STUDIES ON THE PHYSIOLOGICAL SIGNIFICANCE OF MARGINAL RIBOFLAVIN DEFICIENCY IN MAN WITH AN EMPHASIS ON THE METABOLIC ROLE OF RIBOFLAVIN IN RED BLOOD CELL AGEING

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HILARY J. POWERS, B.Sc.

Department of Human Nutrition
London School of Hygiene and Tropical Medicine
University of London

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ABSTRACT

The results of a recently conducted nationwide survey in Britain indicated that a large proportion of the elderly population could be considered to be biochemically riboflavin deficient. The work presented here describes an attempt to assess the physiological significance of marginal riboflavin deficiency in man with particular emphasis on the metabolic importance of riboflavin to red blood cell (RBC) ageing.

Red cells from adults and geriatrics were separated into nine fractions of different mean age by density gradient centrifugation. Haematological measurements including haematocrit, RBC count, reticulocyte count, and haemoglobin (Hb) estimation, were made on whole blood and packed cell samples from all subjects. Glutathione reductase (GR) activity (I.U./g Hb) was measured in all fractions and in unfractionated blood samples from each subject. Biochemical indices selected for study included malonyldialdehyde (MDA) production and percentage haemolysis of RBC in response to peroxidative stress; endogenous haemoglobin derivatives specifically methaemoglobin (MHb), oxyhaemoglobin (OxyHb) and sulphaemoglobin (SHb); and endogenous reduced glutathione (GSH). In addition the effect of daily riboflavin supplements on haematological and biochemical responses of RBC in two biochemically riboflavin deficient subjects was studied.

Studies on RBC distribution suggested that marginal biochemical ariboflavinosis may predispose RBC to premature lysis *in vivo*, particularly in the elderly. Biochemical studies showed that as RBC age GR activity, GSH, and OHb fall and peroxidative haemolysis and MHb rise. These observations implied that older cells with low GR activity are less able to resist oxidative stress than are young cells. On the other hand, more MDA was produced in peroxide stressed younger cells than in older cells which may reflect changes in RBC lipid composition as cells age. Although marginal riboflavin deficiency appeared to result in RBC with increased MHb, increased susceptibility to peroxidative haemolysis and reduced concentrations of GSH these effects were not statistically significant.

Finally, a mechanism was proposed which implicated GR activity as a determinant of RBC survival and a computer programme devised to describe the mechanism. Results of the study suggested that GR activity is a determinant of RBC survival or is closely linked with the activity of another such enzyme.

TO MY FATHER

ACKNOWLEDGEMENTS

The work summarised here has been carried out in the Department of Human Nutrition at the London School of Hygiene and Tropical Medicine.

Thanks are due to the Medical Research Council for making it financially possible to carry out this course of study and to the Central Research Fund for their assistance in purchasing a Beckmann SW 27.1 swinging bucket rotor.

I am most grateful to Professor J.C. Waterlow for the opportunity of working in his Department, and for the continual support he has given throughout the period of study. I would also like to thank Dr J. Stephen for the interest that she has always shown in my work and the willingness with which she has discussed and criticised the project.

Thanks are due also to Professor A.E. Dugdale, who, through his expertise made it possible to develop the computer model of RBC survival, and to Dr J. Morris who kindly permitted me to include among the subjects studied elderly patients under her care.

I would particularly like to thank my supervisor, Dr D.I. Thurnham for his endless patience and cooperation and the sound advice which he so readily gave.

Finally I would like to thank all my colleagues in the Department of Human Nutrition, who, by their constant encouragement and friendship made the course of my study such a happy one.

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LIST OF ABBREVIATIONS

Term	Abbreviation
Adenosine triphosphate	ATP
Adenosine triphosphatase	ATPase
Aspartate aminotransferase	AST
Bovine Serum Albumin	BSA
Change in optical density	ΔOD
Correlation coefficient	r
Density	g/ml
Erythrocyte glutathione reductase	EGR
Ethylene diaminetetra-acetic acid	EDTA
Fatty acids	F.A.
Flavin adenine dinucleotide	FAD
Flavin mononucleotide	FMN
Glucose-6-phosphate dehydrogenase	G6PD
Glutathione, oxidised	GSSG
Glutathione peroxidase	GSHPx
Glutathione, reduced	GSH
Glutathione reductase	GR
Gram	g
Gram-atom	mol
Gravitational force	g
Haemoglobin	Hb
Hexokinase	HX
Hydrogen ion concentration (-log.10)	pH
Hydrogen peroxide	H_2O_2
International Units	I.U.
Iron	Fe

Kilogram	Kg
Logarithm (base 10)	$\log_{10}$
Malonyldialdehyde	MDA
Methaemoglobin	MHb
Microgram	µg
Micromolar (concentration)	µM
Millilitre	ml
Millimolar (concentration)	mM
Millimol	mmol
Minute	min
Molar (concentration)	M
Nanometre	nm
NADH-methaemoglobin reductase	NADH-MHb-R
NADPH-methaemoglobin reductase	NADPH-MHb-R
Nicotinamide adenine dinucleotide	NAD
Nicotinamide adenine dinucleotide, oxidised	NAD^+
Nicotinamide adenine dinucleotide, reduced	NADH
Nicotinamide adenine dinucleotide phosphate	NADP
Nicotinamide adenine dinucleotide phosphate, oxidised	$NADP^+$
Nicotinamide adenine dinucleotide phosphate, reduced	NADPH
Oxyhaemoglobin	OxyHb
Packed cell volume	PCV
Per	/
Peroxide	ROOH
Potassium cation	k^+
Percent	%
Red Blood Cell	RBC

Refractive Index	R.I.
Singlet oxygen	O_2^1
Sodium, cation	Na^+
Standard error of the mean	SEM
Sulphaemoglobin	SHb
Superoxide anion	$O_2^{\overline{\cdot}}$
Thiobarbituric acid	TBA
Trichloracetic acid	TCA
Unsaturated fatty acid	UFA
Weight/volume	w/v
White Blood Cell	WBC

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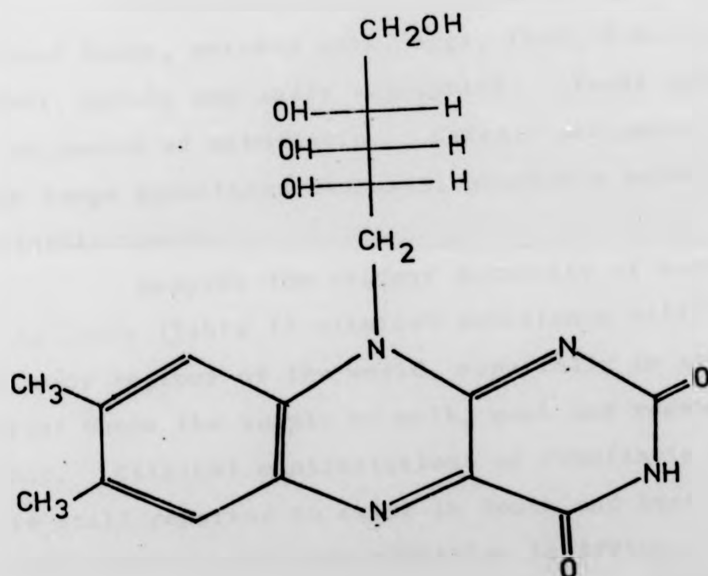
INTRODUCTION

A. The Structure and Occurrence of Riboflavin

The vitamins form a mixed group of chemical compounds for which at present no satisfactory definition exists. When the vitamins were originally discovered, they were so named due to the observation that they constituted essential items in the diet, and to the mistaken belief that they were all amines. They are presently recognized as being organic substances, required in low concentrations in the diet, and often metabolically active as enzyme prosthetic groups, or coenzymes. The system of designation of vitamins by letters, adopted at the time of their discovery has been retained but modified in accordance with the accumulation of new facts related to vitamin activity of different compounds. Although the chemical structures of the presently recognized vitamins have been elucidated many are still known only by their accepted trivial names or even by their old letter designation. The chemical structure of vitamin B_2, commonly known as riboflavin, was elucidated in 1935. It is recognized as being an isoalloxazine derivative with a ribitol side chain. Figure 1 shows the structural formula of riboflavin. This compound consists of orange-yellow crystals with a m.pt. of about 280°C, stable at room temperature, and soluble in water. Riboflavin is

Figure 1 The Structure of Riboflavin



unstable in the presence of alkalis or when exposed to light or u.v. irradiation, which is of significance when considering the effective riboflavin content of foods.

Riboflavin is essential to growth and its active derivatives are involved in the oxidative metabolism of carbohydrates, fats and proteins.

Riboflavin occurs in a wide range of plant and animal foods, notably milk, eggs, fish, kidney, liver, heart, muscle and leafy vegetables. Yeast provides a rich source of riboflavin. Cereals and pulses, due to the large quantities consumed, provide a potentially valuable source.

Despite the evident diversity of sources of riboflavin (Table I) clinical deficiency still exists in many regions of the world, especially in tropical areas where the supply of milk, meat and vegetables is poor. Clinical manifestations of riboflavin deficiency are still reported to exist in South and East Asia, Latin America, and some countries in Africa. Nutritional deficiencies occur predominantly in groups of low socio-economic status. It would be naive to suppose that poor dietary habits alone can explain the incidence of malnutrition but, dietary intakes are generally accepted as the principal factor in determining the prevalence of nutritional deficiency and recommended intakes for nutrients have been quantified as part of an attempt to identify potential nutritional problems. Recommended intakes of particular nutrients have been defined

Table 1: Natural Sources of Riboflavin

from: "Human Nutrition and Dietetics" by Davidson, Passmore, Brock and Truswell (1975).

Food	Description	Range (mg/100 g edible portion)
Good and Moderate Sources		
Wheat bran	Bran layer only	0.5
Wheat and barley	Whole grain	0.12-0.25
Wheat germ	Germ fraction, steam heated and finely ground	0.25
Pulses	Various, fresh	0.1-0.3
Fish	Various, fresh & cured	0.2-0.4
Beef, mutton & pork	Raw	0.1-0.3
Liver & kidney		2.0-3.3
Eggs	Fresh	0.3-0.5
Cheese		0.3-0.5
Cocoa	Powder	0.3-0.4
Chocolate	Plain	0.2
Brewers' yeast		1.3-4.0
Yeast extract e.g. Marmite		5.0-6.0
Wheat germ e.g. Bemax		0.67
Meat extract e.g. Bovril, Oxo		1.8-2.6
Milk	Fresh cow's	0.15
Green leafy vegetables*		0.05-0.30
Maize	Whole	0.10
Oatmeal		0.15
Millets		0.10-0.15
Wheat flour	Wholemeal (i.e. containing germ and fine bran	0.10-0.15
Nuts		0.2
Fruit	Dried	0.1
Beers		0.05-0.10
Poor Sources		
Maize	Meal	0.02-0.1
Rice	Lightly milled	0.05-0.1
Rice	Highly milled	0.03-0.05
Wheat flour	70% extraction	0.03-0.05
Fruits	Fresh, tropical & temperate	0.01-0.1
Potato	All seasons	0.05
Vegetables	Various 30% green	0.09

* Green vegetables and fruit in tropical countries are in general richer in riboflavin than those in temperate regions.

(FAO/WHO 1967; DHSS, 1969) as the amounts sufficient or more than sufficient for the nutritional needs of practically all healthy persons in a population. The possibility of increased requirements due to infection, metabolic abnormality or disease are not taken into account and the recommended intake for one nutrient is given on the assumption that all other nutrients are taken in adequate amounts. It cannot be assumed that intake of nutrients far in excess of recommended intakes will not be harmful. Riboflavin however, as a water-soluble vitamin is readily excreted in the urine if present in the diet far in excess of recommended intakes and toxic effects of excess intake have not been reported. Although recommended intakes are generally expressed on a daily basis it is known that the body has the ability to store all nutrients for a few days at least, and can tolerate daily variations in intake. With these points in mind FAO/WHO (1974) have recommended a daily riboflavin intake of 0.60 mg /1000 kcals for an adult male. On this basis the recommended intakes for different sections of a population have been calculated, and these are presented in Table II. There is no reason to suppose that an increased understanding of the importance of riboflavin in metabolism and a recognition of new factors which may influence its utilization will not result in changes in the recommended intakes shown. It can be seen (Table 2) that recommended riboflavin intakes for women are higher during pregnancy

Table 2: Recommended intakes of Protein, Energy and Riboflavin

Age	Body Weight	Energy (1)		Protein (1,2)	Ribo-flavin (3)
	Kg	Kc	MJ	g	mg
Children					
1	7.3	820	3.4	14	0.5
1-3	13.4	1360	5.7	16	0.8
4-6	20.2	1830	7.6	20	1.1
7-9	28.1	2190	9.2	25	1.3
Male adolescents					
10-12	36.9	2600	10.9	30	1.6
13-15	51.3	2900	12.1	37	1.7
16-19	62.9	3070	12.8	38	1.8
Female adolescents					
10-12	38.0	2350	9.8	29	1.4
13-15	49.9	2490	10.4	31	1.5
16-19	54.4	2310	9.7	30	1.4
Adult man (moderately active)	65.0	3000	12.6	37	1.8
Adult woman (moderately active)	53.0	2200	9.2	29	1.3
Pregnancy (later half)		+ 350	+1.5	38	+0.2
Lactation (first 6 months)		+ 550	+2.3	46	+0.4

From: FAO (1974)

(1) FAO (1972)

(2) As egg or milk protein

(3) FAO (1965)

and lactation; this reflects the estimated increase in requirements during these periods. The elderly, on the other hand, have not been distinguished from the normal adult population in terms of their recommended intakes for riboflavin.

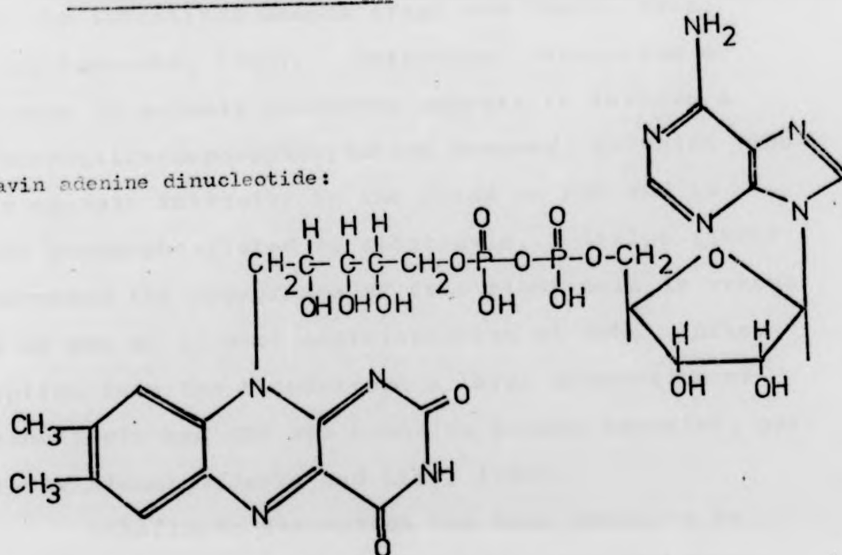
Recommended intakes refer to the amount of nutrient available for metabolic needs; this implies that in relating dietary intakes to recommended intakes account must be taken of possible losses during transport, storage, and cooking of foods. Some losses in riboflavin occur during the processing and cooking of foods and riboflavin, which is light sensitive, may be destroyed in foods exposed to sunlight for long periods of time (DHSS, 1969; FAO/WHO, 1967). Milk, when pasteurized, loses only about 5% of its riboflavin but exposure of milk to sunlight results in rapid losses such that within 4 hours, 71% of the vitamin is lost. Processing of foods such as canning, freezing, dehydration, and irradiation, techniques in common use in many countries, appear to have little effect on the riboflavin content of meat, fish, fruits or vegetables. On the other hand, the practice of sun-drying fish, meat, fruits or vegetables, as seen in some tropical countries, causes significant destruction of riboflavin. As much of the riboflavin present in grain products is contained in the germ and bran, milling processes reduce their riboflavin content dramatically. For example, polished rice contains only about 59% of the riboflavin originally present in brown rice (Dimler *et al.*, 1960).

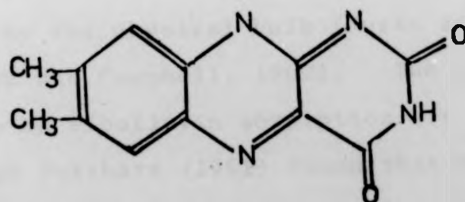
B. <u>Uptake, Storage and Distribution in Tissues</u>

The metabolic utilization of riboflavin appears to depend on its conversion to two phosphorylated derivatives, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Figure 2). Riboflavin in the tissues is found predominantly as the phosphorylated derivatives, rarely as riboflavin *per se*. These phosphorylated derivatives function as prosthetic groups for a wide range of enzymes. The role of flavoproteins in metabolism will be considered in the following section.

Evidence has accumulated for a site-specific saturable intestinal absorption process for riboflavin in man. That absorption of riboflavin in man is localised in the small intestine has been demonstrated by several workers, who administered riboflavin to human subjects by various routes, and measured the urinary recovery (levy and Jusko, 1966; Campbell and Morrison, 1963). Saturability of the intestinal absorption of riboflavin has been demonstrated by the oral administration of FMN equivalent to 5-300 mg of riboflavin of which decreasing proportions were recovered as riboflaving in the urine as the dose increased (Jusko and Levy, 1967). The precise mechanism for intestinal absorption of riboflavin in man has not yet been elucidated as most of the work in this context has been carried out in the rat. In animals, however, riboflavin either formed from FMN in the intestinal lumen or given directly appears to be phosphorylated to

Figure 2 The Structure of Flavin Mononucleotide(FMN) and Flavin Adenine Dinucleotide (FAD)





FMN in the intestinal mucosa (Yagi and Okuda, 1958; Chen and Yamauchi, 1960). Intestinal absorption of riboflavin in animals therefore appears to involve a phosphorylation-dephosphorylation process, in which riboflavin appears initially in the blood as FMN and is rapidly dephosphorylated to riboflavin. Stripp (1965) demonstrated the appearance of free riboflavin in venous blood of man after oral administration of FMN. After absorption into the bloodstream a large proportion of the riboflavin and FMN are bound to plasma proteins, particularly albumin (Jusko and Levy, 1969).

Riboflavin absorption has been shown to be influenced by a number of other metabolites and drugs. There is no evidence however that human requirements of riboflavin are influenced by the carbohydrate/fat ratio. In general food appears to enhance riboflavin absorption, probably due to an increased intestinal transit time caused by the physical bulk (Jusko and Levy, 1969; Morrison and Campbell, 1960). The possible effects of glucose on riboflavin absorption are as yet unclear, although Fukahara (1961) found that the presence of glucose decreased the intestinal absorption of riboflavin in rabbits. The situation in man has received little attention. Although thyroxine has been implicated in the metabolism of riboflavin (Rivlin, 1970a) there is little evidence to suggest that it influences significantly the intestinal absorption in man. There have been conflicting

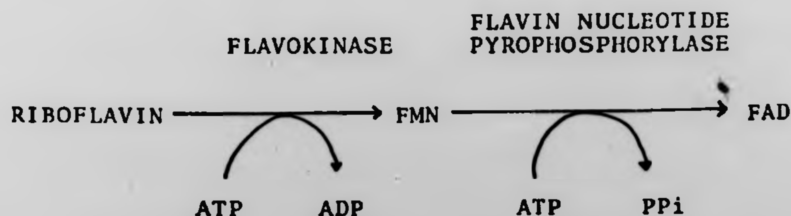
reports concerning the effects of steroid hormones on riboflavin absorption. A study in man (Brummer, Markkanen and Kalliomaki, 1961) showed that the administration of corticosteroids had no effect on urinary recoveries of riboflavin. Another group of workers, however, have shown that estradiol affects riboflavin absorption in male chickens (Cordona and Payne, 1967). Antibiotics such as tetracyclines, penicillin and streptomycin apparently influence synthetic and degradative microorganisms in the intestinal tracts of animals and may thus have a sparing effect on marginal riboflavin deficient diets (Review: Goldsmith, 1965). The effects of antibiotics on riboflavin absorption in man are as yet undefined.

Elimination of riboflavin from man occurs primarily through the urine and stools, in the form of the free vitamin (Jusko and Levy, 1967).

The distribution of riboflavin in the tissues of man has been poorly documented. Considerable work has been carried out to determine the relative composition of flavin compounds in rat tissues and results have shown that the liver, heart and kidney contain most of the riboflavin, as FAD (Cerletti and Ipata, 1960; Yang and McCormick, 1967). Alterations in the distribution of FMN and FAD as a result of riboflavin deficiency have been observed in animals, with large differences in the rates at which particular flavin-dependent enzymes fall

in activity (Burch, Lowry, Padilla and Combs, 1956; Glatzle, Weber and Wiss, 1968). It has been possible to make accurate measurements of flavin compounds in RBC, serum, and WBC of humans. Burch, Bessey and Lowry (1948) detected levels of free riboflavin and FMN in sera of 0.8 μg/100 ml sera, 2.4 μg FAD/100 ml sera, and 22.4 μg total riboflavin/100 ml RBC.

Within most tissues there appears to be enzymes present responsible for the synthesis of flavin prosthetic groups, and phosphatases and nucleotidases capable of catalysing the hydrolysis of FAD to FMN and of FMN to riboflavin (Mandula and Beutler, 1970; McCormick and Russel, 1962). Studies prompted by the observed pattern of flavins following uptake of a given flavin have indicated that the sequence of in vivo formation of prosthetic groups from riboflavin involves the initial formation of FMN followed by FAD (DeLuca and Kaplan, 1958; Mandula and Beutler, 1970).



In summary, there are facilities for the efficient uptake and utilization of riboflavin in its diverse roles, and in situations where dietary riboflavin is low the body

Table 3

Table 3: Properties of Flavoproteins and Metalloflavoproteins

Prosthetic groups Flavin	Other	Physiological acceptor
2(?)FAD	...	O_2
2FMN	...	O_2
2FAD	...	O_2
2FAD	...	O_2
2FMN	...	?
?	?	O_2
?	?	O_2
FAD	TPP, Mg^{+2}	O_2
FMN	...	O_2
FMN	...	O_2
FAD	...	H_2O_2
2nFAD	...	
		ETF
FAD	?	ETF
FAD	...	Respiratory chain
FAD(?)	...	$NADP^+$
2FAD	-S-S-	NAD^+
FAD	$2Mg^{+2}$ (not for electron transfer)	Cytochrome b_5
FAD	...	Cytochrome c
FMN	...	Cytochrome c
FAD	...	2-Methylnaphthoquinone
FMN	...	(menadione)
FAD	?	Oxidized glutathione
2FAD	8Fe 2Mo	O_2(cyt c)
2FAD	8Fe 2Mo	O_2(cyt c), NAD^+
2FAD	16Fe 4Mo	NAD^+
2FAD	8Fe 2Mo	O_2, respiratory chain
1FAD +1FMN	2Fe	O_2, NAD^+ for dihydroorotate
		O_2, orotate for NADH
1FMN	8Fe	Respiratory chain (not cytochrome c!)
1FMN	16-18 Fe	Ferricyanide
1FMN	2-3Fe	Ferricyanide, CoO
1FMN	4Fe	Cytochrome c

eins

Enzyme
D-Amino acid oxidase
L-Amino acid oxidase
Glucose oxidase
"Old yellow enzyme"
Diamine oxidase
Monoamine oxidase
Pyruvate oxidase
Glycolate oxidase
Luciferase
NADH peroxidase
Acyl CoA dehydrogenase (3 separate enzymes known)
Sarcosine dehydrogenase (oxidase)
Electron-transferring flavoprotein (ETF)
NADPH-ferredoxin reductase
Lipoyl dehydrogenase
NADH-cytochrome b_5 reductase
NADPH-cytochrome c reductase
NADPH-quinone reductase
NADPH-glutathione reductase
Xanthine oxidase (dehydrogenase)
Aldehyde dehydrogenase (oxidase)
Dihydroorotic dehydrogenase
NADH dehydrogenase

Source	Molecular weight
Liver, kidney	~100,000
Kidney	138,000
Snake venoms	~140,000
Moulds	154,000
Yeast	105,000
Liver, kidney, brain	?
Liver, kidney,brain probably mitochondrial	?
Lactobacillus delbrueckii	?
Plants	~100,000
Photobacterium fisheri	76,000
Streptococcus faecalis	120,000
Liver, heart mitochondria	200,000(?)
Liver, kidney mitochondria	?
Liver, heart mitochondria	70,000
Plants, adrenal microsomes	35,000
All respiratory particles	100,000
Liver microsomes and mitochondria	40,000
Microsomes	68,000
Yeast	78,000
Cytoplasm of animal cells, plants	56,000
	~50,000
E. coli, plants	?
Milk	300,000
Mammalian liver	
Avian liver or kidney	
Mammalian liver	280,000
Zymobacterium oroticum	125,000
Heart mitochondria	200,000 (particle)
	~500,000
	~250,000
	80,000

Table 3 contd.

Table 3 (continued)

Prosthetic groups: Flavin	Prosthetic groups: Other	Physiological acceptor
1FAD*	8Fe	Respiratory chain
1FAD*	4Fe	PMS, ferricyanide
nFAD	40nFe	
1FAD	4Fe	PMS
1FAD	1Fe	Ferricyanide, PMS
?FAD	?Mo	NO_3^-
2FMN	1 heme	Respiratory chain through cyt c
?FMN	?Zn^{+2}	Same as above

Enzyme	Source	Molecular weight
Succinate dehydrogenase	Beef heart mitochondria	~300,000
	Beef heart mitochondria, yeast	250,000
	M. lactilyticus	
Choline dehydrogenase	Mammalian liver mitochondria	800,000
α-Glycerophosphate dehydrogenase	Pig brain mitochondria	2×10^6(!)
NADP-nitrate reductase	*Neurospora crassa*	
L-Lactate dehydrogenase	Aerobic yeast respiratory particles	200,000
D-Lactate dehydrogenase	Aerobic yeast respiratory particles	?

* FAD linked covalently to protein and can be released only by enzymatic treatment.

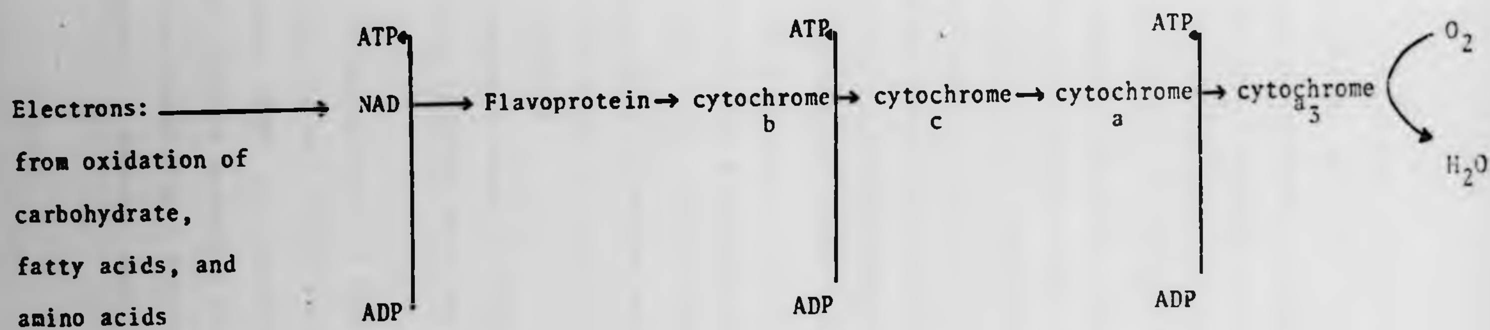
appears to show some adaptation to this stress by a re-distribution of flavins to those areas where the need is most critical. This point will be taken up in more detail in Section D.

C. The Importance of Riboflavin to Metabolism

i) Riboflavin in tissue metabolism

The forms of riboflavin in the tissues of man and animals that have been shown to be metabolically active are FMN and FAD. It is these derivatives which form the prosthetic groups of certain enzymes concerned with oxidation and reduction processes, being integrally associated with the enzymes concerned. The enzymes, together with their respective prosthetic groups are termed flavoproteins. Roles for flavoproteins have been found in carcinogenesis, teratogenesis and haematological disorders. These roles will be described later in this section. Table 3 shows the diversity of the reactions in which flavoproteins fu ction as prosthetic groups. Flavoproteins are considered to be active in three major areas of oxidative metabolism: the mitochrondrial electron transport chain, the NAD(P) linked dehydrogenation of some low energy substrates, and the oxygen-linked dehydrogenation of substrates such as amino acids (Wada and Snell, 1961; Mann, 1968; Straub, 1939). Flavoproteins

Figure 3: Respiratory Chain Phosphorylation (adapted from Lehninger, 'Bioenergetics', 1965)



where: ATP = Adenosine triphosphate

ADP = Adenosine diphosphate

are essential components of the respiratory chain, a final common pathway by which all electrons derived from the catabolism of proteins, carbohydrates, and fats flow to oxygen, the final oxidant in aerobic tissues. Electrons enter the respiratory chain, or electron transport chain, via one of two flavoproteins. One of these accepts electrons from NADH, the other from succinate. For each pair of hydrogen atoms removed from the Krebs cycle two electrons are supplied to the electron transport chain and ultimately reduce one atom of oxygen. The decline in free energy at each electron transfer depends on the relative oxidation-reduction potentials of the two carriers involved in the transfer. Where the decrease in free energy is sufficiently large the phosphorylation of ADP to ATP may take place. In fact, the free energy decrease that occurs during the process of electron transfer from NADH to oxygen accounts for almost all the free energy decrease in the oxidation of glucose in aerobic biological systems.

Figure 3 shows a scheme representing the respiratory chain coupled with the phosphorylation of ADP to ATP.

The production of pyruvate from hexoses is a characteristic of all cells employing the Emden-Meyerhof-Parnas pathway (EMP) for carbohydrate metabolism. In most animal tissues pyruvate is oxidised to acetyl CoA in a reaction involving a multienzyme pyruvate dehydrogenase complex. FAD has been identified as one component

of this complex. FAD has also been shown to function as a prosthetic group during the oxidation of fatty acids in the mitochondria of animal tissues. Flavins have been ascribed roles in amino acid metabolism, as cofactors for amino acid oxidases in which case the flavoproteins carry hydrogen directly to molecular oxygen, and for the synthesis of the amino acid methionine (Wellner and Meister, 1961; Smith, 1962).

ii) Riboflavin in RBC metabolism

In the previous section the role of riboflavin in tissue metabolism was briefly described. The ultimate dependence of the oxidation of complex molecules on the oxygen-requiring electron transport chain was explained. However one of the characteristics of the mature RBC which distinguishes it from many other actively metabolizing tissues is the lack of any mitochondria. The lack of mitochondria in RBC is significant, as it means that the RBC have relatively limited metabolic activity at least as regards carbohydrate oxidation.

The enucleated mature RBC in the peripheral circulation is incapable of *de novo* synthesis of proteins, fats, or carbohydrates and is dependent throughout its lifespan on the enzymes with which it is endowed when it is released from the bone marrow as a reticulocyte. The chief source of energy in the RBC is the oxidative breakdown of glucose through the anaerobic EMP, whereas the

Figure 4

Figure 4: The Hexose Monophosphate Shunt

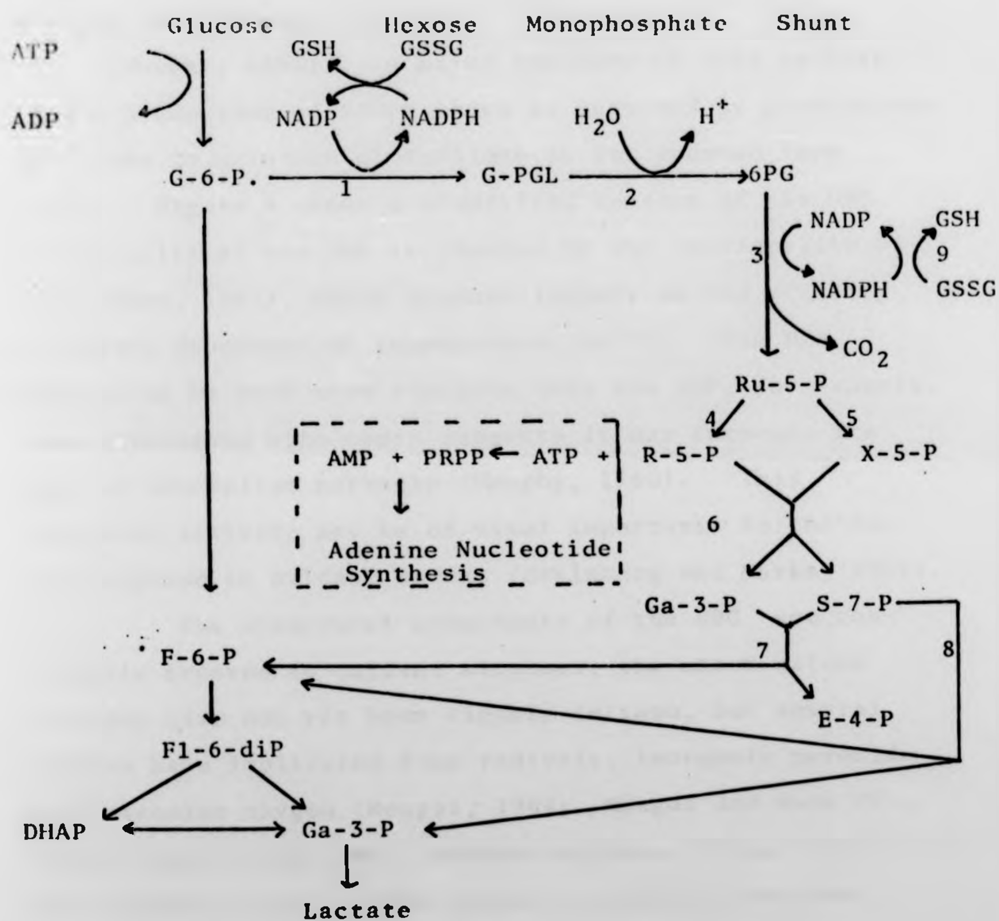
Numbers refer to the following enzymes:

1. glucose-6-phosphate dehydrogenase
2. 6-phosphogluconolactonase (or lactonase)
3. 6-phosphogluconate dehydrogenase
4. xylulose-5-phosphate epimerase
5. ribose-5-phosphate isomerase
6. transketolase
7. transaldolase
8. transketolase (as for 6)
9. glutathione reductase.

Abbreviations:

G-6-P, glucose 6-phosphate
6-PGL, 6-phosphogluconolactone
6-PG, 6-phosphogluconate
Ru-5-P, ribulose 5-phosphate
R-5-P, ribose 5-phosphate
X-5-P, xylulose 5-phosphate
Ga-3-P, glyceraldehyde 3-phosphate
S-7-P, sedoheptulose 7-phosphate
F-6-P, fructose 6-phosphate
E-4-P, erythrose 4-phosphate
PRPP, 5-phosphoribosyl-1-pyrophosphate
DHAP, dihydroxy acetone phosphate
GSH, reduced glutathione
GSSG, oxidised glutathione

Figure 4: The Hexose Monophosphate Shunt (HMS)
(from: Eaton and Brewer, 1974).



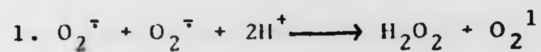
pentose phosphate pathway, or hexose monophosphate shunt (HMS) is estimated as metabolising only about 10% of the glucose entering the cell

(Murphy, 1960). A major function of this pathway is the production of NADPH which is required by glutathione reductase to maintain glutathione in its reduced form (GSH). Figure 4 shows a simplified version of the HMS. The activity of the HMS is limited by the availability of NADP (Rose, 1961), which depends largely on the activity of flavin dependent GR (Mannervick, 1977). The HMS appears to be much more flexible than the EMP, for example, when stimulated with redox reagents it may increase its rate of metabolism markedly (Murphy, 1960). This potential activity may be of vital importance to the RBC when exposed to oxidant stress (Szeinberg and Marks, 1961).

The structural components of the RBC are continually exposed to oxidant stresses, the exact nature of which have not yet been clearly defined, but several workers have implicated free radicals, inorganic peroxide and molecular oxygen (Mengel, 1968; Mengel and Kann Jr., 1966; Mengel, Kann, Jr., Heyman and Metz, 1965; Stocks and Dormandy, 1970). Two types of radicals have been identified in biological systems: free radicals associated with protein moieties and polymeric ring compounds containing unpaired electrons (Commoner, Townsend and Pake, 1954). Evidence has been presented by a number of workers for the likely formation of superoxide free radicals ($O_2^{\cdot}$) and singlet oxygen (O_2^1) in aerobically

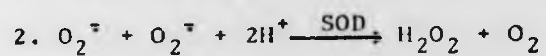
Figure 5: Selected Oxidant Producing Reactions of $O_2^{\cdot -}$

(adapted from Leipzig, Brewer and Kruckeberg, 1974)



1. $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2^1$

   Spontaneous dismutation of $O_2^{\cdot -}$



2. $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

   Catalysis by superoxide dismutase

3. $O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$

   The Haber-Weiss reaction, resulting in the formation of $OH^{\cdot}$

where: $O_2^{\cdot -}$ = superoxide radical

O_2^1 = singlet oxygen

$OH^{\cdot}$ = hydroxyl free radical

metabolising tissues (Flohé and Zimmerman, 1974). Hydrogen peroxide (H_2O_2) *per se* may be produced by the action of xanthine oxidase or hypoxanthine and by the action of superoxide dismutase on superoxide radicals and hydrogen ions (Walls and Hochstein, 1974). Also, the spontaneous dismutation of superoxide radicals can generate H_2O_2 (Loschen, 1975). Figure 5 shows some selected oxidant-producing reactions of $O_2^{\bar{\cdot}}$.

Although there appear to be physiological roles for these species, such as in amino acid synthesis (Serif and Kirkwood, 1958), there is increasing evidence that lipids and proteins are particularly susceptible to degenerative attack from hydrogen peroxide and highly reactive radicals (Flohe and Zimmerman, 1974; Dormandy, 1969; Mengel, Kann Jr. and Meriwether, 1967). It has been demonstrated that the lipids of RBC membranes may be oxidised to form lipid peroxides when exposed to H_2O_2-generating systems (Dodge, Cohen, Kayden and Phillips, 1967), and the resultant lipid peroxides are themselves toxic (Desai and Tappel, 1963; Little and O'Brien, 1967, Chio and Tappel, 1969). Lipid peroxidation may induce structural changes in the membrane which lead to more fragile RBC (Dodge, Cohen, Kayden and Phillips, 1967).

Clearly the RBC requires a system of defence in the the face of oxidant attack and GSH appears to play a major role in the protective systems (Mills and Randall, 1958; Cohen and Hochstein, 1961). GSH is an essential substrate for glutathione peroxidase (GSHPx) activity, an enzyme known to assist in the removal of H_2O_2 and of toxic lipid

Figure 6: A Proposed Role for Glutathione Peroxidase In Lipid Metabolism

LIPIDS

where: G.R. = Glutathione Reductase
GSHPx = Glutathione Peroxidase
ROH = Hydroxylated Lipid
ROOH = Peroxidised Lipid
GSH = Reduced glutathione
GSSG = Oxidized glutathione

peroxides (O'Brien and Little, 1967). Figure 6 shows a proposed role for GSHPx.

There continues to be a certain amount of controversy over the relative importance of GSHPx and catalase in the detoxification of H_2O_2 but there is evidence that both systems are active in removing H_2O_2 (Cohen and Hochstein, 1963). It is relevant to mention, however, that Jacob and his colleagues (Jacob, Ingbar and Jandl, 1965) showed that in acatalasemic individuals the RBC HMS is stimulated about twenty times more than that of normal RBC when exposed to oxidant stress. This reflects the attempt by the cells to counteract the increased demand on the GSHPx system by an increased rate of production of GSH. If GSH production within the RBC is allowed to fall below a critical level it might be predicted that GSHPx would be unable to fulfil its antioxidant function and the RBC may lyse. In conditions of glucose 6 phosphate dehydrogenase (G6PD) deficiency in man, it has been demonstrated that RBC are highly susceptible to oxidative damage and haemolyse _in vivo_ when exposed to oxidant stress (Schrier _et al._, 1958; Hochstein, 1971;) Such RBC, when exposed to low levels of H_2O_2 _in vitro_ for long periods are unable to maintain their GSH levels (Cohen, 1975; Cohen and Hochstein, 1961). These observations have been interpreted as being due primarily to the inability of the cells to maintain NADPH levels and therefore to a limit on the activity of GR and the production of GSH.

An 'adaptive' mechanism has been reported in subjects with G6PD deficiency in which GR binds more tightly to FAD than in normal subjects. Thus, although NADPH might be expected to be in short supply, the activity of GR does not fall even when dietary intakes of riboflavin are low (Thurnham, 1972).

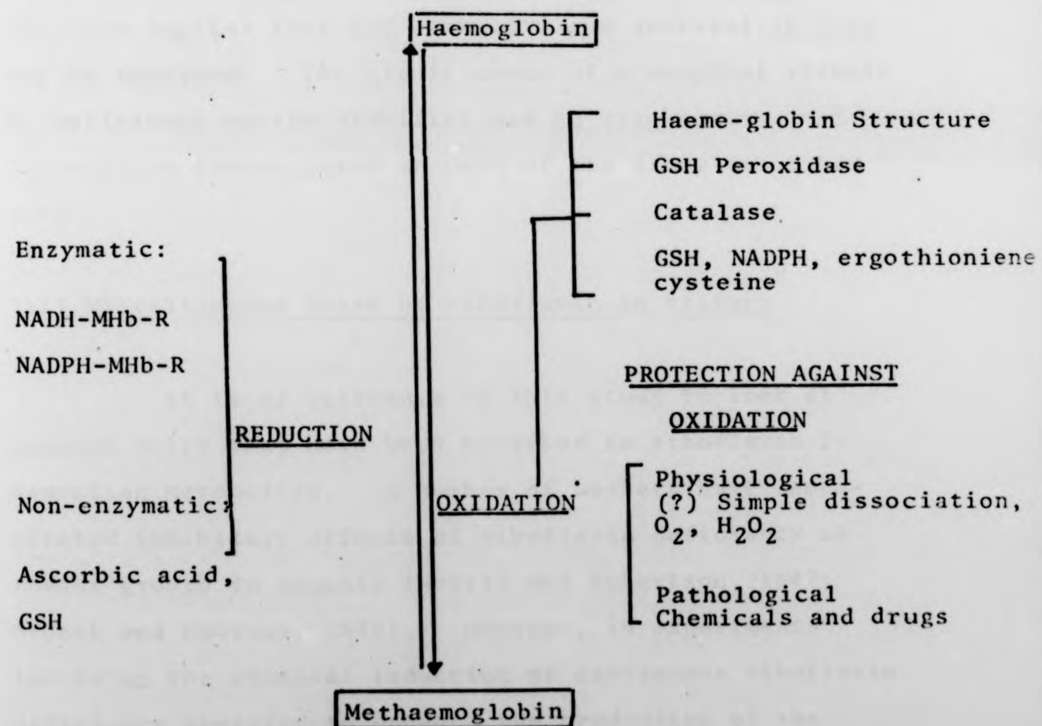
GSH also appears to participate in mechanisms designed to maintain Hb in its oxygen-carrying form. Hb is liable to oxidise to a form incapable of carrying oxygen, methaemoglobin (MHb), in which the sixth coordination position of the ferric haem iron is occupied by a water molecule or a hydroxyl ion (Jaffé and Neumann, 1964).

There is evidence that GSH may undergo slow non-enzymic oxidation to GSSG with the concomitant reduction of MHb to Hb (Morrison and William, 1938). The significance of this effect *in vivo* however has been doubted (Townes and Lovell, 1961; Mohler, *et al.* (1970). There is also evidence for two enzymes capable of catalysing the reduction of MHb. The NADPH-FAD dependent reductase appears though to be of less physiological significance than the NADH-FMN dependent reductase (Matsuki *et al.*, 1978). Figure 7 shows a scheme describing proposed mechanisms for the maintenance of Hb in its functional form.

It has been suggested that H_2O_2 may be involved in the oxidation of Hb to MHb (Cohen and Hochstein, 1964),

Figure 7: Schematic Representation of the Metabolic Processes Presumably Involved in the Oxidation of Haemoglobin to Methaemoglobin, the Protection against Oxidation of Haemoglobin to Methaemoglobin and the Reduction of Methaemoglobin to Haemoglobin in Human Erythrocytes

(from 'The Red Blood Cell', Surgenor, Ed., 1975).



where: NADH - MHb-R = Reduced nicotinamide adenine dinucleotide-dependent methaemoglobin reductase

NADPH-MHb-R = Reduced nicotinamide adenine dinucleotide phosphate-dependent methaemoglobin reductase

GSH = reduced glutathione

which implies that GSHPx may help to protect OHb by destroying the H_2O_2.

GSH it seems, is of particular importance as an antioxidant metabolite within RBC. An impairment in the regeneration or utilization of GSH by the RBC might be expected to increase the susceptibility of the cell to oxidant damage. In riboflavin deficiency GR activity in vitro is reduced (Tillotson and Sauberlich, 1971) and as GR is the major route for GSH production in RBC this implies that RBC stability and survival in vivo may be impaired. The significance of a marginal vitamin B_2 deficiency on the stability and in vivo survival of RBC will be investigated as part of the study described here.

iii) Miscellaneous roles of riboflavin in tissues

It is of relevance to this study to look at defined roles that have been ascribed to riboflavin in mammalian metabolism. A number of workers have demonstrated inhibitory effects of riboflavin deficiency on tumour growth in animals (Morris and Robertson, 1947; Stoerk and Emerson, 1949). However, in experiments involving the chemical induction of carcinomas riboflavin deficiency appeared to enhance the production of the tumours (Tannenbaum and Silverstone, 1953). In man a possible relationship between carcinogenesis and riboflavin status has not yet been established.

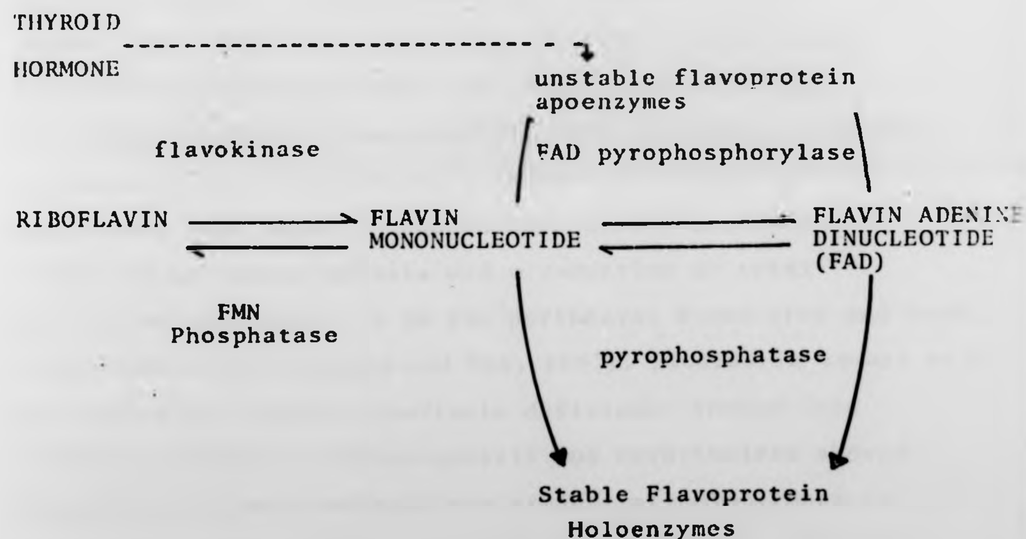
Recent studies in animals have indicated that riboflavin deficiency during pregnancy may result in a wide range of congenital malformations in the progeny (Gilman, Perry and Hill, 1952; Nelson, Baird, Wright and Evans, 1956). It has been suggested that a precipitating factor in this phenomenon is a reduction in activity in the electron transport chain in the foetal tissues during gestation. The observation that foetal flavoprotein enzymes are reduced in activity during severe maternal riboflavin deficiency supports this suggestion (Miller, Poncet and Takacs, 1962).

There is accumulating evidence that riboflavin and thyroid hormone metabolism may be related. It has been shown (Rivlin and Langdon, 1966), that FMN- and FAD-requiring enzymes are decreased in activity in hypothyroidism and increased in activity in hyperthyroidism. Rivlin (1970b) presented a scheme describing the postulated role of thyroid hormone in stimulating the synthesis of the flavin prosthetic groups FMN and FAD. This scheme is shown in Figure 8. Work in animals has also suggested that riboflavin may be involved in the regulation of thyroid hormone action. Riboflavin deficiency apparently reduces the hepatic deiodination of thyroxine and appears to limit the effectiveness of thyroid hormone as an inducer of flavoprotein enzymes (Galton and Ingbar, 1965).

Riboflavin status also appears to be related to erythrokinetics. Some possible mechanisms which may

Figure 8: Postulated Role of Thyroid Hormone in Stimulating the Synthesis of Flavoprotein Apoenzymes and in Stimulating the Formation of FMN and FAD

(From Rivlin, 1970)



relate riboflavin to RBC integrity have already been described, and several workers have postulated a role for riboflavin in erythropoiesis. Sirivich, Driskell and Frieden (1977) demonstrated that riboflavin deficiency in rats resulted in reduced activity of NADH-oxido-reductase, which is FMN dependent, in the duodenum, liver and kidney. They suggested that riboflavin deficiency may inhibit the absorption of iron by altering the integrity of the duodenal mucosa. Also, Halvey and Guggenheim (1958) showed that riboflavin deficiency in rats reduced tetrahydrofolate synthesis, which may limit erythropoiesis through its effect on deoxynucleic acid synthesis. Studies on animals with experimentally induced deficiency of riboflavin have shown a reduced bone marrow activity with resulting erythroid aplasia and a reduction or total absence of reticulocytes in the peripheral blood (Foy and Kondi, 1968; Endicott, Kornberg and Ott, 1947). Studies in humans with galactoflavin induced riboflavin deficiency showed less dramatic changes in erythropoiesis but nevertheless showed hypoplasia of the erythroid series and reticulocytopaenia (Alfrey and Lane, 1970; Lane and Alfrey, 1965). Foy and Kondi (1953) reported the successful treatment of a true red cell aplasia in man with riboflavin. Foy, Kondi and Macdougall (1961) have postulated an indirect action of riboflavin on marrow erythropoiesis through the action of corticosteroids.

The precise mechanisms describing the relationship between riboflavin and erythropoiesis, RBC survival,

carcinogenesis, teratogenesis and thyroid hormone action need yet to be clarified. Part of the work described here constitutes an attempt to clarify the role of riboflavin in erythrokinetics.

D. Riboflavin Deficiency

i) Clinical manifestations

Although the occurence of riboflavin deficiency has been reported in several populations, particularly in children and during pregnancy and lactation it rarely occurs in isolation and is more commonly observed in conjunction with other vitamin deficiencies (Hoorn, Flikweert and Westerink, 1975; Hill, et al., 1977). It is not easy, therefore, to ascribe clinical abnormalities to riboflavin deficiency alone. However, as a result of studies in man employing riboflavin antagonists, and experimental deficiency studies, certain clinical findings are generally accepted as being indicative of riboflavin deficiency (Lane, Mengel and Doherty, 1960; Lane and Brindley, 1964). Early stages of deficiency are associated with angular stomatitis, which may be followed by glossitis, seborrhoeic dermatitis, cheilosis and dermatitis of the eyes, ears, and genitalia (Joliffe, Fein and Rosenblum, 1939; Sydenstricker, Geeslin, Templeton and Weaver, 1939). Vascularization of the cornea has been described in some subjects and cataract formation

may be a manifestation of severe deficiency (Sydenstricker et al., 1939). The anaemia that may develop in riboflavin deficiency is characterised by reduced reticulocytosis and bone marrow hypoplasia, the red cells being normochromic and normocytic (Lane and Alfrey, 1965; Alfrey and Lane, 1970). Haemolytic anaemia is not normally associated with riboflavin deficiency, at least with the degree of riboflavin depletion achieved with riboflavin antagonists.

ii) Metabolic responses to deficiency

As would be expected, riboflavin deficiency results in a reduction in activity of a number of flavin-dependent enzymes. Liver flavoproteins have received a certain amount of attention, a result of which has been the demonstration that enzymes such as xanthine oxidase, glycollic acid oxidase, succinic dehydrogenase and NADPH-cytochrome C reductase are reduced in activity in riboflavin-depleted animals (Burch, Lowry, Padilla and Combs, 1956; Rivlin, 1970a). Studies in riboflavin-deficient rats have suggested that riboflavin deficiency may interfere with the metabolism of folate. For example, Honda (1968) showed that riboflavin deficiency in rats resulted in an impaired conversion of folic acid to N-methyltetrahydrofolate. The observed sensitivity to riboflavin depletion of one FAD-dependent enzyme in erythrocytes, GR, led to the development of a functional test to assist in the measurement of riboflavin status (Glatzle, Weber and Wiss, 1968; Sharada and Bamji, 1972).

Some adaptive mechanisms appear to exist in animals and humans for ensuring a supply of flavoproteins for their most essential functions in situations of ribo-glavin depletion. For example, during riboflavin deficiency in rats hepatic FMN falls relatively faster than FAD, which may be accounted for by the observed increase in activity of hepatic FAD pyrophosphorylase (Prosky, et al., 1964; Fass and Rivlin, 1969). As FAD functions as the prosthetic group of many more enzymes than FMN this may be an adaptive response to limiting riboflavin such that more of the flavoprotein-requiring processes can be maintained (Rivlin, 1970b).

Riboflavin deficiency in man may result from either inadequate intake or an impaired absorption or utilization. Ideally, a diagnosis of riboflavin deficiency should be made on the basis of combined dietary, clinical, and biochemical data. However such data on one individual is often not available and it may be necessary to rely on very limited information in order to establish riboflavin status. The presence of one or more of the clinical signs described above may suggest riboflavin deficiency, and when such signs are accompanied by a history of a dietary intake of less than 0.6 mg daily (FAO, 1974) over a long period the likelihood of there being riboflavin defiency is high. Urinary excretion of riboflavin has been shown to correlate well with intake and tissue content such that on low dietary intakes urinary

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excretion may be low (Davis, Oldham and Roberts, 1946; Horwitt Harvey, Hills and Liebert,1950). These observations have enabled minimum requirements for riboflavin to be obtained from depletion-repletion studies in man (Keys et al., 1944; Williams, Mason, Cusick and Wilder, 1943). As a consequence of such studies in man levels of excretion have been defined for subjects of different ages (Table 4). However, urinary riboflavin levels may be influenced by nitrogen balance, antibiotics and daily riboflavin intake (Cohsolazio et al., 1971; Windmueller, Anderson and Mickelsen, 1964). Therefore the value of urinary measurements in isolation is debatable. The measurement of riboflavin and flavin nucleotide levels in whole blood, plasma, and erythrocytes has been made possible through the development of fluorometric techniques (Burch Bessey and Lowry, 1948; Clarke, 1969), but as a useful index of riboflavin status these methods are open to criticism. Large variations in serum riboflavin which closely mirror dietary intake, unrelated to age or sex, have been reported, and a poor correlation has been observed between erythrocyte riboflavin and dietary intake (Suvarnakich, Mann and Stare, 1952; Beal and Van Buskirk, 1960). In contrast, Bessey and his colleagues (Bessey, Horwitt and Love, 1956) were able to show a good positive correlation between red cell riboflavin levels and dietary intake, suggesting that measurements of red cell riboflavin *per se* may be used to substantiate evidence for riboflavin deficiency obtained from other dietary or clinical sources. The

Table 4: Guidelines for the Interpretation of Erythrocyte Levels[b] and Urinary Excretion[a] of Riboflavin (Sauberlich, Skala and Dowdy, 1974).

a. Less than acceptable (at risk)

Subjects	Deficient (high risk)	Low (medium risk)	Acceptable (low risk)
	μg/g creatinine		
1-3 yrs	< 150	150-499	≥ 500
4-6 yrs	< 100	100-299	≥ 300
7-9 yrs	< 85	85-269	≥ 270
10-15 yrs	< 70	70-199	≥ 200
Adults	< 27	27-79	≥ 80
Pregnant, 2nd trimester	< 39	39-119	≥ 120
Pregnant, 3rd trimester	< 30	30-89	≥ 90

a. Other Interpretive Guidelines

Adults:			
μg/24 hr	< 40	40-119	≥ 120
μg/6 hr	< 10	10-29	≥ 30
a. Load Test (return in adults of 5 mg riboflavin dose)			
μg in 4 hr	< 1000	1000-1399	≥ 1400
b. Erythrocyte Riboflavin			
μg/100 ml cells	< 10.0	10.0-14.9	≥ 15.0

most recent and most satisfactory biochemical method for evaluating riboflavin status is the measurement of erythrocyte glutathione reductase activity. This enzyme catalyses the following reaction:

$$NADPH + H^{+} + GSSG \rightarrow NADP^{+} + 2GSH$$

where GSSG is oxidised glutathione. The reaction is measured in vitro in the presence of excess NADPH and GSSG, with and without added excess FAD. The decrease in absorbance of NADPH at 334 nm is followed over a specified time, and the results expressed in one of two ways, either as the basic, unstimulated activity of EGR (I.U.) or as an activation coefficient (AC)

where $$AC = \frac{\text{Change in absorbance with FAD/10'}}{\text{Change in absorbance without FAD/10'}}$$

The in vitro stimulation of enzyme activity by FAD is greatest in those patients whose riboflavin status is lowest (Tillotson and Baker, 1972; Bamji, 1969).

There is some disagreement over the AC value that should be adopted as a threshold level for the indication of riboflavin deficiency. However, controlled studies of human riboflavin deficiency and GR activity have suggested that an AC of greater than 1.30 may be used as evidence for inadequate riboflavin nutrition (Tillotson and Baker, 1972; Thurnham, personal communication). This method has the advantage of being a measure of functional riboflavin status rather than red

cell or plasma concentration *per se* and as such is an improvement over previously adopted methods for estimating riboflavin deficiency as it is not immediately influenced by dietary intake. The procedure is simple and requires only small quantities of blood, the results are reproducible, the enzyme is reasonably stable and samples can be stored for several months before analysis (Glatzle, Weber and Wiss, 1968; Glatzle, Körner, Christeller and Wiss, 1970; Thurnham, Migasena, Pavapootanon, 1970).

The non-specific nature of the clinical signs and symptoms of a dietary inadequacy of riboflavin has necessitated the development of more reliable methods for diagnosing riboflavin deficiency. Urine, plasma, and, to a lesser extent, RBC riboflavin concentrations are influenced to a large extent by dietary intake. EGR activity is more stable over periods of fluctuating intake and thus is more representative of body reserves.

E. Nutrition of the Elderly

In the considerations of riboflavin so far, no emphasis has been placed on any particular category of individuals within the population. However, although overt malnutrition is no longer seen in many of the Western countries there is still evidence that it exists among the elderly (Hurdle and Williams, 1966; Griffiths et al., 1967; Andrews and Brook, 1966; Exton-Smith, Hodkinson

and Stanton, 1966).

In order to assess the impact that poor nutritional status has on the old person it is necessary to understand the normal physiological changes that occur with increasing age. A number of workers (Korenchevsky, 1961; Shock, 1962; Rockstein and Sussman, 1973; Brozek, 1952), have studied the structural and functional changes occurring in human beings which can be attributed to senescence and not to any particular pathological condition. Studies showed that a number of different organ systems undergo a decrease in functional efficiency. The muscles undergo considerable wasting due to the inability of muscles to regenerate, and this, together with a decrease in elasticity, and calcification of ligaments, shrinkage and sclerosis of tendons, and changes in the vertebral column, contribute to the reduced mobility seen in many old people. As regards the nervous system, there is a loss in the total number of brain cells and fibres, and a hardening of the blood vessels of the brain with increasing age (Brody, 1973). The cardiac output of the heart is significantly reduced in the elderly, probably as a result of the observed increased resistance of the arterial vessels to blood flow, and the lung capacity has also been shown to decrease in old age. Changes in the digestive system, particularly a reduction in the peristaltic activity of the intestine and colon, contribute to the digestive problems of the elderly. The urinogenital systems and the endocrine system have been shown to undergo a process of functional impairment with advancing age, with

a steady decrease in the basal metabolic rate reflecting the decrease in secretion of thyroid hormone in the elderly. One of the most obvious manifestations of these changes is the relative inability of the elderly to respond to mechanical and environmental stress. It is important to realize, however, that although the structural and functional changes briefly mentioned here are general changes associated with ageing, individual variation may be considerable both in the rate at which they take place and the relative degeneration seen in different organ systems (Rockstein, 1975).

Malnutrition in the elderly must be seen not as an inevitable consequence of ageing but more as a result of the combined effects of a changing socioeconomic role with its concomitant social experience, and the structural and functional changes outlined above, which may lead to altered dietary habits and impaired absorption and metabolism of nutrients.

The Department of Health and Social Security in the United Kingdom has published a table of recommended intakes of nutrients for the elderly. The recommendations take into account that on average, energy expenditure declines with old age, and the recommended intakes of calories and nutrients are adjusted accordingly (Table 5, DHSS, 1969). However, Durnin and his colleagues (Durnin _et al._, 1966) were able to show that many people maintain a high level of energy expenditure in old age and thus have calorie intakes considerably higher than the recommended values.

Table 5: Recommended Daily Intakes of Energy and Nutrients for Elderly People in the UK

(From DHSS Report, 1969).

	Men		Women	
	65-74	75 and over	55-74	75 and over
Energy (kcal)	2350	2100	2050	1900
(MJ)	9.8	8.8	8.6	8.0
Protein (g)	59	53	51	48
Calcium (mg)	500	500	500	500
Iron (mg)	10	10	10	10
Thiamine (mg)	0.9	0.8	0.8	0.7
Riboflavin (mg)	1.7	1.7	1.3	1.3
Nicotinic acid (mg)	18	18	15	15
Ascorbic acid (mg)	30	30	30	30
Vitamin A (µg retinol equiv.)	750	750	750	750
Vitamin D (µg cholecalciferol)	2.5	2.5	2.5	2.5

Nutritional surveys into the dietary practices of elderly people have taken two forms, cross-sectional studies and longitudinal studies. In 1965 Exton-Smith and Stanton (Exton-Smith and Stanton, 1965) carried out an investigation into the diets of old people living alone in two London boroughs. When the 60 female subjects were grouped according to age, a clear decrease in intakes of all nutrients could be seen in women aged 80 years or more, when compared with women between 65 and 80 years of age (Table 6). The results of this and another survey (Exton-Smith, Stanton and Windsor, 1972) suggested that low intakes of nutrients are associated with impaired health in old age.

Although cross-sectional studies have their value, any attempt to assess the effects on health of low dietary intakes of certain nutrients necessitates following the same person over a long period of time and comparing the medical, biochemical and dietary data obtained. One of the most rigorous attempts to study the nutritional status of the elderly in a longitudinal manner is that supported by the DHSS, initiated in 1967 and still in progress (DHSS, 1972). Biochemical evidence from the surveys has indicated that there may be two nutrients of particular concern in the nutrition of the elderly: riboflavin and ascorbic acid status (Thurnham and Stephen, 1975; Thurnham, 1977). Clinical diagnoses have included both protein-energy malnutrition and specific vitamin deficiencies (DHSS, 1972). From a consideration of

Table 6: Cross-Sectional Study: Fall in Intake of Nutrients during the Eighth Decade

(from Exton-Smith, 1965)

Calories and Nutrients	Fall in Intake (%)
Calories	19
Protein	24
Fat	30
Carbohydrate	8
Calcium	18
Iron	29
Vitamin C	31

several nutritional studies carried out in various parts of the world (Morgan, Gillum and Williams, 1955; Watkin, 1968; Batata et al., 1967; Griffiths, 1968; Forbes and Reina, 1970; DHSS, 1970; Hoorn, Flikweert and Westerink, 1975) a number of general statements concerning the nutritional deficiencies of the elderly can be made. Protein-energy malnutrition, anaemia, and thiamin, riboflavin, folate, vitamin B_{12}, ascorbic acid and vitamin D deficiency may, particularly at the subclinical level, accompany old age in some individuals. Considerable variations in the prevalence and severity of clinical and subclinical malnutrition have been reported. Clearly, many factors, both intrinsic and extrinsic play some role in this situation.

The diagnosis of a biochemical deficiency of a nutrient usually relies on arbitrarily defined limits established for younger adults, and the relevance to the elderly population of these adopted limits is questionable.

If any attempt is to be made to prevent the occurrence of malnutrition in the elderly, particularly in those groups shown to be more at risk than others, it might be expedient to make use of the observations that poverty, social isolation, and physical disabilities are important factors influencing dietary habits (Brockington and Lempert, 1967; Exton-Smith, Stanton and Windsor, 1972). Large scale supplementation practices may not be the most effective way of dealing with the malnutrition seen to exist among the elderly.

In the U.K. today there are about 7½ million people over 65. In other words the elderly constitute the largest single 'at risk' group vulnerable to malnutrition. Clearly there is a need to understand the principles of normal ageing, to identify any potential nutritional problems, and to assess the physiological significance of nutritional deficiencies defined by various methods.

F. RBC Survival in Man: Techniques of Assessment

Until recently, the RBC was the only mammalian cell whose lifespan could be determined with any degree of accuracy. Several disease states are associated with erythrokinetic abnormalities, manifesting themselves as anaemias (Perrine, 1973; Gross, 1976). Anaemia may develop as a result of three major types of defect; those affecting proliferation, those affecting maturation, and those affecting the survival of the circulating RBC (Barrett, Cline and Berlin, 1966; Foy and Kondi, 1968). The measurement of RBC survival in man has been an important tool in the elucidation of some of the processes of RBC production and sequestration and as such has helped to explain some of the anaemias characteristic of particular disease states.

There have been a number of attempts to measure RBC life span in animals and man, but only with the introduction of labelling techniques have such studies achieved

clinical usefulness (Berlin, Hewitt and Lotz, 1959). The methods for measurement of RBC lifespan can be divided into three main groups; those that label a cohort of cells of the same age; those that label circulating RBC randomly, and those that measure RBC lifespan indirectly. Although methods falling into the latter group only yield values for mean RBC lifespan they have been useful in the past in the recognition of gross erythrokinetic abnormalities. The latter methods depend on the measurement of the rate of excretion from the body of breakdown products of Hb such as carbon monoxide and bilirubin, or of the measurement of the rate of RBC synthesis by a radioiron technique or by reticulocyte counts (Lewis and Gershow, 1961; Huff et al., 1950; Corburn, Williams and Kahn, 1966).

Ashby (1919) reported a technique of differential agglutination in order to measure the lifespan of donor cells in the circulation of a recipient. Donor red cells, compatible but serologically distinguishable, are transfused into a recipient and in samples of blood obtained at intervals from the recipient, the number of transferred red cells can be counted after agglutinating the recipient's cells. This technique is time-consuming, and is generally limited to giving O type blood to an A type recipient, or MM blood to an MN recipient, thus its practical value is restricted (Weiner, 1942; Adner, Foconi and Sjolins, 1963). However, studies employing this technique have been an important factor in the classification of haemolytic

disease according to whether the abnormality is intrinsic or extrinsic to the RBC.

The methods in most common use at present for investigating RBC survival *in vivo* employ radioactive tracers, and it has become possible to define quite precisely the requisite conditions for measuring RBC lifespan by labelling techniques. The RBC alone should acquire a readily-detectable label that is either bound to or constitutes a part of the cell. Some methods rely on labelling RBC at random, such labels should show no preference for cells of any particular age. Other labels, notably Fe^{59}, are designed to be incorporated into RBC during their production, in which case they should be available for a short period of time and should not be metabolised in any way before incorporation. A label should not leak from the RBC or modify the survival of the cell. Neither should it be reutilized after destruction of the cell. No method has yet been developed which fulfils all these criteria, however, some corrections can be made in calculations for departure from ideal labelling conditions and current labelling methods are at least reproducible and provide some information regarding the nature of RBC survival rather than simply providing a mean value of RBC lifespan. Current cohort-labelling methods involve the incorporation of labelled glycine or iron into developing RBC, and the quantitative determination of label remaining in the circulating cells. These methods are complicated by the fact that labelled

glycine is detectable in the precursor pool long after administration of the label, and of the labelled Fe, 80-90% in a senescent cohort of cells is incorporated into new cells (Berlin Hewitt and Lotz, 1954; Burwell, Brickley and Finch, 1953). The principal random-label methods utilize isotopically labelled diisopropylfluorophosphate (DFP) and ^{51}Cr, and depend on an age-independent labelling of the RBC. DFP binds irreversibly to the RBC and is not reutilized after cell destruction (Cohen and Warringa, 1954). Hjort and his colleagues however demonstrated that there is elution of labelled DFP from intact surviving cells in the 7-10 days following administration (Hjort, Paputchis and Cheney, 1960). This fact, together with the large amount of work involved in the preparation of samples for counting β-emitting isotopes of DFP, has limited the popularity of the method for routine clinical use. The most commonly used technique for measuring RBC survival in man is that employing ^{51}Cr as a label. Although ^{51}Cr does not appear to be relabelled to any significant extent there is convincing evidence that it elutes from normal red cells at a rate of about 1% per day, and there is no commonly accepted method to correct for this elution (Ebaugh, Emerson and Ross, 1953; Strumia, Dugan and Colwell, 1962). Also, some workers have suggested that ^{51}Cr does not label RBC in a truly random fashion, as younger cells appear to take up more label than older cells (Danon, Marikovsky and Gasko, 1966).

Despite the various drawbacks of each of these methods much useful information has been obtained concerning the normal processes of RBC production and destruction and the anaemias associated with various deficiency and disease states have been characterised (Lane and Burka, 1976; Jacob and Jandl, 1962b; Brewer, Tarlov and Kellermeyer, 1961).

Studies in this department using density gradient separation of RBC according to age have indicated that this may be a useful tool in identifying erythrokinetic abnormalities in man without recourse to isotopic labelling (Hassan and Thurnham, 1977). The principle of this method rests on the observation that RBC become more dense as they age, and during the course of ultracentrifugation cells become suspended in a density gradient at their own density (Prentice and Bishop, 1965; Leif and Vinograd, 1964). Fractions of RBC of different mean ages can be prepared, and the relative concentrations of RBC in the different fractions is a reflection of the age distribution of cells in the original sample. The precise conditions used for RBC separation determine the usefulness of this technique for studies of RBC survival, but its main attributes lie in the fact that a single blood sample from a subject will provide some indication of an erythrokinetic abnormality, and the time-consuming, potentially hazardous practice of employing radioactive labels in humans can be avoided.

G. Age-Related Changes in RBC and Factors Implicated as Determinants of RBC Survival

The RBC has taken a prominent position in studies of tissue ageing. Several workers have felt that a greater understanding of the processes leading to the RBC destruction would be useful in elucidating the mechanisms of tissue senescence in general (Bessis, 1964).

The RBC in man has a mean lifespan of about 120 days and from the time it enters the peripheral circulation as a reticulocyte until its sequestration in the reticuloendothelial system it undergoes a number of structural and metabolic changes (Rigas and Koler, 1961a; Danon and Marikovsky, 1964; Bernstein, 1959). It is not unreasonable to assume that the biochemical and biophysical changes concerned with RBC ageing ultimately determine the removal of the cells from the circulation and their destruction.

For studies of RBC ageing it has been necessary to develop techniques for the separation of RBC according to age and such techniques have relied on the practical application of observed structural and physiological differences in young and old cells. Marks and Johnson, (1958) demonstrated that old cells are more osmotically fragile than young cells and as a consequence of this observation other workers have employed differential osmotic fragility to separate cells according to age

(Simon and Topper, 1957; Levy, Walter and Sass, 1959). Other workers, inspired by the reported age-related change in surface charge of RBC have attempted to separate RBC using an electrophoretic procedure (Danon and Marikovsky, 1961; Yaari, 1969). The majority of studies into RBC ageing however have been motivated by the observation that the RBC becomes more dense as it ages (Borun, Figuera and Perry, 1957; Rigas and Koler, 1961b; Hoffman, 1958; Coopersmith and Ingram, 1958). A crude separation of RBC according to age can be achieved by slow centrifugation in an isosmotic solution in the absence of a gradient. Rigas and Koler, (1961b) and Sass, Vorsanger and Spear, (1964) successfully prepared reticulocyte-rich and reticulocyte-depleted samples of RBC in this way. Although centrifugation of blood in the absence of a gradient may be useful for detecting gross age-related changes in RBC such methods provide little information about the time-scale of such changes and are unlikely to reveal small but consistent changes that occur during the RBC lifespan. Hence a few workers have preferred to adopt density gradient centrifiguation procedures in order to improve resolution of RBC of different ages (Brok, Ramot, Zwang and Danon, 1966; Danon and Marikovsky, 1964; Leif and Vinograd, 1964). Many of the early studies were performed on animals, and the validity of density gradient centrifugation for separation of RBC according to age was demonstrated by cohort labelling. By means of radioactive iron a newly-formed cohort of cells can be labelled and followed through

their lifespan. When ^{59}Fe labelling is carried out in conjunction with centrifugation at various time intervals it has been shown that the radioactivity appears first in the top layer and gradually moves through to the bottom of a column of cells (Borun, Figuera and Perry, 1957; Piomelli, Lurinsky and Wasserman, 1967; Coopersmith and Ingram, 1958). The nature of the gradients employed and the conditions of centrifugation and separation of the fractions from the gradient determine the degree of resolution achieved and the quality and quantity of information obtained. The amount of information concerning biophysical and chemical changes in RBC as they age is accumulating steadily. The biophysical changes include a diameter decrease, a decrease in osmotic resistance, a decrease in reversible deformability or mechanical resistance, and a reduction in surface charge density (Danon and Marikovsky, 1961; Marks and Johnson, 1958). Lipid analyses of RBC of different ages have indicated that a small loss of total lipid and cholesterol occur during the lifespan of the cell (Westerman, Pierce and Jensen, 1963). Older cells have been shown to have an altered ionic distribution when compared with younger cells, specifically, an increase in Na^+ and a decrease in K^+ occurs, reflecting a reduction in efficiency of the Na/K pump (Bernstein, 1959), which may contribute to the increased osmotic fragility of older cells. The same author has demonstrated a slight increase in oxygen utilization

during the ageing of the mature RBC (Bernstein, 1959).

Several workers have observed changes in the levels and activities of some enzymes and metabolic intermediates during the lifespan of the RBC and it has been suggested that death of the normal erythrocyte is the result of a failure of cellular homeostasis resulting from metabolic inefficiency (Marks and Johnson, 1958; Prankerd, 1958; Bishop and Van Gastel, 1969; Brewer, Tarlov, Kellermeyer and Alving, 1962; Rigas and Koler, 1961a). Among those enzymes reported to decrease in activity in ageing cells are G6PD, 6PGD, flavokinase, aldolase, MHb-R., cholinesterase, catalase, AST, and pyruvate kinase (Brok, Ramot, Zwang and Danon, 1966; Chapman and Schaumburg, 1967; Sass, Vorsanger and Spear, 1964; Allison and Burn, 1955; Ramot, Brok-Simoni and Ben-Bassat, 1969; Fischer and Walter, 1971; Bonsignore et al., 1964). Some workers have been sufficiently convinced by the relationship between RBC age and activity of certain enzymes, particularly AST, to propose these enzymes as useful indices for determining the mean age of a population of RBC (Bartos and Desforges, 1967; Bishop and Van Gastel, 1969; Sass, Vorsanger and Spear, 1964). Glutathione reductase activity has been investigated by two workers who have reported no decrease in activity as cells aged (Brewer, 1969; Bonsignore et al., 1964). There is evidence to suggest that mHb increases in concentration in older cells (Brewer, Tarlov and Kellermeyer, 1962; Keitt, Smith and Jandl, 1966). There are reports

that there are no differences in the concentration of GSH in old and young cells (Harris and Kellermeyer, 1974; Bonsignore et al., 1964). However, Rigas and Koler (1961a) have demonstrated a fall in GSH content in older cells of subjects with Hb-H disease. Creatinine has been shown to be higher in a younger cell population than an older cell population and it has been suggested that creatinine may therefore be used as a sensitive index of the mean age of a RBC population (Griffiths and Fitzpatrick, 1967). Several workers have investigated the possible relationship between glycolysis and RBC ageing and all results have shown an overall fall in glucose consumption with increasing RBC age (Chapman and Schaumburg, 1967; Bernstein, 1959; Gomperts, 1967). The more comprehensive studies of glycolysis have demonstrated a decrease in activity of HX and aldolase and a reduction in ATP concentration in older cells (Bernstein, 1959; Chapman and Schaumburg, 1967). The HMS has received attention from some workers interested in the cellular energetics of the ageing RBC. The HMS pathway metabolizes about 10% of the glucose made available to the cell and is a source of potential energy and high reductive capacity in the form of NADPH (Grimes, 1963). The increased mHb demonstrated in older cells may be related to a decreased ability of the HMS to generate NADPH and GSH (Rigas and Koler, 1961a). Hb has been shown to undergo another alteration with increasing RBC age, namely Haemoglobin A_3, in which one

SH group on the β chain forms a mixed disulphide with GSSG increases in concentration as the red cell ages (Meyering, Israels, Sebens and Huisman, 1960). It is possible that this increase in Hb A_3 is related to the equilibrium between reduced and oxidised glutathione, and may reflect the observed fall in activity of the HMS enzyme, G6PD, in ageing RBC (Carson, 1960). The Hb in old cells has also been shown to exhibit an increased affinity for oxygen in older cells (Edwards and Rigas, 1967). Edwards, Koler, Rigas and Pitcairn (1961) demonstrated a greater oxyhaemoglobin (OxyHb) saturation of old cells that had been equilibrated in an O_2/CO_2 mixture, when compared with young cells. They suggested that the alteration in the O_2 dissociating properties of Hb in old cells may be the result of an age-dependent intramolecular structural alteration of Hb. It has been proposed that the increased oxygen affinity of Hb in older cells, and the concomitant decreased 'haeme-haeme' interactions are related to the observed decrease in 2,3 diphosphoglyceric acid (2,3-diPG) in older cells (Edwards and Rigas, 1967; Bernstein, 1959).

It is a fact that haemolysis in the circulation is a rare occurrence under physiological conditions and senescent cells are normally sequestered in the reticuloendothelia system (Weiss and Tavassoli, 1970). The structural and metabolic changes taking place during the lifespan of the RBC are generally assumed to ultimately determine its sequestration, but the question as to whether the structural

alterations precede or follow the metabolic changes is as yet unanswered.

Glycolytic activity has been implicated as a determinant of RBC survival (Chapman and Schaumburg, 1965; Chapman and Schaumburg, 1967). On the other hand, it has been suggested that the capacity of the HMS pathway to generate NADPH and to maintain levels of GSH, determine RBC survival. G6PD has been proposed as of central importance to RBC survival; several workers have shown that G6PD deficient RBC are less able to protect themselves from oxidative stress, either in the form of H_2O_2 or oxidant drugs and undergo an oxidant-induced haemolysis to an extent not seen in normal cells (Walls and Hochstein, 1964; Kellermeye *et al.*, 1961; Beutler, Dern and Alving, 1954).

Other workers have adopted a different perspective and have concerned themselves with physical changes that take place in the physiologically normal ageing erythrocyte. Some workers have considered that the reduced ATP concentration in older cells and the associated reduction in reversible deformability is of primary importance in determining RBC survival (Burton, 1970; Weed, LaCelle and Merill, 1969). It has also been suggested as a result of observations made on the surface properties of young and old RBC that a reduction in negative surface charge together with an increased membrane antigen density on older erythrocytes may enable macrophages to recognize impaired

membranes and lead to the senescence of the old cells (Skutelsky, Marikovsky and Danon, 1974; Gattegno, Bladier and Cornillot, 1974; Danon, 1975).

In conclusion, many investigations have shown that the normal erythrocyte undergoes several metabolic and biophysical changes during its lifespan, but there has been no satisfactory explanation of the factors which ultimately enable the reticuloendothelial system to recognize senescent cells and to remove them from the circulation.

H. Mathematical Modelling of Biological Systems

As the studies of biological systems have progressed they have revealed themselves as exceedingly complex, and despite the rapidly increasing volume of literature concerning biochemical details there has been little success in attempts to describe and explain how these relate to the properties of an organism. Mathematically-based models of biological systems have received increasing attention over the past 20 years as a means of overcoming some of the conceptual and practical limitations of complex sy tems (Payne and W eeler, 1967; Müller, 1967; Stolwijke, 1967; Ganzoni, Späti, Bühler and Bühlman, 1973).

Locker (1967) conceived of models in science as developing from an interrelationship of intellect and ideas or reason and perception; through this interrelationship a concept is created and the model represents the

image of the concept. In other words, models may be designed to relate existing knowledge of events, relationships, and mechanisms, to proposed theories, in order to develop an analysis of a particular system.

All models of scientific phenomena may be considered as analogue models, and may take the form of simulation by computer programme. The simulated computer model may be used to confirm or reject proposed mechanisms or assumptions, it can suggest experiments and may predict their outcome. Experimental work may produce quantitative information about the components of a system and the observed effects of modifying the activity or concentration of the components on one another. Experimental work may also provide information on such variables as pools and fluxes. A computer may be programmed to deal with vast amounts of data relating to a system and can be used to predict the effects on the components and dynamics of the system of varying the number, type, and values of its components. The computer in effect makes empirical observations in response to an imposed alteration of conditions on a system; it does not explain the observations, it merely describes the system quantitatively. The computer is necessarily restricted to processing in a defined way the information presented to it. The predictions it makes are therefore specific but from the empirical data it generates, generalizations may be made. Mathematical interpretations have been made of a variety of biological systems, including energy balance and

homeostasis (Payne and Dugdale, 1977), and erythrocyte osmotic fragility (Maeda et al., 1977).

Purpose of The Study

In the light of existing knowledge concerning the diverse functions of riboflavin in the tissues of man and the prevalence of biochemical riboflavin deficiency in the elderly in the U.K., an attempt has been made to determine the physiological significance of biochemical riboflavin deficiency in man.

Since the erythrocyte FAD-dependent enzyme, GR, functions to produce GSH which has been implicated as an important factor in the maintenance of RBC integrity, erythrocyte systems which require GSH were studied in an effort to identify metabolic lesions in riboflavin-depleted RBC.

Finally, the possible relationships between riboflavin status, GR activity in ageing cells, and RBC survival were investigated and a computer model of RBC survival was designed to aid in the investigation.

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Finally, the possible relationships between riboflavin status, GR activity in ageing cells, and RBC survival were investigated and a computer model of RBC survival was designed to aid in the investigation.

MATERIALS AND METHODS

All chemicals were obtained from the British Drug House (B.D.H.) unless otherwise stated.

A. Subjects Used for Study

The subjects chosen for study were elderly subjects either hospitalized at St Pancras Hospital for Tropical Diseases (H.T.D.), or attending the Geriatric Day Centre at this hospital, and staff and students of the Department of Human Nutrition at the London School of Hygiene and Tropical Medicine. All subjects were screened for biochemical ariboflavinosis (Section Fb) and categorized as below:

i. Adults < 65 years, normal biochemical riboflavin status

ii. Adults < 65 years, marginal biochemical ariboflavinosis

iii. Adults ≥ 65 years (elderly), normal biochemical riboflavin status

iv. Adults ≥ 65 years, marginal biochemical ariboflavinosis.

An activation coefficient for EGR of ≥ 1.30 was taken as indicative of marginal biochemical ariboflavinosis.

A recent medical history was obtained and any subject known to have recently or be still taking vitamin supplements or drugs that might interfere with riboflavin

metabolism or erythrokinetics was not included in the investigation.

B. Collection of Blood Samples and Preparation for Analysis

Venous blood samples were taken from the antecubital vein into disposable syringes, dispensed immediately into heparinised glass tubes and mixed gently for a few minutes on a rollermixer. Red blood cells (RBC) were obtained by centrifugation of whole blood at 5,000 g for 10 minutes at room temperature and the plasma removed by Pasteur pipette. Cells were washed twice in 0.15 M-NaCl unless indicated otherwise.

C. Haematological Measurements

i. Determination of packed cell volume

The packed cell volume (PCV) of whole blood, or packed cells, was determined using a microhaematocrit method. Samples were taken up into heparinised haematocrit capillary tubes (Harshaw Chemicals Ltd) one end of which was sealed in a small flame and the tubes centrifuged at 12,000 g for 3 minutes in a Hawksley microhaematocrit centrifuge. A reader was employed to give measurements of PCVs.

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ii. Total RBC counts

Total RBC counts were made on whole blood and, in some instances on red cell fractions using an electronic counter (Model D1, Coulter Diagnostics Ltd). Blood was diluted in isoton 50,000 times by mixing 1 µl in 50 ml. A threshold value of 8 was adopted as optimal for counting and the Coulter counter presented RBC counts in 0.1 ml of the dilute preparation.

Calculation:

$$\text{RBC/litre} = \frac{50{,}000 \times X \times 10^3}{0.1}$$

where X is the number of counts/0.1 ml of diluted RBC and 50,000 is the dilution of the RBC sample.

iii. Reticulocyte count

Reticulocytes are immature erythrocytes that still retain some of their nuclear material which can be demonstrated by staining with basophilic dyes. For example the reticular material takes up brilliant cresyl blue and reticulocytes can be recognized by characteristic deep violet filaments.

The stain was prepared as follows. Brilliant cresyl blue (1.0 g) was dissolved in 100 ml of a citrate-saline solution containing 1 part of 102 mM sodium citrate to 4 parts of 0.15-M NaCl and filtered before use.

Method: 3 drops of dye solution were mixed

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Method: 3 drops of dye solution were mixed

with 3 drops of whole blood and incubated at 37°C for 20 minutes in a water bath. The precise volume of other red cell preparations added to the dye solution was directly related to the RBC count. After incubation the sedimented cells were resuspended, smears prepared on clean glass slides, dried and observed under oil immersion without fixing or counterstaining. Reticulocyte counts were made on successive fields until about 50 reticulocytes had been recorded. The total cells in e ery tenth field were counted and the number of reticulocytes expressed as a percentage of the estimated total cells in a l fields observed.

iv. Haemoglobin estimation (Crosby and Houchin, 1957; Cannan, 1958).

Haemoglobin (Hb) was estimated in haemolysates by a Cyanmethaemoglobin method. All forms of Hb in solution react with cyanide which comp exes with Hb in the 6th coordination position of iron to form cyanmethaemoglobin (MHbCN) which shows a characteristic band of absorbance at 540 nm. A 0.1 ml sample of haemolysate was diluted with 3.5 ml of Drabkins solution containing 11.9 mM-$NaHCO_3$, 0.77 mM-KCN and 0.6 mM $K_3Fe(CN)_6$. All assays were done in duplicate and the absorbance at 540 nm read against a reagent blank. Cyanmethaemoglobin standards, containing 57.2 mg/100 ml of Hb were also measured.

Calculated:

$$\text{Hbmg/100 ml} = \frac{\text{ODT} \times 57.2 \times 36}{\text{ODs}}$$

where ODT is the absorbance at 540 nm of the test solution
ODs is the absorbance at 540 nm of the standard
36 represents the dilution factor.

v. Calculation of 'absolute' haematological values

The mean corpuscular Hb concentration (MCHC) was determined for some blood samples.
viz.:

$$\text{MCHC \%} = \frac{\text{Hbg \%}}{\text{PCV}} .$$

D. Red Blood Cell Separation into Fractions of Different Mean Ages by Density Gradient Centrifugation

Principle: The rationale behind density gradient centrifugation is that since RBCs become more dense as they age,when they are centrifuged at high speed on a density gradient they position themselves in the gradient at their own density.

Methods

Preliminary experiments involved the use of Bovine Serum Albumin gradients on which RBC were applied. However these gradients did not result in a clear age-related separation of RBC and the bulk of the work

described in this thesis entailed separating cells on gradients prepared from mixtures of Ficoll/Triosil.

a) <u>Separation of RBCs on discontinuous density gradients of bovine serum albumin (BSA)</u>

i. Preparation of BSA solutions (Leif and Vinograd, 1964)

10.8 g of BSA powder were added to 20 ml of distilled water with 5 g of Amberlite Monobed mixed resin. MB-3. Mixing was carried out at $4^{o}C$ for 10 hrs on a magnetic stirrer. After mixing the BSA solution was centrifuged at $4^{o}C$ at 2000 g for 15 minutes, and the BSA decanted from the resin into a plastic bottle containing 2.5 g Amberlite resin. The mixture was agitated continuously for 2 hours after which t me the BSA was again decanted from the resin and centrifuged at 20,000 rpm in a fixed angle rotor for 2 hours. The top layer was removed with a Pasteur pipette and the clear solution decanted. The following salts were added per 100 g of BSA solution: 0.2391g Na_2CO_3, 0.303 g NaCl, 0.1124 g $MgCl_2.6H_2O$ and 0.0286 g KCl. The specific gravity of the final stock solution was measured using a micropipette. The micropipette was filled with distilled water to a mark on the glass and weighed and a similar measurement was made with the micropipette filled to the same mark with the stock BSA solution. The stock solution was diluted with distilled water to specified densities. Four solutions were thus prepared with densities of 1.080, 1.088, 1.096 and 1.105 g/ml.

ii. Preparation of RBC for application on the gradient:

Samples of packed cells were washed twice and finally suspended in an equal volume of Krebs/Ringer/ Phosphate buffer (KRP) containing 0.1 % (w/v) of albumin. The KRP buffer was prepared by mixing 100 parts of 0.154 M NaCl, 4 parts 0.154 M-KCl, 3 parts 0.110 M $CaCl_2$ and 1 part 0.154 M-$MgSO_4.7H_2O$. 20 parts of 0.1 M sodium phosphate buffer were added slowly with continuous mixing to prevent salt precipitation. The buffer was prepared by dissolving 17.8 g hydrated disodium monohydrogen phosphate ($NaH_2PO_4.2H_2O$). The pH of the final KRP solution was adjusted to pH 7.0 with 0.1 N-HCl or 0.1 N-NaOH. The solut on was stored at 4^oC. BSA solution (see above) was added to a final concentration of 0.1 g%. After the second washing packed cells were resuspended in an equal volume of albuminised KRP.

iii. Preparation of gradient and application of cells:

0.75 ml of each BSA solution were layered into 10 ml polypropylene centrifuge tubes (MSE) with the densest solution at the bottom of each tube. 5 ml of a suspension of RBC were layered onto each gradient and each tube was filled within 2 mm of the top with liquid paraffin.

iv. Conditions of centrifugation:

Tubes were centrifuged at 40,000 g for 2 hours in a fixed angle rotor.

v. Separation of fractions:

The layer of liquid paraffin at the top of each tube was removed with a Pasteur pipette and discarded. The remaining material was removed in successive 1 ml aliquots with graduated pipettes and each aliquot was washed twice in KRP solutions containing 0.1% albumin.

b) Separation of RBC on discontinuous density gradients of Ficoll/Triosil mixtures

Ficoll/Triosil mixtures have been successfully employed in separating RBC according to their ages. A method used by Turner Fisher and Harris (1974) was modified as described below to suit the requirements of the study.

Ficoll is a high molecular weight polysaccharide, while Triosil is a solution of salts of metrizoic acid.

i. Preparation of gradient for a 5 fraction separation of RBC:

Ficoll/Triosil solutions of densities 1.100 g/ml, 1.110 g/ml, 1.115 g/ml, 1.130 g/ml, and 1.140 g/ml were prepared in distilled water. Ficoll (Pharmacia, London) was added to distilled water in the following concentrations: 16% (w/v), 20.5%, 23.0%, 30%, and 35%, and left overnight at $4^{o}C$ to dissolve. A 34% solution of Triosil (Vestric Ltd, Runcorn, Cheshire) was prepared by mixing 20 ml of Triosil with 24 ml of DW. Triosil and Ficoll solutions were mixed in the proportion of 2:3 (v:v).

Solutions were stored at $4^{o}C$ for a maximum of 2 weeks.

3.3 ml of progressively less dense solutions were carefully layered into 17.5 ml cellulose nitrate tubes.

ii. Preparation of gradient for a 9-fraction separation of RBC:

Ficoll/Triosil solutions of the following densities were prepared: 1.00 g/ml, 1.107 g/ml, 1.113 g/ml, 1.119 g/ml, 1.123 g/ml, 1.125 g/ml, 1.129 g/ml, 1.135 g/ml, and 1.140 g/ml. The initial Ficoll solutions contained (w/v) 16%, 19.2, 22, 25, 26.8, 27.8, 29.6, 33 and 35 g% Ficoll respectively. Ficoll was left overnight at $4^{o}C$ to solubilize and added to solutions of 34% Triosil in the proportion of 3:2 (v:v).

1.8 ml volumes of solutions of progressively decreasing density were layered into cellulose nitrate tubes.

iii. Preparation of RBC for application onto gradients:

Packed RBC were washed twice in 0.85% (0.15 M) NaCl and resuspended in the isotonic saline to a haematocrit of about 60%.

iv. Application of cells and centrifugation conditions:

1.0 ml aliquots of RBC were layered onto the 9-solution gradients or 1.5 ml of RBC were layered onto

the 5-solution gradients. Liquid paraffin was added to within 2 mm of the top of each tube, and tubes were centrifuged at 65,000 g (av) for 2 hours at 4°C on a Beckmann SW27.1 swing-out rotor.

v. Separation of RBC fractions from the gradients:

The layer of liquid paraffin at the top of each gradient was removed with a Pasteur pipette, and the fluffy white layer of leucocytes that formed above the least dense gradient solution was also removed. Successive bands of RBC at the interfaces of the gradient solutions were removed with disposable pipettes. The gradient solutions on either side of a particular band of RBC, up to a point midway between Ficoll/Triosil solution interfaces, were included in that fraction of RBC. Cells that could not be removed from the bottom of the tube by gentle shaking in isotonic saline were not included in the densest RBC fraction. Fractions were washed at least twice in a buffered isotonic saline solution prepared by mixing 17.6 ml 0.5 M-KH_2PO_4 and 60.8 ml 0.5 M-K_2HPO_4 and diluting to 1 litre in distilled water. 1 litre of 0.15 M-NaCl was prepared in distilled water and the pH adjusted to pH 7.4 using 0.1 N-NaOH. 100 ml of the phosphate buffer were added to 1 litre of the isotonic sodium chloride solution and the final solution mixed well.

vi. Reproducibility of gradient preparations:

The reproducibility of the preparations was determined by measuring the refractive index of 5 solutions of each density prepared on the same day. A refractometer was used with distilled water as a blank.

E. Red Blood Cell Survival Studies

In order to determine possible effects of marginal riboflavin deficiency on erythrokinetics, a method was devised to calculate the proportion of RBC of different ages in a sample. The total volume of intact RBC in each washed fraction recovered from the gradient was measured to the nearest 0.01 ml and a portion of the cells removed for haemolysis in distilled water at a known dilution. Each haemolysate was centrifuged at 5,000 g for 15 minutes and the clear haemolysate decanted from the RBC stroma. The Hb concentration in the haemolysate was measured (Methods: C.iv.). The derivation of the RBC distribution in a sample of blood was based on the observation that the Hb content of a human erythrocyte remains constant throughout its lifespan (Murphy, 1973), which implied that the Hb distribution of a particular sample through a gradient would reflect the RBC distribution of the same sample through the gradient. Thus:

$$\text{Total Hb in a fraction} = Hb_H \times D_H \times V_c \text{ ml}$$

where: Hb_H is the Hb mg/ml in the haemolysate

D_H is the dilution of the RBC in distilled water in the haemolysate

V_c is the volume (ml) of the fraction.

The Hb content of each fraction was expressed as a percentage of the total Hb recovered from the gradient.

F. Enzymatic Analyses

a) Aspartate Aminotransferase (AST) (Wilkinson, Baron, Moss and Walker, 1972; Rosalki and Wilkinson, 1959)

Principle: The enzymatic analysis for AST activity depends on the following reactions:

$$\text{L-Aspartic acid} + \alpha\text{-oxoglutaric acid} \xrightarrow{\text{AST}} \text{oxaloacetic acid} + \alpha\text{-glutamic acid} \quad \text{equation (1)}$$

$$\text{oxaloacetic acid} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{malic acid} + \text{NAD}^+ \quad \text{equation (2)}$$

where MDH is malate dehydrogenase.

The oxidation of NADH occurring during the course of the reactions may be followed spectrophotometrically by measuring the rate of fall in absorbance at 340 nm at 37°C. Pyridoxal phosphate (PP) forms a prosthetic group of AST and, in an assay system in which all other substrates are present in excess, will limit the

rate of NADH oxidation. Consequently the activity of AST is measured under two sets of conditions: the basic activity is measured in the absence of PP while stimulated activity is measured in the presence of added excess PP.

Reagents: 0.1 M-KH_2PO_4 buffer was prepared in 83 mM-NaOH and the pH adjusted to 7.4 using 0.1 N-NaOH. PP and NADH were prepared in 0.1 M-KH_2PO_4 buffer, PP was stored at -20°C in the dark, NADH was prepared on the day of assay.

L-aspartic acid solution was prepared by dissolving 25 g in 250 ml distilled water with 175 ml N-NaOH by gentle heating. 50 ml of 1.0 M-KH_2PO_4 were added and the pH adjusted to 7.4 with N-NaOH. The buffered solution was then diluted to a final volume of 500 ml in distilled water and stored either at 4°C overnight or at -20°C for up to 4 months.

Method: The activity of AST in 0.1 ml of haemolysate was measured in a system consisting of 0.118 mM-NADH, 16.7 U MDH/100 ml (Sigma Chemicals), 0.127 M-L-aspartic acid and 0.1 M-KH_2PO_4 buffer in a total volume of 3.0 ml in a reaction initiated by 6.67 mM-α-oxoglutaric acid in the presence and absence of 126 μM-PP. On most occasions at least 10 haemolysates were measured on the same day and an automatic dilution procedure was adopted. Table 7 shows the appropriate buffered reagent solutions

Table 7: Buffered Reagent Solutions Prepared for Determinations of AST Activity

Number of Samples	15	20	25	30
Reagent		(ml)		
0.1 M-KH_2PO_4 buffer	85	110	140	170
3.53 mM-NADH	6.1	7.9	10	12.2
0.38 M-L-Aspartic acid	61	79	100	122
Malate dehydrogenase 5 u/ml	6.1	7.9	10	12.2

prepared for the assay of up to 30 samples in duplicate. Aliquots of a haemolysate (0.1 ml) were diluted with 2.6 ml of buffered reagent solution and dispensed into 2 cuvettes containing 0.1 ml of 3.77 mM-PP and 2 cuvettes containing 0.1 ml of 0.1 M-KH_2PO_4 buffer pH 7.4.

The contents of the cuvettes were incubated for 5 minutes at 37°C prior to the addition of 0.2 ml 0.1 M-α-oxoglutaric acid (prepared in 0.1 M-KH_2PO_4, pH 7.4). The fall in absorbance at 340 nm was recorded over a 10 minute period.

b) <u>Activity of erythrocyte glutathione reductase (EGR)</u> (modified from Glatzle, Korner, Christeller and Wiss, 1970).

Principle: EGR catalyses the following conversion:

$NADPH + H^+ + GSSG$ $NADP^+ + 2GSH$. equation (3)

The activity of the enzyme is measured spectrophotometrically by following the rate of oxidat on of NADPH at 334 nm at 37°C. Flavin adenine dinucleotide (FAD) functions as a prosthetic group for EGR and complete activation of the EGR apoenzyme requires preincubation with FAD. The activity of EGR in the assay system is measured in the absence and presence of added excess FAD.

Method: The reaction was conducted in 0.1 M-KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 2.5 mM-K_2EDTA. The enzyme activity in 0.1 ml of an haemolysate was measured in

0.1 M-phosphate buffer and 0.889 mM-GSSG (prepared fresh in 0.034 N-NaOH), with or without 3 μm-FAD (stored in the dark at $-20^{o}C$) in a reaction initiated by 80 μm-NADPH (prepared fresh in 0.12 M-$NaHCO_3$). In general at least 10 assays were conducted at any one time and a Fisons automatic diluter was used to mix 0.1 ml aliquots of haemolysate with 3.35 ml of diluent buffer/GSSG solution (Table 8) and dispense it into cuvettes containing 50 μl of either 216 μM-FAD or 0.1 M-potassium phosphate buffer. Cuvettes were incubated for 5 minutes at $37^{o}C$ and the reaction initiated by 50 μl of 5.76 mM-NADPH. The decrease in absorbance at 334 nm was recorded over 10 minutes at $37^{o}C$.

Calculations: For both AST and EGR the principles of the calculations were the same. Enzyme activity was expressed as International Units per gram of Hb (IU/g Hb) where one I.U. is defined as the number of μmoles of NAD(P)H oxidised per minute in the assay system.

$$IU/g\ Hb = \frac{\Delta OD \times V_c}{\varepsilon \times X}$$

where ΔOD is the fall in absorbance at 334 m (NADPH) or 340 nm (NADH) per minute

V_c is the volume of the cuvette contents

ε is the mMolar extinction coefficient for NAD(P)H at 334 nm (6.1) or 340 nm(6.22)

X is the amount of Hb (g) in 0.1 ml of haemolysate.

The fall in absorbance was measured over the steepest part of the absorbance curve.

Table 8: Preparation of Phosphate Buffer/GSSG Diluent for Automated Dilution

Number of Assays	5	10	15	20	30
0.1 M-potassium phosphate buffer + EDTA (ml)	82.5	165	247.5	330	495
32 mM-GSSG (ml)	2.5	5	7.5	10	15

An Olivetti Underwood programme designed to calculate activities of various RBC enzymes from unprocessed data was generally used. (Beutler, 1971)

The degree of stimulation of enzyme activity by added excess coenzyme was expressed as an activation coefficient (AC):

$$AC = \frac{\text{Change in OD over 10' with added coenzyme}}{\text{Change in OD over 10' without added coenzyme}}$$

A.C. values for AST ≥ 2.00 were considered to indicate subclinical B_6 deficiency. Similarly, A.C. values ≥ 1.30 were taken as indicative of biochemical ariboflavinosis.

C. Determination of Michaelis-Menten Constants (km) for EGR

Determination of the saturation kinetics of EGR for NADPH and GSSG depended on the measurement of EGR activity under various concentrations of one substrate while keeping the concentration of the other substrate and FAD constant and optimum.

Km(NADPH):

The essential aspects of the measurement of EGR activity in an haemolysate have been described (Part b) of this section). The determination of km (NADPH) entailed measuring GR activity at different concentrations of NADPH while keeping the concentration of GSSG and FAD constant at 0.89 mMolar and 3 μMolar respectively. The concentrations

of NADPH in the assay system were 167, 83.5, 41.8, 16.7 and 8.4 μMolar, and the activity of EGR at each concentration was measured in triplicate. The actual concentration of each NADPH solution was determined by measuring the absorbance at 334 m.

Km(GSSG):

The activity of EGR was measured over a range of GSSG concentrations while keeping the final concentrations of NADPH and FAD constant at 125 μMolar and 3 μMolar respectively. The GSSG concentrations adopted were 1.13, 0.57, 0.23, 0.11, and 0.06 mMolar.

The actual concentration of each GSSG solution used was determined using an enzymatic method. The method relied on the catalysis by EGR of the reduction of GSSG with the concomitant oxidation of NADPH (equation 3). The amount of GSSG reduced bears a stoichiometric relationship to the amount of NADPH oxidised and this can be followed spectrophotometrically at 334 nm, at 37°C. The assay system consisted of 50 U/ml GR, 3 μM-FAD, and 125 μM NADPH, in 0.1 M K_2HPO_4/KH_2PO_4 buffer pH 7.4 in a final volume of 3.76 ml, and the reaction was initiated by the addition of GSSG solution. The enzyme was incubated with FAD and NADPH for 5 minutes and any fall in absorbance at 334 nm was recorded on a recorder. GSSG was added to the system and the fall in absorbance at 334 nm recorded until the reaction had run to completion.

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Calculations: Straight lines were fitted to the initial absorbance slope before addition of GSSG and the final slope obtained after the reaction had been completed and the difference in absorbance between them at the time of addition of GSSG represented the amount of NADPH oxidised and hence the amount of GSSG in the test solution.

$$\text{Concentration of GSSG in test solution} = \frac{\Delta OD \times V_c}{6.10 \; V_s} \text{ mMolar}$$

where ΔOD is the fall in absorbance at 334 nm

6.10 is the extinction coefficient of 1 mM-NADPH

V_c is the volume of the cuvette contents

V_s is the volume of GSSG solution added to the cuvette.

Calculation of Km:

Km is defined as the substrate concentration which gives half the maximum rate of enzyme activity. The Lineweaver-Burk reciprocal plot was employed, for which the reciprocals of the varying Molar substrate concentrations and the rates of enzyme activity were plotted on the horizontal and vertical axis respectively. The regression lines were derived for each set of data. The slope of each plot represented Km/Vmax and the intercept on the rate axis gave 1/Vmax.

Thus: Km = Vmax x gradient.

G. Biochemical Measurements in Red Blood Cells

a) Reduced Glutathione (GSH) estimation
(Beutler, Duron and Kelly, 1963)

Principle: GSH contains virtually all of the non-protein sulphydryl in human erythrocytes and hence in a protein-free extract of RBC any sulphydryl can be attributed to GSH. 55'-Dithiobis(2-nitrobenzoic acid) (DTNB) is readily reduced by sulphydryl compounds to form a yellow anion which absorbs visible light at 412 nm.

Method

The method used was a modification of that described by Beutler Duron and Kelly (1963). A 0.1 ml sample of packed cells were haemolysed in 2 ml of distilled water. Two 0.1 ml aliquots of the haemolysate were removed for Hb estimation (Methods: C.iv). Protein was precipitated in the remaining solution by the addition of 3 ml of a metaphosphoric acid precipitating solution containing 1.67 g% metaphosphoric acid, 4.9 mM-K_2EDTA, and 6.1 M-NaCl. The mixture was left at room temperature for 5 minutes to allow complete precipitation of the protein and then filtered. The clean filtrate was either assayed immediately or frozen overnight at -20°C and assayed the following day. The RBC extract was mixed with 0.3 M-Na_2HPO_4 solution and 1.01 mM-DTNB (in 1% sodium citrate, stored at 4°C) in the proportions 4:2:1. Blank

solutions were prepared containing no RBC extract. The absorbance of the assay solutions at 412 nm was measured in an Sp500 spectrophotometer and the maximum absorbance recorded (after approximately 2 minutes).

Calculation

The GSH concentration in erythrocytes was expressed in µmoles/g Hb, and calculated thus:

$$\text{GSH } \mu\text{moles/gHb} = \frac{\text{OD412}}{13600} \times D_F \times D_H \times \frac{1000}{\text{Hb}}$$

where OD412 is the absorbance of the assay solution

D_F is the dilution of the filtrate in the assay cuvette

D_H is the dilution of the haemolysate in the precipitating solution

Hb is the concentration of Hb (g/ml) in the haemolysate

13600 is the molar extinction coefficient of GSH.

b) Estimation of Hb Derivatives
(Evelyn and Malloy, 1938)

Principle

The estimation of Hb derivatives in blood depends on the measurement of the degree of absorbance of U.V. light at different wavelengths. Methaemoglobin (MHb) shows a characteristic absorbance band at 635 nm which is

virtually completely abolished by the addition of NaCN and the consequent conversion of meths into cyanmethaemoglobin (MHbCN). The absorbance band at 620 nm, characteristic of sulphaemoglobin (SHb) is unaffected by the presence of NaCN, thus virtually all the absorbance at 620 nm is due to SHb. Any absorbance by oxyhaemoglobin OxyHb) and MHb can be corrected for. The total Hb present in a solution is estimated by conversion of all forms of Hb to MHbCN by NaCN in the presence of potassium ferricyanide ($K_3Fe(CN)_6$), and measurement of absorbance at 540 nm. The concentration of OxyHb in the original blood sample is obtained by subtracting the calculated concentrations of MHb and SHb from the total Hb estimated.

Method

0.02 ml of washed packed cells were diluted in 3 ml of 0.017 M-KH_2PO_4 buffer adjusted to pH 6.6 with 0.1 M-K_2HPO_4, and the derivatives measured on the freshly prepared sample or on a sample frozen overnight at -20°C under liquid paraffin and filtered before use. 1 ml aliquots were dispensed into cuvettes with a 1 cm pathlength and the absorbance read against a distilled water blank at 635 nm (1i). 0.02 ml of a neutralised solution of 1.02 M-NaCN (neutralised with 2 M-acetic acid) were added to each cuvette and the absorbance read at 635 nm (l_2) after 2 minutes. 0.02 ml of concentrated NH_4OH were then added to each cuvette and the absorbance was read after 2 minutes against a distilled water blank at 620 nm (l_3). 0.6 ml aliquots of the solution were then diluted in 2.4 ml of

0.07 M-K_2HPO_4 buffer pH 6.6, and cyanmethaemoglobin formed by the addition of 0.01 ml of 0.61 M-$K_3Fe(CN)_6$ and 0.01 ml of 2.04 M-NaCN. The absorbance of the final solution was measured at 540 nm (l_4) against a reagent blank.

Calculation

The concentration of Hb derivatives and total Hb were expressed as g% of packed cells and calculated as follows:

i. Total Hb (T) $= \frac{100 \times l_4}{2.38}$

ii. MHb $= \frac{100(l_1 - l_2)}{2.77}$

iii. SHb $= \frac{100L3 - (8.5 \text{ MHb} + 4.4T)}{100}$

The corrected value for total Hb (T_c) was calculated as:

iv. T_c $= T + 0.22$ SHb

and v. OxyHb $= T_c - (\text{MHb} + \text{SHb})$

The relative concentrations were expressed as a percentage of the total (corrected) Hb estimated.

c) Malonyldialdehyde Estimation in RBC exposed to H_2O_2 (Stocks and Dormandy, 1970).

Principle

Malonyldialdehyde (MDA) has been identified has a breakdown product of some lipid peroxides and as such

has been used as an indicator of the degree of lipid peroxidation in various tissues (Dahle, Hill and Holman, 1962; Gutteridge, 1977). Human erythrocytes, when exposed to H_2O_2 may exhibit peroxidation of the constituent unsaturated fatty acids of membrane lipids. When catalase is inhibited by sodium azide, GSHPx constitutes the predominant antioxidant and may (i) remove H_2O_2, (ii) remove lipid peroxides. MDA, which is water-soluble, can be estimated in protein-free extracts of RBC by virtue of the formation of a pink chromogen with thiobarbituric acid (TBA) which absorbs light at 532 nm. The presence of other substances which may absorb light at 532 nm is accounted for by measuring the absorbance of all samples at 532 nm and 600 nm and subtracting the readings obtained at 600 nm from those obtained at 532 nm.

Method

Packed RBC were washed twice in isotonic phosphate-saline buffer pH 7.4, and the haematocrit measured (Methods: c.i). The cells were resuspended (Methods: D.b.v) containing 2 mm sodium azide. 2.5 ml aliquots were taken and incubated with equal volumes of 20 mM-H_2O_2 at 37°C in a shaking water bath for 1 hour. A glass marble was placed at the neck of each 25 ml conical flask used for the incubation. Sample blanks were prepared without H_2O_2 and reagent blanks containing no RBC. Haemolysates were prepared of each sample, using equal volumes of suspension

and distilled water. After incubation 4 ml of each suspension were removed and the protein precipitated with a TCA arsenite solution (o.43 M-TCA solution containing 25 mM-sodium arsenite). The mixture was centrifuged at 5000 g for 15 minutes and the clear supernatent removed. Assays were carried out on fresh extracts or after freezing at -20°C overnight. 6.94 mM-TBA was added to the extract in the proportion of 1:4 respectively, and test and blank solutions boiled for 15 minutes. After cooling the absorbance of each solution was recorded at 500, 532, and 600 nm against the appropriate reagent blank. The Hb concentration in the haemolysates, freed of stroma, was recorded.

Calculations

MDA was expressed as nmoles/g Hb, and calculated as follows:

$$MDA = \frac{OD \times D_A \times D_T \times 10^*}{0.156 \times Hb \times 7} \text{ nmoles/g Hb}$$

where D_A = dilution of suspension in acid

D_T = dilution of extract in TBA

OD = difference between absorbance at 532 + 600 nm

* Accounts for the fact that only 70% of the cell is water and corrects the dilution factors.

Hb is the concentration of Hb (g/ml) in the haemolysate.

0.156 is the μMolar extinction coefficient of MDA.

d) Haemolysis of RBC exposed to H_2O_2

Suspensions of RBC were incubated with and without H_2O_2 as described in part 3 of this section. After 1 hour incubation the intact RBC were pelleted by centrifugation at 5000 g for 15 minutes. 0.1 ml aliquots were removed from each supernatent for Hb estimation (Methods: c.iv). The Hb concentration in total haemolysates of each sample was similarly estimated.

Calculation

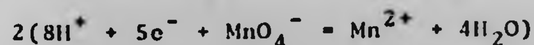
$$\text{Percentage haemolysis} = \frac{Hb_S}{Hb_T} \times 100$$

where Hb_S is the Hb concentration (mg/ml) in the sample supernatent

Hb_T is the Hb concentration in a 100% haemolysate of the sample.

H. Estimation of H_2O_2 in Solution

When potassium permanganate is added to an acidified solution of H_2O_2 it acts as an oxidising agent. Permanganate ions MnO_4^- have such a strong purple colour that they act as their own indicators and with H_2O_2 are reduced to colourless manganous ions. Thus, in the presence of excess acid,

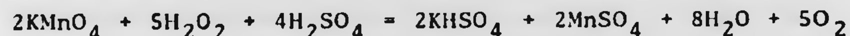


$$2(8H^+ + 5e^- + MnO_4^- = Mn^{2+} + 4H_2O)$$

when an atom in any complex molecule is subjected to a change in polarity (oxidised or reduced) the equivalent weight of the compound is the gram molecular weight divided by the change in polarity of the oxidised or reduced element. Thus, the equivalent weight of $KMnO_4$ is

$$\frac{\text{Molecular weight}}{5} .$$

When $KMnO_4$ is titrated with H_2O_2 in excess acid the following reaction takes place:



$$2KMnO_4 + 5H_2O_2 + 4H_2SO_4 = 2KHSO_4 + 2MnSO_4 + 8H_2O + 5O_2$$

Method

1 ml of a dilute solution of H_2O_2 was diluted further in about 3 ml of 7.5 N-H_2SO_4. 100 µl aliquots of 0.005 N-$KMnO_4$ were added to this solution with continuous mixing using a Compu-Pet (General Diagnostics) until the solution retained a pink colour for at least 3 minutes.

Calculation

$$1000 \text{ ml N-}KMnO_4 \equiv \frac{H_2O_2}{2} = 17.01 \text{ g } H_2O_2$$

$$\text{thus } 1000 \text{ ml } 0.005 \text{ N-}KMnO_4 \equiv 17.01 \times 0.005$$
$$= 0.085 \text{ g } H_2O_2$$

$$\text{and } 1 \text{ ml of } 0.005 \text{ N-}KMnO_4 \equiv 0.085 \text{ mg } H_2O_2 .$$

Thus if X ml of 0.005 N$KMnO_4$ are required to reduce the H_2O_2 in solution, that solution contains

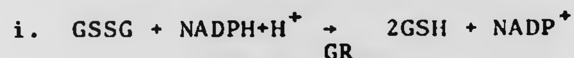
X x 0.085 mg H_2O_2 .

I. Estimation of Total Peroxides in Erythrocytes

(Modified from Heath and Tappel, 1976)

Principle

A GSHPx system is active in RBC in order to protect the cells against oxidant attack by H_2O_2 and degenerative attack by the products of lipid peroxidation. The GSHPx system is integrally linked to the activity of GR, which is responsible for the continuous generation of GSH with a concomitant oxidation of NADPH.



i. $GSSG + NADPH{+}H^+ \xrightarrow{GR} 2GSH + NADP^+$

ii. $ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$

The concentration of total peroxides in a solution may be estimated by measuring the degree of oxidation of NADPH in a linked enzyme system in which peroxides constitute the limiting factor to enzyme activity.

The assay consists of two phases; incubation of the test solution with NADPH, GSHPx, and GSH, followed by addition of GR and a further incubation period. The oxidation of NADPH during the two incubation periods is determined by following the absorbance of the solution at 334 nm.

Method

Suspensions of RBC were incubated in the absence and presence of 10 mM-H_2O_2 (Methods: G.c) for 1 hour at 37°C. Suspensions were frozen at -20°C under nitrogen overnight, and on thawing the stroma removed by centrifugation for 15 minutes at 5000 g. To 0.5 ml of haemolysate were added 0.5 ml of 0.124 M-Tris-HCl buffer pH 7.6 followed by 50 µl 2mM-NADPH, 10 µl 250U/ml GSHPx (the Boehringer Corp. London) and 100 µl GSH 4.25 mM. The absorbance of this solution at 334 nm was measured (T1) and the solutions incubated for a carefully-timed 10 minutes at 37°C after which the absorbance was reread (T2). 50 µl of GR 100 U/ml (the Boehringer Corporation, London) were added to each cuvette, the contents mixed thoroughly and the cuvettes incubated for a further 10 minutes. The absorbance of the solution was again read at 334 nm (T3). The Hb concentration in the haemolysates was determined using the cyanmethaemoblobin method (Methods: C.iv).

Calculation

The amount of NADPH oxidised in the reaction system bears a stoichiometric relationship to the amount of peroxide initially present in the solution. The fall in absorbance at 334 nm indicates the amount of NADPH that has been oxidised. Thus:

$$\text{Concentration of ROOH present in assay solution} = \frac{\Delta OD}{6.1} \text{ mMolar}$$

where ΔOD is the fall in absorbance at 334 nm
and 6.1 is the Extinction Coefficient of a mMolar solution of NADPH.

J. Oral Supplementation of Subjects with Subclinical Riboflavin Deficiency with Riboflavin

Two adult subjects diagnosed as marginally biochemically riboflavin deficient were given oral supplements of riboflavin and a number of haematological and biochemical measurements were made on the erythrocytes.

One subject was given 1 mg of riboflavin daily for 11 days. Blood samples were taken at regular short intervals and red cells separated into fractions of different mean ages (Methods: D.b). Haematological measurements, including RBC counts, reticulocyte counts, and packed cell volume determinations were made on unfractionated blood samples. The activities of EGR and AST were measured in fractions of RBC of different mean ages and in unfractionated blood.

The second subject was given daily supplements of 2 mg of riboflavin for 11 days, and thereafter 3 mg on alternate days. After 81 days supplementation was ceased. Blood samples were taken at regular short intervals and RBC separated into fractions of different mean ages. Haematological and enzymatic measurements were made as described above, and in addition the following

biochemical measurements were made: concentration of red cell GSH, haemolysis and MDA formation in response to peroxidative stress, and relative proportions of Hb derivatives in erythrocytes.

RESULTS

A. Separation of RBC into Fractions of Different Mean Ages

i) Separation of RBC on gradients of bovine serum albumin

Attempts to produce a clear age-related separation of RBC on gradients of bovine serum albumin (BSA) were not successful. There were no obvious bands of RBC at the interfaces of the gradient solutions, and considerable clumping of RBC was evident in the BSA solutions.

ii) Separation of RBC into five fractions on Ficoll/Triosil gradients

RBC centrifuged on a gradient prepared from 5 Ficoll/Triosil solutions formed clearly-defined bands at the interfaces of the gradient solutions (Figure 9). Some cells have moved through the gradient to lie at the bottom of the centrifuge tube. A few of the cells are suspended in the Ficoll/Triosil solutions and some agglutination appears to have taken place. Agglutination of RBC was not apparent in fractions of cells that had been washed free of the suspending Ficoll/Triosil solution.

Figure 9 Separation of RBC on a Density Gradient of 5 Solutions of Ficoll/ Triosil

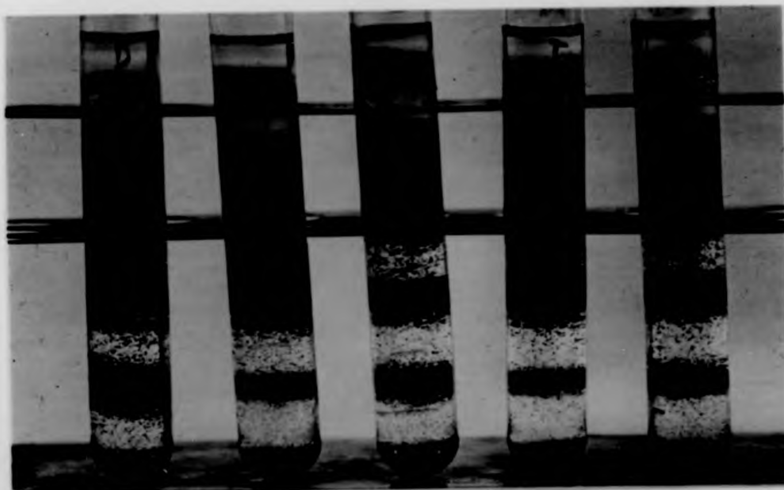


Figure IO

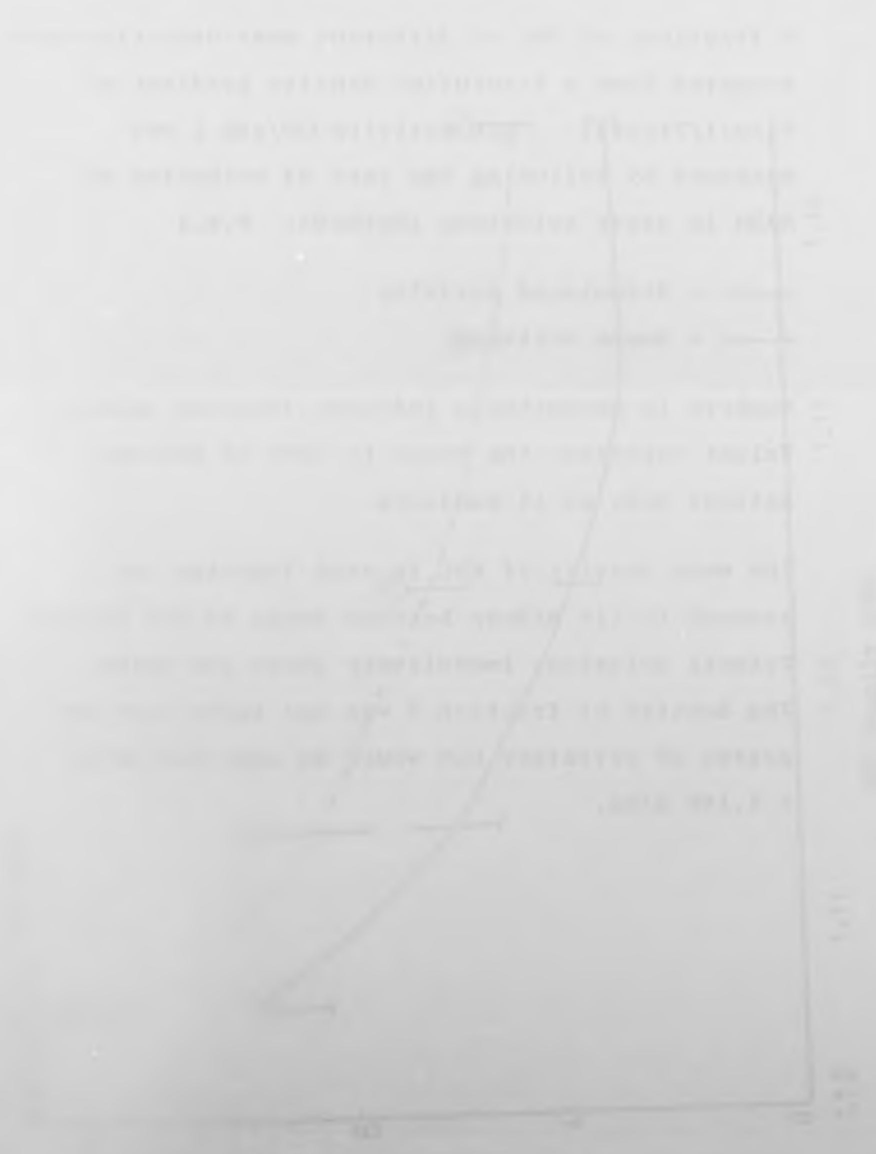


Figure 10: Aspartate Amino Transferase Activity in RBC of Different Densities Obtained from Normal Adults

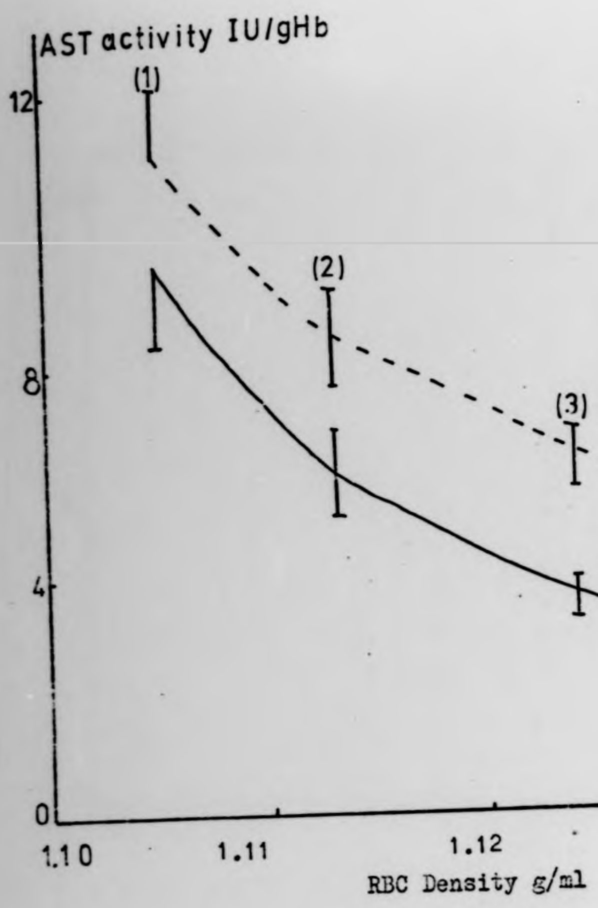
5 fractions of RBC of different mean densities were prepared from a 5-solution density gradient of Ficoll/Triosil. AST activity (IU/gHb) was measured by following the rate of oxidation of NADH in assay solutions (Methods: F.a.)

----- = Stimulated Activity

——— = Basic Activity

Numbers in parentheses indicate fraction numbers. Values represent the means ($\pm$ SEM) of determinations made on 14 subjects.

The mean density of RBC in each fraction was assumed to lie midway between those of the Ficoll/Triosil solutions immediately above and below. The density of fraction 5 was not known with any degree of certainty but would be expected to be $>$ 1.140 g/ml.



AST activity IU/gHb
12
8
4
0
(1)
(2)
(3)
1.10
1.11
1.12
RBC Density g/ml

Figure 10 Aspartate Amino Transferase Activity in RBC of Different Densities

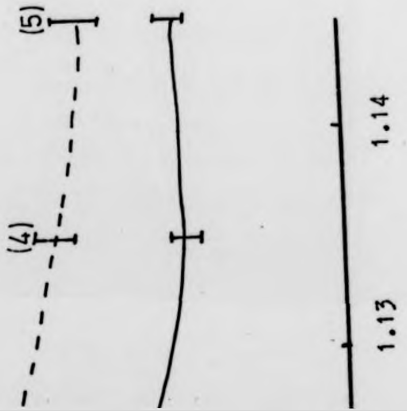


Figure II

Figure 11: Activity of Aspartate Amino Transferase (IU/cell) in Relation to RBC density

----- = Stimulated activity

______ = Basic activity

Values represent means ± SEM for RBC of 6 subjects. The mean activity of AST in a cell fraction was calculated as:

$$\frac{A \times Hb_T}{RBC_T}$$

where A = specific activity (IU/g Hb) of AST in the fraction

HB_T = Total Hb (g) in the fraction

RBC_T = Total number of RBC in the fraction.

(For other details see Figure 10).

Figure 11: Activity of Aspartate Amino Transferase (IU/cell) in Relation to RBC density

----- = Stimulated activity

——— = Basic activity

Values represent means ± SEM for RBC of 6 subjects. The mean activity of AST in a cell fraction was calculated as:

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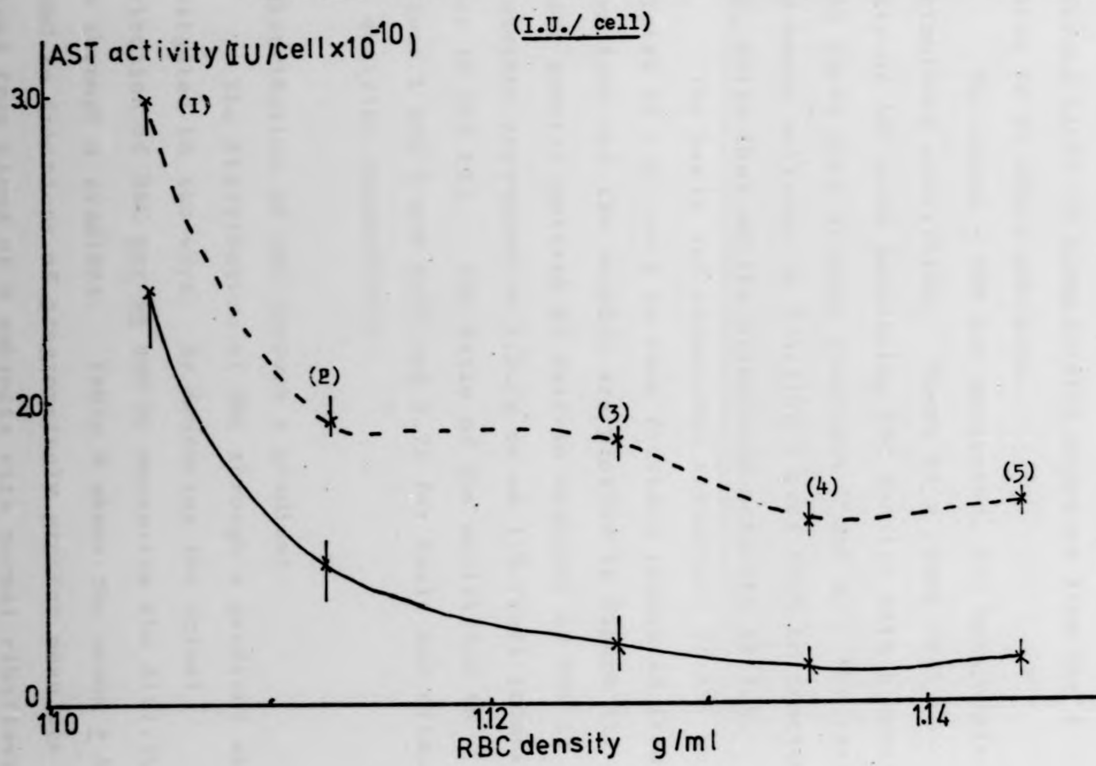
HB_T = Total Hb (g) in the fraction

RBC_T = Total number of RBC in the fraction.

(For other details see Figure 10).

Figure II Activity of Aspartate Amino Transferase in Relation to RBC density

(I.U./ cell)



iii) Aspartate Amino Transferase activity in fractions of RBC recovered from the gradient

Figure 10 shows the activity of aspartate amino-transferase (AST) in haemolysates prepared from the 5 fractions in 14 adult subjects.

The means ± SEM are presented, for both basic and stimulated activities. There is a clear fall in activity of AST with increasing RBC density with a particularly rapid fall between fractions 1 and 2. The ratio of the basic activity in fraction 1 over that in fraction 5 is 3.2, while that of the stimulated activity is 2.5.

The basic and stimulated activities of AST were calculated as I.U./cell in each fraction recovered from the gradient and the results are plotted in Figure 11. The same general pattern of fall in activity of AST is seen whether expressed as I.U./g or as I.U./cell (compare Figures 10 and 11). The ratio of the activities in fractions 1 and 5 are 2.10 and 1.78 for basic and stimulated activity respectively.

The distribution of RBC through a gradient:

The distribution of RBC through a gradient was investigated in two ways: by measuring the actual distribution of RBC *per se* and by measuring the distribution of Hb through a gradient. Table 9 shows the means ± SEM for RBC in fractions of progressively greater mean age obtained from blood of 6 subjects with normal riboflavin

Table 9: Total Number of RBC/Fraction, in Density Gradients of 5 Solutions of Ficoll/Triosil

The means and standard errors of the means from 6 healthy subjects are given where total RBC/Fraction

= RBC/ml of packed cells in fraction x Volume (ml) of packed cells.

Fraction	Mean density (g/ml)	RBC x 10^9
1	1.105	0.99 $\pm$ 0.26
2	1.113	1.70 $\pm$ 0.41
3	1.124	9.41 $\pm$ 0.24
4	1.135	2.84 $\pm$ 0.83
5	1.145	1.55 $\pm$ 0.39

Figure I2

Figure 12: Distribution of Haemoglobin and RBC on a 5-solution density gradient

5 fractions of RBC of progressively greater mean density were obtained from a 5-solution density gradient. The total RBC and Hb in each fraction is expressed as a percentage of the total RBC and Hb recovered from the gradient.

----- = Hb distribution

——— = RBC distribution

Values represent means ± SEM for 6 normal adults.

Figure 12 Distribution of Haemoglobin and RBC on a 5-Solution Density Gradient

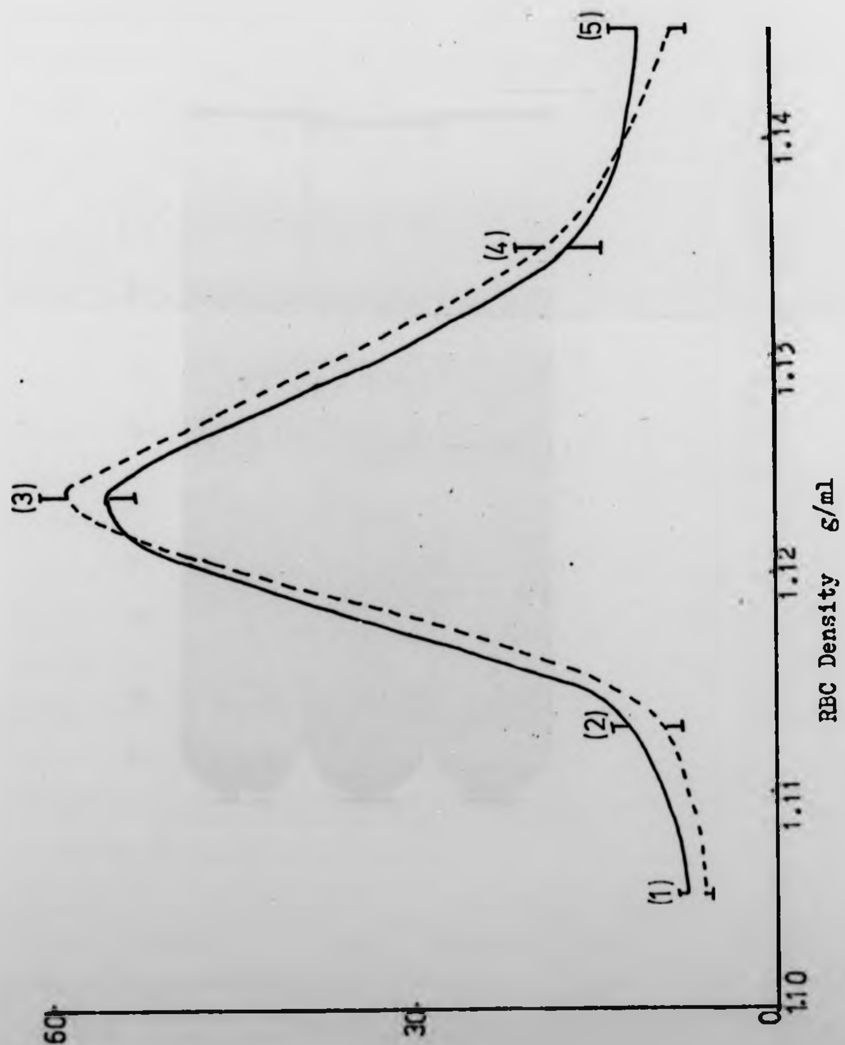


Figure 13 RBC Distribution Through a 9 Solution Density Gradient.

status. The total number of cells in each fraction was calculated as a percentage of the total number of RBC recovered from the gradient and the data expressed in this way are presented in Figure 12. The density gradient is not linear and therefore the distribution of cells would not be expected to conform to a normal distribution. The greatest proportion of cells appear to be suspended in the gradient at an estimated density of 1.124 g/ml, the lightest and most dense fractions represent 7 and 11% respectively of the total number of cells recovered.

The Hb content of RBC in each fraction was calculated as a percentage of the total Hb recovered from the gradient and the means for all fractions are also shown on Figure 12. The distribution of red cells through the gradient was the same whether expressed as a percentage of the total RBC or a percentage of the total Hb represented by each fraction (t-test).

For the remainder of the study RBC distribution through a density gradient was expressed as the percentage of the total Hb recovered from the gradient represented by each fraction.

iv) Separation of RBC into nine fractions on a density gradient of Ficoll/Triosil

Figure 13 shows a typical separation of RBC on a density gradient comprising 9 solutions. There is a dense band of RBC at each interface of the gradient solutions and some suspension of cells in the solutions themselves.

Effect of time on separation of cells:

In order to determine optimum conditions for resolution of cells according to their age samples of RBC from the same subject were subjected to different periods of centrifugation, the speed of centrifugation being constant at 65,000 g (av). The preparation of the gradient and the application of cells onto the gradient was identical in all experiments (Methods: D.b). Table 10 shows the effect of centrifuging for periods of 0.5 hrs to 5.5 hrs on the Hb distribution through a gradient. The values shown represent the means of 2 experiments made on the same subject on two consecutive days. The total Hb recovered from the gradient was expressed as a percentage of that applied to the gradient and thus reflects the percentage recovery of RBC.

As the time of centrifuging increased two effects on the separation of RBC were observed: the distribution profile was altered and the percentage recovery of cells from the gradient was reduced. Centrifuging for up to 1.0 hr resulted in a considerable proportion of the cells remaining in the lightest, youngest fraction of cells with a consequent deficit in the denser fractions. Centrifuging for 1.5 and 2.0 hrs resulted in 2 very similar RBC distribution profiles (Table 10) which it is believed indicates that cells remain at their own density on the gradient between 1.5 and 2.0 hrs. With increased times of centrifuging however, the Hb distribution profile

Table 10: Effect of Duration of Centrifugation on Hb Distribution through a Gradient and Recovery of RBC from the Gradient

Values represent the means and standard errors of two experiments made on the same subject on consecutive days.

Duration of Centrifugation (hours)	1	2	3	4	5	6	7	8	9	% Hb Recovery
0.5	27.0 ± 0.11	32.2 ± 1.32	24.8 ± 0.11	13.4 ± 0.90	1.4 ± 0.86	1.15 ± 0.86	1.8 ± 0.10	1.5 ± 0.06	0.8 ± 0.13	100 ± 0.50
1.0	6.0 ± 0.10	25.7 ± 1.21	35.3 ± 0.06	12.9 ± 1.03	3.2 ± 0.76	11.4 ± 0.09	3.0 ± 0.09	0.9 ± 0.13	1.5 ± 0.15	90 ± 0
1.5	1.0 ± 0.05	6.6 ± 1.7	30.3 ± 0.08	14.3 ± 1.0	9.9 ± 0.70	13.4 ± 0.05	13.8 ± 0.20	5.4 ± 0.05	7.7 ± 0.10	90 ± 0.71
2.0	1.5 ± 0.06	6.2 ± 1.92	32.1 ± 0.10	16.5 ± 1.08	8.1 ± 0.71	11.6 ± 0.04	12.0 ± 0.12	6.1 ± 0.08	6.0 ± 0.11	88 ± 1.41
3.0	1.5 ± 0.08	3.3 ± 1.66	10.1 ± 0.29	16.0 ± 0.84	12.8 ± 0.91	6.1 ± 0.06	19.5 ± 0.11	21.8 ± 0.11	9.2 ± 0.11	84 ± 2.83
5.5	0 ± 0	0 ± 0	0.7 ± 0.10	1.0 ± 0.12	1.3 ± 0.09	1.8 ± 0.10	2.0 ± 0.06	2.2 ± 0.04	90.9 ± 0.06	51 ± 2.83

Table 11: Effect of Duration of Centrifugation on AST Activity Measured in Fractions of RBC Recovered From a Density Gradient

Values represent the means and standard errors obtained from 2 experiments on the blood of one subject taken on consecutive days. Activities are expressed as I.U./g Hb. A_1/A_9 is the ratio of AST activities in Fractions 1 and 9.

Duration of Centrifugation (hours)	Fraction number									$\frac{A_1}{A_9}$
	1	2	3	4	5	6	7	8	9	
0.5	3.7 ± 0.33	3.4 ± 0.48	3.8 ± 0.46	3.8 ± 0.42	3.9 ± 0.40	3.4 ± 0.26	3.3 ± 0.29	2.4 ± 0.14	3.5 ± 0.27	1.48
1.0	5.2 ± 0.44	4.4 ± 0.48	4.5 ± 0.46	3.0 ± 0.32	3.9 ± 0.28	2.4 ± 0.20	3.0 ± 0.24	3.3 ± 0.21	3.3 ± 0.26	1.58
2.0	9.3 ± 0.56	6.4 ± 0.14	4.3 ± 0.32	3.5 ± 0.06	3.0 ± 0.19	3.4 ± 0.51	3.2 ± 0.29	3.0 ± 0.14	3.2 ± 0.08	2.91
3.0	9.0 ± 0.63	8.4 ± 0.60	5.8 ± 0.53	4.7 ± 0.44	4.0 ± 0.39	3.2 ± 0.30	2.9 ± 0.20	3.5 ± 0.36	3.8 ± 0.34	2.37

changed further so that a progressively greater proportion of the cells on the gradient moved downwards towards the denser part of the gradient. Finally, after 5.5 hrs virtually all the cells that had been applied to the gradient appeared at the bottom of the centrifuge tube. These prolonged centrifugations of 3.0 and 5.5 hrs were accompanied by a dramatic decrease in the percentage recovery of Hb from the gradient due to the fact that cells stuck more and more firmly to the base of the centrifuge tube and others haemolysed.

The effect of centrifuging red cells for different times on activity of AST in each fraction is shown in Table 11. The activities given represent means ≠ SEM obtained from 2 experiments made on consecutive days on fresh red cells obtained from the same subject. The ratio of the activity measured in Fraction 1 over that in Fraction 9 was calculated for each time of centrifuging and the results are included in the table.

The most obvious effect of increasing the time of centrifuging on the AST activities in the fractions was seen in Fraction 1 in which a progressive increase in mean activity was observed from 0.5 hrs up to 2.0 hrs. The ratio of the activities in Fraction 1 and 9 reflects the degree of resolution of the cells into young and old cells and the largest value for this ratio was obtained after 2 hrs centrifugation. It may be relevant to notice that at 3.0 hrs there was a marked increase in activity in

Fractions 8 and 9 when compared with that in Fraction 7, an effect not noticeable to the same extent at any of the other times.

All the subsequent experiments which required separation of RBC into fractions of different mean ages employed a 2 hr-centrifugation period at 65,000 g (av).

Validation of the chronological separation by a continuous density gradient technique:

The possibility existed that the bands of RBC observed at the interfaces of the Ficoll/Triosil solutions were artefacts due to a barrier property at the interfaces which impeded the migration of RBC. Therefore, RBC were separated on a continuous density gradient prepared on an automatic density gradient maker (made by courtesy of the technical workshop, L.S.H.T.M.), using two Ficoll/Triosil solutions of densities 1.10 and 1.140 g/ml. The mode of application of cells to the gradient, and the time and speed of centrifuging were unchanged from the standard procedure adopted (Methods: D.b).

After centrifuging, the gradient solution, in which the RBC were suspended, was removed in 1.8 ml aliquots and the RBC washed twice in isotonic saline. Haemolysates of the washed fractions were measured for Hb content and AST activities. A sample of blood from the same subject was separated on a discontinuous density gradient by the standard method (Methods: D) and the AST activity in an haemolysate of each fraction measured.

Figure I4

Figure 14: A Comparison of a Discontinuous Density Gradient and a Continuous Density Gradient

Discontinuous (---) and continuous (——) density gradients of Ficoll/Triosil were prepared. The refractive indices of 1.8 ml aliquots of progressively more dense solutions were measured. The means ± SEM of 2 experiments are shown.

FIGURE 14

A COMPARISON OF A DISCONTINUOUS DENSITY GRADIENT AND A CONTINUOUS DENSITY GRADIENT

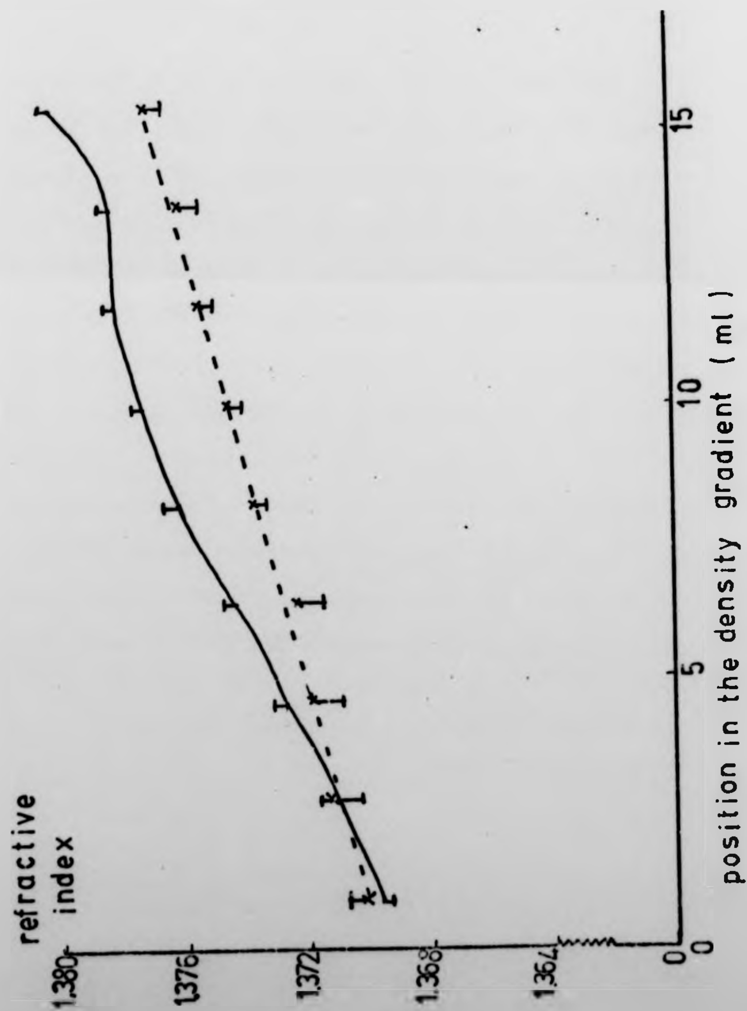


Figure 15

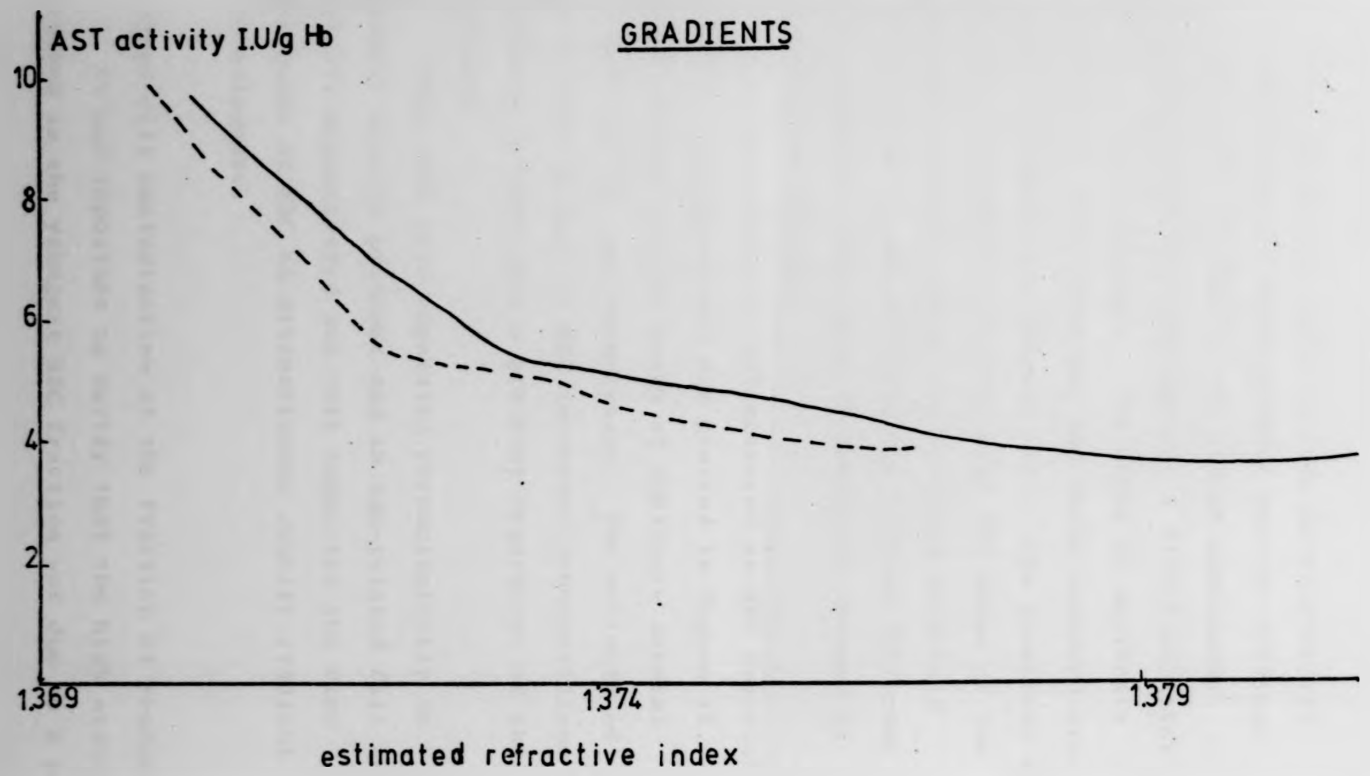
Figure 15: Activity of AST in Fractions of RBC of Different Ages Prepared from Discontinuous and Continuous Density Gradients

The activity of AST in fractions of RBC obtained from a discontinuous (——) and a continuous (---) density gradient.

The mean density of the RBC in each fraction recovered from the gradients is assumed to be proportional to the refractive index of the gradient solution at the position in the gradient at which the cells were suspended. Therefore RBC recovered from the discontinuous gradient will have a density proportional to the refractive index midway between those of the solutions on either side. The refractive index of RBC in Fraction 9 is ascribed an arbitrary value > 1.380. The mean density of RBC recovered from the continuous gradient is assumed to be proportional to the refractive index of the solution in which the cells were suspended.

FIGURE 15 ACTIVITY OF AST IN FRACTIONS OF RBC OF DIFFERENT DENSITIES

FIGURE 15 ACTIVITY OF AST IN FRACTIONS OF RBC OF DIFFERENT DENSITIES PREPARED FROM DISCONTINUOUS AND CONTINUOUS DENSITY GRADIENTS



The refractive index of each Ficoll/Triosil solution comprising the discontinuous density gradient and of each 1.8 ml aliquot of the linear continuous density gradient was measured against a distilled water blank using a refractometer. The means of duplicate determinations on each solution, and their standard errors, are presented graphically (Figure 14). The linearity of the continuous gradient contrasts with the shape of the discontinuous gradient, which was designed to detect small differences in RBC distribution between different groups of subjects. The range of densities spanned by both gradients is similar.

The activities of AST measured in RBC fractions obtained from both gradients are plotted in Figure 15. The values plotted are the means of duplicate determinations made on the same haemolysate. The activity of AST can be seen to fall in RBC recovered from positions progressively further down a gradient regardless of the gradient used.

Thus, RBC were separated chronologically on a continuous density gradient and an age-related fall in AST activity demonstrated and this supported the view that the bands of RBC on discontinuous density gradient were not artefacts.

White blood cell contamination of the fraction of youngest cells:

It was important to verify that the high activity of AST observed in the youngest RBC fraction was due to a property

Table 12: White Blood Cell Contamination of Fraction 1 Recovered from Density Gradients

The WBC concentration was measured in a 1 in 4 haemolysate of packed red cells recovered as Fraction 1 on a density gradient. The haemolysate was prepared in isotonic saline with 3 drops of Zaponin.

Subject	WBC/ml Haemolysate prepared from Fraction 1	Mean WBC/ml haemolysate
HJP	2.04×10^3	
PM	2.0×10^4	7.9×10^3
LE	1.63×10^3	

Table 13: Effect of Lysed WBC on the Activity of AST in Fraction 1 Recovered from a Density Gradient

Subject	AST Activity I.U./g Hb (1)	(2)
HJP	4.84	5.56
PM	7.68	7.50
LE	6.44	6.34

(1) and (2) indicate haemolysates prepared in distilled water or with Zaponin respectively.
Haemolysates prepared with Zaponin contain unlysed WBC (see Table 12). The difference between the activities of AST measured in the two haemolysates was investigated using the paired t-test. The differences are not consistent and not statistically significant.

of the RBC and not to WBC contamination of the fraction.

A RBC suspension of Fraction 1 was obtained in the usual manner (Methods: D) and aliquots taken for the preparation of two haemolysates.

1. A 1:3 haemolysate in distilled water in which all cells including any WBC would be expected to lyse.

2. A 1:3 haemolysate prepared in isotonic saline with 3 drops of Zaponin (Coulter Diagnostics) designed to lyse RBC and leave WBC intact. WBC were counted within 5 minutes of adding the Zaponin in a haemolysate diluted 1:24 in Isoton (Coulter Diagnostics).

A mean WBC concentration of 7.9 x 10^3/ml haemolysate was obtained in 3 haemolysates prepared from RBC of Fraction 1 from 3 different individuals (Table 12).

Each haemolysate was centrifuged at 5,000 g for 10 minutes to remove cell debris and the Hb content and AST activity measured. The results are shown in Table 13. The values represent mean AST activities of duplicate determinations made on 3 subjects with the standard error of the sample mean. The difference between the activities measured in the two preparations was examined by the paired t-test. There is no significant difference in the activities measured in haemolysates prepared in distilled water or Zaponin. The degree of WBC contamination occurring in haemolysates prepared from Fraction 1 does not appear to affect significantly the activity of AST in the haemolysate.

Table 14: Reproducibility of the Fractionation Procedure

Fraction	Hb % Total			Mean ± SEM
	1	2	3	
1	1.3	1.5	1.4	1.4 ± 0.046
2	20.6	27.1	23.2	23.6 ± 1.54
3	37.1	34.4	36.8	37.1 ± 0.17
4	17.6	13.9	15.2	15.6 ± 1.09
5	5.9	3.4	4.6	4.63 ± 0.30
6	5.0	5.2	5.8	5.33 ± 0.24
7	7.3	6.5	6.8	6.80 ± 0.23
8	2.1	1.9	2.3	2.10 ± 0.12
9	3.0	2.7	3.9	3.20 ± 0.36

Three blood samples were taken from the same subject at three difference times during the same day (1, 2, and 3). RBC were separated on 3 individually prepared Ficoll/ Triosil gradients, using fresh solutions on each occasion. The Hb distribution through each gradient and the mean ± SEM are shown.

Reproducibility of fractionation:

The reproducibility of the fractionation procedure was assessed by taking a sample of blood from an individual at 3 periods during the same day and carrying out a fractionation immediately using separately-prepared gradient solutions. The RBC distribution profile was determined for each separation (Methods: E) and the results are presented in Table 14. The mean values and standard errors for the percentage of Hb in each fraction are also given. The standard errors are well below 10% of the means for all but Fraction 9 which shows a standard error of 11%.

B. The Activity of Two Enzymes in RBC of Different Ages

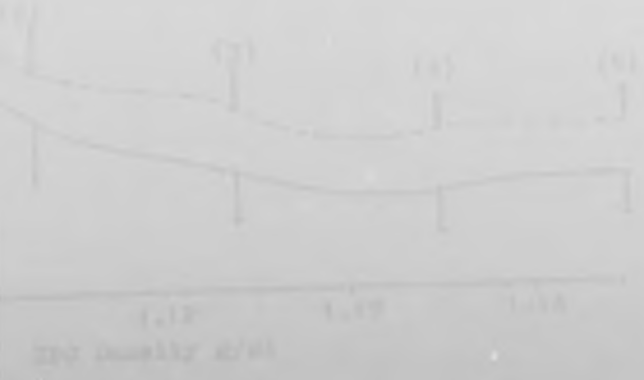
i. Glutathione Reductase

The activity of GR, expressed as IU/g Hb was measured in the 4 population groups under study, in fractions obtained from 5-solution and 9-solution density gradients. Figure 16 shows the fall in basic and stimulated activity of GR with increasing RBC age from adults with normal riboflavin status. Data were obtained using density gradients comprising 5 solutions (Figure 16A) and 9 solutions (Figure 16B). Means $\pm$ SEM are given.

Both the basic and stimulated activities show a rapid fall between the first two fractions followed by a period of exponential decline, regardless of the gradient used, and then a period of constant enzyme activity. However, a small but consistent rise in activity in the older cells is seen in samples separated into 9 fractions (16B). The significance of the observed difference in activity between Fractions 7 and 9 in Figure 16B was investigated using the paired t-test. Although the mean differences for both basic and stimulated activity appears rather small on the figure it was observed in every subject studied and is significant at $p < 0.001$ for both basic and stimulated activity.

The ratio of the activity of GR in Fraction 1 compared with that in Fraction 7 (the fraction of lowest

Figure 16



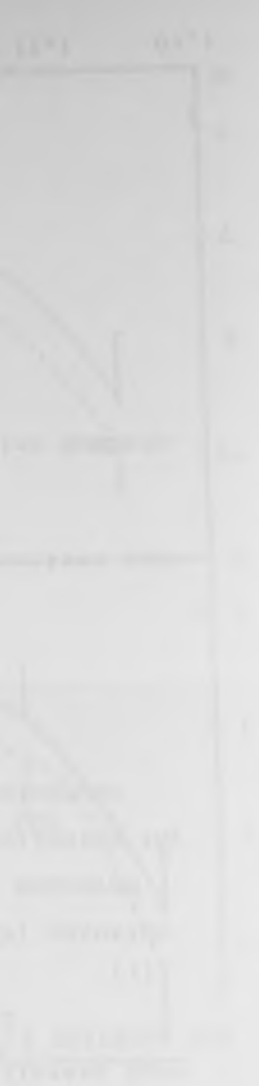


Figure 16 EGR Activity in RBC of Different Ages from Normal Human Subjects

EGR activity I.U /gHb

(B)

11
9
7
5
0

(1) (2) (3) (4) (5) (6) (7) (8) (9)

(A)

11
9
7
5
0

(1) (2) (3) (4) (5)

1.10 1.11 1.12 1.13 1.14

RBC Density g/ml

Figure 16: EGR Activity in RBC of Different Ages from normal Adult Subjects

RBC were separated on 5- (A) and 9- (B) solution density gradients. EGR activity was measured in haemolysates of each fraction by following the rate of oxidation of NADPH in assay solutions (Methods: F.b.).

----- = Stimulated activity IU/g Hb

——— = Basic activity IU/g Hb.

Values are the means ± SEM of determinations made on 14 (16A) and 12 (16B) subjects.

Figures in parentheses indicate fraction numbers.

Figure 16 EGR Activity in RBC of Different Ages from Normal Human Subjects

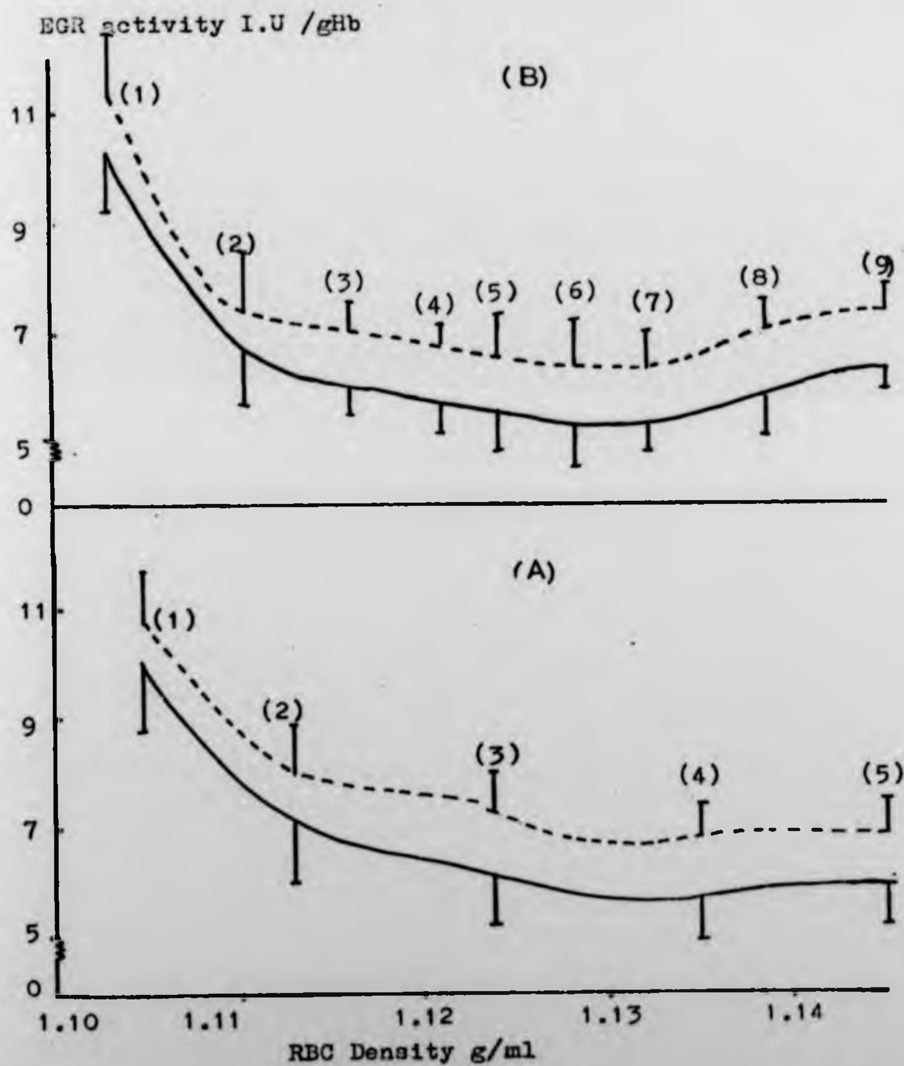


Figure I7

Figure 17: Activation Coefficient of EGR in RBC of Different Ages

RBC were separated on 5- (17A) and 9- (17B) solution density gradients, and the activation coefficient for EGR measured in haemolysates from each fraction.

The activation coefficient represents:

$$\frac{\text{EGR activity (IU/g Hb) in the presence of added FAD}}{\text{EGR activity (IU/g Hb) in the absence of added FAD}}$$

Values represent the means $\pm$ SEM of determinations made on 14 (17A) and 12 (17B) normal adult subjects.

Figures in parentheses indicate fraction numbers.

FIGURE 17 ACTIVATION COEFFICIENT OF EGR IN RBC OF DIFFERENT AGES FROM NORMAL SUBJECTS

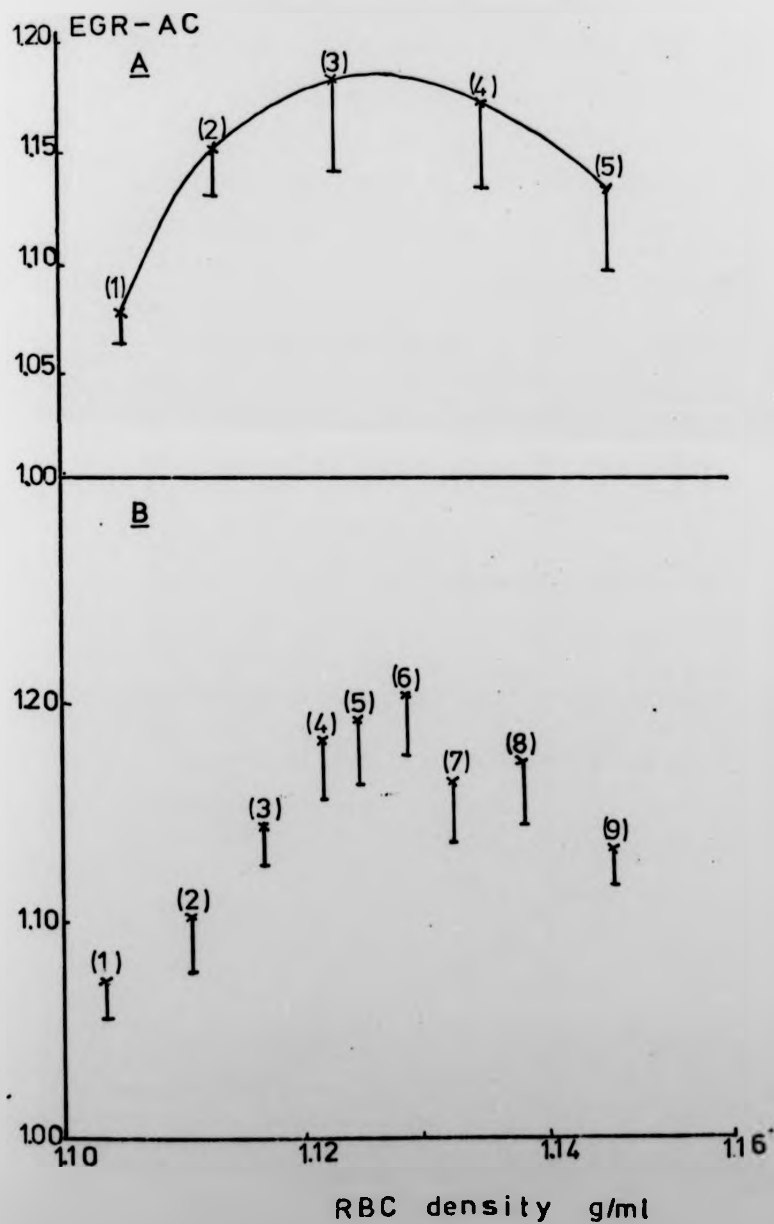


Figure 18

Figure 18: EGR Activity in RBC of Different Ages From Normal Elderly Subjects

EGR activity (IU/g Hb) was measured in fractions of RBC of different mean ages (see the legend for Figure 16B for details).

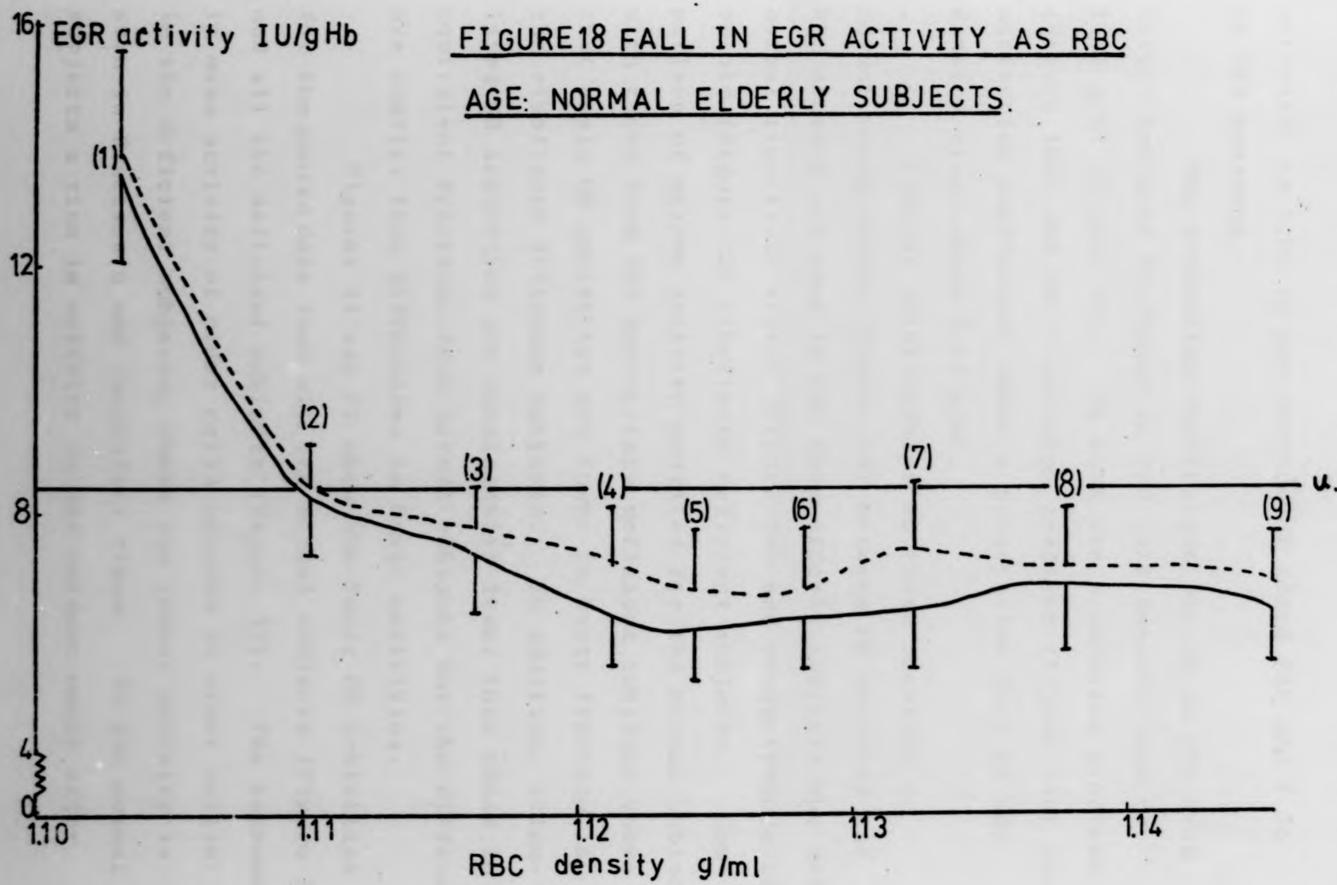
Values represent means ± SEM of determinations made on 7 subjects.

Line (u) indicates unfractionated RBC basic EGR activity.

----- = Stimulated activity

——— = Basic activity.

Figures in parentheses indicate fraction numbers.


EGR activity IU/gHb
FIGURE 18 FALL IN EGR ACTIVITY AS RBC
AGE: NORMAL ELDERLY SUBJECTS.
16
12
8
4
0
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
u.
1.10
1.11
1.12
1.13
1.14
RBC density g/ml

activity) is 1.97 in the absence of added FAD and 1.76 in its presence.

The activation coefficient for GR in RBC from normal subjects increased as red cell density rose to 1.13 g/ml (Figure 17). On both the 5-solution gradient (Figure 16A) and the 9-solution gradient (Figure 16B) the activation coefficient shows a progressive fall as RBC density rises above 1.13 g/ml.

Similar relationships to those observed on normal young adults (Figure 16) between GR activity and RBC density are seen in RBC from elderly subjects who had normal riboflavin status (Figure 18) and young (Figure 19) or old (Figure 20) riboflavin deficient subjects. Comparison of enzyme activity profiles for the normal subjects with those from the appropriate deficient subjects shows that basic GR activities are lower in every fraction in the riboflavin deficient subjects. In addition, stimulated GR activities are consistently lower than those in equivalent fractions from normal subjects but the differences are smaller than differences in basic activities.

Figures 21 and 22 show the basic GR activities for the pooled data from all the normal subjects (Figure 21) and all the deficient subjects (Figure 22). The increase in mean activity of older cells appears to start earlier in the deficient subjects, where the lowest activity is seen in Fraction 6 and thereafter rises. In the normal subjects a rise in activity is not evident until after

Figure 19

Figure 19: <u>EGR Activity in RBC of Different Ages From Adults with Marginal Riboflavin Deficiency</u>

EGR activity was measured in RBC fractions of different mean densities (for details see the legend for Figure 16B).

Line (u) indicates unfractionated RBC basic EGR activity.

----- = Stimulated activity

——— = Basic activity

Values represent means ± SEM of determinations made on 4 subjects.

Figures in parentheses indicate fraction numbers.

FIGURE19 <u>FALL IN EGR ACTIVITY AS RBC AGE:</u>

FIGURE 19 FALL IN EGR ACTIVITY AS RBC AGE: MARGINALLY RIBOFLAVIN-DEFICIENT ADULTS

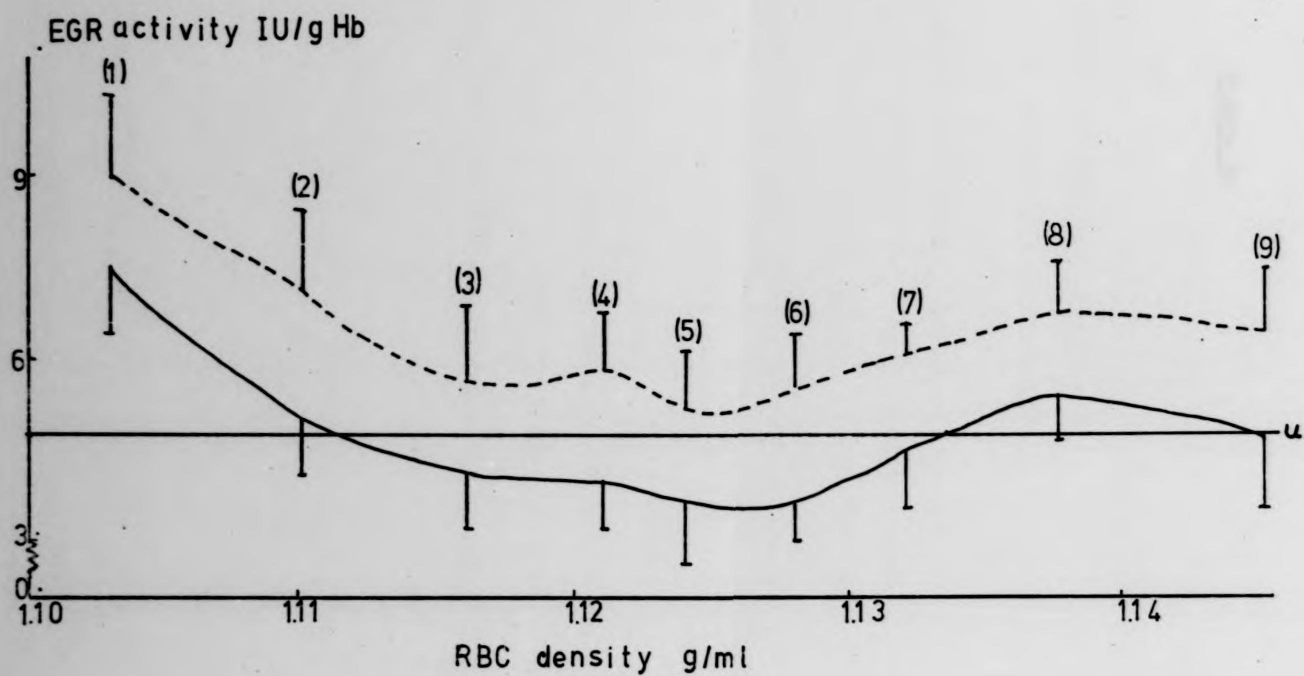


Figure 20

Figure 20: <u>EGR Activity in RBC of Different Ages From Elderly Subjects with Marginal Riboflavin Deficiency</u>

EGR activity was measured in fractions of RBC of different mean densities (see legend of Figure 16B for details).

Values represent means ± SEM for 5 subjects.

Line (u) indicates unfractionated RBC basic EGR activity.

----- = Stimulated activity

——— = Basic activity

Figures in parentheses indicate fraction numbers.

FIGURE 20 FALL IN EGR ACTIVITY AS RBC

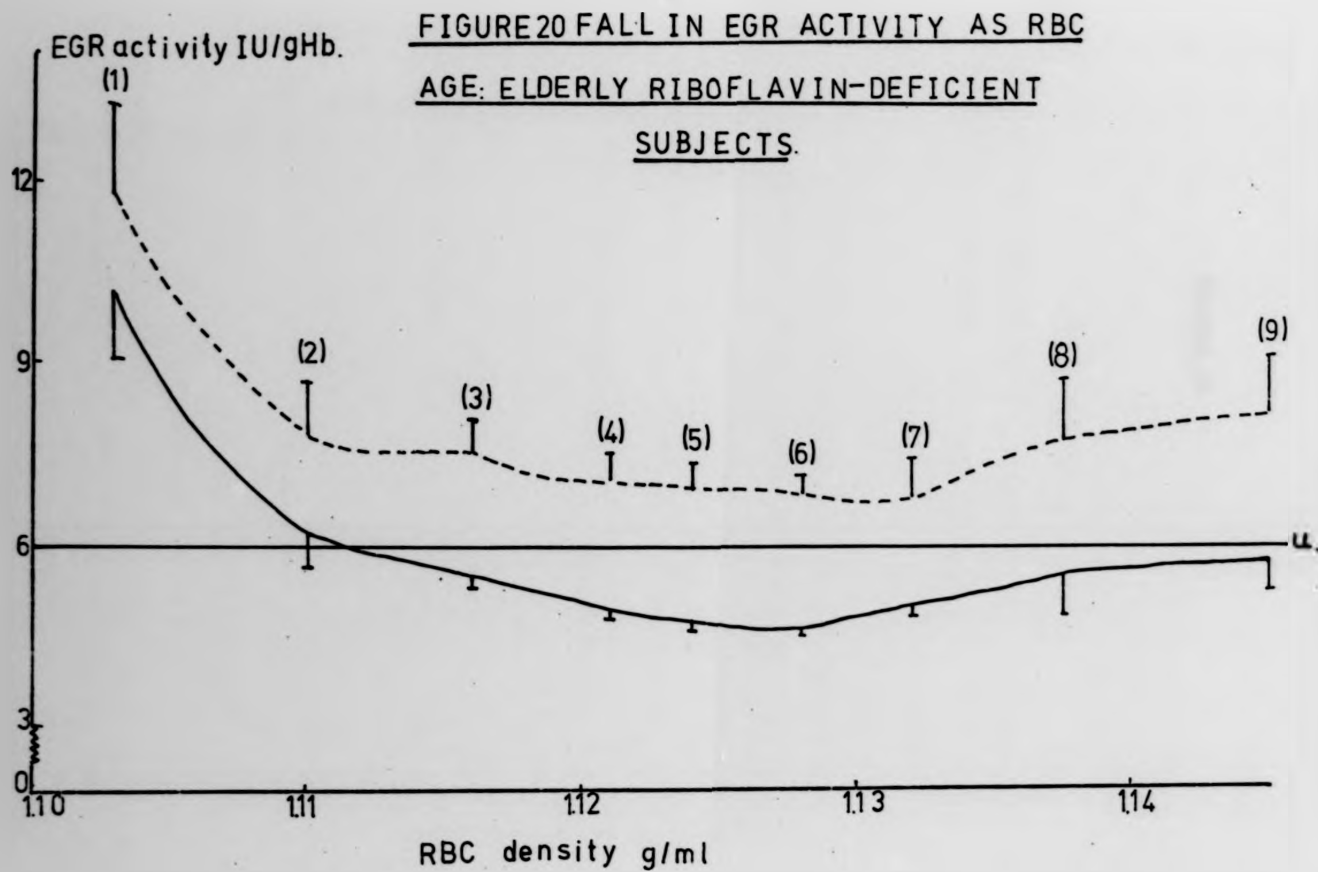


FIGURE 20 FALL IN EGR ACTIVITY AS RBC AGE: ELDERLY RIBOFLAVIN-DEFICIENT SUBJECTS.

Figure 2I

Figure 21: Relationship Between RBC Age and EGR Activity in Blood from Normal Adults

The pooled basic EGR activity data from Figures 16B and 18 are presented (see legend for Figure 16B for details).

The values represent means ± SEM for 19 subjects.

Line (u) indicates unfractionated RBC basic EGR activity.

The significance of the final increase in activity between Fractions 7 and 9 is shown (paired t-test) ($p < 0.001$).

FIGURE 21 RELATIONSHIP BETWEEN RBC AGE AND EGR ACTIVITY: NORMAL SUBJECTS

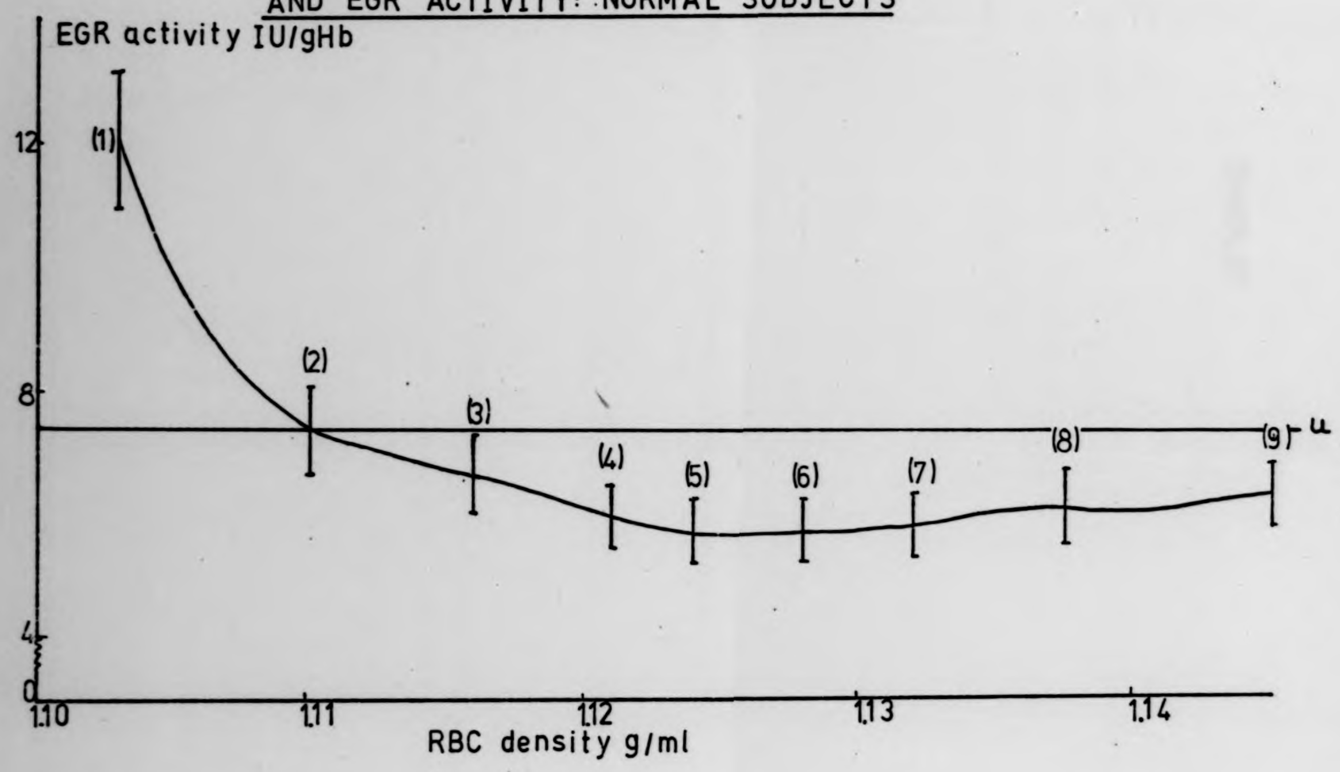


Figure 22

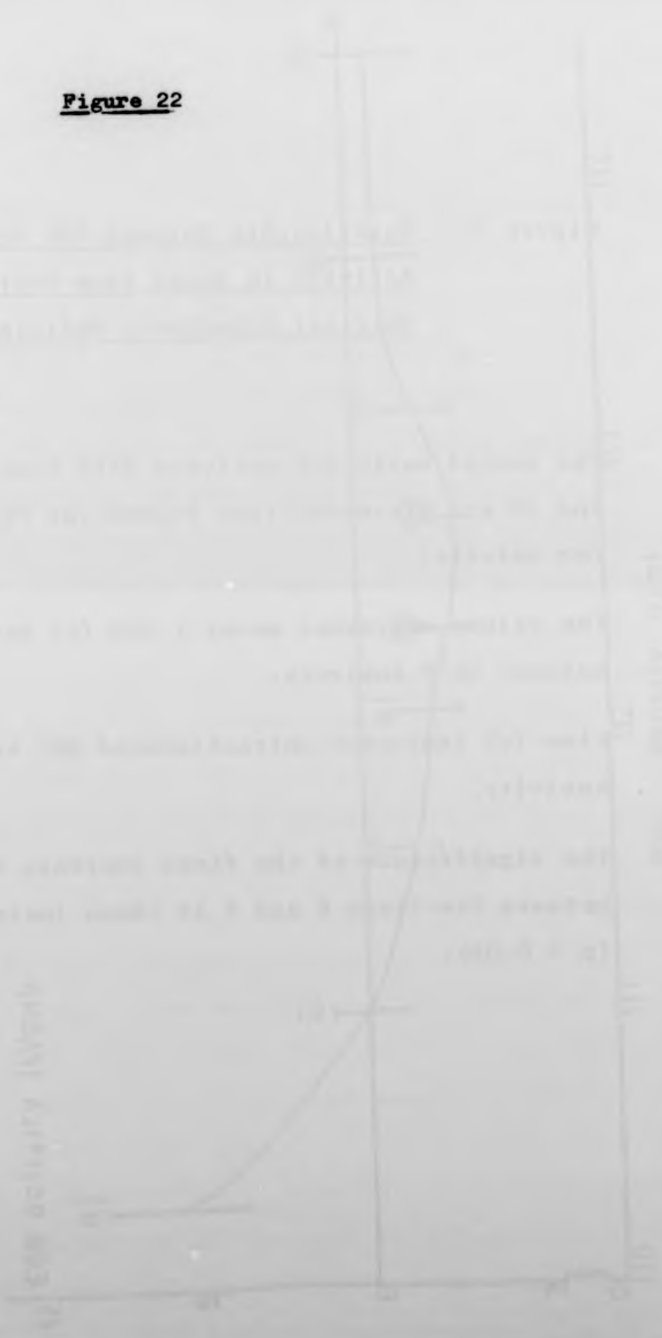


Figure 22: Relationship Between RBC Age and EGR Activity in Blood from Subjects with Marginal Riboflavin Deficiency

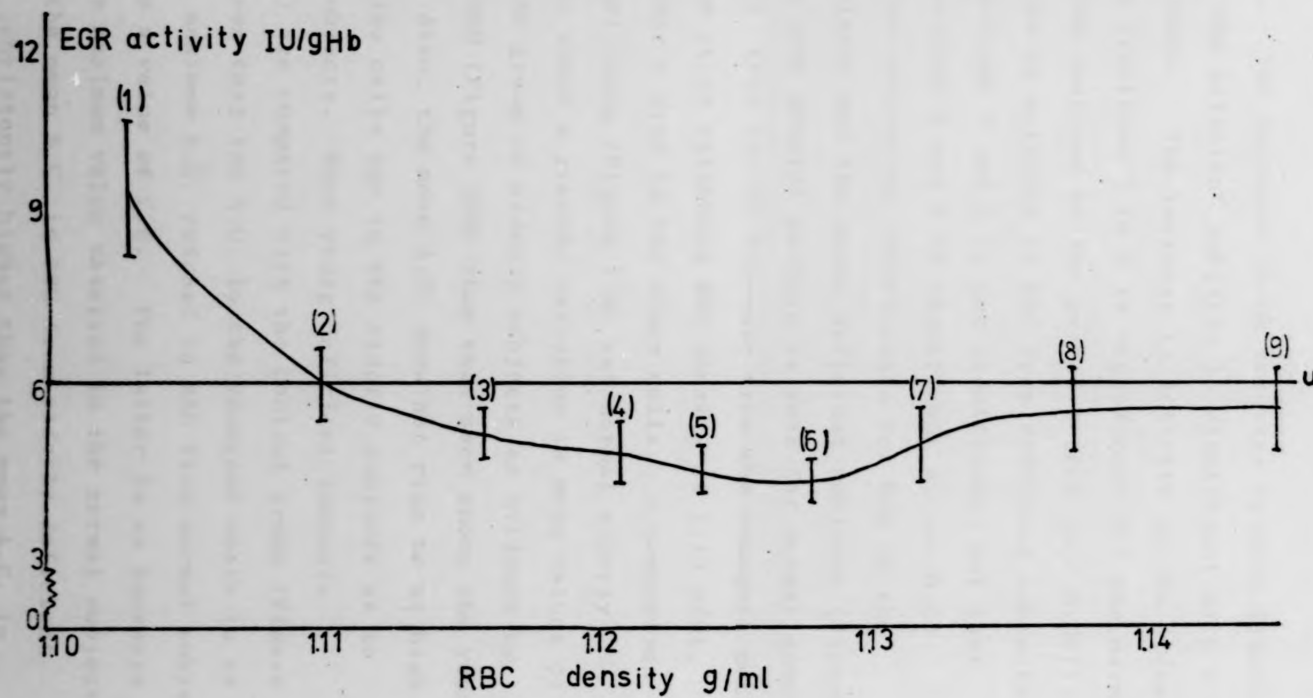
The pooled basic EGR activity data from Figures 19 and 20 are presented (see legend for Figure 16B for details).

The values represent means ± SEM for determinations on 9 subjects.

Line (u) indicates unfractionated RBC basic EGR activity.

The significance of the final increase in activity between Fractions 6 and 9 is shown (paired t-test) ($p < 0.05$).

FIGURE 22 RELATIONSHIP BETWEEN RBC AGE AND EGR ACTIVITY: MARGINALLY RIBOFLAVIN-DEFICIENT SUBJECTS



Fraction 7. The increase in GR activity between Fractions 6 and 7 of the deficient subjects is significant at $p < 0.02$ (paired t-test). The increase in activity in the oldest cells, from Fractions 7 to 9 is significant for the normal subjects when analysed by the paired t-test ($p < 0.001$). The late rise in activity in RBC from deficient subjects between Fractions 7 and 9 is not significant, but that between Fractions 6 and 9 is significant at $p < 0.05$.

The activation coefficients for EGR in the elderly subjects and the young deficient subjects (Figure 23) follows the same general pattern as seen for normal adults (Figure 17B), that is, an increase from the youngest cells to a maximum at an estimated RBC density of 1.13 g/ml, and thereafter a drop in the older cells. A comparison of the normal young (Figure 17B) and normal elderly (Figure 23B) shows a greater variation in mean values of A.C. within the group of elderly subjects as evidence by the larger SEM (Figure 23B) than that seen among the younger subjects. Also, the mean A.C. does not rise to as high a value as the cells age in the elderly subjects as in the young subjects. When young deficient subjects (Figure 23A) are compared with the control group (Figure 17B) it can be seen that the A.C. in the youngest cells is as high as the maximum A.C. reached in RBC from normal subjects and rises to a value of 1.6. The latter is an increase of 33% over the maximum value observed in the normal subjects. Similarly, the mean A.C. in RBC from elderly deficient subjects is consistently higher than the mean A.C. in

Figure 23

Figure 23: Activiation Coefficient of EGR in RBC of Different Ages

EGR activation coefficient was measured in RBC of different mean ages in blood from 4 young subjects with marginal riboflavin deficiency (A), 7 elderly subjects with normal riboflavin status (B) and 5 elderly subjects with marginal riboflavin deficiency (C).

(See legend for Figure 17 for details).

Figure 23 Activation Coefficient of Erythrocyte Glutathione Reductase in RBC of Different Ages

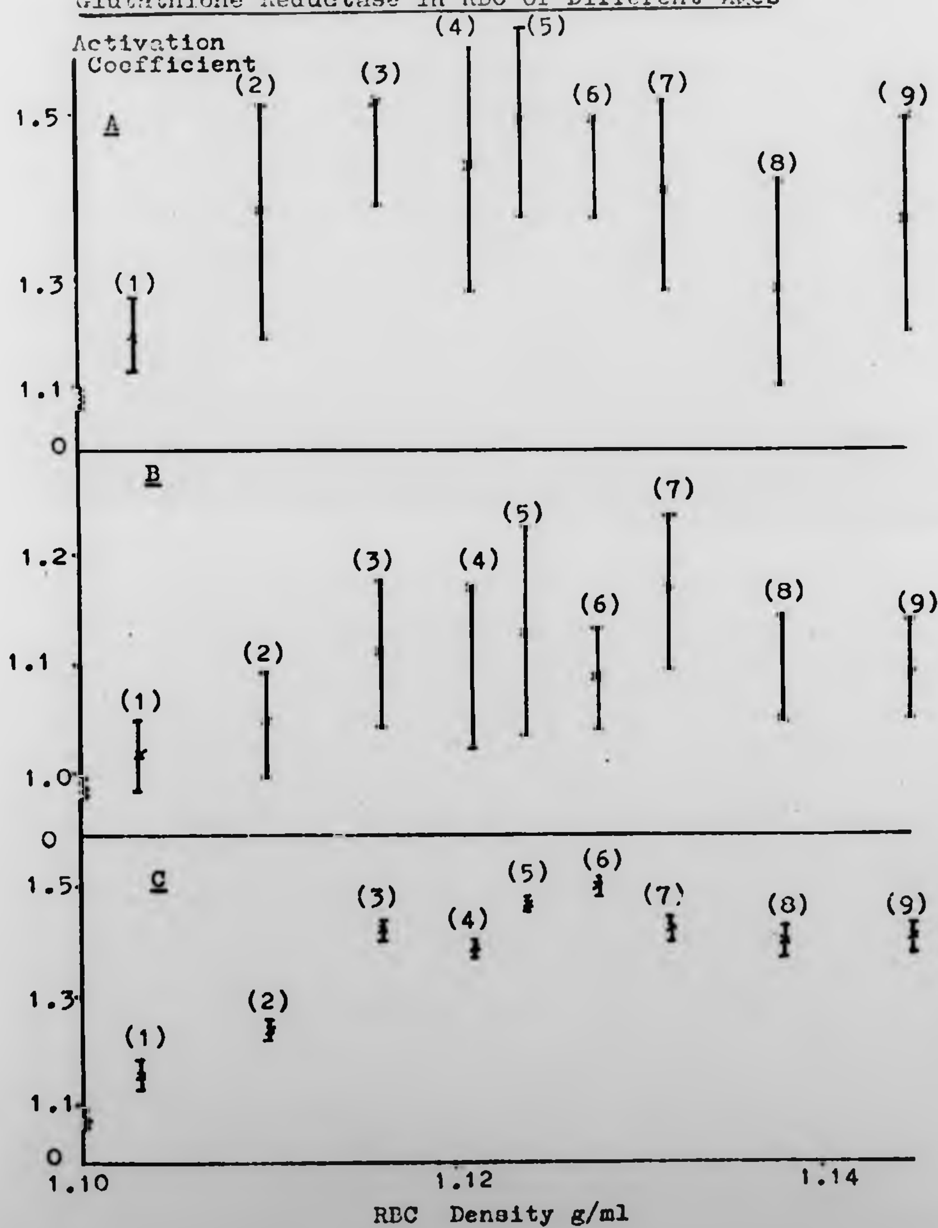


Figure 24

Figure 24: Effect of Marginal Riboflavin Deficiency on the Amount of Unsaturated GR Apoenzyme in RBC

GR activity was measured in RBC of different mean ages (see legend for Figure 16B for details) and the amount of unsaturated GR apoenzyme calculated as:

> GR activity (IU/g Hb) in the presence of added FAD - GR activity (IU/g Hb) in the absence of added FAD.

——— = Apoenzyme of normal subjects (19)

----- = Apoenzyme of subjects with marginal riboflavin deficiency (9).

Means ± SEM are shown. The differences in the amount of unsaturated apoenzyme in equivalent fractions from normal and riboflavin deficient subjects was tested (t-test), and the levels of significance are shown.

Figures in parentheses indicate fraction numbers.

Figure 24 Effect of Marginal Riboflavin Deficiency on the Amount of Unsaturated GR Apoenzyme in RBC

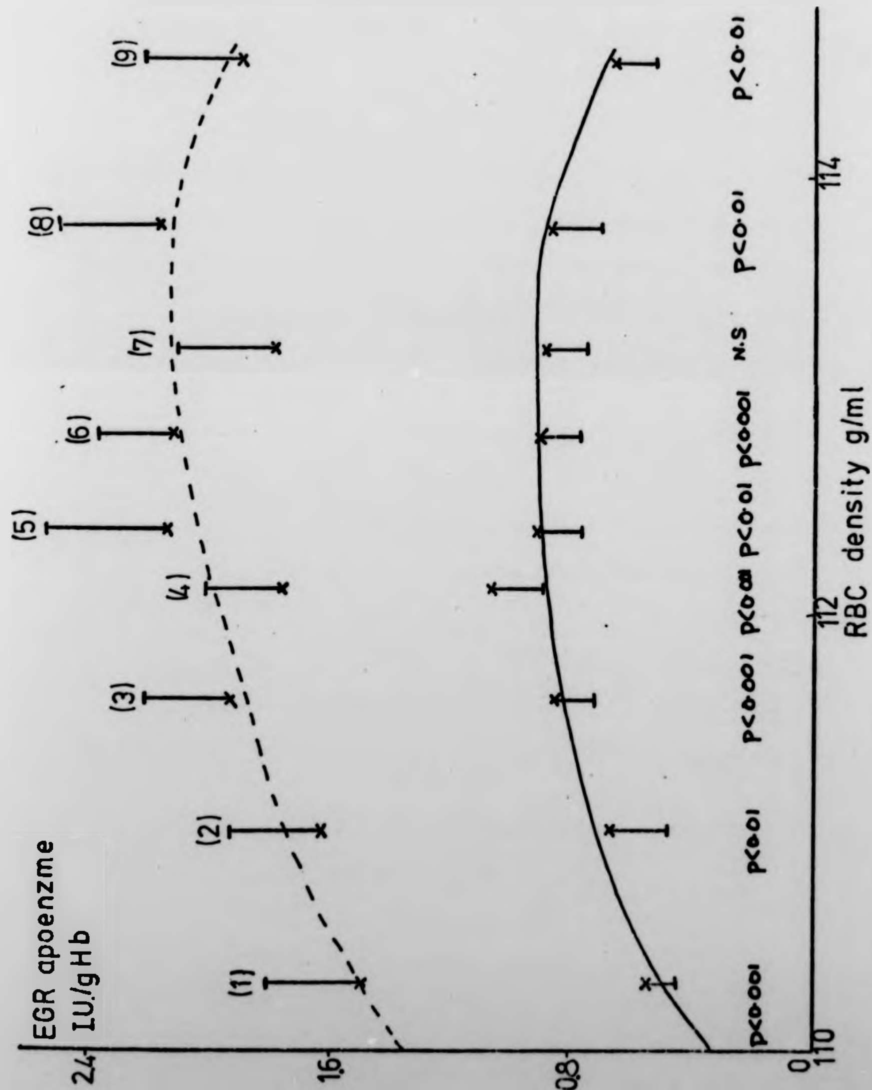


Figure 25

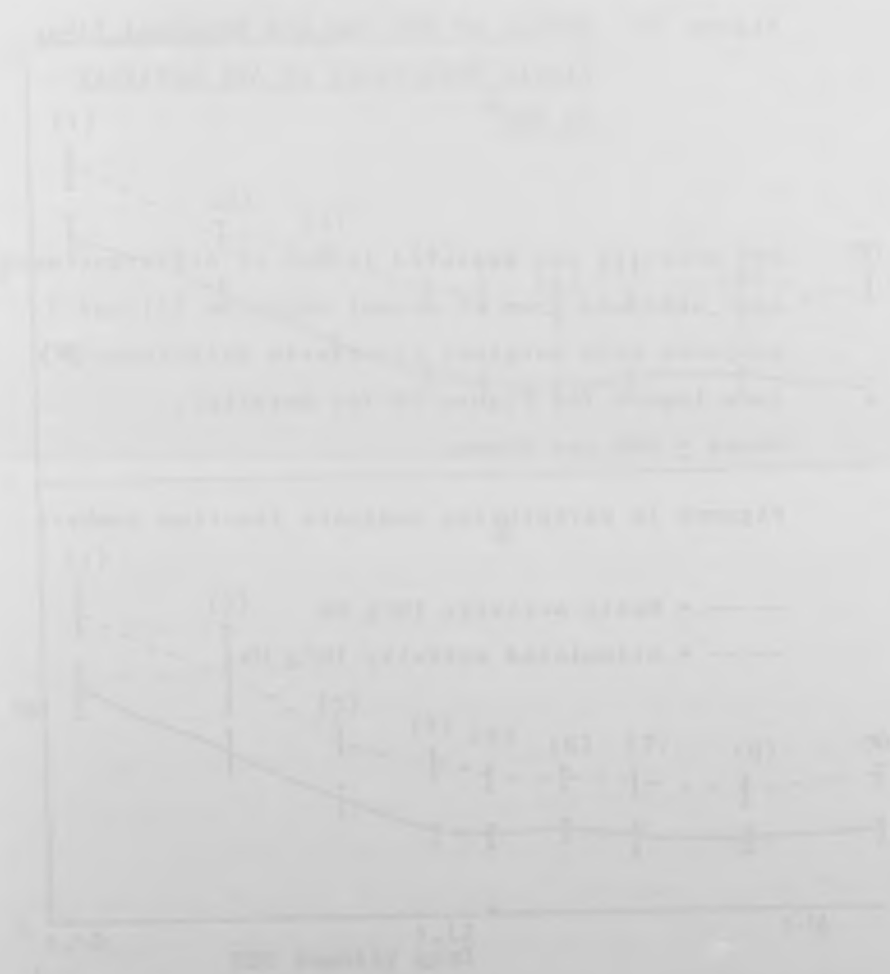


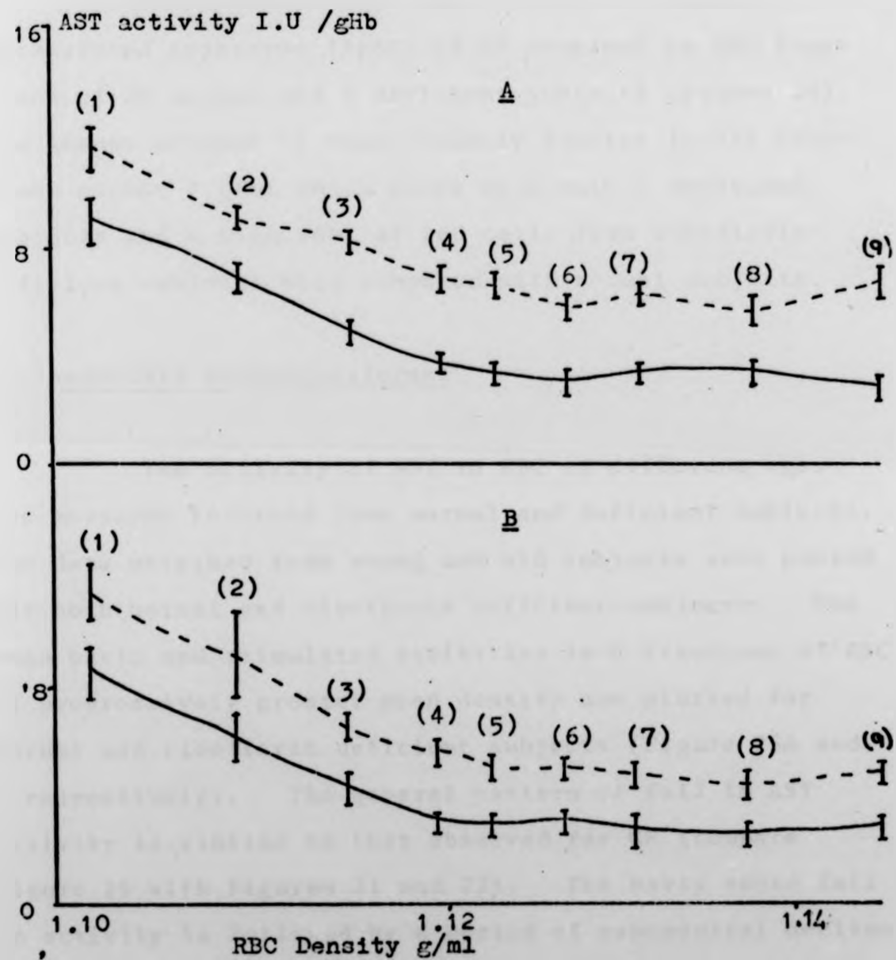
Figure 25: Effect of RBC Age and Marginal Riboflavin Deficiency on AST Activity in RBC

AST activity was measured in RBC of different mean ages obtained from 15 normal subjects (A) and 3 subjects with marginal riboflavin deficiency (B) (see legend for Figure 10 for details).
Means $\pm$ SEM are shown.

Figures in parentheses indicate fraction numbers.

——— = Basic activity IU/g Hb
----- = Stimulated activity IU/g Hb.

Figure 25 Effect of RBC Age and Marginal Riboflavin Deficiency on the activity of Aspartate Amino Transferase in RBC



fractions of RBC from normal elderly subjects over the entire range of RBC densities (Figures 23C, 23B).

A comparison was made between the amount of unsaturated apoenzyme (ApoE) of GR observed in RBC fractions of 20 normal and 9 deficient subjects (Figure 24). The amount of ApoE is significantly greater in all fractions except 7 (for which there were only 7 deficient subjects and a high SEM) of red cells from riboflavin-deficient subjects when compared with normal subjects.

ii. Aspartate Aminotransferase

The activity of AST in RBC of different ages was measured in blood from normal and deficient subjects. The data obtained from young and old subjects were pooled for both normal and riboflavin deficient subjects. The mean basic and stimulated activities in 9 fractions of RBC of progressively greater mean density are plotted for normal and riboflavin deficient subjects (Figure 25A and B respectively). The general pattern of fall in AST activity is similar to that observed for GR (compare Figure 25 with Figures 21 and 22). The early rapid fall in activity is followed by a period of exponential decline which develops into a period of constant activity in the older cells. A final small increase in activity in Fraction 9 of the riboflavin deficient subjects is not statistically significant. The fall in activity of AST

Figure 26

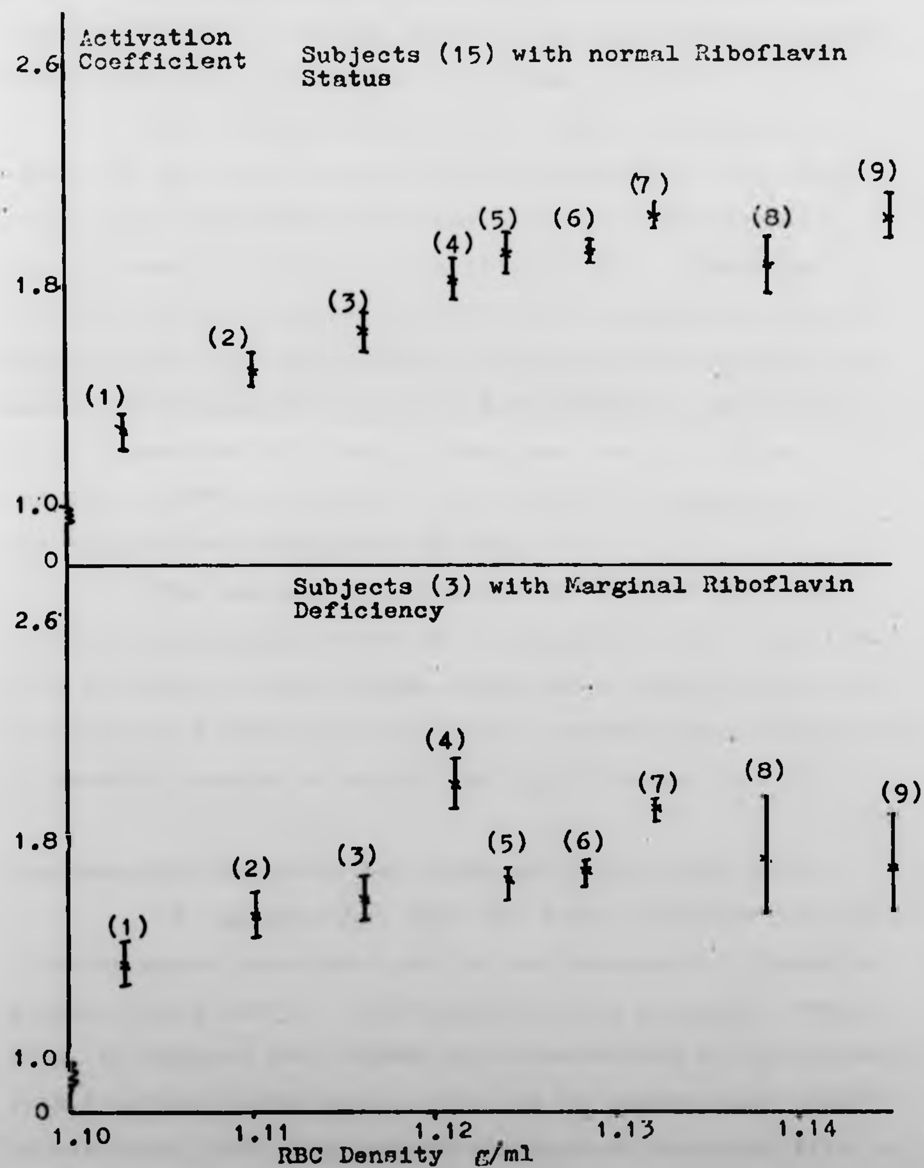
Figure 26: Effect of RBC Age and Riboflavin Status on the Activation Coefficient for AST in RBC

AST activity was measured in fractions of RBC of different mean ages obtained from 15 subjects with normal riboflavin status (A) and 3 subjects with marginal riboflavin deficiency (B) (see legend for Figure 10 for details). The activation coefficient was calculated as:

$$\frac{\text{AST activity (IU/g Hb) in the presence of added pyridoxal phosphate}}{\text{AST activity (IU/g Hb) in the absence of added pyridoxal phosphate}}$$

Means and SEM are shown. Figures in parentheses indicate fraction numbers.

Figure 26 Effect of RBC Age and Riboflavin Status on the Activation Coefficient of Aspartate Amino Transferase in RBC



between young and old cells was calculated as the ratio of the activity in Fraction 1 over that in Fraction 9. The mean value calculated for riboflavin deficient subjects is 2.44 $\pm$ 0.2 (SEM) which is not significantly different from that calculated for normal subjects.

The influence of red cell age and riboflavin status on the A.C. for AST was investigated. For both normal and riboflavin deficient subjects an increase in A.C. is seen as red cells age (Figure 26). The mean increase is less regular in RBC from riboflavin deficient subjects but this is probably attributable to the very low number of samples (3). It is interesting to note that the progressive fall in A.C. observed for EGR (Figures 17B and 23) in RBC densities > 1.13 g/ml is not apparent for the activation coefficient of AST.

The maximum A.C. reached for AST in RBC from riboflavin-deficient subjects is slightly lower than that seen in subjects with normal riboflavin status (Figure 26) but as only 3 riboflavin deficient subjects were studied it is probably unwise to attach any significance to this.

Contamination of dense RBC fractions with young cells:

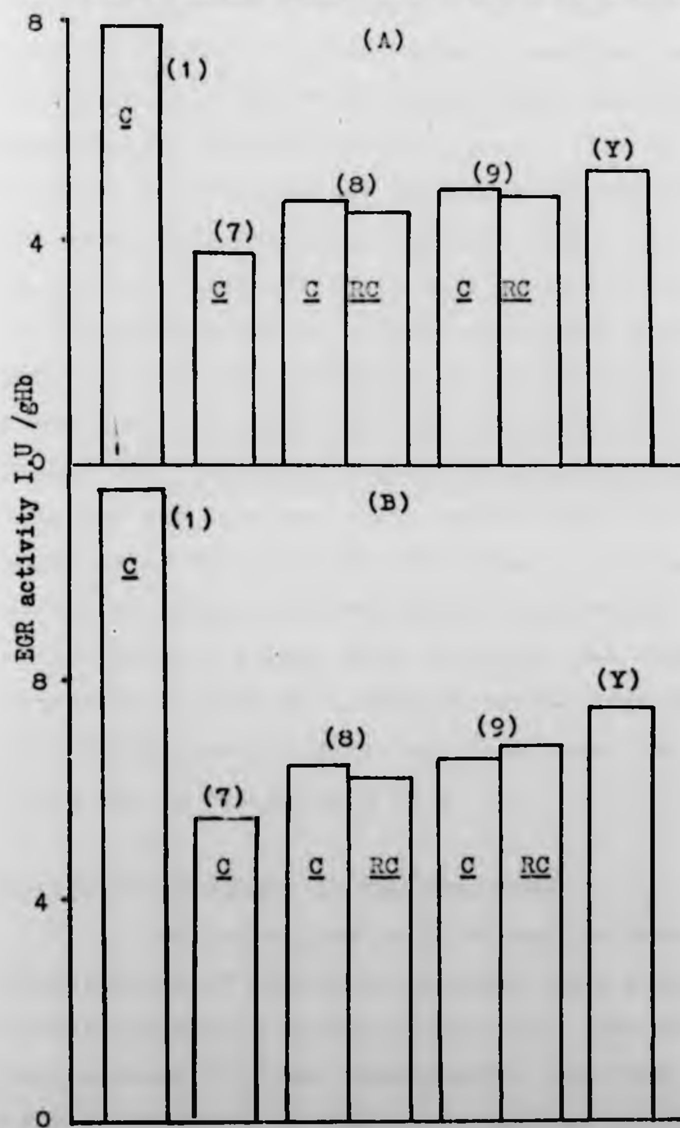
It was possible that the final increase in EGR activity might have been due to the presence of chronologically young cells. An experiment was designed, therefore, to measure the degree of contamination of the densest cell fractions with young cells and to assess what contribution such cells might make to the observed secondary rise in

Figure 27

Figure 27: <u>Effect of Removing Less Dense Cells from Fractions 8 and 9 on the GR Activity in these Fractions</u>

GR activity was measured in Fractions 7, 8 and 9 prepared in the standard way (C) and in Fractions 8 and 9 after recentrifuging (RC) to remove contaminating less dense cells (Y). The experiment was performed on RBC from 2 subjects A and B. The GR activity measured in Fraction 1 from each subject is included for comparison.

Figure 27 Effect of Removing Less Dense Cells from Fractions 8 and 9 on the activity of EGR.



EGR activity in fractions containing the dense cells. Washed samples of Fractions 8 and 9 were obtained from a gradient in the standard manner, combined, washed free of Ficoll/Triosil and recentrifuged on a fresh Ficoll/Triosil gradient for 1 hr at 65,000 g (av). The majority of cells migrated to the expected positions for Fractions 8 and 9 but some cells remained suspended higher in the gradient. No definite bands of cells were observed other than those of Fractions 8 and 9. Three fractions of cells were obtained from the gradient: Fractions 8, 9 and one prepared from all other cells on the gradient (Fraction Y, Figure 27). The EGR activity in an haemolysate of each fraction was measured and compared with the activities measured in the original fractions 1, 7, 8 and 9 (Figure 27). Recentrifugation resulted in the separation of a portion of cells having a higher mean activity than that measured in Fractions 7, 8 or 9. Removal of the less dense cells did not significantly affect the GR activity in the recentrifuged RBC in Fractions 8 or 9.

Reticulocyte counts in RBC fractions:

Reticulocytes were counted in washed packed-cell preparations of fractions obtained from 6 subjects with normal riboflavin status (Table 15). The highest reticulocyte count (4%) was consistently observed in Fraction 1, of each gradient, a smaller proportion (0.19%) appeared in Fraction 2, whereas no reticulocytes were seen in any other fraction. When expressed as a percentage of the total number of reticulocytes on the gradient Fraction 1 contained 86 $\pm$ 1.5%, Fraction 2 contained 14 $\pm$ 1.5%.

Table 15: Distribution of Reticulocytes in a Density Gradient

Fraction	Reticulocytes	
	% in Fraction	% of total reticulocytes counted
1	4.0 ± 0.75	86 ± 1.52
2	0.19 ± 0.03	14 ± 1.52
u	0.91 ± 0.09	-

The reticulocytes counted in Fractions 1 and 2 and in unfractionated RBC (u) are expressed as a percentage of the total cells in each fraction. The reticulocytes in Fractions 1 and 2 are also expressed as a percentage of the total number on the gradient. The means ± SEM of determinations made on 6 adults with normal riboflavin status are shown.

Kinetics of EGR in young and old RBC

Km NADPH

The activity of endogenous EGR in assay solutions containing different concentrations of NADPH was investigated in haemolysates prepared from young and old RBC. Figure 28 shows a typical saturation curve for GR with NADPH. The activity of GR is expressed as the fall in absorbance at 334 nm of the assay solution over 10 minutes. The activity of GR increased steadily with increasing concentrations of NADPH up to 80 μmolar above which point a plateau developed. A Lineweaver-Burk reciprocal plot of enzyme activity and NADPH concentration was constructed from the data of Figure 28 and a regression analysis of the data produced the straight line shown on Figure 29. From the gradient of this slope and the intercept on the y axis, the Km value was calculated (Methods: F.c). Table 16 shows the values for Km (NADPH) calculated for young, old, and unfractionated RBC. No difference could be detected in the values of Km (NADPH) in the 3 preparations of cells. In other words, the saturation kinetics of GR for NADPH did not appear to be influenced by the age of the RBC.

Km GSSG

Km (GSSG) was also determined in haemolysates prepared from unfractionated blood, young cells and old

Figure 28

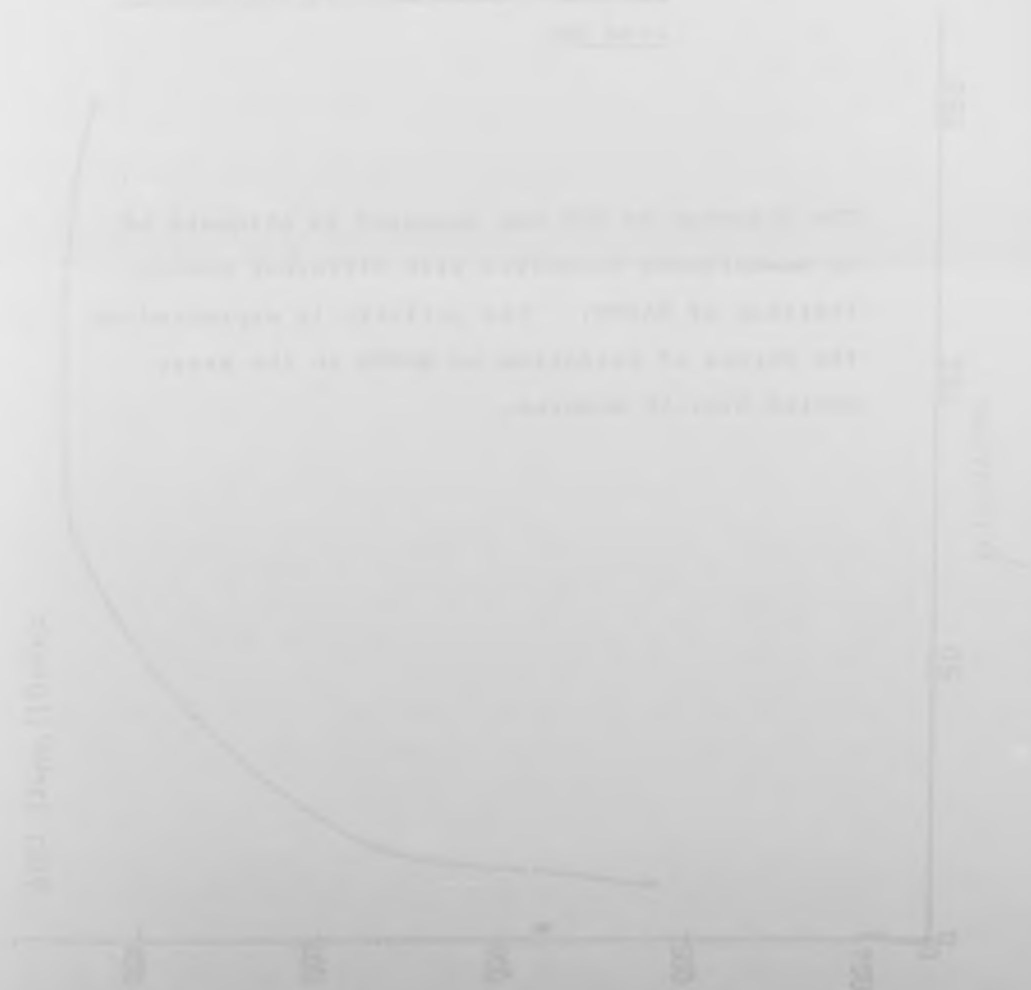


Figure 28: Activity of EGR with Different Concentrations of NADPH in Unfractionated RBC

The activity of EGR was measured in aliquots of an haemolysate incubated with different concentrations of NADPH. The activity is expressed as the degree of oxidation of NADPH in the assay system over 10 minutes.

FIGURE 28 ACTIVITY OF EGR WITH DIFFERENT CONCENTRATIONS OF NADPH: UNFRACTIONATED RBC

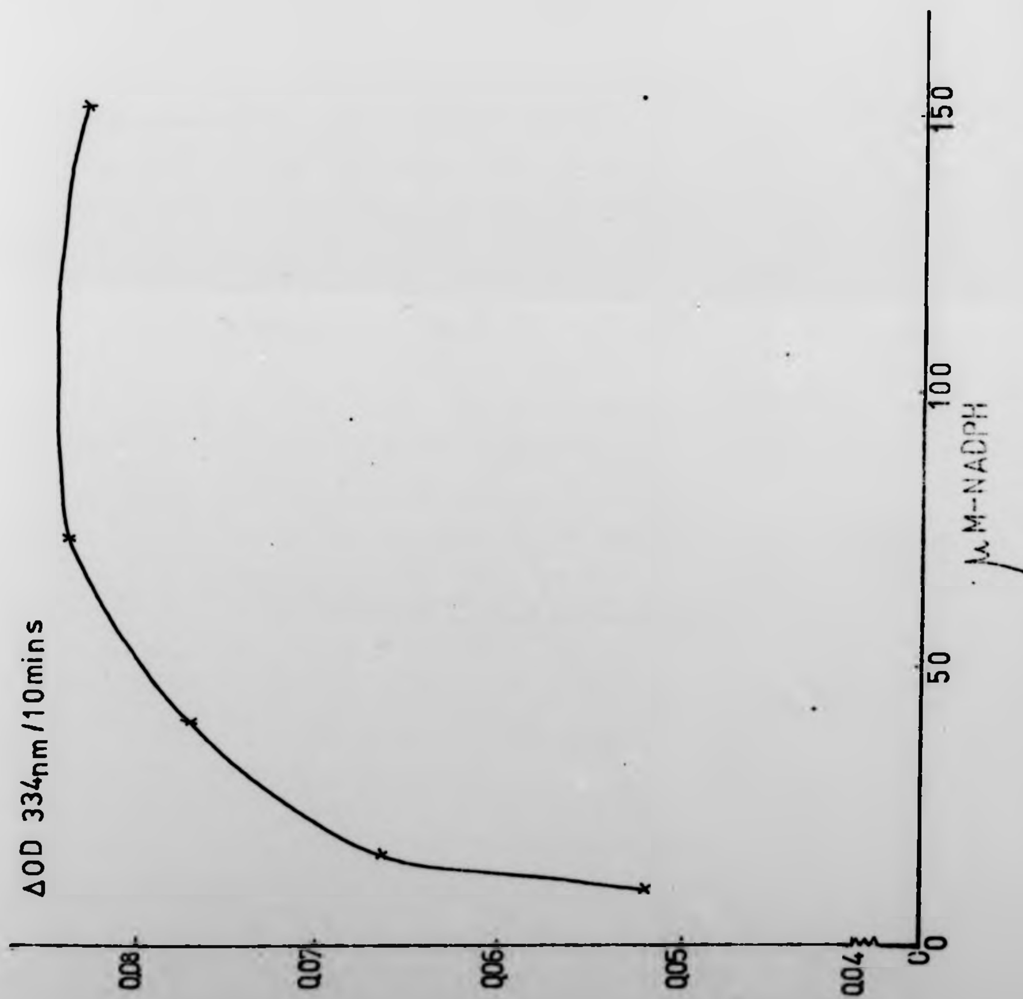


Figure 29

Figure 29: <u>Lineweaver-Burk Plot for EGR Activity with Different Concentrations of NADPH</u>

The reciprocal of the maximum enzyme activity measured over a range of substrate concentrations was plotted against the reciprocal of the concentration of NADHP in the assay system. A regression analysis gave:

b = 0.7614, a = 11.0280, r = 0.9806.

Therefore if

$$K . \frac{1}{V_{max}} = b$$

$$K \times 11.0280 = 0.7614 \times 10^{-4}$$

and $K_m = 6.9 \times 10^{-6}$ M-NADPH.

FIGURE 29 LINEWEAVER-BURKE PLOT FOR EGR ACTIVITY AT VARYING CONCENTRATIONS OF NADPH

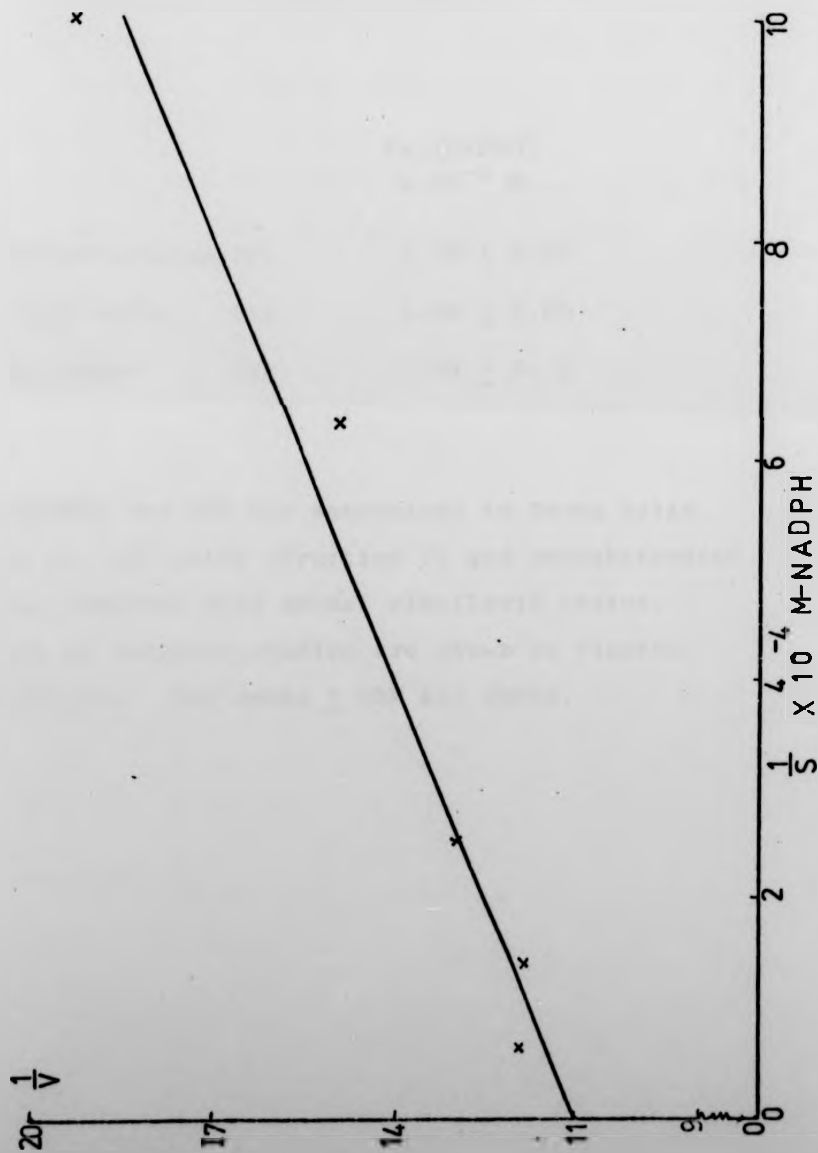


Table 16: Influence of RBC Age on the Saturation Kinetics for NADPH of Unresolved EGR

		Km (NADPH) $\times 10^{-6}$ M
Unfractionated	(9)	4.29 ± 0.63
Young cells	(5)	4.30 ± 0.80
Old cells	(5)	4.04 ± 0.78

The Km (NADPH) for EGR was determined in young cells (Fraction 1), old cells (Fraction 7) and unfractionated cells from subjects with normal riboflavin status. The number of subjects studied are shown as figures in parentheses. The means ± SEM are shown.

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cells. Figure 30 shows a typical saturation curve for GR activity with different concentrations of GSSG. The activity of GR rose steadily up to a concentration of about 0.8 mM-GSSG, above which a plateau was observed. The corresponding Lineweaver-Burk reciprocal plot is shown in Figure 31. The Km (GSSG) did not appear to be influenced by RBC age (Table 17), implying that the saturation kinetics of unresolved GR for its substrate GSSG remains constant during the ageing of the RBC.

Figure 30

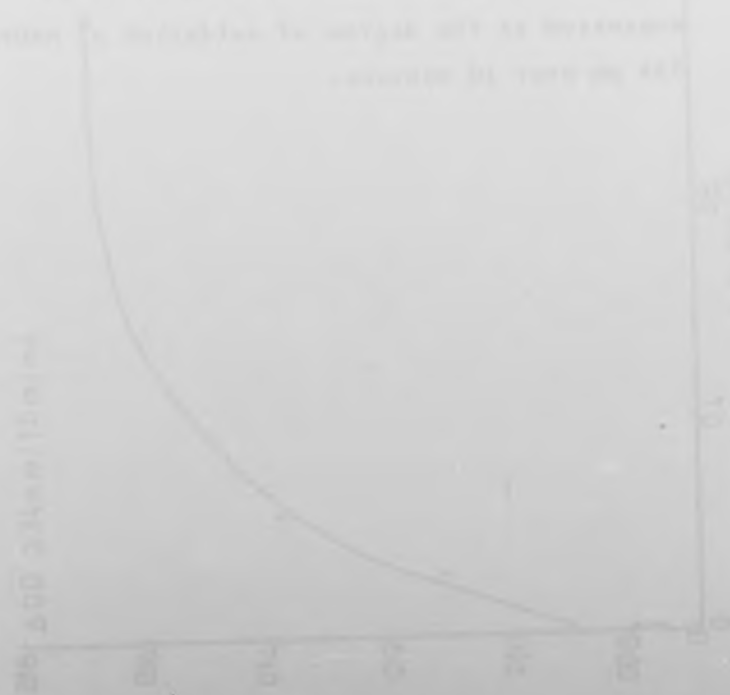

Figure 30: Activity of EGR with Different Concentrations of GSSG in Unfractionated RBC

GR activity was measured in aliquots of an haemolysate incubated with different concentrations of GSSG. The activity of GR is expressed as the degree of oxidation of NADPH at 334 nm over 10 minutes.

FIGURE 30 ACTIVITY OF EGR WITH DIFFERENT CONCENTRATIONS OF GSSG: UNFRACTIONATED RBC

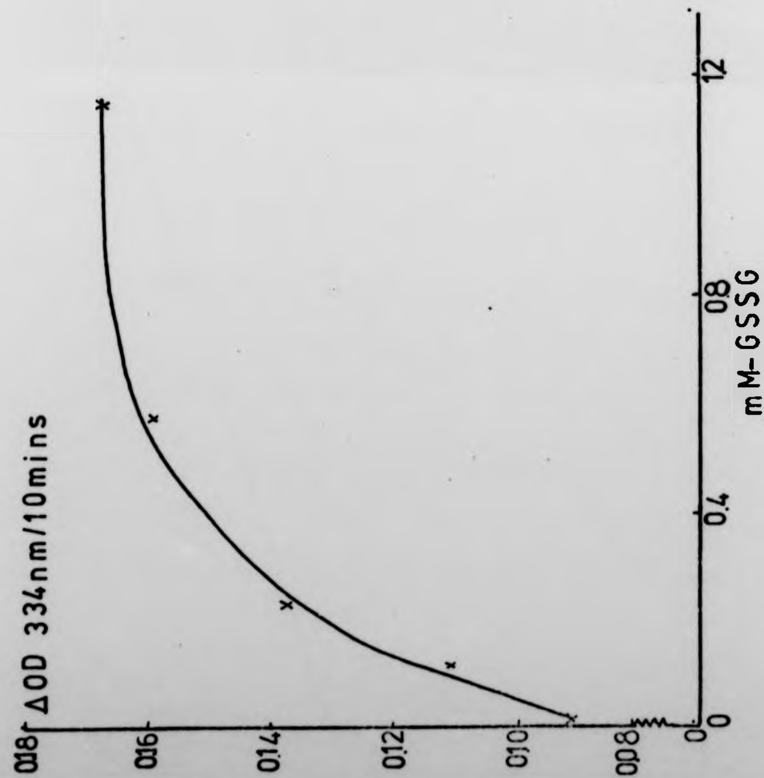

Figure 31

Figure 31: <u>Lineweaver-Burk Plot for Activity of EGR with Varying Concentrations of GSSG</u>

The figure was constructed as described for Figure 29, using GSSG concentrations. A regression analysis gave:

$b = 0.2800, \quad a = 5.9212, \quad r = 0.9944.$

Therefore, if $K.\frac{1}{V_{max}} = b$

$$K \times 5.9212 = 0.2800 \times 10^{-3}$$

and $\underline{K_m = 4.73 \times 10^{-5}\ \text{M-GSSG.}}$

FIGURE 31 LINEWEAVER—BURKE PLOT FOR ACTIVITY OF EGR WITH VARYING CONCENTRATIONS OF GSSG: UNFRACTIONATED RBC

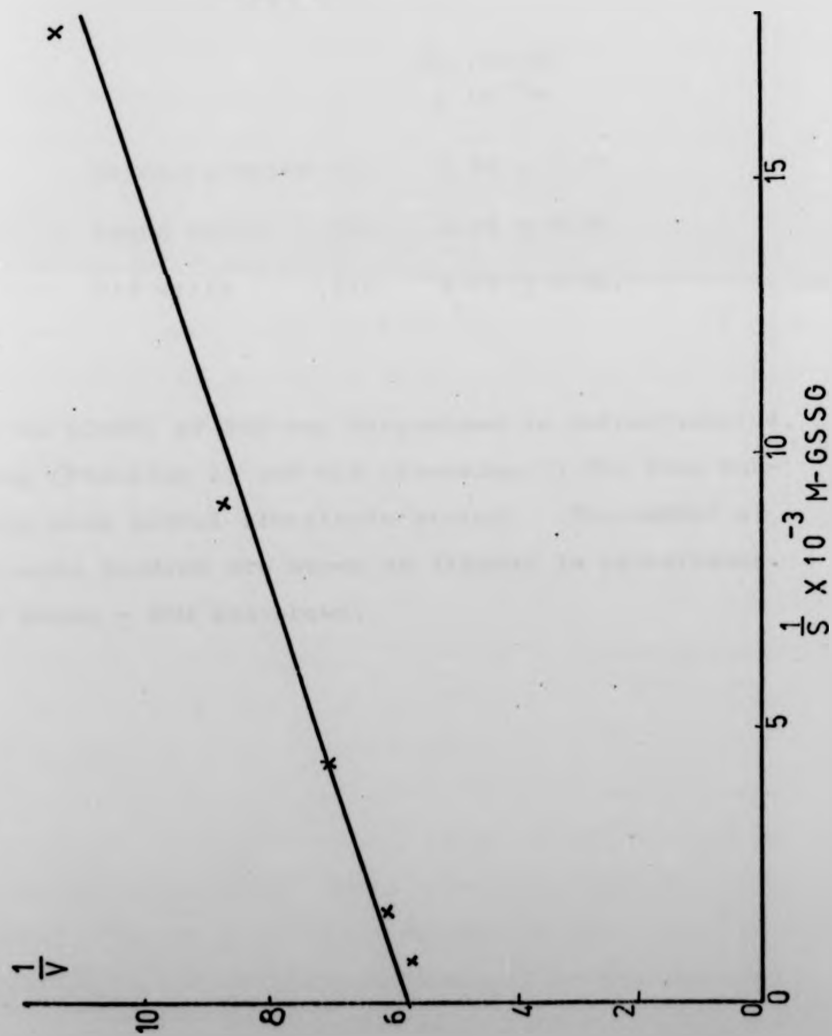

Table 17: Effect of RBC Age on the Saturation Kinetics for GSSG of Unresolved EGR

		Km (GSSG) x 10^{-5}M
Unfractionated	(7)	5.33 ± 0.77
Young cells	(5)	5.44 ± 0.94
Old cells	(5)	5.02 ± 0.80

The Km (GSSG) of EGR was determined in unfractionated, young (Fraction 1) and old (Fraction 7) RBC from subjects with normal riboflavin status. The number of subjects studied are shown as figures in parentheses. The means ± SEM are shown.

C. Distribution of Red Blood Cells According to their Age

A sample of whole blood from a human subject consists of a heterogeneous population of RBC. The proportions of cells of different ages within a blood sample can be illustrated by a RBC distribution profile through a density gradient.

Preliminary studies of RBC separated on a density gradient of 5 solutions indicated that marginally riboflaving-deficient subjects had proportionately fewer old cells than normal subjects (Figure 32). An analysis of the difference in the proportion of Hb in equivalent fractions from the two groups of subjects indicated that, despite the low numbers of samples Fractions 1, 2 and 4 were significantly different such that blood from riboflavin-deficient subjects had proportionally more young cells and fewer old cells than blood from normal subjects (t-test: $p < 0.05$ for Fractions 1, 2 and 4).

A more precise study of the RBC distribution profiles was made on erythrocytes separated on a density gradient of 9 solutions. There was a consistent trend towards a reduction in the proportion of old cells with a concomitant increase in the proportion of young cells in blood from riboflavin-deficient subjects when compared with the corresponding normal controls (Figures 33 and 34). The effect of marginal riboflavin deficiency was most clearly seen among elderly subjects where a significant reduction in the proportion of old cells was observed

Figure 32

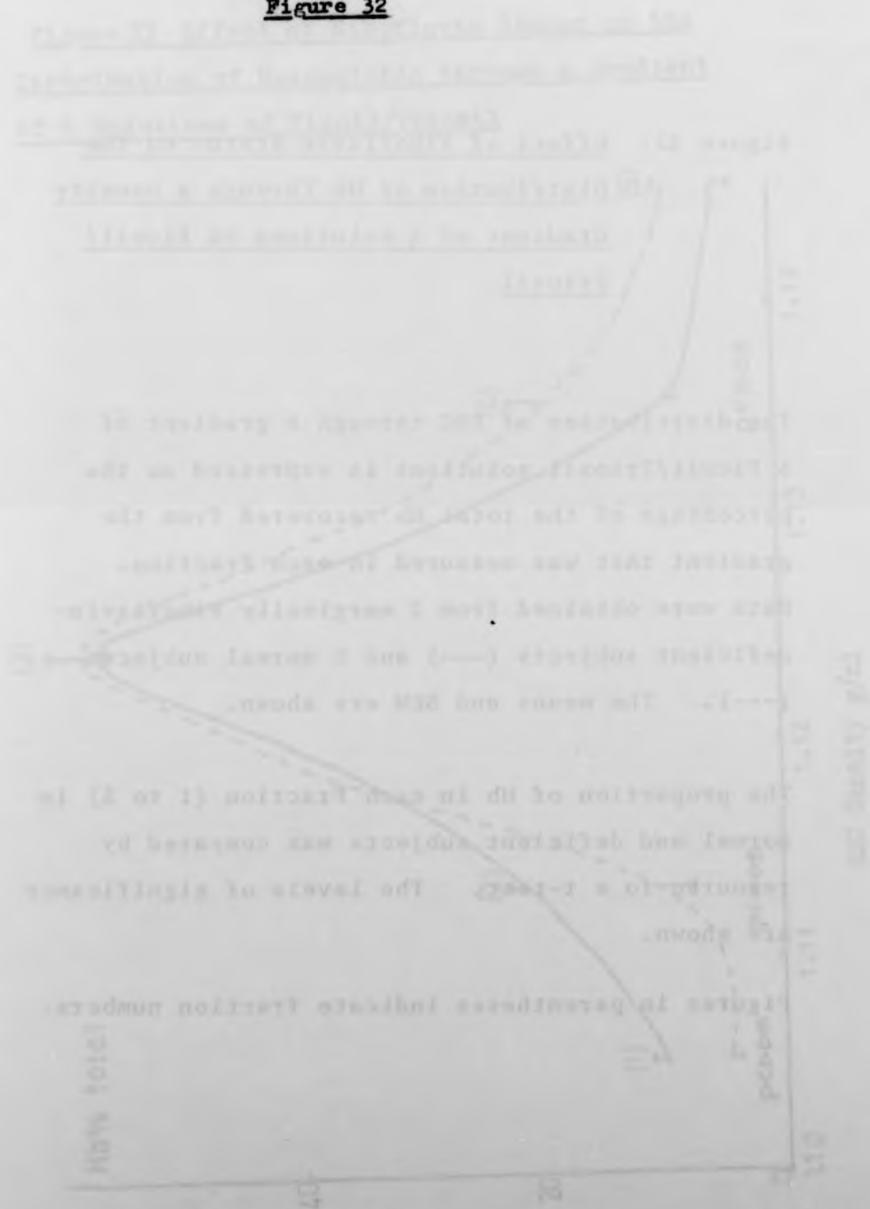

Figure 32: Effect of Riboflavin Status on the Distribution of Hb Through a Density Gradient of 5 solutions of Ficoll/ Triosil

The distribution of RBC through a gradient of 5 Ficoll/Triosil solutions is expressed as the percentage of the total Hb recovered from the gradient that was measured in each fraction. Data were obtained from 2 marginally riboflavin-deficient subjects (——) and 9 normal subjects (---). The means and SEM are shown.

The proportion of Hb in each Fraction (1 to 5) in normal and deficient subjects was compared by recourse to a t-test. The levels of significance are shown.

Figures in parentheses indicate fraction numbers.

Figure 32 Effect of Riboflavin Status on the Distribution of Haemoglobin through a gradient of 5 Solutions of Ficoll/Triosil

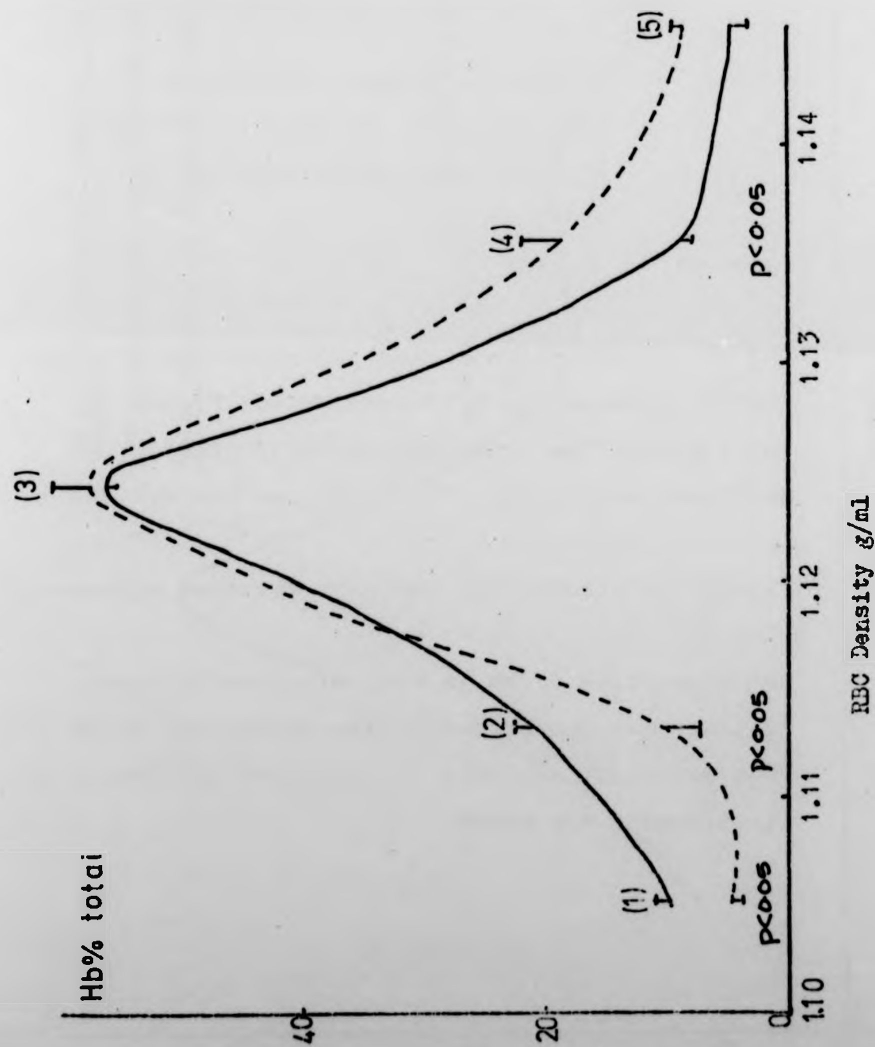

Figure 33

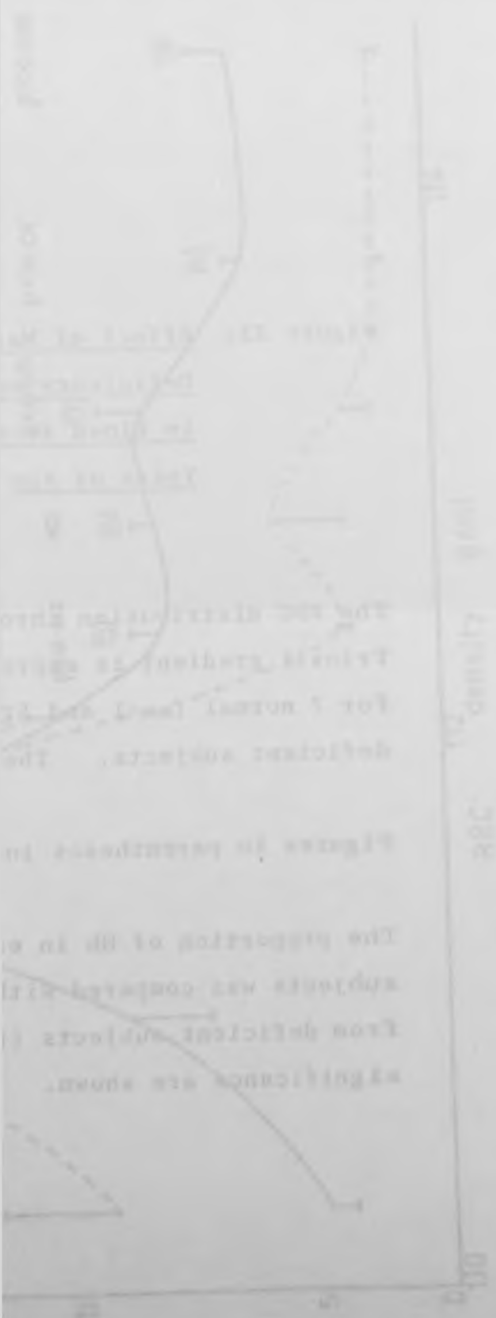

Figure 23: Effect of Mg

Deficiency

in blood

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for 7 normal (---) and (

deficient subjects. The

Figures in parentheses in

The proportion of Hb in e

subjects was compared with

from deficient subjects (

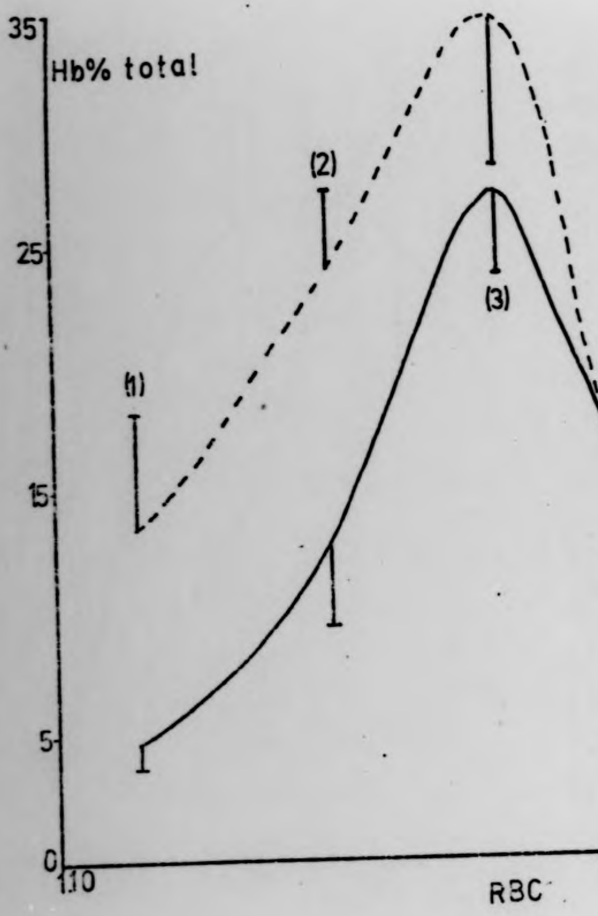
significance are shown.

Figure 33: Effect of Marginal Riboflavin Deficiency on the RBC Distribution in Blood from Human Subjects ≥ 65 Years of Age

The RBC distribution through a 9-solution Ficoll/ Triosil gradient is expressed as for Figure 32, for 7 normal (---) and 5 marginally riboflavin deficient subjects. The means and SEM are shown.

Figures in parentheses indicate fraction numbers.

The proportion of Hb in each fraction in normal subjects was compared with the equivalent value from deficient subjects (t-test) and the levels of significance are shown.



35
Hb% total
(2)
25
(3)
(1)
15
5
0
1.10
RBC

Figure 33 Effect of Marginal Riboflavin Deficiency on RBC Distribution in Human Subjects ≥ 65 years of age.

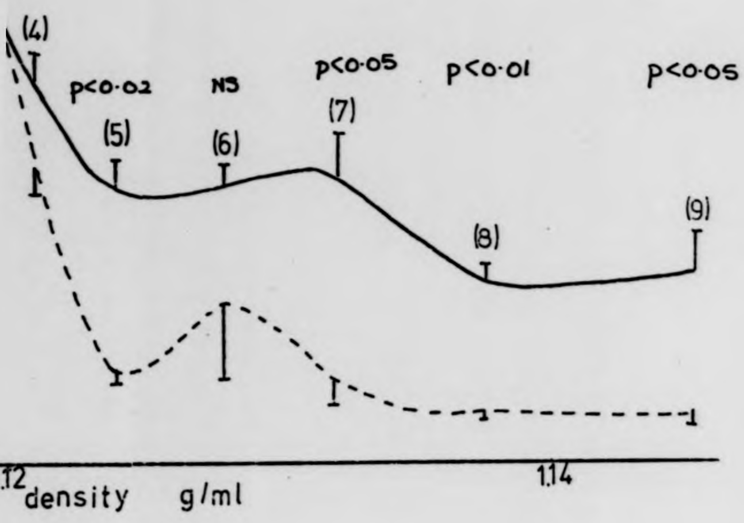


Figure 34

Figure 34: Effect of Marginal Riboflavin Deficiency on RBC Distribution in Blood from Adults < 65 Years of Age

The distribution of RBC through a 9-solution Ficoll/Triosil gradient is expressed as for Figure 32, for 11 normal subjects (---) and 4 marginally riboflavin deficient subjects (——). The means and SEM are shown.

The figures in parentheses indicate fraction numbers.

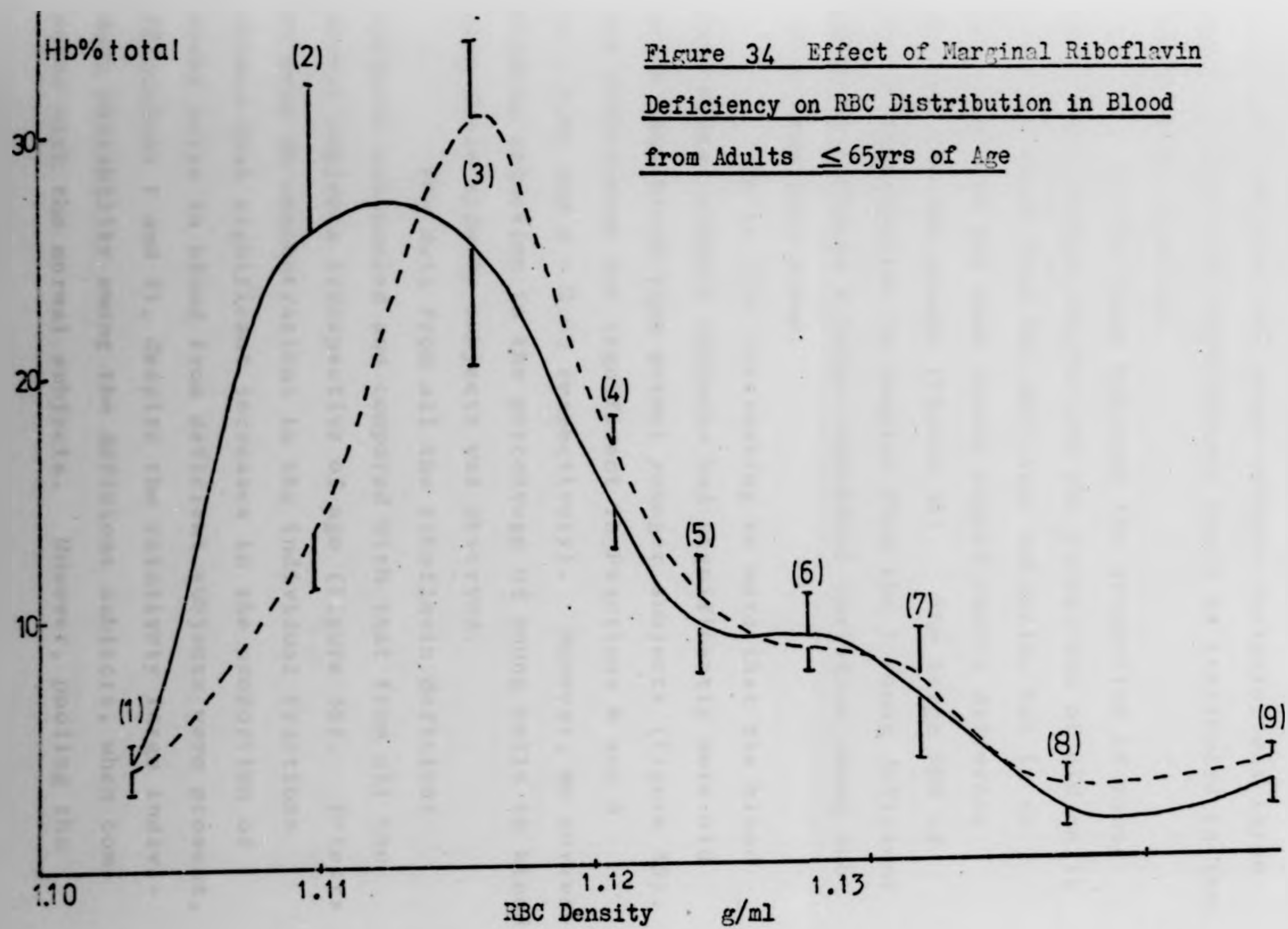


Figure 34 Effect of Marginal Riboflavin Deficiency on RBC Distribution in Blood from Adults ≤ 65yrs of Age

(t-test: Figure 33). Although there appeared to be a greater proportion of young cells in blood from the elderly deficient subjects the inter-subject variation was large and no statistical significance could be attributed to the differences observed.

In the young subjects the proportion of young cells was likewise higher and the proportion of old cells lower in blood from the deficient subjects, but in no fraction were the mean values significantly different between the two groups (Figure 34). The large SEM of the Hb distribution in samples from the 4 young deficient subjects reflects a large individual variation among subjects from this group.

It is also interesting to note that the blood from normal elderly subjects had significantly more old cells than blood from normal younger subjects (Figure 35). The differences are significant in Fractions 8 and 9 ($p < 0.01$ and $p < 0.05$ respectively). However, no corresponding reduction in the percentage of young cells in blood from these elderly subjects was observed.

The data from all the riboflavin deficient subjects was pooled and compared with that from all the normal subjects irrespective of age (Figure 36). T-tests on mean Hb concentrations in the individual fractions showed that significant increases in the proportion of young cells in blood from deficient subjects were present, (Fractions 1 and 2), despite the relatively large individual variability among the deficient subjects, when compared with the normal subjects. However, pooling the

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Figure 35

Figure 35: <u>The Influence of Human Age on RBC Distribution in Blood</u>

The RBC distribution through a 9-solution Ficoll/Triosil gradient is expressed as for Figure 32, for 11 normal subjects < 65 years of age (---) and 7 normal subjects ≥ 65 years of age (——). Means and SEM are shown.

Numbers in parentheses indicate fraction numbers.

The proportion of Hb in each fraction from subjects ≥ 65 years of age was compared with that in fractions from subjects < 65 years of age (t-test) and the levels of significance are shown.

Figure 35 The Influence of Human Age on RBC distribution in Blood.

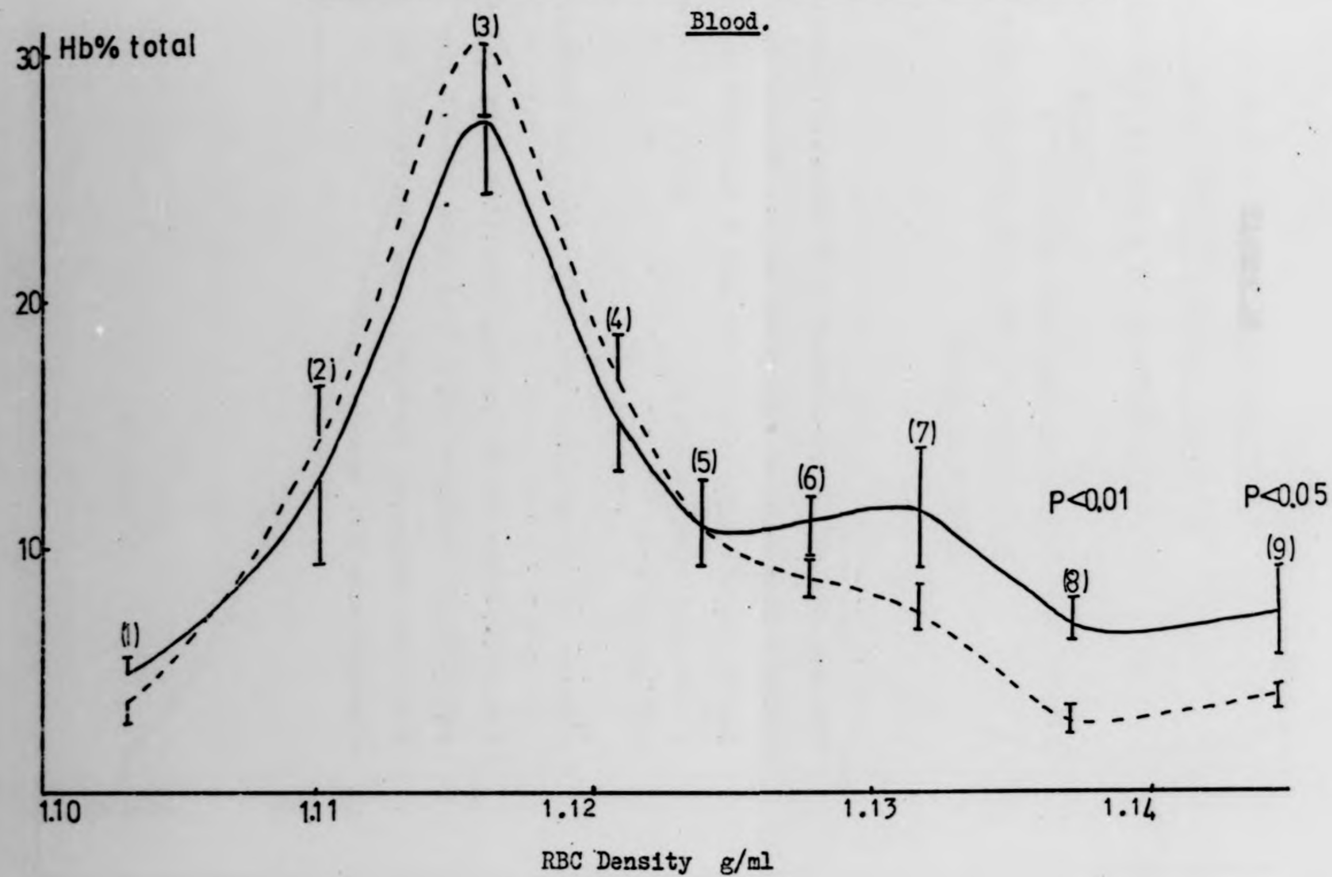


Figure 36

Figure 36: Effect of Marginal Riboflavin Deficiency on RBC Distribution in a Sample of Blood

The RBC distribution through a 9-solution Ficoll/ Triosil gradient is expressed as for Figure 32, for 20 normal subjects (---) and 9 marginally riboflavin deficient subjects (——). Means and SEM are shown.

Figures in parentheses indicate fraction numbers.

The proportion of Hb in each fraction from normal subjects is compared with that from riboflavin deficient subjects (t-test), and the levels of significance are shown.

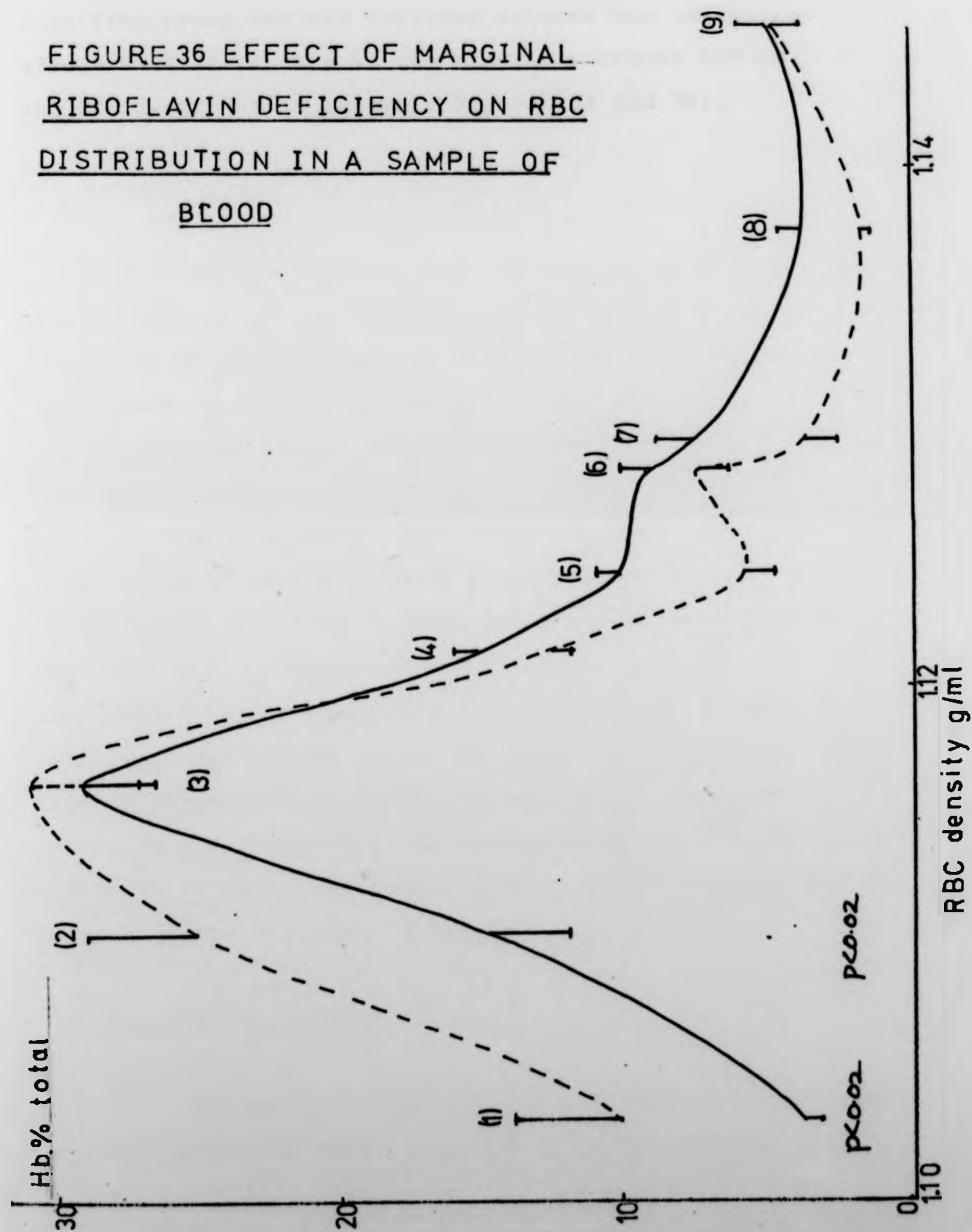


FIGURE 36 EFFECT OF MARGINAL RIBOFLAVIN DEFICIENCY ON RBC DISTRIBUTION IN A SAMPLE OF BLOOD

data from young and old subjects diluted out the marked effect that deficiency in the elderly subjects had on the RBC distribution (compare Figures 33 and 36).

D. Effect of Marginal Riboflavin Deficiency on Some Biochemical and Haematological Indices in Unfractionated Blood of Human Subjects

i. Haematological indices

The mean packed cell volumes of whole blood samples taken from 22 normal subjects was 41.2% with a standard error of 0.71% in comparison with 39.9 $\pm$ 1.08% in 7 marginally riboflavin-deficient subjects. Thus there appeared to be no significant effect of marginal riboflavin deficiency on the PCV (t-test).

As marginally riboflavin-deficient subjects were found to have a reduced proportion of old cells in their blood (Figures 33 and 34), reticulocyte counts were made on whole blood samples from 9 marginally riboflavin-deficient subjects and from 15 subjects with normal riboflavin status (Table 18). All reticulocyte counts fell within the normal range (Dacie and Lewis, 1963) and no significant difference was observed between the two groups of subjects (t-test), although the mean count was slightly higher in the deficient subjects.

ii. Biochemical indices

As GSH is believed to play a role as a protector against oxidation within the RBC (Mills and Randall, 1958;

Table 18: Reticulocyte Concentrations in Whole Blood Samples from Normal and Riboflavin-Deficient Subjects

	Reticulocytes % total cells	
	Normal Subjects	Marginally Riboflavin-Deficient Subjects
	(15)	(9)
Mean ± SEM	0.41 ± 0.04	0.45 ± 0.09
Range	0.20 - 0.79	0.20 - 1.06

The figures in parentheses indicate the number of subjects investigated.

Table 19: Effect of Marginal Riboflavin Deficiency on GSH Concentrations in RBC

	GSH µmoles/g Hb	
	Normal Subjects (13)	Marginally Riboflavin Deficient Subjects (5)
Mean ± SEM	7.54 ± 0.57	6.21 ± 0.68
Range	3.5 - 10.2	3.4 - 7.7

Figures in parentheses indicate the number of subjects investigated.

Benesch and Benesch, 1954), it was considered relevant to this study to investigate (i) GSH concentration *per se*, (ii) lipid oxidation, and (iii) Hb oxidation in RBC from normal and marginally riboflavin-deficient subjects. A later section (Results: Section F) will describe investigations into the extent of lipid oxidation, data presented here describe the effects of riboflavin deficiency on GSH concentrations and the relative concentrations of Hb derivatives in unfractionated RBC.

GSH concentrations in unfractionated RBC:

GSH concentrations were determined in samples of washed packed RBC from normal and deficient subjects. The concentrations, expressed as μmoles/g Hb, were not significantly different in the two groups of subjects (Table 19), although the RBC of marginally deficient subjects had a lower mean GSH concentration than the RBC of normal subjects.

Haemoglobin derivatives in unfractionated RBC:

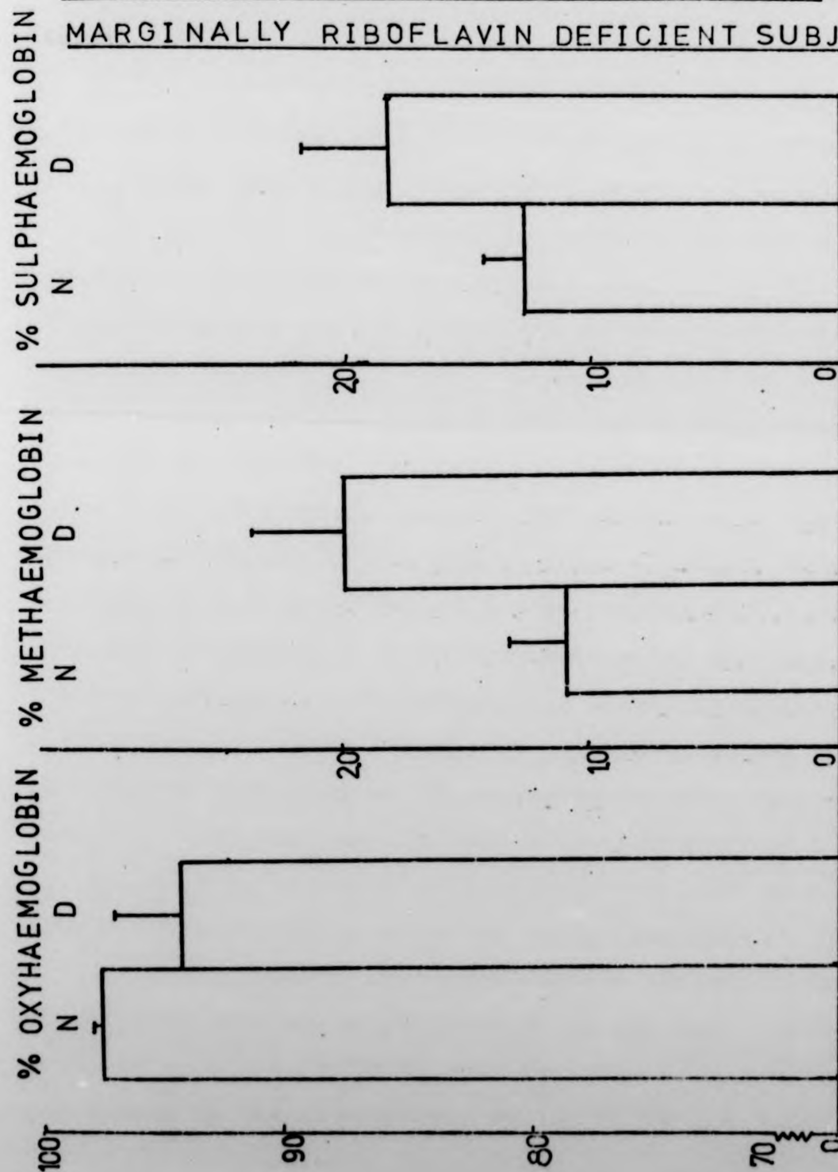
The concentrations of MHb, OxyHb, and SHb in whole blood samples were determined in a number of normal and marginally riboflavin-deficient subjects. The concentrations were expressed as a percentage of the total Hb measured in a sample (Figure 37). In all subjects the methaemoglobin and sulphaemoglobin were present in concentrations of less than 3% in the blood. There was a tendency for marginally riboflavin-deficient subjects to have

Figure 37

Figure 37: <u>Haemoglobin Derivatives as a Percentage of the Total Hb in Whole Blood Samples from Normal and Marginally Riboflavin-Deficient Subjects</u>

The amount of methaemoglobin, sulphaemoglobin and oxyhaemoglobin was measured and expressed as a percentage of the total haemoglobin in a whole blood sample from normal (N) and marginally riboflavin-deficient subjects (D). The means and standard errors for 12 normal subjects and 5 riboflavin-deficient subjects are shown.

FIGURE 37 HAEMOGLOBIN DERIVATIVES IN WHOLE BLOOD SAMPLES FROM NORMAL AND MARGINALLY RIBOFLAVIN DEFICIENT SUBJECTS



slightly more MHb and SHb in their blood, and consequently less OxyHb than normal subjects, nevertheless the differences were not consistent and not statistically significant (t-test).

E. Biochemical and Haematological Changes in RBC as they Age

The fact that the density of the RBC increases as the cell ages has been used as the basis for separation of cells into fractions of different mean ages (Rigas and Koler, 1961b; Danon and Marikovsky, 1964). The Hb content of the human erythrocyte is reported to remain unchanged during ageing of the cell (Murphy, 1973) and RBC distribution studies described earlier (Results: Section B) support this view. RBC separated into fractions of progressively greater mean age would therefore be expected to show some increase in the MCHC if the separation had a truly chronological basis and caused no permanent density alteration in the RBC. Table 20 shows data obtained from 4 fractions of RBC of progressively greater mean age (prepared as described in Results: Section F) in 5 normal subjects. The RBC in the oldest cell fraction, which comprised about 18% of the total RBC, shows a MCHC 121% higher than that of the youngest 15% of cells (Fraction 7). The increase in MCHC between Fractions I and IV was found to be highly significant ($p < 0.001$) when the data were subjected to a paired t-test analysis.

Experiments described earlier (Results: Section C) established that GR activity falls as RBC age. GSH, produced as a result of GR activity, has been ascribed roles both in the maintenance of the Hb in its functional

Table 20: MCHC in RBC of Progressively Increasing Age

Fraction	MCHC	
1	32.1 ± 0.37	
2	34.9 ± 0.85	
3	34.7 ± 0.83	$P < 0.001$
4	38.8 ± 1.32	
Unfractionated	33.6 ± 0.71	

The means ± SEM are given for data obtained from 5 normal subjects.

MCHC (%) was calculated as Hb g%/PCV.

The mean values in Fraction 1 (young cells) and Fraction 4 (old cells) were compared by a paired t-test analysis and the level of significance calculated is shown in the table.

Table 21: Effect of RBC Age on GSH Concentrations in Normal and Marginally Riboflavin-Deficient Subjects

Fraction	RBC Density g/ml	Normal subjects (9)	Marginally riboflavin deficient subjects (5)
1	1.103	9.00 ± 0.79	7.24 ± 0.35
2	1.110	6.27 ± 0.57	6.50 ± 0.55
3	1.116	5.74 ± 0.45	6.34 ± 0.43
4	1.121	5.32 ± 0.35	5.94 ± 0.43
5	1.124	5.10 ± 0.48	5.50 ± 0.39
6	1.128	5.03 ± 0.54	5.12 ± 0.42
7	1.132	4.37 ± 0.54	3.70 ± 0.57
8	1.1375	3.73 ± 0.44	3.58 ± 0.33
9	1.145	3.64 ± 0.46	3.0 ± 0.23

Figures in parentheses show the number of subjects studied.

The Means ± SEM are given.

GSH concentrations are expressed as µmoles/g Hb.

The Mean EGR A.C. of normal subjects was 1.18 ± 0.02

and of deficient subjects 1.41 ± 0.04.

Figure 38

Figure 38 Relation Between RBC Density and GSH concentration in Blood from Normal and Marginally Riboflavin-Deficient Subjects

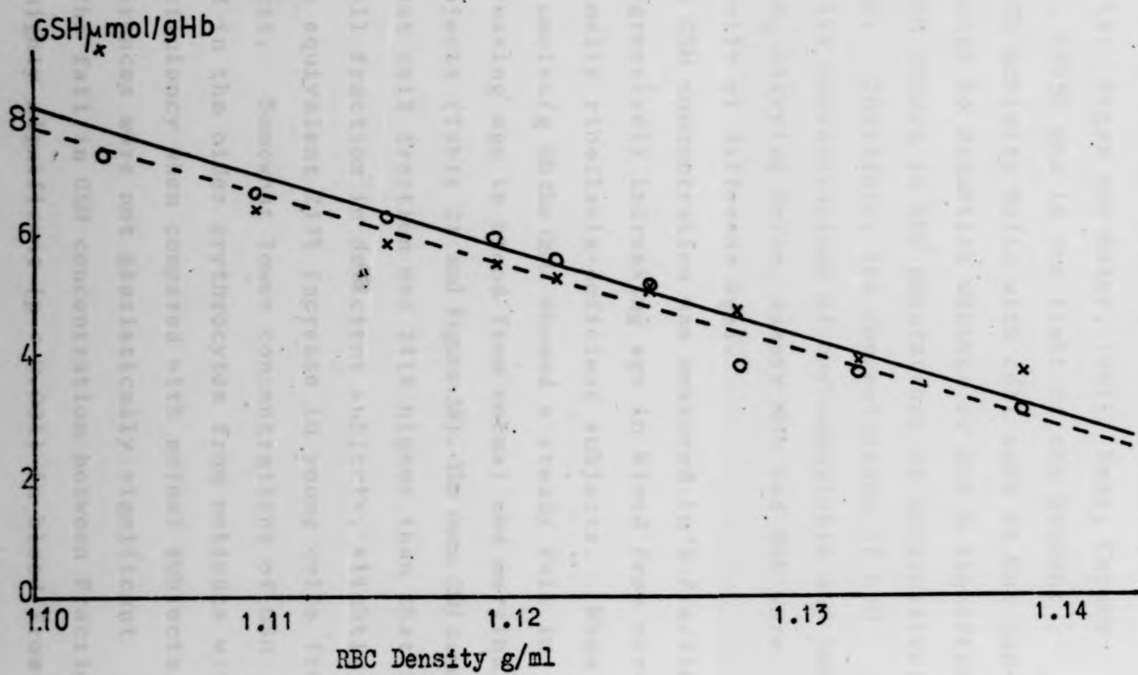


Figure 38: Relation Between RBC Density and GSH Concentration in Blood from Normal and Marginally Riboflavin-Deficient Subjects

GSH was measured in fractions of RBC of different ages in blood from 9 normal subjects (xxx——) and 5 riboflavin-deficient subjects (ooo---). Regression analysis was performed on both sets of data:

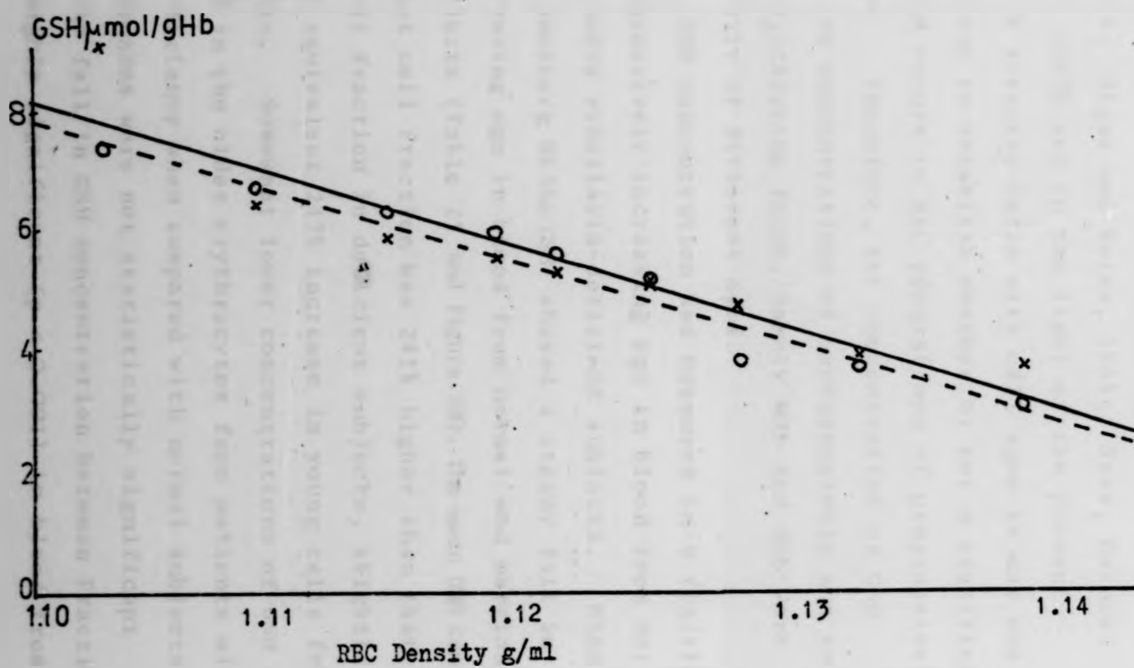
Normal subjects:

GSH μmoles/g Hb = RBC density g/ml (-112.84)+132.19
  $r = -0.926$, $p = 0.0003$.

Riboflavin-deficient subjects:

GSH μmoles/g Hb = RBC density g/ml (-108.11)+126.73
  $r = -0.971$, $p = < 0.0001$.

Figure 38 Relation Between RBC Density and GSH concentration in Blood from Normal and Marginally Riboflavin-Deficient Subjects



oxygen-carrying state (Jaffe and Neumann, 1968; Allen and Jandl, 1961). Reports concerning the effect of RBC age on GSH concentrations have not been consistent (Prankerd, 1958; Rigas and Koler, 1961; Sass, Caruso and O'Connell, 1965) and in the light of the present finding that GR activity falls with cell ages it was considered necessary to establish whether or not a significant decrease in GSH occurs in RBC populations of progressively increasing age. Therefore, the concentration of GSH and the relative concentrations of oxyhaemoglobin and two oxidised non O_2-carrying forms, namely MHb and SHb were measured in cells of different ages.

The GSH concentration was measured in 9 fractions of RBC of progressively increasing age in blood from normal and marginally riboflavin-deficient subjects. When expressed as μmoles/g Hb the GSH showed a steady fall in cells of increasing age in blood from normal and marginally deficient subjects (Table 21 and Figure 38). The mean GSH concentration in the youngest cell fraction was 241% higher than that in the oldest cell fraction in deficient subjects, slightly less than the equivalent 247% increase in young cells from normal subjects. Somewhat lower concentrations of GSH were measured in the older erythrocytes from patients with riboflavin deficiency when compared with normal subjects, but the differences were not statistically significant (t-test). The fall in GSH concentration between Fractions 1 and 9 was highly significant ($p < 0.001$) in blood from

Figure 39

Figure 39: <u>Relation Between RBC Age and Relative Concentrations of Hb Derivatives in Blood From Normal and Marginally Riboflavin-Deficient Subjects</u>

The relative concentrations of MHb, SHb, and OxyHb in RBC are expressed as a percentage of the total Hb measured. Regression analyses were performed on the Hb data and RBC density. The regression equations are as follows:

Normal subjects (xxx——):

A: %OxyHb = RBC density g/ml (-305.36) + 435.92
$r = -0.847$, $p = 0.0040$

B: %MHb = RBC density g/ml (180.12) - 198.471
$r = 0.881$, $p = 0.0017$

C: %SHb = RBC density g/ml (132.68) - 145.76
$r = 0.787$, $p = 0.012$

Marginally riboflavin-deficient subjects (ooo---):

A: %OxyHb = RBC density g/ml (-426.72) + 569.16
$r = -0.934$, $p = 0.0020$

b: %MHb = RBC density g/ml (365.87) - 404.24
$r = 0.943$, $p = 0.0001$

C: %SHb = RBC density g/ml (121.25) - 132.41
$r = 0.873$, $p = 0.0021$

Figure 39 Relation Between RBC Age and concentrations of Haemoglobin Derivatives in Blood from Normal and Marginally Riboflavin Deficient Subjects

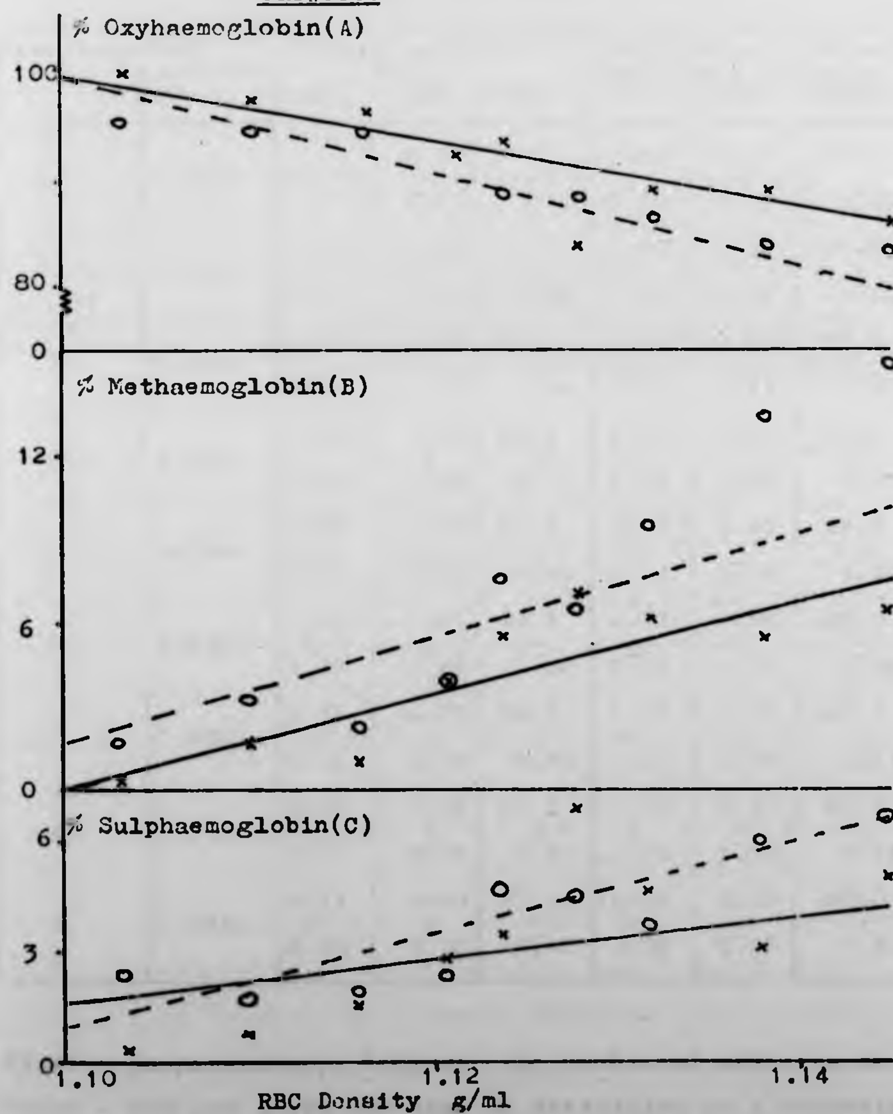


Table 22: Effect of RBC Age and Riboflavin Status on The Relative Concentrations of MHb, SHb, and OxyHb in Blood

Fractions	RBC density g/ml	Normal subjects (4)			Subclinically Riboflavin Deficient Subjects (3)		
		MHb	SHb	OxyHb	MHb	SHb	OxyHb
1	1.103	0 ± 0	0.3 ± 0.15	99.7 ± 0	1.50 ± 0.79	2.53 ± 1.01	95.6 ± 0.40
2	1.110	1.40 ± 0.43	0.68 ± 0.28	97.9 ± 0.21	3.30 ± 0.70	1.80 ± 0.75	94.6 ± 1.50
3	1.116	1.00 ± 0.37	1.63 ± 0.38	97.1 ± 0.18	2.40 ± 0.82	2.00 ± 0.82	95.6 ± 1.61
4	1.121	3.85 ± 1.54	3.38 ± 1.34	92.6 ± 0.77	4.07 ± 1.84	2.43 ± 1.04	93.5 ± 2.57
5	1.124	3.70 ± 0.47	3.70 ± 1.03	92.6 ± 0.23	5.53 ± 2.12	4.80 ± 2.18	89.6 ± 3.64
6	1.128	7.53 ± 2.39	7.05 ± 1.89	85.4 ± 1.14	6.73 ± 3.04	4.60 ± 1.79	89.0 ± 3.59
7	1.132	6.25 ± 1.71	4.73 ± 1.45	89.9 ± 0.85	9.70 ± 4.53	3.70 ± 1.74	87.2 ± 6.17
8	1.1375	5.50 ± 1.22	3.30 ± 0.98	91.2 ± 0.61	13.70 ± 4.33	6.23 ± 1.92	80.4 ± 3.74
9	1.145	6.73 ± 0.83	5.60 ± 2.10	87.7 ± 0.42	16.20 ± 3.39	6.80 ± 2.27	80.0 ± 4.83

Figures in parentheses indicate the number of subjects studied. Means ± SEM are given for each Hb derivative as a percentage of the total Hb in a sample.

Mean EGR-AC of normal subjects was 1.17 ± 0.04 and of the riboflavin deficient subjects was 1.47 ± 0.07.

both normal and deficient subjects. There were also highly significant negative correlations between RBC density and GSH concentration in both normal ($p = 0.0003$) and deficient ($p = < 0.0001$) subjects.

A clear increase in the concentration of MHb was observed in the cells from the denser fractions when compared with the younger-cell fractions (Figure 39 and Table 22), concomitant with a decrease in the concentration of oxyhaemoglobin. The MHb concentration in Fraction 9 was significantly higher than in Fraction 1 for both normal ($p < 0.01$) and deficient ($p < 0.05$) subjects. The sulphaemoglobin concentrations in the different fractions were erratic but the concentration was consistently higher in the older cells than in the younger cells. A highly significant negative correlation was observed between OxyHb concentration and RBC density of normal subjects ($p = 0.004$) and riboflavin-deficient subjects ($p = 0.0002$) and equally significant positive correlations between MHb concentration and RBC density in normal ($p = 0.0017$) and deficient ($p = 0.0001$) subjects.

The MHb concentration in Fraction 9 of normal subjects was significantly lower than that measured in Fraction 9 of riboflavin-deficient subjects ($p < 0.05$). Mean OxyHb concentrations on the other hand were lower in deficient subjects at Fractions 1 ($p < 0.001$) and 8 ($p < 0.05$), when compared with normal subjects, but no other differences were significant (t-test) (Table 22).

Figure 40

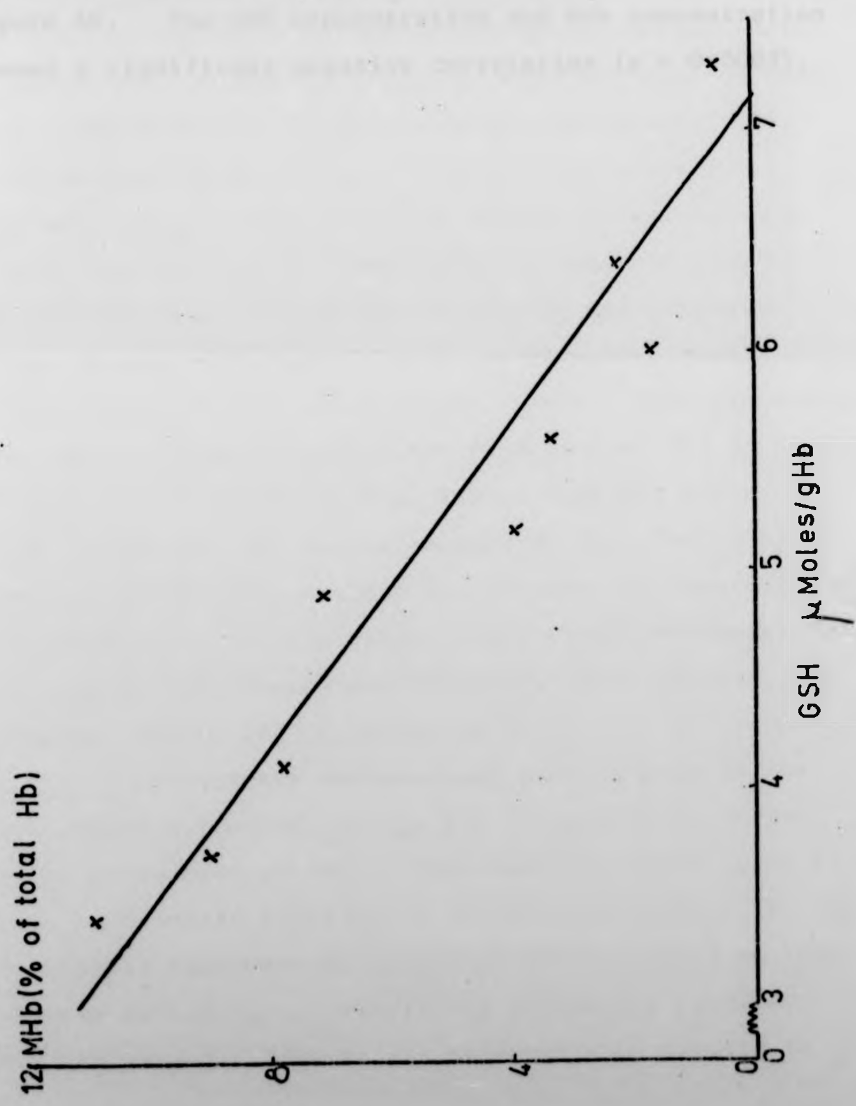
Figure 40: <u>Relationship Between GSH Concentrations and MHb in RBC</u>

A regression analysis was performed on the GSH concentration (μmoles/g Hb) and % MHb in RBC of different ages from the pooled data of 5 riboflavin-deficient and 9 normal subjects.

%mHb = μmoles/g Hb of GSH (-2.682) + 19.067

$r = 0.960$, $p < 0.0001$.

FIGURE 40 RELATIONSHIP BETWEEN GSH AND % METHAEMOGLOBIN IN RBC



Mean GSH and MHb concentrations for each fraction were calculated using all the data available from both normal and deficient subjects. A regression analysis was performed on the 9 mean values and this is shown in Figure 40. The GSH concentration and MHb concentration showed a significant negative correlation ($p = 0.0002$).

F. Some Physiological and Biochemical Responses of RBC on Exposure to H_2O_2

The unsaturated lipids of biological structures are liable to undergo oxidative changes resulting in the formation of a number of reactive compounds such as peroxides and aldehydes (Kibrick, Safier and Skupp, 1959; Fontaine and Valli, 1977). Since the autoxidation of lipids *in vitro* always produces malonyldialdehyde (MDA) it has been assumed that MDA is also formed *in vivo* in the autoxidation of cell lipids (Stocks and Dormandy, 1970; Stocks, Offerman, Modell and Dormandy, 1972; Dodge, Cohen, Kayden and Phillips, 1967). Very few workers have been able to establish the formation of MDA *in vivo* (Walker, Rudra and Dickerson, 1973; Kibrick, Safier and Skupp, 1959) but RBC can be induced to form MDA *in vitro* when exposed to H_2O_2 and the MDA produced is considered by many workers to be indicative of the extent of endogenous lipid oxidation (Stocks and Dormandy, 1970; Stocks, Offerman, Modell and Dormandy, 1972).

EGR appears to have some part to play in the antioxidant mechanisms of the RBC in that it is active in the production of GSH, a substrate for GSHPx which is known to detoxify potentially harmful peroxides. It was of interest therefore to determine whether RBC from human subjects with marginal riboflavin deficiency produced more MDA than RBC from normal subjects when exposed to

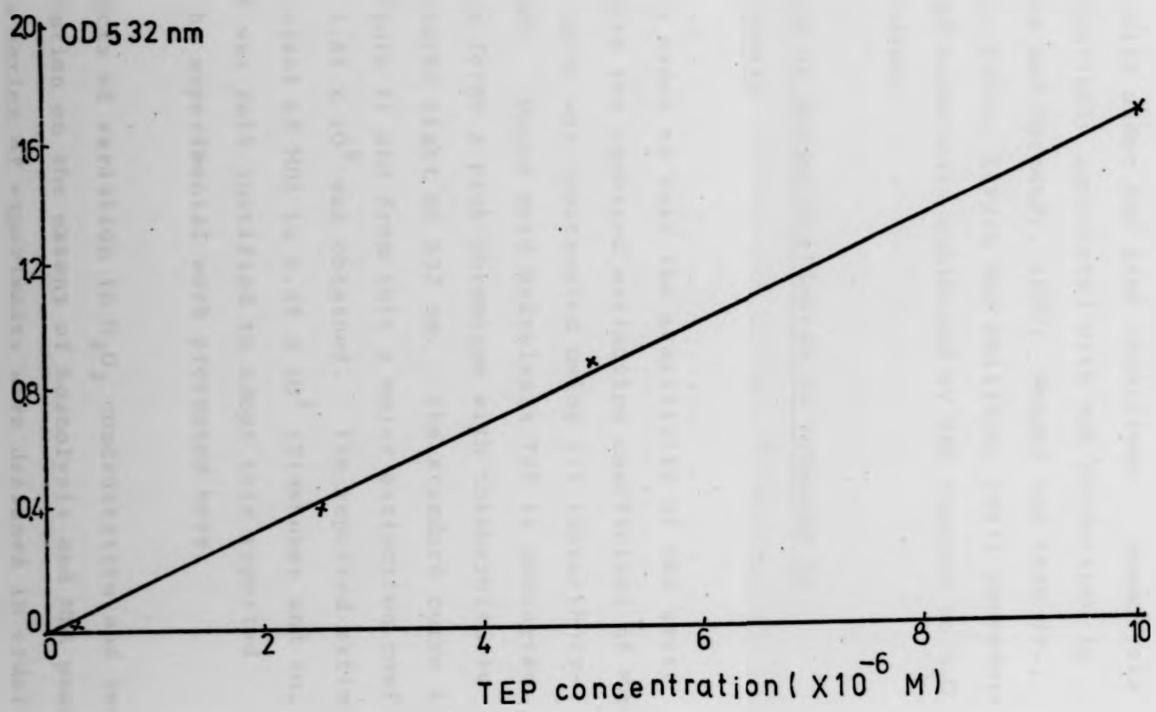
Figure 4I

Figure 41: Standard Curve for Malonyldialdehyde

1-1 tetraethoxypropane (TEP) on hydrolysis is quantitatively converted to MDA. The latter reacts with thiobarbituric acid and the product was measured at 532 nm.

A regression analysis was performed on the absorbance data and the concentration of TEP used.

FIGURE 41 STANDARD CURVE FOR MALONYLDIALDEHYDE



the same degree of oxidant stress *in vitro*. Also, as old RBC have been shown to have low GR activity it was postulated that they would produce more MDA *in vitro* than young cells under the same conditions. Haemolysis of RBC is invariably associated with MDA production *in vitro* (Stocks and Dormandy, 1970; Mengel and Kann Jr., 1966; Dodge, Cohen, Kayden and Phillips, 1967) therefore the extent of haemolysis exhibited by RBC exposed to H_2O_2 was also studied.

i. Production of malonyldialdehyde in response to oxidant stress

In order to test the sensitivity of the assay and to confirm the reported extinction coefficient of MDA a standard curve was constructed using 1-1 tetraethoxypropane (TEP). Under acid hydrolysis TEP is converted to MDA which forms a pink chromogen with thiobarbituric acid and absorbs light at 532 nm. The standard curve is shown in Figure 41 and from this a molar extinction coefficient of 1.66×10^5 was obtained. The reported extinction coefficient of MDA is 1.56×10^5 (Sinnhuber and Yu, 1958). It was felt justified to adopt this reported value for the experimental work presented here.

1. The effects of variation in H_2O_2 concentration and length of incubation on the extent of haemolysis and MDA production:

A series of experiments were designed in order to clarify the relationship between the incubation conditions

Figure 42

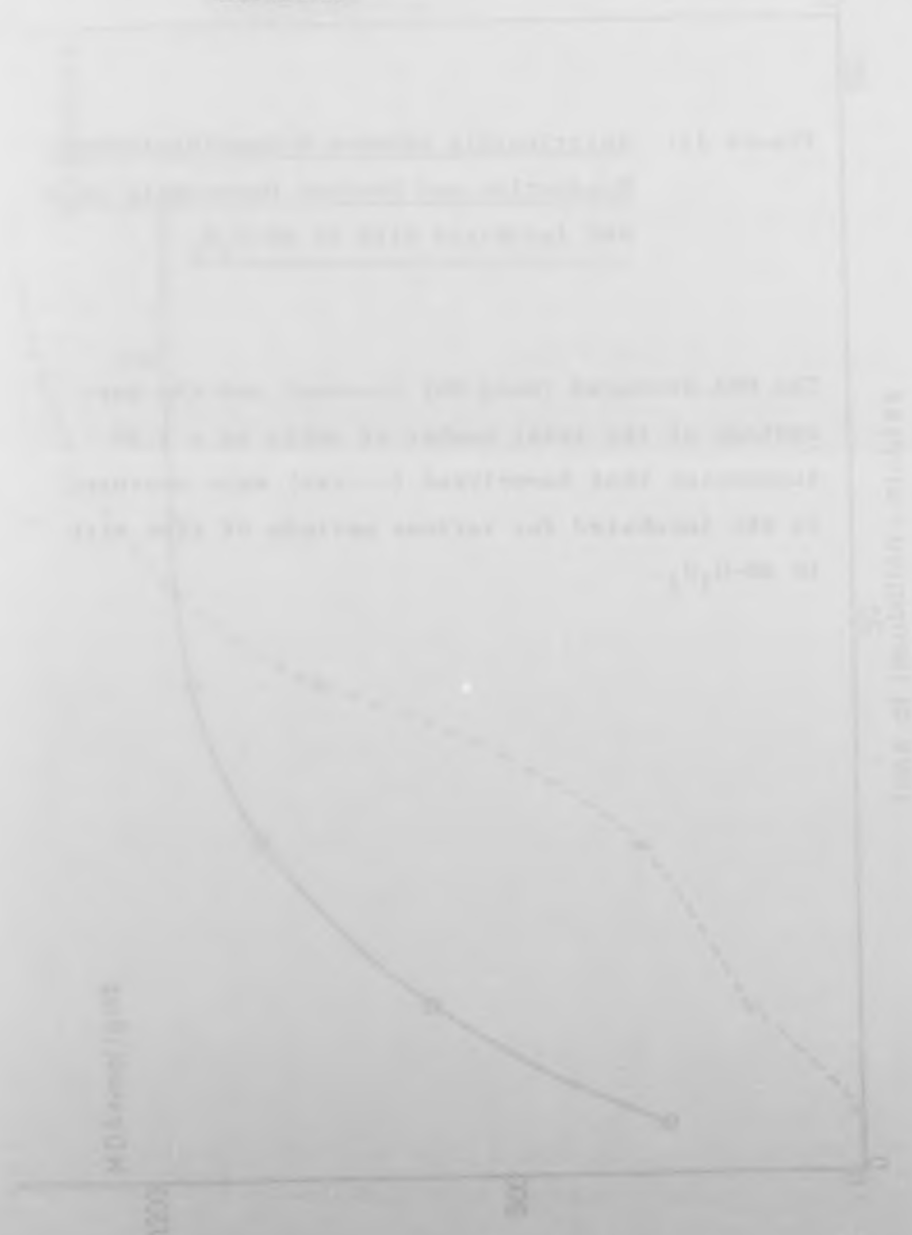


Figure 42: Relationship between Malonyldialdehyde Production and Percent Haemolysis of RBC Incubated with 10 mM-H_2O_2

The MDA produced (nm/g Hb) (——ooo) and the percentage of the total number of cells in a 2.5% suspension that haemolysed (---xxx) were measured in RBC incubated for various periods of time with 10 mM-H_2O_2.

Figure 42 Relationship Between Malonyldialdehyde Production and % Haemolysis of RBC Incubated with 10mM H_2O_2

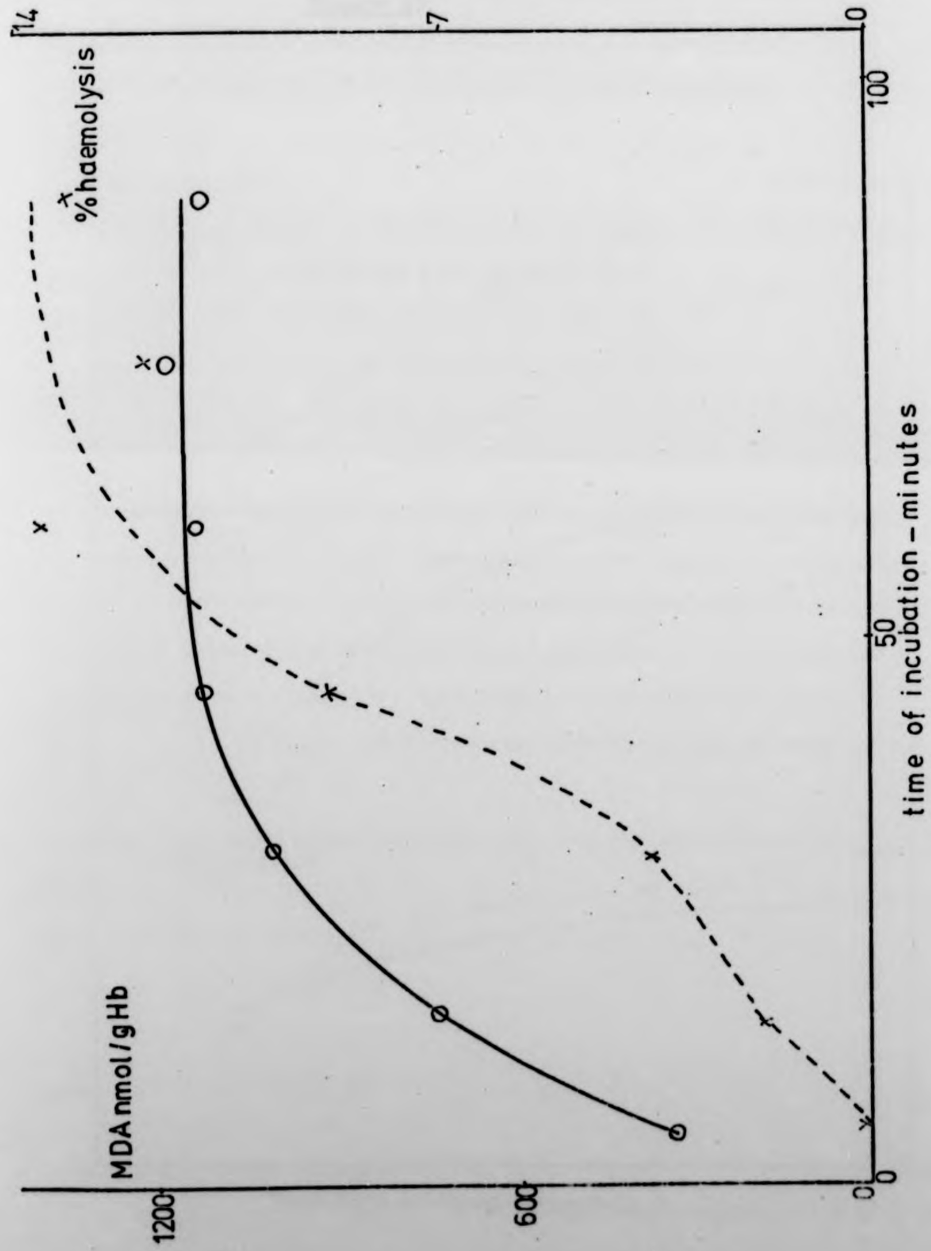
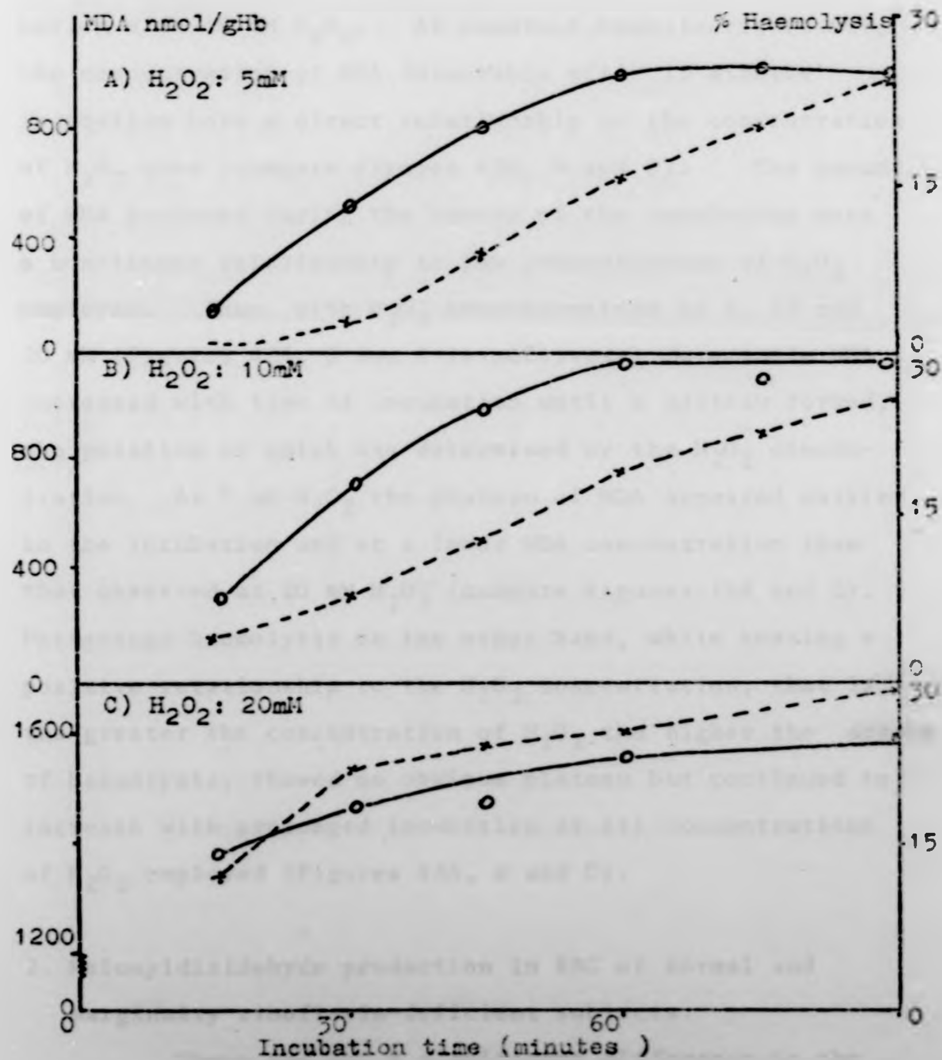


Figure 43

Figure 43. <u>Relationship Between Malonyldialdehyde Production and Haemolysis During Incubation with Different Concentrations of H_2O_2</u>

RBC suspended in a phosphate buffer containing sodium azide were incubated for 90 minutes with different concentrations of H_2O_2. MDA was measured in red cell extracts at intervals during the incubation (——ooo) and the percentage of cells that haemolysed was measured (---xxx).

Figure 43 Relationship Between Malonyldialdehyde Production and Haemolysis of RBC of Different Ages Incubated with Different Concentrations of H_2O_2



and the resultant haemolysis and MDA production in RBC. A progressive increase in incubation time resulted in a progressive increase in MDA production and haemolysis (Figure 42). MDA was detected prior to any detectable haemolysis and formed a plateau after 45 minutes' incubation with 10 mM H_2O_2. At constant haematocrit (2.5%), the concentration of MDA detectable after 15 minutes' incubation bore a direct relationship to the concentration of H_2O_2 used (compare Figures 43A, B and C). The amount of MDA produced during the course of the incubation bore a non-linear relationship to the concentration of H_2O_2 employed. Thus, with H_2O_2 concentrations of 5, 10 and 20 mM (Figures 43A, B and C respectively) detectable MDA increased with time of incubation until a plateau formed, the position of which was determined by the H_2O_2 concentration. At 5 mM-H_2O_2 the plateau of MDA appeared earlier in the incubation and at a lower MDA concentration than that observed at 20 mM H_2O_2 (compare Figures 43A and C). Percentage haemolysis on the other hand, while bearing a positive relationship to the H_2O_2 concentration, that is, the greater the concentration of H_2O_2 the higher the degree of haemolysis, showed no obvious plateau but continued to increase with prolonged incubation at all concentrations of H_2O_2 employed (Figures 43A, B and C).

2. Malonyldialdehyde production in RBC of normal and marginally riboflavin-deficient subjects:

There was not a significant difference in the

Table 23: MDA Production in RBC Incubated with $10\ mM\text{-}H_2O_2$ in the Presence of Sodium Azide

MDA nmol /gHb

Normal subjects		Marginally Riboflavin-Deficient Subjects	
C.W.	1045 ± 16.42	J.A.	1170 ± 19.64
N.F.	977 ± 18.09	J.S.	1007 ± 32.09
D.T.	1260 ± 19.46	M.S.	1045 ± 40.21
L.B.	1256 ± 24.09	J.G.	1018 ± 37.16
P.J.	649 ± 32.11	M.G.	854 ± 19.40
H.P.	1000 ± 31.41		
A.B.	755 ± 30.32		
S.P.	766 ± 22.14		
M.G.	900 ± 20.93		
M.S.	1211 ± 21.63		
Mean ± SEM		Mean ± SEM	
981 ± 68.7		1018 ± 50.4	

The mean EGR-AC for the normal subjects is 1.17 ± 0.03 (SEM), and that for the marginally riboflavin-deficient subjects 1.39 ± 0.04.

amount of MDA detected in RBC from normal subjects or riboflavin-deficient subjects (t-test) after 1 hr incubation with 10 mM H_2O_2 in the presence of sodium azide (Table 23). Any MDA formed during the incubation of RBC without H_2O_2 was accounted for in the calculations, thus the values presented indicate MDA production due to oxidative stress (i.e. H_2O_2) specifically. No MDA was detected in RBC prior to incubation.

Over the relatively small range of activation coefficients for EGR (1.10 - 1.42) no correlation was observed between the concentration of MDA produced and EGR-AC.

3. Malonyldialdehyde production in fractions of RBC of different mean ages:

In view of the observed fall in GR activity during the ageing of RBC an investigation was made into the relative susceptibility of RBC of different ages to oxidant attack by H_2O_2.

RBC in a sample of blood were separated into 9 distinct fractions according to their age (Methods: Section D), and, after removing aliquots for enzymatic analysis fractions were pooled as follows:

Fractions 1 and 2	formed Fraction I
Fraction 3	formed Fraction II
Fractions 4, 5 and 6	formed Fraction III
Fractions 7, 8 and 9	formed Fraction IV

Figure 44

Figure 44: Malonyldialdehyde Production in RBC of Different Mean Ages During Incubation with H_2O_2

MDA (nmoles/g Hb) was measured in 4 fractions of progressively older RBC (Fractions I → IV), that had been incubated for 1 hr with 10 mM-H_2O_2, at a final haematocrit of 2.5%. Any MDA formed during incubation of cells without H_2O_2 was subtracted from the appropriate fractions. The concentrations of MDA measured in different fractions were compared (t-test) and the significance levels are shown.

Results are the means ± SEM for 7 normal subjects.

Figure 44 Malonyldialdehyde Production in RBC of Different Mean Ages During Incubation with H_2O_2: Normal Subjects.

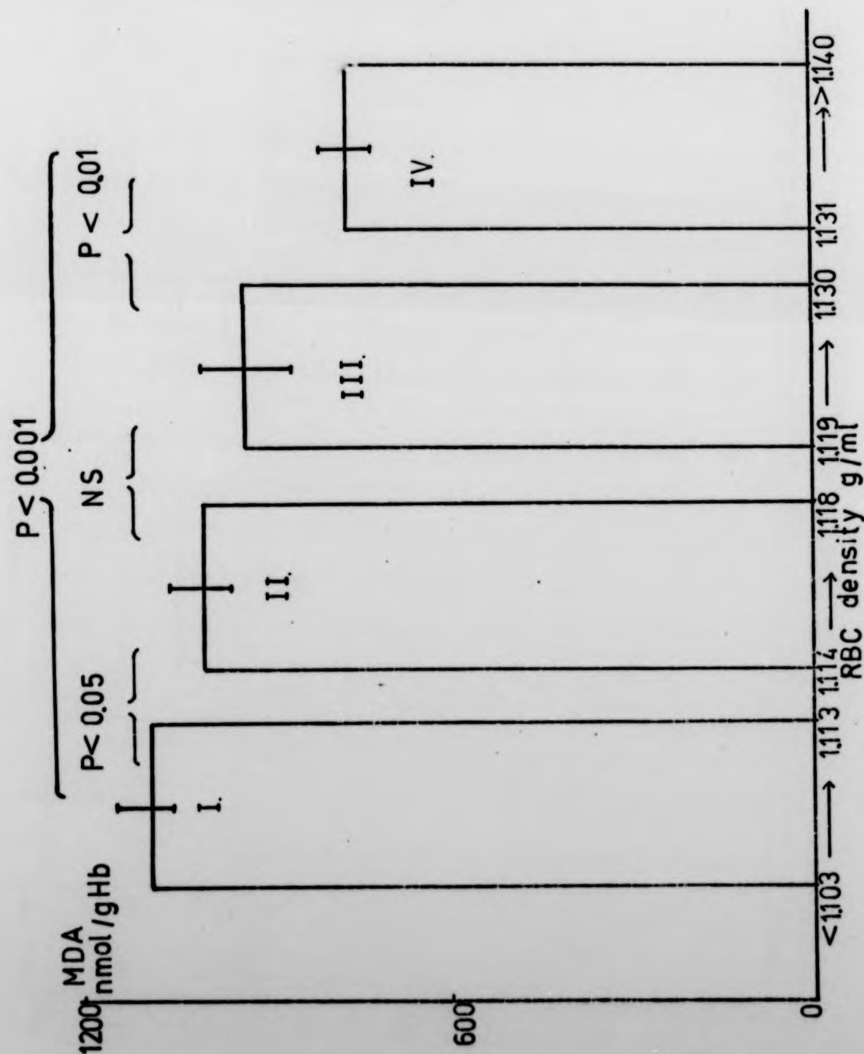


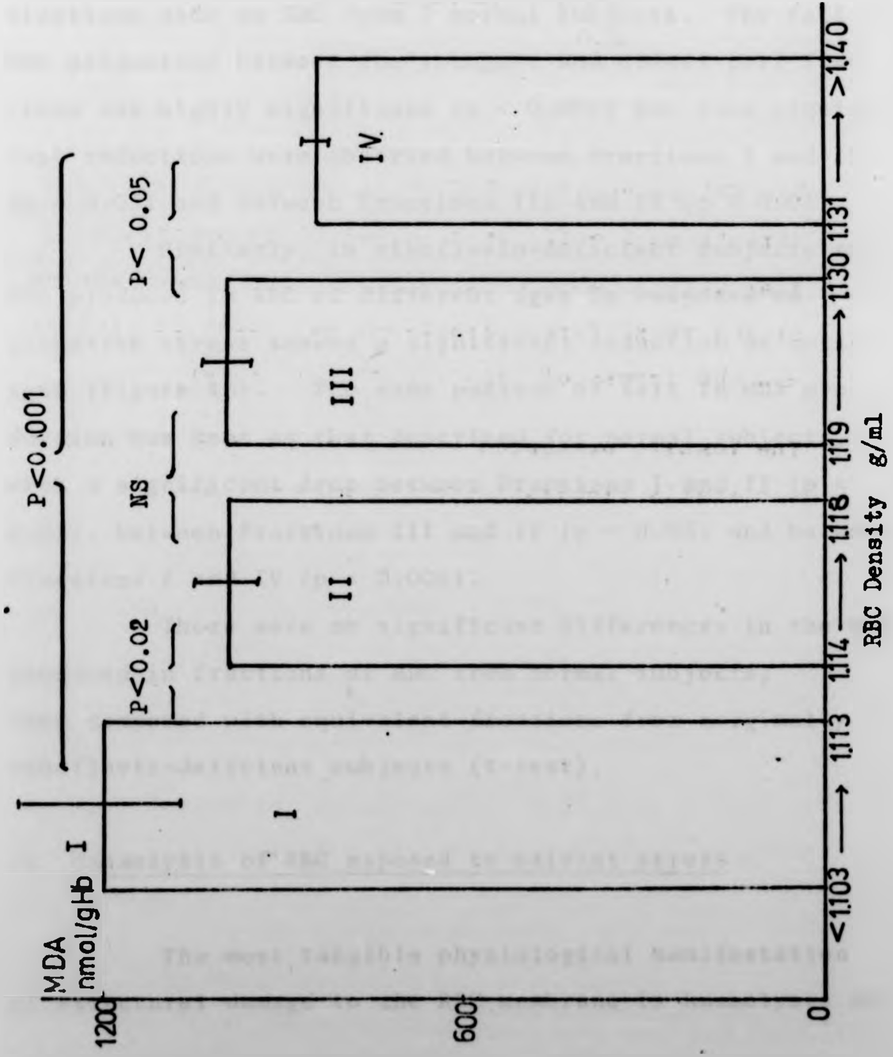
Figure 45

Figure 45: Malonyldialdehyde Production in RBC of Different Ages During Incubation with H_2O_2

The MDA produced in RBC of progressively mean age (Fractions I → IV) as a result of incubation for 1 hr with 10 mM-H_2O_2 is shown. The concentration of MDA in different fractions was compared (t-test) and the levels of significance are shown.

The results presented are the means ± SEM from 5 marginally riboflavin-deficient subjects.

Figure 45 Malonyldialdehyde Production in RBC of Different Ages During Incubation with H_2O_2



Suspensions of each fraction were incubated for 1 hr with 10 mM H_2O_2 at 37°C in the presence of azide (Methods

The MDA detected in RBC from normal subjects after incubation showed a consistent reduction in RBC as they aged (Figure 44). Each value shown is the mean of determinations made on RBC from 7 normal subjects. The fall in MDA production between the youngest and oldest cell fractions was highly significant ($p < 0.001$) but less significant reductions were observed between Fractions I and II ($p < 0.05$) and between Fractions III and IV ($p < 0.01$).

Similarly, in riboflavin-deficient subjects the MDA produced in RBC of different ages in response to oxidative stress showed a significant reduction as cells aged (Figure 45). The same pattern of fall in MDA production was seen as that described for normal subjects, with a significant drop between Fractions I and II ($p < 0.02$), between Fractions III and IV ($p < 0.05$) and between Fractions I and IV ($p < 0.001$).

There were no significant differences in the MDA produced in fractions of RBC from normal subjects, when compared with equivalent fractions from marginally riboflavin-deficient subjects (t-test).

ii. Haemolysis of RBC exposed to oxidant stress

The most tangible physiological manifestation of structural damage to the RBC membrane is haemolysis of

Table 24: The Extent of Haemolysis of RBC During a 1 hour Incubation with 10 mM-H_2O_2

% Haemolysis

Normal subjects		Marginally riboflavin deficient subjects	
N.F.	15	M.S.	7
D.T.	9	C.W.	16
L.D.	17	T.A.	9
M.S.	0	J.S.	18
H.J.P.	4	J.G.	14
N.F.	13	M.G.	13
J.C.	9		
M.C.	15		
L.	18		
A.B.	10		
M.G.	20		
Mean $\pm$ SEM		Mean $\pm$ SEM	
12.20 $\pm$ 1.84		12.83 $\pm$ 1.75	

The mean EGR-AC for normal subjects is 1.16 $\pm$ 0.03 (SEM) and that for marginally riboflavin-deficient subjects 1.39 $\pm$ 0.03.

Table 25: Effect of RBC Age on Susceptibility to Haemolysis During Incubation of RBC from Normal Subjects in the Presence and Absence of 10 mM-H_2O_2

	% Haemolysis							
Fraction	I		II		III		IV	
10 mM-H_2O_2	-	+	-	+	-	+	-	+
M.G.	0.5	18.5	0.5	21.0	0.9	20.2	1.2	24.0
M.C.	0.6	3.6	0.4	9.5	0.5	8.7	0.9	10.2
N.F.	0.3	14.0	0	14.0	1.2	15.5	1.0	21.2
C.W.	0	5.0	0.4	5.5	0.7	9.2	1.0	10.0
H.P.	0.4	4.5	0.5	6.2	0.7	7.6	0.9	10.8
J.C.	0	5.0	0.6	8.6	1.0	14.0	1.2	14.2
D.T.	0.3	3.3	0	4.5	0.6	6.9	1.0	9.8
Mean	0.3	7.7	0.34	9.9	0.82	11.73	1.03	14.31
SEM	0.08	2.11	0.08	2.04	0.09	1.74	0.05	2.08
			$p < 0.05$		$p < 0.02$		$p < 0.001$	

The % haemolysis of RBC incubated with (+) and without (-) 10 mM-H_2O_2 for 1 hr is shown for 7 normal subjects. Values are the means of determinations made on 2 or 3 suspensions prepared from the same RBC. The probability values indicate the level of significance at which haemolysis in the presence of H_2O_2 was greater than that in Fraction I (paired t-test). The mean EGR-AC is 1.18 $\pm$ 0.030 (SEM).

the cell. It was postulated that during the course of the incubation of red cells with H_2O_2 those cells with the least effective antioxidant systems would be those cells most likely to haemolyse. Consequently, concomitant with the measurement of MDA production in H_2O_2-stressed RBC was an assessment of RBC fragility.

1. Haemolysis of unfractionated RBC:

The degree of haemolysis exhibited by RBC OF normal subjects and that shown by RBC from marginally riboflavin-deficient subjects were not significantly different (Table 24). Values shown are the means of determinations made on 2 or 3 suspensions of RBC from each subject. Any haemolysis taking place in RBC incubated without H_2O_2 was accounted for in the calculations so that the degree of haemolysis shown is that due to H_2O_2 alone.

2. Haemolysis of RBC of different mean ages:

Older cells incubated with H_2O_2 showed a consistently greater degree of haemolysis than young cells from the same subject (Table 25). The degree of haemolysis in cells incubated in the absence of H_2O_2 was age-related such that the youngest cells showed the least haemolysis and the oldest cells exhibited the most haemolysis (Table 25). The significance of the small but consistent increase in haemolysis in the older cells in the presence of H_2O_2

Table 26: Effect of RBC Age on the Extent of Haemolysis of Cells from Riboflavin-Deficient Subjects Incubated in the Presence and Absence of 10 mM H_2O_2

	% Haemolysis							
Fraction	I		II		III		IV	
10 mM H_2O_2	-	+	-	+	-	+	-	+
Subject								
M.S.	0.6	6.6	0.9	14.2	1.0	12.1	1.0	19.5
T.A.	0	7.0	0	12.0	0.7	14.2	1.2	14.6
C.W.	0.5	6.5	0.7	3.6	0.2	5.7	1.1	14.5
J.G.	0	12.0	0.3	14.5	0.7	17.7	0.9	20.0
J.S.	0.5	4.5	0	7.5	0.9	12.0	1.5	14.0
Mean	0.32	7.32	0.38	10.36	0.70	12.34	1.14	16.50
SEM	0.12	1.12	0.17	1.88	0.13	1.74	0.10	1.22
			$p < 0.05$		$p < 0.05$		$p < 0.001$	

RBC were obtained from subjects with a mean EGR-AC of 1.41 ± 0.04 (SEM). The % haemolysis of the cells after 1 hr incubation with (+) and without (-) 10 mM-H_2O_2 was measured for cells of different ages; the values are the means of determinations made on 2 or 3 suspensions of cells from the same fraction. The amount of haemolysis taking place in each fraction in the presence of H_2O_2 was compared with that taking place in Fraction 1, and the levels of significance are shown.

was tested using a paired t-test and was significantly greater in Fractions II ($p < 0.05$), III ($p < 0.02$) and IV ($p < 0.001$) when compared with Fraction 1.

Suspensions of RBC from marginally riboflavin-deficient subjects were investigated in a similar fashion and the same age-related haemolysis was observed (Table 26). The increase in haemolysis over that in Fraction 1 was significant for Fractions II ($p < 0.05$), III ($p < 0.05$) and IV ($p < 0.001$).

The RBC in Fractions II, III and IV of deficient subjects showed greater haemolysis than RBC in equivalent fractions from normal subjects but the differences could not be considered statistically significant (t-test).

iii. The response of the glutathione peroxidase antioxidant system of the RBC on exposure to H_2O_2

1. GSH concentration:

In view of the observed decrease in activity of GR *in vitro* in older cells and the decreased concentration of GSH it was postulated that GSH *per se* might be a factor limiting the efficiency of the antioxidant potential of GSHPx during exposure of RBC to oxidant stress. It was of interest therefore to determine the ability of RBC to maintain concentrations of GSH in the face of high concentrations of H_2O_2.

The procedure for the estimation of GSH was essentially the same as that described earlier (Methods: G)

Table 27: Effect of Zaponin on the Measurement of GSH in Protein-Free Extracts of RBC

	GSH µmol/ml Haemolysate	
Zaponin	-	+
Subject		
1	0.058	0.058
2	0.063	0.064
3	0.071	0.070
4	0.059	0.058
5	0.076	0.074
Mean	0.065	0.065
SEM	0.003	0.003

GSH was measured in extracts of haemolysates to which 1 drop of Zaponin (+) or distilled water (-) had been added. The concentrations of GSH (µMoles/ml haemolysate) from 5 subjects were compared (t-test) and the levels of significance are shown.

although the preparation of cells for the assay was somewhat modified. Thus, 1.5 ml aliquots of RBC suspended in a phosphate buffer containing sodium azide, at a haematocrit of 5.0% , were added to equal volumes of H_2O_2 solutions of different concentrations and either mixed for 2 minutes at room temperature or incubated for 1 hr at 37°C. One drop of a solution of Zaponin was added to the suspensions to haemolyse the cells and after a further 2 minutes precisely, the protein was precipitated. The GSH concentration in each filtered RBC extract and in an extract of RBC prior to the addition of H_2O_2 was measured. Hb estimations were made on completely haemolysed samples of RBC. All GSH concentrations were expressed as μmoles/g Hb.

Effect of Zaponin on the measurement of GSH:

In order to determine whether Zaponin might interfere with the assay for GSH, GSH was determined in protein-free extracts of haemolysates to which 1 drop of Zaponin or distilled water had been added (Table 27). A paired t-test analysis of the results showed that Zaponin had no significant effect on the determination of GSH in solution.

<u>Experiments with unfractionated blood</u>

Immediate oxidation of GSH:

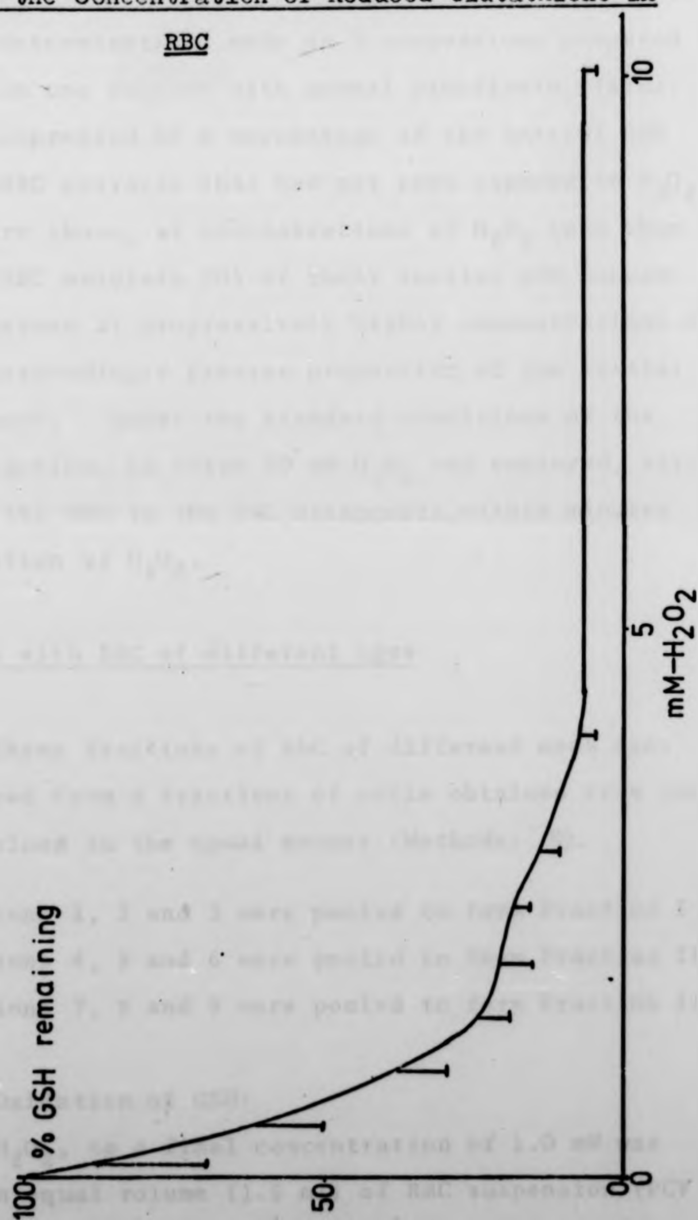
The effect of adding H_2O_2 of different concentrations to RBC, on the GSH concentration in RBC extracts prepared within 5 minutes of the addition of H_2O_2, is shown

Figure 46

Figure 46: <u>Effect of Different Concentrations of H_2O_2 on the GSH Concentration in RBC</u>

GSH (μmoles/g Hb) was measured in RBC extracts prepared within 3 minutes of the addition of different concentrations of H_2O_2 to RBC suspensions. The GSH is expressed as a percentage of the GSH measured in RBC to which no H_2O_2 was added. The means $\pm$ SEM of RBC extracts prepared in triplicate are shown. All estimations were made on a single blood sample obtained from a subject with normal riboflavin status.

Figure 46 Effects of Different Concentrations of H_2O_2 on the Concentration of Reduced Glutathione in RBC



in Figure 46. The results presented are the mean values ($\pm$ SEM) of determinations made on 3 suspensions prepared from RBC from one subject with normal riboflavin status. The GSH is expressed as a percentage of the initial GSH present in RBC extracts that had not been exposed to H_2O_2. As the figure shows, at concentrations of H_2O_2 less than 1.0 mM the RBC maintain 50% of their initial GSH concentrations whereas at progressively higher concentrations of H_2O_2 a correspondingly greater proportion of the initial GSH disappears. Under the standard conditions of the MDA investigation, in which 10 mM-H_2O_2 was employed, virtually all the GSH in the RBC disappears within minutes of the addition of H_2O_2.

Experiments with RBC of different ages

Three fractions of RBC of different mean ages were prepared from 9 fractions of cells obtained from one sample of blood in the usual manner (Methods: D).

Fractions 1, 2 and 3 were pooled to form Fraction I
Fractions 4, 5 and 6 were pooled to form Fraction II
Fractions 7, 8 and 9 were pooled to form Fraction III

Immediate Oxidation of GSH:

H_2O_2, to a final concentration of 1.0 mM was added to an equal volume (1.5 ml) of RBC suspension (PCV 5.0%). The GSH concentrations in RBC extracts was

Figure 47

Figure 47: Effect of RBC Age on the GSH Concentration in RBC Exposed Briefly to Oxidant Stress

GSH was measured in RBC of progressively greater mean age (Fractions I → III) to which H_2O_2 was added to a final concentration of 1.0 mM. A protein-free extract was prepared within 3 minutes of the addition of H_2O_2 and the GSH measured immediately. The GSH is expressed as a percentage loss as a result of the exposure to H_2O_2. The data were obtained from RBC from 5 subjects with normal riboflavin status.

Figure 47 Effect of RBC Age on the GSH concentrations in RBC Exposed Briefly to Oxidant Stress

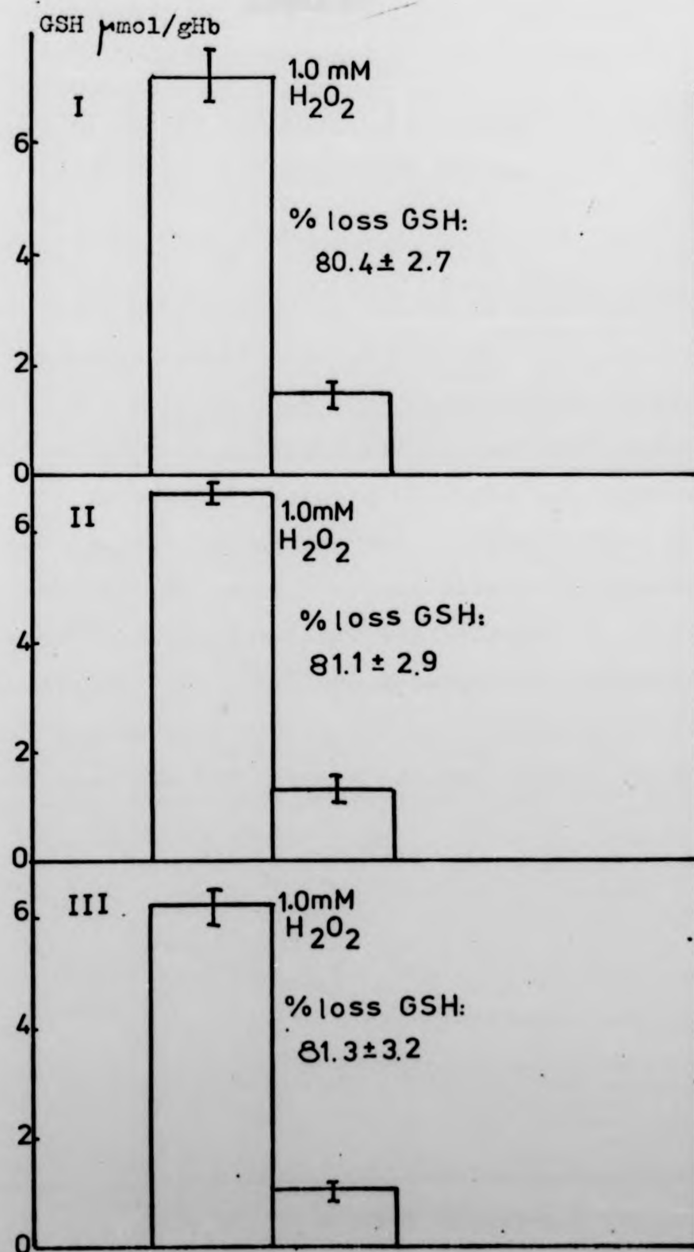


Figure 47 Effect of RBC Age on the GSH concentrations in RBC Exposed Briefly to Oxidant Stress

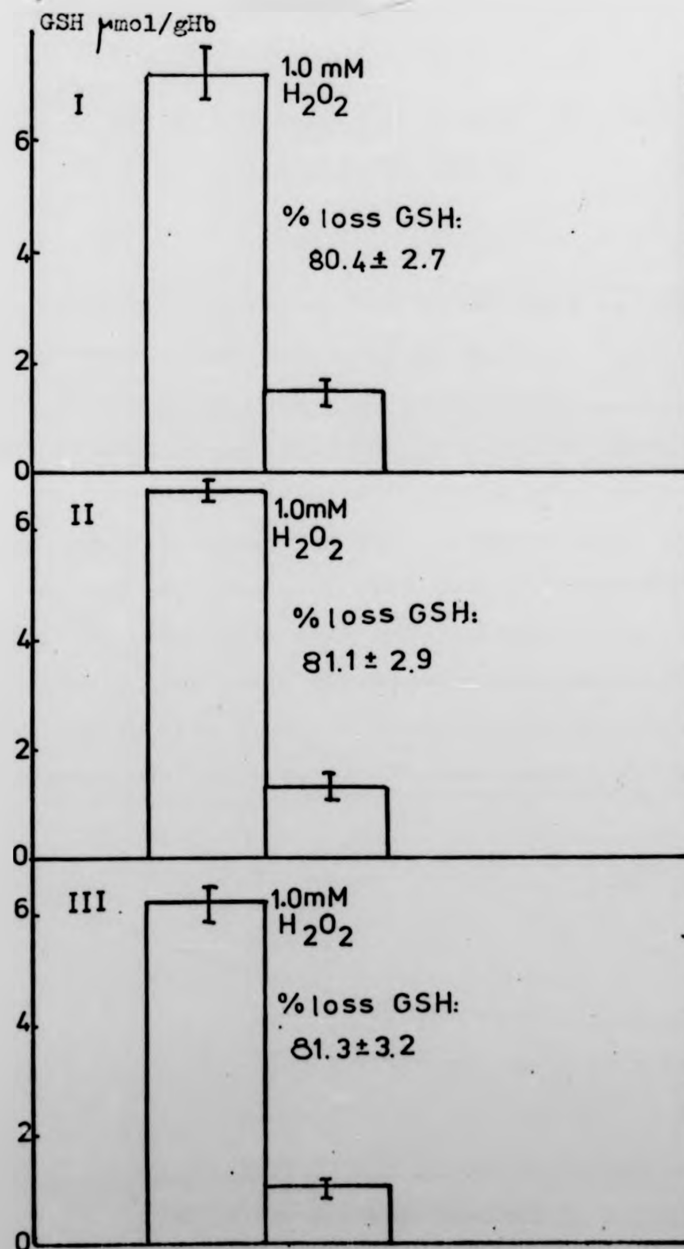


Figure 48

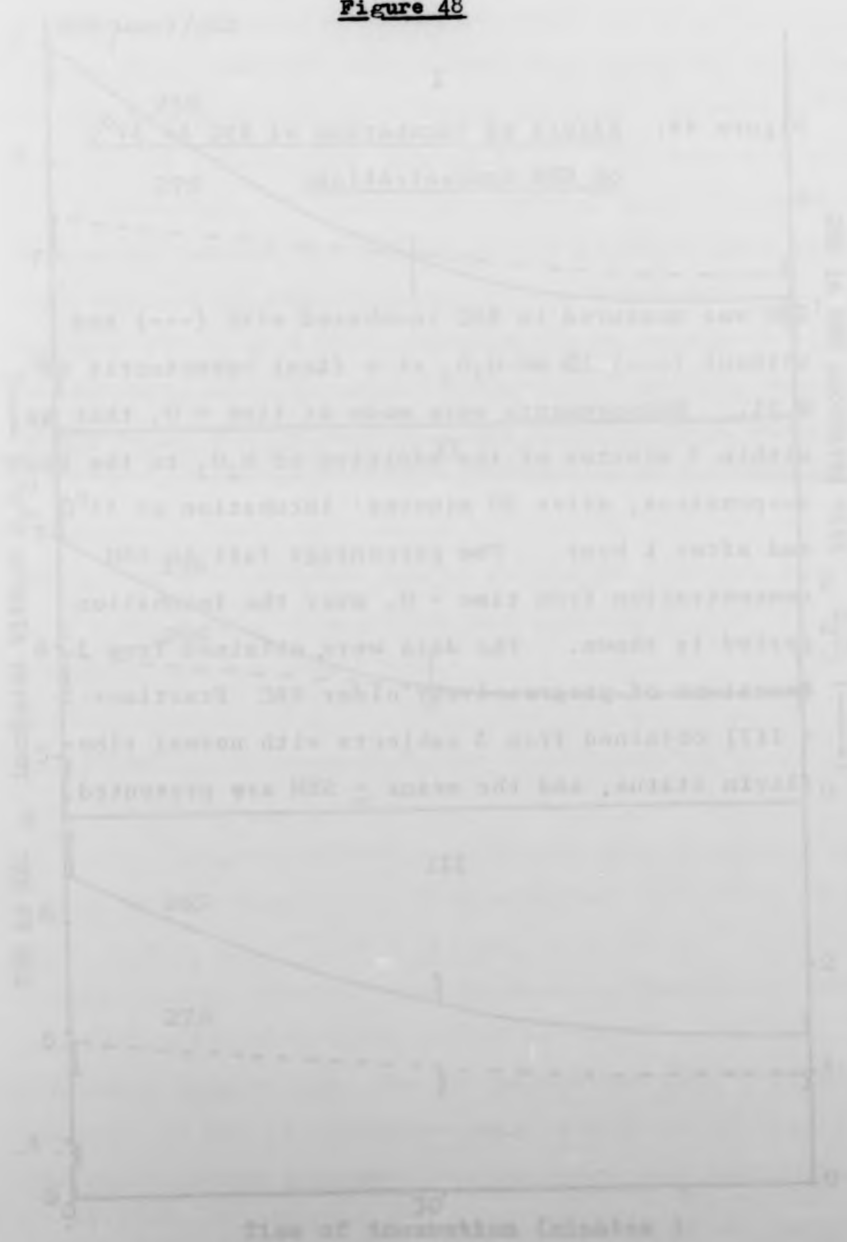
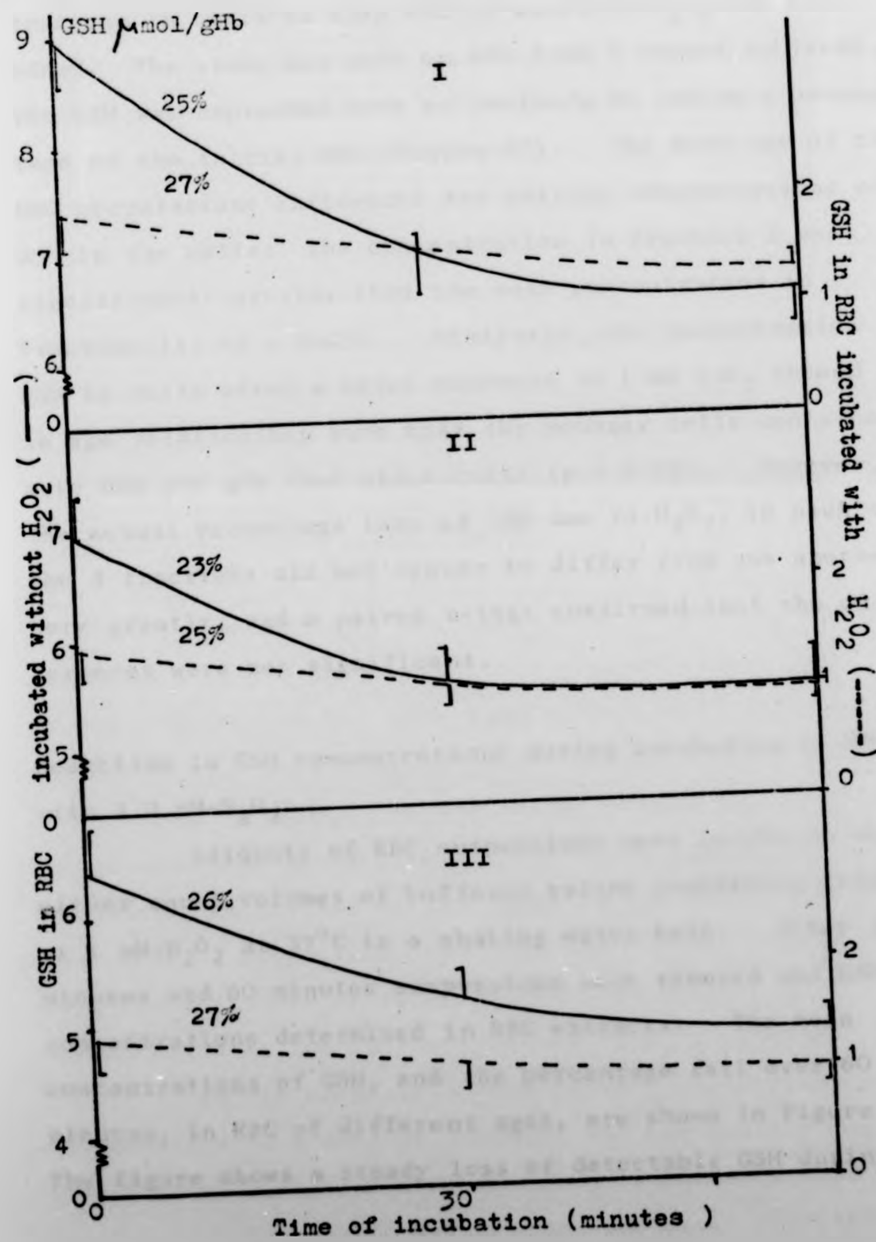


Figure 48: Effect of Incubation of RBC At 37°C on GSH Concentrations

GSH was measured in RBC incubated with (---) and without (——) 1 mM-H_2O_2 at a final haematocrit of 2.5%. Measurements were made at time = 0, that is, within 3 minutes of the addition of H_2O_2 to the test suspensions, after 30 minutes' incubation at 37°C and after 1 hour. The percentage fall in GSH concentration from time = 0, over the incubation period is shown. The data were obtained from 3 fractions of progressively older RBC Fractions I → III) obtained from 3 subjects with normal riboflavin status, and the means ± SEM are presented.

Figure 48 Effect of Incubation of RBC at 37 C on Concentrations of Reduced Glutathione



measured spectrophotometrically and compared with concentrations in extracts from RBC to which no H_2O_2 had been added. The study was made on RBC from 5 normal subjects. The GSH was expressed both as µmoles/g Hb and as a percentage of the initial GSH (Figure 47). The mean age of the RBC preparations influenced the initial concentrations of GSH in the cells; the concentration in Fraction 1 was significantly greater than the mean concentration in Fraction III ($p < 0.05$). Similarly, the concentration of GSH in cells after a brief exposure to 1 mM H_2O_2 showed an age relationship such that the younger cells contained more GSH per gHb than older cells ($p < 0.05$). However, the actual percentage loss of GSH due to H_2O_2, in each of the 3 fractions did not appear to differ from one another very greatly, and a paired t-test confirmed that the differences were not significant.

Reduction in GSH concentrations during incubation of RBC with 1.0 mM-H_2O_2:

Aliquots of RBC suspensions were incubated with either equal volumes of buffered saline containing azide or 1 mM-H_2O_2 at 37°C in a shaking water bath. After 30 minutes and 60 minutes suspensions were removed and GSH concentrations determined in RBC extracts. The mean concentrations of GSH, and the percentage fall over 60 minutes, in RBC of different ages, are shown in Figure 48. The figure shows a steady loss of detectable GSH during

the incubation both in the presence and absence of H_2O_2. RBC exposed to 1 mM-H_2O_2 showed an immediate loss of GSH in the order of 20% but thereafter the proportion of GSH that disappeared during a 1 hr incubation period was the same in blank and test solutions.

At each stage in the incubation the concentration of GSH in the older cells was less than that in the younger cells, but the rate of disappearance of GSH did not appear to be related to the mean age of the RBC, under the conditions described here.

iv. Removal of H_2O_2 from RBC within 5 minutes of its addition to a RBC suspension

When RBC were incubated with 10 mM-H_2O_2 the older cells of a sample haemolysed to a greater extent than younger cells from the same sample (Tables 25 and 26). Virtually all the GSH was oxidised within 5 minutes of addition of 10 mM-H_2O_2 to the cells (Figure 46). It was postulated that the degree of haemolysis taking place in RBC during incubation would depend on the amount of H_2O_2 remaining in suspension after all the GSH had been oxidised. H_2O_2 was therefore measured in RBC suspensions, by a method based on the microestimation of H_2O_2 (Methods: H)

RBC in suspension were totally haemolysed by the addition of 1 drop of Zaponin, and the protein was precipitated immediately by 2N-H_2SO_4. Mixtures were centrifuged at 3,000 g for 15 minutes and the clear supernatent

Table 28: Effect of Zaponin on the Estimation of H_2O_2 by Microtitration

$mM-H_2O_2$ (by dilution)	Calculated H_2O_2 added mM ZAPONIN	
	-	+
20	20.5	20.5
10	10.25	10.30
5	5.20	5.10
2.5	2.10	2.10

H_2O_2 was estimated in solutions of known H_2O_2 concentration to which 1 drop of Zaponin (+) had been added and solutions to which no Zaponin had been added (-). The H_2O_2 measured by the microtitration method is referred to as 'calculated' H_2O_2.

Table 29: Effect of Protein-Free Extracts of RBC on the Determination of H_2O_2

$mM-H_2O_2$ added to extract (calculated by dilution)	$mM-H_2O_2$ added to extract (calculated by titration)
20	21.0
10	10.10
5	4.98
2.5	2.30

H_2O_2 was measured in RBC extracts to which known concentrations of H_2O_2 were added.

decanted off. Hb was determined in a totally haemolysed preparation of each suspension studied.

It was necessary to ensure that the presence of Zaponin did not interfere with the assay for H_2O_2. H_2O_2 solutions of different concentrations were prepared and aliquots of each solution taken and treated with 1 drop of Zaponin. The H_2O_2 in 1 ml aliquots of each solution was then assayed by titration with 0.005 N-$KMnO_4$. That Zaponin *per se* had no effect on the estimation of H_2O_2 can be seen from Table 28. The calculated concentrations of H_2O_2 do not differ significantly in the presence or absence of Zaponin.

A preliminary experiment was also conducted to determine whether RBC extracts contained any substances that would interfere with the $KMnO_4$ estimation of H_2O_2. Protein-free extracts of RBC were prepared, 1 ml aliquots of H_2O_2 solutions of different concentrations were added to the extracts and the resulting solutions assayed for peroxide. Table 29 shows that the concentrations of H_2O_2 added to the RBC extracts, calculated by titration, were extremely close to the known concentrations of these solutions, as prepared by dilution.

Thus, there appeared to be no reason why the estimation of H_2O_2 in RBC extracts derived from RBC haemolysed with Zaponin were not valid.

The concentration of total peroxide in suspensions of RBC of different mean ages measured within 5 minutes

Table 30: Effect of RBC Age on Their Ability to Detoxify H_2O_2

Subject	Peroxide (mMolar) Estimated by Titration			
	FRACTION			
	I	II	III	U.
D.H.	10.5	12.8	13.4	13.2
P.O.	11.0	14.8	14.5	14.9
H.P.	8.8	9.3	9.3	8.8
J.R.	12.4	12.8	14.8	14.9
D.T.	11.4	13.0	13.0	12.8
A.B.	12.4	13.5	14.5	14.4

N.S. $\quad p < 0.01$

$p < 0.01$

1.5 ml aliquots of 20 mM-H_2O_2 were added to equal volumes of progressively older RBC (Fractions I to III) and to unfractionated RBC (U.). A protein-free extract was prepared within 5 minutes of the addition of H_2O_2 and the concentration of the peroxide remaining was estimated titrimetrically.
A paired t-test was performed on data from different Fractions and the significance shown.

of the addition of H_2O_2 are shown in Table 30. The values given represent the means of determinations made on 3 suspensions prepared from each sample of RBC. The concentration of H_2O_2 remaining in the suspension showed a progressive increase as the cells aged. Although the magnitude of the difference in peroxide concentration between young and old cells was not constant the increase was consistently observed and was significant at $p < 0.01$ between Fractions II and III, and between Fractions I and III (paired t-test).

G. The Estimation of Total Peroxides in Blood

Previous experiments showed an inverse relationship between cell age and the amount of MDA formed when RBC were incubated with 10 mM-H_2O_2 (Results: F). The production of MDA during the exposure of RBC to H_2O_2 is an index of susceptibility of the cells to form peroxide and the low concentrations of MDA in old cells suggest that there is little peroxide formed in these cells under the conditions of incubation (Figure 44). An attempt was made, therefore, to measure total peroxides in 3 fractions of RBC of different mean ages that had been incubated with 10 mM-H_2O_2, and a preliminary experiment done to determine whether the enzymic method selected could detect inorganic peroxide (Table 31). The control solution contained all reagents except peroxide. GR was added to all tubes at T_2 to reduce GSSG formed during the removal of peroxides. The fall in absorbance of NADPH at 340 nm between T_2 and T_3 is an indirect measure of the concentration of peroxide in the assay system. The fall in absorbance at 340 nm was quite large in the presence of both 5 and 10 mM-H_2O_2, the very low absorbance at T_3 indicated that all the NADPH had been oxidised. The control solution exhibited a fall in absorbance of 0.066 OD units, despite the absence of peroxide, which probably represented autoxidation of GSH.

Table 32 shows the extent of the fall in absorbance at 340 nm in assay solutions containing 0.1 ml of

Table 31: Enzymatic Determination of Peroxide in Solution

Initial H_2O_2 mM	OD_{340} T_2	OD_{340} T_3	ΔOD_{340}
5	0.431	0.014	0.417
10	0.525	0.028	0.497
Control 0	0.489	0.423	0.066

5 mM and 10 mM-H_2O_2 were assayed by the enzymatic method for measuring tissue peroxide (Methods I)
The results represent the fall in absorbance of NADPH at 340 nm.
The reagent control contained all reagents except H_2O_2 and was included to measure the autoxidation of GSH in the assay system.

Table 32: Detection of Peroxide in Haemolysates by an Enzymatic Method

Subject	ΔOD_{340} nm/10 minutes				
	BL.C.	BL.	T.C.	T.	ΔOD (T-BL)
HJP	0	0.095	0	0.055	-0.040
DIT	0	0.113	0	0.062	-0.051
PD	0	0.107	0	0.101	-0.006
AB	0	0.100	0	0.102	+0.002
Reagent Control			0.072		

Suspensions of RBC in a buffered saline solution that contained azide to inhibit catalase were incubated for 1 hr at 37°C with and without 10 mM-H_2O_2 to obtain test and blank haemolysates respectively. RBC suspensions were haemolysed by a single freezing (-20°C for 16 hrs) and thawing and 0.1 ml aliquots of the centrifuges haemolysates were incubated for 10 minutes at 37°C in a 1.21 ml assay system to generate GSSG (Methods: I). 5 U GR were then added and the subsequent fall in absorbance of NADPH at 340 nm over 10 min. incubation was recorded. Blank and test controls (BL.C. and T.C. respectively), consisted of haemolysates in buffer with no other reagents. The reagent control contained all reagents but no haemolysate. ΔOD(T-Bl) is the Δ in absorbance at 340 nm obtained by subtracting the blank reading from its appropriate test reading.

Table 33: Determination of Peroxide in Haemolysates Obtained from RBC of Different Ages

	Fraction	Subject	DH	JR	PD	HJP	DIT	B	Reagent Control
ΔOD_{340} nm/10 minutes	I	Bl.C.	0	0	0	0	0	0	0.067
		Bl.	.020	0	0	0	0	0	
		T.C.	0	0	0	0	0	0	
		T.	.188	.075	-	.121	.092	.099	
	II	Bl.C.	0	0	0	0	0	0	
		Bl.	.020	0	.060	0	0	0	
		T.C.	0	0	0	0	0	0	
		T.	.228	.030	-	.074	.058	.086	
	III	Bl.C.	0	0	0	0	0	0	
		Bl.	.080	.010	0	0	0	0	
		T.C.	0	0	0	0	0	0	
		T.	.154	.206	-	.130	.095	.104	
	Un-fraction-ated	Bl.C.	0	0	0	0	0	0	
		Bl.	.020	0	.010	0	0	0	
		T.C.	0	0	0	0	0	0	
		T.	.198	.095	-	.045	.100	.093	

Four Ficoll/Triosil solutions of the following densities were prepared: 1) 1.100, 2) 1.113, 3) 1.125, and 4) 1.145 g/ml, and 1 ml aliquots of packed RBC separated in the usual manner (Methods: D). Three bands of cells were removed from between the interfaces of solutions 1 and 2, 2 and 3, and 3 and 4. Washed RBC were incubated as described for Table 34 and haemolysates prepared. Assay systems used 0.5 ml haemolysate and 0.5 ml of buffer for the measurement of peroxides (see Table 32 for other details). The fall in absorbance at 340 nm during the incubation period was recorded for Blank (Bl), test (T), Control blank (Bl.C.) and Control test (T.C.) systems. The fall in absorbance of the reagent control, which contained no haemolysate, was also recorded.

haemolysate and 0.9 ml of Tris buffer, pH 7.6. The reagent control, which contained all reagents but no haemolysate, showed a fall in absorbance of 0.072 OD units, whereas the test and blank controls, which were prepared from RBC that had been incubated with or without 10 mM-H_2O_2, and to which no other reagents were added, underwent no change in absorbance at 340 nm during the period of assay. The test and blank solutions on the other hand, which contained haemolysates obtained from RBC that had been incubated with and without 10 mM-H_2O_2 respectively, and all assay reagents, did show an appreciable decrease in absorbance at 340 nm in excess of that shown by the reagent control. However, for no subject was the decrease significantly greater in the test solution than in the appropriate blank. In fact, for 3 subjects the decrease in absorbance was higher in the blank solutions than the test solutions.

The test solutions were expected to contain more peroxide than the blank haemolysates since the RBC from which the former were obtained had been exposed to H_2O_2. It was possible that insufficient haemolysate had been added to the assay system, therefore a further experiment was carried out in which the proportion of haemolysate in the assay system was increased 5-fold. The investigation was repeated in assay systems prepared from RBC of different mean ages which had been incubated in the presence and absence of 10 mM-H_2O_2 as described in Table 32. The results are presented in Table 33. All values given are

Table 34: Hb Concentration in Haemolysates Used in the Experiment Described in Table 33

Fractions	1		2		3		Unfractionated	
	µM Hb in Haemolysate							
Subject	Blank	Test	Blank	Test	Blank	Test	Blank	Test
D.H.	138	16.2	170	24.2	153	24.2	123	16.2
J.R.	158	62.4	161	77.7	164	30.8	149	48.6
H.J.P.	118	25.9	130.5	22.7	124	20.3	109	24.2
D.I.T.	113	34.1	123	51.1	98	48.6	117.6	24.2
A.B.	131	25.9	116	28.3	88.3	31.7	132	25.9

the means of determinations made on 2 haemolysates prepared from different RBC suspensions. The reagent control showed the same fall in absorbance at 340 nm as was observed previously (Tables 31 and 32) and the control blanks and control tests again showed no change in absorbance at 340 nm during the period of assay. With the increased amount of haemolysate in the assay medium however, the change in absorbance in the blank solutions was negligible, while there was a relatively large change observed in the test solutions which was anticipated. However, the fact that there was a negligible fall in absorbance in blank solutions from all the different RBC haemolysates, whereas reagent controls had shown a consistent fall and the only change was the increase in haemolysate, suggested that this unexpected behaviour may have been in some way associated with the increased amount of Hb in the blank solutions.

The Hb concentration in fresh aliquots of haemolysates that had been used in the assay system described in Table 32 was therefore measured. All the haemolysates used in the assay were obtained by a single freezing and thawing of the RBC; however it was found that Hb concentration in test haemolysates was consistently much lower than that in the corresponding blank haemolysates (Table 34) suggesting either incomplete haemolysis or denaturation of Hb. There was no evidence of any Hb precipitation in suspensions of RBC that had been incubated with H_2O_2 which

Table 35: Effect of Different Concentrations of Hb on the Measurement of Peroxide in Haemolysates

μM-Hb in cuvette	OD_{340}		ΔOD_{340}
	T_2	T_3	$(T_2 - T_3)$
87.8	1.30	1.30	0
67.4	1.29	1.29	0
62.4	1.30	1.30	0
40.8	1.22	1.22	0
26.1	0.87	0.66	0.21
13.1	0.51	0.26	0.252
5.0	0.70	0.422	0.278
Reagent Control	0.361	0.269	0.092

A freshly prepared RBC suspension was haemolysed without prior incubation with H_2O_2 by rapid freezing and thawing, the red cell stroma removed, and the clear supernatent diluted to give a range of Hb concentrations. An enzymatic assay for peroxide was performed in duplicate on each haemolysate, as described in Table 32. The absorbance at 340 nm was recorded just prior to addition of GR (T_2) and after 10 mins incubation with GR (T_3). The absorbance readings, and the final concentration of Hb in the assay system are shown.

suggested that the low Hb concentration in blank solutions may have been due to incomplete haemolysis.

The influence of Hb on the assay system was investigated in an experiment described in Table 35. A suspension of RBC not previously exposed to peroxide, was diluted to progressively lower Hb concentrations in a buffered saline solution containing azide to ensure comparability with previous experiments where azide was included. The suspensions were frozen overnight at $-20^{o}C$ and thawed the following day. The buffy layer and cell debris were removed by centrifuging and the clear supernatents assayed for peroxides. The Hb concentration of each haemolysate was determined in duplicate and it was found that the apparent concentration of peroxide in the assay system was indirectly associated with the concentration of Hb (Table 35). At concentrations of Hb greater than 40 μMolar there was apparently no peroxide in the system despite the fact that the reagent control, which was identical except for the absence of Hb, showed its usual behaviour (see Tables 31, 32 and 33). At Hb concentrations equal to or less than 40 μMolar the apparent concentration of peroxide within the system was greater than that observed in the reagent control and there was a progressive increase as the Hb concentration fell.

It should be pointed out that the haemolysates were freshly prepared, that none of the solutions had been in contact with peroxide, and therefore peroxides should not have been present in the system.

Table 36: Peroxide Determination in Haemolysates of Known Hb Concentration

Subject	Hb µmolar	ΔOD 340 nm Control Blank	Blank	Control Test	Test
DH	35.7	0	0.220	0.004	0.190
PD	29.4	0.005	0.249	0.001	0.210
JR	27.2	0	0.240	0	0.210

Haemolysates were prepared from RBC as described in Table 34. The fall in absorbance at 340 nm in assay systems containing only haemolysates in buffer (Controls) and in systems containing haemolysates and substrates for the enzyme assay (blank and test) was measured over a 10 minute incubation period. (Details as for Table 32.) The final Hb concentrations in the assay systems are shown.

One further experiment was performed in which Hb concentrations of all solutions to be used were the same. This entailed diluting each haemolysate prepared from RBC which had been incubated without H_2O_2 (BL) until the Hb concentration equalled that of the equivalent test solution. Table 36 shows results of peroxide estimations made on RBC incubated with and without 10 mM-H_2O_2 from 3 subjects. The degree of oxidation of NADPH together with the Hb concentrations of all solutions assayed, are shown. No oxidation of NADPH was observed in the control blank and control test solutions. The concentrations of Hb in the test solutions were similar to those in the experiment described in Table 33, and similar results were obtained. The Hb concentrations in the blank solutions, however, were considerably less than those used in the experiment described in Table 35 and the effect of this was to increase the degree of oxidation of NADPH to a similar extent to that seen in the test solutions.

These results suggested that the Hb concentration in the assay solution was having an indirect effect on the stability of the reagents and affecting the apparent concentration of peroxide present in the blank and test solutions. In other words, the peroxide detected in the test systems was probably unrelated to the prior exposure of the red cells to peroxide.

H. Effects of Riboflavin Supplementation on Some Biochemical and Haematological Properties of RBC

Studies of RBC of different ages suggested that old RBC with low GR activity are more susceptible to the effects of oxidant stress *in vivo* (p.159) and *in vitro* (p.176) than are young cells, and that marginally riboflavin-deficient subjects have a reduced proportion of old cells when compared with normal subjects. The effects of riboflavin repletion on GR activity of unfractionated RBC have been fairly well documented (Glatzle, Körner, Christeller and Wiss, 1970; Beutler, 1969a,b)but there is no published information regarding the effects of dietary riboflavin repletion on the activity of GR in RBC of different ages. Intact RBC have been shown to synthesize FAD *in vitro* (Mandula and Beutler, 1970), but it is not known whether young and old cells can synthesize FAD with equal ability.

In the following experiment, red blood cell GR activity and susceptibility to oxidant stress was examined in cells of different ages from a marginally B_2-deficient subject receiving oral B_2 supplementation. The effects of riboflavin supplementation on the relative proportions of old and young RBC in the samples were also investigated.

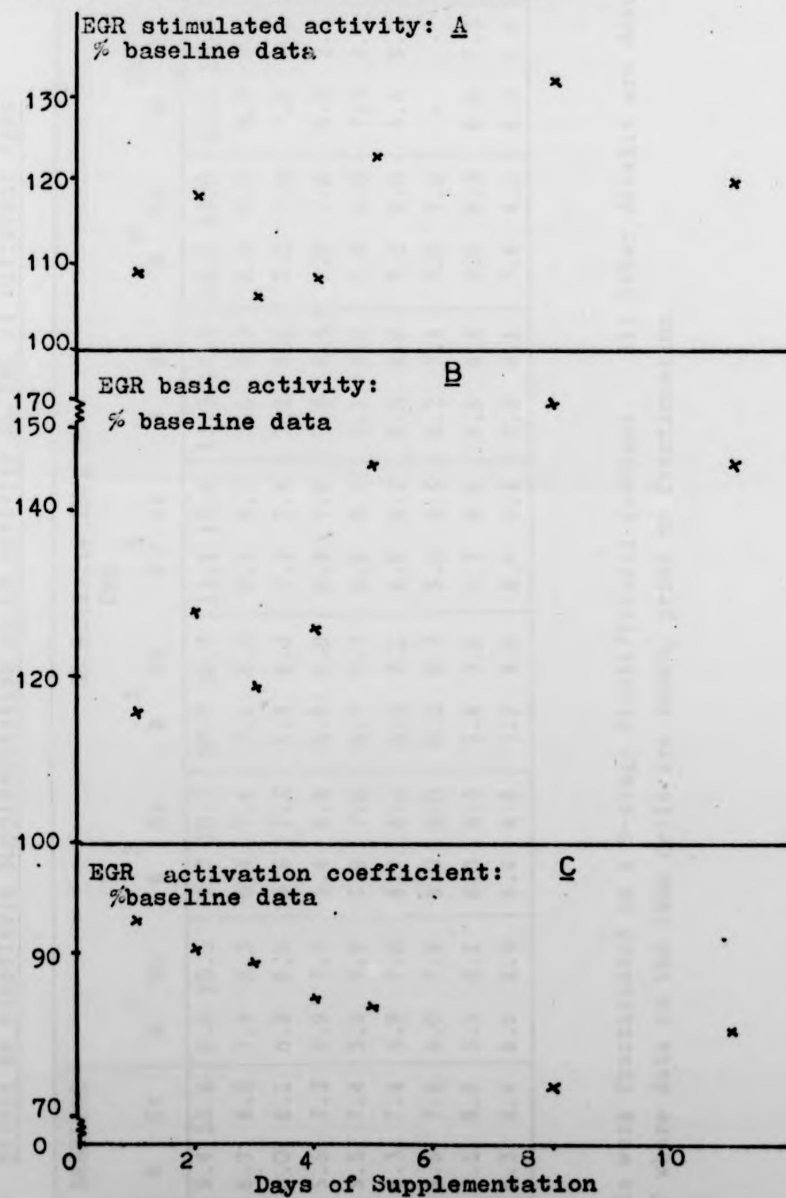
A preliminary short-term experiment was carried out during which the effects of 1 mg riboflavin per day on GR and AST activity in young and old cells was studied and

Table 37: Effect of an Oral Supplementation of 1 mg Riboflavin per Day on EGR Activity and EGR-AC in a Marginally Riboflavin-Deficient Subject

		(DAY)							
	Baseline	1	2	3	4	5	8	11	32
IU_B	5.7 ± 0.49	6.6	7.3	6.8	7.2	8.3	9.7	8.3	6.5
IU_{St}	7.9 ± 0.68	8.6	9.3	8.4	8.5	9.7	10.4	9.5	7.6
AC	1.39 ± 0.10	1.3	1.27	1.24	1.18	1.17	1.07	1.14	1.17

EGR activity (I.U./g Hb) was measured with ($I.U._{St}$) and without ($I.U._B$) added FAD in haemolysates of unfractionated RBC during a period of riboflavin supplementation. The assay was carried out in a system containing 0.89 mM-GSSG, 2.0 mM-EDTA, 0.1 M potassium phosphate buffer pH 7.4, in a reaction initiated by 80 μM-NADPH and followed at 334 nm at 37°C with or without 3 μM-FAD. The subject received 1 mg B_2/day until day 11 when no further supplement was given. The baseline value represents the mean (± SEM) of 3 measurements made on 3 separate occasions prior to supplementation. The enzyme activity and activation coefficient are shown.

Figure 49 Effect of Riboflavin Supplementation on EGR Activity and Activation Coefficient in Blood from a Marginally Riboflavin Deficient Subject



('baseline data' refers to pre-supplementation value)

Table 38: Effect of Riboflavin Supplementation on GR Activity in RBC of Different Ages

FRACTION	BASELINE		GR Activity IU/g Hb Day															
			1		2		3		4		5		8		11		32	
	B	St	B	St	B	St	B	St	B	St	B	St	B	St	B	St	B	St
1	9.4	10.6	9.9	10.3	9.7	10.7	10.7	10.7	11.1	12.6	13.3	12.4	12.2	13.0	15.1	16.3	13.0	13.8
2	6.9	8.8	7.5	9.3	6.4	7.4	7.1	8.0	8.1	8.7	8.1	8.8	8.4	8.7	8.3	9.2	7.6	8.4
3	6.0	8.1	6.9	9.5	5.5	7.3	5.4	6.2	7.2	7.4	7.2	8.0	7.3	7.8	7.0	8.2	7.1	8.3
4	5.5	7.3	5.9	7.7	5.4	6.8	5.4	6.9	6.8	7.6	7.3	8.5	7.0	7.6	6.8	7.3	5.8	7.1
5	5.5	7.4	5.9	7.9	5.9	7.6	5.7	7.7	6.8	8.7	7.1	8.0	7.4	8.0	7.3	8.2	6.3	7.9
6	5.3	7.4	5.9	7.6	5.7	8.2	5.8	7.1	6.9	9.2	6.6	8.0	7.2	8.0	6.6	8.0	5.9	7.7
7	5.5	7.6	6.0	7.9	6.0	8.0	6.2	8.2	5.9	8.3	6.2	7.4	7.0	7.8	-	-	5.7	7.6
8	6.1	8.9	5.8	8.1	6.0	8.3	5.8	7.9	5.7	8.1	6.5	8.8	8.0	9.3	6.9	7.3	6.1	7.7
9	6.3	8.4	6.6	8.4	6.4	8.9	7.7	8.9	8.4	9.3	7.2	8.1	7.6	8.2	6.3	7.5	6.2	8.1

Red cells were fractionated on a 9-stage Ficoll/Triosil gradient. All other details are described in Table 37 where data on the same cells are shown, prior to fractionation.

Table 39: Effect of Oral Supplementation of Riboflavin on the Activation Coefficient of EGR in Fractions of RBC of Different Mean Ages

Fraction	Base-line	Activation Coefficient Day of Experiment							
		1	2	3	4	5	8	11	32
1	1.13	1.04	1.10	1.10	1.14	0.93	1.07	1.08	1.06
2	1.28	1.24	1.16	1.13	1.07	1.09	1.04	1.11	1.11
3	1.35*	1.38*	1.33*	1.15	1.03	1.11	1.07	1.17	1.17
4	1.33*	1.31*	1.26	1.28	1.12	1.16	1.09	1.07	1.22
5	1.35*	1.34*	1.29	1.35*	1.28	1.13	1.08	1.12	1.25
6	1.40*	1.29	1.44*	1.22	1.33*	1.21	1.13	1.21	1.32*
7	1.39*	1.32*	1.33*	1.32*	1.41*	1.19	1.11	-	1.33*
8	1.46*	1.40*	1.38*	1.36*	1.42*	1.35*	1.16	1.10	1.27
9	1.33*	1.27	1.28	1.16	1.11	1.13	1.12	1.19	1.31*

EGR activity was measured in the presence (stimulated) and absence (basic) of added FAD in haemolysates of RBC of different ages during a period of riboflavin supplementation. (Details of the method are given in Table 37.)

The activation coefficient was calculated as:

$$\frac{\text{stimulated EGR activity}}{\text{basic EGR activity}}$$

The supplement was given from day 1 until day 11 inclusive; the baseline data were obtained prior to supplementation.

* denotes an EGR-AC ≥ 1.30.

compared with a baseline value consisting of the mean of 3 measurements made prior to supplementation.

The basic and stimulated activities of GR, and the activation coefficient in unfractionated RBC showed an immediate response to the supplement and over the 11 days the basic GR activity rose to 180% of the baseline value and the stimulated activity to 132% (Figure 49 and Table 37). Concomitant with the rise in EGR activity was a reduction in AC to 77% of the baseline value. During the post-supplementation period GR activities fell and the AC started to rise again. Thus, by day 32 from the initiation of the experiment the basic and stimulated activities had fallen again to 114% and 96% respectively of the baseline value and the AC had risen to 84% of the baseline value.

Studies of RBC of different ages showed an immediate response by all cells to supplementation such that after 24 hours from the first dose of riboflavin the GR activity had risen (Table 38) with a concomitant reduction in the AC (Table 39). If an AC of ≥ 1.30 is taken as a reflection of riboflavin depletion, Table 39 illustrates that the higher the AC of a fraction of cells prior to supplementation then the longer it takes for cells in that fraction to recover from their depleted state. On day 32, 21 days after cessation of supplementation, it was interesting to note that the older cell fractions had again reverted to a riboflavin-depleted state, with AC ≥ 1.30, whereas the AC in the youngest 3 fractions had

Table 40: Effect of Riboflavin Supplementation on the Amount of Unsaturated ApoEnzyme of EGR in Fractions of RBC of Different Mean Ages

Fraction	Base-line	Day 1	2	3	4	5	8	11	32
1	1.2	0.4	1.0	0	1.5	0	0.8	1.2	0.8
2	1.9	1.8	1.0	0.9	0.6	0.7	0.3	0.9	0.8
3	2.1	2.6	1.8	0.8	0.2	0.8	0.5	1.2	1.2
4	1.8	1.8	1.2	1.5	0.8	0.8	0.6	0.5	1.3
5	1.9	2.0	1.7	2.0	1.9	0.9	0.6	0.9	1.6
6	2.1	1.7	2.5	1.3	2.3	1.4	1.1	1.4	2.1
7	2.1	1.6	2.0	2.0	2.4	1.2	0.8	-	1.9
8	2.8	2.3	2.3	2.1	2.4	2.3	1.3	0.7	1.6
9	2.1	1.8	1.5	1.2	0.9	0.9	0.6	1.2	1.9

EGR activity was measured with (stimulated) and without (basic) added FAD in haemolysates of RBC of different ages during a period of riboflavin supplementation. (For details see Table 37). Unsaturated apoenzyme was calculated as: stimulated EGR activity (IU/g Hb) - basic EGR activity (IU/g Hb).

Table 41: AST Activity and Activation Coefficient in RBC of Different Ages During Riboflavin Supplementation

Fraction	Baseline			Day 1			Day 2			Day 3		
	B	St	AC	B	St	AC	B	St	AC	B	St	AC
1	6.9	9.0	1.4	8.7	9.1	1.0	5.6	8.3	1.5	5.3	8.9	1.7
2	3.5	7.5	2.1	5.0	7.5	1.5	4.7	5.6	1.2	4.3	5.6	1.2
3	2.1	3.2	1.5	4.0	6.3	1.6	2.6	5.4	2.1	3.0	5.5	1.8
4	2.4	4.5	1.9	2.8	5.7	2.0	2.4	3.8	1.6	2.5	5.5	2.2
5	1.3	3.0	7.3	2.2	4.8	2.2	2.3	3.9	1.7	2.0	3.7	1.9
6	2.3	5.0	2.2	2.2	5.6	2.5	2.8	3.4	1.2	1.9	4.1	2.2
7	2.8	6.0	2.1	1.3	4.9	2.7	1.6	3.1	1.9	2.1	4.4	2.1
8	4.7	5.0	1.1	2.3	5.1	2.2	3.4	3.7	1.1	2.3	4.3	1.9
9	2.4	4.9	2.0	2.3	5.0	2.2	1.3	4.3	3.3	1.2	3.1	2.6
Unfractionated	2.4	5.1	2.1	2.7	5.4	2.0	2.7	5.0	1.9	2.7	5.8	2.1

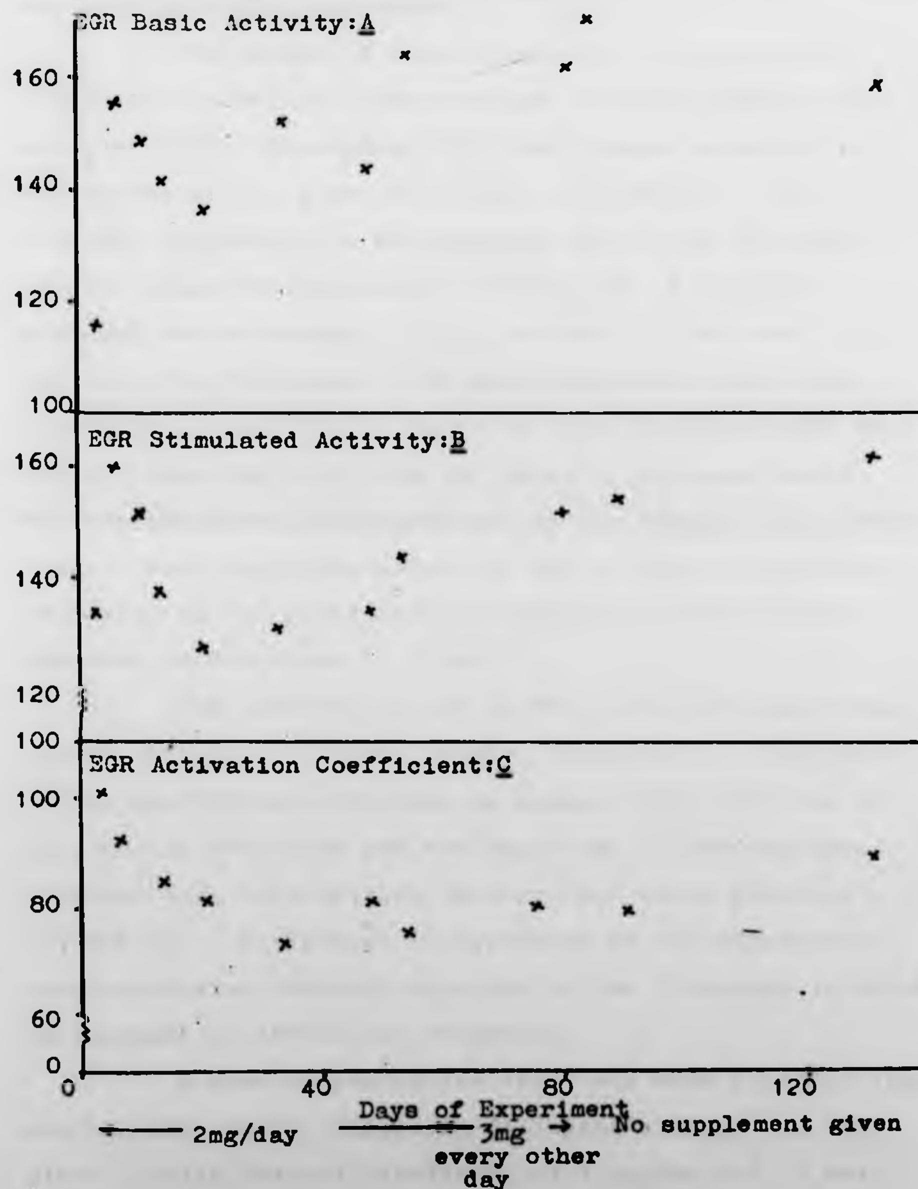
Fraction	Day 4			Day 5			Day 8			Day 11			Day 32		
	B	St	AC	B	St	AC	B	St	AC	B	St	AC	B	St	AC
1	7.8	11.9	1.5	6.8	9.9	1.5	5.7	9.3	1.6	7.9	10.7	1.4	5.6	4.5	0.8
2	4.6	7.2	1.6	3.9	6.6	1.7	4.6	8.6	1.9	4.6	6.9	1.4	5.1	5.1	1.0
3	2.8	5.3	1.9	2.8	5.6	2.0	3.8	6.9	1.8	3.3	4.6	1.4	3.3	4.5	1.4
4	2.3	4.8	2.1	2.5	5.3	2.1	2.9	5.9	2.0	2.4	4.4	1.8	2.4	4.6	1.9
5	2.4	4.8	2.0	2.3	4.9	2.1	3.0	5.5	1.8	1.8	3.8	2.1	2.1	3.6	1.7
6	2.0	4.6	2.3	2.1	4.3	2.1	2.5	5.4	2.2	2.1	3.6	1.7	2.4	4.0	1.7
7	1.8	4.4	2.4	2.6	6.9	2.7	2.3	4.9	2.1	2.9	3.9	1.3	1.8	3.5	1.9
8	2.5	4.4	1.8	2.8	5.3	1.9	3.4	4.9	1.4	-	-	-	2.8	3.2	1.1
9	2.0	2.7	1.4	4.1	8.2	2.0	2.6	5.0	1.9	2.4	3.2	1.3	2.0	3.5	1.8
Unfractionated	2.0	3.8	1.9	3.6	7.0	1.9	3.7	6.6	1.8	2.9	5.3	1.8	2.6	3.9	1.5

The basic (B) and Stimulated (St) activities of AST, and AST activation coefficient (AC) were measured in RBC of different ages during a period of riboflavin supplementation (from day 1 to 11) and post supplementation (day 32). The baseline data represent the means of 3 determinations made within 1 month prior to supplementation.

The assay system consisted of 0.125 M-aspartic acid, 0.1 ml haemolysate, 0.5 U malate dehydrogenase, and 0.117 M-NADH in 0.1 M-KH_2PO_4 buffer pH 7.4. The reaction was initiated by 6.7 mM oxoglutaric acid and NADH oxidation followed at 340 nm at 35°C.

Enzyme activities are expressed as IU/g Hb.

Figure 50 Effect of Riboflavin Supplementation on EGR Activity and Activation Coefficient



The activity of EGR was measured in RBC in vitro with (stimulated) and without (basic) added FAD during a period of riboflavin supplementation. Values are expressed as a % of the baseline value (prior to supplementation)

remained virtually unchanged.

The amount of unsaturated GR i.e. apoenzyme (Table 40) in RBC of different ages showed a trend during supplementation consistent with the changes observed in holoenzyme activity and AC (Tables 38 and 39). There is a slight suggestion in the baseline data that the amount of free apoenzyme increases with the age of the cell, although the difference is only evident in the very young and very old fractions. On supplementation with riboflavin the younger cells appear to take up riboflavin more rapidly than the older ones as shown by the more rapid fall in apoenzyme concentrations in the younger cells with time. Post-supplementation, by day 32 the apoenzyme had increased in the older cells, whereas no such rise was observed in Fractions 1, 2 and 3.

The activity of AST in RBC during the supplementation showed no striking changes (Table 41). The activation coefficients did show an overall drop over the 11 days in all fractions but the magnitude of the decreases observed were considerably smaller than those observed for EGR-AC. A response to cessation of the riboflavin supplementation was only observed in two fractions.in which an increase in AST-AC was observed.

A more comprehensive study was made on blood from another marginally riboflavin-deficient subject who was given a daily dose of riboflavin of 2 mg/day for 50 days and then 3 mg every other day for the next 30 days.

Figure 50 shows the effect of this regime on GR

Table 42: Effect of Riboflavin Supplementation on EGR Activity and Activation Coefficient in RBC of Different Ages

GR activity IU/g Hb

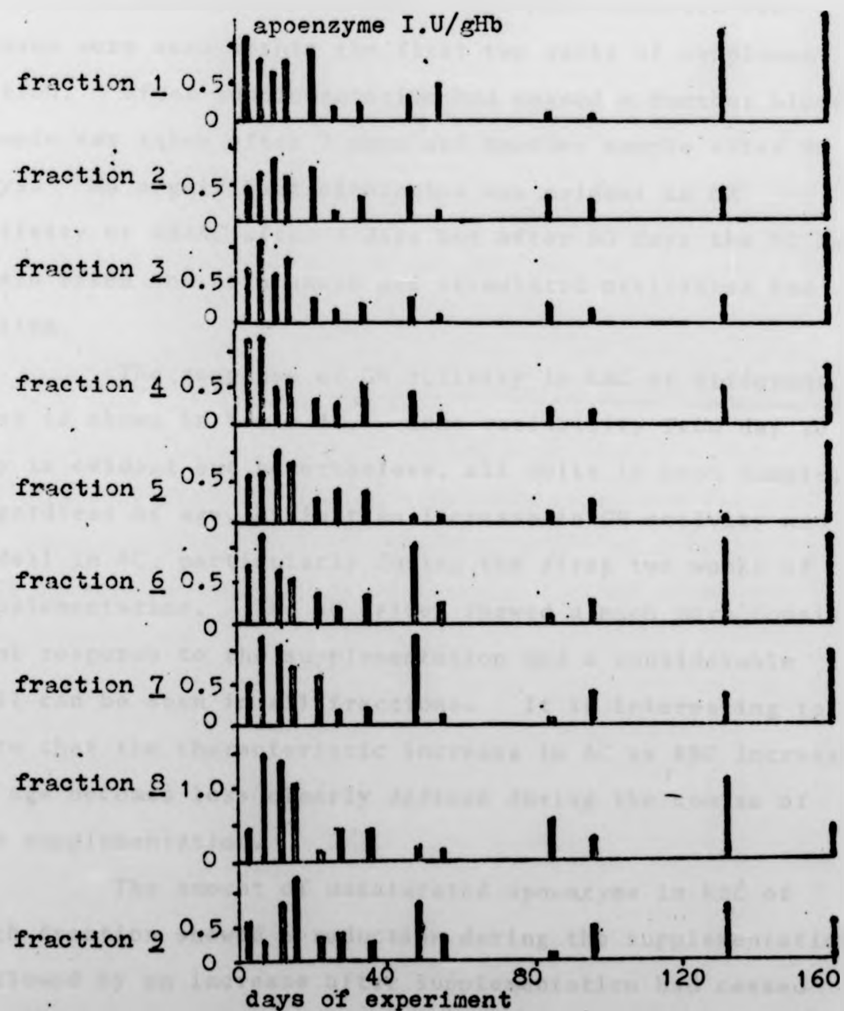
Fraction		Baseline	Day of Study 4	7	11	14	21	26	34	48	54	81	84	96	132
1	B	6.6	7.2	9.9	6.1	6.9	14.2	7.4	14.8	6.5	11.1	14.5	13.8	7.8	8.7
	St	8.1	8.0	10.8	6.8	7.7	15.2	7.5	15.0	6.8	11.6	14.5	13.5	7.6	10.0
	AC	1.23	1.11	1.09	1.12	1.11	1.02	1.0	1.01	1.05	1.0	1.0	1.0	1.0	1.17
2	B	5.3	5.5	6.4	6.0	5.5	4.6	4.9	5.6	7.7	7.5	5.7	7.3	4.5	5.8
	St	6.3	6.2	7.1	7.5	6.1	5.4	4.9	5.9	8.1	7.7	6.1	7.6	4.7	6.2
	AC	1.19	1.13	1.11	1.13	1.11	1.18	1.0	1.05	1.05	1.02	1.03	1.04	1.04	1.08
3	B	5.1	5.0	6.6	6.3	5.5	4.4	4.6	3.9	5.4	6.2	6.9	6.8	4.2	6.0
	St	6.0	6.6	7.4	7.0	6.3	4.7	4.7	4.1	5.7	6.3	7.7	7.0	4.3	6.3
	AC	1.18	1.32	1.12	1.11	1.13	1.07	1.07	1.05	1.05	1.02	1.12	1.03	1.03	1.05
4	B	4.6	4.8	5.5	6.1	5.5	6.1	4.5	4.5	5.8	6.7	7.0	6.0	4.1	4.8
	St	5.8	5.8	6.8	6.7	6.2	6.4	4.8	5.0	6.2	6.8	7.9	6.2	4.2	5.3
	AC	1.26	1.21	1.23	1.11	1.12	1.05	1.06	1.09	1.07	1.01	1.13	1.03	1.0	1.09
5	B	4.4	4.9	7.1	6.7	5.4	5.2	5.1	3.6	6.1	6.7	6.2	6.8	5.5	5.6
	St	5.6	5.6	7.8	8.1	6.2	5.5	5.5	4.0	6.1	6.8	6.5	6.9	5.8	5.7
	AC	1.27	1.14	1.11	1.21	1.15	1.08	1.08	1.10	1.0	1.02	1.05	1.01	1.11	1.02
6	B	4.3	4.9	6.6	6.5	6.3	4.8	5.7	5.5	5.5	6.6	5.6	5.9	4.3	6.1
	St	5.6	6.1	8.4	7.3	6.8	5.2	5.9	5.9	6.6	6.9	6.0	5.8	4.6	7.2
	AC	1.3	1.24	1.26	1.12	1.07	1.09	1.09	1.06	1.11	1.02	1.07	1.0	1.08	1.16
7	B	4.6	5.6	-	6.7	6.2	5.9	5.5	4.4	5.7	6.8	6.0	6.7	4.5	5.9
	St	6.3	7.8	-	7.9	7.0	6.6	5.7	4.6	7.0	6.9	6.2	6.4	4.9	6.3
	AC	1.37	1.39	-	1.18	1.13	1.19	1.04	1.05	1.23	1.02	1.03	1.0	1.08	1.22
8	B	4.5	5.2	-	7.8	7.3	5.0	7.0	5.0	7.2	6.2	7.4	6.0	5.4	6.2
	St	5.6	6.7	-	8.8	8.4	5.6	7.4	5.4	7.8	6.4	7.5	6.6	5.7	7.4
	AC	1.24	1.29	-	1.13	1.15	1.12	1.06	1.09	1.08	1.04	1.01	1.1	1.08	1.19
9	B	5.1	-	-	7.6	7.3	5.9	5.8	4.7	6.4	6.2	6.2	7.4	5.1	6.6
	St	6.8	-	-	8.3	8.4	6.7	6.2	4.9	7.3	6.5	6.5	7.4	5.7	7.4
	AC	1.33	-	-	1.09	1.15	1.14	1.08	1.05	1.15	1.06	1.05	1.0	1.1	1.13
U	B	4.3	5.0	6.7	6.4	6.1	5.9	4.9	6.6	6.2	7.1	7.0	7.4	4.6	6.9
	St	5.0	6.7	8.5	7.6	6.9	6.4	5.1	6.8	6.7	7.2	7.6	7.7	4.9	8.1
	AC	1.35	1.34	1.26	1.19	1.13	1.09	1.04	1.03	1.08	1.02	1.09	1.04	1.08	1.18

EGR activity was measured with (St) and without (B) added FAD in RBC of different ages, during a period of *in vivo* riboflavin supplementation and EGR-AC calculated. (For assay details see Table 37).

The supplementation regime consisted of: 2 mg riboflavin/day from day 1 to 50, followed by 3 mg every second day until day 80 when the supplementation ceased.

The mean values of 3 determinations made on different samples within the 3 weeks prior to supplementation are shown as the baseline data.

Figure 51 Effect of Riboflavin Supplementation on the Amount of Unsaturated Apoenzyme of EGR in RBC of Different Ages.



EGR activity was measured with(stimulated) and without (basic) added FAD in RBC of different ages during riboflavin supplementation.(for details see table 42). Unsaturated apoenzyme was calculated as: stimulated-basic activity(IU/gHb

activity and AC in unfractionated RBC. The same trends are seen as were described for the previous case, that is, an increase in basic and stimulated activity and a fall in the activation coefficient. The most dramatic responses were seen within the first two weeks of supplementation. After supplementation had ceased a further blood sample was taken after 3 days and another sample after 90 days. No significant alteration was evident in GR activity or GR-AC after 3 days but after 90 days the AC had again risen and both basic and stimulated activities had fallen.

The response of GR activity in RBC of different ages is shown in Table 42. Some variability from day to day is evident but nevertheless, all cells in each sample, regardless of age, exhibit an increase in GR activity and a fall in AC, particularly during the first two weeks of supplementation. The AC values showed a much more consistent response to the supplementation and a considerable fall can be seen in all fractions. It is interesting to note that the characteristic increase in AC as RBC increase in age becomes less clearly defined during the course of the supplementation.

The amount of unsaturated apoenzyme in RBC of each fraction showed a reduction during the supplementation, followed by an increase after supplementation had ceased (Figure 51). The general pattern of response of RBC GR apoenzyme was the same regardless of the age of the cell.

Table 43: Effect of Riboflavin Supplementation on the PCV and MCHC of Blood from a Marginally Riboflavin-Deficient Subject

	Duration of Experiment (days)										
	Baseline	4	7	11	14	21	26	34	48	54	81
MCHC	32 ± 0.36	34	35	35	36	31	35	33	33	34	34
PCV	35 ± 0.13	35	36	38	38	39	38	37	38	41	41

The MCHC and PCV were determined in blood from a marginally riboflavin-deficient subject during a period of supplementation. (For details of supplementation see Table 42.) The baseline data represent the mean ± SEM of 3 measurements made on separate occasions during the 3 weeks prior to supplementation.

Table 44: The Relation Between EGR-AC of Unfractionated RBC and RBC Distribution Through a Density Gradient During Riboflavin Supplementation

Day of Experiment	EGR-AC	% Hb Fractions			
		1+2	3+4	5+6	7+8
Baseline	1.35	28.3	48.9	15.8	3.7
4	1.34	51.5	42.3	9.8	1.5
7	1.26	23.5	48.1	19.4	4.7
11	1.19	23.9	64.1	5.1	1.7
14	1.13	28.3	53.4	10.6	5.5
21	1.09	6.7	64.5	18.8	6.3
26	1.06	23.6	53.3	12.8	8.1
34	1.03	8.3	54.3	25.9	6.3
48	1.08	55.4	34.4	5.0	3.3
54	1.02	42.8	42.5	12.5	5.9
81	1.09	32.8	46.8	10.8	5.2
84	1.06	33.4	50.6	6.5	5.2
96	1.08	40.3	42.6	10.4	4.3
112	1.19	17.1	62.7	16.9	2.0
132	1.18	35.1	32.3	22.8	7.0

At intervals during a period of riboflavin supplementation RBC were separated into 9 fractions of different mean ages, and the concentration of Hb in each fraction expressed as a % of the total Hb recovered from the gradient (Methods: E) Data from individual fractions were pooled as shown in the table, and a regression analysis performed on the EGR-AC of unfractionated RBC and, during the period of supplementation up to day 34, on the proportion of the Hb in pooled fractions. A significant correlation was obtained between EGR-AC and the proportion of young cells (Fractions 1 and 2).

Regression equation:

% young cells = EGR-AC (-71.908) + 81.414 .

$r = 0.733$, $p = < 0.05$.

(For details of the enzyme assay and derivation of EGR-AC see Table 37.)

Table 45: Effect of Riboflavin Supplementation on GSH Concentrations in RBC of Different Ages

Fraction	GSH μMoles/g Hb				
		Day			
	Baseline	4	34	54	120
1	6.32	6.40	10.64	7.84	6.40
2	5.76	5.83	8.53	7.06	5.90
3	5.62	5.62	7.66	6.72	5.90
4	5.60	5.71	7.04	6.35	6.40
5	5.40	5.43	6.72	5.76	5.72
6	3.82	3.97	6.63	5.37	5.01
7	3.04	3.14	4.99	4.08	4.02
8	3.06	3.05	4.63	3.74	3.63
9	2.98	2.96	3.75	3.45	3.15
		$p<0.02$	$p<0.001$	$p<0.001$	$p<0.01$

GSH concentration was measured (Methods: G) in RBC of different ages during a period of riboflavin supplementation. (For details of regime see Table 42.) GSH is expressed as μMoles/g Hb. A paired t-test was performed on baseline data in comparison with data obtained on all other days, and the levels of significance are shown.

During the first three weeks the apoenzyme showed a dramatic reduction and remained low for the remainder of the supplementation.

Haematological changes:

PCV and MCHC were measured in unfractionated blood on 3 separate occasions prior to supplementation, and the means were used as baseline values. The whole blood PCV increased steadily over the course of the supplementation, from 35 up to 41 on day 81 (Table 43). Similarly, a small increase was observed in the MCHC of unfractionated blood, from 32 prior to supplementation up to 34 on day 81.

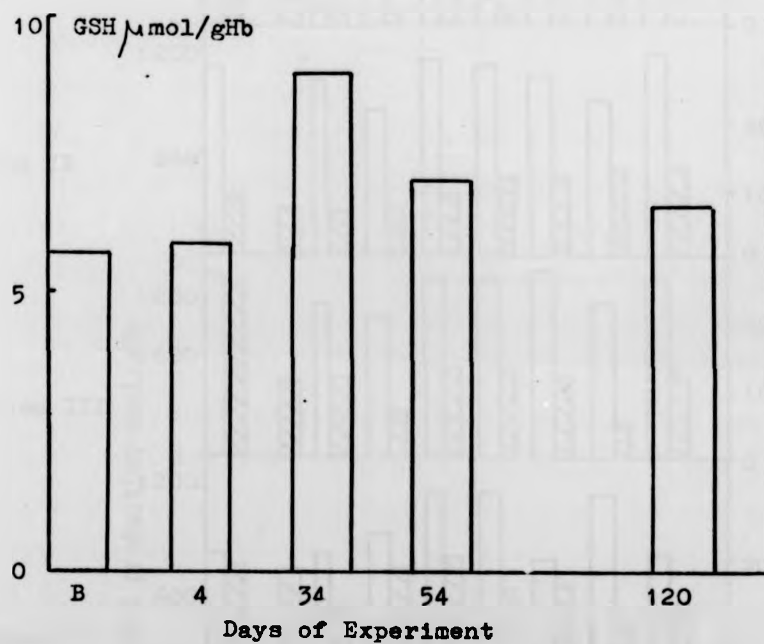
RBC distribution:

During the first 34 days of supplementation the proportion of young cells in the samples of blood taken showed a significant reduction (Table 44). The percentage of cells comprising Fractions 1 and 2 showed a significant correlation with the EGR-AC of the unfractionated blood ($r = 0.728$, $p < 0.05$). After day 34 a sudden and very marked increase in the proportion of cells in Fractions 1 and 2 rose above that observed at the initiation of the experiment.

GSH concentrations:

On selected days the GSH concentrations were measured in RBC of different ages (Table 45) and in

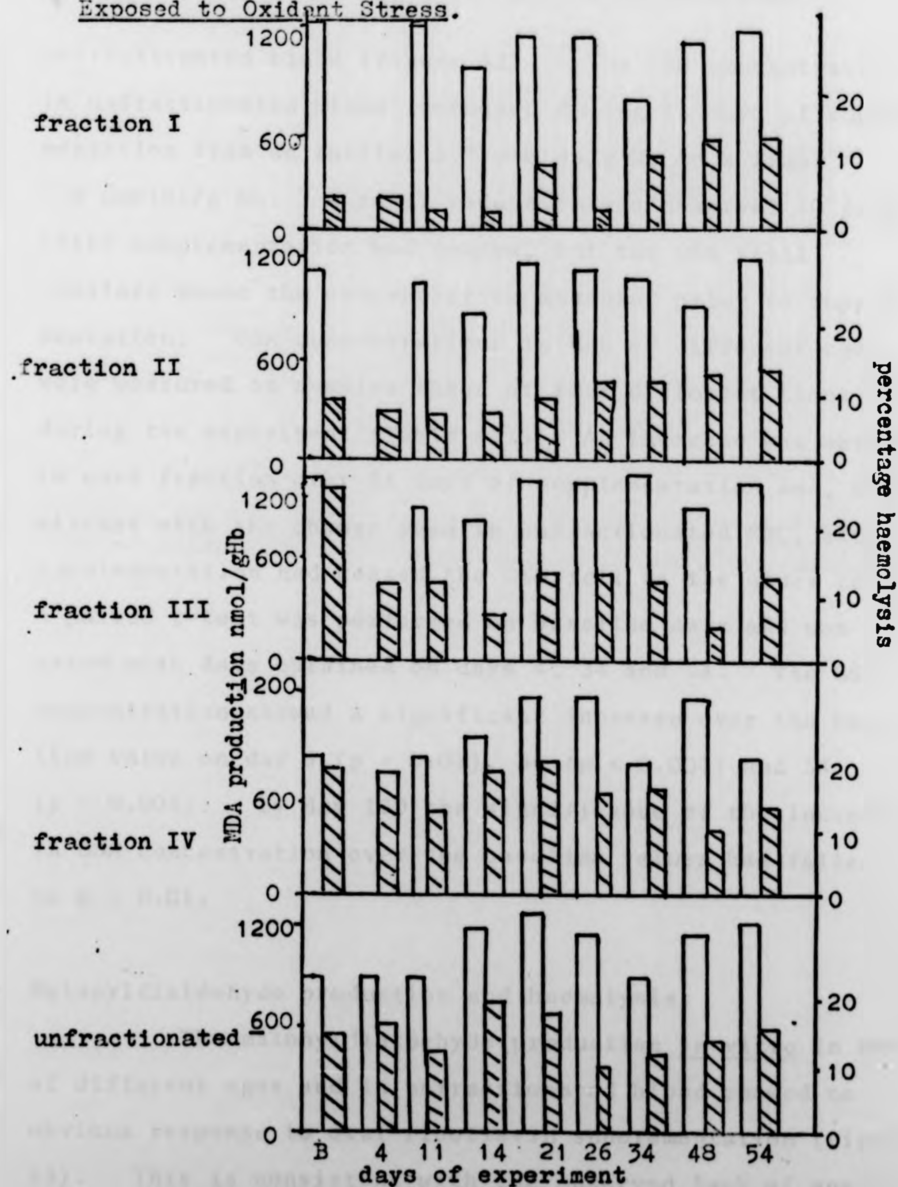
Figure 52 Effect of Riboflavin Supplementation on RBC GSH Concentrations



GSH concentration was measured in unfractionated RBC During a period of Riboflavin Supplementation (For details of regime see Table 42)

B = Baseline data

Figure 53 Effect of Riboflavin supplementation on Malonyldialdehyde Production and Haemolysis of RBC Exposed to Oxidant Stress.



At intervals during a period of riboflavin supplementation RBC were separated into fractions of progressively greater mean age (fractions I-IV) and suspensions of the cells incubated with 10mM-H_2O_2 (see figure 44). MDA and % haemolysis of RBC were measured.

unfractionated blood (Figure 52). The GSH concentration in unfractionated blood increased during 54 days of supplementation from an initial 5.7 µmoles/g Hb to a final 7.0 µmoles/g Hb. A small reduction was observed 40 days after supplementation had ceased, but the GSH still remained above the concentration measured prior to supplementation. GSH concentrations in RBC of different ages were measured on samples taken at four different times during the experiment (Table 45). An increase was observed in each fraction over 54 days of supplementation and, consistent with the change seen in unfractionated RBC, after supplementation had ceased the GSH fell in the older cells. A paired t-test was performed on baseline data and compared with data obtained on days 4, 34 and 54. The GSH concentration showed a significant increase over the baseline value on day 4 ($p < 0.02$), 34 ($p < 0.001$) and 54 ($p < 0.001$). By day 120 the significance of the increase in GSH concentration over the baseline values had fallen to $p < 0.01$.

Malonyldialdehyde production and haemolysis:

The malonyldialdehyde production *in vitro* in RBC of different ages and in unfractionated blood showed no obvious response to oral riboflavin supplementation (Figure 53). This is consistent with the observed lack of any differences in the amount of MDA produced in RBC from normal and marginally riboflavin-deficient subjects (p.170)

Lower concentrations of MDA were detected in older cells than young cells in each sample of blood tested. Similarly the degree of haemolysis exhibited by RBC was not significantly altered by riboflavin supplementation in either RBC of different ages or in unfractionated blood. During the first 34 days of supplementation the older cells underwent a greater degree of haemolysis than the young cells. However, in two blood samples taken whilst supplementation was still in progress (days 48 and 54) the cells in Fraction I showed a sudden and marked increase in haemolysis. This increase in haemolysis coincided with the dramatic rise in the proportion of young cells in these samples (Table 44).

I. A Computer Model for RBC Survival

A number of erythrocyte enzymes have been shown to fall in activity with increasing RBC age and it has been suggested that the lifespan of the RBC may be determined by a reduction in activity of certain key enzymes (Allison and Burn, 1955; Brok, Ramot, Zwang and Danon, 1966). Although it is known that GSH plays an important role in the maintenance of RBC integrity (Allen and Jandl, 1961; Scheuch et al., 1961) and that EGR is active in the production of GSH, this enzyme has received little attention in studies concerning the possible determinants of RBC survival. These studies showed that with increasing age of a cohort of RBC the activity of GR initially falls exponentially then plateaus and finally there is an increase in activity in the oldest cells (Figures 16B). The activity of AST behaves similarly apart from the increase in the oldest cells of a cohort. (Figure 25)

In order to explain the observed pattern of fall in GR activity a mechanism is proposed and a computer programme was derived to simulate a fall in GR activity and determine its effect on RBC survival of a cohort of cells.

Preliminary calculations were made to determine the mean age of RBC in each fraction recovered from a gradient. The Hb concentration in each fraction is a measure of the proportions of cells of different ages in the respective fractions and reflects the relative

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Figure 54

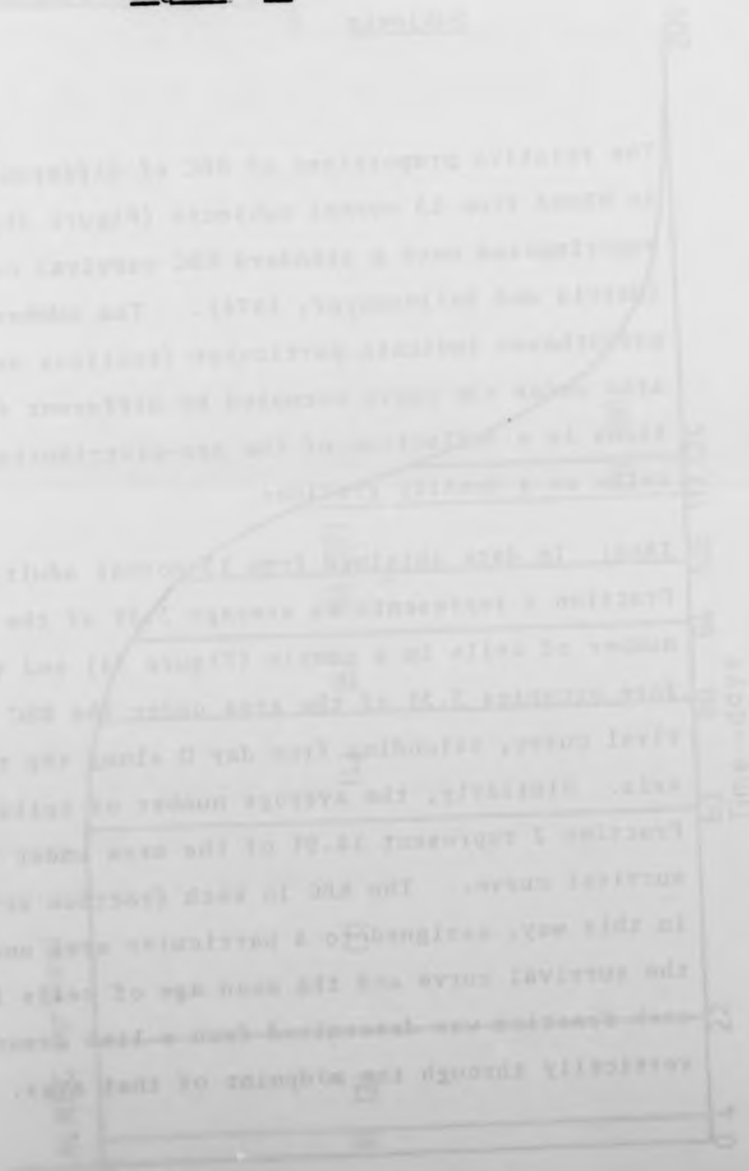


Figure 54: Relative Proportions of RBC of Different Ages in Blood from Normal Subjects

The relative proportions of RBC of different ages in blood from 13 normal subjects (Figure 34) were superimposed onto a standard RBC survival curve (Harris and Kellermeyer, 1974). The numbers in parentheses indicate particular fractions and the area under the curve occupied by different fractions is a reflection of the age-distribution of cells on a density gradient.

Thus: In data obtained from 13 normal adults Fraction 1 represents on average 3.3% of the total number of cells in a sample (Figure 34) and therefore occupies 3.3% of the area under the RBC survival curve, extending from day 0 along the time axis. Similarly, the average number of cells in Fraction 2 represent 14.9% of the area under the survival curve. The RBC in each fraction were, in this way, assigned to a particular area under the survival curve and the mean age of cells in each fraction was determined from a line drawn vertically through the midpoint of that area.

Figure 54 Relative Proportions of RBC of Different Ages in Blood from Normal Subjects.

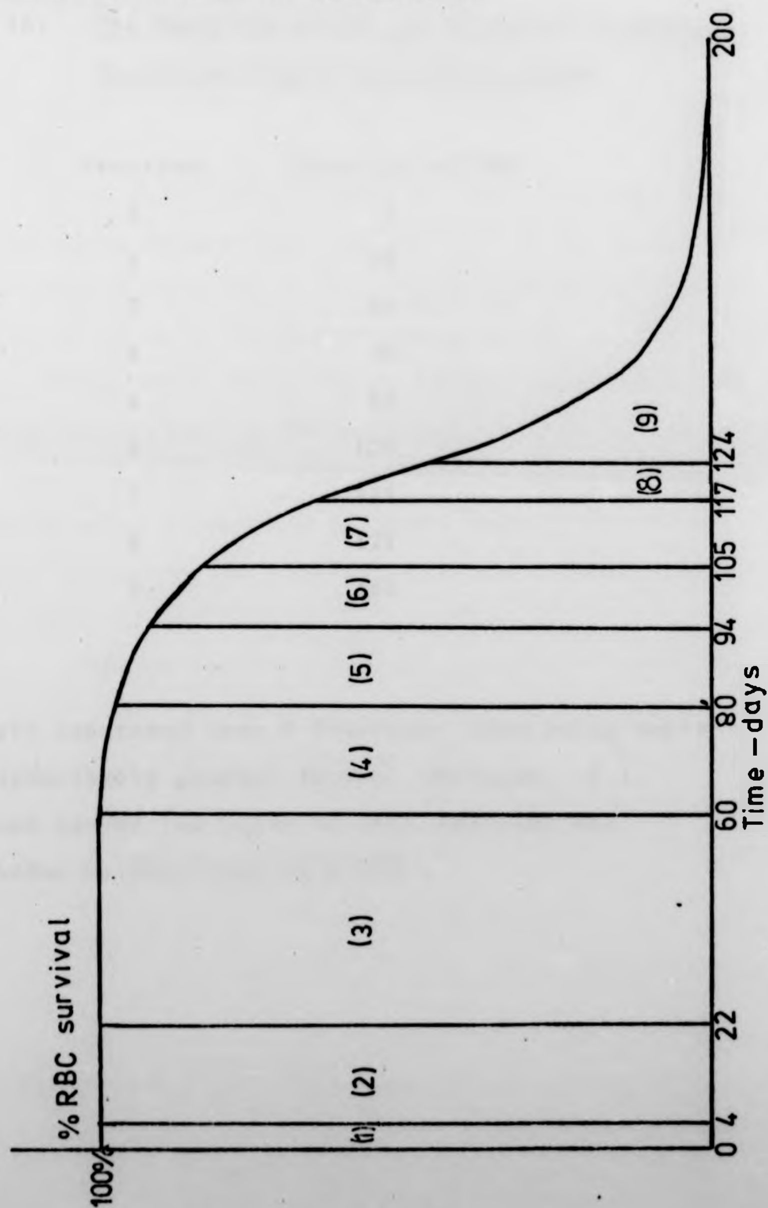


Table 46: The Mean Age of RBC in Different Fractions Recovered from a Density Gradient

Fraction	Mean Age of RBC
1	2
2	13
3	41
4	70
5	87
6	100
7	111
8	121
9	162

RBC were separated into 9 fractions containing cells of progressively greater density (Methods: A.). The mean age of the cells in each fraction was calculated as described on p.226 .

proportions of cells of different ages in a sample of blood. Thus, by superimposing these proportions of cells in each fraction, which were determined experimentally, onto the standard survival curve of a cohort (Figure 54) the mean age of each fraction of RBC can be determined.

The mean ages of cells in each fraction are shown in Table 46 and the profiles for basic enzyme activity of GR and AST against mean cell age were reconstructed using this information (Figure 55). The late secondary rise in activity of GR in RBC older than 100 days and the absence of any such rise in AST activity is clear from the figure. The activity profiles of GR in the presence of excess FAD followed exactly the same pattern, including the significant rise in enzyme activity in old cells (Figure 55).

Fundamental to the model proposed to explain this pattern of activity of GR is the assumption that GR activity is a determinant of RBC survival or is closely related to another such enzyme. If it is assumed that GR activity falls in a single erythrocyte logarithmically with time then:

$$A_t = A_o \exp(-kt) \qquad \text{(Equation 1)}$$

where A_t and A_o are activities at time t and 0 respectively and k is a rate constant for the fall in enzyme activity. If GR activity is essential to the survival of the RBC or

Figure 55

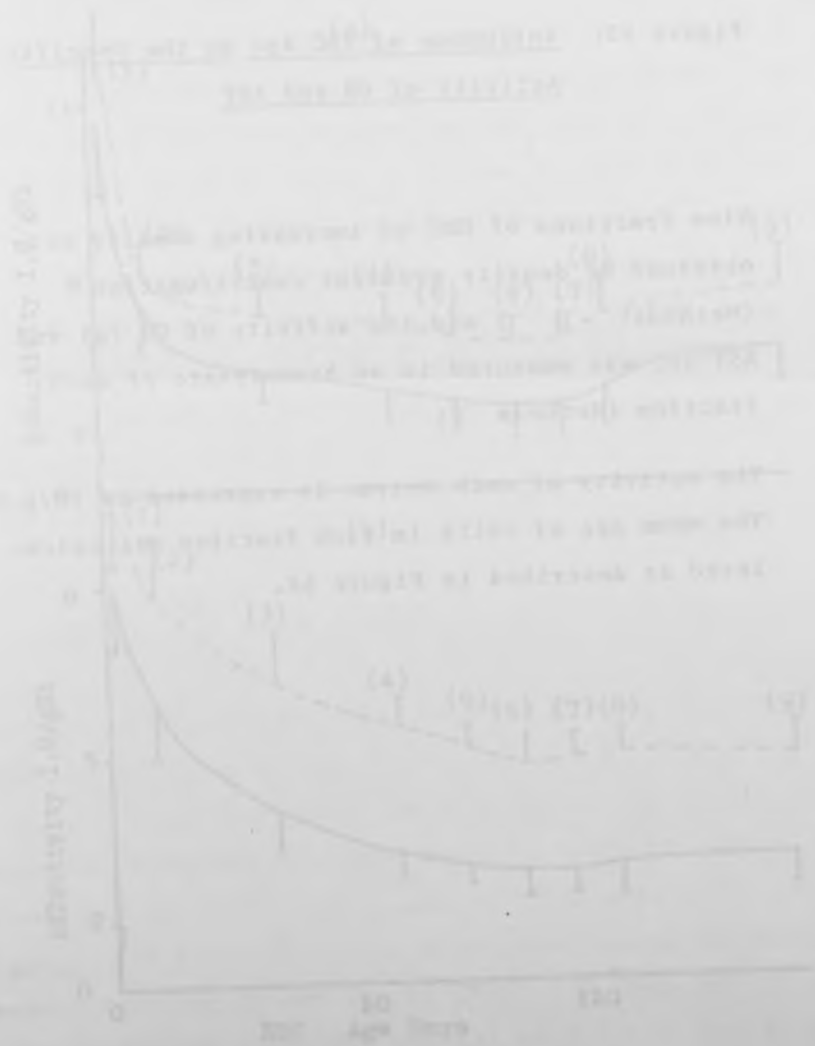
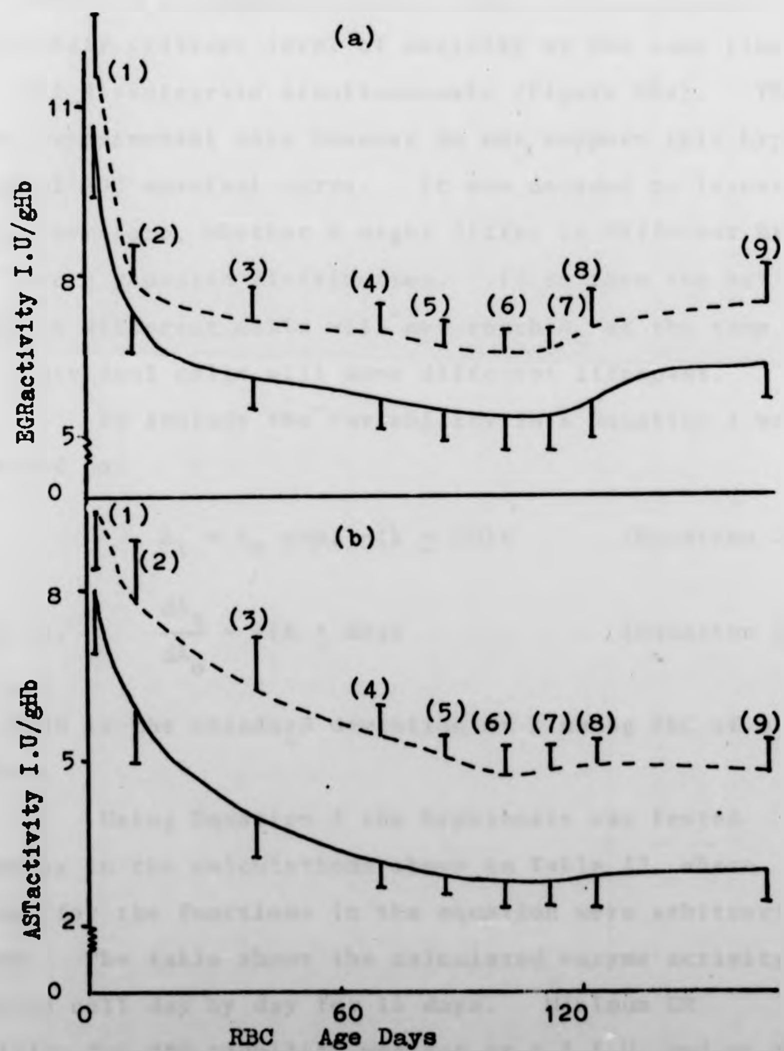


Figure 55: Influence of RBC Age on the Specific Activity of GR and AST

Nine fractions of RBC of increasing density were obtained by density gradient centrifugation (Methods: D) and the activity of GR (a) and AST (b) was measured in an haemolysate of each fraction (Methods F)

The activity of each enzyme is expressed as IU/g Hb. The mean age of cells in each fraction was calculated as described in Figure 54.

Figure 55 Influence of RBC Age on the Specific Activity of Erythrocyte Glutathione Reductase and Aspartate Amino Transferase



is closely linked with the activity of another such enzyme then there must be a critical level of activity below which the cell is not viable. Let this level be A_c. If all the cells in a cohort have the same value for k then Equation 1 suggests that all cells in a cohort will reach their critical level of activity at the same time and will disintegrate simultaneously (Figure 56a). The known experimental data however do not support this hypothetical RBC survival curve. It was decided to investigate, therefore, whether k might differ in different RBC and have a gaussian distribution. If so then the activity of GR in different cells will not reach A_c at the same time and individual cells will have different lifespans.

To include the variability in k Equation 1 was modified to:

$$A_t = A_o \exp. -(k \pm SD)t \qquad \text{(Equation 2)}$$

that is,

$$\frac{dA_t}{dA_o} = -(k \pm SD)t \qquad \text{(Equation 3)}$$

where SD is the standard deviation of k among RBC of a cohort.

Using Equation 2 the hypothesis was tested manually in the calculations shown in Table 47, where values for the functions in the equation were arbitrarily fixed. The table shows the calculated enzyme activity in each cell day by day for 15 days. Minimum GR activity for RBC viability was set at 4.5 I.U. and as the

Table 47: Relationship Between RBC Survival and GR Activity: A Hypothetical Study

K	N	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
4	1	10	9.6	9.2	8.8	8.5	8.2	7.8	7.5	7.2	6.9	6.6	6.4	6.1	5.9	5.7	5.5
6	2	10	9.4	8.8	8.3	7.8	7.3	6.9	6.5	6.1	5.7	5.4	5.1	4.8	4.5		
6	3	10	9.4	8.8	8.3	7.8	7.3	6.9	6.5	6.1	5.7	5.4	5.1	4.8	4.5		
8	4	10	9.2	8.5	7.8	7.2	6.6	6.1	5.6	5.1	4.7						
8	5	10	9.2	8.5	7.8	7.2	6.6	6.1	5.6	5.1	4.7						
8	6	10	9.2	8.5	7.8	7.2	6.6	6.1	5.6	5.1	4.7						
8	7	10	9.2	8.5	7.8	7.2	6.6	6.1	5.6	5.1	4.7						
10	8	10	9.0	8.1	7.3	6.6	5.9	5.3	4.8								
10	9	10	9.0	8.1	7.3	6.6	5.9	5.3	4.8								
12	10	10	8.8	7.7	6.8	6.0	5.3	4.6									
	ΣA	100	92	85	78	72	66	61	48	40	37	17.4	16.6	15.7	10.4	5.7	5.5
	N_T	10	10	10	10	10	10	10	9	7	7	3	3	3	2	1	1
	$\bar{A}$	10	9.2	8.5	7.8	7.2	6.6	6.1	5.3	5.7	5.3	5.8	5.5	5.3	5.2	5.7	5.5

It is assumed that in a cohort of 10 RBC the activity of GR in all cells is initially 10 I.U. and falls at a rate of 8 $\pm$ 2 (SD)% per day. It is also assumed that when the GR activity in an individual cell falls to 4.5 the cell disintegrates. The mean activity in surviving cells and the number of cells surviving over 15 days were calculated,

where, ΣA is the sum of EGR activity in all surviving cells;

N_T is the number of cells of the cohort surviving at any time;

and $\bar{A}$ is the mean EGR activity in surviving cells over the lifespan of the cohort.

activity fell below this figure the cell was no longer viable and ceased to be included in the calculations. The number of surviving cells and their mean activity on each day are shown. Over the first 7 days GR activity falls exponentially but from the 8th day onwards most of the values are increased. That is, the enzyme activity curve obtained using Equation 2 and arbitrary figures is very similar in shape to the data derived experimentally.

A more sophisticated analysis was therefore undertaken using a computer programme to model RBC survival and GR activity during the lifespan of a cohort of 10,000 cells. Values based on experimental data but excluding the high activities in the reticulocyte-rich fraction (1) were substituted in Equation 2 for A_o, A_c, k and SD of k and the percentage of RBC remaining intact at any time together with the mean enzyme activity of the surviving cells were calculated. A random number generator was used to form a series of values of k with a given mean and standard deviation. The high activities in Fraction 1, which were attributed to the high concentrations of reticulocytes, were excluded since reticulocytes are immature RBC which only exist in the peripheral circulation for about 2 days.

The computer program was designed to analyse the data it was given in a manner similar to that shown in Table 47 except that a much larger number of cells were used and k was expressed in absolute terms as fall in

Figure 56

Figure 56: Relationship Between RBC Survival and GR Activity as Predicted by a Computer Model

A computer program was designed to model RBC survival and GR activity during the lifespan of a cohort of 10,000 cells. Values based on experimental data but excluding the high activities in the reticulocyte-rich Fraction 1 were substituted in equation 2 (p.230) for A_o, A_c, K and SD of k. The predicted enzyme activities and survival of RBC are shown for when k is the same for all cells (56a), k has a normal distribution (56b) or when A_o and A_c have normal distributions (56c, 56d, respectively).

Figure 56 Glutathione Reductase Activity and RBC Survival as Predicted by a Computer Model.

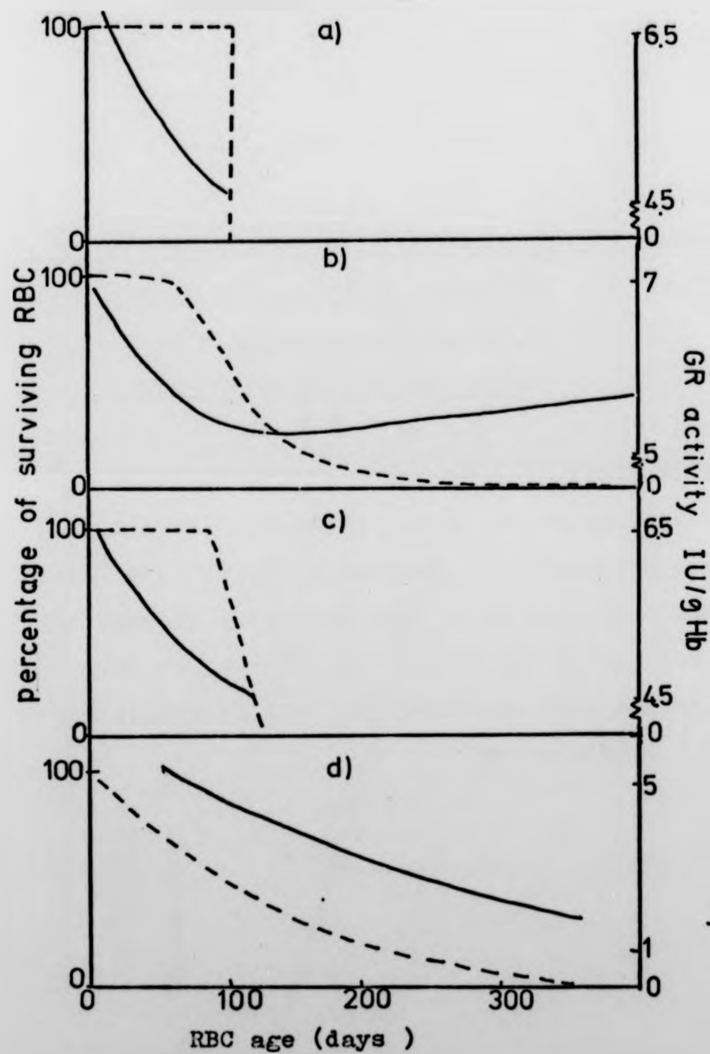


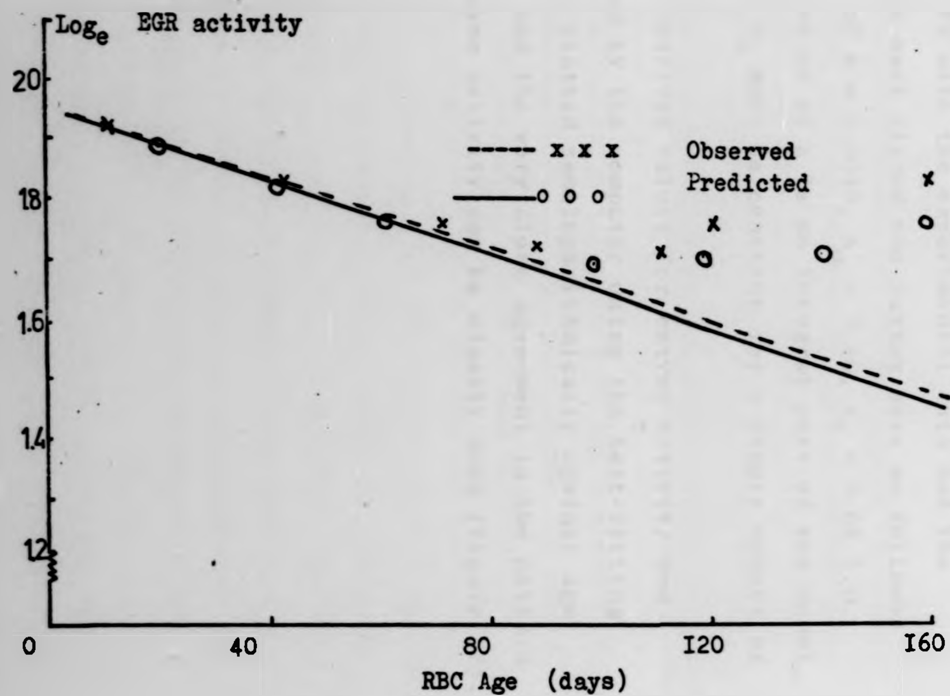
Figure 57

Figure 57: Relationship Between GR Activity and RBC Age: A Comparison of Data Obtained Experimentally with that Predicted by a Computer Model

A semilogarithmic plot is shown of GR activity data obtained experimentally (Figure 55a) and that predicted by a computer model (Figure 56b). A regression analysis was performed on data obtained over the first 60 days of the lifespan of a cohort of RBC.

Figure 57 Relationship between EGRactivity and RBC Age: a Comparison

Figure 57 Relationship between EGRactivity and RBC Age: a Comparison of data obtained experimentally with that Predicted by a Computer Model



units of enzyme activity per day. Figure 56 shows the enzyme activities and predicted survival of red cells when k is the same for all cells (56a), or when k has a normal distribution (56b) or when A_o and A_c have normal distributions (56c, 56d, respectively). Only Figure 56b shows similarity with the experimental data and the constants which best fitted the latter were as follows: $k = 0.004$, SD of $k = 0.0013$, $A_o = 7.10$, $A_c = 4.65$ I.U. The variable nature of k is an integral part of the model, whereas A_o and A_c must be constant for a single cohort of cells.

The observed values for enzyme activity and those predicted by the computer using the best-fitting constants were plotted semilogarithmically against age in Figure and the very close agreement in the pattern of fall in enzyme activity can be clearly seen (Figure 57).

DISCUSSION

1. SEPARATION OF RBC INTO FRACTIONS OF DIFFERENT MEAN AGES

A. Choice of Density Gradient Medium

Many workers interested in RBC ageing processes have, with varying degrees of success, separated RBC into fractions of different mean age by centrifugation techniques (Danon and Marikovsky, 1964; Fischer and Walter, 1975; Marks, Johnson and Hirschberg, 1958; Borun, Figueroa and Perry, 1957). Rigas and Koler (1961b) demonstrated very clearly that ultracentrifugation of RBC achieves far better resolution according to age than simple low speed centrifugation, and recent advances in the development of suitable density gradient media (Hinton and Mullock, 1975) have led to an increase in the use of density gradients for analytical RBC separation studies (Piomelli, Lurinsky and Wasserman, 1967; Prentice and Bishop, 1965; Turner, Fisher and Harris, 1974). In attempting to select a gradient material for RBC separation it was necessary to consider the density range of possible gradient solutions; the viscosity, osmolarity, and toxicity; the ease of preparation of the density gradients and the ease with which the material could be removed from the separated RBC. Preliminary attempts to separate RBC on density gradients of BSA (Leif and Vinograd, 1964) were not successful.

This failure is attributed to the fact that the volume of sample added to the gradient was too great. It has been shown experimentally (Brakke, 1964) that a density gradient has a maximum sample capacity; when the sample concentration on a gradient is too high a streaming occurs and the resolution of the sample is disturbed. Also, too high a sample concentration can overload a gradient and cause a broadening of separated bands and loss of resolution. However, even with a reduced sample size to improve the degree of resolution of RBC on a BSA gradient, the preparation of the buffered BSA solutions is an extremely time-consuming process. On the other hand, separation of RBC on gradients of Ficoll/Triosil solutions achieved good resolution and RBC recovery (Figure 13), thus confirming the value of these materials for the separation of living cells (Hinton and Mullock, 1975).

B. Confirmation of the Age-Related Separation of RBC

The activity of AST has been shown to fall with increasing RBC age (Chapman and Schaumburg, 1967; Sass and Spear, 1958). AST activity was therefore adopted as a marker to indicate the degree of resolution of RBC into fractions of differing mean age. The fall in activity of AST as IU/g Hb or as IU/RBC in fractions of RBC separated on Ficoll/Triosil gradients confirmed that the separation was indeed chronological (Figures 10 and 11).

A two-hour centrifugation period was adopted on the basis of the improved resolution of RBC, determined by measuring

$$\frac{\text{AST IU/g Hb Fraction (1)}}{\text{AST IU/g Hb Fraction (9)}}$$

and the relatively small loss of intact cells from the gradient under these conditions (Table 11). The progressively smaller recovery of cells from the gradient with longer periods of centrifugation (Table 10) suggested that RBC suffered mechanically or that osmotic damage occurred, with prolonged centrifugation at high speed.

The dramatic fall in reticulocyte concentration between Fractions 1 and 2, and the absence of any reticulocytes in fractions of more dense RBC also lends support to the validity of the separation procedure (Table 15). Finally, any possibility that the banding of RBC at the interfaces of the gradient solutions was artefactual was discounted as a result of the close similarity between AST activities determined in fractions of RBC separated on discontinuous and continuous density gradients (Figure 15).

C. Contamination of RBC with WBC

WBC in samples of blood separated on a density gradient appeared as a white fluffy layer at the paraffin oil/gradient interface. Although care was taken not to include them in the youngest fraction of RBC separated from

the gradient it was not possible to exclude them totally. Nevertheless, the WBC in Fraction I did not appear to contribute significantly to the activity of AST measured in this fraction (Table 13). It was therefore felt that the high activity of AST in the youngest cell fraction could be justifiably attributed to the high concentration of reticulocytes. Several workers have proposed that the rapid fail in activity of some erythrocyte enzymes in the least dense region of a density gradient reflects the loss of the ability of the mature non-reticulated RBC to synthesise macromolecules de novo (Turner, Fisher and Harris, 1974; Allison and Burn, 1955) but this will be discussed more fully later (p. 249)

2. THE EFFECTS OF SUBJECT AGE AND RIBOFLAVIN STATUS ON THE AGE DISTRIBUTION OF RBC IN BLOOD

A. Effects of Riboflavin Deficiency on RBC Survival

A preliminary study indicated that the distribution of Hb through a density gradient was a valid indicator of the age-related RBC distribution in a sample (Figure 12). This was predictable as the MCH in RBC from human subjects has been shown to remain constant as RBC age (Murphy, 1973). Therefore, using Hb concentration in the fractions as a measure of RBC number, the relative proportions of RBC of different ages were investigated in samples of blood from normal and riboflavin-deficient subjects.

A reduction in the proportion of old red cells and an increased proportion of young cells in adult or geriatric subjects with marginal riboflavin deficiency when compared with normal subjects was observed (Figures 32, 33, 34 and 36). These results could be interpreted as being indicative of a reduced RBC survival time *in vivo*.

Several reports in the literature suggest that an association exists between severe riboflavin deficiency and anaemia in man (Foy and Kondi, 1961; Alfrey and Lane, 1970). In particular, experimentally-induced riboflavin deficiency in man is associated with hypoplastic or aplastic anaemia which is reversed specifically by the administration

of riboflavin (Alfrey and Lane, 1970).

Unfortunately such studies have failed to define clearly the degree of riboflavin deficiency achieved experimentally and it can only be assumed that the treatment used resulted in gross riboflavin depletion due to a combination of the administration of a riboflavin-deficient diet and a riboflavin antagonist, galactoflavin. The precise biochemical lesion responsible for hypoplastic anaemia in robflavin deficiency is not known, although a number of hypotheses have been advanced. Alfrey and Lane (1970) suggested that the disorder is at the level of the erythroid precursors. Foy and Kondi (1968) on the other hand have implicated adrenal insufficiency in the anaemia since they were able to reverse the anaemia in baboons by administering prednisone. More recent studies on riboflavin deficiency in rats support the suggestion that an associated anaemia may be due to decreased hepatic iron mobilization and absorption of iron from food (Sirivech, Driskell and Frieden, 1977; Hassan, 1978).

The importance of this anaemia of riboflavin deficiency in clinical medicine however may not be very great. There are no known reports of chronic anaemia associated with pure riboflavin deficiency that is not experimentally-induced. This may be because

i. riboflavin deficiency occurs primarily in association with other nutritional deficiencies and although anaemia may be evident the complexity of the nutritional

deficiency and the therapies utilized make interpretation of the aetiology of the anaemia difficult.

ii. The degree of deficiency of riboflavin that has been reported in field studies cannot compare with the extreme depletion possible with the use of riboflavin antagonists.

iii. Riboflavin antagonists may also bind more rapidly at points in the metabolism which would normally be protected from depletion by physiologically protective mechanisms.

Although the anaemia of severe riboflavin deficiency was not reported to be associated with increased haemolysis of RBC *in vivo* (Alfrey and Lane, 1970), results obtained in the study presented here suggest that a marginal riboflavin deficiency may be associated with the premature destruction of RBC *in vivo*. The significance of the effect of the marginal riboflavin deficiency on RBC survival appeared to be greater in the elderly subjects, but this may be merely a reflection of the apparently longer survival time of RBC in normal elderly subjects when compared with younger subjects (Figure 35). (A discussion of this observation will follow later in this section.) In no subject with riboflavin deficiency was the reduction in the proportion of old RBC accompanied by a detectable reticulocytosis (Table 18) or a significantly reduced PCV. It may be that a small increase in the percentage of reticulocytes would not be detected by the manual

counting technique. G6PD deficiency, which is associated with a reduced RBC survival time *in vivo* likewise is not associated with reticulocytosis unless stimulated by a haemolytic episode (Brewer, Tarlov and Kellermeyer, 1961). Routine haematological investigations might not be expected therefore to detect any abnormality in the erythrokinetics of marginally riboflavin-deficient subjects and this may be one reason why riboflavin deficiency in man is not generally associated with a reduced RBC survival time.

It is well recognized that the reduced proportion of old cells measured in riboflavin-deficient subjects could have been artefactual and not a true indication of a reduced *in vivo* RBC survival time. However, there was no difference in the percentage of cells from normal or deficient subjects recovered from the density gradients suggesting that riboflavin deficiency did not result in an enhanced destruction of cells during manipulation *in vitro* and supporting the view that the reduction in old cells in blood from riboflavin-deficient subjects was not an experimental artefact.

There is only indirect evidence in the literature for a reduced RBC survival time *in vivo* and poor riboflavin status. For example, Carson, Brewer and Ickes (1965) demonstrated that the RBC of a human subject with a deficiency of EGR activity were more susceptible to the *in vivo* haemolytic effects of 8-aminoquinoline. The same

RBC when transferred into a recipient with normal GR activity exhibited an abnormally short Cr^{51} half-life indicating that the defect was intrinsic to the RBC. A less direct association between riboflavin and RBC survival has been described by Pitcher and Williams (1963). 34 out of 41 subjects with liver disease were shown to have a reduced RBC survival time, as measured by the Cr^{51} half-life, accompanied by abnormally low GSH levels in 43% of the subjects.

The precise mechanism for the apparent premature destruction of RBC from marginally riboflavin deficient subjects remains to be elucidated. However, the wealth of reports implicating GSH as an important factor in the maintenance of RBC integrity (Jacob and Jandl, 1962b; Rigas and Koler, 1961a) suggest that the defect is intra-corpuscular and related to the reduced activities of GR in the deficient subjects rather than extra-corpuscular. It is possible that the reduced RBC survival is a result of a lytic plasma factor. There are cases reported in the literature in which some factor in the plasma of certain individuals results in an acquired sensitivity of RBC to lysis in vivo (Dacie et al., 1953). However, whole blood samples from riboflavin-deficient subjects involved in the study described here were regularly stored for 24 hours at 4°C in the absence of A.C.D. and no abnormal haemolysis was observed. This indicated that autohaemolysis was normal and suggested that a lytic plasma factor

was not the major cause of the observed abnormal RBC survival.

It is postulated that the apparent premature destruction of RBC in riboflavin deficient subjects is causally associated with a reduction in the activity of GR in old RBC accompanied by cumulative degenerative changes in the structure and metabolism of ageing RBC. This problem is discussed in greater detail in Section 3 (p.249) in the light of physiological and biochemical observations made on ageing RBC.

B. Effects of Subject Age on RBC Survival

RBC survival studies on elderly and young subjects indicated a significantly higher proportion of old RBC in blood from elderly subjects (Figure 35). It was interesting to note that RBC from these hospitalised elderly subjects had consistently very low GR activation coefficients reflecting good riboflavin status.

The question of whether the RBC survival time in elderly subjects differs from that in young subjects is open to debate. Takaku (1959) reported that ageing is associated with a reduced RBC lifespan, whereas Casassa, Cerrato and Turco (1957) suggested that the survival time of RBC in aged people is prolonged. On the other hand, a RBC $T_{\frac{1}{2}}$ ^{51}Cr study in 37 men and women over 70 years showed no difference in red cell survival from young

controls (Hurdle and Rosin, 1962). Unfortunately, because chromium elutes from red cells and therefore curves of disappearance of labelled red cells from the circulation may be due to more than one factor, the T½ ^{51}Cr has no simple relationship to the mean red cell lifespan (The International Committee for Standardization in Haematology, 1971). Also, there is considerable variability in the T½ ^{51}Cr values reported by different workers for normal subjects, partly due to the use of different labelling techniques and partly due to differences in the method of calculation of results. Thus, results from chromium labelling studies must be regarded with caution and conflicting reports of RBC survival in elderly subjects may not be resolved until methods and interpretation of data have been standardised.

Human ageing is associated with a gradual but significant reduction in basal oxygen consumption paralleled by an apparent reduction in the creatinine coefficient, a measure of muscle metabolism (Albanese, 1963). The apparently prolonged RBC survival time in the elderly observed in this study may be a reflection of a general age-induced reduction in turnover rates of various metabolites.

As long ago as 1917 it was demonstrated that increasing the environmental temperature of poikilothermic animals significantly shortens their lifespan (Loeb and Northrup, 1917). These workers went on to argue that with an increase in temperature there was an associated increase

in the rate of metabolism and this was the primary cause of premature death. In fact a 'wear and tear' theory of ageing has a long history and this theory assumes that an organism 'wears out' with use in much the same way as inanimate objects. Clearly such a theory is open to criticism but it may be useful to consider its implications as regards RBC survival in the elderly.

A reduction in the rate of utilization of enzymes in metabolic processes may be associated with an increased retention of structural and functional integrity of the enzymes. Thus it has been demonstrated in riboflavin-deficient rats that the enzyme glutathione peroxidase, requiring GSH as a substrate, shows very little fall in activity as the cells age by comparison with normal rats. GSH concentrations in the riboflavin-deficient rats were low and may have limited the rate at which GSHPx could operate (Hassan, 1978). Also, as has been shown (Figures 16 and 18) the GR activity of young and old RBC from healthy elderly subjects is higher than the activity in RBC of the same density in younger adults, which again may indicate a decreased utilization of the enzyme.

It has been reported that cardiac output falls in the elderly (Brandfonbrener, Landowne and Shock, 1955). This could be interpreted to suggest that an individual RBC will pass through a particular point in the circulation less frequently than an equivalent RBC in a young adult. In certain parts of the circulation, for example the spleen,

the BRC is exposed to mechanical and biochemical stresses, and a reduction in the frequency with which the cell experiences such stresses may lead to an increased survival time.

It is also possible that higher activities of GR in RBC of elderly subjects may be a contributory factor in prolonging RBC survival. In the course of these studies an hypothesis has been advanced (Results I) in which it is suggested that GR activity is a major determinant of RBC survival. It is inherent in this hypothesis that RBC endowed with high activities of GR may survive longer.

The reduction in RBC survival time as a result of ariboflavinosis which appeared greater in the elderly than in younger subjects (compare Figures 33 and 34) may have been due to the apparently relatively longer survival time of RBC in the control elderly subjects.

3. ENZYME ACTIVITY AND THE AGEING RED BLOOD CELL

A. The Age-Related Activity of AST and EGR

Among the many physical and chemical alterations associated with ageing in the human RBC during its lifespan, is the reduction in specific activity of certain enzymes (Chapman and Schaumburg, 1967; Sass, Vorsanger and Spear, 1964; Turner, Fisher and Harris, 1974).

The ratio of the specific activity of AST in young to old cells was found to have a mean of 2.71 $\pm$ 0.21 (SEM) in the absence of added cofactor, and 1.84 $\pm$ 0.23 (SEM) in its presence (Figure 25). Due to the differences in separation techniques employed and therefore the relative mean ages of young and old RBC fractions a direct comparison of these ratios with those of other workers is not possible. However, the reduction in the ratio of enzyme activity in young to old cells in the presence of added cofactor supports a similar observation made by Fischer and Walter (1971) who found that the forms of the enzyme AST in older cells are of 3 types: enzyme which is active, enzyme which is inactive but can be reactivated by added cofactor and enzyme that is 'lost' or permanently inactive. The reduced activity of AST associated with old RBC may be partly due to the consequence of a reduced level of cofactor but primarily protein modification.

In the case of GR activity, however, Bonsignore, _et al._(1964) reported that the activity did not fall with increasing age of the RBC. In contrast, the results reported here (Figures 16, 18, 19, 20, 21 and 22) did show a fall in GR activity with increasing RBC age, and the data were confirmed by other workers who published at the same time (Powers and Thurnham, 1976; Ganzoni, Barras and Marti, 1976).

The relative activities in young and old red cells when measured with and without the appropriate coenzyme however, showed differences when compared with AST. The mean activity ratio between young and old cells for basic EGR activity was 1.97 while the FAD-stimulated activity ratio was 1.76. The difference between these two values is smaller than found in the case of AST. A reduction in the amount of FAD in older cells can only be partly responsible for the observed fall in specific activity of GR as cells age and a process of protein modification must also be postulated. The observed increase in the amount of unsaturated apoenzyme (Figure 24) and EGR-AC (Figures 17 and 23) as the youngest cells of a cohort age do indicate a loss of some FAD, but the final drop in apoenzyme and EGR-AC in the oldest cells may be due to an increased loss of GR activity, due to irreversible protein modification, rather than an increase in the FAD in these cells. This explanation is supported by the fact that the age-related loss of activity of AST is markedly

reduced in the presence of added cofactor and that the final drop in AST-AC in the oldest cells is very small and is not consistent (Figures 25 and 26).

The fact that the activity of GR in the presence of added FAD showed a progressive reduction in RBC as they aged in all subjects studied, suggests that either enzyme protein is actually lost from cells as they age or the enzyme undergoes some structural modification which impairs its function. There is some evidence that two other erythrocyte enzymes, G6PD (Fornaini et al., 1969) and pyruvate kinase (Paglia and Valentine, 1970) undergo structural modifications as a function of RBC age. It is possible that as RBC age a changing intracellular environment leads to an alteration in the function of some enzyme proteins. In order to determine whether qualitative changes occur in the GR of ageing RBC, the saturation kinetics of EGR for NADPH and GSSG were investigated in old, young and unfractionated RBC. The Km values for these two substrates did not appear to be age related (Tables 16 and 17). However, in order to complete the kinetic study it will be necessary to separate the apoenzyme from bound FAD and to study the affinity of the resolved apoenzyme for FAD over a wide range of concentrations of the latter. Such an investigation may help to elucidate the biochemical basis of the observed fall in GR activity in ageing RBC by providing information concerning any alteration of substrate-binding properties of the enzyme in cells as they age.

B. EGR Activity and RBC Survival

The highly significant although small rise in mean GR activity in old cells of normal adults (Figure 16B) could not be fully explained by the presence of contaminating young cells in the dense RBC fractions. The total absence of reticulocytes in the dense RBC fractions and the minimal effect that separating out contaminating younger cells from old RBC fractions had on GR activity (Figure 27) suggests that the observed increase in GR activity in old RBC is not artefactual. Ganzoni, Barras and Marti (1976) in their study of 5 erythrocyte enzymes in rats illustrated that the activity of GR did decrease but that none of the other enzymes increased noticeably in the oldest cells. However, these authors made no reference to the increase in their script and only reported a reduction in GR activity in ageing rat erythrocytes.

The work reported here and also that of Ganzoni, Barras and Marti (1976) showed no significant increase in AST activity of the oldest cells. In an attempt to explain this difference, the possibility was investigated of there being a system able to select out old RBC with lowest GR activities thus raising mean EGR activity in the oldest fractions. The validity of such a suggestion was examined using a mathematical approach and will be discussed in more detail in Section 7.

The apparent relationship between riboflavin

status and RBC survival (Figures 33 and 34) may be a function of the GSH concentration in the RBC. Reduced glutathione has been shown by several workers to play an important role in the maintenance of RBC integrity. For example, Jacob and Jandl (1962a) demonstrated that sulphydryl-inhibited RBC are removed rapidly from the human circulation by selective sequestration in the spleen. These same authors have also shown (1962b) that when membrane sulphydryl groups are blocked _in vitro_ the cation gradients across the cell membrane are disrupted with a resulting cellular swelling and haemolysis.

Furthermore, much evidence for the importance of GSH for RBC survival comes indirectly from studies on the RBC of subjects with G6PD deficiency. G6PD deficiency is generally associated with a slight reduction in RBC life-span, a reduction in RBC GSH concentration, an elevated _in vitro_ specific GR activity and a marked sensitivity to drug-induced haemolysis _in vivo_ (Brewer, Tarlov and Kellermeyer, 1961; Beutler, Robson and Buttenwieser, 1957). The increase in GR activity is due to considerably reduced free apoenzyme concentrations (Flatz, 1970; Thurnham, 1972) and may be a genetic modification that ensures adequate GR enzyme activity for all the available NADPH.

In the light of such observations it was not surprising to find that the concentration of GSH (when expressed as μMoles/g Hb) falls with increasing age of the erythrocyte (Figure 38). There have been a number of reports to the

contrary including those by Rigas and Koler (1961a) and Prankerd (1958) who failed to find a reduction in GSH concentrations as RBC age. However, these groups of workers, by expressing GSH concentrations per volume of cells, failed to take into account the fall that occurs in MCV as RBC age (Keitel, 1958) which would tend to mask any age-related reduction in GSH when expressed in this way. Sass, Caruso and O'Connell (1964) made this point very clearly when they demonstrated that a fall in GSH concentrations during RBC ageing could not be detected when GSH was expressed per volume of cells, but could be detected when expressed per grams of Hb.

The maintenance of GSH may be important to RBC integrity for several reasons: (i) sulphydryl groups in GSH may be essential for maintaining the sulphydryls of the RBC membrane (Jacob and Jandl, 1961b); (ii) sulphydryl groups participate in oxidation-reduction reactions in the RBC, notably those involving GSHPx which functions to reduce potentially harmful peroxides in the cell (Cohen and Hochstein, 1963); (iii) GSSG can inhibit hexokinase activity (Eldjarn and Bremer, 1962; Beutler and Teeple, 1969).

The importance of GR therefore, appears to lie in its ability to maintain glutathione in its reduced form, not only for its metabolic functions but possibly also as a structural component of the cell. The anti-oxidant role of GSH will be discussed more fully in Section 4.

It is possible that the reduced proportion of old RBC in riboflavin-deficient subjects may be due to an inability to supply GSH rapidly enough to maintain the metabolic requirements of old cells. That fact that GSH concentrations in unfractionated RBC and old RBC *in vitro* was not significantly lower in deficient subjects than normal controls (Table 19) may mean that the minimum GSH concentration is a useful index of RBC viability. RBC with very low concentrations of GSH may be more fragile *in vivo*, rapidly removed from the circulation and are not detectable *in vitro*. Therefore, the observed reduction in RBC survival in riboflavin-deficient subjects may be ascribed to a reduction in the ability to maintain GSH at a concentration sufficient to meet the metabolic demand so that old cells in particular, with low GR activity, are unable to maintain their structural and functional integrity in the face of mechanical and oxidative stresses of the circulation.

4. RIBOFLAVIN AND HAEMOGLOBIN STABILITY

Riboflavin, in its FAD form, acts as a prosthetic group for EGR which functions to generate GSH within the RBC. The work of several research groups has suggested that the maintenance of GSH concentrations, via active EGR, may be a factor determining the structural integrity of red cells (Carson, Brewer and Ickes, 1961; Hassan and Thurnham, 1977; Mortensen, 1964). The observed reduction in the lifespan of RBC in marginally riboflavin-deficient subjects supports this suggestion (Figures 33 and 34).

It is well recognized that the RBC is exposed to both chemical and physical stresses in its passage through the circulation. The chemical stresses are predominantly oxidant in nature, consisting of peroxides and a diverse array of free radicals. Free radicals are highly reactive cellular components formed from atoms or molecules in which an electron pair is for a brief moment separated into independent electrons. Such radicals will oxidatively attack other molecules in their vicinity to reform the electron pair. In biological systems the molecule that most commonly generates free radicals is oxygen (Demopoulos, 1973) and the RBC, by its very function of transporting oxygen, may be particularly at risk from free radical damage. Unsaturated lipids, which constitute a vital component of the RBC membrane, appear to be prime

targets for free radical attack (Tappel, 1965). There are several reports to suggest that H_2O_2 may be produced *in vivo*, either during normal metabolism or by oxidant drugs such as menadione (Cohen and Hochstein, 1964; Cohen and Hochstein, 1961; Winterbourn, 1979), and several studies have demonstrated that H_2O_2, generated *in vitro*, may result in haemolysis of the RBC (Stocks and Dormandy, 1970; Dodge, Cohen, Kayden and Phillips, 1967). The experiments described in Figures 38 and 39, and Table 24 suggest that as red cells age they accumulate structural and functional lesions and that the severity of the lesions may be influenced by the riboflavin status of the individual.

The most vital function of the RBC is to transport oxygen to the tissues; for this the cell requires haemoglobin in a form capable of binding oxygen under high pressure and of releasing it under low oxygen pressure. However, during the course of its survival in the peripheral circulation the Hb is in a state of equilibrium between oxidised and reduced forms. Methaemoglobin is an oxidised form of Hb incapable of binding oxygen and its concentration in a RBC at any particular time is determines by the equilibrium between the rate of its formation and reduction.

In unfractionated RBC from all normal subjects the MHb constituted less than 2.5% of the total Hb

(Table 22, Figure 37), which agrees well with reports that a normally-metabolizing RBC contains only 1-2% MHb (Evelyn and Malloy, 1938; Kravitz, Elegant, Kaiser and Kagan, 1956).

In RBC from normal and marginally riboflavin-deficient subjects a clear increase was observed in the percentage of MHb, with a concomitant decrease in the percentage of Oxyhaemoglobin, with progressively increasing RBC age (Figure 39). This is in agreement with the work of others who were able to show that MHb concentrations in old erythrocytes exceed that in young erythrocytes (Brewer, Tarlov, Kellermeyer and Alving, 1962; Keitt, Smith and Jandl, 1966). This suggests that as a red cell ages it becomes progressively less able to protect its Hb from changes that may ultimately lead to its denaturation and destruction.

The highly significant negative correlation between GSH concentrations and percentage MHb in ageing RBC (Figure 40) gives some support to proposals that have been made concerning the role of GSH as a protective factor in Hb oxidation (Mills and Randall, 1958; Hill, Haut, Cartwright and Wintrobe, 1964). Also, the significantly higher percentage of MHb in old cells of riboflavin-deficient subjects (Figure 39) and the slightly elevated level of MHb and reduced concentrations of GSH in unfractionated blood of deficient subjects when compared with normal controls (Tables 19 and 22) reinforces such proposals.

GSH is a substrate for GSHPx and several workers have shown that GSHPx has a role in the protection of HB against oxidation. For example, Mills and Randall (1958) demonstrated that GSHPx can inhibit Hb oxidation *in vitro* induced by ascorbic acid. GSHPx functions to reduce peroxide and these workers postulated the formation of H_2O_2 during the coupled oxidation of ascorbic acid and Hb and the removal of this potentially harmful H_2O_2 by GSHPx.

Recently, preliminary experiments in human RBC have indicated that GSHPx activity falls as RBC age (Spooner, personal communication) and this has also been shown in rats (Hassan, 1978). One can speculate that this may be due to protein modification of the enzyme during RBC ageing *in vivo* and implies that old RBC may be particularly at risk from the degenerative effects of oxidative attack.

A detailed study of oxidative haemolysis and precipitation of Hb in RBC exposed to oxidant drugs suggested that GSH may play a directly protective role in preventing the process of Hb oxidation by its ability to form mixed disulphides with Hb, which apparently helps to maintain the normal configuration of Hb and slow down its precipitation. Whether the oxidation is spontaneous however, or accelerated by oxidant drugs, the sequence of events leading ultimately to the precipitation of Hb as Heinz bodies appears to be as follows: (Allen and Jandl, 1961):

i. oxidation of glutathione;

ii. oxidation of protein sulphydryls;

iii. protein precipitation.

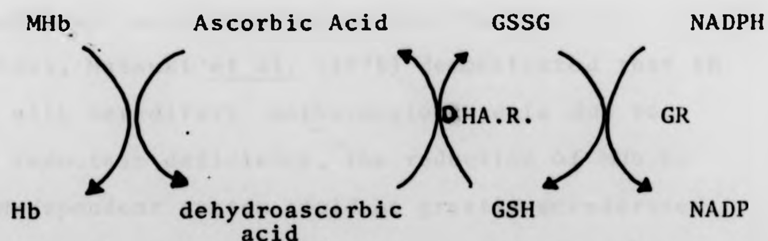
During the oxidative precipitation of Hb, insoluble brown or green pigments, including sulphaemoglobin, are formed which are considered to be forms of irreversibly denatured Hb (Jandl, Engle and Allen, 1960). It was found that sulphaemoglobin was present in greater concentrations in older than younger cells of all subjects (Figure 39) and elevated concentrations were also present in unfractionated blood from deficient subjects when compared with normal controls (Figure 37). In other words, red cell ageing and riboflavin deficiency appear to be related to an increased proportion of the oxidised forms of Hb which have also been associated with the oxidant effects of certain drugs (Allen and Jandl, 1961).

The functioning of glyceraldehyde 3 phosphate dehydrogenase is also necessary to provide reducing conditions in the RBC. This enzyme has recently been shown to depend on GSH for its function; it may contain GSH as a prosthetic group and is rich in sulphydryl groups and can be inhibited in its activity by sulphydryl-binding substances (Krimsky and Racker, 1952; Koeppe, Boyer and Stulberg, 1956).

Low GR activity is associated with an increase in the concentrations of oxidised forms of Hb (Figure 39). Slightly reduced concentrations of GSH and/or slightly increased concentrations of MHb do not necessarily affect cell stability but nevertheless give some indication of

the oxidant status of the red cell.

Riboflavin in its coenzyme form, FAD, is necessary for the generation of GSH via GR activity. GSH has been shown to reduce MHb *in vitro* but the reaction is so slow it is unlikely to be physiologically important (Jaffé and Neumann, 1968). Also, ascorbic acid can reduce MHb *in vivo* and *in vitro* (Gibson, 1943; Barcroft, Gibson, Harrison and McMurray, 1945) and the dehydroascorbic acid produced is reduced through a GSH-dependent reductase (Christine, *et al*., 1956; Hughes and Maton, 1968), viz:



where: DHA.R. = dehydroascorbic acid reductase
GR = Glutathione reductase.

The non-enzymic reduction of Hb by ascorbic acid, however, occurs extremely slowly and may not be of physiological significance.

Methaemoglobin reductase in various forms has been identified in the RBC by several workers. Two general types have been identified: NADH-dependent

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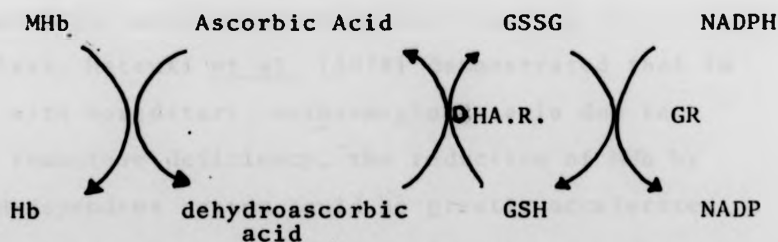

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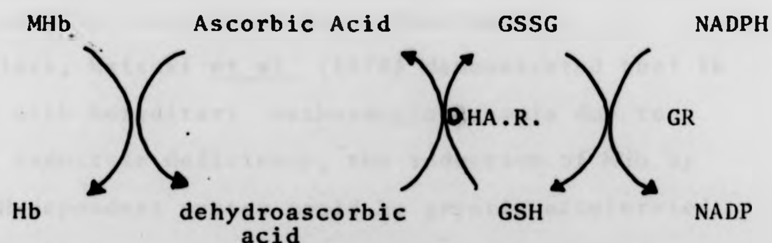

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reductase, which is reported to account for about 61% of the total MHb reducing capacity of the RBC (Scott, Duncan and Ekstrand, 1965) and NADPH-dependent reductase.

One group of workers (Yubisui _et al._, 1977) purified an NADPH-dependent reducing system which they believed to be flavin-dependent. However, a deficiency of G6PD, an enzyme responsible for the maintenance of NADPH concentrations in the RBC, is not generally associated with methaemoglobinaemia (Brewer, Tarlov, Kellermeyer and Alving, 1962). In addition, Sass,Caruso and Farhangi (1967) could not detect methaemoglobinaemia in a subject with partial NADPH-MHb reductase deficiency, thus this system is not generally considered to be important _in vivo_. Nevertheless, Matsuki _et al._ (1978) demonstrated that in subjects with hereditary methaemoglobinaemia due to NADH-MHB reductase deficiency, the reduction of MHb by the NADPH-dependent system could be greatly accelerated by riboflavin _in vitro_. Riboflavin may therefore have therapeutic value in the treatment of patients with hereditary methaemoglobinaemia and methaemoglobinaemia induced by oxidant drugs.

Several investigators have isolated and partially purified NADPH-dependent enzymes with MHb reductase activity (Huennekens, Caffrey, Basford and Gabrio, 1957; Scott, Duncan and Ekstrand, 1965). It is however not yet clear whether this activity can be attributed to one enzyme or whether FAD is indeed a prosthetic group. Nevertheless

the work of Matsuki <u>et al.</u> (1978) does suggest that a flavin is involved in NADPH-MHb reductase activity.

The precise characteristics of the NADH-dependent MHb reductase (NADH-MHb.R) system are also a subject of some contention. Flavin was an essential component of the NADH-MHb.R system purified by Scott and McGraw (1962) but was not found to be a constant component of NADH-MHb.R purified by other workers (Sugita, Nomura and Yoneyama, 1971; Hultquist and Passon, 1971). Variations in assay conditions and the degree of purity of preparations may account for some of the differences in properties of the enzymes.

The importance of riboflavin for the maintenance of Hb in its oxygen-carrying form may lie in the fact that at least four separate systems involved in protecting Hb from oxidation or converting MHb back to oxyhaemoglobin appear to be influenced by the availability of riboflavin. These systems include GSHPx peroxidase, the non-enzymic action of GSH, NADH-MHb reductase and NADPH-MHb reductase. Although taken individually they may not appear to be of physiological importance, an impairment of more than one system may well lead to increased levels of MHb. Methaemoglobin <u>per se</u> does not seem to have a significantly deleterious effect on the integrity of the RBC (Jaffé, 1959) but increased levels of MHb, associated with riboflavin depletion of the RBC may be indicative of a more general impairment of the antioxidant systems of the cell.

5. OXIDATION OF RBC LIPIDS AND HAEMOLYSIS

Erythrocytes from normal and marginally riboflavin-deficient subjects haemolysed when incubated *in vitro* with H_2O_2. The degree of haemolysis in unfractionated samples of RBC did not appear to correlate with GR activity over the small range of GR activities measured. However, old cells showed significantly greater haemolysis than young cells from the same sample of blood. These results suggest that the susceptibility of RBC to peroxidative haemolysis within a sample may be influenced by the EGR activity which in turn may influence the amount of GSH available for the peroxide-reducing enzyme, GSHPx.

Malonyldialdehyde (MDA) is a breakdown product of lipid peroxides and its concentration in tissues is considered to reflect the degree of lipid peroxidation in a tissue. However, the amount of MDA detected /g Hb in cells exposed to oxidant stress *in vitro* may not be a satisfactory indication of the amount of lipid peroxidation occurring in RBC of different ages. This matter will be considered in depth later in this section.

Unsaturated fatty acids (UFA) in any mixture of lipids or in tissues are susceptible to autoxidation induced by UV irradiation or molecular oxygen (Mengel, Kann, Heyman and Metz, 1965), and workers have employed different techniques to estimate the degree to which this occurs in systems *in vitro* (Dodge, Cohen, Kayden and

Phillips, 1967; Dahle, Hill and Holman, 1962). Among the methods used are:

i. Measurement of total peroxides by iodometric titration, giving a 'peroxide-value' (PV).

ii. Measurement of the rate of oxygen consumption.

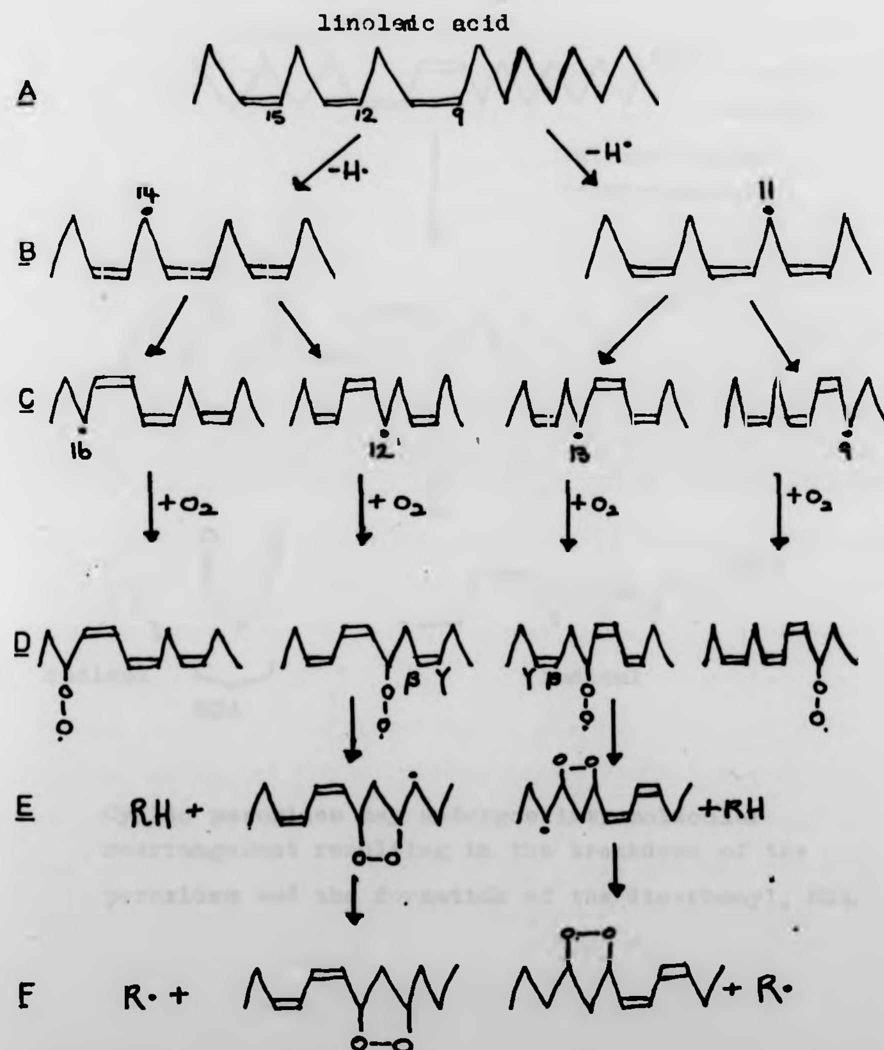
iii. Measurement of the concentration of conjugated dienes and trienes.

iv. Measurement of MDA using thiobarbituric acid (Stocks and Dormandy, 1970; Mengel and Kann, Jr., 1966; Dodge, Cohen, Kayden and Phillips, 1967).

The close association that has been observed between conjugated diene concentration, oxygen uptake and MDA concentration during in vitro autoxidation of UFA (Dahle, Hill and Holman, 1962) and the powerful inhibitory effect of 3 antioxidants in preventing MDA formation in RBC in vitro (Stocks and Dormandy, 1970) support the generally accepted view that MDA production is a reflection of oxidative changes in the constituent UFA in a biological system.

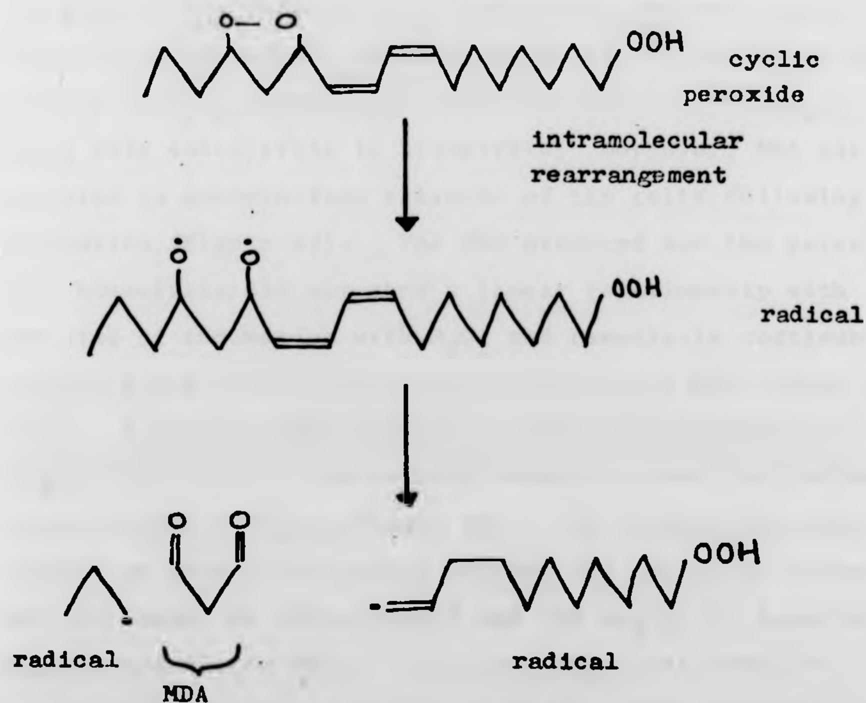
Dahle, Hill and Holman (1962) proposed a mechanism for UFA autoxidation and MDA production on the basis of experiments with methylene-interrupted fatty acid methyl esters (Figures 58 and 59). They showed that MDA only results from the breakdown of lipid peroxides formed from UFA with ≥ 3 double bonds. Thus, although MDA estimation in crude mixtures of FA can have qualitative and comparative significance the amount of MDA produced is directly related to the degree of unsaturation of the

Figure 58 Proposed Mechanism for Cyclic Peroxide Formation During Autoxidation of Linoleate (from Dahle, Hill, and Holman, 1962)



where: A Linolenic acid; B free radical; C conjugated free radical; D conjugated diene peroxy radical; E cyclic peroxide radical; and F cyclic peroxide. RH and R represent free radical scavengers and the equivalent free radical respectively.

Figure 59 Proposed Mechanism for Malonylaldehyde Formation from Cyclic Peroxides.
(from Dahle, Hill, and Holman, 1962)



Cyclic peroxides may undergoe intramolecular rearrangement resulting in the breakdown of the peroxides and the formation of the dicarbonyl, MDA.

constituent FA and their relative concentrations.

Preliminary experiments with unfractionated RBC designed to investigate the relationship between susceptibility to haemolysis and the amount of MDA formed in RBC exposed to H_2O_2 showed that cells incubated with H_2O_2 *in vitro* were susceptible to haemolysis; moreover, MDA was detected in protein-free extracts of the cells following incubation (Figure 42). The MDA produced and the percentage haemolysis did not show a linear relationship with the time of incubation with H_2O_2 and haemolysis continued to take place after the concentration of MDA had ceased to rise. A direct relationship was observed between the MDA concentration or percentage haemolysis and the initial concentration of H_2O_2 (Figure 43). The results therefore suggest an interrelationship between the oxidative stress, and the amount of MDA produced and the degree of haemolysis experienced by the RBC. It is possible that when the amount of MDA produced reaches a plateau this reflects the depletion of the oxidising species and the point at which a limit is imposed on the peroxidation system. The lower the concentration of H_2O_2 the greater the likelihood of the oxidising species being exhausted during the 1 hour incubation period.

A limiting concentration of available H_2O_2 may have been the reason for the failure to demonstrate any correlation between EGR-AC and either the amount of MDA detectable following incubation of unfractionated RBC with H_2O_2 (Table 23) or the degree of haemolysis (Table 24).

Hassan (1978) in recent studies using RBC from control and riboflavin-deficient rats was able to show a positive correlation between MDA formation and EGR-AC. This might be expected since GR is necessary for the formation of GSH in the RBC and GSHPx, which requires GSH as a substrate, can reduce both lipid peroxides and hydrogen peroxide (Christopherson, 1968; Cohen and Hochstein, 1963; O'Brien and Little, 1967).

In the incubation medium used in the present study the enzyme activity of catalase, an erythrocyte enzyme capable of reducing high concentrations (> 10^{-6} M) of H_2O_2 (Cohen and Hochstein, 1963) was inhibited by sodium azide thereby putting great demands on GSHPx to detoxify the peroxides. RBC from marginally riboflavin-deficient subjects, however, do not contain significantly lower concentrations of GSH than RBC from normal subjects (Table 19). The absence of a significant elevation in MDA production and percentage haemolysis in peroxide-stressed RBC *in vitro* from marginally riboflavin-deficient subjects may be because the peroxide-reducing potential of RBC is not significantly impaired. It may also be relevant to note that vitamin E (α-topocopherol) has been ascribed a role in the protection of RBC lipid against oxidation (Mengel, Kann, Keyman and Metz, 1965; Dodge, Cohen, Kayden and Phillips, 1967; Mengel and Kann, Jr., 1966).

There is no evidence concerning the vitamin E status of subjects used in this study and although severe

deficiency is most unlikely differences in vitamin E status among individuals might have obscured the possibility of detecting small increases in MDA production attributable to a marginal riboflavin deficiency. For example, in a study of the influence of haemolytic syndromes on MDA production *in vitro* Stocks, Offerman, Modell and Dormandy (1972) presented data relating low serum tocopherol concentrations (< 0.5 mg/100 ml) with increased production of MDA in the *in vitro* test.

The failure to detect elevated levels of MDA production and percentage haemolysis in unfractionated RBC from marginally riboflavin-deficient subjects may not be incompatible with the observation that RBC survival is slightly reduced in these subjects. The H_2O_2 incubation system, while providing a useful method for testing the response of RBC to severe oxidative stress, may not represent an exaggeration of natural stress mechanisms. Endogenously RBC are exposed to continual chemical stresses and also to mechanical stress (Leblond, 1971; Weiss and Tavassoli, 1970). The spleen for example imposes both chemical and mechanical stresses on the cells as they pass through it and the reduced survival time of RBC in animals and man with a range of haematological disorders has been shown to be at least partially corrected by splenectomy (Pohl, Bugajer-Gleitman, Lachmann und Moser, 1976; Ferretti, 1967).

RBC from vitamin E-deficient lead-poisoned rats

showed an elevated mechanical fragility *in vitro* when measured by the filterability of erythrocytes through polycarbonate filters, but an increased resistance to peroxidative haemolysis *in vitro*. Moreover, vitamin E deficiency and lead poisoning interacted *in vivo* to produce a severe haemolytic anaemia (Levander, Morris, Higgs and Ferretti, 1975). It appears that the haemolytic anaemia *in vivo* is a result of a toughening effect of lead on red cell membranes such that they were less susceptible to *in vitro* peroxidative haemolysis but more vulnerable to *in vivo* sequestration in the spleen. Thus, the H_2O_2 incubation system used in this study may not adequately reflect the stresses to which RBC are subjected *in vivo*.

Increasing mean age of RBC incubated with 10 mM H_2O_2 was associated with an increase in the percentage of cells that haemolysed (Tables 25 and 26) and a decrease in the concentration of MDA measured by the TBA test (Figures 44 and 45). In addition, increasing RBC age was also accompanied by a reduction in GSH concentration (Figure 38), a reduction in GR activity (Figure 21) and an increase in the percentage of MHb in the cell (Figure 39). The observed increase in the susceptibility to peroxidative haemolysis with increasing age *in vitro* therefore may reflect a decrease in the efficiency of the cell to protect itself against oxidants including a reduced capacity of the GR/GSHPx system to detoxify peroxide.

Studies described earlier (Figure 43) demonstrated that with unfractionated RBC an increased concentration of H_2O_2 in the incubation medium resulted in an increased haemolysis. An increase in the concentration of H_2O_2 in the incubation medium also resulted in an increase in the concentration of MDA (Figure 43). In addition, MDA formation appeared to precede haemolysis, as shown in Figure 42. Despite the apparent reduction in the amount of MDA formed in old cells when compared with young cells, it is possible that the increased degree of haemolysis in old cells was due to structural impairment of RBC membranes and enzymes, caused by the accumulation of lipid peroxides and their breakdown products. The effects were greater as cells got older possibly as a result of the reduced efficiency of the GR/SGHPx system.

Dick, Dick and Tosteson (1969) reported an inhibition of sheep red cell membrane Na^+ and K^+-dependent ATPase by GSSG at slightly above physiological concentrations. It is possible that a reduction in GSH concentrations in old cells may be accompanied by an increase in GSSG concentrations which may result in partial inhibition of ATPase and an increased osmotic fragility. During the incubation of RBC with 10 mM-H_2O_2 _in vitro_ however the GSH was virtually all oxidised within a few minutes of the addition of H_2O_2 (Figure 46) which implies that the differential haemolysis seen in young and old cells may not primarily be due to ATPase inhibition _in vitro_.

The effect of lipid oxidation on fragility of RBC *in vitro* may not be confined to an impairment of the structural integrity of the membrane as lipid peroxides and their breakdown products can damage proteins, particularly sulphydryl enzymes. For example, Desai and Tappel (1963) demonstrated that linolenic acid could bind to cytochrome-c resulting in a decreased solubility and an oxidation of 20% of the constituent amino acids. Chio and Tappel (1969) showed that MDA could cross-link with amino groups of ribonuclease *in vitro* leading to inactivation of this enzyme. Inactivation of enzymes of glycolysis or the HMS, by lipid peroxides formed *in vitro* may impair vital ATP and NAD(P)H-producing steps and antioxidant systems, both of which would lead to a final disruption of the cell.

The explanation for the observed differential haemolysis of young and old cells *in vitro* rests primarily on the assumption that the lipids of old RBC are more susceptible to peroxidation. However, consistently less MDA (nmol/gHb) was measured in old cells exposed to H_2O_2 *in vitro* which would suggest that the degree of peroxidation was less in old cells than young cells. The observations are not incompatible however for two reasons:

1. MDA is derived primarily from peroxidised fatty acids containing ≥ 3 double bonds and it is known that quantitative differences exist in the lipid composition of young and old cells.

Not only does the total phospholipid and cholesterol content of the RBC decrease during its lifespan but old cells contain a significantly greater proportion of linoleic acid and a smaller proportion of arachidonic acid (Van Gastel, Van Den Berg, DeGier and Van Deenen, 1965; Westerman, Pierce and Jensen, 1963). Linoleic acid, while undergoing peroxidation does not form MDA (Dahle, Hill and Holman, 1962). Thus it is possible that the reduction in detectable MDA in old cells after incubation with H_2O_2 is partly due to the decreased proportion of the appropriate lipid peroxide to form MDA in old cells.

2. MDA is also known to bind cellular components, thus it may be that in old cells there is less available than in young cells to bind with TBA.

The reaction of lipid peroxides and MDA with proteins and phospholipids therefore has relevance not only to the structural and functional impairment it may precipitate but also to the effects this may have on the detection of MDA *in vitro*. Desai and Tappel (1963) demonstrated the formation of a peroxidised linolenate-cytochrome-C compound *in vitro* which showed no TBA activity but a high peroxide value. Also Goldstein and McDonagh (1975) showed that MDA forms Schiff bases when cross-linked to the free amino-groups of phospholipid *in vivo*; this compound appeared in chloroform-isopropanol extracts of RBC and was not TBA-reactive. High concentrations (4 - 6%) of MDA have been shown to react with mitochondrial proteins

with the formation of cross-linkages (Schauenstein, Esterbauer and Zollner, 1977), and it may be that similar cross-linkages could occur with the proteins of the RBC membrane. MDA linked to proteins may not be available for reaction with TBA in a protein-free extract of RBC and the stabilization of MDA by cross-linking with phospholipids may also reduce the amount of MDA available for reaction with TBA. Although there is no obvious reason as to why peroxides or MDA produced in progressively older RBC would be more likely to cross-link with membrane constituents it is interesting to note that the Schiff bases observed by Goldstein and McDonagh (1975) in RBC from patients receiving the oxidant drug Dapsone were mainly detected in the old RBC population.

Mechanism for Haemolysis:

In order to understand more clearly the processes leading to MDA formation and haemolysis a series of experiments were designed in order to study the effects of H_2O_2 on RBC GSH concentrations and the effect of RBC age on the ability to detoxify H_2O_2. Results showed that when RBC were exposed to H_2O_2 the amount of GSH oxidised increased rapidly as the H_2O_2 concentration increased between 0 and 10 mM (Figure 46). At 1.0 mM-H_2O_2 about 80% of the endogenous GSH was oxidised within 3 minutes and this appeared to be independent of cell age (Figure 47). In experiments to measure the oxidation of GSH in the presence of 1 mM-H_2O_2, after the first 3 minutes, oxidation occurred at the same

rate as that in cells not incubated with H_2O_2 (controls) and again was independent of cell age (Figure 48). This suggests that under these conditions all the H_2O_2 is reduced before the GSH required by GSHPx is exhausted, and that once the cells have successfully removed H_2O_2 further incubation will not result in overt haemolysis until all the remaining GSH has been oxidised. However, older cells, containing lower initial concentrations of GSH would presumably reach their GSH-depleted state before younger cells and haemolyse earlier.

It is believed that under the conditions used in the study of malonyldialdehyde production and haemolysis of RBC the factor which determined whether cells haemolysed was the concentration of H_2O_2 remaining after all the GSH had been oxidised. It was found that the concentration of peroxides in older RBC, measured within 5 minutes of the addition of 10 mM-H_2O_2 was consistently and significantly higher than in young RBC (Table 30). This is most probably attributable to the limitation that low GSH concentrations in old cells placed on the activity of GSHPx such that proportionately less H_2O_2 could be reduced by this system. The differential haemolysis observed in young and old cells may therefore be a direct consequence of the lower concentration of GSH which presumably resulted from lower GR activity in the old cells.

Measurement of Peroxides in RBC

An enzymatic method was used to assay peroxides in RBC. In the method GSHPx is linked with GR and one assumes that 1 mole of NADPH oxidised by the activity of GR is equivalent to 1 mole of peroxide present in the initial haemolysate.

Unfortunately autoxidation of GSH is difficult to control in this assay (Heath and Tappel, 1976) and from the results (Table 35) it seemed as if haemolysate also could stimulate the autoxidation, particularly when present at concentrations below 30 μM. An experiment was performed in which the haemolysate was freshly prepared without previous exposure to H_2O_2 and it was found that the apparent concentration of peroxides present was inversely proportional to the concentration of Hb in the haemolysate.

When care was taken to control the concentration of Hb in haemolysates under study the amount of NADPH oxidised during the assay, in haemolysates prepared from RBC previously incubated with peroxide, was no greater than in haemolysates prepared from RBC previously incubated without H_2O_2 (Table 36). These results suggested that incubation of RBC for 1 hour with 10 mM-H_2O_2 did not result in peroxidation of membrane lipid. However, under identical incubation conditions MDA, a breakdown product of lipid peroxides, was produced in the RBC (Table 23).

The influence of Hb on the enzymatic assay for peroxide may be due to reaction with the components of the

assay system. For example, there is evidence to suggest that when OxyHb : GSH concentrations are high *in vitro* the GSH may be oxidised to GSSG, and GSSG has been shown to form mixed disulphides with Hb (Beutler, Robson and Buttenweiser, 1957; Allen and Jandl, 1961; Huisman and Dozy, 1962). In the peroxide assay system if GSSG, formed by enzymic or non-enzymic oxidation, became bound to Hb there would be a restriction on the activity of GR.

This may explain the apparent inhibitory effect shown by concentrations of Hb > 30 μM on the oxidation of NADPH. On the other hand, low concentrations of metal ions have been shown to catalyse lipid peroxidation *in vitro* (Schneider, Smith and Hunter, 1964; Wills, 1965; Tappel, 1954). Although EDTA was present in the assay system it is conceivable that the enhancement of NADPH oxidation by low concentrations of Hb was partly due to trace ferric ions. Another complicating factor is that Hb *per se* may catalyse the oxidation of unsaturated fatty acids as Tappel (1955) demonstrated. In other words, Hb may interfere with the *in vitro* determination of lipid peroxides both by a catalytic effect on lipid oxidation and/or by affecting the concentration of GSH, which, either enzymatically or non-enzymatically can give rise to GSSG.

Quite apart from the influence that Hb appeared to have on the assay system it is possible that lipid peroxide formed in RBC during incubation with H_2O_2 was not contained in the stroma-free haemolysates used for assay.

It was not clear at the onset of the study whether or not lipid peroxides formed in RBC *in vitro* remain as an integral part of the RBC membrane. Lipid peroxides can bind to the protein moieties of the RBC membrane (Desai and Tappel, 1963) and in this form would be removed during the preparation of stroma-free haemolysates for peroxide assay.

Several reports have emphasized the limited stability of lipid peroxides and their tendency to progress through chain reactions to a variety of end products (Dormandy, 1969; Tappel, 1973; Pryor, 1973). A number of workers have reported the formation of lipid peroxides *in vitro* however, the techniques most commonly used for their detection rely on the measurement of breakdown products or other fatty acid derivatives formed during lipid peroxidation (Kibrick, Safier and Skupp, 1959; Dodge, Cohen, Kayden and Phillips, 1967; Fontaine and Valli, 1977). Similarly, although there is increasing evidence that lipid peroxidation occurs in tissues *in vivo* this evidence does not come from the measurement of peroxides *per se* but other products of the peroxidation process (DiLuzio, 1973; Tappel, 1965; Tappel, 1973; Walker, Rudra and Dickerson, 1973; Mengel and Kann, Jr., 1966).

The failure to detect lipid peroxides in haemolysates may therefore be due either to the intrinsic instability of the peroxides or to binding of peroxides to

proteins of the RBC membrane. It might be more fruitful to concentrate on methods for the measurement of conjugated dienes and trienes in the phospholipid fraction of cell membranes in order to detect a differential in the oxidation of lipids in young and old cells exposed to H_2O_2 _in vitro_.

The apparent resistance to freeze/thaw haemolysis of RBC that have been incubated under conditions known to give rise to MDA (Table 34) may be due to cross-linking of the MDA with constituents of the membrane. It is interesting to note that the formation of Schiff bases between MDA and proteins of the mitochondrial membrane is associated with an increased resistance towards detergents (Schauenstein, Esterbauer and Zollner, 1977). The increased resistance to freeze/thaw haemolysis of RBC containing MDA may be related to a formation of cross-linkages with membrane proteins and a consequent stabilization of the membrane against rupture at low temperature. In order to quantify the possible relationship between MDA concentrations in RBC extracts, the formation of fluorescent Schiff bases in RBC membranes and susceptibility of cells to rupture on freeze/thaw haemolysis the following experiment could be done. RBC would be incubated for 1 hour with 10 mM-H_2O_2 then:

1. The MDA concentration determined in protein-free extracts of RBC.
2. Schiff bases measured in chloroform-isopropanol extracts of RBC.

3. The susceptibility of the cells to rupture on freeze/thaw haemolysis, measured.

This experiment would also give some indication as to whether Schiff bases were interfering with the measurement of MDA in the studies discussed above.

6. BIOCHEMICAL AND HAEMATOLOGICAL RESPONSES OF RBC TO RIBOFLAVIN SUPPLEMENTATION IN VIVO

The value of the functional enzymatic assay as a reflection of riboflavin status was confirmed by 2 experiments in which marginally riboflavin-deficient subjects were given regular oral doses of riboflavin and the activity of EGR measured in unfractionated RBC.

Short-term Supplementation:

The addition of 1 mg riboflavin daily for 11 days increased the activity of GR *in vitro* when measured with and without FAD. Concomitant with the increase in GR activity was a fall in EGR-AC (Figure 49). A similar effect of riboflavin supplementation on basic and stimulated EGR activity *in vitro* was reported by Beutler (1970). This author interpreted his observations as being indicative of the existence of 3 forms of GR in RBC: i) active enzyme, ii) enzyme which can readily be activated *in vitro* by FAD, iii) enzyme which can be activated *in vivo* by the administration of riboflavin but which resists *in vitro* activation by FAD. The mechanisms responsible for the different activation characteristics *in vitro* and *in vivo* for the latter form of GR were not elaborated upon. However, it is possible that a prolonged incubation period with GAD *in vivo* activates forms of GR which, as a result of protein modification, have altered FAD-binding properties. It is also possible, as Beutler (1970) points out,

that long-term administration of riboflavin may influence the synthesis of GR apoenzyme by developing erythroblasts.

However, as the increase in activity of EGR occurred within 24 hours of supplementation it must have been due predominantly to activation of preformed apoenzyme.

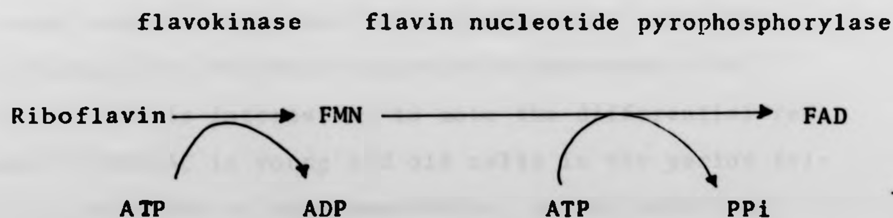
The reduction in basic GR activity that took place during the 3 weeks after supplementation ended is presumably indicative of a reduced availability of FAD to maintain turnover of FAD within the RBC. In addition, there was a fall in stimulated activity (Figure 49) which may support the idea that some of the GR in the circulation can only be activated by the prolonged presence *in vivo* of high concentrations of FAD. It might however merely indicate accelerated protein breakdown when FAD is limiting.

The decrease in EGR-AC during supplementation (Figure 49) is a reflection of the relatively greater effect of riboflavin administration on basic activity *in vivo* than on stimulated activity *in vivo*. This supports the idea that the major effect of riboflavin supplementation is to activate preformed apoenzyme.

Biochemical riboflavin status is commonly assessed on the basis of the EGR-AC response of this value to riboflavin supplementation. The activity of GR expressed as IU/g Hb does not show such a regular response to supplementation as EGR-AC (compare Tables 38 and 39) probably due to the greater number of factors affecting

precision in the measurement of enzyme activity. When the AC is calculated, many experimental variations become unimportant as they affect measurement of basic and stimulated activities equally.

The fact that all RBC regardless of their age showed a progressive increase in basic activity (Table 38) and a reduction in EGR-AC (Table 39) when measured *in vitro* suggests that both reticulocytes and mature RBC are capable of synthesising FAD. Mature RBC have been shown to synthesise riboflavin nucleotides from riboflavin (Mandula and Beutler, 1970) and it is believed that the mechanism is the same as that described for plants (Giri, Krishnaswamy and Appaji Rao, 1958; Giri, Appaji Rao, Cama and Kumar, 1960) viz.:



Although those RBC with the lowest baseline GR activity (that is, older cells) showed the greatest overall response to riboflavin supplementation, in terms of an increase in basic and stimulated GR activity and a decrease in EGR-AC, this cannot necessarily be interpreted as meaning that old RBC exhibit a faster rate of incorporation

of riboflavin and synthesis of FAD. On the contrary, the slightly higher level of unsaturated apoenzyme in old cells than young cells, both prior to supplementation and after supplementation ended, and the more rapid fall in unsaturated apoenzyme in young cells than old cells during the course of the supplementation (Table 40) suggest that there may be a decline in the rate of FAD turnover as RBC age. This may also be accompanied by a decline in the rate of incorporation of riboflavin and synthesis of FAD in the cells. To determine whether a differential does exist in the ability of young and old cells to incorporate riboflavin and synthesise FAD, experiments involving the incorporation of radioactive riboflavin into young and old cells _in vitro_ could be carried out. In fact, Pispa and Huttenen (1966) have already demonstrated that rat reticulocytes incorporate riboflavin into FAD more effectively than mature RBC.

It is interesting to note the differential response of EGR-AC in young and old cells in the period following cessation of supplementation, during which an increase in EGR-AC occurred only in older RBC. The absence of a similar increase in the young cells appears to have been due to a parallel fall in both basic and stimulated activities rather than a maintenance of GR activity. At this stage it is not possible to interpret the apparent differences in response of EGR-AC during the period post-supplementation as being indicative of a

preferential loss or uptake of riboflavin in young and old cells. However, it may be that during a period of riboflavin depletion i.e. the period post-supplementation, developing erythroblasts show a reduced synthesis of GR apoenzyme and this of course would reduce all measurements of EGR activity.

The lack of any consistent response in AST activity to riboflavin supplementation and the period post-supplementation is consistent with the view that riboflavin affects the activity of the flavin-dependent GR specifically (Table 41) and confirms the usefulness of the enzyme method for the assessment of riboflavin status.

Long-term Supplementation:

Additional information concerning effects of riboflavin on RBC metabolism was obtained from the long-term supplementation experiment. Confirmatory evidence was obtained for the existence of unsaturated apoenzyme in the RBC which can be activated by the administration of riboflavin (Table 42; Figure 51).

The lack of any age-related increase in EGR-AC (Table 42) or differences in GR-apoenzyme concentration (Figure 51) at the end of the period of supplementation is believed to reflect the virtual complete saturation of GR with FAD in all cells. The fact that a decline in GR activity was nevertheless still apparent between

young and old cells supports the suggestion made earlier that the availability of FAD is not the sole factor determining the fall in GR activity as cells age but that protein modification must also be implicated.

Although these studies of GR activity in subjects receiving oral supplements of riboflavin provided information regarding the ease with which cells of different ages could be saturated by FAD they gave no indication as to whether increasing riboflavin intake of marginally riboflavin-deficient subjects had any physiological implications. The long-term supplementation experiment therefore included an investigation into the effects of riboflavin supplementation on haematological values and biochemical indices which might reflect the overall stability of the cell.

The increases in PCV and MCHC of unfractionated RBC during supplementation although not very great (Table 43) suggests that riboflavin influences RBC production and/or destruction. A falling PCV is generally considered to indicate that the body is unable to maintain cell synthesis at a rate equivalent to cell destruction. A rising PCV on the other hand indicates that synthesis is occurring at a faster rate than RBC destruction. The PCV prior to supplementation may therefore not have been optimal and RBC synthesis may be controlled by riboflavin availability. Marginal riboflavin deficiency is associated with a reduction in the proportion of old cells and

an increase in the proportion of young cells in a sample, by comparison with the RBC distribution in subjects with normal riboflavin status (Figures 33 and 34). As young cells have a lower MCHC than old cells (Table 20) an increased proportion of young cells under conditions of normal Hb synthesis would be expected to lower the MCHC not to raise it. However, riboflavin has also been implicated in iron metabolism (Sirivech, Driskell and Frieden, 1977) and as iron is required for the normal synthesis of Hb, it is quite possible that the slight increase in MCHC values was due to an improved iron mobilization and Hb synthesis. It should also be mentioned at this point that a significantly higher MCHC was found in elderly subjects who consumed vitamin-enriched breakfast cereals by comparison with elderly subjects not eating these products (Thurnham, 1979).

During the course of the supplementation there was a significant increase in GSH concentrations in the red cells (Table 45). There is considerable evidence to suggest that GSH is important in maintaining RBC integrity (Allen and Jandl, 1961; Gross, 1976; O'Brien and Little, 1967) and in support of this was the apparent increase in red cell lifespan during the supplementation period (Table 44). During the first 5 weeks of supplementation there was a reduction in the proportion of cells in Fractions 1 and 2. These fractions contain the younger cells and the reduction showed a significant correlation with EGR-AC (Table 44). The reduction in the number of

young cells, coincident with riboflavin supplementation, supports what was said above that there was probably a lengthening of red cell lifespan, i.e. an increase in the proportion of old cells. It also supports the suggestion made earlier that the increased proportion of young RBC found in the blood from marginally riboflavin-deficient subjects is causally related to the riboflavin status.

Confidence in the above conclusion, however, was disturbed when a dramatic increase in the proportion of young cells appeared in the blood between days 34 and 54 of the supplementation period (Table 44). During the same period a marked increase in peroxidative haemolysis was also observed in the young RBC and the unfractionated RBC (Figure 53). Mel, Prenant and Monanhas (1977) showed that a reticulocytosis induced in animals by bleeding resulted in the release into the peripheral circulation of a class of 'immature' reticulocytes with impaired stability as indicated by a reduction in deformability compared with mature RBC. The increased susceptibility to peroxidative haemolysis by the younger cells in the blood may therefore be a reflection of some idiopathically-induced increase in fragility of cells which resulted in premature haemolysis _in vivo_ and a compensatory erythropoiesis.

7. GLUTATHIONE REDUCTASE ACTIVITY AS A DETERMINANT OF RBC SURVIVAL

The basic and stimulated specific activities of GR declines as RBC age, but in very old cells there is a significant rise in activity (Figure 21). The activity of AST also declines as RBC age but no rise is observed in old cells (Figure 25).

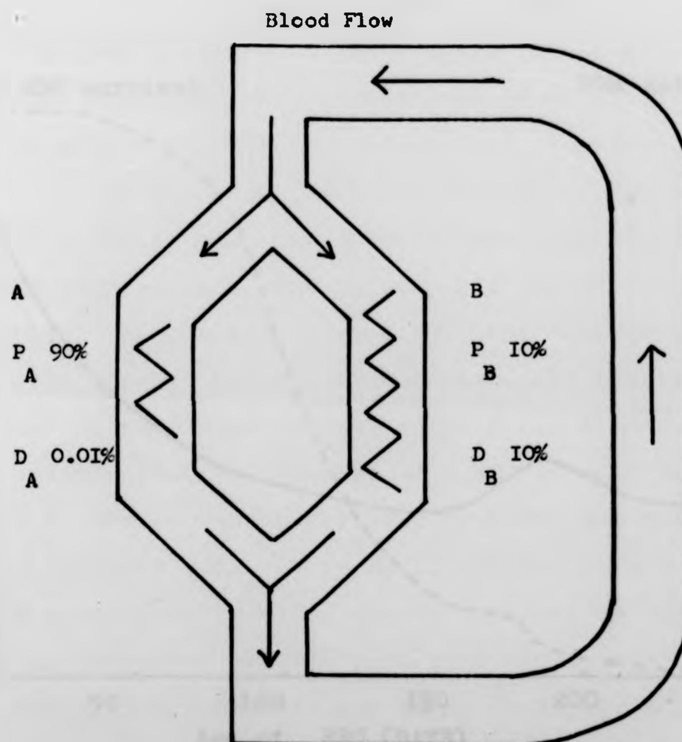
A model, based on a simple mathematical function to explain the observations was investigated (Results, I). Inherent to the model was the assumption that GR activity was a determinant of RBC survival or was closely linked with the activity of another such enzyme, and the very close similarity between predicted and observed GR activities and between the predicted RBC survival curve and published curves (compare Figures 55 and 56) supported the initial hypothesis.

The model incorporated the suggestion that the rate of decline in activity of GR was not the same in all cells of a cohort, but had a monotonic distribution, and that cells disintegrated when GR activity fell below a critical level. The rise in GR activity in old cells was attributed to a selection out of the population of those cells of a cohort with GR activity below the critical level, leaving cells in the oldest fractions recovered from a density gradient with progressively higher mean activities.

The variations in k (the rate of decline in GR activity) can be explained either by intrinsic differences in the stresses to which RBC are subjected during multiple passages through the circulation or to the existence of isoenzymes of GR. There is evidence that only 2 forms of GR exist in the RBC (Long, 1967) and it is unlikely that the existence of 2 isoenzymes could account for the Gaussian distribution of k which is necessary for the model to fit the reported findings. It is possible that the very rapid fall in GR activity between Fractions 1 and 2 of a centrifuged sample of blood is due to the presence of a short-lived isoenzyme in reticulocytes and that the variation in k among non-reticulated erythrocytes is due to variations in the stresses to which RBC are subjected in the circulation. The latter possibility was modelled using a simplified scheme of the circulation.

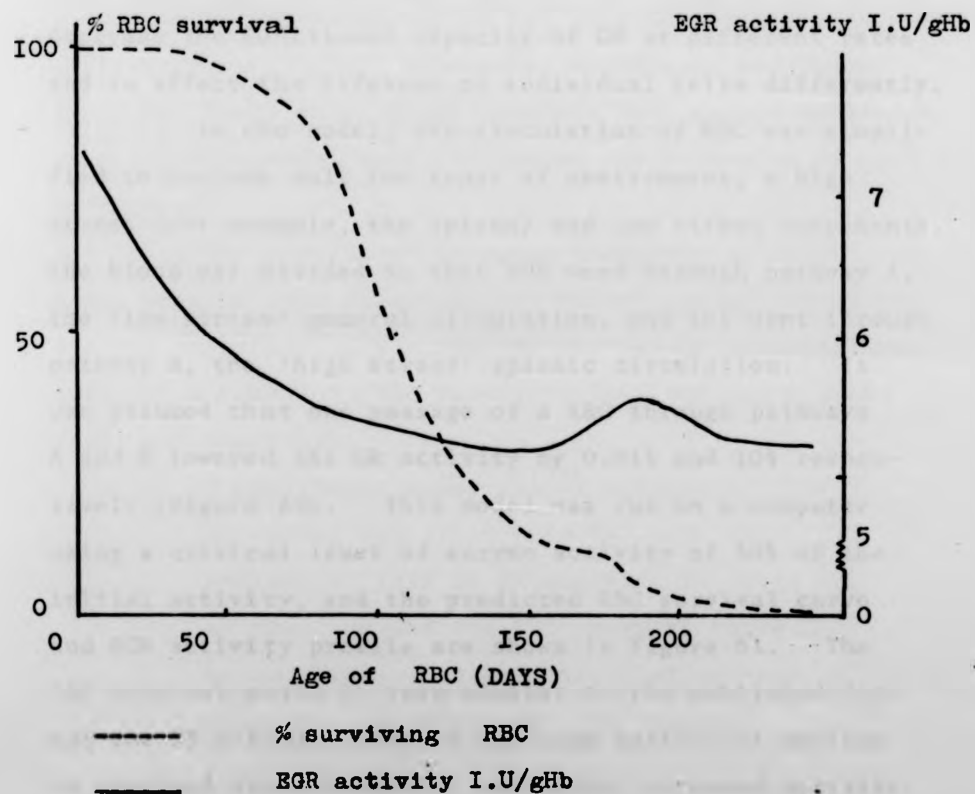
There is evidence that the splenic circulation imposes greater stresses on RBC than other parts of the general circulation (Weiss and Tavassoli, 1970). Thus in the repetitive passage of a RBC through the mammalian circulation it is subjected to relatively high stress (the splenic circulation) and low stress (most other parts of the circulation). On each passage through the circulation the probability of a RBC going through the high stress or low stress pathway is governed by the flow rates through each pathway but, subordinate to this, the route taken by an individual RBC is a random event. Mechanical and chemical stress is associated with an

Figure 60 A Simplified Model Of The Circulation



The flow of blood through the spleen, a high stress pathway (B) and the rest of the circulation, a low stress pathway (A) was modelled. P_A and P_B represent the % of the blood that flows through each pathway, D_A and D_B the % loss of activity of EGR assumed to occur when a RBC passes through each pathway.

Figure 61 RBC Survival and EGR Activity as predicted by a Simple Computer Model of the Circulation



increased load being placed on enzyme systems (Jacob, Ingbar and Jandl, 1965) and with a possible loss of the structural integrity of enzyme protein (Tappel, 1973; Desai and Tappel, 1963). Therefore, RBC may be subjected serially and randomly to two or more levels of stress which decrease the functional capacity of GR at different rates and so affect the lifespan of individual cells differently.

In the model, the circulation of RBC was simplified to include only two types of environment, a high stress (for example, the spleen) and low stress components. The blood was divided so that 90% went through pathway A, the 'low stress' general circulation, and 10% went through pathway B, the 'high stress' splenic circulation. It was assumed that one passage of a RBC through pathways A and B lowered the GR activity by 0.01% and 10% respectively (Figure 60). This model was run on a computer using a critical level of enzyme activity of 50% of the initial activity, and the predicted RBC survival curve and EGR activity profile are shown in Figure 61. The RBC survival curve is very similar to the published data and the GR activity follows the same pattern of decline as observed experimentally, including increased activity in some of the old cells.

While it would be unwise to draw strictly quantitative conclusions from such a simplified model of the circulation the results support known facts concerning the effects of splenectomy or splenomegaly on RBC survival

(Pohl, Bugajer-Gleitman, Lachmann und Moser, 1976; Jandl, Greenberg, Yonemoto and Castle, 1956). The increased stress to which RBC are subject within the spleen means that most RBC would reach their critical level of GR activity and disintegrate there. A splenic sensing mechanism for senescent cells has been postulated by several workers (Danon, 1975; Skutelsky, Marikovsky and Danon, 1974) but there is no clear evidence that such a mechanism exists; this model does not demand the existence of such a mechanism.

The results of this two-compartment circulation model suggest that environmental factors alone can account for the variation in the rate of fall in GR activity among cells of a cohort. The justifications for the present model are therefore:

1. A hypothesis using simple mathematics has accounted quantitatively for both the pattern of fall in GR activity in cells as they age and the RBC survival curve.
2. An erythrocyte enzyme, AST, that is not essential for RBC survival, does not show the late rise in activity in old cells, characteristic of GR activity.
3. A known physiological mechanism (destruction of the majority of RBC in the spleen) can help to account for 'k' the different rates of decline in enzyme activity in different cells.

The observed pattern of fall in GR activity and the pattern of RBC survival in the elderly normal subjects and the marginally riboflavin-deficient subjects support the model. In marginally riboflavin-deficient subjects the rise in GR activity starts from Fraction 6 whereas in normal subjects no significant rise is detectable until Fraction 7 (compare Figures 16B and 19); this suggests that cells from marginally riboflavin-deficient subjects reach their critical level of GR activity earlier than in normal subjects. This is compatible with the lower GR activity in every fraction of riboflavin-deficient subjects when compared with normal subjects and the slightly reduced RBC survival time in deficient subjects (Figures 32, 33 and 34). Normal elderly subjects on the other hand showed an increased proportion of old cells and a higher activity of GR in every fraction when compared with normal adults (Figures 35 and 18). Also the rise in activity of GR in old cells from these elderly subjects was less significant than that seen in normal adults (compare Figures 16B and 18). RBC entering the peripheral circulation with high GR activity might be expected to retain the activity above a critical level longer, also, a reduced cardiac output in the elderly (Brandfonbrener, Landowne and Shock, 1955) implies a reduced frequency of passages through the 'high stress' circulation; a combination of these factors may explain the apparently prolonged lifespan of RBC in elderly subjects. If RBC survive longer in the circulation

a rise in GR activity due to loss of some cells may not take place until late in the lifespan of the cohort. The practical limits to the degree of resolution of old RBC into different fractions may obscure a late rise in activity of GR in old cells.

The results of several lines of research have indicated that GSH has an important role in the maintenance of RBC integrity (Allen and Jandl, 1961; Mortensen, 1964; Scheuch, et al., 1961) therefore it is not unreasonable to propose GR as a determinant of RBC survival. The model may have value in determining the critical enzyme which limits RBC survival under varying physiological and pathological conditions.

CONCLUSIONS AND IDEAS FOR FUTURE STUDIES

Human red blood cells were successfully separated into fractions of different mean ages, employing a discontinuous density gradient. AST, an enzyme known to fall in activity with increasing RBC age, declined in activity through the density gradient, thus confirming the chronological nature of the red cell separation. An age-related decline in specific activity of EGR, a flavin-dependent enzyme, was also demonstrated *in vitro*, both in the presence and absence of excess cofactor, FAD. This was interpreted as indicating that with increasing RBC age there may be both a reduced level of cofactor available for GR activity and more importantly a structural modification of enzyme protein leading, in effect, to a 'loss' of potentially active enzyme.

Blood samples from marginally riboflavin-deficient subjects contained a reduced proportion of old cells and an increased proportion of young cells compared with blood from normal subjects, suggesting that marginal riboflavin deficiency is associated with a decrease in the lifespan of RBC. This effect was most apparent among the elderly subjects but this may have been due to the apparently longer survival time of RBC in normal elderly subjects when compared with normal young subjects. The reduction in cardiac output characteristic of human ageing and therefore the reduction in the frequency with which

red cells are subjected to particularly stressful areas of the circulation, such as the spleen, may explain the apparently longer survival time of RBC in normal elderly subjects.

Red blood cell ageing, regardless of riboflavin status, was accompanied by a number of biochemical and physiological changes including a reduction in the concentration of GSH, an increase in the concentration of MHb and an increase in the susceptibility of cells to peroxidative haemolysis in vitro. GSH is a product of GR activity and therefore might be expected to fall concomitant with the decline in GR activity as red cells age. GSH and FAD have both been ascribed roles in the maintenance of Hb in its oxygen-carrying form, and therefore the observed decline in GSH concentration and the possibility that RBC FAD concentration also declines as red cells age may have contributed to the elevated concentration of MHb in older RBC. As part of a study into the possible mechanisms for peroxidative haemolysis it was demonstrated that when RBC are exposed to H_2O_2 young cells are able to detoxify the H_2O_2 more rapidly than old cells. The reduced concentration of GSH in old cells may have resulted in a reduction in the efficiency of the GSHPx system in removing H_2O_2 therefore leading to an increased susceptibility to the haemolysing effect of H_2O_2 in vitro.

When compared with normal subjects, marginal riboflavin deficiency was associated with a consistently

lower GR activity and a slightly increased susceptibility to peroxidative haemolysis by RBC in each fraction recovered from the density gradient. A significantly higher concentration of MHb was demonstrated in the old cells of marginally riboflavin-deficient subjects in comparison with old cells from normal subjects. These differences were considered to indicate that those degenerative changes associated with riboflavin-dependent enzymes which take place as RBC age are accelerated in riboflavin deficiency. These differences were also evident in unfractionated blood from riboflavin-deficient subjects and controls with normal riboflavin status.

Two riboflavin supplementation experiments were performed in which subjects with marginal riboflavin deficiency were administered regular oral doses of riboflavin and the biochemical and haematological responses of RBC monitored. Cells of all ages responded to the supplement in that GR activity, both basic and stimulated, rose during the course of supplementation indicating that riboflavin can be incorporated into young and old cells. However, the more rapid fall in unsaturated GR apoenzyme observed in young cells than in old cells suggested that the turnover of FAD may be slower in old cells, either due to an impaired conversion of riboflavin to FAD or to a leakage of FAD from older cells. Concomitant with the increase in GR activity was a significant increase in GSH concentration in cells of all ages.

The riboflavin supplementation appeared to

'normalise' the RBC survival profile, as demonstrated by the progressive reduction in the proportion of young cells and an increase in the proportion of old cells, supporting the suggestion that riboflavin deficiency may reduce the survival time of RBC *in vivo*.

The measurement of MDA, a breakdown product of lipid peroxidation, did not appear to be a useful means of assessing the concentration of lipid peroxides in cells of different ages. With increasing RBC age, a reduction was observed in the concentration of MDA produced as a response to oxidant stress *in vitro*. This may have been due to quantitative changes that take place in the lipid components of RBC as they age and also to the reaction of MDA with lipid and/or protein constituents of the RBC membrane. On the other hand, MDA production in unfractionated RBC did appear to reflect the degree of peroxidative stress to which cells were subjected *in vitro* in that MDA produced increased with increasing exposure of RBC to H_2O_2. No differences were observed between the amount of MDA produced in RBC from normal or marginally riboflavin-deficient subjects, possibly because old cells, which would be expected to show accumulated oxidant damage, appear to be removed from the circulation prematurely in marginally riboflavin-deficient subjects.

An enzymatic method for the detection of total peroxides in blood proved unsuccessful. This was attributed to the fact that Hb interfered with the assay,

possibly due to its ability to bind with and to modify components of the assay system. It was also thought possible that lipid peroxides formed in vitro remained as an integral component of the RBC membrane and were therefore not present in stroma-free haemolysates used in the assay.

In both normal and marginally riboflavin-deficient subjects studied, a significant rise in activity of GR was observed in the oldest cells recovered from a density gradient by comparison with penultimate fractions. This may have been due to a selection out of the circulation of those cells with GR activity below a minimum activity for survival and therefore a progressive increase in the mean GR activity of the oldest surviving cells. This hypothesis was tested using a mathematical approach and a computer model which simulated RBC survival and GR activity in cells of a cohort. In developing the model it was assumed that GR activity was a major determinant of RBC survival or was closely related to the activity of another such enzyme. The close fit between the predicted RBC survival curve and published data and between the predicted GR activity of cells in a cohort and the experiment data, including the rise in old cells supported the original hypothesis. The hypothesis was tested further using a simple model of the circulation in which RBC were assumed to experience two levels of stress, a 'high stress' splenic circulation and a 'low stress' general circulation, which

reduced GR activity to different extents. The computer model simulated RBC survival and GR activity in a cohort of cells; a close fit was obtained between observed data and that predicted by the model, lending further support to the original hypothesis.

In summary, marginal riboflavin deficiency appears to be associated with an acceleration of the oxidant processes taking place within the red cell as it ages, and a small reduction in the survival time of RBC *in vivo*. GR activity may be a primary determinant of RBC survival such that a critical level of activity exists below which the RBC will not survive. However, in otherwise healthy individuals the biochemical effects of marginal riboflavin deficiency are not considered to be of physiological importance. When riboflavin deficiency is accompanied by folic acid, vitamin E or vitamin B_{12} deficiency, as might occur among the elderly population, an impairment of RBC production and survival may result.

Plan for Future Work

A number of interesting areas of study have emerged from the work presented here.

It was suggested that the observed decline in GR activity in RBC as they age may have been due to a decreased availability of FAD in old cells, enzyme protein modification, or a combination of both. In order to investigate the former possibility it would be necessary

to determine fluorimetrically the concentration of FAD in RBC of different ages. Conformation changes in GR in ageing RBC could be approached kinetically. That is, if large enough quantities of blood could be obtained then GR apoenzyme could be prepared from young and old RBC and the affinity of the enzyme for different concentrations of FAD could be investigated.

Results of the supplementation experiment suggested that the incorporation of riboflavin into FAD may be slower in older cells. In order to establish whether this is indeed the case experiments could be performed in which the rate of uptake of radioactive riboflavin was measured in young and old cells. It might also be instructive to determine the activities of flavokinase and FMN phosphorylase, the two erythrocyte enzymes considered to be responsible for the conversion of riboflavin into FAD, in young and old cells.

In order to establish whether the lipids of RBC become more susceptible to peroxidation as the cells age alternative methods of assessment need to be attempted such as the estimation of dienes and trienes in lipid extracts of cells, or the direct enzymatic estimation of peroxides in preparations of RBC membranes. As GSHPx was ascribed an important role in the protection of RBC against oxidative damage it would be interesting to measure the activity of this enzyme in RBC as they age, in blood from both normal and marginally riboflavin-deficient subjects.

Preliminary studies indicated that GR activity may be a primary determinant of RBC survival, however more work is required on this point. It would be instructive to investigate in detail the pattern of decline in a number of different erythrocyte enzymes as RBC age, particularly those that have been implicated as determinants of RBC survival, to determine whether other enzymes show the same increase in activity in old cells. It might also be possible to measure the activity of GR in RBC before and after passing through the splenic circulation to test the hypothesis that the stress imposed on RBC in the spleen effects a reduction in EGR activity.

Finally, more work is required to elucidate the possible role of riboflavin in iron metabolism in man, particularly in its relation to the processes of red cell production from the bone marrow.

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APPENDIX

Second European Nutrition Conference, Munich 1976
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Influence of Red Cell Age on the Measurement of Riboflavin Status[1]

Hilary J. Powers and D.I. Thurnham

Department of Human Nutrition, London School of Hygiene and Tropical Medicine, London

Introduction

Erythrocytes are produced continuously by the bone marrow, and each has a finite circulation time, hence a blood sample will contain red cells of different ages. Increasing age of the erythrocyte is accompanied by increasing density and a decrease in activity of some enzymes (*Allison and Burn*, 1955). Erythrocyte glutathione reductase (EGR) is dependent for its activity on flavin adenine dinucleotide (FAD), and the degree of saturation of a tissue by FAD is expressed by an activation coefficient (AC), which is a measure of riboflavin status.

DHSS surveys in elderly people in the UK have shown a large proportion of subjects with biochemical ariboflavinosis, i.e. AC $\geq$ 1.30 (*Thurnham and Stephen*, 1975). Experiments were done on samples from elderly and younger people to determine whether differences in the survival time of erythrocytes and changes in the enzyme activity accounted for the apparent ariboflavinosis.

Materials and Methods

Carefully washed, packed red cells were layered onto density gradients of a Ficoll/Triosil mixture. Ultracentrifugation at 80,000 *g* for 2 h at 4 °C resulted in nine discrete bands of red cells of differing mean age. Fractions were separated by Pasteur pipettes, washed with saline and haemolysed in distilled water. EGR activity in the haemolysate (0.1–2.0 mg haemoglobin) was measured in 0.1 *M* potassium phosphate buffer (pH 7.4), 0.92 m*M* GSSG, 20 m*M* EDTA with or without 8 μ*M* FAD in a reaction initiated by 80 μ*M* NADPH and followed at 334 nm and 35 °C in a total volume of 3.6 ml.

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Table I. EGR activity and haemoglobin in red cell fractions of differing mean age

Subjects	Mean red cell density, g/ml									
	1.103	1.110	1.116	1.121	1.124	1.127	1.132	1.1375	1.145	unfractionated
Young (5)										
IU_B	11.6	8.6	7.5	6.7	6.6	6.4	6.6	6.9	8.2	8.3
IU_S	13.1	10.1	9.3	8.6	8.2	8.1	7.7	8.7	9.8	10.4
AC	1.13	1.17	1.24	1.28	1.24	1.27	1.17	1.26	1.20	1.25
Hb%	2.6	19.9	23.7	15.0	14.8	8.2	8.1	2.7	4.3	
Elderly (5)										
IU_B	15.9	9.2	8.7	7.5	7.2	7.4	7.7	7.6	7.4	9.4
IU_S	15.9	9.3	9.3	8.0	7.6	7.8	8.8	7.5	7.7	9.9
AC	1.0	1.01	1.07	1.07	1.06	1.05	1.14	0.99	1.04	1.05
Hb%	2.5	7.4	22.0	18.1	13.3	9.1	14.5	9.4	9.7	
Elderly deficient (2)										
IU_B	9.4	7.7	6.1	5.4	4.9	4.5	5.5	4.5	5.6	7.1
IU_S	11.3	9.9	8.7	7.6	7.5	7.0	7.9	7.1	8.6	10.2
AC	1.20	1.29	1.43	1.41	1.53	1.56	1.44	1.5	1.54	1.49
Hb%	17.1	28.4	31.5	12.2	3.5	3.8	2.3	1.6	1.6	

Number of subjects in parentheses. Results shown are mean values. IU = International units (μmoles NADPH oxidised min^{-1}, g Hb^{-1}) measured without (IU_B) and with (IU_S) FAD; AC = activation coefficient (IU_S/IU_B); Hb% = haemoglobin concentration expressed as percentage of total.

Results and Discussion

The results (table I) show that there was a fall in both basic and stimulated EGR activity with increasing erythrocyte age and a corresponding increase in AC in all subjects. The percentage of haemoglobin indicates the proportion of cells in each fraction. No significant differences between any of the three groups were found in the rate of decrease of enzyme activity or the distribution of cells between fractions.

The study has shown that red cell age influences the AC. It seems probable that washing procedures which efficiently remove white cells will also remove selectively more of the younger red cells, affecting the final AC produced, and lead to over-estimation of riboflavin deficiency. Further studies to determine the contribution of white cells in whole blood EGR measurements and the effect of washing procedures are under examination.

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H.J. Powers, Department of Human Nutrition, London School of Hygiene and Tropical Medicine, Keppel Street, *London WC1E 7HT* (England)

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