BIOCHEMICAL AND HAEMATOLOGICAL ASPECTS OF ETHANOL METABOLISM IN HUMANS

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ABSTRACT

Macrocytosis or raised mean cell volume (MCV) (which as measured by the Coulter S counter) is one of the results of alcohol abuse. There is a need to identify (chronic) alcoholics by laboratory tests. The obvious measurement of blood alcohol is not suitable as ethanol is so rapidly cleared from the body. It is usually undetectable 2 - 3 hours after drinking. To this the following battery of tests: MCV, fast haemoglobin, gamma glutamyl transferase and thiamine, have been examined.

These tests which were performed, on a population consisting of 115 random hospital patients, 14 patients attending diabetic clinic and 13 'normal' volunteers. For ethical reasons it was not possible to obtain samples from known alcoholics. Instead those samples which contained red cells above 92 fl of MCV were suspected of including alcoholics and correlated with other parameter which may be assumed to be elevated in alcoholics.

The results showed that there were 23 abnormal findings likely to be associated with heavy drinkings in 70 bloods selected for high MCV.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

General

Gly Hb	glycosylated haemoglobin
Hb	haemoglobin
Hb A	adult haemoglobin
IEF	isoelectric focussing
MCV	mean cell volume (mean corpuscular volume)
PCV	packed cell volume
RBC	red blood cell
THF	tetrahydrofolate

Chemicals, enzymes

ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetate (potassium salt)
GDH	glycerolphosphate dehydrogenase
GGPNA	gamma glutamyl-p-nitroanilide
GGTP	gamma glutamyl transpeptidase (gamma glutamyl transferase)
HMFF	hydroxymethylfurfural
NADH	reduced nicotinamide adenine dinucleotide
TBA	2-thio barbituric acid
TCA	trichloroacetic acid
TIM	triose phosphate isomerase
TK	transketolase
TPP	thiamine pyrophosphate
Tris	tris (hydroxymethyl) aminomethane

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CHAPTER 1

INTRODUCTION

Excessive drinking is becoming increasingly recognised as a major social, economic and medical problem in New Zealand. There is no accurate measure of the number of alcoholics, but experts in the field suggest that there are at least 53,000. In addition, there are estimated to be over 200,000 excessive drinkers (New Zealand Official Year Book, 1981). For 1977, the per capita consumption was 8.54 litres of absolute alcohol per annum. This figure shows a dramatic increase over previous consumption levels such as 5.5 litres/annum in 1965 and 3.0 litres/annum in 1945 (Batt, 1978). People who drink excessively usually have a poor work performance and absenteeism which constitutes a loss to the work-force. They are more frequently involved in crime and in disruption to family life. Alcohol may account for many of the road accidents because increasing blood alcohol levels result in a progressive impairment of driving performance.

The consumption of excessive alcohol is known to cause many medical problems. With a wide distribution through the body organs and diffusion into individual cells, one can readily appreciate that many cellular functions are affected by alcohol. The number of hospital admissions attributable to alcohol consumption is not known in New Zealand, but various reports from overseas suggest that anything from 8.7-27.0 percent of patients are excessive drinkers (Crawford, 1979).

Any toxin, such as alcohol, can interfere in the production of blood cells in the main haematopoietic organ, the bone marrow. From clinical studies, alcohol is implicated in nearly all the processes involved; of stimulation of stem cells; of mitosis; synthesis of proteins and the development of the cell membrane into the mature erythrocyte (Hillman, 1975). The principal abnormality found in the blood of heavy drinkers is an increase in the mean cell volume (Wu et al, 1974).

The effect of alcohol on the liver has been studied most extensively because of its primary responsibility for metabolizing alcohol and it may be affected most frequently of all the organs. Enzyme induction, fat infiltration and toxic damage are the main effects caused

by alcohol on liver (Belfrage et al, 1973). Elevated levels of liver enzymes occurring in the blood depend on increased levels within the cell and subsequent released on tissue injury and such changes have been observed to occur in response to alcohol intake (Morgan et al, 1981).

During the early stages of alcohol consumption, excessive accumulation of lipids in the liver occurs with an associated enhanced mortality (Lieber, 1975). From experiments in baboons, Lieber (1975) has shown that alcohol, independent of dietary deficiencies, accounts for fat infiltration and collagen accumulation to leading to cirrhosis.

It was reported by Zieve (1958) that alcoholic liver disease may lead to premature destruction of red cells by haemolysis. Cooper (1980) concluded that such haemolysis involved an interplay between several factors. Congestive splenomegaly, with its attendant conditioning of red cell membranes to form spherocytes, leads ultimately to destruction of the cells in the spleen. Target cell formation, resulting from a proportional transfer of cholesterol and lecithin from plasma lipoproteins to red cell membranes, increases the surface area of the cell and thereby counteracts the effects of splenic conditioning minimizing haemolysis. Spur cells may also be formed by a selective transfer of cholesterol from plasma lipoproteins to red cell membranes. Such membranes have decreased fluidity with impairment of deformability and distorted membrane architecture, so increasing the spleen's damaging effect on red cells. All these abnormalities of membrane lipids may be accounted for by liver dysfunction.

Blood with a high mean corpuscular volume contains a greater proportion of macrocytic cells which may be a consequence of asynchronism between nuclear and cytoplasmic maturation (Wintrobe, 1961). During the development of the erythrocyte, before denucleation, the unclumped appearance of the nuclear chromatin, typical of an uncommitted blast cell, persists even though synthesis of haemoglobin in the cytoplasm is evident. The adequate development of nucleo-proteins require sufficient quantities of folic acid and vitamin B_{12}, as both are required in the transfer of one-carbon fragments into nucleic acids. The methyl groups are transferred from N-5-methyl tetrahydrofolic acid into methionine within the developing erythrocyte with subsequent release of tetrahydrofolate for recycling. A vitamin B_{12} dependent enzyme, homocysteine-5-methyl THF transferase is required to catalyse this reaction (Herbert et al. 1976).

Racker et al (1953) and Horecker et al (1953) reported that transketolase was responsible for the metabolism of pentose phosphate with thiamine pyrophosphate as a cofactor. Wolfe et al (1958) found that the transketolase activity was markedly reduced due to a deficiency of thiamine in a group of alcoholic patients with Wernicke's encephalopathy. Subsequently, red cell transketolase has been used to detect thiamine deficiency in man (Brin, 1963). It has been postulated that thiamine has a role in nervous tissue that is distinct from its function as a coenzyme (Cooper et al, 1976). Cooper et al (1976) found that in subacute necrotizing encephalopathy, an inhibitor prevented the synthesis of thiamine triphosphate from thiamine diphosphate and as a result, thiamine triphosphate was deficient in the brain. It is clear that thiamine deficiency can cause brain damage and transketolase activity can be used to test thiamine deficiency.

Acetaldehyde, the first intermediate in ethanol metabolism, is known to be highly toxic (Stowell, 1977), and that in spite of high blood ethanol concentration and a relatively high rate of metabolism, blood acetaldehyde concentration are extremely low (Couchman & Crow, 1980). Stowell (1977) suggested that these low blood concentrations may be due to a binding mechanism. Recently, Stevens et al (1981) reported that acetaldehyde formed an adduct with haemoglobin during heavy drinking in a similar manner to that of glucose in diabetics. This adduct formation may play a role in binding acetaldehyde and reducing blood acetaldehyde concentration below a point at which cellular damage could occur. Stevens et al (1981) suggested that the presence of the adduct would correspond to an integrated measure of prior alcohol consumption. It was decided to examine such adduct formation in vitro and in vivo.

CHAPTER 2

HAEMOGLOBIN ADDUCTS

2-1. INTRODUCTION.

Haemoglobin (Hb) is the major protein in the erythrocyte and is produced during the last stages of red cell maturation. It can be modified *in vivo* by reaction with small molecules such as glucose and urea, both of which can occur in excessive amounts in the body in certain medical conditions.

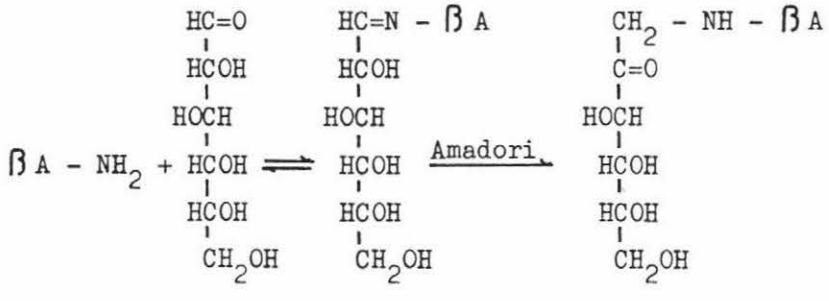
2-2. GLYCOSYLATION

The first modification to be discovered was glycosylation which was found to occur in diabetics (McDonald *et al*, 1978). Glucose can form adducts with the N-terminal valine of beta chains of haemoglobin A (Koenig *et al*, 1977) and the negatively charged adducts can be isolated chromatographically as fast minor components of haemoglobin (Trivelli *et al*, 1971). Human red blood cell haemolysate contains the following four minor Hb components:-

Hb A_{1a1}	$\alpha_2(\beta\text{-N-CHO-P})_2$	0.2 %
Hb A_{1a2}	$\alpha_2(\beta\text{-N-glucose 6 phosphate})_2$	0.2 %
Hb A_{1b}	$\alpha_2(\beta\text{-N-CHO})_2$	0.5 %
Hb A_{1c}	$\alpha_2(\beta\text{-N-glucose})_2$	4.0 %

These components are collectively referred to as the Hb A_1 fraction, and can be resolved using cation-exchange chromatography.

Hb A_{1c} is formed by the reaction of glucose with the NH_2-terminal amino group of the β chain to form an aldimine linkage, which subsequently undergoes an Amadori rearrangement to form the more stable ketoamine linkage (Bunn *et al*, 1978). The reaction is shown as follows:-



Hb Glucose Aldimine (Schiff base) Ketoamine (Hb A_{1c})

It may be elevated 2-3 fold in patients with diabetes mellitus, and has been used increasingly as a means of monitoring hyperglycaemia (Cole et al, 1977).

2-3. CARBAMYLATION

The second modification was discovered by Flückiger et al, (1981) who reported that the increase in Hb A_1 and Hb A_{1c} in uraemia was largely caused by carbamylation of haemoglobin, usually from a condensation of urea-derived cyanate with the N-terminal amino groups. The reaction is shown as follows:-

$$H_2N - \overset{\overset{O}{\|}}{C} - NH_2 \rightleftharpoons NH = C = O$$

Urea Cyanate

$$R - NH_2 + HN = C = O \longrightarrow R - \overset{\overset{H}{|}}{N} - \overset{\overset{O}{\|}}{C} - NH_2$$

Amino group Cyanate Carbamylation of amino group

2-4. ACETALDEHYDE ADDUCT

Another modification of Hb has been suggested by Stevens et al (1981). Acetaldehyde, like reducing sugars, may form adducts with valine, lysine and tyrosine. The suggested chemical reaction is shown as follows:-

$$\beta A - NH_2 + CH_3 - \overset{\overset{H}{|}}{C} = O \qquad CH_3 - \overset{\overset{H}{|}}{C} = N - \beta A \qquad CH_3 - \overset{\overset{H_2}{\|}}{C} - NH - \beta A$$

Hb Acetaldehyde Acetaldehyde adduct

It was suggested that a single exposure of red cells or haemolysate to 30-300 µmol/l acetaldehyde led to a stable modification of 0.2-1.0 % of the total haemoglobin.

2-5. ASPIRIN ADDUCT

In a seminar on glycosylated proteins held on 29th May, 1982 in Massey University, some authorities claimed that excess aspirin could increase the amount of fast Hb by acetylating the terminal amino group.

2-6. THE DETERMINATION OF HAEMOGLOBIN ADDUCTS

The presence of haemoglobin adducts have been determined in various ways. Kynoch et al (1977) separated the haemoglobin components by column chromatography on a cation exchange resin. The faster moving glycosylated haemoglobin eluted from the column ahead of the slower moving haemoglobin, and could be measured colorimetrically.

Electrophoresis has also been used to distinguish haemoglobin when there has been amino acid substitution in the peptide chain (Jeppsson, 1977). Isoelectricfocussing yields greater resolution of components and may separate the haemoglobin adducts. Lastly, the glucose moiety attached to the haemoglobin molecule may be converted to hydroxymethylfurfural and measured spectrophotometrically (Flückiger et al, 1976).

These three methods are examined in detail.

2-7. METHODS

2-7-1. Column chromatography

The ion-exchange resin Biorex 70 is a non-spherical copolymer of methacrylic acid and divinylbenzene having a functional group of carboxylic acid enabling the separation of fast moving haemoglobins, Hb A_{1a1}, Hb A_{1a2}, Hb A_{1b}, and Hb A_{1c} from the slower moving haemoglobins Hb A and Hb A_2. The binding of haemoglobin to the resin depends on the sodium ion concentration and pH of the eluting buffer. Cyanide is used in the buffer to stabilise the haemoglobin in its cyanmet Hb form. The methods used for the separations were based on those described by Kynoch et al (1977) and Chou et al (1978).

2-7-1-a. Preparation of resin.

Bio-rex 70, 200-400 mesh (Bio-Rad Laboratories, California, U.S.A.), a weakly acidic carboxylic cation exchanger, was precycled and equilibrated by immersion of 100 g of resin in 1 litre of 0.5 mol/l NaOH for 30 min; washed with distilled water until neutral pH; immersed in 0.5 mol/l HCl for 30 min, and washed again. The treated resin was suspended in pH 6.7 buffer, and allowed to settle for 60 min when the fine particles

remaining in suspension were removed. A 60 X 2 cm column of the resin was prepared and pH 6.7 buffer run through until pH and conductivity of the effluent was the same as the original buffer.

2-7-1-b. Buffers

(i) pH 6.7, 0.102 mol/l

4.59 g $NaH_2PO_4.H_2O$, 1.19 g Na_2HPO_4 and 0.65 g KCN to 1 litre of distilled water.

(ii) pH 6.4, 0.346 mol/l

14.35 g $NaH_2PO_4.H_2O$, 6.52 g Na_2HPO_4 to 1 litre of distilled water.

2-7-1-c. Preparation of haemolysate

A haemolysate was prepared by a modification of Trivelli et al (1971) as follows: Red cells were washed three times at 20°C with five volumes of saline. The packed cells were then lysed with two volumes of distilled water and one volume of toluene, refrigerated at 4°C overnight and then centrifuged at 27,000 g for 30 min at 4°C to remove cell stroma.

2-7-1-d. Column chromatography of haemoglobin

A 0.5 ml aliquot of haemolysate was placed on top of the column without disturbing the resin. After running the haemoglobin into the gel, 4.0 ml of pH 6.7 buffer was carefully added and the column connected to a buffer reservoir situated 30 cm higher. The eluate was collected in aliquots of 10 ml at a flow rate of 2.0 ml per minute and the absorbance was measured in a 1 cm cuvette on a Hitachi 101 spectrophotometer at 415 nm.

A change to pH 6.4 buffer enabled elution of the slower haemoglobins.

For total haemoglobin, 0.25 ml of the original haemolysate was diluted to 100 ml with pH 6.7 buffer and the absorbance measured at 415 nm as before.

2-7-1-e. Regeneration of resin

The resin could be regenerated after elution of haemoglobins by re-equilibration in the pH 6.7 buffer.

2-7-1-f. Measurement of haemoglobin

The absorption of cyanmethaemoglobin is maximal at 415 nm where the sensitivity is 10 times greater than the conventional wavelength of 540 nm (Fig. 2-1-1). At 415 nm, Beer's law was satisfied up to an absorbance of 1.0 (Fig. 2-1-2).

2-7-1-g. Elution of haemoglobin

All the fast Hb is eluted in the first 80 ml of pH 6.7 buffer (Fig. 2-1-3). The slow haemoglobin does not appear to move from the top of the column until the buffer is changed to pH 6.4 and 0.346 mol/l.

2-7-1-h. Reproducibility

The percentage of fast haemoglobin was calculated from:-

$$\% \text{ fast Hb} = \frac{A_{415} \text{ fast Hb} \times 100}{A_{415} \text{ total Hb} \times 2}$$

The mean value of 5 estimations with the same specimen was 10.96 % (Standard deviation 0.63) where the absorbance of total Hb was 0.60.

The individual results are shown below:-

	1	2	3	4	5
A_{415}	0.122	0.122	0.139	0.134	0.139
% Hb	10.2	10.2	11.6	11.2	11.6

FIGURE 2-1-1. ABSORBANCE OF CYANMETHAEMOGLOBIN AT DIFFERENT WAVELENGTHS FROM 225-600nm

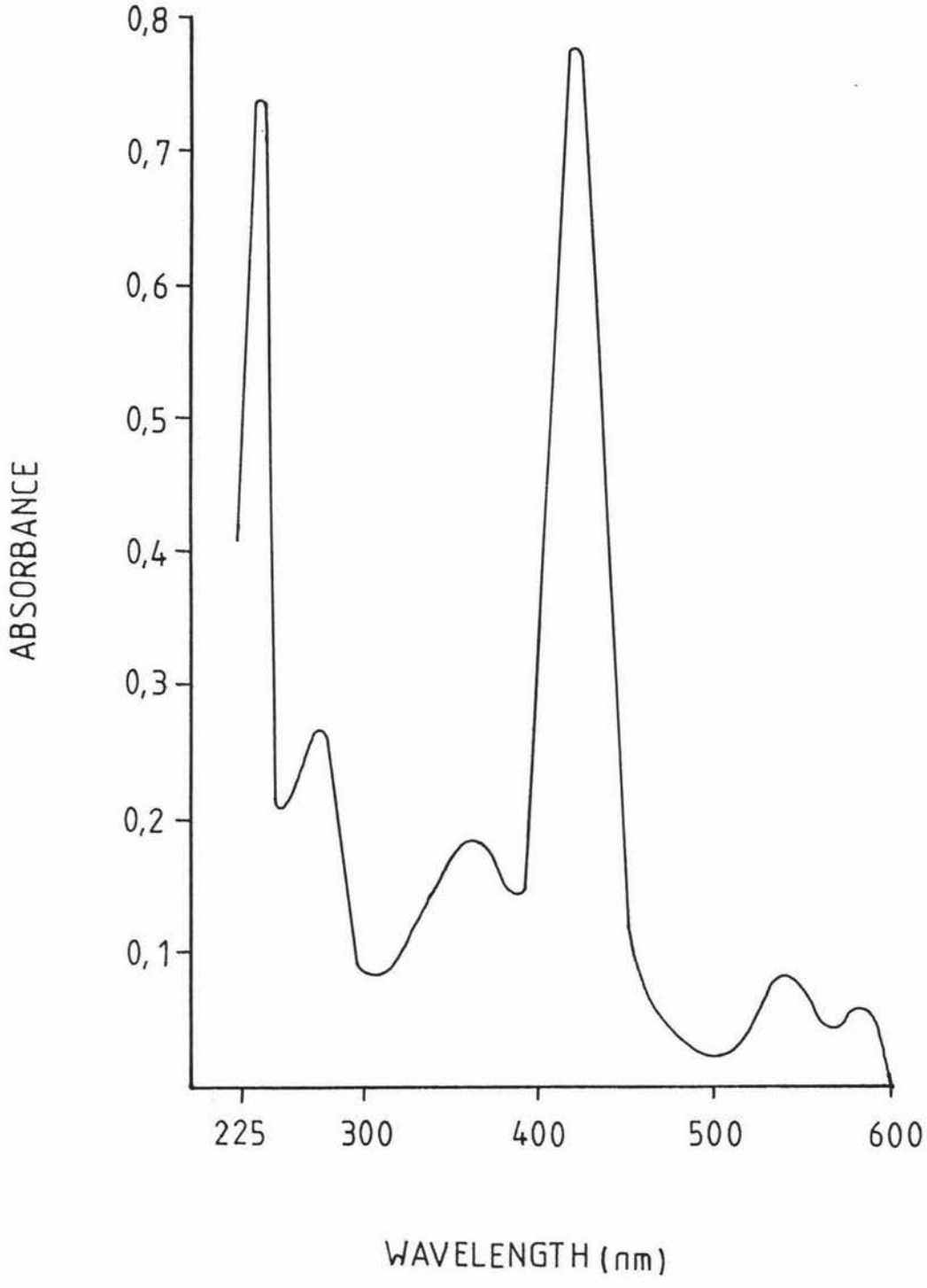


FIGURE 2-1-2, ABSORBANCE OF CYANMETHAEMOGLOBIN AT 415 nm

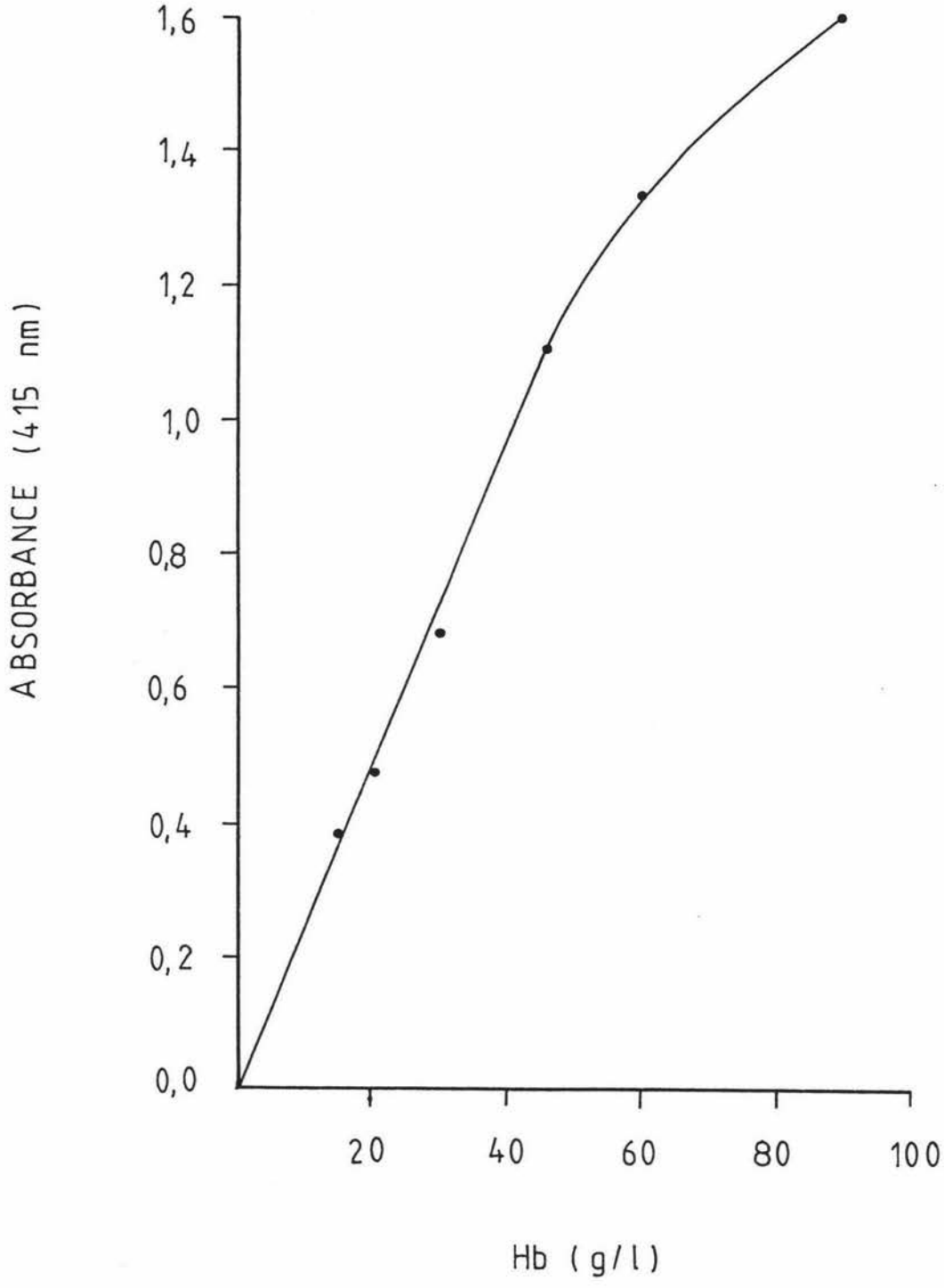
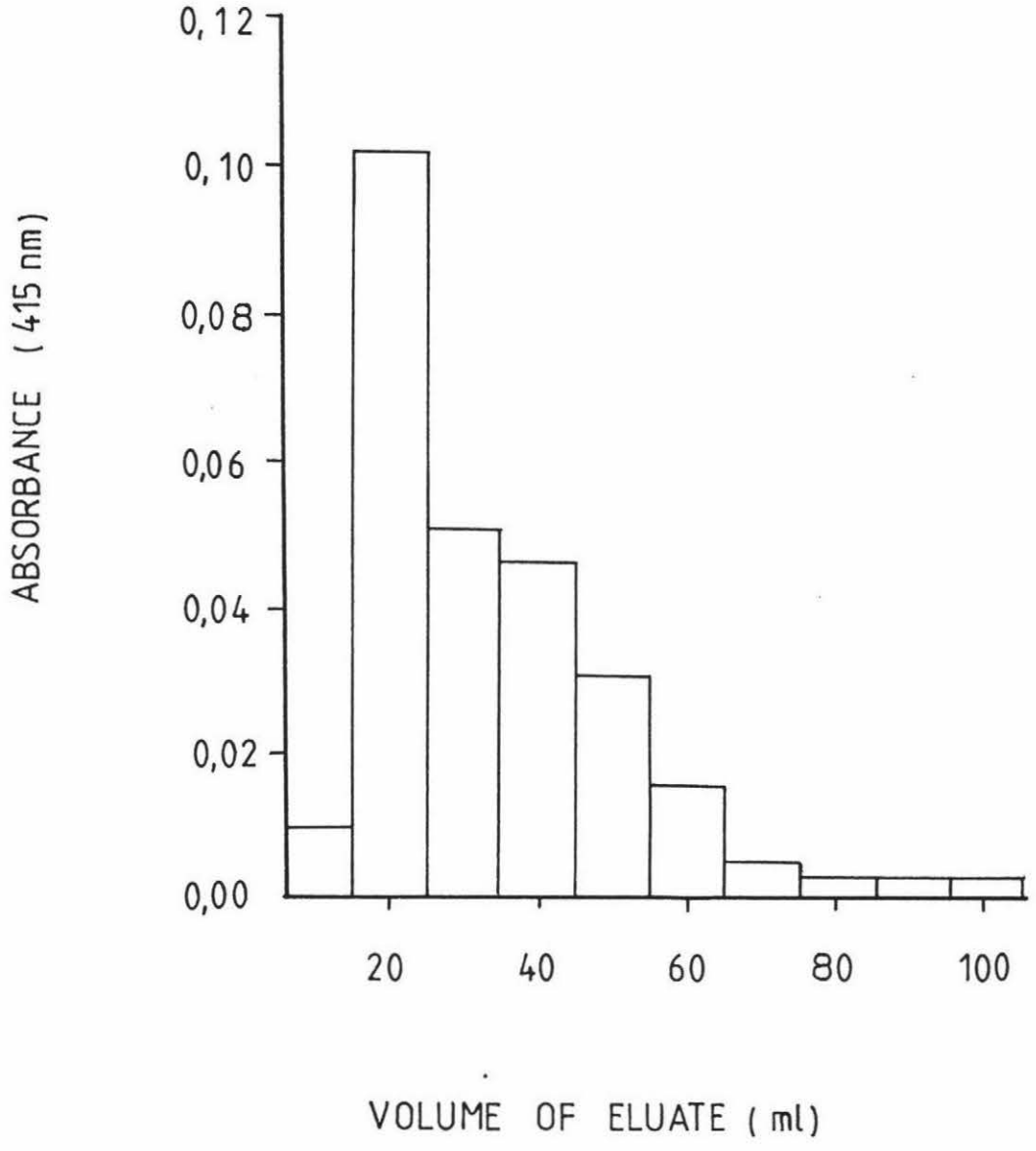


FIGURE 2-1-3. THE ELUTION PROFILE OF FAST HAEMOGLOBIN FROM A BIOREX 70 COLUMN WITH pH 6,7 BUFFER



2-7-2. Isoelectric focussing

The migration of haemoglobin is observed in an electric field on polyacrylamide gel containing a pH gradient developed by rearrangement of the carrier ampholyte molecules according to their isoelectric points. These ampholytes are complex mixtures of amphoteric substances which are prepared by the copolymerization of polyamines with suitable reactants to introduce acid groups. Each haemoglobin fraction migrates until its isoelectric point matches that of the gel. At that location, its charge is lost and the haemoglobin focuses into a sharp, stationary band.

The methods used were based on those of Jeppsson (1977) and Winter et al (1977).

2-7-2-a. Haemolysates

The following haemolysates at concentrations of 5 mg Hb/ml were used:-

(i) Fresh normal Hb

(ii) Fast haemoglobin fraction from column chromatography concentrated to half its volume in a Diaflo ultrafilter XM50 (Amicon, Mass. U.S.A.)

(iii) Slow haemoglobin fraction from column chromatography and concentrated x2 as above.

(iv) Fast haemoglobin fraction dialysed overnight at 4°C against two changes of distilled water, and then freeze-dried.

(v) Slow haemoglobin fraction processed in the same way as (iv).

2-7-2-b. Preparation of gels

A pH 5.5-7.7 gel was prepared as follows:-

10.0 ml acrylamide (29.1 % w/v), 10.0 ml Bis (0.9 % w/v), and 7.0 ml glycerol (87 % v/v) were mixed with 0.2 ml ampholine (pH 4-6), 0.2 ml ampholine (pH 5-7), 2.25 ml ampholine (pH 6-8), and 0.75 ml ampholine (pH 7-9). The solution was made up to 60 ml with distilled water and degassed under vacuum. The solution was carefully mixed with 1.5 ml ammonium persulphate (1 % w/v) avoiding air bubbles and poured into a glass mould 245 mm x 115 mm x 2 mm. A gel formed which was ready for use after 4 hours at room temperature. Commercially prepared gels were purchased from L.K.B., ampholine PAG plate pH 5.5-8.5.

2-7-2-c. Electrophoresis

Rectangles of filter paper 0.5 mm x 5.0 mm, to which were added 20 µl of haemolysate, were placed on a polyacrylamide gel. Migration and focussing of Hb was performed using a constant power of 12.5 watts at 300 volts and 42 milliamp. at the beginning and 1800 volts and 6.9 milliamp. at the end of migration. The plates were maintained at 4°C for the focussing period of 120 min.

2-7-2-d. Measurement of pH gradient

After focussing, the pH gradient was measured as follows. A narrow strip of the blank gel across the electrodes 1 cm wide and 9 cm long was cut and divided into 9 equal segments and placed into tubes containing 1 ml of distilled water at 4°C. The pH of each tube was then measured with a pH meter, (Tables 2-2-1 and 2-2-2) and the pH gradients plotted (Figures 2-2-1 and 2-2-2). The remaining gel was fixed and stained overnight with Coomassie Brilliant Blue G-250 (0.3 g in 4.1 % perchloric acid), then destained and stored in 4.1 % perchloric acid. The staining patterns are shown in Figures 2-2-3 and 2-2-4.

TABLE 2-2-1

pH gradient of the pH 5.5-8.5 gel

No. of the gel	pH
0	8.5
1	7.8
2	7.4
3	7.1
4	6.9
5	6.7
6	6.4
7	5.9
8	5.6

TABLE 2-2-2

pH gradient of the pH 5.5-7.7 gel

No. of the gel	pH
0	9.3
1	9.1
2	7.7
3	7.2
4	6.9
5	6.8
6	6.6
7	5.5
8	5.0

FIGURE 2-2-1, THE pH GRADIENT ON POLYACRYLAMIDE GEL PURCHASED FROM LKB & USED IN ISOELECTRIC FOCUSSING

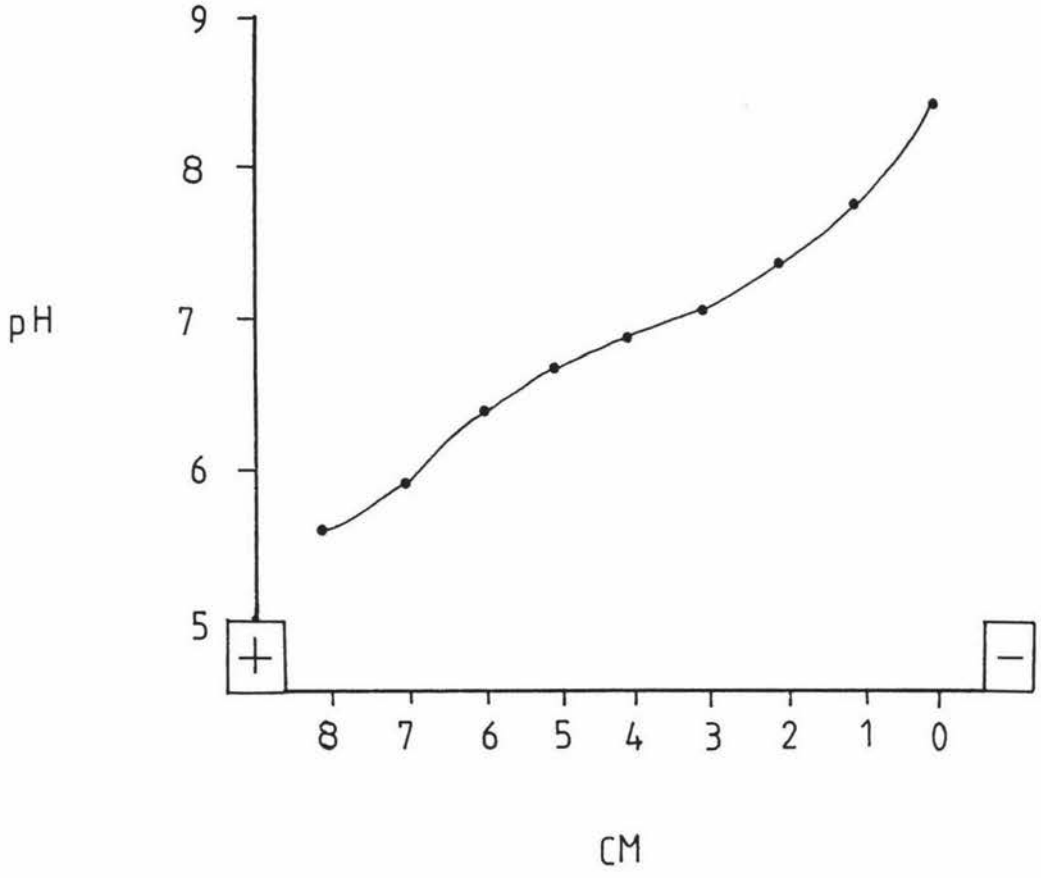


FIGURE 2-2-2, THE pH GRADIENT ON POLYACRYLAMIDE GEL PREPARED IN THE LABORATORY & USED IN ISOELECTRIC FOCUSSING

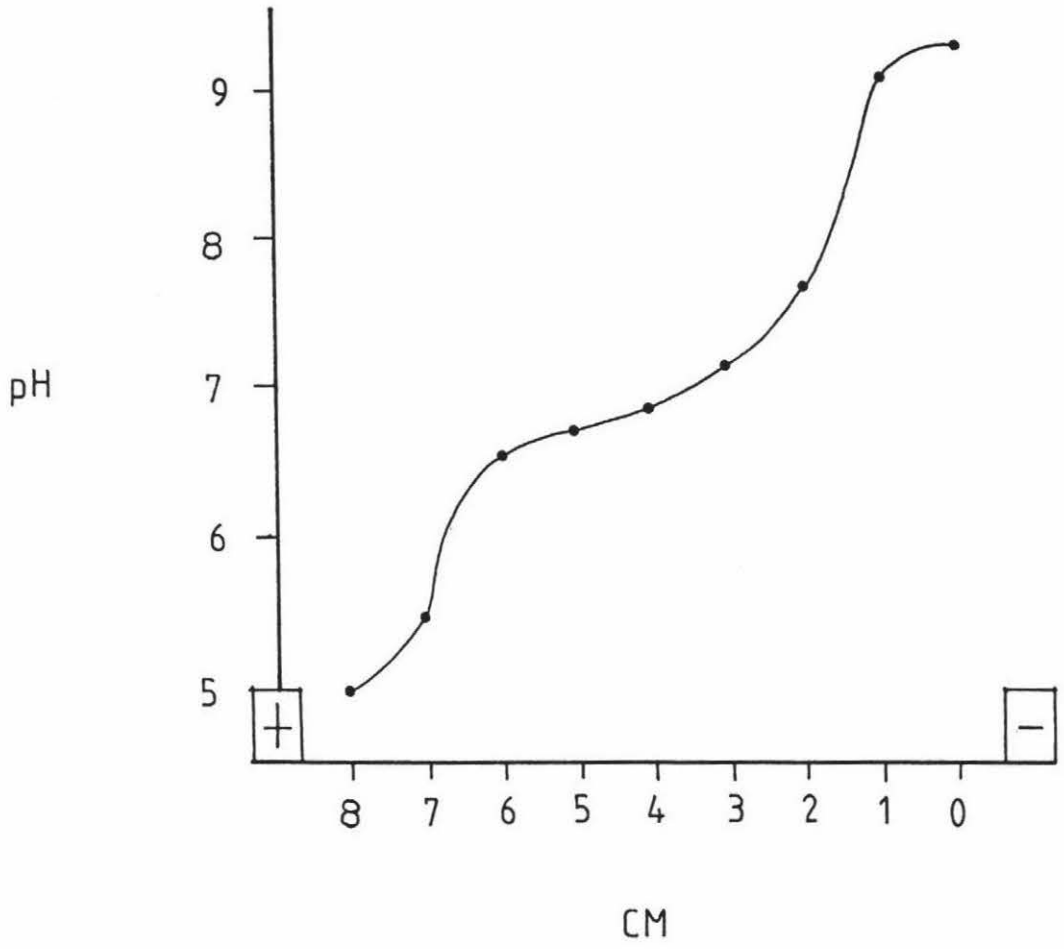


FIGURE 2-2-3, ISOELECTRIC FOCUSSING OF HUMAN HAEMOGLOBINS ON POLYACRYLAMIDE GEL PURCHASED FROM LKB

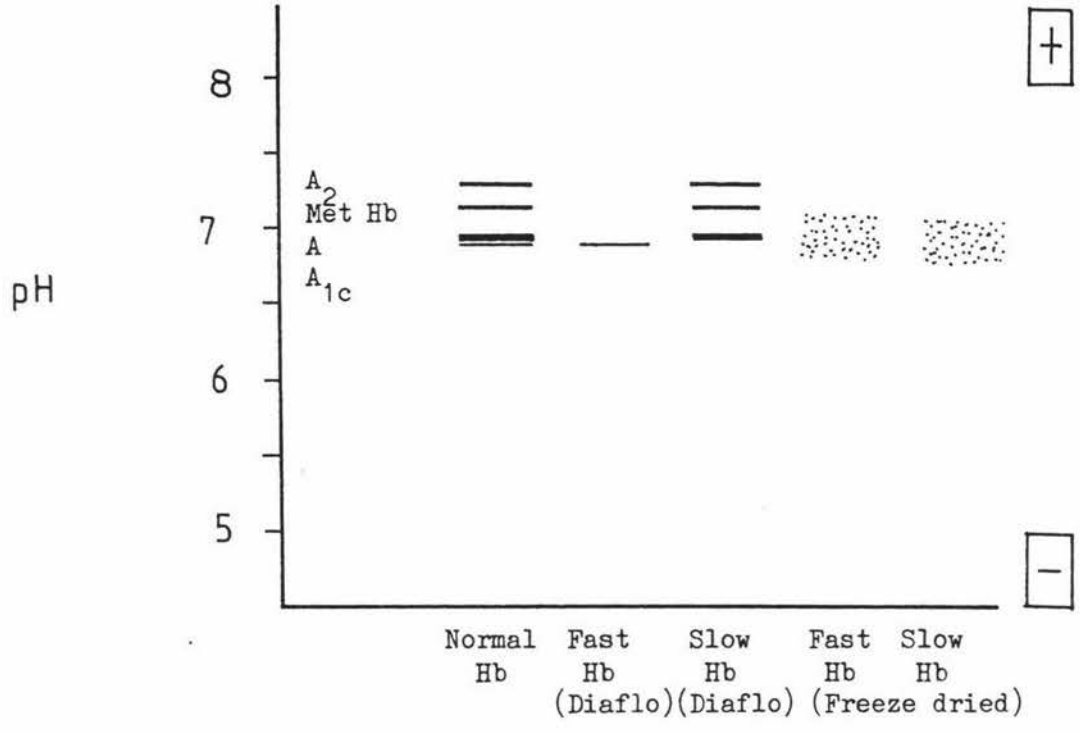
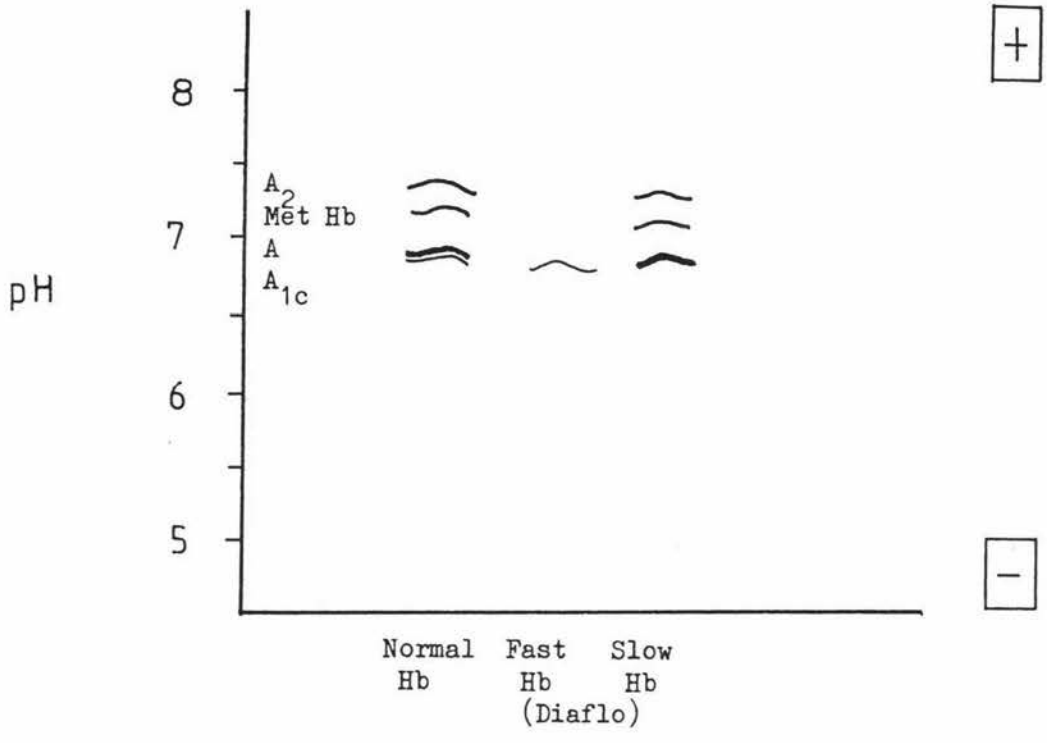


FIGURE 2-2-4, ISOELECTRIC FOCUSSING OF HUMAN HAEMOGLOBINS ON POLYACRYLAMIDE GEL PREPARED IN THE LABORATORY



2-7-3. Hydroxymethylfurfural assay

The glucose moiety of glycosylated haemoglobin can be converted to hydroxymethylfurfural (HMFF) by heating with oxalic acid for 60 min in an autoclave at 124 KPa (18 lb/in^2). The adduct formed by reacting 2-thiobarbituric acid (TBA) with HMFF, has an absorption peak at 443 nm and can be measured spectrophotometrically.

2-7-3-a. Production of hydroxymethylfurfural from haemoglobin

The methods used were based on those described by Parker et al (1981) and Subramaniam et al (1980). To 1.0 ml of pooled haemoglobin solutions (10 mg/ml, and 20 mg/ml) was added 1.0 ml of 0.5 mol /l oxalic acid and autoclaved for various times at 124 KPa corresponding to 124°C. Standards were prepared from 50 µmol /l and 100 µmole/l fructose in place of the pooled haemoglobin solution.

The solutions were cooled to room temperature and proteins precipitated with 1.0 ml of 40 % trichloroacetic acid and filtered through glass wool. To 1.5 ml eluate was added 0.5 ml 0.05 mol /l TBA and incubated at 40°C for 30 min. After cooling for 15 min the absorbance was measured at 443 nm in a Hitachi 101 spectrophotometer.

Hydroxymethylfurfural (Sigma) solutions were prepared in 0.9 % sodium chloride and standardised spectrophotometrically using the absorbance at 284 nm and the extinction coefficient of 16700 (Parker et al 1981).

The effect of heating time on fructose standards and haemoglobin solutions shows that maximal HMFF production occurs only after 60-100 minutes at 124°C (Fig. 2-3-1). Tests with standards showed that the HMFF formed was stable during heating for 60 minutes (Fig. 2-3-2).

2-7-3-b. Effect of antioxidants on HMFF formation from haemoglobin

Various antioxidants were added separately to 1 ml 0.5 mol /l oxalic acid as shown below before autoclaving with 1 ml of pooled haemoglobin (10 mg/ml) for 60 min. The TBA was added as previously described.

FIGURE 2-3-1, RATE OF PRODUCTION OF HMFF FROM THE NORMAL HAEMOLYSATE POOL AND FRUCTOSE STANDARDS

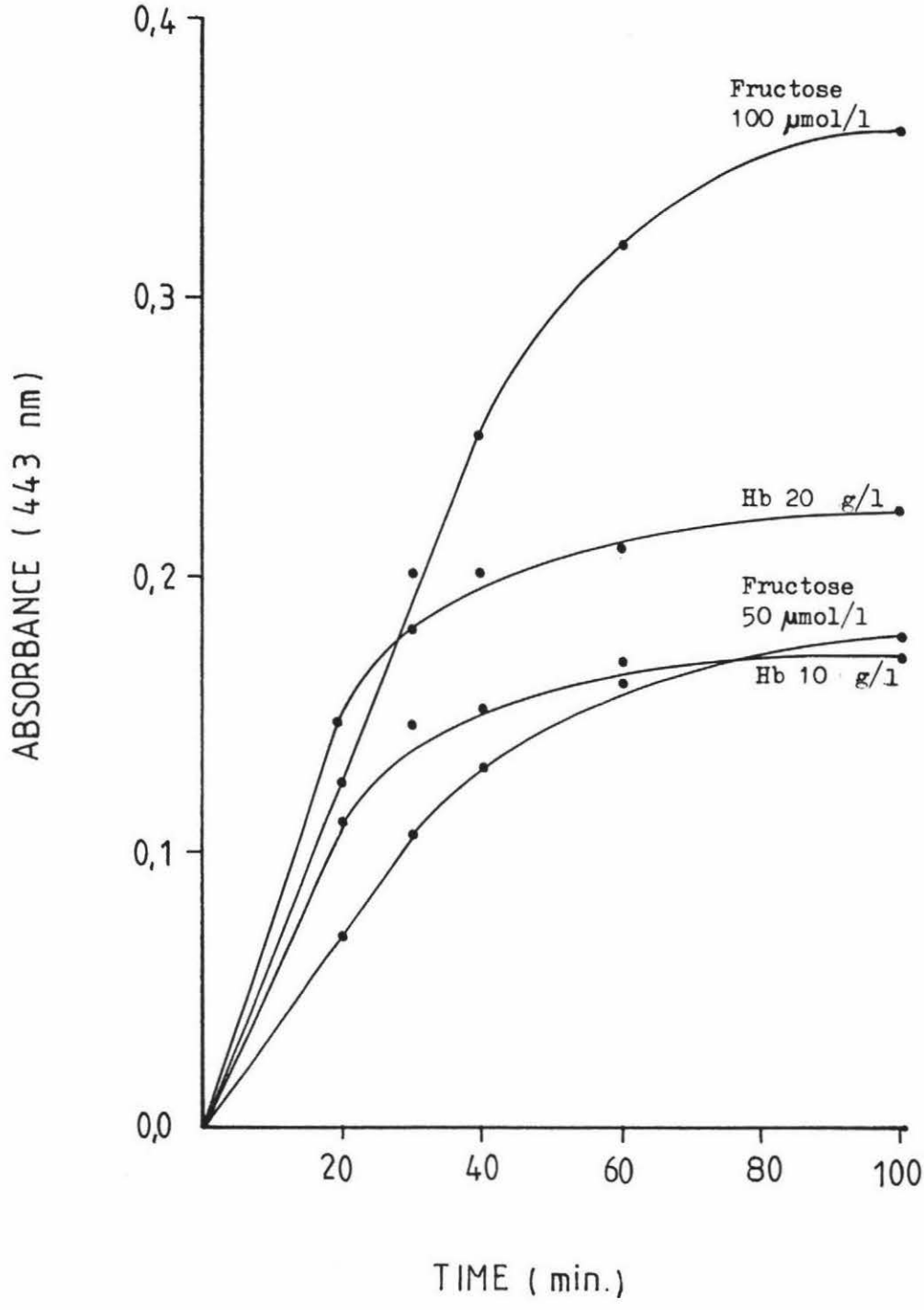
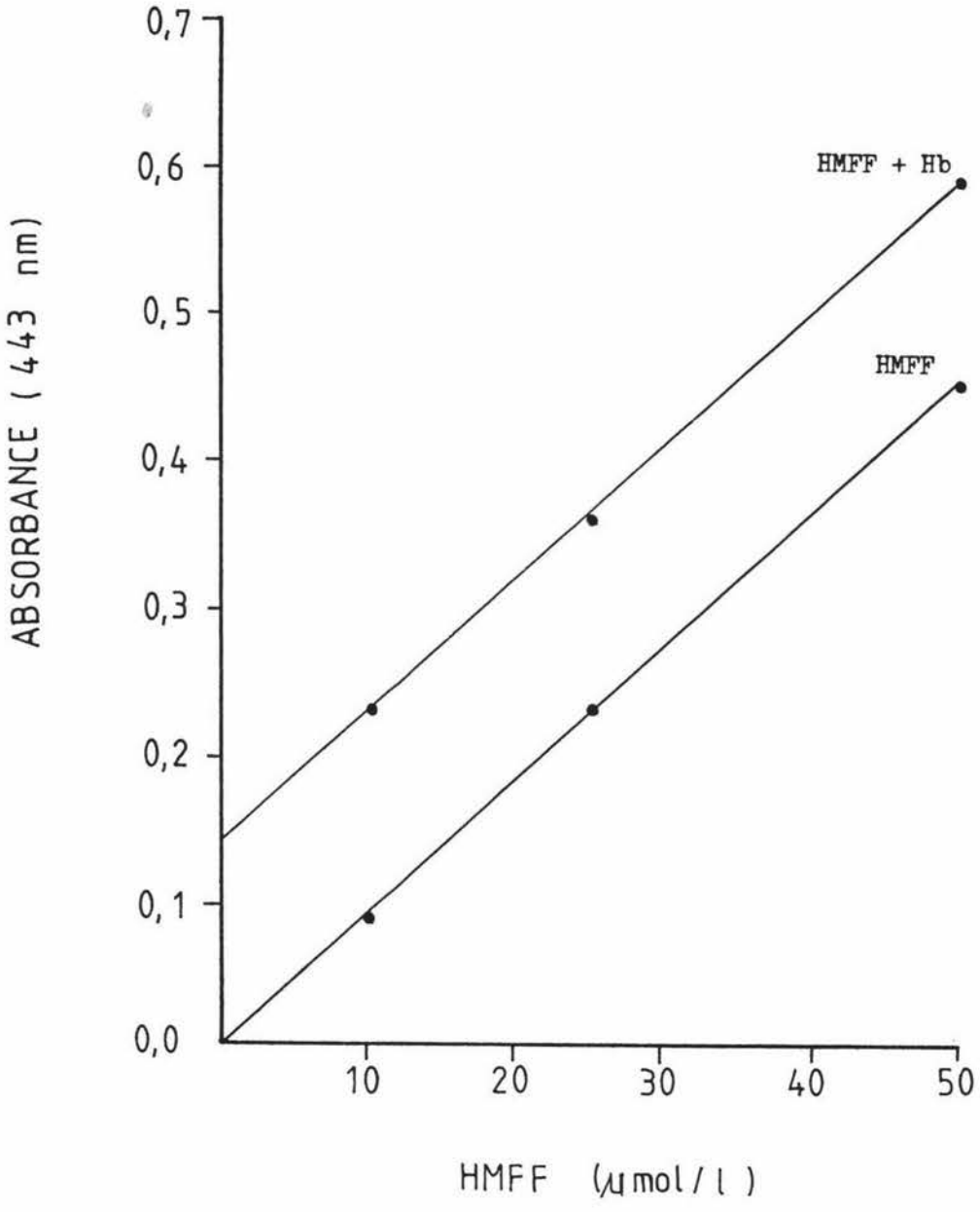


FIGURE 2-3-2. THE ABSORPTION OF HMFF & HMFF + Hb AT 443 nm



Antioxidants + oxalic acid	Final concentration	Absorbance at 443 nm
Hb alone	- %	0.162
10 mg sodium metabisulphate	1.0	0.120
5 mg phenol	0.5	0.130
5 mg 2-mercapto ethanol	0.5	0.095
10 mg ascorbic acid	1.0	A yellowish precipitate formed after autoclaving.
10 mg ferrous sulphate	1.0	A bluish green precipitate formed after autoclaving.
5 mg hydroxybutylated toluene	0.5	The chemical did not dissolve even after heating.

Some of the antioxidants were unsuitable for the test because of precipitation and pigment formation or insolubility. The remaining antioxidants gave a reduced absorbance of the final product so that there was no advantage in their use.

2-7-3-c. Precision of the method

The concentration of HMFF in 5 aliquots of the same haemoglobin solution was determined after heating with oxalic acid for 60 minutes at 124 KPa, then reacting with TBA as previously described. The results, recorded below, gave a mean value of 17.0 nmol /ml with a standard deviation of 0.69.

Test	Absorbance at 443 nm	HMFF nmole/ml
1	0.162	18.0
2	0.150	16.7
3	0.145	16.1
4	0.152	16.9
5	0.154	17.1
HMFF 50 nmol /ml	0.450	-

2-8. ACETALDEHYDE ADDUCT

2-8-1. Introduction

It has been proposed by Stevens et al (1981) that acetaldehyde formed an adduct with haemoglobin in a similar manner to glucose.

Attempts were made to prepare an acetaldehyde adduct in vitro:-

(i) Using a modification of the procedure described by Stevens et al (1981), 2.5 mmole of normal haemoglobin was incubated for 2 hours at 37°C with 30 mmole acetaldehyde. The solution was dialysed overnight at 4°C against two changes of distilled water then freeze dried. The resulting powder was only partially soluble in distilled water and subsequently discarded.

(ii) The dialysate from the above experiment was concentrated in a Diaflow XM50 ultrafiltered. The resulting concentrate showed brown precipitation and was again discarded.

(iii) The incubation solution of (i) together with the slow haemoglobin fraction obtained from column chromatography and treated in the same way were subjected to isoelectric focussing without dialyzing out the acetaldehyde. The results were indistinguishable from the untreated samples (Fig. 2-5-1).

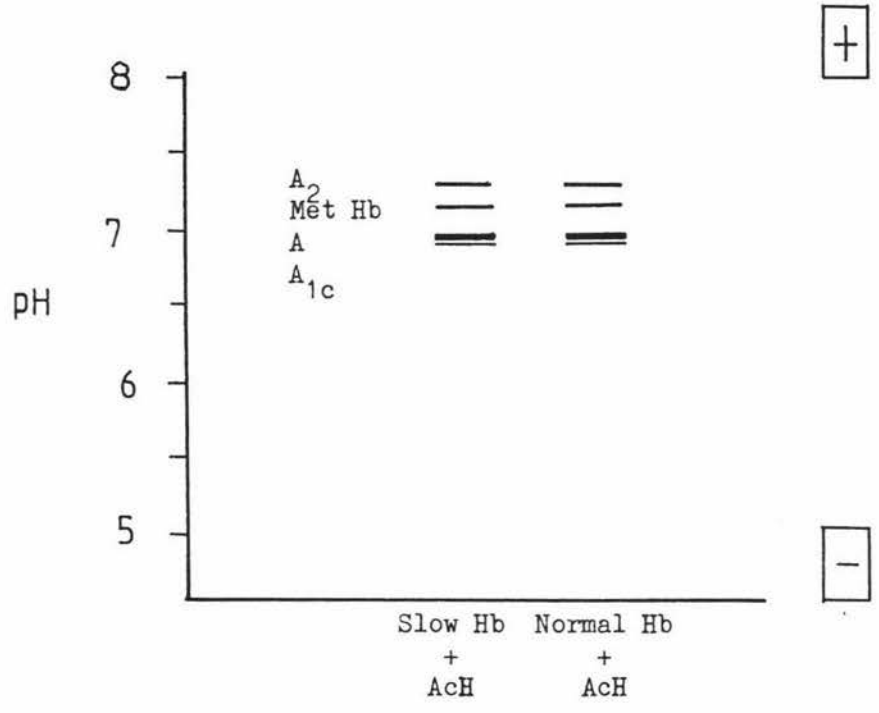
2-9. CONCLUSIONS

Column chromatography on Biorex 70 resin showed that only fast haemoglobin eluted from the column with pH 6.7, 0.102 mol/l buffer. Slower haemoglobins did not elute until the buffer was changed to pH 6.4, 0.346 mol/l. The resin was readily regenerated for re-use. Measurement of dilute haemoglobin solutions spectrophotometrically showed greater sensitivity at 415 nm.

Haemoglobins A, A_2 and A_{1c} could be separated and identified qualitatively by isoelectric focussing. Glucose adduct could be measured quantitatively by conversion to hydroxymethylfurfural and the method appeared to be sensitive and reproducible.

Attempts to prepare acetaldehyde adduct *in vitro* were unsuccessful so that it was decided to look for the presence of adduct *in vivo*. The presence of fast haemoglobin determined by column chromatography in excess of that attributable to glucose by the HMFF method should be an indicator of other haemoglobin adducts.

FIGURE 2-5-1, ISOELECTRIC FOCUSSING OF HAEMOGLOBINS TO WHICH ACETALDEHYDE HAD BEEN ADDED (LKB pH 5,5 -8,5 GEL)



CHAPTER 3

BIOCHEMICAL AND HAEMATOLOGICAL MARKERS OF ALCOHOL ABUSE

3-1. INTRODUCTION

Because of ethical contraints which prevented access to medical records, blood samples from known alcohol abusers could not be obtained directly, but unidentified samples from the routine haematological workload were available for study.

Several biochemical and haematological tests have been utilised for detecting the alcoholic (Holt et al, 1981). The mean corpuscular volume of the red cell increases with heavy drinking even in the absence of folic acid or vitamin B_{12} deficiency (McPhedran et al, 1973; Wu et al, 1974).

An early indication of liver damage is the leakage from the cells into the plasma of certain enzymes (Tietz, 1970). The liver enzymes aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT) are usually normal in the less acutely ill, are elevated in bone and muscle disease, and rise in volunteers given large amounts of alcohol (Lancet ed., 1980). However, the diagnostic sensitivity of these enzymes for alcoholism is low, because of increased individual resistance to the toxic effects of alcohol or decreased hepatic reserve of these enzymes (Clark & Kricka, 1981). Such tests must be interpreted with caution as they are non-specific and other drugs and diseases may cause raised values. The measurement of serum GGTP levels for the diagnosis of alcohol abuse has diagnostic sensitivity and is convenient to perform (Clark & Kricka, 1981). The specific sensitivity of this enzyme in the detection of hepatic involvement in the alcoholic or heavy drinker, partly results from induction of hepatic microsomes by alcohol, and is also related to the predominently microsomal location of GGTP in the liver, since hepatic microsomal injury is one of the earliest effects of alcohol toxicity (Rosalki et al, 1972).

The ratio of alpha-amino-n-butyric acid (AANB) to leucine in the plasma might be a useful indicator of long-term alcohol consumption as suggested by Holt et al (1981), but hepato-cellular disease in general,

rather than alcohol consumption alone, increased this ratio. This test is expensive for routine use, requiring an amino-acid analyser.

Plasma transferrin may be a good indicator of alcohol abuse as it appears to reflect liver disease and may detect low to moderate alcohol consumption (Holt et al,1981). However, the electrofocussing plus immunofixation technique is complex for routine laboratory analysis. Thiamine deficiency may also be found in some patients with an alcohol problem and the erythrocyte transketolase has been used as a non-specific indicator of this deficiency (McLaren et al,1981).

It has been suggested that combining the results of several tests may be useful for detecting alcoholics in an unselected population (Morgan et al,1981). Consequently MCV, GGTP and transketolase activity were selected for further study.

3-2. MCV

The size of the red cell is expressed by the term "Mean Cell Volume" (MCV). Before the advent of automatic counting systems, MCV was calculated from $\frac{\text{PCV per litre}}{\text{RBC per litre}} \times 10^{15}$ fl. Manual red cell counts suffered from technical errors (inaccurate apparatus, indifferent technique) and statistical errors due to the uneven distribution of the suspension of red cells in the counting chamber. The PCV was obtained by various centrifugation methods and suffered from incomplete packing of red cells. This incomplete packing introduced an error "plasma trapping".

The introduction of electronic cell counters, such as the Coulter S, increased the accuracy of the red cell count and the MCV. The principle of the instrument is based on the fact that blood is a poor conductor of electricity, whereas certain diluents are good conductors. A constant electric current is maintained between two electrodes, and the blood cell creates an impulse while passing between these electrodes. The amplitude of the impulse is proportional to the cell size. The cell count is determined from the number of pulses while a mean cell volume is obtained from the sum of the pulse heights divided by the cell count.

A haematocrit (PCV) may be calculated from the red-cell count multiplied by the sum of pulse amplitudes, but Dacie and Lewis (1975) claimed that this indirectly derived PCV was frequently 1.5-3 % lower than that obtained by micro-haematocrit centrifugation, presumably because of errors due to plasma trapped in the centrifuged blood. It was recommended that Coulter S counter be calibrated by setting the observed value for haematocrit 3 per cent lower so reducing the values obtained for mean corpuscular volume.

The normal ranges quoted in the literature for MCV can be divided into two distinct groups depending on whether this correction was made:-

Corrected group

Reference	Method	No.	Male normal range	No.	Female normal range
Chanarin et al,(1973)	Coulter S	32	80.4-90.0 fl	32	81.9-89.7 fl
Davidson et al,(1978)	Coulter S	100	81.0-95.4	100	81.2-96.2
Chalmers et al,(1979)	Coulter S	1596	84.7-87.5	797	85.1-87.1

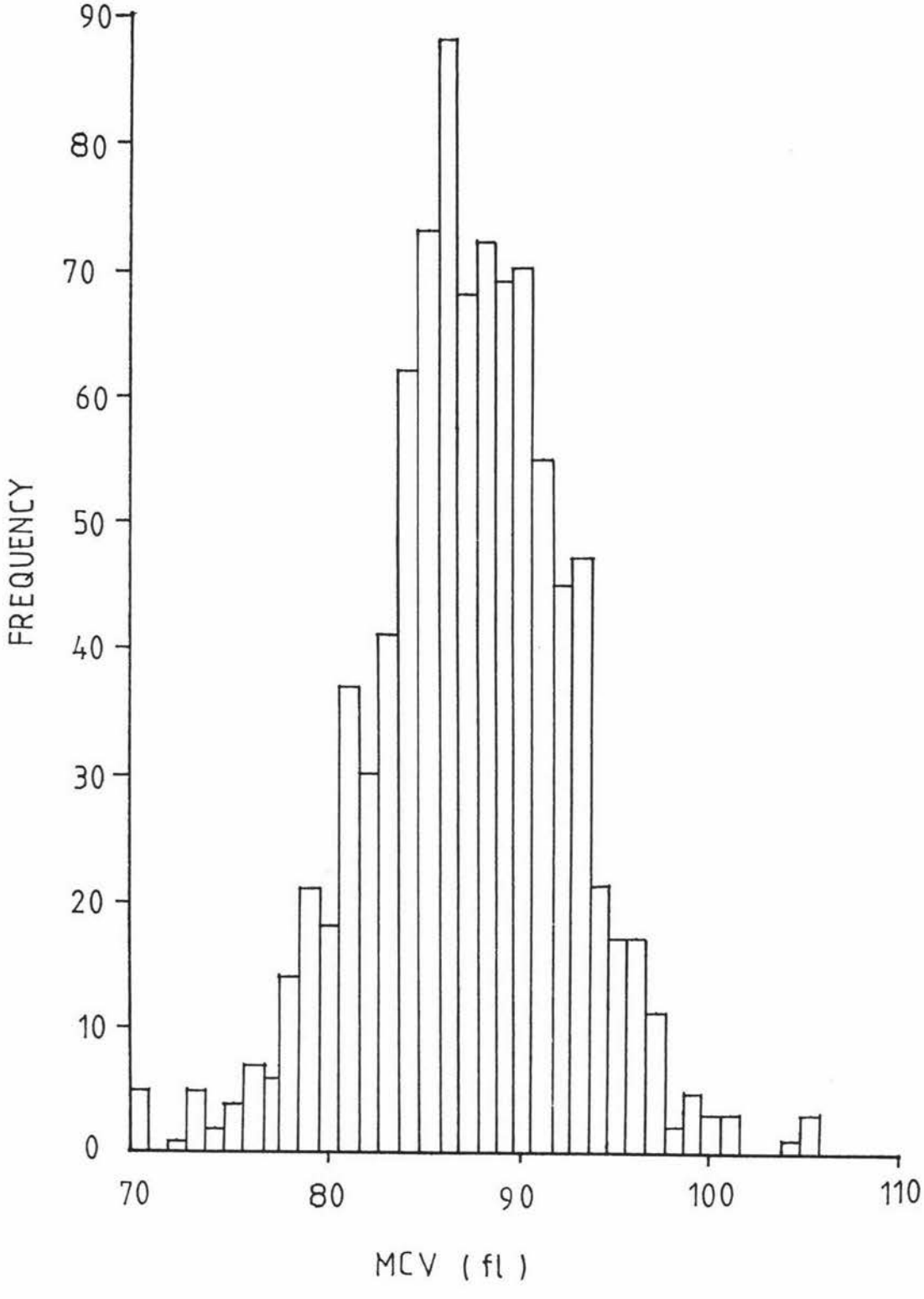
Uncorrected group

Reference	Method	No.	Male normal range	No.	Female normal range
Okuno (1972)	Undefined	-	82.2-100.6 fl	-	81.9-100.7
Helman et al,(1975)	Undefined	1000	81.9-96.9	1000	83.0-97.8
Giorno et al,(1980)	Haemac 630	638	79.5-100.7	1106	76.3-99.2
Papoz et al,(1981)	Undefined	604	81.0-101.0	391	81.0-99.0

The significance of applying plasma trapping correction lies in the upper dividing line between a normal MCV and a macrocytic MCV. The MCV and haemoglobin concentration on all blood samples for this study were measured on a Coulter counter model (S) at the Palmerston North Hospital. The instrument was standardised with a '4C Normal' (Coulter Diagnostic, U.S.A.) haematology reference control. Correction for 3 % plasma trapping was applied. The normal range was expected to be 77-91 fl on the basis of reported experience with this counter.

In a survey of 975 blood samples taken for routine analysis at the Palmerston North Hospital haematology laboratory (Couchman, unpublished data), it was found that 19 % had MCVs greater than 91 fl. Patients with haemoglobins less than 120 g/l and cord bloods, which have a high MCV, had been excluded from analysis (Fig. 3-1).

FIGURE 3-1, FREQUENCY HISTOGRAM FOR MCV,



3-3. DETERMINATION OF GAMMA GLUTAMYL TRANSPEPTIDASE

3-3-1. Principle

The enzyme gamma glutamyl transpeptidase (EC 2.3.2.2) catalyzes the transfer of the gamma glutamyl group from gamma glutamyl peptides to suitable acceptors (Rosalki & Tarlow, 1974). The substrate employed in this method is gamma glutamyl-p-nitroanilide, with glycylglycine serving as acceptor. Buffering is provided by Tris and by glycylglycine. Serum or plasma is added to the buffer-acceptor solution, and the reaction initiated by addition of substrate in HCl solution. The increase in absorbance at 405 nm due to the p-nitroaniline formed in the reaction is measured. The method investigated in this thesis was based on that described by Tietz (1976).

3-3-2. Preparation of beef liver gamma glutamyl transpeptidase (GGTP)

Fresh beef liver (50 g) was homogenised in 100 ml of 50 mmol phosphate buffer, pH 7.4, at 4°C, then centrifuged at 7000 g for 30 min at 4°C. The supernatant was assayed and diluted with the same buffer until the enzyme activity was 200-250 IU and stored at -20°C in aliquots of 1 ml until use.

3-3-3. Assay procedure

To 2.7 ml Tris-glycylglycine buffer pH 8.5* prewarmed to 30°C in a 10 mm cuvette was added 100 µl plasma. The enzyme reaction was initiated by adding 0.2 ml of L-gamma-glutamyl-p-nitroanilide (104 mmol /l), mixing rapidly, and then recording the change in absorbance at 405 nm over 3 min. Each run included a blank of 100 µl of distilled water in place of the plasma used to correct for non-enzyme hydrolysis of the substrate.

* Tris-glycylglycine buffer pH 8.5 - 115 mmol Tris/l and 138 mmol glycylglycine/l, 0.25 ml of dimethyl sulphoxide to each ml of substrate to maintain the solubility of the reagent.

3-3-4. The calculation of the enzyme activity

The absorption coefficient for p-nitroaniline is 9.87 $l^{-1}mmol^{-1}cm^{-1}$. Enzyme activity is given by the difference in absorption per minute multiplied by the absorption coefficient absorption coefficient so that the activity of the enzyme in international units is given by

$$\frac{\Delta A}{Time} \times \frac{10^3}{9.87} \times \frac{3.0}{0.1}$$

where $\frac{\Delta A}{min}$ = observed rate of reaction

3.0 = total reaction volume in ml

0.1 = specimen volume in ml

9.87 = absorption coefficient = 9.87 $l^{-1}mmol^{-1}cm^{-1}$

10^3 = µmol in 1.0 mmol

The minimum detectable change in absorbance was 0.001/min corresponding an activity of 3 IU.

The typical graph of change in absorbance is shown in Fig. 3-2.

3-3-5. Precision of the method

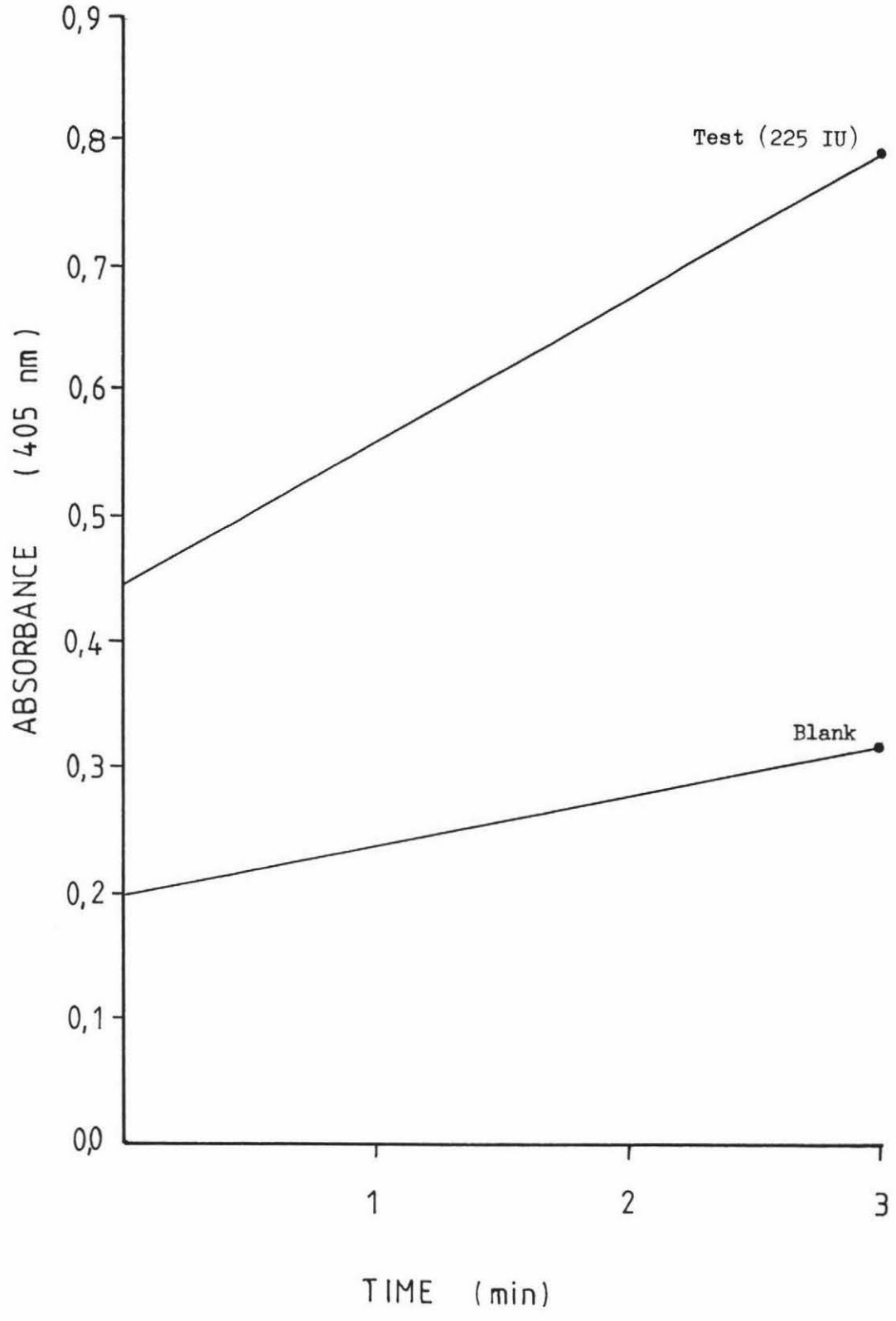
The enzyme (GGTP) activity was determined five times on the same sample of beef liver extract. The results, recorded below, gave a mean value of 231 IU with a standard deviation of 6.4.

Test	GGTP activity IU
1	223
2	236
3	236
4	235
5	225

3-3-6. Normal values

Tietz (1976) proposed upper values for this enzyme of 50 for males and 30 IU for females when measured at 37°C. If the tests were carried at a 30°C, he proposed that these results be divided by 1.35 to give upper limits of 37 and 22 IU for males and females respectively. None of the specimens from 13 normal volunteers showed enzyme activity greater than 3 IU (Appendix Table 1).

FIGURE 3-2. THE CHANGE IN ABSORBANCE OF P-NITRO-ANILINE DUE TO GGTP ACTIVITY



3-4. THIAMINE DEFICIENCY

3-4-1. Introduction

Warnock et al (1978) reported that transketolase apoenzyme could be reconstituted by the addition of thiamine pyrophosphate (TPP) or samples containing TPP, yielding the holoenzyme with activity proportional to the amount of TPP added.

3-4-2. Principle

Transketolase (TK) (EC 2.2.1.1) , in conjunction with its required coenzyme, thiamine pyrophosphate (TPP), catalyzes two reactions in the hexose monophosphate shunt.

(i) $\text{Xylulose-5-PO}_4 + \text{ribose-5-PO}_4 \xrightleftharpoons{\text{TK-TPP}} \text{sedoheptulose-7-PO}_4 + \text{glyceraldehyde-3-PO}_4$

(ii) $\text{Xylulose-5-PO}_4 + \text{erythrose-4-PO}_4 \xrightleftharpoons{\text{TK-TPP}} \text{fructose-6-PO}_4 + \text{glyceraldehyde-3-PO}_4$

The glyceraldehyde-3-PO_4 produced from reactions (i) and (ii) can be degraded to dihydroxyacetone by adding triose-phosphate isomerase (TIM) and glycerophosphate dehydrogenase (GDH) to the reaction mixture:-

(iii) $\text{Glyceraldehyde-3-PO}_4 \xrightleftharpoons{\text{TIM}} \text{dihydroxyacetone-PO}_4$

The dihydroxyacetone-PO_4 formed can be changed to glycerol-1-PO_4 by GDH and the coenzyme NADH and the formation of NAD^+ measured by the decrease in absorption at 340 nm.

(iv) $\text{Dihydroxyacetone-PO}_4 + \text{NADH} \xrightleftharpoons{\text{GDH}} \text{glycerol-1-PO}_4 + \text{NAD}^+$

Adequate TPP is necessary in reactions (i) and (ii) and deficiency results in a lowering of production of NAD^+.

The method investigated was described by Vo-Khactu et al (1974) and Williams (1976).

3-4-3. Method

Blood specimens were drawn by venipuncture into evacuated tubes containing EDTA. The blood was centrifuged at 800 *g* for 10 min 4°C and the plasma removed. The packed cells were washed with five volumes of normal saline (0.15 mol/l). This wash procedure was repeated twice. The packed cells were lysed with two volumes of water and one volume of toluene, mixed and refrigerated at 4°C overnight. The haemolysate was centrifuged at 27,000 *g* for 30 min 4°C, and the aqueous supernatant was stored at -20°C until analysis. The final haemoglobin concentration was adjusted to 25-45 g/l.

To 3 ml of 100 mmol/l Tris buffer pH 7.5 in a 10 mm cuvette was added 0.1 ml haemolysate, 0.1 ml ribose-5-phosphate (120 mmol/l), 0.1 ml distilled water, and 0.1 ml NADH (5.1 mmol/l). A standard measure of thiamine activity was prepared from the same solutions by adding 0.1 ml TPP (10 mmol/l) in place of distilled water. Both cuvettes were placed in a spectrophotometer (Cecil CE-292) for 15 min to warm up to 37°C. The reaction was started by adding 10 µl of glycerophosphate dehydrogenase/ triose-phosphate isomerase (2 mg/ml) reagent and the change in absorbance at 340 nm with unit time recorded.

3-4-4. Calculation of the enzyme activity

Enzyme activity was calculated from the difference in absorption per minute multiplied by the volume of mixture divided by the volume of haemolysate and absorbance of 1 µmol of NADH/NADPH at 340 nm so that the activity of the enzyme in international units is given by

$$\frac{1000 \times \Delta A \times Vc}{A_{NAD} \times V_H \times Hb}$$

where ΔA = change in absorbance per minute

Vc = volume in cuvette = 3.41 ml

V_H = volume of haemolysate used = 0.1 ml

A_{NAD} = absorption coefficient for NAD/NADPH = 6.22 $l^{-1}\mu mol^{-1}cm^{-1}$

The effect of adding additional thiamine pyrophosphate was calculated from the difference in enzyme activity before and after saturation with TPP divided by the rate of TPP saturated enzyme:-

$$\frac{TK_{sat} - TK}{TK_{sat}} \times 100\ \%$$

where TK_{sat} = transketolase activity after saturation with TPP in vitro

TK = transketolase activity of the haemolysate without addition of TPP

3-4-5. Normal ranges

The normal ranges given by Williams (1976) are :-

Enzyme activity 0.42-1.22 IU/g Hb

Thiamine pyrophosphate effect 0-25 %

3-4-6. Results in the normal plasma

The results on plasma from normal subjects ranged from 0.56-1.00 IU/g Hb with a mean of 0.77 and a standard deviation of 0.14 (Appendix Table 4-1). The addition of TPP did not alter the activity.

3-4-7. Conclusions

The three tests described, proved to be readily performed and reliable and were used to screen for excessive alcohol consumption in hospital patients described in the next chapter.

CHAPTER 4

RESULTS IN NORMAL VOLUNTEERS AND HOSPITAL PATIENTS

4-1. Source of blood samples

4-1-1. Normal volunteers

Blood samples were taken in 'Vacutainers' containing potassium EDTA from volunteers on the staff of Massey University. There were 11 males and 2 females. Haemoglobin and MCV were not estimated on these samples.

The results of all other tests in these subjects are recorded in Appendix Table 1.

4-1-2. Hospital patients

Blood samples, which had been taken in 'Vacutainers' containing potassium EDTA, were selected from the routine workload of the haematology department of Palmerston North Hospital on the following basis:-

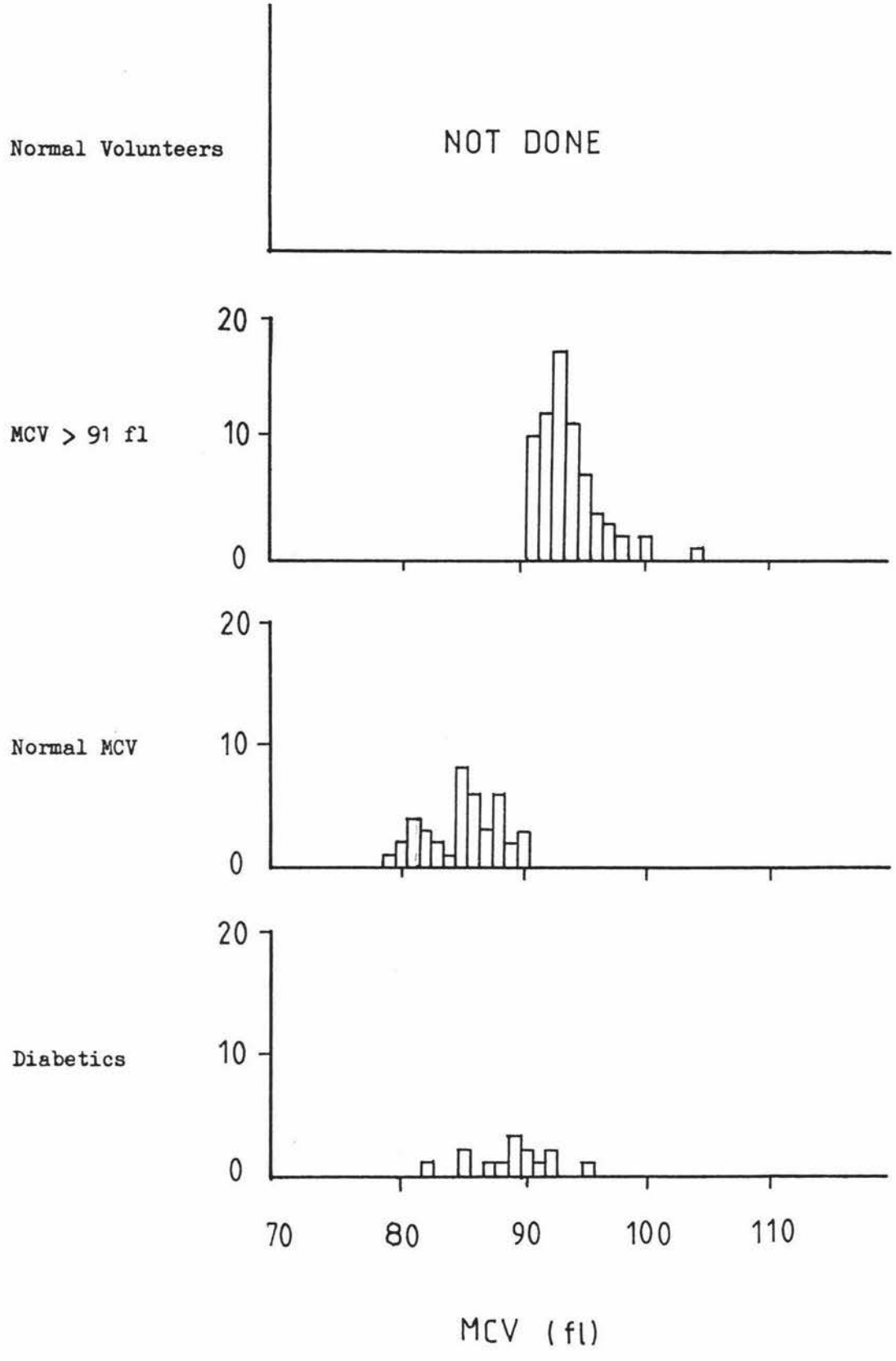
(i) 70 samples were selected with a mean corpuscular volume greater than 91 fl and normal haemoglobin. There were 34 males and 36 females with haemoglobin concentrations from 120 to 182 g/l within the normal range. All results are recorded in Appendix Table 2.

(ii) 45 samples were selected at random with a normal MCV and haemoglobin. There were 29 males and 16 females with MCVs from 79-90 fl and haemoglobin concentrations from 124-170 g/l. All results are recorded in Appendix Table 3.

(iii) Blood was obtained from 14 patients attending the diabetic clinic at Palmerston North Hospital. Neither the clinical details or sex of this group were available. Their MCVs ranged from 83-96 fl and haemoglobins from 110-160 g/l. All results are recorded in Appendix Table 4.

Histograms for mean corpuscular volume on all samples are shown in Fig. 4-1.

FIGURE 4-1, HISTOGRAMS FOR MCV FOR THE THREE GROUPS OF SUBJECTS



4-2. Fast haemoglobin

The percentages of fast haemoglobin, as determined by column chromatography described in Chapter 2, are recorded in Appendix Tables 1 to 4 and illustrated in histograms of Fig. 4-2.

The range of results found in blood samples with normal MCV were from 4.0–10.0 % corresponding to the range 6.0–9.0 % for normals described by Kynoch et al (1977). The results from normal volunteers was also within this range. In the samples with MCVs >91, the range was extended up to 12.0 %. While the mean values from this group are not very different from normal, the standard deviation is significantly wider. The range in diabetic patients extends up to 15.0 % due to excessive amounts of glucose in the blood of such patients.

4-3. Hydroxymethylfurfural

The results of HMFF estimations by the method described in Chapter 2, are recorded in Appendix Tables 1 to 4 and illustrated as histograms in Fig. 4-3.

Results ranged from 15–23 in normal volunteers and 14–27 in bloods with a normal MCV. In bloods with a high MCV, there were more HMFF results at the high end of the range 27–29 than would be expected although the mean value was not significantly greater than for normal MCV bloods. Specimens from diabetics showed increased HMFF values as expected due to high plasma glucose levels.

4-4. The relationship between fast haemoglobin and HMFF results

As would be expected, there was a high degree of correlation between fast haemoglobin and HMFF results in diabetic patients ($r = 0.724$, $t = 3.64$, $df = 12$, $p < 0.0025$) Fig. 4-4.

The overall correlation in normal and high MCV bloods is similar and scarcely attains statistical significance (Figures 4-5 and 4-6).

FIGURE 4-2, HISTOGRAMS FOR FAST Hb FOR THE THREE GROUPS OF SUBJECTS

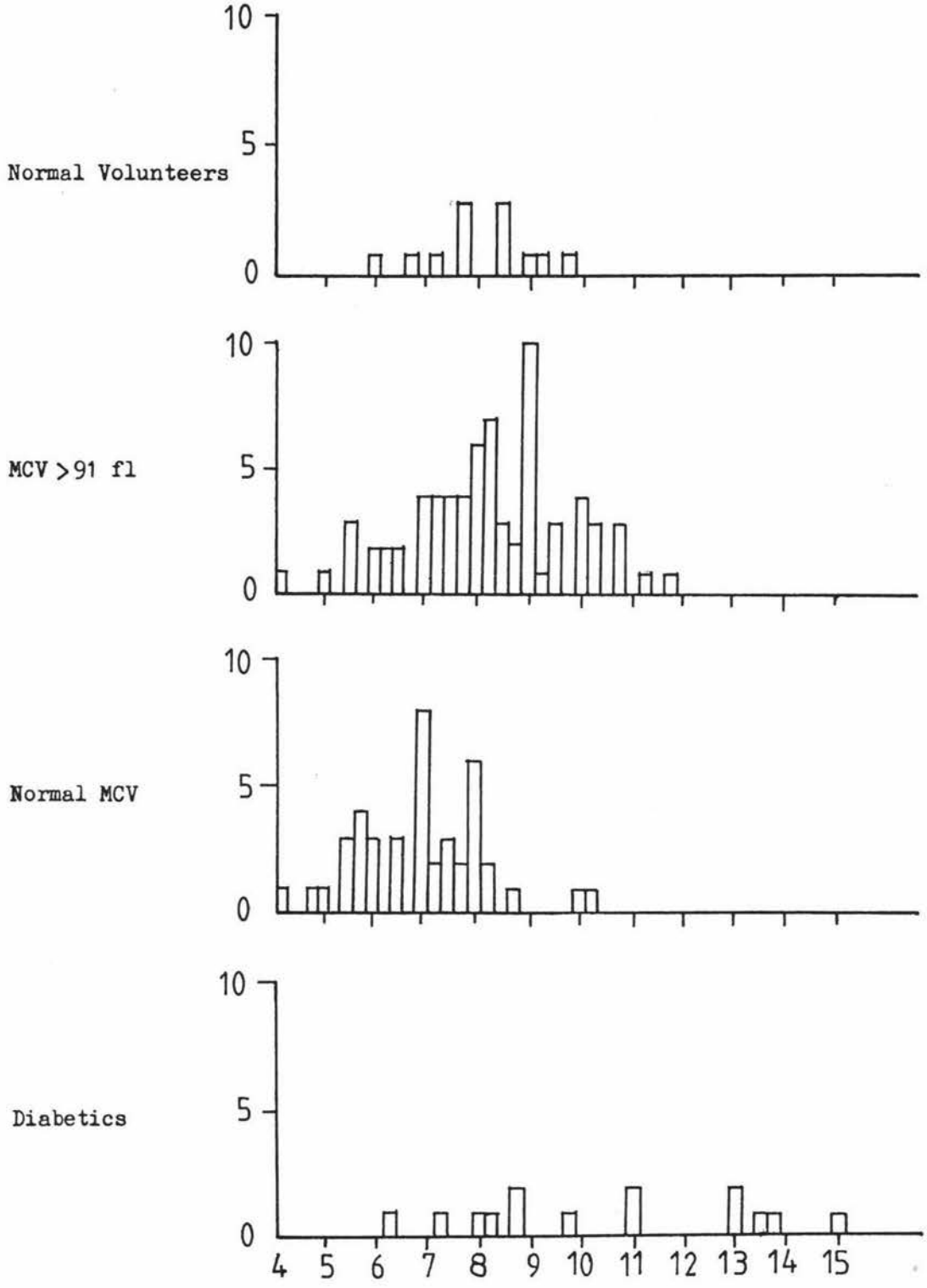


FIGURE 4-3, HISTOGRAMS FOR HMFF FOR THE THREE GROUPS OF SUBJECTS

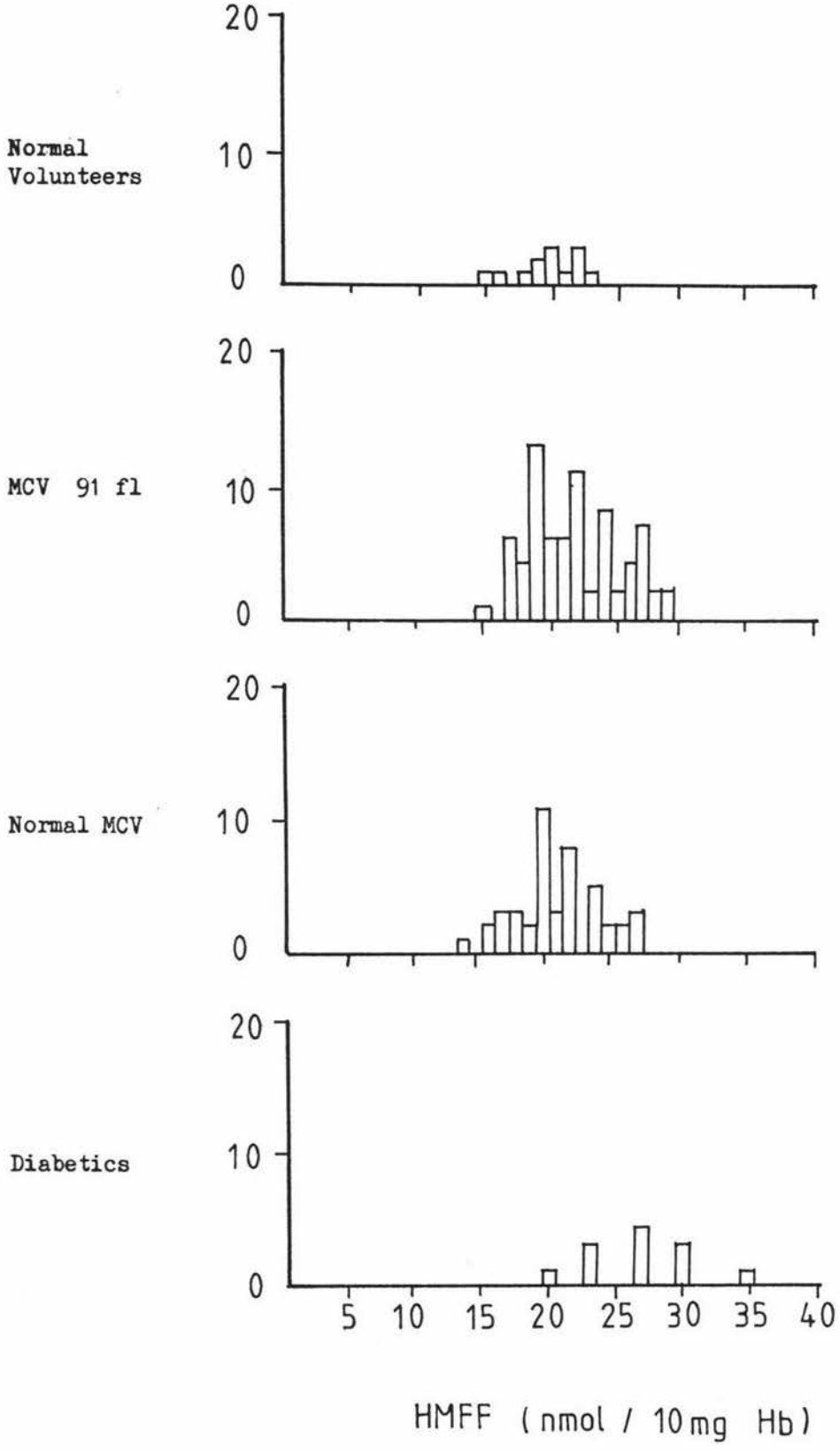


FIGURE 4-4, CORRELATION CURVE BETWEEN HMFF & FAST Hb FOR THE DIABETICS

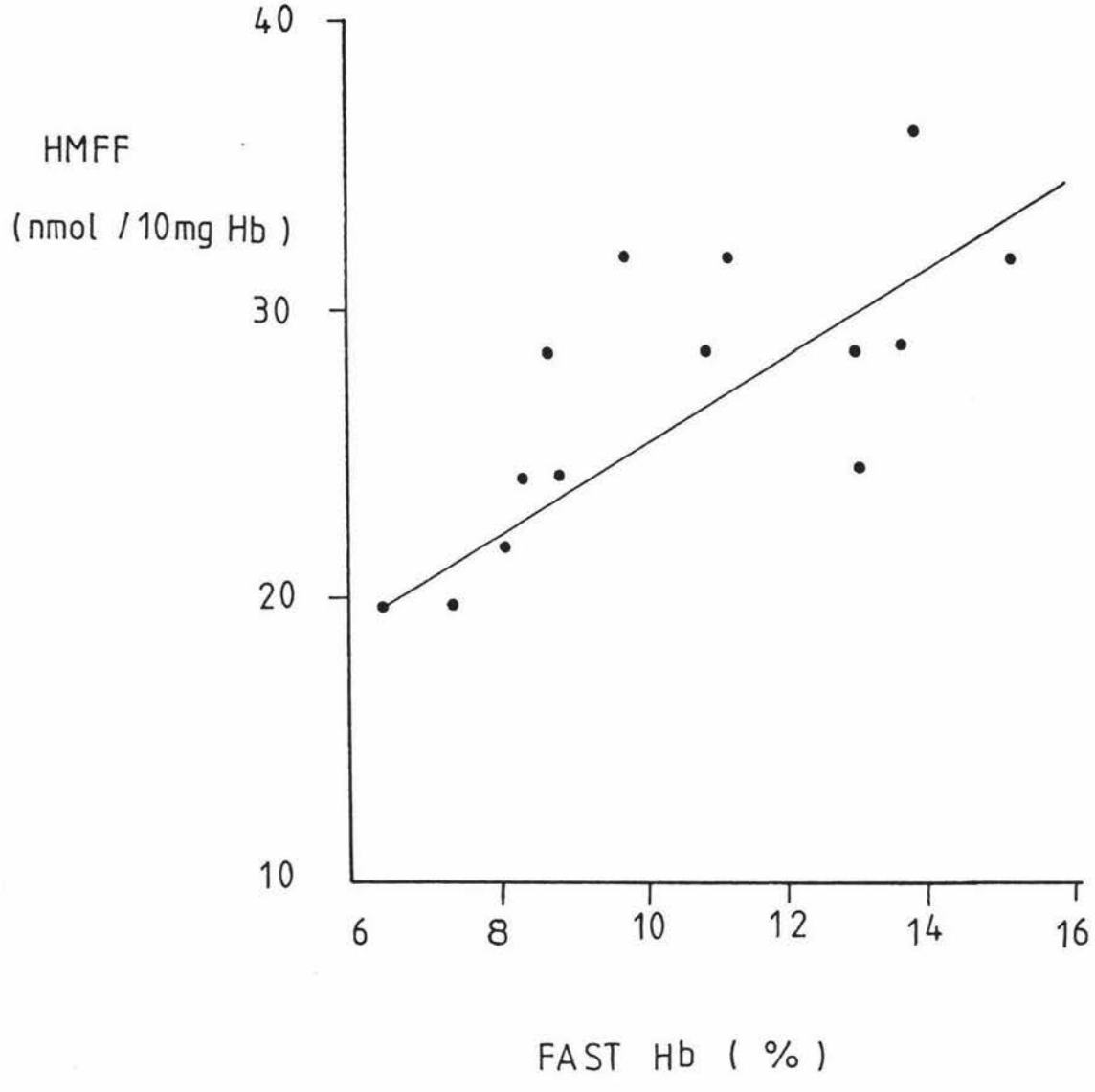


FIGURE 4-5, CORRELATION CURVE BETWEEN HMFF & FAST Hb FOR NORMAL MCV GROUP

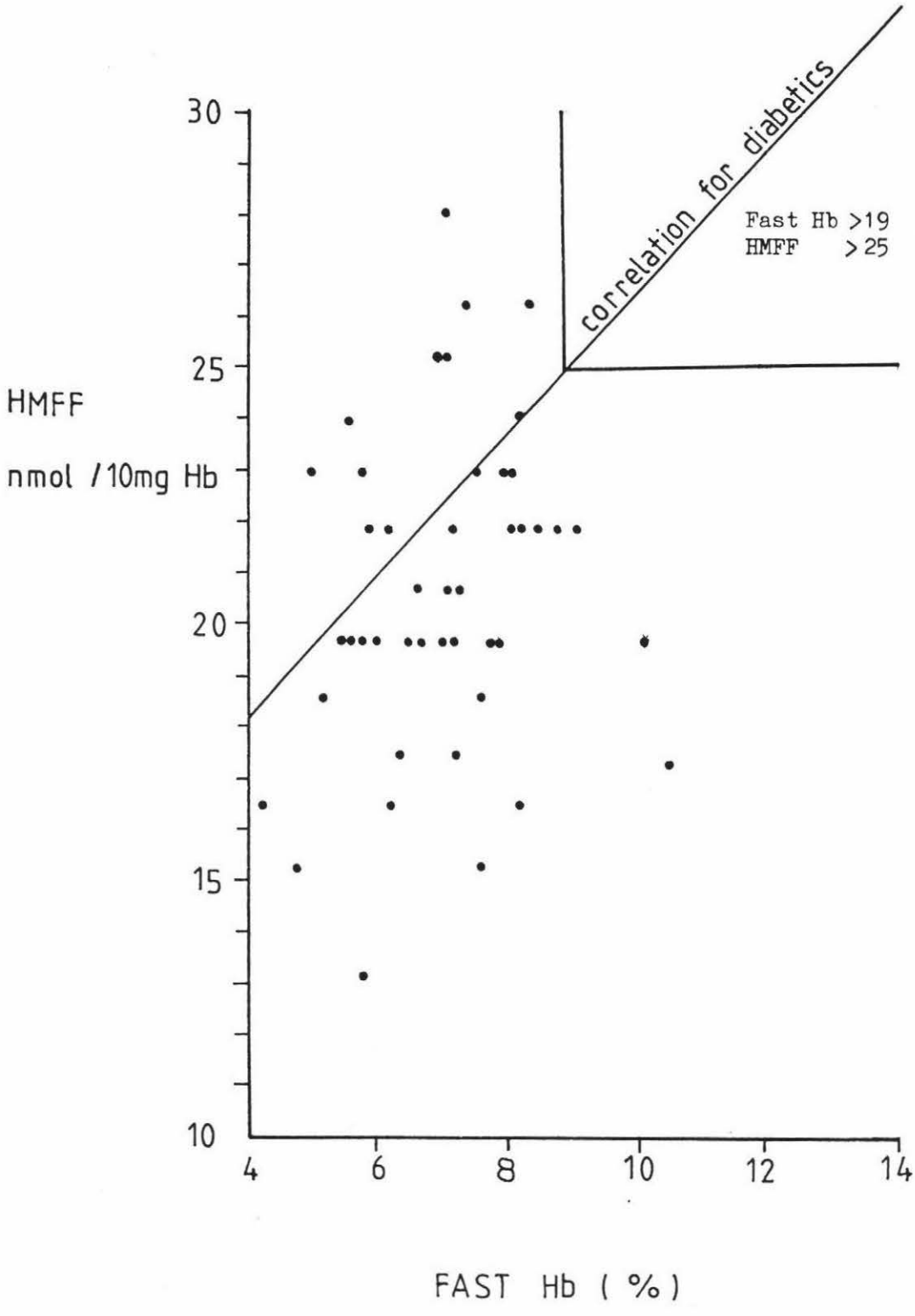
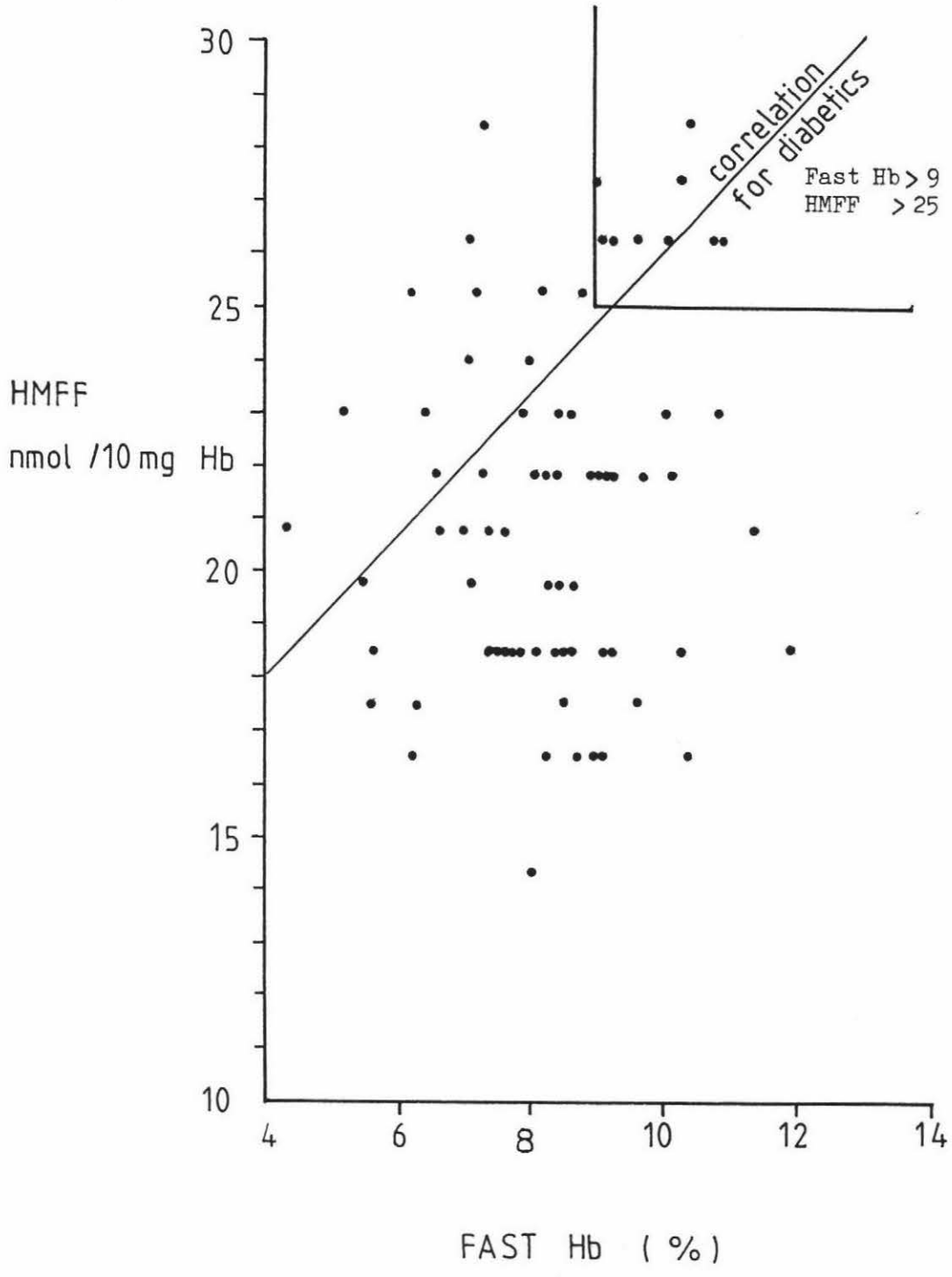


FIGURE 4-6, CORRELATION CURVE BETWEEN HMFF & FAST Hb FOR HIGH MCV GROUP



However there are 9 blood specimens in the high MCV group with a fast haemoglobin >9.0 % and HMFF >25 whereas there are no such cases in the normal MCV group. These 9 blood specimens are tabulated below:-

Sample number	HMFF	% Fast Hb
2	26	9.1
5	26	10.9
28	27	10.3
38	28	10.4
49	27	9.0
64	26	10.1
66	26	9.6
67	26	9.1
68	26	10.8

The HMFF values correpond to those expected from the level of fast haemoglobin due to glycosylation of the haemoglobin molecule.

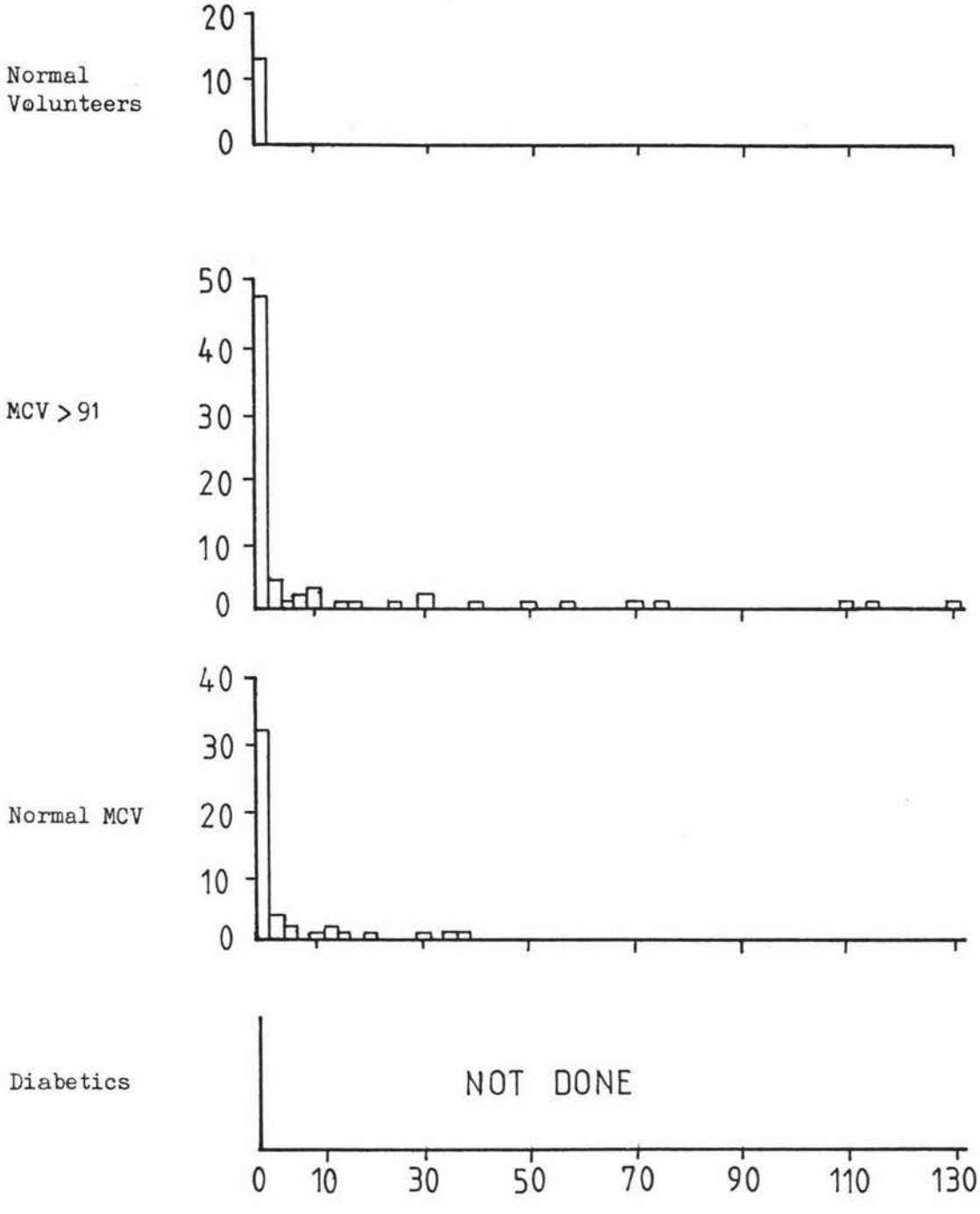
There were a further 2 specimens (numbers 31 and 46) with raised HMFF results of 26 and 28 respectively, but with normal levels of fast haemoglobin as measured by column chromatography (7.1 and 7.4 %).

4-5. Gamma glutamyl transpeptidase

The plasma GGTP levels, determined by the method described in Chapter 2 are recorded in Appendix Tables 1 to 3, and illustrated by histograms in Fig. 4-7. None of the samples from normal volunteers showed levels greater than 3 IU. The results in bloods with a normal MCV ranged up to 39 IU and up to 130 IU in bloods from the high MCV group. The upper limits described in the literature are 37 IU for males and 27 IU for females. This would suggest liver damage in 8 patients from the high MCV group. These samples in the high MCV group were:-

Sample number	Sex	GGTP IU
3	F	58
12	F	70
28	F	75
30	M	115
51	F	51
55	M	40
59	M	130
60	M	111

FIGURE 4-7, HISTOGRAMS OF GGTP



4-6. Thiamine deficiency

The results of the transketolase assay described in Chapter 2 are recorded in Appendix Tables 1 to 3, and illustrated by histograms in Fig. 4-8. A change in activity of the enzyme when thiamine pyrophosphate is added is an indication of thiamine deficiency in the blood sample. Fig. 4-9 shows the effect of such an addition to the blood samples with high MCV. Only when the transketolase activity is below 0.5 IU is this effect apparent and is almost linear. None of the normal volunteers appeared to be thiamine deficient.

Specimens which increased transketolase activity when thiamine pyrophosphate was added were:-

	Specimen number	Sex	TK	TPP effect
High MCV	6	M	0.56	9
	19	F	0.40	43
	25	M	0.17	64
	26	F	0.23	67
	31	M	0.28	50
	32	M	0.19	60
	37	F	0.47	55
	49	F	0.20	66
Normal MCV	9	M	0.17	76
	29	M	0.38	50

FIGURE 4-8, HISTOGRAMS OF TRANSKETOLASE ACTIVITY

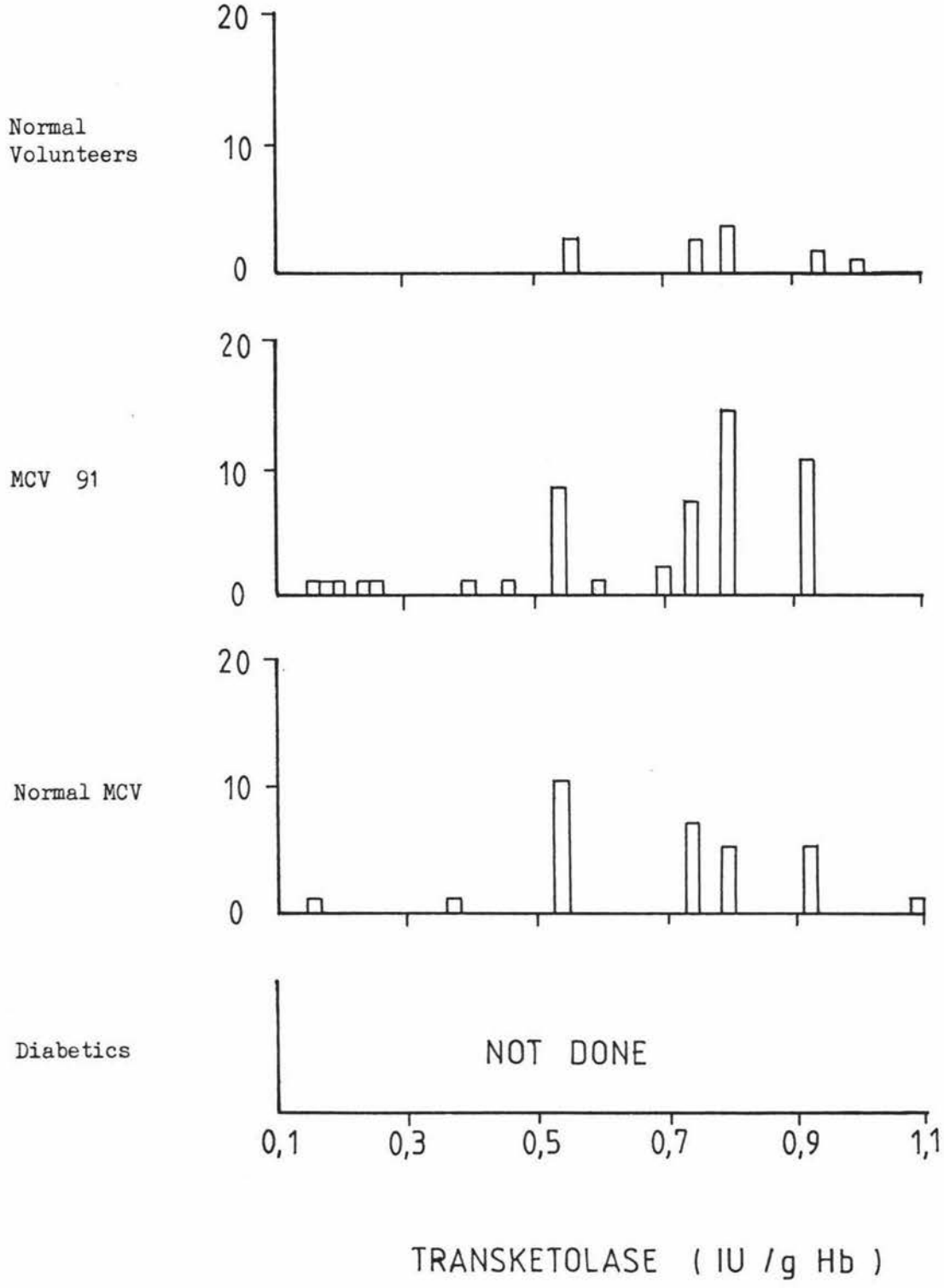
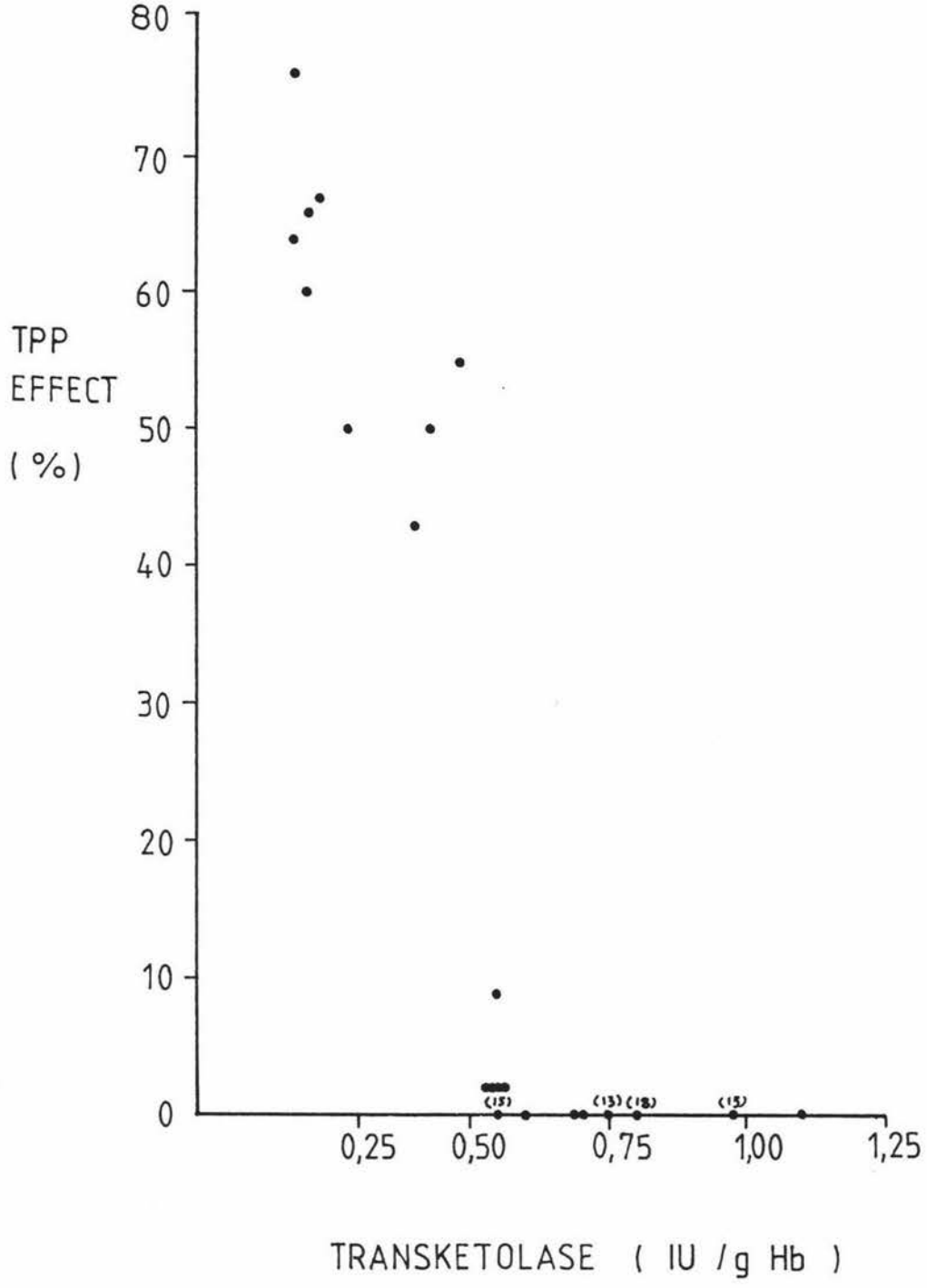


FIGURE 4-9, CORRELATION BETWEEN TRANSKETOLASE ACTIVITY & THE EFFECT OF ADDING EXCESS THIAMINE PYROPHOSPHATE FOR THE HIGH MCV GROUP BLOODS



4-7. Combination of data

Abnormalities were found in 23 out of 70 specimens with a mean corpuscular volume greater than 91 fl. These cases, listed below, show that none of the abnormalities coincide in the same patient. The only abnormalities found in 45 bloods with a normal mean corpuscular volume were two with thiamine deficiency.

Specimens with abnormalities in the high MCV group were:-

HMFF ⩾ 25	GGTP ⩾ 40 IU	Thiamine deficiency
2	-	-
-	3	-
5	-	-
-	-	6
-	12	-
-	-	19
-	-	25
-	-	26
28	28	-
-	30	-
-	-	31
-	-	32
-	-	37
38	-	-
-	-	49
-	51	-
-	55	-
-	59	-
-	60	-
64	-	-
66	-	-
67	-	-
68	-	-

CHAPTER 5

DISCUSSION

The original aim of the investigation was to determine whether acetaldehyde in the blood of heavy drinkers formed an adduct with haemoglobin, thereby increasing the percentage of fast haemoglobin.

Any reasonably active aldehyde or ketone can be attached covalently to proteins by reductive alkylation at the amino groups, and borohydride, cyanoborohydride and dimethylamine borane are reducing agents of choice for these reductive alkylations (Geoghegan *et al*, 1981). To obtain acetaldehyde adduct *in vitro*, it appears to be necessary to add sodium borohydride (Stevens *et al*, 1981). Preliminary attempts to prepare acetaldehyde adducts without the addition of such agents were unsuccessful and it was decided to place more emphasis on the search for adduct *in vivo*.

In the studies of blood samples taken from normal volunteers and hospital patients, it was hoped to find the acetaldehyde adduct in samples which had an increase in fast haemoglobin as measured by column chromatography. Increased levels of fast haemoglobin were found only in 9 blood specimens from the high MCV group, but in all of these cases, they could be attributed to glucose as the hydroxymethylfurfural test was also raised to the level predicted by the correlation between the two tests in bloods from diabetic patients.

The colorimetric HMFF assay is specific for ketoamine linked glucose (Bunn *et al*, 1978), however, it is difficult to standardise because it can give falsely high positive results due to *de novo* formation of HMFF in samples containing high concentrations of glucose (Bunn, 1981). Parker *et al* (1981) recommended that a haemolysate blank should be included in the test because the amount of colour generated in the blank was independent of the haemolysate when the haemoglobin concentration was adjusted to 10 g/l but dependent on the length of incubation time in the autoclave. They also suggested that approximately 4 nmol of HMFF should be subtracted from the results of the test. Falsely high readings can also be given by a wide variety of aromatic carbonyl compounds which may react with TBA to produce yellow pigments (Keeney & Bassette, 1959). Among the high MCV bloods, two had a high HMFF levels and a normal fast haemoglobin. Abraham *et al* (1978) also found high HMFF values with normal levels of fast haemoglobin as

determined by column chromatography but were unable to offer any explanation for these findings. A later study by Subramaniam et al (1980) showed similar results and they suggested that there are some proportions of glycosylated Hb A that the column method does not measure. None of the normal MCV bloods showed elevated fast Hb or HMFF levels.

There are too few cases in this category to be certain of excluding the possibility of adduct formation. In any case, the length of time in which these patients had been in hospital and hence not consuming alcohol is not known, but many have been sufficiently long for any adduct to disappear. Further studies will have to be made on known alcoholics or heavy drinkers in order to validate these points.

Evidence for liver damage caused by excessive alcohol consumption may be found by an increased level of liver enzyme in the plasma. There were 8 patients in the high MCV group with an increase in GGTP. While liver disease may also result from the use of drugs such as the anti-epileptics, dilantin or phenobarbitone (Rosalki et al, 1971). Such causes cannot be excluded but it would appear that the most likely cause in the present study is alcohol abuse and the combination of elevated GGTP levels and mean corpuscular volume is quite likely to reflect the daily alcohol intake (Papoz et al, 1981).

Thiamine deficiency is related to the neurological disorder, Wernicke-Korsakoff syndrome seen in some chronic alcoholics. Thiamine deficiency was seen in eight subjects with a high MCV and two subjects with a normal MCV, however it was felt that the relatively large number of cases found in this study of general hospital patients was more likely to be attributable to other causes. Some of the samples selected could have come from patients undergoing chemo- or radiotherapy for cancer. The anorexia of such patients coupled with potent and harsh chemical therapy may cause significant malnutrition (Warnock et al, 1978). If this is the case, then there may well be concomitant folate deficiency resulting in a pre-megaloblastic anaemia with macrocytic cells.

These results suggest that an increased MCV is associated with a metabolic disorder as yet undefined. The increase in MCV in alcoholics has been reported by Wu et al (1974) and provides a useful laboratory screening test for this disorder (Clark & Kricka, 1981). However, it is not a specific test although it is usually associated with a degree of

anaemia. This present study has shown that 4 out of 14 bloods in the diabetic group had increased MCV; supporting the findings of Davidson et al (1981).

Zieve (1958) reported a syndrome associated with alcoholic fatty liver and cirrhosis with macrocytosis and haemolytic anaemia. The hyper-triglyceridemia of this syndrome appears to have no influence on red cell survival, but Cooper (1980) has suggested that this disorder is due to hypersplenic haemolysis. However, a proportional transfer of cholesterol and lecithin from lipoproteins to red cell membranes in liver disease results in an increase in surface area of the red cells (Cooper 1980). While Zieve's syndrome may be the end result of a long period of excessive drinking, there are undoubtedly intermediate manifestations of the disease, with intermediate degrees of red cell abnormality and compensated red cell destruction. Cases such as these may account for the non anaemic macrocytosis of alcoholism.

If the mean corpuscular volume increases as a result of lipid changes in the red cell membrane, this may be an indication of similar changes in the membranes of other organs such as the central nervous system. It has been suggested that tolerance develops to the effects of ethanol due to a change in the lipid composition of the membranes within the central nervous system (Littleton, 1980). Work on the central nervous system is limited in humans, but the red cell provides a readily obtainable source of membranes in humans and in some ways behaves much like myelin.

While lipid incorporation is necessary in maintaining the integrity of the cell membrane, shape is also important in the proper functioning of the erythrocyte. The normal biconcave shape of the erythrocyte is maintained by a major protein spectrin (Lux, 1979). Palek et al (1981) reported that spectrin together with actin and polypeptide 4.1 formed a two-dimensional submembrane skeleton in the normal red cell to provide a structural support to the membrane and to modulate cell shape, membrane stability, deformability and a lateral mobility of certain transmembrane proteins. Any impairment of this deformability and distortion of membrane architecture can lead to accelerated conditioning and destruction of red cells in the spleen (Cooper, 1980). Acetaldehyde was also found to react with spectrin and

actin in the erythrocyte membrane and produced stomatocyte changes in cell morphology characteristic of those found in alcoholics (Gaines et al, 1977). Therefore a measurement of spectrin levels in red cells of alcoholics with macrocytosis may be significant. Kansu (1980) using an immunological method to study spectrin loss during red cell lysis in vitro showed that spectrin could be extracted from washed erythrocyte ghosts and a specific antibody raised enabling estimation by immunological methods.

It did not prove possible in this study, to identify acetaldehyde adduct formation as such. Only those subjects with increased liver enzymes in the plasma might be classified as heavy alcohol consumers and none of these had an increase in fast haemoglobin. Nevertheless, a significant finding is the large number of abnormalities associated with an increase in mean corpuscular volume which may or may not be related to alcohol consumption and this alone warrants further investigation.

Application of the tests described in this thesis showed a total of 23 abnormal findings likely to be associated with heavy drinking in 70 bloods selected for high MCV whereas there were only 2 such abnormalities in the group of 45 patients selected with a normal MCV.

Many of the questions posed here may be answered by examination of the medical records of patients with a high mean corpuscular volume. From the evidence discussed above, it appears that an increase in mean corpuscular volume is an indicator of metabolic abnormality as yet undefined and may not be associated with alcohol consumption in the majority of cases.

APPENDIX I

APPENDIX TABLE 1

Results in normal volunteers

Subject No.	Sex	Hb g/ l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
1	M	-	8.6	⟨3	23.00	0.94	0
2	M	-	8.1	⟨3	21.90	0.75	0
3	M	-	9.0	⟨3	20.80	0.56	0
4	M	-	9.8	⟨3	15.33	0.56	0
5	M	-	7.5	⟨3	17.52	0.56	0
6	M	-	6.2	⟨3	14.23	0.75	0
7	M	-	8.6	⟨3	21.90	0.80	0
8	M	-	7.9	⟨3	19.71	0.80	0
9	M	-	8.7	⟨3	18.61	0.75	0
10	F	-	9.4	⟨3	19.71	0.80	0
11	F	-	6.9	⟨3	21.90	1.00	0
12	M	-	7.9	⟨3	19.71	0.94	0
13	M	-	7.8	⟨3	18.61	0.80	0

APPENDIX TABLE 2

Hospital patients with a mean corpuscular volume greater than 91 fl

Subject No.	Sex	MCV fl	Hb g/ l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
1	F	94	127	7.4	⟨3	18.61	0.75	0
2	M	94	155	9.1	⟨3	26.28	0.80	0
3	F	92	120	10.0	58	23.00	0.94	0
4	M	95	144	8.4	⟨3	18.61	0.80	0
5	F	94	155	10.9	⟨3	26.28	0.75	0
6	M	93	155	11.3	⟨3	20.80	0.56	9
7	F	92	137	8.5	⟨3	17.52	0.56	2
8	F	92	126	9.2	⟨3	18.61	0.80	0
9	F	93	141	9.2	⟨3	18.61	0.94	0
10	F	95	135	8.6	⟨3	18.61	0.62	0
11	F	99	145	9.6	⟨3	17.52	0.80	0
12	F	92	120	8.7	70	19.71	0.80	0
13	M	92	142	7.6	⟨3	18.61	0.75	0
14	M	94	130	8.0	⟨3	14.23	0.56	2
15	M	96	140	10.2	⟨3	18.61	0.56	0
16	M	93	156	9.1	⟨3	16.42	0.80	0
17	F	94	124	10.3	⟨3	16.42	0.94	0
18	M	93	149	11.9	31	18.61	0.94	0
19	F	96	N	8.2	⟨3	16.42	0.40	43
20	F	95	N	9.1	⟨3	18.61	0.75	0
21	F	96	N	8.7	⟨3	16.42	0.80	0
22	M	92	153	5.6	⟨3	18.61	0.94	0
23	M	94	140	6.7	⟨3	20.80	0.56	0
24	F	97	126	6.2	⟨3	16.42	0.80	0
25	M	94	151	8.1	⟨3	21.90	0.17	64
26	F	95	120	8.3	⟨3	21.90	0.23	67
27	F	94	124	6.3	⟨3	17.52	0.75	0
28	F	94	123	10.3	75	27.38	0.80	0
29	F	99	127	9.0	⟨3	16.42	0.94	0
30	M	93	132	5.6	115	17.52	0.75	0

N = Normal

APPENDIX TABLE 2 (Cont'd)

Subject No.	Sex	MCV fl	Hb g/l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
31	M	95	133	7.1	<3	26.28	0.28	50
32	M	93	182	8.5	<3	18.61	0.19	60
33	M	98	138	7.0	<3	20.80	0.80	0
34	M	100	145	8.6	<3	23.00	0.80	0
35	M	97	129	7.4	<3	20.80	0.94	0
36	M	94	157	7.6	<3	20.80	0.56	2
37	F	96	128	6.4	<3	23.00	0.47	55
38	M	95	170	10.4	<3	28.47	0.80	0
39	F	95	131	7.1	<3	24.09	0.75	0
40	M	96	164	7.1	<3	19.71	0.94	0
41	M	105	140	9.3	<3	21.90	0.56	0
42	F	92	140	6.6	9	21.90	0.94	0
43	M	96	153	5.5	<3	19.71	0.94	0
44	F	95	135	4.3	<3	20.74	0.56	0
45	M	97	140	5.2	<3	23.00	0.80	0
46	M	101	140	7.4	<3	28.47	0.80	0
47	M	101	140	7.9	<3	23.00	0.81	0
48	F	94	128	8.9	<3	21.90	0.71	0
49	F	96	127	9.0	<3	27.38	0.20	66
50	M	92	155	6.2	<3	25.19	0.71	0
51	F	93	120	8.1	51	18.61	-	-
52	F	95	142	7.8	11	18.61	-	-
53	F	94	120	8.3	25	19.71	-	-
54	F	93	134	8.3	6	19.71	-	-
55	M	95	158	9.1	40	21.90	-	-
56	M	93	140	10.1	8	21.90	-	-
57	F	94	145	10.8	15	23.00	-	-
58	M	98	145	7.6	4	25.19	-	-
59	M	92	152	8.4	130	21.90	-	-
60	M	92	149	8.5	111	23.00	-	-

APPENDIX TABLE 2 (Cont'd)

Subject No.	Sex	MCV fl	Hb g/l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
61	F	97	133	9.7	8	21.90	-	-
62	F	95	130	8.8	5	25.19	-	-
63	F	94	133	8.0	11	24.09	-	-
64	M	94	154	10.1	5	26.28	-	-
65	F	98	123	9.2	33	21.90	-	-
66	F	94	138	9.6	11	26.28	-	-
67	F	93	121	9.1	/3	26.28	-	-
68	M	94	146	10.8	/3	26.28	-	-
69	F	93	139	8.2	/3	25.19	-	-
70	M	93	142	7.3	19	21.90	-	-

APPENDIX TABLE 3

Hospital patients selected for normal MCV

Subject No.	Sex	MCV fl	Hb g/l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
1	F	84	N	8.2	⟨3	16.42	0.56	2
2	M	80	N	8.2	⟨3	24.09	0.75	0
3	M	80	N	10.5	⟨3	17.52	0.56	0
4	M	86	N	10.1	⟨3	19.71	0.80	0
5	M	81	N	9.1	⟨3	21.90	0.75	0
6	M	82	N	6.4	⟨3	17.52	0.80	0
7	M	86	154	5.8	⟨3	13.14	0.56	0
8	F	83	131	4.2	⟨3	16.42	0.56	0
9	M	82	145	7.3	⟨3	20.80	0.17	76
10	F	84	176	5.5	⟨3	19.71	0.56	0
11	M	86	155	5.8	16	23.00	0.94	0
12	M	89	150	7.6	⟨3	15.33	0.75	0
13	F	88	138	6.2	⟨3	21.90	0.75	0
14	F	86	145	6.2	16	16.42	0.94	0
15	M	79	143	4.8	⟨3	15.33	0.94	0
16	M	81	139	7.4	⟨3	26.28	1.10	0
17	M	89	169	7.2	39	19.71	0.94	0
18	M	82	142	6.0	⟨3	19.71	0.75	0
19	M	86	145	7.6	⟨3	18.61	0.94	0
20	F	81	131	5.8	⟨3	19.71	0.56	0
21	F	81	135	5.6	⟨3	19.71	0.56	0
22	F	85	125	5.2	⟨3	18.61	0.80	0
23	F	83	128	5.0	⟨3	22.97	0.56	0
24	M	82	152	5.6	⟨3	24.09	0.56	0
25	M	90	140	6.7	⟨3	19.71	0.80	0
26	M	83	150	6.5	⟨3	19.71	0.80	0
27	M	91	141	7.1	⟨3	20.80	0.75	0
28	F	91	133	7.6	⟨3	23.00	0.56	0
29	M	91	160	5.9	⟨3	21.90	0.38	50
30	M	89	155	8.2	7	21.90	-	-

N = Normal

APPENDIX TABLE 3 (Cont'd)

Subject No.	Sex	MCV fl	Hb g/l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
31	F	88	135	7.2	30	17.52	0.38	50
32	F	88	145	8.5	7	21.90	-	-
33	M	87	147	7.1	17	19.71	-	-
34	F	89	135	7.9	<3	19.71	-	-
35	M	90	152	8.8	37	21.90	-	-
36	M	87	140	7.1	4	25.19	-	-
37	F	87	143	8.4	4	26.28	-	-
38	M	87	153	7.1	4	23.00	-	-
39	M	86	146	6.7	13	20.80	-	-
40	F	87	141	8.1	<3	21.90	-	-
41	F	89	137	7.2	<3	21.90	-	-
42	M	86	162	7.0	<3	25.19	-	-
43	M	87	160	8.0	<3	23.00	-	-
44	M	89	143	8.0	22	23.00	-	-
45	M	86	N	7.9	<3	19.71	0.75	0

N = Normal

APPENDIX TABLE 4

Diabetic patients

Subject No.	Sex	MCV fl	Hb g/l	Fast Hb %	GGTP IU	HMFF nmol/10mg Hb	Transketolase IU/g Hb	TPP effect %
1	-	93	111	8.5	-	24.09	-	-
2	-	96	130	6.5	-	19.71	-	-
3	-	89	112	11.0	-	28.47	-	-
4	-	91	159	15.2	-	31.76	-	-
5	-	83	127	8.9	-	24.09	-	-
6	-	86	160	8.8	-	28.47	-	-
7	-	90	176	9.9	-	31.76	-	-
8	-	90	158	7.4	-	19.71	-	-
9	-	88	137	11.3	-	31.76	-	-
10	-	92	125	8.1	-	21.90	-	-
11	-	90	155	13.8	-	36.14	-	-
12	-	93	149	13.0	-	28.47	-	-
13	-	86	150	13.0	-	24.09	-	-
14	-	91	125	13.6	-	28.47	-	-

APPENDIX II

Materials	Suppliers
Acrylamide	BDH Laboratory Chemicals
Ampholine (pH 4-6), (pH 5-7), (pH 6-8), (pH 7-9)	Merck
Ampholine PAG plate	LKB-Produkter AB
Beef liver	Local slaughter center
Bio-Rex 70	Bio-Rad Laboratories
Gamma glutamyl-p-nitroanilide	Sigma
Glycerophosphate dehydrogenase	Sigma
Hydroxymethylfurfuraldehyde (HMFF)	Sigma
Ribose-5-phosphate	Sigma
Thiamine pyrophosphate	Sigma
Thiobarbituric acid (TBA)	Sigma
Triose-phosphate isomerase	Sigma

All other chemicals and solvents were reagent grade or better and supplied by Sigma Chemical Company, BDH Laboratory Chemicals or May and Baker Ltd.

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