THE ROLE OF VITAMIN C DEFICIENCY
IN CHRONIC ALCOHOLISM

MAUREEN A. O'KEANE

A Thesis submitted for the degree of Master of Science of the University of Glasgow.

DEPARTMENT OF MATERIA MEDICA

STOBHILL GENERAL HOSPITAL

GLASGOW N.1.

SEPTEMBER 1971.

ProQuest Number: 10647080

ProQuest 10647080

ACKNOWLEDGEMENT

SUMMARY

Gastric erosions caused by home-brewed lager.

A 22 year old male was admitted to hospital because of melaena due to acute gastric erosions. In the three weeks prior to his admission, he had drunk several gallons of home-brewed lager. Investigations revealed that this was contaminated with acetaldehyde and acetic acid which had been produced by an acetobacter organism present in the yeast. Experiments were carried out on the effect of home-brewed lager and beer on the stomachs of the guinea pigs. The incidence of erosive gastritis was significantly increased when the animals were given similarly contaminated home-brewed lager. This observation is of importance since the home-brewing of beer and lager has become common since its legalisation in 1964.

A clinical study was carried out to determine the leucocyte ascorbic acid levels and the iron status of fifty alcoholics admitted to medical and psychiatric wards. In addition, the dietary intake of vitamin C and iron was estimated.

The age range of the group was 24-70 years and this included nine females, age range 34-50 years. None of the patients had overt liver disease and none were intoxicated at the time the blood sample was taken.

All the alcoholic patients had an inadequate intake of iron (15 mg/day) but the haemoglobin, erythrocyte

protoporphyrin and serum iron levels showed that over 55%
of the patients showed no signs of anaemia. In the absence
of liver disease, the incidence of anaemia in alcoholics
was lower than expected.

L.A.A. was estimated by the 2,4 dinitrophenyl
method and the average daily intake of vitamin C was
calculated from the diet history. The alcoholic group
was matched with a normal group of patients according to
age (within five years) and sex. The results show that,
as a group, alcoholics are sub-clinically scorbutic. Their
vitamin C levels, 18.8 ± 11.01 (mean $\pm$ S.D,) $\mu g/10^8$ WBC are
significantly lower (p 0.0005) than those of the matched
group, 27.41 ± 7.59 (mean $\pm$ S.D.) $\mu g/10^8$ WBC. The L.A.A.
levels in different age groups are not significantly
different from each other but in every age group the
alcoholic group has a significantly lower vitamin C level
when compared with the control group. When the diet
histories were studied, it was clear that the main reason
for low L.A.A. levels was inadequate intake of vitamin C.

Having established that alcoholics are generally
deficient in vitamin C, an investigation was carried out
to ascertain if this condition could directly affect the
metabolism of ethanol. An experiment was designed to
measure the induction of hepatic alcohol dehydrogenase
by alcohol in sub-clinically scorbutic guinea pigs.

However , the criteria of sub-clinical scurvy
(i.e. a state where there is a diminished level of ascorbic
acid in the body without apparent manifestations of scurvy)
had to be established at the outset. The leucocyte

ascorbic acid levels and the vitamin C levels in the liver, stomach and upper small intestine of guinea pigs were measured by the usual 2,4 dinitrophenyl hydrazine method. These levels show that guinea pigs are sub-clinically scorbutic after two weeks on a scorbutic diet.

Only male guinea pigs were used in the experiments. The levels of ADH were measured in the guinea pig livers after daily injections of 2 ml of 30% ethanol in saline for two days, one week, two weeks and three weeks. The guinea pigs in the scorbutic groups were always maintained on the scorbutic diet for two weeks.

The results show that ADH is maximally induced within two days and that there is a highly significant increase in ADH levels in sub-clinically scorbutic guinea pigs fed ethanol. The potentiation of ADH induction in sub-clinically scorbutic states could explain the increased tolerance of alcoholics for alcohol since sub-clinical scurvy was present in the majority of the cases of chronic alcoholism.

The level of NAD was measured in guinea pigs injected with 30% alcohol for two weeks and fed the scorbutic diet. The levels of NAD are higher in sub-clinically scorbutic animals fed ethanol than in normal animals fed ethanol.

CONTENTS

CHAPTER V   The levels of alcohol dehydrogenase

in sub-clinically scorbutic guinea pigs

CHAPTER I

INTRODUCTION.

ALCOHOL :

Alcoholic beverages have been used since the dawn of history. The oldest alcoholic drinks were fermented beverages of relatively low alcohol content, i.e. beer and wines. When the Arabs introduced the then recent science of distilling into Europe, in the Middle Ages, the Alchemists believed that alcohol was the long sought elixir of life. Alcohol was therefore held to be a remedy for practically all diseases. However, it is now recognised that the therapeutic value of alcohol is much more limited than its social value.

Ethanol plays at least five distinct roles in the body. The most widely appreciated is its effect on the central nervous system. In addition, ethanol is oxidised and utilised as a source of calories. Its metabolism in time yields acetaldehyde and acetate which exert pharmacologic and metabolic effects of their own. Ethanol may be hepato-toxic, leading to liver cell damage and, in some cases, eventually cirrhosis. Finally, alcohol acts as a drug similar to phenobarbital since it can induce microsomal drug detoxifying enzymes (1).

Alcohol is not a stimulant but rather is a primary and continuous depressant of the central nervous system. The apparent stimulation results from the unrestrained activity of various parts of the brain which have been freed from inhibition as a consequence of the depression of inhibitory control mechanisms.

Alcohol is absorbed from the stomach, small intestine and colon. Absorption of alcohol from the stomach is rapid

at first but it then decreases to a very slow rate although
the gastric concentration of alcohol is still high. If
the emptying of the stomach is delayed, the subsequent
absorption of alcohol from the stomach will also be delayed (2).
Absorption from the small intestine is rapid and complete
and is independent of both the concentration of alcohol and
the presence of food in the stomach or intestine. After
absorption , alcohol is fairly uniformly distributed
throughout all tissues and fluids of the body.

Of the alcohol entering the body, between 90 and
98% undergoes oxidation. The rest is excreted unchanged
in the urine. The rate of oxidation of alcohol is a linear
function of time and is only moderately increased by
increasing the blood concentration. In the adult, the
average rate of metabolism is 7g. / hour. The initial
oxidation of alcohol occurs in the liver and the important
steps in the metabolism are outlined in Fig. 1. The
acetate which is carried away from the liver by the blood
is ultimately broken down into carbon dioxide and water (3).
Some of the acetate may go into the formation of fat or
cholesterol. Acetate can also be incorporated into amino
acids via other intermediate steps. About 90% of the
total amount of alcohol broken down in the body under normal
conditions undergoes partial oxidation to acetate in the
liver. The remaining 10% is broken down directly into
carbon dioxide in different organs.

During the first two steps of the metabolism
of 1gm. of alcohol - to acetaldehyde and acetyl CoA -
approximately 2.6 kilocalories are liberated and are available
to the body. Acetyl CoA or acetate enters the general

Fig. 1.

The main pathways are shown by heavy arrows.

$$NAD \quad NADH \qquad NAD \quad NADH$$

$$CH_3CH_2OH \xrightarrow{\quad (1) \quad} CH_3CHO \xrightarrow{\quad (2) \quad} CH_3COOH \Longrightarrow \text{To blood.}$$

Ethanol   Acetaldehyde   Acetic acid

Acetyl .CoA

$CH_3CO.CH_2COOH$ Tricarboxylic acid

Aceto-acetate   cycle

Synthesis

To blood

Carbon dioxide

$CH_3CH(OH)CH_2COOH$

β-hydroxybutyric acid
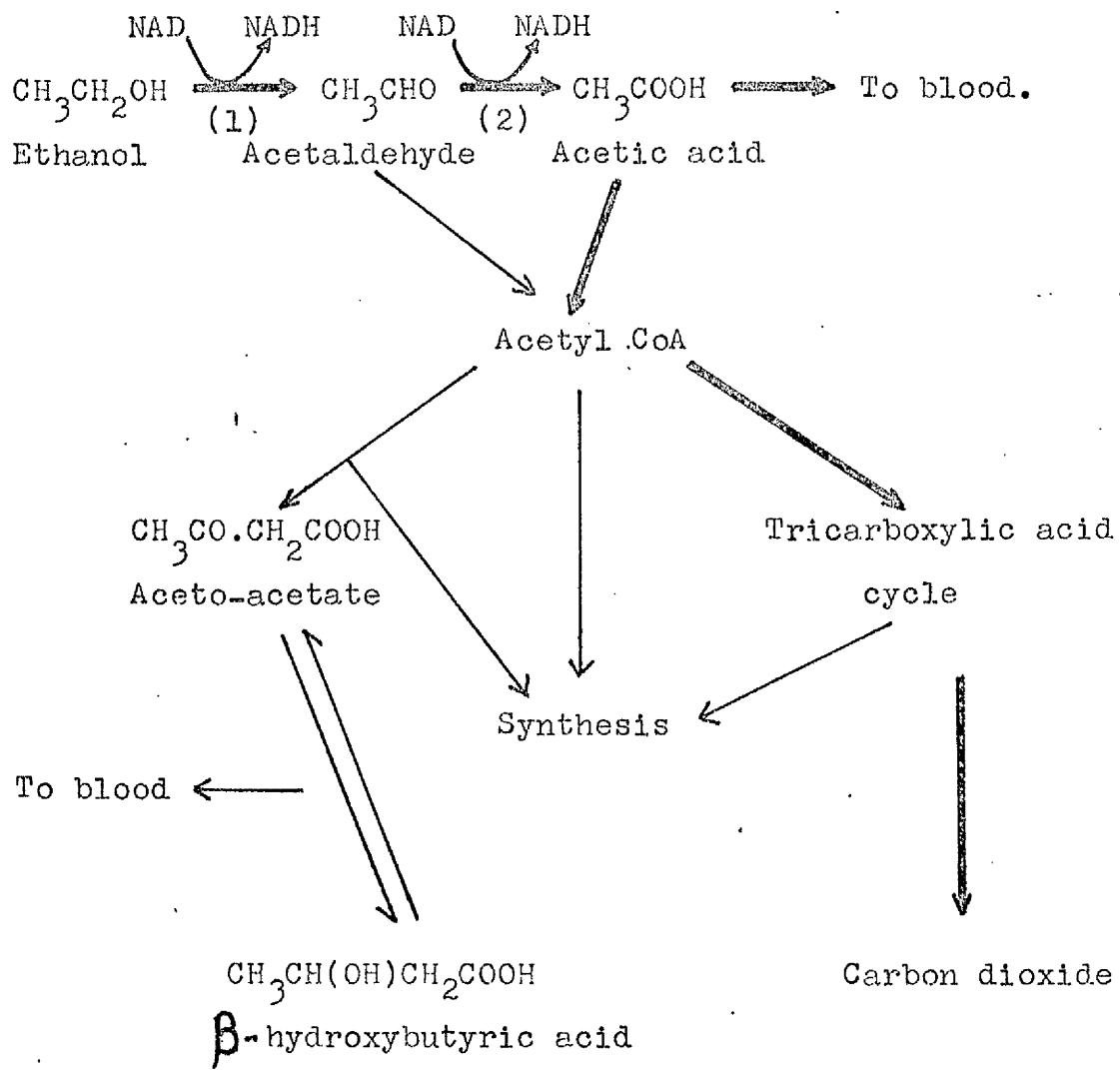
(1)   Alcohol dehydrogenase. (ADH).

(2)   Acetaldehyde dehydrogenase.

metabolic pool in the body and, provided that these substances
are oxidised completely, another 4.5 kilocalories are
available to the body.   Thus the energy released by ethyl
alcohol oxidation is approximately 7.1 kcal / g.   Alcohol
is a ready source of energy that is utilised more rapidly
than most foods since it is quickly absorbed from the
gastrointestinal tract and requires no preliminary digestion.
Some alcoholic beverages also contain protein and carbo-
hydrates but none contain any vitamins.   Man is born with
a very effective system which is able to break down alcohol
and utilise the energy released thereby.

The enzyme alcohol dehydrogenase (ADH) was first
isolated and crystallised from horse liver by Bonnichen and
Wassen in 1948 and its properties were thoroughly studied
by Theorell and his co-workers.   In the presence of
alcohol dehydrogenase ethanol reacting with nicotinamide
adenine dinucleotide (NAD) is converted to acetaldehyde with
the production of reduced nicotinamide adenine dinucleotide
(NADH).   Alcohol dehydrogenase is a zinc containing enzyme
which is dependent upon free sulphydryl groups in the
protein for its activity (4).   It is present in the cytoplasm
of liver cells and has a pH optimum of 10.8.   Human liver
ADH exhibits a broad substrate specificity e.g. 1,3 propane-
diol and 2-methoxyethanol are oxidised by ADH.   Alcohol
dehydrogenase has a high affinity for NAD since alcohol is
preferentially oxidised in the cell.   Those substrates
which are oxidised at a slower rate when alcohol is present
are the ones that need NAD for their breakdown.   All the
substrates which are oxidised in the tricarboxylic acid

cycle belong to this group.

Several authors (5, 6) have reported an exceptionally active enzyme variant of human alcohol dehydrogenase which occurs in 20% of a Swiss and 4% of a London population. This form of the enzyme can be distinguished from the normal ADH because its pH optimum is 8.5. For many years the ADH system was thought to be the only one capable of oxidising ethanol. However, recently the oxidation of ethanol to acetaldehyde by the microsomal fraction of the liver has been reported (7). Several reaction mechanisms have been proposed. The system requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen and has a pH optimum of 7.4. Roach et al. (8) stated that this microsomal system is mediated through hydrogen peroxide dependent systems, one of which is catalase. However, Lieber and de Carli (9) believe that the microsomal ethanol oxidising system is NADPH dependent but does not involve catalase. The importance of this system in the overall ethanol oxidation in the body is not clear. Certainly the existence of a microsomal ethanol oxidising system, especially its capacity to increase adaptively in activity after ethanol feeding, may explain various effects of ethanol, including proliferation of smooth endoplasmic reticulum, induction of other hepatic microsomal drug-detoxifying enzymes and the metabolic tolerance to ethanol which develops in alcoholics.

In man there are four common hepatic disorders that are thought to be related to the ingestion of ethanol or ethanol-containing beverages because of the fact that these

disorders are more common in the drinker than in the non-drinker.   These disorders are fatty infiltration of the liver, acute alcoholic hepatitis, Laennec's cirrhosis and haemachromatosis (10).

The interactions of alcohol and living material have been well studied, well analysed and should be well understood.   That the latter is not completely so does not detract in any way from the benifits accessible to mankind from the prudent use of alcohol.

VITAMIN C :

Scurvy was the first deficiency disease to be recognised as such. As early as 1720, Kramer observed that medicines gave no relief to this condition, but ——————— "if you can get green vegetables, if you can prepare a sufficient quantity of fresh antiscorbutic juices, if you have oranges, lemons, citrons or their pulp and juice preserved with whey in cask so that you can make a lemonade or rather give to the quantity of 3 or 5 ounces of their juice in whey, you will, without other assistance, cure this dreadful evil."

In his exhaustive treatise on scurvy, Lind (1757) showed experimentally, in human subjects, that oranges and lemons incorporated in the diet could prevent the onset of the disease. In 1804 it became compulsory in Britain to issue a daily ration of lemon or lime juice to all British sailors. This led to the almost complete eradication of scurvy in the British navy. Holst and Frölich (1907) showed that experimental scurvy could be produced in guinea pigs and this provided a bioassay for the antiscorbutic vitamin. In 1921 Zilva discovered that vitamin C could be easily destroyed by oxidation and could best be protected by reducing agents. In 1928 Szent-Györgyi isolated a powerful reducing agent in crystalline form from the adrenal cortex. He believed it to be a hexuronic acid derivative having the empirical formula $C_6H_8O_6$. He also isolated this compound from cabbage and orange juice and recognised this crystalline material as probably being identical to the reducing agent reported by Zilva in vitamin C concentrates. King and Waugh (1932) finally succeeded in isolating a crystalline compound from lemon juice concentrates,

identified it as hexuronic acid and demonstrated its anti-
scorbutic properties.   A few weeks later, Svirbely and
Szent-Györgyi (1932) announced that hexuronic acid obtained
from adrenal glands, cabbage and oranges was also highly
antiscorbutic.   Shortly afterwards, the exact chemical
constitution of the antiscorbutic hexuronic acid was
established independently by workers in several laboratories.
It was also synthesised chemically.   Because of its
physiological role in the prevention of scurvy, the compound
was called ascorbic acid.

Ascorbic acid is reversibly oxidisable in the body
producing dehydroascorbic acid, which possesses full
vitamin activity.   The physiological functions of the
vitamin are probably related to the oxidation-reduction
system.   The structural formulae involved are :-

$$
\begin{array}{ccc}
O\!=\!\!C\!-\! & & O\!=\!\!C\!-\! \\
| & & | \\
OH\!-\!C & & O\!=\!\!C \\
\| & O & | \quad O \\
OH\!-\!C & & O\!=\!\!C \\
| & & | \\
H\!-\!C\!-\! & & H\!-\!C\!-\! \\
| & & | \\
OH\!-\!C\!-\!H & & OH\!-\!C\!-\!H \\
| & & | \\
CH_2OH & & CH_2OH
\end{array}
$$

$$\rightleftharpoons$$
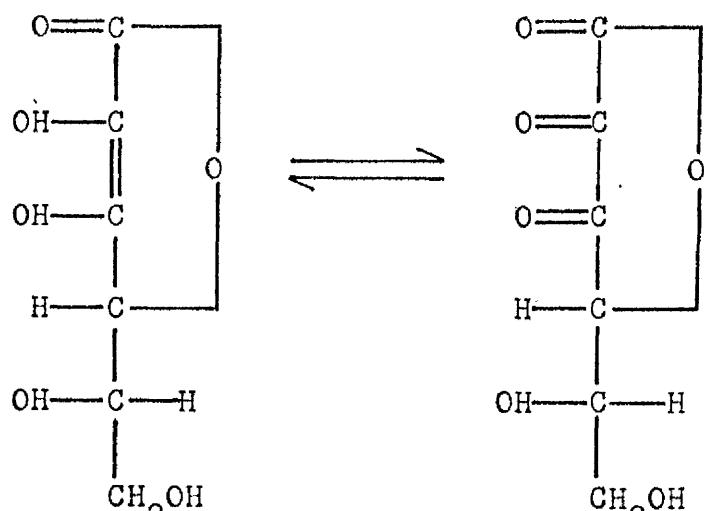
Ascorbic acid   Dehydroacsorbic acid

Ascorbic acid is an optically active compound.   Hughes and
Hurley (11) have shown that when guinea pigs on a low,
controlled intake of ascorbic acid (0.3 mgs / 100 gms body
weight) were given a daily oral dose of L-ascorbic acid or
of its isomer D-isoascorbic acid (both 1.5 mgs / 100 gms
body weight), the ascorbic acid was deposited in the tissues
while D-isoascorbic acid was not.   They concluded that in
guinea pigs ascorbic acid is absorbed from the gastrointestinal
tract much more readily than isoascorbic acid and ascorbic
acid is either more readily abstracted from the blood or
is more readily retained by the tissues, or both, than is
isoascorbic acid.   Hughes and Jones (12) found that the
presence of isoascorbic acid in the drinking water overcomes
its low absorption efficiency and results in substantial
tissue levels.   The appearance of the normally accepted
symptoms of scurvy may, in this way, be suppressed by iso-
ascorbic acid although the growth rate does not equal that
of ascorbic acid sufficient guinea pigs.   Although iso-
ascorbic acid and other compounds closely related chemically
to ascorbic acid do possess some antiscorbutic activity,
none compares in potency with the natural vitamin.

In the strict sense of the word, vitamin C may be
said to posses few pharmacological actions.   Prolonged
high dosage of ascorbic acid does not have any qualitative
or quantitative effect on the body (13, 14).   In the
scorbutic individual however, administration of the vitamin
leads to a rapid alleviation of the symptoms.

Ascorbic acid and dehydroascorbic acid form a
redox system with semidehydroascorbic acid as a highly

reactive intermediate (15).   Almost all metabolic processes
the disturbance of which give rise to scurvy, involve
reactions in which ascorbic acid is oxidised.   The formation
and maintenance of collagen is dependent on a normal
ascorbic acid level (16).   Similarly dependent on ascorbic
acid is the hydroxylation of the side chain of dopamine
which results in the formation of noradrenaline.

Some authors (17, 18) have suggested that ascorbic
acid is involved in the hydroxylations occurring in steroid
synthesis in the adrenals because the secretion of these
steroids is reduced in ascorbic acid deficient patients.
Others (19) have proposed that scurvy appears to involve
such severe stress which results in an increase in adreno-
cortical secretion, that the synthesis rate of cortisol
and corticosterone is incapable of matching the rate of
release of these steroids.

Ascorbic acid is also concerned in tyrosine metabolism,
as a reducing agent in the conversion of folic to tetra-
hydrofolic acid and in the incorporation of iron into
ferritin (20).   These latter properties may account for
the anaemia of scurvy which has been reported by various
authors (21, 22).   Ascorbic acid also serves some definite
metabolic function in platelet aggregation (23).

Ascorbic acid is involved in carbohydrate metabolism,
as evidenced by the fact that scorbutic animals exhibit
hyperglycaemia and low hepatic glycogen content and are
resistant to insulin.

In contrast to the reactions dependent on ascorbic
acid, the hydroxylation of tryptophan to 5-hydroxytryptophan,

the precursor of serotonin, is mediated by dehydroascorbic acid which is thereby reduced to ascorbic acid (24). The regeneration of ascorbic acid from dehydroascorbic acid in the tissues plays an important metabolic role. Ascorbic acid is synthesised by higher plants and all animals except primates and guinea pigs. Animals which do not form ascorbic acid lack the enzyme L-gulonolactone oxidase, required to convert L-gulonolactone to L-ascorbic acid. Ascorbic acid is absorbed in the gastrointestinal tract in the same way as glucose and other carbohydrates (25). Equilibrium between ascorbic acid absorbed from the diet and that present in the tissues is attained in less than four hours. The normal level of ascorbic acid in leucocytes is 20 - 50 ug. / 10^8 W.B.C. (26). The leucocyte ascorbic acid levels depend on the degree of tissue saturation and on dietary intake. With an ascorbic acid concentration of 20 mgs / kg. body weight, the daily turnover is about 1 mg. / kg., corresponding to a half life of 16 days (27). In man, the main route of excretion is through the urine with trace amounts expelled in the faeces and respiratory carbon dioxide. In urine, ascorbic acid appears mainly unchanged but part is hydrolysed to diketogulonic acid and appears finally as oxalic acid (25). Other metabolites are probably derivatives of L-threose or L-threonic acid (28,29).

The British Medical Association Committee on Nutrition (1950) recommend a daily intake of vitamin C of 30 mgs per person per 24 hours to provide a good margin of safety for the prevention of scurvy. It has been suggested that up to 10% of the households in Britain and

an even greater proportion of individuals may have a vitamin C intake which is permanently below this prescribed level (30).

The most striking manifestation of ascorbic acid deficiency is a marked tendency to bleeding with the appearance of extensive patches of haemorrhage under the skin and in the gums, muscles, fatty tissues and internal organs. Hansky (31) found that 15 of a sample of 31 patients in whom the cause of bleeding remained undetermined, had a low vitamin C intake. Other secondary features of of ascorbic acid deficiency are impairment of connective tissue formation, leading to poor wound healing, roughening of the skin (hyperkeratosis) and anaemia.

The present work reports nutritional studies, with particular reference to the role of vitamin C, on alcoholics and on guinea pigs which had been fed ethanol. An investigation of the effect of home-brewed material (lager) on the gastric mucosa of healthy guinea pigs and of man was also undertaken and the findings are presented in a succeeding Chapter.

CHAPTER II

Gastric erosions caused by home-brewed lager.

The fermentation of sugars by yeast to produce ethanol has been known since antiquity. Ethanol is the sole product of yeast fermentation under anaerobic conditions. Other fermentative products i.e. acetaldehyde and acetic acid, will be produced if the yeast is infected by certain other bacteria.

Seven years ago, restrictions governing the home-brewing of beer were removed and, since then, home-brewing has become ever more popular. Many types of brewing kits have become available. Each includes fresh hops, yeast, malt or malt extract and salts, which are required for fermentation. The main attractions of these kits are the ease of operation and their low cost.

These studies were prompted by the admission of a patient with melaena, who had recently ingested large amounts of home-brewed lager.

CASE REPORT.

A 22-year old electrician was admitted to Stobhill General Hospital in October 1970 with abdominal pain, nausea and vomiting and melaena. There was no history of previous dyspeptic symptoms nor of aspirin ingestion. A few weeks previously, he had brewed lager at home, using a kit which is widely sold. He made about five gallons of this mixture and had drunk most of it himself over a period of three weeks.

On examination, he was not shocked ; blood pressure 120 / 60 ; pulse 105 per minute. Apart from generalised pallor, there was no clinical abnormality. His haemoglobin on admission was 11.8 g.% and his P.C.V. was 35%. Over the

the subsequent few days, his haemoglobin fell to 9.8 g% and his P.C.V. to 33%. A barium meal showed no abnormality of the oesophagus, stomach or duodenum. Fibroscopy five days after admission revealed that the gastric rugal pattern was normal, the mucosa was not injected but one superficial erosion was seen in the anterior wall of the pyloric canal. The patient had no recurrence of his symptoms.

The clinical diagnosis was one of erosive gastritis. It was considered that this could have been due to the ingestion of the home-brewed lager and studies were carried out to investigate this possibility.

MATERIALS AND METHODS.

Five gallons of beer and five gallons of lager were brewed in the laboratory from a kit similar to that used by the patient (Fig. 2.1). A sample of laboratory-brewed beer and lager, the patient's lager and commercial pale ale and lager were analysed by gas-liquid chromatography in a Pye 104 Chromatograph. This work was conducted in the Department of Forensic Medicine in the University of Glasgow, by Dr. Hamilton Smith. Resolution of the components was achieved on a column 5 ft. long packed with chromosorb 101. The operating temperature was 170°C and the rate of nitrogen gas flow was 60 ml / minute. The internal standard was n-propanol, at a concentration of 8 mg / 100 ml. water. Samples of the patient's lager, laboratory-brewed beer and lager and fresh yeast straight from a lager kit were analysed bacteriologically by Mr. Peter Owen of the Department of Microbiology, University of Glasgow.

Figure 2.1.   Photograph of the kits used to brew lager and beer.

ANIMAL STUDIES.

Thirty-six male guinea pigs - weight range 330 -
580 gms - were fed on the standard guinea pig diet 18
(Table 2.1) based on the recommendations laid down by the
U.F.A.W. handbook (1957) (32). The guinea pigs were divided
into three groups each group comprising twelve animals
(Table 2.2). One group was given measured aliquots of tap
water as their sole drinking fluid, another group was given
measured aliquots of laboratory-brewed lager and a third
group was given measured aliquots of laboratory-brewed beer.
In addition, each animal was given a small amount of the
appropriate liquid into their stomach. On days 1 and 2
they were each given 2 mls by intubation tube; on day 3
they were given 4 mls and on day 4 they were given 5 ml.
The animals did not fast before force-feeding but were lightly
anaesthetised with ether. The animals were killed on day 5.

In a second experiment, thirty-six male guinea pigs -
weight range 250-680 gms - were divided into three groups.
The experiment was repeated exactly as before but the animals
were not force-fed (Table 2.2). The guinea pigs were killed
by ether anaesthesia and the stomachs were dissected. The
gastric mucosa were examined carefully by the naked eye for
the presence of bleeding points (defined as the obvious
continued oozing of blood from a localised point on the
mucosa after careful swabbing). The gastric mucosa of each
animal was also tested for the presence of blood by the
orthotolidine method (33). The mucosa was tested by placing
drops of the reagent on the mucosa of the whole stomach after
removal from the body. In each case the number of seconds

TABLE 2.1   COMPOSITION OF NORMAL DIET FOR GUINEA PIGS.

Ingredients	%
Grass meal	30
Bran	15
Groundnut cake	15
Linseed cake	10
Barley	20
Meat and bone meal	8
Salt	1
Ground limestone	1

TABLE 2.2   GUINEA PIG EXPERIMENTAL GROUPS.

(12 animals in each group)

Guinea Pig Diets		
Group	Food	Liquid
A	Diet 18 + Cabbage daily	Water
B	Diet 18 + Cabbage daily	Lager - Hosp. brew
C	Diet 18 + Cabbage daily	Beer - Hosp. brew

taken for the blue colour to develop was estimated by stop-watch.

One stomach from each group of animals in the first experiment, i.e. with intubation, was examined histopatho-logically. The stomach was not sectioned serially but only random sections were examined. The slides were stained with Haematoxylin and Eosin.

RESULTS.

The results of Gas-Liquid Chromatography analysis are shown in Fig. 2.2. The peak heights are not proportional to the concentrations of the components of the mixture. The contaminants were present only in trace amounts and therefore their percentage composition in the lager could not be determined. The ethanol content of all the lager samples was approximately the same. Both the patient's lager and the laboratory-brewed lager contained traces of acetaldehyde and acetic acid. The patient's lager had more of these contaminants than had the laboratory brew. The laboratory brew also contained traces of ethyl acetate which could have been formed by the mass action esterification of acetic acid. In contrast, the laboratory-brewed beer was free from any contaminating substance.

These contaminants are formed by different fermentative pathways (Fig. 2.3). The main fermentative pathway is that shown across the top of Figure 3, and occurs when yeast is growing and multiplying under anaerobic conditions. If there are any extraneous bacteria present, ethanol will be metabolised by different routes. These pathways require enzymes which are not present in yeast.

Fig. 2.2   G.L.C. ANALYSIS OF BEERS AND LAGERS.

AMYL ALCOHOL

ETHYL ACETATE
ACETIC ACID
ETHANOL 3%
ACETALDEHYDE
                        HOSPITAL LAGER

ETHANOL 3.4%
                        PATIENT'S LAGER

ETHANOL 3.2%
                        TENNENT'S LAGER

ETHANOL 3.6%
                        HOSPITAL BEER

ETHANOL 2.6%
                        McEWAN'S PALE ALE
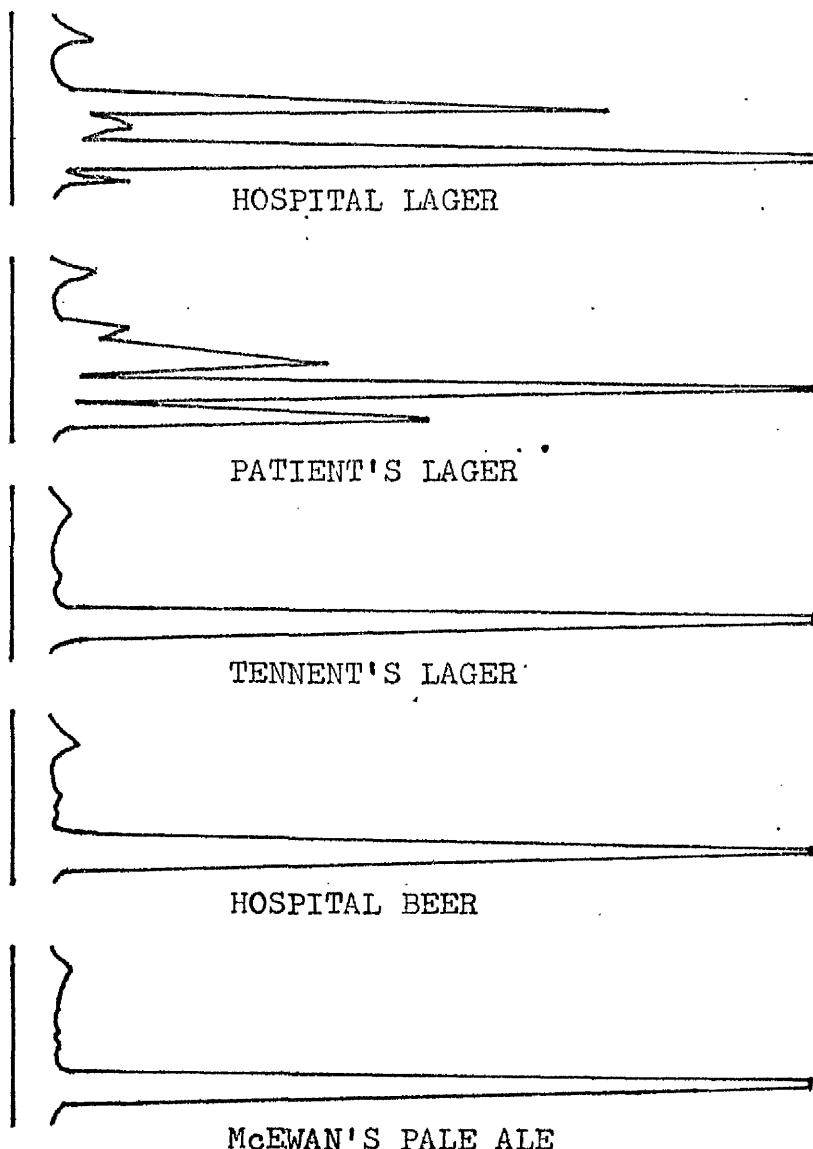
Fig. 2.3   SOME PATHWAYS OF ETHANOL FERMENTATION.
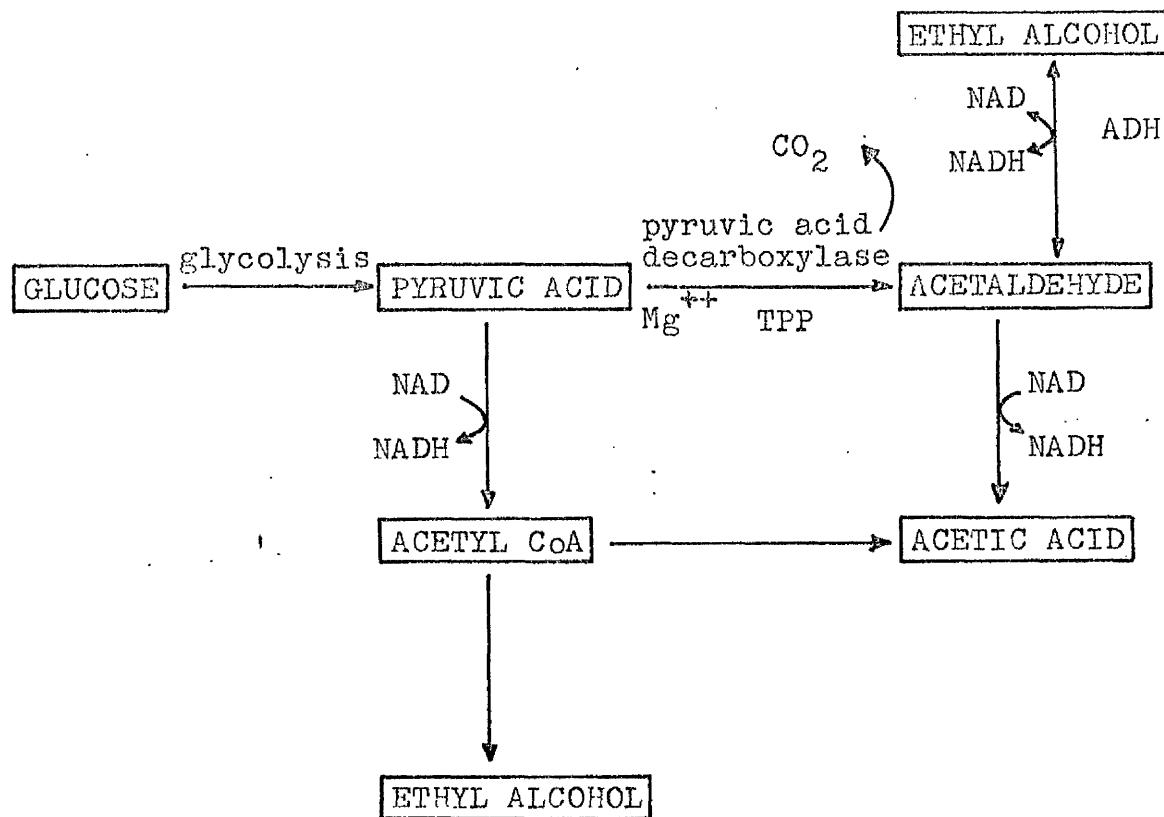
Acetic acid can be produced via acetyl CoA or directly from acetaldehyde. Direct conversion of acetaldehyde to acetic acid occurs only in alkaline medium.

The laboratory-brewed beer was free from bacterial contaminants, but both the patient's lager and the laboratory-brewed lager had an infection of Acetobacter melanogenus which is known to produce acetic acid from ethanol. The dry lager yeast also had this infection.

On histopathological examination, the sections of the stomachs from the beer and lager drinking animals showed much greater congestion than the stomachs of the water-drinking animals (Figs 2.4, 2.5, 2.6). The congestion was superficial and there was no cellular infection. The irritating effect

of the lager is clearly seen in Fig. 2.7. This mucosal
reddening was present in most of the beer and lager drinking
animals. There was no visual difference in the extent of
mucosal reddening between the beer and lager drinking
animals. Obvious bleeding points were found significantly
more often in the alcohol drinking animals which were also
receiving aliquots by intubation tube. When the animals
were not force-fed, there was no significant difference in
the number of visible bleeding points (Table 2.3).

The results of the orthotolidine tests on the gastric
mucosa are shown in Table 2.4. The differences between
these patterns were analysed by the Mann-Whitney test
(Table 2.5). The contaminated lager was significantly more
irritant than the beer ($p < 0.02$) in intubated animals. In
the second experiment, when the animals were drinking the
fluids but were not being force-fed, there was a significant
difference ($p < 0.05$) between the lager-drinking group and
the water-drinking group, but not between the beer and water
drinking groups.

Fig. 2.4   Section of stomach of a water-drinking
animal.   The gastric lumen appears at the top of the
photograph.   The stomach was stained with H and E,
thus blood vessels appear orange in colour.
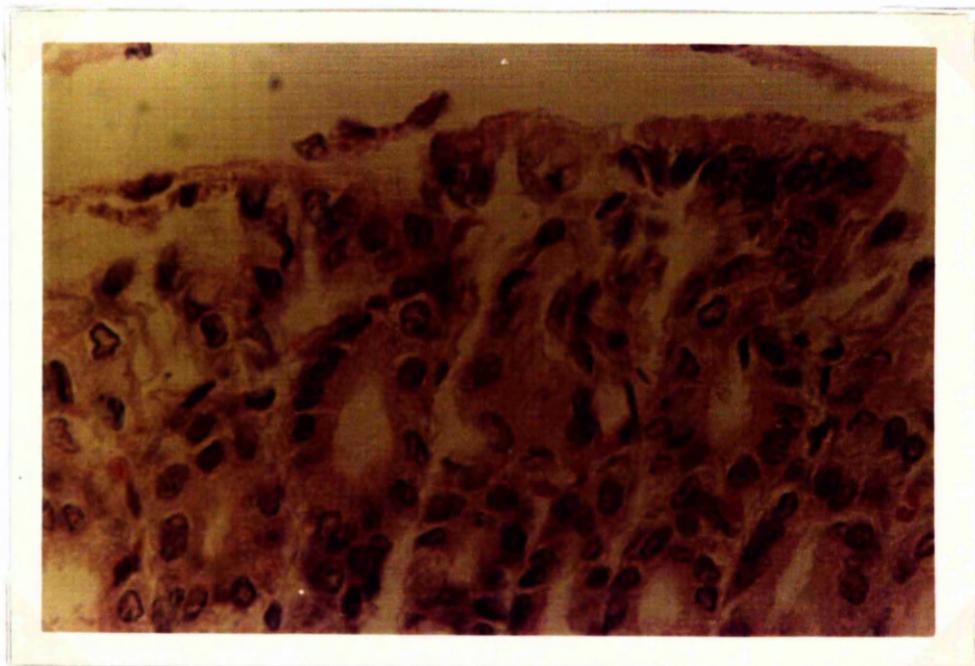
Fig. 2.5   Section of stomach from a lager-drinking
animal (hospital-brewed).   The gastric lumen appears
at the top right hand side of the photograph.   Note
the large increase in the number of blood vessels as
compared with Fig. 2.4.
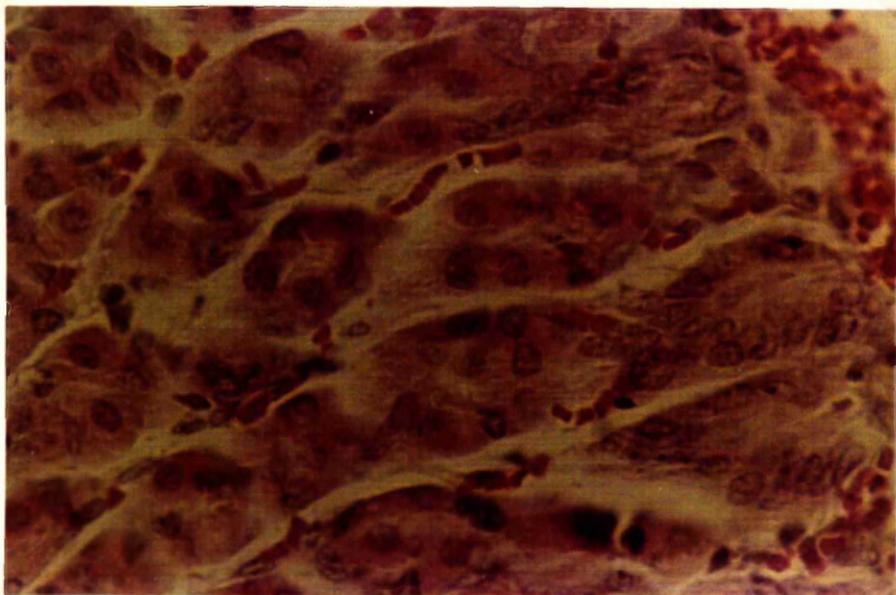
Fig. 2.6   Section of the stomach from a beer-drinking
animal (hospital-brewed), the lumen appearing at the right
hand side of the figure.   This stomach is also congested.
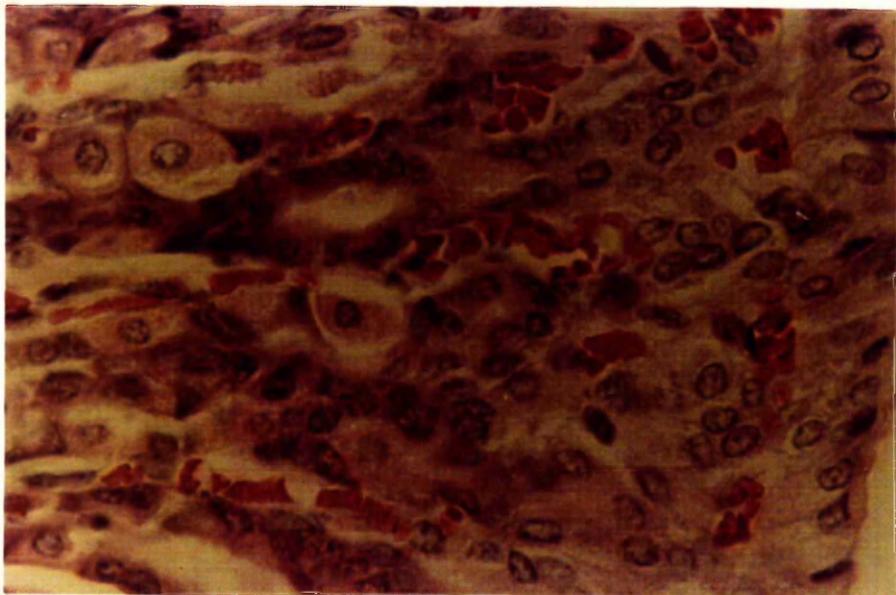
Fig. 2.7   Guinea pig stomachs.
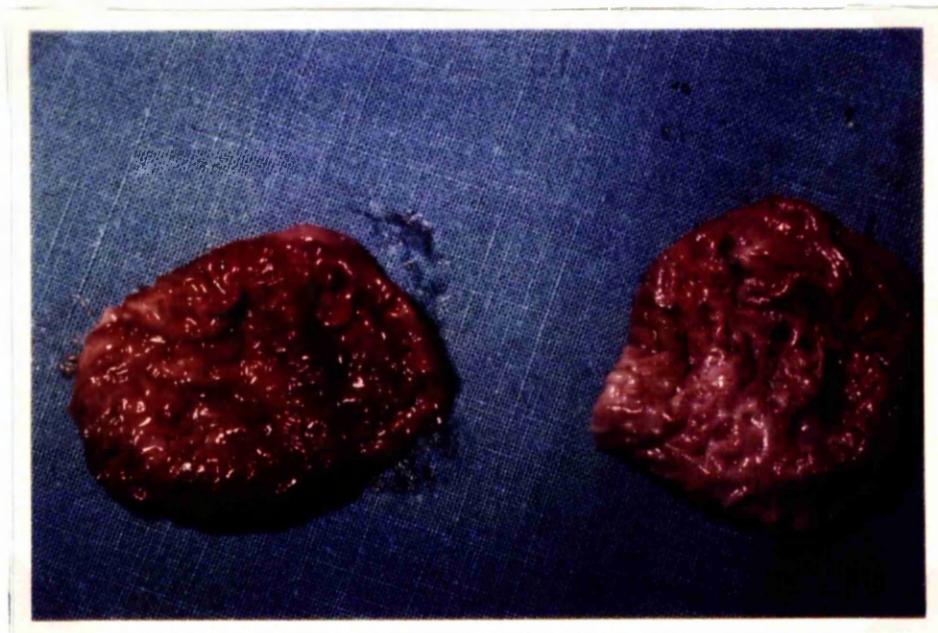
The pale mucosa on the right came from a guinea pig which
drank only water.   The inflamed mucosa on the left came
from a guinea pig which drank hospital-brewed lager and was
given additional aliquots of this lager by gastric
intubation.

TABLE 2.3

Number of guinea pigs with visible bleeding points on gastric mucosa. All animals fed on Diet 18 + cabbage.		
Drinking fluid	12 animals/group with intubation.	12 animals/group without intubation.
Beer	6	5
Lager	7	5
Water	2	4

TABLE 2.4

	With intubation			Without intubation		
Comparison of bleeding of gastric mucosa by the orthotolidine test on guinea pigs' stomachs. All animals fed Diet 18 + cabbage.. Each cross represents one guinea pig.						
	Beer	Lager	Water	Beer	Lager	Water
Negative -	++		+++++	++++	++	+++++++
Positive -						
30 secs -	++++++	+++++	++++	+++++	++++	++++
25 secs -	++	++	+	++	++++++	+
20 secs -	+	++++		+		
10 secs -						
0 secs -						

TABLE 2.5

Results of Mann-Whitney tests.

(from orthotolidine tests on guinea pig stomachs - Table 2.4)

---

(1) With intubation :

	U value	p
Lager and water	16	<0.02
Beer and water	35	0.1
Lager and beer	36	N.S

(2) Without intubation :

	U value	p
Lager and water	32	<0.05
Beer and water	50	N.S
Lager and beer	55.5	N.S

(N.S : Not Significant)

DISCUSSION.

The results suggest that the laboratory-brewed lager is more irritant to the gastric mucosa than the laboratory-brewed beer. This difference could be attributed to the harmful contaminants, acetaldehyde and acetic acid, present in the lager. These contaminants were present only in trace amounts, but acetaldehyde is extremely toxic.

Augur (34) showed that 9% (w/v) ethanol or 100 mM acetic acid can cause congestion in the stomachs of dogs with Heidenhaem pouches. In addition, it is known that the long continued use of strong alcoholic beverages in man may result in chronic gastritis in approximately one out of three individuals (35). These figures are valid as long as the alcoholic beverages are not contaminated with other harmful substances. The laboratory-brewed beer was not contaminated and did not produce any significant irritation of the gastric mucosa of guinea pigs, over a period of four days, in contrast to the contaminated beverages.

Home-brewing of beer, lager and also wine has become very popular since the introduction of these kits. The main advantage is the low cost, but it now appears that home-brewing is not without danger, particularly in the bacterial contamination of the yeast, which occurred in all three samples of the lager yeast. If the yeast is contaminated, the liquid will taste and smell sour and this should be a deterrent to the consumer. These findings suggest that the consumer requires to be protected either by Public Health legislation or by greater quality control measures on the part of the producer.

CHAPTER III

A clinical and nutritional study of alcoholics.

According to the World Health Organisation,
" alcoholics are those excessive drinkers whose dependence
upon alcohol has attained such a degree that it interferes
with their bodily and mental health, their interpersonal
relationships and their smooth social and economic
functioning or who show early signs of such developments".
This definition could be applied to all patients interviewed
in this study.

It has been apparent for many years that alcoholics,
in addition to consuming ethanol, do not as a rule consume
a diet that bears any close resemblance to that of non-
alcoholics (10).   It is also true that just as ethanol
consumption will modify nutritional requirements, diet
modifies the effect of ethanol.

Alcoholic patients admitted to the Medical Unit
at Glasgow Western Infirmary have often been noted to
have a low level of Leucocyte Ascorbic Acid (LAA).   The
cause of this may be dietary in many cases but another
possible explanation for this is the accelerated oxidative
catabolism of ascorbic acid as occurs in the siderotic
Bantu (36).   It was decided to test these theories by
measuring leucocyte ascorbic acid and comparing this level
with the dietary intake of vitamin C obtained from an
analysis of the average daily food and drink intake of the
patients.

Both alcohol and vitamin C have an effect on
iron metabolism.   Alcohol stimulates the absorption of iron
from the small intestine but it has a detrimental effect

on haemopoiesis (37).   In ascorbic acid deficiency, iron
absorption is greater than normal in response to an
increased erythropoietic activity, but the metabolism of
storage iron is abnormal (38).   Therefore either prolonged
alcohol ingestion or vitamin C deficiency has frequently
been associated with anaemia.

In order to assess the effect of ethanol at a
known level of leucocyte ascorbic acid, the serum iron, the
total iron binding capacity and the erythrocyte porphyrins
both erythrocyte protoporphyrin and erythrocyte
coproporphyrin levels, were measured along with the
leucocyte ascorbic acid level in alcoholic patients.   The
dietary intake of iron was also assessed from an analysis
of the diet histories.

Materials and Methods.

A survey of fifty alcoholics, ages ranging from
24 to 70 years, was carried out.   The group included nine
female patients, age range 34 - 50 years.   Most of the
patients were seen in hospital but some were interviewed
at a voluntary care centre for alcoholics.   None of the
patients had overt liver disease.   The blood sample for
LAA, packed cell volume (P.C.V.), serum iron, total iron
binding capacity and erythrocyte porphyrins estimation
was withdrawn as soon as possible after admission to
hospital.   Haemoglobin levels were estimated in 40 of the
patients.   None of the patients were intoxicated at the
time the blood sample was taken.   A complete dietary
history was recorded from each patient.   The alcoholic
group was matched according to age (within five years)

and sex with a normal group of patients.

Leucocyte ascorbic acid was estimated by the
2,4 dinitro-phenol method (39), which is described over-
leaf and the average daily intake of vitamin C and iron
was calculated from the diet history. Serum iron and
total iron binding capacity were measured by the method
of Ramsay (40) and erythrocyte porphyrins, both
erythrocyte protoporphyrin and erythrocyte coproporphyrin,
were measured by the method of Rimington (41).

Method for the measurement of leucocyte ascorbic acid.

All estimations of leucocyte ascorbic acid
must be started within four hours of withdrawal of the
blood from the patient. Three to four millilitres of
fresh whole blood are added to a bottle containing 12.5 ml
of a mixture of 200 ml of 0.9% saline / 50 ml of 6%
dextran ($\bar{M}_W$ = 125,000) / 2 ml of 10% sequestrene (42),
mixed thoroughly and allowed to stand for one half hour at
room temperature, during which time most of the erythro-
cytes have separated. 10 ml of the supernatant fluid
are taken for the estimation and the remainder is used for
a leucocyte count. The 10 ml of supernatant fluid are
spun at 3,000g for 15 minutes to bring down the white cells
and platelets. The supernatant is discarded and the
cellular deposits are ground with 1.3 ml of 5% trichloro-
acetic acid. At this stage, the estimation can be left
overnight at 2 - 4°C.

The specimen is then centrifuged at 3,000g for
ten minutes to remove cell debris and 1 ml of the resulting
supernatant fluid is incubated with 0.3 ml of 100 vols

of 2.2 g of 2,4 dinitrophenyl hydrazine in 10 N sulphuric acid / 5 vols of 5% thiourea / 5 vols of 0.6% copper sulphate reagent (42) at $37^{\circ}C$ for four hours. After cooling in ice-water, 1.5 ml of 65% sulphuric acid is added and the absorbance is read against a reagent blank at 520 mμ in a spectrophotometer. A calibration graph can be prepared by using concentrations of 0.0, 0.5, 1.0, 2.5, 3, 4 and 5 ug ascorbic acid / ml in 5% trichloro-acetic acid, these being treated identically to the test solution. The test readings can then be read off the straight line graph thus obtained. The ascorbic acid content of the leucocytes is expressed as μg of ascorbic acid / 10^8 leucocytes and is calculated as follows :

$$\frac{\text{Graph reading x 1.3 x 100}}{\text{Leucocyte count in hundreds /c.mm.}}$$

= μg ascorbic acid / 10^8 leucocytes.

RESULTS.

.The range of leucocyte ascorbic acid levels in the alcoholic group is 0-50 μg/10^8 WBC (Fig. 3.1) with a mean of 18.18 $\pm$ 11.01 (mean $\pm$ S.D.) μg/10^8 WBC. The range in the control group is 11-46 μg/10^8 WBC (Fig. 3.2) with a mean of 27.41 $\pm$ 7.59 (mean $\pm$ S.D.) μg/10^8 WBC. Thus the vitamin C levels of the alcoholics are significantly lower (p < 0.0005) than those of the matched group (Fig. 3.3).

It is seen in Table 3.1 that this vitamin C deficiency occurs in every age group in the alcoholic sample, and in both sexes. The vitamin C levels in different age groups are not significantly different from each other, but in every age group, the alcoholic patients

Fig. 3.1

No. of Patients.

NORMAL RANGE

FEMALES

MALES

(Every rectangle denotes
one alcoholic patient).

Leucocyte ascorbic acid (μg/10^8 WBC)
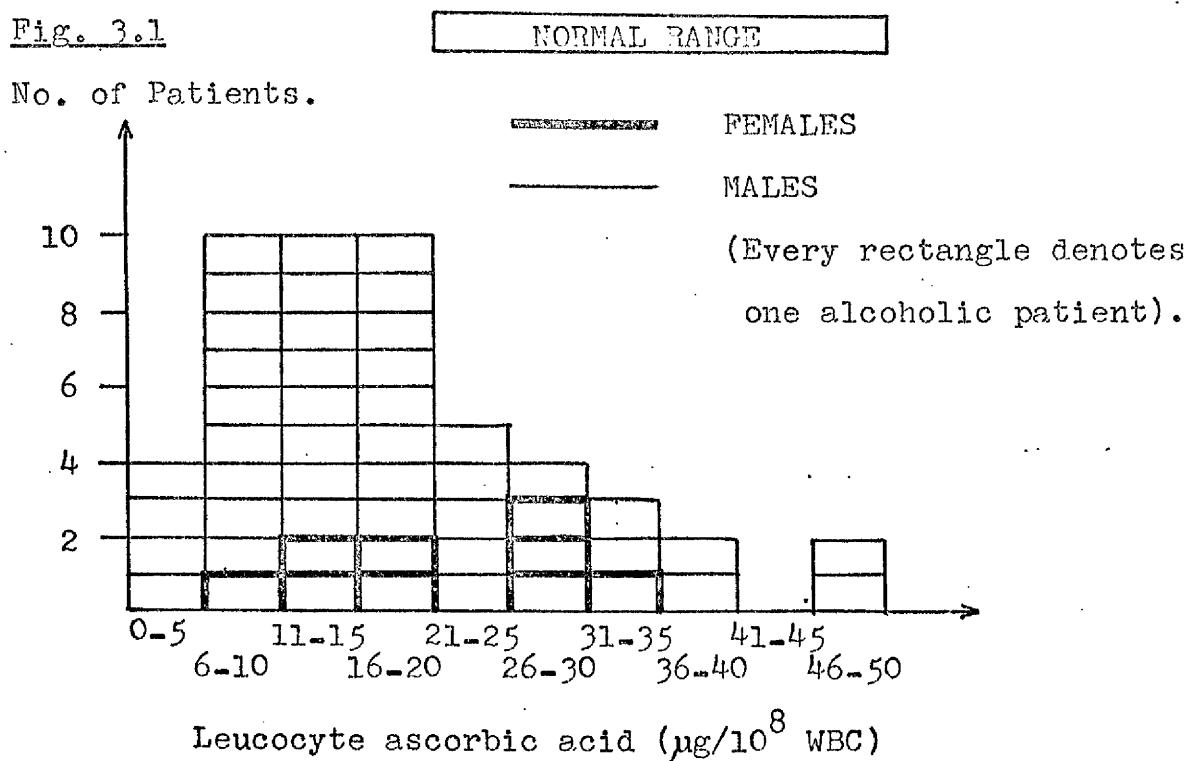
Fig. 3.2   Leucocyte ascorbic acid levels in compared,
normal patients (Every rectangle denotes
one patient).

No. of Patients.

FEMALES

MALES

Leucocyte ascorbic acid (μg/10^8 WBC)

Fig. 3.3   Leucocyte ascorbic acid levels in normal
and alcoholic humans.

L.A.A. $(\mu g/10^8$ WBC$)$

No. of samples = 50

Students 't'. = 4.684

p   <   0.0005
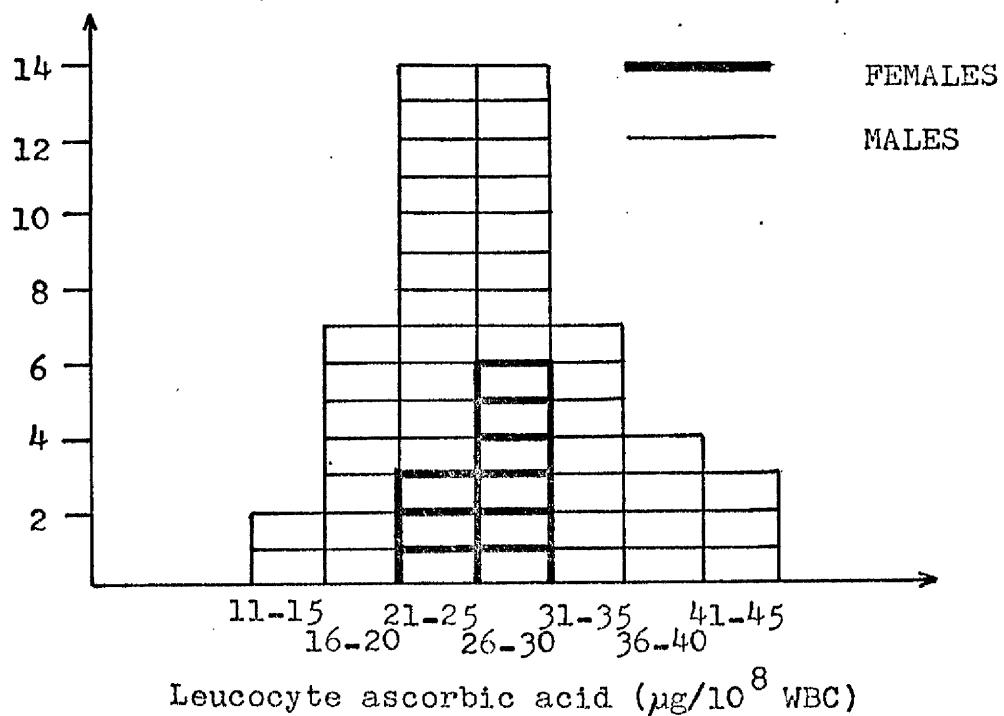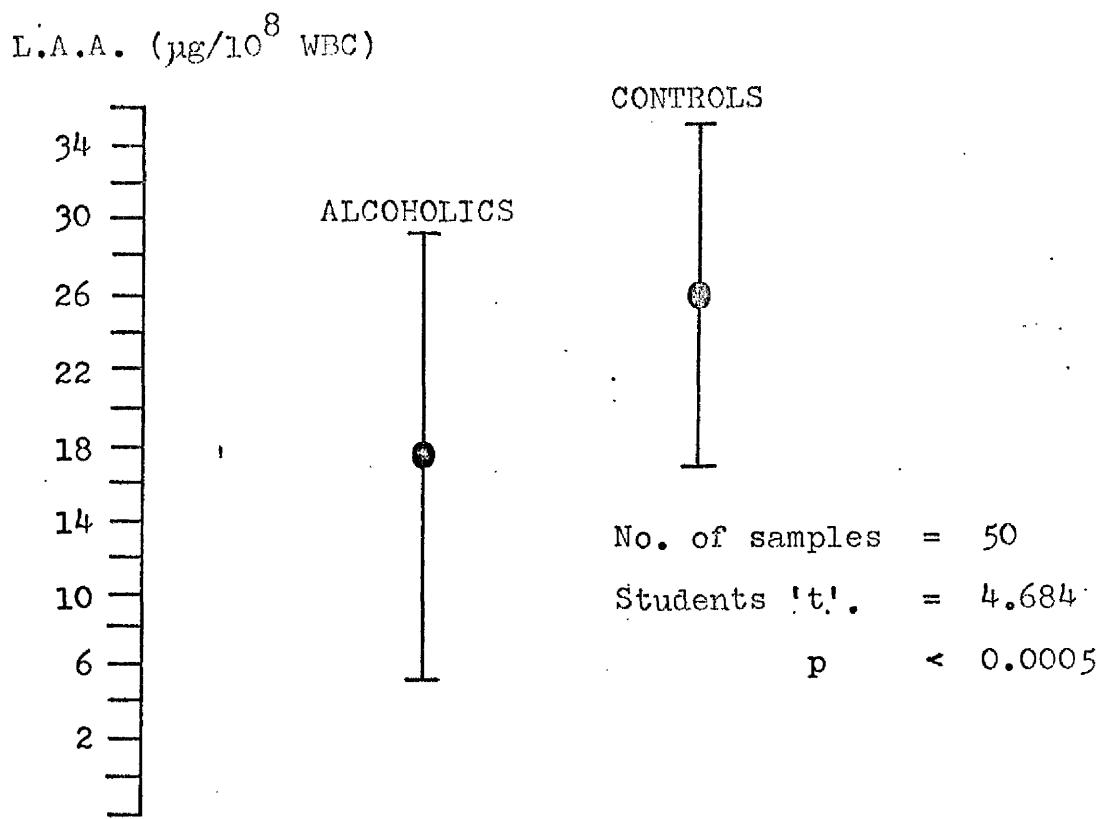
TABLE 3.1   Leucocyte ascorbic acid levels in normal and
alcoholic humans in different age groups.

Age Group	Alcoholics $(\mu g/10^8$ WBC ; mean $\pm$ S.D.$)$	Controls	p
Over 45 (25)	17.2 ± 11.9	25.1 ± 7.7	< 0.005
Under 45 (25)	19.4 ± 10.0	27.7 ± 7.9	< 0.005
Under 30 (9)	16.8 ± 10.6	27.7 ± 9.4	< 0.025
Females (9)	21.3 ± 8.2	26.2 ± 1.8	< 0.05

Fig. 3.4   L.A.A. and diet.

L.A.A
$(\mu g/10^8$ WBC)

Vitamin C intake (mg/24 hours)
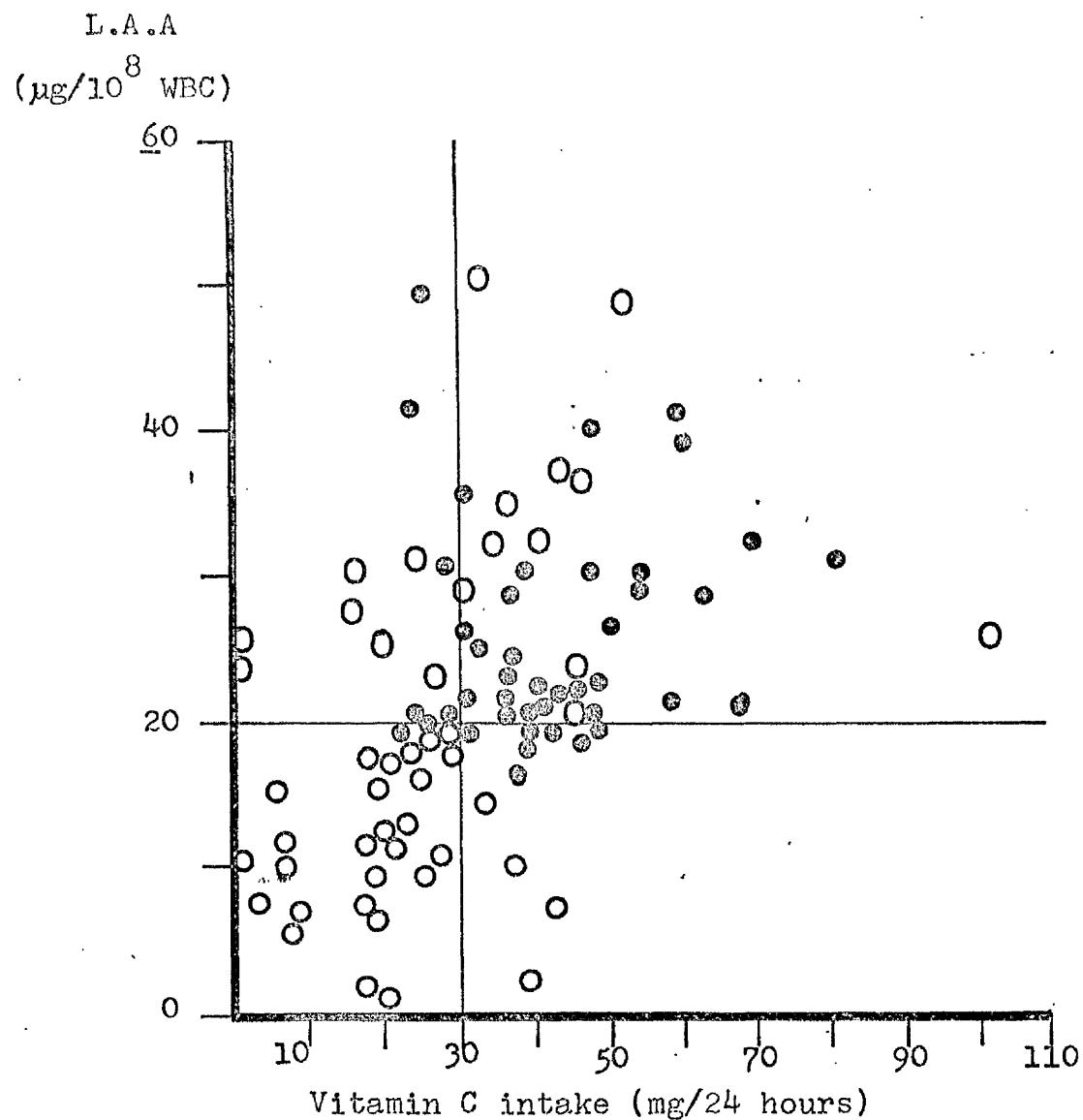
O   Represents one alcoholic patient.

●   Represents one control patient.

L.A.A. of 20 $\mu g/10^8$ WBC is the lower limit of normal range

Vitamin C intake of 30 mg/24 hours is recommended by the B.M.A.

have a significantly lower L.A.A. level than the control
group.

The comparison of vitamin C intake with leucocyte
ascorbic acid in both the alcoholic and control patients
is shown in Fig. 3.4. The British Medical Association
recommend an intake of vitamin C of at least 30 mg per
person per day to maintain adequate health and prevent any
signs of scurvy. Half of the alcoholic patients (25 out
of 50) have a low leucocyte ascorbic acid level because of
insufficient intake. Only two subjects in the control
group have a low leucocyte ascorbic acid because their
intake is less than 30 mg per day. Five alcoholic
patients and seven control patients have an adequate intake
of vitamin C but their leucocyte ascorbic acid levels are
below 20 $\mu g/10^8$ WBC. These patients may break down vitamin
C faster than normal or may not absorb it adequately.
Only eleven alcoholic patients compared with 29 out of 50
control subjects have an adequate vitamin C intake and a
leucocyte ascorbic acid level above 20 $\mu g/10^8$ WBC.

The results of haemoglobin and packed cell volume
measurements and white blood cell counts in normal and
alcoholic patients are summarised in Table 3.2.

The normal levels of erythrocyte coproporphyrin
(E.C.P.) for both males and females are 1.78 $\pm$ 1.3 $\mu g/100$ ml
red blood cells (R.B.C.). The accepted range is
0 - 4.0 $\mu g/100$ ml of red blood cells (43). The erythrocyte
coproporphyrin levels found in forty alcoholics were
1.8 $\pm$ 1.6 (mean $\pm$ S.D.) $\mu g/100$ ml of R.B.C. The range was
0 - 7.6 $\mu g/100$ ml R.B.C. The erythrocyte coproporphyrin

TABLE 3.2

	Sex	Normal level (mean ± S.D.)	Alcoholic level (mean ± S.D.)	p
Haemoglobin	Male (35)	100% (16g/100 ml blood) ± 25%	100% ± 9.9%	N.S.
Haemoglobin	Female (5)	100% (14g/100 ml blood) ± 28.6%	86.4% ± 15.9%	N.S.
Packed cell volume	Male (39)	46.2% ± 3%	45.6% ± 4.6%	N.S.
Packed cell volume	Female (8)	40.6% ± 4.8%	37.3% ± 9.2%	N.S.
White blood cell counts	Male and Female (28)	7,500 ± 2,500 WBC/c.mm.	7,550 ± 2,600 WBC/c.mm.	N.S.

level was above 4 µg/100 ml R.B.C. in only four alcoholic patients. Therefore the E.C.P. levels in normal and alcoholic patients are not significantly different.

The normal values for erythrocyte protoporphyrin (E.P.P.) for males and females are 24.03 ± 12.8 (mean ± S.D.) µg/ 100 ml R.B.C. The values found in forty alcoholic patients were 43.3 ± 34.65 (mean ± S.D.) µg/100 ml. R.B.C. with a range of 11-161 ug/100 ml.R.B.C. In the alcoholic group, 14 out of 36 patients had an E.P.P. greater than 35 µg/100 ml. These results must be considered with the serum iron and total iron binding capacity (T.I.B.C.) results.

Serum iron levels were measured in 34 male alcoholics. The mean of these levels was 120 ± 75.7 (mean ± S.D.) µg% and the range of 20-358 µg% is significantly different from the normal range for men which is 90-140 µg%. Twelve patients had a serum iron level lower than 90 µg%. This could arise from insufficient intake of iron. When each diet history was analysed, it was found that every alcoholic patient had an intake less than the recommended intake of 15 mg of iron per day. Eight patients (approximately 25%) had a serum iron level greater than 140 µg%. Of these, five patients had a serum iron level in excess of 200 µg%.

The range of total iron binding capacities in alcoholic patients was 180-558 µg% compared with a range of 250-400 µg% in normal patients.

The serum iron results and the T.I.B.C. results were used to evaluate the percentage saturation of iron
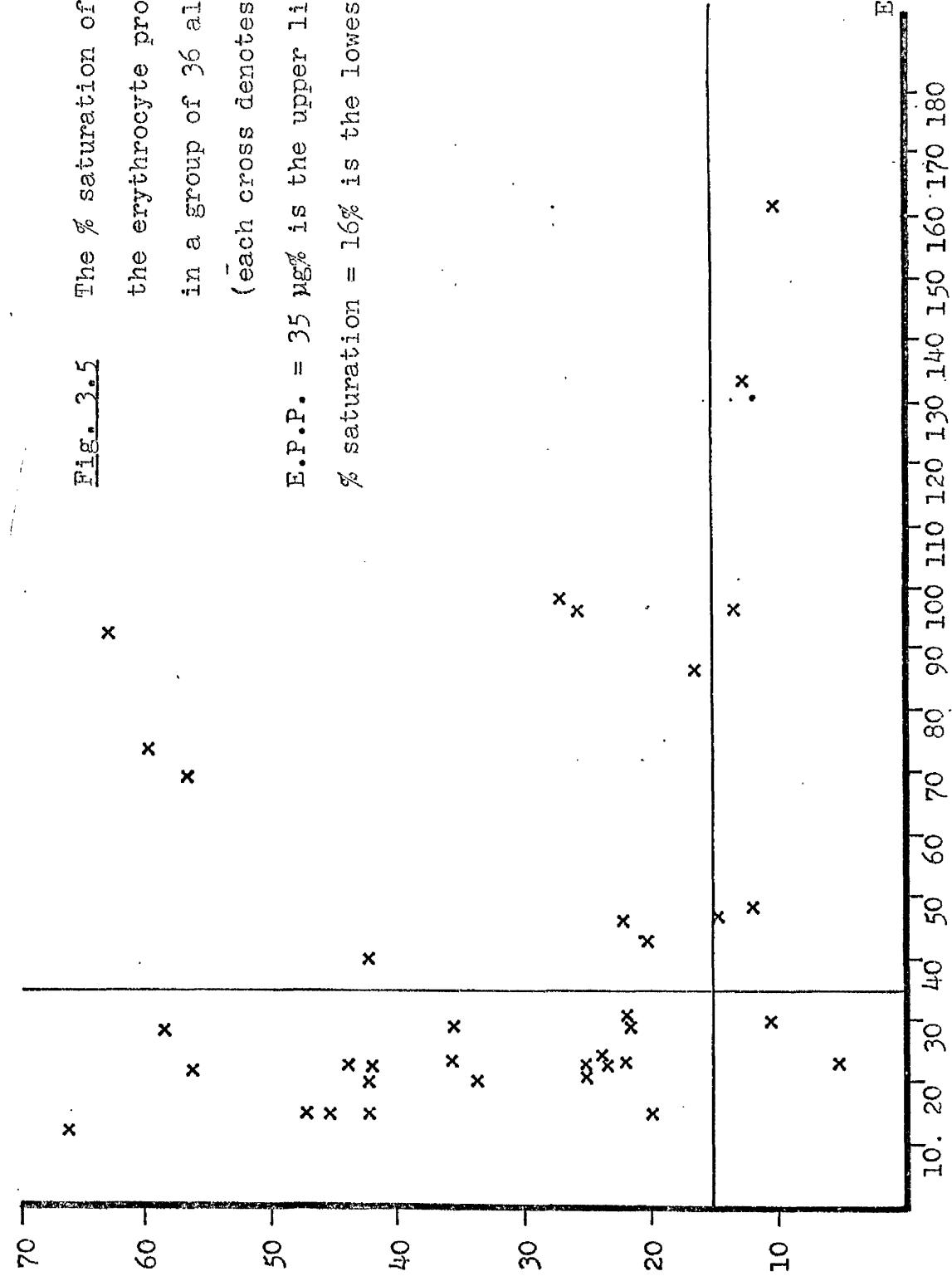
Fig. 3.5   The % saturation of iron compared with
           the erythrocyte protoporphyrin levels
           in a group of 36 alcoholic patients.
           (each cross denotes one alcoholic patient)

E.P.P. = 35 µg% is the upper limit of normal range.

% saturation = 16% is the lowest acceptable value.

Fig. 3.6   The correlation of % saturation of iron with erythrocyte protoporphyrin levels
in 36 alcoholic patients.

% saturation
of iron.

36 samples, p < 0.05.

10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180

(EPP : μg%)
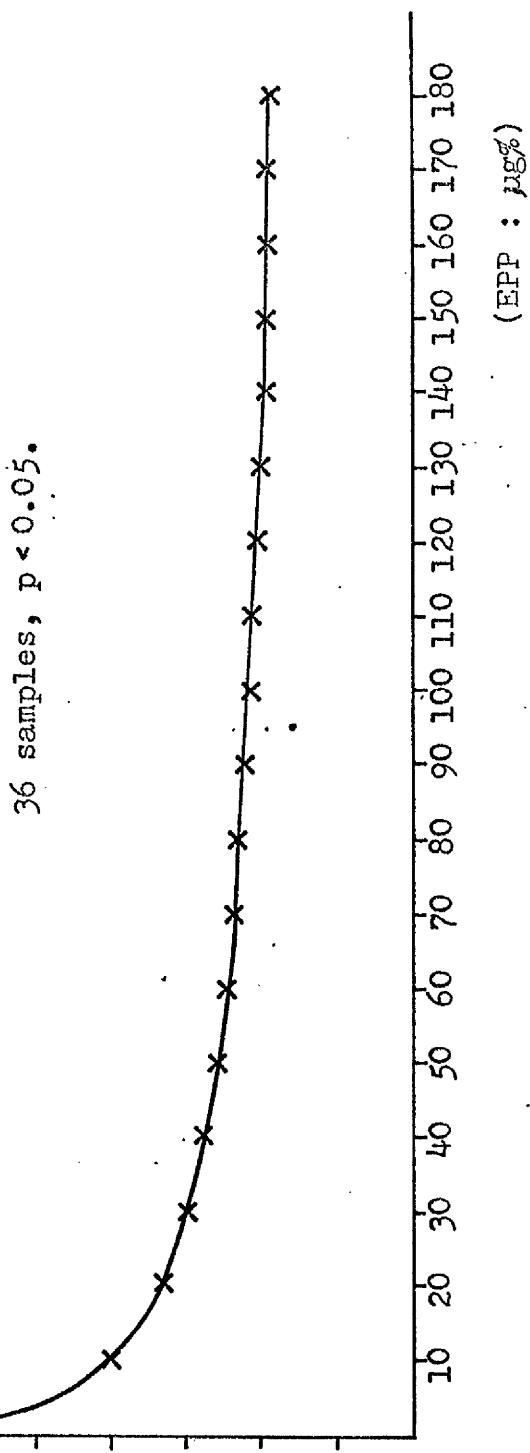
in the blood of the alcoholic patients.   The % saturation

of iron was compared with the E.P.P. levels (Fig. 3.5) to

establish the incidence of anaemia in this alcoholic

group.   The figure shows five cases of iron deficiency

anaemia, i.e. a saturation of the total iron binding

capacity of less than 16% and an E.P.P. level higher than

35 µg/100 ml R.B.C.   The normal saturation of iron is

about 33%.   Over 50% of the patients show no signs of

abnormal iron metabolism since their iron saturation is

greater than 16% and the E.P.P. values are between 0-35

µg/100 ml R.B.C.   There is a statistically significant

correlation (p <0.05) between the % saturation of iron

and the E.P.P. levels in 36 estimations (Fig. 3.6).

The asymptotic curve has the equation $y = ax^b$ where :

a = 70.13 and b = -0.25, and the coefficient of correlation

is -0.28.

DISCUSSION.

The results show that, as a group, alcoholics are

sub-clinically scorbutic and that this is caused by

insufficient dietary intake of vitamin C.   There is no

evidence from these results that the catabolism of vitamin

C is increased in alcoholics nor is there any reason to

suggest that alcoholics require more than 30 mg of vitamin

C per 24 hours.

The aetiology of the anaemia caused by prolonged

alcoholic ingestion has been variously ascribed to gastro-

intestinal blood loss, dietary deficiency of iron and

folic acid, increased haemolysis and depressed haemopoiesis.

The subject has been reviewed by Kimber et al (44).
Similarly, approximately 90% of patients with scurvy have
anaemia with a haemoglobin level of below 12 g/100 ml of
blood (45).   The cause of this is not clear but the anaemia
of scurvy has been attributed to the same factors as those
described above.

It has been shown that alcoholics are not folate
deficient (46), but in this survey, all alcoholics had an
inadequate intake of iron.   The incidence of anaemia in
these alcoholics is lower than expected from the dietary
results, since only five alcoholic patients had iron
deficiency anaemia.

The enzyme ferrochelatase catalyses the insertion
of iron into protoporphyrin to produce haem.   Therefore
the level of protoporphyrin in the blood is elevated if
there is a deficiency of iron or a depression of the
activity of ferrochelatase.   It is known that alcohol
depresses ferrochelatase activity (43).   In this study
there are five patients with raised E.P.P. levels but their
percentage saturation of iron is greater than 16%.   This
anaemia is due to depressed haemopoeisis.

Five patients have a serum iron level of over
200 µg% which is indicative of active hepatocellular
damage although none of the alcoholics had a clinical
diagnosis of hepatocellular failure.

The haemoglobin and the P.C.V. levels show no
difference between the normal and the alcoholic patient.
55% of these alcoholic patients show no sign of anaemia
on the basis of these haemoglobin levels.   Therefore,

in the absence of liver disease, the incidence of anaemia
produced by prolonged alcohol ingestion is much lower
than expected.

———————————

CHAPTER IV

The criteria of sub-clinical scurvy in guinea pigs.

Sub-clinically scorbutic guinea pigs were formerly defined as guinea pigs on a low ascorbic acid diet without any signs of frank scurvy. Clinical signs of scurvy in guinea pigs consist principally of emaciation, atrophy of mucosal surfaces and petechial haemorrhages on the serous surfaces (32). This definition of sub-clinical scurvy is very vague and thus it was decided to establish biochemical criteria for the condition by measuring vitamin C levels in leucocytes and in other tissues (liver, stomach and upper small intestine) of guinea pigs fed on a normal or a scorbutic diet. Previous attempts to measure ascorbic acid levels in guinea pig leucocytes were unsuccessful because of the difficulty in separating the buffy layer.

In this chapter a method is described for the separation of guinea pig blood buffy layer, and this technique was subsequently used for measurement of leucocyte ascorbic acid in guinea pigs.

Materials and Methods.

Six male guinea pigs, weight range 150-250 g. were caged individually in cages with a covering of peat and wood straw. They were fed diet 18 (Table 4.1) for two weeks. Their sole drinking fluid was tap water and they were not fed cabbage. After two weeks the animals were killed by ether anaesthesia. Between three and four millilitres of blood were withdrawn by cardiac puncture just before death and this was used for L.A.A. estimation as described overleaf. The vitamin C content of the liver, stomach

TABLE 4.1   Normal diet given to guinea pigs.

Ingredients	%	Ingredients	%
Grass meal	30	Barley	20
Bran	15	Meat and bone meal	8
Groundnut cake	15	Salt	1
Linseed cake	10	Ground limestone	1

This diet has 164 mg/kg of ascorbic acid. According to the Handbook of Biological Data, guinea pigs, both male and female, are estimated to require 100-200 mg of ascorbic acid /kg of diet for normal growth.

TABLE 4.2   Ascorbic acid deficient diet.

Ingredients	%	Ingredients	%
Skimmed milk powder	30	Vegetable oil	8
Ground rolled oats	39	Cod liver oil	2
Fine wheat bran	20	Salt	1

This diet has 81 mg of ascorbic /kg of diet.

and upper small intestine was measured in each guinea pig
by the method described overleaf.

Eighteen male guinea pigs, weight range 150-250 g. were
fed a scorbutic diet (Table 4.2) and given tap water
ad libitum.   Six guinea pigs were killed by ether
anaesthesia at the end of one week, another six were killed
after two weeks and the remaining six animals were killed
at the end of three weeks.   The leucocyte ascorbic acid
and the vitamin C level in the liver, stomach and upper
small intestine was measured in each animal.

The percentage water content of each guinea pig
tissue was measured by leaving a weighed sample of the
tissue in an oven at $110^{\circ}C$ for several days until the dry
weight was constant.

The optimum molecular weight dextran for the separation
of guinea pig buffy layer.

Vitamin C is absorbed slowly from the plasma into
the white blood cells (42) and so the best diluent for the
measurement of leucocyte ascorbic acid is that which
allows an effective separation of the bulk of the red
blood cells from the white blood cells in the shortest
possible time.   The guinea pig buffy layer does not
separate in the dextran solution used for human L.A.A.
estimations because of the differences in density of the
blood cells in guinea pig and human blood.

In order to find the optimum molecular weight
dextran for separating the guinea pig buffy layer, a range
of dextran solutions were tested.   The dextrans tested
were :

T_{10} weight average molecular weight ($\bar{M}_w$)   10,000

T_{40} "   "   "   "   40,000

T_{250} "   "   "   "   250,000

T_{500} "   "   "   "   500,000

T_{2000} "   "   "   "   2,000,000

T_{125} "   "   "   "   125,000

The latter (T_{125}) is used for separating the buffy layer of human blood. The solid dextrans were supplied by Pharmacia (Uppsala, Sweden). The solutions were all prepared in the same way viz. with 200 ml of 0.9% saline/ 50 ml of a 6% solution of the dextran in 0.9% saline/ 2 ml of 10% sequestrene in 0.9% saline.

A 3 ml aliquot of fresh blood, obtained by direct cardiac puncture from several guinea pigs and then pooled, was added to 12.5 ml of each dextran solution. The blood was allowed to settle for thirty minutes. There was no visible separation in the tubes containing T_{10}, T_{40} and T_{125} solutions but there was a very clear separation in the tubes containing T_{250} and T_{500} solutions. At the end of thirty minutes haematocrit measurements were taken from each tube. In guinea pig blood, the normal ratio of white blood cells to red blood cells is approximately 1 : 500. The ratio after separating the blood in T_{500} is approximately 1 : 1. After separation in T_{250}, the ratio is roughly 1 : 2.

Therefore, from the visual observations and the haematocrit results, the optimum molecular weight dextran for separating guinea pig blood cells is T_{500}. The separation is complete in thirty minutes.

After separation in 12.5 ml of the T_{500} dextran / saline / sequestrene solution, the supernatant layer is used for guinea pig leucocyte ascorbic acid estimations. The procedure and calculation is then exactly the same as for the estimation of L.A.A. in human blood (Chapter III, page 30).

Estimation of vitamin C levels in guinea pig tissues.

The vitamin C content of the liver, stomach and upper small intestine of guinea pigs are all measured in the same manner.

The tissue, 1 g, is homogenised in a piston homogenizer with distilled water (2 ml). 19 ml of distilled water are added to 1 ml of the homogenate. Then, equal volumes of the diluted homogenate and 10% trichloroacetic acid (T.C.A.) are mixed together in order to denature the protein in the tissue and allow all the ascorbic acid to be dissolved.

Thus, the final concentration of T.C.A. in the solution is 5% and the final dilution of the tissue is 1 : 120. At this stage, the estimation can be left overnight at 2-4°C.

The solutions are then centrifuged to remove cell debris and 1 ml of the resulting supernatant fluid is incubated with 0.3 ml of 100 vols of 2.2 g of 2,4 dinitrophenyl hydrazine in 10 N sulphuric acid / 5 vols of 5% thiourea in distilled water / 5 vols of 0.6% copper sulphate reagent for four hours at 37°C. After cooling in ice-water, 1.5 ml of 65% sulphuric acid are added and the absorbance is read, against a reagent blank, at 520 mu

in a spectrophotometer.

A calibration graph is prepared using concentrations of 0.0, 0.5, 1.0, 2.5, 3.0, 4.0 and 5.0 µg of ascorbic acid / ml in 5% T.C.A., these being treated identically to the test solutions. The test readings can then be read off the straight line graph thus obtained.

The ascorbic acid content of the tissues is expressed as µg of ascorbic acid / g of wet weight of tissue and is calculated as follows :

Conc.(graph reading) x diln. = µg of ascorbic acid/g wet wt. If the percentage water content of the tissues is known, the result can be expressed as µg of ascorbic acid / g dry weight of tissue and is calculated :

$$\frac{\text{Conc.(graph reading) x dilution}}{(1 - \% \text{ water content})} = \text{µg / g dry wt.}$$

Results.

The physiological conditions of the guinea pigs fed the scorbutic diet deteriorated during the three weeks of the experiment. There were no signs of scurvy after one week and the guinea pigs were normally active. There was however, some loss of coat hair especially about the nose. After two weeks, the animals still looked healthy but they had lost a lot of coat hair. At the end of the third week, the animals were rather inactive and they had lost weight and a lot of coat hair.

The normal range of vitamin C levels, expressed as µg/g wet weight, in the various guinea pig tissues is shown in Table 4.3. The percentage water content of

Tissue	Normal range of ascorbic acid (μg/g of wet wt.) (mean ± S.D.)	% water content (mean ± S.D.)	Normal range of ascorbic acid (μg/g of dry wt.) (mean ± S.D.)
Liver	265.8 ± 108.9	73.0 ± 1.2	985.9 ± 361.3
Stomach	123.5 ± 19.5	79.9 ± 1.3	614.8 ± 86.6
Upper small intestine	145.5 ± 19.9	81.4 ± 3.4	785.6 ± 107.4

TABLE 4.3.   Ascorbic acid levels in various guinea pig tissues.

the same guinea pig tissues is also shown in this Table.
These percentages were used to calculate the range of
ascorbic acid levels in the dry weight of the guinea pig
tissues (Table 4.3).

The leucocyte ascorbic acid levels showing the
decrease in the levels of vitamin C when the guinea pigs
were fed the scorbutic diet for a maximum of three weeks
are shown in Fig. 4.1.   There is no significant change
in the guinea pig L.A.A. levels beyond the first week.
The straight line graph obtained from these results is
drawn in Fig. 4.1.

The levels of ascorbic acid in the guinea pig
stomach decreases rapidly when the scorbutic diet is the
only food available to the animals (Fig. 4.2).   As with
the L.A.A. results, there is no significant change in the
in the levels after the initial decrease which occurred in
one week.   The vitamin C levels in the stomach decrease
exponentially with time (Fig. 4.2).   The liver ascorbic
acid levels showing the onset of sub-clinical scurvy in
guinea pigs are shown in Fig. 4.3.   There is a significant
change in the ascorbic acid levels after each week.   The
exponential curve ($y = ae^{bx}$), obtained from these results,
is also shown in Fig. 4.3.   The decrease in the vitamin C
levels found in the upper small intestine (Fig. 4.4)
mirrors the results obtained for the liver.   The decrease
is again exponential (Fig. 4.4).

Fig. 4.1   Leucocyte ascorbic acid levels,
(μg/10^8 WBC ; mean $\pm$ S.D.)
showing the onset of sub-
clinical scurvy in
guinea pigs
(39 animals) fed the
scorbutic diet.
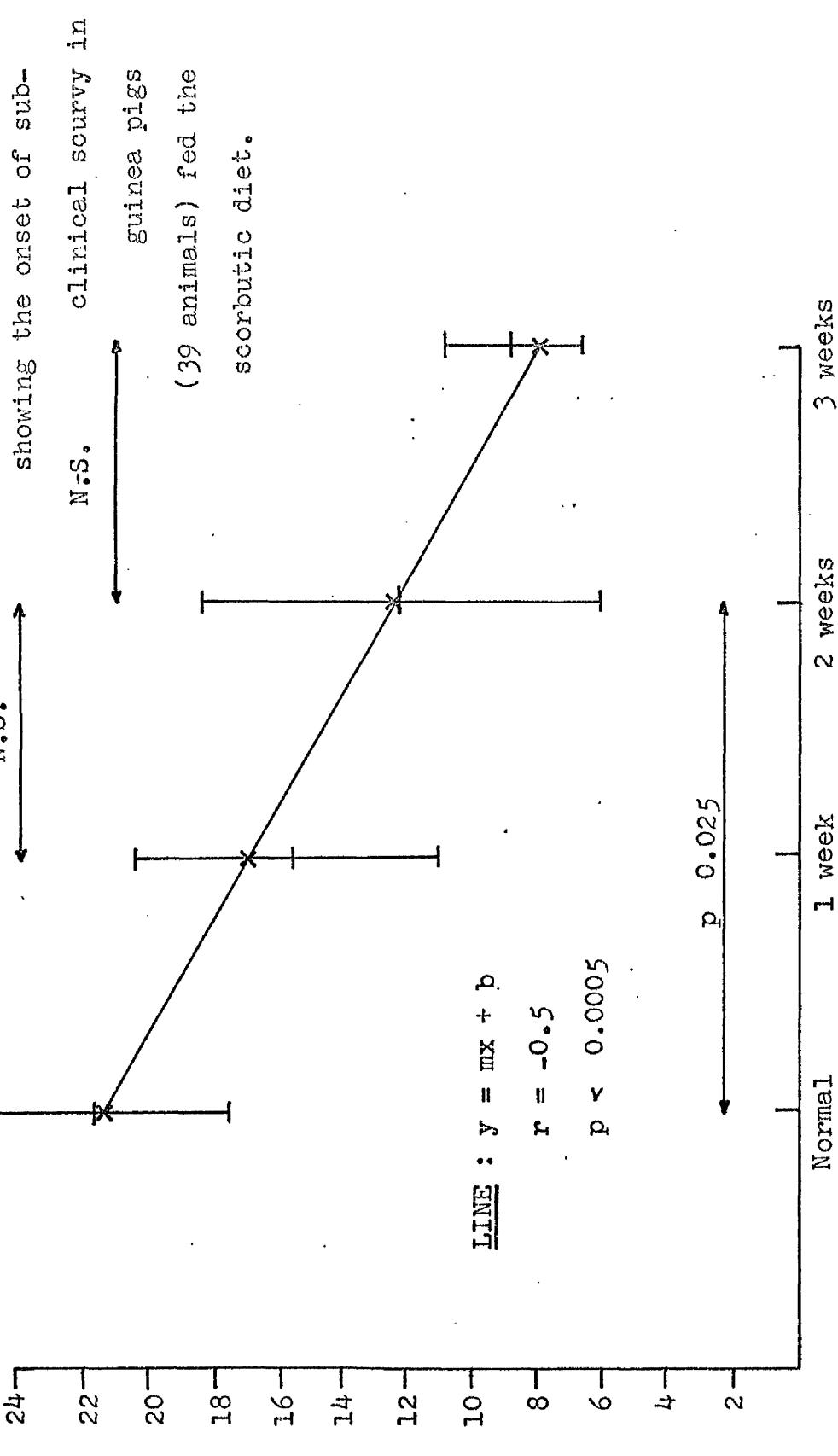
Fig. 4.2 Stomach ascorbic acid levels (µg/g wet weight : mean ± S.D.) showing the onset of sub-clinical scurvy in guinea pigs (21 animals) fed the scorbutic diet.
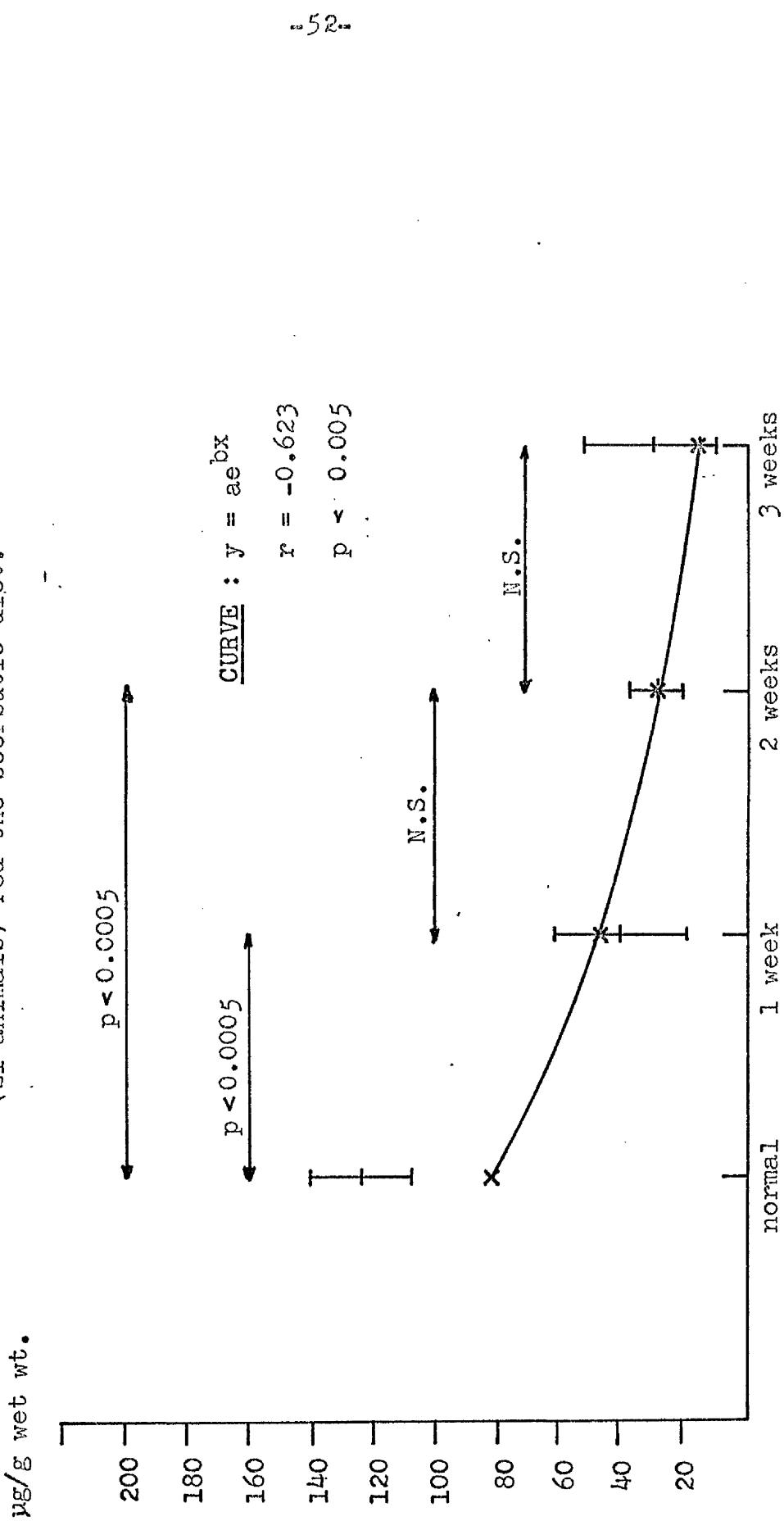
Fig. 4.3   Liver ascorbic acid levels (µg/g wet weight :
mean $\pm$ S.D.).   To show the onset of sub-clinical
in guinea pigs (21 animals) fed a scorbutic diet.

µg/g wet wt.

CURVE : $y = ae^{bx}$

$r = -0.838$

$p < 0.0005$

$p \ll 0.0005$

$p \ll 0.0125$

$p < 0.005$

$p < 0.05$

Normal   1 week   2 weeks   3 weeks
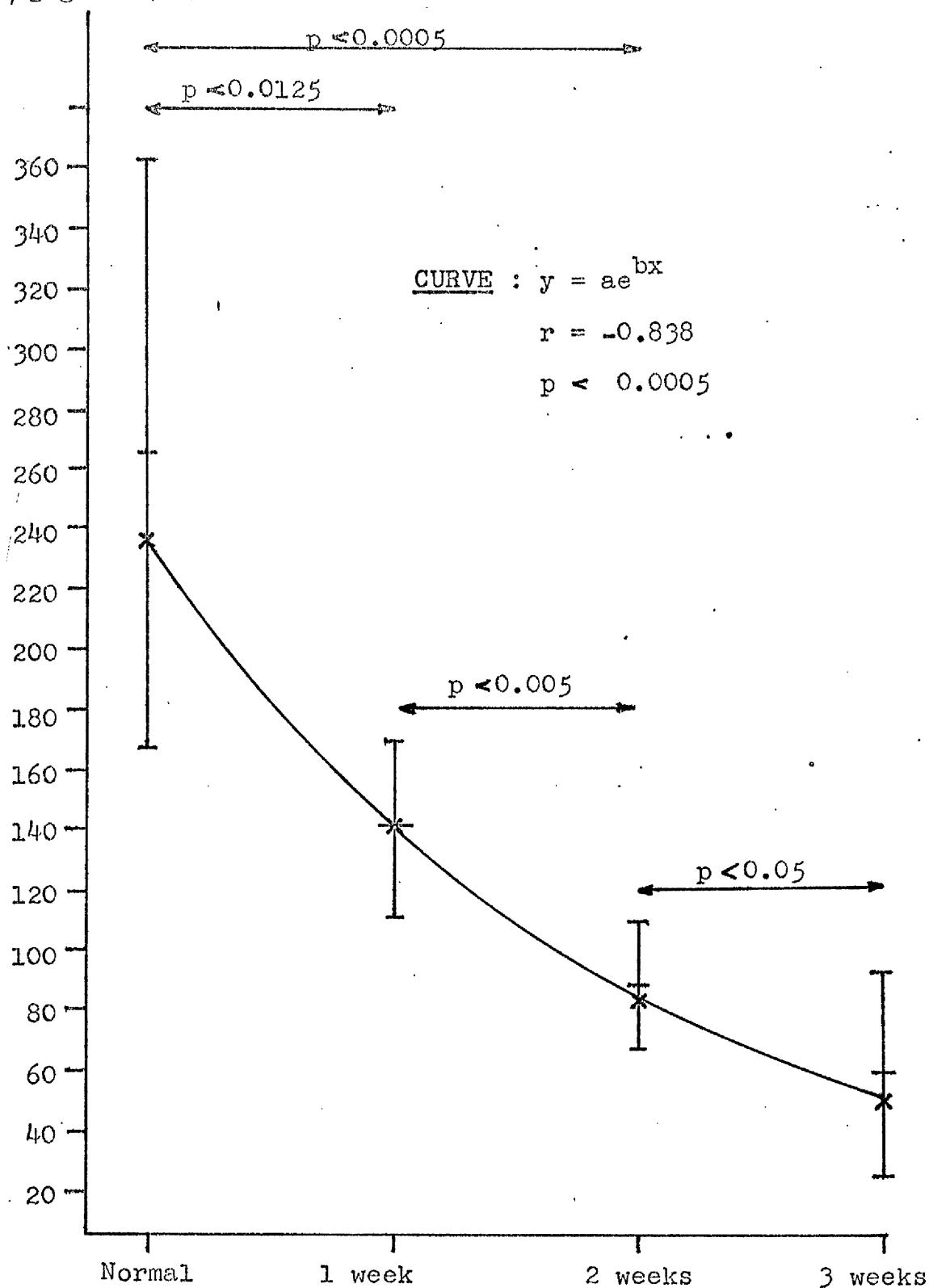
Fig. 4.4   Upper small intestine ascorbic acid levels (μg/g wet wt. : mean ± S.D.).

To show the onset of sub-clinical scurvy in guinea pigs (16 animals) fed a scorbutic diet.

CURVE : $y = ae^{bx}$

$r = -0.821$

$p < 0.0005$
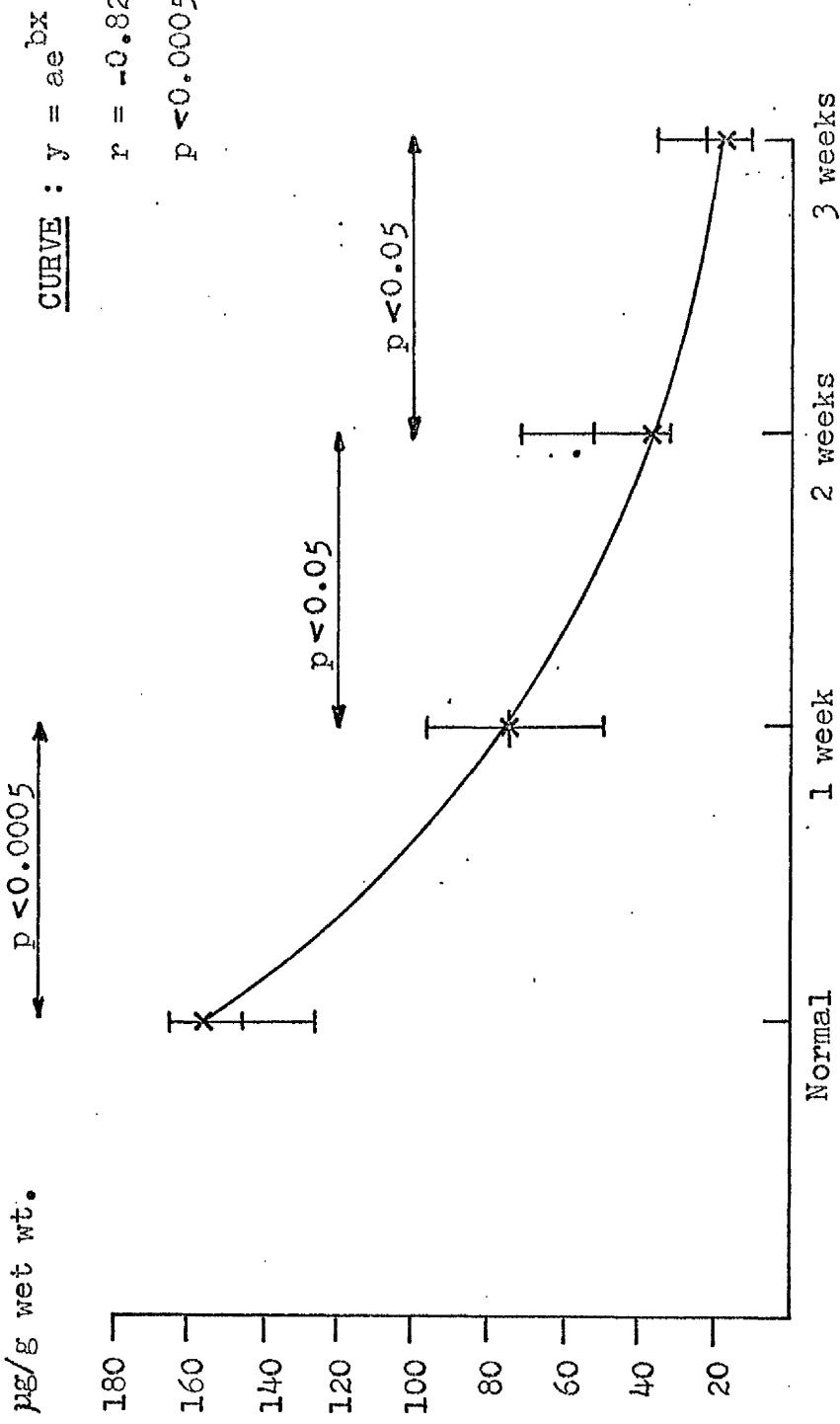
Discussion.

The vitamin C levels in the tissues depend on dietary intake and the rate of absorption. Thus there is a wide variation in the tissue levels of ascorbic acid. The liver is richer in vitamin C than the stomach and the upper small intestine. This was expected since vitamin C is essential for several reactions which occur in the liver such as the formation of the highly reactive semidehydro-ascorbic acid (15). However, vitamin C has no known function in the stomach or upper small intestine. These differences in vitamin C levels also occur in the dried tissues.

Both the physiological results and the tissue levels indicate that sub-clinical scurvy develops when the guinea pig has been fed this scorbutic diet for two weeks. Frank scurvy will develop in about three weeks. By contrast, man requires three to four months to show symptoms of scurvy. This difference is due to the fact that in man, ascorbic acid has a half-life of sixteen days whereas in the guinea pig the half-life of ascorbic acid is only four days (27). Also in man, about 1.0 mg/kg/day is required to maintain a body pool of 20 mg/kg, but in guinea pigs an average of 9.3mg/kg/day are required to maintain a body pool of 54 mg/kg. Therefore, a smaller dose of ascorbic acid, when compared on an equivalent weight basis, is required to maintain the body pool of the vitamin in man than in the guinea pig.

Equilibrium between ascorbic acid absorbed from the diet and that present in the tissues is reached in less

than four hours. Leucocyte ascorbic acid levels parallel tissue levels and although there is a wide variation in these results, they clearly indicate the onset of sub-clinical scurvy in guinea pigs. Tissue levels, especially liver levels, are also a sensitive index of the vitamin C status of the animal.

Thus these quantitative measurements in the blood and tissues are used in future experiments to define the sub-clinically scorbutic state in guinea pigs.

CHAPTER V

The levels of alcohol dehydrogenase in sub-clinically scorbutic guinea pigs.

It is an old observation that alcoholics have an upper limit of tolerance which is usually three to four times the amount of alcohol that can be consumed by the occasional drinker. The reason for this elevated bio- chemical tolerance has often been sought in an adaptive increase in the enzymatic processes involved in the oxidation of alcohol. Some recent studies (47, 48) have been concerned with the effect of chronic alcohol administration on the activity of liver alcohol dehydrogenase (ADH) in experimental animals. The data obtained from these studies are somewhat at variance with each other. Reports of in vitro studies on ethanol oxidation or activity of ADH and catalase in the liver of rats pretreated with ethanol have also been contradictory (49).

Nutrition affects the rate of ethanol metabolism. It has been shown that the rate of ethanol metabolism is diminished in fasted animals (50). Many experiments, designed to study the biochemical effects of alcohol, have been completed using laboratory animals. In these experiments, the animals are usually fed a well defined standard diet with all the supplements of vitamins and trace metals. However, the results of these experiments should be applied with caution since the diet consumed by alcoholics is very varied and often inadequate in some essential components.

Since it has been shown that, as a group,

alcoholics are sub-clinically scorbutic (Chapter III),
attempts were made to discover if this condition could
directly affect their metabolism of ethanol.   Thus an
experiment was designed to measure the levels of alcohol
dehydrogenase (ADH) in sub-clinically scorbutic guinea
pigs.

The metabolic pathway of ethanol oxidation in
guinea pigs is the same as that in man.   Ethanol is
converted to acetaldehyde in the presence of ADH with
nicotinamide adenine dinucleotide (NAD) as an essential
co-factor.   ADH is the first enzyme and is the rate
limiting enzyme in this metabolic pathway.   ADH is
induced in the liver when alcohol is taken.

Materials and methods.

Only male guinea pigs, weight 240-450 g, were
used in these experiments.

Experiment 1.

. Sixteen male guinea pigs were divided into two
groups each comprising eight animals.   One group was
given diet 18 and cabbage and the other was given the
scorbutic diet (Table 4.2).   Both groups were given tap
water as their sole drinking fluid.   The guinea pigs were
caged individually and were given fresh aliquots of food
and liquid every day.   They were maintained in these
groups for twelve days.   On the next two consecutive days,
six animals from each group were injected intraperintoneally
with 2 ml of 30% ethanol (Burrough's 95% ethyl alcohol)
in saline (v/v) and given 10% ethanol in tap water as
their sole drinking fluid.   At the end of two weeks, the

guinea pigs were killed by cervical dislocation and the liver rapidly excised for the measurement of ADH by the method described overleaf.   The guinea pigs were injected with alcohol on the last day of the experiment and were killed 90 minutes from the time of injection.

A blood sample was taken from all the water drinking animals and half of the alcohol drinking animals for L.A.A. estimation by the method described in Chapter IV. The part of the liver not used for ADH measurements was taken from half of the guinea pigs and stored at -70°C. until the liver ascorbic acid levels were measured by the dinitrophenyl method described in Chapter IV.

Experiment 2.

This experiment was conducted exactly as above but the guinea pigs were maintained in the first groups for only one week.   During the second week, the animals were split into four groups, since six animals in each of the original groups were injected daily with ethanol and given ethanol as their sole drinking fluid in the same doses as in Experiment 1.   At the end of the second week, all the guinea pigs were killed by cervical dislocation and the liver was rapidly excised for the measurement of ADH.

Blood samples were obtained for L.A.A. measurements and the livers were stored for liver ascorbic acid measurements as before.

Experiment 3.

Sixteen male guinea pigs were divided into the following four groups   :

TABLE 5.1

	Food	Liquid	No. of animals
1.	Diet 18 + cabbage	Tap water	2
2.	Diet 18 + cabbage	10% ethanol in tap water (v/v)	6
3.	Scorbutic diet	Tap water	2
4.	Scorbutic diet	10% ethanol in tap water (v/v)	6

The experiment was carried out as before. The guinea pigs in the alcohol groups were injected intra-peritoneally with 2 ml of 30% ethanol in saline daily for two weeks. At the end of this period, ADH levels were measured in all the guinea pigs, blood samples were obtained for L.A.A. estimations and livers were stored from half the guinea pigs for ascorbic acid measurements.

Experiment 4.

In this experiment, sixteen male guinea pigs were split into two groups with eight animals in each. All the animals were fed diet 18 and given cabbage every day. Four animals were given water as their sole drinking fluid while the other twelve were given 10% ethanol in tap water. The latter group were injected daily with 2 ml of 30% ethanol in saline. The guinea pigs were maintained in these groups for one week. After this, the guinea pigs were divided into the four groups described in Table 5.1. and the animals receiving alcohol were injected daily. The guinea pigs were maintained in these

four groups for two weeks.   At the end of three weeks,
the ADH levels in the liver were measured along with the
L.A.A. and the liver ascorbic acid levels.

Experiment 5.

Two dozen male guinea pigs, weight range 234- 275g,
were divided into the same groups as described in Table 5.1.
There were six animals in each group.   The guinea pigs
in the alcohol drinking groups were injected intra-
peritoneally with 2 ml of 30% ethanol in saline.   The
animals were maintained in these groups for two weeks.
At the end of two weeks, the guinea pigs were killed by
cervical dislocation and the livers rapidly excised for
the measurement of nicotinamide adenine dinucleotide
(NAD) by the method of Jedeiken and Weinhouse (51).

Method for the measurement of ADH in guinea pig liver.

The guinea pig is killed by cervical dislocation
and the liver is rapidly excised. The liver sample, 1 g,
is homogenised with 3 ml of 0.1M sodium pyrophosphate
buffer (pH = 9.0) in a piston homogeniser.   The homogenate
is then sonicated for exactly one minute at medium power
and amplitude 4 using an M.S.E. 10 k.watt ultrasonic
disintegrator.   Sonication breaks down the cell walls
and allows all the ADH to be released from the cell.
After sonication, the samples are spun down at 10,000 g
at $4^{o}C$ for thirty minutes to remove most of the cell
debris and then the supernatant fraction is re-spun at
100,000 g at $4^{o}C$ in an ultracentrifuge for one hour (52).
All the ADH is contained in the clear supernatant fraction.

The levels of ADH are estimated indirectly on a

spectrophotometer (Pye-Unicam S.P. 8000). The increase
in optical density at 366 mμ indicates the rate of formation
of reduced nicotinamide adenine dinucleotide (NADH),
which depends quantitatively on the amount of ADH in the
cell. The determination of the activity depends on
the reaction :

$$C_2H_5OH + NAD \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+$$
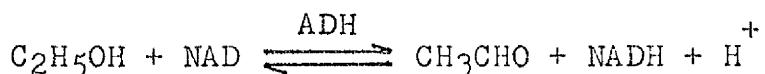
In alkaline solution, and by trapping the acetaldehyde
with semicarbazide, the equilibrium is shifted almost
quantitatively to the right.

The assay mixture contains :

0.1M sodium pyrophosphate buffer	2.57 ml
2.2M semicarbazide solution	0.10
96% ethanol	0.10
0.03M NAD solution	0.20
0.3M glutathione solution	0.01

This solution is mixed and the reaction is started by
the addition of 0.02 ml of the supernatant, which is the
enzyme containing solution. The solution is again mixed
and the optical density at 366 mμ and 25°C is read every
minute for five minutes. The mean change in extinction
(ΔE/min) is calculated from these readings.

The calculation is completed as follows :

$$\frac{\Delta E/min.V.}{\epsilon.d.v.} = \text{Units/ml enzyme sample solution.}$$

Where :

$\Delta E/\text{min}$ = optical density difference/minute.

V = assay volume in ml. (3 ml).

ε = extinction coeff. of NADH (3.3cm^2/mu at 366mu).

d = light pathway in cm. (1 cm).

v = sample volume in ml. (0.02 ml).

From this, it follows that :

$\Delta E/\text{min} \times 45.5$ = units/ml enzyme sample solution.

and the specific activity is calculated :

$$\frac{\Delta E/\text{min} \times 45.5}{\text{mg. protein taken}} = \text{units/mg. protein}$$

The protein content of the solution is measured by the Lowry method (54). One enzyme unit is the amount of enzyme which catalyses the transformation of 1 μ.mole of substrate per minute. (The abbreviation mu is used to denote milli-unit).

Results.

.ADH was maximally induced in guinea pigs fed ethanol within two days. The levels of alcohol dehydrogenase in the groups fed alcohol with either diet 18 or the scorbutic diet did not differ significantly beyond two days (Fig. 5.1). Consequently, all the results were pooled and only four groups were considered. Figure 5.2 shows the ADH levels in each of the collective groups.

The NAD levels measured in the guinea pig livers are shown in Fig. 5.3. The levels of L.A.A. measured in guinea pig blood and the liver ascorbic acid levels are shown in Table 5.2.

TABLE 5.2 Leucocyte and liver ascorbic acid levels in guinea pigs.

Group	No. of animals	L.A.A. levels (μg/10^8 WBC; mean ± S.D.)	No. of animals	Liver ascorbic acid levels (μg/g; mean ± S.D.)
Water + Diet 18	10	43.84 ± 17.06	6	148.25 ± 41.92
Alcohol + Diet 18	13	45.50 ± 15.50	10	184.50 ± 58.88
Water + Scorbutic diet	12	15.73 ± 5.26	6	38.80 ± 6.68
Alcohol + Scorbutic diet	11	12.31 ± 5.6	10	51.13 ± 12.30

Fig. 5.1. ADH levels in guinea pigs given 2 ml of 30% ethanol in saline daily and 10% ethanol as their sole drinking fluid, for different times (6 animals each group).
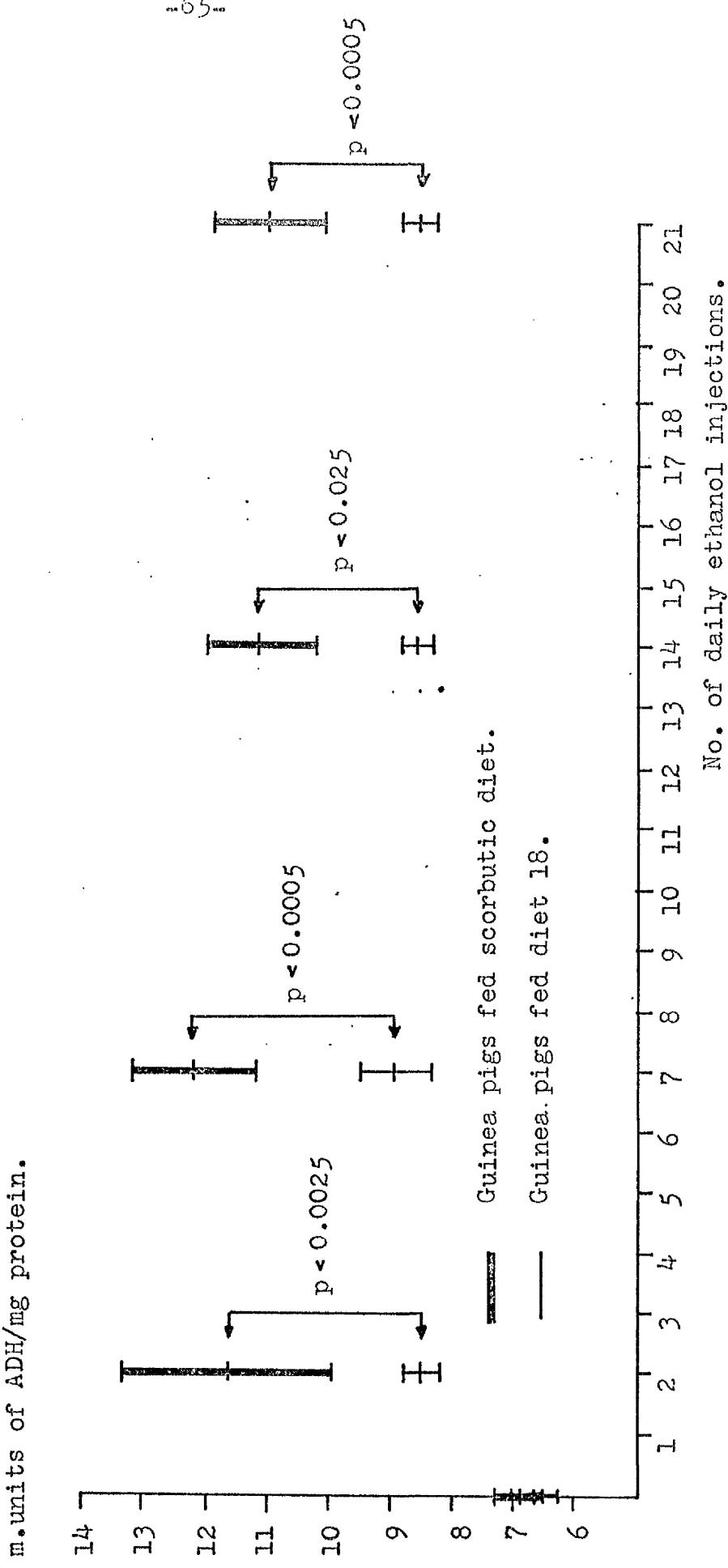
Fig. 5.2   ADH levels in guinea pig livers

m.units of ADH
/mg protein.
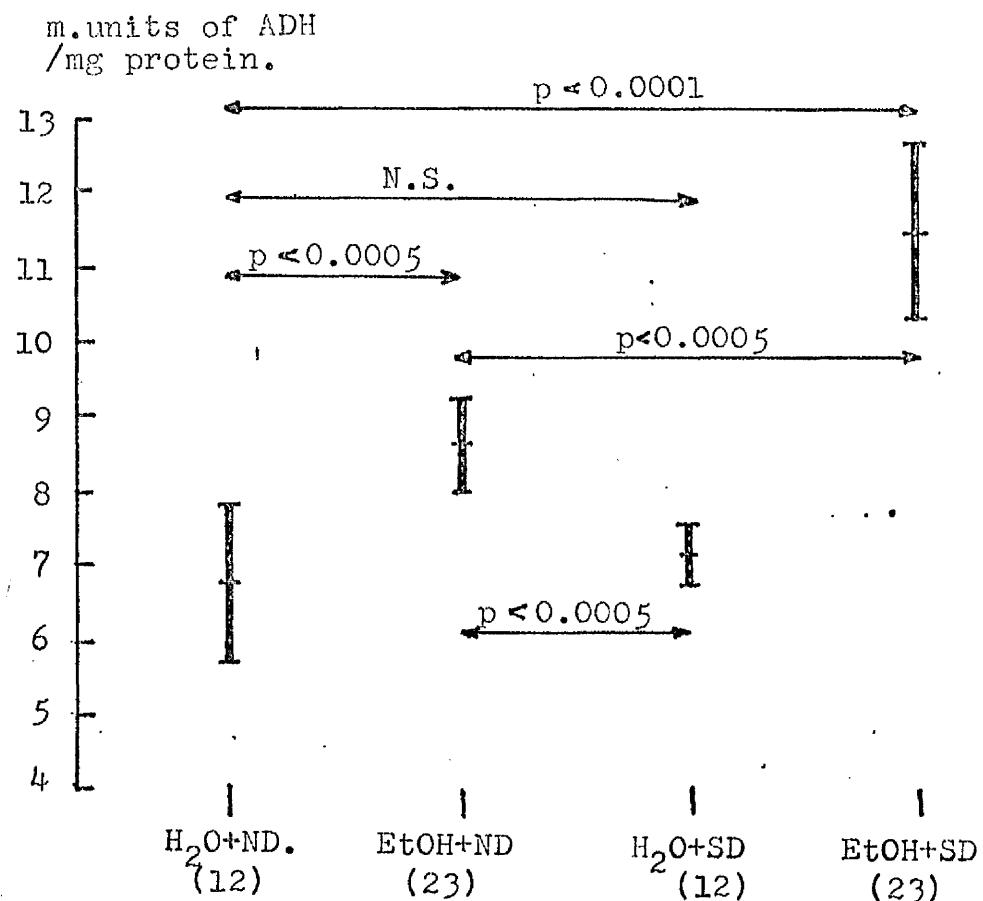
H$_2$O + ND : Tap water with diet 18.

EtOH + ND : 10% ethanol and ethanol injected with diet 18.

H$_2$O + SD : Tap water with scorbutic diet.

EtOH + SD : 10% ethanol and ethanol injected with
              scorbutic diet.

Fig. 5.3   The levels of NAD in guinea pig liver.
The animals which were receiving ethanol were injected
daily for two weeks with 2 ml of 30% ethanol in saline
and given 10% ethanol in tap water as their sole drinking
fluid.
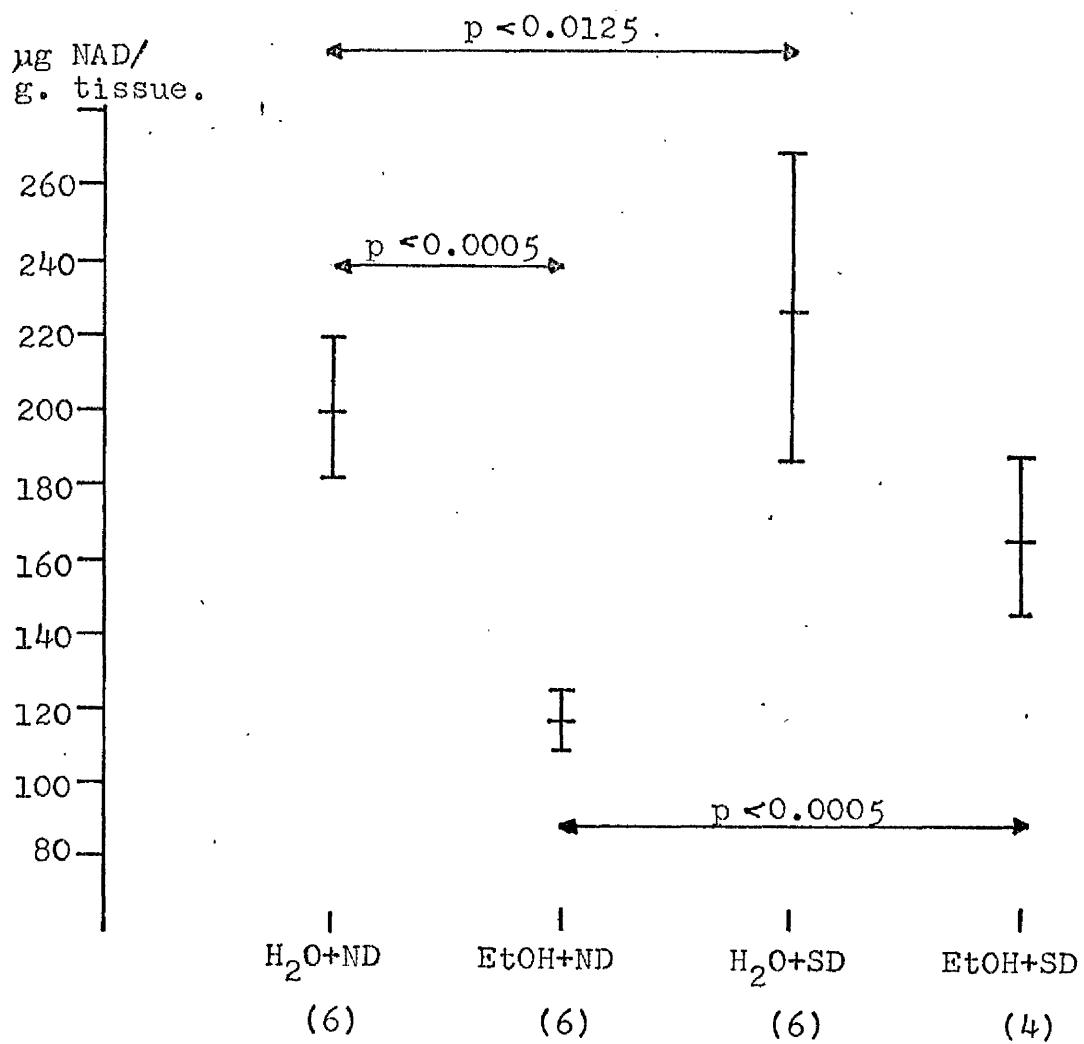
Discussion.

      Under these conditions of ethanol feeding, ADH
is maximally induced in guinea pigs within two days. The
levels of L.A.A. and liver ascorbic acid show that the
guinea pigs fed the scorbutic diet are sub-clinically
scorbutic. Thus there is a dramatic and highly significant
increase in the ADH levels of sub-clinically scorbutic
guinea pigs fed ethanol. Since hepatic metabolism is by
far the most significant mechanism for the elimination of
ingested ethanol in both man and guinea pig, an increase
in such an important enzyme could partly explain the
tolerance of alcoholics for alcohol.

      Since ADH is the rate limiting enzyme in the path-
way, it has been presumed that factors which affect the
rate of ethanol metabolism _in vivo_ do so through alterations
in the rate of the initial oxidation reaction. The main
factors which determine the rate of the ADH-catalysed
dehydrogenation of ethanol are the concentration of the
substrate, the level of ADH and the availability of NAD.
The concentration of the substrate influences the rate of
this reaction only at levels below which the enzyme is
'saturated'. It has been shown in dogs that the rate of
ethanol metabolism is proportional to the concentration
of ethanol in blood at levels below 10 mg% ; above this
concentration, saturation of the enzyme occurs and the rate
of ethanol metabolism is independent of the ethanol
concentration (56). Since ADH is saturated at such low
substrate concentrations, the availability of the enzyme
becomes most important in the determination of the rate of

ethanol dehydrogenation at ethanol concentrations usually
employed in animal and human studies.   Thus, in sub-
clinically scorbutic states, where the levels of ADH are
increased, the rate of alcohol metabolism will be
significantly increased.

The preceding statement is likely to be true
only if one assumes an optimal supply of NAD to participate
in the dehydrogenation reaction.   With the oxidation of
ethanol to acetaldehyde and the formation of acetyl CoA
from acetaldehyde, NAD is reduced to NADH.   If the supply
is not to be exhausted, NADH must be constantly re-oxidised
to NAD via some other oxidation-reduction reaction.   It
is known that there is some disparity between the rates
of production of NADH and its oxidation to NAD as evidenced
by the increase in the ratio NADH : NAD which occurs in
the liver during ethanol metabolism (57).   Since the sum
of NAD and NADH is always constant in a cell, the results
in this study, that is, a decrease in NAD levels when
ethanol is injected, also indicate such a disparity.

Thus, metabolic systems involved with the oxidation
of hepatic NADH may secondarily affect the rate of ethanol
metabolism by influencing the availability of the oxidised
co-factor.   Dehydroascorbic acid is reduced to ascorbic
acid in several reactions which occur in the cell.
During this reaction, NADH is re-oxidised to NAD (Fig. 5.4).
In the sub-clinically scorbutic state the amount of NAD
present in the cell is increased.   Thus, in sub-clinical
scorbutic states, more NAD is available for alcohol
metabolism.   The results show that when ethanol is fed to

Fig. 5.4

Ethanol ⇌ Acetaldehyde → Acetyl CoA

NAD   NADH   NAD   NADH

Ascorbic acid ← ——————— Dehydroascorbic acid

————————
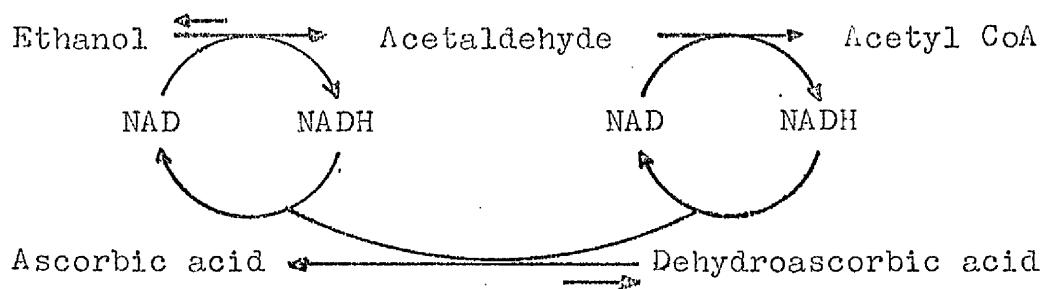
the sub-clinically scorbutic animal, there is a significant
increase in the level of NAD (p < 0.0005). Thus, this whole
cycle is encouraged by a lack of ascorbic acid and it has
already been shown that alcoholics do lack ascorbic acid.

————————

REFERENCES :

1. Rubin E., and Lieber C.S.,

    Annals of Int. Med. 69. 5, (1968).

2. Cooke A.R.,

    Am. J. of digestive dis. 15. 5, (1970).

3. Forsander O.A.,

    J. biol. Chem. 235. 34, (1960).

4. Westerfield W.W.,

    Am. J. clin. Nutr. 9. 426, (1961).

5. Blair H.A., and Vallee B.L.,

    Biochem. 5. 6, (1966).

6. von Wartburg J.P., and Schurch P.M.,

    Ann. N.Y. Acad. Sci. 151. 936, (1968).

7. Orme-Johnson W.H., and Ziegler B.M.,

    Biochem. Biophys. Res. Commun. 21. 78, (1965).

8. Roach M.K., et al.

    Biochem. Biophys. Res. Commun. 36. 4, (1969).

9. Lieber C.S., and De Carli L.M.,

    J. biol. Chem. 245. 10, (1970).

10. Scheig R.,

    Am. J. clin. Nutr. 23. 4, (1970).

11. Hughes R.E., and Hurley R.J.,

    Br. J. Nutr. 23. 211, (1969).

12. Hughes R.E., and Jones P.R.,

    Nutr. Report International 1. 5, (1970).

13. Lowry O.H., Bessey O.A., and Burch H.B.,

    Proc. Soc. Exp. Biol. and Med. 80. 361, (1970).

14. Takeniouchi K. et al.

    J. Vitaminology 12. 49, (1966).

15. Schneider W., and Standinger H.,

    Klin. Wachr. 42. 879, (1964).

16. Robertson W. van B.,

    Ann. N.Y. Acad. Sci. 92. 159, (1961).

17. Levin et al.

    J. biol. Chem. 235. 2080, (1960).

18. Chalopin et al.

    Wld. Rev. Nutr. Diet. 6. 165, (1966).

19. Hodges J.R., and Hotston R.T.,

    Br. J. Pharmacol. 39. 1, (1970).

20. Mazur A.,

    Ann. N.Y. Acad. Sci. 92. 223, (1961).

21. Goldberg A.

    Quart. J. Med. 32. 125, (1963).

22. Bothwell et al.

    Brit. J. Haemat. 10. 50, (1964).

23. Sahud M.A., and Aggeler P.M.,

    Proc. Soc. Exp. Biol. and Med. 134. 1, (1970).

24. Cooper J.R.,

    Ann. N.Y. Acad. Sci. 92. 208, (1961).

25. Woodruff C.W.,

    Beaton and McHenry, (Eds) Nutrition Vol. 2. 265.

    Academic Press, N.Y. 1964.

26. Gibson et al.

    Br. Med. J. 1. 1152, (1966).

27. Hellman L., and Burns J.J.,

    J. biol. Chem. 230. 923, (1958).

28. Tolbert et al.,

   Am. J. clin. Nutr. 20. 3, (1966).

29. Baker et al.,

   Am. J. clin. Nutr. 19. 6, (1966).

30. Allen R.S.L., and Brook M.,

   Br. J. Nutr. 22. 555, (1968).

31. Hansky J., and Allmand F.,

   Austr. Ann. Med. 18. 248, (1969).

32. Universities Federation of Animal Welfare (1957);

   Handbook on the Care and Management of Laboratory

   Animals, p 233. London.

33. Steingold A., and Roberts A.A.,

   Gut 2. 75, (1961).

34. Augur N.A.

   Gastroenterology 58. 3, (1970).

35. Goodman and Gilman,

   The Pharmacological Basis of Therapeutics. 2nd. Ed.

   Chapter 8. (1955).

36. Lynch et al.

   Am. J. clin. Nutr. 20. 641, (1967).

37. Jandl J.M.,

   J. clin. Invest. 34. 390, (1955).

38. Bothwell et al.

   Br. J. Haemat. 20. 155, (1971).

39. Gibson et al.,

   See Ref 26.

40. Ramsay W.M.N.,

   Biochem. J. 57. 17, (1954).

41. Rimmington C.,

   Broadsheet No. 36. N.S.

   Association of Clinical Pathologists. (1961).

42. Denson K.W., and Bowers E.F.,

Clin. Sci. 21. 157, (1961).

43. Moore M.R.,

Ph.D. Thesis, Univ. of Glasgow. 1970.

44. Kimber et al.,

Quart. J. Med. 34. 33, (1965).

45. Booth J.B., and Todd G.B.,

Br. J. Hosp. Med. Review, 1970.

46. Williams I.R., and Girdwood R.H.,

Scot. Med. J. 15. 285, (1970).

47. Dajani R.M., Danielski J. and Orten J.M.,

J. Nutr. 80. 196, (1963).

48. Figueroa R.B., and Klotzy A.P.,

Am. J. clin. Nutr. 11. 235, (1962).

49. See Ref. 47.

50. Forsander et al.,

Biochem. J. 94. 259, (1964).

51. Jedeiken L.A., and Weinhouse S.,

J. biol. Chem. 213. 271, (1955).

52. Greenberger et al.,

Lab. Invest. 14. 3, (1965).

53. Mannheim Boehringer Information Pamphlet.

54. Lowry et al.,

J. biol. Chem. 193. 265, (1951).

55. Marshall E.K., and Frity W.F.,

J. Pharmacol. and Exp. Therap. 109. 431, (1953).

56. Smith M.E., and Newman H.W.,

J. biol. Chem. 234. 1544, (1959).