman; 100 ml: 5 gm: 200 ml) by liquid scintillation spectrometry on a Beckman LS-250 spectrometer. Thin-layer chromatography (TLC) with zonal scraping was carried out on aliquots of the fractions using the conditions described below for the  $^3\text{H}$ -TIQ substrates. Recovery was  $70 \pm 5\%$ .

The substrate TIQs were produced in 1M NaOAc pH 6 buffer by condensation of 7- $^3\text{H}$ -d,1-NE with excess aldehyde, as described by Cohen and Collins (1970). Reaction with pre-distilled AcD gave as the major product, 1-methyl-4,6,7-trihydroxy-1,2,3,4-TIQ (TIQ I), while formaldehyde (HCHO) yielded 4,6,7-trihydroxy-1,2,3,4-TIQ (TIQ II) (Fig. 1). The extent of reaction was determined by iodochrome monitoring and by TLC. Iodochrome monitoring consisted of oxidizing open-chain amines (e.g. CAs) with iodine to a pink color, while cyclized amines such as TIQs turn yellow in solution. Aqueous solutions (2.5 cc) of each compound (1 mg/ml) were tested at pH 7.0 by addition of 0.1 ml  $\text{Na}_2\text{S}_2\text{O}_3$  0.05N followed in two minutes by 0.2 ml  $\text{Na}_2\text{S}_2\text{O}_3$  0.05 N. The TIQs were then

separated from unreacted aldehyde by adsorption on aluminum oxide ( $\text{Al}_2\text{O}_3$ ) columns at pH 8.3, washing twice with distilled water, followed by elution with 1N HCl. The eluates were then readjusted to pH 7.4 and again were subjected to TLC with zonal scraping for proof of purity, prior to addition to the homogenates.  $^3\text{H}$ -NE was prepurified on  $\text{Al}_2\text{O}_3$  in the same manner.

TLC glass plates (20 x 20 cm) were coated with 0.25 mm Adsorbosil-1, air-dried 20 minutes, and heat-activated for 30 minutes at  $110^\circ\text{C}$ . Development was in sec-butanol: formic acid: water (15:3:2) under nitrogen atmosphere in a tank lined with solvent-saturated filter paper (TLC System A). A second TLC system (TLC System B) was also employed, using Brinkman cellulose MN 300 plates, 0.5 mm thick, developed in methanol: n-butanol: benzene: water (40:30:20:10). Visualizing spray reagents were potassium ferricyanide (4,4 mg/ml in 0.2 M phosphate buffer, pH 8.3) followed by ferric chloride (2.5% in water freshly mixed with 1.5 volumes of acetone). Unlabelled TIQ standards were co-chromatographed with the radioactive reaction products. These standards had been synthesized in this laboratory by established organic procedures and their identities confirmed by elemental analysis and nuclear magnetic resonance (Collins and Kernozek, 1972).

## II. IN VIVO ACETALDEHYDE AND ETHANOL BLOOD LEVELS

Non-fasted Holtzman Sprague-Dawley rats (300-400 gm) were given EtOH ( 2 gm/kg i.p.; 25% v/v) 1 hour after pyrogallol (PG) or saline. Tail blood samples (20  $\mu$ l) were taken at various intervals after EtOH injection by cutting the distal inch of the tail with a razor blade and milking it. AcD and EtOH blood levels were then estimated on a Barber-Coleman 5320 flame ionization gas chromatograph (GC) by the method of Coldwell et al (1971). In this technique, 2 cm square disks of #1 Whatman filter paper were soaked in 0.2% sodium fluoride and then air-dried. After placement of disks in 30 cc serum bottles, blood samples were blown into the disks from heparinized 20  $\mu$ l pipettes. With the pipette left in, the bottle was then closed with a rubber septum-centered stopper. Equilibration of vapors was achieved in a shaking water bath at 35°C for 15 minutes.

As the modifications of the GC technique, 2 cc headspace gas samples were injected into the GC, and 6' x  $\frac{1}{4}$ " metal columns packed with Porapak QS (80/100 mesh, Waters Associates) were used. Conditions employed for Porapak were gas flows (ml/min) for nitrogen, hydrogen, air, 100, 30, 300, respectively; column temperature, 145°C., injector temperature, 200°C; detector temperature, 210°C; retention times were determined ( Fig 2 ).

All other compounds tested for their effect on EtOH and AcD levels were injected into the animals in the same manner as PG, being dissolved immediately before injection in distilled water, in order to minimize any changes in aqueous solution.

### III. IN VITRO DISAPPEARANCE OF ACETALDEHYDE

Before testing for inhibition of the semi-purified ALDH, and exploratory experiment was performed to examine whether pre-injection with PG would decrease the rate of disappearance of AcD added to rat liver in vitro. AcD 0.33 mM and NAD 0.33 mM were injected through the rubber stopper of a 30 cc serum bottle containing 1 ml rat liver homogenate (1:1 v/v in pH 7.4 sodium pyrophosphate 0.1 M). The liver tissue was obtained from rats injected one hour pre-sacrifice with PG (250 mg/kg) or saline (control). Incubation of the homogenate was for 30 minutes in a 37°C. shaking water bath. Samples of vapor were taken at 5, 10, 15 and 30 minutes and measured by gas chromatography. For each sample, the level of AcD at 30 minutes was subtracted from the maximal level of AcD observed.

This experiment was conducted to determine by gas chromatography if AcD disappearance (oxidation) was slowed by in vivo PG. The results indicated that indeed it was, thus substantiating the in vivo experiments with injected AcD, reported by Collins et al, 1974 . Therefore, it seemed feasible to further examine liver ALDH (vide infra).

IV. IN VITRO ASSAY OF ALDEHYDE DEHYDROGENASE\*

The intramitochondrial assay for ALDH was done using the method of Dietrich and Erwin (1971). Immediately after the decapitation of four male Holtzman rats weighing 350 gm each, the livers were removed and cut into small pieces, fibrous structures being removed at this time. All procedures were carried out at or below 4°C. The tissue was made into a 10% v/v homogenate in 0.32 M sucrose with 10 strokes of a teflon homogenizer. After centrifugation for 10 minutes at 750xg in 50 cc polyallomer tubes, the precipitate was discarded and the supernatant was centrifuged at 20,200xg for 15 minutes. The resulting supernatant was discarded and the mitochondrial pellet resuspended to the original volume by manual homogenization with the teflon homogenizer. Centrifugation at 20,200xg for 15 minutes was again done. The precipitate was resuspended to a final volume of 100 ml in 0.05M NaH<sub>2</sub>PO<sub>4</sub> pH 7.4. This mixture was sonicated in a 250 ml beaker for four 1-minute periods with 3-minute cooling intervals between each, using a Branson sonifier model LS-75, setting 7, amperage 7. The sonicate was centrifuged at 37,000xg for 15 minutes, saving the clear supernatant at 0°C for the ALDH assay.

The assay was conducted using a Beckman ACTA C111 Recording Spectrophotometer, the absorbance of the NADH formed from NAD being monitored using the tungsten lamp at a wavelength of 340 nm. Settings were as follows: dynode voltage 600, scan speed 1 nm/sec., chart expansion 50 nm/inch, span 0.1, dwell 2.5 sec., interval 15 sec., shutter standard and reference open.

The concentration of protein in the enzyme preparation was performed according to Lowry (1962). The reagents used were: (A) Na<sub>2</sub>CO<sub>3</sub>

\* Carried out in the laboratory of Dr. Boris Tabakoff, Dept. of Biochemistry, Chicago Medical School.

2% in 0.1 N NaOH; (B)  $\text{CuSO}_4 \cdot \text{H}_2\text{O}$  0.5% in 1% sodium citrate; (C) reagent (B) 1 ml plus reagent (A) 50 ml; (D) Folin commercial reagent diluted with water to give a solution of 1N. To each test tube is added 0.05 ml sample, 0.65 ml water, and 1 ml reagent C. The tube is mixed by vortex and is left at room temperature for 10 min. Reagent D (0.1 ml) is added rapidly to each tube which is vortexed. Samples may be read after 30 min at an excitation wavelength of 750nm on a Beckman Acta C111 spectrophotometer. Control samples were made from solutions of bovine serum albumin.

All solutions were kept in ice immediately after preparation.

The assay mixture contained 0.2 ml enzyme (0.50 mg/ml), 0.1 ml inhibitor, and the following components given with their final concentrations: 0.1 ml NAD 0.001 M, 0.1 ml  $\text{NaH}_2\text{PO}_4$  0.05M pH 7.0, 0.1 ml propionaldehyde substrate 0.002 M, brought up to a final volume of 1.0 ml with distilled water.

Although the pH optimum for ALDH is 9.6-10.0, the pH of 7.0 was used to minimize the oxidation of PG and other compounds tested for inhibition. The slight oxidation of PG that occurs in the first 5 min (3-minute warm-up time and 2-minute reaction time) was subtracted by using a control sample containing PG with no propionaldehyde substrate. Most of the oxidation of PG in the 25°C experimental conditions used seemed to occur after 10 minutes, as evidenced by an increasing peak of absorbance at the wavelength setting used. This, however, occurred too late to affect the experimental results.

Quartz cuvettes of 1.5 ml volume were used, being rinsed once with EtOH and thrice with water after each reaction. After a 3-minute adaptation period in the dark at 25°C in the machine, the reaction was started by the addition of propionaldehyde, followed by mixing each

reaction twice with a clean Pasteur pipette. The maximum velocity in O.D. units for the first 100 seconds of reaction time was recorded, and converted into moles NADH/min/mg protein by using the conversion factor of  $32.8 \times 10^{-9}$ . This conversion factor included the extinction coefficient for NADH, which is 6220 moles per liter. The average velocity of three samples at each concentration of inhibitor, while varying NAD and propionaldehyde concentrations, was computed and plotted in a double reciprocal graph of velocity versus substrate concentration (Lineweaver and Burk, 1934). Linear regression analysis, computed on an Olivetti Programma 101, gave the corresponding values of  $1/V_{max}$  and  $-1/K_m$ .

The structures of compounds tested for inhibition of ALDH are shown in Fig. 12.



V. CHRONIC ALCOHOL DRINKING MODELS

The animal model used here for voluntary alcohol ingestion was reported by Freund in 1969 using mice. The diet is shown in Table 1.

Two hundred male ICR-DUB mice (Scientific Small Animals Farms) weighing 25-35 gm, were housed separately\*, given daily water and 1.5 gm of Purine rodent lab chow (about 1/3 normal voluntary intake). After nine days, weights were found to be reduced 25-30%, and animals were then started on a diet of ad lib EtOH in Metrecal or on isocaloric sucrose-Metrecal solution, either diet being the sole source of food. Animals were observed for gross signs of intoxication for 5 days, at which time they were withdrawn from EtOH-Metrecal diets onto isocaloric sucrose-Metrecal diets. They were then sacrificed after 1-2 days of withdrawal. Animals had been graded behaviorally as to their degree of intoxication and withdrawal severity, as shown in Table 2. Animals were sacrificed at the proper times to represent each of the following groups: (1) weight-reduced, (2) weight-reduced plus intoxicated with EtOH, (3) weight-reduced, intoxicated, plus withdrawn onto sucrose, (4) weight-reduced plus sucrose feeding for the entire experiment, and (5) controls, which were fed lab chow ad lib and then sacrificed before weight-reduction or special diets.

Since it was discovered that EtOH-drinking mice (Tables 12 and 14) drank a lesser daily volume and regained less of their reduced weight

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\* Performed in the laboratory of Dr. Charles Scudder, Loyola University of Chicago.

than did the sucrose drinkers, it was decided that pair-feeding would be conducted in the next experiment, which involved rabbits. This involved measuring the daily intake of the EtOH-drinkers drinking ad lib, and then feeding no more than that amount to the sucrose controls on the following day. Thus, the question of differential nutrition between the experimental and control groups would be ruled out as a source of problems.

Ten-week-old male New Zealand white rabbits were housed singly and for one week were fed water and Purina rabbit chow ad lib.

For 80 days, six animals were fed liquid diets as the sole source of calories. Cage bottoms were of mesh, allowing feces to fall from the cage and not be reingested. EtOH diets were composed of Metrecal Shape chocolate drink plus 95% EtOH. The three corresponding control animals were given isocaloric sucrose substituted for EtOH, and each was pair-fed the average volume of diet consumed by the three experimental animals on the previous day. Freshly mixed food was offered daily in pottery bowls to avoid the problems previously encountered: spoilage, which occurred to some extent with the mouse experiment, and tube plugging due to sedimentation, which also occurred frequently in the mouse experiment, even with larger-bore tube openings, thus precipitating withdrawal or starvation as a complication of technique. Gas chromatographic testing of the EtOH-Metrecal solutions in open pottery bowls showed negligible loss of EtOH to evaporation over 24 hr at room temperature.

Blood samples were taken from the ear vein of each rabbit, including controls, at about 11 A.M. daily or every other day. The samples were then measured for concentrations of EtOH and AcD with the

flame-ionization detector of the Barber-Colman GC Model 5320 as described previously for rat experiments.

Animals were changed to clean cages weekly. Cage-cleaning, feeding, blood-sampling and weekly weighing were all performed in a random order among the animals in order to minimize differences in handling.

Each experimental animal drank EtOH-Metrecal ad libitum from 500 ml daily servings, which none of them over completed. In this diet, EtOH initially comprised 35% of the calories, or 6.0% volume/total volume (v/tv) concentration. The EtOH concentration was slowly increased by steps over eighty days until it reached 20% v/tv, or 71% of total calories (Table 14).

All animals were allowed to eat until the end of the experiment, and thus withdrawal effects were not sought. After 80 days, animals were sacrificed by decapitation and the livers removed, washed and placed in formalin. Livers were then sectioned for light microscopy and stained with hematoxylin and eosin.

As the results indicate, only minimal intoxication was seen, so attempts to induce withdrawal seemed futile, contrary to the mouse experiment.

## RESULTS

### A. SYNTHESIS AND THIN-LAYER CHROMATOGRAPHY OF TETRAHYDROISOQUINOLINES

Table 3 shows the results of iodochrome monitoring of CA-aldehyde condensation reactions. The reaction times for HCHO plus CAs were several times faster than those for AcD plus CAs. NE reacted more rapidly with either aldehyde than did EPI. The fastest reaction was that of NE plus HCHO (5 minutes), while EPI took 35 minutes to completely react with AcD at the conditions used.

The results of thin-layer chromatography of NE and related compounds are listed in Table 4. Two separate solvent systems were employed to confirm the results. Standard compounds included NE, its 3-O-methylated product normetanephrine (NM), TIQ I (NE-AcD), TIQ II (NE-HCHO), and the 6-O-Methylated products for each of the TIQs. The degree of migration was measured by  $R_f$ , the ratio of the distance between the compound and the origin, divided by the distance between the solvent front and the origin.

From the highest to the lowest migrating substances, the results were: System A, NM, 6-O-Me-TIQ I, NE, TIQ I, 6-O-Me-TIQ II, and TIQ II; System B, NM, TIQ I, 6-O-Me-TIQ II, 6-O-Me-TIQ I, NE, and TIQ II. With the exception of TIQ I on System B, each catechol compound was separated from its corresponding O-methyl compound by an  $R_f$  value of 0.06 to 0.10.

### B. IN VITRO CATABOLISM OF TETRAHYDROISOQUINOLINES

The in vitro catabolic products of  $^3\text{H}$ -NE or  $^3\text{H}$ -TIQs were studied following the two hour incubation. A decrease in adsorption of radioactive label to the catechol-binding  $\text{Al}_2\text{O}_3$  column indicated a conversion to non-catechol metabolites.

Metabolism of the two TIQs was qualitatively different from that

of their NE "precursor." Non-catechol portions from incubations of brain or liver tissue with TIQ II consistently showed only one radioactive TLC zone which co-chromatographed with a synthesized standard of the expected 0-methylated metabolite, 4,7-dihydroxy-6-methoxy-TIQ. Similarly, non-catechol effluent from incubations of TIQ I with either tissue demonstrated a single radioactive zone-co-chromatographing with a synthetic standard of the expected 1-methyl-6-0-methylated product (Fig. 10).

When examined by TLC, the non-catechol portions from NE incubations, contained radioactive normetanephrine and radioactive 0-methylated products migrating with standards of 3-methoxy-4-hydroxyphenylglycol and vanillylmandelic acid; these deaminated non-catechol products are included in the total percentages for NE products in Table 5.

For either TIQ, the catechol portions contained radioactivity apparently associated only with unreacted substrates. Thus, it appeared in these studies that formation of possible products of MAO action on these TIQs, as had occurred with NE, was insignificant for these alkaloids.

As shown in Table 5, 0-methylation in brain homogenates proceeded to a similar degree for all three catechol substrates. Metabolism of TIQ I ( $21.5 \pm 6.1\%$ ), however, was slightly but significantly less than that of NE ( $33.6 \pm 4.6\%$ ). Liver, on the other hand, was found to metabolize these substrates in a somewhat reverse order, TIQ I > TIQ II > NE ( $78.2 \pm 8.3$ ,  $58.1 \pm 4.5$  and  $35.3 \pm 4.6\%$ , respectively).

Comparing the two TIQs, the AcD-derived TIQ I was a better substrate for liver COMT than was the HCHO-derived TIQ II, while the two were not appreciably different in brain tissue. Pyrogallol inhibited the 0-methylation in liver of TIQ II (89.4%) more than that of TIQ I (60.4%), but the relative extents of inhibition were reversed in brain

tissue (TIQ I, 90.7%; TIQ II, 73.1%). (Fig. 3 and 4)

The conversion in vitro of the substrates to glucuronide products was apparently ruled out by incubation of the homogenate products with B-glucuronidase for 4 hours resulting in no change in the TLC and column radioactivity patterns. Possible oxidation of the substrates to quinone products was countered by the addition to the incubation mixtures of ascorbic acid.

C. EFFECT OF PYROGALLOL ON IN VITRO ETHANOL AND ACETALDEHYDE BLOOD LEVELS

Using the microtechnique described (vide supra), separation of AcD, EtOH, and acetone was attained on a Porapak QS column (Fig. 2). Relative retention times were as follows: AcD 1.0, EtOH 2.1, Acetone 2.6.

Peak heights were found to be directly proportional to peak volumes (measured by planimetry) in the concentrations used, so that peak heights were used for conversion to AcD or EtOH concentrations. Figure 5 shows Porapak calibration curves prepared for the estimation of blood AcD and EtOH, respectively. Acetone was not seen in the blood of rats injected with EtOH or AcD, although it has been reported to exist under such conditions (Coldwell et al, 1971).

Table 6 illustrates the effect of PG pre-injection on the in vivo blood levels of AcD and EtOH in rats injected with EtOH 2.0 g/kg. PG potentiated the AcD levels, the concentrations, of AcD being increased significantly ( $p < 0.05$ ) at 60, 120, 180 and 270 min. Comparing saline-injected rats to PG-injected rats, the AcD blood concentrations at these times were increased ( $\mu\text{g/ml}$ ) by PG from  $2.6 \pm 0.5$  to  $4.8 \pm 1.2$ , from  $2.0 \pm 0.4$  to  $4.1 \pm 1.6$ , from  $1.0 \pm 0.2$  to  $3.6 \pm 1.2$ , and from  $0.3 \pm 0.1$  to  $1.9 \pm 0.4$ , respectively.

EtOH levels were increased slightly by PG, from a maximum saline level of  $1700 \pm 400 \mu\text{g/ml}$  to  $1900 \pm \mu\text{g/ml}$ . The increase of EtOH concentration caused by PG was not significant when the saline and PG values were compared either maximum vs. maximum (1700 vs. 1900) or time vs. time (60 minutes saline vs. 60 minutes PG, 120 vs. 120, etc).

The administration of PG to rats was noticed to result in darkening of the blood from the normal bright red color seen following saline injection, to dark red, purple or reddish-brown.

D. EFFECT OF PYROGALLOL, PYROGALLOL ANALOGUES AND METABOLITES, AND DIETHYLDITHIOCARBAMATE ON ALDEHYDE DEHYDROGENASE IN VITRO

The results of a preliminary screening experiment on the effect of PG on AcD metabolism in vitro is shown in Fig. 11. The rate of disappearance of AcD in rat liver was diminished after pre-injection with PG. The AcD peak height was significantly increased ( $p < 0.01$ ) by PG at 15 and 30 minutes.

A sample spectrophotometric recording of the ALDH assay is shown in Fig. 6, denoting typical results seen upon testing an inhibitor of the enzyme. The effect of PG on ALDH activity in vitro is demonstrated in the two graphs of Fig. 7 and 8. The double reciprocal plots of activity versus NAD or propionaldehyde concentrations indicate a mixed inhibition by PG with respect to both compounds.

The binding of PG was reversible, as demonstrated by the dilution method. Aliquots of PG plus enzyme which were allowed to stand for 5 minutes to permit possible binding of PG to enzyme, and were added to the assay mixture. Thus, if PG were irreversibly bound to enzyme, the PG-enzyme aliquot would not inhibit the assay activity. However, such evidence for irreversibility was not seen (Table 7). Instead, PG-enzyme aliquots added to the assay caused inhibition of activity equal to the inhibition caused by the same concentration of PG added alone. Thus, the binding of PG was apparently of the reversible nature.

Various control samples were employed to demonstrate the verity of the observed inhibition by PG (Table 7). Several samples contained all assay components except one, ruling out artifactual results. Other samples were pre-incubated with PG for 3,5,10 or 15 minutes at room temperature before propionaldehyde was added, as compared to the normal



3 minute warm-up to room temperature. The results from these samples showed that pre-incubation for up to 15 minutes does not effect the inhibition of ALDH by PG. Thus, a possible inhibitory role for an aqueous degradation product of PG is apparently disallowed.

In Table 8, the effect on ALDH of PG and structural analogues is seen. The strongest inhibitors appear to be PG, 1,2,4-trihydroxybenzene, hydroquinone, RO 4-4602, diethyldithiocarbamate and 3-methoxycatechol, all of which caused > 15% inhibition at  $5 \times 10^{-4}$  M. The remaining compounds, gallic acid, 2,3,-dimethoxyphenol, propylgallate, shikimic acid, 3,4-dihydroxytoluene, and tetrahydroxyquinone, inhibited either mildly or not at all.

Table 9 compares the effects of various drugs on ALDH activity in vitro, to their in vivo effects on EtOH-derived AcD blood levels. PG, DDC, and RO 4-4602 were both in vitro inhibitors and in vivo potentiators in these experiments, while hydroquinone, 3-methoxycatechol and 1,2,4-trihydroxybenzene had in vitro but not in vivo effects. Gallic acid and 2,3-dimethoxyphenol had no observable effects on either system.

## E. CHRONIC ALCOHOL DRINKING MODELS

### 1. MOUSE EXPERIMENTS

The behavioral effects on male mice of ingestion of the EtOH-Metrecal diet are indicated in Table 2. All animals became intoxicated to some degree, approximately one-fourth reaching the ataxic stage, one-fourth the comatose stage, and one-half achieving intermediate severity of intoxication. The study of Freund (1969), by comparison, resulted in only intermediate and maximal stages of severity of intoxication, with 50% of animals in each group. All of Freund's animals surpassed the initial ataxic stage of intoxication.

A major behavioral difference in the two studies on mice was in the extent of withdrawal symptoms observed, as shown in Table 2. None of the mice died during convulsion, as opposed to 10% of those of Freund; in fact, only one of the 28 mice in the experiments described here even reached the convulsive stage, compared to 60% of Freund's animals that had tonic-clonic convulsions. The second stage of withdrawal, that of tail-beating, etc., was achieved by 2 of 28 and 3 of 10 of the mice in this study and Freund's study, respectively. Both of the animals reaching the second stage of withdrawal in our experiment exhibited tail-beating, stereotypy, retropulsion, broad-based gait and the tail-to-nose maneuver, the latter commonly known as the "Straub tail!"

Possible toxicities or hyponutritive qualities of the Metrecal diets were examined by measuring the weight changes (Table 10) and the number of deaths (Table 11) during chronic Metrecal drinking in mice. Approximately 3% (5 of 180) of mice died during the phase of 25-30% weight reduction before the Metrecal diet was instituted. Another 8

animals expired during the five days of drinking the EtOH-Metrecal combination. Three of these animals died with their limbs extended, possibly denoting death during convulsion, according to Freund (1969). No mice died during the withdrawal of EtOH onto isocaloric sucrose or while on the sucrose-Metrecal diet that followed weight reduction.

The change in the weights of mice while on the two Metrecal diets is shown in Table 12. Measuring the change in body weight from the end of the weight reduction period to the end of the Metrecal regimens, the EtOH group was seen to lose an additional 4.4% while those drinking sucrose regained 14.6% of their weight.

The daily volume of Metrecal diet consumed ad lib by mice, in Table 13, shows a doubling of consumed liquid diet by the sucrose group compared to the consumption of the EtOH group.

## 2. RABBIT EXPERIMENT

In the rabbit experiment, EtOH consumption (Table 14) almost doubled following the gradual doubling of EtOH-derived calories from 35% to 71%. The greatest changes in consumption were seen to occur in the lower ranges of EtOH concentration. Increasing EtOH from 6.0 to 7.5% v/v and from 7.5 to 10.0% v/v caused the highest changes of about 5-6 ml/day. Concentrations of EtOH above 10.0% did not contribute as much to an increase in consumption.

Table 15, indicates the physical changes seen while the animals were fed either of the liquid diets. Massive loss of body hair was seen in two-thirds of both the EtOH- and the sucrose-drinking groups. The hair loss was heaviest on the underside and on the legs, and began to occur within 14 days after the experiment was begun. A nutritional basis may have existed for this hair loss, since fur mites could not be detected from microscopic examination of skin scrapings from affected areas of any animal performed by Dr. Fred Buddingh of the Animal Research Facilities, Loyola University.

Weight loss or sub-normal weight gain was seen in both the EtOH and sucrose groups (Table 12). Actually, five of these six animals gained a small percentage of their original ten-week-old weight during the 80-day experiment. However, their litter mates eating normal lab chow and water ad lib increased their body weights by 70%.

The only death occurred in a sucrose control rabbit on day 76. This animal had shown extensive hair loss over a period of weeks and finally became listless and ceased to eat 2 days before its death. None of the ethanol-treated animals expired (Table 11).

Gross intoxication was never observed in the ethanol-drinking

rabbit (except for a few days early in the experiment). Animals were observed inside and outside of the cages for ataxia and for stuporous or comatose qualities.

a) Blood levels of ethanol and acetaldehyde

Ethanol and acetaldehyde blood levels are shown in Fig. 9. Neither compound was ever observed in the blood of sucrose animals. In the ethanol group, ethanol levels ranged from 0-100 mg/100 ml of blood (mg%) during most of the experiment, although the blood ethanol of animal 1 rose to 200-400 mg% while drinking the 10.0% ethanol solution and returned to normal within 10 days. It was during this period that we observed increased ataxia, daytime eye-closure, and warmth and redness of the ears in this animal.

The pattern of blood acetaldehyde levels usually resembled the blood ethanol variations, the acetaldehyde fluctuating from 0-30  $\mu\text{g/ml}$  but usually below 9  $\mu\text{g/ml}$ . However, when 17.5% and 20.0% ethanol solutions were fed to the animals, large increases occurred in the blood levels of both EtOH and AcD.

b) Liver Pathology (Figs. 13 and 14)

Hematoxylin - and eosin - stained liver sections performed by Dr. Buddingh, showed severe fatty changes throughout the livers of animals on the EtOH-Metrecal diet. The livers of sucrose-Metrecal-fed animals displayed negligible or no fatty change, while the animals on normal lab chow had normal hepatic architecture. No other hepatic pathology, such as hepatocyte swelling, was observed. No electron microscopy was performed.

## DISCUSSION

### A. OVERVIEW

These studies were concerned with several related aspects of the biochemical bases of alcohol dependence. TIQs, hypothesized to be aberrant CA "metabolites" during alcoholism, were investigated as in vitro substrates for the important CA-metabolizing enzymes, COMT and MAO. Secondly, when in vivo rat intoxication experiments were initiated, it was noted that PG pre-injection caused AcD blood levels to be significantly elevated. The mechanism of this elevation was explored briefly in vivo and in greater detail in vitro with purified rat liver ALDH. Concurrently, chronic alcoholic mouse and rabbit models were studied.

Various parameters were gauged in these studies, including signs of gross intoxication, rabbit liver cytoarchitecture, and rabbit blood levels of AcD and EtOH.

B. IN VITRO CATABOLISM OF TETRAHYDROISOQUINOLINES

In the TIQ catabolic experiments with liver tissue, O-methylated products derived from NE were always formed to a lesser extent than were O-methylated products from either TIQ. These findings were in accord with the report of the metabolism by rat liver COMT of the 1-methyl-6,7-dihydroxy-TIQ, salsolinol (analogous to TIQ I without the aliphatic hydroxy group). A. Collins et al (1973), found salsolinol to be a better substrate in vitro for COMT than the parent amine, DA. Salsolinol was also seen to serve as a moderate competitive inhibitor of COMT in vitro (A. Collins et al, 1973). In our work, a further observation can be made that the 1-methyl group of TIQ I seemed to increase O-methylation in liver compared to TIQ II, but made no significant difference in the brain metabolism of the two alkaloids. Although TIQ I is methylated on the 1-carbon atom, it is a 2<sup>o</sup> amine; our results can be compared to those of Creveling et al (1970) which indicated a higher affinity of COMT for the 2<sup>o</sup> amine, E, than for the 1<sup>o</sup> amine, NE. However, Axelrod and Tomchick (1958) found the substrate specificity of COMT to be roughly equal for E and NE.

Pyrogallol inhibition was quite variable in its extent and was never complete. In an earlier work by Crout (1961), 90-100% inhibition of semi-purified COMT was achieved after injection of PG 200 mg/kg, some 50 mg/kg below our level. PG inhibition of COMT in our system never reached 100%, possibly for some or all of the following reasons: (1) PG is itself a known substrate for COMT and was partially consumed during the experiments; (2) PG may be oxidized to an inactive quinone during the long incubations, despite the addition of ascorbic acid; (3) different COMT isoenzymes act in both tissues and respond differently to the

inhibitor (although Rock et al (1970) reported this not to be the case); (4) other O-methylating enzymes which are PG-insensitive acted upon the substrates; or (5) endogenous activators of COMT or inhibitors of the actions of PG might have been present in the homogenates.

It was also noted that inhibition of O-methylation was consistently 10%-40% greater for the TIQ substrates than for NE. Differences in the relative affinities of the various substrates and their respective products for the COMT-SAM complex may be responsible for this latter effect.

Although 6-O-methylation was apparently seen and is expected because of the analogy to the predominant meta-O-methylation Fig 12 of the parent CAs cited in the literature, O-methylation of the TIQs on the 7- rather than the 6-hydroxyl group is possible. Creveling et al (1972) have reported 7-O-methylation of (DA-related) 6,7-dihydroxy-TIQs by highly purified COMT preparations. In order for a 7-methoxy-4,6-dihydroxy-TIQ to have been present in our homogenates, its TLC migration would have to be identical to that of its 6-O-methyl isomer, which is possible. A. Collins et al (1973) also commented on the lack of separation of various TIQs by TLC. However, the possibility of 7-O-methylation of the 4-hydroxylated (NE-derived) TIQs should be examined with gas chromatographic systems which separate the 6- and 7- isomers.

It has been mentioned in Results that MAO action on these TIQs appeared to be insignificant in our homogenates. However, the product of MAO action on TIQs is unknown, whether it be ring-breaking to allow deamination or some other means. But one simple TIQ thus far, salsolinol, has been reported to inhibit MAO competitively (A. Collins et al, 1973), as has a tetrahydro-beta-carboline (Mc Isaac et al, 1972). The question



of whether these compounds are structurally altered during this competition remains to be determined. It is possible that MAO products of TIQs did form under our conditions and co-migrated with other TIQs, thus masking their identity. It was our original intent to look for such products of TIQs, but unfortunately the structures of such postulated products or intermediates are presently unknown, and thus the organic synthesis of standards was not attempted. More than likely, however, added oxygen, the limiting factor reported for MAO by some investigators (Achee et al, 1972), was possibly necessary to allow maximum MAO activity (although the presence of deaminated metabolites of NE in our system was evidence for the existence of some MAO activity in our homogenates).

In the studies reported here, the formation of an O-quinone from a 4,6,7-trihydroxy-TIQ by non-enzymatic oxidation was probably eliminated by the addition of the antioxidant, ascorbic acid. Moreover, simple quinone standards were found to migrate close to the origin in the TLC systems used, and little radioactivity was seen in that area. Conjugation by glucuronyl transferase can also occur, theoretically, but only in vivo, according to Axelrod (1958), since they found that in vitro systems require cofactor addition. In support of this, the incubation of our homogenate products with beta-glucuronidase produced no clear changes in the TLC or column radioactivity patterns.

In regard to exploratory in vivo studies, the facile O-methylation of CA-derived TIQs should be taken into account by investigators concerned with TIQ detection and isolation. Difficulties experienced in detecting TIQs in vivo could be due to their active conversion to O-methylated (and perhaps conjugated) metabolites. Indeed, gas chromatographic studies by Bigdelli and Collins, (1973) show that, in rats acutely

intoxicated with ETOH, TIQ derivatives are detectable in brain and adrenal tissue when COMT is inhibited and AcD is elevated with PG.

Physiologically, such CA-aldehyde condensation products may enhance NE and DA activity by acting as competitive COMT inhibitors or by reducing available COMT cofactor, SAM. Single doses of L-DOPA have been reported to act in the latter manner, that is, as a cofactor depletor (Wortman et al, 1970). Hence, a metabolic relationship might exist between the formation of TIQs from aldehydes and CAs, and certain neurological aspects of chronic alcohol toxicity or of the alcohol withdrawal syndrome.

### C. PYROGALLOL AND IN VIVO ACETALDEHYDE AND EtOH BLOOD LEVELS

The mechanism by which PG potentiates blood AcD levels following injection of EtOH or AcD may be a complex one. Several possibilities were examined, leaving us with the belief that inhibition of ALDH was a logical mechanism, even though PG had never before been reported to possess such a property.

Among the possible mechanisms of AcD potentiation, it was first thought that the primary known property of PG, (1) COMT inhibition, was predominant reason. Possibly an indirect mechanism of action of PG, that of inhibition of COMT and subsequent competitive inhibition of aldehyde dehydrogenase by the presumably potentiated catecholaldehydes, was at the root of the AcD elevation. Since in vivo detection methods for the catecholaldehydes are not yet developed, direct evidence of their potentiation was not achievable. However, administration of other known COMT inhibitors -- tropolone, pyrocatechol (Molinoff and Axelrod, 1970), and 3,5-dihydroxy-4-methoxybenzoic acid (Nikodejevic et al, 1970)-- was found to have no significant effects on AcD levels. Thus, inhibition of COMT appears to be unrelated to the ability of PG to elevate EtOH-derived AcD levels (Collins et al, 1974). (2) It was considered possible that the O-methylated products of PG, 3-methoxycatechol and 2,3-dimethoxyphenol (Archer et al, 1960), may be the actual elevators of AcD blood levels. However, these compounds did not affect blood AcD when given one hour before the administration the EtOH . (3) A possible quinone oxidation product of PG could be active in affecting AcD levels. PG is known to oxidize in aqueous solutions at room temperature, especially at alkaline pH. Thus, the effect of hydroquinone pre-injection was examined, but this compound was also ineffective in elevating EtOH derived AcD. There-

fore, if PG is converted to any appreciable extent in vivo to the quinone form, it was probably not in this form that PG exerted its action on AcD levels. Purpurogallin was not tested in vivo.

(4) Another possibility considered was an anti-oxidant or a general cytotoxic effect of PG. It seems unlikely, however, that PG would specifically affect the oxidation of AcD without slowing that of EtOH as well. As for the idea of cytotoxicity, the activities of EtOH catabolic enzymes seemed unaffected, since the EtOH turnover pattern appeared to be unchanged by PG pretreatment. Furthermore, Crout et al (1960) examined the metabolism of tyramine, which is metabolized by enzymes other than COMT, and found that PG pretreatment appears to have no general cytotoxic or omnienzyme-inhibitory power.

(5) PG potentiation of blood AcD levels may be via stimulation of EtOH oxidation. PG is suspected to be a potent  $H_2O_2$ -stimulating agent in aqueous solution (Heikkila, 1972). EtOH oxidation via hepatic catalase, "peroxidatic oxidation," (Thurman et al, 1972; Oshno et al, 1973) would be stimulated by this newly-generated cofactor  $H_2O_2$ . If modulation of catalase activity is involved, PG may be an important drug for detailed studies on the extent of the role of this enzyme in EtOH oxidation, a hitherto controversial topic. Speaking against this postulated mechanism of PG action would be the fact that such "induction" occurs very soon after only a single dose of PG, which seems unlikely for enzyme induction or activation. Actually, enzyme inhibition, unlike induction, could occur much more rapidly and would not require synthesis of new enzyme.

Moreover, EtOH turnover appeared to be unchanged after PG injection, arguing against an enhanced rate of EtOH catabolism. Also, a large increase in AcD blood level was seen after injection of AcD alone fol-

lowing PG, implying that a great part, if not all, of the PG-induced AcD elevation is due to an action of PG on AcD catabolism (Collins et al, 1974 ).

Finally, aminotriazole, shown to inhibit hepatic catalase effectively at the dose given (Margoliash and Novogrodsky, 1958), did not lower the PG-potentiated AcD blood level (Collins et al 1974 ). This again implies that activation of catalase appeared to play no major role in the action of PG.

It has been argued recently by Feytmans and Leighton (1973), that the role of peroxidation of EtOH is minor in the rat after acute administration, since inhibition of ADH activity by pyrazole effects a strong inhibition of EtOH oxidation. However, these authors admit that  $H_2O_2$  generation is the rate-limiting factor for peroxidation, while the catalase content of the liver is in excess even to metabolize all the EtOH the rat can oxidize. Thus, a compound which generates large amounts of  $H_2O_2$ , as PG may, could increase the EtOH-oxidizing activity of catalase to a significant degree. The lack of effect of aminotriazole on the PG-induced elevation of AcD may only mean that aminotriazole dosage was not large enough to overcome the greatly stimulated catalase activity created by PG-derived  $H_2O_2$ . Although aminotriazole 1 gm/kg has been reported to cause 90-100% inhibition of hepatic catalase (Margoliash and Novogrodsky, 1958), it may not do so under conditions of vigorously increased  $H_2O_2$  levels. This question should be examined experimentally, possibly using higher dosage of aminotriazole and measuring blood  $H_2O_2$ .

(6) An inhibition of aldehyde catabolism, probably through ALDH, may well be a significant mode of action of PG. As such, it would explain the similarities in the effects on blood AcD and EtOH levels of

both PG and the known ALDH inhibitor diethyldithiocarbamate ( Collins et al., 1974).

D. EFFECT OF PYROGALLOL ON ALDEHYDE DEHYDROGENASE IN VITRO

The elevation by PG of AcD levels in EtOH- or AcD-treated rats leads one to speculate that induction of EtOH catabolism or inhibition of AcD catabolism may be occurring, as discussed. Massive induction of ADH by PG in the one-hour pre-EtOH period seems highly unlikely, especially since added cofactor (NAD) would probably be required for such an occurrence. The induction of the catalase system of EtOH catabolism, perhaps by  $H_2O_2$  generation, is unlikely, since pretreatment with the catalase inhibitor aminotriazole was reported not to change the effect of PG (Collins et al, 1974). PG has been seen, though, to be a  $H_2O_2$ -generating compound (Heikkila, 1972), probably through the oxidation of two of its hydroxyl groups. This possibility requires examination, possibly by measurement of PG-derived  $H_2O_2$  injection on EtOH-derived blood EtOH and AcD levels. The difficulty in the latter scheme would be in achieving high levels of  $H_2O_2$  to simulate the proposed continuing generation of  $H_2O_2$  of PG, without reaching lethal levels of  $H_2O_2$  that might occur after a single large injection.

The inhibition of aldehyde reductase seems an unlikely mechanism to be at work, since the enzyme is found in low concentration in the liver and appears to contribute insignificantly to the overall metabolism of aldehydes in the body (Tabakoff and Erwin, 1970). However, under conditions of extremely high AcD levels such as those created by PG, aldehyde reductase may be playing a much larger role than normal. Possibly some of the EtOH levels seen after EtOH injection were derived from the AcD reverting to its precursor. But the absence of appreciably increased EtOH levels (Collins et al, 1974) following injection of PG and AcD argues against this.

Inhibition of ALDH by PG was, in fact, seen in the in vitro studies in this study. Pyrogallol, similarly to propranolol (Duncan 1973), reversibly inhibited ALDH, giving mixed inhibition with respect to both aldehyde and  $\text{NAD}^+$  cofactor. The nature of enzyme inhibition by PG has never been totally explicable, even in the well-known case of CA catabolism. Bacq (1936) originally attributed the NE pressor-potentiating properties of PG to its antioxidant properties. It is unlikely that anti-oxidation is the means of inhibition of ALDH by PG, especially now that it has been shown that the commercial antioxidant, propylgallate, had no effect on ALDH activity.

The report by Crout (1961) on COMT inhibition by PG stressed that structural analogy, PG being a "double catechol" in effect, did not explain the mixed competition that was observed. In vitro, competitive and partially non-competitive properties were seen. Inhibition of COMT by PG preinjection lasted 30-60 minutes after a single injection of 200 mg/kg, or even longer after several smaller doses every half-hour for several hours.

In our work, the millimolar concentrations calculated for the  $\text{ID}_{50}$  values of pyrogallol and other compounds might be interpreted to indicate only weak inhibition of ALDH. However, millimolar levels of NAD and propionaldehyde were required in these experiments to achieve detectable rates, since pH 7.0, used to prevent PG oxidation, has been shown to yield only one-fifth the activity seen at the optimal pH of 9.6-10.0 (Erwin and Dietrich, 1966). Thus, the  $\text{ID}_{50}$  values for PG may be in the micromolar rather than millimolar range had micromolar concentrations of aldehyde been used, as in other studies (Duncan, 1973). This may explain the apparent discrepancy between the potent AcD-elevating effect of PG



in vivo and its seemingly weaker ALDH inhibitory power in vitro.

PG is known to affect other enzyme systems (Webb, 1966), although the kinetics are usually not reported. Dehydroshikimate reductase from pea seedlings is inhibited by PG, as well as by gallic and shikimic acids. Shikimic acid is non-aromatic but structurally similar to gallic acid. Shikimic acid showed no inhibition of ALDH in our study, possibly because of the carboxyl group or perhaps because the ring is cyclohexyl rather than phenyl, and the three hydroxyls are non-planar.

The enzyme histidine decarboxylase from guinea pig kidney can also be inhibited by PG as well as by catechol, tyrosine, tryptophan, and DOPA. In that case, structural analogy seems to play very little part, especially since tryptophan and indole lack even the hydroxyl group of the other compounds. Finally, PG has been found to have a slight inhibitory effect on lamb kidney D-amino acid oxidase at concentrations where catechol, hydroquinone, and similar compounds served as stronger inhibitors.

PG has been reported to cause striking hepatotoxicity in animals but the mechanism of this action is not quite clear (Webb, 1966)

Very little else is known of the biochemical pharmacology of PG, but all facts should be recognized if ever therapeutic uses of this drug are proposed. The LD<sub>50</sub> was calculated to be 566 mg/kg in normal mice, and 490 mg/kg in hyperthyroid mice (Webb, 1966). Since thyroid hormones have been found to be COMT inhibitors in vitro (D'Iorio, 1963), perhaps their increased levels in hyperthyroidism cause an inhibition of PG methylation by COMT, thus increasing PG half-life and toxicity. PG is also known to act as a mitotic inhibitor of oligochaete cleavage, the mechanism of which is unclear, but the same property belongs to others

with quinone structures. In fact, many of the physiobiochemical effects of PG have not been studied directly but can only be inferred from known properties of quinones, benzoquinone (called hydroquinone in reduced form) being the most commonly tested.

The methemoglobinemia induced by quinones significantly in vitro is insignificant in vivo, implying that erythrocytes can reduce quinones effectively (Webb, 1966). Apparently, then, the darkened blood observed after PG injection of rats in these experiments was due to products of PG or to some mechanism other than methemoglobinemia.

Oxidative phosphorylation is inhibited by various quinones, but little evidence is available as to whether electron shunts, phosphorus transfer, quinone displacement, or sulfhydryl reaction is the mechanism responsible. Although the vitamins K belong to the quinone family, none of the PG-related quinones have been seen to inhibit vitamin K synthesis (Webb, 1966). Such bleeding difficulties, even had they been inductible by PG, would have had little bearing on the short-term experiments done in this study.

In animals, the CNS stimulation by quinones is followed by depression of the CNS and of respiration (Webb, 1966). This resembles the effect of PG, that of pressor response soon followed by tachyphylaxis (Wylie et al., 1960). It is possible that some of the physiologic darkening of the blood in rats treated with PG was due to cyanosis secondary to respiratory depression. It was noted that the rate of respiration seemed to be increased, but quantitation of the respiratory depth was not done. Unlike animals, humans who have overdosed with catechol, hydroquinone, and others, show only depression with no initial stimulation. This depression has been found not to be the result of asphyxia,

methemoglobinemia, or hypoglycemia (Webb, 1966). As can be seen, much work on the pharmacology of the quinones and of PG in particular needs to be performed.

The compound RO 4-4602, very important in Parkinson's disease therapy as a decarboxylase inhibitor and also shown to be a COMT inhibitor (Baldessarini, 1972), has now been shown to be an in vitro ALDH inhibitor of approximately equivalent strength to pyrogallol. The presence of the gem-trihydroxybenzene nucleus of both drugs may account for their similar actions. The lesser AcD-potentiation in vivo caused by RO 4-4602 compared to PG (unpublished results), may be due to more rapid catabolism, excretion, or distribution of RO 4-4602, due to the serylhydrazine residue. RO 4-4602 was one of only three compounds that affected both AcD levels in vivo and ALDH activity in vitro, the others being pyrogallol and diethyldithiocarbamate. Several compounds had in vitro but not in vivo effects at the concentrations tested, including hydroquinone, 3-methoxycatechol, and gallic acid, while the in vitro inhibitor 1,2,4-trihydroxybenzene, was not tested in vivo, but 6-hydroxy-DA was tested (The 1,2,4-trihydroxybenzene moiety is present) and caused no increases in blood AcD in EtOH intoxicated rats (Collins *et al*, 1974). Interestingly, all in vivo AcD-potentiators were found to have ALDH inhibitory powers in vitro (Table 9). However, the order of potency in vivo did not correspond to the in vitro strengths.

From the results of these experiments, the PG-induced enhancement of pressor responses to CAs may now be thought of as arising from two possible actions of PG: COMT inhibition and ALDH inhibition. Increased levels of biogenic amine-derived aldehydes may be induced by PG administration. It has been suggested that these aldehydes have pharmacol-

ogic properties of their own (Sabelli and Giardina, 1971; Barondes, 1962) through (a) release of stored amines, (b) shifts in metabolic pathways, (c) incorporation into storage vesicles, (d) condensation with endogenous amines. Thus, some of the pharmacologic actions of PG may be mediated by certain endogenous aldehydes.

It is possible that a non-toxic PG containing compound similar to RO 4-4602 could be employed by clinical investigators as substitutes for disulfiram (Antabuse) therapy in alcoholics. Various toxic manifestations of Antabuse treatment (Fox, 1973), including death, may be reduced by using a different inhibitor of ALDH.

Another likely use for PG is in experiments where condensation products of aldehydes and CAs (e.g. tetrahydroisoquinolines or TIQs) are sought after EtOH administration. By maximizing levels of TIQ precursors, e.g. Norepinephrine and AcD, while simultaneously blocking O-methylation of the TIQs, levels of TIQs in tissues may be raised to increase the ease of their detection. In fact, such experiments utilizing the twin actions of PG have already been reported (Collins and Bigdeli, 1974).

## E. CHRONIC ALCOHOL DRINKING MODELS

### 1. MOUSE EXPERIMENTS

The intoxicating effects of the EtOH-Metrecal protocol on male mice indicated that these animals appeared more susceptible to the effects of EtOH than were female mice or male rabbits. The withdrawal effects, however, were less marked than were those seen by Freund. These discrepancies might be attributable to strain or sex differences, or to specific factors associated with feeding.

First of all, clogging of the metal tubes, through which mice drank from inverted glass bottles, appeared to be a factor. The sediment from Metrecal, although prepared fresh and shaken well, often clogged the tube and starved the animals, in effect, until the next feeding the following morning. Examination of the bottles at 12 A.M. to 2 A.M. on various nights still did not prevent this occurrences. Thus, animals may not have drunk as much as they would have without clogging. Freund did not report such a complication in his study. This problem may have led to some of the deaths seen during EtOH drinking, especially in mice that were found dead with hind limbs extended, which according to Freund, denotes death during convulsion. Use of larger bore feeding tubes did not decrease the clogging, even in our rabbit experiment, so open pottery crocks were used with the rabbits.

A second factor contributing to our lack of success with intoxication and withdrawal signs in mice may well be that we, as well as Freund, were observing effects of Metrecal toxicity or of malnutrition. This factor has been examined by Mendelson. Data leading to this conclusion include the facts that (a) mice were dying during the weight reduction, (b) more mice died during EtOH drinking, possibly due to the

combined effects of weight reduction and EtOH, (c) EtOH drinkers continued to lose weight while the isocaloric sucrose group gained almost 20% more than the EtOH group, and (d) EtOH drinkers consumed only 50% of the volume, and therefore of the calories, of their sucrose counterparts. These data lead to the conclusions that (a) drastic weight reduction should be avoided, (b) pair-fed controls are required to rule out caloric discrepancies, and (c) some of the behavioral effects attributed to EtOH in our experiment may be due to toxicity of Metrecal or to malnutrition, as has been suggested by Mendelson (1971).

## 2. RABBIT EXPERIMENTS

Although the number of rabbits used (3 ethanol, 3 sucrose) is limited, several observations can be made regarding the feasibility of this ethanol-Metrecal diet in rabbits.

Four factors in the design of this experiment are worthy of note:

- (1) The choice of either alcohol drinking or starvation, rather than force-feeding, was implemented. This may allow various systems within the animal to regulate its alcohol and caloric intakes. Thus, the human alcoholic pattern is more closely approximated. Further, the occurrence of gastrointestinal ulcerations and other results of force-feeding may be avoided, but the question of alcohol preference was begged in this protocol.
- (2) Pair-fed controls were used to eliminate the effects of malnutrition often seen from depression of appetite by ethanol. The importance of pair-feeding is demonstrated in Freund's report on the mouse-Metrecal model, wherein animals on the ad lib ethanol diet drank less than one-half the amount of fluid as those drinking isocaloric sucrose ad lib (Table 12). This fact, on top of the 20% weight reduction effected in Freund's mouse experiments, possibly contributed to the intoxication and withdrawal effects, even though, as Freund notes, the clinical symptomatology induced by extreme starvation is quite different from the ethanol withdrawal syndrome. In our study, ethanol and sucrose groups were control-fed not only for caloric concentrations, but for total daily caloric in-take as well.
- (3) A possible weak point in our study may be the massive concentration of ethanol attained in the experimental diet. The gradual increase from 6% to 20% v/tv ethanol was instituted because no significant gross intoxication was observed at the lower ethanol concentrations. It is still possible that behavioral alterations did occur at night while the

animals were not watched. However, the ability to catabolize ethanol and acetaldehyde seemed to be accelerated in all ethanol-treated animals as evidenced by the cyclical blood level curves of these compounds, despite increasing intake, at all but the two highest concentrations of diet ethanol (Fig. 9). Even these high concentrations failed to produce intoxication. Apparently, enhanced EtOH metabolism and/or possible adaptation of the nervous system may have prevented gross behavioral changes.

(4) Large pottery bowls were used as food containers rather than glass bottles or cylinders with steel drinking tubes. The latter, in our hands, had a great tendency to clog up due to the sedimentation of particles from the chocolate Metrecal itself. In the experiments with mice, this clogging may have resulted in several deaths from either starvation or alcohol withdrawal. The large surface area of the pottery bowls was not seen to increase evaporation of ethanol, since less than 2% evaporation of ethanol in 24 hours was observed when the supernatant of the 500 xg centrifugate was assayed by gas chromatography.

The pathological changes in the animals brought up the possibility of nutritional deficiencies and/or toxicity of Metrecal itself. The lack of normal weight gain and the loss of body hair throughout the experimental period occurred in both the ethanol and sucrose groups. This points to the lack of proper nutrients for rabbits in this diet protocol.

The severely fatty livers seen only in the ethanol group indicates the role of alcohol, and not of malnutrition, in producing such fatty change. Ruebner et al (1972) also found that substitution by isocaloric carbohydrates for ethanol did not reproduce the fatty livers in monkeys fed Freund's diet at a diet ethanol concentration of 7.9%



v/tv. In addition, choline supplements to the diet in Ruebner's work did not reverse the fatty changes, suggesting that these changes are not due to the lack of lipotropic factors or to dietary imbalance, but to EtOH itself.

The single death that occurred four days before the end of the study was that of a sucrose control animal. This further implicates the lack of proper nutrients for rabbits in this diet, especially when daily volumes are limited by pair-feeding. The liver of this dead animal appeared no different from those of the other animals in the sucrose group, indicating that the cause of death was not reflected in the cyto-architecture of the liver by light microscopy. It is possible, though, that further deaths in both experimental and control animals may have occurred had the length of the study been extended. It is also possible that EtOH served some protective function in animals imbibing it, thus prolonging their lives. It was not clear whether the single death resulted from:

- (1) Prolonged nutritional deficiency from the composition or amount of the diet;
- (2) Possible hepatotoxicity of Metrecal as suggested by Mendelson, which was not noticeable by light microscopy in our study, but may still be contributory;
- (3) An illness unrelated to diet;
- (4) The last factor, the absence of bulk, may be underscored by citing the percent fiber found in Purina Lab Chows for mice (3.8%) versus that for rabbits (14.6%) (Purina, 1972). The lack of fiber in a liquid diet may lead to abnormalities of digestive processes upon which proper nutrition depends.

Ethanol and acetaldehyde blood levels in rabbits were hard to compare to those of other studies, primarily because of the differences in dose, species, age, sex, route of administration, and other factors. The blood levels in our studies usually were in the range of 0-100 mg% EtOH and 0-9  $\mu\text{g/ml}$  AcD. Majchrowicz (1973), feeding EtOH to rats by intragastric intubation for up to eight days, showed that blood EtOH and AcD concentrations ranged from 100-500 mg% and 1-3  $\mu\text{g/ml}$ , respectively. Freund's mice reached blood levels of EtOH of at least 400 mg% while drinking EtOH-Metrecal ad lib for five days. Very few EtOH metabolism experiments with rabbits, however, have been reported. Duritz and Truitt (1966) observed peak blood EtOH and AcD levels of 380 mg% and 4  $\mu\text{g/ml}$ , respectively, after acute EtOH injection (4.0 g/kg i.v.) in rabbits. Both the rate of alcohol metabolism and the range of blood alcohol concentrations were lower in rabbits than in rats and mice in a study cited by Israel and Mardones (1971). These authors calculated rabbit EtOH metabolism to be less than one-third that of rats and the blood alcohol was found to range from 30-100 mg% under conditions that gave rats levels of 0-300 mg%.

Blood AcD concentration seemed to be related to blood EtOH levels in our rabbits, but other investigators (Mello and Mendelson, 1970) found no dose or dose-time relationship between blood EtOH and blood AcD, and also saw that mean blood AcD concentrations were relatively constant through all dose ranges of EtOH, possibly due to rapid induction of ALDH.

Lack of gross intoxication in our animals, even with high blood EtOH levels, may involve enhanced EtOH metabolism by the liver or by the nervous system. Raskin and Sokoloff (1972) have demonstrated a minute amount of alcohol dehydrogenase in the rat brain which undergoes an

adaptive increase in its activity during chronic EtOH ( 20 days) ingestion. In human alcoholics studied by Mello and Mendelson (1970), blood alcohol level was rarely found to be an adequate predictor of behavior, possibly inferring nervous system adaptation.

Relatively similar blood AcD and EtOH levels despite increasing EtOH consumption may be related to an increase in the rate of EtOH metabolism, although this effect is still controversial, as indicated in a review by Hawkins and Kalant (1972). These authors point to two processes that may be involved in the effect of prolonged EtOH intake on the levels of liver alcohol dehydrogenase: an adaptive process involving a specific induction of ADH, and a non-specific decrease of enzyme levels as a manifestation of depressed protein synthesis resulting from liver impairment.

The three-month duration of this experiment may contribute to some of our findings, since the age of the animal is known to influence the blood EtOH concentration and the rate of its disappearance. These differences apparently are not due to altered liver ADH activity, but may be caused by age-dependent changes that may alter the compartmentalization (e.g. body fat) NAD/NADH<sub>2</sub> ratios, or rate of metabolism of EtOH by processes other than ADH (Hollstedt and Rydberg, 1970). The very high levels of EtOH and AcD seen near the end of the experiment, however, may well be due to general debilitation caused by prolonged malnutrition. Further, malnutrition states, such as ascorbic acid deficiency, are known to themselves contribute to changes in ADH and NAD/NADH<sub>2</sub> ratios (O'Keane et al, 1972)

F. SUMMARY

These studies have uncovered the important role of PG as a dual enzyme inhibitor, favoring catecholamine-derived isoquinoline formation in in vivo studies. The first report of gas chromatographic evidence of isoquinoline biosynthesis after ethanol intoxication (Collins and Bigdeli, 1974) has recently emerged. These authors utilized the effects of PG revealed in the present studies.

Pyrogallol can now be postulated to enhance isoquinoline levels in vivo through at least three biochemical mechanisms, two of which were demonstrated for the first time in these studies:

(1) The oxidation of acetaldehyde via aldehyde dehydrogenase is inhibited, thus maximizing concentrations of one isoquinoline precursor.

(2) The O-methylation and possibly inactivation of isoquinolines are decreased.

(3) Combined with the well-known inhibition by pyrogallol of the catechol O-methyltransferase degradation of norepinephrine and dopamine, these first two effects lead to elevated isoquinoline levels and maximal possibility of detection.

Compounds structurally related to PG were found to inhibit aldehyde dehydrogenase in vitro and elevate ethanol derived acetaldehyde levels in vivo, properties which may lend these drugs to clinical application as disulfiram-like deterrents in alcoholics.

Animal models of chronic alcoholism using a liquid (Metrecal) diet were attempted. This protocol produced intoxication and slight withdrawal symptoms in mice. Rabbits, despite developing elevated blood acetaldehyde and ethanol concentrations, displayed virtually no gross

intoxication while demonstrating fatty liver changes. Malnutritional aspects of this diet were possible factors contributing to the pathologies seen.

TABLE 1

## Composition of liquid diet\*

	<u>EtOH (95%)</u>		<u>Metrecal</u>		<u>Sucrose 87% w/tv</u>		<u>Water added</u>	
	<u>v/tv</u>	<u>cal%</u>	<u>v/tv</u>	<u>cal%</u>	<u>v/tv</u>	<u>cal%</u>	<u>v/tv</u>	<u>cal/ml diet</u>
EtOH	6	35	61	65	—	—	33	0.9
Sucrose	—	—	61	65	9	35	30	0.9

\* Freund, 1969. Diet used in this study with mice and initially with rabbits.

TABLE 2

Maximal Stages of Intoxication and Withdrawal reached  
in mice

<u>Intoxication</u>	<u>No. mice</u>	
	<u>J.R.</u>	<u>Freund</u>
1. ataxic, but rapid gait	12/48	0/10
2. between 1 and 3	25/48	5/10
3. coma, no righting reflex	11/48	5/10
 <u>Withdrawal</u>		
1. overactivity, tremors	6/28	0/10
2. tail-beating, tail-to-nose, retropulsion, broad gait, stereotypy	2/28	3/10
3. tonic-clonic convulsion	1/28	6/10
4. death during convulsion	0/28	1/10

J.R.= results of present study

Freund= results of G. Freund (1969)

TABLE 3

Reaction times of NE and EP1 with aldehydes to form TIQs,  
determined by iodochrome monitoring

	Reaction time (min)	
	<u>AcD</u>	<u>HCHO</u>
NE	20	5
EP1	35	7



TABLE 4

R<sub>f</sub> values for NE and NE-derived TIQs by thin-layer chromatography in two solvent systems

	<u>System A</u>	<u>System B</u>
NE	0.40	0.22
NM	0.46	0.31
TIQ I	0.33	0.29
6-O-Me-TIQ I	0.43	0.26
TIQ II	0.18	0.21
6-O-Me-TIQ II	0.28	0.27

System A: sec-butanol:formic acid: water (15:3:2),

Adsorbosil-1 TLC plate

System B: methanol:n-butanol:benzene: water (40:30:20:10),

cellulose MN 300 plate

TABLE 5

Effect of Pyrogallol on the O-methylation of Noradrenaline and two tetrahydroisoquinoline derivatives in rat brain and liver homogenates

<sup>3</sup> H-Compound	<u>Brain</u>			<u>Liver</u>		
	Saline	% O-Methylation* PG	% Inhib. due to PG	Saline	PG	% Inhib. due to PG
NE (5)**	33.6 ± 4.6 <sup>a</sup>	15.5 ± 3.0	53.9	35.3 ± 4.6 <sup>b,c</sup>	17.6 ± 3.4	50.2
TIQ I (8)	21.5 ± 6.1 <sup>a</sup>	2.0 ± 1.0	90.7	78.2 ± 8.3 <sup>b,d</sup>	31.0 ± 4.6	60.4
TIQ II (8)	29.3 ± 4.5	7.9 ± 3.2	73.1	58.1 ± 4.5 <sup>c,d</sup>	6.2 ± 5.1	89.4

\* Per cent of added substrate that was O-methylated, out of total radioactivity, corrected for recovery, (recovery = 70 ± 5 per cent). Results expressed as mean ± S.D.

\*\* Number of determinations for each saline and each PG experiment in parentheses.

Statistical significance by student's unpaired t-test:

a - p < 0.005

b, c, d - p < 0.001

TABLE 6

Effect of PG on EtOH-derived AcD and EtOH blood levels ( $\mu\text{g/ml}$ )

<u>Time (min)</u>	<u>Saline</u>		<u>PG</u>	
	<u>AcD<sup>a</sup></u>	<u>EtOH<sup>b</sup></u>	<u>AcD<sup>a</sup></u>	<u>EtOH<sup>b</sup></u>
30	1.2 $\pm$ 0.3	1200 $\pm$ 300	2.0 $\pm$ 0.8	1000 $\pm$ 300
60	2.6 $\pm$ 0.5	1600 $\pm$ 330	4.8 $\pm$ 1.2	1900 $\pm$ 550
120	2.0 $\pm$ 0.4	1700 $\pm$ 400	4.1 $\pm$ 1.6	1900 $\pm$ 470
180	1.0 $\pm$ 0.2	1200 $\pm$ 380	3.6 $\pm$ 1.2	1300 $\pm$ 410
270	0.3 $\pm$ 0.1	650 $\pm$ 220	1.9 $\pm$ 0.4	600 $\pm$ 280
360	0.1 $\pm$ 0.1	300 $\pm$ 200	0.5 $\pm$ 0.3	450 $\pm$ 210

Each figure represents mean  $\pm$  S.D. of 4 rats following PG (250 mg/kg i.p.) 1 hour before EtOH (2.0 g/kg i.p.)

<sup>a</sup>  $p < 0.05$ , PG compared to saline, at 60, 120, 180 and 270 min.

<sup>b</sup> N.S. at any time, PG-compared to saline. Student's unpaired t-test.

TABLE 7

Reversibility and deletion controls in studies on the effect of PG on ALDH activity

Conditions	Pre-Incubation with PG	Activity
(1) NAD, Enz, Prop.	-	12.5
(2) (1) plus PG <sup>3</sup>	3	6.3
(3) " " "	5	6.3
(4) " " "	10	6.3
(5) " " "	15	6.3
(6) NAD, Enz.	-	0.5
(7) NAD, Prop.	-	0.2
(8) Enz., Prop.	-	0.0
(9) NAD, Enz., PG	3	0.3
(10) NAD, Prop., PG	3	0.1
(11) Enz., Prop., PG	3	0.0
(12) PG-Enz., aliquot <sup>4</sup>	3	6.3

1 moles NADH/min/mg protein  $\times 10^{-11}$

2 Enz.= enzyme, Prop.= propionaldehyde

3 PG = pyrogallol,  $10^{-4}$  M

4 Reversibility experiment (see text)

TABLE 8

Effect of pyrogallol and related compounds on in vitro activity of rat liver mitochondrial aldehyde dehydrogenase

<u>COMPOUND</u>	<u>M x 10<sup>-4</sup></u>	<u>ACTIVITY</u>	<u>% INHIBITION</u>
Pyrogallol (PG)	0	12.5	0
	0.5	11.7	6.4
	5.0	8.8	27.0
	10.0	6.3	49.5
1,2,4-Trihydroxybenzene	0.5	7.7	38.5
	5.0	6.3	49.5
	10.0	5.0	60.0
Hydroquinone	0.5	9.3	26.2
	5.0	7.3	41.9
	10.0	5.8	54.1
RO 4-4602	0.5	11.8	5.4
	5.0	8.0	36.8
	10.0	6.5	46.0
Diethyldithiocarbamate (DDC)	0.5	11.6	6.8
	5.0	9.2	26.7
	10.0	8.3	33.3
3-Methoxycatechol	0.5	12.5	0
	5.0	10.3	17.2
	10.0	8.6	31.4
Gallic Acid	0.5	12.5	0
	5.0	12.5	0
	10.0	10.6	15.6
2,3-Dimethoxyphenol	0.5	12.5	0
	5.0	12.0	4.3
	10.0	11.6	6.9
Propylgallate	0.5	12.5	0
	5.0	12.5	0
	10.0	11.7	6.2
d,1-Shikimic Acid	0.5	12.5	0
	5.0	12.5	0
	10.0	12.1	3.4

TABLE 8 (continued)

	<u>M x 10<sup>-4</sup></u>	<u>ACTIVITY</u>	<u>% INHIBITION</u>
3,4-Dihydroxytoluene	0.5	12.5	0
	5.0	12.5	0
	10.0	12.5	0
Tetrahydroxyquinone	0.5	12.5	0
	5.0	12.5	0
	10.0	12.5	0

TABLE 9

Comparison of in vitro ALDH inhibition and in vivo potentiation of EtOH- derived AcD blood levels.

<u>Category</u>	<u>Compound</u>	<u>In vitro</u>	<u>In vivo*</u>
1	PG	+	+
2	DDC	+	+
1,3	RO 4-4602	+	+
4	1,2,4-trihydroxybenzene	+	**
4	Hydroquinone	+	0
5	3-methoxy-Catechol	+	0
5	2,3-dimethoxy phenol	0	0
4	Gallic acid	0	0

\* Collins et al, 1974.

\*\* Lethal at dose used for other compounds.

In vitro= + = inhibits ALDH over 15% at  $5 \times 10^{-4}$  M.

In vivo= + = increases blood AcD post-injection of EtOH (3g/kg) compared to saline.

Categories:

1= COMT inhibitor; 2=ALDH inhibitor; 3= DOPA decarboxylase

inhibitor; 4= similar structure to PG; 5= product of COMT action on PG

TABLE 10

Weight change of rabbits drinking Metrecal diets

<u>DIET</u>	<u>% change from initial weight</u>	<u>Average Group change</u>
EtOH	+ 5	-0.67 $\pm$ 2.94
"	-10	
"	+ 3	
Sucrose	+ 7	+6.33 $\pm$ 0.94
"	+ 7	
"	+ 5	
Lab chow	+73	+73.0 $\pm$ 0.00



TABLE 11

Animal deaths during chronic alcohol experiments

	<u>Mice</u>	<u>Rabbits</u>
Weight-reduction	5/180	-
EtOH-drinking	8/ 80*	0/3
Withdrawal	0/ 28	-
Sucrose-drinking	0/ 20	1/3

\* 3 died with limbs extended, possibly denoting convulsions.

TABLE 12

Weight change in mice during Metrecal diets

<u>Diet (with Metrecal)</u>	<u>% change from initial weight* (Ave.)</u>
Sucrose-(n=29)	+ 14.6 $\pm$ 4.2
EtOH - (n=42)	- 4.4 $\pm$ 2.9

\* following 9 days of weight reduction

p < 0.01, student's unpaired t-test

TABLE 13

Daily volume of Metrecal diet consumed ad lib by mice

<u>Diet</u>	<u>Volume, ml (aver., 5 days)</u>
Sucrose (n=29)	16.3 $\pm$ 2.1
EtOH (n=42)	8.2 $\pm$ 3.7

p < 0.01, Student's unpaired t-test.

Values expressed as average  $\pm$  S.D.

TABLE 14

EtOH consumption pattern of rabbits

<u>% EtOH (v/v)</u>	<u>% total calories from EtOH</u>	<u>Days at this level</u>	<u>EtOH consumption, ml (average, n=3)</u>
6.0	35	12	15.8
7.5	39	4	21.9
10.0	46	19	26.7
12.5	54	10	25.8
15.0	59	10	25.0
17.5	63	6	27.1
20.0	71	19	29.1

TABLE 15

Physical effects of liquid diet on rabbits

	<u>Hair loss</u>	<u>Death</u>	<u>Weight change %*</u>
EtOH	2/3	0/3	- 1
Sucrose	2/3	1/3	+ 7
Lab Chow	0/1	0/1	+ 73

\* Per cent of weight at start of diet administration.

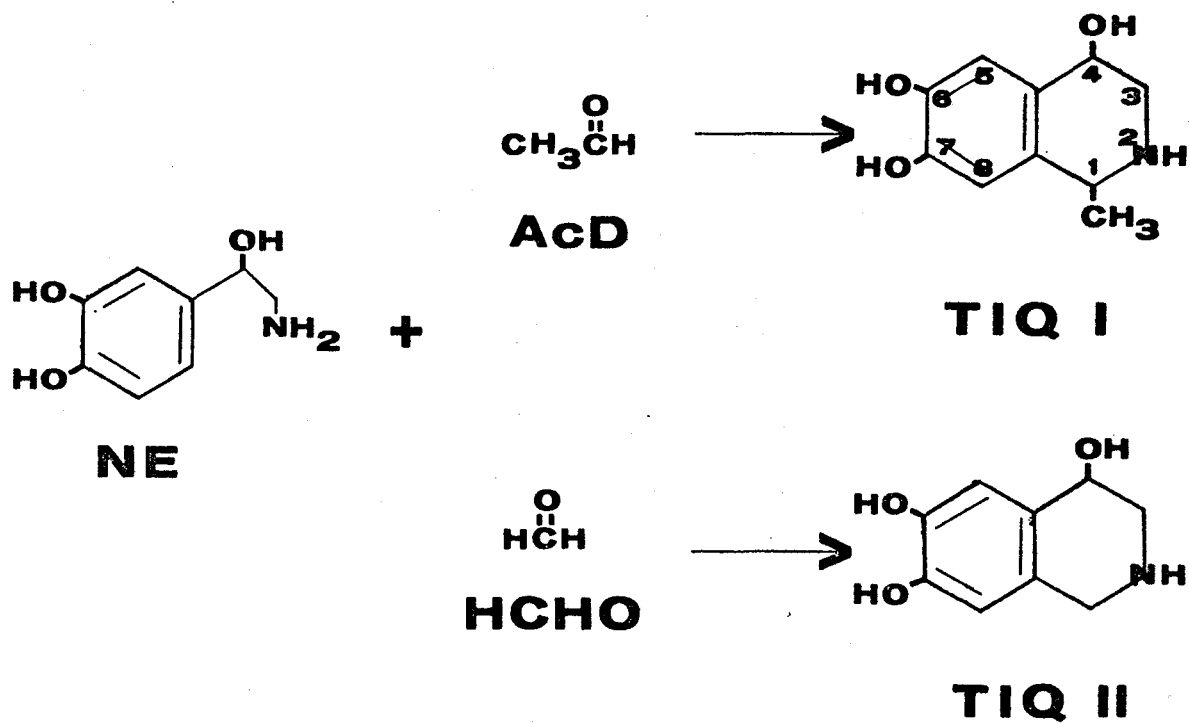


FIG. 1. Condensation of norepinephrine with aldehydes to form tetrahydroisoquinoline alkaloids.

NE=norepinephrine, AcD=acetaldehyde, HCHO=formaldehyde, TIQ=1,2,3,4-tetrahydroisoquinoline.

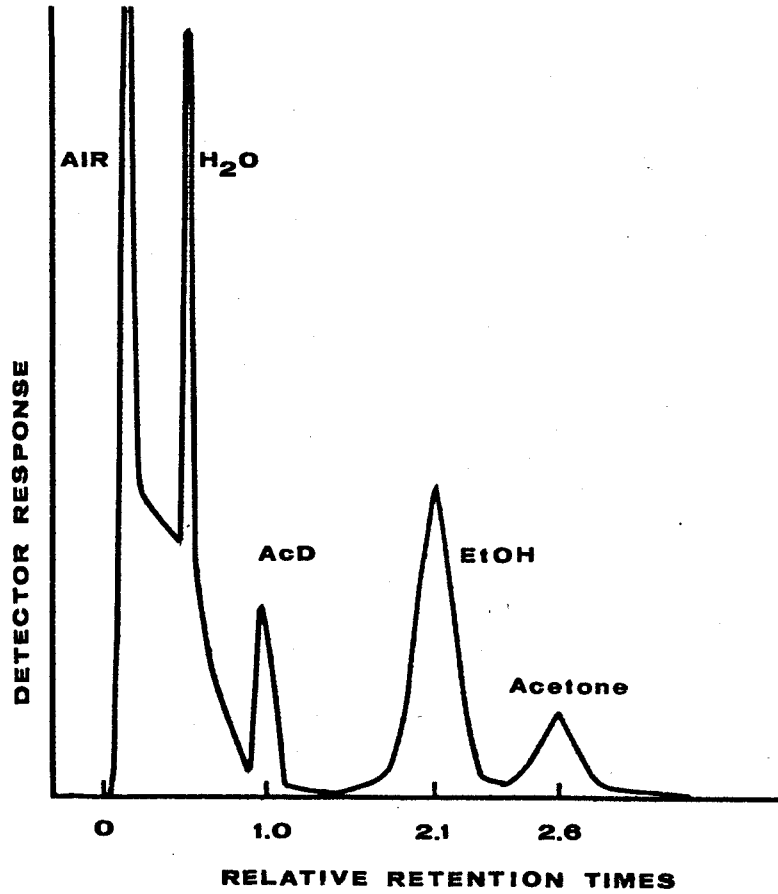


FIG. 2. Gas chromatogram of air, water (H<sub>2</sub>O), acetaldehyde (AcD), ethanol (EtOH) and acetone, using Porapak Q-S column in Varian 2100 gas chromatograph.

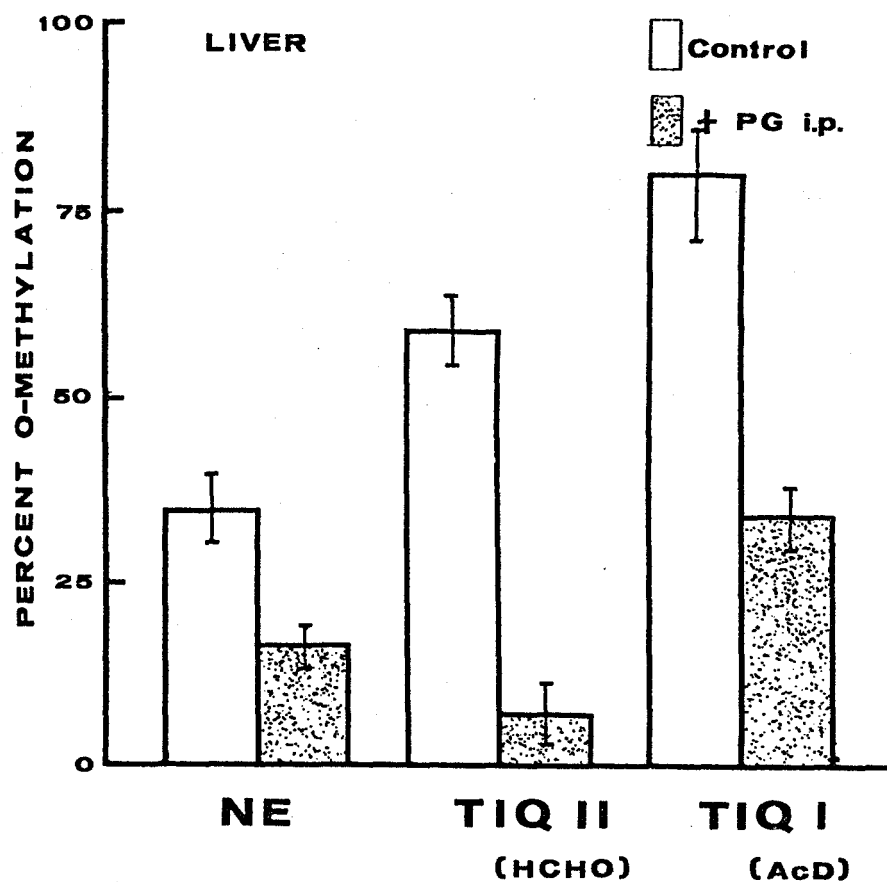


FIG. 3. Metabolism of TIQs in rat liver homogenate.

Results expressed in terms of percent non-catechol (O-methylated) products formed, out of total recovered radioactivity.



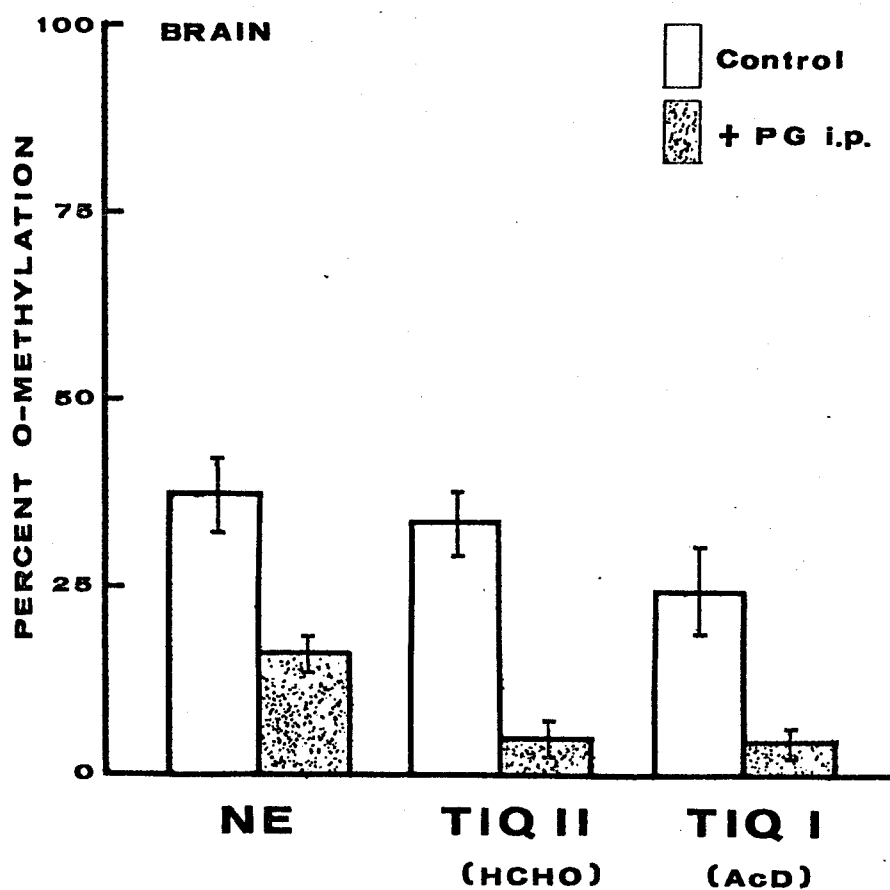


FIG. 4. Metabolism of TIQs in rat brain homogenate

Results expressed in terms of percent non-catechol (O-methylated) products formed, out of total recovered radioactivity.

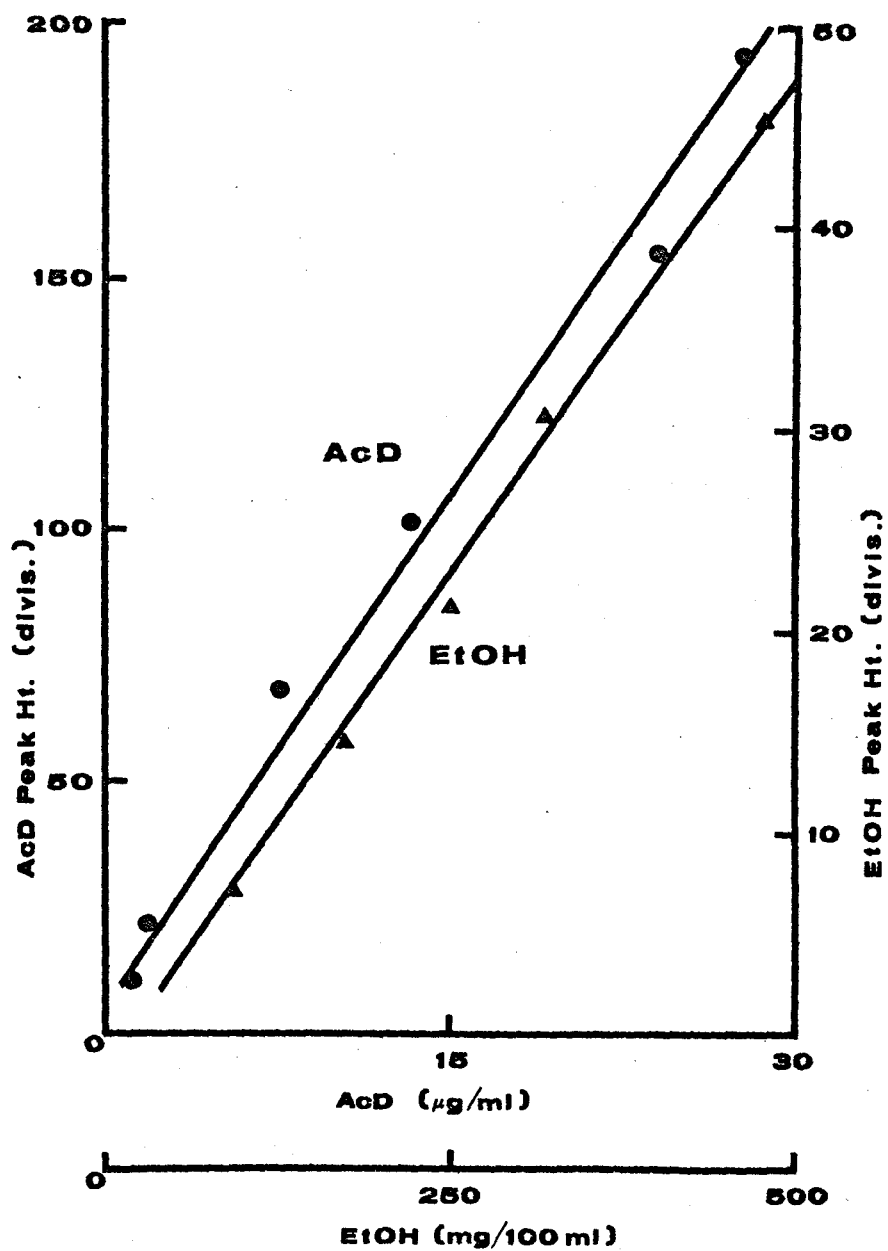


FIG. 5. Calibration curve for EtOH and AcD in whole blood determined by the micromethod of Coldwell *et al* (1971).

Peak heights expressed in divisions on recording paper ( $n=4$  per point)

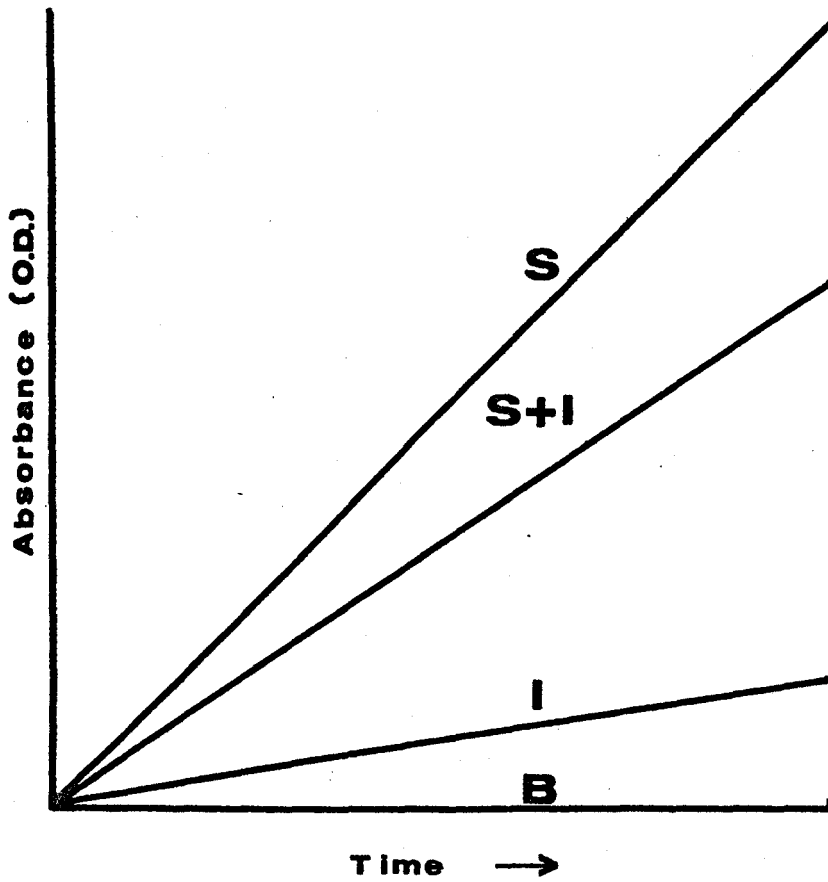


FIG. 6. Representative spectrophotometric recording in aldehyde dehydrogenase assay.

S= substrate alone added; S + I = substrate plus inhibitor;  
I= inhibitor alone added; B= blank control, no substrate or inhibitor added. Absorbance is expressed in optical density units at 340 nm. Time scale is 2 min. Total.

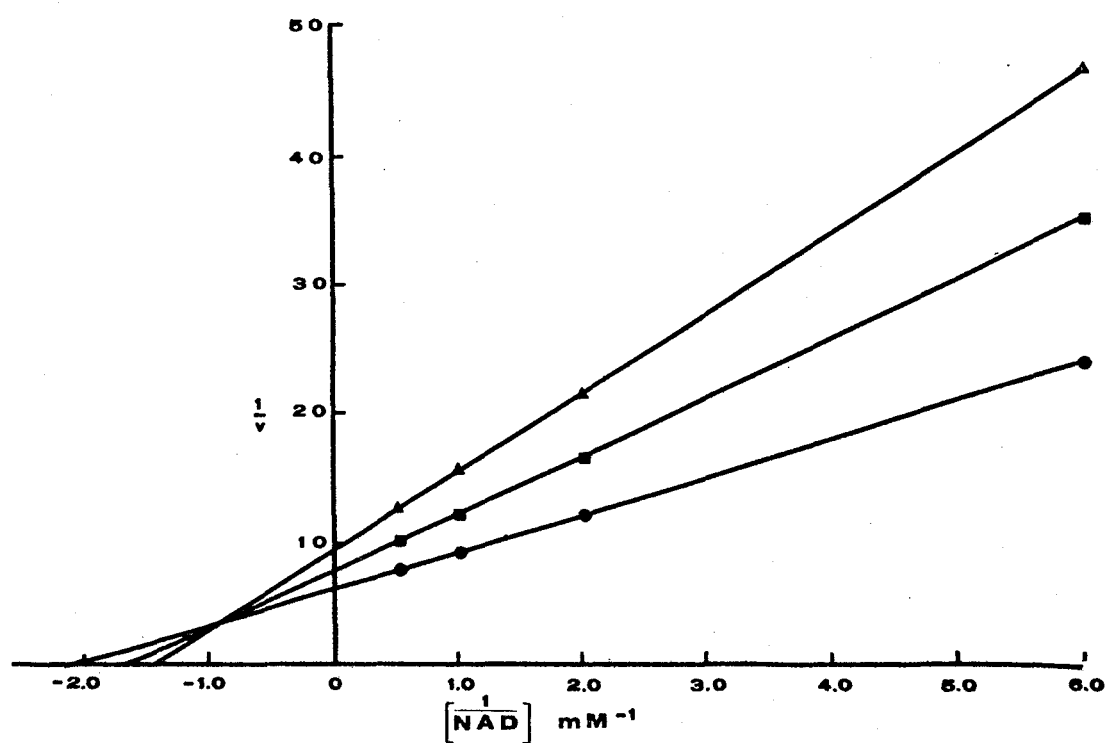


FIG. 7. Double reciprocal plot of the inhibition of rat liver mitochondrial ALDH by PG with respect to NAD cofactor.

PG concentrations: ●, none; ■, 100 μM; ▲, 500 μM.

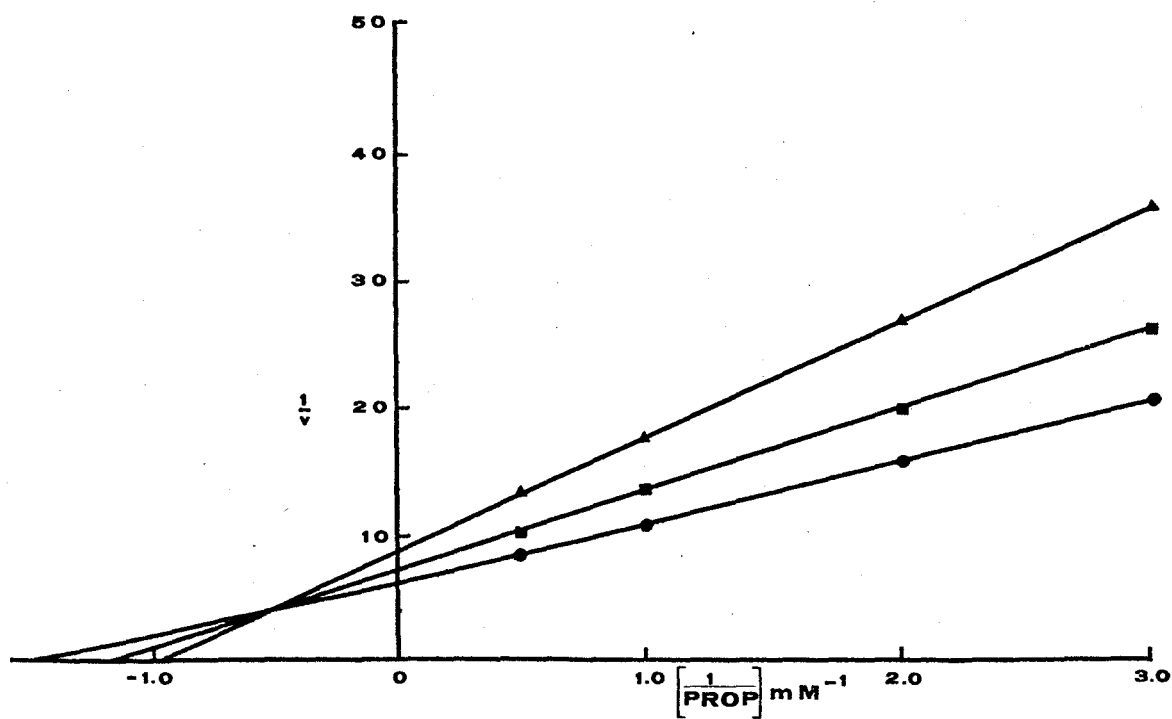


FIG. 8. Double reciprocal plot of the inhibition of rat liver mitochondrial ALDH by PG, with respect to the substrate propionaldehyde.

PG concentrations: ●, none; ■, 100  $\mu\text{M}$ ; ▲, 500  $\mu\text{M}$ .  
PROP= propionaldehyde.

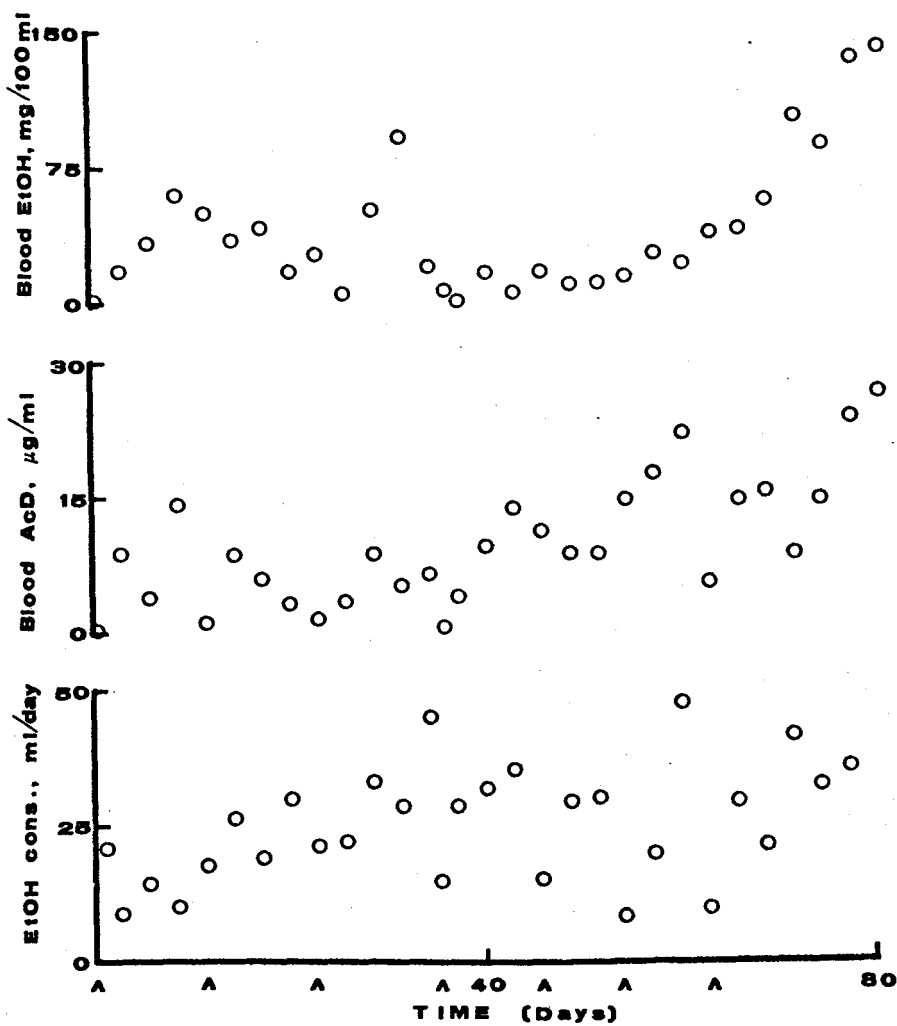


FIG. 9. Ethanol and acetaldehyde blood levels and daily ethanol consumption in a representative rabbit drinking Metrecal/ethanol.

^ indicates increased ethanol concentration in diet (6.0, 7.5, 10.0, 12.5, 15.0, 17.5, and 20.0 % v/v, respectively).

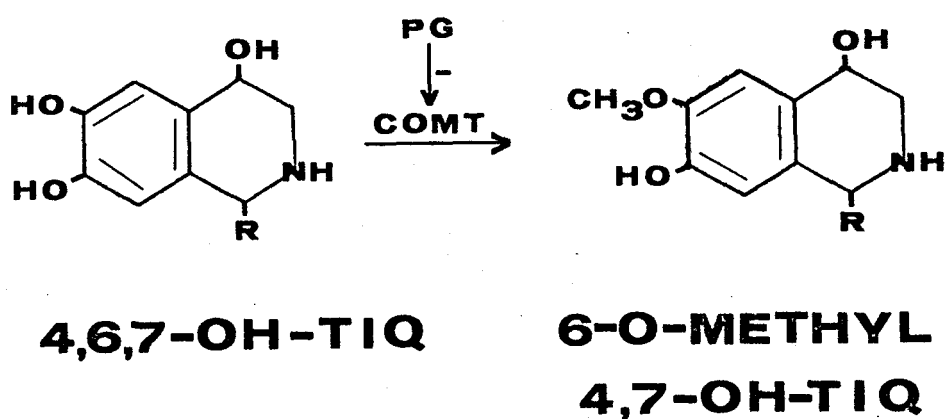
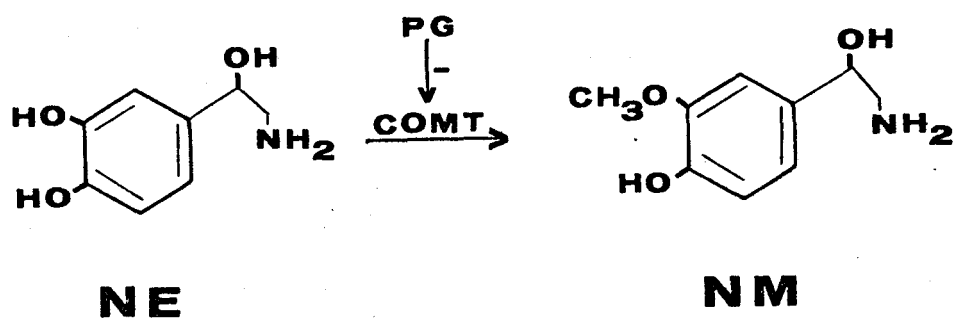


FIG. 10. Analogous metabolism by COMT of NE and NE-derived TIQs, inhibitable by PG.

R= H or CH<sub>3</sub>; NE= norepinephrine; NM= normetanephrine; COMT= catechol O-methyltransferase; PG= pyrogallol; TIQ= 1,2,3,4-tetrahydroisoquinoline.

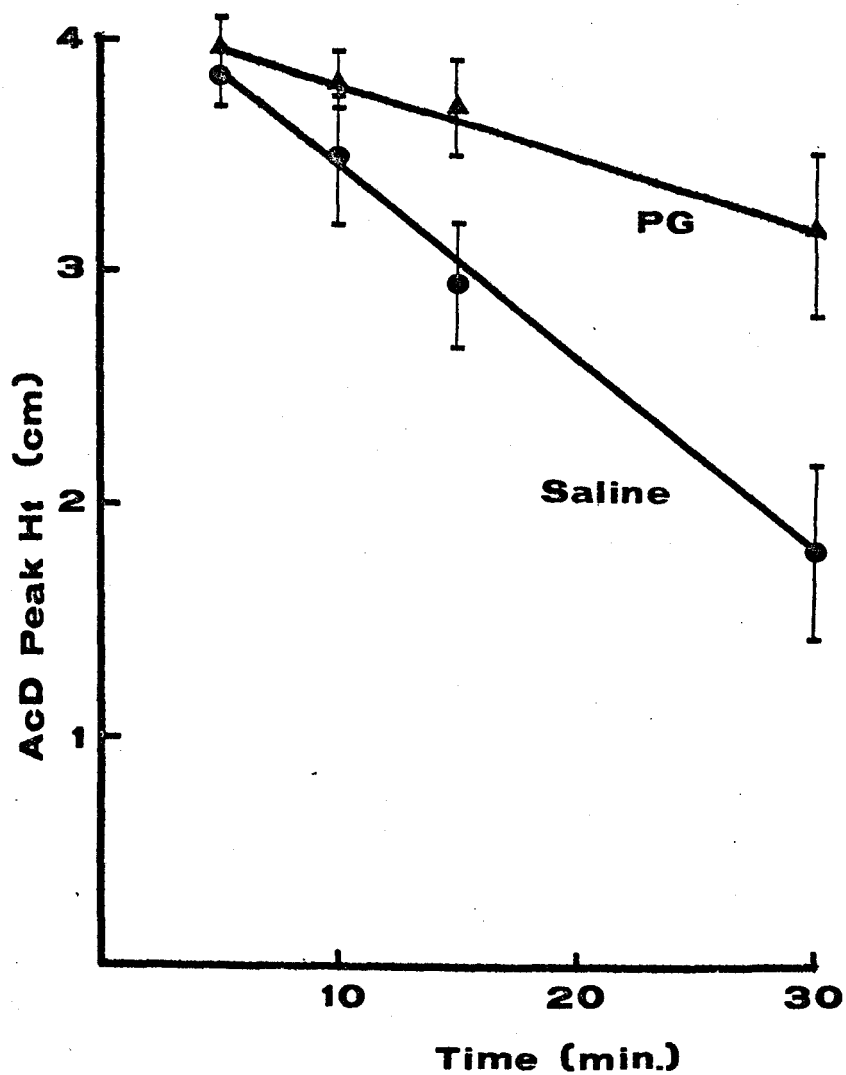


FIG. 11. Effect of pre-injection of PG or saline on AcD disappearance in rat liver homogenate.

Doses: PG 250 mg/kg or saline 0.9%, 1 ml i.p. 1 hr. pre-sacrifice.  
Homogenate: 1 ml rat liver homogenate 1:1 in pH 7 pyrophosphate buffer.  
AcD 0.33 mM and NAD 0.33 mM added.  
Peak heights expressed in cm. as mean  $\pm$  S.D. (n=3).



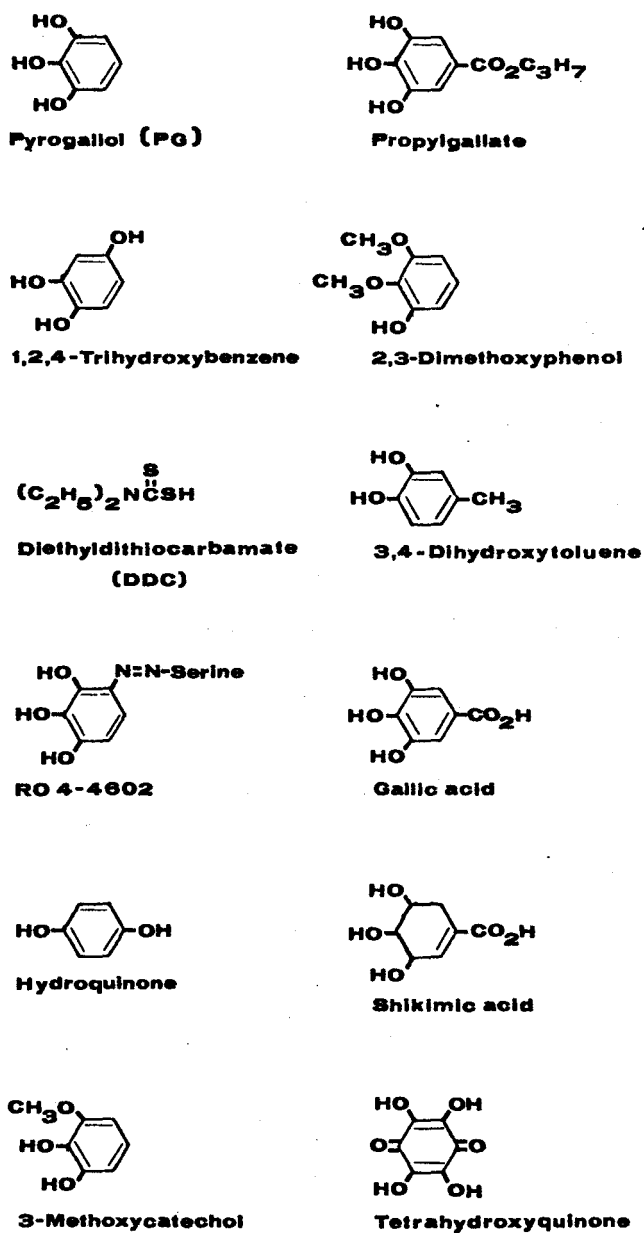


FIG. 12. Structures of PG and related compounds tested for effect on in vitro activity of rat liver mitochondrial ALDH.

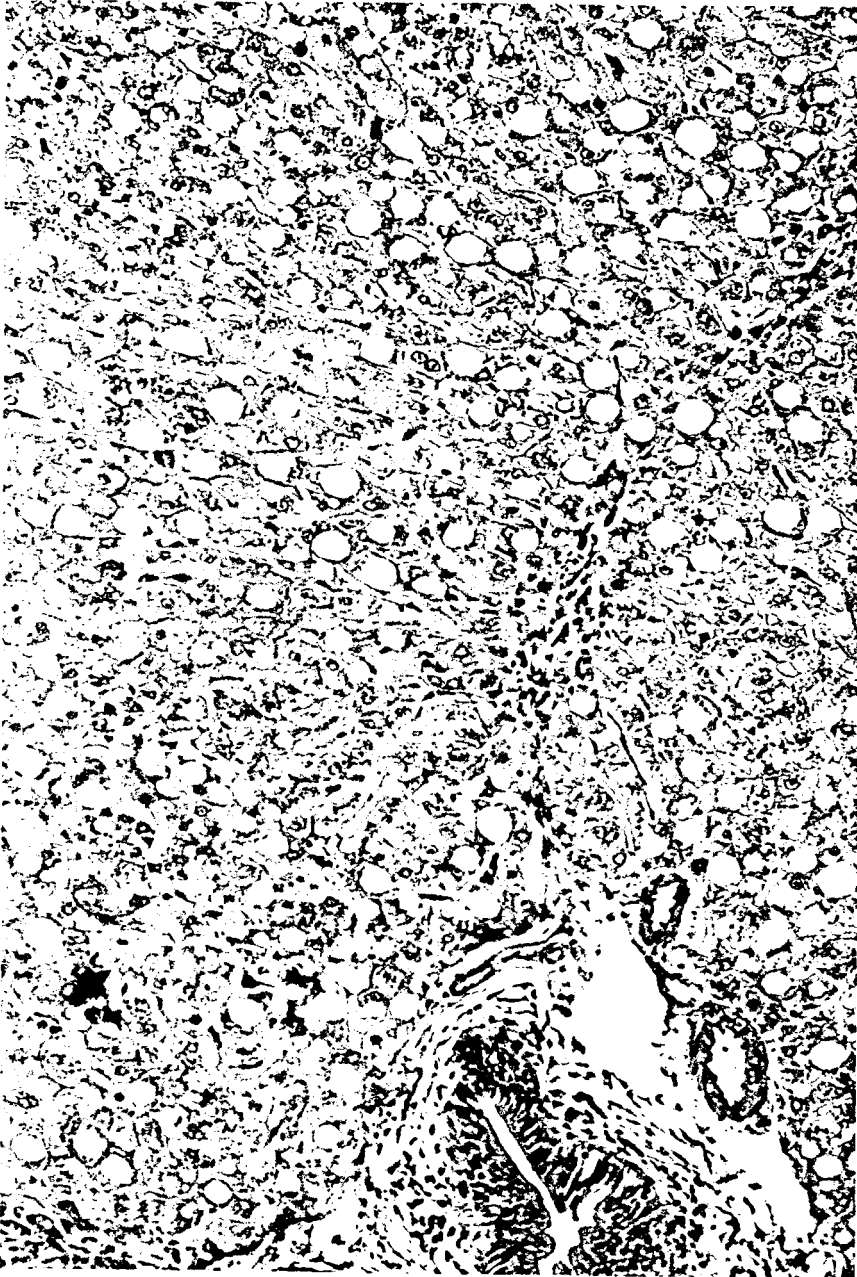


FIG. 13. Liver section of rabbit after 80 days of EtOH/Metrecal diet, showing abundant fat vacuoles.

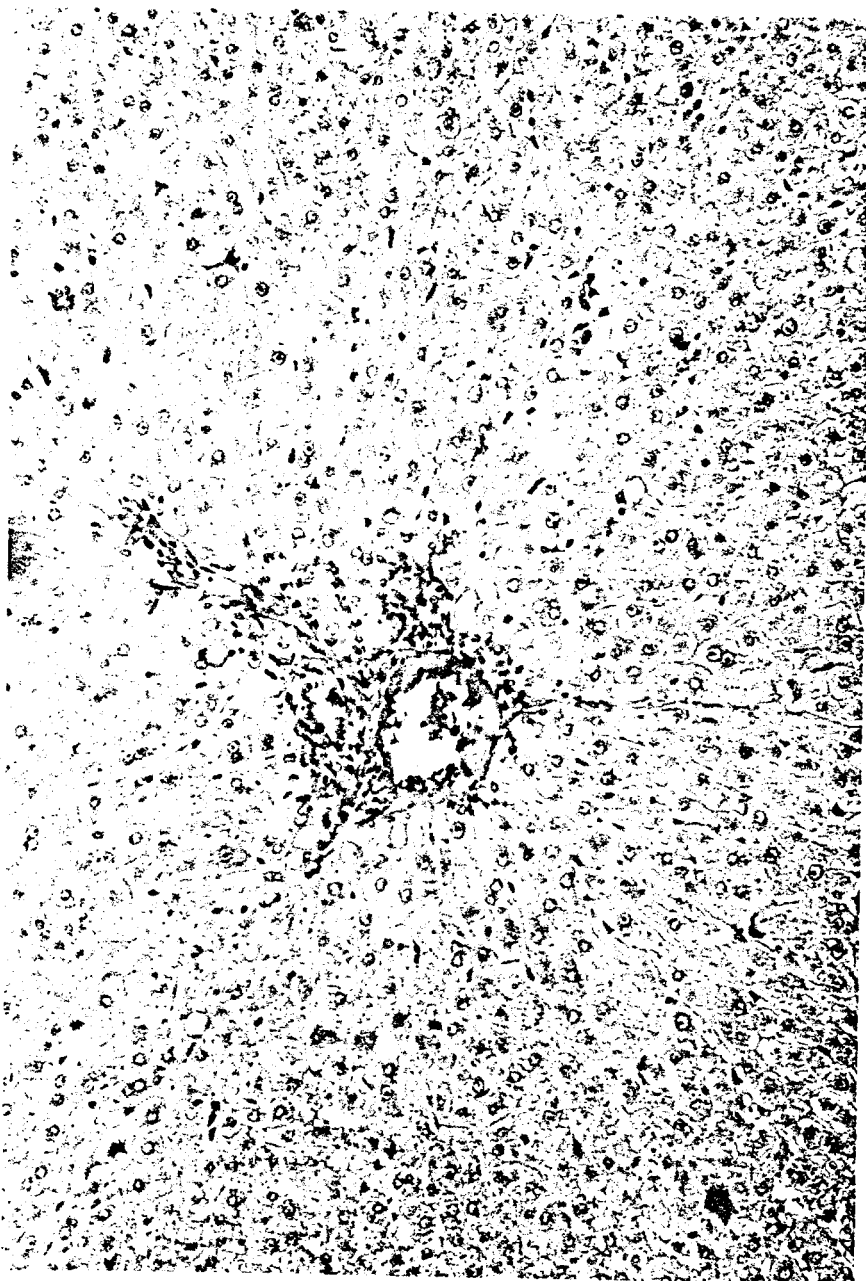


FIG. 14. Liver section of rabbit after 80 days of Sucrose/Metrecal diet, showing relatively normal cell structure with slight fatty changes.

ABBREVIATIONS

AcD	acetaldehyde
ADH	alcohol dehydrogenase
AH	aluminum hydroxide
ALDH	aldehyde dehydrogenase
ATP	adenosine triphosphate
CA	catecholamine
CAMP	cyclic adenosine 3',5'-monophosphate
COMT	catechol O-methyltransferase
DA	dopamine
DBH	dopamine beta-hydroxylase
DDC	diethyldithiocarbamate
DOPA	dihydroxyphenylalanine
DS	disulfiram
E	epinephrine
EtOH	ethanol
gm	gram
HCHO	formaldehyde
hr	hour
5HT	serotonin (5-hydroxytryptamine)
K	potassium
kg	kilogram
MAO	monoamine oxidase
Mg	magnesium
mg	milligram
min	minute

ABBREVIATIONS (continued)

ml	milliliter
Na	sodium
NE	norepinephrine
O-Me-TIQ	6-O-methylated form of corresponding TIQ
PG	pyrogallol
PNMT	phenylethanolamine N-methyltransferase
SAM	S-adenosylmethionine
TH	tyrosine hydroxylase
THP	tetrahydropapaveroline
TIQ	tetrahydroisoquinoline
TIQ I	1-methyl-4,6,7-trihydroxy-TIQ
TIQ II	4,6,7-trihydroxy-TIQ

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APPROVAL SHEET

The dissertation submitted by Joel Allen Rubenstein has been read and approved by the following Committee:

Dr. Michael Collins, Chairman  
Assistant Professor, Department of Biochemistry  
and Biophysics, Loyola

Dr. Joseph Bernsohn, Professor, Departments of Biochemistry and Biophysics and Pharmacology, Loyola

Dr. Robert Jacobs, Assistant Professor, Department of Pharmacology, Loyola

Dr. Mary Druse Manteuffel, Assistant Professor,  
Department of Biochemistry and Biophysics, Loyola

Dr. Charles Scudder, Associate Professor, Department of Pharmacology, Loyola

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 17, 1974

Michael A. Collins, Ph.D.