ETHANOL METABOLISM IN HUMANS

A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Science
in
Biochemistry
at Massey University

Kenneth G. Couchman
1974

SUMMARY

Alcohol metabolism in humans has been studied by examining blood, urine and breath samples taken at frequent intervals for 3 hours after an alcohol load of 0.5ml/kg in a fasting condition.

A gas chromatographic method was developed for the simultaneous estimation of acetaldehyde, ethanol and acetone levels in blood and urine specimens and various column packings were investigated. Porapak Q was the most suitable material and the method finally adopted used the headspace gas phase over urine or perchlorate precipitated blood specimens to which had been added sodium sulphate to displace the volatile components from the aqueous phase. Protein precipitation was necessary in order to prevent the loss of acetaldehyde from the blood samples. A gas sampling valve was fitted to enable similar determinations in breath samples but was not used in this study.

Assays by ezymatic methods were developed for lactate, pyruvate, β-hydroxybutyrate, glucose and glycerol utilising the changes in concentration of NADH which was measured by fluorometry and the merits of converting NAD^+ to a fluorescent compound was examined. Twenty male and eight females volunteered for the study. Blood samples were obtained from an intravenous catheter, a procedure supervised by a physician. Blood alcohol levels were monitored by breath tests with an electrochemical device, (an Alcolimiter) for detecting ethanol.

There were considerable variations in the peak alcohol levels and in the time taken for equilibration in the body. Estimates of

the rate of metabolism of ethanol and of body content were in agreement with those published in the literature. Breath testing was found to be a satisfactory means of estimating blood alcohol concentration in the post-absorptive phase. The Alcolimiter gave readings that were on an average 10mg/100ml low, but this could be corrected by recalibration. Blood acetaldehyde levels rose to 0.1mg/100ml and occasionally to 0.2mg/100ml.

A fall in blood pyruvate level, which remained low throughout the test period, was seen to coincide with the increase in blood alcohol. There was a tendancy for lactate to rise at the same time. This was not consistent, but the ratio of lactate to pyruvate increased 2 or 3-fold in most cases which reflected the change in cytoplasmic redox ratio.

Small increases were observed in blood glucose even though the alcoholic drink was free of sugar. There were increases in blood glycerol levels in all subjects and some of these were quite large. These findings were contrary to some reports which have appeared in the literature.

The excretion of electrolytes was examined in the urine but the results were difficult to interpret.

Alcohol concentrations in urine samples were measured and compared to the blood levels and the diuretic effect of alcohol was noted. These findings, together with those reported in the literature have been discussed together with their significance in interpreting disturbances of metabolism when alcohol is consumed.

More assays are thought to be required including those for blood acetate, blood triglycerides with free fatty acids and some hormones. It is considered that the use of labelled compounds could add a new dimension to the *in vivo* investigations on human volunteers.

ACKNOWLEDGMENTS

I am indebted to my supervisor, Professor R. D. Batt for his continuing interest in the study and help with the preparation of the manuscript for the thesis. I am also grateful to Dr L. Bieder for his medical supervision of the work with volunteers and assistance in formulating the protocol. My appreciation is due and acknowledged to Terry Braggins for technical assistance, Dr R. Greenway both for advice and the assays of electrolytes, Dr R. Brooks for help with estimations using atomic absorption spectroscopy; and to all the members of the team working on alcohol research in the department for continual advice and discussion of the project.

The thesis was typed and copied by Ms Marion Trevor to whom I am indebted for her patience and care at all stages of preparing the final manuscript.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION

The present investigation is part of a continuing and expanding research programme which has been initiated to define the role of acetaldehyde during alcohol metabolism in human subjects. The first studies at Massey University were centered around the enzyme mechanisms responsible for alcohol metabolism in sheep liver (Hendtlass, PhD., thesis, 1973). More recently, this work was expanded to include the isolation and characterisation of mitochondrial and cytoplasmic dehydrogenases from liver extracts (K. E. Crow, unpublished results).

Although there is a large amount of published work on alcohol metabolism, the attention given to acetaldehyde utilisation has been small by comparison. This may be due in part to the considerable difficulties in estimating acetaldehyde reflected in the wide differences in levels reported in the literature. These have varied from 0.1 to 30.0 mg./100ml. (Truitt and Walsh, 1971, p. 164). Early spectrophotometric studies on distillates from blood samples lacked specificity and were prone to contamination by chemical oxidation products of ethanol during sample preparation which probably accounted for the higher values. The development of gas chromatography has provided a highly specific method for the detection and quantitative estimation of acetaldehyde in the presence of other volatile compounds and recent studies have shown that the blood levels after alcohol consumption are not usually greater than 0.2 mg/100ml.

Even at this order of magnitude, it is still not clear whether acetaldehyde has been produced directly from alcohol or as an artefact during sample preparation (Truitt, 1970), and further studies are required to establish definitive blood levels. Acetaldehyde has been shown to be extremely toxic, when it was infused to give blood levels from 0.2 to 0.7 mg/100ml., the symptoms produced were similar to those attributable to alcohol (Asmussen et al, 1948). Truitt and Walsh (1971) reported that it was 35 times more potent than ethanol in producing hypnosis in mice, but they also considered that the relative blood levels with alcohol may also be important.

Ethanol is metabolised in the liver by oxidation to acetaldehyde and then to acetate and the major pathway for these reactions are catalysed primarily by the enzymes, alcohol and alehyde dehydrogenase. At the same time, NAD^+ is reduced to NADH creating an imbalance in the normal ratios between these two co-factors, which is reflected in the various metabolic pathways. In glycolysis, the direction of the reaction:-

$$\text{PYRUVATE} + \text{NADH} + H^+ \rightleftharpoons \text{LACTATE} + NAD^+$$

is favoured from left to right with the ratio of lactate : pyruvate increasing. The formation of acetyl CoA from pyruvate, which required NAD^+, is reduced with a consequent decrease in energy production from the tricarboxylic acid cycle. This reduction in energy production can be compensated less efficiently by an increase in substrate level phosphorylation with a rapid depletion of the glycogen stores and glucose and a resulting hypoglycaemia. An initial hyperglycaemia has been attributed to release of catecholamines

by acetaldehyde. The decrease in availability of NAD^+ also favours the reaction from left to right of:-

ACETOACETATE + NADH + H^+ $\rightleftharpoons$ β HYDROXYBUTYRATE + NAD^+

which is catalysed by the enzyme, β-hydroxybutyrate dehydrogenase located in mitochondria. Peripheral blood levels of these two substances can reflect changes in the intramitochondrial redox ratio.

There have been conflicting reports in the literature on the effects of ethanol on fat metabolism. Crouse et al. (1968) have shown a fall in blood glycerol and free fatty acid levels whilst Maickel and Brodie (1963) demonstrated an increase. Truitt et al. (1966), have shown an elevation of plasma free fatty acids and hepatic triglycerides in the rat after infusing with acetaldehyde and they suggested that these increases can be attributed to the release of catecholamines.

Alcohol is said to inhibit the release of anti-diuretic hormone from the neurohypophysis resulting in an increase in urinary volume. Such a diuresis is accompanied by a decrease in the excretion of electrolytes with a corresponding increase in plasma levels. At the same time, there is an increase in the excretion of calcium and magnesium (Flink, 1971), presumably due to a temporary unresponsiveness to parathyroid hormone (Estep 1969) mediated through the action of alcohol or acetaldehyde.

In addition to the release of catecholamines, acetaldehyde has been implicated in disturbances of biogenic amine metabolism and there are hypotheses that by diversion of the metabolic pathways, or condensation with neuroamines, alkaloid like precursors may be formed which could be of significance in addiction (Davis and Walsh, 1971). It

seems likely that acetaldehyde plays a major role in promoting a number of metabolic disturbances and it was planned to study these simultaneously in human subjects following a test dose of 0.5ml of ethanol per kilogram body weight. The study was planned to include the determination of the redox state of the body by estimating levels of the pairs, lactate/pyruvate and hydroxybutyrate/acetoacetate, disturbances in glycolysis and gluconeogenesis from blood glucose levels, changes in fat metabolism from blood glycerol levels and the diuretic, electrolyte and mineral effects from estimations on urine samples. At the same time, alcohol and acetaldehyde was to be determined in the blood, breath and urine.

Although most of these metabolic changes have been examined previously and reported in the literature, there have been few attempts to follow all of these simultaneously. There are various reasons for this, but most have been due to the relative insensitivity of techniques available at the time. The introduction of gas chromatography and the use of stable porous polymers for separations has considerably eased the problems of measuring acetaldehyde and ethanol levels. Similarly, fluorometric procedures have increased the sensitivity and reduced the volume of reagents and sample required for the estimation of various organic metabolites by enzymatic methods. By linking these advances in technology to a standardised test procedure supervised by a medical practitioner, simultaneous investigations were possible on multiple blood samples obtained from an indwelling intravenous catheter.

CHAPTER 2

THE ESTIMATION OF ACETALDEHYDE, ACETONE AND ETHANOL IN BREATH, URINE AND BLOOD SAMPLES

2.1.1 Introduction

To study *in vivo* alcohol metabolism, it was necessary to develop techniques for the measurement of ethanol and acetaldehyde in blood, urine and expired air. In addition, it was considered to be of value to detect acetone resulting from fasting or hypoglycaemia and to be able to distinguish ethanol from methanol. The most satisfactory method for estimating all of these volatile compounds was gas liquid chromatography.

The minimum quantities of these compounds to be estimated were the endogenous levels of blood. For acetaldehyde this has been reported as 0.05 - 0.4 mg/100ml., ethanol less than 0.15 mg/100ml., and acetone 0.2 - 0.3 mg/100ml (Geigy, 1970). Methanol is not normally present. These levels will rise on the consumption of ethanol. For acetaldehyde and acetone, the expected range was 0.1 - 1.0 mg/100ml., with blood ethanol levels up to 150 mg/100ml. Air from the alveoli will be in equilibrium with the blood so that ethanol up to 100 ug/100ml., and acetaldehyde to 1.0 ug/100ml., of air was expected (Freund and O'Hollaren, 1965).

2.1.2 Sample Preparation

Whole or diluted blood samples may be injected in volumes of 1 - 5 ul., into the gas chromatograph. However, preliminary experiments showed that at the injector port temperatures of 100 - 150°C, coagulation of the sample in the needle occurred regularly necessitating frequent and tedious cleaning. Additionally, the charred deposits retained in the injector port provided a site for the adsorption of substances from successive samples with secondary "ghost" peaks. To eliminate these it was found necessary to clean the injector port after every 5 injections which was not only a time consuming procedure but also affected reproducibility.

Deproteinised samples could be used but this procedure would lead to the introduction of inorganic salts into the injector port. Irreversible binding of acetaldehyde to the protein precipitate was also a possibility but was not examined.

Volatile constituents will equilibriate with the air space above a blood sample. This headspace gas is then suitable for direct injection on to the gas chromatograph column, usually at a volume of 500 ul (Freund and O'Hollaren, 1965).

2.1.3 Gas Liquid Chromatograph

A "Varian" model 2740 gas liquid chromatograph was used. This had two columns which can be operated independantly with hydrogen flame ionisation detectors. The output from the electrometers were fed either through a "Varian" model 477

integrator, or to a twin pen recorder. Switching enabled independant or simultaneous use of the columns although only one could be integrated at a time.

Stainless steel columns 5 feet long by ⅛ inch outside diameter were used. Together with the injector insert, they were washed before use in the following sequence:- water; concentrated nitric acid; water; concentrated ammonia and acetone. The columns were then washed through with 10 mg. of orthophosphoric acid per millilitre of acetone, to deposit a layer of insoluble phosphate on the metal wall. This was said to eliminate secondary adsorption effects and tailing when using highly polar compounds (Hrivnak, 1970). Figure 2.1, page 8, illustrates the results of such treatment on the water elution peak from an unpacked column.

2.1.4 Integration

The integrator functioned by detecting the rising slope of an eluting peak, fixing the baseline level and accumulating counts at a rate determined by the difference of the incoming signal from that of the baseline and the accumulated count was printed when the slope became zero. While not integrating, the baseline was automatically tracked at a pre-set rate, but faster tracking was possible by manual intervention.

Restraints were imposed on the profile of the chromatogram and hence on the column operating conditions for accurate integration. Most commonly, peaks were superimposed on a slowly descending baseline and sufficient time for automatic

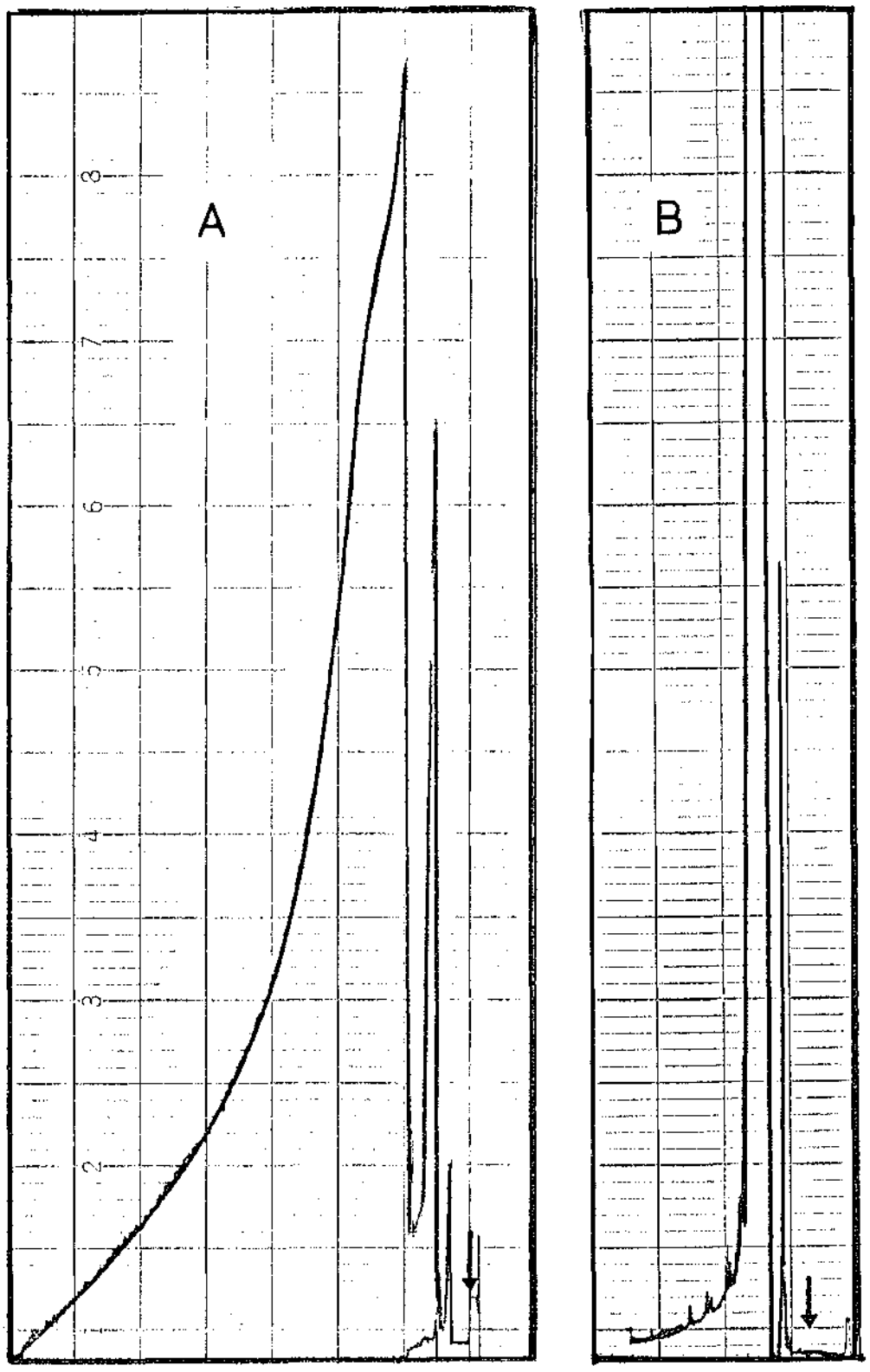

Figure 2.1

A gas chromatogram of 0.5 µl. distilled water on unpacked, 5' x $\frac{1}{8}$" stainless steel columns. (A) before treatment; (B) after treatment with phosphoric acid. Injector 105°C., oven 150°C., detector 180°C., carrier gas 20 ml/minute, range 10^{-12}, attenuation x 1, chart speed 2 cm/minute

or manual correction of the baseline value between peaks was necessary (figure 2.2, page 10).

2.1.5 Syringes

A "Hamilton" 5 µl., syringe type 5A-RN-GP was used for liquid injections and a 500 ul., gas-tight syringe type 500A-RN-GSG for head space gas.

The syringes were cleaned with 5% potassium hydroxide followed by water, acetone, ether, acetone and again water, in that order. These syringes were found to be a major source of contamination leading to spurious peaks. They were checked frequently by using them to inject 1 µl., distilled water into the gas chromatograph. If a peak other than water was detected which could not be removed by repeatedly rinsing the syringe, it was dismantled and the seal between the barrel and the needle renewed according to the maker's instructions, then flushed with water. One syringe was kept exclusively for distilled water which helped to differentiate syringe from septum, injector port or column contamination. The 500 µl., syringe was cleaned with acetone followed by ether. The plunger was dismantled from the barrel and wiped clean followed by drying in a stream of air. One syringe was kept exclusively for air injections.

2.1.6 Distilled Water

A bulk supply was redistilled in an all glass apparatus over

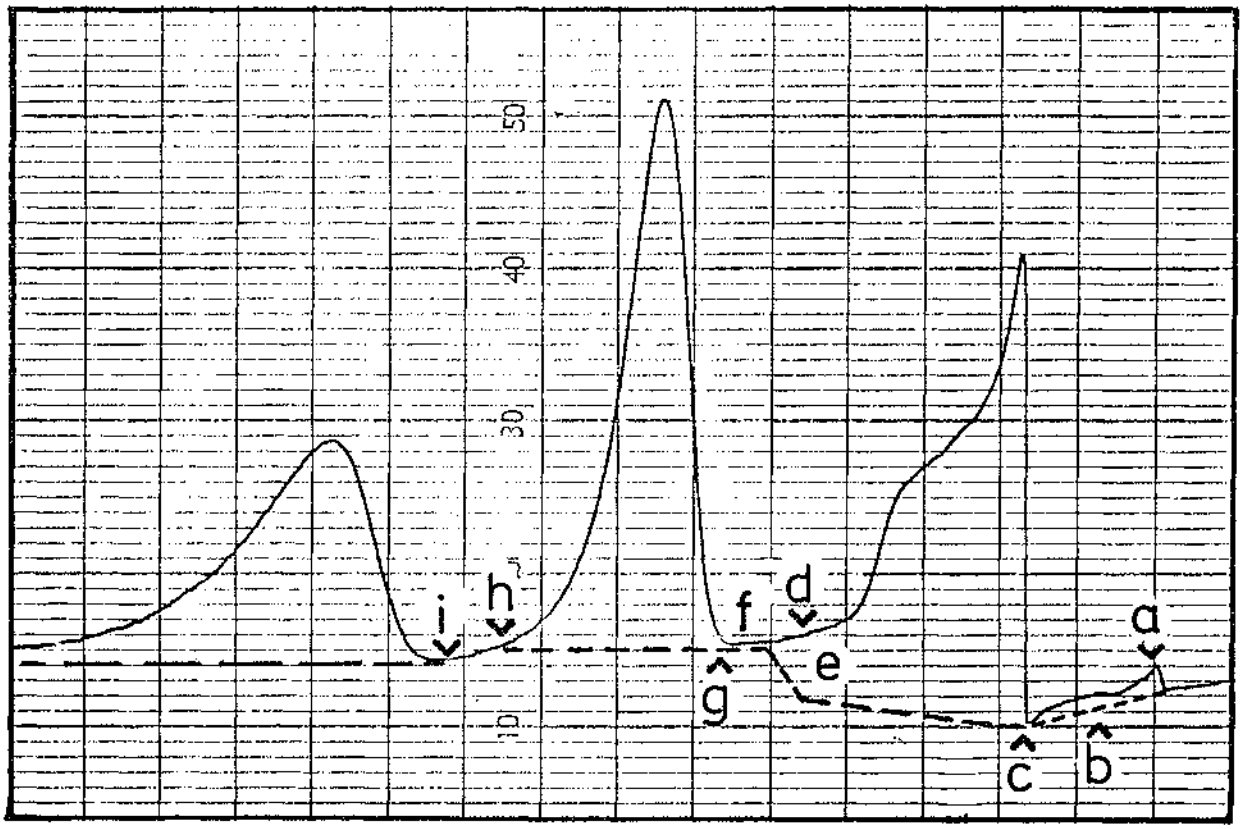

Figure 2.2

A gas chromatogram illustrating integrator function.

(a) injection
(b) automatic baseline tracking
(c) integration commences
(d) integration stops and the results printed when the slope is zero
(e) manual tracking of the baseline (dotted line)
(f) automatic baseline tracking
(g) integration commences
(h) signal negative relative to baseline, integration stops
(i) zero slope detected, peak integral printed

permanganate. On storage, acetaldehyde and ethanol as well as other volatiles could be absorbed from the atmosphere of the laboratory, but these were readily removed by boiling. Acetaldehyde was the principal contaminant and its use was subsequently restricted in the open laboratory and concentrated stock solutions stored in a separate cold room from other standards.

2.1.7 Internal Standardisation

Injection of precise microlitre volumes into the gas chromatograph was difficult to achieve. Commonly, the 5 µl., syringe used had a needle volume of 0.7 - 0.8 µl., from which approximately 0.1 µl., of solution evaporated in the injector port in addition to that expelled by the plunger. To circumvent these problems, an additional volatile substance, not normally encountered in the specimens to be analysed, was added in a fixed proportion to mixtures of acetaldehyde, ethanol and acetone of known concentration to determine the ratio of the peak areas. Such internal standards should have similar properties to those volatile substances being measured and should also elute near but not overlap them. Acetonitrile, ethyl methyl ketone and iso-propanol are such compounds. Although iso-propanol occurs in some congeners of alcoholic beverages, blood levels were not expected to be significant. It was found that internal standardisation did not compensate for errors due to blockage of the gas tight syringe needle. The resulting smaller peak areas were not in the same proportion so that it was necessary to repeat such faulty sampling.

2.1.8 Standards

The following compounds were redistilled to remove contaminants and stock solutions of 500 mg/100ml., prepared as indicated and stored in stoppered glass bottles at 4°C.

	mg/ml.	boiling point	1/20 dilution mg/ml.	volume of 1/20 to make up to 250 ml.
acetaldehyde	780	21°	39.0	31.3
ethanol	790	56°	39.5	31.6
methanol	788	78°	39.4	31.6
ethyl methyl ketone	791	65°	39.5	31.6
iso-propanol	804	80°	40.2	32.2
acetonitrile	780	81°	38.8	32.2

In addition, ethanol was checked by freezing point osmometry as suggested by Redetski (1973). A 0.1 moles/litre ethanol stock solution equal to 100 milliosmols/Kg., water, was prepared from 6.2 ml., of 95% ethanol to one litre of water, standardised against a 100 milliosmol sodium chloride solution (3.086 g/l. at 20 C.) and stored in sealed vials. For use, the undiluted ethanol, a ½ and a ¼ dilution gave standards of 460, 230 and 115 mg/100ml., respectively.

2.1.9 Determination of Optium Operating Conditions

The separation of peaks on a chromatogram is dependent upon the efficiency and resolution of the column. These factors

can be estimated from measurements made on the chromatogram. Efficiency, given by the number of theoretical plates, is approximated by the expression:-

$$16\left(\frac{\text{retention time in units of length}}{\text{peak width at the base}}\right)^2$$

This can also be expressed as a function of column length as the height equivalent to a theoretical plate (HETP), where:-

$$\text{HETP} = \frac{\text{column length}}{\text{theoretical plates}}$$

Apart from the physical nature of the column packing material, efficiency and resolution are determined by the carrier gas flow rate and the temperature of the column. A plot of HETP against carrier gas flow rates as proposed by Deemter (1956), can be used to compare the efficiency of columns at different temperatures. The highest efficiency is given by the gas velocity giving the lowest HETP.

One microlitre samples of distilled water containing 1mg/100ml., acetaldehyde and 5mg/100ml., ethanol were analysed. The injector port temperature was 155°C, and the detector 175°C. The column temperature was varied from 100 - 180°C, in steps of 20°, only 5 minutes being required for stabilisation. Carrier gas flow rates were varied by altering the pressure control valve which had previously been calibrated for flow rate at the detector at each temperature. Attenuation and chart speed were adjusted to give peaks which were on scale and of suitable width for accurate measurement.

Headspace gas samples were prepared by pipetting 1.0 ml. of

a standard mixture of acetaldehyde and ethanol into a 25 ml., screw capped bottle and left to equilibrate for 15 minutes at room temperature. The aluminium cap and rubber liner was pierced with a 21 gauge hypodermic needle through which the needle of a gas tight syringe could be passed, 500 ul. samples were injected onto the column.

2.2 COLUMN PACKINGS

2.2.1 Porapak Q (Waters Associated Inc.)

Proapaks are porous polymers of ethylvinylbenzene cross-linked with divinylbenzene to form a uniform structure of distinct pore size which does not require liquid coating (Hollis, 1966). Porapak Q has been used for the analysis of blood samples by various workers, including those listed below:-

	Baker et al. 1969	Waterhouse 1972	Cooper 1971
Column temperature °C.	100	110	170
Retention times (minutes):-			
methanol	5.0	2.5	1.3
acetaldehyde	10.0	4.0	-
ethanol	15.0	3.0	2.2
internal standard	acetonitrile	n-propanol	iso-propanol
retention time (minutes)	23.0	27.0	3.5

The freshly washed, treated columns were filled with approximately 2.7 grams of Porapak using a vacuum water pump and a vibrator to pack the particles. The column was then preconditioned in the gas chromatograph at 200°C, overnight with a slow flow of nitrogen carrier gas while disconnected from the detector.

Three main peaks were detected and attributed to water, acetaldehyde and ethanol in that order. The Van Deemter plots for acetaldehyde and ethanol are shown in figure 2.3 (page 16) and these suggested optimal conditions of 140 - 160°C column temperature and carrier gas flow rates of 30 - 50 ml./minute. Inspection of the chromatograms showed that:-

(a) at 160-180° tailing of the water peak was particularly apparent, figure 2.4, page 17, and the three components were only completely resolved over a narrow optium range of flow rates, 30 - 40 ml/minute.

(b) at 120-140° resolution was good at flow rates below 45 ml/minute but peak shapes were poor below 20 ml/minute.

(c) at 100° tailing of the ethanol peak prolonged the total analysis time beyond 12 minutes.

Methanol, acetone and various volatile substances suitable for internal standards were added to the acetaldehyde/ethanol mixture at concentrations of 5 mg/100ml. At a column temperature of 140°C, and a carrier gas flow rate of 40 ml/minute, the methanol peak overlapped that of acetaldehyde. Reduction of temperature to 130° resolved these two components adequately to give retention times of:-

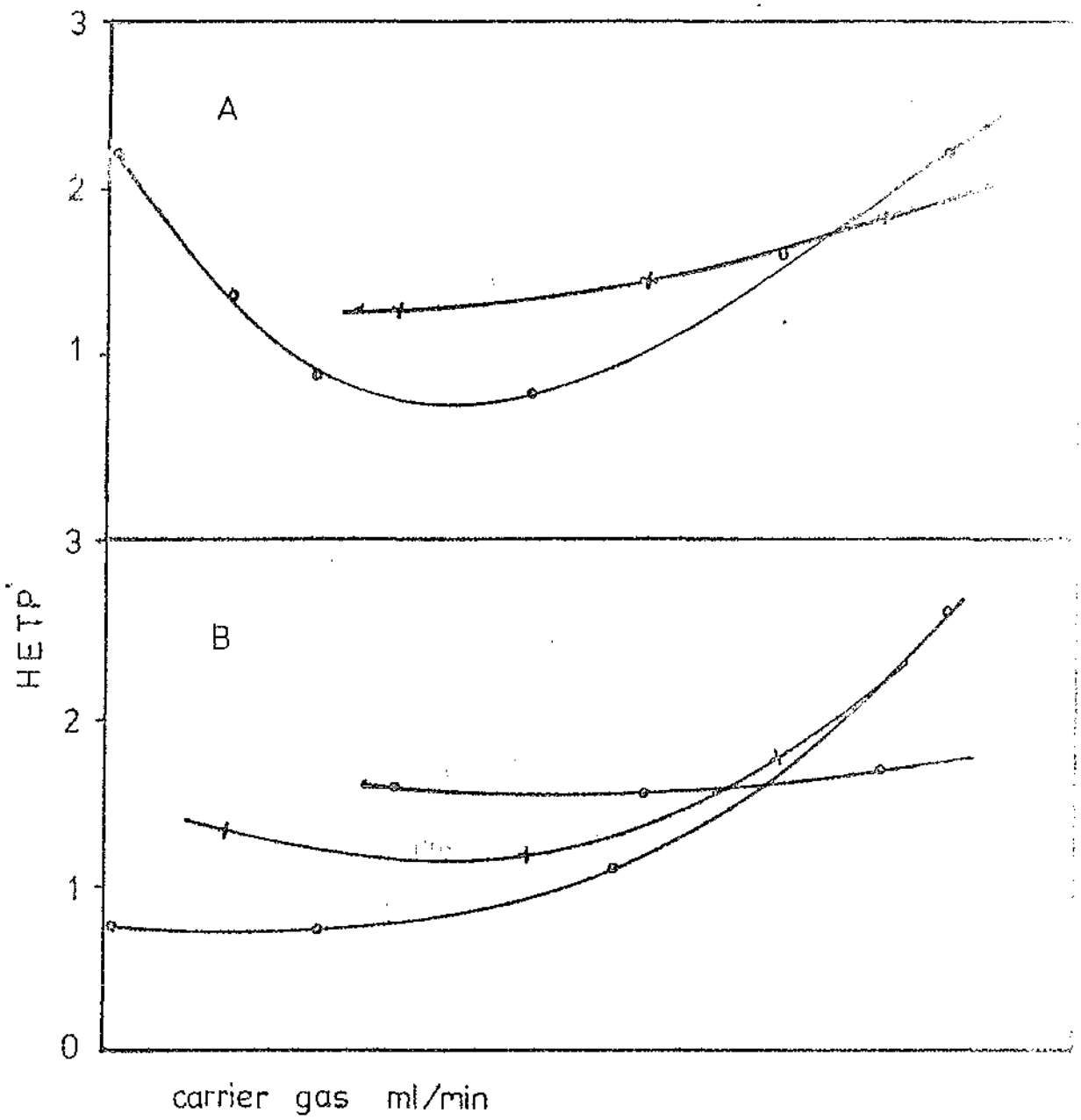

Figure 2.3

Van Deemter plots at different temperatures, of carrier gas flow rate against height equivalent to a theoretical plate (HETP) for

(A) acetaldehyde and

(B) ethanol

Column 5' x $\frac{1}{8}$" stainless steel packed with Porapak Q.

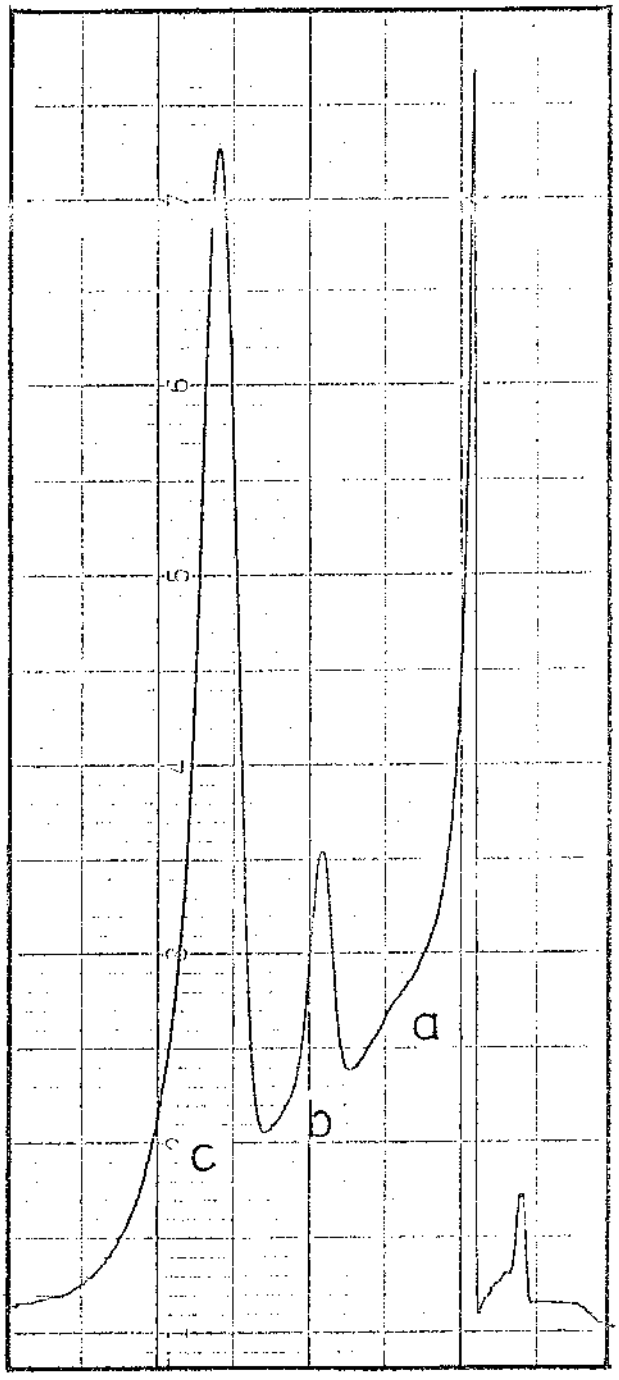

Figure 2.4

A gas chromatogram of 1 µl. acetaldehyde, 1 mg/100ml. plus 5 mg/100ml. ethanol on Porapak Q at 180°C, showing marked tailing of the water peak (a); acetaldehyde, (b) and ethanol, (c), superimposed on the tail. Carrier gas 55 ml/minute, chart speed, 4 cm/minute.

methanol	1.1 minutes
acetaldehyde	1.6 minutes
ethanol	3.2 minutes
acetonitrile	4.5 minutes
acetone	6.0 minutes
isopropanol	6.7 minutes
ethyl methyl ketone	16.0 minutes

The total analysis time was 7 minutes using acetonitrile as the internal standard. Dilutions of a standard solution containing 5.0 mg/100ml., of acetaldehyde and 100mg/100ml., ethanol were made to give a range of concentrations from 0.01 and 0.20 mg/100ml., respectively. One microlitre volumes were injected into the vaporiser at 150°, a column temperature of 130° and a carrier gas flow rate of 40 ml/minute. The electrometer was set on range 10^{-11} and attenuation x1. The integrator settings were: peak width at ½ height, 30 seconds; slope sensitivity control at 6 μV/second; baseline correction rate of 3 μV/second and a counting rate of 125 per second.

The detector response was linear for acetaldehyde and ethanol (figure 2.5, page 19). The average acetaldehyde : ethanol detector ratio was 1.44 : 1.0. When the ethanol peak area was used as an internal standard, acetaldehyde could be estimated within $\pm$ 4% down to a level of 0.1 mg/100ml. Below this the error increased to 60% or more due largely to inaccuracies of peak area integration. To obtain sufficient counts in the peak area below concentrations of 1.0 mg/100ml., the integrator count rate was increased to 1 000 per second.

The inaccuracies of acetaldehyde determination were largely

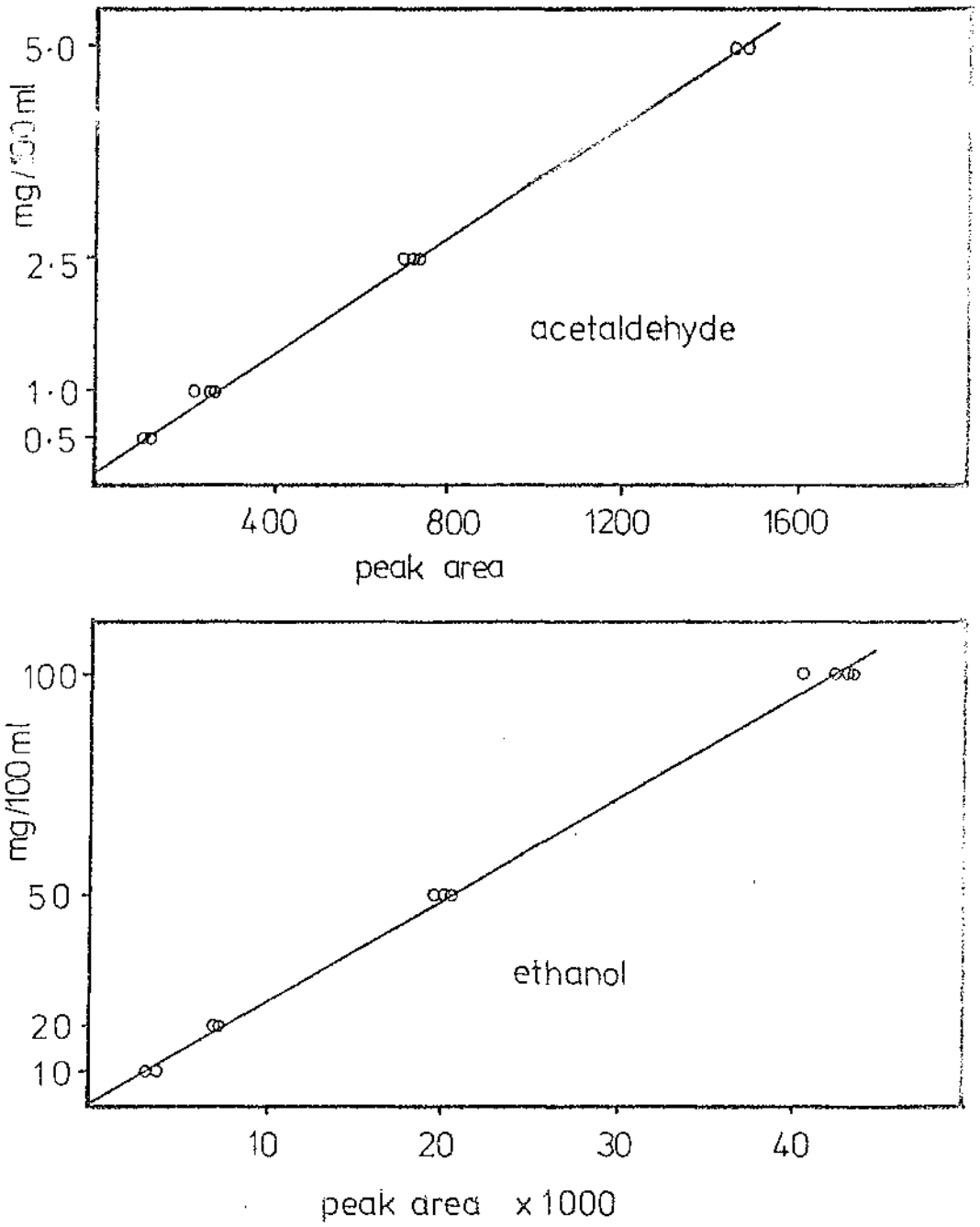

Figure 2.5

Calibration curves for acetaldehyde and ethanol, peak area against concentration. Porapak Q at 130°C.

due to the water peak that appeared immediately before it. When headspace gas was used, this water peak was minimal and levels down to 0.1 mg/100ml., could be determined. Sensitivity could be increased tenfold by equilibrating the sample at 55°C, as suggested by Duritz and Truitt (1964), although at this temperature, water vapour displaced acetaldehyde non-specifically bound to the column, equivalent to 0.01 mg/100ml. Non-specific binding of all substances occurred with increasing use and was dependant upon the quantities injected, but acetaldehyde was the most common contaminant in this way. Dressman (1970) suggested that this could be removed by "steam cleaning" with multiple injections of 5^{-10} µl. distilled water. Syringe contamination was more of a problem. After injecting headspace from an aqueous solution containing 10 mg/100ml., acetaldehyde and 100mg/100ml., ethanol, further injections of headspace over water from the same syringe resulted in an acetaldehyde peak corresponding to 0.2 mg/100ml., and ethanol of 1.0mg/100ml. Dismantling and cleaning of the syringe with remaking the teflon seals was then required.

It was concluded that Porapak Q and headspace gas was suitable for estimating both acetaldehyde and ethanol in aqueous solutions. At low concentrations, frequent checks were necessary in order to recognise syringe or column contamination. An analysis could be completed in seven minutes.

2.2.2 Carbowax 1500 (5%) on Chromosorb W-DMCS

Chromosorb (Varian), is an inert support prepared from

diatomaceous earth, acid washed and treated with demethyl-chlorosilane in order to reduce surface active sites which could increase tailing or adsorption of the components being analysed. Carbowax 1500 (Mann Research Labs. Inc.) is the Union Carbide Company trade name for a polyethylene glycol of 500 - 600 molecular weight. It is a soft wax with the consistency of grease which is 75% soluble in water and has been used previously by Boiteau and Moussion (1968) and Duritz and Truitt (1964) for the separation of alcohols by gas chromatography. Very short retention times at low column temperatures were found.

One gram of carbowax was dissolved in 20ml. of toluene to give a 5% concentration and then 5 grams of Chromosorb added to make a slurry. The excess solvent was removed on a sintered glass filter and the material dried in an air stream with continuous stirring. It was then packed into a column and conditioned in the gas chromatograph at 130°C. overnight with a slow flow of carrier gas.

Injection of 1 ul., of distilled water gave three main peaks (figure 2.6, page 22). The first of these, (a), had the same retention time as acetaldehyde at temperatures ranging from 60 to 130°C., and it was not possible to differentiate a 1 mg/100ml., solution because of this. The peak was possibly acetaldehyde resulting from the decomposition of polyethylene glycol by the dissolved oxygen in the water as implied by Persinger and Shank (1973). The remaining peaks, (b) and (c) can be attributed to water or decomposition products and would interfere with the analyses because of tailing between them.

Injection of headspace gas gave rise to three peaks in

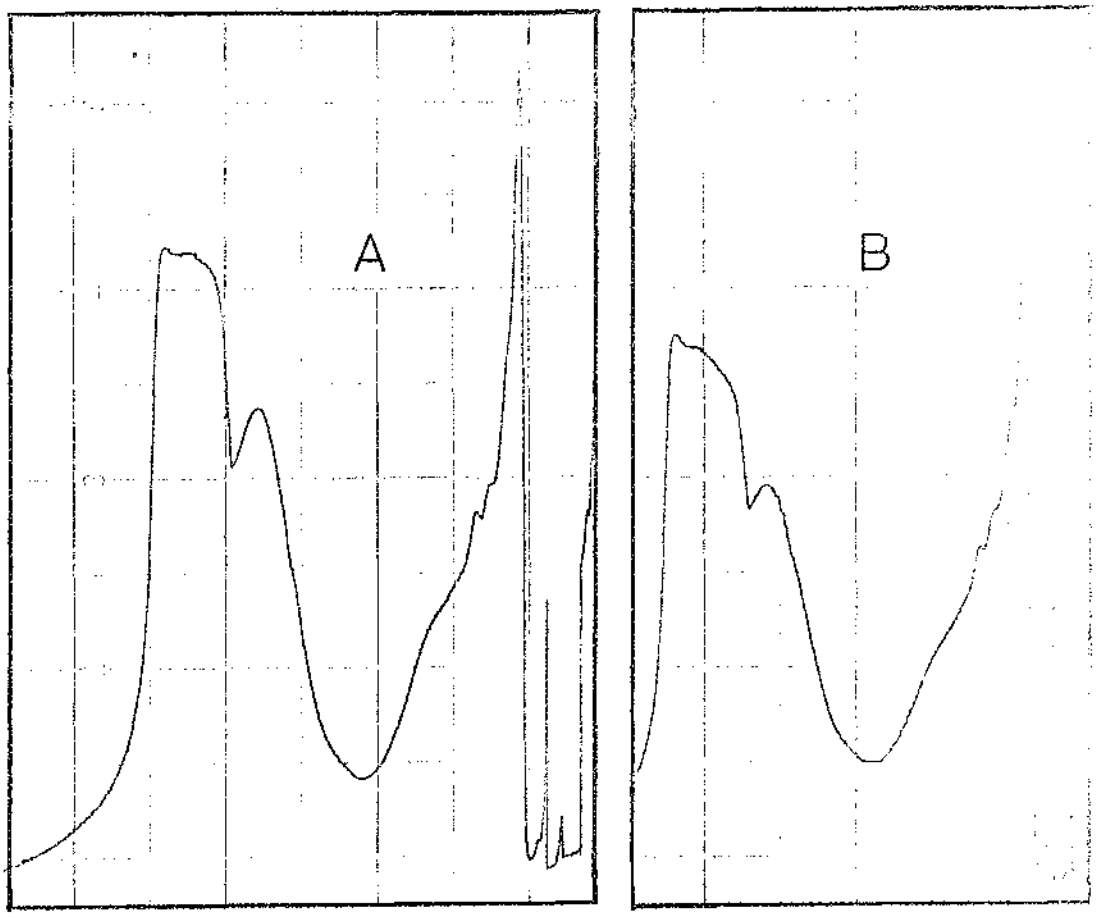

Figure 2.6

A gas chromatogram showing the inability to distinguish between (A) 1 µl. distilled water and (B) 1 µl. of 1 mg/100ml. acetaldehyde by a column containing 5% Carbowax 1500 on Chromosorb W-DMCS at 80°C. Carrier gas 30 ml/minutes, range 10^{-11} attenuation x 1, chart speed 1 cm/min.

addition to the volatile components (figure 2.7, page 24). The first, (a), broad and relatively low peak was attributed to the disruption of the carrier gas flow. The second, (b), a tall sharp peak was absent if nitrogen was injected suggesting that it was oxygen or carbon dioxide. The third peak, (c), was small but sharp and its variation in size from room air to headspace from water samples suggested that this might be a water vapour effect.

Van Deemter plots showed greatest efficiency at 100 - 200°C, but at these temperatures, acetaldehyde eluted immediately after a trough in the baseline which precluded accurate integration of the peak. This trough was eliminated at temperatures below 100°. At 80° and a carrier gas flow rate of 30 ml/minute, separation of acetaldehyde, ethanol and ethyl methyl ketone was obtained. Under these conditions, an analysis could be completed in two minutes, the retention times were:-

acetaldehyde	24 seconds
acetone	30 seconds
ethyl methyl ketone	43 seconds
ethanol	56 seconds
methanol	not detected
iso-propanol	eluted with the ethanol peak

The optium settings for the gas chromatograph controls were: range of 10^{-11} and x1 attenuation; integrator peak width at half height set at 3 seconds; slope sensitivity control at 6 μV/second; baseline correction rate of 3 μV/second and a counting rate of 1 000 per second. Linear responses were obtained for acetaldehyde from 0.5 to 5.0 mg/100ml., and for ethanol from 10 to 100 mg/100ml. The integrator did not

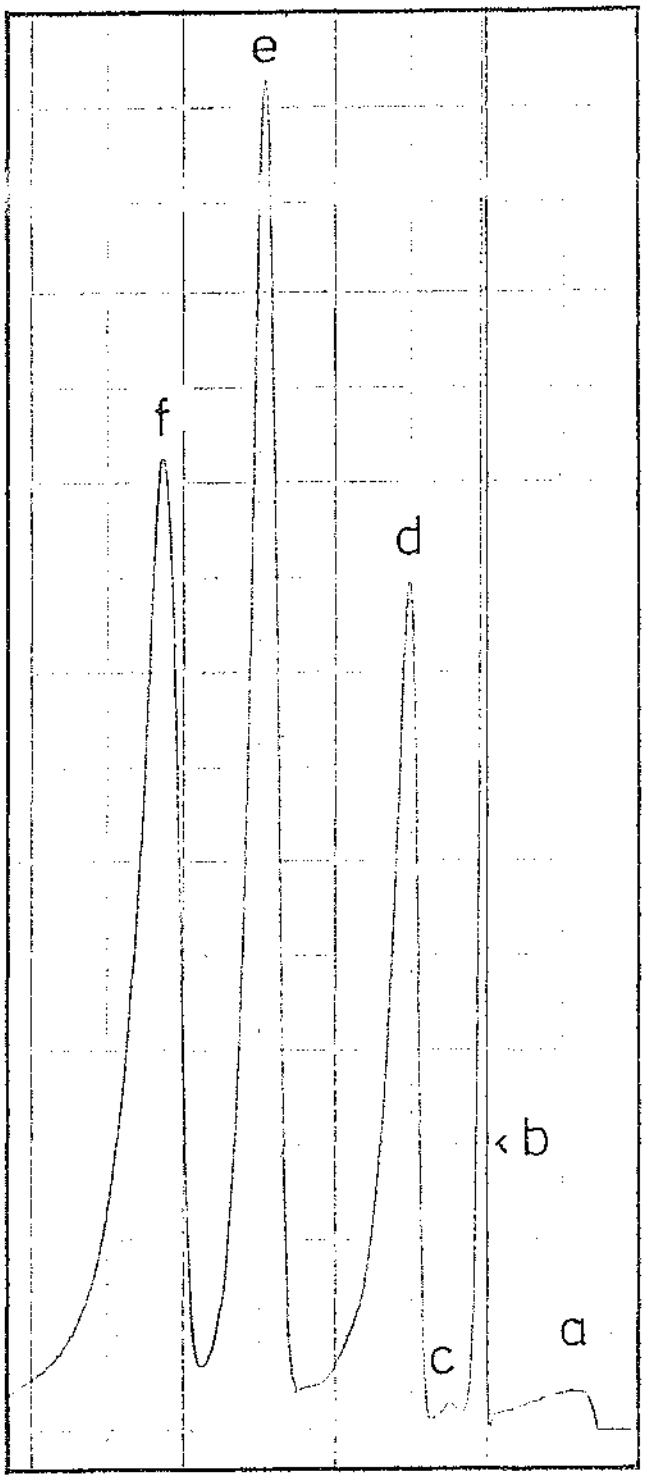

Figure 2.7

Separation of acetaldehyde, ethyl methyl ketone and ethanol in nitrogen gas by 5% Carbowax 1500 on Chromosorb W-DMCS at 80°C. Range 10^{-11} x chart speed 6 cm/minute. The peaks are

(a) disruption of carrier gas flow; (b) oxygen or carbon dioxide;
(c) possibly water vapour; (d) acetaldehyde 5mg/100ml.;
(e) ethyl methyl ketone 5 mg/100ml. (f) ethanol 100 mg/100ml.

detect ethanol peaks below 5 mg/100ml., when the settings gave the optium results for higher concentrations. Injections of headspace over water resulted in a small peak corresponding to 0.025 mg.100ml., acetaldehyde and was probably due to water vapour in the sample, so that this limited the minimum level of acetaldehyde detectable.

Equilibration at higher temperatures increased the peak areas of the test solutions and also of the blank, so that there was no net gain in sensitivity for acetaldehyde. The best compromise was found at 40°C., where the peak area of acetaldehyde at 0.1 mg/100ml., was four times that of the blank and ethanol could be detected down to 0.3 mg/100ml. At higher temperatures, a peak appeared with a two minute retention time, attributed to water, which lengthened the total analysis time.

In conclusion, Carbowax 1500 offered a rapid method for estimating etnanol, but was less reliable for acetaldehyde at the levels likely to be encountered in human blood samples.

2.2.3 Carbowax 400 (10%) on Chromosorb W-DMCS

Other carbowaxes have been used in gas chromatography, but there are problems in defining the exact nature of the commercial product. The Union Carbide Company markets polyethylene glycols under the name of Carbowax with a serial number suffix which is related to molecular weight, for example, 1500 has a molecular weight of 500 - 600 and Carbowax 1540, of 1300 - 1600 (Bennett, 1963). Curry et al.

(1966) used polyethylene glycol 400 on celite at a temperature of 85°C., with similar retention times to those found below.

A 10% coating of Carbowax 400 (Mann Research Labs. Inc.) on Chromosorb was prepared as previously described. This material was liquid at room temperature, tending to solidify on cooling, and smelt strongly of acetaldehyde. Marked instability of the gas chromatograph was found at temperatures between 50 and 110°C., and there was a tendency for the base-line to fall rapidly with continuing use. Operation was only possible with low carrier gas flow rates, less than 10 ml/minute, as above this it was not possible to adjust the baseline to zero.

Injections of headspace gas equilibrated at room temperature, a range setting of 10^{-11} and attentuation of x 1 resulted in an ethanol retention time of two minutes. The minimum amount that could be detected was 10mg/100ml., as smaller quantities gave peaks that were lost amongst a multitude of irregular peaks which eluted at about the same time. Later experience suggested that these were probably due to water vapour. Acetaldehyde at 1 mg/100ml., could not be detected under these conditions. It was concluded that this material was unsuitable for the present purpose.

2.2.4 Carbowax 1540 (5%) on Chromosorb W-DMCS

Carbowax 1540 is another polyethylene glycol available (Mann Research Labs. Inc.) with a larger molecular size, 1300 - 1600, than Carbowax 1500. This is partially soluble in water, but has a solid consistency like candle wax and can

be fractured. It was hoped that the larger molecular size would be more stable to decomposition while retaining the retention time characteristics of the lower molecular weight material.

An extremely stable baseline was found and the maximum range setting, 10^{-12} could be used. Unfortunately, acetaldehyde could not be resolved sufficiently from peaks of unknown origin eluting on either side of it. In addition, the appearance of a water vapour peak with a retention time three times that of ethanol would have prolonged the total analysis time to that given by Porapak Q.

2.2.5 Conclusions

A comparison has been made between the gas liquid chromatographic materials, Porapak Q and coatings of various polyethylene glycols on an inert support. Only Carbowax 1500 and Porapak Q were suitable for the analysis of volatile substances likely to be found in the blood, urine or breath of individuals consuming alcohol. The material offering the most sensitive technique was Porapak Q but it had a longer total analysis time than that of 5% Carbowax 1500 on Chromosorb W. This latter material was not suitable for accurate estimations of small amounts of acetaldehyde because of its decomposition to acetaldehyde and to the close proximity of the acetone peak, but it was useful for qualitative work where speed was important.

Both materials at their optimum operating temperatures and carrier gas flow rates were used in subsequent experiments.

2.3 APPLICATION TO BLOOD AND URINE SAMPLES

2.3.1 Introduction

In the previous chapter, the use of gas liquid chromatography for estimating the volatile substances in biological fluids was discussed and the relative merits of various column packing materials examined. It was suggested that the best way of determining these volatiles was to analyse the vapour phase above samples.

Duritz and Truitt (1964) showed that when whole blood was equilibriated at 55°C. in an enclosed space, the levels of acetaldehyde, but not ethanol, declined rapidly but that this did not occur if the sample had previously been deproteinised by the zinc sulphate/barium hydroxide method. However in a later paper, Truitt (1970) showed that acetaldehyde increased with incubation and doubt arose as to the validity of this technique. No mention was made of possible displacement of acetaldehyde from the Carbowax 1500 column used. Both of these papers state that 1.0ml. of blood was deproteinised by adding 0.5 ml. of saline and then 0.25 ml. of 5% zinc sulphate followed two minutes later by 0.25 ml. of 0.3 M barium hydroxide. These volumes of precipitant are insufficient to deproteinise 1.0 ml. of blood but would be adequate for 0.1 ml. volumes.

Boiteau and Moussion (1968) also used headspace gas from a

25 ml. bottle containing a fluoride impregnated filter paper disk on which 20 ul. of blood was absorbed and equilibrated at 55°C. Coldwell et al. (1971) who subsequently used the paper disk technique, described an increase in acetaldehyde of approximately 2 µg/ml. in a sample containing 40 µg/ml., but only when stored at 4°C for 24 hours. In view of these findings, it was considered that some of these experiments should be repeated.

2.3.2 Effect of blood volume

Various factors are likely to affect the concentration of volatile constituents in the headspace gas phase and one of these is the ratio of sample to container volume. To test these effects, different volumes of blood containing 1.0 mg/100ml. acetaldehyde, 10 mg/100ml. ethanol and 1.0 mg/100ml. of the internal standard, ethyl methyl ketone, were pipetted into 25 ml. screw capped bottles which were immersed in a water bath at 55°C. Samples of the gas phase were taken at various time intervals and injected without delay into the gas chromatograph.

A plot of peak area against volume after a 20 minute incubation (figure 2.8, page 30) showed that maximum concentrations were not obtained until a blood volume of 1.0ml. was used, or a ratio of blood to air of 1 : 25.

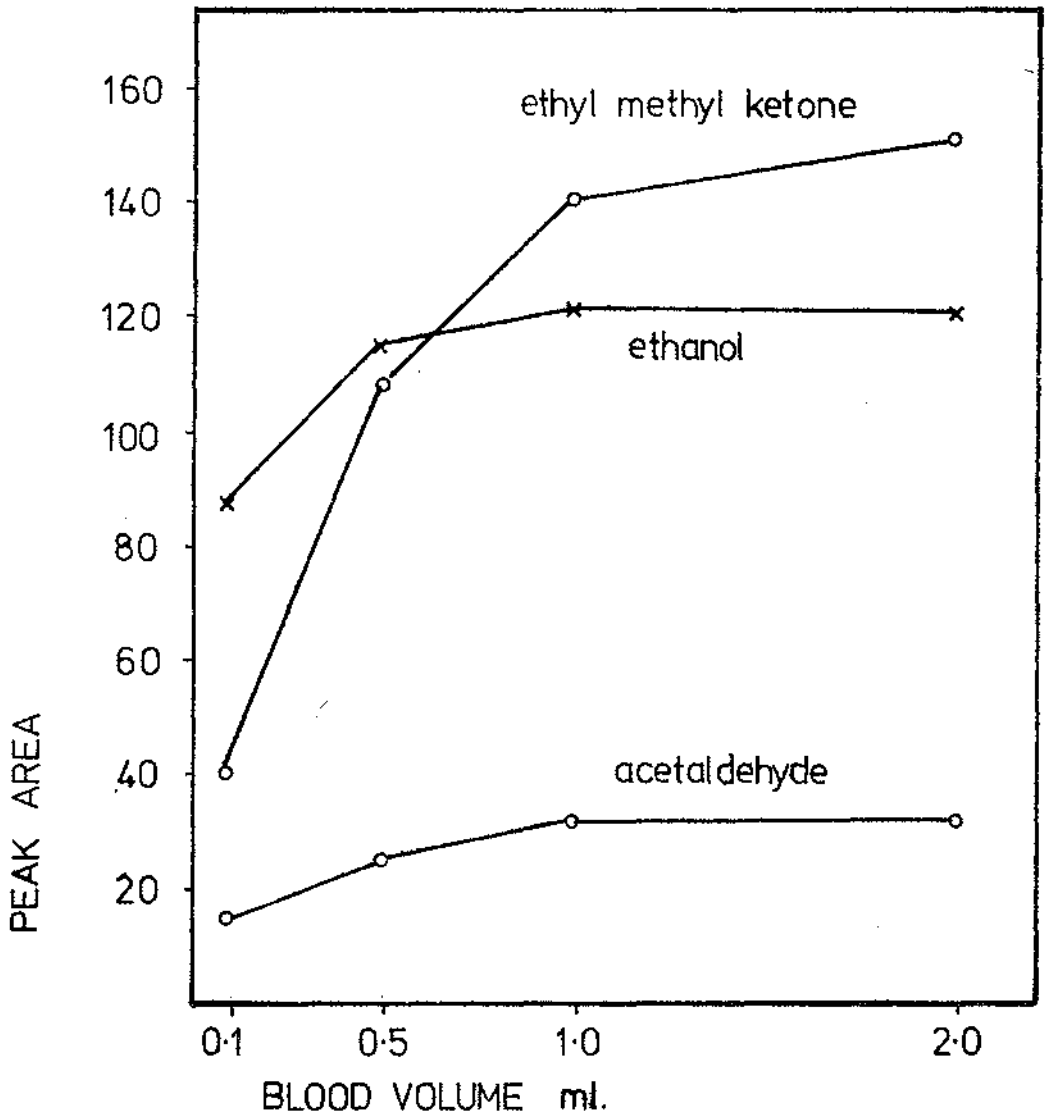

Figure 2.8

The effect of blood volume on the concentration of acetaldehyde, ethyl methyl ketone and ethanol in the headspace gas from a sealed 25 ml. container equilibrated at 55°C.

2.3.3 Stability of blood sample

A bottle containing 2.0 ml. of the blood used above was incubated at 55°C. over a ninety minute period. The head-space was analysed at frequent intervals and the results plotted (figure 2.9, page 32). Maximum levels were not attained until 10 minutes had elapsed; thereafter, acetaldehyde showed a steady decline until 20 minutes when it started to rise at a rapid and uniform rate. Ethanol declined continuously from 15 minutes while ethyl methyl ketone levels remained constant. It was noted that the increase in acetaldehyde coincided with haemolysis of the blood specimen which was several days old. The experiment was repeated using a freshly drawn blood sample and in this case acetaldehyde continued to decline until almost zero levels were reached; no haemolysis took place. It was shown that acetaldehyde added to fresh blood at a concentration of 1.0 mg/100ml. decreased to one tenth of this value over a three hour period at room temperature whereas ethanol at 10 mg/100ml. remained unchanged.

It appeared that acetaldehyde was either metabolised or bound within the intact erythrocyte and when disruption of the cell membranes took place, acetaldehyde appeared as a result of the metabolism of ethanol. This problem was not apparent when 50 ul. of blood was used, presumably because most of the constituents were liberated from the sample leaving a concentration below which enzymic action was too slow to be detected over the 30 minute observation period.

An internal standard was used to compensate for inaccuracies

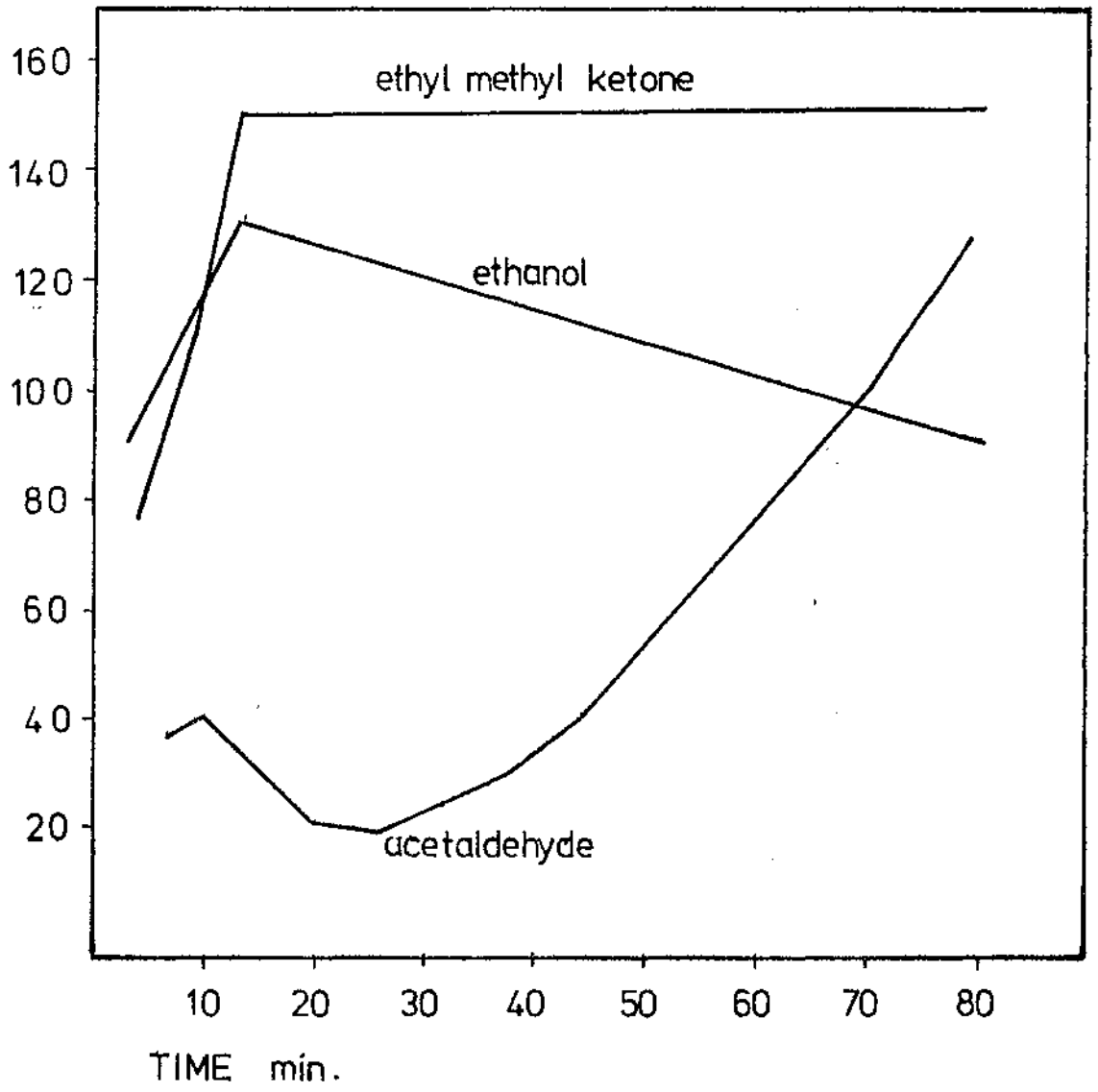

Figure 2.9

The effect of incubation at 55°C. on the levels of acetaldehyde, ethyl methyl ketone and ethanol in the headspace gas.

of volumes injected into the gas chromatograph due to the operating pressure in the injector port and this introduced further problems. The change in concentration of the standard in the headspace gas with the volume of the aqueous phase should parallel that of the components being measured. For example, the estimated concentration of ethyl methyl ketone doubled when the volume was increased from 50 to 100 ul. while that for ethanol increased by only 1.5 and acetaldehyde did not change appreciably. These difficulties may have little adverse practical effect if a more suitable internal standard is used for ethanol without the application of a correction for acetaldehyde. However, the main factor in using any such method is its sensitivity. With the gas chromatograph operating in the maximum range of 10^{-12}, the peak areas of a 0.1 mg/100ml. acetaldehyde standard were poorly reproduced and not proportional to concentration because of variations in the baseline setting and background noise levels. This was clearly unsatisfactory when account was taken that most estimations on normal volunteers would be of this order.

2.3.4 Precipitated blood samples

If the variations in acetaldehyde and ethanol levels were due to enzymic action, it seemed likely that protein precipitation would prevent this. To 1.0 ml. of blood containing 0.1 mg/100ml. of acetaldehyde and 50 mg/100ml. ethanol, was added 0.25 ml. of 5% zinc sulphate and 0.25 ml. of 3 M barium hydroxide, following the modification of the Somogyi precipitation method used by Duritz and Truitt (1964). Instead of

adding 0.5 ml. of saline as a diluent, 0.5 ml. of 100 mg/100ml. acetonitrile in normal saline was used instead. This served the dual role of diluent and internal standard. The mixture was contained in a 25 ml. screw-capped container and did not initially appear to be deproteinised. On incubation at 55°C. the haemoglobin pigment slowly turned brown indicating denaturation. Analysis of the headspace gas during this time showed that acetaldehyde increased and ethanol decreased while the internal standard remained constant over a 45 minute observation period.

A fresh preparation was treated with the reagents in the same way but centrifuged. The clear, unhaemolysd supernatant was incubated at 55°C. and slowly became opaque indicating completion of the deproteinisation. No change was seen in the concentration of the three components over a three hour period. This suggested that the acetaldehyde production reported by Truitt (1970) was the result of incompletely deproteinised blood. A further experiment was carried out using the correct proportions of zinc sulphate and barium hydroxide to whole blood and deproteinisation was completed immediately. No change was seen in the concentration of the volatile constituents over a two hour incubation period at 55°C.

A more convenient method of protein precipitation and the one used for the preparation of blood samples for enzymatic assays, used perchlorate. It was found that the addition of an equal volume of blood to ice-cold 1.0 M perchlorate provided a protein free supernatant after centrifugation. Incubation of the whole precipitate as before did not result in the alteration of the concentration of acetaldehyde or ethanol in

the headspace gas after a 15 minute equilibration period. As accurate measurement of blood volume was not possible by hypodermic syringe, bottles containing 3.0 ml. of perchlorate were weighed and reweighed following the addition of approximately 3.0 ml. of blood.

Blood weight was converted to volume by dividing by the weight per millilitre of normal blood (1.06 grams.). If the blood volume was over 3.0 ml. further perchlorate could be added and if below 3.0 ml. a correction factor was applied.

2.3.5 Headspace gas enrichment

Incubation of the sample at 55°C. increased the concentration of the volatile substances approximately six-fold from the room temperature levels. An alternative procedure suggested by Savory et al. (1968) was to add a dehydrated salt to displace volatile substances from the water phase. The method finally adopted used 0.5 ml. of perchlorate supernatant in a 5.0 ml. screw capped container to which was added 100 ul. of 50 mg/100ml. acetonitrile as an internal standard. Anhydrous sodium sulphate was added and the bottle incubated in a waterbath at 34°C. Warming was necessary because when room temperatures were low, solidification of the salt by supercooling occurred causing a marked loss of volatile material in the gas phase. The choice of 34°C. was one of convenience; it was, in fact, the water-bath temperature required for another procedure. At 55°C. the concentration of water vapour in the gas phase was such that the water peak on the gas chromatograph tailed into that of acetaldehyde.

Porapak Q was the most suitable column material but water vapour eluted a small quantity of adsorbed acetaldehyde.

The effect of sodium sulphate concentration was determined by using a standard perchlorate supernatant and varying the quantity of salt. The results showed that the volatile substances in the headspace increased up to the point of salt saturation of the aqueous phase and thereafter levelled off until there was complete dehydration of the specimen. This plateau made sample preparation easier in that the weight of sodium sulphate could be approximated by measuring the volume in a calibrated tube. The method gave a twofold increase in peak areas over that involving heating at 55°C. alone.

2.3.6 Gas chromatograph operation

The optium settings for the gas chromatograph operating with a 5' x ⅛" stainless steel column containing Porapak Q were as follows:-

Injector temperature, 150°C; column oven 130°C; detector, 150°C; carrier gas flow rate, 40 ml/minute; range, 10^{-12}, attenuation x 4. The following integrator settings were used:-

peak width, 30 seconds; slope sensitivity, 0.6 μV/sec.; baseline correction rate, 0.3 μV/sec.; counting rate 125 Hz. This enabled integration of ethanol levels up to 300 mg/100ml. without alteration of the attenuator. During operation, the baseline was set using the fast digital baseline correction

button 15 seconds prior to the elution of the acetaldehyde peak. Thereafter this correction was automatic. After the acetaldehyde peak, the recorder attenuator was used to keep the remaining peaks on the chart paper. This was not essential but provided a useful reference for future work, especially as a check on integration and peak identification. Acetone was eluted on the descending slope of the internal standard so that a larger error was found with this component. An alternative internal standard could have been used to overcome this problem but only at the expense of an extended analysis time.

2.3.7 Standards

A standard perchlorate supernatant was prepared as follows. The requisite quantities of acetaldehyde, acetone and ethanol stock solutions for 10 ml. of blood were made up to a volume of 8.5 ml. with distilled water. This corrected for the average water content of blood which was 85%. To this was added 10 ml. of perchlorate. This was necessary because the ratios of the peak areas were different if distilled water was used in place of the perchlorate. Such solutions were made to cover a range of acetaldehyde and acetone concentrations of 0.05 to 1.0 mg/100ml. together with ethanol from 10 to 200 mg/100ml. These were treated in the same way as perchlorate supernatants from blood samples and the ratio to the internal standard determined in each case. The results are plotted in figure 2.10 (page 38), and show that ethanol was linear up to 250 mg/100ml. At low concentrations, the acetaldehyde curve

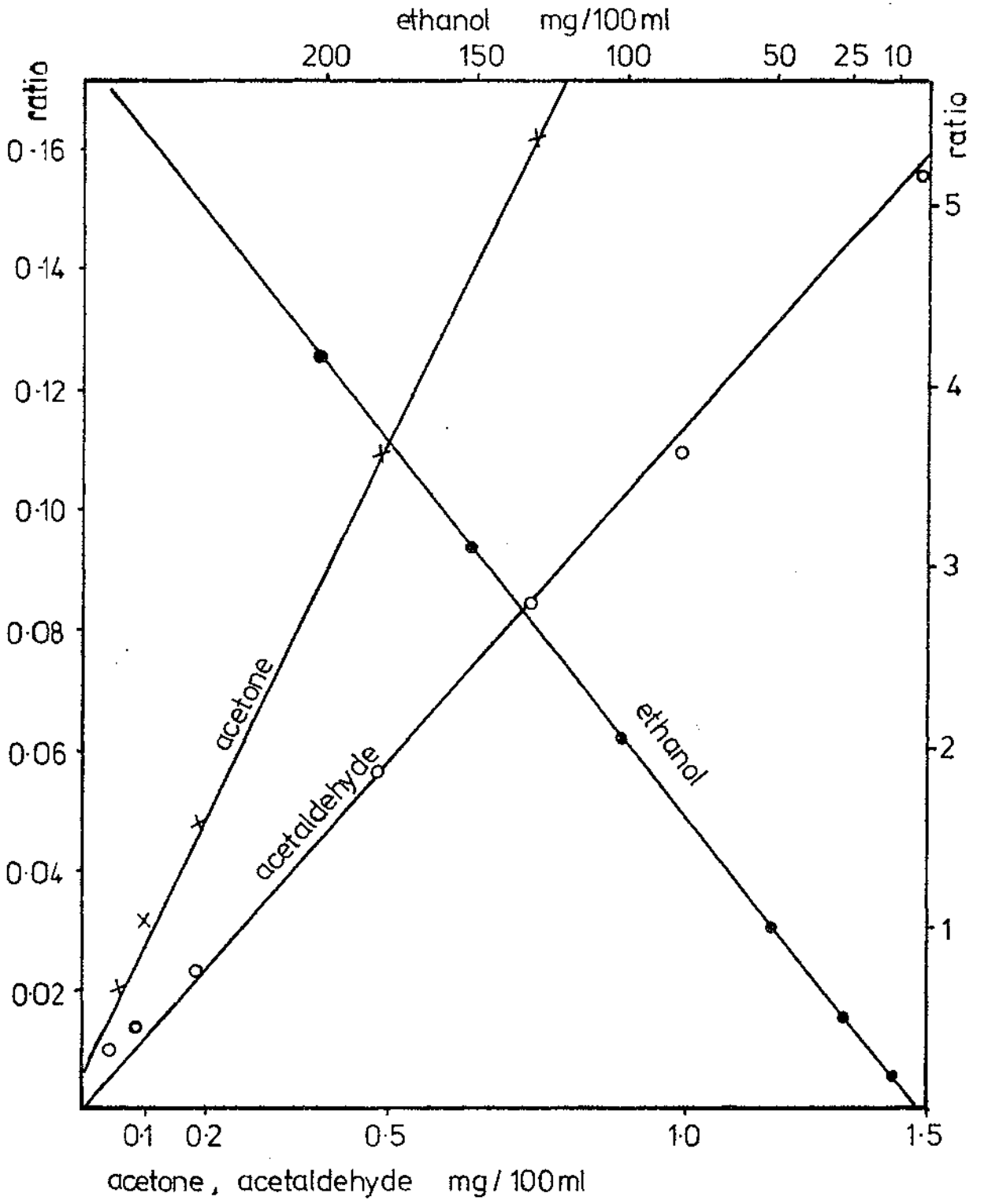

Figure 2.10

Calibration curves for acetaldehyde, ethanol and acetone in headspace gas, concentration against ratio of the peak areas to internal standard. Porapak Q at 130°C.

did not pass through the zero origin. This was not fully explained by the small peak eluted by water alone or by contamination of the other solutions by acetaldehyde. Low results at the high end of the scale can be explained by early integration caused by the elution of the high ethanol concentrations in those particular standards. Acetone levels were determined throughout the range with a wider variability because of the close proximity of the internal standard as already explained. In practice, acetaldehyde levels, while not being determined very precisely, were sufficiently accurate for the general trends to be found and corresponded closely to values from an alternative assay procedure developed by K. E. Crow (personal communication).

2.3.8 Urinary alcohol

Urine samples could be tested in the same manner as for blood perchlorate supernatants except that the standards were made up to 20 ml. with distilled water and did not contain perchlorate.

2.3.9 Conclusions

The use of perchlorate supernatants from blood samples appeared to be a satisfactory method for stabilising the acetaldehyde content and the sodium sulphate method for increasing the concentration of volatile constituents in the headspace gas was found to be more satisfactory than heating to 55°C. This

technique was particularly developed for the determination of acetaldehyde and is more complicated than that required for the estimation of ethanol levels only.

2.4 BREATH TESTING BY GAS CHROMATOGRAPHY

Breath specimens were collected by breathing through a 250 ml. separating funnel. At the end of expiration, the tap was closed and a rubber stopper placed in the open end. Samples were withdrawn with a gas-tight syringe through a hypodermic needle inserted in this stopper. The introduction of precise volumes of air into the injector port of the gas chromatograph could not be ensured because of the high carrier gas operating pressure and in the absence of an internal standard, it was not possible to estimate alcohol levels accurately.

A more satisfactory means of introduction of breath samples was clearly required and subsequently a six port gas sampling valve was purchased. This enabled a sample loop (which could be filled at atmospheric pressure) to be switched into the column carrier gas flow. The valve was a standard optional accessory for the Varian 2740 gas chromatograph and provision had been made in the instrument for its installation behind the front panel. The operating button projected from the front and on being pushed, the carrier gas flow was immediately diverted around the sample loop and into one of the columns. The loop was attached to fittings on the side of the instrument and was provided with external pathways for filling. These

connections were made of capillary tubing and to avoid excessive back pressure in the lungs, a stream splitter was needed in the breath flow to vent most of the sample to the atmosphere. The volume passing through the loop could be monitored by attaching a soap bubble flowmeter to the sample loop vent orifice. At any time during the expiration, it could be switched into the chromatograph and the alcohol concentration analysed.

It was found that condensation of ethanol occurred in the sample loop with a subsequent contamination of later breath samples. A small heating box was fitted to maintain it between 50 and 60°C. so that together with continuous carrier gas flushing, this problem was eliminated. It was then possible to obtain reproducible peak areas for simulated breath alcohol standards. Unfortunately, due to the time required for the supply of the valve and the development of the technique, the apparatus was not ready for use until the work reported in this thesis had been completed.

2.5 Breath testing by electro-chemical analysis

The construction and use of the "Alco-limiter" manufactured by Energetics Science Inc., New York, has been previously described by Stowell (1973). Briefly, the breath sample, collected in a heated container within the instrument, is pumped through a fuel cell and the current produced by the electrolytic conversion of ethanol to acetic acid is proportional to the concentration of ethanol in the air. Six standard size 'C' 1.5 volt batteries were used to supply

current to the electronic circuits and pump motor. Although it was recommended that alkaline batteries be used, normal heavy duty batteries were found to be adequate. The fuel cell and sample container were heated by an external 12 volt supply which could be obtained from a car battery or by a transformer from the mains.

The fuel cell could readily be replaced. When new, zero readings were obtained on subjects who had not been drinking and the recovery after testing a subject with a high alcohol level was rapid. As the fuel cell aged, the basal level of non-drinking subjects increased to a reading of 10 mg/100ml. and at the same time, the return to zero after a positive test was delayed for five minutes or more. Throughout the life of the cell, it was necessary to increase indicated alcohol levels by about 3-5 mg/100ml. at intervals of 2 - 3 weeks by means of a variable resistor.

The instrument was calibrated by blowing air through approximately 100 ml. of a standard alcohol solution in a 250 ml. Dreschel bottle. Harger et al. (1956) demonstrated that air equilibrioted with 121 mg/100ml. ethanol at 34°C. yields a gas mixture equivalent to the expired alveolar air from a subject with a blood alcohol level of 100 mg/100ml. Standards were made from a stock 60.5 mg/ml. ethanol solution by taking the number of millilitres equivalent to one tenth of the number of milligrams/100 ml. blood alcohol required, and diluting to 500 ml. with distilled water. For example, 10 ml. of stock solution made up to 500 ml. will give the equivalent of a blood alcohol level of 100mg/100ml.

It was expected that the air bubbles would require to be broken up by means of a diffusing filter in order to ensure complete equilibriation with the aqueous phase. This was

not found to be necessary and a simple open ended tube terminating near the bottom of the bottle was sufficient. The tubing connecting with the Alco-limiter was dried between sampling to avoid condensation.

The effect of temperatures between 20 and 45°C. on a simulated breath sample of 100 mg/100ml. was determined by the Alco-limiter. It was shown, figure 2.11,(page 44) that on either side of the usual breath temperature of 34°C. there was a temperature coefficient of about 5 mg/100 ml. per degree Celsius.

This instrument was used for approximately 20 to 30 tests for each pair of subjects and the calibration was checked on the day prior to the test.

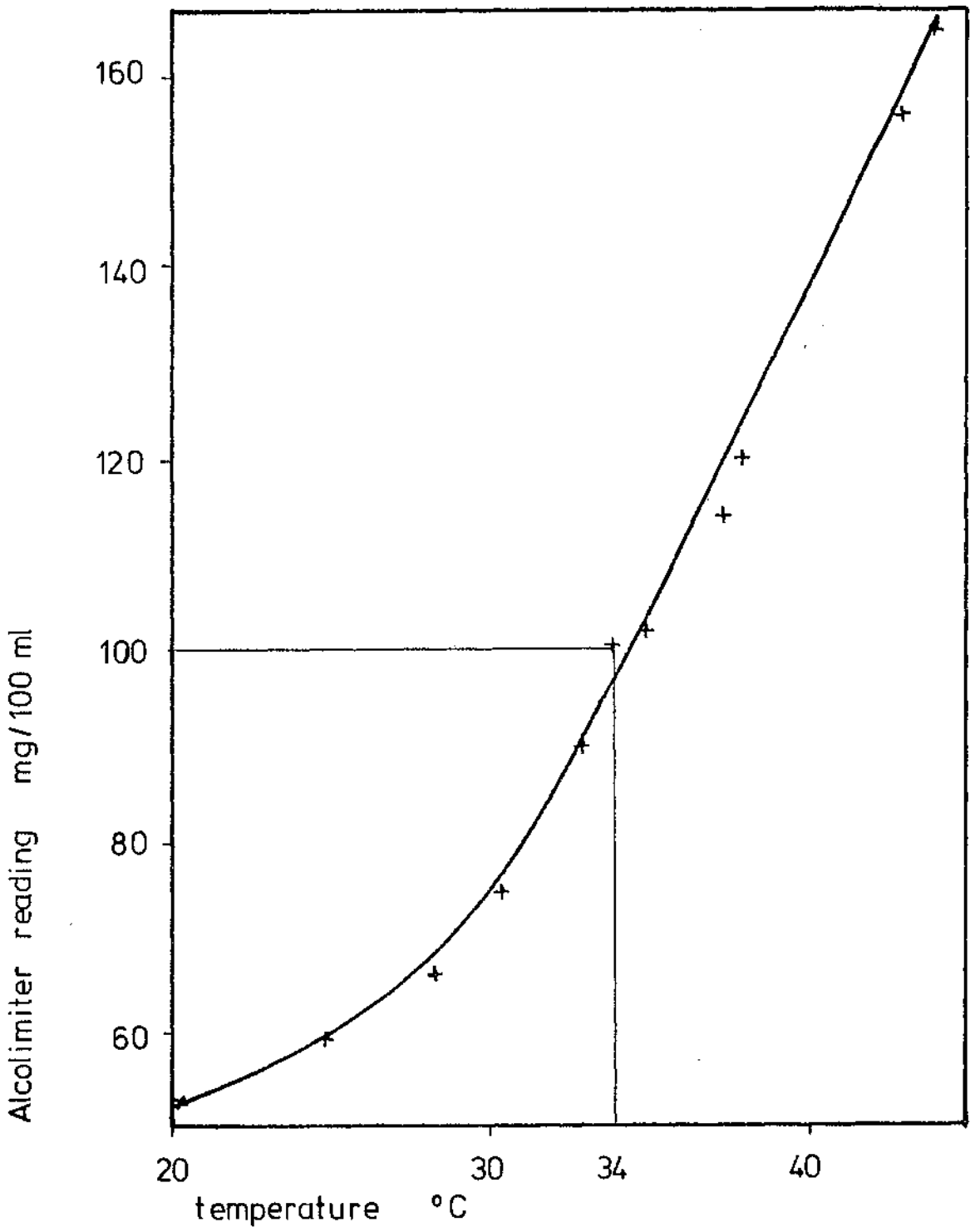

Figure 2.11

Variation in the indicated blood alcohol concentration with the temperature of a simulated breath sample, determined by the Alcolimiter.

Chapter 3

THE ESTIMATION OF NON VOLATILE CONSTITUENTS IN BODY FLUIDS

3.1 Introduction

In preliminary studies, it was shown (Stowell, 1973) that the most satisfactory methods for determining the ratios of lactate/pyruvate and hydroxybutyrate/acetoacetate, were enzymatic asseys. Even so, the colorimetric estimations involved relatively large quantities of blood and the resulting perchlorate supernatant had to be neutralised before use. The change in optical density of the solution was in general small. It was decided that fluorometry should be employed as advocated by Olsen (1971), so that far smaller quantities of NADH could be detected with a subsequent scaling down of quantities of reagents and blood samples. As a preliminary study to these methods, the fluorometry of NADH was examined as well as the possibility of the conversion of NAD^+ to a fluorescent product as suggested by the fluorometer instrument manufacturers (G. K. Turner Associates, Palo Alto, California).

3.2 Reagents

Buffers, standards, enzymes and co-factors are listed in Appendix I. The buffers were prepared freshly each week and filtered through a Buchner funnel with a hard Whatman

paper (No. 50) to remove small particles which interfere with the fluorescence measurements. This interference was particularly noticeable at high sensitivity levels as used for the pyruvate assay and caused a fast drift of instrument readings. The standards were freshly diluted from the stock solutions and the co-factors were weighed out and dissolved in the appropriate buffers immediately prior to use. Enzyme solutions were diluted where necessary in 2.1 M ammonium sulphate and the requisite volumes were measured by microlitre pipettes.

3.3 Fluorometry

3.3.1 Instrumentation

The fluorometer used in the following studies was a 'Turner' model 430 which had both emission and excitation monochromators and a xenon light source. A blank control not only adjusted the machine dark current but could be used to subtract reagent fluorescence. A range switch enabled adjustment of sensitivity by precise multiples in addition to a continuously variable control and a high/low switch giving a maximum gain of approximately x 500 000.

A six position sample turret was used utilising 12 x 75 mm. test tubes. Beckton-Dickinson RTU disposable culture tubes were found to be particularly suitable for these assays; they are cheap, of constant size and wall thickness and relatively free from glass defects. The tubes were cleaned

by immersing overnight in a proprietry cleaning solution such as 'Pyroneg', rinsed with water and immersed in concentrated nitric acid for 1-2 hours. Following washing with distilled water, the tubes were inverted in a plastic coated wire rack and dried in a stream of warm air. These tubes could be washed and re-used many times. A polarising screen in front of the emission monochromator minimised effects of imperfections in the glass.

3.3.2 Measurement of NADH

NADH when excited by light of 350 nm. has a native fluorescence with an emission peak at 460 nm. Using solutions in pH 7.0 buffer, it was found that fluorescence was linear below 10^{-4} moles/litre; reproducibility was poor at the 10^{-7} level and the minimum practicable working concentration was 10^{-6} moles/l.

It is possible to estimate the concentration of NADH to be measured because one mole of NAD^+ is reduced to NADH for every mole of lactate or β- hydroxybutyrate being assayed. Taking β- hydroxybutyrate as an example, the normal blood levels are 1×10^{-5} to 2×10^{-4} moles/litre. This concentration is halved on deproteinsation with perchlorate and there is a further 20 fold dilution when 50 μl. of the supernatant is added to 1.0ml. of the assay mixture. The final concentration in the cuvette is 2.5×10^{-7} to 5×10^{-6} moles per litre which is just within the sensitivity range of the fluorometer.

3.3.3 Measurement of NAD^+

Pyruvate and acetoacetate is assayed from the reduction of fluorescence of NADH, the product in this case being NAD^+ which is not normally fluorescent, is equal to the concentration of the original substrate. Taking pyruvate, for example, the normal blood levels are 1×10^{-5} to 1×10^{-4} moles/litre. By applying the same dilution factors as above, the NAD^+ produced in the cuvette is 2.5×10^{-7} to 2.5×10^{-6} moles/litre.

In 1957, Lowry et al. published a method in which NAD^+ was converted into a fluorescent compound. Residual NADH was first destroyed by acidification and the NAD^+ converted to a fluorescent compound with strong alkali. The parameters of this technique were examined to see whether it would give a better assay procedure for pyruvate. Sodium hydroxide was used in the original technique, but it was found that when this was added to the assay mixture in sufficient concentration, precipitation of sodium phosphates occurred. The use of potassium hydroxide as the alkaline reagent circumvented this problem, and the concentration required was found not to be critical in the range of 5-12 molar solutions. The maximum fluorescence was attained after 70 minutes at room temperature and had an emission peak at 460 nm. with an excitation wavelength of 360 nm. The fluorescence in the presence of strong alkali was unstable when exposed to ultraviolet light, but was minimised by diluting the solution fivefold with distilled water. Using this method, it was possible to measure concentrations of NAD^+ down to 1×10^{-7} moles/litre.

3.4 Lactate

Lactate was measured by the increase in NADH concentration during conversion to pyruvate by the enzyme, lactic dehydrogenase, at pH 9.5. A 1.0 molar hydrazine buffer served to trap the ketone and effectively neutralized the perchlorate, which was diluted twenty fold.

Aliquots (50 µl.) of perchlorate supernatant or standard were added to 1.0 ml. of hydrazine buffer containing NAD^+ and enzyme. After 45 minutes at room temperature, the fluorescence of the NADH was measured following dilution with 4.0 ml. of 0.1 molar hydrochloric acid.

The optimal concentration of NAD^+ was determined by using a constant amount of lactic acid (2.0 mmoles/litre) and enzyme (2 µl./ml. reagent mix) and varying the NAD^+ concentration from 0.1 to 2.5 mg/ml. Maximum fluorescence was not achieved in 40 minutes by concentrations below 1.0 mg/ml. and there appeared to be suppression of fluorescence at 2.5 mg/ml. A level of 1.0 mg/ml. was used in subsequent tests.

The enzyme concentration was determined in a similar manner and the optium was found to be 2.0 µl/ml. (3.7 units), the reaction being much slower at lower concentrations. A standard curve was prepared under these conditions and was linear from 0.1 to 1.5 mmoles/litre on a fluorometer range setting of x 30 and the sensitivity switch at high. The variable sensitivity control could be adjusted so that the 1.5 mmoles/litre standard read 15 on the 0-33 scale. No fluorescence was seen in the blanks prepared from blood

perchlorate supernatants.

The effect of perchlorate was tested on standard solutions. No significant difference was seen with up to twice the normal concentration in the cuvette. At four times the concentration, the fluorescence was proportionally lower throughout the range of standards. The method finally used was based on these findings and is detailed in Appendix I.

3.5 D (-) β Hydroxybutyrate

β- hydroxybutyrate was measured in a similar manner to lactate. The enzyme catalyzing the reaction was β- hydroxybutyrate dehydrogenase and a hydrazine buffer, pH 9.5, removed acetoacetate as a hydrazone and ensured the reaction proceeded to completion.

The assay was carried out in an identical way to that used for lactate. The optimal amount of NAD^+ was found to be 0.25 mg/ml. and enzyme 2.5 µl/ml. (0.05 units) with an incubation period of 60 minutes at room temperature. The fluorescence was read without dilution using the x 300 range and sensitivity switch on high. The reading of a 0.1 mmoles/litre standard was set to 10 on the 0-33 scale to enable direct readings of hydroxybutyrate levels. The method used is detailed in Appendix I.

3.6 Pyruvate

Pyruvate was measured by the decrease in fluorescence of NADH during reduction to lactate at pH 7.0, catalysed by the enzyme lactate dehydrogenase. This enzyme was obtained pyruvate kinase free.

The optimal concentration of enzyme was determined by varying the amount in a standard volume of pH 7.0 phosphate buffer in the presence of 1×10^{-6} moles/litre NADH and 50 ul. of a standard solution equivalent to a blood level of 0.1 mmoles/litre pyruvate. The maximum range setting (x 1 000) of the fluorometer was used. The NADH solution was set to read 100 with the sensitivity control and buffer only, to zero with the blank control. Under these conditions, the fluorescence of NADH was seen to decrease at a linear rate which was accelerated by adding the enzyme. In the presence of pyruvate, a very rapid decrease in fluorescence was observed followed by a return to the former rate after 5 minutes (figure 3.1, page 52). No difference was seen between 2 to 25 ul. of enzyme which had been diluted tenfold in 2.1 molar ammonium sulphate (0.4 to 5.0 units).

The effect of NADH concentration was determined on a range of pyruvate standards and the change in fluorescence plotted against pyruvate concentration (figure 3.2, page 53). A non linear curve was produced when the concentration was too low, whilst at too high an NADH concentration, the change in fluorescence was decreased; the optium was found to be 4×10^{-6} moles/litre.

A plot of the change in fluorescence against time for each

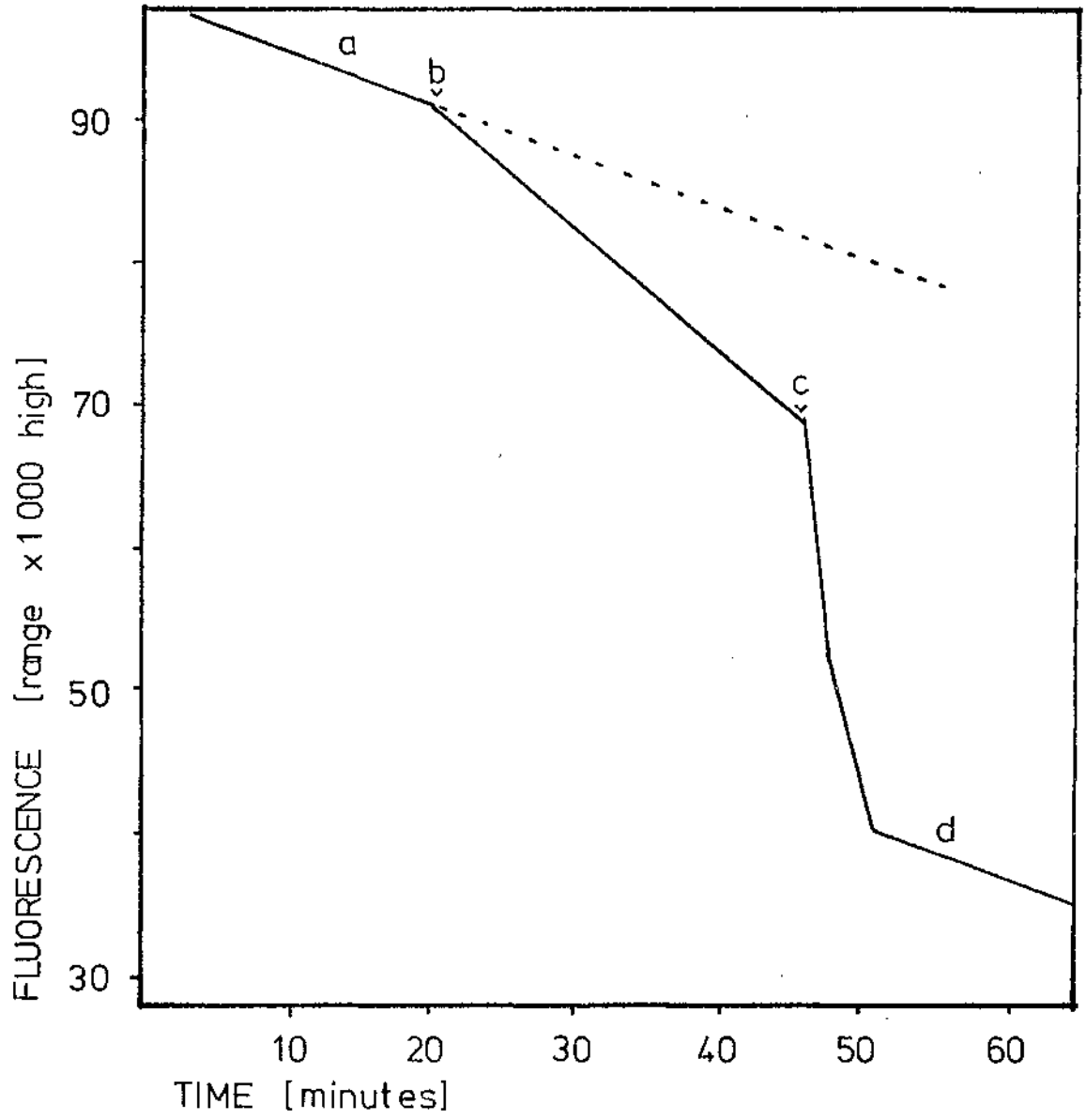

Figure 3.1

Change in fluorescence of a 1 x 10^{-6} M. NADH solution.

(a) natural re-oxidation in phospate buffer, pH 7.0

(b) addition of lactic dehydrogenase

(c) addition of sodium pyruvate

(d) reaction completed, slope equal to natural rate of re-oxidation

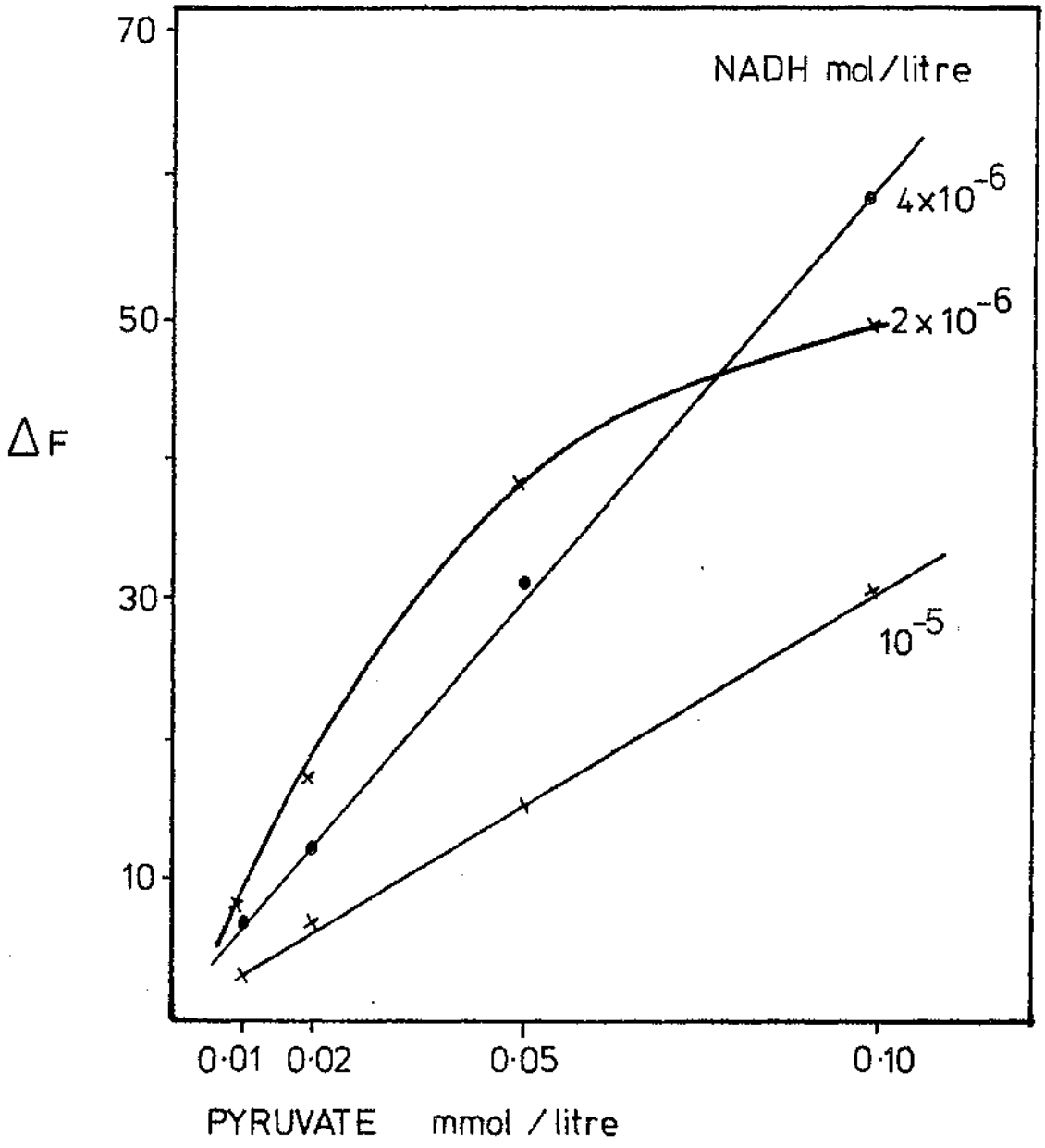

Figure 3.2

The effect of NADH concentration on the assay of pyruvate. Concentration against change in fluorescence (ΔF).

of the pyruvate standards showed that the reaction was complete in 20 minutes at room temperature (figure 3.3, page 55). The standard curves were linear for each of the time intervals with an optimum apparently being reached at 15 minutes where the fitted slope passed through the origin. The addition of perchlorate at four times the normal level had no significant effect, but a visible precipitate of perchlorate occured which could be centrifuged down without interfering with the readings.

3.7 Pyruvate assay by direct measurement of NAD^+

The possibility of utilising the conversion of NAD^+ to a fluorescent compound in order to enable direct measurement was examined. Using the reaction conditions previously described and the same range of pyruvate standards, remaining NADH in 1.0 ml. of the reaction mixture was destroyed by adding 0.3 ml. of 2 M hydrochloric acid. After two minutes, 1.0 ml. of 16 M potassium hydroxide was added and the fluorescence allowed to develop in the dark for 90 minutes at room temperature.

Using the undiluted preparation and exposing to the ultraviolet light only as long as necessary to obtain a reading on the chart recorder, the fluorescence of the cuvette without pyruvate was set to zero by the blank control and the 0.1 mmoles/litre standard to 100 by the sensitivity control at a range setting of x 100. A linear relationship was observed between the range of standards. By diluting the alkaline product five times with distilled water, the

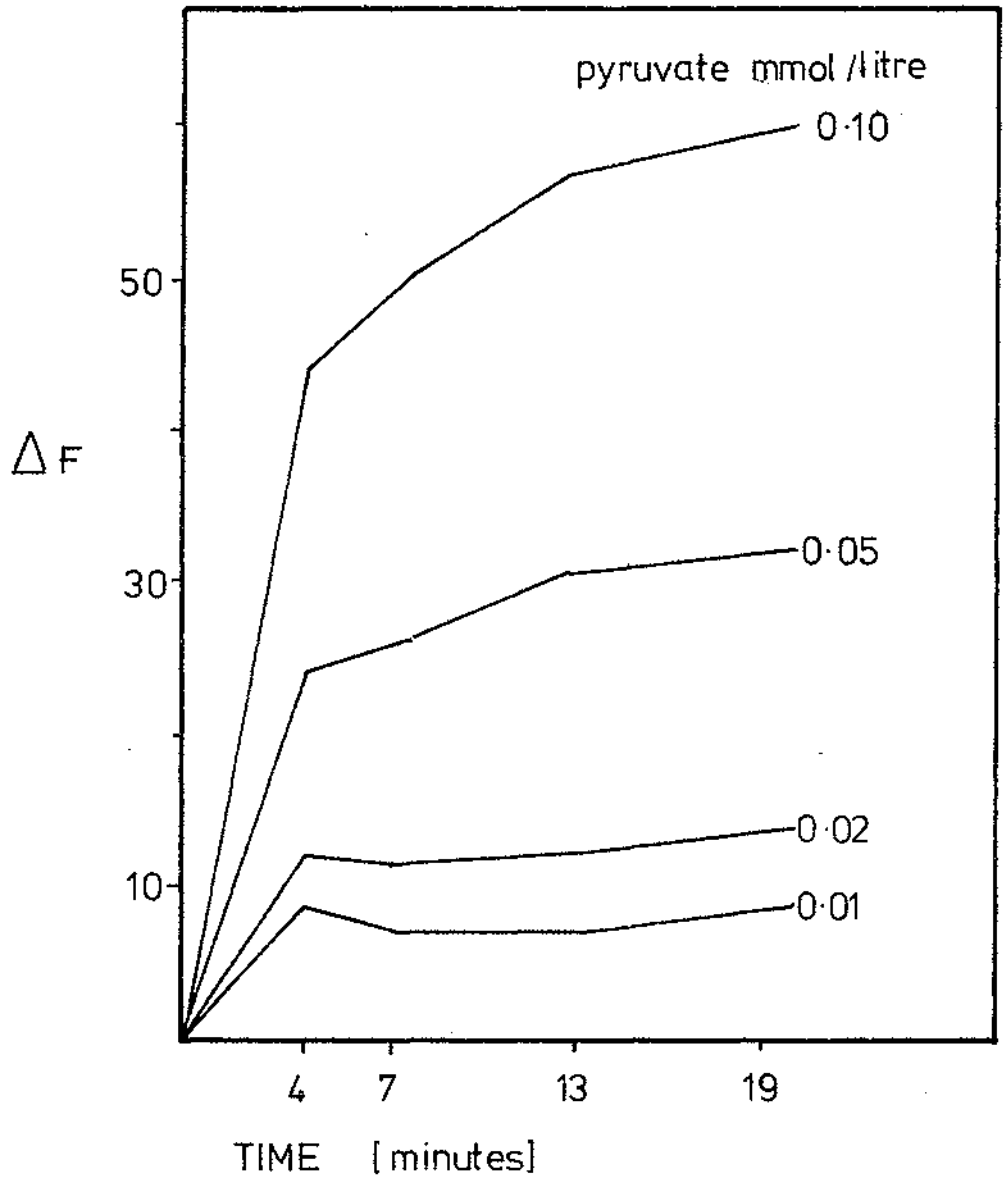

Figure 3.3

A plot of change in fluorescence, Δ F, against time for pyruvate concentrations from 0.01 to 0.10 mmoles/litre.

fluorescence was stable to ultra-violet light. This product, diluted or undiluted, was stable for at least 24 hours in the dark.

A high fluorescence was found in the blank which contained the products from the destruction of NADH only. Lowry et al. (1957) suggested that this residual fluorescence was due either to NAD^+ in the NADH preparation or to the slow re-oxidation in solution. It was recommended that solutions of NADH be made in tris buffer, pH 9.0, in order to minimise this effect. Using this buffer, a small decrease in fluorescence of a 10^{-6} moles/litre NADH was seen over 60 minutes but no further decrease over the next hour. In pH 7.0 phosphate buffer, the decrease in fluorescence was faster and continued over a two hour period and a solution one week old contained 3% more NAD^+ than with a fresh preparation.

The method described above was applied to blood samples. Cuvettes containing NADH and blood perchlorate supernatants or standards were set up without enzyme for the blanks, and with enzyme for the tests. The resulting fluorescence was recorded using the x 10 range at high sensitivity and zero was set to a buffer solution only. The results are recorded below:-

Standard mmoles/l	blank	test	test-blank
0	18	18	0
0.01	18	20	2
0.02	18	24	6
0.05	18	35	17
0.10	18	54	36

Blood sample	blank	test	test-blank	Pyruvate mmoles/l
1	13	41	28	0.078
2	15	48	33	0.093
3	16	49	33	0.093
4	12	39	27	0.077

From these results it can be seen that each of the blanks were different and hence it would be necessary to set up two tubes for every sample tested, subsequently deriving pyruvate levels from a standard curve.

In order to determine whether perchlorate concentration had any effect on the variability of the blanks in these tests, aliquots of a single blood sample were treated with equal volumes of 0.5, 1.0 and 2.0 moles/litre perchlorate. The supernatant from the first was brown in colour, but when treated with alkali, developed a lower fluorescence than the other two. The excitation and emission maxima were identical to those obtained with alkali treated NAD^+ so that it might be inferred that the method was detecting endogenous NAD^+.

With these difficulties, it was decided that the method had no advantages over measurements based on the reduction in fluorescence of NADH; this was used throughout the study and is detailed in Appendix I.

3.8 Acetoacetate

Concentrations of acetoacetate were measured in a similar manner to pyruvate except that the reduction to β-hydroxybutyrate was catalysed by β-hydroxybutyrate dehydrogenase at pH 7.0. Acetoacetic acid crystals were prepared by hydrolysis of ethyl acetoacetate as described by Krueger (1952). Standard solutions were made by rapidly filtering some of the crystals from the ether extraction phase through fine mesh stainless steel gauze. They were dried in a dessicator under vaccuum for ten minutes and weighed in a tube containing silica gel. The crystals were then washed off the gauze into the appropriate volume of deionised water.

The assay in the presence of 0.05 mmoles/litre NADH and 10 µl. enzyme at room temperature for 30 minutes, showed only a small decrease in fluorescence for a 0.2 mmoles/litre standard at maximum fluorometer sensitivity. Previous assays by Stowell (1973) and Mellanby and Williamson (1965) used less enzyme and higher levels of NADH (0.2 mmoles/litre). If this concentration of NADH was required to ensure completion of the reaction, then the decrease in NADH in the assay by a normal blood level of acetoacetate would be from 0.2 to 0.195 mmoles/litre. A trial with standard NADH solutions showed that this was not possible with any degree of accuracy by fluorometry. To complicate matters, it was expected that acetoacetate concentration would fall during ethanol metabolism due to the decrease in NAD^+ levels by the actions of alcohol and aldehyde dehydrogenase systems. Stowell (1973) found that recoveries of acetoacetate added to blood samples were less than 75% and also that the method was subject to errors of the order of $\pm$ 30% at low concentrations. These factors, together with problems in preparing standards and lability in blood and perchlorate supernatants, suggested that the information to be obtained from this test would not warrant continued intensive work on the assay.

3.9 Glycerol

A two stage enzymatic technique was followed according to Laurell and Tibbling (1966) and is described in Appendix I. Plasma samples were deproteinised by the zinc sulphate/barium hydroxide technique because it was found that the fluorescence

developed using perchlorate supernatants had no relationship to added glycerol. Glycerol was first converted to glycerol-3-phosphate in the presence of ATP which was catalysed by the enzyme, glycerol kinase. The glycerol-3-phosphate was oxidised to dihydroxyacetone phosphate and the proton acceptor was NAD^+. The enzyme catalysing this reaction was glycerol phosphate dehydrogenase and the amount of NADH produced was proportional to the amount of glycerol.

3.10 Glucose

The method used was the procedure described in the G. K. Turner "Manual of Fluorometric Procedures" based on the method of Phillips and Elevitch (1968). This technique was used unmodified and is described in Appendix I. The analysis was performed on diluted plasma. Hydrogen peroxide was produced by the action of glucose oxidase on glucose. In a second stage, horse radish peroxidase catalysed the transfer of oxygen to the acceptor, homovanillic acid, which fluoresced strongly in its oxidised form.

3.11 Assays of Calcium and Magnesium by Atomic Absorption

The simplest method for the determination of calcium and magnesium levels in urine is by atomic absorption spectrophotometry. In this method, the element in the flame absorbs the radiation emitted by a hollow cathode lamp. The characteristic radiation of the element being measured is produced by collision of the metal with the neon gas in the lamp. A combination calcium/magnesium lamp was used which emitted a spectral line for calcium at 422.67 nm. and 285.21 nm. for

magnesium. The urine specimens were diluted 25 times in 0.26% strontium chloride which dissociated the calcium and magnesium from the phosphate complexes, as strontium forms a more stable complex. The diluted urine was then atomised and sprayed into the acetylene/air flame.

3.12 Assays of Sodium, Potassium and Chloride

These estimations were performed by Dr R. M. Greenway with the assistance of Mr T. Braggins on an Autoanalyser incorporating a flame photometer.

Chapter 4

RESULTS

4.1 Subjects

The ages and drinking habits of the participants in the study are included in table 4.1 (page 62). There were 20 males and 8 females in the age range of 19 to 61 years, of whom thirteen were below 25 years of age and ten between 25 and 34 years. Most of them were either university staff members (technical and academic) or students, both graduate and undergraduate. One male, case 11, drank regularly 200 ml. of spirits a night and seven subjects drank more than 2 jugs of beer a week whilst four consumed less then one bottle per week. All of the females were very light drinkers, taking only an occasional sherry or spirits and two were near teetotallers (cases 27 and 28).

4.2 Effects of Alcohol

The alcohol load was given in each case after a light breakfast and, in most cases, this produced a light headed or dizzy feeling within ten minutes of finishing the drink. These sensations became more pronounced if the subjects stood up. Typically, they became talkative and relaxed;

TABLE 4.1

The age, sex and drinking habits of the subjects with their interpretation of the effects of the alcohol dose

Subject	Age	Sex	Obvious Alcohol Effect	Jugs of Beer Per Week	Spirits	Glasses of Sherry
1	29	M	+	2		
2	25	M	none	1		
3	29	M	+	1		
4	22	M	+	1		
5	26	M	+	0.5		
6	19	M	+	0.25		
7	19	M	none	2		
8	32	M	+	occasional		
9	43	M	+	occasional		
10	23	M	+	1		
11	61	M	none	0	200ml/day	
12	48	M	+	1		
13	36	M	+	1		
14	21	M	+	1		
15	20	M	+	1		
16	23	M	none	4		
17	19	M	+	2	occasional	
18	20	M	+	2	occasional	
19	22	M	+	4		
20	31	M	none	5		
21	39	F	none			1/day
22	21	F	+		occasional	1/week
23	28	F	+		occasional	occasional
24	30	F	+			1/day
25	31	F	+			3/week
26	30	F	+			3/week
27	20	F	+	near teetotal		
28	22	F	+	near teetotal		

had difficulty in constructing a sentence or in concentrating upon a single subject and their gait became unsteady. This passed off after about one hour when most felt pleasantly relaxed and were inclined to go to sleep. At the termination of the study, they felt fatigue, which tended to persist throughout the remainder of the day even though they all had a midday meal. Six subjects did not show these effects (table 4.1, page 62).

No definite trends were seen in blood pressures or pulse rates. In general these settled to lower levels throughout the three hour test period but the levels were frequently raised before the consumption of alcohol and could, more properly, be related to some apprehension about the test.

4.3 The absorption, equilibriation and elimination of alcohol

4.3.1 Absorption

The blood alcohol curves (table 1, Appendix 2) were divided into three distinct patterns based on the time at which the maximum blood level was attained. The first, (a), consisting of 9 subjects, showed a peak alcohol level at 15 minutes; the second, (b), with 6 subjects and a peak at 30 minutes; and the third, (c), with 12 subjects showed a maximum blood alcohol level at 60 minutes (figure 4.1, page 64). Case 25 was omitted because the intravenous catheter was blocked in the early stages and samples were not obtained. Very high

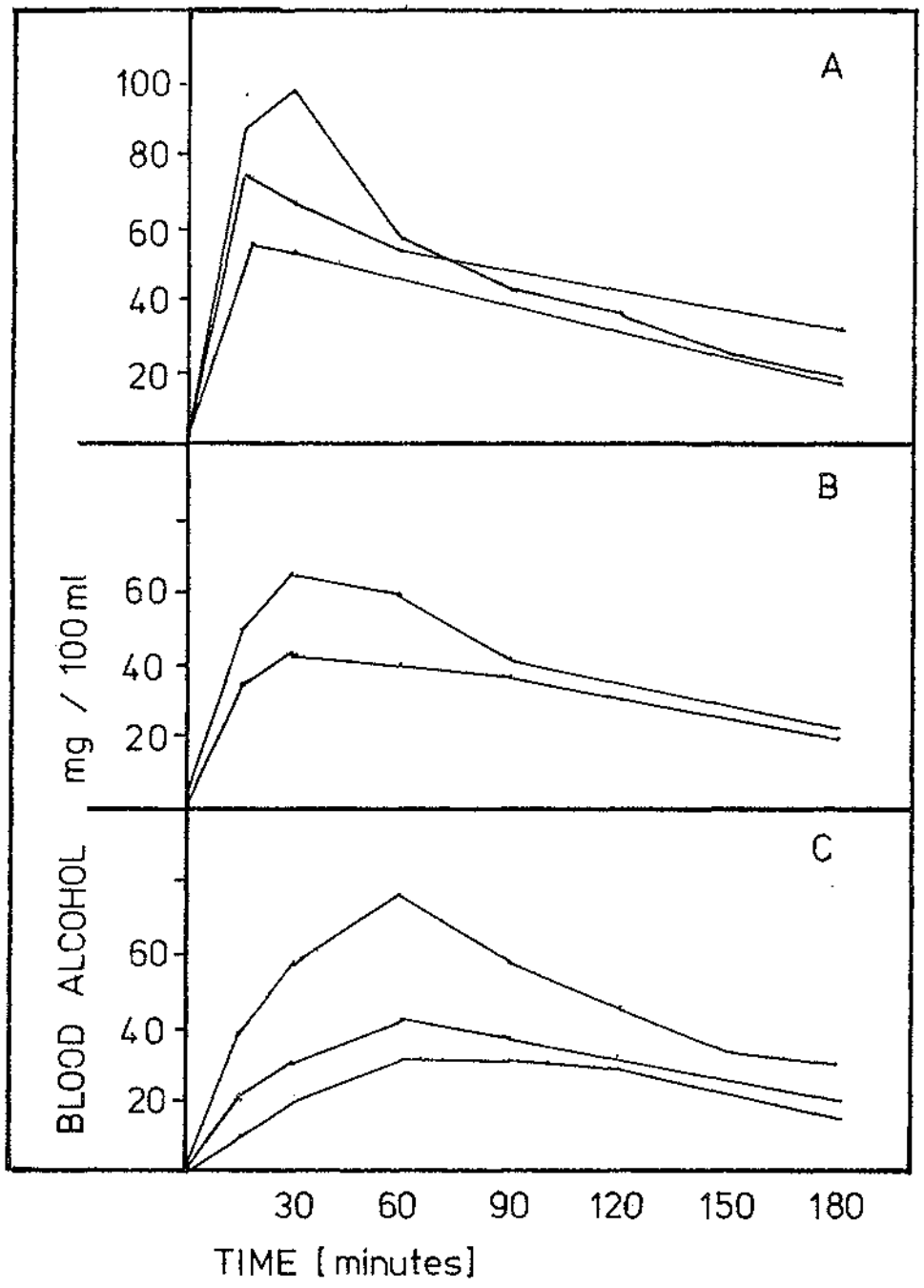

Figure 4.1

Graphs illustrating the variation of the rate of absorption and equilibration of alcohol; (a) fast absorption with peak alcohol levels at 15 minutes (b) peaks at 30 minutes; (c) peak at 60 minutes or longer

initial levels were reached in case 11, over 90mg/100ml. This was in an individual who was used to drinking spirits and did not dilute the vodka with as much soda water as the other subjects and, in effect, he drank a more concentrated solution of alcohol.

4.3.2 Equilibriation

The time when alcohol had equilibriated throughout the body water was estimated from the commencement of a linear descending graph, the so-called β slope (Widmark, 1931, cited by Wallgren and Barry, 1970), which related to the rate of metabolism of alcohol in the body. Examination of the blood alcohol curves showed that this time was variable and usually took 30 to 90 minutes after drinking, but in four cases, was not complete until after 120 minutes (Table 4.2, page 66).

4.3.3 Elimination

The rate of metabolism of ethanol by the body was determined by fitting a straight line to the later points on the blood alcohol curve. This was done in conjunction with the estimations of section 4.4 as only extrapolation to the ordinate of the true β slope will give a reliable estimate of body water content. These rates of metabolism are included in table 4.2 (page 66) and show a range of 7.3 - 16.0 mg/100ml/hour with a mean of 12.2 mg/100ml/hour.

TABLE 4.2

Results of the application of the Widmark formulae, where:-

p is the body weight in Kg.

Co is the intersect of the ordinate by the extrapolated β slope in mg/l.

r is the Widmark factor for body water

β is the rate of elimination of alcohol from the blood in mg/l.

Subject Number	P	Alcohol Dose Grams.	Co	r	β	Peak Alcohol Pattern	Equilibriation Time (Minutes)
1	80	34	520	0.82	110	b	90
2	68	29	620	0.68	140	b	60
3	85	36	680	0.62	142	a	30
4	88	37	520	0.82	100	c	60
5	81	34	640	0.66	128	a	60
6	70	30	490	0.87	110	c	90
7	77	32	540	0.77	93	c	90
8	81	34	560	0.75	137	c	120
9	65	28	600	0.72	130	b	60
10	75	32	590	0.72	73	c	60
11	82	35	660	0 65	157	a	90
12	67	29	480	0.91	95	b	60
13	81	34				c	
14	74	31	620	0.67	135	b	90
15	78	33	600	0.70	138	a	30
16	69	30	570	0 77	95	c	60
17	65	29	680	0.66	154	a	60
18	77	34	620	0.72	94	a	60
19	74	31	550	0.77	143	a	30
20	70	30	540	0.79	105	c	90
21	54	23	730	0.58	110	c	120
22	51	23	670	0.67	100	a	60
23	72	31	710	0.60	140	c	60
24	62	27	700	0.63	160	b	30
25	58	25	710	0.59	157		90
26	60	25	660	0.63	107	c	120
27	60	25	610	0.69	150	a	30
28	58	25	660	0.64	100	c	120

It was not possible to determine a rate for case 13 as none of the extrapolated slopes intersected the ordinate at a point which gave a realistic body water ratio. It was found part way through the test, that case 19 had been drinking heavily the previous night and had an initial alcohol level of 30 mg/100ml. An estimate of his probable alcohol level at midnight was made by extrapolation from that at 9 a.m. with a line parallel to the β slope which gave a blood alcohol level of 160 mg/100ml. This could have been achieved by the consumption of three jugs of beer, and corresponded closely to the estimate of the subject of the amount consumed between 10 p.m. and midnight.

4.4 Body Water Estimates

Widmark (cited by Wallgren and Barry, 1970, page 44) has reported detailed studies on the kinetics of the absorption and elimination of alcohol in humans. Using his formulae, the total body water content may be determined by the Widmark factor, r, from:-

$$r = \frac{Ao}{Co \times p}$$

Where:

Ao = the amount of alcohol ingested in milligrams.

Co = the intersect of the extrapolated B slope with the ordinate in milligrams/litre.

p = body weight in Kg.

The results of these calculations appear in Table 4.2 (page 66).

As previously mentioned, it was not possible to determine a β slope on case 13. If the points at 90, 120 and 150 minutes were extrapolated to the ordinate, the value of Co would have been 900 and r = 0.47, an unrealistically low figure. Co for case 19 was determined by subtracting 320, the initial alcohol level in mg/l. from the estimated Co of 870.

The values of r for males ranged from 0.62 to 0.91 with a mean of 0.74 and for females the range was 0.53 to 0.69 with a mean of 0.63.

4.5 Acetaldehyde (Table 2, Appendix 2)

Blood acetaldehyde levels reached a maximum of 0.1 mg/100ml., in the majority of cases by 30 minutes. There was a trend in some towards a bimodal curve with a second peak at 90-120 minutes, but the majority maintained a constant level throughout. In case 4, none was detected and in case 6, the levels remained low increasing to 0.06 mg/100 ml. towards the end of the test period. Case 19, who had been previously drinking, showed the highest level in the males reaching 0.2 mg/100ml. between 15 and 60 minutes. In case 21, one point at 60 minutes was 0.22 mg/100ml., but the remainder were just above 0.1 mg/100ml.

4.6 Acetone (Table 3, Appendix 2)

Fasting blood acetone levels gave a mean of 0.087 with a

range of 0.04 to 0.14 mg/100ml. Two results were outside this range; case 6 at 0.25 mg/100ml. and case 19 at 0.21 mg/100ml. After consumption of alcohol, these levels increased and remained high throughout the test period. There were minor fluctuations, some of them in parallel with acetaldehyde levels. The results from 60-180 minutes were averaged for each subject and ranged from 0.076 to 0.218 with a mean of 0.137 mg/100ml.

4.7.1 Lactate (Table 4, Appendix 2)

There were three distinct patterns of blood lactate levels. The most common (16 out of 28 cases) showed an increase at 15 minutes following alcohol consumption with a maximum at 30-60 minutes. This was followed by a decrease over the next two hours to normal values and gave curves similar to those for blood alcohol levels. The second pattern (3 cases) showed a slow rise from initial low levels, 0.60 - 0.76 mmoles/1 with a peak occurring at 120 minutes. The third group (8 cases) showed no definite response to alcohol. In four of these, the lactate levels were high initially, 1.3 to 1.55 mmoles/l. and declined to normal values during the test period.

4.7.2 Pyruvate (Table 5, Appendix 2)

In 25 out of 28 cases, the initial values were between 0.065

and 0.14 mmoles/l. These dropped by approximately 50% in the first 15 minutes to give a range between 0.04 and 0.075 mmoles/l. Typically, the lower level was maintained throughout the test period with no significant changes; the range at 180 minutes was 0.035 to 0.085 mmoles/l. In the remaining three cases, the initial sharp fall was not observed. One of these, case 19, had previously been drinking and his initial level was already 0.045 mmoles/l. so that it could have been unlikely to observe a further fall. The other two cases, 23 and 24, were remarkably similar, showing a slight drop at 15 minutes followed by an increase above the initial level at 60 minutes and then a slow decline to reach 0.05 and 0.078 mmoles/l. by 180 minutes.

4.7.3 B-hydroxybutyrate (Table 6, Appendix 2)

Initial levels ranged from 0.010 to 0.088 mmoles/l. with a mean of 0.031. Fifteen minutes after drinking alcohol, these had risen to a mean of 0.090 mmoles/l. and a range of 0.040 to 0.160 mmoles/l. Statistical analysis between fasting and the 15 minute levels, (omitting the extreme values of 0.160 and 1.37) gave a regression coefficient of 1.04 ($P < 0.005$, d.f.23, mean fasting = 0.031, mean 15 min. = 0.090). A similar analysis between the 15 and either the 150 or 180 minute levels, omitting the extreme values as before, gave a regression coefficient of 0.49 (P 0.01-0.02 d.f.23. 15 min., mean = 0.090, 180 min., mean = 0.080).

4.7.4 Redox ratios (Table 7, Appendix 2)

The ratio lactate/pyruvate is a measure of the redox state of the cytosol. Initial values showed a mean of 10 and a range from 7 to 16. In most cases, this ratio increased 15 minutes after ethanol to maximum or near maximum values ranging between 16 and 41. There was a tendency for the ratio to decline by 180 minutes but the levels were still high at this time with a mean of 20 and range 10-37.

Two cases did not show any significant change. Case 23 showed a slow decline in lactate from 0.95 to 0.68 mmoles/l., and at the same time pyruvate concentrations decreased from 0.088 to 0.069 mmoles/l., with the ratio remaining constant. Case 24 showed a rise in lactate from 0.78 to 1.14 mmoles/l. at 30 minutes but the pyruvate level remained substantially unaltered throughout the study at about 0.1 mmole/l.

4.8 Glucose (Table 8, Appendix 2)

The first seven subjects were given vodka diluted with lemonade containing 11.5% sucrose. Their blood glucose levels rose significantly from a mean of 4.4 to 6.0 mmoles/l. in the first 15 minutes, falling to 4.3 mmoles/l. by 150-180 minutes.

The remaining subjects had vodka in soda water without sugar. Their blood glucose levels increased from the mean fasting values of 4.6 mmoles/l. (range 3.6-6.2) to a mean of 5.2 mmoles/l. (range 4.2-6.5) and this increase was statistically

significant ($t = 4.6$, d.f. 39, $P < 0.001$). Similar values were obtained for the 30 minute samples, but by 150-180 minutes they had falled to a mean of 4.8 and a range of 3.8-5.8 mmoles/l. with a significant decrease in variance at the 2.5% level ($F = 2.8$, d.f. 20, 20).

4.9 Glycerol (Table 9, Appendix 2)

Initial blood glycerol levels ranged from 0.02 to 0.10 mmoles/l., in 26 out of 28 cases. There were two quite distinct patterns during the alcohol studies each with two subpatterns. In the majority of cases, (18 out of 28), the initial level doubled by 30 minutes and either remained elevated between 0.13 and 0.2 mmoles/l., or declined to the pre-test state. The remaining 10 cases increased their glycerol level above 0.3 mmoles/l., by 30 minutes and either remained elevated at this level or declined over the next two hours below 0.2 mmoles/l. Two of such cases showed an upswing again at about 2 hours after drinking the vodka.

4.10 Urine Alcohol Levels (Table 10, Appendix 2)

For the first 30 minutes or so after drinking and while the alcohol was being absorbed from the stomach and the small intestine, urinary alcohol levels were lower than the blood values. During the equilibriation phase, the urine levels could be 1.5 or more times higher than the blood levels.

An estimate of the commencement of the post absorptive phase could be made by inspection of the alcohol curves and was judged to begin at the time when breath and urine levels paralleled the β slope of the blood level. The urine/blood ratios were averaged within this phase for all subjects to give a range from 1.06 to 1.49 with a mean of 1.26 (table 4.3, page 74). These results are similar to those of Kaye and Cardona (1969), who tested 148 random bloods and urines and found a mean of 1.28 and a range from 0.21 to 2.66.

4.11 Diuretic Effect of Alcohol (Table 11, Appendix 2)

The total urine volume collected over the three hour test period averaged 900ml., with a range from 277 to 1573ml. Case 19 excreted only 277 ml., and was probably dehydrated due to his previous drinking episode. Cases 1 and 13 excreted substantially larger volumes than the rest, 1400 and 1570 ml. respectively. No urine was obtained from case 17, which was thought to be due to psychological problems involved in providing a specimen rather than non-production of urine.

The urine flow was largest 30-60 minutes after drinking and coincided with a decrease in specific gravity to 1000 in all cases (Table 12, Appendix 2). As urine flow decreased, the specific gravity returned to normal. There was no association between peak urine flow and the time of attaining a maximum blood alcohol concentration. This could vary considerably. For example, compare case 3, with a peak blood alcohol at 15 minutes and a maximum diuresis at 120 minutes, to case 8, with a blood peak at 90 minutes and urine flow at 30 minutes.

TABLE 4.3

The ratios, urine/blood alcohol level during the absorptive and post-absorptive phase.

Subject	Time (Minutes) after drinking							Average During Post Absorptive Phase
	15	30	60	90	120	150	180	
1		0 76	1.25	1.19		1.20	1.26	1.22
2		1.05	1.29	1.33	1.37	1.29	1.44	1.34
3				1.40	1.20	1.30		1.30
4	0.32	1.00	0.78	1.30		1.26	1.50	1.35
5	0.20	0.90	1.14	1.46	1.40	1.30		1.33
6	0.38	0.93	1.80	1.44	1.07	1.10	1.14	1.19
7			1.50	1.13				1.13
8	0.90	1.00	1.25	1.33	1.31	1.27		1.30
9	0.34	0.98	1.02	1.18	1.14	1.27		1.15
10	0.40	0.70	1.20	1.12	1.23	1.20	1.03	1.16
11	0.20	0.90	1.49	1.44	1.24	1.64	1.60	1.48
12	0.26	0.90	1.42	1.45	1.31	1.20		1.32
13	0.47	0.98	0.92	1.19	1.20	1.36		1.25
14	0.12	0.73	1.10	1.36	1.36	1.36		1.36
15	0.35	1.07	1.37	1.22	1.18	1.23		1.21
16	0.52	1.33	1.26	1.35	1.22	1.06		1.22
17								
18	0.13	0.70	1.50	1.33	1.26	1.21		1.33
19		0.98	1.20		1.05			1.13
20		1.60	1.40	1.40				1.40
21	0.40	1.07	1.14	1.13	1.07	1.07		1.07
22		1.23	1.32					1.32
23		0.87	1.26	1.24	1.25	1.16		1.23
24		0.83	1.28	1.19	1.13	1.06		1.17
25			1.07	1.05				1.06
26		0.76	1.30		0.87			
27	0.22	1.19	1.60	1.50	1.38			1.49
28			1.20	1.37	1.28			1.33

4.12 The Excretion of Ca^{++}, Mg^{++}, Na^{+}, K^{+}, and Cl^{-} in the Urine

The amount excreted in each specimen was determined by multiplying concentration by volume ÷ 1 000 (Tables 13-17, Appendix 2). The changes in amounts excreted were not great because concentration was inversely proportional to urine volume and as the total collection period varied from 90-180 minutes, the average amount excreted in 30 minutes was used to compare subjects (Table 4.4, page 76). Of the seventeen subjects in whom urine samples at 15 minutes were obtained, the amounts excreted in those samples were two times or more the amounts in the next 15 minute sample, with the exception of cases 5, 8 and 14. This may be explained by the collection procedure. The bladder was voided prior to drinking and timing started on cessation, so that this first specimen was collected over at least a 30 minute period. In addition, the higher concentrations in these specimens were undoubtedly due to "washing out" of the previous sample accumulated overnight in the bladder. When this effect was ignored, patterns of excretion related to peak diuresis time were observed. These patterns are summarised in Table 4.5 (page 77) which shows that in the majority of cases there was a rise and fall in amount excreted with peak at or near maximum diuresis. The correlation and partial correlation coefficients between pairs were:-

r_{NaCl}	=	0.952	eliminating K	=	0.951
r_{KCl}	=	0.591	eliminating Na	=	0.585
r_{NaK}	=	0.453	eliminating Cl	=	0.444
r_{CaMg}	=	0.410			
r_{NaMg}	=	0.220	n.s.		
r_{NaCa}	=	0.282	n.s.		

TABLE 4.4

The average excretion in urine per 30 minutes of Cl^-, Na^+, K^+, Ca^{++}, and Mg^{++}.

Subject	Cl mmoles	Na mmoles	K mmoles	Ca μ moles	Mg μ moles
1	5.9	5.1	2.0	476	228
2	5.2	4 4	1.8	388	296
3	2.2	2.0	1.3	114	166
4	4.8	3.0	7.0	355	147
5	6.7	6.0	4.4	422	264
6	5.0	5.3	2 3	298	68
7	6.2	4.8	2.8	144	210
8	3.3	2.9	0.7	352	180
9	5.6	4.7	2.3	224	132
10	3.2	3.2	1.7	193	98
11	6.3	5.7	2.6	100	75
12	4.6	4.1	2.1	266	195
13	4.6	4.2	2.5	226	126
14	3.2	3.4	2.0	156	142
15	4.8	4.5	4.2	252	148
16	6.8	6.5	3.1	170	250
18	7.0	5.6	3.2	170	148
19	3.5	2.9	2.7	142	137
20	5.8	4.6	3.5	143	163
21	3.2	3.2	0.8	112	112
22	1.1	1.2	1.0	30	110
23	4.3	4.1	2.3	72	204
24	3.0	3.0	1.5	266	210
25	3.5	3.0	1.9	102	205
26	1.3	0.8	0.6	202	185
27	5.5	4.9	2.8	215	200
28	6.0	4.9	3.9	190	220

TABLE 4.5

Patterns of excretion of electrolytes and minerals.

* = a rise and fall with a peak near the time of maximum diuresis.

F = a steady decrease.

R = a steady increase.

\- = not determined.

Subject	Cl	Na	K	Ca	Mg
1	*	*	NC	*	*
2	*	*	*	*	*
3	*	*	*	*	*
4	*	*	*	*	*
5	*	*	*	*	*
6	R	R	R	*	R
7	-	-	-	-	-
8	*	*	NC	*	*
9	*	*	*	*	*
10	R	R	R	R	R
11	NC	NC	R	*	*
12	*	*	*	*	-
13	R	R	R	R	R
14	NC	NC	NC	F	F
15	*	*	*	*	*
16	*	*	*	*	*
18	*	*	*	*	*
19	-	-	-	-	-
20	*	*	*	*	*
21	*	*	*	*	*
22	NC	NC	F	R	R
23	NC	NC	F	-	F
24	R	R	R	F	F
25	F	F	F	F	F
26	NC	NC	NC	*	*
27	*	*	*	*	*
28	R	R	*	R	R

4.13 Breath versus Blood Alcohol Levels

Breath tests by the Alcolimiter were compared with the interpolated blood alcohol levels at the time of the test. Readings were most consistent in the post absorptive phase (about one hour post drinking) and ranged from 6% to 41% below the blood level, the average being -22%:-

Case No.	Breath values % below blood alcohol levels (values averaged)
1	11
2	12
3	30
4	40
5	19
6	27
7	21
8	33
9	20
10	12
11	23
12	20
13	12
14	23
15	21
16	30
17	14
18	30
19	21
20	25
21	41
22	16
23	11
24	6
25	26
26	36
27	16
28	22

Results obtained before this phase were extremely variable and could be either above or below the blood levels and usually bore no relationship to the post absorptive levels. The complete data are tabulated in Appendix 2, Table 18.

Chapter 5

DISCUSSION

5.1 Effects

The effects of a single dose of ethanol have been listed by Maling (1970, p. 279). Amongst these are dizziness, mild euphoria, release of inhibitions and loud profuse speech, all occurring at blood alcohol levels between 20 and 100mg/100ml. The first, dizziness or light headedness, was the initial symptom in the present study and occurred within 15 minutes of drinking and at this time the average blood level was 25 mg/100ml. Sixty minutes later the blood levels had risen to 45 mg/100ml. and this symptom had largely disappeared although other signs of inebriation were present. This illustrates the "Mellanby" phenomenon (Mellanby, 1919) where the blood alcohol level at which a symptom appears is lower than when it disappears, or stated in another way, the effect is greater when blood alcohol levels are rising than when they are descending. This has been suggested by Kalant et al. (1971) to be an example of tolerance developing during the effects of a single dose of alcohol. The cause of this effect is not known, but Maling (1970) has suggested that it could be to variations in alcohol concentration in different parts of the body. However, there are equally rapid changes in the concentrations of other body constituents, as shown in this study such as the fall in pyruvate or increase in β-hydroxybutyrate which reflect the changing redox ratio

in the cytoplasm and mitochondria of cells metabolising alcohol. Accordingly, there could well be a biochemical explanation for this phenomenon. Six subjects apparently experienced no such effect from alcohol, either early light-headedness or late effects. This was not solely related to previous drinking habits (Table 4.1, page 62), although three of the heaviest drinkers were in this category. It must be borne in mind however, that stated drinking habits may not be reliable and the evidence given is largely subjective in any case.

No other significant physiological changes were noted. The blood pressures and pulse rates were variable and tended to decrease throughout the test from a higher level which could have been due to some apprehension about the test. Reports vary in the literature on this point; e.g., Stein et al. (1960) showed an increased cardiac output and blood flow through the liver but the blood pressure was unaffected. Gould et al. (1972) did not note any significant change in heart rate or systemic arterial pressure after 90 ml. of whisky, but Allison et al. (1971) reported an increase in diastolic pressure and pulse rate after one ounce of 95% alcohol, while Reisby et al. (1969) reported a fall in blood pressure. It is quite likely that the conditions under which the tests are performed and the preparation of the subjects are most significant in promoting these effects.

Perhaps greater note could have been made of flushing as an indicator of peripheral dilatation, but in most cases this was difficult to determine with accuracy. Wolff (1972) measured this by optical densitometry of earlobes and showed a flush to commence 2-7 minutes after drinking with a peak

at 30-37 minutes, coinciding with the appearance of alcohol in the blood and to peak blood alcohol levels. The flush was much less pronounced in subjects of Caucasoid origin than Mongoloid and was the same in infants as well as adults so that any adaptation with age was unlikely.

The "infectious" behaviour of social drinking was illustrated by the behaviour patterns of each pair of subjects. The sessions varied from the hilarious and vociferous to those which were quiet and soporific. The atmosphere could change on a visit by a colleague not involved in the study when humour might be swept aside depending upon circumstances. This sort of behaviour pattern makes subjective measurements impracticable.

5.2 Absorption, Equilibration and Elimination of Alcohol and the Estimation of total Body Water

Alcohol is absorbed from both the stomach and the duodenum, but at a much higher rate from the latter (Kalant et al. 1971, page 240). From the intestinal tract it passes to the liver via the portal vein. The liver is interposed between the intestinal tract and the general circulation so that most absorbed alcohol passes initially through the liver into the circulation via the hepatic veins which drain into the inferior vena cava. The alcohol then proceeds through the heart and lungs to the arteries and recirculation through the liver is via the hepatic artery.

The most rapid absorption rates will be achieved by rapid gastric emptying into the duodenum and carbonated beverage

was given with the vodka in order to achieve this. From the blood-stream, alcohol diffuses into all the body water compartments until equilibrium has been reached. Alcohol is only sparingly soluble in fat so that there is no significant uptake in the adipose tissue of the body.

The observed blood alcohol curve results from a combination of absorption and equilibration factors. The absorption of alcohol into the blood has been described as the influx and its elimination, the efflux (Kalant, 1971). The initial absorption slope is where influx is greater than efflux. If these two are equal, then there is no apparent movement up or down of the blood alcohol level. A typical example of this was case 6 where the blood levels remained at 38 mg/100ml. between 30 and 80 minutes. When influx is less than efflux, the alcohol level fails. Widmark (cited by Wallgren and Barry, 1970, page 44) defined four phases of the alcohol curve; absorptive; plateau; equilibration and liver elimination. A fifth stage exists where the blood alcohol levels are low and the elimination is non-linear. This occurs below 20 mg/100ml. where the alcohol dehydrogenase enzyme system is unsaturated with a consequent decrease in oxidation.

The results in this study have demonstrated all of these features. It was apparent that until equilibration was completed, it was not possible to estimate the β slope. This slope might be used to determine either the body water content (Pawan and Hoult, 1963) or to estimate the amount of alcohol consumed for legal or forensic purposes. If blood testing is carried out over too short a period of time, or there are insufficient estimations, it would be practically impossible to determine either body water

contents or estimate likely amounts of alcohol consumed. In five of the subjects, equilibration was not complete until 90 minutes after drinking and in four it took at least 120 minutes. Delayed equilibration would lead to underestimates of body water content or overestimates of the total amount of alcohol consumed; at the same time, calculation of a β value could be very inaccurate. When the blood alcohol level dropped to approximately 25 mg/100 ml. the rate of metabolism slowed in the majority of cases so a sufficiently high dose of alcohol must be given to maintain blood levels above this value throughout a three hour period. These points are illustrated in the following examples:

1. Case 11

   This person was a known heavy drinker whose β slope would have been overestimated at 28 mg/100ml./hour or even greater if the study had been terminated at 2 hours instead of 3. Alternatively, taking Co at 860 and a normal body water of 0.72, the estimated amount of alcohol consumed would have been 51 grams or 150 ml. of Vodka, whereas the actual amount consumed was 100 ml.

2. Case 28

   If this test had been terminated at 2 hours, the β slope would have appeared to be 4 mg/100ml./hour and the amount of alcohol consumed, assuming a normal body water for females of 0.60, 19 grams or 55 ml. of Vodka whereas the true rates were 10 mg/100ml./hr. and 72 ml. Vodka.

In the calculations for the Widmark factor 'r', body water

is overestimated because a correction for the blood solids has not been applied. The water content of blood averages 850g/l. (Geigy, 1970) so that the solids comprise 20% of the total on a weight for weight basis. If this 20% correction is made for the observed averages for 'r', then for males 0.72 is lowered to 0.58 and for females, 0.63 to 0.50, and these figures correspond to the total water as a percentage of body weight of 59% for males and 51% for females measured by dilution of deuterium oxide (Geigy, 1970).

The values of 'r' and the observed average β slope in this study of 12.2 mg/100ml./hr. and a range of 7.3-16.0 mg/100ml./hr. corresponds closely to the results of 13 studies reviewed by Wallgren and Barry (1970, p. 47-48). These gave mean β values from 12 to 17 mg/100ml./hr. with individual slopes ranging from 2-29 mg/100ml./hr.; the upper limit of one range was 40 in a study of 922 subjects.

Whether the rates can be increased by prolonged use of alcohol has been the subject of much discussion. Wallgren and Barry (1970, page 519-522), reviewing the literature, stated that only a tentative conclusion may be drawn that increased elimination occurs in alcoholics. The main problem in testing such hypotheses adequately has been the lack of care to ensure that proper conditions apply when deriving the β slope and r values. Errors could readily arise as can be seen from the data obtained in the present study.

Iber et al. (1969), reported average β values of 49 mg/100ml./hr. in 30 alcoholics compared to 25 mg/100ml./hr. in 15 control subjects. Their control β level was higher than most other workers and it is also difficult to reconcile

the stated blood alcohol levels of 90-140 mg/100ml. with a dose of only 0.5 g/kg body weight. Goldberg (1943) showed constant r values and differences between non-drinkers, moderate and heavy drinkers of β slopes which were 12.4, 13.8 and 15.9 mg/100ml./hr., respectively. The total number of subjects tested were few, 9, 16 and 14 respectively but the differences were statistically significant ($P = < 0.001$). Wallgren and Barry (1970, p. 490) quote several references to subjects consuming 400 grams of alcohol per day over extended periods which would require a rate of metabolism of the order of 35 mg/100ml./hr. in a man of average weight. Lederman (1956, p. 104), in a review of alcohol consumption and its metabolism, considered such individuals to be examples of the extreme range of the distribution of β slopes about the normal. If this is the case, an alternative explanation to that of development of increased metabolism on exposure, might be that the population of alcoholics include an excess of subjects whose β slopes are normally in the upper percentile of the distribution range.

5.3 Acetaldehyde

Blood acetaldehyde levels reached 0.1-0.2 mg/100ml. on the average and remained at these levels while alcohol values decreased from 60 to 20 mg/100ml. Majchrowicz and Mendelson (1970), using direct injection of the supernatant of a zinc sulphate/barium hydroxide precipitate of blood into the gas chromatograph, found acetaldehyde levels between 0.11 and 0.15 mg/100ml. in subjects who have consumed bourbon whisky

and about half of these levels when grain alcohol was consumed. The values were constant throughout a range of blood alcohols up to 400 mg/100ml. even over an extended period of time.

Much work has been carried out by various workers on the observations by Truitt (1970) on the release of acetaldehyde from blood samples, but the possibility that deproteinisation may have been inadequate, as discussed in chapter 2, seems to have been overlooked. The production of acetaldehyde from partially denatured or haemolysed blood, which increases with temperature and may be inhibited with sodium nitrate or azide (Curry, personal communication), strongly suggests enzymic oxidation. However, Sippel (1972) has put forward the hypothesis that non-enzymic oxidation of ethanol in the perchlorate precipitate is due to a free radical chain reaction involving the autoxidation of ascorbic acid and this may be prevented by adding an antioxidant such as thiourea. This work seems to have been done at 56°C. only.

During confirmatory studies of acetaldehyde levels by an enzymatic method on perchlorate supernatants from this thesis, Crow (personal communication) found that the acetaldehyde was largely present in the red cells with very little in the plasma. As ascorbic acid is present in both red cells and plasma, acetaldehyde should have been found equally in both if non-enzymic oxidation was occurring. Both mechanisms could be contributing to the production of acetaldehyde with higher temperatures favouring the non enzymatic pathway. Such observations support the view that the acetaldehyde levels found in this study are true blood values at the time of venesection. However, it is not clear why acetaldehyde is not evenly distributed throughout cells

and plasma. Possibly, it is bound to the cell membranes or to haemoglobin and the disappearance of acetaldehyde added to whole blood reported in chapter 2 of this study may support this suggestion.

Acetaldehyde production is the first step in the oxidation of ethanol and the reaction is catalysed by alcohol dehydrogenase:-

$$\text{ETHANOL} + \text{NAD}^+ \rightleftharpoons \text{NADH} + \text{H}^+ + \text{ACETALDEHYDE}$$

Aldehyde dehydrogenase catalyses further oxidation to acetate:-

$$\text{ACETALDEHYDE} + \text{NAD}^+ \rightleftharpoons \text{NADH} + \text{H}^+ + \text{ACETATE}$$

If the formation and not the removal of acetaldehyde is the rate limiting step in this oxidation, as considered by Lundquist (1971), then acetaldehyde concentrations in the blood would not be expected to increase as the alcohol level is raised unless either ethanol metabolism is increased or the rate of acetaldehyde oxidation is inhibited. Hald and Jacobsen (1948) showed that tetraethylthiuramdisulphide (Disulfiram or Antabuse) inhibited aldehyde dehydrogenase and caused raised blood levels of acetaldehyde. Casier and Polet (1958), however, claimed that only small quantities were formed and attributed most of the toxic effects to disulfiram itself or one of its metabolites and also to accumulated free alcohol in the body. Truitt and Duritz (1967) showed a five-fold increase in blood acetaldehyde levels in rats given ethanol and disulfiram but later work by Truitt (1970) threw doubt on the specificity of the method he used for detecting acetaldehyde.

Whilst blood levels might be important in some symptoms of ethanol intoxication, such as increased heart rate and

dilatation of peripheral vessels (Asmussen et al. 1948), intracellular acetaldehyde formed by local oxidation of the circulating ethanol might be of greater importance. There is the possibility that if acetaldehyde is bound to red cell membranes as previously suggested, a similar reaction may occur in other tissue cells.

The presence of alcohol dehydrogenase in tissues other than the liver has been shown by various workers (Hawkins and Kalant, 1971) and it has been found in rat brain in minute amounts by Raskin and Sokoloff (1968). But there have apparently been no similar studies on the hormone producing organs such as the adrenal, thyroid, pituitary, ovary or testes.

Acetaldehyde may be of greater importance in its pharmacological effects than ethanol especially in its effects on neuroamine metabolism either by competitive inhibition of aldehyde dehydrogenase (Lahti and Majchrowicz, 1974) or by direct condensation with neuroamines to form simple alkaloid derivatives having addictive properties (see review by Davies and Walsh, 1971).

5.4 Ketogenesis

The light breakfast which the volunteers had about an hour before testing may well account for the lower blood acetone levels, before alcohol consumption, than those reported by Levey et al. (1964). The preparation of their subjects was not precisely recorded, but their use of the term "basal" might imply an overnight fast:-

Levey et al.,	mean 0.29	range 0.16-0.51 mg/100ml.
Present study	mean 0.087	range 0.04-0.14 mg/100ml.

The mean and range of the β-hydroxybutyrate levels correspond closely with the non-fasting levels of Bergemeyer and Bernt (cited in Geigy, 1970):-

	Fasting		Non-fasting		
	mean	range	mean	range	
Bergmeyer and Bernt	0.090	0.056-0.164	0.035	0.013-0.095	mg/100ml.
Present study			0.031	0.010-0.088	mg/100ml.

After alcohol consumption, levels of both acetone and β-hydroxybutyrate increased. These may be explained from known rates and pathways of ethanol metabolism. Ethanol is oxidised in the liver to acetaldehyde at an average rate of 26 mmoles/litre/hour but acetaldehyde levels did not exceed 0.04 mmoles/litre. Accordingly, the turnover rate to acetate, catalysed by aldehyde dehydrogenase, is high. Further oxidation of acetate via the tricarboxylic acid cycle is reduced by an alteration in the redox ratio (see section 5.5), but although Eskelson et al. (1970) have shown acetate derived from ethanol can be a precursor for cholesterol, presumably through the formation of fatty acyl CoA esters and mevalonate, Lundquist et al. (1963) have shown that acetate was released into the bloodstream with only a small conversion to acetyl CoA. The acetate is utilised as a source of energy in various tissues of the body by conversion to acetyl CoA and then to CO_2 via the tricarboxylic acid cycle (Ballard, 1972). Partial depression of this cycle presumably leads to the condensation of two acetyl CoA molecules to give acetoacetyl CoA and, subsequently acetoacetic acid by deacylation. This is then reduced to

β-hydroxybutyrate because of the change in ratio of NAD^+ to NADH; this conversion is discussed further under redox systems in section 5.5.

Acetone can arise from the spontaneous decarboxylation of acetoacetic acid and a raised level indicates an increased formation of acetoacetate. There appeared to be no direct correlation between acetone and β-hydroxybutyrate levels but this is not surprising as there would probably be considerable individual variations in the ratios of β-hydroxybutyrate:acetoacetate.

Regression analysis on the β-hydroxybutyrate data showed that a prediction could be made of the 15 minute level from the fasting figure. The analysis between the 15 and 180 minute levels confirmed the visual interpretation of the graphs in that low levels at 15 minutes continued to increase while high levels declined towards the end of the test period.

Another source of ketone bodies could come from the beta-oxidation of fatty acids. However, a reduced activity of the tricarboxylic acid cycle by an altered redox ratio would also inhibit beta-oxidation.

5.5 Lactate, Pyruvate and the Redox Ratio

The first step in the metabolism of ethanol involves NAD^+ as a coenzyme and hydrogen acceptor during oxidation to acetaldehyde and again in the next reaction to acetate, so that an NAD^+ molecule is reduced twice in the overall conversion of ethanol to acetate. The rate of reduction of NAD^+ in man averages 52 mmoles/litre/hour. During the

conversion of ethanol there is a dramatic change in redox levels in the body and reduced NAD^+ is formed to an extent where the rate of re-oxidation by the respiratory chain becomes the limiting factor in overall ethanol metabolism. This change can only be estimated indirectly, normally by measurement of metabolites which form redox pairs, such as lactate/pyruvate, which reflect cytosol levels and β-hydroxybutyrate/acetoacetate for mitochondrial levels of NAD^+ and reduced NAD^+. Peripheral blood samples reflect the changes in redox levels of the tissues through which it has passed (Forsander, 1970). Ideally, the changes occurring in the liver can only be measured in blood obtained by catheterisation of the hepatic vein. Excess lactate from the liver will be metabolised in the peripheral tissues and affect the redox levels of those tissues. There is no direct contribution to lactate or pyruvate by ethanol and the change in the ratio of lactate:pyruvate is due to a large extent to the conversion of pyruvate to lactate, which is well illustrated in this study. The lactate levels vary considerably and some of these would seem to be high as a result of ischaemia rather than ethanol consumption.

The change in redox ratios has been shown to influence both energy production and synthetic pathways (Forsander, 1970). The overall activity of the tricarboxylic acid cycle is reduced which has been shown by depression of CO_2 production. The low pyruvate levels lead to low oxaloacetic acid levels by a reduction in the pyruvate carboxylase reaction (Krebs et al., 1969). Biogenic amine metabolism may also be altered by the shift in the redox ratio leading to excretion of less oxidised forms (Asaad et al., 1974), as an alternative to the hypothesis that acetaldehyde is a competitive inhibitor

of the appropriate enzyme mechanisms (Hawkins and Kalant, 1972). There is also a similar effect on the steroid hormones where there is a shift to the hydroxy, or reduced state, from the keto, or oxidised state.

5.6 Carbohydrate Metabolism

Blood glucose levels increased rapidly after consumption of ethanol irrespective of whether the drink contained sugar or not. Vartia and Forsander (1960) showed a similar rise, falling to hypoglycaemic levels by the following morning. They also showed that this decrease was inversely proportional to the amount of ethanol consumed. Freinkel et al. (1963) showed that this decrease occured 4-6 hours after drinking so that confirmation was not possible in the present study with volunteers where all blood sampling was concluded at three hours.

The effects of ethanol on carbohydrate metabolism have been reviewed by Axelrod (1973) who suggested that while gluconeogenesis was inhibited by ethanol, glycolysis was stimulated. Consequently, if the glycogen stores were adequate, a transient rise in blood glucose could occur with a frank hyperglycaemia if glucose was administered at the same time. Where the glycogen stores were inadequate, as would occur during fasting or carbohydrate deprivation, hypoglycaemia would result.

The mechanisms of these actions are related to decreases in pyruvate levels. Gluconeogenesis from lactate is inhibited,

and also from amino acids by a reduced activity of the tricarboxylic acid cycle, and there is also increased glycolysis to maintain energy production with a consequent depletion in circulating glucose,(Krebs et al. 1969). Simultaneously, the hormones controlling blood glucose levels; insulin; glucagon; growth hormone; cortisol and epinephrine are all affected either by a direct action of ethanol or by the change in redox ratios (Axelrod, 1973).

The hypoglycaemic effect was illustrated by case 19 who had been drinking the evening prior to the test. His initial blood glucose level at 9 a.m. was 2.8 mmoles/l., in spite of a large meal consisting largely of carbohydrate at about 1 a.m. The alcohol given in the test was sufficient to restore the blood glucose to normal levels throughout the next two hours although the test was ended prematurely because the subject felt unwell. This is a good example of the counterbalancing effects of another dose of alcohol on a "hangover".

5.7 Fat Metabolism

The increased redox ratio following ethanol ingestion can lead to a diversion of dihydroxyacetone phosphate and glyceraldehyde 3 phosphate in the glycolytic pathway to glycerol, providing a means for the reoxidation of reduced NAD^+ and this is the basis of enhanced ethanol metabolism by fructose (Tygstrup et al., 1965). It seems unlikely that this mechanism could wholly account for the dramatic increase in blood glycerol levels in some subjects without

a simultaneous fall in glucose level. An alternative source of glycerol results from the hydrolysis of triacylglycerols by adipose tissue lipase which has been shown to be activated by catecholamine release (Maickel et al., 1963). Catecholamines have been shown to be elevated on drinking alcohol (Forman, 1958).

The increased glycerol levels found in the present study were contrary to those of Feinman and Lieber (1967), who showed a marked drop in glycerol and free fatty acid levels following ethanol ingestion. However their experimental conditions were completely different as their subjects drank alcohol slowly over a two hour period with a consequent fall in glycerol before relatively high blood alcohol levels were attained. This could be attributed to an apparent suppression of free fatty acid mobilisation by acetate produced from ethanol metabolism (Crouse et al., 1968). Lieber (1974) gives examples from the literature of either a decrease or an increase in free fatty acid levels and argues that experimental conditions may largely determine whether fatty acids are mobilised by catecholamine release or by compensating fatty acid synthesis from the acetate formed.

5.8 Diuresis

The diuretic effects of alcohol noted in this study were similar to those of Eggleton (1942) who showed that the onset was delayed 20-30 minutes after drinking and that the peak diuresis was unrelated to peak blood alcohol concentrations.

Her observations on the effects of variation in absorption of alcohol were only evident when testing the same individual. Comparison of urine volume with time of attaining a peak blood alcohol concentration in a group of individuals did not show any association in either her study or in this present one. Diuresis is apparently related to rising blood alcohol levels and a further response can be stimulated by another dose of alcohol. On the other hand, maintaining a constant blood level does not maintain the diuresis. The volume of urine excreted is also dependent upon the quantity of alcohol ingested and upon room temperature, being greater when it is colder. This could account for a large volume with case 1, who was the first subject tested and before adequate heating was installed in the study room. A similar explanation for the large volume from case 13 could not be made; this subject was tested concurrently with case 12 who only excreted 680 ml. Ogata (1963) showed that the diuretic effect of water or alcohol was excessive in 19 of 30 alcoholics, but not in 15 non-alcoholics. Evidence of reversion to normal after a period of abstinence was not very strong so that the possibility exists that alcoholics may be drawn from a small population of individuals who have a greater diuretic sensitivity to alcohol.

The diuretic effect of alcohol has been attributed to inhibition of secretion of antidiuretic hormone from the neurohypophysis and it is claimed that it can be suppressed by injection of a postpituitary extract (Eggleton, 1946; Rubini, 1955).

5.9 The Elimination of Electrolytes and Minerals in the Urine

Good data from voluntary urine specimens is difficult to obtain and this is illustrated in the present study. One subject could not pass any urine at all and this failure to do so was unlikely to have been due to urine not being produced. Incomplete emptying of the bladder will lead to carry-over into the next sample with a consequent error in rate and quantity excreted. This is a possible explanation for the discrepancies in some subjects such as case 1, where 3 ml. was collected in the first 30 minutes and 880 ml. in the next.

It is not possible to comment on the effect of alcohol alone from the present data as there are no baseline levels for comparison. For example, water will effect a diuresis and Eggleton (1946) has shown that this and alcohol will reduce chloride output whereas other diuretics will increase it. Kalbfleich et al. (1963) collected basal urine samples over one hour with an initial water load of 20 ml./kg., and replacement of the fluid volume excreted. These workers clearly demonstrated an increase in calcium and magnesium excretion rate in μmoles/minute and at the same time a decrease in potassium after giving 30 ml. of 100% ethanol. No significant changes were seen in sodium and chloride elimination in contrast to the work of Rubini et al. (1955).

The averages excreted in 30 minutes (Table 4.4, p. 76) reflects quite accurately the general level of elimination by each subject which shows that the conservation measures

of the kidney against gross losses of electrolyte or minerals are efficient. The mechanism of this is thought to be brought about by hormonal control of tubular reabsorption. For example, serum calcium levels are controlled by parathyroid hormone which can inhibit reabsorption in the tubules. Alcohol can cause an unresponsiveness in this hormone to tissue levels of calcium and result in its increased elimination (Estep et al., 1968). This may be due to the ketosis during alcohol consumption as fasting also causes a loss in calcium (Jones et al., 1966). Calcium and magnesium elimination are thought to be related (Flink, 1971) and this is seen in the low but significant correlation coefficient between the two. Magnesium deficiency is of great importance in chronic alcoholism and many of the signs and symptoms are common to both diseases (Flink, 1971) and loss in the urine may be of significance particularly where there is a dietary deficiency.

However, Beard and Knott (1971) have pointed out that a diuresis occurs only as long as the blood alcohol increases and that there is normally a water retention and a decrease in electrolyte excretion. This is apparently common in alcoholics in the absence of malnutrition and an aberrant distribution of electrolyte between the intra and extra cellular spaces may account for the malfunctioning of nervous and cardiovascular systems.

5.10 Blood Alcohol Estimates by Breath Testing

Alcohol in the pulmonary capillary plasma will be in

equilibrium with the air of the alveoli. It should, therefore, be possible to estimate the alcohol level in pulmonary arterial blood by measurement of expired air. In the post absorptive phase, the difference between arterial and venous blood will be minimal so that breath and venous levels should be similar. The breath test will reflect brain alcohol levels, by virtue of its equilibrium with pulmonary arterial blood, much more accurately than venous blood obtained from the ante cubital fossa.

In practice, many factors are present which will lead to discrepancies between arterial and venous blood. It has been shown in this study, that even under near ideal test conditions where alcohol was consumed both rapidly, on an empty stomach and with a carbonated beverage to hasten gastric emptying, the post absorptive phase could be prolonged to ninety minutes after drinking. Under different conditions, equilibration could easily be delayed much longer.

Expired breath initially contains tidal air which was not in contact with the alveoli and this must be exhaled before alveolar air is obtained. It has been shown that to obtain alveolar air, a continuous expiration against minimal pressure for at least 6 seconds is necessary (Dubowski, 1974). This has been allowed for in the design of the Alcolimiter by the inclusion of an electronically timed pressure sensitive switch. Assuming adequate collection of alveolar air which was initially at normal body temperature, the temperature will drop to that of the mouth which is about $34^{\circ}C$. Simultaneously, the alcohol concentration will also fall. There will also be some re-equilibration with the saliva which contains alcohol at a higher concentration than that of the plasma because it has a higher water content. The extent of this is difficult to establish and

would be, in part, dependent on the amount of moisture in the mouth and bronchi.

It is not surprising that the blood:breath ratio of 1:2 100 determined by Harger et al. (1950) and now widely accepted by forensic scientists for the estimation of blood alcohol levels from breath samples, is different from the in vivo studies of Jones et al. (1974). These workers showed that this ratio varied between individuals and gave a mean value of 1:2 350 and at the same time confirmed the in vitro ratio of 1:2 100. The results of the present study show individual variations about a mean value which was generally lower than the blood level.

Another factor leading to wider variations between breath and blood values, is the temperature of the breath as it leaves the mouth. This has a range of 32.4-35.7°C. under normal conditions (Dubowski, 1974) and could conceivably be extended in hot and cold climates or in subjects with hypo- or hyperthermia. It was shown with a simulator breath solution giving a blood alcohol reading of 100 mg/100ml. on the Alcolimiter, that there was a difference of 5.4 mg/100ml./ degree celsius around the 34°C. level (figure 2.11, page 44). Further designs of instruments to measure breath alcohol may incorporate a temperature compensation device.

Some of the above points can be clarified by the use of a gas chromatograph which has been adapted for measuring the alcohol in breath samples (Section 2.4, page 40) and this will enable the determination of blood:breath ratios. A thermistor has also been incorporated into the mouthpiece for the determination of breath temperatures.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The present investigations were initiated to obtain biochemical baseline data on human volunteers who had consumed alcohol. It is intended to extend this work to groups other than normal young adult males and females and, if possible, use the findings for comparative clinical studies.

The estimations used were selected for reasons outlined in chapter 1 and clearly more metabolites could be determined on blood, breath and urine samples if required. Most of the metabolites estimated in the present study have already been examined in human volunteer groups by other workers, but there are no reports in the literature of investigations where they have all been followed simultaneously in an individual who has consumed alcohol. A unique feature of this study has been the application of acetaldehyde estimations in blood samples while alcohol and body metabolite levels were being followed.

Differences have been noted from the work of others in this field and it is suggested that experimental conditions may have a large effect on the nature of the results obtained. The rate of drinking, type of beverage and conditions under which it was consumed could have widely differing effects upon the parameters measured. Absorption of alcohol would be delayed if taken with a meal or consumed over a longer period of time and a lower rate

of equilibration with the body water compartments would result. Under these conditions, the classical absorption and elimination curve would be altered in an unpredictable way with fluctuations up and down throughout the period of study. Such fluctuations would influence the extent to which diuresis occurred; this is marked only during rising body alcohol levels. Redox ratios and the control of various metabolic pathways are also likely to be affected. The ingestion of food prior to or during drinking could well affect the availability of glucose or the ability of the body to reoxidise NADH with changes in redox ratios being offset. Variation in alcohol absorption patterns could account for the observed difference in glycerol levels found in this study compared with the results of other workers. Alternatively the enzymatic technique for estimating glycerol may be at fault, but the significance of the raised levels might be confirmed if the free fatty acids were also found to be increased. Taking blood samples from alcoholics during a "drying out" phase from high blood alcohol levels is unlikely to give information on metabolite levels which may be directly compared with this study. An exception could be with blood acetaldehyde levels which may well be higher.

The electrolyte and mineral studies gave little information of significance and it is not proposed to include such estimations in further studies. In any case, electrolyte excretion would be greatly affected by the dietary intake and estimations on single blood samples would determine whether there was a deficiency or not. The causes of such deficiencies can only be determined by proper balance studies in which intake and

excretion in both urine and faeces are measured.

Although one of the objectives of this study was to develop a technique for measuring blood acetaldehyde, it became clear during the investigation that other factors were involved apart from the techniques of sample preparation and instrumentation. A reason for the apparent absence of acetaldehyde in the plasma must be found if circulating levels are to be of any significance. The apparent reversible binding to erythrocytes, which may act as a carrier, could be of importance in work on the clinical effects of alcohol. The fate of acetaldehyde in whole blood, or its origin as an artefact during sample preparation may be determined by the use of radioactive labelled compounds in vitro. The presence of comparatively high levels of acetaldehyde in breath samples would lend support to the possibility of arterial and venous blood level differences and modifications to the gas chromatograph to enable breath testing have been made to investigate this possibility. Peripheral production of acetaldehyde from alcohol in various organs may be of greater importance than previously recognised and this possibility requires study.

There are several ways in which future work can be directed depending upon resources available. The group of volunteers could be enlarged in an effort to find differences which may provide pointers to the cause of addiction, but this approach is likely to be unrewarding unless a cohort analysis was intended over a long period of time. Alternatively, the single alcohol dose studies could be applied directly to alcoholics who have abstained from drinking for several days to enable comparisons with

the present study; alcohol could be given under normal drinking conditions or different alcoholic drinks could be compared to see whether the congeners have any effect, particularly on acetaldehyde levels. Further investigations into likely adaptive processes during a long period of drinking is warranted. It is postulated in these circumstances, that as the blood alcohol level declines, an abnormal situation arises which is corrected by further consumption of alcohol. Such a hypothesis is supported by the finding that alcohol alone will raise the blood glucose level in alcoholic hypoglycaemia and it would be of interest to determine whether insulin has a major role in this process. From the present study, extension of the range of metabolite assays to include blood acetate, triglycerides and free fatty acids would be worthwhile.

It is clear that such studies could receive a new dimension of significance if labelled compounds could be used in the investions on human volunteers. Mass spectrometry would enable the use of stable isotope labels such as deuterium or ^{13}C, so that in vivo experiments would be possible in humans. However animal experiments may be required, for example, to insert cannulae in the hepatic artery and vein so that acetaldehyde and acetate levels of blood entering and leaving the liver may be calculated. Levels of metabolites in body samples do not provide information on the behaviour of the individual substances investigated. The rates of formation and removal may well be altered in different circumstances when alcohol is ingested and the use of labelled ethanol could provide direct evidence of such changes. In addition, the precursor role of alcohol and

acetaldehyde to other body components is an area which is yet ill defined. The role of acetaldehyde in forming addictive substances by interaction with body amines is a possibility which could effectively be studied if labelled compounds were used in the *in vivo* studies.

The subject of this thesis represents the development of a basic study in continuing investigations on alcohol and acetaldehyde in humans.

APPENDIX I

A WORKBOOK OF PROCEDURES USED IN THE STUDY OF ALCOHOL METABOLISM IN HUMAN VOLUNTEERS

LIST OF CONTENTS

PREPARATION

Two subjects were tested at the same time which required the following preparations to be made the day prior to the test.

Containers

Test tubes	7 x 1 cm., labelled and stoppered with rubber bungs.
	16 for whole blood
	16 for separated plasma
Cuvettes	7 x 1 cm. for fluorometric assays.
	120 approx. (Becton-Dickinson, RTV culture tube 12 x 75 mm.)
Bottles	5 ml., screw capped with rubber liner, labelled.
	16 for perchlorate supernatants
	28 for headspace gas preparation
Bottles	25 ml., screw capped with rubber liners, labelled.
	16 containing 3.0 ml. of M. $HClO_4$., weighed
	12 for storage of urine specimens
Bottles	300 ml., labelled, stoppered.
	12 for collection of urine samples

Alcolimiter

This was calibrated to 100 mg/100ml. with breath blown through a freshly prepared alcohol solution equilibrated in a water bath to $34^{\circ} \pm 0.2$. This solution was made by diluting 10 ml. of stock 60.5 mg/ml. ethanol to 500 ml. with distilled water.

Gas chromatograph

The Porapak Q column was washed by injections of 5 µl. distilled water into the vaporiser under normal operating conditions. Each injection was followed on the chart recorder and repeated until the water elution tail was minimal and no acetaldehyde or other volatile eluted.

Reagents

Phosphate and hydrazine buffers were freshly prepared and the following reagents weighed into 5 ml. screw capped bottles and stored in the refrigerator until use:-

NAD^+ 25 mg.		for hydroxybutyrate
NAD^+ 20 mg.		for lactate
NADH approx. 3 mg.		for pyruvate
Horse radish peroxidase	1.6 mg.	for glucose
Homovanillic acid	4.0 mg.	for glucose
L-cysteine	4.8 mg.	for glycerol
ATP	2.5 mg.	for glycerol
NAD^+	4.5 mg.	for glycerol

MATERIALS

Buffers

1.0 M Phosphate, pH 7.0 for pyruvate and glucose

K_2HPO_4	12.09 g.
KH_2PO_4	4.08 g.
	0.2 g.

deionised water to 100 ml.

1.1 M Hydrazine pH 9.0 for lactate and hydroxybutyrate

Hydrazine sulphate	1.3 g.
Hydrazine hydrate	5.0 ml.
	0.2 g.

deionised water to 100 ml.

TRIS pH 9.0 diluent for NADH

HCl 0.1 M	7.5 ml.
TRIS	0.6 g.

deionised water to 100 ml.

1.0 M Hydrazine hydrochloride + Magnesium for glycerol

Hydrazine hydrochloride	10.5 g.

This was dissolved in approximately 70 ml. of deionised water and the pH adjusted to 9.4 with approximately 12 ml. of 15M KOH. To this was added 0.1 ml. of 30% magnesium chloride solution and the volume made up to 100 ml. with deionised distilled water.

Note: All buffers were filtered to remove dust particles before being used in fluorometry.

Stock Standards

L (+) Lactic acid	10 mmoles/l.	96 mg.
Sodium pyruvate	1.0 mmoles/l.	11 mg.
β hydroxybutyrate	1.0 mmoles/l.	24 mg. of D-L form

These quantities were dissolved in 100 ml. of N.10 HCl and stored at 4°C. for no longer than two months.

Glucose 50 mmoles/l.

0.99 g. to 100ml. of 0.1% benzoic acid, stored at 4°C.

Glycerol 20 mmoles/l.

This standard was purified and dehydrated by distillation under vacuum and made up to a 20 mmoles/l. by weighing.

Standards for gas chromatography

An initial 1/20 dilution of the 100% concentration reagents were made, followed by further dilution of the volume indicated below to 500 ml. with deionised water to make stock solutions containing 500 mg/100ml.:-

Acetaldehyde	31.2 ml.
Ethanol	31.6 ml.
Acetone	31.6 ml.
Acetonitrile	32.3 ml.

Enzymes and Co-Factors

NAD^+ β nicotinamide adenine dinucleotide

NADH reduced form, both grade III (Sigma)

Lactic dehydrogenase (E.C.1.1.1.27) type I, 100 units/mg. (Sigma)

Lactic dehydrogenase pyruvate kinase free, 300-600 units/mg. (Sigma)

D-β-hydroxybutyrate dehydrogenase (E.C.1.1.1.30) type II, 3-5 units/mg. (Sigma)

glycerophosphate dehydrogenase (E.C.1.1.1.8) type III, 500 units/mg. (Sigma)

Glycerokinase (E.C.2.7.1.30), 80-100 units/mg. (Sigma)

Glucose oxidase liquid, fungal, approximately 30% solids. 750 Baker units/ml. (Hughes and Hughes)

Horse radish peroxidase type I (Sigma), approximately 60 purpurogallin units/mg.

TREATMENT OF VOLUNTEERS

Preparation

Volunteers were asked to have no more than one cup of tea or coffee with milk and sugar if preferred, and one slice of thinly buttered toast for breakfast at 7.30 a.m. on the morning of the test. On arrival at the metabolic studies room, medical history, weights and blood pressures were taken during a rest period between 8.30-9 a.m. A "Butterfly-19, Int" intermittent infusion set (Abbott), which comprised a thin wall siliconed needle of 18 G bore with 3½ inches of tubing and reseal injection site was inserted into a vein near the cubital fossa and retained *in situ* by adhesive plaster. The needle was kept open by flushing with 0.5 ml. of a dilute heparin solution (250 units/ml. in isotonic saline) after each sample was withdrawn. Blood samples were taken with a hypodermic syringe by puncturing the resealing site and the first 2-3 ml. of blood containing heparin solution was discarded.

At 9 a.m. one volunteer was given 0.5 ml. of alcohol per kilogram body weight. Vodka was used at 75 proof spirits (N.Z.) and the quantity in millilitres was determined by dividing half the body weight by 0.4. The weight of alcohol given was calculated by multiplying this volume by 0.34. The vodka was diluted with 250 ml. of soda water and was consumed over a 10 minute period. The second volunteer was given the alcohol 5 minutes later.

Collection of Specimens

Blood samples were taken before drinking and then 15, 30,

60, 90, 120, 150 and 180 minutes after drinking had finished. The zero time urine sample was discarded and further specimens were collected immediately after the blood sample was taken until the diuresis was complete. Pulse and blood pressures were taken prior to the blood samples.

Blood Samples

Eight millilitres of blood was obtained without venous stasis. Approximately 3.0 ml. was immediately blown through the needle into the bottle containing ice cold perchlorate and thoroughly mixed. The needle was then removed and the remaining blood transferred to a 7 x 1 cm. tube containing one drop of heparin (5 000 U/ml.) and stored on ice until separation of the plasma by centrifugation.

The bottles containing blood and perchlorate were reweighed and the weight of added blood determined. Weight was converted to volume by dividing by 1.06. Where the volume of blood was greater than 3.0 ml. a corresponding volume of perchlorate was added. Where the volume of blood was less than 3.0 ml. a dilution factor was calculated from:-

$$\frac{\text{Total Volume} \times 0.5}{\text{Volume of Blood}}$$

Results of assays in which the perchlorate supernatant was used, were multiplied by this factor.

Urine Samples

The time of collection was noted together with volume. Specific gravity was measured by a hydrometer and a 20 ml. aliquot taken for further analysis.

Storage

All specimens were kept on ice or in a refrigerator for the following two days whilst the various tests were being performed. They were subsequently stored at -20^{o} until required again.

GAS CHROMATOGRAPHY

Standards

1.0 ml. each of acetaldehyde and acetone stock 500mg/100ml. solutions in a volumetric flask were made up to 50 ml. with deionised distilled water to make a 10 mg/100 ml. solution. The standards were prepared as follows:-

ethanol 500 mg/100ml.	1.0	2.0	3.0	0.5	1.0	2.0
acetaldehyde/acetone 10 mg/100ml.	0.1	0.2	0.5	0.1	0.2	0.5
Deionised water ml.	7.4	6.3	5.0	9.4	8.8	7.5
Molar perchlorate ml.	10.0	10.0	10.0	-	-	-

Concentration in:-	Blood			Urine		
Equivalent to:						
ethanol mg/100ml.	50	100	150	25	50	100
acetaldehyde/acetone mg/100ml.	0.1	0.2	0.5	0.1	0.2	0.5

Preparation of headspace gas

To a 5.0 ml. screw capped container was added 100 µl. of 50 mg/100 ml. acetonitrile and 0.5ml. of standard, perchlorate supernatant or urine. Dried sodium sulphate was added from a calibrated test tube, 0.4-0.6 g., and the bottle quickly capped and placed up to its neck in a waterbath at 34°C. mixing the contents vigorously at frequent intervals, for at least 15 minutes.

Headspace gas was removed by a gas-tight syringe through a 19 gauge needle piercing the screw cap and 500 µl. was then injected

immediately into the gas chromatograph.

Gas Chromatograph operating conditions

Column temperature	130°C
Vaporiser	150°C
Detector	150°C
Column	5' x ⅛" stainless steel, treated with phosphoric acid and containing Porapak Q
Carrier gas flow	40 ml./minute at 30 P.S.I.
Hydrogen flow	30 ml./minute
Breathing air	300 ml./minute
Electrometer	Flame ionisation
Range	10^{-12}
Attenuation	x 4
Integrator settings:-	
peak width	30"
slope sensitivity	5
baseline correction	5
count rate	125 Hz.
Chart recorder	0.2 mV and 15 cm./hour

Integrator Operation

The baseline was set to 10 on the recorder scale with the gas chromatograph bucking control. The DBC fast button was pressed until the negative and positive lights alternated to establish a baseline. The integrator was switched to automatic and the sample injected. The descending water elution peak was followed until a small peak which appeared on this slope was passed. Integration was triggered by

switching off and then back to automatic the integration switch and the peak area was printed out. The fast DBC button was pressed until the slope indicator began to return from negative to zero prior to the acetaldehyde peak. This peak was integrated by triggering the printout when the negative baseline light came on and the fast DBC button was held until the slope detector indicated zero. The remaining peaks were automatically integrated. Peaks were kept on the chart scale by the use of the integrator attenuation switch. It was sometimes necessary to initiate acetone integration by temporarily increasing slope sensitivity.

Calculations

The ratios of standards/acetonitrile were plotted against concentration and a straight line fitted. A multiplication factor was determined for each component by taking a point on the line and dividing the concentration by the corresponding ratio. The concentration in the unknown sample was obtained by multiplying the ratio unknown/acetonitrile by these factors.

ASSAYS BY ENZYMATIC PROCEDURES

Standard lactate, pyruvate and hydroxybutyrate

The stock solution was diluted with deionised water 1:10 and the following standards prepared on the day of use to give concentrations equivalent to a blood perchlorate supernatant:-

1/10 dilution of stock ml.	1.0	1.0	1.0
Deionised water, ml.	-	1.0	3.0
Concentration equivalent of:-			
Lactate mmoles/l.	2.0	1.0	0.5
Pyruvate mmoles/l.	0.2	0.1	0.05
β hydroxybutyrate mmoles/l.	0.2	0.1	0.05

Lactate assay

Reagent mixture sufficient for 20 tests was prepared from:-
20 ml. hydrazine buffer, pH 9.0
20 mg. NAD^+
40 µl. lactic dehydrogenase, 25 mg/ml.

Deionised water for the blank, 50 µl. of the standards and 50 µl. of perchlorate supernatants were pipetted into 7 x 1 cm. cuvettes. Reagent mixture, 1.0 ml. was added, mixed and left at room temperature for 45 minutes. The reaction was stopped by adding 4.0 ml. of 0.1 M HCl to all tubes.

The fluorometer was set to an excitation wavelength of 350 nm. and 460 nm. emission and the range to x 30 high. Zero was set with the blank cuvette in position one of the

multiple sample turret by the blank control and the 1.0 mmoles/l. standard (position 2) was adjusted to read 10 on the 0-33 scale with the sensitivity control. Linearity was confirmed with the 0.5 mmoles/l. standard. The test cuvettes were read, checking the blank and standard each time the sample turret was refilled.

Calculation: Lactate mmoles/l. = $\frac{\text{reading}}{10}$

Normal range: 0.5-1.1 mmoles/l.

Pyruvate assay

NADH was dissolved in Tris buffer, pH 9.0 to give a concentration of 1.140 mg/ml. Reagent mixture was prepared sufficient for 25 tests:-

50 ml. 1.0M phosphate buffer, pH 7.0
0.25 ml. NADH solution
25 µl. lactic dehydrogenase diluted 1/35 in 2.1M ammonium sulphate

Reagent mixture (100 µl.) for the blank and 100 µl. of the standards or perchlorate supernatants were pipetted into 7 x 1 cm. cuvettes. Reagent mixture, 2.0 ml., was added, mixed and incubated at room temperature for 15 minutes.

With the same fluorometer settings but on the x 100 range, the blank cuvette was set to 100 with the sensitivity control and the 0.2 mmoles/l. standard to 10 with the blank control. The remaining standards and tests were read maintaining these settings as there was a slow downward drift due to natural re-oxidation of NADH.

A calibration curve was drawn by fitting the best line to

the readings of the standards and pyruvate concentration was determined from the corresponding fluorescence reading.

Normal range: 0.05-0.11 mmoles/l.

β-Hydroxybutyrate assay

Reagent mixture for 25 tests was prepared from:-
50 ml. Hydrazine buffer, pH 7.0
12.5 mg. NAD^+
100 ul. β-hydroxybutyrate dehydrogenase

The procedure was similar to the pyruvate assay except that the reaction time was 60 minutes. Using the same fluorometer wavelengths but on the x 300, high, range, the blank was set to zero and the 0.2 mmoles/l. standard to 20 on the 0-33 scale. Linearity was confirmed with the remaining standards and the test cuvettes read, maintaining the blank and standard readings.

Calculation: β-Hydroxybutyrate mmoles/l. = $\frac{\text{reading}}{10}$

Normal range: 0.01-0.95 mmoles/l.

Glucose assay

The 1.0 M phosphate buffer, pH 7.0 was diluted tenfold to 0.1 M with deionised water. The following standards were prepared from the stock solution:-

Volume of 50 mmoles/l. stock glucose, ml.	0.6	1.0	2.0
0.1 M phosphate buffer, ml.	9.4	9.0	8.0
Glucose concentration mmoles/l.	3	5	10

The reagent mixture was prepared by dissolving 1.6 mg. of

horseradish peroxidase in 4.0 ml. of 0.1 M phosphate buffer, adding 10 ul. of glucose oxidase and 4 mg. of homovanillic acid and mixing gently to dissolve.

The standards and plasma were diluted 1/40 by taking 50 µl. to 2.0 ml. buffer. 50 µl. of this dilution was pipetted into 7 x 1 cm. cuvettes and 0.2 ml. of reagent mixture added, mixed and incubated at 37°C for 30 minutes. The reaction was stopped by adding 4.0 ml. of 0.1 N NaOH.

Fluorometer setting were: Excitation, 325 nm., emission 425 nm., range x 3, high.

The blank was set to zero and the 5 mmoles/l. standard to 10 on the 0-33 scale. The remaining standards and tests were read and linearity confirmed.
Calculation: Glucose mmoles/l. = reading x 5
Normal range: 3.5-5.5 mmoles/l.

Glycerol assay

Standards were prepared by diluting 2.0 ml. of stock 20 mmoles/l. to 100 ml. with deionised water to give 0.4 mmoles/l. A portion of this was diluted with an equal volume of water for the 0.2 mmoles/l. standard and an equal volume again for the 0.1 mmoles/l.

Plasma was deproteinised by adding 0.1 ml. to 0.5 ml. of 5% zinc sulphate solution and to this mixture 0.5 ml. of 0.3N barium hydroxide. The clear supernatant was recovered by centrifugation. The standard solutions and a water blank were treated in a similar manner. To a clean 7 x 1 cm. cuvette was transferred 0.2 ml. of this supernatant and 0.1 ml. of reagent mixture and left at room temperature for

60 minutes. The reaction was stopped by adding 2.0 ml. of 0.01 N NaOH containing EDTA to each tube.

The reagent mixture sufficient for 20 tests was prepared freshly before use from:-

4.8 mg.	L-cysteine
2.5 mg.	ATP
4.5 mg.	NAD^+
2.0 ml.	Hydrazine hydrochloride buffer containing $MgCl_2$
10 ul.	glycerokinase diluted 1/25 in 2.1 M ammonium sulphate
10 ul.	glucose phosphate dehydrogenase

The fluorometer was set to 350 nm. emission and 460 nm. excitation with the range at x 300, high. The blank was set to zero and the 0.2 mmoles/l. standard to 20 on the 0-33 scale. The remaining standards and tests were read and linearity confirmed. The 0.4 mmoles/l. standard was read on the 0-100 scale with the range setting on x 100 high.

Calculation: Glycerol mmoles/l. = $\frac{\text{reading}}{10}$

Normal range: 0.03-0.13 mmoles/l.

TABLE 1

Blood Alcohol mg/100 ml.

	Minutes After Drinking							
Subject	0	15	30	60	90	120	150	180
1	0	34	42	40	36	30	25	19
2	0	40	54	48	41	35	27	20
3	0	84	62	54	46	41	32	–
4	0	20	29	41	37	32	29	20
5	0	61	55	51	44	39	34	24
6	0	32	38	37	37	34	29	26
7	0	33	45	44	40	–	31	–
8	0	11	19	32	33	29	22	15
9	0	50	51	49	40	36	26	22
10	0	24	35	51	49	43	40	37
11	0	89	97	57	43	37	25	20
12	0	47	50	38	33	29	24	19
13	0	38	58	76	57	46	33	31
14	0	49	64	59	42	36	30	22
15	0	60	55	48	40	33	26	23
16	0	25	36	47	43	37	35	28
17	0	64	59	53	44	36	29	26
18	0	75	66	53	49	43	38	35
19	32	82	80	73	65	59	51	–
20	0	32	36	40	38	34	26	23
21	0	37	61	64	61	52	46	41
22	0	74	59	57	51	–	41	–
23	0	32	54	58	50	44	36	29
24	0	56	64	54	46	38	31	26
25	0	–	–	54	48	39	31	24
26	0	31	51	62	53	45	39	34
27	0	56	54	46	38	32	23	18
28	0	26	–	50	48	46	40	36

TABLE 2

Blood Acetaldehyde mg/100 ml.

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	0.05	0 11	0 11	0.11	0.11	0.09	0.09	0.06
2	0	0.09	0.11	0.07	0.03	0.03	0 05	0.06
3	0	0.14	0.08	0.12	0.08	0.09	-	-
4	0	0	0	0	0	0	0	0
5	0	0	0	0.08	0.08	0.14	0.03	0.03
6	0	0.03	0.02	0.01	0.01	0.06	0.03	0.07
7	0	0.11	0.09	0.07	0.10	-	0.07	-
8	0.03	0.05	0.06	0.10	0.09	0.11	0.11	0.05
9	0.01	0.05	0.11	0.05	0.09	0.05	0.05	0.06
10	0	0.09	0.06	0.06	0.07	0.10	0.10	0.11
11	0.03	0.10	0.11	0.04	0.16	0.08	0.07	0.05
12	0.01	0.09	0.09	0.08	0.09	0.09	0.10	0.03
13	0.02	0.06	0.06	0.10	0.10	0.08	0.07	0.07
14	0.01	0.10	0.10	0.07	0.06	0.16	0.09	0.05
15	0	0.09	0.08	0.10	0.08	0.06	0.05	0.07
16	0.03	0.02	0.05	0.13	0.06	0.08	0.08	0.05
17	0.01	0.11	0.10	0.12	0.09	0.08	0.08	0.04
18	0.01	0.09	0.15	0.15	0.16	0.10	0.10	0.09
19	0.05	0.20	0.14	0.22	0.12	0.11	0.12	-
20	0.01	0.07	0.09	0.08	0.10	0.10	0.09	0.07
21	0.02	0.08	0.07	0.22	0.13	0.14	0.08	0.15
22	0	0.18	0.11	0.13	0.11	-	0.08	-
23	0.01	0.06	0.09	0.13	0.10	0.06	0.09	0.04
24	0.01	0.12	0.13	0.10	0.09	0.11	0.08	0.05
25	0.01	-	-	0.14	0.08	0.06	0.08	0.09
26	0.01	0.10	0.10	0.18	0.12	0.13	0.15	0.10
27	0	0.17	0.13	0 14	0.10	0.07	0.06	0.06
28	0	0.05	-	0.11	0.15	0.14	0.12	0.14

TABLE 3

Blood Acetone mg/100 ml.

Subject	Minutes since Drinking							
	0	15	30	60	90	120	150	180
1	-	-	-	-	-	-	-	-
2	0.06	0.09	0.16	0.07	0.05	0.11	0.07	0.08
3	0.14	0.09	0.08	0.11	0.14	0 16	0.12	-
4	0.11	0.16	0.17	0.10	0.10	0.09	0.10	0.13
5	0.08	0.14	0.08	0 16	0 27	0.14	0 05	0 07
6	0.25	0.12	0.14	0.10	0 09	0 12	0 11	0.11
7	0.05	0.11	0 16	0 12	0 19	-	0 15	-
8	0.06	0.10	0.11	0 10	0 12	0.14	0 16	0.12
9	0.07	-	-	0.13	0 08	0 14	0.15	0.04
10	-	0 14	-	-	0.09	-	-	0.11
11	0.13	0.22	0.19	0.20	0.16	0.18	0.23	0.23
12	0.06	-	0.10	0.09	0.09	0.10	0 08	0.07
13	0.06	0.08	0.10	-	0.11	0.09	0.09	0.09
14	0.14	0.14	0.22	0.16	0.14	0.18	0.15	0.18
15	0.11	0.19	0.16	0.18	0.21	0.21	0.26	0.23
16	0.12	0.09	0.14	0.14	0.11	0.15	0.11	0.12
17	0.12	0.18	0.17	0.17	0.17	0.17	0.18	0.15
18	0.07	-	-	0.10	0.11	-	0.15	0.18
19	0.21	0.26	0.28	0.25	0.26	0.27	0.25	-
20	0	0	0	0	0	0	0	0.06
21	0.04	0	0	0	0.19	0.13	0.13	0.15
22	0.13	0.15	0.17	0.19	0.21	-	0.20	-
23	0	0.08	0.21	0	0	0	0	0
24	0.06	0.13	0.15	0.16	0.15	0.18	0.14	0.14
25	0.05	-	-	0.13	0.15	0.14	0.16	0.17
26	0.09	0.11	0.10	0.15	0.08	0.15	0.17	0.11
27	0.06	0.05	0.11	0.10	0.08	0.09	0.07	0.12
28	0.10	0.13	-	0.13	0.13	0.15	0.15	0.15

TABLE 4

Blood Lactate mmoles/l.

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	1.25	1.27	1.50	1.60	1.40	1.38	1.35	1.98
2	1.05	1.28	1.45	1.32	0.96	1.03	0.79	0.85
3	0.70	1.30	1.22	1.27	1.23	1.08	1.00	-
4	1.20	1.34	1.52	1.52	1.22	1.05	1.00	1.00
5	1.34	1.50	1.54	1.46	1.30	1.34	1.14	1.00
6	1.17	0.94	0.90	0.90	0.87	0.98	0.85	0.85
7	0.90	1.02	1.17	1.24	1.14	-	0.80	-
8	1.50	1.40	1.30	1.00	1.00	0.95	0.80	0.90
9	1.55	1.40	1.35	1.20	1.35	1.25	1.30	1.25
10	0.60	0.64	0.70	0.90	0.84	1.10	0.83	0.75
11	0.75	1.18	1.30	1.40	1.56	1.44	1.33	1.28
12	0.80	1.10	1.20	0.70	0.90	0.65	0.55	0.45
13	0.66	0.75	0.86	1.19	1.15	1.25	1.30	1.20
14	0.93	0.90	0.94	1 06	0.94	0.92	0.94	1.06
15	0.95	1.60	1.26	1.10	1.25	1.02	1.02	0.98
16	0.80	0.87	1.04	1.20	1.11	0.96	1.00	0.85
17	0.84	1.44	0.76	0.79	0 61	1.35	0.90	1.33
18	1.09	0.82	0.93	0.93	0.62	0.58	1.06	1.15
19	0.74	0.88	0.88	0.94	1.04	1.11	0 97	-
20	0.82	0.98	1.28	1.04	1.29	1.20	1.15	1.13
21	1.40	1.25	1.43	1.68	1.33	1.10	1.08	1.42
22	0.93	0.93	1.20	1.42	1.34	-	0.81	-
23	0.95	0.90	0.92	1.00	0.85	0.82	0 68	0.79
24	0.78	0.97	1.14	1.10	1.15	0.96	0.86	0.83
25	1.04	-	-	1.40	1.52	1.17	1.36	1.08
26	1.20	1.18	1.30	1.43	1.14	1.00	1.02	1.00
27	1.30	1.28	1.20	1.18	1.04	1.02	1.00	1.10
28	1.02	1.33	-	1.16	1.20	1.06	1.15	1.18

TABLE 5

Blood Pyruvate mmoles/l.

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	0.11	0.07	0.07	0.08	0.08	0.08	0.07	0.05
2	0.11	0.06	0.06	0.06	0.05	0.05	0.04	0.04
3	0.07	0.04	0.05	0.05	0.06	0.06	0.06	-
4	0.11	0.09	0.10	0.09	0.07	0.07	0.06	0.06
5	0.11	0.08	0.07	0.07	0.07	0.07	0.06	0.06
6	0.10	0.07	0.08	0.08	0.06	0.05	0.05	0.05
7	0.10	0.07	0.08	0.09	0.08	-	0.06	-
8	0.16	0.06	0.05	0.04	0.06	0.05	0.06	0.04
9	0.14	0.05	0.05	0.05	0.06	0.08	0.07	0.06
10	0.07	0.05	0.05	0.05	0.04	0.04	0.04	0.04
11	0.10	0.04	0.04	0.03	0.04	0.04	0.04	0.04
12	0.12	0.06	0.06	0.05	0.08	0.05	0.05	0.05
13	0.09	0.06	-	0.04	0.05	0.04	0.05	0.04
14	0.10	0.06	0.07	0.05	0.05	0.05	0.06	0.06
15	0.09	0.08	0.07	0.08	0.08	0.06	0.07	0.08
16	0.08	0.05	0.05	0.07	0.06	0.05	0.04	0.04
17	0.11	0.06	0.04	0.03	0.04	0.03	0.04	0.04
18	0.12	0.05	0.05	0.04	0.03	0.04	0.03	0.05
19	0.05	0.05	0.05	0.06	0.05	0.05	0.05	-
20	0.09	0.07	0.07	0.06	0.06	0.06	0.06	0.06
21	0.12	0.06	0.07	0 07	0.07	0.07	0.08	0.08
22	0.09	0.06	0 07	0.07	0.08	-	0.04	-
23	0.09	0.08	0.08	0.10	0 08	0 07	0 07	0.05
24	0.12	0.11	0.11	0.13	0.11	0.10	0 11	0.08
25	0.10	-	-	0.06	0 05	0 05	0.06	0 04
26	0.10	0.05	0.08	0.07	0 05	0 05	0.05	0.04
27	0.14	0.08	0.08	0.10	0.08	0 08	0.08	0.08
28	0.10	0.08	-	0.07	0 07	0.06	0 06	0.06

TABLE 6

Blood β-hydroxybutyrate mmoles/l.

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	0.03	0.07	0.08	0.05	0.04	0.04	0.04	0.06
2	0.01	0.0	0.04	0.05	0.04	0.06	0.05	0.05
3	0.06	0.11	0.08	0.07	0.07	0.06	0.06	-
4	0.03	0.07	0.06	0.09	0.08	0.07	0.07	0.09
5	0.02	0.08	0.08	0.09	0.08	0.06	0.06	0.08
6	0.01	0.07	0.06	0.06	0.05	0.05	0.04	0.04
7	0.04	0.11	0.08	0.10	0.09	-	0.09	-
8	0.03	0.16	0.16	0.10	0.12	0.10	0.08	0.06
9	0.05	0.09	0.11	0.10	0.12	0.11	0.10	0.08
10	0.02	0.05	0.05	0.06	0.06	0.05	0.04	0.06
11	0.04	0.07	0.05	0.05	0.04	0.06	0.05	0.06
12	0.05	0.13	0.14	0.10	0.12	0.12	0.08	0.08
13	0.04	0.11	0.17	0.14	0.12	0.13	0.15	0.10
14	0.02	0.07	0.08	0.08	0.10	0.11	0.11	0.12
15	0.02	0.12	0.15	0.13	0.14	0.13	0.12	0.12
16	0.02	0.08	0.07	0.07	0.05	0.05	0.05	0.03
17	0.03	0.08	0.12	0.14	0.11	0.15	0.10	0.09
18	0.02	0.06	0.10	0.12	0.09	0.09	0.08	0.06
19	0.88	1.37	1.15	1.36	1.13	1.00	0.84	-
20	0.02	0.05	0.06	0.06	0.07	0.06	0.06	0.05
21	0.02	0.08	0.08	0.07	0.08	0.09	0.11	0.08
22	0.04	0.11	0.12	0.19	0.18	-	0.10	-
23	0.02	0.07	0.08	0.07	0.06	0.05	0.04	0.04
24	0.04	0.09	0.14	0.19	0.16	0.14	0.10	0.09
25	0.02	-	-	0.08	0 08	0.09	0.12	0.12
26	0.04	0.06	0.05	0.05	0.06	0.07	0 07	0.06
27	0.02	0.10	0.10	0.09	0.08	0.06	0.09	0.08
28	0 03	0.08	-	0.07	0.12	0.11	0.11	0.11

TABLE 7

Ratio Lactate/Pyruvate

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	12	20	21	20	19	18	20	18
2	10	23	25	23	19	21	20	21
3	10	30	30	24	22	20	18	-
4	11	15	16	17	17	16	16	17
5	12	19	19	19	17	18	17	17
6	12	13	12	12	15	21	19	17
7	9	16	15	14	13	-	14	-
8	10	24	25	26	17	18	15	22
9	11	27	28	27	22	16	19	22
10	9	14	16	20	22	27	22	21
11	7	29	32	41	38	35	31	30
12	7	18	19	13	12	14	11	10
13	8	13	-	32	22	29	25	31
14	9	15	14	21	19	19	16	16
15	10	19	18	14	16	17	14	12
16	10	18	21	18	19	20	26	21
17	7	23	19	25	15	31	22	37
18	9	16	20	23	19	13	33	24
19	16	16	18	16	20	23	18	-
20	9	14	19	17	21	20	18	19
21	12	20	20	25	19	15	14	18
22	11	17	18	22	17	-	21	-
23	11	12	12	10	11	11	10	15
24	7	9	11	8	11	10	8	11
25	11	-	-	23	32	22	25	25
26	12	23	16	22	22	20	21	28
27	10	16	16	12	13	13	13	14
28	10	17	-	17	18	19	21	18

TABLE 8

Blood Glucose mmoles/l.

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	5.1	7.3	7.0	5.7	5 3	4.6	4.7	4.7
2	4.0	5.8	6.0	4.4	3 8	3.9	4 1	4.1
3	4.1	7.2	6.8	6.1	5 0	4 8	3.8	-
4	4.8	4.8	4.6	5.6	4 2	4 4	4.7	4.7
5	4.0	6.9	6.4	4.2	3.9	4.5	3.9	4.3
6	4.3	4.8	4.7	4.4	4.0	4.3	4.3	4.1
7	4.4	5.3	3.9	3.7	3.8	-	4.3	-
8	5.8	5.7	4.2	4.8	5.1	4.7	5.0	5.0
9	5.2	5.6	5.2	5.9	5.1	5.4	5.6	5.8
10	3.6	4.2	3.5	4.2	4.0	4.1	4.8	4.7
11	5.3	5.9	5.7	4.8	-	5.0	-	4.7
12	6.2	6.4	5.4	5.7	5.1	5.3	5.0	4.6
13	5.1	5.8	6.9	6.0	6.1	4.7	5.8	4.7
14	3.6	5.2	5.6	5.2	4.5	4.8	5.1	5.0
15	4.3	4.2	5.7	4.2	4.7	4.8	5.6	4.6
16	4.9	4.7	4.8	5.3	5.1	4.2	5.0	5.3
17	3.9	4.4	4.8	4.7	4.2	5.4	4.0	5.0
18	4.2	4.2	5.3	4.0	4.6	4.2	4.2	4.1
19	2.8	4.9	4.4	4.9	4.2	4.3	5.2	-
20	4.9	4.8	5.2	5.2	5.1	4.7	4.2	4.4
21	5.0	4.7	4.4	5.7	6.4	4.8	5.6	4.6
22	5.3	5.7	6.5	6.1	4.6	-	5.0	-
23	3.1	3.9	4.7	4.0	4.2	3.8	4.0	3.1
24	5.2	5.7	5.4	5.4	4.9	5.0	5.0	5.4
25	3.8	-	-	4.3	4.2	3.8	4.0	3.9
26	4.7	6.5	5.2	3.9	3.6	3.6	-	4.3
27	4.2	5.2	5.8	4.8	4.6	4.4	4.2	3.8
28	3.9	4.6	-	4.0	4.0	3.9	3.4	4.6

TABLE 9

Blood Glycerol mmoles/l.

Subject	Minutes since Drinking 0	15	30	60	90	120	150	180
1	0.10	0.23	0.31	0.24	0.19	0.11	-	0.07
2	0.10	0.46	0.58	0.34	0.27	0.17	0.21	0.27
3	0.12	0.17	0.37	0.26	0.24	0.19	0.12	-
4	0.04	-	0.13	0.08	0.04	0.04	0.04	0.04
5	0.05	0.14	0.15	0.08	0.06	0.08	0.06	0.08
6	0.06	0.15	0.39	0.27	0.27	0.32	0.40	0.31
7	0.05	0.06	-	-	-	-	0.05	-
8	0.04	0.07	0.10	0.11	0.13	0.19	0.15	0.20
9	0.03	0.04	0.11	0.10	0.08	0.10	0.12	0.18
10	0.09	0.09	0.20	0.15	0.16	0.19	0.14	0.15
11	0.17	0.34	0.77	0.69	-	1.00	-	0.61
12	0.04	0.30	0.42	0.31	0.29	0.41	0.42	0.49
13	0.06	0.20	0.23	0.32	0.26	0.21	0.22	0.30
14	0.02	0 03	0.06	0.05	0.03	0 04	0.04	0 03
15	0.04	0.05	0 11	0 08	0.07	0 08	0.09	0 07
16	0.09	0 06	0.09	0.09	0 08	0.06	0.06	0.05
17	0.07	0.16	0.36	0.16	0.17	0.15	0 11	0.28
18	0.06	0.27	0.34	0.40	0.46	0.36	0.33	0.46
19	0.05	0.14	0.28	0.20	0.10	0.09	0.11	-
20	0.02	0.04	0.09	0.04	0.10	0.10	0.09	0.07
21	0.08	0.11	0.08	0.08	0.10	0.10	0.08	0.14
22	0.18	0.10	0.16	0.15	0.14	-	0.10	-
23	0.04	0.04	0.12	0.09	0.10	0.10	0.08	0.09
24	0.07	0.09	0.15	0.15	0.15	0.12	0.14	0.15
25	0.06	-	-	0.08	0.06	0.07	0.08	0.05
26	0.06	0.07	0.08	0.11	0.07	0.07	0.10	0.08
27	0.07	0.06	0.08	0.08	0.07	0.08	0.09	0.17
28	0.09	0.08	-	0.18	0.20	0.17	0.16	0.17

TABLE 10

Urine Alcohol mg/100 ml.

	Minutes since Drinking							
Subject	0	15	30	60	90	120	150	180
1	-	-	32	50	43	-	30	24
2	-	-	57	62	55	48	35	29
3	-	-	-	-	64	50	42	-
4	-	6	29	32	48	-	36	30
5	-	14	50	58	64	56	44	-
6	-	12	35	68	53	37	32	30
7	-	-	-	65	45	-	-	42
8	-	10	19	40	44	38	28	-
9	-	17	50	50	47	41	33	-
10	-	9	25	62	55	53	48	38
11	-	20	90	85	62	46	41	32
12	-	12	45	54	48	38	29	-
13	-	18	57	70	68	55	45	-
14	-	6	47	67	57	49	41	-
15	-	21	59	66	49	39	32	-
16	-	13	48	59	58	45	37	-
17	-	-	-	-	-	-	-	-
18	-	10	46	80	65	54	46	-
19	-	-	78	87	-	62	-	-
20	-	-	59	56	54	-	-	-
21	-	14	66	73	69	56	49	-
22	-	-	73	75	-	68	-	-
23	-	-	47	73	62	55	42	-
24	-	-	53	69	55	43	33	-
25	-	-	25	58	51	-	33	-
26	-	-	39	81	-	39	-	-
27	-	12	63	74	59	44	-	-
28	-	21	65	60	64	59	-	-

TABLE 11

Urine Volume (ml)

	Minutes after drinking							
Subject	15	30	60	90	120	150	180	Total
1	0	3	880	350	0	10	170	1 413
2	0	400	410	160	100	50	60	1 180
3	0	0	0	240	390	260	0	890
4	150	100	300	350	0	140	65	1 105
5	25	100	350	180	55	160	-	870
6	80	120	280	150	100	50	80	860
7	0	0	370	280	0	0	200	850
8	130	320	270	80	35	25	-	860
9	90	150	340	120	90	50	-	840
10	38	20	200	400	250	65	55	1 028
11	130	50	180	76	50	50	35	571
12	55	20	160	300	100	45	-	680
13	300	300	450	400	55	68	-	1 573
14	35	150	400	200	65	40	-	890
15	140	200	320	85	35	27	-	807
16	75	130	570	170	70	50	-	1 065
18	140	55	55	350	350	85	-	1 035
19	0	32	170	0	75	-	-	277
20	0	250	400	270	-	-	-	920
21	28	50	420	310	30	20	-	860
22	0	130	315	0	180	-	-	625
23	0	90	180	300	100	40	-	710
24	0	680	220	30	30	30	-	990
25	0	80	400	250	5	-	-	735
26	50	300	500	0	10	-	-	860
27	40	25	350	300	45	-	-	760
28	125	155	300	300	150	-	-	1 030

TABLE 12

Urine Specific Gravity

	Minutes After Drinking						
Subject	15	30	60	90	120	150	180
1			1 000	1 000			1 010
2		1 000	1 000	1 000	1 005	1 011	1 015
3				1 002	1 000	1 000	
4	1 013	1 002	1 000	1 000		1 005	1 012
5	1 015	1 007	1 002	1 000	1 000	1 005	
6	1 015	1 002	1 000	1 000	1 006	1 010	1 010
7			1 003	1 000			1 008
8	1 002	1 000	1 000	1 005	1 012	1 015	
9	1 009	1 000	1 000	1 002	1 007	1 010	
10	1 016		1 000	1 000	1 000	1 005	1 007
11	1 012	1 007	1 000	1 006	1 015	1 012	
12	1 015		1 000	1 000	1 006	1 012	
13	1 000	1 000	1 000	1 000	1 006	1 010	
14	1 020	1 005	1 000	1 000	1 007	1 010	
15	1 012	1 000	1 000	1 007	1 015		
16	1 012	1 000	1 000	1 000	1 010	1 015	
18	1 006	1 010	1 010	1 000	1 000	1 005	
19			1 000		1 015		
20		1 000	1 000	1 000			
21		1 006	1 000	1 000			
22		1 005	1 000				
23		1 016	1 000	1 000	1 004	1 012	
24		1 000	1 000	1 018	1 020	1 020	
25		1 014	1 000	1 000			
26		1 007	1 000	1 000			
27	1 015	1 011	1 000	1 000	1 010		
28	1 008	1 000	1 000	1 000	1 003		

TABLE 13

Urine Sodium (mmoles)

	Minutes After Drinking							
Subject	15	30	60	90	120	150	180	Total
1	-	0.1	12.1	4.3	-	0.7	13.5	30.7
2	-	6.6	3.2	2.9	4 1	3 9	5.6	26.2
3	-	-	-	5.3	2.7	2.0	-	10.0
4	5.6	1.8	3.0	2.4	-	3.2	2.3	18.3
5	2.3	5.2	10.9	4.2	1 6	5.8	-	30.0
6	6.9	1.7	3.7	3.8	5.0	4.0	6.8	31.8
7	-	-	13.1	4.8	-	-	15 9	30.0
8	2.7	1.9	2.1	3.0	2.3	2.3	-	14.3
9	5.9	2.4	3.7	3.6	4.4	3.3	-	23.3
10	4.1	1.9	4.2	1.3	2.5	2.4	2.9	19 4
11	11.5	3.5	3.6	4.2	4 4	4.3	3.1	34.5
12	4.9	1.8	4.6	1.0	4.8	3.3	-	20.4
13	5.4	2.2	3.0	1.0	3.6	5.7	-	20.9
14	2.8	4.4	2.3	2.0	2.8	2.5	-	16.8
15	9.6	1.8	2.2	3.6	2.5	2.4	-	22.3
16	6.0	2.4	7.0	5.5	6.5	5.3	-	32.6
18	7.4	4.1	4.0	6.9	3.0	2.7	-	28.2
19	-	2.0	3.5	-	6.2	-	-	11.7
20	-	2.5	5.3	6.1	-	-	-	13.9
21	2.9	1.7	4.3	2.9	2.0	2.0	-	15.8
22	-	1.7	1.5	-	1.7	-	-	4.9
23	-	7.0	3.2	3.6	3.4	3.3	-	20.5
24	-	4.7	1.3	2.7	2.5	3.5	-	14.8
25	-	5.4	3.5	2.7	0.2	-	-	11.8
26	1.0	0.6	1.0	-	0.5	-	-	3.1
27	4.1	1.6	4.9	5.1	3.7	-	-	19.4
28	5.7	1.9	3.8	4.2	3.9	-	-	19.6

TABLE 14

Urine Chlorides (mmoles)

	Minutes After Drinking							
Subject	15	30	60	90	120	150	180	Total
1	-	0 1	14.5	5.3	-	0 8	14.7	35 4
2	-	8.4	4.1	3.4	4.4	4.4	6.2	30.9
3	-	-	-	6.6	2.7	2.0	-	11 2
4	10.9	3.0	5.3	3.8	-	3.7	2.2	28.9
5	2.7	6.4	12.0	4.7	1.7	6.0	-	33.4
6	6.9	2.2	4.0	3.5	4.8	3.3	5.4	30.1
7	-	-	13.4	5.6	-	-	18.1	37.1
8	3.1	2.1	2.5	3.2	2.8	2.6	-	16.3
9	7.1	3.0	4.6	4.0	5.2	3.8	-	27.7
10	4.1	1.9	4.3	1.4	2.5	2.2	2.9	19.3
11	12.8	3.9	4.0	4.6	4.6	4.5	3.3	37.6
12	6.0	2.1	5.1	1.3	5.2	3.5	-	23.1
13	6.4	3.5	2.6	1.5	3.6	5.4	-	23.0
14	3.0	3.3	2.2	2.0	2.9	2.8	-	16.2
15	11.6	2.6	2.3	3.4	2.3	2.1	-	24.2
16	7.0	2.8	7.6	5.6	6.2	4.8	-	34.0
18	9.2	4.7	4.8	8.7	4.2	3.1	-	34.9
19	-	2.4	4.2	-	7.2	-	-	13.8
20	-	3.3	7.3	6.9	-	-	-	17.5
21	2.9	1.8	4.4	3.1	2.1	1.7	-	15.9
22	-	1.7	1.4	-	1.4	-	-	4.5
23	-	7.4	3.6	3.8	3.5	3.5	-	21.7
24	-	5.4	1.4	2.8	2.4	3.1	-	15.0
25	-	6.1	4.2	3.3	0.3	-	-	13.8
26	1.6	1.1	1.7	-	0.8	-	-	5.1
27	4.4	2.0	5.9	5.6	3.9	-	-	21.9
28	6.8	2.5	4.6	5.2	4.8	-	-	23.9

TABLE 15

Urine Potassium (mmoles)

	Minutes After Drinking							
Subject	15	30	60	90	120	150	180	Total
1	-	0.04	4.0	1.2	-	0.3	6.3	11.8
2	-	1.8	0.9	0.9	1.7	2.0	3.7	11.0
3	-	-	-	4.7	1.1	0.9	-	6.7
4	16.4	4.0	6.4	5.3	-	5.9	3.9	41.9
5	2.0	4.2	8.4	3.1	0.9	3.5	-	22.2
6	3.7	1.1	1.9	1.6	2.0	1.3	2.1	13.7
7	-	-	7.9	2.5	-	-	6.6	17.0
8	-	0.7	0.5	0.8	0.8	0.8	-	3.6
9	3.4	1.2	1.9	1.4	2.1	1.3	-	11.3
10	3.0	1.1	2.4	0.9	1.1	0.8	1.0	10.3
11	6.7	1.2	1.1	1.4	1.6	1.9	1.5	15.4
12	4.6	0.8	1.8	0.5	1.6	1.3	-	10.6
13	5.3	1.4	1.6	0.8	1.3	2.2	-	12.6
14	2.2	2.3	1.6	1.1	1.4	1.4	-	10.0
15	9.2	2.1	2.1	2.7	2 6	2.2	-	20.9
16	3.7	1.1	2.6	2.0	2.8	3.0	-	15.3
18	4.5	1.9	2.1	5.2	2.8	1.6	-	16.1
19	-	1.3	3.0	-	6.5	-	-	10.7
20	-	2.0	5.4	3.2	-	-	-	10.6
21	1.0	0.4	0.9	0.8	0.4	0.4	-	4.0
22	-	1.9	1.1	-	0.9	-	-	4.0
23	-	5.2	2.0	1.7	1.3	1.3	-	11.5
24	-	3.4	0.7	1.1	0.9	1.4	-	7.5
25	-	4.7	1.9	1.0	0.1	-	-	7.7
26	0.6	0.8	0.6	-	0.4	-	-	2.3
27	3.3	1.0	3.8	1.9	1.2	-	-	11.2
28	5.1	1.8	2.1	3.6	2.8	-	-	15.4

TABLE 16

Urine Calcium (μ moles)

Subject	Minutes After Drinking 15	30	60	90	120	150	180	Total (mmoles)
1	–	13	1 593	483	0	43	723	2.86
2	–	700	512	330	355	222	213	2.33
3	–	–	–	314	148	114	–	0.57
4	532	231	450	396	–	342	182	2.13
5	156	383	805	324	107	333	–	2.11
6	260	150	316	387	330	190	155	1.79
7	–	–	300	87	–	338	–	0.72*
8	203	259	456	356	271	212	–	1.76
9	265	113	170	203	203	165	–	1.12
10	228	125	250	100	173	138	145	1.16
11	189	73	68	74	98	63	40	0.60
12	330	200	341	129	313	20	–	1.33
13	264	45	135	224	189	275	–	1.13
14	158	162	132	112	122	91	–	0.78
15	525	130	202	236	92	80	–	1.26
16	152	56	177	167	175	125	–	0.85
18	271	151	138	158	88	43	–	0.85
19	–	106	110	–	352	–	–	0.57
20	–	78	176	176	–	–	–	0.43
21	140	74	139	76	71	60	–	0.56
22	–	33	39	–	45	–	–	0.12
23	–	151	86	60*	33	30*	–	0.36
24	–	292	275	360	180	222	–	1.33
25	–	182	100	120	8	–	–	0.41
26	250	150	325	–	88	–	–	0.81
27	262	94	116	225	168	–	–	0.86
28	285	74	144	99	158	–	–	0.76

* estimated

TABLE 17

Urine Magnesium (μ moles)

Subject	Minutes After Drinking 15	30	60	90	120	150	180	Total (mmoles)
1	-	6	721	245	-	14	391	1.37
2	-	552	430	253	284	86	181	1.78
3	-	-	-	487	199	132	-	0 83
4	132	78	180	185	-	172	125	0 88
5	89	290	490	180	83	184	-	1.32
6	6	22	59	114	101	110	32	0.41
7	-	-	518	132	-	-	616	1.26
8	104	131	216	179	147	117	-	0.90
9	126	71	99	136	126	109	-	0.66
10	127	58	116	48	82	80	86	0.59
11	129	52	59	58	72	52	31	0.45
12	244	136	155	42	201	-	-	0.78
13	135	30	63	84	115	197	-	0.63
14	177	149	72	66	126	113	-	0.71
15	308	66	93	126	77	62	-	0.74
16	300	94	279	208	203	160	-	1.25
18	273	160	143	39	28	91	-	0.74
19	-	110	116	-	324	-	-	0.55
20	-	118	240	132	-	-	-	0.49
21	142	82	122	84	66	64	-	0.56
22	-	107	117	-	218	-	-	0.44
23	-	288	207	180	179	163	-	1.02
24	-	279	227	231	152	161	-	1.05
25	-	380	240	190	13	-	-	0.82
26	-	234	370	-	139	-	-	0.74
27	184	77	172	204	158	-	-	0.80
28	316	96	111	165	190	-	-	0.88

TABLE 18

(results after the line represent the post-absorptive phase)

Case 1

Time (min.)	Alcolimiter values	Blood Alcohol Values	Differences (breath:blood values)
30	45	42	+3
53	45	40	+5
55	45	40	+5
75	38	38	0
89	32	36	-4
104	27	33	-6
116	25	31	-6
129	24	29	-5
148	22	26	-4
178	20	19	+1
186	17	17	0

Case 2

20	80		45	+35	
28	74		52	+22	
38	50		52	-2	
54	45	54	48	-3	+6
65	49	48	47	+2	+1
82	42	43	43	-1	0
90	34	35	41	-7	-6
102	32		38	-6	
118	25	27	33	-8	-6
133	23	25	31	-8	-6
157	21	22	25	-4	-3
168	20	20	22	-2	-2
185	16	18	19	-3	-1

Case 3

Time (min.)	Alcoliniter Values			Blood Alcohol Values	Differences (breath: blood values)		
11	49	50		55	-6	-5	
15	49	54		84	-35	-30	
26	49			68	-19		
31	43	43		61	-18	-18	
58	42	44		54	-12	-10	
72	38	36		51	-13	-15	
81	35	35		49	-14	-14	
92	31	32		46	-15	-14	
117	28	30		40	-12	-10	
128	25	25		38	-13	-13	
142	19	19		34	-15	-15	

Case 4

19	25			23	+2		
28	26			28	-2		
42	30			34	-4		
55	30			39	-9		
67	26			41	-15		
79	28			39	-11		
103	24			35	-11		
114	20			33	-13		
137	19			29	-10		
163	11	15		25	-14	-10	
189	11	11		21	-10	-10	

Case 5

11	106			45	+60		
22	48			59	-11		
37	46			57	-11		
51	43			53	-10		
69	41			50	-9		
82	39			47	-8		
99	40			43	-3		
110	36			41	-5		
128	30			37	-7		
146	28	28		33	-5	-5	
170	19	20		28	-9	-8	
190	20	16	18	23	-3	-7	-5

Case 6

Time (min.)	Alcolimiter Values			Blood Alcohol Values	Differences (breath:blood values)		
28	40			37	+3		
41	36	36		38	-2	-2	
57	36	35		37	-1	-2	
73	40	40		37	+3	+3	
87	30	35	30	37	-7	-2	-7
113	23	24		35	-12	-11	
137	26	28		32	-6	-4	
160	19	19		28	-9	-9	
176	18	17		26	-8	-9	

Case 7

37	62			35	+27		
45	49	50		44	+5	+6	
60	46			44	+2		
75	36	38	40	42	-6	-4	-2
113	28	30		37	-9	-7	
127	28	28		34	-6	-6	
147	22	25		31	-9	-6	
163	19	20		29	-10	-9	
183	18			26	-8		

Case 8

36	30			22	+8		
47	35	35		26	+9	+9	
57	36			30	+6		
76	27	28		32	-5	-4	
97	30	26	30	32	-2	-6	-2
115	24	20	21	30	-6	-10	-9
128	18	19		27	-9	-8	
154	12			21	-9		
171	10			17	-7		

Case 9

Time (min.)	Alcolimiter Values		Blood Alcohol Values	Differences (breath: blood values)	
29	50		51	-1	
40	42		49	-7	
54	44	44	47	0	0
69	39		45	-6	
95	33	32	40	-7	-8
116	25	28	35	-10	-7
138	23	24	30	-7	-6
157	17		26	-9	
180	20		21	-1	

Case 10

35	55		37	+18	
44	56		42	+14	
53	47	50	48	-1	+2
67	52	52	51	+1	+1
103	44		46	-2	
118	38	38	44	-6	-6
154	33	34	40	-7	-6
176	29	29	37	-8	-8

Case 11

37	68	60	82	-14	-22
50	51		68	-17	
77	40	40	49	-9	-9
100	32	32	40	-8	-8
109	25	27	38	-13	-11
126	24	25	33	-9	-8
159	19	19	24	-5	-5
180	16	16	19	-3	-3

Case 12

Time (min.)	Alcolimiter Values		Blood Alcohol Values	Differences (breath:blood values)	
45	45		44	+1	
60	40		38	+2	
95	32	32	33	-1	-1
110	28	25	30	-2	-5
135	18		26	-8	
160	14		22	-8	
175	12		20	-8	

Case 13

22	45		47	-2	
43	61		65	-4	
67	56		72	-16	
98	45	48	54	-9	-6
112	37	42	49	-12	-7
135	36		40	-4	
160	35	33	32	+3	+1
175	25	25	30	-5	-5

Case 14

108	31	31	38	-7	-7
130	22	25	33	-11	-8
145	22		30	-8	
170	20		24	-4	

Case 15

85	30		41	-11	
108	28	28	36	-8	-8
125	26	26	32	-6	-6
145	22		27	-5	

Case 16

Time (min.)	Alcolimiter Values		Blood Alcohol Values	Differences (breath:blood values)	
35	46	41	37	+9	+4
57	40		45	-5	
75	36	36	45	-9	-9
96	31		42	-11	
125	26		37	-11	
140	23		35	-12	
172	15		29	-14	

Case 17

28	55	55	61	-6	-6
44	52	52	56	-4	-4
58	45		53	-8	
97	34		43	-9	
166	22		27	-5	

Case 18

27	67	68	-1	
54	46	56	-10	
76	36	50	-14	
158	26	38	-12	

Case 19

18	100	82	+18	
29	70	80	-10	
43	70	77	-7	
45	68	77	-9	
65	55	72	-17	
80	55	68	-13	
98	47	64	-17	
117	50	59	-9	
147	36	52	-16	
154	33	50	-17	

Case 20

Time (min.)	Alcolimiter Values	Blood Alcohol Levels	Differences (breath:blood values)
14	63	30	+17
25	59	34	+15
41	53	37	+16
52	49	39	+10
72	40	39	+9
88	33	38	-5
114	25	34	-9
147	21	28	-7
170	18	24	-6

Case 21

27	46		55	-9	
44	48	50	63	-15	-13
74	45		63	-18	
110	44		55	-11	
127	30	32	50	-20	-18
155	23		45	-22	
157	26	28	45	-19	-17
178	24		41	-16	

Case 22

25	68	65	+3
40	59	58	+1
80	50	53	-3
103	42	49	-7
127	35	45	-10
145	35	42	-7
151	33	41	-8

Case 23

50	65	57	+8
73	52	55	-3
87	51	51	0
123	35	43	-8
143	30	33	-3
180	22	29	-7

Case 24

Time (min.)	Alcolimiter Values		Blood Alcohol Levels	Differences (breath:blood values)	
46	58		58	0	
69	50		52	-2	
110	36		41	-5	
140	30		33	-3	

Case 25

18	44		-		
48	50		-		
70	43		52	-9	
112	30		41	-11	
136	25		35	-10	
162	20		29	-9	
177	18		25	-7	

Case 26

41	75		55	+20	
71	53		59	-6	
106	38		49	-11	
132	30		43	-13	
162	24		37	-13	
173	20		35	-15	

Case 27

45	45	46	50	-5	-4
70	38		44	-6	
100	30		36	-6	
135	22		27	-5	
155	15		22	-7	

Case 28

45	55	58	42	+13	+15
75	48		49	-1	
105	40		47	-7	
138	36		43	-7	
160	28		39	-11	

REFERENCES

ALLISON, R.D., J.C. KRAMER, G.M. ROTH (1971)
Angiology 22, 211-222. Effects of Alcohol and Nitroglycerin on Vascular Responses in Man

ASAAD, M.M., H. BARRY, D.C. CLARKE, B.N. DIXIT (1974)
Br. J. Pharmac. 50, 277-282. Effect of Ethanol on the Oxidative Metabolism of Tryptamine by Rat Liver Homogenate

ASMUSSEN, E., J. HALD, V. LARSEN (1948)
Acta. Pharmacol. 4, 311-320. The Pharmacological Action of Acetaldehyde on the Human Organism

AXELROD, D.R. (1973)
In "The Biology of Alcoholism" volume III, chapter 8, p. 291-302 published by Plenum Press. Metabolic and Endocrine Aberrations in Alcoholism

BAKER, R.N., A.L. ALENTY, J.F. ZACK (1969)
J. Chrom. Sc. 7, 312-314. Simultaneous Determination of Lower Alcohols, Acetone and Acetaldehyde in Blood by Gas Chromatography

BALLARD, F.J. (1972)
Am. J. Clin. Nutr. 25 773-779. Supply and Utilisation of Acetate in Mammals

BENNETT, H. (1963)
In "Industrial Waxes", Chemical Publishing Co.

BOITEAU, H.L., C. MOUSSION (1968)
Ann. Biol. Clin. 26 907. Cited by Coldwell et al. (1971)

CASIER, H., H. POLET (1958)
Arch. Int. Pharmacodyn. CXIII 439-496. Influence du disulfiram (Antabus) sur le metabolisme de L'alcool ethylique marque chez la souris

COLDWELL, B.B., G. SOLOMONRAJ, H.L. TRENHOLM, G.S. WIBERG (1971)
Clin. Toxicol. 4, 99-113. The Gas Chromatographic Estimation of Ethanol, Acetaldehyde, and Acetone in Ethanol Metabolism Studies

COOPER, J.D. (1971)
Clin. Chim. Acta 33, 483-485. Determination of Blood Ethanol by GLC

CRUSE, J.R., C.D. GERSON, L.M. DE CARLI, C.S. LIEBER (1968)
J. Lipid Res. 9, 509-512. The Role of Acetate in the Reduction of Plasma Free Fatty Acids produced by Ethanol in Man.

CURRY, A.S., W.G. WALKER, G.S. SIMPSON (1966)
Analyst 91, 472-743. Determination of Ethanol in Blood by Gas Liquid Chromatography

DAVIS, V.E., M.J. WALSH (1971)
In "The Biological Basis of Alcoholism" ed., Israel and Mardones, Pub., Wiley, Interscience. Chapter 3, The Effect of Ethanol on Neuroamine Metabolism

DEEMTER, J.J. VAN, F.J. ZUIDERWEG, A. KLINKENBERG (1956)
Chem. Eng. Sci. 5, 271

DRESSMAN, R.C. (1970)
J. Chrom. Sci. 8, 265. Elimination of "Memory Peaks" Encountered in Aqueous Injection Gas Chromatography

DUBOWSKI, K.M. (1974)
Clin. Chem. 20, 294-299. Biological Aspects of Breath-Alcohol Analysis

DURITZ, G., E.B. TRUITT (1964)
Quart. J. Stud. Alc. 25, 498-509. A Rapid Method for the Simultaneous Determination of Acetaldehyde and Ethanol in Blood Using Gas Chromatography

EGGLETON, M.G. (1942)
J. Physiol. 101, 172-191. The Diuretic Action of Alcohol in Man

EGGLETON, M.G., I.G. SMITH (1946)
J. Physiol. 104, 435-442. The Effects of Ethyl Alcohol and Some Other Diuretics on Chloride Excretion in Man

ESKELSON, C.D., C. CAZEE, J.C. TOWNE, B.R. WALSKE (1970)
Biochem. Pharacol. 19, 1419-1327. Cholesterolgenesis in vitro from Ethanol

ESTEP, H., W.A. SHAW, G. WATLINGTON, R. HOBE, W. HOLLAND, St G. TUCKER (1969)
J. Clin. Endocrinol. 29, 842-848. Hypocalcaemia due to Hypomagnesia and Reversible Parathyroid Hormone Unresponsiveness

FEINMAN, L., C.S. LIEBER (1967)
Amer. J. Clin. Nut. 20, 400-403. Effect of Ethanol on Plasma Glycerol in Man

FLINK, E.B. (1971)
In "The Biology of Alcoholism" Vol. I, Chapter 12. Mineral Metabolism in Alcoholism. ed., Kissin and Begleiter. Pub., Plenum Press, New York

FORSANDER, O.A. (1970)
Q. J. Stud. Alcohol. 31, 550-570. Influence of Ethanol on the Redox State of the Liver

FREINKEL, N., D.L. SINGER, R.A. ARKY, S.J. BLEICHER, J.B. ANDERSON, C.K. SILBERT (1963)
J. Clin. Invest. 42, 1112-1113. Alcohol Hypoglycaemia, I: Carbohydrate Metabolism of Patients with Clinical Alcohol Hypoglycaemia and the Experimental Reproduction of the Syndrome with pure Ethanol

FREUND, G., P. O'HOLLAREN (1965)
J. Lipid. Res. 6, 471-477. Acetaldehyde Concentrations in Alveolar Air Following a Standard Dose of Ethanol in Man

GEIGY (1970)
Scientific Tables. 7th edition, ed., Diem and Lentner. Published J.R. Geigy, Basle

GOLDBERG, L. (1943)
Acta. Physiol. Scand. 5, Suppl. 16, 128. Quantitative Studies on Alcohol Tolerance in Man

GOULD, L., M. ZAHIR, A. DE MARTINO, R.F. GOMPRECHT, F. JAYNAL (1972)
Q. J. Stud. Alcohol. 33, 714-721. Haemodynamic Effect of Ethanol in Patients with Cardiac Disease

HALD, J., E. JACOBSEN (1948)
Acta. Pharmacol. Toxicol. 4, 305-310. The Formation of Acetaldehyde After Ingestion of Antabuse and Alcohol

HARGER, R.N., B.B. RANEY, E.G. BRIDWELL, M.F. KITCHEL (1950)
J. Biol. Chem. 183, 197. The Partition Ratio of Alcohol Between Air and Water, Urine and Blood; Estimation and Identification of Alcohol in these Liquids from Analysis of Air Equilibrated with them

HARGER, R.N., R.B. FORNEY, R.S. BAKER (1956)
Q. J. Stud. Alcohol. 17, 1. Estimation of the Level of Blood Alcohol from Analysis of Breath, II: Use of Rebreathed Air

HAWKINS, R.D., H. KALANT (1971)
Pharmacol. Reviews. 24, 67-157. The Metabolism of Ethanol and its Metabolic Effects

HOLLIS. O.L. (1966)
Anal. Chem. 38, 309. Introduction of Porapak Q

HRIVNAK, J. (1970)
J. Chrom. Sci. 8, 602-603. Use of Phosphoric Acid Additive for Gas Chromatographic Analysis on Open Tubular Columns

IBER, F.L., N. CARULLI, R.M.H. KATER (1969)
Fed. Proc. 28, 626. The Kinetics of Alcohol Removal from the Blood of Man: Comparison in Recently Drinking Alcoholics and Non-alcoholics

JONES, A.W., T.P. JONES, B.M. WRIGHT (1974)
Paper presented at the 6th International Conference of Alcohol, Drugs and Traffic Safety, Toronto. "In vivo and in vitro Studies of the Alcohol Partition Ratio"

JONES, J.E., M.J. ALBRINK, P.C. DAVIDSON, E.B. FLINK (1966)
Am. J. Clin. Nutr. 19, 320. Fasting and Re-feeding of Various Suboptimal Isocaloric Diets. Effects on Nitrogen and Mineral Balances in Obese Patients

KALANT, H. (1971)
In "The Biology of Alcoholism" Volume I, Chapter 1. Plenum Press

KALANT, H., A.E. LEBLANC, R.J. GIBBINS (1971)
In "The Biological Basis of Alcoholism" ed. Israel and Mardones. Chapter 9, Tolerance to, and Dependance on, Ethanol

KALBFLEISCH, J.M., R.D. LINDEMANN, H.E. GINN, W.O. SMITH (1963)
J. Clin. Invest. 42, 1471-1475. Effects of Ethanol Administration on Urinary Excretion of Magnesium and other Electrolytes in Alcoholic and Normal Subjects

KAYE, S., E. CARDONA (1969)
Am. J. Clin. Path. 52, 577-584. Errors of Converting a Urine Alcohol Value into a Blood Alcohol Value

KREBS, H.E., R.A. FREEDLAND, R. HEMS, M. STUBBS (1969)
Biochem. J. 112 117-124. Inhibition of Hepatic Gluconeogenesis by Ethanol

KRUEGER, R.C. (1952)
Am. Chem. Soc. J. 74, 5536. Crystalline Acetoacetic acid

LAHTI, R.A., E. MAJCHROWICZ (1974)
Q. J. Stud. Alcohol. 35, 1-14. Ethanol and Acetaldehyde Effects on Metabolism and Binding of Biogenic Amines

LAURELL, S., G. TIBBLING (1966)
Clin. Chim. Acta. 13, 317. An Enzymatic Fluorometric Micromethod for the Determination of Glycerol

LEDERMANN, S. (1956)
In "Alcool, Alcoolisme, Alcoolisation; Donnes Scientifique de Caractere Physiologique, Economique et Social". Institut National D'Etudes Demographique. Travaux et Documents. Cahier No. 29. Presses Universitaires, Paris

LEVEY, S., O.J. BALCHUM, V. MEDRANO, R. JUNG (1964)
J. Lab. Clin. Med. 63, 574-584. Studies of Metabolic Products in Expired Air, II: Acetone

LIEBER, C.S. (1974)
Lipids 9, 103-116. Effects of Ethanol Upon Lipid Metabolism

LOWRY, O.H., N.R. ROBERTS, J.I. KAPPHAHN (1957)
J. Biol. Chem. 224, 1047-1064. The Fluorometric Measurement of the Pyridine Nucleotides

LUNDQUIST, F., I. SVENDSON, P.H. PETERSEN (1963)
Biochem. J. 86, 119-124. The Metabolism of Ethanol in Rat Liver Suspensions

LUNDQUIST, F. (1971)
In "The Biological Basis of Alcoholism" ed., Israel and Nardones. Chapter 1, The Metabolism of Alcohol

MAICKEL, R.P., B.B. BRODIE (1963)
Ann., N.Y. Acad. Sci. 104, 1059-1064. Interaction of Drugs with the Pituitary Adrenocortical System in the Production of the Fatty Liver

MAJCHOWICZ, E., J. MENDELSON (1970)
Science 168, 1100-1102. Blood Concentration of Acetaldehyde and Ethanol in Chronic Alcoholics

MALING, H.M. (1970)
In "Internation Encyclopaedia of Pharmacology and Therapeutics" Section 20, Vol. II, page 279. Alcohols and Derivatives. Pergamon Press

MELLANBY, E. (1919)
Alcohol, its Absorption into and Disappearance from the Blood under Different Conditions. Special Report Series No. 31. Medical Research Committee, London

MELLANBY, J., D.H. WILLIAMSON (1965)
In "Methods of Enzymatic Analysis" p. 454-458. ed., Bergmeyer, H.V. Pub., Academic Press

McCOLLISTER, R.J., E.B. FLINK, N.D. LEWIS (1963)
Am. J. Clin. Path. 12, 415-420. Urinary Excretion of Magnesium in Man Following the Ingestion of Alcohol

OGATA, M. (1963)
Q. J. Stud. Alcohol. 24, 398-411. Clinical and Experimental Studies on Water Metabolism in Alcoholism

OLSEN, C. (1971)
Clin. Chim. Acta. 33, 293-300. An Enzymatic Fluorometric Micromethod for the Determination of Acetoacetate, B-hydroxybutyrate Pyruvate and Lactate

PAWAN, G.L.S., W.H. HOULT (1963)
Biochem, J. 87, 6P. The Determination of Total Body Water in Man by Ethanol Dilution

PERMAN, E.S. (1958)
Acta. Physiol Scand. 44, 241-247 The Effect of Ethyl Alcohol on the Secretion from the Adrenal Medulla in Man

PERSINGER, H.E., J.T. SHAH (1973)
J. Chrom. Sci. 11, 190-191. The Chemistry of Polyethylene Glycols used in Gas Liquid Chromatography

PHILLIPS, R.E., F.R. ELEVITCH (1968)
Am. J. Clin. Path. 49, No. 5. An Enzymatic Fluorometric Method for the Determination of Glucose in Plasma

RASKIN, N.H., L. SOKOLOFF (1968)
Science 162, 131. Brain Alcohol Dehydrogenase

REDETSKI, H.M. (1973)
Q. J. Stud. Alcohol. 34, 206-208. Use of the Osmometer for Preparation, Analysis and Control of Standard Solutions of Alcohol

REISBY, N., A. THEILGAARD (1969)
Acta. Psychiat. Scand. Suppl. No. 208, 1-204. The Interaction of Alcohol and Meprobamate in Man

RUBINI, M.E., C.R. KLEEMAN, E. LAMDIN (1955)
J. Clin. Invest. 34, 439. Studies on Alcohol Diuresis I: The Effect of Ethyl Alcohol Ingestion on Water, Electrolytes and Acid-Base Metabolism

SAVORY, J., F. SUNDERMAN, N. ROSZEL, P. MUSHAK (1968)
Clin. Chem. 14, 132-144. An Improved Procedure for the Determination of Serum Ethanol by Gas Chromatography

SIPPEL, H.W. (1973)
Acta. Chem. Scand. 27, 541-550. Non-enzymatic Oxidation in Biological Extracts

STEIN, S.W., W.H. ABELMANN, C.S. LIEBER, G.R. CHERRICK (1960)
J. Clin. Invest., 39, 1032. Hepatic and Systemic Blood Flow in Man During Intravenous Infusion of Alcohol

STOWELL, A. (1973)
Determination of Pyruvate, Lactate, Acetoacetate and B-hydroxybutyrate in Human Blood and the Electrochemical Analysis of Ethanol in Air. Dissertation for fulfillment of BSc Hons., Massey University

TRUITT, E.B., G. DURITZ (1966)
In "The Biochemical Factors in Alcoholism" ed., R.J. Maickel. Pub., Pergamon Press. "The role of Acetaldehyde in the Actions of Ethanol".

TRUITT, E.B. (1970)
Q. J. Stud. Alcohol. 31, 1-12. Ethanol Induced Release of Acetaldehyde from Blood and its Effect on the Determination of Acetaldehyde

TRUITT, E.B., M.J. WALSH (1971)
In "The Biology of Alcoholism" vol I, chapter 5 The role of Acetaldehyde in the Actions of Ethanol ed., Kissin and Begleiter. Pub., Plenum Press

TYGSTRUP, N., K. WINKER, F. LUNDQUIST (1965)
J. Clin. Invest. 44, 817-830. The Mechanism of the Fructose Effect on the Ethanol Metabolism of the Human Liver

VARTIA, O.K., O.A. FORSANDER (1960)
Q. J. Stud. Alcohol 21, 597-604. Blood Sugar Values in Hangover

WALLGREN, H., H. BARRY (1970)
"The Actions of Alcohol". Pub., by Elsevier

WATERHOUSE, M. (1972)
Estimation of Ethanol in Blood. Dissertation for partial fulfillment of BSc., Hons., Massey University

WOLFF, P.H. (1972)
Science 175, 449-450. Ethnic Differences in Alcohol Sensitivity