ETHANOL AND ACETALDEHYDE METABOLISM IN SHEEP

A thesis presented in partial
fulfilment of the requirements
for the degree of

Doctor of Philosophy
in Biochemistry

at

Massey University,

New Zealand.

Jane HENDTLASS

1973

ABSTRACT

Ethanol and acetaldehyde metabolism in sheep has been studied in three different types of experiments:in purified enzyme systems, in liver homogenates and in the intact animals. Particular emphasis has been placed on the aldehyde oxidase enzyme from sheep liver, a molybdo-flavoprotein with a broad specificity which includes aldehydes, quinines and N^1-methyl nicotinamide. This thesis describes a method for preparing an enzyme solution in which sheep liver aldehyde oxidase constitutes 85% of the total protein present. Investigations of its physical and kinetic properties show that the sheep liver enzyme differs from the aldehyde oxidases previously prepared from pig and rabbit livers. In addition, an antibody to sheep liver aldehyde oxidase has been prepared from rabbit serum and has been shown to act as a specific, competitive inhibitor of the enzyme. This has been used to assess the contribution that aldehyde oxidase makes to acetaldehyde oxidation in sheep liver homogenates under several different conditions.

The effects of steroids on ethanol and acetaldehyde metabolism has been investigated, special interest being taken in the effects of progesterone. Progesterone stimulates sheep liver aldehyde oxidase activity *in vitro* and inhibits sheep liver aldehyde dehydrogenase. Administration of progesterone to castrated sheep *in vivo* increased the rates of ethanol and acetaldehyde oxidation, and aldehyde oxidase has been identified as a factor in decreasing acetaldehyde concentrations in the homogenates of livers from these animals during the metabolism of exogenous ethanol. Low endogenous ethanol concentrations in peripheral venous blood of sheep are positively correlated with high progesterone levels in sheep due to its experimental administration, and to pregnancy and the oestrus cycle.

Studies of the effects of disulphiram on ethanol and acetaldehyde metabolism have shown that the compound inhibits sheep liver aldehyde oxidase and aldehyde dehydrogenase enzymes *in vitro*, increases endogenous concentrations of acetaldehyde in peripheral venous blood, and causes acetaldehyde accumulation during ethanol metabolism *in vivo*. When diazepam is present together with disulphiram it provides protection from all but one of the effects shown by disulphiram alone. It does not alter the disulphiram inhibition of sheep liver aldehyde dehydrogenase. Amitryptyline is an inhibitor of both aldehyde oxidase and aldehyde dehydrogenase enzymes. It seems to increase the aldehyde

oxidase response to disulphiram, and its *in vivo* administration causes acetaldehyde accumulation in peripheral blood during and in the absence of metabolism of exogenous ethanol.

Investigations into the effects of ethanol on ethanol and acetaldehyde metabolism in sheep have shown that ethanol increases the activity of aldehyde oxidase *in vitro* and its chronic administration accelerates acetaldehyde oxidation *in vivo*. A supplementary study of the interrelationships between the relative concentrations of NADH and NAD^+, and ethanol and acetaldehyde metabolism shows that aldehyde oxidase participation in acetaldehyde oxidation is dependent on the NAD^+ concentrations, and that acetaldehyde oxidation can account for much of the NADH accumulation that occurs during ethanol metabolism *in vivo*.

Acetaldehyde oxidation during ethanol metabolism in sheep can be diverted through the aldehyde oxidase catalyzed pathway, avoiding dependence on the NAD^+-linked aldehyde dehydrogenase enzyme. The results in this thesis have shown that aldehyde oxidase can catalyze up to two-thirds of acetaldehyde oxidation in sheep liver when NAD^+ is limited, and that the pathway is dependent on the endocrine state and the pattern of ethanol consumption of the animal.

ACKNOWLEDGEMENTS

The author wishes to thank her supervisors, Prof.R.D.Batt and Dr.R.M.Greenway, for their help and advice throughout the course of this study.

In addition she expresses sincere appreciation of the help, advice and technical assistance rendered by other members of the staff of the Department of Chemistry and Biochemistry, Massey University; in particular, thanks are due to Miss L.Burson who willingly performed many duties outside her usual sphere of work.

The staff of the Animal Physiology Unit, especially Mr.L.Robertson, were generous in giving freely of their time and knowledge in experiments involving live animals.

The author also thanks Mrs.E.Self for typing the manuscript and remaining patient during inevitable delays.

Finally the patience and perseverance of Mr.R.E.Hendtlass must be recognised, for without these this thesis would not have been.

Upon the advice of Prof.R.D.Batt, this thesis is presented in the form of several papers to facilitate its later publication.

LIST OF CONTENTS

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

a.m.	ante meridian
by vol.	by volume
CoQ	coenzyme Q
D	diffusion constant
Δ	change in
DCI	2,6-dichlorophenol indophenol
DM	dry matter
E.C.No.	Enzyme Commission Number
EDTA	ethylenediamine-tetra-acetate
FAD	flavin adenine dinucleotide
g	gravity
HCl	hydrochloric acid
K	degrees Kelvin
K_i	inhibitor constant
K_m	Michaelis constant
LD_{50}	lethal dose for 50% of a population
M	molecular weight
MeB	methylene blue
mmoles	millimoles
mg%	$mg/100cm^3$
N	number of samples
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NE	not estimated
OD	optical density
p	relative density
$p<$	probability less than
pH_{max}	optimum pH
R	universal gas constant
R_f	distance travelled relative to the front
RNA	ribonucleic acid
s	sedimentation coefficient
S	Svedburgs
SD	standard deviation

SE_M	standard error of the mean
-S-S-	sulphydryl group
tert.	tertiary
T	absolute temperature
$\bar{v}$	partial specific volume
vol.	volume
w/v	weight/volume
v/v	by volume
'zero'	taken at zero time

Chapter 1

INTRODUCTION

Acetaldehyde can be detected in low concentrations in normal blood (Gee & Chaikoff, 1926; Supniewski, 1926; Briggs, 1926-27; Hine et al, 1952) and urine (Briggs, 1926-27). It is normally formed by oxidation of the ethanol arising from gastrointestinal fermentation (Krebs & Perkins, 1970; Blomstrand, 1971) or the drinking of alcoholic beverages (Hartroft, 1967). Although acetaldehyde is formed in several metabolic reactions, Jacobsen (1950) has concluded that precursors other than ethanol make quantitatively insignificant contributions to the acetaldehyde levels in the blood.

The liver is considered to be the main, but not the only, site for both the synthesis and degradation of acetaldehyde (Lubin & Westerfeld, 1945; Hald & Larsen, 1949; Hunter & Lowry, 1956; Hedlund & Kiessling, 1969). Some acetaldehyde metabolism can take place extrahepatically (Larsen, 1959), primarily in the brain (Ridge, 1963) and muscle tissue (Harting, 1951).

Catabolism of ethanol and acetaldehyde gives rise to free acetic acid (Lundquist et al, 1963; Hedlund & Kiessling, 1969; Williamson et al, 1969) but some other minor products are formed. These must be taken into account in considering the addictive and toxic properties of ethanol.

The following account of acetaldehyde metabolism covers the most significant known mechanisms for acetaldehyde synthesis and degradation. Acetaldehyde is the only metabolite of ethanol foreign to the general metabolic pool (Westerfeld, 1961). It has frequently been implemented in explanations of ethanol toxicity (Akabane, 1960; Akabane et al, 1964d; Truitt & Duritz, 1967; Akabane, 1970) and a brief description of its pharmacology is included.

METABOLISM OF ACETALDEHYDE

I) ENZYMATIC SYNTHESIS OF ACETALDEHYDE IN MAMMALS

A number of enzymes have been described which catalyze the formation of acetaldehyde. These are listed in Table 1 and described below.

Alcohol Dehydrogenase: (E.C.No.1.1.1.1.) Alcohol dehydrogenase is the most studied enzyme catalyzing the formation of acetaldehyde from ethanol (Goodman & Tephly, 1969; Lester & Benson, 1969; Lieber & DeCarli, 1969). It contains zinc as a prosthetic group and uses NAD^+ bound α-stereoisometrically (Levy & Vennesland, 1957) as the hydrogen acceptor (Theorell, 1965). The $K_{m(ethanol)}$ for horse liver alcohol

TABLE 1 Enzymatic Formation of Acetaldehyde

Enzyme	Reaction	Location
Alcohol Dehydrogenase	alcohol+NAD^+ ⇌ aldehyde+NADH+H^+	Liver Kidney
Microsomal Mixed Function Oxidase Microsomal Ethanol Oxidising System	alcohol+NADPH+H^++O_2 ⇌ aldehyde +$NADP^+$+$2H_2O$	Liver
Catalase	alcohol+ H_2O_2 ⇌ aldehyde+$2H_2O$	Erythrocytes Liver
Threonine Aldolase	L.threonine ⇌ acetaldehyde+glycine	Liver Kidney
Allothreonine Aldolase	L.allothreonine ⇌ acetaldehyde+ glycine	Liver
Desoxyribose Phosphate Aldolase	desoxyribose-5-phosphate ⇌ glyceraldehyde-3-phosphate +acetaldehyde	Intestine
β-Alanine Decarboxylase	β-alanine ⇌ acetaldehyde+CO_2+NH_3	Brain Liver
Pyruvate Dehydrogenase	pyruvate ⇌ acetaldehyde+CO_2	Heart

dehydrogenase is of the order of 1mM (Goldstein, 1970). Accordingly, the enzyme is still 83% saturated at an ethanol concentration of 10mM (48mg%). This explains why zero-order kinetics are observed for ethanol disappearance *in vivo* (Dundee *et al*, 1971). Below concentrations near 2.5mM (12mg%) the rate of ethanol oxidation decays exponentially (Marshall & Fritz, 1953; Lester, 1962).

Alcohol dehydrogenase is present normally in mammalian liver, probably to oxidise the ethanol formed from fermentation reactions in the gastrointestinal tract (Krebs & Perkins, 1970) although some ethanol may also be formed during *in vivo* metabolism (Racker, 1952; Pihl & Fritzson, 1955; Karasek & Greenburg, 1957; McManus *et al*, 1966; Krebs & Perkins, 1970). The basal concentration of ethanol in human venous blood has been variously reported as 0.001mM (Blomstrand, 1971), 0.0012-0.019mM (Krebs & Perkins, 1970), 0.1-0.2mM (Lester, 1962) and 1mM (Hine *et al*, 1952).

Liver alcohol dehydrogenase is considered to be the main enzyme catalyzing the oxidation of ingested ethanol. Hartroft (1967) has claimed that ethanol constitutes 10% of the total calorie intake of the "average" American male and, because of the number of people who include relatively large amounts of ethanol in the daily diet, it is difficult to claim that the substance is an abnormal dietary constituent.

Microsomal Enzyme Systems: Two distinct enzyme systems which catalyze ethanol oxidation to acetaldehyde in microsomes have been described.

The Mixed Function Oxidase has been implicated in the metabolism of various drugs (Orme-Johnson & Ziegler, 1965; Roach, 1969; Hilton & Sartorelli, 1970). This enzyme can be induced by either the chronic administration of ethanol (Rubin & Lieber, 1968) or by drugs such as phenobarbital (Rubin *et al*, 1968; Reinhard & Spector, 1970), and the induction is said to explain the increased drug tolerance noted in sober alcoholics (Rubin *et al*, 1968).

The other microsomal system which catalyzes the oxidation of ethanol is referred to as the Microsomal Ethanol Oxidising System (Lieber & DeCarli, 1968). This differs from the mixed function oxidase in its substrate specificity and response to inhibitors (Lieber, 1970), but it can also be induced by chronic ethanol administration (Lieber & DeCarli, 1968; Lieber & DeCarli, 1970).

Both enzyme systems show optimum activities at pH 7 (Lieber, 1970) and require O_2 and NADPH as cofactors. Under normal conditions, the contribution of these enzymes to total acetaldehyde formation is

probably small (Khanna et al, 1970; Krebs & Perkins, 1970), and the contribution may not even be very significant in cases where there are continuing high levels of alcohol intake (Khanna & Kalant, 1970; Mezey, 1972). It should be noted that there is not general agreement on the occurrence of ethanol oxidising enzymes in microsomes. Isselbacher & Carter (1970) queried the actual existence of the two enzymes.

Catalase: (E.C.No.1.11.1.6.) Catalase in erythrocytes and liver has been demonstrated to have a peroxidase function with ethanol (Keilin & Hartree, 1936). Parks and others (1961) claim that some acetaldehyde is formed in vivo by this catalase action but, in general, authors do not consider that the amount produced in this reaction is very large (Jacobsen, 1952; Kinard et al, 1956; Lester & Benson, 1970).

Other Enzymes Synthesising Acetaldehyde: Five other enzymes have been described which are able to form acetaldehyde from precursors other than ethanol. According to different research workers acetaldehyde can arise from threonine or allothreonine (Karasek & Greenburg, 1957), desoxyribose (Racker, 1952), β- alanine (Pihl & Fritzson, 1955) and pyruvate (McManus et al, 1966).

It should be noted however that if acetaldehyde is formed in vivo in the absence or at low concentrations of ethanol, the equilibrium constant of alcohol dehydrogenase is such that most of the acetaldehyde would be rapidly reduced to ethanol.

II) ENZYMATIC DEGRADATION OF ACETALDEHYDE IN MAMMALS

A number of enzymes have been described which catalyze the conversion of acetaldehyde into other substances. These are listed in Table 2 and described below.

Alcohol Dehydrogenase: (E.C.No.1.1.1.1.) The equilibrium constant of alcohol dehydrogenase favours the reduction of acetaldehyde to ethanol (Backlin, 1958). Acetaldehyde arising in the absence of high concentrations of ethanol will therefore be reduced to ethanol by this enzyme.

Aldehyde Dehydrogenase: (E.C.No.1.2.1.3.) Aldehyde dehydrogenase is an NAD^+-linked enzyme which was partially purified by Racker in 1949. Since then full descriptions of the enzyme have been reported by a number of workers (Levy & Vennesland, 1957; Deitrich & Hellerman, 1963; Stoppani et al, 1966). The enzyme is relatively non-specific for aldehydes (Deitrich, 1962) and is strongly inhibited by sulphydryl binding agents such as arsenite (Stoppani & Milstein, 1957; Deitrich, 1967). Inhibition of the enzyme by steroids has been described by Maxwell & Topper (1961; Maxwell, 1962).

TABLE 2 Enzymatic Conversion of Acetaldehyde to other Compounds

Enzyme	Reaction	Location
Alcohol Dehydrogenase	acetaldehyde+NADH+H^+ $\rightleftharpoons$ alcohol+NAD^+	Liver Kidney
Aldehyde Dehydrogenase	acetaldehyde+NAD^+ $\longrightarrow$ acetate+NADH+H^+	Liver
Glyceraldehyde-3-Phosphate Dehydrogenase	acetaldehyde+NAD^++P_I $\rightleftharpoons$ acetylphosphate +NADH+H^+	Muscle
Aldehyde Oxidase	acetaldehyde+O_2 $\rightleftharpoons$ acetate+H_2O_2	General Distribution
Xanthine Oxidase	acetaldehyde+O_2 $\rightleftharpoons$ acetate+H_2O_2	General Distribution
Pyruvate Carboxylase	acetaldehyde+pyruvate $\rightleftharpoons$ acetoin+CO_2	Heart Muscle Brain
Threonine Aldolase	acetaldehyde+glycine $\rightleftharpoons$ L.threonine	Liver Kidney
Salsolinol Reaction	acetaldehyde+dopamine $\rightleftharpoons$ salsolinol	Brain
α-Ketoglutarate Oxidase	acetaldehyde+ α-ketoglutarate $\rightleftharpoons$ 5-hydroxy-4-ketohexanoic acid	Heart
Carbonic Anhydrase	acetaldehyde+H_2O $\rightleftharpoons$ hydrated acetaldehyde	Erythrocytes

Aldehyde dehydrogenase activities have now been assigned to two cytoplasmic proteins which are said to have different specificities (Raison et al, 1966). A mitochondrial enzyme with aldehyde dehydrogenase activity has also been described (Walkenstein & Weinhouse, 1953; Horton & Packer, 1970) with kinetic properties which differ from those of the cytoplasmic enzyme (Glenn & Vanko, 1959).

The aldehyde dehydrogenase activities are probably able to catalyze up to 85% of total acetaldehyde metabolism (Richert & Westerfeld, 1957). However other enzymes must be capable of contributing to acetaldehyde removal since depletion of 90% of the liver aldehyde dehydrogenase activity has been shown to have little effect on *in vivo* acetaldehyde metabolism (Westerfeld, 1961). Liver aldehyde dehydrogenase activity has been shown to increase with chronic ethanol administration (Dajani & Orten, 1960; Dajani et al, 1963). In some strains of mice this liver activity has been positively correlated with a noted perference for alcohol (Sheppard et al, 1970).

Alcohol dehydrogenase can couple with aldehyde dehydrogenase (Racker, 1949) to catalyze the dismutation reaction between acetaldehyde and pyruvate described by Dixon and Lutwak-Mann (1937). Acetic acid and lactic acid are the products of this reaction.

Glyceraldehyde-3-Phosphate Dehydrogenase: (E.C.No.1.2.1.12) Acetaldehyde oxidation is catalyzed by glyceraldehyde-3-phosphate dehydrogenase at 10^{-5} times the rate at which it catalyzes glyceraldehyde-3-phosphate oxidation (Harting, 1951). The enzyme contains sulphydryl (Pihl & Lange, 1962) and glutathione (Krimsky & Racker, 1952) groups, and it is inhibited by disulphiram (Nygaard & Sumner, 1952). Since the enzyme is said to constitute 7-10% of total muscle protein, by weight (Nygaard & Sumner, 1952), it must be important in the relatively little extrahepatic acetaldehyde metabolism which occurs.

Aldehyde Oxidase: (E.C.No.1.2.3.1.) Aldehyde oxidase is a molybdoflavoprotein first recognised in pig liver (Gordon et al, 1940) and since then it has been detected in most mammalian tissue (Deitrich, 1966). It has a low specificity for aldehydes and the rabbit enzyme (Rajagopalan et al, 1962) can also oxidise quinines (Knox, 1946) and N^1-methyl nicotinamide (Rajagopalan & Handler, 1964a).

Pig and rabbit aldehyde oxidases can couple substrate oxidation to molecular oxygen or cytochrome c reduction ("oxidase" reaction) and to various non-physiological hydrogen acceptors ("dehydrogenase" reaction).

Dietary deficiency in molybdenum leads to reduction of aldehyde oxidase activity *in vivo* (Richert & Westerfeld, 1957). It is considered

that the molybdenum in the enzyme molecule acts as the primary electron acceptor (Aleman et al, 1965).

Studies show that FAD (Kand et al, 1972) and iron (Handler et al, 1964; Rajagopalan & Handler, 1964b) play important roles as secondary electron acceptors, and CoQ has also been implicated (Rajagopalan et al, 1962).

Xanthine Oxidase: (E.C.No.1.2.3.2.) Xanthine oxidase is a molybdoflavoprotein similar to aldehyde oxidase with the additional function of being able to catalyze purine oxidation. It has been purified from milk (Corran et al, 1939) and liver (Kielly, 1955) and detected in intestinal tissue, blood, lung, spleen and kidney (Richert & Westerfeld, 1951; Westerfeld & Richert, 1951; DeRenzo et al, 1953; Mahler et al, 1954a). Some species and location differences have been reported (Richert & Westerfeld, 1951; Corran et al, 1939).

Bovine liver xanthine oxidase oxidises acetaldehyde at 28% of the rate at which hypoxanthine is converted to xanthine (Corran et al, 1959). Dietary deficiency of molybdenum reduces xanthine oxidase activity in vivo (DeRenzo et al, 1953; Richert & Westerfeld, 1953) but the enzyme activity is increased under conditions of vitamin E deficiency (Dinning, 1952). Vitamin E is said to convert the Type D ("dehydrogenase") form of the enzyme to the Type O ("oxidase") form (Cattigoni & Dinning, 1971) and it is possible that this may be a regulatory mechanism.

Pyruvate Carboxylase: (E.C.No.6.4.1.1.) A condensing reaction between pyruvate and acetaldehyde, catalyzed by pyruvate carboxylase, was first described by Green and his coworkers in 1941. The enzyme responsible for the reaction has been detected in brain and liver (Green et al, 1941; Westerfeld, 1949). The reaction yields acetoin, and is dependent on thiamine pyrophosphate (Green et al, 1941). Since alcoholics are often thiamine deficient (Williams et al, 1943), and show signs of the deficiency particularly during withdrawal (Delaney et al, 1966; Kershaw, 1967), considerable interest has been shown in this enzyme and a detailed review of the work was published in 1955 (Järnefelt, 1955). However, it has been demonstrated that acetaldehyde metabolism is not generally impaired in thiamine deficiency (Stotz & Westerfeld, 1944; Lubin & Westerfeld, 1945) and acetoin does not appear as a major metabolite during the in vivo metabolism of acetaldehyde (Lubin & Westerfeld, 1945).

Threonine Aldolase: (E.C.No.4.1.2.5.) Acetaldehyde has been shown to condense with glycine in an aldolase reaction (Karasek & Greenburg, 1957). The extent to which the reaction takes place depends on available glycine and the significance of the reaction in acetaldehyde metabolism in mammals has been questioned (Lundquist et al, 1959). Sheep have

particularly high levels of this enzyme.

Salsolinol Reaction: Recently it has been reported that dopamine and acetaldehyde can condense enzymatically (Davis et al, 1970) to form salsolinol, which is a morphine-like compound. This compound has no known addictive properties (Davis & Walsh, 1970a; Yamanaka et al, 1970) but much interest is being shown in similar reactions between acetaldehyde and other amines in vivo.

α-Ketoglutarate Oxidase: In 1966, Bloom and others (Bloom & Westerfeld, 1966; Bloom et al, 1966) reported the condensation of acetaldehyde with oxoglutarate to give the compound, 5-hydroxy-4-ketohexanoic acid. This compound is excreted in the urine and it is suggested that α-ketoglutarate oxidase catalyzes the reaction (Bloom et al, 1966).

Carbonic Anhydrase: (E.C.No.4.2.1.1.) Erythrocyte carbonic anhydrase can catalyze the hydration of acetaldehyde to form a gem diol, $CH_3CH(OH)_2$ (Pocker & Meany, 1965; Fridovich, 1966). An oxidation mechanism for acetaldehyde involving the removal of a hydride ion and a proton or a pair of hydrogen atoms from the gem diol can be envisaged, but it has been shown that aldehyde oxidase and xanthine oxidase are specific for non-hydrated acetaldehyde (Handler et al, 1964; Fridovich, 1966).

PHARMACOLOGY OF ACETALDEHYDE

Toxicity: It is generally agreed that acetaldehyde is more toxic than ethanol; the level of acetaldehyde in mammalian serum reported to cause death of 50% of the animals is 5-12mM(24-56mg%)(Stotz et al, 1944; Asmussen et al, 1948; Westerfeld, 1955) whereas ethanol is usually considered to be lethal at about 100mM(440mg%)(Dilts, 1970). Death results from respiratory arrest (Supniewski, 1926). In alcoholics the acetaldehyde: ethanol ratio in blood is lower than that found in moderate drinkers, and the figure for moderate drinkers is itself lower than that for normal abstainers (Westerfeld, 1955). These findings suggest that acetaldehyde tolerance may be significant in ethanol addiction.

Sympathomimetic Reaction to Acetaldehyde: The sympathomimetic reaction in mammals is probably the most studied pharmacological property of acetaldehyde. It is caused by acetaldehyde induced release of epinephrine and norepinephrine from the adrenal medulla (Perman, 1962a; Mendelson et al, 1969; Ogata et al, 1971) and from peripheral catecholamine stores probably in the adrenergic nerve endings (Kumar & Sheth, 1962; Akabane et al, 1964a; Møller, 1971a; Møller, 1971b). This release does not require nervous intervention (Akabane et al, 1964d). Serotonin secretion by the adrenal medulla is also stimulated by acetaldehyde (Duritz & Truitt, 1963; Duritz & Truitt, 1966).

Elevated blood catecholamine levels resulting from acetaldehyde stimulated release are maintained by acetaldehyde inhibition of brain and liver monoamine oxidase (Towne, 1964; Lahti, 1968; Møller, 1971b). This enzyme catalyzes the initial step in catecholamine degradation.

Adrenal Cortical Response to Acetaldehyde: Acetaldehyde and ethanol both cause a decrease in the cholesterol content in the adrenal gland, and an increase in its weight, seeming to indicate modified secretory function of the adrenal cortex (Akabane et al, 1964c). The resulting elevated blood cortisol levels may cause the hyperglycaemic response to alcohol that is sometimes observed (Perman, 1962b; Akabane, 1970). Aldosterone synthesis seems decreased (Saruta et al, 1971) and this could explain the altered sodium and chloride excretion observed under alcohol stress (Akabane et al, 1964c).

Acetaldehyde Effect on Mitochondrial Respiration: Acetaldehyde is reported to act as a potent inhibitor of mitochondrial respiration (Wingard & Teague, 1958; Perman, 1962b) though the mechanism for this action is under dispute. Pyruvate metabolism appears to be impaired, either by direct inhibition by the acetaldehyde (Kiessling, 1962) or because of the NAD^+ depletion caused by acetaldehyde oxidation (Beer & Quastel, 1958; Rehak & Truitt, 1958; Truitt & Duritz, 1967).

OUTLINE OF THE WORK PRESENTED IN THIS THESIS

The object of the investigation was to define in more detail the metabolism of ethanol and acetaldehyde in mammals. This metabolism was studied at three levels: the enzymatic level, in the liver homogenate system and under in vivo conditions. Although a large amount of scientific information is available concerning the metabolism of ethanol in mammals, the metabolism of acetaldehyde has been neglected, by comparison. Any studies of this ethanol oxidation product should be carried out in conjunction with studies on ethanol utilisation.

The sheep was chosen as the experimental animal as it was considered a suitable species for the type of experimental work envisaged. Sheep are ruminants but absorption and metabolism of ethanol from the gastro-intestinal tract are similar to nonruminants (Leroy, 1961). Two species of rumen bacteria can form ethanol (Bryant, 1959) so that it can occur in concentrations of 11-13mM in the rumen when fed a readily fermentable carbohydrate diet (Allison et al, 1964). Normally it occurs in concentrations around 0.1mM in the bovine rumen, less than in the contents of the rat stomach (Krebs & Perkins, 1970). Sheep and bovine rumen flora are essentially similar (Hungate, 1966). Intraruminal ethanol oxidation proceeds to acetic acid. It reaches equilibrium when

10-25% of the ethanol has been metabolised (Moomaw & Hungate, 1963).

In this study particular emphasis has been placed on the properties of the sheep aldehyde oxidase enzyme and its involvement in acetaldehyde oxidation. Further, the effect of some steroid hormones on both the aldehyde oxidase enzyme and on overall ethanol and acetaldehyde metabolism has been investigated.

Various drugs used as deterrents in the treatment of alcoholism have been studied in the sheep as they affect acetaldehyde metabolism and should give some information on the similarities between the ruminant and the nonruminant in this field. The interaction between these drugs and others has been previously reported (MacCallum, 1969) and is studied in some detail.

Investigation of the way chronic ingestion of low doses of ethanol can influence sheep aldehyde oxidase and general acetaldehyde metabolism may help to assess the effect that ethanol as a foodstuff can have on the pathway for alcohol oxidation.

Chapter 2

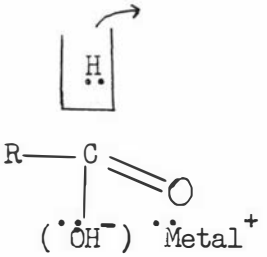
ALDEHYDE OXIDASE

Aldehyde oxidase was first described and partially purified from pig liver in 1940 (Gordon et al, 1940). It was distinguished from the previously known xanthine oxidase (Corran et al, 1939) by its inability to catalyze purine oxidation at pH 7.0. Xanthine oxidase can catalyze both aldehyde and purine oxidations and is physically similar to the aldehyde oxidase enzyme.

The aldehyde oxidase in rabbit liver was described in 1946 (Knox, 1946). It differed from the pig-liver enzyme in its ability to catalyze quinine and N^1-methyl nicotinamide oxidation. It has a higher pH_{max} for aldehydes and it can catalyze some xanthine oxidation at pH 7.0 (Rajagopalan & Handler, 1964c). This rabbit aldehyde oxidase has been highly purified (Rajagopalan et al, 1962) and both its physical and kinetic properties have been described in detail.

Pig and rabbit aldehyde oxidases and the xanthine oxidases from milk and liver have several physical features in common. They are all flavoproteins with flavin adenine dinucleotide as the prosthetic group. (One aldehyde oxidase has been described, from horse liver, which is not a flavoprotein (Järnefelt, 1955)). All contain molybdenum as a metallic electron acceptor, and have molecular weights in the range 200,000 - 300,000. These enzymes all have "oxidase" - coupled reduction of molecular oxygen - and "dehydrogenase" - coupled reduction of other electron acceptors - actions. The pig aldehyde oxidase differs from the others in its lack of non-haem iron as an additional prosthetic group (Igo et al, 1961).

Xanthine oxidase and aldehyde oxidase are specific for non-hydrated acetaldehyde (Handler et al, 1964; Fridovich, 1966) and oxidation proceeds by firstly the removal of a hydride ion and then a subsequent replacement with a hydroxide ion from the medium:



(Handler et al, 1964)

Aldehyde oxidase has been postulated to contain an internal electron transport system analogous to that in the respiratory chain (Rajagopalan & Handler, 1964b). Molybdenum with a covalently-bound sulphydryl

group, acts at the substrate binding site as the hydride ion acceptor (Rajagopalan & Handler, 1964c); the molybdenum accepts electrons and becomes reduced to Mo^{+5} state (Aleman et al, 1965). Molybdenum is also said to react in the substrate hydroxylation step (Fridovich, 1962). It seems that sulphur does not play a direct part in the electron transport system (Mahler, 1956).

Inhibitor studies, using the various hydrogen acceptors which can couple with aldehyde oxidase, have led to the description of a sequence in which other prosthetic groups accept electrons from molybdenum within the enzyme molecule (Handler et al, 1964; Rajagopalan & Handler, 1964b).

$$\text{Substrate} \dashrightarrow \underset{\text{-S-S-}}{Mo^{+6}} \dashrightarrow \text{FAD} \dashrightarrow \text{CoQ} \dashrightarrow \text{Fe} \dashrightarrow O_2$$

The order is essentially the same as that described by Bray, Palmer, Beinert & Ehrenberg (Bray et al, 1964; Ehrenberg & Bray, 1965) for xanthine oxidase except that CoQ is added in the sequence. CoQ is not present in xanthine oxidase but is reported to play an active role as a prosthetic group in aldehyde oxidase. It acts as an electron acceptor and is probably responsible for the large peak in the difference absorption spectrum (oxidised-reduced) of the purified enzyme at 275 nm (Rajagopalan et al, 1962).

In an attempt to determine the contribution which aldehyde oxidase makes to the enzymic oxidation of acetaldehyde during ethanol metabolism in sheep, it was decided to firstly purify and characterise the sheep liver enzyme. This chapter describes the purification procedure developed and the general properties of the isolated enzyme. It also includes a description of the preparation and properties of an antibody specific to the enzyme. The antibody acts as a specific inhibitor of sheep liver aldehyde oxidase and can be used in in vitro studies with crude liver homogenates to inactivate aldehyde oxidase and determine the effect this has on overall ethanol and acetaldehyde metabolism.

METHODS AND MATERIALS

I) ENZYME ASSAYS WITH VARIOUS HYDROGEN ACCEPTORS:

Aldehyde oxidase couples with a number of hydrogen acceptors (Rajagopalan & Handler, 1964b). Assays for its activity have been based on several of these. Inhibitors and activators were incubated with the enzyme for five minutes at the reaction temperature unless otherwise stated. Activators increased the rate of substrate oxidation in the

system, but except for molybdenum, were not essential to observe oxidase activity.

Methylene Blue: In most cases aldehyde oxidase was assayed by coupling with methylene blue. The assay system was incubated in evacuated Thunberg tubes flushed with oxygen-free nitrogen. Components of the reaction mixture are given in Table 3. Reactions were started by adding both the substrate and methylene blue to the temperature-equilibrated mixture from a side arm, and the time taken for methylene blue to be completely decolorised at 37°C was recorded. Coupling of methylene blue reduction with acetaldehyde oxidation can be catalyzed by both aldehyde oxidase and xanthine oxidase enzymes. Concurrent assay for xanthine oxidase using hypoxanthine (5.2mM) as substrate made it possible to estimate the xanthine oxidase contribution.

Bovine liver xanthine oxidase catalyzed the oxidation of acetaldehyde at 28% of the rate of hypoxanthine (Corran et al, 1939).

One unit of enzyme is defined as that amount required to reduce 1 µg methylene blue in one minute.

The aldehyde oxidase reaction could be assayed more accurately by following methylene blue reduction in a spectrophotometer. Special tubes, which could be evacuated, were used and the reaction mixture was the same as that described in Table 3. Substrate and methylene blue were added from a side arm to start the reaction and the rate of change in the optical density at 660 nm was measured using a Bausch and Lomb spectrophotometer. The reaction system was maintained at 22°C. A standard curve for measuring the oxidised methylene blue concentration was prepared by reducing oxidised methylene blue with a small amount of solid sodium dithionite. The decrease in optical density at 660 nm was recorded for several methylene blue concentrations. (Fig.1).

"Dehydrogenase" activity was measured using this methylene blue assay.

Oxygen: Aldehyde oxidase activity can be measured manometrically in Warburg manometers. The reaction mixture, as described in Table 3, was equilibrated at 37°C and substrate was added from a side arm to start the reaction. Results of this assay have been quoted in terms of oxygen uptake (µl/min).

"Oxidase" activity was measured by this method. At pH 8.5, one 'methylene blue' unit of aldehyde oxidase was calculated to catalyze the uptake of 0.15 µl O_2/min under standard assay conditions.

TABLE 3 Composition of the Assay Mixtures used in the Determination of Aldehyde Oxidase Activity

Hydrogen Acceptor:	
Methylene Blue:	(cm^3)
substrate[a]	1.0
methylene blue (0.05 g/ cm^3)	0.2
enzyme solution (40 -800 units/cm^3)	1.0
buffer (borate, pH 8.5)	0.5
molybdenum trioxide (10^{-3}M)	0.5
water/inhibitor/activator	1.0

Hydrogen Acceptor:	
Oxygen:	(cm^3)
substrate[a]	0.5
enzyme solution(40 -800 units/cm^3)	0.5
buffer (borate, pH 8.5)[b]	0.5
molybdenum trioxide(10^{-3}M)	0.5
water/inhibitor/activator	1.0
water	1.0

2,6-Dichlorophenol Indophenol:	
substrate[a]	0.5
enzyme solution (40 -800 units/cm^3)	0.5
buffer (borate, pH 8.5)[b]	0.5
molybdenum trioxide(10^{-3}M)	0.5
water/inhibitor/activator	1.0
2,6-dichlorophenol indophenol (0.03%)	0.3

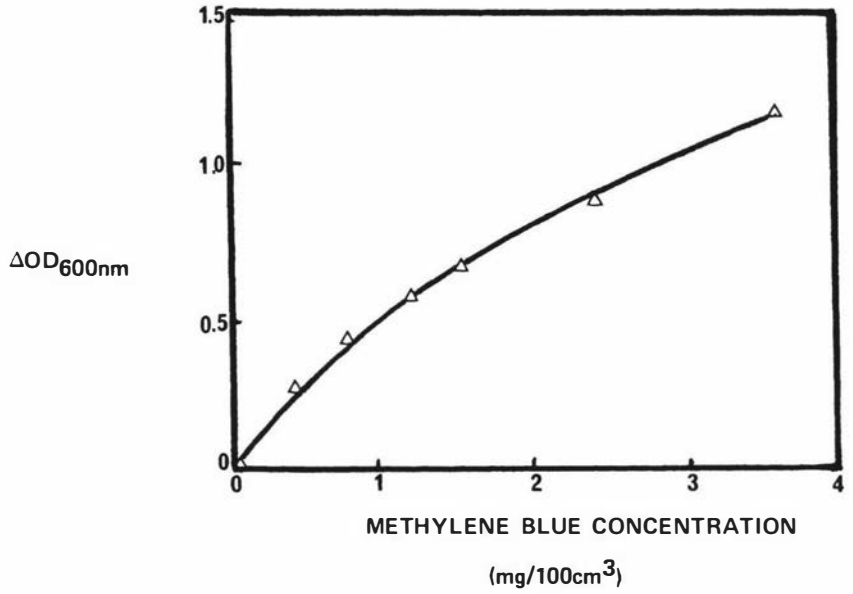
Cytochrome c:	
substrate[a]	0.5
enzyme solution(40 -800 units/cm^3)	0.5
buffer (borate, pH 8.5)[b]	0.4
molybdenum trioxide (10^{-3}M)	0.5
water/inhibitor/activator	1.0
cytochrome c($3x10^{-5}$M)	1.5

Ferricyanide:	
substrate[a]	0.5
enzyme solution (40 -800 units/cm^3)	0.5
buffer (borate, pH 8.5)[b]	0.4
molybdenum trioxide (10^{-3}M)	0.5
water/inhibitor/activator	1.0
potassium ferricyanide (2×10^{-3}M)	1.5

[a]Acetaldehyde (23mM) was used as substrate unless otherwise stated.

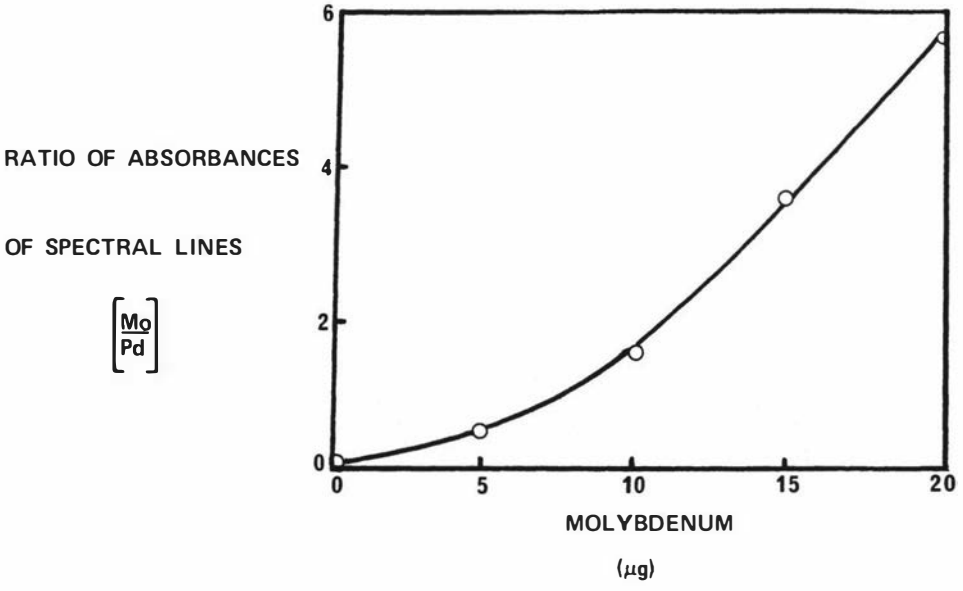
[b]Borate buffer was prepared from sodium borate and hydrochloric acid as described by Dawson et al (1969).

FIGURE 1 STANDARD CURVE FOR THE DETERMINATION OF CHANGES IN OXIDISED METHYLENE BLUE CONCENTRATION



A known concentration of oxidised methylene blue was fully reduced with a small amount of solid sodium dithionite, and the decrease in optical density at 600nm (ΔOD_{600nm}) was recorded.

FIGURE 2 STANDARD CURVE FOR THE EMISSION SPECTROGRAPHIC ESTIMATION OF MOLYBDENUM



Samples containing a known amount of molybdenum and a standard amount of palladium were arced at 7mA. The ratio of the absorbances of known molybdenum and palladium spectral lines was determined for each sample.

2,6-Dichlorophenol Indophenol: Spectrophotometric measurement of the rate of 2,6-dichlorophenol indophenol reduction was performed in a Hitachi 101 spectrophotometer. The initial rate of change in optical density at 600 nm was measured when substrate was added to the aerobic reaction mixture shown in Table 3. The temperature was maintained at 22°C. One "methylene blue" unit of aldehyde oxidase catalyzed a change in OD_{600nm} of 0.018/min under standard assay conditions.

Cytochrome c: Cytochrome c reduction was also followed in a Hitachi 101 spectrophotometer, by measuring changes in optical density at 550 nm. The initial rate of change at 22°C when substrate was added to the reaction mixture tabulated in Table 3 was used to assess the aldehyde oxidase activity with this hydrogen acceptor. One "methylene blue" unit of aldehyde oxidase catalyzed a change in OD_{550nm} of 0.023/min under standard assay conditions.

Ferricyanide: Assays based on the rate of ferricyanide reduction catalyzed by aldehyde oxidase were carried out in a Hitachi 101 spectrophotometer by measuring the initial rate of change in optical density at 420 nm after the addition of substrate to the temperature equilibrated mixture (22°C). The reaction mixtures for this assay are also given in Table 3. One "methylene blue" unit of aldehyde oxidase catalyzed a change in OD_{420nm} of 0.013/min under standard assay conditions.

II) OTHER METHODS

Protein Estimation: Protein concentrations were estimated by the spectrophotometric method of Lowry, Rosebrough, Farr & Randall (Lowry et al, 1951). A linear relationship between protein concentration and absorbance at 500 nm was demonstrated under the test conditions using bovine serum albumin solutions. An increase in OD_{500nm} of 0.81 corresponded to a protein concentration of 1 mg/cm^3.

Purity and Molecular Weight Determinations: Tests of homogeneity of the aldehyde oxidase preparations were carried out using disc electrophoretic separation methods with 7.5% polyacrylamide gel (Davis, 1964). Tris/glycine buffer (pH 8.3) was used as the medium and the run made at 250 volts. Normally amido black (0.1% w/v) in 1% (v/v) aqueous acetic acid was used to stain the gel for protein after separation.

The Spinco Model E analytical ultracentrifuge fitted with an AnR-D rotor was used for the more precise purity estimates and the determination of sedimentation coefficients and molecular weights.

Absorption Spectra: Absorption spectra of the purified enzyme preparations were obtained from a Unicam S.P.800 recording spectrophotometer. Cells of width 0.2 cm and a light path of 1.0 cm were used. A small amount of solid dithionite was added as a reducing agent when necessary.

Emission Spectrography: The molybdenum content of samples from dried aldehyde oxidase preparations was determined by emission spectrography, using palladium as an internal standard. Arcing was carried out at 7 mA and the required lines were located relative to an iron emission spectrograph on the same plate. A standard curve of the ratios of the absorbances of the molybdenum and palladium lines at known molybdenum levels (Fig.2) allowed calculation of the molybdenum present in each protein sample.

Preparation of Calcium Phosphate Gel: Calcium phosphate gel was prepared by mixing calcium chloride with trisodium phosphate as described by Dixon & Webb (1964). It was aged in the dark for four weeks and used at a concentration of 6.62 mg/cm^3 (DM)(in glass distilled water).

RESULTS:

I) ENZYME PURIFICATION: SHEEP LIVER ALDEHYDE OXIDASE: The results from a typical purification run are tabulated in Table 4. Fresh sheep liver (obtained less than 1.5 h from slaughter) was used for enzyme isolations after the finding that the storage of intact liver in the deep freeze (-20°C) for 4 days caused a considerable reduction in activity (82%).

Sheep liver (634 g) was homogenised in chilled distilled water (950 cm^3) for two 1 minute periods. The homogenate was centrifuged (14,600g, 0°C, 60 min) and the supernatant was filtered through glass wool to remove lipid material giving a filtrate with a pH of 6.4. The specific activity of the supernatant varied from animal to animal over a range of 0.05 to 5.00 units/mg protein. Only 2.5% of the aldehyde oxidase activity was discarded in the residue.

Freezing of the liver extract supernatant (-20°C, 8 h) resulted in 35% loss of activity. By contrast, the enzyme was quite stable in solution at 4°C for the same time. The extract lost 50% of the enzyme activity when stored at +20°C for 8 hours.

The next step in the purification procedure was an acidification by adding 1M HCl dropwise, reducing the pH to 5.5. This solution was kept at 4°C for fifteen minutes and centrifuged (14,600g, 0°C, 30min). All the activity remained in the supernatant with only 60% of the protein. Below pH 5.2 the aldehyde oxidase was precipitated and inactivated.

Without further adjusting the pH, solid ammonium sulphate was added to the supernatant at 0°C to 30% saturation. Stirring was continued in the cold for thirty minutes and the solution then centrifuged (14,600g, 0°C, 20 min). The concentration of ammonium sulphate was then raised in

TABLE 4 Purification Procedure for Sheep Liver Aldehyde Oxidase

Step (Fraction)	Volume (cm^3)	Activity[a] (units/cm^3)	Protein (mg/cm^3)	Yield (%)	Specific Activity (units/mg)	Purification (x)
Original supernatant	485	117 (15.7)	101.2	100	1.15	1
Precipitation at pH 5.5	138	445 (10)	21.1	108	21.1	18.3
Ammonium sulphate fractionation 30%-45% saturated (dialysed)	128	772 (6.1)	21.1	174	36.8	32.0
Treatment with calcium phosphate gel : Gel wash (0.05M-0.2M)	98	109 (0)	3.04	21.6	35.9	31.2
Alkaline ammonium sulphate fractionation (30-40% saturated)	17.1	245 (0)	4.15	16.8	59	51.4

Weight of liver used to prepare homogenate : 634 g.

The final precipitate was dissolved in 0.05M phosphate buffer, pH 8.1

[a]Corresponding xanthine oxidase activities are given in brackets.

the supernatant with stirring, to 45% saturation.

After centrifuging, the precipitate was taken up in 128 cm^3 of a solution of molybdenum trioxide (10^{-3}M).

Although some aldehyde activity remained in solution up to 60% saturation with ammonium sulphate, the higher salt concentration precipitated a considerable amount of the xanthine oxidase activity. Enzyme preparations intended for kinetic work were prepared from the fraction precipitating between thirty and forty per cent saturation with ammonium sulphate to keep xanthine oxidase activity at a low level.

The ammonium sulphate fractionation gave considerable activation of the oxidase but this disappeared during the next calcium phosphate step. Rajagopalan & Handler (1964c) have reported some increase in aldehyde oxidase activity in the presence of quaternary nitrogen but no explanation was given for this effect.

The active enzyme solution prepared as described above was dialysed against aqueous molybdenum trioxide solution (10^{-6}M) overnight (4°C) and again centrifuged (23,500g, 0°C, 20 min).

Calcium phosphate gel solution (1494 mg calcium phosphate: 2710 mg protein) was mixed with the clear brown dialysate and the solution left to stand (4°C, 45 min). It was then centrifuged (550g, 5 min), the supernatant discarded and the precipitate washed eight timeswith 0.05M phosphate buffer (pH 6.5). The washings were discarded. The gel was then washed twice with 0.2M phosphate buffer (pH 6.5); these washings contained 21.6% of the original activity. A step similar to this had been used in the purification of rabbit aldehyde oxidase described by Rajagopalan & others(1962).The low yield of activity was offset by the complete separation of xanthine oxidase activity from the aldehyde oxidase (Table 4).

In the next step, the enzyme solution was raised to pH 10.5 with 4M NH_4OH and immediately taken to 30% saturation with solid ammonium sulphate. After standing at 4°C for 30 minutes, the mixture was centrifuged (23,500g, 0°C, 10 min). The supernatant was adjusted to 40% saturation with ammonium sulphate and the resulting precipitate, obtained by centrifugation, was dissolved in 17 cm^3 0.05M phosphate buffer (pH 8.1). At this stage the enzyme solution contained between 50 and 150 times the specific activity of the aldehyde oxidase in the original supernatant. In the best preparation, the specific activity was 111 units/mg protein but this was stable for only one day at 4°C; it subsequently decreased to 12-13 units/mg protein and was stable at that level for 4-7 days at the same temperature.

II) PHYSICAL PROPERTIES: SHEEP LIVER ALDEHYDE OXIDASE: Polyacrylamide gel electrophoresis of the purified protein solution (specific activity equivalent to 111 units/mg protein) showed a single major protein band with one minor slower moving component (Fig.3). This could have been an active dimer of the enzyme similar to those reported in xanthine oxidase preparations (Nelson & Handler, 1968). The following method was developed to locate the aldehyde oxidase and xanthine oxidase activities in the gel.

After electrophoresis, the gel was removed from its tube and immersed in the following solution in a Thunberg tube:

methylene blue (0.05g/100 cm^3)	0.3 cm^3
buffer (borate, pH 8.5)	0.5 cm^3
molybdenum trioxide (10^{-3}M)	0.5 cm^3
water	7.0 cm^3

Substrate (1.0 cm^3, acetaldehyde 23mM and/or hypoxanthine 5.2mM) was placed in the side-arm of the Thunberg tube.

After repeated evacuation and flushing with oxygen-free nitrogen, the substrate was tipped into the solution in which the gel was immersed. The system was incubated for one hour until a white line of reduced methylene blue was distinguishable from the blue oxidised compound which had diffused through the gel (Fig.4). White lines indicated the sites of enzyme activity in the gel.

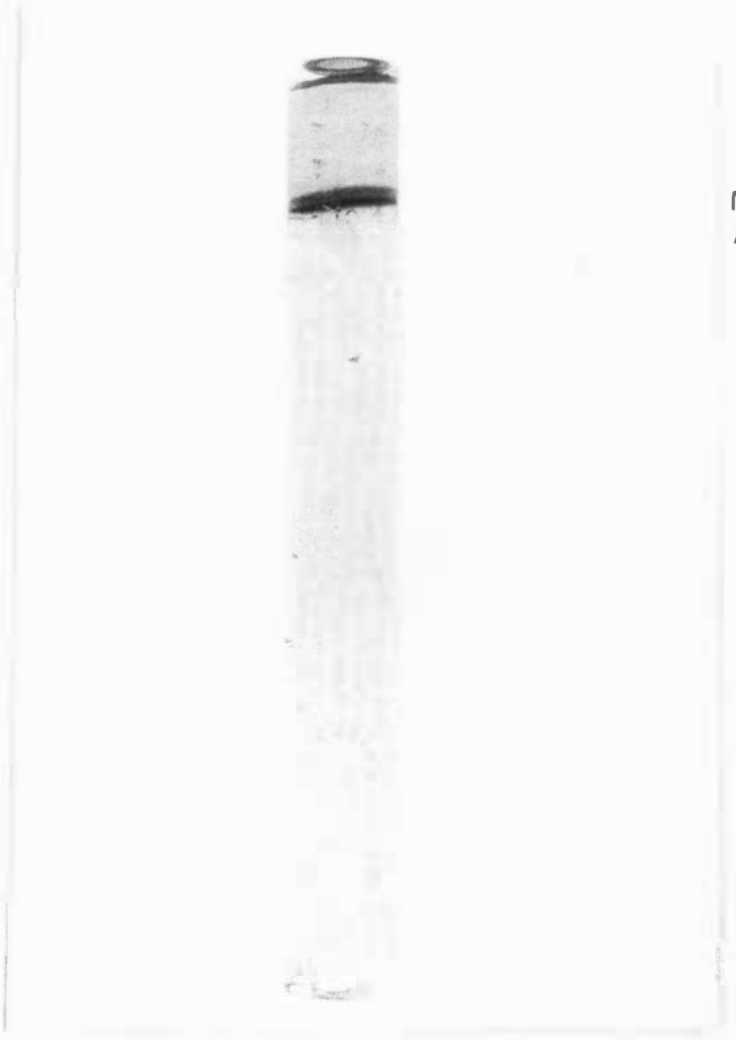
The fluid was removed from the Thunberg tube on a water-pump, taking care not to release the vacuum, and the gel was left in the tube. The white activity line was stable in the gel for about four hours depending on the amount of enzyme activity present. It was not possible to take satisfactory photographs because of the requirement to keep the gel in the Thunberg tube under vacuum.

The R_f values for aldehyde oxidase and xanthine oxidase, obtained by this method, were 0.18 and 0.34 respectively when electrophoresis was performed in tris/glycine buffer, pH 8.3, at 250 volts.

Homogeneity of the protein preparations were determined by ultracentrifugal analyses. In a typical run it was found that the monomeric form of the enzyme constituted 85% of the total protein in solution and confirmed the presence of a minor component of higher molecular weight (Fig.5).

The sedimentation coefficient of the protein, determined using the boundary method, was 12.2 Svedbergs at 19.5°C in 0.05M phosphate buffer,

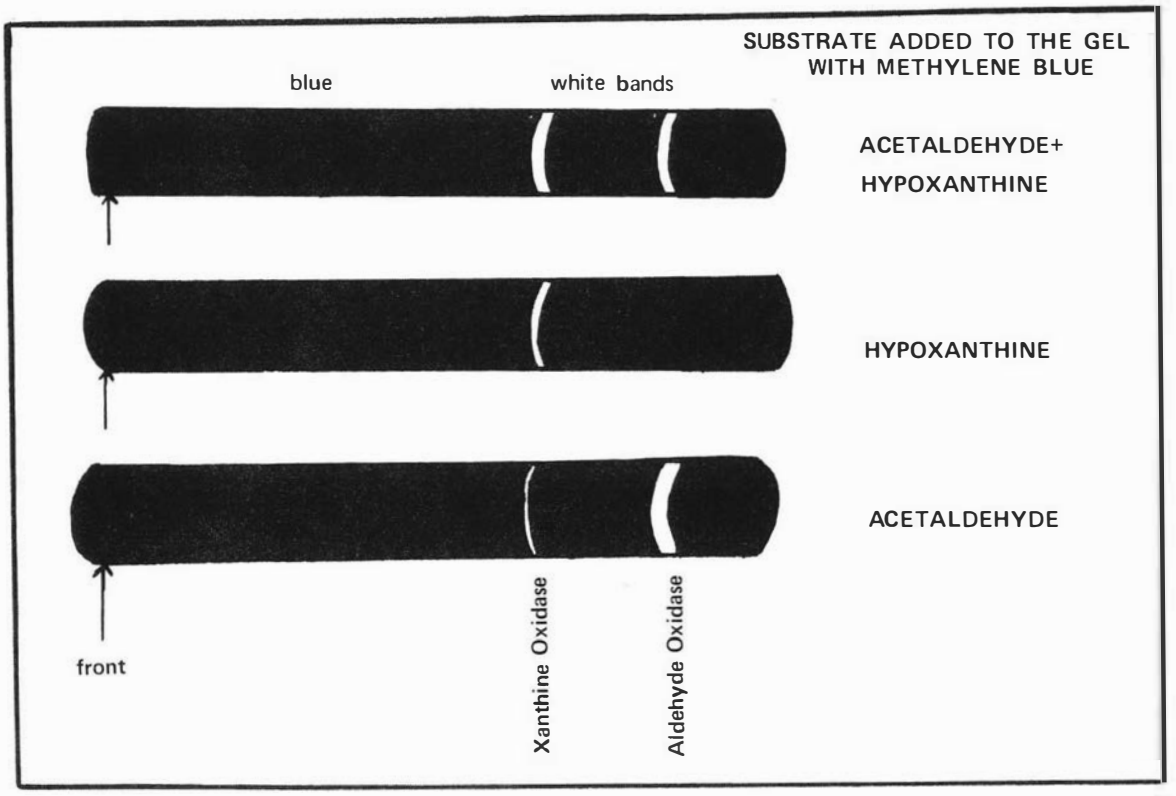
FIGURE 3 ELECTROPHORESIS OF A PURIFIED SHEEP LIVER ALDEHYDE OXIDASE PREPARATION



The enzyme preparation had a specific activity equivalent to 12.2 units/mg protein and a protein concentration of 12.4 mg/cm^3.

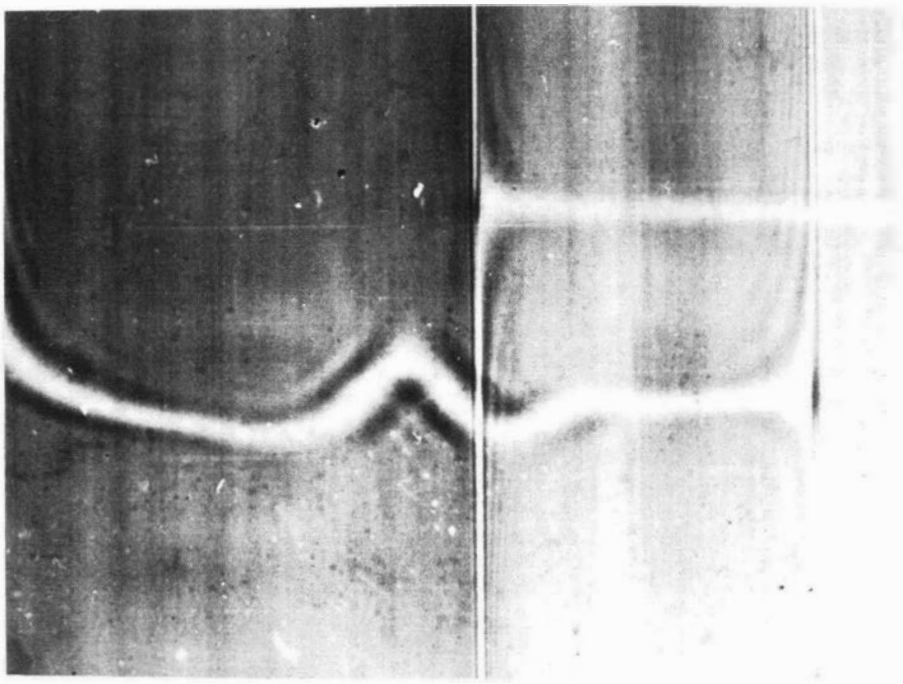
The proteins in the sample (5μl) were separated in 7.5% polyacrylamide gel by electrophoresis in Tris/glycine buffer, pH 8.3, at 250 volts. Proteins were then located in the gel with Amido Black stain (0.1% in 1% acetic acid).

FIGURE 4 DIAGRAMS TO SHOW THE PATTERNS OBTAINED WHEN SHEEP LIVER ALDEHYDE OXIDASE AND XANTHINE OXIDASE WERE SEPARATED BY GEL ELECTROPHORESIS AND STAINED WITH A METHYLENE BLUE ACTIVITY STAIN



Substrate and methylene blue were added to the gel in pH8.5 buffer after the electrophoresis had been performed. The system was incubated anaerobically at 37^{o}C for two hours, and the staining solution was then removed under vacuum. White bands were evident in the blue gel, showing where methylene blue reduction had occurred.

FIGURE 5 SEDIMENTATION PATTERN OF A PURIFIED SHEEP LIVER ALDEHYDE OXIDASE PREPARATION (12.4 mg/cm^3 PROTEIN IN 0.05M PHOSPHATE BUFFER) IN THE ANALYTICAL ULTRACENTRIFUGE



This photograph was taken 48 minutes after attainment of maximum speed (52,640 r.p.m) at 15.5°C.

pH 8.1 (Protein conc.= 12.4mg/cm^3). This value with corrections gave the following figure:-

$$s_{20,w} = 12.5 \text{ S.}$$

A synthetic boundary cell was used to determine the diffusion coefficient of the protein at known times, during an ultracentrifuge run. From these, the diffusion coefficient at zero time was calculated to be $0.5 \times 10^{-6} m^2/sec$.

The molecular weight of sheep liver aldehyde oxidase was estimated by substitution in the formula:

$$M = \frac{R \ T \ s}{D(1 - \bar{v}p)}$$

$= 214{,}000$ Dalton

$R = 8.314 \times 10^7$ erg/mole/K
$T = 292.5$ K
$s = 12.21 \times 10^{-13}$ sec
$D = 5 \times 10^{-7} m^2/sec$
$\bar{v} = 0.74$ (Nelson & Handler, 1968)
$p = 0.98$

Estimation of the molybdenum and flavin concentrations in the purified enzyme preparation gave a molybdenum:flavin ratio of 1:2 (Table 5).

From these figures it was calculated that one gram mole of molybdenum would be present in 211,000g protein, which agreed closely with the molecular weight previously determined.

Absorption spectra of the oxidised and reduced forms of the purified enzyme are shown in Figure 6. Excess acetaldehyde changed the spectrum of the oxidised enzyme to that obtained for the dithionite-reduced protein. It would seem therefore that complete reduction of enzyme occurred in the presence of substrate, as demonstrated with the rabbit aldehyde oxidase (Handler et al, 1964). The flavin in xanthine oxidase is only partially reduced by substrate initially (Mahler, 1956).

The difference spectrum (oxidised-reduced) of the protein showed the peaks typical of flavoproteins at 350 nm and 450 nm. A large maximum observed at 275 nm suggested the presence of CoQ in the enzyme. A similar conclusion has been suggested for the rabbit liver aldehyde oxidase by Rajagopalan and his coworkers (1962).

III) KINETIC PROPERTIES: SHEEP LIVER ALDEHYDE OXIDASE:

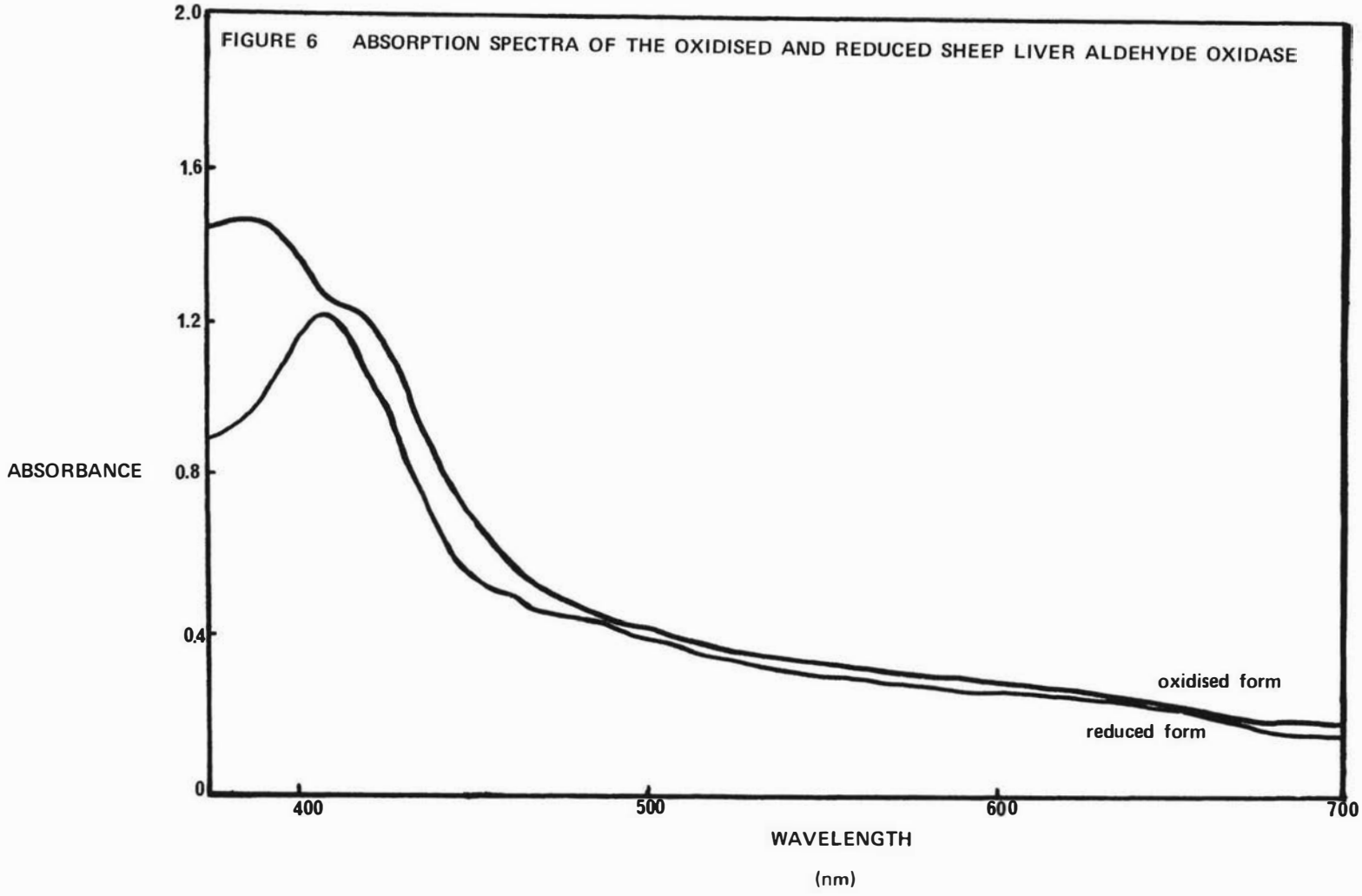
pH Optimum: The optimum pH for sheep liver aldehyde oxidase activity was pH 8.6 and was the same for both the "dehydrogenase" and "oxidase" activities (Fig.7). Rabbit aldehyde oxidase preparations showed a similar pH_{max} (Knox, 1946), but apparently some variations occur

TABLE 5 Estimation of the Molybdenum and Flavin Components of Purified Aldehyde Oxidase Preparations

Preparation Number	Specific Activity (units/mg protein)	Protein Concentration (mg/cm^3)	Molybdenum[a] Concentration^{-1} (g protein/ g.mole molybdenum)	Flavin[b] Concentration^{-1} (g protein/ g.mole flavin)
1	108	7.14	2×10^5	0.96×10^5
2	111	13.9	2.14×10^5	1.04×10^5
3	12.2	12.4	2.18×10^5	1.08×10^5

a) Molybdenum was determined in an emission spectrograph on oven-dried samples (24 h, 60°C) using palladium as an internal standard.

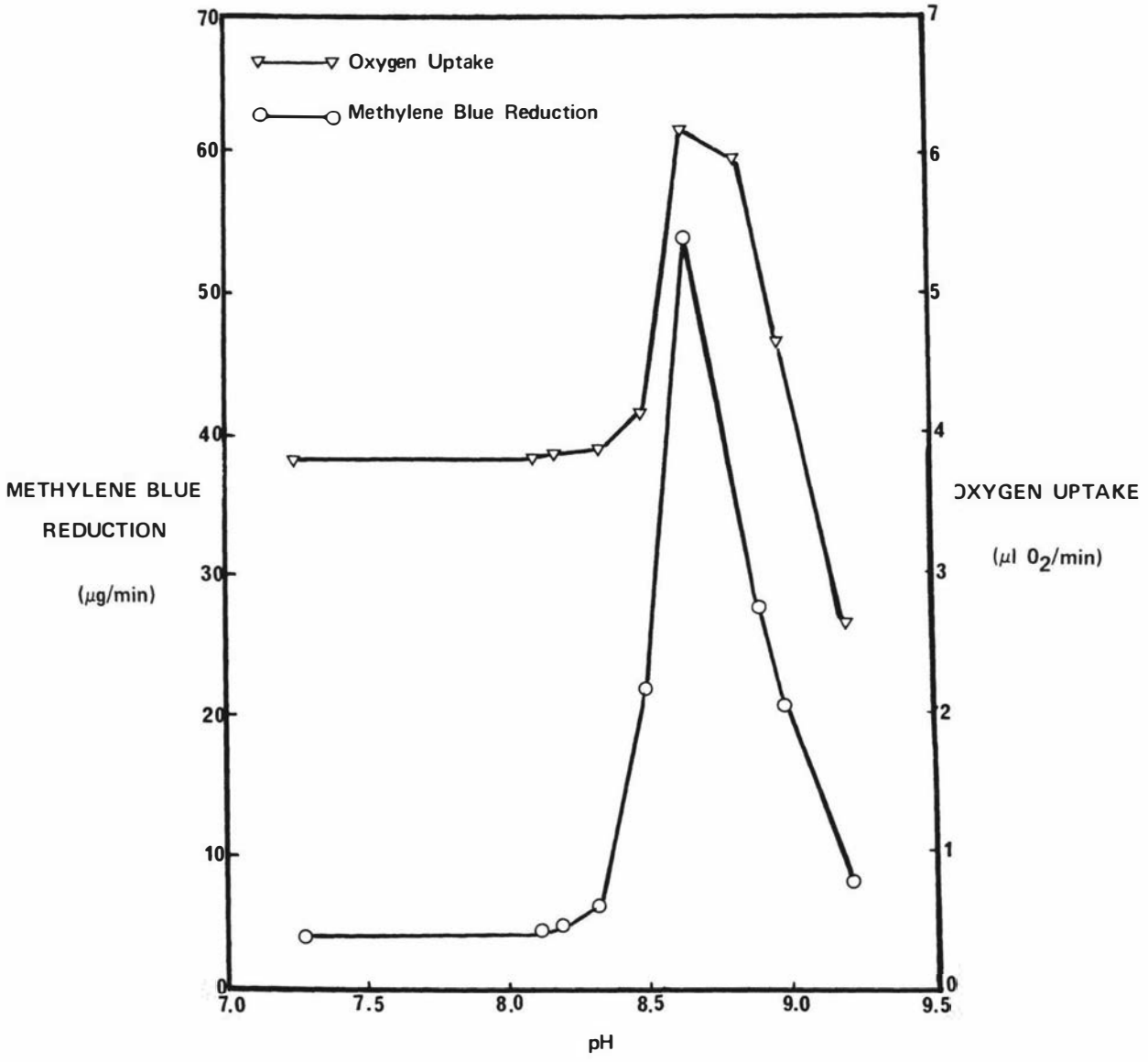
b) Flavin was estimated from the difference (oxidised-reduced) in the optical density of the solution at 450 nm. A molar extinction coefficient of 1.13×10^4 was assumed (Rajagopalan & Handler, 1964c).



Protein was dissolved in 0.05M phosphate buffer, pH 8.1, to give a concentration of 12.4 mg/cm^3. The specific activity of the solution was 12.2 in the example shown.

Excess acetaldehyde (23mM) or a small amount of solid dithionite was added to reduce the enzyme in solution. Both resulted in the same changes in the absorption spectrum.

FIGURE 7 DETERMINATION OF THE OPTIMUM pH FOR SHEEP LIVER ALDEHYDE OXIDASE ACTIVITY USING ACETALDEHYDE AS SUBSTRATE



"Dehydrogenase" activity was assayed using methylene blue reduction rate as described in the text. The assay mixture contained 51 units of aldehyde oxidase activity.

"Oxidase" activity was assayed by measuring the rate of oxygen uptake in Warburg manometers as described in the text. The assay mixture contained 41 units of aldehyde oxidase activity.

Acetaldehyde (23mM) was used as the substrate, and reactions were carried out at 37°C.

depending on the substrate used (Knox, 1946; Rajagopalan & Handler, 1964c).

Specificity: Various compounds were tested as substratesfor the sheep liver aldehyde oxidase (Table 6). The enzyme can reduce several electron acceptors (Gordon et al, 1940; Rajagopalan & Handler, 1964b) and the oxidation rate of each substrate depended on the assay system used.

Both aromatic and aliphatic aldehydes were oxidised, including formaldehyde (gas chromatographically tested for homogeneity). Although it is possible that this activity is an adaption to the production of one-carbon compounds in the rumen (Hungate, 1966), pig aldehyde oxidase also oxidised formaldehyde (Palmer, 1963) at about the same relative rate. No report of formaldehyde oxidation by rabbit aldehyde oxidase has been found.

Sheep aldehyde oxidase resembled the rabbit enzyme in that it oxidised quinines (Knox, 1946) but it could not couple N^1-methyl nicotinamide oxidation to reduction of cytochrome c or molecular oxygen (Rajagopalan et al, 1962). Purines, ethanol and pyruvate were not oxidised in any of the assay systems at pH 8.5.

Michaelis Constant: The Km value of the enzyme for acetaldehyde as the substrate was 2.4mM. Both oxygen uptake and methylene blue reducing systems have similar affinities as for acetaldehyde (Fig.8), but some other substrates were oxidised more readily in either the "oxidase" (eg. formaldehyde) or "dehydrogenase" (N^1-methyl nicotinamide) assays (Table 6).

Inhibition and Activation: The compounds assayed for their effect on the various sheep liver aldehyde oxidase systems are given in Table 7. Their depended on the hydrogen acceptor used for the assay.

Quinacrine, triton X-100 and menadione inhibited sheep liver aldehyde oxidase but their relative effects differed from those observed in rabbit aldehyde oxidase depending on the electron acceptor used (Rajagopalan & Handler, 1964b). Quinacrine is a common flavoprotein inhibitor (Haas, 1944) which also affects pig liver aldehyde oxidase (Mahler et al, 1954b); triton X-100 and menadione inhibitions have been used to indicate the presence of CoQ in similar enzyme systems (Rajagopalan et al, 1962).

8-hydroxy quinoline is a metal binding agent which inhibits only the "oxidase" reaction of sheep liver aldehyde oxidase as it does in the pig enzyme (Mahler et al, 1954b)and in xanthine oxidase (Doisy et al, 1955).

TABLE 6 Specificity of Sheep Liver Aldehyde Oxidase with Various Hydrogen Acceptors

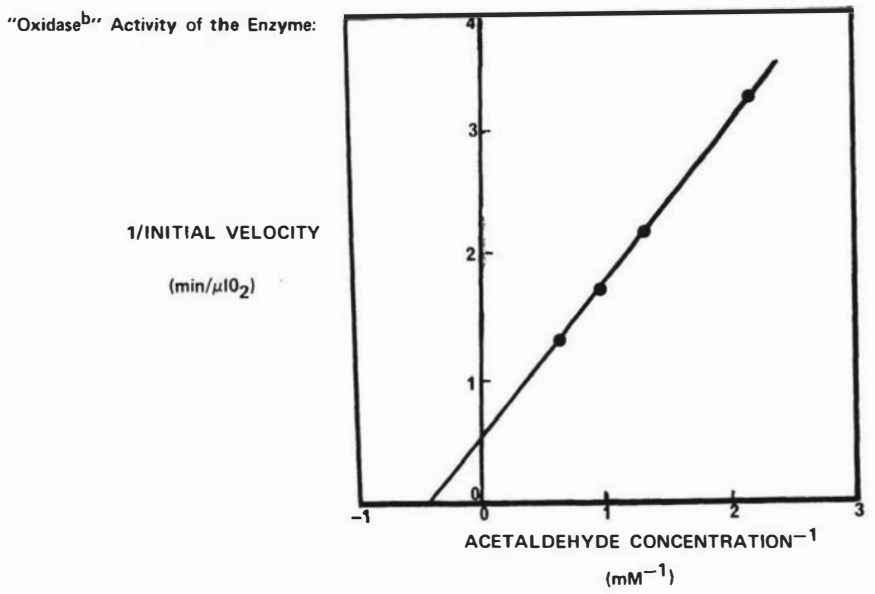
Substrate	Final Concentration (mM)	Relative Rates				
		Oxygen Uptake	Methylene Blue Reduction.	Dichlorophenol Indophenol	Cytochrome c.	Ferricyanide
Acetaldehyde	3.3	100	100	100	100	100
Formaldehyde	4.9	114	26	45	133	28
Benzaldehyde	1.7	82	31	99	75	28
N^{1}-methyl nicotinamide	6.9	0	72	81	0	17
Cinchonidine	(satd)	33	21	32	62	20
Quinine hydrochloride	0.2	58	12	52	56	24
Ethanol	3.1	0	0	0	0	0
Pyruvate	1.7	0	0	0	0	0
Hypoxanthine	0.7	0	0	0	0	0

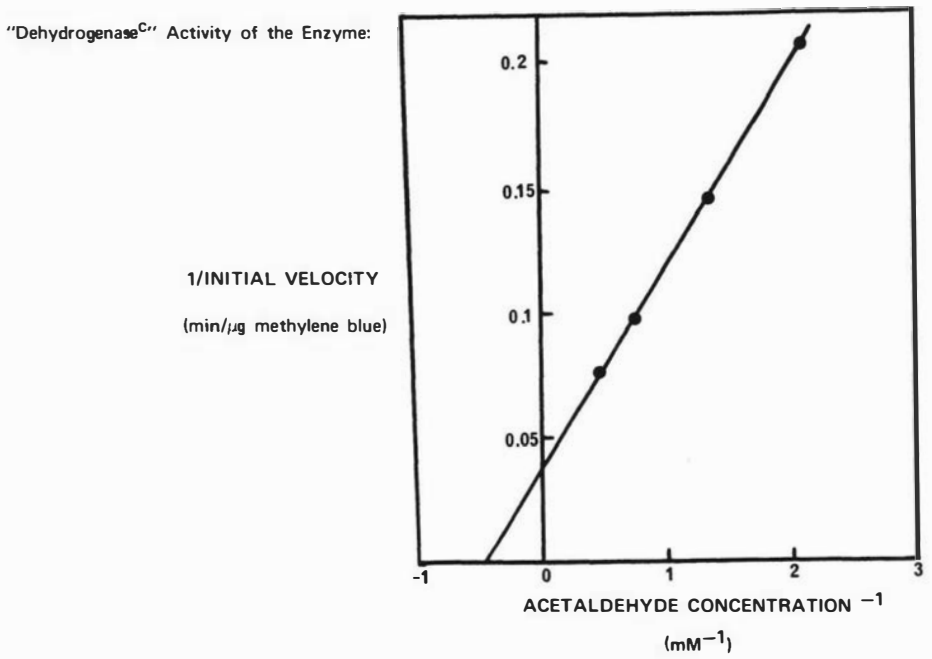
Assay systems have been described in the text. All reactions were carried out at pH 8.5.

The oxidation rate of each substrate has been compared with the rate of acetaldehyde oxidation, arbitarily placed at 100, for each assay system. In each assay 88 units of enzyme (mean specific activity = 20.2 units/mg protein) were used.

Each assay was performed in duplicate, using two different enzyme preparations, and the mean value is quoted.

FIGURE 8 DETERMINATION OF THE MICHAELIS CONSTANT[a] OF SHEEP LIVER ALDEHYDE OXIDASE FOR ACETALDEHYDE AS SUBSTRATE





a) A Lineweaver–Burke plot was used to determine the K_m of sheep liver aldehyde oxidase. Each assay was performed in duplicate with two different enzyme preparations, and the mean activities have been plotted.

b) Oxygen uptake rates were measured manometrically as described in the text. Initial acetaldehyde concentrations were assayed gas chromatographically (Chapter 3) in duplicate systems that had been temperature equilibrated with the assay flasks.

c) Methylene blue reduction was followed spectrophotometrically as described in the text. No correction was made for possible evaporation of acetaldehyde prior to starting the assay.

TABLE 7 The Effect of Several Compounds on the Rate of Reduction of Hydrogen Acceptors Catalyzed by Sheep Liver Aldehyde Oxidase

Inhibitor	Final Concentration (mM)	Relative Rates			
		Oxygen Uptake	Methylene Blue Reduction	Dichlorophenol Indophenol	Cytochrome c.	Ferricyanide
Menadione	0.07	31	0	26	38	94
Triton X-100	0.39	18	111	115	46	98
Quinacrine	2.5	33	40	NE	NE	NE
8-Hydroxy Quincline	0.09	32	91	93	19	93
Disulphiram	1.25	55	105	98	57	78
Calcium Carbimide	1.6	20	94	102	16	91
Metronidazole	0.15	50	12	67	67	32
Acetate	14.0	97	100	100	87	100
Ascorbic Acid	7.1	50	59	NE	NE	NE

Assays were carried out at pH 8.5 as described in the text, using acetaldehyde (23mM) as substrate.

The rate of oxidation of acetaldehyde alone has been arbitarily placed at 100 for each assay system. In each assay 88 units of enzyme (mean specific activity = 20.2 units/mg protein) were used.

Each assay was performed in duplicate, using two different enzyme preparations, and the mean value is quoted.

Disulphiram, calcium carbimide and metronidazole are drugs used to deter alcoholics from drinking (Jacobsen & Martenson-Larson, 1949; Ferguson, 1956; Semer, 1966). They can cause accumulation of acetaldehyde in the body following intake of ethanol. Assay of the disulphiram effect on sheep liver aldehyde oxidase activity showed that inhibition was restricted to the "oxidase" action of the enzyme. Inhibition was non-competitive, and the inhibitor constant (defined by Dixon & Webb, 1964) was 1.6×10^{-3}M. Calcium carbimide inhibition of the "oxidase" reaction of sheep aldehyde oxidase has provided an explanation for clinical accumulation of acetaldehyde observed when ethanol and calcium carbimide are taken concurrently (Kjeldgaard, 1949). Metronidazole inhibited all assay systems for sheep liver aldehyde oxidase.

No product inhibition by acetate was apparent in the presence of excess acetaldehyde. Ascorbate affected both "oxidase" and "dehydrogenase" activities. This was different from its effect on milk and liver xanthine oxidases (Doisy et al, 1955).

In view of the inhibition of rabbit aldehyde oxidase previously reported (Rajagopalan et al, 1962) several steroids were assayed for their effect on sheep liver aldehyde oxidase activity (Table 8). Little or no effect was recorded for several of the hormones investigated, but inhibition was observed in the presence of tetrahydrocortisol, oestriol and cortisone, depending on the hydrogen acceptor used. Oestradiol-17β and cortisone stimulated only the "dehydrogenase" reaction of the enzyme, and androsterone only the "oxidase" reaction. Progesterone was the only steroid investigated to stimulate both the "dehydrogenase" and "oxidase" reactions catalyzed by sheep liver aldehyde oxidase.

Ethanol in low concentrations stimulated aldehyde oxidase from sheep liver (Table 9) but no reports of similar action were found for other flavoproteins. Assaysof the rate of oxygen uptake were performed at various ethanol concentrations (Fig.9) to assess the extent of this effect. It was interesting to note the extent of the increase of enzyme activity at only 26 mM ethanol concentrations. This work has been extended in an in vivo study reported in Chapter 6.

IV) IMMUNOLOGY

Attempts to find a specific inhibitor for aldehyde oxidase, led to the preparation of the rabbit antibody to the sheep liver enzyme.

A male rabbit (New Zeeland, white) was injected with 9.8mg purified enzyme prepared from ewe liver. This had been mixed with Freund's

TABLE 8 Assay of Sheep Liver Aldehyde Oxidase using Oxygen Uptake and Methylene Blue Reduction in the Presence of Several Steroids

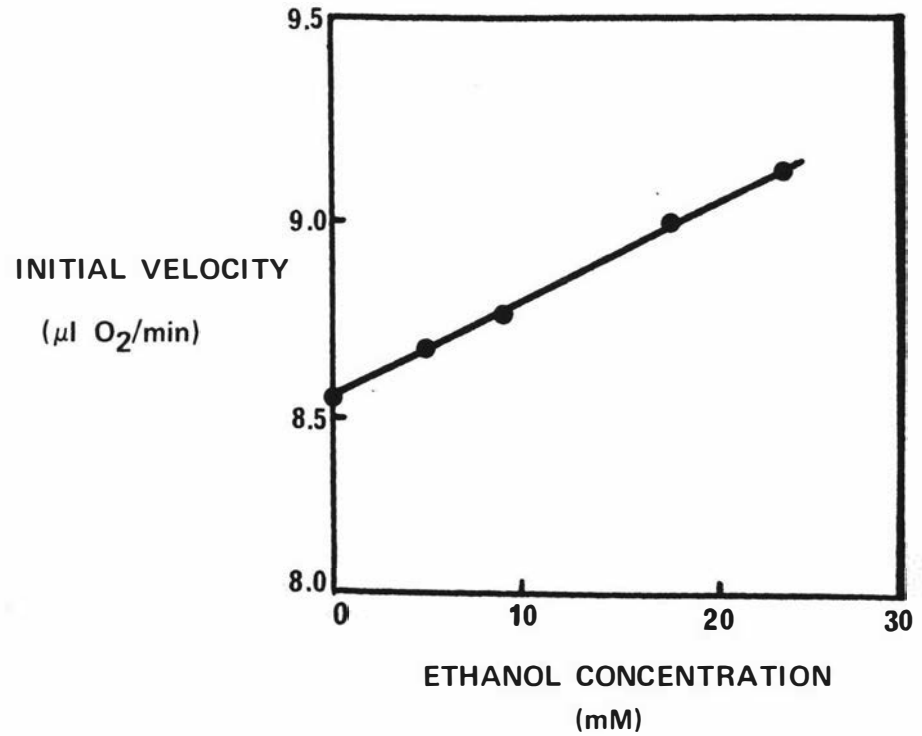
Steroid	Final Concentration (mM)	Relative Rate	
		Oxygen Uptake	Methylene Blue Reduction
-	-	100	100
Pregnanediol	0.65	100	100
Tetrahydrocortisol	0.17	64	88
Androsterone	0.14	124	100
Oestriol	1.12	87	83
Testosterone	0.82	88	100
Cortisone	0.74	63	145
Tetrahydrocortisone	0.64	89	116
Oestradiol-17β	1.75	100	189
Progesterone	0.37	160	142

Assays were carried out at 37°C and pH 8.5 as described in the text.
Acetaldehyde (23mM) was used as substrate. The rate of oxidation of acetaldehyde in the absence of steroids has been arbitarily placed at 100, for each assay system.
In each assay 54 units of enzyme (mean specific activity = 18.3 units/mg protein) were used.
Each assay was performed in duplicate, using two different enzyme preparations, and the mean value is quoted.

TABLE 9 **Effect of Ethanol on the Rate of Reduction of Several Hydrogen Acceptors catalyzed by Sheep Liver Aldehyde Oxidase[a]**

Hydrogen Acceptor	% Increase in Activity in the Presence of Ethanol (26mM)
Molecular Oxygen	112
Methylene Blue	131
Cytochrome c	111

FIGURE 9 **EFFECT OF VARIOUS ETHANOL CONCENTRATIONS ON THE RATE OF OXYGEN UPTAKE CATALYZED BY SHEEP LIVER ALDEHYDE OXIDASE[a]**



a) Assays were carried out at pH 8.5, using acetaldehyde (23mM) substrate as described in the text. Each assay was performed in duplicate with two different enzyme preparations and the mean increase in activity is quoted. Fifty-six units of aldehyde oxidase activity (mean specific activity = 20.2) were used in each assay.

complete adjuvant (1:1 by vol) and was equally divided between subcutaneous and intramuscular sites following the method developed by Newstead & Ormsby (Newstead & Ormsby, 1970). The application was repeated after seven days, and boosters containing no adjuvant were administered after twelve and sixteen weeks.

Three months after the first injection of antigen (enzyme preparation) the rabbit was bled from the ear using a suction method (Sanders, 1966). Blood (50 cm^3) was then collected every two or three weeks over a period of four months.

Serum was prepared, and fractionated by 50% saturation with solid ammonium sulphate (Clausen, 1969). The γ-globulin precipitate was collected, dissolved in 0.9% (w/v) NaCl and dialysed against cold physiological saline for three days until the $BaCl_2$ test showed no sulphate ions remained in solution. The protein concentration of the preparation was 13.9 mg/cm^3. A blank preparation similarly prepared from the blood of an uninoculated rabbit contained 18.2 mg/cm^3 protein.

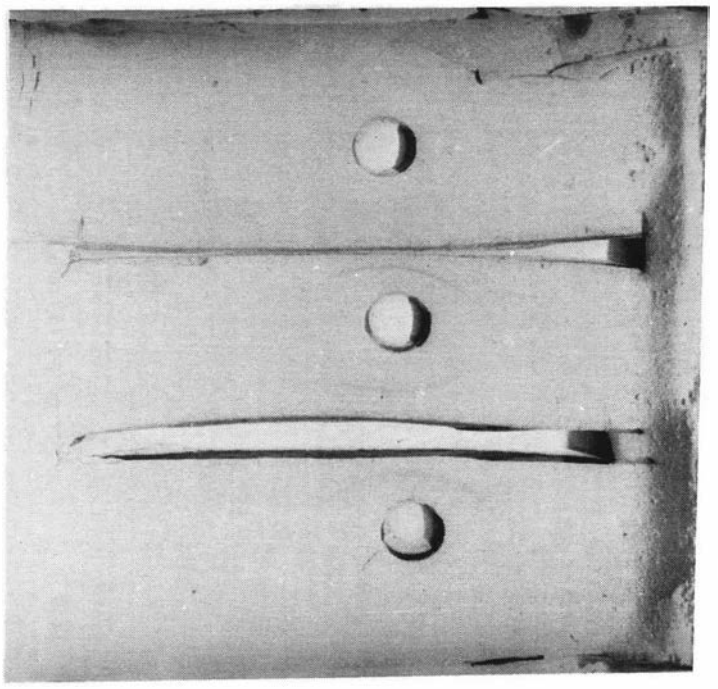
The γ-globulin preparations required immunochemical and enzyme kinetic definition of their specificity.

Electrophoresis of aldehyde oxidase preparations from ewe and wether livers and an impure solution containing xanthine oxidase but no aldehyde oxidase (5-10 μl) was performed in a 1% agar slab (pH 8.4, 250 volts). The low mobility of aldehyde oxidase necessitated electrophoretic runs of up to 3 hours. Central troughs were cut out following electrophoresis, and filled with the γ-globulin preparation (0.2 cm^3) (Fig.10). This was allowed to diffuse through the gel for 6 hours (20°C) and the slab was then washed (0.9% (w/v) NaCl, 20°C, 16 hours) (Clausen, 1969). The resultant precipitin lines showed the antibody was immunologically active with both ewe and wether aldehyde oxidase preparations. It did not precipitate xanthine oxidase or other components of the crude solution.

Methylene blue reducing activity in the presence of anti-aldehyde oxidase γ-globulin was inhibited in aldehyde oxidase only (Table 10). Xanthine oxidase activity was slightly stimulated. The blank γ-globulin preparation also showed slightly stimulatory properties for both aldehyde oxidase and xanthine oxidase assays.

Assessment of the effect of anti-aldehyde oxidase γ-globulin on the reduction of molecular oxygen by the enzyme showed that complete inhibition was effective for about 10 minutes (Fig.11). Activity was slowly restored from this time, but did not reach that initial velocity

FIGURE 10 IMMUNOELECTROPHORESIS OF ALDEHYDE OXIDASE PREPARATIONS FROM EWE AND WETHER LIVERS AND A XANTHINE OXIDASE PREPARATION



a) The xanthine oxidase preparation was prepared from the protein not precipitated by 50% saturation with ammonium sulphate at pH5.5 obtained during the standard preparation procedure for aldehyde oxidase. The solution contained 21.6mg/cm^3 protein, after dialysis against molybdenum trioxide solution (10^{-6}M) overnight.

b) Ewe aldehyde oxidase was prepared as described in the text, to the dialysed ammonium sulphate precipitated fraction. The preparation contained 10.2mg protein/cm^3, after dialysis against molybdenum trioxide solution (10^{-6}M) overnight.

c) Wether aldehyde oxidase preparation also followed the method described in the text, to the dialysed ammonium sulphate precipitated fraction. The preparation contained 9.1mg protein/cm^3, after dialysis against molybdenum trioxide solution (10^{-6}M) overnight.

Enzyme preparations (5μl) were electrophoresed in 1% agar (pH8.4; 250 volts) for three hours. Diffusion of the anti-aldehyde oxidase γ–globulin (0.2cm^3) was continued for 16h (20^{o}C) and the gel was then washed in physiological saline (16h; 20^{o}C).

Precipitin lines developed between the anti-aldehyde oxidase γ–globulin and both aldehyde oxidase preparations.

TABLE 10 Effect of Anti-Aldehyde Oxidase γ-Globulin Preparation on Sheep Liver Aldehyde Oxidase and Xanthine Oxidase Methylene Blue Reducing Activity

Enzyme	Relative Activity
Aldehyde Oxidase	100
" " + 1 cm^3 γ-globulin : 25 units enzyme	0
" " + 1 cm^3 blank γ-globulin : 25 units enzyme	111
Xanthine Oxidase	100
" " + 1 cm^3 γ-globulin : 0.8 units enzyme	110
" " + 1 cm^3 blank γ-globulin : 0.8 units enzyme	110

Assays were performed at pH 8.5 using acetaldehyde (23mM) or hypoxanthine (5.2mM) substrate as described in the text.

Enzyme activity in the absence of any γ-globulin preparation has been arbitarily placed at 100. This was equivalent to a mean rate of oxygen uptake equal to 5.6 $\mu l O_2$/min. Each assay was performed in duplicate, using two different enzyme preparations.

FIGURE 11 OXYGEN UPTAKE CATALYZED BY SHEEP LIVER ALDEHYDE OXIDASE[a] IN THE PRESENCE OF ANTI–ALDEHYDE OXIDASE γ–GLOBULIN OR BLANK γ–GLOBULIN PREPARATIONS

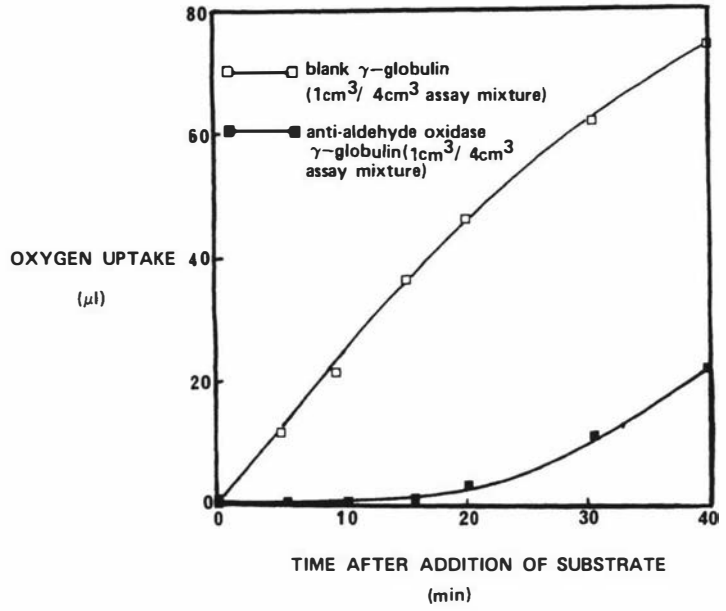
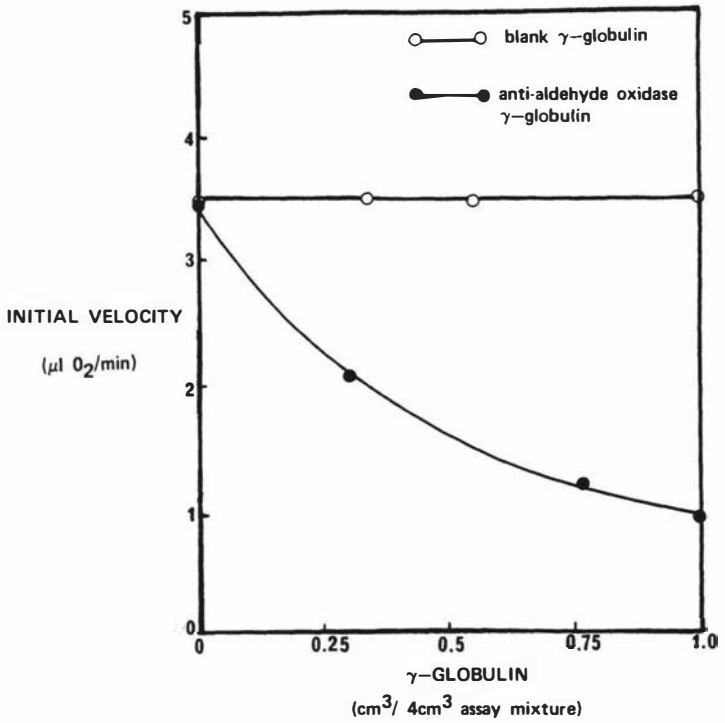


FIGURE 12 EFFECT OF VARYING THE γ–GLOBULIN CONCENTRATION ON THE RATE OF OXYGEN UPTAKE CATALYZED BY SHEEP LIVER ALDEHYDE OXIDASE[a]



a) Assays were carried out at pH 8.5 using acetaldehyde (23mM) as substrate as described in the text. Each assay mixture contained 49 methylene blue units of aldehyde oxidase (mean specific activity: 18.3) and assays were performed in duplicate with two different enzyme preparations. The value plotted is the mean result in each case.

The anti-aldehyde oxidase γ–globulin preparation had a protein concentration of 13.9mg/cm^3 and the blank γ–globulin preparation had a protein concentration of 18.2 mg/cm^3. Each was incubated with the enzyme for five minutes (37^{o}C) prior to addition of the substrate.

shown by the enzyme in the presence of blank γ-globulin. The effect of varying the γ-globulin concentration has been shown in Figure 12. The inhibitor constant was equivalent to 1 mg/cm^3 protein and inhibition appeared to be competitive. The blank γ-globulin had little effect on the initial velocity at any concentration investigated.

When the specificity and kinetic properties of the anti-aldehyde oxidase γ-globulin had been established, its effect on ethanol metabolism in liver homogenates was tested (Fig.13). Aldehyde oxidase was soluble in this system. No exogenous NAD^+ was added, therefore ethanol oxidation was limited by the NAD^+ reoxidation rate.

Ethanol concentrations in the homogenate were the same with both antibody and blank γ-globulin preparations. In the presence of the antibody, acetaldehyde accumulated concurrently with ethanol degradation. However, when blank γ-globulin was used little acetaldehyde accumulated, presumably because it was oxidised by active aldehyde oxidase present. The antibody seemed to be effective in inhibiting aldehyde oxidase activity in liver homogenates.

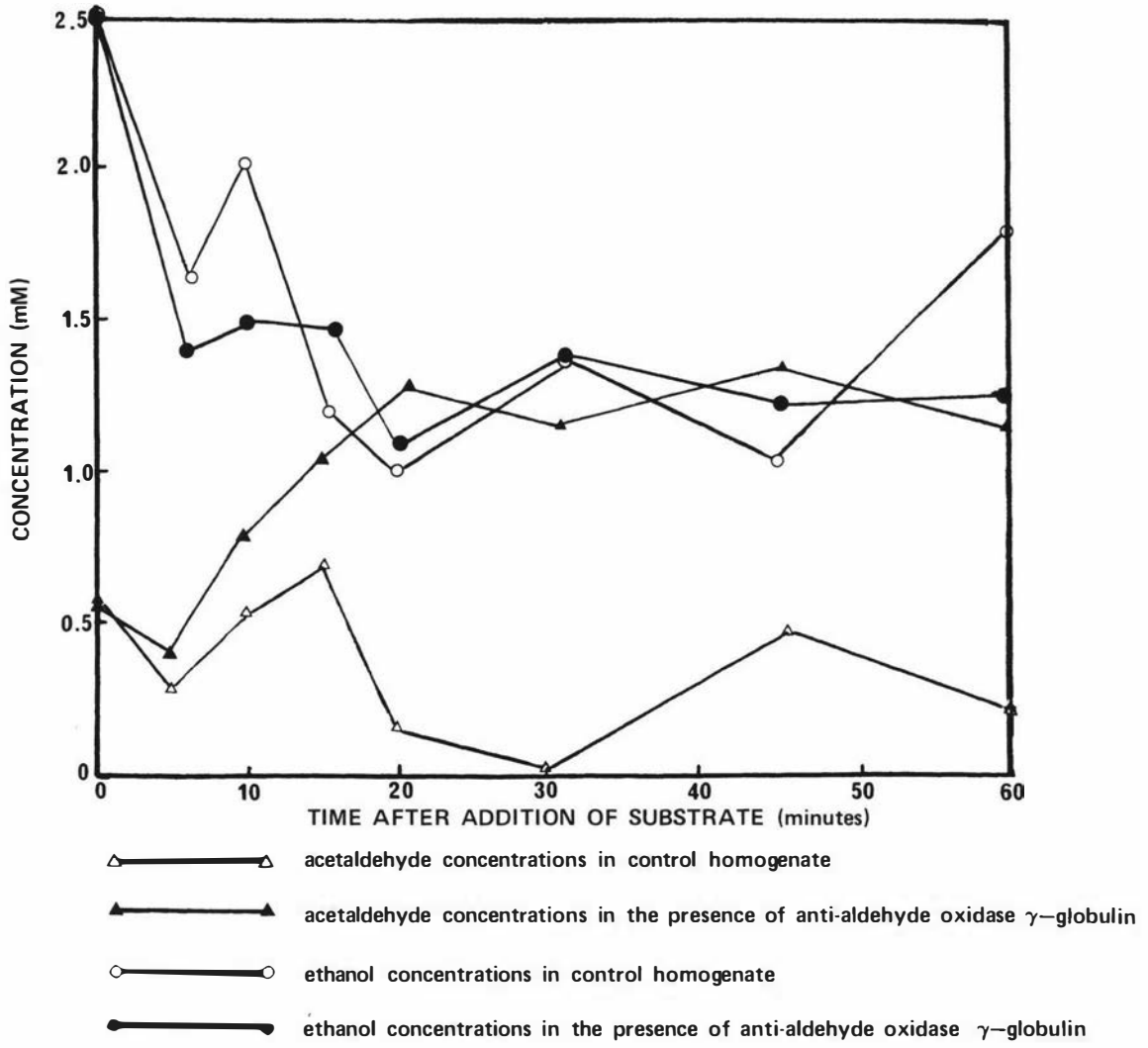
DISCUSSION

Comparison of the properties of the sheep liver aldehyde oxidase prepared in this study with the properties of other aldehyde oxidising flavoproteins showed it had some features in common with each of the others (Table 11).

The protein resembled the rabbit enzyme in its location in the cytoplasm and the apparent inclusion of CoQ as a prosthetic group (Rajagopalan *et al*, 1962). It also had a similar pH_{max} (Knox, 1946), affinity for acetaldehyde substrate (Rajagopalan & Handler, 1964c), and substrate specificity (Knox, 1946). Pig aldehyde oxidase resembled this sheep liver enzyme in the relative concentrations of molybdenum and flavin in the molecule (Gordon *et al*, 1940). It had a similar molecular weight, but was localised in the mitochondria (Igo *et al*, 1961) and did not oxidise quinines. The disulphiram inhibition found in this study of sheep aldehyde oxidase resembled that for the bovine xanthine oxidase (Hunter & Lowry, 1956). By contrast, rabbit aldehyde oxidase was competitively inhibited by disulphiram (Kjeldgaard, 1949) and the K_i (2×10^{-7} M)(Hunter & Lowry, 1956) was much lower than that for the sheep enzyme.

The variable effect of steroids seemed to differentiate the sheep liver aldehyde oxidase from other aldehyde oxidising flavoproteins. Progesterone and œstradiol-17β were found to stimulate the sheep enzyme

FIGURE 13 EFFECT OF ANTI–ALDEHYDE OXIDASE γ–GLOBULIN PREPARATION ON ETHANOL AND ACETALDEHYDE LEVELS IN SHEEP LIVER HOMOGENATES DURING METABOLISM OF EXOGENOUS ETHANOL



Liver was homogenised in 0.1M phosphate buffer (pH7.4) as described by Lundquist and his associates (1963). γ–globulin (in physiological saline) was incubated with the homogenate (37°C) for five minutes. Ethanol (65 mM, 0.1M phosphate buffer, pH7.4) was injected into the mixture through a resealing stopper and samples ($1cm^3$) were withdrawn through this stopper at the times stated. The 'zero' sample was taken 10 seconds after addition of the ethanol.

Each $0.5cm^3$ duplicate homogenate sample was deproteinised with $Ba(OH)_2$($0.5cm^3$, 0.3M) solution containing n-propanol (0. $3cm^3/l$) as an internal standard. $ZnSO_4.6H_2O$(5% w/v) was added and the solutions were refrigerated (4°C) until required. Ethanol and acetaldehyde determinations were performed on the gas chromatograph. A detailed description of this procedure is presented in Chapter 3.

TABLE 11 Comparison of Some Characteristics of Aldehyde Oxidase and Xanthine Oxidase Enzymes

	Sheep Liver Aldehyde Oxidase	Pig Liver Aldehyde Oxidase	Rabbit Liver Xanthine Oxidase	Milk Xanthine Oxidase	Bovine Liver Xanthine Oxidase
Molecular Weight ($\times 10^5$)	2.14	2.5 (Mahler et al, 1954b)	3.00 (Handler et al, 1964)	3.00 (Nelson & Handler 1968)	2.8 (Handler et al, 1964)
$s_{(20,w)}$	12.5	10.6 (Palmer, 1963)	12.2 (Rajagopalan et al, 1962)	11 (Bray et al, 1966)	11.7 (Nelson & Handler, 1968)
Molybdenum (mole/mole protein)	1	1 (Gordon et al, 1940)	2 (Handler et al, 1964)	2 (Avis et al, 1954)	2 (Kielly, 1955)
Flavin (mole/mole protein)	2	2 (Gordon et al, 1940)	2 (Handler et al, 1964)	2 (Avis et al, 1954)	2 (Kielly, 1955)
pH_{max} (substrate)	8.6 (Acetaldehyde)	7.2 (Acetaldehyde) (Gordon et al, 1940)	8.4 (cinchonidine) (Knox, 1946)	8.3 (xanthine) (Bray, 1963)	8 (xanthine) (Kielly, 1955)
K_m (mM) (Acetaldehyde)	2.4	100 (Palmer, 1963)	1.0 (Rajagopalan & Handler, 1964a)	36 (Fridovich, 1966)	?
Substrates:-					
Aldehydes	+	+	+	+	+
Purines	-	very slight	very slight	+	+
Quinines	+	-	+	-	-
N^1-methyl nicotinamide	'Dehydrogenase' only	very slight	+	-	-
Cellular Distribution	Supernatant	mitochondria (Igo et al, 1961)	supernatant (Rajagopalan et al, 1962)	microsomes (Bray, 1963)	supernatant (Bray, 1963)

although they inhibited rabbit aldehyde oxidase (Rajagopalan et al, 1962). Because of its possible physiological significance this property has been studied more closely in a later chapter. An aldehyde dehydrogenase from rabbit liver was also sensitive to steroids (Maxwell & Topper, 1961) but it was inhibited by progesterone, and oestradiol-17β had no effect.

While an electron chain system was indicated by the different reaction shown with each hydrogen acceptor, the sites of oxidation and inhibition within the sheep aldehyde oxidase chain seem to differ from the sites in the rabbit sequence postulated by Rajagopalan & Handler (1964b). The results from this study fit in reasonably satisfactorily with the electron transport sequence postulated for the rabbit aldehyde oxidase (Handler et al, 1964).

Menadione and triton X-100, CoQ inhibitors (Rajagopalan et al, 1962), acted subsequent to the ferricyanide reducing site in the sheep enzyme. Therefore ferricyanide appeared to couple with FAD or molybdenum reoxidation. This is shown in the postulated sequence in Figure 15.

Methylene blue and 2,6-dichlorophenol indophenol reactions were subject to inhibition by quinacrine, an FAD inhibitor, and the CoQ inhibitors, menadione and triton X-100. Their reductions therefore seemed coupled with CoQ reoxidation.

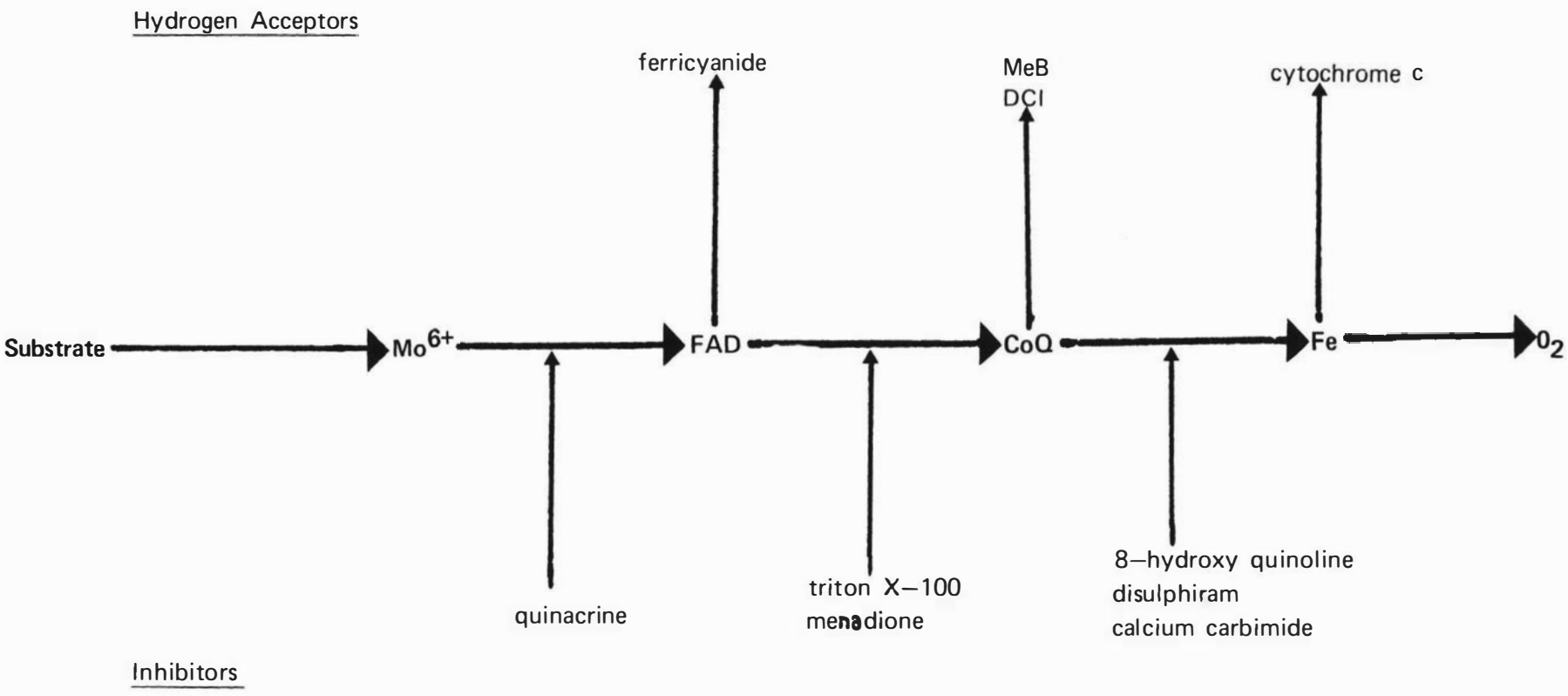
Those inhibitors which appear to act on the iron moiety, 8-hydroxy quinoline and disulphiram (Jokivartio, 1950; Doisy et al, 1955), inhibited only cytochrome c and oxygen reduction suggesting that this is the final intraenzymic step in the electron transfer sequence.

Calcium carbimide appeared to inhibit sheep aldehyde oxidase at the iron transfer site. Rabbit aldehyde oxidase was not affected by calcium carbimide at all (Kjeldgaard, 1949). Metronidazole inhibition of sheep aldehyde oxidase affects coupling with all the hydrogen acceptors studied. It may not act at a specific site within the enzyme molecule but rather as a general inhibitor of electron transfer.

N^1-methyl nicotinamide was active as a substrate only when assayed in the methylene blue, ferricyanide and 2,6-dichlorophenol indophenol systems. Electron egress from the transport system appeared to occur prior to the iron prosthetic group. In contrast, formaldehyde oxidation was coupled principally to oxygen uptake and cytochrome c reduction. Iron appeared to act as the initial acceptor of electrons from formaldehyde.

This work set out to purify and describe sheep liver aldehyde

FIGURE 14 POSTULATED SEQUENCE OF SITES OF ELECTRON EGRESS AND INHIBITION OF ELECTRON TRANSPORT WITHIN THE SHEEP LIVER ALDEHYDE OXIDASE MOLECULE



This differs from that previously postulated for rabbit liver aldehyde oxidase in the sites for methylene blue (MeB) and 2, 6–dichlorophenol indophenol (DCI) reduction.

oxidase for use in later work. It has shown this enzyme to differ considerably from the previously known aldehyde oxidising flavoproteins, while retaining the basic high molecular weight structure and containing flavin and molybdenum.

Chapter 3

ESTIMATION OF ETHANOL AND ACETALDEHYDE IN BIOLOGICAL FLUIDS

Methods for estimating ethanol and acetaldehyde in biological fluids have become more important as the medico-legal interest in these has increased, and research centred on the addictive properties of ethanol has expanded. Acetaldehyde was estimated in separate samples by different methods until the advent of gas chromatographic procedures. Now it is possible to measure both ethanol and acetaldehyde concentrations accurately in a single blood sample.

Ethanol Determinations: The original medico-legal method for ethanol determinations was based on the treatment of the distillate from a biological sample with added potassium dichromate. The excess dichromate was assayed iodometrically (Widmark, 1922). This method was simplified in 1948 (Ryan et al, 1948) and Bennett (1971) has now published a method which measures the optical density of an ether-dichromate solution. In the modified procedure, ethanol is converted to diethyl ether with nitrous acid. Adaptation of the dichromate method to allow analysis of alveolar air (Begg et al, 1964) has had important legal implications but the accuracy is too low to allow its use in research studies where reproducibility and high levels of accuracy are essential.

Despite its lack of specificity, the dichromate method for estimating ethanol concentrations was not contested until 1950, when Burbridge and his associates introduced a microdiffusion procedure to give greater specificity but this has not been used extensively.

In 1951, enzymatic oxidation of ethanol was coupled to reduction of NAD^+ (Bonnischen & Theorell, 1951) giving NADH which shows strong absorbance in the near ultraviolet region with a maximum at 340nm.

The original enzymatic method measured the increase in optical density at 340nm when ethanol in distillates was oxidised in the presence of alcohol dehydrogenase. Increased sensitivity was obtained when the fluorescence rather than the light absorbance of NADH was measured (Ellis & Hill, 1969; Perez et al, 1971). The use of alcohol dehydrogenase to reduce NAD^+ in the presence of ethanol has been the basis of several attempts to automate ethanol analysis (Huet, 1966; Ellis & Hill, 1969; Mueller & Lang, 1971). Other methods have been reported that couple NAD^+ reduction to phenazine methosulphate (Roos, 1971) and nitro-blue tetrazolium (Rosalki, 1970). It is well known that a number of aliphatic alcohols and aldehydes interfere with these

enzymatic procedures.

Acetaldehyde Determinations: The estimation of acetaldehyde in blood was first described by Gee and Chaikoff in 1926. The insoluble dimedon derivative of acetaldehyde was formed, collected and weighed. A later method used bisulphite to obtain an estimate of the contribution that acetaldehyde made to the total dichromate oxidation described in the ethanol determination methods (Janke & Stephan, 1935).

The Stotz method for the estimation of acetaldehyde in biological fluid was based on the distillation of the substance from a tungstic acid filtrate, the acetaldehyde being collected in bisulphite solution. The optical density of this solution was measured after reaction with p-hydroxybiphenyl (Stotz, 1943). A similar method which dispenses with the distillation procedure has since been developed (Lester & Greenburg, 1950).

A microdiffusion method in which the acetaldehyde was trapped in a semicarbazide solution with measurement of the optical density of the semicarbazone at 224nm (Burbridge et al, 1950a; Burbridge et al, 1950b) has been used by some workers, but other aliphatic aldehydes present interfere with the acetaldehyde reaction.

An automated method for the chemical estimation of blood acetaldehyde has been described using a Technicon Autoanalyser (Duncombe & Shaw, 1966; Taylor & Northmore, 1967).

Enzymatic methods for estimating acetaldehyde have included use of yeast aldehyde dehydrogenase to catalyze the reduction of NAD^+ by acetaldehyde (Lundquist, 1958). The optical density of the NADH was measured at 340nm. Alcohol dehydrogenase has also been used to reduce acetaldehyde to ethanol, the decrease in NADH concentration being measured (Bergenmeyer, 1965).

Gas Chromatography: The introduction of gas chromatographic methods for the separation and estimation of the volatile components of serum was first described in 1958 (Fox, 1958). In the original method a preliminary fractional distillation of the serum was carried out and the distilled components were separated on a column packed with firebrick: tricresyl phosphate: glycerol (60:22:18 by weight).

In 1962, Lester described the estimation of ethanol and acetaldehyde from a single sample. The method used Carbowax 1500 (5% on Haloport 60-F) to separate the ethanol and acetaldehyde in the vapour phase over deproteinised blood.

An improved method used Polypak 2 (80-120 mesh) to separate ethanol and acetaldehyde in the filtrate from deproteinised blood (Roach &

Creaven, 1968) and an internal standard (tert.butanol) was used to correlate the results where different sample volumes were used.

Ethanol and acetaldehyde from intestinal contents (Blomstrand, 1971) and in fermented beverages (O'Keane et al, 1971) have been separated (by gas chromatography) using Chromosorb 101 without a stationary phase. Diethyl ether arising from the preliminary oxidation of ethanol by nitrous acid has also been estimated gas chromatographically using Chromosorb 101 as the packing (Gessener, 1971).

A comprehensive review of the gas chromatographic methods up to 1971 (Coldwell et al, 1971), has concluded that Haloport 60-F (60-80 mesh) (Duritz & Truitt, 1964) provided the most satisfactory separation of ethanol, acetaldehyde and acetone. This packing has now been superceded by Porapak Q which is a polymer of ethyl vinyl benzene-divinyl benzene. It was introduced in 1967 (Colehour, 1967) and has been used to separate the ethanol and acetaldehyde from both alveolar air (Jansson & Larsson, 1969) and from blood (Truitt, 1970).

The method adopted for the present studies uses Porapak Q for the gas chromatographic estimation of ethanol, acetaldehyde and acetone in blood and the method has also been used with liver homogenates.

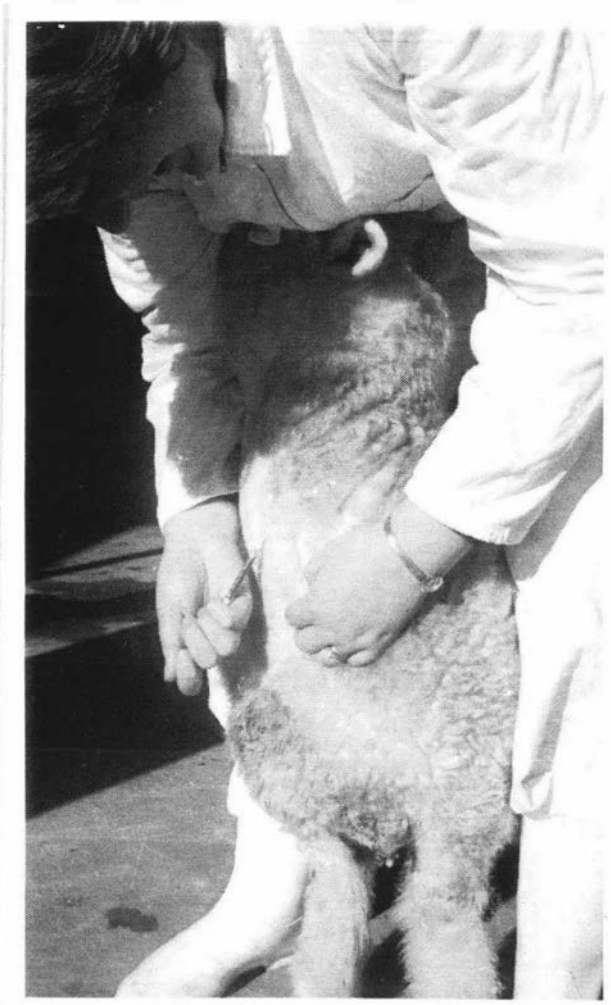
DETAILS OF THE METHOD

Collection of Blood: Blood was collected in a $1cm^3$, calibrated, disposable syringe fitted with a 1.5", 20-gauge needle, which had been flushed with heparin before use. The blood was divided into $0.5cm^3$ duplicate samples and put into $2cm^3$ vials which already contained $0.5cm^3$ ice-cold diluent. The diluent contained n-propanol ($0.2cm^3/l$), which acted as an internal standard, and mercuric chloride (2mg/l) as an antibacterial agent.

Blood samples were normally taken from the left jugular vein of the sheep (Fig.15) and therefore comprise peripheral venous blood. Ethanol and acetaldehyde concentrations in the portal vein and the rumen artery blood were shown to be much higher than those in the jugular vein (Table 12). It was therefore important to specify the site of blood collection.

Errors occur in measuring blood and diluent volumes. With syringes, maximum errors of $0.05cm^3$ in $0.5cm^3$ (10%) were obtained, while the error in the diluent volume was about $0.02cm^3$ in $0.5cm^3$ (4%). Therefore the errors incurred in obtaining a diluted blood sample were about $\pm$ 7%.

FIGURE 15 COLLECTION OF BLOOD FROM THE LEFT JUGULAR VEIN OF A SHEEP



Blood was collected in a $1cm^3$ heparinised syringe fitted with a 1½" 20–gauge needle.

TABLE 12 Comparison of Ethanol and Acetaldehyde Levels in Jugular, Portal and Rumen Arterial Blood from Sheep

	Ethanol (mM)	Acetaldehyde (mM)
Jugular	0.34	0.02
Portal	0.65	0.05
Rumen Artery	0.66	0.06

Ethanol assays used the alcohol dehydrogenase method (Bonnischen & Theorell, 1951). The change of optical density at 340nm was linear over the range from 0-1.40 mM ethanol.

Acetaldehyde was assayed by the method of Burbridge et al, (1950a). The optical density at 224nm was linear through the range of 0.01-0.70 mM acetaldehyde.
Blood was taken from a ewe, anaesthetised with halothane gas, and analysed immediately. Each assay was performed in triplicate and the mean value is quoted.

All experiments reported in this thesis used sheep starved for twelve hours. Ethanol, acetaldehyde and acetone concentrations in peripheral venous blood were shown to vary throughout the day (Fig.16) with a maximum at 0.5 - 1.0 hour after feeding common to the three animals studied. To avoid unnecessary errors, sampling and feeding times were standardised to 9.00a.m. and 9.30a.m. respectively. Sheep were fed a standard maintenance diet of chopped hay (digestibility 53% (Davey, 1972)), and commercial sheep nuts.

Storage of Blood Samples: The blood was immediately added to ice-cold diluent in a vial, which was sealed and refrigerated at 4°C until required for analysis.

Some deterioration was shown to occur during prolonged storage under these conditions but this did not occur until after 10 days (Fig.17). The blood was therefore routinely analysed within that time.

Ethanol represents less than half of the alcohols formed in putrefying blood (Bogusz et al, 1970). Since n-propanol is also formed it could cause some errors in the estimation of the composition of aged blood when it was used as an internal standard.

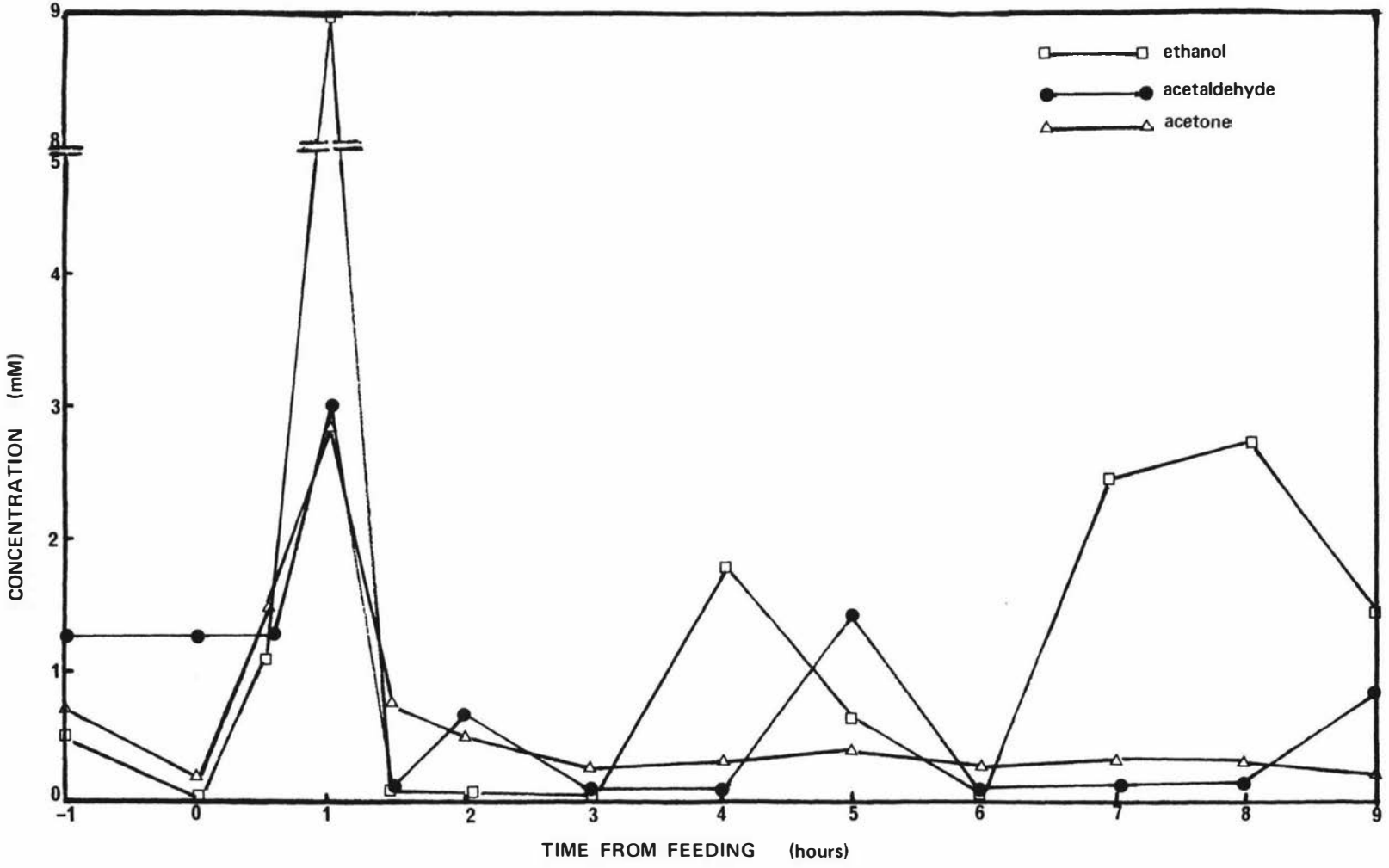
Estimation of Ethanol, Acetaldehyde and Acetone in Blood Samples: Normally the determinations of ethanol, acetaldehyde and acetone concentrations were performed on a Varian Aerograph 1700 gas chromatograph. This was fitted with a flame ionisation detector, and incorporated an 1/8",8' stainless steel column packed with Porapak Q (50-80 mesh), fitted for "off-column" injection. The column was preconditioned overnight at 150°C.

The injection temperature was kept at 143°C and the detector at 183°C. The oven temperature was maintained at 106°C and dry nitrogen (flow rate, 0.44cm^3/sec) was used as the carrier gas. Attenuation was set at 1-4x depending on concentrations in the sample, and the chart recorder speed was 12cm/h.

To estimate the ethanol and acetaldehyde in a sample, the prepared blood solution (5µl) was injected directly into the injection port. Non-volatile material precipitated in the trap which was cleaned after every three or four samples.

Volatile compounds in the diluted blood samples were separated sufficiently to allow quantitative estimation of ethanol, acetaldehyde and acetone concentrations (Fig.18). Methanol and formaldehyde peaks could also be identified in sheep blood but their overlap prevented their quantitative determination. Use of an internal standard (n-propanol)

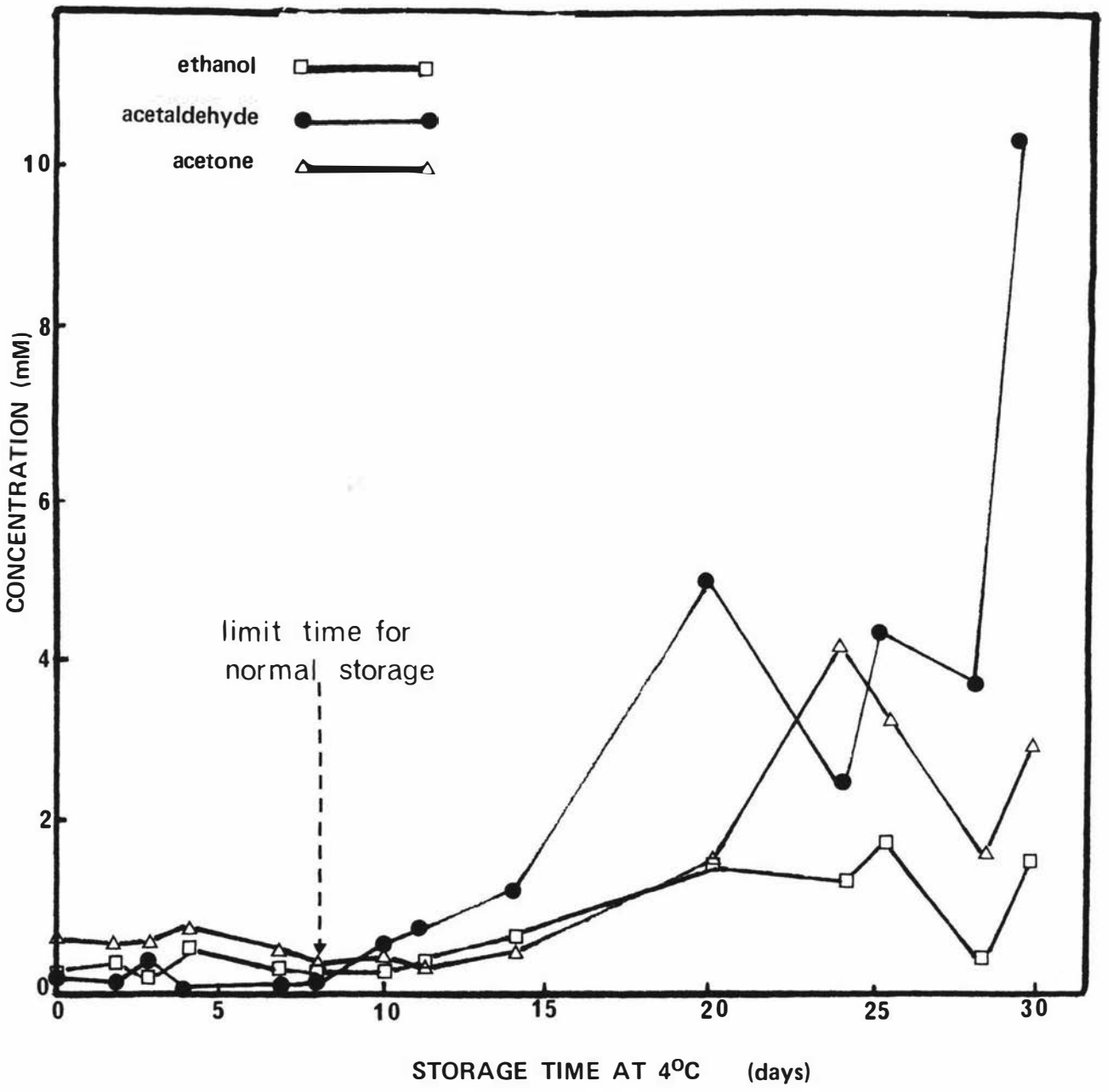
FIGURE 16 TYPICAL VARIATION IN ENDOGENOUS ACETALDEHYDE, ETHANOL AND ACETONE LEVELS IN SHEEP BLOOD (LEFT JUGULAR) THROUGHOUT THE DAY



The ewe was fed at zero time with 554g chopped hay (digestibility 53% (Davey, 1972)) and 82g commercial sheep nuts.

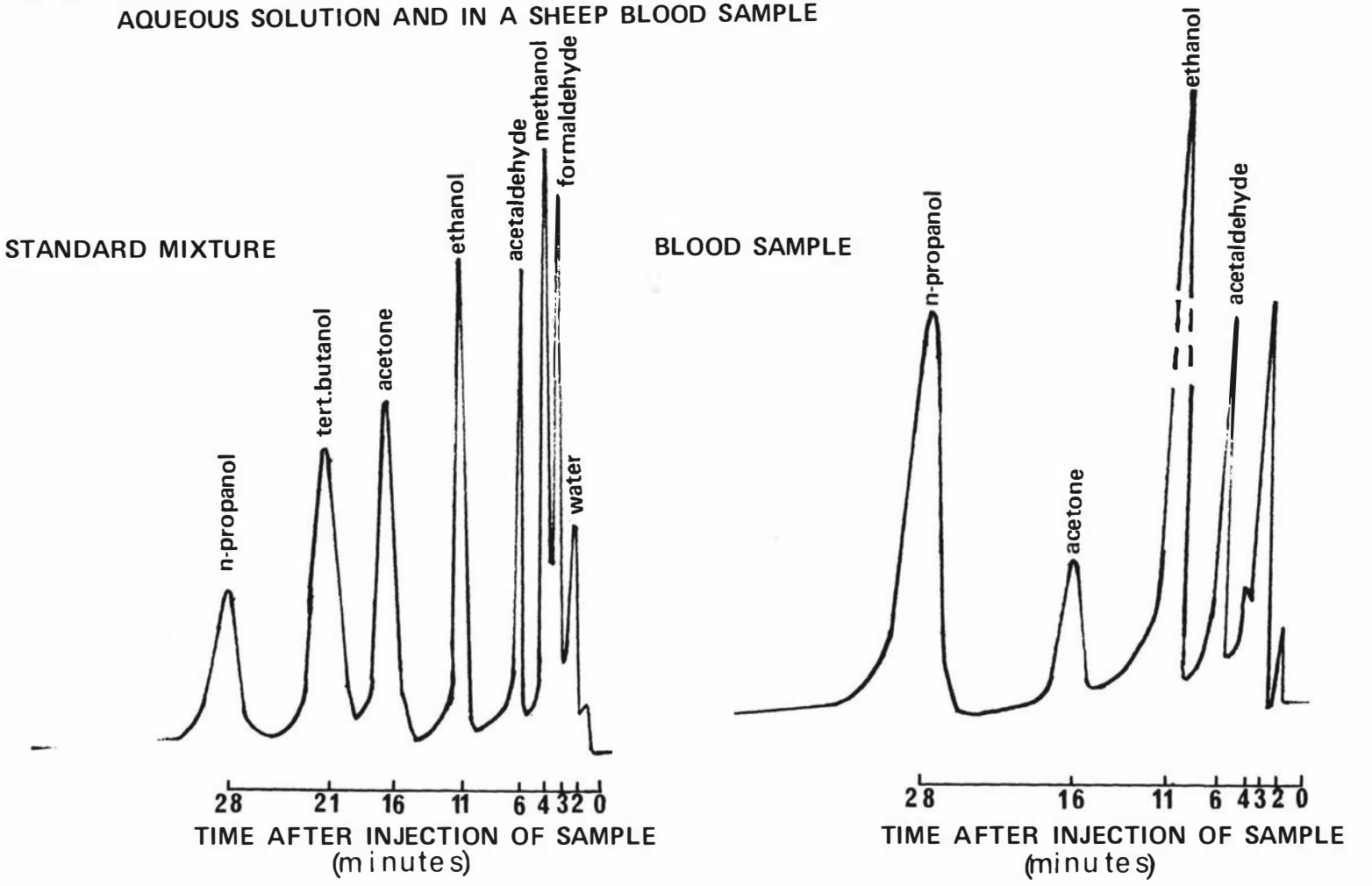
Blood samples were taken from the left jugular vein as described in the text. They were assayed in duplicate for acetaldehyde, ethanol and acetaldehyde gas chromatographically.

FIGURE 17 VARIATIONS IN ACETALDEHYDE AND ETHANOL CONCENTRATIONS IN A TYPICAL BLOOD SAMPLE DURING STORAGE AT 4°C.



Blood was diluted 1:1 (by vol.) with aqueous n-propanol ($0.2cm^3/l$ containing $HgCl_2$(2mg/l)). Each solution was assayed gas chromatographically in duplicate for ethanol, acetaldehyde and acetone concentrations.

FIGURE 18 TYPICAL TRACES OF THE GAS CHROMATOGRAPHIC SEPARATION OF VOLATILE COMPOUNDS IN STANDARD AQUEOUS SOLUTION AND IN A SHEEP BLOOD SAMPLE



The components of each sample (5μl) were separated on a 8', 1/8" stainless steel column packed with Porapak Q(60–80 mesh), fitted for "off-column" injection, and maintained at 106^{o}C. The injection temperature was kept at 143^{o}C and the temperature of the detector was 183^{o}C. The chart recorder moved the paper at the rate of 12cm/h. Carrier gas passed through the column at a flow rate of 0.44cm^{3}/sec.

The blood sample was diluted 1:1 (by vol.) with diluent (n-propanol, 0.2cm^{3}/l containing $HgCl_2$ (2mg/l)) and stored at 4^{o}C prior to analysis.

made the volume of sample injected on to the gas chromatograph uncritical since all peak areas were expressed relative to the n-propanol peak area.

The total errors from duplicate estimations of the composition of a single blood sample have been calculated in Table 13. The largest errors were obtained with acetaldehyde, at this stage, because it was not completely separated from methanol. For all the compounds estimated, the major errors arose in the planimetric method for measuring peak areas. Very small concentrations (0.01-0.05mM) can incur an error of ±50% due to reading the planimeter to ± 1 unit. This was decreased to about ±6% for ethanol concentrations above 2.6mM and acetaldehyde concentrations above 1.55mM.

Calibration curves for the gas chromatographic measurement of ethanol, acetaldehyde and acetone concentrations (Fig.19) were prepared using standard aqueous solutions containing n-propanol as the internal standard. (The detector response was shown to be the same for both blood and aqueous solutions.)

$$\text{The response ratio} = \frac{\text{area under compound peak}}{\text{area under n-propanol peak}} = 1 \text{ when}$$

ethanol concentration = 4.52mM
acetaldehyde concentration = 3.54mM
acetone concentration = 2.92mM

The curves were linear in the range 10^{-1} -10^{-5}M concentrations and the constant blank readings, equivalent to0.09mM ethanol, 0.56mM acetaldehyde and 0.06mM acetone, were shown to be caused by components of the diluent solution.

Infusion of Ethanol or Acetaldehyde into Sheep: Ethanol (20mmoles) or acetaldehyde (7mmoles) was administered intravenously into sheep in 10cm^3 sterile physiological saline. The infusion was complete in thirty seconds and zero time was taken from its completion. The site of infusion was very low in the left jugular vein and blood samples were taken from at least 6" above the injection site.

Dundee and his associates (1971) have reported that intravenous infusion delays the equilibration of arterial and venous blood ethanol levels in humans for up to fifteen minutes while equilibration throughout the body fluid can take thirty to sixty minutes. This would seem to be due to differences in blood flow through various tissues.

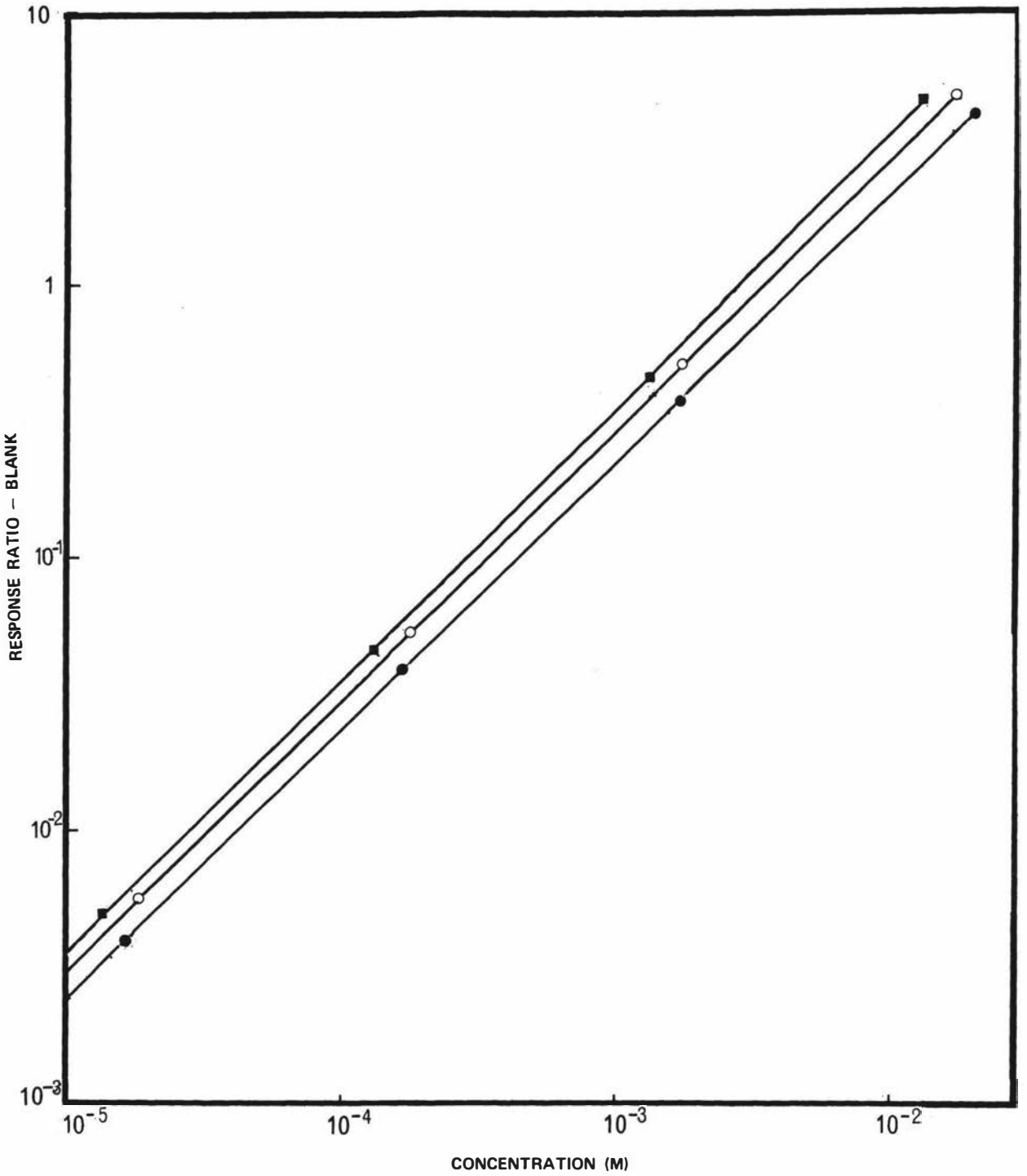
Liver Homogenate Preparation and Analysis: Homogenates were prepared from sheep liver less than 1h from slaughter. Liver samples (8g) were

TABLE 13 Errors incurred in the Estimation of Ethanol Acetaldehyde and Acetone Concentrations by Gas Chromatography

Duplicate number	Acetaldehyde (mM)	Ethanol (mM)	Acetone (mM)
1	0.43	1.29	0.18
"	0.61	1.29	0.18
"	0.61	1.32	0.22
"	0.71	1.32	0.22
2	0.46	1.33	0.18
"	0.67	1.21	0.23
"	0.67	1.21	0.23
"	?	1.29	0.18
Mean=	0.59	1.28	0.20
Range=	0.43-0.71	1.21-1.33	0.18-0.23
S.D. =	0.11	0.04	0.02
SE_M	0.04	0.02	0.01

Normally single estimations of each duplicate were used and the results meaned.

FIGURE 19 CALIBRATION CURVES FOR THE GAS CHROMATOGRAPHIC ESTIMATION OF ETHANOL, ACETALDEHYDE AND ACETONE CONCENTRATIONS



$$\text{Response Ratio} = \frac{\text{area under compound peak}}{\text{area under n-propanol peak}}$$

Each compound was in aqueous solution. Assays were performed in triplicate and each point shown is the mean result for the assay.

homogenised in 40cm^3 0.1M phosphate buffer (pH7.4) in a Potter Elvejham homogeniser (Potter & Elvejham, 1936) according to the method of Lunquist, Svendsen & Peterson (1963).

The homogenate (9.5cm^3) was equilibrated (37°C, 5min) in a tube fitted with a resealing stopper, and any exogenous compounds were added in buffer (0.1M phosphate, pH7.4). Substrates (0.5cm^3 in 0.1M phosphate buffer, pH7.4) were injected through the stopper and samples (0.5cm^3) were withdrawn at the times stated. The samples were immediately placed in vials which already contained 0. 5cm^3 ice-cold diluent. This diluent was an aqueous solution of n-propanol (0.3cm^3/l) and barium hydroxide (0.3M). A 5% (w/v) solution of zinc sulphate (0. 5cm^3) was added to the vial, which was stoppered and refrigerated immediately.

Gas chromatographic analysis of the homogenate samples followed the method used for blood. No change in ethanol, acetaldehyde or acetone concentrations was observed during storage for seven days and the extra components in the samples did not seem to affect gas chromatographic separation of the volatile compounds or the sensitivity of the detector.

DISCUSSION

The methods used in estimating ethanol, acetaldehyde and acetone concentrations in this study were designed to reduce to a minimum the errors which were inherent in the collection, storage and analysis of samples.

Sampling from the jugular vein provided the means for taking individual samples at short intervals of time; five minute intervals between samples were possible under most circumstances. Measurement of the endogenous concentrations of ethanol, acetaldehyde and acetone in blood could have been better achieved by using the portal vein between the digestive tract and the liver, but this was not used as it necessitated entering the abdominal cavity, with a general anaesthetic and consequent alteration of metabolism.

Ethanol metabolism varies markedly between animals in one species, and even within the one animal from day to day (Marshall & Fritz, 1953). It therefore seemed reasonable to reduce this variation by using each animal as its own control; a statistically reliable number of samples were taken from each animal both before and during any given treatment. Sheep used as blanks were included in each experiment.

Gas chromatography allowed for the simultaneous identification and estimation of micro amounts of ethanol, acetaldehyde and acetone in single samples. This has eliminated those errors associated with the use

of different samples with different methods for the estimation of each compound. The gas chromatographic method is certainly the most popular and practicable estimation method for studies which involve the measurement of concentrations of several volatile compounds in a single sample.

Chapter 4

EFFECT OF PROGESTERONE ON ETHANOL AND ACETALDEHYDE METABOLISM

Many reports of the effect of hormones on ethanol metabolism have appeared in the literature. Thyroid hormone is claimed to increase ethanol metabolism in rats (Kinard et al, 1962; McCarthy & Lovenberg, 1968; Rawat & Lundquist, 1968; Ylikahri & Mäenpaa, 1968) although it appears to decrease the rate of ethanol oxidation in man (Trystrup et al, 1965; Stokes & Lasley, 1967). Glucagon is reported to increase ethanol metabolism in mammals by Nelson and Jensen (1961) but Kinard (1967) could not confirm these results. Insulin seems to increase ethanol metabolism and there is an associated increase in plasma free fatty acids (Rawat, 1969).

At the enzyme level, thyroid hormone has been shown to decrease the activity of alcohol dehydrogenase from rat liver (Ylikahri & Mäenpaa, 1968).

Progesterone and oestradiol-17β have been reported to inhibit rabbit liver aldehyde oxidase (Rajagopalan et al, 1962) and aldehyde dehydrogenase (Maxwell & Topper, 1961), with the aldehyde oxidase inhibition being attributed to interference with the electron transfer system in the enzyme. Aldehyde dehydrogenase inhibition can be reversed by urea and it is likely that progesterone affects the enzyme by causing a change in configuration (Maxwell, 1962).

The sex-associated difference in sheep liver aldehyde oxidase activity described in this work was found by chance and further study was encouraged by the possibility that acetaldehyde metabolism may be associated with the sex-linked differences exhibited by alcoholics (Wilkinson et al, 1971). For example, it has been reported that female patients take thirteen years to become physically dependent on alcohol, from their first hospitalization caused by drink, while for men the corresponding figure is nineteen years.

In this study the effect of progesterone and other steroids on sheep liver aldehyde oxidase has been examined and progesterone has been shown to affect the total aldehyde oxidase activity in the liver. It also influences the endogenous levels and the metabolism of ethanol and acetaldehyde in vivo.

METHODS

The initial survey of sheep liver activities of aldehyde oxidase and xanthine oxidase used fresh livers obtained from the local abbatoirs.

Livers were kept on ice prior to use and assays were performed within two hours of slaughter. Sampling was carried out at intervals during an eight month period (October, 1970 - May, 1971) when the abbatoirs were operating.

Liver Extract: Extract was prepared by homogenising liver (300g) in ice-cold distilled water (450g). This was performed in a Waring blendor for two 1 minute periods, the homogenate centrifuged (14,600g; 0°C; 60min), and the supernatant collected.

Partial Purification of Aldehyde Oxidase: Liver extract was taken to pH5.5 by adding hydrochloric acid (1M) dropwise. After standing (4°C; 15min) the preparation was centrifuged (14,600g; 0°C; 30min) and solid ammonium sulphate added to the supernatant to give 30% saturation. The solution was stirred at 0°C for 30 minutes and then centrifuged (14,600g; 0°C; 20min). More ammonium sulphate was added to the supernatant to give 40% saturation. After again stirring and centrifuging the precipitate was dissolved in a solution of 10^{-3}M molybdenum trioxide. This was then dialysed against aqueous molybdenum trioxide (10^{-6}M) for 12 hours at 4°C.

The specific activity of the enzyme preparation increased 30-40 fold during these steps. No alcohol dehydrogenase activity was present in the preparation and xanthine oxidase activity was less than 5% than in the original extract.

Assays were performed as described in Chapter 2. One unit of aldehyde oxidase activity catalyzed the reduction of 1 µg methylene blue in one minute.

Partial Purification of Aldehyde Dehydrogenase: The preparation of sheep liver aldehyde dehydrogenase has been described in the Appendix I. The method involved extraction of an acetone powder from liver with an EDTA solution (1M, pH 7.0). An ethanol precipitate (0.4-1.4 vol. ethanol; -10°C) obtained from the EDTA extract was dissolved in EDTA solution (1M, pH 7.0) and when this solution was acidified (pH 4.5), the activity remained in the supernatant. Precipitation steps using RNA (5%) and an ammonium sulphate fractionation between 50-60% saturation, further increased the activity of the enzyme.

This procedure increased the specific activity of aldehyde dehydrogenase 53 times and no alcohol dehydrogenase, aldehyde oxidase or xanthine oxidase activity remained in the preparation.

Assays of aldehyde dehydrogenase activity were carried out by following the reduction of NAD^+ at pH 9.3 (pyrophosphate buffer, 0.01M),

observing the change in optical density at 340nm (Racker, 1949). One unit of this enzyme was taken as the amount of enzyme required to change the optical density at 340nm by 0.001 in one minute. The standard assay conditions have been described in Appendix I.

In vivo experiments with progesterone were performed on 28.1kg (mean), one year old, Southdown sheep which had been neonatally castrated (i.e. wethers). A maintenance diet of chopped hay and commercial sheep nuts was provided.

Progesterone (60mg) was suspended in peanut oil and administered subcutaneously in the rear loin each day. A control animal was injected with oil containing no progesterone.

Collection and analysis of blood from the jugular vein has been described in Chapter 3. Samples were analysed for ethanol, acetaldehyde and acetone by gas chromatography. In in vivo metabolism experiments ethanol (20 mmoles) or acetaldehyde (7 mmoles) was administered intravenously in $10cm^3$ sterile saline.

Liver Homogenate: The preparation of liver homogenates, which has already been described (Chapter 3), followed the method described by Lundquist and his associates (1963).

Statistics: The significances of all differences quoted were measured using the t-test.

RESULTS

Aldehyde Oxidase and Xanthine Oxidase Activities in Sheep Livers: Aldehyde oxidase activity in ewe liver varied depending on the date of sampling (Table 14). The activity in the March-May ("late") period was much higher than in the October-February ("early") period ($p < 0.001$). Wether liver aldehyde oxidase levels did not differ from the "early" ewe liver activity and were lower ($p < 0.001$) than the "late" ewe liver activities. Differences in xanthine oxidase activity followed the changes in the aldehyde oxidase activity but were not large enough to be significant.

Mapping the aldehyde oxidase activity of individual livers on a histogram (Fig.20) has shown that "early" ewe and wether samples followed J-type curves with low maxima. Most wether samples fell in the 50-100 units/g liver range but the "early" ewe liver activities were mostly in the 0-50 units/g liver range. Activities of ewe livers samples between March and May ranged from 0-346 units/g liver.

Steroid Effect on Sheep Liver Aldehyde Oxidase: The effects of various steroids on sheep liver aldehyde oxidase activity were investigated.

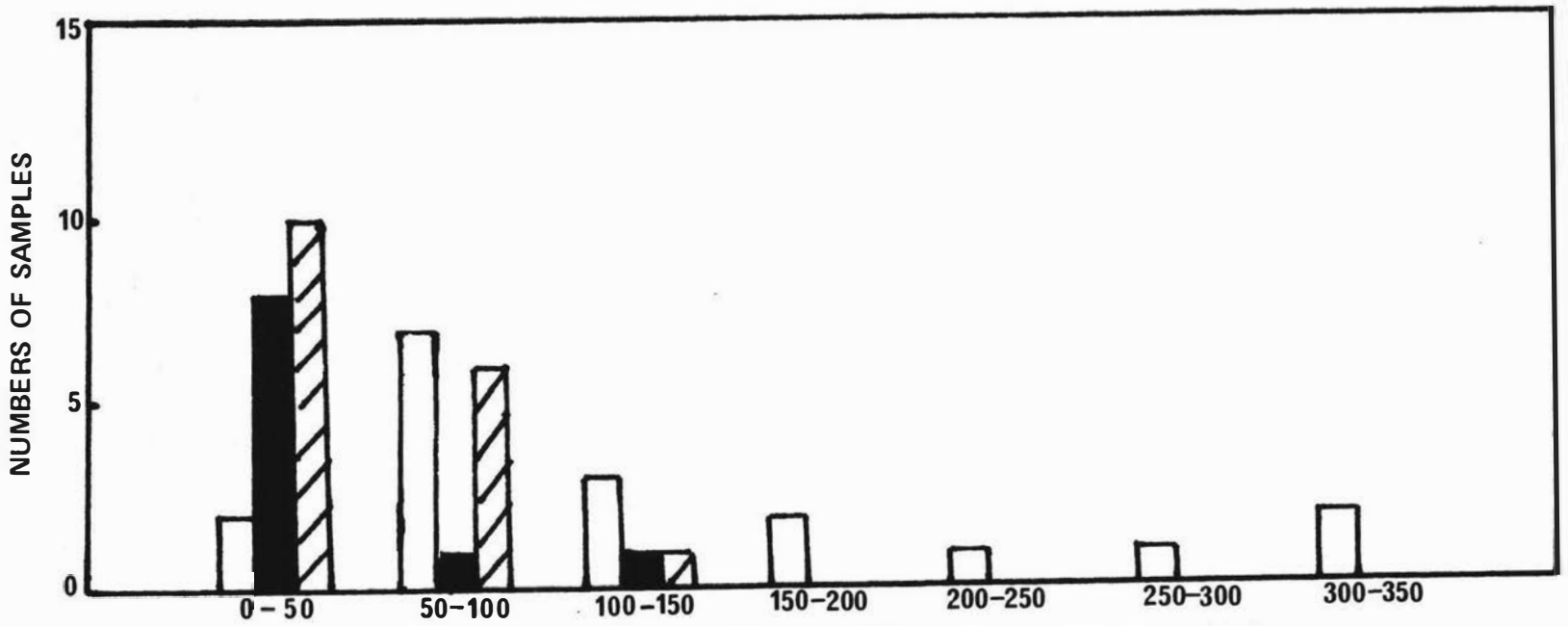
TABLE 14 Aldehyde Oxidase and Xanthine Oxidase Activities in Ewe and Wether Livers

	(N)	Aldehyde Oxidase Activities (units/g wet weight liver $\pm SE_M$)	Xanthine Oxidase Activities (units/g wet weight liver $\pm SE_M$)
Total Ewe	(31)	63.7 ± 14.5	43.5 ± 5.9
Oct.-Feb.	(10)	25.3 ± 9.4	33.5 ± 5.6
Mar.-May	(21)	145.0 ± 18.2	62.2 ± 7.6
Total Wether	(16)	30.2 ± 1.8	31.7 ± 8.4

Aldehyde oxidase was assayed in the "methylene blue" system described in Chapter 2, using acetaldehyde (23mM) as substrate.

Xanthine oxidase was assayed in the "methylene blue" system described in Chapter 2, using hypoxanthine (5.2mM) as substrate.

FIGURE 20 HISTOGRAM TO SHOW THE DISTRIBUTION OF ALDEHYDE OXIDASE ACTIVITIES IN THE LIVERS OF INDIVIDUAL SHEEP



activities in the livers from ewes sampled between March and May in the sampling period

activities in livers from ewes sampled between October and February in the sampling period

activities in livers from wethers sampled throughout the sampling period

Assays for aldehyde oxidase activity followed the method described in Chapter 2, using methylene blue as the hydrogen acceptor. One unit of enzyme catalyzed the reduction of 1μg methylene blue in one minute.

Liver extracts were prepared by homogenising 10g fresh liver in 15 cm^3 chilled, distilled water in a Waring blendor (for two 1 minute periods). The homogenate was centrifuged (14,600g, 0^{o}C, 60 min) and the resulting supernatant was used in assays for aldehyde oxidase activity. Each assay was performed in duplicate.

Among the compounds studied (Table 15), progesterone (0.32mM) and androsterone (0.14mM) stimulated oxygen uptake catalyzed by sheep liver aldehyde oxidase preparations, and progesterone (0.32mM), oestradiol -17β (1.75mM) and cortisone (0.74mM) increased the rate of methylene blue reduction catalyzed by the enzyme (Chapter 2, Table 8). Progesterone was the only steroid in the study to stimulate both the "oxidase" and "dehydrogenase" reactions of sheep liver aldehyde oxidase.

The electron acceptors which couple with aldehyde oxidase showed universal stimulation by progesterone (Table 16) and it seemed likely that activition was associated with facilitation of general electron transfer within the aldehyde oxidase molecule, or with the first two electron acceptors, molybdenum and FAD. When progesterone concentration in the system was plotted against the observed aldehyde oxidase activity (Fig. 21) a logarhythmic relationship was noted. This pattern of aldehyde oxidase response was unusual and is difficult to explain as due to simple enzyme activation. The possibility that progesterone itself was being oxidised has not been studied.

Steroid Effect on Sheep Liver Aldehyde Dehydrogenase: The NAD^+-dependent aldehyde dehydrogenase enzyme was active (18,800 ± 3262 (SE_M) NAD^+ units/g liver) in twelve ewe livers assayed during the March-May sampling period. Progesterone (0.32mM) caused a 60% inhibition of the partially purified sheep liver aldehyde dehydrogenase. Maxwell & Topper (1961) have reported that progesterone also inhibited the rabbit liver enzyme.

Administration of Progesterone to Sheep: Ethanol, acetaldehyde and acetone are endogenous constituents of sheep blood. In a study to determine the effect of progesterone on the levels of these substances, daily samples were taken from wethers both before and during progesterone treatment. The results showed that endogenous ethanol levels were reduced by hormone treatment (Table 17) despite strict adherence to a standard diet programme. No significant changes in acetaldehyde or acetone blood levels were observed.

The clearance of intravenously infused acetaldehyde showed that little accumulation of the compound occurred either before or during progesterone treatment (Fig.22). The initial reaction reducing acetaldhyde to ethanol was less active in the treated sheep. A large amount of ethanol seemed to be synthesised particularly forty-five minutes after the acetaldehyde had been infused into the sheep. Both treated and untreated animals showed this effect and it could be associated with the establishment of a new equilibrium between reduced and oxidised hydrogen

TABLE 15 Assay of Sheep Liver Aldehyde Oxidase Catalyzed Oxygen Uptake in the Presence of Several Steroids.

Steroid	Final Concentration (mM)	Relative Rate
Nil	-	100
Progesterone	0.32	160
Pregnanediol	0.65	100
Oestradiol - 17β	1.75	100
Tetrahydrocortisone	0.64	89
Tetrahydrocortisol	0.17	64
Androsterone	0.14	124
Cortisone	0.74	63
Oestriol	0.12	87
Testosterone	0.82	88

Assays were performed at pH 8.5 as described in Chapter 2 with acetaldehyde (23 mM) as the substrate. The rate of oxidation of acetaldehyde in the absence of steroids has been arbitarily placed at 100. This was equivalent to a rate of oxygen uptake equal to 3.6 $\mu l O_2$/min, under standard assay conditions.

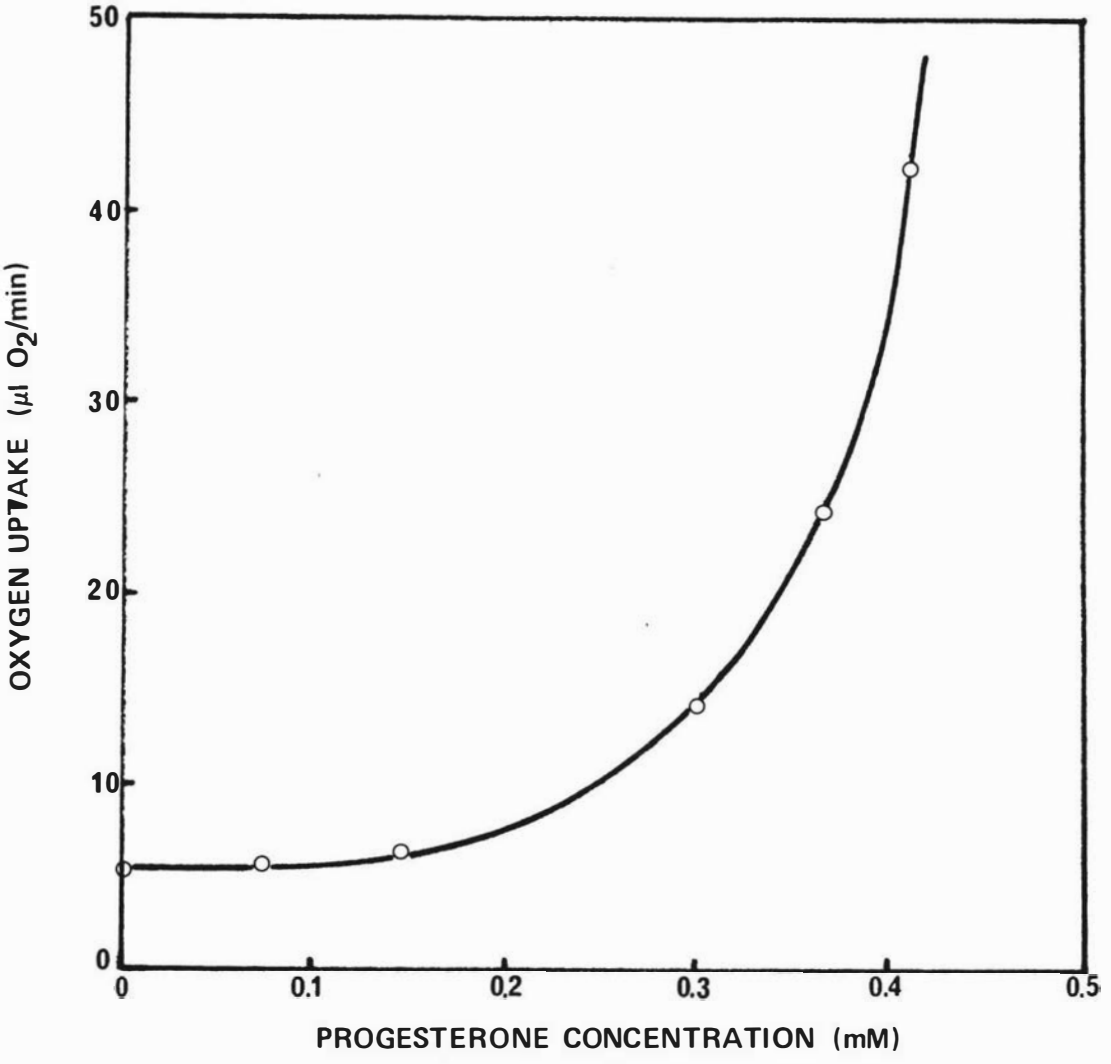
TABLE 16 The Effect of Progesterone (0.32 mM) on the Coupling of Acetaldehyde Oxidation to Various Electron Acceptors by Sheep Liver Aldehyde Oxidase

Electron Acceptor	Control	Progesterone
Methylene Blue	100	142
Oxygen	100	160
2-6 Dichlorophenol Indophenol	100	183
Cytochrome c	100	156
Ferricyanide	100	135

All assays were performed under the standard conditions described in Chapter 2 using acetaldehyde (23 mM) as substrate. The rate of oxidation of acetaldehyde in the absence of progesterone has been arbitarily placed at 100, for each assay system. In each assay 54 units of enzyme (mean specific activity = 18.3 units/mg protein) were used.

Each assay was performed in duplicate, using two different enzyme preparations, and the mean value is quoted.

FIGURE 21 EFFECT OF VARYING THE PROGESTERONE CONCENTRATION ON SHEEP LIVER ALDEHYDE OXIDASE ACTIVITY



Aldehyde oxidase activity was assayed by measuring the initial rate of oxygen uptake using the method described in Chapter 2. Each assay contained forty units of aldehyde oxidase (mean specific activity = 18.3 units/mg protein) and was performed in duplicate using two different enzyme preparations. The result shown is the mean.

TABLE 17 Endogenous Constituents of Peripheral Venous Blood before and during Progesterone Treatment

	(N)	Acetaldehyde (mM ± SE_M)	Ethanol (mM ± SE_M)	Acetone (mM ± SE_M)
Sheep No.1				
Before Progesterone	(25)	0.34 ±0.10	7.35 ± 2.75	0.41 ±0.06
With Progesterone	(15)	0.34 ±0.16	2.23 ± 0.77	0.34 ±0.07
Sheep No.2				
Before Progesterone	(17)	0.24 ± .05	8.56 ± 3.65	0.47 ±0.04
With Progesterone	(16)	0.15 ± .09	1.53 ± 0.47	0.33 ±0.08
Sheep No.3 (control)				
Before Oil	(13)	0.19 ±0.06	7.88 ± 2.76	0.36 ±0.04
With Oil	(18)	0.13 ±0.11	7.09 ± 1.03	0.31 ±0.03

Wethers were maintained on a standard diet, and blood samples ($1cm^3$) were taken prior to feeding each day. They were diluted (1:1 by vol.) with diluent (n-propanol, $0.2cm^3/l$; $HgCl_2$, 2mg/l) and stored at 4^oC.

Assays for acetaldehyde, ethanol and acetone were performed on the gas chromatograph (Chapter 3).

Each animal was allowed two days on each treatment, before any blood samples were analysed.

FIGURE 22 CLEARANCE PATTERN OF ACETALDEHYDE FROM THE PERIPHERAL VENOUS BLOOD OF SHEEP FOLLOWING ITS INTRAVENOUS INFUSION

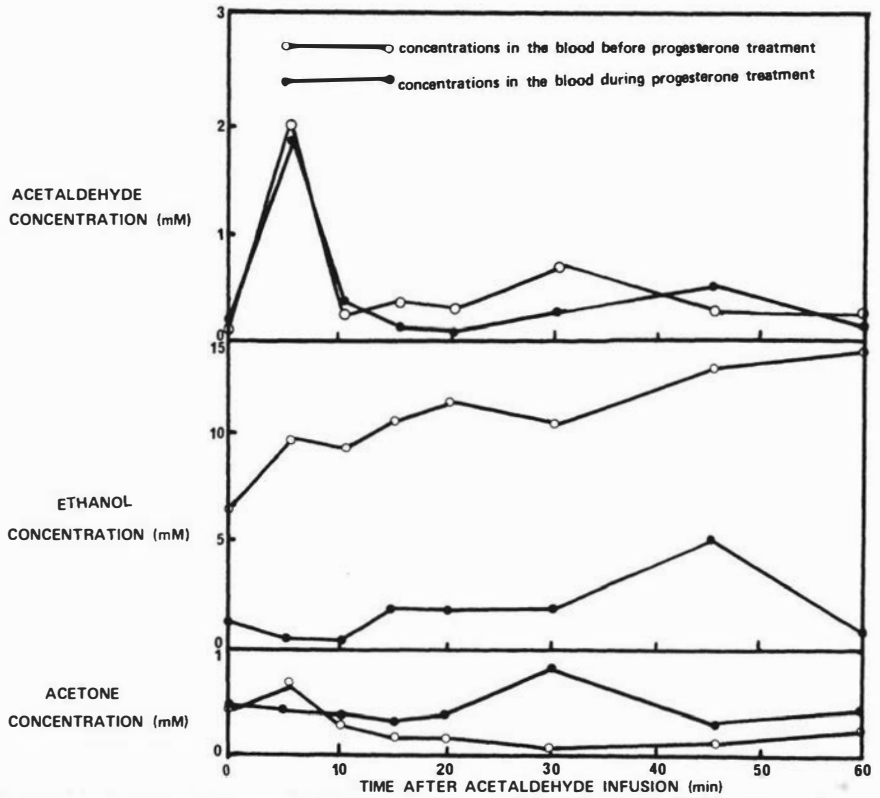
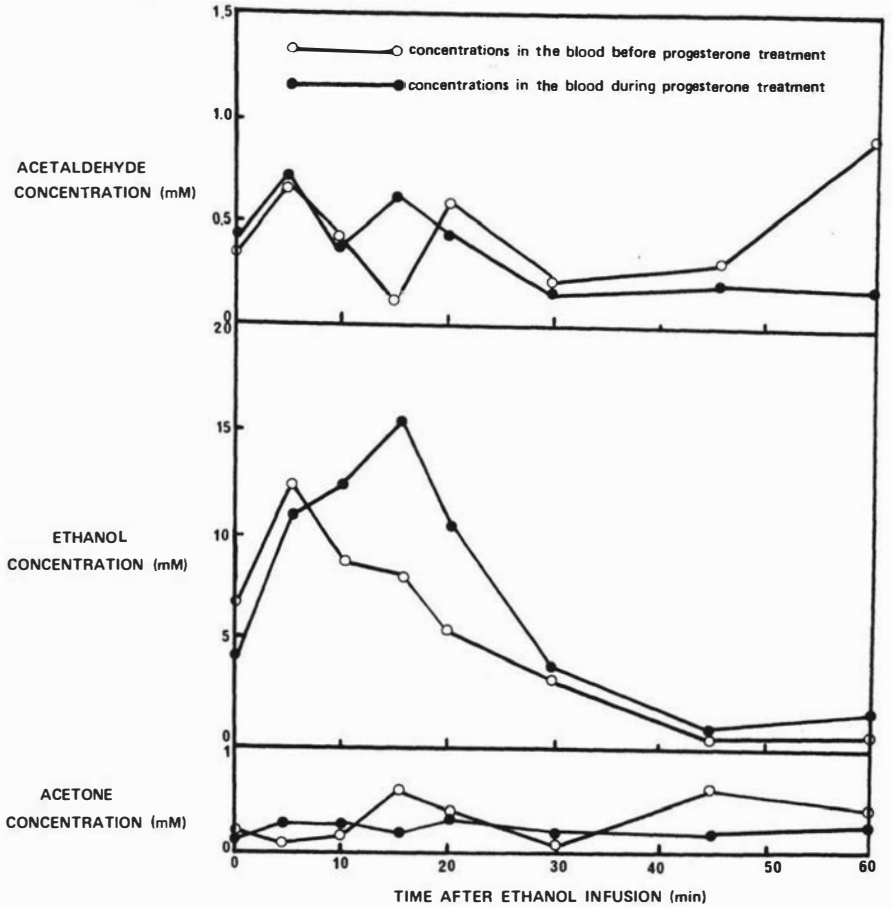


FIGURE 23 CLEARANCE PATTERN OF ETHANOL FROM THE PERIPHERAL VENOUS BLOOD OF SHEEP FOLLOWING ITS INTRAVENOUS INFUSION



Acetaldehyde (7mmoles) or ethanol (20mmoles) was infused into the lower left jugular vein in 10cm^3 physiological saline immediately after the 'zero' blood sample had been taken. Infusion was complete in 30 seconds and time was measured from its completion. Each experiment was performed on both sheep under treatment with progesterone and on two control wethers. The mean concentrations at each time interval after infusion have been plotted.

Blood samples (1cm^3) were taken from the left jugular vein of the wethers at the times indicated. Each was divided into two 0.5 cm^3, duplicate samples and diluted (1:1 by vol.) with diluent as described in Chapter 3. The samples were stored at 4^{o}C until they could be analysed on the gas chromatograph.

acceptors. The size of the increase would preclude the possibility that it arose directly from the infused acetaldehyde.

The clearance of ethanol from peripheral venous blood is shown graphically in Figure 23; the delay in reaching a maximum has been reported in humans by Dundee and others (1971) and is probably associated with delayed equilibration of the ethanol throughout the blood stream. Progesterone increased the mean ethanol disappearance rate from 0.30mM/min (N=10) to 0.51mM/min (N=6) but there was no change in the rate in the control animal. Overall, less acetaldehyde accumulated during ethanol oxidation in the progesterone-treated animals despite more rapid ethanol removal. It is possible that this is due to a stimulation of the aldehyde oxidase enzyme.

At the conclusion of these progesterone experiments the animals were slaughtered and liver homogenates were prepared for use in the experiments described below.

Liver Homogenates: Homogenates of the livers from progesterone-treated and control wethers were assayed for alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidase and aldehyde oxidase activity (Table 18). Only aldehyde oxidase showed any marked difference in activity between the progesterone-treated and untreated animals. Aldehyde oxidase activity in the liver appeared to have been increased as a result of the *in vivo* administration of progesterone. The daily dose of progesterone (60mg) was only six times that required to prevent ovulation in ewes (Dutt & Casiola, 1948; Woody *et al*, 1967).

The homogenates prepared from the livers of progesterone-treated and control wethers were further used in an experiment designed to show the effect that *in vivo* progesterone administration had on ethanol metabolism *in vitro*. Ethanol was introduced into the temperature-equilibrated ($37^{o}C$) system as described in Chapter 3, and samples were withdrawn from the mixture at the times stated.

Very similar ethanol concentration changes were recorded in the homogenates of livers from treated and untreated animals (Fig.24). However, the acetaldehyde accumulation in the homogenates of livers from the control wethers did not seem to occur in those from the progesterone-treated sheep.

An anti-aldehyde oxidase γ-globulin solution, prepared from rabbit serum as described in Chapter 2, was used to demonstrate the role of aldehyde oxidase in producing those differences in ethanol metabolism which seem to result from administration of progesterone *in vivo*.

TABLE 18 Activities of Various Enzymes in Livers from Wethers Treated with Progesterone

	Enzymes[a]			
	Alcohol[b] Dehydrogenase (μmoles ethanol/ min/g liver)	Aldehyde[c] Dehydrogenase (μmoles acetaldehyde/ min/g liver)	Xanthine[d] Oxidase (μmoles hypo-xanthine/ min/g liver)	Aldehyde[d] Oxidase (μmoles Acetaldehyde /min/g liver)
Sheep No.1	8.1	2.3	0.04	0.59
Sheep No.2	5.3	3.7	0	1.09
Sheep No.3 (control)	7.4	2.8	0	0.03
Sheep No.4[e] (control)	8.6	1.2	0.03	0.03

a) Assays were performed at pH 7.4 (0.1M phosphate buffer). Each assay was performed in duplicate and the mean value is quoted.

b) Alcohol dehydrogenase was assayed by measuring the rate of change in optical density at 340 nm when ethanol oxidation was coupled to NAD^+ reduction (Trenholm et al, 1970).

c) Aldehyde dehydrogenase was assayed according to the method described in Appendix I (Racker, 1949).

d) Methylene blue was used to assay for xanthine oxidase and aldehyde oxidase (Chapter 2).

e) Sheep No. 4 was a control wether not subjected to administration of oil.

It was shown that this specific inhibitor of the sheep aldehyde oxidase enzyme had little effect on ethanol metabolism in homogenates of livers from untreated sheep (Fig.25). However, the γ-globulin preparation did seem to increase the acetaldehyde concentrations in the homogenates of livers from treated animals during ethanol metabolism, to levels similar to those recorded for the controls. Higher aldehyde oxidase activities in the livers from wethers treated with progesterone *in vivo* appeared to result in decreased acetaldehyde levels in the homogenates of those livers during ethanol metabolism *in vitro*.

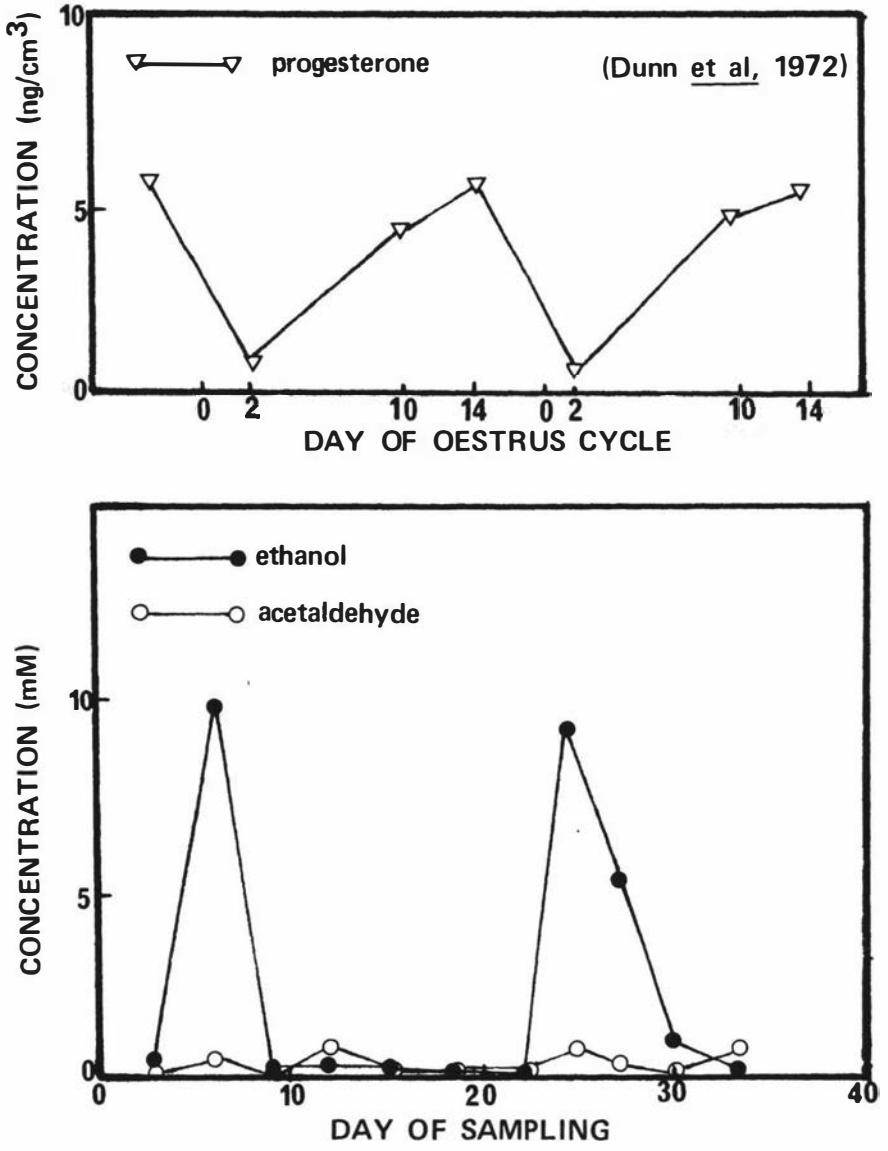
Oestral Variation in Endogenous Levels of Ethanol and Acetaldehyde: The oestral cycle in the ewe lasts seventeen days (Deane *et al*, 1966). The day for onset of oestrus is normally designated day 0 when progesterone levels are low allowing ovulation to occur on day 1. They rise rapidly until day 9, and slowly until day 14. On day 15, the progesterone level falls markedly over twenty four hours in preparation for the next cycle (Dingle *et al*, 1968). Values from a recent publication by Dunn and his associates (1972) have been plotted in Figure 26.

Endogenous ethanol and acetaldehyde levels in peripheral venous blood from a normally cycling ewe have been plotted below this with two seventeen day cycles correlated with variations in the blood ethanol concentrations. If it is considered that the maximum ethanol levels occur on day 0, as would be suggested from the ethanol levels in progesterone-treated wethers, as inverse relationship between progesterone and ethanol concentrations could be claimed. Acetaldehyde concentrations did not vary noticeably during the sampling period.

Endogenous Levels of Ethanol and Acetaldehyde during Pregnancy: The endogenous concentrations of ethanol and acetaldehyde were assayed in the venous blood of a pregnant ewe which eventually delivered twins. Pregnancy lasts for twenty one weeks in the sheep (Short, 1961) and in this study time has been counted back from parturition since the date of conception was not known. A slow rise in blood ethanol was evident by week 14 (Fig.27) but levels were variable and comparatively low until one week after parturition. At this time endogenous ethanol concentrations had increased to twenty times the level at week 7, and they decreased again the following week.

Individual levels of ethanol and acetaldehyde in blood have been plotted in Figure 28 for several weeks before and after parturition. The striking increase in blood ethanol concentration occurred within twenty four hours of weaning, the lamb remaining with the ewe for five days.

FIGURE 26 COMPARISON OF THE VARIATION IN ENDOGENOUS ACETALDEHYDE AND ETHANOL LEVELS WITH THE LEVELS OF PROGESTERONE IN PERIPHERAL VENOUS BLOOD OF A NON–PREGNANT EWE REPORTED IN THE LITERATURE



Blood was taken from the left jugular vein of the ewe and diluted 1:1 (by vol.) with diluent (n-propanol, 0.2cm^3/l; $HgCl_2$, 2mg/l). It was stored at 4^{o}C until analysis.

Assays of the ethanol and acetaldehyde concentrations in each sample were performed gas chromatographically as described in Chapter 3.

Absolute values for acetaldehyde and ethanol concentrations in the blood have been graphed, for every third day of sampling.

FIGURE 27 MEAN ENDOGENOUS ACETALDEHYDE AND ETHANOL LEVELS IN THE PERIPHERAL VENOUS BLOOD OF A EWE DURING AND FOLLOWING PREGNANCY

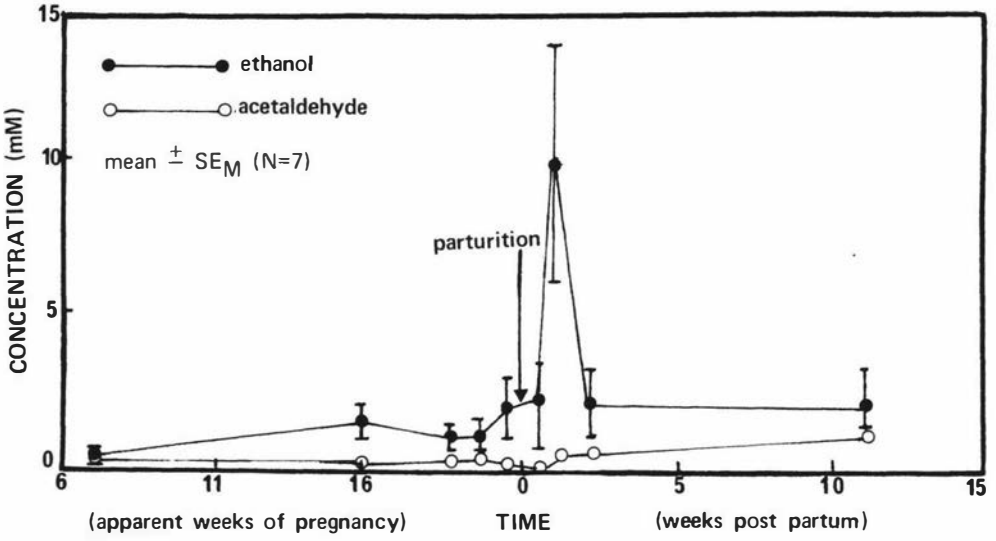
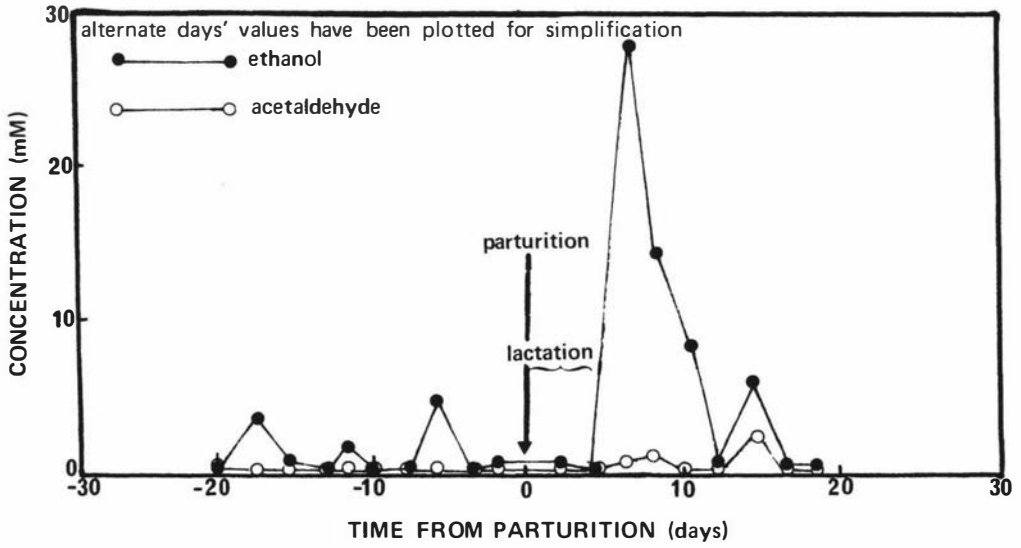


FIGURE 28 ABSOLUTE ENDOGENOUS ACETALDEHYDE AND ETHANOL LEVELS IN THE PERIPHERAL VENOUS BLOOD OF A PREGNANT EWE FOR THREE WEEKS BEFORE AND AFTER PARTURITION



Blood samples were taken daily from the left jugular vein of the ewe and diluted 1:1 (by vol.) with diluent (n-propanol, 0.2cm^3/l; $HgCl_2$, 2mg/l). They were stored at 4^{o}C until analysis.

Assays of the acetaldehyde and ethanol concentration in each sample were performed gas chromatographically as described in Chapter 3.

Progesterone levels rise during pregnancy to about the maximum oestral value (Short & Moore, 1959) and sheep synthesise about 2.7 mg total progesterone per day, in contrast to human production (250mg/day) during pregnancy. The corpus luteum normally produces the hormone but during pregnancy an extraovarian synthetic site is said to be involved (Short & Moore, 1959; Short, 1961).

Progesterone in the peripheral blood of the ewe falls rapidly within a few hours of birth (Short & Moore, 1959). The demonstrated increase in blood ethanol following parturition, seemed to be delayed by lactation and it is possible that the hormones responsible for lactation (oxytocin and lactating hormone) might interfere with other endocrine influences on ethanol metabolism.

DISCUSSION

High progesterone levels in sheep have been shown to correlate well with high liver aldehyde oxidase activities. The enhancement of enzyme activity was found to be independent of the progesterone concentration in the enzyme assay system and it occurred under normal physiological conditions as well in experimental animals where higher progesterone levels had been administered. Purified sheep liver aldehyde oxidase was also stimulated directly by progesterone, but the increased activity observed was insufficient to account for the aldehyde oxidase activities or the accelerated acetaldehyde metabolism observed in livers from progesterone-treated animals. It seemed that progesterone was causing some indirect increase in total liver aldehyde oxidase activity perhaps by enzyme induction or release from a regulatory control.

High levels of progesterone in the sheep were also associated with low endogenous ethanol concentrations in peripheral venous blood, and accelerated ethanol metabolism *in vivo*. This occurred when there was no apparent change in liver alcohol dehydrogenase activity during the administration of progesterone to experimental animals. It is possible that progesterone plays a physiological role by diverting acetaldehyde oxidation through the aldehyde oxidase pathway. Such a change in metabolism could accelerate ethanol oxidation by removing the product of alcohol dehydrogenase activity without involving NAD^+. This interpretation could also provide an explanation of the report that ethanol does not alter the redox state of pregnant women (Kesäniemi & Kurppa, 1971), since these women have elevated progesterone levels.

Chapter 5

EFFECT OF DISULPHIRAM AND OTHER COMPOUNDS ON ETHANOL AND ACETALDEHYDE METABOLISM

A number of compounds produce unpleasant clinical effects when consumed at the same time as ethanol. Among those in routine pharmacological use, tolazoline (a vasodilator), furazolidine (an antibacterial agent), chloral hydrate (a hypnotic), barbiturates (hypnotics and sedatives) and metronidazole (an antitrichomoniachal agent) have this effect (Forney & Hughes, 1968; Creaven & Roach, 1969; Trenholm et al, 1970; Evans & Caterall, 1971). Other compounds which cause an adverse reaction to ethanol are disulphiram (used in the rubber industry), cyanamide (an agricultural fertilizer) and an unknown component of the fungus Coprinus atramentarius. Flushing, giddiness, accelerated pulse rate, and increased respiratory rate and volume are predominant symptoms of this adverse effect (Hald et al, 1948).

Disulphiram (Hald et al, 1948), metronidazole (Semer et al, 1966) and cyanamide (Akabane, 1970) or its calcium derivative, calcium carbimide (Ferguson, 1956), have been used as deterrents to drinking in the treatment of alcoholics.

This study was planned to show the effect of disulphiram on ethanol and acetaldehyde metabolism in sheep. The effects of diazepam and amitryptyline, alone or in conjunction with disulphiram, were also examined and some preliminary experiments using metronidazole were performed.

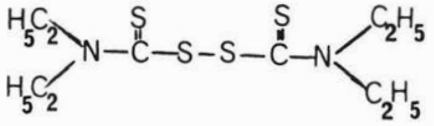
Disulphiram: Disulphiram (structure, Fig.29) is a widely used deterrent drug which is only slowly eliminated from the body (Hald et al, 1948; Hald & Jacobsen, 1948b; Eldjarn, 1950; Martindale, 1958). The compound is toxic in large doses, resulting in anorexia, muscle wasting, decreased body temperature and paralysis. The LD_{50} for rats is 8.6g/kg (Child, 1950; Child et al, 1951).

The normal therapeutic dose for adults, 0.5-1.5 g/day, causes little toxicity (Hald et al, 1948) though some side effects may arise; fatigue, gastrointestinal upsets, anxiety and decreased mental acuity and sexual potency (Child, 1950; Child et al, 1951; Graham, 1951). Increased blood lactate (Jokivartio, 1950) and decreased liver respiration in vitro (Edwards, 1949) caused by disulphiram have been claimed to account for some of these effects, and it has been reported that denaturation of serum protein (Burnett & Reading, 1969), depressed

FIGURE 29 STRUCTURES OF SOME COMPOUNDS THAT AFFECT ACETALDEHYDE AND ETHANOL METABOLISM

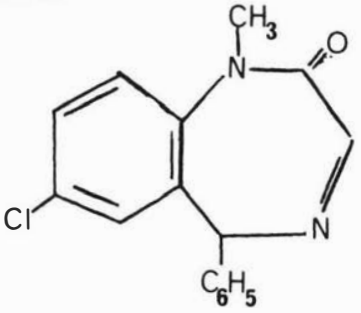
Disulphiram

Trade Name "Antabuse"



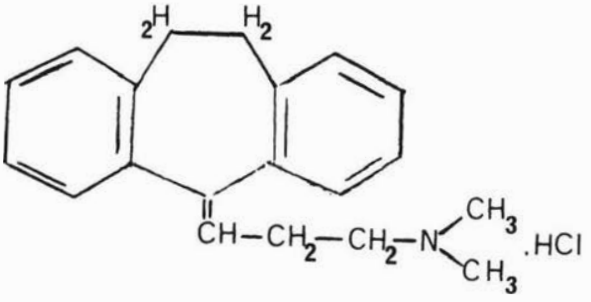
Diazepam

Trade Name "Valium"



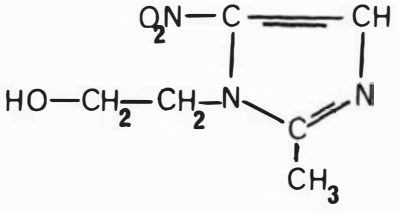
Amitryptyline Hydrochloride

Trade Name "Tryptanol"



Metronidazole

Trade Name "Flagyl"



cardiac activity (Ikomi, 1956a) and decreased thyroid secretion (Wase & Christensen, 1954) may also result.

Disulphiram inhibits several enzymes, including dopamine-β-hydroxylase and microsomal mixed function oxidases (Vessell et al, 1971). The disulphiram inhibition of those enzymes catalyzing aldehyde oxidation, aldehyde oxidase (Kjeldgaard, 1949), aldehyde dehydrogenase (Graham, 1951) and xanthine oxidase (Hunter & Lowry, 1956), appears to cause acetaldehyde accumulation after ingestion of ethanol. Two to ten-fold increases in blood acetaldehyde concentrations during ethanol metabolism when disulphiram is present have been attributed entirely to decreased acetaldehyde oxidation, not to an increased rate of ethanol metabolism (Hald & Jacobsen, 1948a; Hald et al, 1949; Barrera et al, 1950; Hine et al, 1950; Jacobsen, 1950; Newman & Petzold, 1951; Hine et al, 1952; Ikomi, 1956b).

Although most of the toxic reaction to ethanol in disulphiram-treated patients is caused by the high acetaldehyde concentrations that arise in the body, several of the effects are not directly attributable to acetaldehyde (Hald & Jacobsen, 1948a). It has been suggested that a toxic ethanol-disulphiram compound (Fujiwara & Niigata, 1953) or a quaternary ammonium compound (Burnett & Reading, 1969) may be responsible for these.

Diazepam and Amitryptyline: Diazepam and amitryptyline have been shown to alter the clinical reaction to ethanol in the presence of disulphiram (MacCallum, 1969).

Diazepam (structure, Fig. 29) is a tranquiliser often used to treat withdrawal symptoms and severe ethanol intoxication in alcoholics (Glatt, 1970; Guerro-Figueroa et al, 1970; Lundquist, 1970a; Burns, 1972) but it does not affect ethanol metabolism in these people (Forney & Hughes, 1968).

Amitryptyline is a central nervous system stimulant (Forney & Hughes, 1968) usually administered as amitryptyline hydrochloride (structure, Fig.29). There appear to be few reports of the effect of amitryptyline on ethanol metabolism but it does decrease the duration of ethanol-induced sleep in mice (Khanna & Kalant, 1970).

Metronidazole: Metronidazole (structure, Fig. 29) has been used by several workers in attempts to deter alcoholics from drinking ethanol (Semer et al, 1966; Lehmann & Ban, 1967; Friedland & Vraisberg, 1968). Some authors have queried its effectiveness (Goodwin, 1967; Linton & Hain, 1967; Gallant et al, 1968; Gelder & Edwards, 1968; Strassman et al, 1970) but the compound has been reported to decrease ethanol

consumption in rats (Campbell et al, 1967). Metronidazole has been shown to inhibit alcohol dehydrogenase and xanthine oxidase from rats (Fried & Fried, 1966) and alcohol dehydrogenase from humans (Edwards & Price, 1967).

METHODS

Enzyme Preparation and Assay: Sheep liver aldehyde oxidase was prepared for use in in vitro studies following the method described in Chapter 4. Dialysis after the ammonium sulphate fractionation steps gave enzyme preparations which had 30-40 times the activity in the original liver extract, and contained no alcohol dehydrogenase or aldehyde dehydrogenase activities. Xanthine oxidase activity was reduced to less than 5% that in the original extract.

Unless otherwise stated, aldehyde oxidase was assayed by following the rate of oxygen uptake using acetaldehyde (23mM) as substrate. The reaction was carried out in borate buffer (pH 8.5) at 37°C (Chapter 2). Inhibitors were incubated with the enzyme for five minutes prior to the addition of substrate.

Sheep liver aldehyde dehydrogenase was purified 53 times using the method described in Appendix I. Preparations contained no alcohol dehydrogenase, aldehyde oxidase or xanthine oxidase activities. The enzyme assays measured the rate of reduction of NAD^+ by following the change in optical density at 340nm. They were performed in pyrophosphate buffer (pH 9.3; 0.01M) at 22°C using acetaldehyde (0.23mM) as the substrate (Appendix I).

In Vivo experiments used ewes (approx.50kg) fed a maintenance diet of chopped hay (digestibility 53% (Davey, 1972)) and commercial sheep nuts. Disulphiram, diazepam and amitryptyline hydrochloride were administered orally, each treatment lasting ten to fourteen days.

The compounds were administered to each animal sequentially in the order:-

control
disulphiram (16mg/kg/day)
disulphiram (16mg/kg/day) and diazepam (0.8mg/kg/day) together
disulphiram (16mg/kg/day)
disulphiram (16mg/kg/day) and amitryptyline hydrochloride (1.2mg/kg/day) together
disulphiram (16mg/kg/day)
control

Diazepam and amitryptyline hydrochloride controls were administered in a separate experiment in the sequence:-

control
diazepam (0.8mg/kg/day)
control
amitryptyline hydrochloride (1.2mg/kg/day).

Metronidazole (8mg/kg/day) was administered in a further separate experiment.

Each treatment was continued for two days before blood samples were taken for analysis. Experiments requiring intravenous administration of exogenous ethanol were performed on the tenth day of each treatment.

Blood samples ($1cm^3$) were taken from the left jugular vein daily, diluted (1:1 by vol.) with diluent (n-propanol, $0.2cm^3/l$; $HgCl_2$, 2mg/l) and stored at 4^oC. Each sample was assayed for acetaldehyde, ethanol and acetone using the gas-chromatographic method described in Chapter 3.

Statistics: The significances of the differences quoted were determined by the t-test.

RESULTS

I) DISULPHIRAM, DIAZEPAM AND AMITRYPTYLINE

A three-pronged approach was adopted in this study of the effects of disulphiram, diazepam and amitryptyline on ethanol and acetaldehyde metabolism in sheep. This involved investigation of

i) Effects on Sheep Liver Enzymes (Aldehyde Oxidase and Aldehyde Dehydrogenase) in vitro
ii) Effects on Endogenous Levels of Acetaldehyde, Ethanol and Acetone in Peripheral Venous Blood in vivo
iii) Effects on the Clearance of Exogenous Ethanol from Peripheral Venous Blood in vivo

i) Effects of Disulphiram, Diazepam and Amitryptyline Hydrochloride on Sheep Liver Enzymes in vitro

Sheep Liver Aldehyde Oxidase: Disulphiram Effect: Inhibition of sheep liver aldehyde oxidase activity by disulphiram had previously been shown (Chapter 2, Table 7) and this effect was specific for the coupling of aldehyde oxidation to reduction of cytochrome c and molecular oxygen. Further study has shown the disulphiram inhibitor constant to be 1.6mM (Fig.30) and inhibition was non-competitive.

Sheep Liver Aldehyde Oxidase: Diazepam Effect: Diazepam alone was shown to have no effect on the "oxidase" reaction of sheep liver aldehyde oxidase in vitro but it did appear to protect the enzyme from

FIGURE 30 INHIBITOR CONSTANT[a] FOR DISULPHIRAM EFFECT ON SHEEP LIVER ALDEHYDE OXIDASE[b]

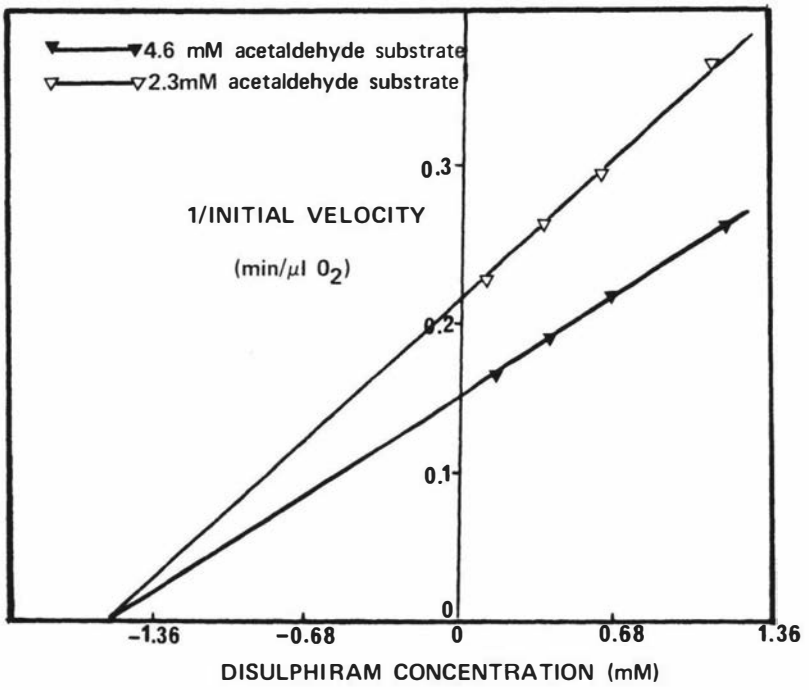
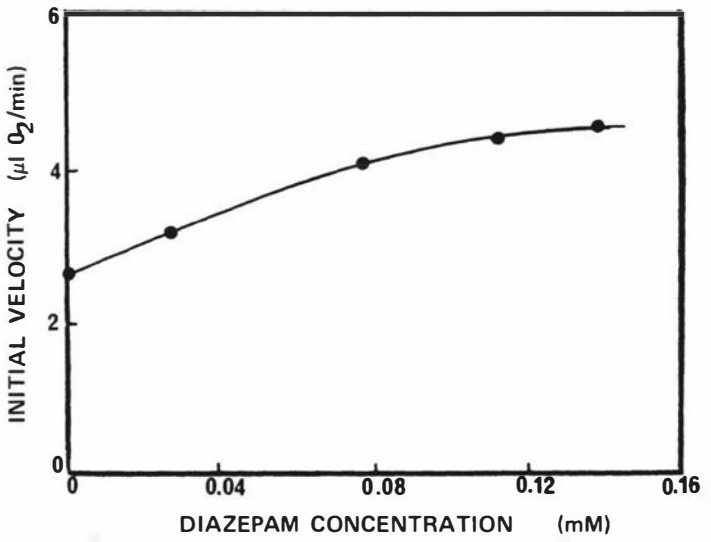


FIGURE 31 GRAPH TO SHOW THE EFFECT OF VARYING DIAZEPAM CONCENTRATIONS ON DISULPHIRAM (1.25mM) INHIBITION OF SHEEP LIVER ALDEHYDE OXIDASE[b]



a) Determination of the Inhibitor Constant used the Dixon method (1953)

b) Assays were performed at pH 8.5 (borate buffer) and 37°C. The assay system used molecular oxygen as the hydrogen acceptor and is described in Chapter 2. Unless otherwise stated acetaldehyde (23mM) was used as the substrate.

Each experiment was performed in duplicate with two different enzyme preparations, and the result plotted is the mean value.

disulphiram inhibition (Table 19).

An experiment to show the effect of varying the diazepam concentration in the assay system, while maintaining the disulphiram concentration constant, demonstrated that complete protection was obtained with diazepam levels which were one-tenth those of disulphiram (Fig.31). It would seem therefore that diazepam does not react directly with the disulphiram to prevent its inhibition of aldehyde oxidase.

Sheep Liver Aldehyde Oxidase: Amitryptyline Effect: Amitryptyline hydrochloride inhibited oxygen uptake catalyzed by sheep liver aldehyde oxidase (Table 19) and was further shown to act non-competitively, with an inhibitor constant of 17.4mM.

Increased inhibition was observed when disulphiram was present in the assay system (Table 19) and a synergystic effect between the two compounds, amitryptyline hydrochloride and disulphiram, seemed possible. Addition of the percentage inhibition shown in separate disulphiram and amitryptyline assays gave values less than the figures recorded when the same concentrations were used together in the aldehyde oxidase assay system (Fig. 32).

Sheep Liver Aldehyde Dehydrogenase: Disulphiram Effect: Sheep liver aldehyde dehydrogenase was inhibited by disulphiram *in vitro* (Table 20), but the concentration required to cause 56% inhibition was about one thousand times that required for a similar effect on the bovine enzyme preparation (Deitrich & Hellerman, 1963).

Sheep Liver Aldehyde Dehydrogenase: Diazepam Effect: Diazepam (0.18mM) appeared to be a weak inhibitor of sheep liver aldehyde dehydrogenase (Table 20), and it did not affect the disulphiram inhibition of the enzyme at a concentration similar to that effective with aldehyde oxidase.

Sheep Liver Aldehyde Dehydrogenase: Amitryptyline Effect: Amitryptyline hydrochloride (9.6mM) was a potent inhibitor of sheep liver aldehyde dehydrogenase activity *in vitro*, both in the presence and absence of disulphiram (Table 20).

ii) Effects of Disulphiram, Diazepam and Amitryptyline Administration *in vivo* on Endogenous Acetaldehyde, Ethanol and Acetone Levels in Peripheral Venous Blood of Sheep

Endogenous Acetaldehyde: Disulphiram administration to sheep more than doubled the concentration of endogenous acetaldehyde in the peripheral venous blood ($p<0.01$) (Fig.33), an increase very similar to that reported in humans under disulphiram treatment (Hine *et al*, 1952).

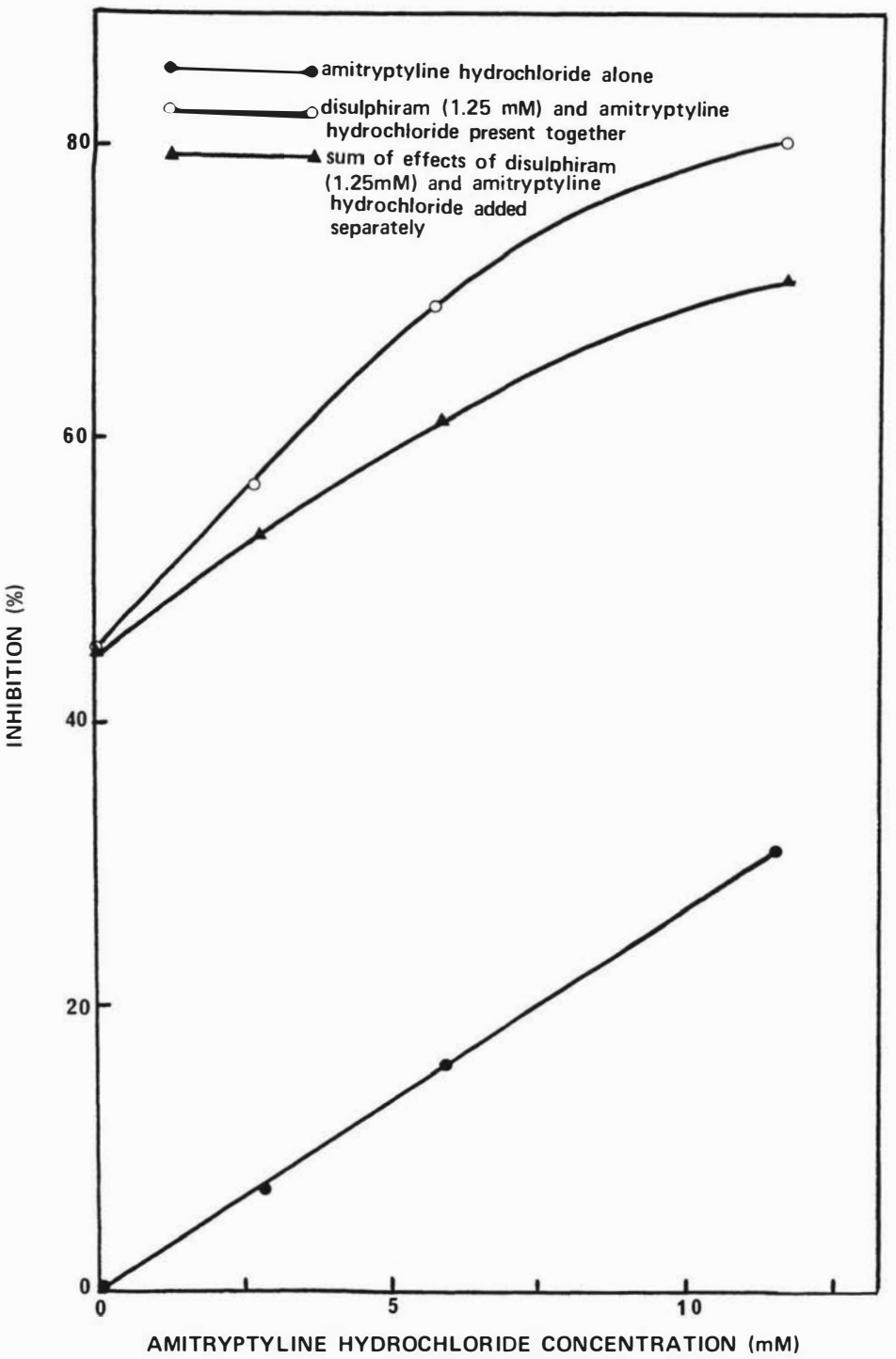
TABLE 19 Effect of Several Compounds on Sheep Liver Aldehyde Oxidase Activity

Compound Added	Relative Activity
Nil	100
Disulphiram (1.25mM)	55
Diazepam (0.13mM)	99
Amitryptyline Hydrochloride (10.5mM)	76
Disulphiram (1.25mM) and Diazepam (0.13mM)	98
Disulphiram (1.25mM) and Amitryptyline Hydrochloride (10.5mM)	20

The enzyme activity in the absence of inhibitors has been arbitarily set at 100, which corresponds to a mean rate of oxygen uptake equal to 0.22 $\mu l O_2$/min/mg protein in the standard assay system (Chapter 2). Assays were performed at pH 8.5 (borate buffer) and 37°C, with acetaldehyde (23mM) as the substrate (Chapter 2).

Each assay was performed in duplicate with two different enzyme preparations, and the result quoted is the mean value.

FIGURE 32 INHIBITION OF SHEEP LIVER ALDEHYDE OXIDASE BY AMITRYPTYLINE HYDROCHLORIDE WITH AND WITHOUT DISULPHIRAM PRESENT



Values are expressed as % decrease in the initial rate of oxygen uptake catalyzed by sheep liver aldehyde oxidase (42 units/assay, mean specific activity =27.6 units/mg. protein) at pH 8.5 (borate buffer) and 37°C, with acetaldehyde (23mM) as substrate (Chapter 2).

Each assay was performed in duplicate with two different enzyme preparations and the result plotted is the mean.

TABLE 20 Effect of Several Compounds on Sheep Liver Aldehyde Dehydrogenase Activity

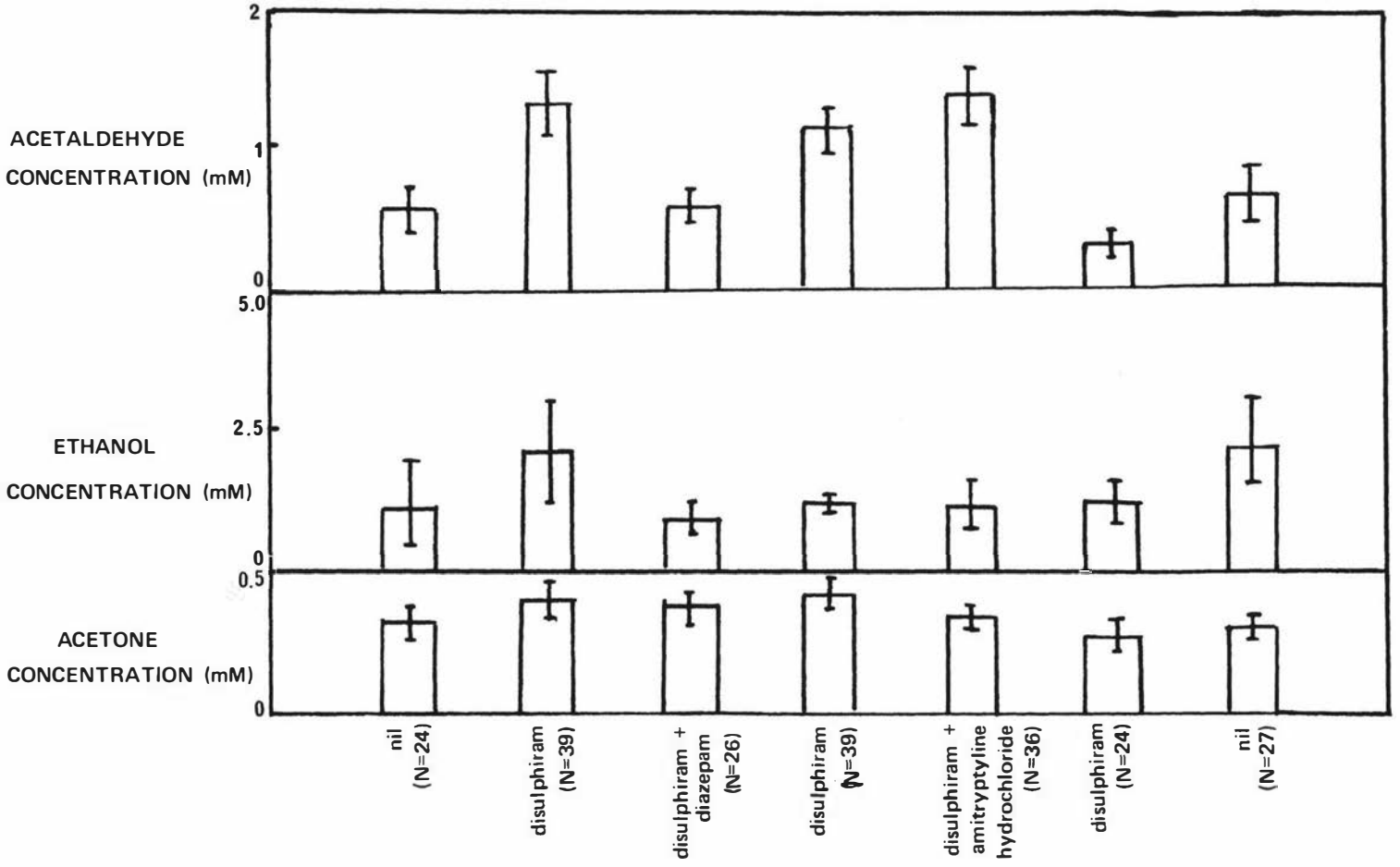
Compound Added	Relative Activity
Nil	100
Disulphiram (1.7mM)	44
Diazepam (0.18mM)	73
Amitryptyline Hydrochloride (9.6mM)	0
Disulphiram (1.7mM) and Diazepam (0.18mM)	42
Disulphiram (1.7mM) and Amitryptyline Hydrochloride (9.6mM)	0

The enzyme activity in the absence of inhibitors has been arbitarily set at 100, which corresponds to a mean change in OD_{340nm} of 0.029/10sec/mg protein.

Assays were performed at pH 9.3 (0.01M pyrophosphate buffer) and 22°C, with acetaldehyde (0.23mM) as the substrate (Appendix I).

Each assay was performed in duplicate with two different enzyme preparations, and the result quoted is the mean value.

FIGURE 33 EFFECTS OF DISULPHIRAM, DIAZEPAM AND AMITRYPTYLINE HYDROCHLORIDE ADMINISTRATION IN VIVO ON ENDOGENOUS ACETALDEHYDE, ETHANOL AND ACETONE LEVELS IN PERIPHERAL VENOUS BLOOD OF SHEEP



Blood samples ($1cm^3$) were taken from the left jugular vein prior to feeding each day. They were diluted 1:1 (by vol) with diluent (n-propanol, $0.2cm^3/l$; $HgCl_2$, 2mg/l) and stored at 4^oC.

Assays for acetaldehyde, ethanol and acetone were performed on the gas chromatograph (Chapter 3).

Each value is the mean of all samples collected from the three ewes receiving treatment and is expressed as mean $\pm SE_M$.

Within twenty-four hours of beginning diazepam administration in conjunction with disulphiram, the endogenous acetaldehyde levels had dropped to their basal values ($p<0.01$) (Fig.33), and when diazepam was withdrawn after ten days administration, endogenous acetaldehyde concentrations again rose ($p<0.01$).

Administration of amitryptyline hydrochloride together with disulphiram had no significant effect on the already high endogenous acetaldehyde levels in the sheep (Fig.33), but its withdrawal resulted in a large decrease in blood acetaldehyde within two days ($p<0.01$),despite maintenance of disulphiram administration. The new acetaldehyde concentration was similar to the basal level and some metabolic compensation may have been made by the animals for reduced acetaldehyde oxidation rates caused by disulphiram and amitryptyline together.

Withdrawal of disulphiram had no effect on the already low endogenous acetaldehyde levels.

Endogenous Ethanol and Acetone: Administration of disulphiram had no significant effect on the endogenous ethanol and acetone concentrations in peripheral venous blood of the sheep (Fig.33). The administration of diazepam or amitryptyline in conjunction with disulphiram did not alter this effect but the final withdrawal of disulphiram did cause some increase in endogenous ethanol concentrations ($p<0.05$).

Effect of Diazepam or Amitryptyline Administered Alone: Control experiments in which diazepam or amitryptyline hydrochloride was administered alone to the sheep, showed that both these compounds affected the endogenous constituents of peripheral venous blood *in vivo*.

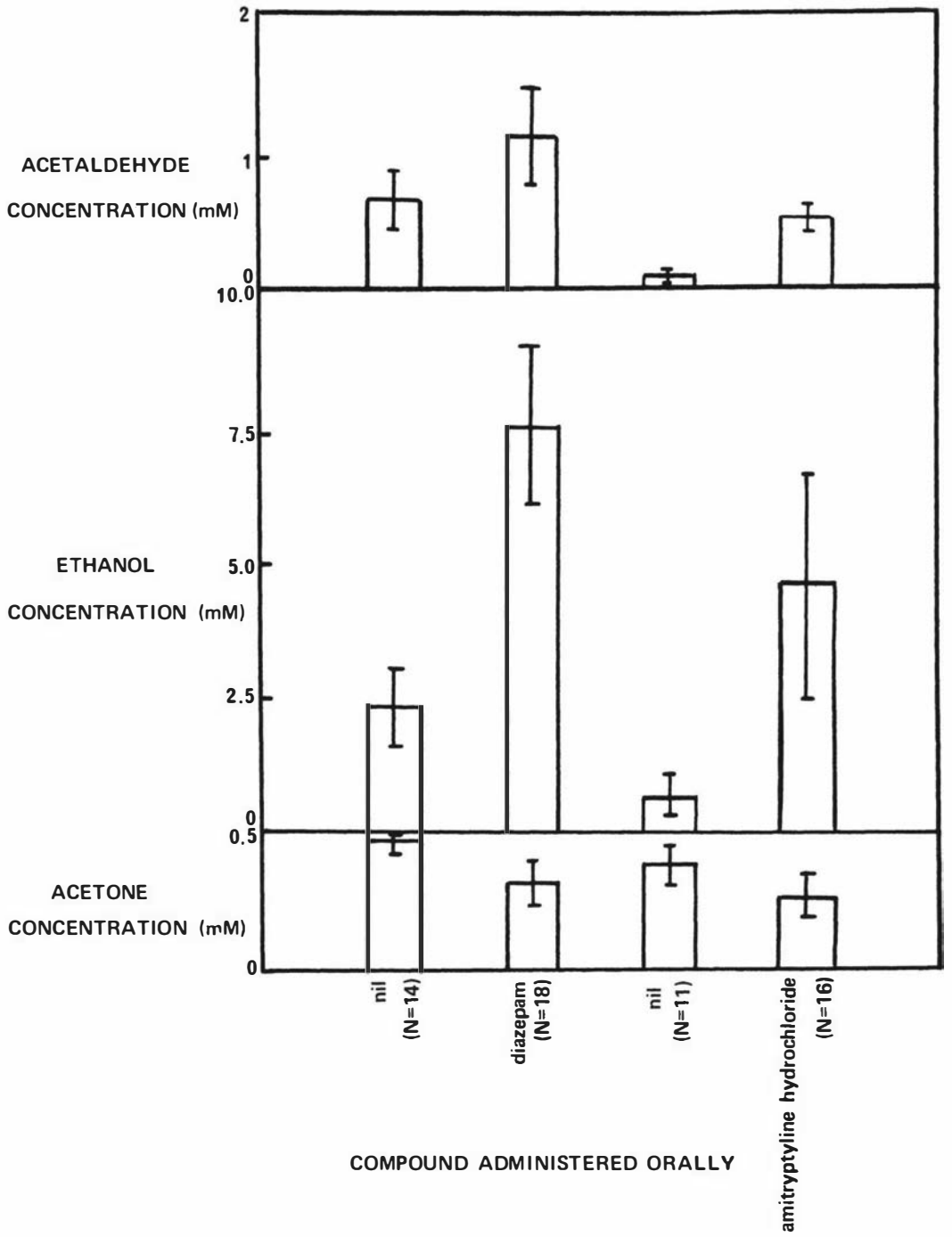
Oral administration of diazepam tripled the endogenous ethanol levels in the animals ($p<0.01$) (Fig.34), the increase being maximum within seventy-two hours of beginning the treatment. Although diazepam did not affect endogenous acetaldehyde or acetone concentrations, its withdrawal decreased the acetaldehyde levels ($p<0.01$) together with the ethanol concentration ($p<0.01$).

It was found that amitryptyline administration increased the endogenous acetaldehyde in venous blood within twenty-fours of beginning treatment ($p<0.01$). An apparent increase in blood ethanol was not considered significant because of variations between individual samples during amitryptyline treatments.

iii) Effects of Disulphiram, Diazepam and Amitryptyline on the Clearance of Exogenous Ethanol from Peripheral Venous Blood of Sheep

A study of the effects of several combinations of compounds on

FIGURE 34 EFFECTS OF DIAZEPAM AND AMITRYPTYLINE HYDROCHLORIDE ADMINISTRATION IN VIVO ON ENDOGENOUS ACETALDEHYDE, ETHANOL AND ACETONE LEVELS IN PERIPHERAL VENOUS BLOOD OF SHEEP



Blood samples ($1cm^3$) were taken from the left jugular vein prior to feeding each day. They were diluted 1:1 (by vol) with diluent (n-propanol, $0.2cm^3/l$; $HgCl_2$,2mg/l) and stored at 4^oC.

Assays for acetaldehyde, ethanol and acetone were performed on the gas chromatograph (Chapter 3).

Each value is the mean of all samples collected from the two ewes receiving treatment and is expressed as mean $\pm$ SE_M.

exogenous ethanol metabolism in sheep was designed to extend and supplement those results obtained in the investigation of the changes in the endogenous components of blood.

Effects of Administration of Disulphiram alone, and in Combination with Diazepam: Acetaldehyde accumulated in the blood of disulphiram-treated sheep during metabolism of exogenous ethanol (Fig.35). This differed from the results obtained for untreated animals, and for those receiving diazepam and disulphiram concurrently. There appeared to be some reduction in the ethanol oxidation rate in sheep under disulphiram treatment, possibly due to displacement of the alcohol dehydrogenase equilibrium, and acetone levels were not affected by administration of either disulphiram or diazepam.

Effects of Administration of Disulphiram alone, and in Combination with Amitryptyline: Acetaldehyde accumulation in sheep during *in vivo* ethanol metabolism was about the same in animals treated with disulphiram alone and those receiving amitryptyline and disulphiram together (Fig. 36). Ethanol disappearance may have been slower during treatment with amitryptyline and disulphiram together, and acetone levels were not influenced by either treatment.

When amitryptyline was withdrawn, acetaldehyde concentrations in blood during ethanol metabolism decreased despite maintenance of disulphiram administration. The acetaldehyde levels were lower than those appearing during ethanol metabolism under previous disulphiram treatments (Fig.35, Fig.36), supporting the previous suggestion that some mechanism exists to adapt acetaldehyde metabolism to the presence of amitryptyline and disulphiram together.

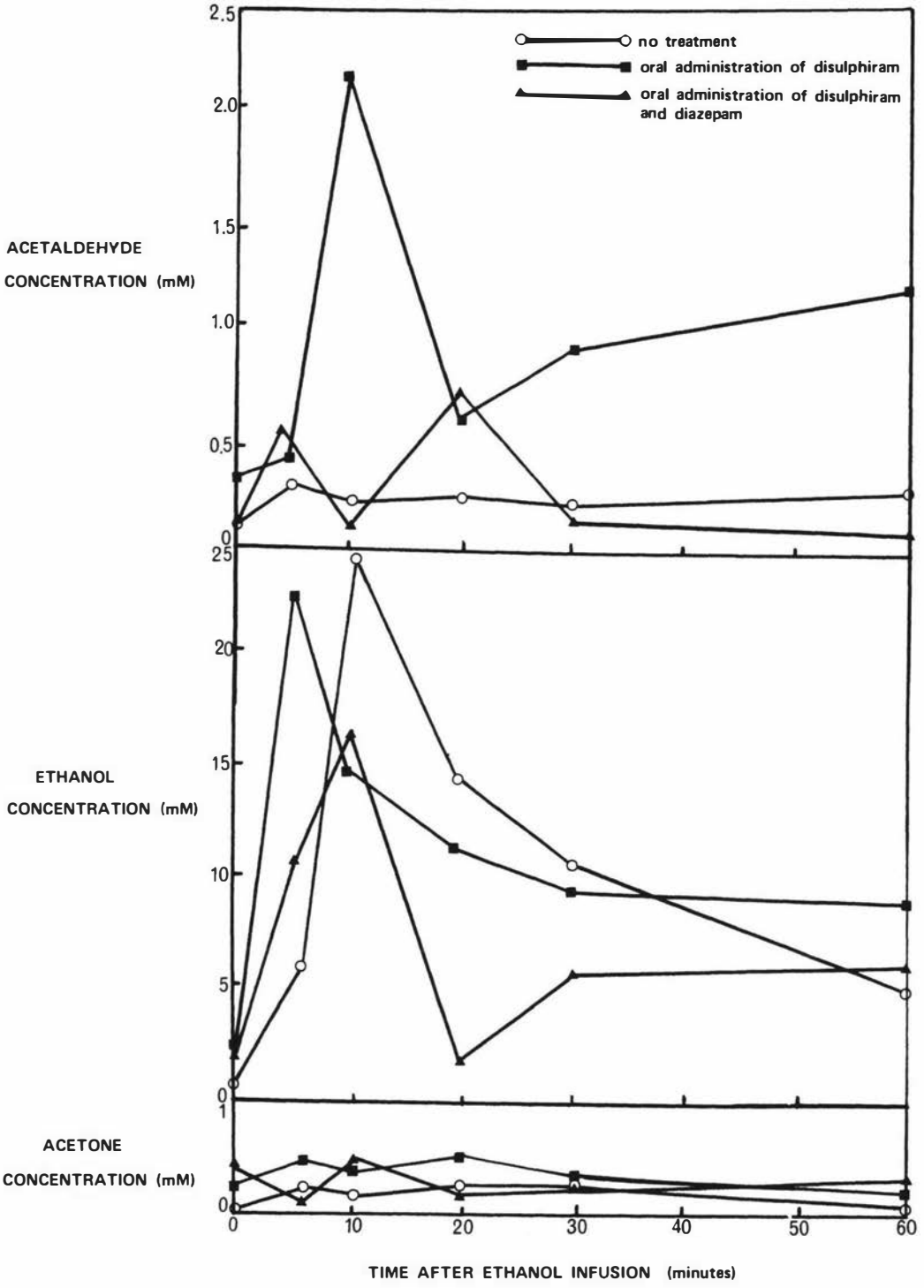
Effects of Administration of Diazepam or Amitryptyline alone: Diazepam administered alone had little effect on acetaldehyde levels in sheep blood during *in vivo* ethanol metabolism. Although its presence did not alter ethanol concentrations either, withdrawal of the diazepam accelerated the change in ethanol concentration from a maximum of 0.7mM/min(N=2) to 1.8mM/min(N=2).

Amitryptyline hydrochloride administered alone caused acetaldehyde accumulation to 1.15mM(N=2) thirty minutes after ethanol administration. It had little effect on ethanol or acetone concentrations in sheep blood during ethanol metabolism *in vivo*.

II) METRONIDAZOLE

Some preliminary experiments were performed to examine the effect of metronidazole on ethanol metabolism.

FIGURE 35 EFFECTS OF ORAL ADMINISTRATION OF DISULPHIRAM, AND DIAZEPAM AND DISULPHIRAM TOGETHER ON IN VIVO CLEARANCE OF EXOGENOUS ETHANOL FROM PERIPHERAL VENOUS BLOOD OF SHEEP



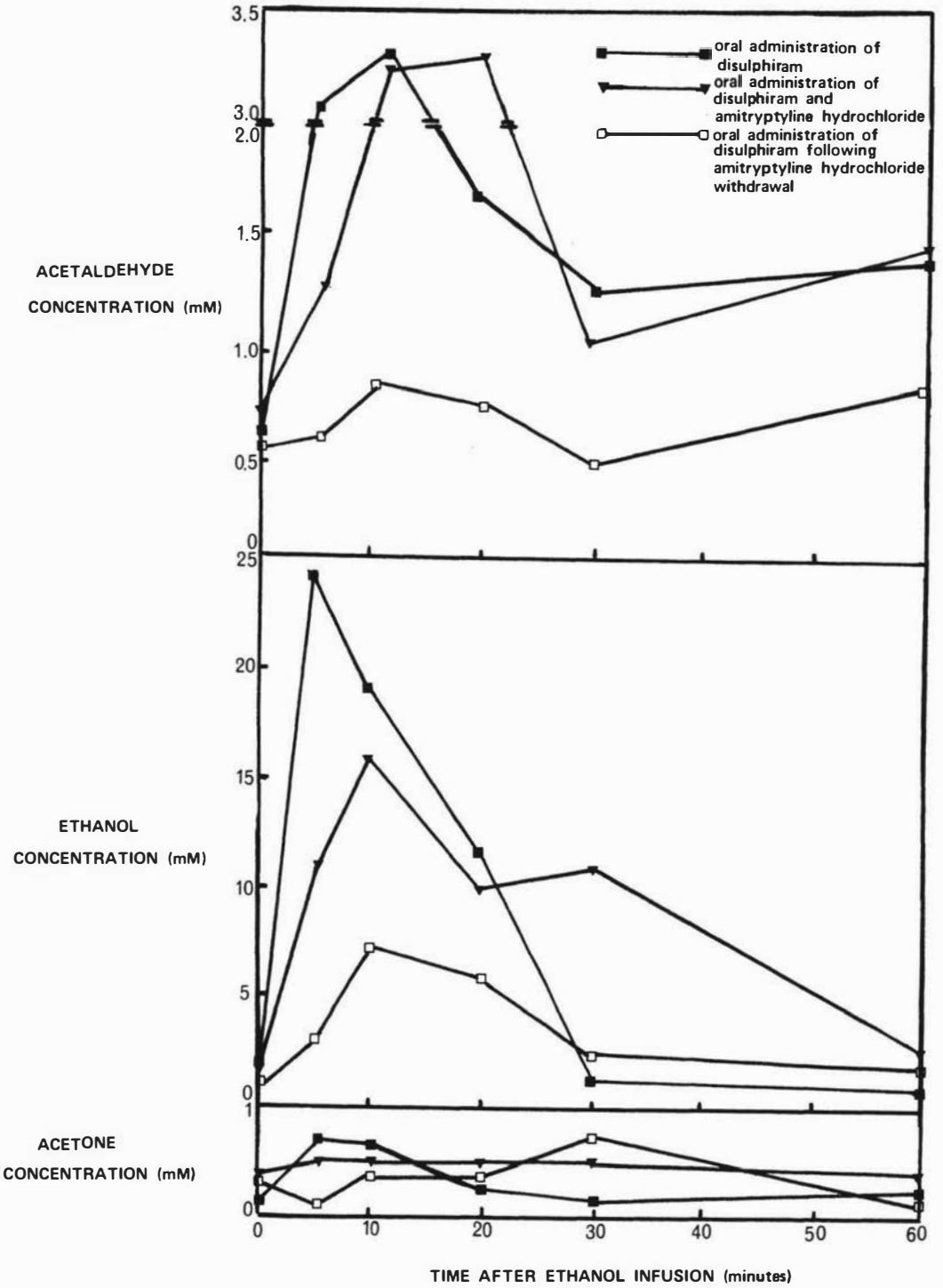
Ethanol (20mmoles) in 10cm^3 sterile physiological saline was infused into the lower left jugular vein immediately after the 'zero' blood sample had been taken. Infusion was complete in less than 30 seconds and time was taken from its completion.

Blood samples (1cm^3) were taken from the left jugular vein of the ewes at the times indicated. Each was divided into two 0.5cm^3, duplicate samples and diluted (1:1 by vol.) with diluent (n-propanol, 0.2cm^3/l; $HgCl_2$, 2mg/l). The samples then were stored at 4°C until analysis.

Assays of the acetaldehyde, ethanol and acetone concentrations in each sample were performed gas chromatographically as described in Chapter 3.

Three ewes were subjected to each treatment and each point plotted is the mean of the concentration of each component at that particular time after ethanol infusion.

FIGURE 36 EFFECTS OF ORAL ADMINISTRATION OF DISULPHIRAM, AND AMITRYPTYLINE HYDROCHLORIDE AND DISULPHIRAM TOGETHER ON IN VIVO CLEARANCE OF EXOGENOUS ETHANOL FROM PERIPHERAL VENOUS BLOOD OF SHEEP



Ethanol (20mmoles) in 10cm^3 sterile physiological saline was infused into the lower left jugular vein immediately after the 'zero' blood sample had been taken. Infusion was complete in less than 30 seconds and time was taken from its completion.

Blood samples (1cm^3) were taken from the left jugular vein of the ewes at the times indicated. Each was divided into two 0.5cm^3, duplicate samples and diluted (1:1 by vol.) with diluent (n-propanol, 0.2cm^3/l; $HgCl_2$, 2mg/l). The samples then were stored at 4^{o}C until analysis.

Assays of the acetaldehyde, ethanol and acetone concentrations in each sample were performed gas chromatographically as described in Chapter 3.

Three ewes were subjected to each treatment and each point plotted is the mean of the concentration of each component at that particular time after ethanol infusion.

i) Effect of Metronidazole on Sheep Liver Aldehyde Oxidase and Aldehyde Dehydrogenase in vitro: Metronidazole inhibited the reactions of all the hydrogen acceptors tested which couple with sheep liver aldehyde oxidase activity (Chapter 2, Table 7).

Sheep liver aldehyde dehydrogenase activity was decreased by 35% in the presence of metronidazole (0.3mM), in contrast to the finding of Fried and Fried (1966) that the compound had no effect on rat liver aldehyde dehydrogenase.

ii) Effect of Metronidazole Administration in vivo on Endogenous Acetaldehyde, Ethanol and Acetone Levels in Peripheral Venous Blood of Sheep: Administration of metronidazole (8mg/kg/day) to ewes was found to elevate ($p<0.01$) the acetaldehyde concentration in peripheral venous blood to some seven times the control level (Table 21). Endogenous ethanol levels also tended to rise but variability in the control ethanol values reduced the significance of these results. Acetone concentrations remained unchanged by metronidazole treatment.

Withdrawal of metronidazole resulted in decreased ($p<0.05$) endogenous acetaldehyde concentrations in the peripheral venous blood of sheep, though they did not revert to their basal level, and ethanol concentrations also tended to be lower.

iii) Effect of Metronidazole on the Clearance of Exogenous Ethanol from Peripheral Venous Blood of Sheep: The influence exerted by metronidazole on the clearance pattern of ethanol from sheep blood is shown in Figure 37. It seemed to have slowed acetaldehyde oxidation over the first twenty minutes after ethanol infusion, and ethanol metabolism may be decreased. This was anticipated from the alcohol dehydrogenase inhibition reported in monogastric animals (Fried & Fried, 1966; Edwards & Price, 1967), and it may have reduced acetaldehyde accumulation at later sampling times. Acetone levels during ethanol metabolism were less variable in the metronidazole-treated animal.

DISCUSSION

The results presented in this chapter of the thesis have shown that disulphiram altered ethanol and acetaldehyde metabolism in sheep, in both *in vitro* and *in vivo* experiments. Sheep liver aldehyde oxidase was subject to inhibition by disulphiram at inhibitor concentrations similar to those reported to inhibit bovine liver xanthine oxidase (Hunter & Lowry, 1956). Disulphiram was a much less potent inhibitor

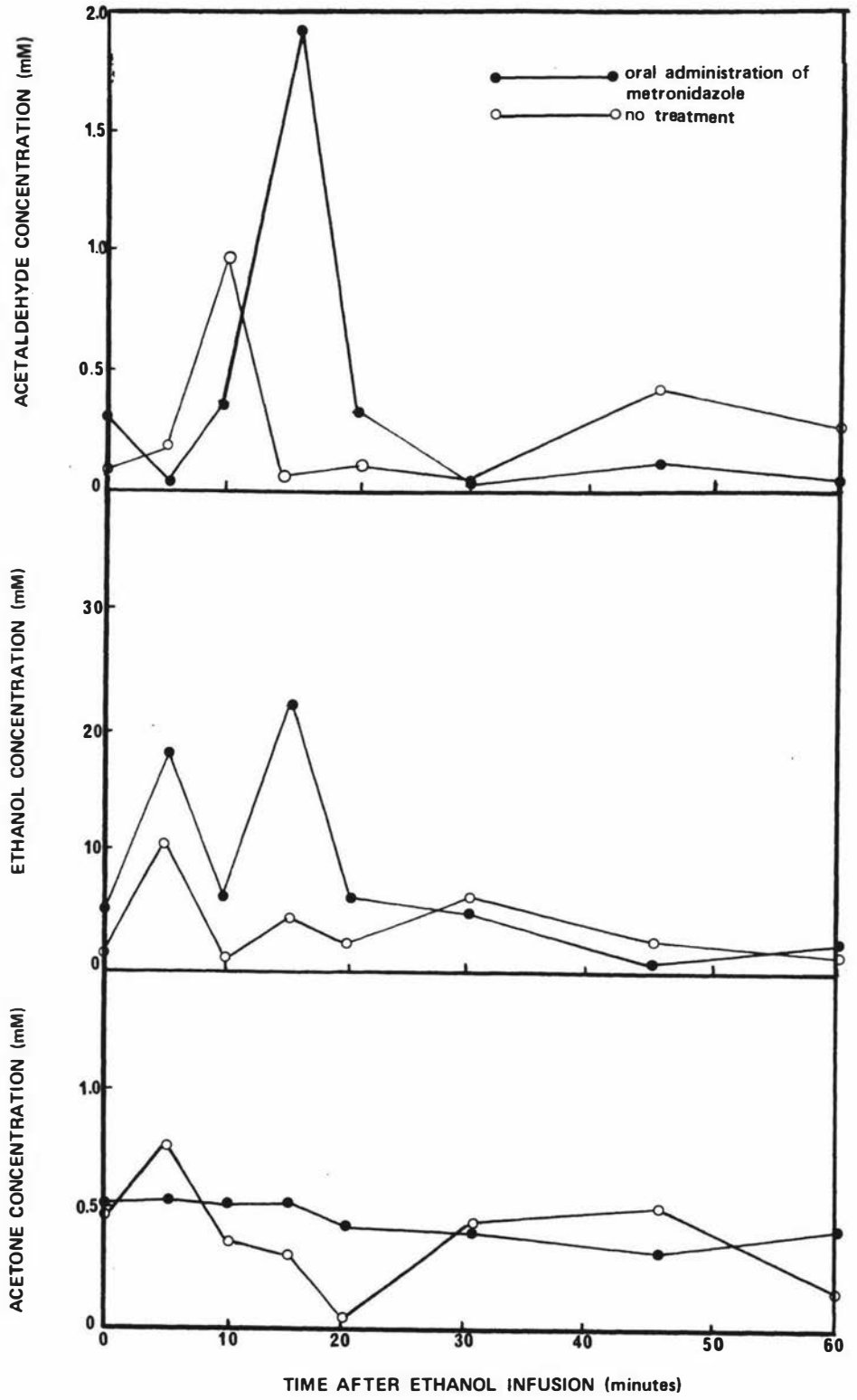
TABLE 21 Effect of Administration of Metronidazole on Endogenous Levels of Ethanol, Acetaldehyde and Acetone in Sheep Blood

Treatment		Mean ± SE_M
Nil (N=8)	Acetaldehyde	= 0.19 ± 0.15 mM
	Ethanol	= 2.66 ± 1.24 mM
	Acetone	= 0.56 ± 0.08 mM
Metronidazole (N=8)	Acetaldehyde	= 1.29 ± 0.56 mM
	Ethanol	= 5.03 ± 1.36 mM
	Acetone	= 0.45 ± 0.07 mM
Nil (N=7)	Acetaldehyde	= 1.01 ± 0.30 mM
	Ethanol	= 2.12 ± 1.24 mM
	Acetone	= 0.43 ± 0.21 mM

Blood samples ($1cm^3$) were taken from the left jugular vein prior to feeding each day. They were diluted (1:1 by vol.) with diluent (n-propanol, $0.2cm^3/l$; $HgCl_2$, 2mg/l) and stored at $4^{\circ}C$.

Assays for acetaldehyde, ethanol and acetone were performed on the gas-chromatograph (Chapter 3).

FIGURE 37 EFFECTS OF ORAL ADMINISTRATION OF METRONIDAZOLE ON IN VIVO CLEARANCE OF EXOGENOUS ETHANOL FROM THE PERIPHERAL VENOUS BLOOD OF SHEEP



Ethanol (20mmoles) in 10cm^3 physiological saline was infused into the lower left jugular vein immediately after the 'zero' blood sample had been taken. Infusion was complete in 30 seconds and time was taken from its completion.

Blood samples (1cm^3) were taken from the left jugular vein of the ewe at the time indicated. Each was divided into two 0.5cm^3, duplicate samples and diluted (1:1 by vol.) with diluent (n-propanol, 0.2 cm^3/l; Hg Cl_2, 2mg/l). The samples were then stored at 4^{o}C until analysis.

Assays of the acetaldehyde, ethanol and acetone concentrations were performed gas chromatographically as described in Chapter 3.

of sheep liver aldehyde dehydrogenase than that reported for bovine liver aldehyde dehydrogenase (Deitrich & Hellerman, 1963).

Endogenous acetaldehyde levels in sheep blood were approximately thirty times those reported by Hine and his associates (1952) for humans, and ethanol concentrations were about the same in both species. Disulphiram administration *in vivo* raised the endogenous acetaldehyde concentration to 2.4 times the controls in these experiments with sheep, three times the controls in the earlier work with humans (Hine *et al*, 1952), and the tendency toward increased ethanol levels was also very similar in sheep and humans. Metabolism of exogenous ethanol *in vivo* by sheep was altered by disulphiram in a manner similar to that reported in humans, and both sheep and humans showed decreased rates of ethanol oxidation and the same proportional increases in acetaldehyde accumulation in the blood.

Therefore, overall ethanol and acetaldehyde metabolism in sheep and its alteration by disulphiram seemed qualitatively very similar to those in humans. Quantitative differences in acetaldehyde concentrations may have arisen from species differences in the substrate/product equilibria of the enzymes involved but this aspect was not investigated.

The results presented here for the effects of diazepam and amitryptyline on the disulphiram-induced changes in sheep ethanol and acetaldehyde metabolism may be extrapolated into the human situation. Diazepam had been previously shown to protect patients under disulphiram treatment from the clinical reaction they should have experienced when they drank alcohol (MacCallum, 1969). It seemed possible from this work with sheep that diazepam may have protected the aldehyde oxidase enzyme from inhibition by disulphiram, and thus acetaldehyde accumulation during ethanol metabolism would be decreased.

MacCallum (1969) also reported that amitryptyline ensured a strong reaction to ethanol in disulphiram-treated patients. In sheep, amitryptyline was shown to inhibit aldehyde oxidising enzymes, to potentiate the inhibition of these enzymes by disulphiram and to cause acetaldehyde accumulation during ethanol metabolism *in vivo*. Similar reactions in humans could result in the intense clinical reaction to ethanol observed during treatment with disulphiram and amitryptyline together.

Studies of the influence that metronidazole exerts on ethanol metabolism in sheep have suggested that the compound decreased the rate of ethanol oxidation *in vivo*. It seemed to cause a rise in

endogenous acetaldehyde concentrations *in vivo* and inhibit aldehyde oxidising enzymes *in vitro*, but there was no accumulation of acetaldehyde during metabolism of exogenous ethanol. It may be possible to explain the variable clinical response observed in humans who consume alcohol when under metronidazole treatment (Friedland & Vraisberg, 1968; Gallant *et al*, 1968) from these results, since decreased ethanol oxidation would prevent much of the *in vivo* acetaldehyde accumulation expected from the purified enzyme studies. A more intensive study of the effects of metronidazole would be most interesting.

Chapter 6

OTHER ASPECTS OF ETHANOL AND ACETALDEHYDE METABOLISM

Chronic consumption of ethanol alters general metabolism, particularly in the liver, and several extensive review articles have been published describing these effects (Lieber, 1970; Lundquist, 1970; Mendelson, 1970). Exposure to ethanol seems to result in fatty infiltration of the liver and increased plasma free fatty acid concentrations (Iseri et al, 1966). Mitochondrial respiration decreases (Kiessling & Tilander, 1963) while blood lactate and 17 β-hydroxy steroid levels increase (Lieber, 1968; Aderand et al, 1970). Hypoglycemia may occur in some people who drink large quantities of ethanol (Akabane et al, 1964b; Murdock, 1971) but this seems to be an unusual side effect (Forney & Hughes, 1968) restricted to individuals with depleted glycogen reserves (Kreisberg et al, 1971) and diabetics (Nakanishi, 1963).

These effects of ethanol administration may be attributed to the changes in the relative concentrations of NADH and NAD^+ that occur in the liver during ethanol metabolism (Majchrowicz et al, 1967). Ethanol oxidation catalyzed by liver alcohol dehydrogenase probably causes some of these increased NADH levels but up to 85% of acetaldehyde metabolism can be catalyzed by NAD^+-dependent aldehyde dehydrogenase (Richert & Westerfeld, 1957) and it seems that acetaldehyde oxidation could account for nearly half of the NADH formation during ethanol metabolism.

Studies of the properties of the FAD-dependent sheep liver aldehyde oxidase, reported in Chapter 2, have revealed its stimulation by even low concentrations of ethanol in the assay system. If aldehyde oxidase was active in in vivo acetaldehyde oxidation, less accumulation of NADH would result and those effects of ethanol metabolism which seem to result from increased NADH levels in the liver may decrease. It might also accelerate ethanol oxidation by making more NAD^+ available, so explaining the increased ethanol oxidation rates observed in alcoholics despite the lack of increased alcohol dehydrogenase activity (Green et al, 1965; Banks et al, 1970; Hillbom & Pikkarainen, 1970; Mezey, 1972) and doubtful activity of the microsomal ethanol oxidising system (Khanna & Kalant, 1970; Mezey, 1972) in these people.

These possibilities prompted a study of the effects of chronic ethanol consumption on in vivo acetaldehyde metabolism in sheep. A further set of experiments was designed to show the effects of ethanol and acetaldehyde metabolism in vivo on the relative concentrations of NADH and NAD^+ in sheep liver, and to allow calculation of the proportion of acetaldehyde oxidation catalyzed by aldehyde oxidase during

in vitro ethanol metabolism by sheep liver homogenates.

METHODS

In vivo experiments were performed with Romney ewes (approx.55kg) fed a maintenance diet of chopped hay (digestibility 53% (Davey, 1972)) and commercial sheep nuts. Normally water was always available but those animals required to consume dilute ethanol solutions (5% by vol.) were offered these as their only source of fluid. Ethanol solutions were made up freshly each day, and the volume consumed over twenty four hours was measured by 9 a.m., prior to feeding.

Those experiments which required infusion of acetaldehyde were performed on sheep which had been starved for twelve hours. Fluid was not available for the duration of the experiment and acetaldehyde (7mmoles) was infused (into the lower left jugular vein) in $10cm^3$ sterile, physiological saline. Infusion was complete in 30 seconds and time was measured from its completion.

Blood samples ($1cm^3$) were taken from the left jugular vein, divided into $0.5cm^3$, duplicate samples and diluted (1:1 by vol.) with diluent (n-propanol, $0.2cm^3/l$; $HgCl_2$, 2mg/l). These solutions were stored at 4°C until they were assayed for acetaldehyde, ethanol and acetone on the gas chromatograph (Chapter 3).

Lactate and Pyruvate Estimations were performed on blood samples ($1cm^3$) taken from the left jugular vein at the same time as those for gas chromatographic analysis. They were deproteinised immediately by adding $1cm^3$ $Ba(OH)_2$ solution (0.3M) followed by $1cm^3$ $ZnSO_4.6H_2O$ solution (5% w/v), and centrifuging (550g; 0°C; 5 min). The supernatants were refrigerated (4°C) for no longer than 90 minutes and then analysed for lactate and pyruvate following a method described by Lundquist, Fugmann, Klaning and Rasmussen (1959).

Pyruvate: The reaction mixture included

buffer (0.1M phosphate, pH7.4)	$1.0cm^3$
NADH (0.01M)	$0.3cm^3$
distilled water	$0.7cm^3$
deproteinised blood solution	$1.0cm^3$

Lactate dehydrogenase ($10mg/25cm^3$) (25 µl) was added to start the reaction. The system was incubated (22°C, 5min) and the decrease in optical density at 340nm was observed on a Hitachi 101 spectrophotometer. A calibration curve was prepared for each experiment from standard pyruvate solutions which accompanied each set of blood assays.

Lactate: The assay system for lactate estimations included

K_2CO_3 (1.2M) + semicarbazide (0.9M; pH 10.3)	1.0cm^3
NAD^+ (0.01M)	0.3cm^3
distilled water	0.7cm^3
deproteinised blood solution	1.0cm^3

Again 25 µl lactate dehydrogenase solution (10mg/25cm^3) was added to start the reaction. The increase in optical density at 340nm was recorded on a Hitachi 101 spectrophotometer after 100min (22°C). The standard graph showed a linear relationship between increase in optical density at 340nm and lactate concentration over the range observed in blood. Standard lactate solutions were assayed with each set of blood samples.

Liver homogenates were prepared from sheep livers within one hour of slaughter. Liver was homogenised in 0.1M phosphate buffer, pH7.4 (1:5 w/v) as described in Chapter 3, and NAD^+ was added in 0.5cm^3 phosphate buffer (0.1M; pH7.4) as required.

Samples (1.0cm^3) were withdrawn from the reaction mixture (initially 10cm^3) through a resealing stopper at the indicated times during incubation (37°C) with ethanol. They were treated with $Ba(OH)_2$ solution (0.3M, containing n-propanol, 0.03cm^3/l) and $ZnSO_4.6H_2O$ solution (5% w/v) as described in Chapter 3, and analyses for ethanol and acetaldehyde were performed on the gas chromatograph.

Anti-sheep liver aldehyde oxidase γ-globulin solutions were prepared as described in Chapter 2. The preparation used had a protein concentration of 13.9mg/cm^3, and 3 cm^3 of this solution were incubated (37°C, 5 min) in 10cm^3 total volume of homogenate reaction mixture, prior to addition of the ethanol.

Statistics: The significances of the differences quoted were measured by the t-test.

RESULTS

The results presented in this chapter have been divided into two sections. The first describes the effect that chronic ingestion of ethanol has on *in vivo* acetaldehyde metabolism in sheep. Section II describes the interrelationships between ethanol and acetaldehyde metabolism and the relative concentrations of NAD^+ and NADH *in vivo* and *in vitro*.

I) EFFECTS OF CHRONIC INGESTION OF DILUTE ETHANOL SOLUTIONS ON ETHANOL AND ACETALDEHYDE METABOLISM IN VIVO

i) Voluntary Intake of Dilute Ethanol Solutions: The volume of 5% (by vol.) ethanol voluntarily consumed by two ewes was compared with the water consumption by control sheep over a period of twenty weeks (Fig.38a; Fig.38b). There was little difference between the volumes of fluid ingested by those animals exposed to ethanol and those exposed to water during the first sixteen weeks of the experiment. After this time one sheep showed a marked increase in its consumption of ethanol but this was not altered when the ethanol solution was later replaced by water.

The ethanol consumption for sheep I was equivalent to the consumption of 230-408 calories per day (Fig.38a) and sheep II consumed between 230-1072 calories per day as ethanol (Fig.38b). If these values are compared with the maintenance requirement for sheep of 1930 calories per day for a 52kg sheep under similar conditions reported in the literature (Coop, 1962), sheep I and II seemed to be receiving about 10-25% and 10-55% respectively of their daily calory requirements as ethanol.

ii) Effect of Chronic Ingestion of Ethanol on Basal Acetaldehyde, Ethanol and Acetone Levels in Peripheral Venous Blood of Sheep:

Basal ethanol concentrations in the peripheral venous blood of those ewes consuming dilute ethanol solutions were increased ($p < 0.01$) from 0.1 ± 0.05mM(mean$\pm$ SE_M; N=5) before ethanol consumption to 3.9 ± 1.2 (mean$\pm SE_M$; N=5) during treatment, in samples taken randomly throughout the experiment. Acetaldehyde and acetone levels in the peripheral venous blood also increased, 2.24 and 1.5 times respectively, but variability in these concentrations during exposure to ethanol made the differences insignificant.

iii) Effect of Chronic Consumption of Ethanol on the Clearance of Exogenous Acetaldehyde from the Peripheral Venous Blood of Sheep:

Acetaldehyde disappearance from the peripheral venous blood of sheep was accelerated by the chronic consumption of dilute ethanol solutions (Fig.39), to be virtually complete within thirty minutes of infusion in those animals exposed to ethanol for 6.5 and 19.5 weeks. The changes in the ethanol levels in the venous blood that occur during acetaldehyde metabolism were also altered by chronic consumption of ethanol. Ethanol concentrations were reduced to below their 'zero' value during acetaldehyde metabolism in those sheep consuming

FIGURE 38a VOLUNTARY CONSUMPTION OF DILUTE ETHANOL SOLUTIONS BY SHEEP I

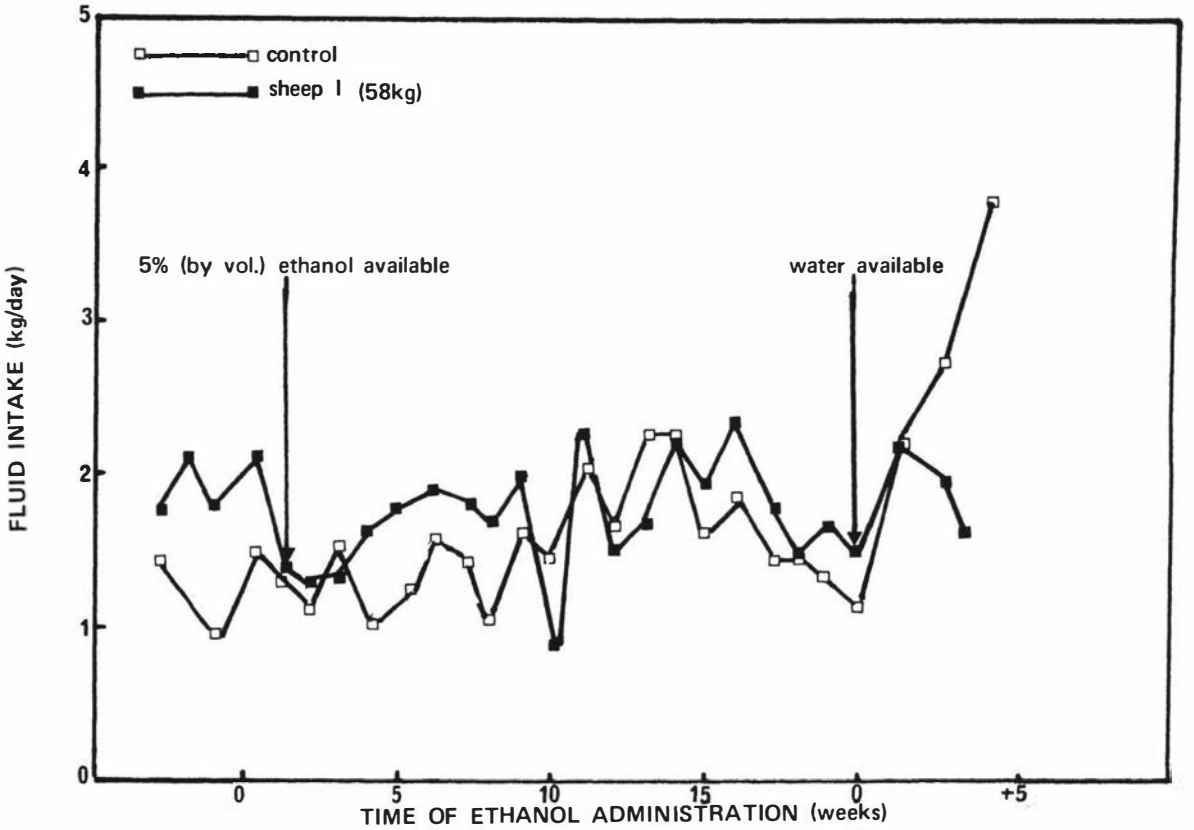
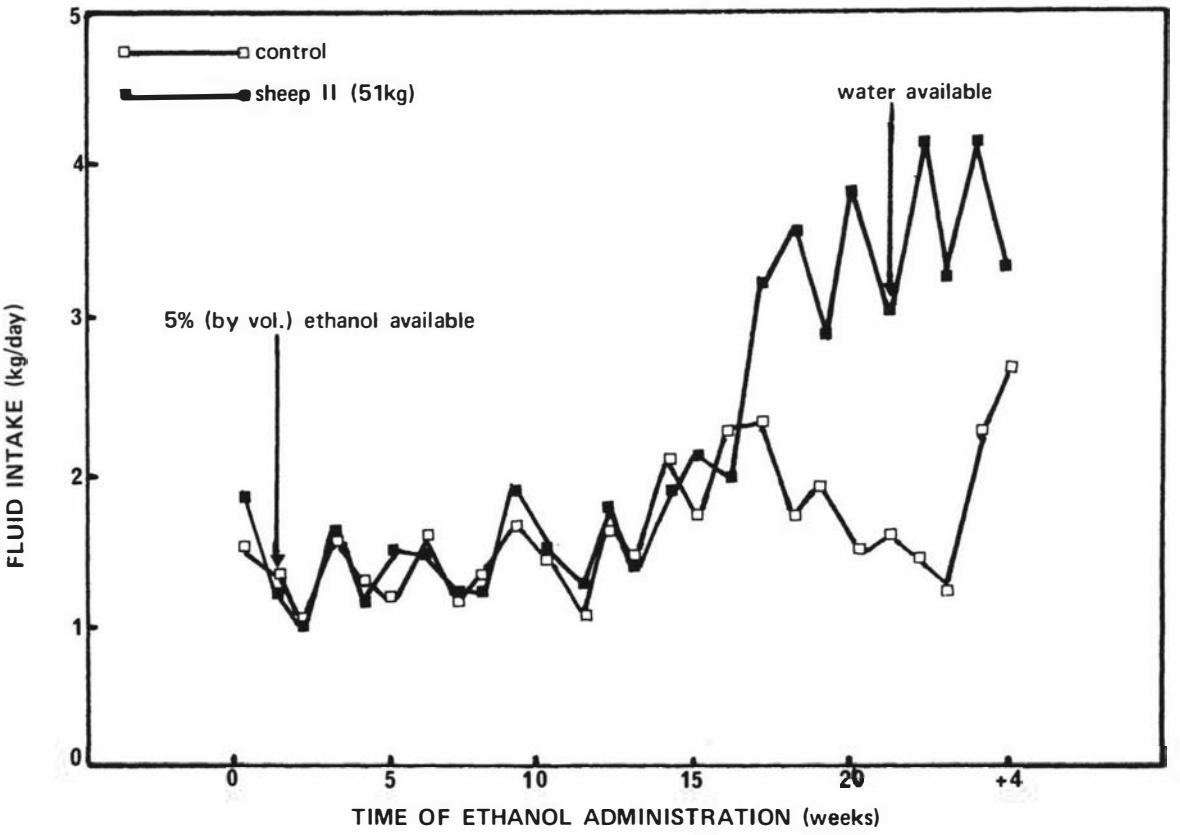


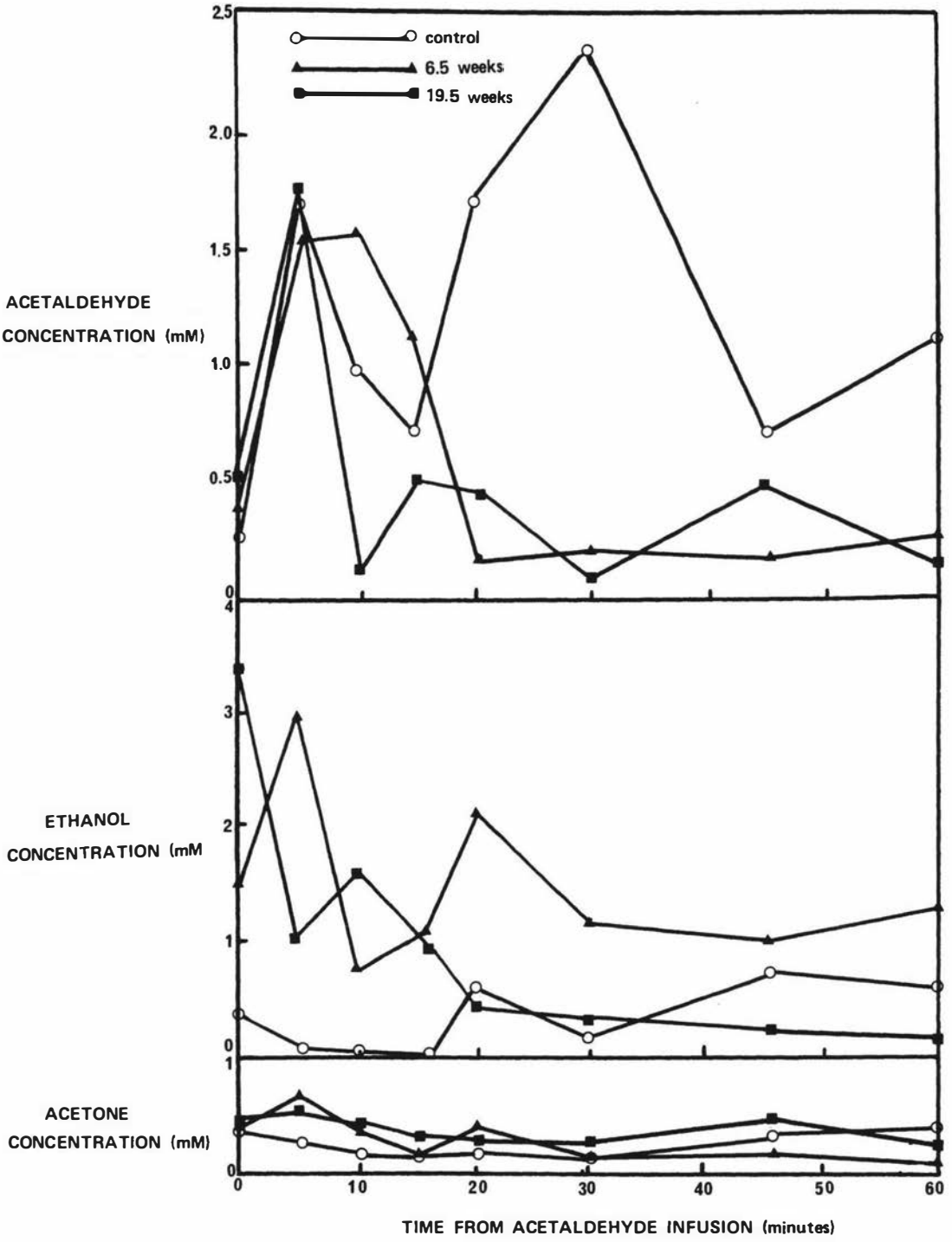
FIGURE 38b VOLUNTARY CONSUMPTION OF DILUTE ETHANOL SOLUTIONS BY SHEEP II



Dilute ethanol (5% by vol.) was available to each animal as their sole fluid source. The control animals had water always available, as did the experimental sheep at the times indicated before and after exposure to ethanol.

The fluid intake was measured each morning at 9 a.m prior to feeding and fresh ethanol solutions were made up daily.

FIGURE 39 EFFECT OF CHRONIC CONSUMPTION OF DILUTE ETHANOL SOLUTIONS ON THE IN VIVO CLEARANCE OF EXOGENOUS ACETALDEHYDE FROM THE PERIPHERAL VENOUS BLOOD OF SHEEP



Two ewes were exposed to dilute ethanol solutions (5% by vol.) as their sole fluid source and the pattern of clearance of exogenous acetaldehyde was determined in each animal at the indicated times after commencing the experiment. Mean results from the two animals have been plotted.

Acetaldehyde (7mmoles) was infused into the lower left jugular vein in 10cm^3 physiological saline immediately after the 'zero' blood sample had been taken.

Blood samples (1cm^3) were taken from the left jugular vein of the ewes at the times indicated. Each was divided into two 0.5cm^3, duplicate samples and diluted (1:1 by vol.) with diluent (n-propanol, 0.2cm^3/l; $HgCl_2$, 2mg/l). They were stored at 4°C until their analysis.

Assays for acetaldehyde, ethanol and acetone were performed gas chromatographically as described in Chapter 3.

ethanol, whereas the control animals showed increased ethanol levels - possibly resulting from reduction of acetaldehyde. Chronic consumption of ethanol appeared to have little effect on the changes in acetone concentration in peripheral venous blood that occurred during acetaldehyde metabolism.

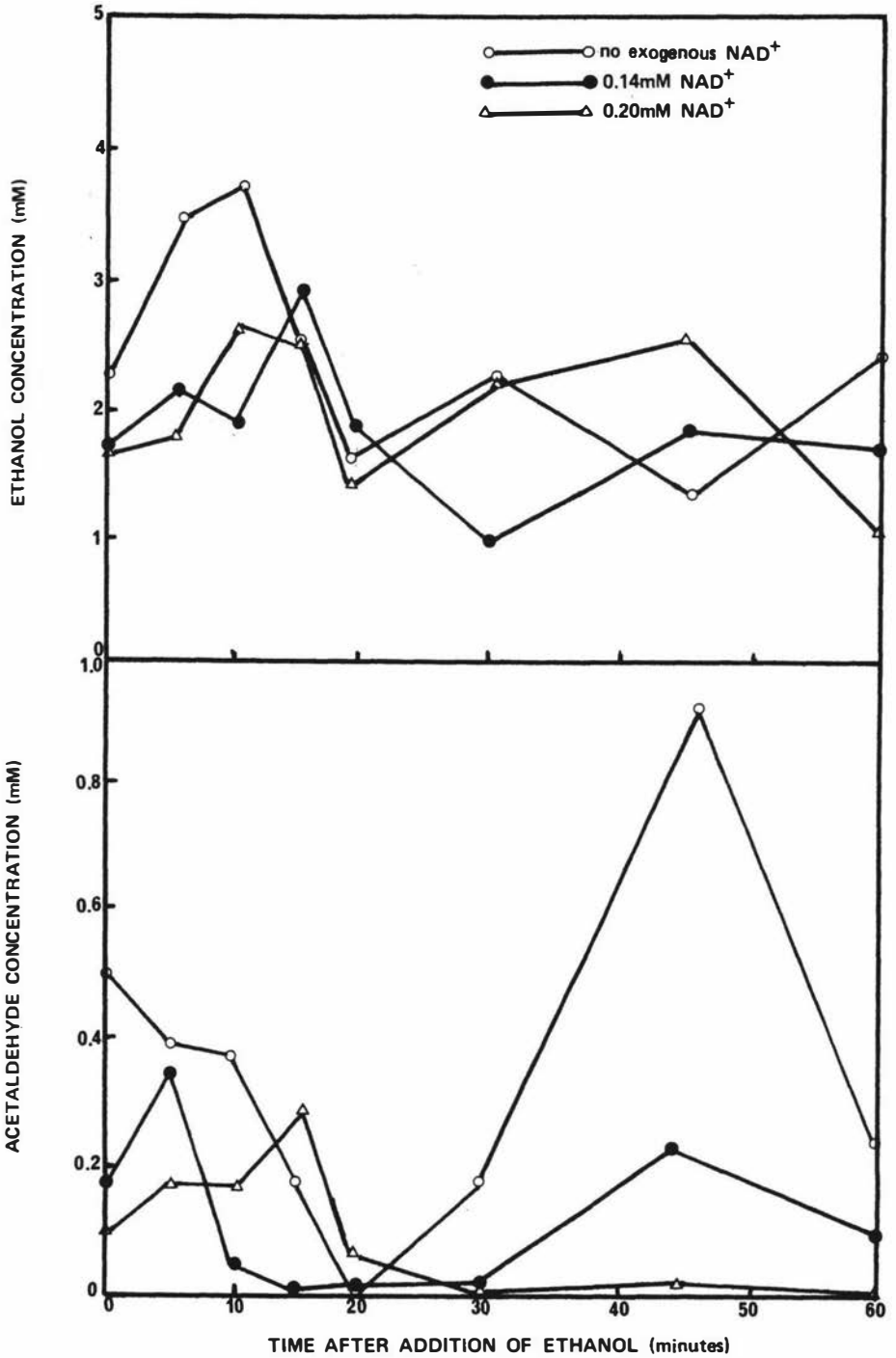
II) RELATIONSHIPS BETWEEN RELATIVE CONCENTRATIONS OF NADH AND NAD^+, AND ETHANOL AND ACETALDEHYDE METABOLISM IN SHEEP LIVER

i) Metabolism of Exogenous Ethanol in Sheep Liver Homogenates: Ethanol metabolism in homogenates prepared from ewe livers was altered depending on the concentration of exogenous NAD^+ present in the system (Fig. 40). Although effects on the changes in ethanol concentration were slight, acetaldehyde accumulated or was reduced to ethanol in those homogenate systems containing no NAD^+, and these effects were lessened by 0.14mM NAD^+ and prevented at 0.20mM NAD^+ levels.

The anti-aldehyde oxidase γ-globulin preparation described in Chapter 2 was used in an attempt to assess the proportion of acetaldehyde oxidation that is catalyzed by aldehyde oxidase during ethanol metabolism in sheep liver homogenates in vitro. When no exogenous NAD^+ was added to the system, the change in ethanol concentration was 2.0mM in twenty minutes and 0.15mM acetaldehyde accumulated in that time (Fig.41). The mean rate of acetaldehyde oxidation can therefore be estimated to be about 0.09mM/min. However, when anti-aldehyde oxidase γ-globulin preparation was added to this system which contained no exogenous NAD^+ the acetaldehyde concentration, twenty minutes after adding the ethanol, was increased to 1.3mM while ethanol levels remained the same. The rate of acetaldehyde oxidation appeared to be reduced to 0.03mM/min by this specific inhibitor of aldehyde oxidase activity, suggesting that aldehyde oxidase was catalyzing 66% of the acetaldehyde oxidation occurring in sheep liver homogenates in the absence of exogenous NAD^+.

When NAD^+ (0.6mM) was added to the liver homogenate system prior to the addition of ethanol (Fig.41) the rate of ethanol oxidation was similar to that occurring when NAD^+ was limited (0.09mM/min). Anti-aldehyde oxidase γ-globulin preparation added to the homogenate had no effect on the changes in either ethanol or acetaldehyde concentrations, that occurred during ethanol metabolism. Aldehyde oxidase may have been inactive when NAD^+ concentrations were sufficient to allow acetaldehyde oxidation by the aldehyde dehydrogenase enzyme system. It seemed that aldehyde oxidase contributed significantly to acetaldehyde oxidation only when NAD^+ was limited.

FIGURE 40 EFFECTS OF VARYING THE NAD^+ CONCENTRATION ON ETHANOL AND ACETALDEHYDE LEVELS IN SHEEP LIVER HOMOGENATES DURING METABOLISM OF EXOGENOUS ETHANOL

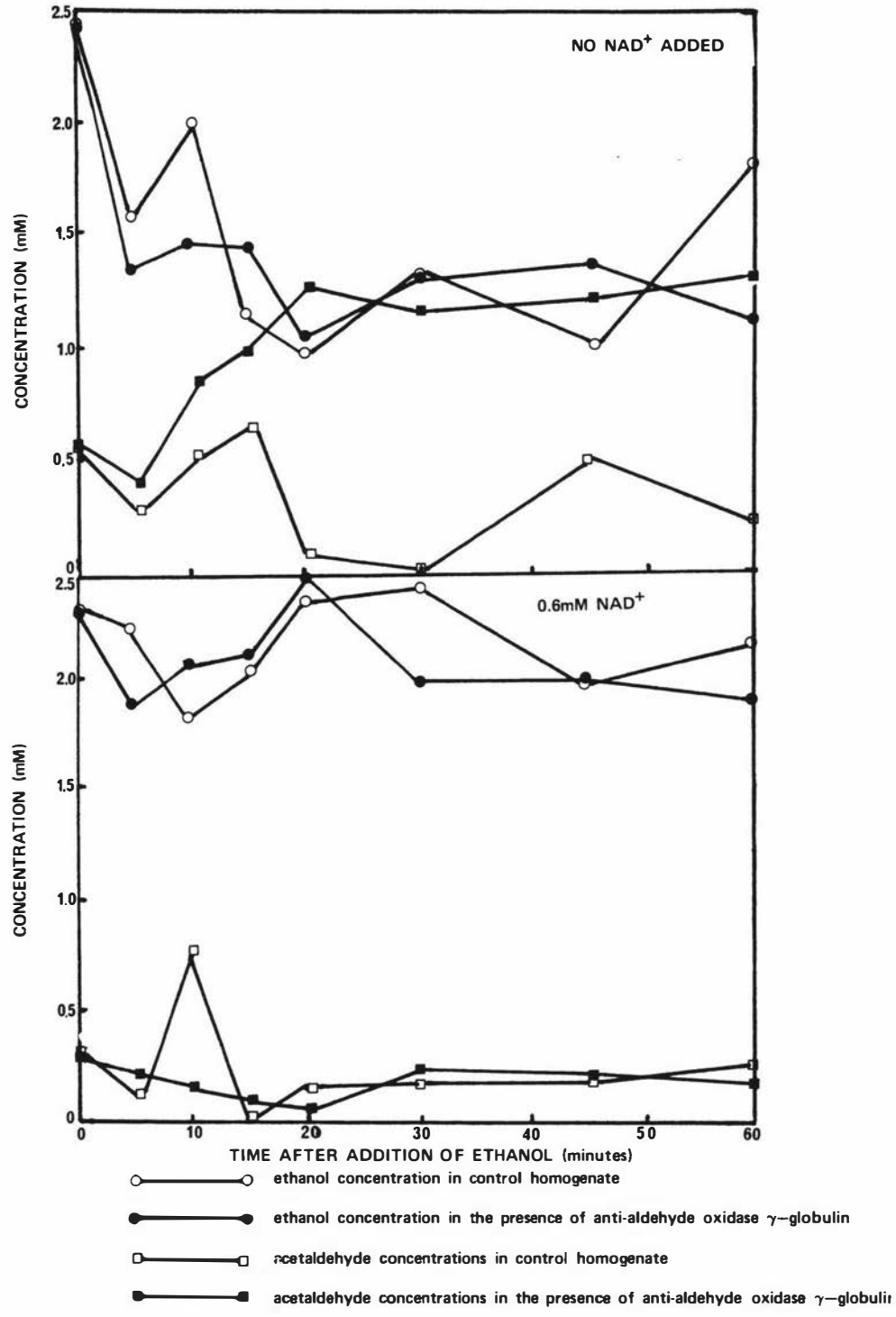


Liver was homogenised in 0.1M phosphate buffer (pH 7.4) as described by Lundquist and his associates (1963). NAD^+ (in 0.1M phosphate buffer, pH7.4) was incubated with each homogenate for five minutes (37°C) prior to addition of ethanol (65mM, in 0.1M phosphate buffer, pH7.4; $0.5cm^3$) through a resealing stopper. Samples ($1cm^3$) were withdrawn through this stopper at the times stated. The 'zero' sample was taken 15 seconds after addition of the ethanol.

Each sample was divided into two $0.5cm^3$, duplicate samples and deproteinised with $0.5cm^3$ $Ba(OH)_2$ solution (0.3M, containing n-propanol, $0.3cm^3/l$.) $ZnSO_4.6H_2O$ ($0.5cm^3$; 5% w/v) was added and the samples were stored at 4°C until they were analysed.

Ethanol and acetaldehyde determinations were performed on the gas chromatograph as described in Chapter 3.

FIGURE 41 EFFECT OF ANTI–ALDEHYDE OXIDASE γ--GLOBULIN PREPARATION ON ETHANOL AND ACETALDEHYDE LEVELS IN SHEEP LIVER HOMOGENATES DURING METABOLISM OF EXOGENOUS ETHANOL



Each experiment was performed with two different liver homogenates and the ethanol and acetaldehyde concentrations plotted are the means.

Liver was homogenised in 0.1M phosphate buffer (pH7.4) as described by Lundquist and his associates (1963). NAD^+ (in 0.1M phosphate buffer, pH7.4) and anti-aldehyde oxidase γ–globulin solution (in physiological saline) were incubated with each homogenate for five minutes (37°C) prior to the addition of ethanol (65mM in 0.1M phosphate buffer, pH7.4; 0.5cm^3) through a resealing stopper. Samples (1cm^3) were withdrawn through this stopper at the times stated. The 'zero' sample was taken 15 seconds after addition of the ethanol.

Each sample was divided into two 0.5cm^3, duplicate samples and deproteinised with 0.5cm^3 $Ba(OH)_2$ solution (0.3M, containing n-propanol, 0.3cm^3/l). Zn SO_4, $6H_2O$ (0.5cm^3; 5% w/v) was added and the samples were stored at 4°C until they were analysed.

Ethanol and acetaldehyde determinations were performed on the gas chromatograph as described in Chapter 3.

Each experiment was performed with two different liver homogenates, and the ethanol and acetaldehyde concentrations plotted are the means.

ii) Changes in the Relative Concentrations of Pyruvate and Lactate in Peripheral Venous Blood of Sheep during Acetaldehyde and Ethanol Metabolism in vivo: The changes that occur in the lactate: pyruvate ratio in venous blood have been reported to reflect the changes in the NADH:NAD^+ ratio that are occuring at the same time in the cytoplasm of the liver cells (Lieber, 1968). Metabolism of exogenous acetaldehyde by sheep in vivo was accompanied by a five-fold increase in the lactate:pyruvate ratio ten minutes after acetaldehyde infusion (Fig.42). This maximum corresponded with the greatest rate of change in the acetaldehyde concentration of the blood. After ten minutes the lactate: pyruvate ratio rapidly decreased to values less than those observed before acetaldehyde infusion. These were the result of low lactate levels in the peripheral venous blood, the concentration changing from 9.8mM to 0.1mM in the interval thirty to sixty minutes after acetaldehyde infusion.

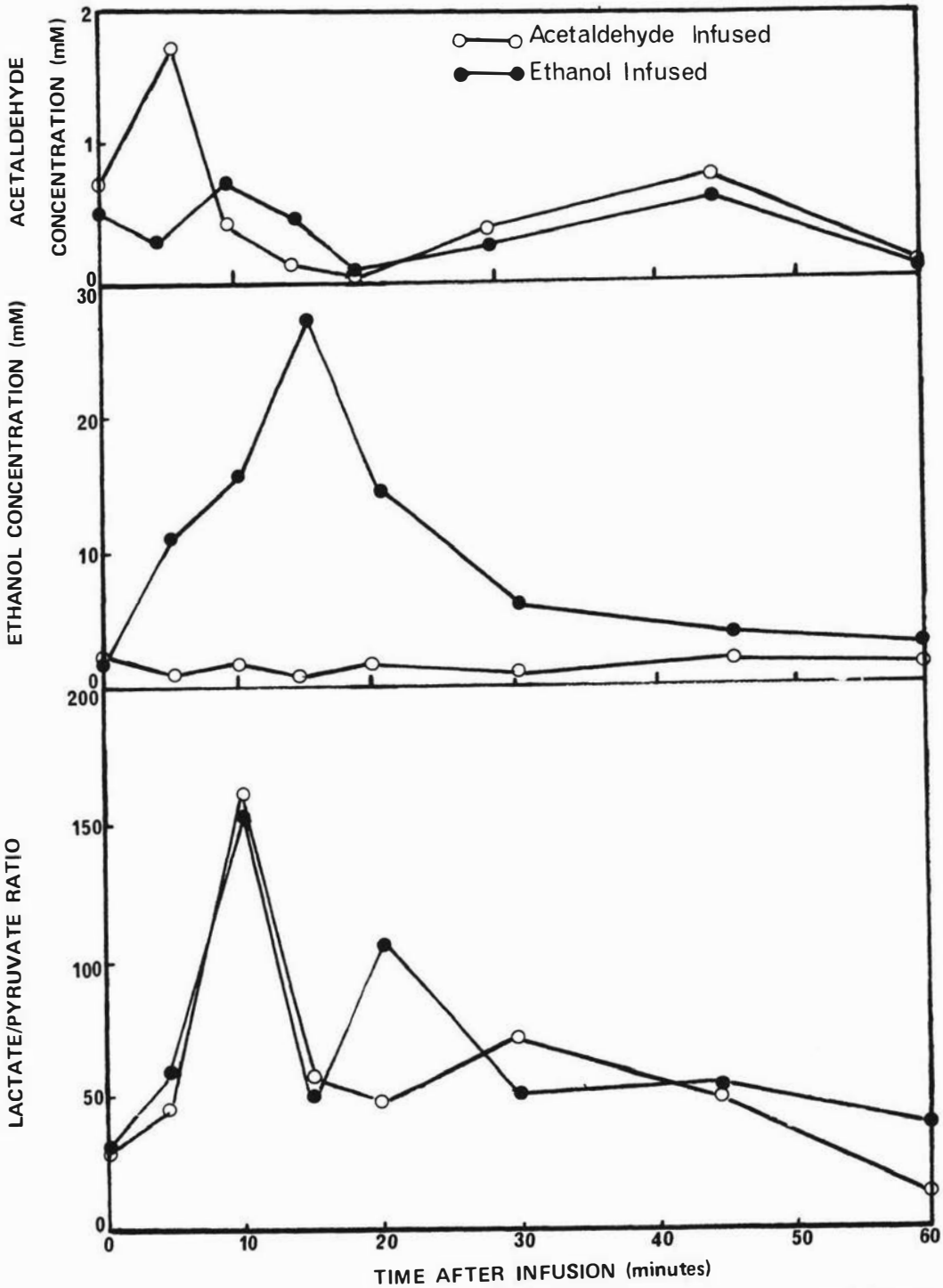
In vivo metabolism of exogenous ethanol by sheep was accompanied by a five-fold rise in the relative concentrations of lactate and pyruvate in peripheral venous blood (Fig.42). This was similar to that observed during acetaldehyde metabolism despite the larger amount of ethanol which was infused. The rapid return to the basal value of the lactate: pyruvate ratio was caused by decreasing lactate concentrations in the blood as the rate of ethanol metabolism decreased, and accompanying increases in the pyruvate levels to seven times the 'zero' concentration sixty minutes after ethanol infusion.

DISCUSSION

A mechanism which increases the rate of acetaldehyde oxidation in sheep liver appeared to be active during the chronic consumption of dilute ethanol solutions. This effect was not observed by Majchrowicz and his co-workers (1968) in experiments with rats which were offered 20% (by vol.) ethanol for four months.

While the ethanol levels in the peripheral venous blood of sheep appeared to be insufficient to directly stimulate aldehyde oxidase activity in the manner described in Chapter 2, the concentrations in the portal vein and the hepatic artery taking blood to the liver may be up to forty times those in the peripheral blood (Krebs & Perkins, 1970). It is possible that the increased rate of acetaldehyde metabolism observed in sheep subjected to chronic consumption of dilute ethanol solutions could result from direct stimulation of the aldehyde oxidase enzyme by ethanol being absorbed from the gut.

FIGURE 42 EFFECT OF IN VIVO METABOLISM OF EXOGENOUS ACETALDEHYDE OR ETHANOL ON THE RELATIVE CONCENTRATIONS OF LACTATE AND PYRUVATE IN THE PERIPHERAL VENOUS BLOOD OF SHEEP



Acetaldehyde (7mmoles) or ethanol (20mmoles) was infused into the lower left jugular vein in 10cm^3 physiological saline immediately after the 'zero' blood sample had been taken. Infusion was complete in 30 seconds and time was measured from its completion. Each experiment was performed on two different wethers and the concentrations plotted are the means for each time interval.

Blood samples were taken from the left jugular vein at the times indicated. They were treated with diluent (n-propanol, 0.2cm^3/l;$HgCl_2$, 2mg/l) or deproteinised ($Ba(OH)_2/ZnSO_4.6H_20$) as described in the text and stored at 4^{o}C.

Assays for acetaldehyde and ethanol were performed gas chromatographically as described in Chapter 3. Pyruvate and lactate analyses used the lactate dehydrogenase method and have been described in the text.

Tolerance of ethanol in humans is increased by exposure to it (Hawkins, 1966) and decreased acetaldehyde levels in the body during ethanol metabolism could be a factor in this adaption. Accumulation of NADH in the liver during ethanol metabolism may also contribute to the toxicity which can accompany metabolism of large amounts of ethanol (Lieber, 1968). The rise in the lactate:pyruvate ratio in peripheral blood that reflects the NADH:NAD^+ ratio in the liver (Lieber, 1968) seemed to result from acetaldehyde oxidation by the sheep during the in vivo metabolism of both ethanol and acetaldehyde.

It seemed that aldehyde oxidase was capable of increased activity both in the presence of ethanol and when NAD^+ concentrations were reduced. This would reduce acetaldehyde accumulation by providing an alternative route for its metabolism. NADH accumulation resulting from aldehyde dehydrogenase catalyzed acetaldehyde oxidation would be lessened and ethanol oxidation accelerated. A possible mechanism for removing the toxic products of ethanol metabolism may involve the aldehyde oxidase bypass for acetaldehyde oxidation.

Chapter 7

CONCLUSION

Ethanol is a natural product of gastrointestinal fermentation and mammals have a complete metabolic pathway for its degradation (Fig.43). Results in this thesis substantiate reports that sheep also form ethanol in the gastrointestinal tract. The alcohol seems to arise from fermentation in the rumen but under normal conditions the rumen ethanol level is about the same as that in the stomach of a non-ruminant (Krebs & Perkins, 1970). These results suggest that the basal blood alcohol levels are similar to those in humans and there seems little reason to suppose that extrapolation from the results of this work with sheep into the human situation should present more problems than other types of generalisation between species.

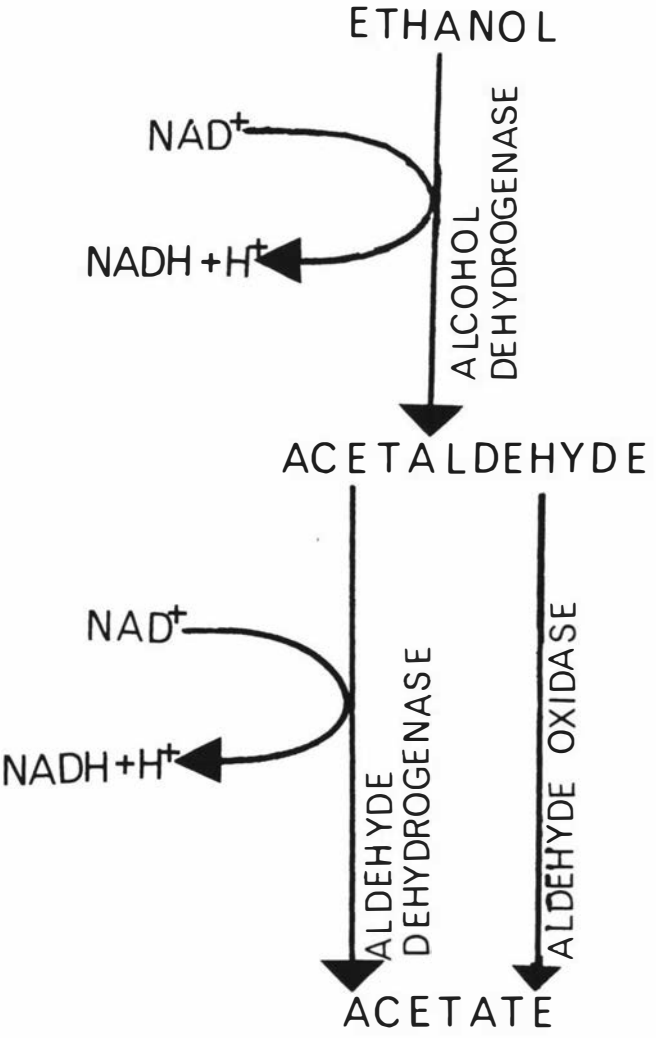
The pathway for ethanol metabolism via acetaldehyde is similar in both ruminants and monogastric animals. Drugs which specifically impair aldehyde oxidation have a similar effect in both types of animal. The main differences appear to be the result of the higher basal blood acetaldehyde levels in sheep which could be influenced by the *in vivo* ethanol:acetaldehyde equilibrium probably maintained by alcohol dehydrogenase (Westerfeld, 1955).

The contribution that aldehyde oxidase makes to acetaldehyde metabolism is maximal under conditions where NAD^+ reoxidation is a limiting factor in the action of aldehyde dehydrogenase. The high affinity for substrate shown by aldehyde dehydrogenase provides an explanation for acetaldehyde rapidly increasing the NADH concentrations in the body. However, the aldehyde oxidase levels are sufficient to explain the acetaldehyde metabolism rates following a 90% depletion of aldehyde dehydrogenase activity in rat liver reported by Westerfeld,(1961).

Aldehyde oxidase could play a vital role in limiting the formation of high levels of acetaldehyde during ethanol metabolism without directly involving NAD^+. Results in this study suggest that some detoxification mechanisms which cope with acute and chronic elevated ethanol levels in sheep are centred on the aldehyde oxidase enzyme.

Chronic ethanol consumption in sheep led to an increase in the *in vivo* rates of acetaldehyde oxidation and *in vitro* studies showed that ethanol itself directly stimulated aldehyde oxidase activity. These factors would prevent the build-up of NADH or acetaldehyde and

FIGURE 43 MAJOR PATHWAYS FOR ETHANOL METABOLISM IN THE LIVER



could be compared to the type of results expected if NAD^+ was added directly to the system. It has been common practice for some time to reduce the alcohol dependence of addicts by exogenous NAD^+ administration (O'Hollaren, 1961; Rappaport, 1969) and it has been found that the treatment reduces both acute and chronic symptoms of ethanol ingestion.

The effects of progesterone on aldehyde oxidase may give some protection from the toxic products of ethanol oxidation to embryos during the crucial stages of development. Progesterone levels are high during the latter half of the oestrus cycle, after release of the ovum, and during pregnancy. Reduced concentrations of acetaldehyde would result at these times through progesterone stimulation of aldehyde oxidase.

The results described have indicated that rates of ethanol and acetaldehyde metabolism in sheep are dependent on the endocrine state and the pattern of ethanol consumption in the animal.

Appendix I

ALDEHYDE DEHYDROGENASE

The pathway for acetaldehyde oxidation in sheep liver has already been shown to depend on the availability of NAD^+, and NADH formation during acetaldehyde metabolism has been assumed to result from the coupling of aldehyde oxidation to NAD^+ reduction by aldehyde dehydrogenase. A preliminary study of the properties of the sheep aldehyde dehydrogenase enzyme was therefore required to extend some aspects of the aldehyde oxidase investigation.

Bovine aldehyde dehydrogenase was partially purified by Racker in 1949. It catalyzed the coupling of aldehyde oxidation to reduction of NAD^+ and could also participate in a dismutation reaction between acetaldehyde and pyruvate, in conjunction with alcohol dehydrogenase.

More recent study of the aldehyde dehydrogenase enzymes from various species has shown them to be sulphydryl proteins (Stoppani & Milstein, 1957; Deitrich, 1967) with a broad specificity for aldehydes (Glenn & Vanko, 1959; Maxwell & Topper, 1961; Deitrich et al, 1962).

This Appendix describes a method for partially purifying sheep liver aldehyde dehydrogenase and includes the results of a preliminary study of some kinetic properties of the enzyme.

METHODS

Enzyme Assay: Assay of aldehyde dehydrogenase activity measured the rate of formation of NADH by following changes in the optical density of the reaction mixture at 340nm (Racker, 1949). The assay mixture included

NAD^+ (0.30M)	$0.2cm^3$
buffer, pH9.3 (0.01M pyrophosphate)	$0.5cm^3$
distilled water	$2.5cm^3$
enzyme solution(100-1000units/cm^3)	$0.3cm^3$

The mixture was equilibrated for 5 minutes at 22°C and the reaction was then started by adding $0.5cm^3$ substrate. The change in OD_{340nm} was measured in a Hitachi 101 spectrophotometer. Unless otherwise stated acetaldehyde (0.26mM) was used as the substrate.

One unit of aldehyde dehydrogenase activity was defined as the amount of enzyme required to catalyze a change in optical density at 340nm of 0.001 absorbance units per minute.

Compounds used in the inhibitor studies were incubated in the assay mixture for 5 minutes (22°C) before the reaction was started.

Protein Estimation: Protein concentrations were estimated by the

spectrophotometric method of Lowry, Rosebrough, Farr and Randall (1951). A linear relationship between protein concentration and absorbance at 500nm was demonstrated under the test conditions using bovine serum albumin solutions. An increase in OD_{500nm} of 0.81 corresponded to a protein concentration of $1mg/cm^3$.

RESULTS

I) PURIFICATION OF SHEEP LIVER ALDEHYDE DEHYDROGENASE

The results of a typical purification run are tabulated in Table 22. Fresh sheep liver (109 g) was homogenised in ice-cold, redistilled acetone ($217\ cm^3$) for three 30 second periods. Further ice-cold acetone ($217\ cm^3$) was added to the homogenate and the mixture was immediately centrifuged (14,600g, 0°C, 15min). The precipitate was dried on blotting paper at 22°C until all the residual acetone had evaporated.

The resulting acetone powder (94 g) was stirred with $282\ cm^3$ EDTA (3.6mM, pH7.0) for two hours (22°C) and then centrifuged (14,600g, 0°C, 30min). The aldehyde dehydrogenase activity was in the supernatant.

The next step in the procedure was an ethanol precipitation achieved by adding $113\ cm^3$ absolute ethanol (-10°C) dropwise to the EDTA extract. The solutions were kept at -10°C in ice/salt baths and after stirring for thirty minutes the mixture was centrifuged (14,600g, -10°C, 15min). The precipitate was discarded and a further $113\ cm^3$ absolute ethanol (-10°C) was added dropwise to the supernatant, with stirring (30min, -10°C).

After centrifuging (14,600g, -10°C, 15min), the precipitate was taken up in $35\ cm^3$ EDTA solution (3.6mM, pH7.0).

An acidification step was performed by adding 0.1M HCl dropwise to the enzyme solution, reducing the pH to 4.4. The mixture was immediately centrifuged (23,500g, 0°C, 10min) and the supernatant was retained.

Sodium hydroxide solution (0.1M) was slowly added to the preparation until pH 5.2 had been reached, and $0.07\ cm^3$ RNA solution (5% (w/v), pH5.2, 0°C) was then added. After centrifuging (23,500g, 0°C, 10min), a further $0.07\ cm^3$ 5% (w/v)RNA solution (pH5.2) was added to the supernatant. The mixture was again centrifuged and the precipitate was taken up in $10\ cm^3$ EDTA solution (3.6mM, pH7.0).

Sodium hydroxide (0.1M) was again added dropwise to the preparation to raise the pH to 6.3, and a protamine sulphate solution (1%w/v) was added slowly to precipitate any excess RNA. When no further precipitation could be observed the mixture was centrifuged (23,500g, 0°C, 10min) and the protamine sulphate procedure was repeated with the

TABLE 22 Purification Procedure for Sheep Liver Aldehyde Dehydrogenase

Step	Volume (cm^3)	Activity (units/cm^3)	Protein Concentration (mg/cm^3)	Yield (%)	Specific Activity (units/mg)	Purification (x)
EDTA extract of acetone powder	282	148	7.43	100	19.8	1
Alcohol Precipitation (0.4-0.8 vol.)	35	200	10	16.8	20	1.1
Precipitation at pH4.4.	33	140	2.15	11.1	65	3.3
Treatment with RNA and Protamine Sulphate	14	672	3.92	22	171	8.9
Ammonium Sulphate Fractionation (50-60% saturated)	2	858	2.72	4.1	318	16.1
Elution from Sephadex G.200	4	390	0.37	3.7	1045	53

Weight of liver used to prepare acetone powder = 109g

The final enzyme solution was prepared in 3.6mM EDTA, pH7.0.

supernatant. Centrifugation left the aldehyde dehydrogenase activity in the supernatant (14 cm^3).

Without adjusting the pH(pH6.3), solid ammonium sulphate was added to the supernatant at 0°C to reach 50% saturation. Stirring was continued for sixty minutes and the solution was then centrifuged (23,500g, 0°C, 10min). The concentration of ammonium sulphate was then raised in the supernatant, with stirring, to 60% saturation.

After centrifuging, the precipitate was taken up in 2.0 cm^3 EDTA solution (3.6mM, pH7.0).

The enzyme solution was passed through a column (1.5x13cm) of Sephadex G.200 which had been prepared in EDTA (3.6mM, pH7.0). Normally the aldehyde dehydrogenase activity appeared in the second and third 2 cm^3 fractions collected after protein had begun to elute. The purification achieved with this step was between one and five times depending on the specific activity that had been achieved in the previous steps.

The enzyme preparation had a specific activity of 1000-1050 units/mg protein, which is about a fifty-fold purification from the acetone powder extract. Deitrich (1962) claimed to have purified the bovine aldehyde dehydrogenase forty three times, achieving a specific activity of 1300 units/mg protein in his preparation. No alcohol dehydrogenase, aldehyde oxidase or xanthine oxidase activities could be detected in the enzyme preparations but some lactate dehydrogenase was present.

II) KINETIC PROPERTIES OF SHEEP LIVER ALDEHYDE DEHYDROGENASE

i) Specificity: Several compounds were tested as substrates for sheep liver aldehyde dehydrogenase (Table 23). Aliphatic aldehydes were rapidly oxidised by the enzyme but no activity was observed with benzaldehyde or cinchonidine as substrate.

Although formaldehyde acted as a substrate for this enzyme preparation the reaction could have been catalyzed by a separate formaldehyde dehydrogenase enzyme (Strittmatter & Ball, 1955).

ii) Michaelis Constant: The K_m for the enzyme for NAD^+ as the substrate was 0.36mM. This was much greater than that reported for bovine aldehyde dehydrogenase by Deitrich (1962) and could not be explained by extraneous alcohol dehydrogenase activity.

iii) Inhibition: The compounds assayed for their effect on aldehyde dehydrogenase activity are given in Table 24. Progesterone inhibited sheep liver aldehyde dehydrogenase as it did the rabbit enzyme

TABLE 23 Specificity of Sheep Liver Aldehyde Dehydrogenase[a]

Substrate	Final Concentration (mM)	Relative Activity
Acetaldehyde	0.23	100
Crotonaldehyde	15	57
Benzaldehyde	10	0
Formaldehyde	25	88
Pyruvic Aldehyde	15	53
Salicylic Aldehyde	10	25
Cinchonidine	Satd.	0

TABLE 24 The Effect of Several Compounds on Sheep Liver Aldehyde Dehydrogenase[a] Activity

Inhibitor	Final Concentration (mM)	Relative Rate
Nil	-	100
Progesterone	0.31	40
Disulfiram	1.7	44
Metronidazole	0.3	65
Calcium Carbimide	3.1	100
Menadione	0.015	48
Triton X-100	0.78	29
8-Hydroxy Quinoline	170	54
Acetate	30	100

a) Assays were carried out at pH9.3 as described in the text, using acetaldehyde (0.23mM) as substrate unless otherwise stated.
The rate of oxidation of acetaldehyde alone has been arbitarily placed at 100. This was equivalent to a change in optical density at 340nm of 0.165/min/mg protein under standard assay conditions.
Each assay was performed in duplicate, using two different enzyme preparations and the mean value is quoted.

(Maxwell & Topper, 1961), and disulphiram inhibition of the sheep enzyme required one thousand times the concentration required by the bovine enzyme for the same effect (Deitrich & Hellerman, 1963). The metronidazole inhibition of the sheep liver enzyme was not observed by Fried and Fried (1966) in their work with the rat liver aldehyde dehydrogenase. Inhibitors of general electron transfer-menadione, triton X-100 and 8-hydroxy quinoline-appeared to inhibit aldehyde dehydrogenase activity while the product of acetaldehyde oxidation, acetate, had no effect.

iv) Optimum pH: Sheep liver aldehyde dehydrogenase was 1.4 times more active at pH9.3 than at pH7.4.

DISCUSSION

Sheep liver aldehyde dehydrogenase prepared in the manner described appeared to differ markedly from the enzyme prepared from bovine liver (Deitrich, 1962). The specificity for aliphatic aldehydes was similar to the cytoplasmic aliphatic aldehyde specific dehydrogenase described in rabbit liver (Raison et al, 1966) which was also sensitive to steroids. The sheep liver enzyme was more active in alkaline than neutral solution which differentiated it from a mitochondrial enzyme observed in rat livers (Glenn & Vanko, 1959).

Previous results in this thesis have suggested that sheep liver aldehyde dehydrogenase was active in catalyzing acetaldehyde metabolism in sheep although it would not have been saturated at the physiological NAD^+ concentrations that have been reported in rat livers (Lundquist et al, 1959).

REFERENCES

ADERAND,W.H.,CRONHOLM,T. & SJOVALL,J.(1970) Biochim.Biophys.Acta 202,343

AKABANE,J.(1960) Med.J.Shinshu Uni. 5,113

AKABANE,J.(1964) Med.J.Shinshu Uni. 9,141

AKABANE,J.(1970) Medicinaa 7,100

AKABANE,J.,NAKANISHI,S.,KOHEI,H.,ASAKAWA,S.,MATSUMURA,R.,OGATA,H. & MIYAZAWA,T.(1964a) Med.J.Shinshu Uni. 9,1

AKABANE,K.,NAKANISHI,S.,KOHEI,H.,MATSUMURA,R. & OGATA,H.(1964b) Med.J.Shinshu Uni. 9,25

AKABANE,J.,NAKANISHI,S.,KOHEI,H.,MATSUMURA,R. & MARYAMA,Y.(1964c) Med.J.Shinshu Uni. 9,59

AKABANE,J.,NAKANISHI,S.,KOHEI,H.,MATSUMURA,R. & OGATA,H.(1964d) Jap.J.Pharmacol. 14,295

ALEMAN,V.,SMITH,S.T.,RAJACOPALAN,K.V. & HANDLER,P.(1965) in Nonhaem Iron Proteins (San Pietro,A.G.,ed.)Kent, Ohio:Antioch Press

ALLISON,M.J.,DOUGHERTY,R.W.,BUCKLIN,J.A. & SNYDER,E.E.(1964) Science 144,54

ASMUSSEN,E.,HALD,J. & LARSEN,V.(1948) Acta Pharmacol. 4,311

AVIS,P.G.,BERGER,F.,BRAY,R.C. & SHOOTER,K.V.(1954) Nature 173,1230

BACKLIN,K.I.(1958) Acta Chem.Scand. 12,1279

BANKS,W.L.,KLINE,E.S. & HIGGINS,E.S.(1970) J.Nutrition 100,581

BARKER,S.B.(1941) J.Biol.Chem. 137,783

BARRERRA,S.E.,OSINSKI,W.A. & DAVIDOFF,E.(1950) Amer.J.Psychiat. 107,8

BEER,C.T. & QUASTEL,J.H.(1958) Can.J.Biochem.Physiol. 36,531

BEGG,T.D.,HILL,I.D. & NICHOLIS,L.C.(1964) Brit.Med.J. 1,9

BENNETT,C.(1971) Amer.J.Med.Technol. 31,217

BERGENMEYER,H.(1965) in Methods in Enzymatic Analysis, 2nd edition, p.290. New York:Academic Press

BLOMSTRAND,R.(1971) Life Sci. 10 PtII,575

BLOOM,R.J.,FULLER,P.G.,WESTERFELD,J.G. & WESTERFELD,W.W.(1966) Biochemistry 5,3211

BLOOM,R.J. & WESTERFELD,W.W.(1966) Biochemistry 5,3204

BOGUSZ,M.,GUMINSKA,M. & MARKIEWICZ,J.(1970) J.Forensic Med. 17,156

BONNISCHEN,R.K. & THEORELL,H.(1951) Scand.J.Clin.Lab.Invest. 3,58

BRAY,R.C.(1963) The Enzymes 7,533

BRAY,R.C.,PALMER,G. & BEINERT,H.(1964) J.Biol.Chem. 239,2667

BRAY,R.C.,CHISHOLM,A.J. & HART,L.I.(1966) in Flavins and Flavoproteins p.117 (Slater,E.C.,ed.)Amsterdam:Elsevier

BRIGGS,A.P.(1926-27) J.Biol.Chem. 71,67

BRYANT,M.P.(1959) Bact.Rev. 23,125

BURBRIDGE,T.N.,HINE,C.H. & SCHICK,A.F.(1950a) J.Lab.Clin.Med. 35,983

BURBRIDGE,T.N.,HINE,C.H.,BOWMAN,K.P.,SIMON,A.,MACKLIN,E. & SCHICK,A.F. (1950b) J.Pharmacol.exp.Therapeut. 98,4

BURNETT,G.B. & READING,H.W.(1969) Lancet i,415

BURNS,C.(1972) Personal Communication

CAMPBELL,B.,TAYLOR,J.T. & HASLETT,W.L.(1967) Proc.Soc.Exp.Biol.Med. 124,191

CATTIGONI,G.L. & DINNING,J.S.(1971) J.Nutrition 101,1327

CHILD,G.P.(1950) J.Pharmacol.exp.Therapeut. 98,5

CHILD,G.P.OSINSKI,W.,BENNETT,R.E. & DAVIDOFF,E.(1951) Amer.J.Psychiat. 107,774

CLAUSEN,J.(1969) in Immunochemical Techniques for the Identification and Estimation of Macromolecules (Work,T.S. & Work,E. eds.) Amsterdam:North Holland

COLDWELL,B.B.,SOLOMONRAJ,G.,TRENHOLM,H.L. & WIBERG,G.S.(1971) Clin.Toxicol. 4,99

COLEHOUR,J.K.(1967) Analyt.Chem. 39,1190

COOP,I.E.(1962) J.Agric.Sci. 58,179

CORRAN,H.S.,DEWAN,J.G.,GORDON,A.H. & GREEN,D.E.(1939) Biochem.J. 33,1694

CREAVEN,P.J. & ROACH,M.K.(1969) J.Pharmacy Pharmacol. 21,332

CRONHOLM,T. & SJOVALL,J.(1970) Eur.J.Biochem. 13,124

DAJANI,R.M.,DANIELSKI,J. & ORTEN,J.M.(1963) J.Nutrition 80,196

DAJANI,R.M. & ORTEN,J.M.(1960) Fed.Proc. 19,47

DAVEY,A.W.F.(1972) Personal Communication

DAVIS,B.J.(1964) Ann.N.Y.Acad.Sci. 121,404

DAVIS,V.E. & WALSH,M.J.(1970a) Science 167,1005

DAVIS,V.E. & WALSH,M.J.(1970b) Science 169,1105

DAVIS,V.E.,WALSH,M.J. & YAMANAKA,Y.(1970) J.Pharmacol.exp.Therapeut. 174,401

DAWSON,R.M.C.,ELLIOTT,D.C.,ELLIOTT,W.H. & JONES,K.M.(1969) in Data for Biochemical Research. London:Oxford University Press

DEANE,H.W.,HAY,M.F.,MOOR,R.M.,ROWSON,L.E.A. & SHORT,R.V.(1966) Acta Endocrinol. 51,245

DEITRICH,R.A.(1962) J.Biol.Chem. 237,560

DEITRICH,R.A.(1966) J.Pharmaceut.Sci. 15,1911

DEITRICH,R.A.(1967) Arch.Biochem.Biophys. 119,253

DEITRICH,R.A. & HELLERMAN,L.(1963) J.Biol.Chem. 238,1683

DEITRICH,R.A., HELLERMAN,L. & WEIN,J.(1962) J.Biol.Chem. 237,560

DELANEY,R.L.,LANKFORD,H.G. & SULLIVAN,J.F.(1966) Proc.Soc.Exp.Biol.Med. 123,675

DE RENZO,E.C.,KALEITA,E.,HEYTLER,P.G.,OLESON,J.J.,HUTCHINGS,B.L. & WILLIAMS,J.H.(1953) Arch.Biochem.Biophys. 45,247

DILTS,N.(1970) Amer.J.Obstet.Gynec. 107,1018

DINGLE,J.T.,HAY,M.F. & MOOR,R.M.(1968) J.Endocrinol.40,325

DINNING,J.S.(1952) J.Biol.Chem. 202,213

DIVATIA,K.J.,HINE,C.H. & BURBRIDGE,T.N.(1952) J.Lab.Clin.Med. 39,974

DIXON,M. & LUTWAK-MANN,C.(1937) Biochem.J. 31,1347

DIXON,M. & WEBB,E.C.(1964) in The Enzymes, 2nd Edition. London:Longmans

DOISY,R.J.,RICHERT,D.A. & WESTERFELD,W.W.(1955) J.Biol.Chem. 217,307

DUNCOMBE,R.E. & SHAW,W.H.C.(1966) in Technicon Symposium Vol.II. Automation in Analytical Chemistry. p.15

DUNDEE,J.W.,ISAAC,M. & TEGGART,J.(1971) Quart.J.Studies on Alcohol 32,741

DUNN,T.G.,HOPWOOD,M.L.,HOUSE,W.A. & FAULKNER,L.C.(1972) Amer.J.Physiol. 222,468

DURITZ,G. & TRUITT,E.B.(1963) Fed.Proc. 22,272

DURITZ,G. & TRUITT,E.B.(1964) Quart.J.Studies on Alcohol 25,498

DURITZ,G. & TRUITT,E.B.(1966) Biochem.Pharmacol. 15,711

DUTT,R.H. & CASIOLA,L.E.(1948) Endocrinol. 43,208

EDWARDS,T.P.(1949) Texas Repts.Biol.Med. 7,684

EDWARDS,J.A. & PRICE,J.(1967) Nature 214,190

EHRENBERG,A. & BRAY,R.C.(1965) Arch.Biochem.Biophys. 109,199

ELDJARN,L.(1950) Scand.J.Clin.Lab.Invest. 2,198

ELLIS.E.W. & HILL,J.B.(1969) Clin.Chem. 15,91

EVANS,B.A. & CATERALL,R.D.(1971) Brit.Med.J. 4,146

FERGUSON,J.K.W.(1956) Can.Med.Ass.J. 74,793

FORNEY,R.B. & HUGHES,F.W.(1968) in Combined Effects of Alcohol and Other Drugs. Springfield;Ill.:Thomas

FOX,J.E.(1958) Proc.Soc.Exp.Biol.Med. 97,236

FRIDOVICH,I.(1962) J.Biol.Chem. 237,584

FRIDOVICH,I.(1966) J.Biol.Chem. 241,3126

FRIED,R. & FRIED,L.W.(1966) Biochem.Pharmacol. 15,1890

FRIEDLAND,P. & VRAISBERG,M.(1968) Diseases of the Nervous System 29,326

FUJIWARA,E. & NIIGATA,V.(1953) Acta Med.Biol. 1,61

GALLANT,D.M.,BISHOP,M.P.,CAMP,E. & TISDALE,C.(1968) Current Therapeut.Res. 10,82

GEE,A.H. & CHAIKOFF,I.L.(1926) J.Biol.Chem. 70,151

GELDER,M.G. & EDWARDS,G.(1968) Brit.J.Psychiat. 114,473

GESSENER,P.K.(1971) Anal.Biochem. 38,499

GLATT,M.(1970) in The Alcoholic and the Help He Needs. New York:Priory Press Ltd.

GLENN,J.L. & VANKO,M.(1959) Arch.Biochem.Biophys. 82,145

GOLDSTEIN,A.(1970) New England J.Med. 283,275

GOODMAN,J.I. & TEPHLY,T.R.(1969) Fed.Proc. 28,546

GOODWIN,D.W.(1967) Amer.J.Psychiat. 123,1276

GORDON,A.H.,GREEN,D.E. & SUBRAMANYON,V.(1940) Biochem.J. 34,764

GRAHAM,W.D.(1951) J.Pharmacy Pharmacol. 3,3160

GREEN,N.J.,COHEN,R.B. & ISSELBACHER,K.J.(1965) Lab.Invest. 14,264

GREEN,D.E.,WESTERFELD,W.W.,VENNESLAND,B. & KNOX,W.E.(1941) J.Biol.Chem. 140,683

GUERRO-FIGUERO,R.,RYE,M.M.,GALLANT,D.M. & BISHOP,M.P.(1970) Neuropharmacol. 9,143

HAAS,E.J.(1944) J.Biol.Chem. 155,321

HALD,J. & JACOBSEN,E.(1948a) Acta Pharmacol. 4,305

HALD,J. & JACOBSEN,E.(1948b) Lancet ii,1001

HALD,J.,JACOBSEN,E. & LARSEN,V.(1948) Acta Pharmacol. 4,285

HALD,J.,JACOBSEN,E. & LARSEN,V.(1949) Acta Pharmacol. 5,179

HALD,J. & LARSEN,V.(1949) Acta Pharmacol. 5,292

HANDLER,P.,RAJAGOPALAN,K.V. & ALEMAN,V.(1964) Fed.Proc. 23,30

HARTING,J.(1951) Fed.Proc. 10,195

HARTROFT,W.S.(1967) Fed.Proc. 26,1432

HAWKINS,R.D.(1966) Can.J.Physiol.Pharmacol. 44,241

HEDLUND,S.-G. & KIESSLING,K.-H.(1969) Acta Pharmacol.Toxicol. 27,381

HILLBOM,H.E. & PIKKARAINEN,P.H.(1970) Biochem.Pharmacol. 19,2097

HILTON,J. & SARTORELLI,A.C.(1970) J.Biol.Chem. 245,4187

HINE,C.H.,ANDERSON,H.H.,MACKLIN,E.A.,BURBRIDGE,T.N.,SIMON,A. & BOWMAN, K.M.(1950) J.Pharmacol.exp.Therapeut. 98,13

HINE,C.H.,BURBRIDGE,T.N.,MACKLIN,E.A.,ANDERSON,H.H. & SIMON,A.(1952) J.Clin.Invest. 31,317

HORTON,A.A. & PACKER,L.(1970) Biochem.J. 116,19P

HUET,M.(1966) in Technicon Symposium Vol.II. Automation in Analytical Chemistry. p.155

HUNGATE,R.E.(1966) in The Rumen and its Microbes. New York:Academic Press

HUNTER,F.E. & LOWRY,O.H.(1956) Pharmacol.Rev. 8,98

IGO,R.P.,MACKLER,B. & DUNCAN,H.(1961) Arch.Biochem.Biophys. 93,435

IKOMI,F.(1956a) Med.J.Shinshu Uni.. 1,257

IKOMI,F.(1956b) Med.J.Shinshu Uni. 1,263

ISERI,O.A.,LIEBER,C.S. & GOTTLIEB,L.S.(1966) Amer.J.Path. 48,535

ISSELBACHER,K.J. & CARTER,E.A.(1970) B.B.Res.Commun. 39,530

JACOBSEN,E.(1950) Biochim.Biophys.Acta 4,330

JACOBSEN,E.(1952) Pharmacol.Rev. 4,107

JACOBSEN,E. & MARTENSON-LARSON,O.(1949) J.Amer.Med.Ass. 139,918

JANKE,A. & STEPHAN,J.(1935) Biochem.Z. 278,30

JANSSON,B.O. & LARSSON,B.T.(1969) J.Lab.Clin.Med. 74,961

JARNEFELT,J.(1955) Ann.Acad.Sci.Fenniciae Ser.A. and Med.Anthropol. 55,7

JOKIVARTIO,E.(1950) Quart.J.Studies on Alcohol 11,183

KAND,M.,BRADY,F.O.,RAJAGOPALAN,K.V. & HANDLER,P.(1972) J.Biol.Chem. 247,765

KARASEK,M.A. & GREENBURG,D.M.(1957) J.Biol.Chem. 227,191

KEILIN,D. & HARTREE,E.F.(1936) Proc.Royal Soc.B. 119,141

KERSHAW,P.W.(1967) Brit.J.Psychiat. 113,387

KESANIEMI,A. & KURPPA,A.(1971) Acta Obstet.Gynec.Scand. 50,167

KHANNA,J.M. & KALANT,H.(1970) Biochem.Pharmacol. 19,2033

KHANNA,J.M.,KALANT,H. & LIN,G.(1970) Biochem.Pharmacol. 19,2493

KIELLY,R.K.(1955) J.Biol.Chem. 216,405

KIESSLING,K.H.(1962) Exp.Cell Res. 17,367

KIESSLING,K.H. & TILANDER,K.(1963) Exp.Cell Res. 30,476

KINARD,F.W.(1967) in Biochemical Factors in Alcoholism (Maickel,R.G.ed.) Oxford:Pergamon

KINARD,F.W.,HAY,M.G. & KINARD,F.W.Jr.(1962) Nature 196,380

KJELDGAARD,N.O.(1949) Acta Pharmacol. 5,397

KNOX,W.E.(1946) J.Biol.Chem. 163,699

KREBS,H.A. & PERKINS,J.R.(1970) Biochem J. 118,635

KREISBERG,R.A.,SIEGAL,A.M. & OWEN,W.C.(1971) J.Clin.Invest. 30,175

KRIMSKY,I. & RACKER,E.(1952) J.Biol.Chem. 198,721

KUMAR,M.A. & SHETH,U.K.(1962) Arch.int.pharmacodyn.CXVIII,188

LAHTI,R.A.(1968) Diss.Abstracts 29,B2327

LARSEN,J.A.(1959) Nature 184,1236

LEHMANN,H.E. & BAN,T.A.(1967) Current Therapeut.Res. 9,419

LEROY,M.(1961) Ann.Nut.L'Alimentation 15,280

LESTER,D.(1962) Quart.J.Studies on Alcohol 23,17

LESTER,D. & BENSON,G.B.(1969) Fed.Proc. 28,546

LESTER,D. & BENSON,G.H.(1970) Science 169,282

LESTER,D. & GREENBURG,L.A.(1950) Quart.J.Studies on Alcohol 11,391

LEVY,H.R. & VENNESLAND,B.(1957) J.Biol.Chem. 228,85

LIEBER,C.S.(1968) Adv.Int.Med. 14,151

LIEBER,C.S.(1970) Gastroenterology 59,930

LIEBER,C.S. & DECARLI,L.M.(1968) Science 162,917

LIEBER,C.S. & DECARLI,L.M.(1969) Clin.Res. 17,306

LIEBER,C.S. & DECARLI,L.M.(1970) J.Biol.Chem. 245,2505

LINTON,P.H. & HAIN,J.D.(1967) Quart.J.Studies on Alcohol 28,544

LOWRY,O.H.,ROSEBROUGH,N.J.,FARR,A.I. & RANDALL,R.J.(1951)
J.Biol.Chem. 193,265

LUBIN,M. & WESTERFELD,W.W.(1945) J.Biol.Chem. 161,503

LUNDQUIST,F.(1958) Biochem.J. 68,172

LUNDQUIST,G.A.R.(1970a) in Alcohol and Alcoholism p.170 (Popham,R.E. ed.)
Toronto: University of Toronto Press

LUNDQUIST,F.(1970b) in Alcohol and Alcoholism p.33 (Popham,R.E. ed.)
Toronto: University of Toronto Press

LUNDQUIST,F.,FUGMANN,V.,KLANING,E. & RASMUSSEN,H.(1959) Biochem.J.72,409

LUNDQUIST,F.,SVENDSEN,I. & PETERSEN,P.H.(1963) Biochem.J. 86,119

MacCALLUM,W.A.G.(1969) Lancet i,313

MAHLER,H.R.(1956) Adv. Enzymol. 17,233

MAHLER,H.R.,MACKLER,B. & GREEN,D.E.(1954a) J.Biol.Chem. 210,149

MAHLER,H.R.,MACKLER,B.,GREEN,D.E. & BOCK,R.M.(1954b) J.Biol.Chem.
210,465

MAJCHROWICZ,E.,BERCAW,B.L.,COLE,W.M. & GREGORY,D.H.(1967) Quart.J.
Studies on Alcohol 28,213

MAJCHROWICZ,E.,LIPTON,M.A.,MEEK,J.L. & HALL,L.(1968) Quart.J.Studies
on Alcohol 29,553

MARCUCCI,F.,GUAITANI,A.,FANELLI,R.,MUSSINI,E. & CARATTINI,S.(1971)
Biochem.Pharmacol. 26,1711

MARSHALL,E.K. & FRITZ,W.F.(1953) J.Pharmacol.exp.Therapeut. 109,431

MARTINDALE,W.(1958) in The Extra Pharmacopoeia, Vol.I, 24th edition.
London:The Pharmaceutical Press

MAXWELL,E.S.(1962) J.Biol.Chem. 237,1699

MAXWELL,E.S. & TOPPER,Y.J.(1961) J Biol.Chem. 236,1032

McCARTHY,K. & LOVENBERG,W.(1968) Fed.Proc. 27,640

McMANUS,I.R.,CONTAG,A.O. & OLSEN,R.E.(1966) J.Biol.Chem. 241,349

MENDELSON,J.H.(1970) New England J.Med. 283,24

MENDELSON,J.H.,OGATA,M. & MELLO,N.K.(1969) Fed.Proc. 28,262

MEZEY,E.(1972) Biochem.Pharmacol. 21,137

MØLLER,S.E.(1971a) Acta Pharmacol.Toxicol. 30,429

MØLLER,S.E.(1971b) Acta Pharmacol.Toxicol. 30,449

MOOMAW,C.R. & HUNGATE,R.E.(1963) J.Bacteriol. 88,721
MUELLER,R.G. & LANG,G.E.(1971) Clin.Chem. 17,458
MURDOCK,H.R.(1971) Amer.J.Clin.Nut. 24,394
NAKANISHI,S.(1963) Med.J.Shinshu Uni. 8,101
NELSON,C.A. & HANDLER,P.(1968) J.Biol.Chem. 243,5368
NELSON,D. & JENSEN,C.E.(1961) Fed.Proc. 20,189
NEWMAN,H.W. & PETZOLD,H.V.(1951) Quart.J.Studies on Alcohol 12,30
NEWSTEAD,D.F. & ORMSBY,J.E.(1970) N.Z.J.Dairy Sci.Technol. 5,52
NYGAARD,A.P. & SUMNER,J.B.(1952) Arch.Biochem.Biophys. 39,119
OGATA,M.,MENDELSON,J.H.,MELLO,N.K. & MAJCHROWICZ,E.(1971)
Psychosom.Med. 33,159
O'HOLLAREN,P.(1961) West.J.Obstet.Gynec. 69,101
O'KEANE,M.,SMITH,S. & GOLDBERG,A.(1971) Lancet, ii,795
ORME-JOHNSON,W.H. & ZIEGLER,D.M.(1965) B.B.Res.Commun. 21,78
PALMER,G.(1963) Biochim.Biophys.Acta 56,444
PARKS,R.E.,TEPHLY,T. & MANNERING,G.J.(1961) Biochem.Pharmacol. 8,172
PEREZ,V.J.,CICERO,T.J. & BAHN,B.A.(1971) Clin.Chem. 17,307
PERMAN,E.S.(1962a) Acta Physiol.Scand. 55,189
PERMAN,E.S.(1962b) Acta Physiol.Scand. 55,203
PIHL,A. & FRITZSON,P.(1955) J.Biol.Chem. 215,345
PIHL,A. & LANGE,R.(1962) J.Biol.Chem. 237,1356
POCKER,Y. & MEANY,J.E.(1965) Biochemistry 4,2535
POTTER,V.R. & ELVEJHAM,C.A.(1936) J.Biol.Chem. 114,495
RACKER,E.(1949) J.Biol.Chem. 177,883
RACKER,E.(1952) J.Biol.Chem. 196,347
RAISON,J.K.,HENSON,G. & HEINITS,K.G.(1966) Biochim.Biophys.Acta 118,285
RAJAGOPALAN,K.V.,FRIDOVICH,I. & HANDLER,P.(1962) J.Biol.Chem. 237,922
RAJAGOPALAN,K.V. & HANDLER,P.(1964a) J.Biol.Chem. 239,1509
RAJAGOPALAN,K.V. & HANDLER,P.(1964b) J.Biol.Chem. 239,2022
RAJAGOPALAN,K.V. & HANDLER,P.(1964c) J.Biol.Chem. 239,2027
RAPPAPORT,M.(1969) Quart.J.Studies on Alcohol 30,570
RAWAT,A.K.(1969) Eur.J.Biochem. 9,93
RAWAT,A.K. & LUNDQUIST,F.(1968) Eur.J.Biochem. 5,13
REHAK,M.J. & TRUITT,E.B.(1958) Quart.J.Studies on Alcohol 19,399
REINHARD,J.F. & SPECTOR,E.(1970) Toxicol.Appl.Pharmacol. 17,12
RICHERT,D.A. & WESTERFELD,W.W.(1951) Proc.Soc.Exp.Biol.Med. 76,252
RICHERT,D.A. & WESTERFELD,W.W.(1953) J.Biol.Chem. 203,915
RICHERT,D.A. & WESTERFELD,W.W.(1957) J.Biol.Chem. 227,533
RIDGE,J.W.(1963) Biochem.J. 88,95

ROACH,M.K.(1969) Fed.Proc. 28,546

ROACH,M.K. & CRAVEN,P.J.(1968) Clin.Chem.Acta 21,275

ROOS,K.J.(1971) Clin.Chem.Acta 31,285

ROSALKI,S.B.(1970) J.Clin.Path. 23,826

RUBIN,E. & LIEBER,C.S.(1968) Science 162,690

RUBIN,C.S.,HUTTERA,F. & LIEBER,C.S.(1968) Science 159,1469

RYAN,M.T.,NOLAN,J. & CONWAY,E.J.(1948) Biochem.J.Proc.Biochem.Soc. 42,LXIV

SANDERS,B.J.(1966) Amer.J.Clin.Path. 46,704

SARUTA,T.,COOK,R. & KAPLAN,N.M.(1971) Proc.Soc.Exp.Biol.Med. 138,353

SEMER,J.M.,FRIEDLAND,P.,VRAISBERG,M. & GREENBURG,A.(1966) Amer.J. Psychiat. 123,722

SHEPPARD,J.R.,ALBERSHEIM,P. & McLEARN,G.(1970) J.Biol.Chem. 245,2876

SHORT,R.V.(1961) in Hormones in Blood, p.379 (Gray,C.H. & Bacharach, A.L.eds.) New York:Academic Press

SHORT,R.V. & MOORE, N.W.(1959) J.Endocrinol. 19,288

STOKES,P.E. & LASLEY,B.(1967) in Biochemical Factors in Alcoholism, p.101 (Maickel,R.G.ed.) London:Pergamon Press

STOPPANI,A.O.M. & MILSTEIN,C.(1957) Biochem.J. 67,406

STOPPANI,A.O.M.,SCHWARCZ,M.N. & FREDA,C.E.(1966) Arch.Biochem.Biophys. 113,464

STOTZ,E.(1943) J.Biol.Chem. 148,585

STOTZ,E. & WESTERFELD,W.W.(1944) J.Biol.Chem. 152,51

STRASSMAN,H.D.,ADAMS,B. & PEARSON,A.W.(1970) Quart.J.Studies on Alcohol 31,394

STRITTMATTER,P. & BALL,E.G.(1955) J.Biol.Chem. 213,445

SUPNIEWSKI,J.V.(1926) J.Pharmacol.exp.Therapeut. 30,429

TAYLOR,A.F. & NORTHMORE,M.E.(1967) in Technicon Symposium Vol.II. Automation in Analytical Chemistry p.263

THEORELL,H.(1965) Harvey Lec.Ser. 61,17

TOWNE,J.C.(1964) Nature 201,709

TRENHOLM,H.L.,MAXWELL,W.B.,PAUL,C.J.,WIBERG,G.S. & COLDWELL,B.D.(1970) Can.J.Biochem. 48,706

TRUITT,E.B.(1970) Quart.J.Studies on Alcohol 31,1

TRUITT,E.B. & DURITZ,G.(1967) in Biochemical Factors in Alcoholism, p.61 (Maickel,R.B. ed.) Oxford:Pergamon Press

TRYSTRUP,N.,WINKLER,K. & LUNDQUIST,F.(1965) J.Clin.Invest. 44,817

VESSELL,E.S.,PASSANANTI,G.T. & LEE,C.H.(1971) Clin.Pharmacol.Therepeut. 12,785

WALKENSTEIN,S.S. & WEINHOUSE,S.(1953) J.Biol.Chem. 200,515

WASE,A.W. & CHRISTENSEN,J.(1954) J.Biol.Chem. 211,75

WESTERFELD,W.W.(1949) Proc.Soc.Exp.Biol.Med. 71,28

WESTERFELD,W.W.(1955) Texas Repts.Biol.Med. 13,559

WESTERFELD,W.W.(1961) Amer.J.Clin.Nut. 9,426

WESTERFELD,W.W. & RICHERT,D.A.(1951) J.Biol.Chem. 192,35

WIDMARK,E.M.P.(1922) Biochem.Z. 131,473

WILKINSON,P.,KORNACZEWSKI,A.,RANKIN,J.G. & SANTAMARIA,J.N.(1971)
Med.J.Aust. 19,1217

WILLIAMS,R.D.,MASON,H.L.,POWER,M.H. & WILDER,R.M.(1943)
Arch.Int.Med. 71,35

WILLIAMSON,J.R.,SCHOLZ,R.,BROWNING,.E.T.,THURMAN,R.G. & FUKAMI,M.N.(1969)
J.Biol.Chem. 244,5044

WINGARD,C. & TEAGUE,R.S.(1958) Arch.int.pharmacodyn. CXVI,54

WOODY,C.O.,FIRST,N.L. & POPE,A.L.(1967) J.Animal.Sci. 26,139

YAMANAKA,Y.,WALSH,M.J. & DAVIS,V.E.(1970) Nature 227,1143

YLIKAHRI,R.H. & MAENPAA,P.H.(1968) Acta Chem.Scand. 22,1707