ERYTHROCYTE PATHOPHYSIOLOGY OF RIBOFLAVIN DEFICIENCY IN RATS

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by

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TO MY WIFE AND CHILDREN
FOR THEIR
PATIENCE AND COOPERATION

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ABSTRACT

Pathophysiology of erythrocytes from riboflavin-deficient rats has been investigated. Riboflavin-deficiency was produced by feeding a riboflavin-deficient diet ad-libitum to weanling male Wistar specified-pathogen-free, albino rats (40-50g) housed individually in wire-bottomed cages. Characteristic signs of severe deficiency were produced in most animals,and included weight loss, hair discolouration and skin lesions. Biochemical status of riboflavin deficiency was assessed enzymatically by measuring the erythrocyte glutathione reductase activity coefficient (EGR-AC) and the association of this index with all other changes which occurred, was investigated. Control animals were fed the riboflavin-deficient diet plus 22mg of riboflavin/kg of diet. Pair-fed animals were given the average amount of food which was eaten by the deficient rats the previous day.

The results reported in this thesis showed that riboflavin-deficiency is directly correlated with red blood cell fragility as measured by either in-vitro exposure of these cells to a H_2O_2-generating system and/or hypotonic saline solutions. The degree of haemolysis obtained was found to be negatively correlated with concentrations of reduced glutathione (GSH) of the riboflavin-deficient blood. It was found also that thyroid hormone prevented the in-vitro haemolysis of red cells from riboflavin-deficient rats exposed to the above system and a preliminary experiment suggested that prior administration of thyroxine in-vivo had a similar effect on red cell integrity when tested subsequently in-vitro.

When erythrocytes from riboflavin-deficient rats were separated into fractions of different mean age, haemoglobin concentrations in the red cell fractions suggested that there was a progressive reduction in the number of young cells as the severity of the riboflavin-deficiency increased and a corresponding increase in the proportion of old cells. There was also a progressive reduction in the number of reticulocytes and plasma iron and an increase in the non-haem and ferritin iron in liver tissue as biochemical riboflavin-deficiency increased. Red cells from the deficient animals were significantly more fragile in all fractions than the comparable fractions from control animals. Other changes which accompanied riboflavin-deficiency were increases in the concentration of erythrocyte peroxides, methaemoglobin and the activities of glutathione peroxidase, NADH-methaemoglobin reductase and NADPH-methaemoglobin reductase.

Riboflavin-deficiency causes a reduction in the activity of glutathione reductase and it is suggested that the increased fragility of red cells from riboflavin-deficient rats may be a consequence of the inability to maintain concentrations of GSH. The reduced concentrations of GSH may also be inadequate to supply glutathione peroxidase with adequate substrate causing increased concentrations of peroxides to accumulate and possibly exert damaging effects on lipid structures, for example, cell membranes. The increase in erythrocyte methaemoglobin may be further evidence of the defence mechanisms against oxidant moieties and the increase in methaemoglobin reductase and glutathione peroxidase, compensatory effects to counteract the oxidant stress.

Many of the changes found in erythrocytes of riboflavin-deficient rats occurred also, to a variable degree, in erythrocytes from iodine-deficient rats. In addition it was confirmed that concentration of plasma thyroxine fell in blood from riboflavin-deficient rats. The interaction of thyroid status and riboflavin-deficiency is causing the changes found in erythrocytes is discussed.

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LIST OF ABBREVIATIONS

Adenosine triphosphate	ATP
Adenosine triphosphatase	ATPase
Aspartate aminotransferase	AST
Centimetre	cm
Change in optical density	VO.D.
Correlation coefficient	r
Density	g/ml
Erythrocyte glutathione reductase	EGR
Erythrocyte glutathione peroxidase	EGP
Ethylenediaminetetra-acetate	EDTA
Fatty acids	F.A.
Figure	Fig.
Flavin adenine dinucleotide	FAD
Flavin mononucleotide	FMN
Free erythrocyte porphyrin	FEP
Glucose-6-phosphate dehydrogenase	G-6-PD
Glutathione, oxidized	GSSG
Glutathione, reduced	GSH
Glutathione reductase	CR
Gram	g
Gram - atom	mol
Gravitational field, unit of (in centrifuge, 9.81 m.S^{-2})	g
Haemoglobin	Hb
Hydrogen ion concentration, minute log of	pH
Hydrogen peroxide	H_2O_2
Hydroxyl anion	OH^-
International units	i.u.
Iron	Fe
Kilogram	Kg

Litre (10^{-3} m^{-3} = dm^3)	l
Logarithm(base 10)	log
Methaemoglobin	MetHb
Microgram	µg
Micromole	µ mol
Millilitre	ml
Millimolar (concentration)	mM or mmol/l
Millimole	mmol
Minute	min
Molar (concentration)	M
NADH-Methaemoglobin reductase	NADH-MR
NADPH-Methaemoglobin reductase	NADPH-MR
Nicotinamide-adenine dinucleotide	NAD
Nicotinamide-adenine dinucleotide, oxidized	NAD^+
Nicotinamide-adenine dinucleotide, reduced	NADH
Nicotinamide-adenine dinucleotide phosphate	NADP
Nicotinamide-adenine dinucleotide phosphate, oxidized	$NADP^+$
Nicotinamide-adenine dinucleotide phosphate, reduced	NADPH
Number	no.
O-Phthalaldehyde	OPT
Osmotic fragility	O.F.
Oxyhaemoglobin	HbO_2
Packed cell volume	PCV
Per	/
Per cent	%
Red Blod Cell	RBC
Singlet oxygen	O_2^1

Standard deviation	SD
Sulfhaemoglobin	SGb
Superoxide anion	$O_2^{\overline{\cdot}}$
Thiobarbituric acid	TBA
Trichloroacetic acid	TCA
Volume	vol.
Volume/Volume	V/V
Weight	wt.
Weight/Volume	W/V
White Blood Cell	WBC

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

I- HISTORICAL REVIEW

1. Discovery of Riboflavin and Effects of Riboflavin Deficiency in Man and Animals

The significance of Blythe's discovery of the yellow-green pigment in milk in 1879 (Spies, Hilman, Cohlan, Kramer and Kanof, 1960) was not appreciated before Gyorgy's studies which led to the discovery of riboflavin. Gyorgy's studies between 1908 and 1933 were stimulated by the appearance of pathological changes in the skin when animals were fed diets lacking some "nutritional essentials" (Gyorgy, 1954). Early investigators found that the "nutritional essentials" could be met by feeding yeast. Furthermore, they believed that the "nutritional essential" was the same substance which prevented and cured beri-beri. As a result of this, the customary procedure in the laboratory was to use yeast as a source of vitamin B and attribute to the antineuritic substance (or thiamin) all "Vitaminic" properties (Sherman and Lanford, 1940). Some years later, however, it became apparent that yeast must contain a heat-resistant, water-soluble growth factor in addition to the heat-labile antineuritic substance, since heated yeast retained some growth supporting properties. The active factor in yeast and other substances was the greenish-yellow, water-soluble material first isolated from milk by Blythe, 1879 (Spies *et al.* 1960). In 1935, Kuhn, Reinemund, Weygand and Strðbele suggested that these yellow fluorescent, water-soluble pigments be named "Flavins" and that a prefix be attached to indicate the food of origin. Accordingly, the term "lactoflavin" was introduced to the flavin isolated from milk, "ovaflavin" from egg white and "hepatoflavin" from liver. Chemical analysis however, soon indicated that all biologically active flavins were derivatives of isoalloxazine with two methyl groups and a sugar (pentose) radical attached. Since the sugar radical is ribose, the term "lactoflavin" and other trivial names were subsequently changed to riboflavin, a designation which was originally proposed by the Council of Pharmacy and Chemistry of the American Medical Association in 1937 and later by the International Commission for the Reform of Biochemical Nomenclature in 1952 (Gyorgy, 1954).

Riboflavin plays an important part in the economy of food utilization both for growth and for maintenance. Pair-feeding experiments showed that the rats fed the same amount of control diets as eaten by animals receiving the riboflavin-deficient diet, grew more rapidly (Sure, 1941; Sure and Dicheck, 1941). In fact, riboflavin-deficient rats consume 15-20% more calories than those required by control animals to maintain the same weight (Luse, Burch and Hunter,1962). It was further demonstrated that, unlike in thiamin deficiency where gross anorexia occurs, the final collapse of the riboflavin-deficient rat is not associated with any great decrease in the food intake (Voris, Black, Swift and French,1942). The most logical explanation for the poor economy in ribo-flavin deficiency is that intermediate products of metabolism are probably wasted through incomplete metabolism.

The absence of riboflavin from the food of both man and animals has been studied by many workers and a variety of lesions have been reported:

1. Growth retardation: Mannering and Elvehjem, 1944; Tillotson and Sauberlich, 1971 and Goldsmith, 1975.

2. Skin changes varying from brown colouration to alopecia and epithelial lesions. Lesions are usually described as a dry, scaling seborrheic dermatoses associated with hair loss. Skin lesions have been reported in rats (Sullivan and Nicholis, 1941; Wolback and Bessey, 1942); mice (Lippincott and Morris, 1941); and monkeys (Waisman, 1944; Mann, Watson, McNally and Goddard, 1952).

3. Fatty livers in dogs (Potter, Axelrod and Elvehjem, 1942); rats (Burch, Lowry, Padilla and Combs, 1956; Rivlin, Menendez and Langdon, 1968, and mice (Hoppel and Tandler, 1975) and in cats (Gershoff, Andrus and Hegsted, 1959).

4. Cataracts in rats (Baum, Michaelree and Brown, 1942); in cats (Gershoff, et al., 1959) and in the ox (Phillpot and Pirie, 1943).

5. Anaemia was reported to occur in riboflavin-deficiency in rats, mice, dogs, foxes, pigs and monkeys (Spector, Maass, Michaud, Elvehjem and Hart, 1943; Wintrobe, Buschke, Follis and Humphreys, 1944; Waisman, 1944; Greenberg and Rinehart, 1956). Other workers have suggested that anaemia occurs only occasionally in rats, however, (Endicott, Kornberg and Otto, 1947; Shukers and Day, 1943; Kornberg, Tabor and Sebrell, 1944) and anaemia was not observed in cats (Gershoff et al., 1959) or guinea pigs (Hara, 1960).

6. Partial paralysis in rats (Shaw and Phillips, 1941; and Lippincott and Morris, 1941); mice (Phillips and Engel, 1938) and monkeys (Mann et al, 1952).

7. Testicular atrophy in rats (Shaw and Phillips, 1941); in monkeys (Mann et al., 1952)

Riboflavin deficiency in humans was not recognized as a clinical entity until it was induced experimentally in human subjects by Sebrell and Butler (1939). Clinical findings consisted of lesions on the lips and at the angles of the mouth and a seborrheic type of dermatitis. The clinical signs are similar to those reported by Stannus in association with Pellagra in Nyasaland in 1912 (Stannus, 1912) but in fact the clinical signs lacked specificity and are associated with deficiencies of several nutrients e.g. iron, vitamin B_6, folic acid, etc. Riboflavin deficiency is still widespread particularly in South Africa, South East Asia and South America.

2- Factors affecting riboflavin deficiency in Rats

It has been reported that the production of riboflavin-deficiency in rats is influenced by many factors, among which, age, sex, strain, the composition of the diet, care of cages, food cups and drinking water supply, temperature and humidity of the animals' quarters, whether the animals are housed one or more to a cage, and even the number of hours of light and dark prevailing in the animal room may have an effect (Lambooy, 1975).

The most important factors in producing riboflavin-deficiency are however:

a - Ensuring that the diet contains as little riboflavin as possible.

b - Minimising coprophagy or faecal contamination of the cage.

a - Composition of the diet

i - Fat: Mannering, Lipton and Elvehjem (1941) using rat growth as their measure of riboflavin requirements showed that high-fat diets (25 and 40% lard) increased the riboflavin requirements. Further studies suggested that hydrogenated cotton seed oil had similar properties (Mannering, Orsini and Elvehjem, 1944). The unhydrogenated cotton seed oil, however, was found by Reiser and Pearson (1949) to restrict the growth of chicks in concentrations as low as 5% of the diet, while others found a diet containing 20% corn oil to have a similar effect on the growth of rats (Beare, Murrey and Campbell, 1957). Both these oils contain a high proportion of unsaturated fatty acids and the general belief at the time of these experiments was that fat depressed the bacterial synthesis of riboflavin in the gut and that unsaturated fatty acids had a greater depressant effect on the bacterial synthesis than saturated fats (Reiser and Pearson, 1949).

ii-<u>Protein</u>: It is not possible to vary the amount of fat in a diet without changing the relative concentration of the other constituents. The effects of the high-fat, low-fat, high protein and low protein on riboflavin requirements of the rat were studied by Czaczkes and Guggenheim (1946). They noted that rats on high-protein, high-fat diets needed at least twice as much riboflavin as rats kept on a control diet containing adequate riboflavin for a standard laboratory diet. These authors believed that the apparently different riboflavin requirements of rats on different diets were due to differences in the amount of riboflavin which was synthesized in the gut and available to the rat in some way.

iii-<u>Carbohydrates</u>: Animal experiments have suggested that exc ss dietary carbohydrate in the form of starch reduces the apparent riboflavin requirements of the rat (Coates, Henry, Kon, Kon, Mawson, Stanier and Thompson, 1946; Ford, Henry, Kon, Porter, Thompson, and Wilby, 1953; Haenel, Ruttloff, and Ackermann, 1959.

Much of this work supported the idea that starch carbohydrate in the diet increased intestinal synthesis of riboflavin. For instance, dextrin and corn starch were reported to increase the amount of riboflavin synthesised in the intestine by providing the intestinal micro-organisms with a favourable medium for such synthesis, or by increasing the number of micro-organisms, or by changing the flora to a type capable of producing greater quantities of the vitamin (Mannering et al., 1944). Coprophagy however, was shown to be the major route by which the intestinally synthesized riboflavin became available to the rat (Barnes, Fiola, McGehee, and Brown, 1957). Incomplete digestion of dextrin and corn starch allows some carbohydrates to reach the lower regions of the tract, where micro-organisms are found in abundance. In these circumstances pellet sizes are increased which appears to increase the likelihood of coprophagy. It is also possible that some bacterially synthesized riboflavin is absorbed directly from the colon.

Sucrose is more readily digested and absorbed than starch, and it does not reach the colon in any great quantities. Stool size on a sucrose diet is reduced and Mannering et al.,(1944) reported that riboflavin requirements of the rat were increased on a sucrose-based diet by comparison with a starch diet. Morgan, Cook and Davison (1938) reported that riboflavin requirements were even lower on a lactose-based diet. Their explanation for these findings was that they were able to show that the caeca of lactose-fed rats contained appreciable amounts of riboflavin. However, the importance of other dietary factors e.g. dietary fibre, which may also have been present, was not appreciated at the time.

The idea that riboflavin synthesized within the gut was available to man and that intestinal synthesis and urinary excretion of riboflavin depended upon the ratio of fat to carbohydrate calories in the diet was still popular even in the 50's when Widdowson and McCance (1954) were working in German orphanages . Also at that time Japanese workers were suggesting that the high vegetable fibre content of Japanese diets promoted intestinal synthesis of riboflavin and thus reduced the dietary riboflavin requirements of the Japanese people (Iinuma, 1955). However, it is now accepted there is very little evidence that intestinally-synthesized riboflavin is available to rats and man (Kasper, 1965; Jusko and Levy, 1970).

b- <u>Coprophagy</u>

The practice of coprophagy, i.e. feeding on excrement is commonly observed among insects and birds and was first recorded in laboratory animals in 1911(see Elvehjem, 1948)when it was reported that rats maintained on a nutritionally poor diet had poor weight gain and became coprophagous. If such animals were fed the faeces of rats on a complete diet, their weight gain was restored (Elvehjem, 1948).

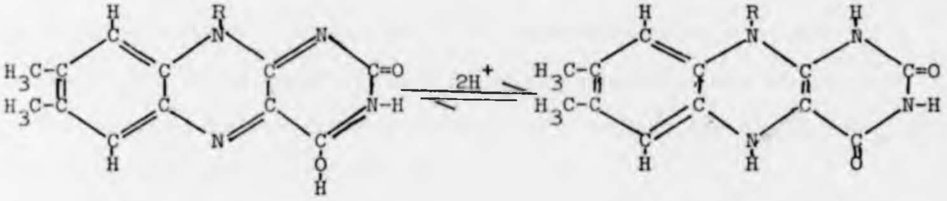
Vitamins are found in the faeces of both ruminants and non-ruminants. In ruminants, intestinal synthesis of vitamins takes place in the rumen and additionally the vitamins become available for absorption. In non-ruminants however, vitamin synthesis occurs primarily in the caecum and absorption from the large intestine is negligible (Michelson, 1956). Nutritional benefits can only be derived therefore from this vitamin-rich source by coprophagy (Michelson, 1956; Barnes, <u>et al</u>., 1957). So, coprophagy delays the ultimate excretion of compounds of dietary origin and their products, and alters the quantitative utilization of nutrients following chemical modification by intestinal flora. This flora in turn may be changed as a result of coprophagy, making bacterial synthesis of nutrients more efficient (Fitzgerald, Gustafsson and McDaniel, 1964).

Attempts have been made by several authors to prevent coprophagy (Barnes et al.,1957; Barnes and Fiola, 1958; Barnes, Fiola and Kwong, 1963; Frape, Wikinson and Chubb, 1970; and Thomas and Roe, 1974). Barnes et al., 1957 estimated that the rat normally recycles from 50-60% of its faeces when fed a complete diet, and more if the diet is nutritionally inadequate. The workers showed that growth rate could be depressed by 15-25% if coprophagy was prevented by faecal collection cups, even when a nutritionally complete diet was provided. Without cups, coprophagy is only slightly reduced by caging on screens as opposed to solid floors (Barnes et al., 1957). By preventing coprophagy, supplementation of the rat's diet with additional nutrients may be reduced and the time required to obtain a pathological condition in metabolic studies may be more precisely determined.

Frape et al.,(1970) described a metabolic cage and a tail cup to improve the precision of experimental outcome with male rats. The tail cup employed was described as an improvement over the method of restraint by leather jacket described by Armstrong and Softly (1966) and also of the tail cup described by Barnes et al.(1957). Even the tail cups described by Frape et al.(1970) were found to be unsuccessful however, unless considerable daily attention was given to the animals (Hassan,1975; Prentice, 1978). Other ways of overcoming the problem of coprophagy such as the use of grids with extra wide spaces and/or the inclusion of the antibiotic succinylsulphathiazole in the diet have been attempted but are only partially successful (Hassan,1975).

3. Role of Riboflavin in the Metabolism

a- Functional forms of riboflavin:



Oxidised form (Functional) R= Ribityl sugar Reduced form (Non-Functional)

Figure (1)

Riboflavin comprises an isoalloxazine nucleus with an attached ribityl sugar. The isoalloxazine nucleus acts as a reversible redox system and the function of riboflavin is to undergo reversible oxidation and reduction as illustrated in Fig. (1). Labelling experiments using ^{14}C riboflavin in rats (Yang and McCormick, 1967) showed that riboflavin is converted into flavin mononucleotide (FMN) especially in the tissues of small intestine, kidney and liver and the FMN is the precursor for the biosynthesis of flavine adenine dinucleotide (FAD). The two forms of riboflavin are active as coenzymes in flavoprotein enzymes and play an important role in many essential metabolic functions (Dixon and Webb,1964; Lipman, 1969). They are particularly important in biological oxidation, where they can accept hydrogen from reduced nicotinamide adenine dinucleotide (NADH and NADPH). Flavoproteins are specially important in the mitochondria where they are an integral part of the electron transport chain.

b- Distribution of riboflavin in the tissues

Riboflavin is actively absorbed across the intestinal mucosa and enzymatically phosphorylated to FMN within the mucosal cells (Yagi and Okuda, 1958; Chen and Yamouchi, 1960). It appears initially in the blood as FMN. In man and rats, dephosphorylation of FMN occurs in the whole blood and this forms the source of the free riboflavin detected in venous blood (Jusko, Levy, Yaffee, and Goodischer, 1970; Nogami, Hanano, Awazu, and Iga, 1970.

Most of the flavin taken in by an organism is stored as co-enzymes largely in association with specific proteins with which they function catalytically in numerous biological oxidations. Maximum concentrations are obtained with intakes of about 40ug per day in rats while maximum growth was attained on about 30ug (Kuhn et al.,1935), but more recently Bro-Rasmussen (1958) concluded that the maximum requirements for the rat were 18μg/day. Male rats grow faster than females, even with suboptimal tissue flavins. Hence, tissues from male animals were found to contain less riboflavin than females with a given suboptimal intake (Decker and Byerrum, 1954).

Early attempts to measure the distribution of riboflavin in the tissues gave only approximate values because of the lack of sufficiently sensitive methods. It was not until fluorimetry was used to measure riboflavin that reliable data became obtainable (Burch, Lowry, DeGubareff and Lowry, 1958). These workers showed that most of the flavin present in the blood was there as FAD and that amounts in white blood cells (100 times) and red cells (10 times) were greater than those present in the serum.

Cerletti and Ipata (1960) using fluorimetric analysis on tissue samples, showed that liver, kidney and heart contained most of the rat's riboflavin, and again, this was mostly in the form of FAD. The kidney was richest in FMN where it formed almost 25% of the total flavins, whereas only 15% of the flavins in the whole body were FMN. Free riboflavin constituted only 2% of the total body flavins. Yang and McCormick (1967) using ^{14}C riboflavin in physiological amounts reported that the liver contained most of the radio activity and that most of this was present as FAD. The amount of radio-activity present in the blood was very low.

c- Effect of riboflavin deficiency on the distribution of riboflavin in tissues

The main effect of riboflavin deficiency on tissue flavin levels is to reduce the absolute quantities present. Several workers showed that within three weeks levels of red carcas riboflavin fell to a little less than half initial values (Burch, Lowry, Padilla and Combs, 1956; Bessey, Lowry, Davis and Dorn, 1958). Secondly, there is a redistribution of flavin within the tissues to compensate for the different metabolic requirements of different enzyme systems. Burch <u>et al</u>. (1956) showed that different tissues contained different amounts of FAD and FMN-dependent enzymes and the activity of these fell at different rates. Changes were much smaller in the kidney and heart, while in the brain they were negligible. FMN is lost more rapidly than FAD in the liver. NADH-dehydrogenase, an FAD-dependent enzyme has the highest activity in all tissues and is significantly decreased only in severe deficiency. By contrast, activity of glutathione reductase falls rapidly from the onset of deficiency (Tillotson and Sauberlich, 1971; Glatzle, Weiser, Weber and Wiss, 1973.

d- Flavoprotein enzymes

It is now known that there are about 80 flavoprotein enzymes (Enzyme Nomenclature, 1972). A few of these have FMN as prosthetic groups but the majority contain FAD. As already mentioned the biological activity of flavin groups in flavoprotein enzyme is due to the ability of the riboflavin molecule to undergo oxidation and reduction, i.e. they act as hydrogen carriers. The prosthetic group of the enzyme is readily reduced by its specific substrate when the latter is activated by combination with the active centre in the enzyme protein.

In general there are two main groups of flavoprotein enzyme divided according to the type of reaction they catalyse (Dixon and Webb, 1964). The first comprises those flavoproteins which react directly with oxygen, reducing the oxygen molecule to hydrogen peroxide (H_2O_2) e.g. xanthine oxidase. Most of this group react *in vitro* with dyes such as indophenol and methylene blue. The second group include those flavoprotein enzymes which do not use oxygen as acceptors but react *in vitro* with other specific acceptors, for example, glutathione reductase which uses oxidised glutathione (GSSG). Others in the second group are important links in the respiratory chain.

Within the red blood cell, there are more than ten flavoprotein enzymes, and probably the most important of these are those enzymes which remove excess electrons from oxidised haemoglobin, that is converting methaemoglobin to haemoglobin, or act as hydrogen carriers in the formation of reduced glutathione (GSH). The latter is part of a chain of enzymes which assist in the detoxification of free radicals, singlet oxygen, H_2O_2 and lipid peroxides. In the red blood cells, oxidation is a continuous threat to the stability and function of circulating red cells which occurs by the action of such endogenously-produced substances as free radicals, singlet oxygen or H_2O_2. The two flavoprotein enzymes which play a role in this protective function are methaemoglobin reductase (MR) and erythrocyte glutathione reductase (EGR)(Fig 2).

e- Riboflavin deficiency and anaemia

The question whether anaemia occurs as a primary rather than a secondary manifestation of riboflavin deficiency has been a matter of controversy for many years. The first suggestion that riboflavin deficiency and anaemia were associated was made by Gyorgy and co-workers (1938) who found that riboflavin administration caused a definite increase in haemoglobin production above the basal level when fed to "standardised anaemic dogs" on a salmon and bread diet. However, alterations of haematological indices as a result of riboflavin deficiency were not found in man in early studies (Horwitt, Hills, Harvey Liebert and Steinberg , 1949). More recently other workers combined a riboflavin-deficient diet with a riboflavin antagonist, galactoflavin, and their patients developed a severe anaemia which was reversed by administration of riboflavin (Alfrey and Lane, 1963: Lane, Alfrey, Mengel, Doherty and Doherty, 1964). In these experiments the first change observed in the peripheral blood was a decrease in reticulocytes which occurred within two weeks of initiation of the riboflavin-deficient diet and antagonist and was followed by a significant fall in haemoglobin concentration. In rats too, Alfrey and Lane (1970) reported that riboflavin deficiency alone caused slight anaemia to develop, and subsequent administration of riboflavin was followed by a reticulocytosis.

Azzam (1966) suggested that there is a slight similarity between anaemia-accompanied protein deficiency, and anaemia-accompanied riboflavin deficiency. In both these anaemias he suggested that there is a common pathway in that protein deprivation may reduce erythropoietin production and riboflavin deficiency may have a similar effect or may be required as a co-factor in the production of red cells. He further suggested erythropoietin titres in the plasma of riboflavin-deficient patients before and after riboflavin administration, as well as the measurement of the target organ response to endogenous erythropoietin in riboflavin deficiency, would be crucial to the elucidation of the exact role of riboflavin in erythropoiesis. Recent work has indicated three other possible ways in which riboflavin deficiency may cause anaemia. Zaman and Verwilghen (1977) suggested that stored iron was lowered in riboflavin-deficient rats and they suggested that this reduction was possibly caused by a defective incorporation of iron. Sirivich, Driskell and Frieden (1977) suggested that the integrity of the duodenal mucosa and absorption of iron may be adversely altered by riboflavin deficiency, for, in addition to finding low concentrations of stored iron and non-haeme Fe, the activity of the enzyme which transports iron across cell membranes, NADH-Oxido-reductase (Ferriductase; FMN-dependent) was decreased in liver, kidney and especially the duodenum. Finally, several workers have reported that riboflavin deficiency in rats reduced tetrahydrofolate synthesis which could affect deoxynucleic acid synthesis and consequently erythropoiesis, and be the possible cause of anaemia (Halvey and Guggenheim, 1958; Honda, 1968).

f- Riboflavin and thyroid hormones

Many investigators have demonstrated that the conversion of riboflavin into its active derivitive FMN and FAD is decreased in hypothyroidism (Rivlin et al., 1968; Rivlin, 1970 a and b; Fazekas, Pinto, Haung, Chaudhuri and Rivlin, 1978). Early studies reported little reaction between the thyroid gland and riboflavin metabolism. Riboflavin administration to normal animals had no effect either upon the histology of the gland, or upon the basal metabolic rate (Drill, 1943). The urinary loss of riboflavin in animals was not dramatically altered by treatment with large doses of thyroxine (Bessey et al., 1958) but when they expressed their data on a body weight basis, the data showed a slight decrease in the riboflavin excretion. Similarly in man, the urinary excretion of riboflavin after a loading dose of riboflavin is less in hyperthyroidism than in normal subjects (Palagiano and Baldassarre 1966). In general, the effects of hypothyroidism are in the same direction as those of riboflavin deficiency but are of a somewhat lesser magnitude.

In riboflavin deficiency a diminished amount of FMN and FAD are available for stabilising flavoprotein apoenzymes. Since the activities i.e. amounts of flavoprotein apoenzymes are increased by thyroid hormone, one might expect that in riboflavin deficiency, the lack of FMN and FAD would limit the effectiveness of thyroid hormones as an enzyme inducer (Fazekas et al., 1978). FAD, however, is necessary for the synthesis of thyroxine and it is possible that riboflavin availability may have ultimate control over thyroid hormone metabolism (Rivlin, 1975).

4. Measurement of Riboflavin Status

a - Non-functional methods

i - Dietary intake of riboflavin

An adequate dietary intake of a nutrient is by definition associated with health and the absence of clinical signs of deficiency (McCormick, 1972). The vitamin status of an individual is the net result of an interaction between intake and obligatory metabolic turnover. To quantitate obligatory turnover, i.e. minimum requirements of riboflavin, controlled depletion/repletion studies were done in man and animals (Sebrell Butler, Wooley and Isbell, 1941;

Williams, Mason, Power, and Wilder, 1943; Horwitt, Harvey, Rothwell, Culter, and Haffron, 1956).

These studies helped to establish minimum dietary requirements in man (FAO/WHO, 1965). Bro-Rasmussen, (1958) summarised the evidence for other species.

Dietary analyses themselves are not very useful for assessing vitamin status. The method is time-consuming and inaccurate and really only of use for surveys of large numbers of subjects where the many errors will tend to eliminate one another.

Careful studies however, on individual subjects receiving carefully controlled intakes have helped to correlate riboflavin concentrations in urine (Pearson, 1967; Yang and McCormick, 1967) and blood (Bessey et al., 1956; Pearson, 1967) and more recently the degree of saturation of erythrocyte glutathione reductase with FAD (Tillotson and Baker, 1972) with various degrees of dietary deficiency.

ii - Riboflavin in urine, plasma and tissues

Measurement of riboflavin studies by analysis of riboflavin in plasma and urine have been very useful but they do have some disadvantages. Both measurements are influenced by recent dietary intake Bertlett, 1955), have not been shown to be related to body stores and precision of measurements becomes poorer as the deficiency increases. In the case of urine, ideally a 24 hour sample should be obtained, but this can be difficult, even in hospitals. Random samples can be taken but many factors can adversely influence urinary excretion of riboflavin, for example, time of collection (Hegsted, Gershoff, Trulson and Jolly, 1956), physical activity (Coon, 1965), urinary volume (Horwitt, Harvey, Hills and Liebert, 1950), dietary creatinine (Plough and Consalazio, 1959), nitrogen balance (Windmueller, Anderson and Mickelsen, 1964), and stress (Sauberlich, Dowdy and Skala, 1974). With plasma the main problem is that the amount required for the assay is unacceptably large in many circumstances.

b- Functional tests

i -General: Functional tests of riboflavin status are those which measure functionally active riboflavin in the tissues. Analysis of riboflavin levels in tissues other than blood is only possible in animal experiments, however, experiments have shown that riboflavin levels in the tissues do reflect nutritional status(Kuhn, Kaltschmitt and Wagner-Jauregg, 1935: Yang and McCormick, 1967). A direct assessment of tissue bound riboflavin was therefore desirable. Circulating concentrations of riboflavin in the plasma fall more rapidly than those in the tissues on depletion (Burch et al., 1956) , therefore, analysis of tissue bound riboflavin is a better measure of marginal riboflavin status than analysis of riboflavin concentration in the plasma. The concentration in red cells however, is a reasonably sensitive and practical index for evaluating functional status. The disadvantage of using erythrocyte flavin levels as a measure of riboflavin status is that a large volume of blood is required.

ii-Enzymatic assay of riboflavin status: The enzyme tests using glutathione reductase is based on the same principles as that introduced for the assessment of thiamine status by (Brin, 1962) and for pyridoxine status by Raica and Sauberlich (1964). Two flavin-dependent enzymes are present in the erythrocytes which can be used as a measure of riboflavin status, NADH-methaemoglobin reductase (NADH-MetHb reductase) and glutathione reductase (EGR). Several workers independently proposed the use of EGR activity as an enzymatic measure of riboflavin-deficiency (Glatzle, Weber and Wiss,1968;

Beutler, 1969; Bamji, 1969). EGR possesses two properties which make it suitable for use as a functional test for riboflavin deficiency. The EGR cofactor, FAD, cannot be removed by dilution and the free apoenzyme is relatively stable both in-vivo and in-vitro (Glatzle et al., 1968). EGR activity is sensitive to riboflavin nutritional status; it is lowered in riboflavin deficiency and can be restored in-vitro with exogenous FAD or by the in-vivo administration of riboflavin to man and animals (Beutler, 1969; Tillotson and Sauberlich, 1971; and Tillotson and Baker, 1972). The ratio of enzyme activity after in-vitro reactivation to that before reactivation serves as a measure of the extent of riboflavin deficiency and is usually quoted as an activity coefficient (AC). The greater the deficiency of riboflavin the greater is the stimulation or AC produced by the in-vitro addition of FAD. Absolute EGR activity can be used as an index of riboflavin status but measurement of AC is much more useful.

The behaviour of this enzyme at different stages of riboflavin deficiency has been correlated with glutathione reductase activity in the tissues (Glatzle. et al., 1973) and other flavin-dependent enzymes enzyme in riboflavin-deficient rats (Prentice and Bates, 1978). In human, glutathione reductase activity was measured during partial riboflavin depletion and subsequent repletion of six male volunteers (Tillotson and Baker, 1972). All these groups reported that the activity coefficient of EGR is a sensitive measurement of riboflavin metabolic studies in humans and animals. The test has proved itself very useful in the field surveys(Glatzle, Korner, Christeller and Wiss, 1970; Bamji, 1969; Thurnham, Migasena and Pavapootanon, 1970: Thurnham, Migasena, Vudhivai and Supawan, 1971; Buzina, Jusic, Brodarec, Milanovic, Brubacher, Viulleumier and Wiss, 1971; Flatz,1970;

Sauberlich, Judd, Nichoalds, Broquist and Darby, 1972; Thurnham and Stephen, 1975), animal studies (Beutler and Srivastava, 1970; Tillotson and Sauberlich, 1971; Glatzle et al., 1973; Bamji and Sharada, 1972; Lankisch, Schroeter, Lege and Vogt, 1973) and hospital studies (Bamji, 1969; Tillotson and Baker, 1972).

5. Role of Riboflavin in Maintenance of Red Cell Stability

a - Riboflavin and red cell metabolism

The erythrocyte is a unit of protoplasm highly specialised for the transport of oxygen and carbon dioxide and survives in the circulation for about 120 days in man, and 60 days in rats, (Granick, 1949). In addition to mature erythrocytes a few reticulocytes (approximately 2%); the youngest cells in the circulation are also present.

The metabolism of erythrocytes, although exceeding complex, may be considered incomplete when compared with that of most other cells. For example, the mature red cell is unable to synthesize protein but does appear to synthesize certain simpler compounds such as reduced glutathione (Prins, Oort, Loos, Zürcher and Beckers, 1963; Dimant, Lamdsberg and London, 1956; Elder and Mortensen, 1956; Szeinberg, Adam, Ramot, Shela and Meyers, 1959), nicotinamide mononucleatide, NAD (Tupula, 1958; Leder and Handler, 1951), and adenosine triphosphate (ATP) (Lowry, Ramot and London, 1960; Lowry, Williams and London, 1961). While it was at one time believed that fat synthesis took place in erythrocytes (Altman, 1953; James, Lovelock and Webb, 1957), recent studies indicate that lipid synthesis occurs only in white cells and reticulocytes (O'Donnell, Ottolenghi, Malkin, Denstedt, and Heard, 1958; Marks and Gelborn, 1959).

Figure (2):

Defense Mechanisms Against Oxidative Stress in the Red Blood Cell

Figure (2);

To show effect of oxidative species (I) on haemoglobin (II) and lipid structure (III) and the role of erythrocyte defense mechanisms, viz: glutathione peroxidase/glutathione reductase system (IV), catalase (V), and methaemoglobin reductase systems (VI) supported by hexose monophosphate shunt (VII) and glycolysis (VIII).

Key:

i- SOD = Superoxide dismutase.
ii- GP = Glutathione peroxidase.
iii- GR = Glutathione reductase.
iv- NADPH-MR
= NADPH-Methaemoglobin reductase.
v- NADH-MR
= NADH-Methaemoglobin reductase.
vi- Lactic acid dehydrogenase.
vii- Catalase.
viii- G-6-PD
= Glucose-6-phosphate dehydrogenase.

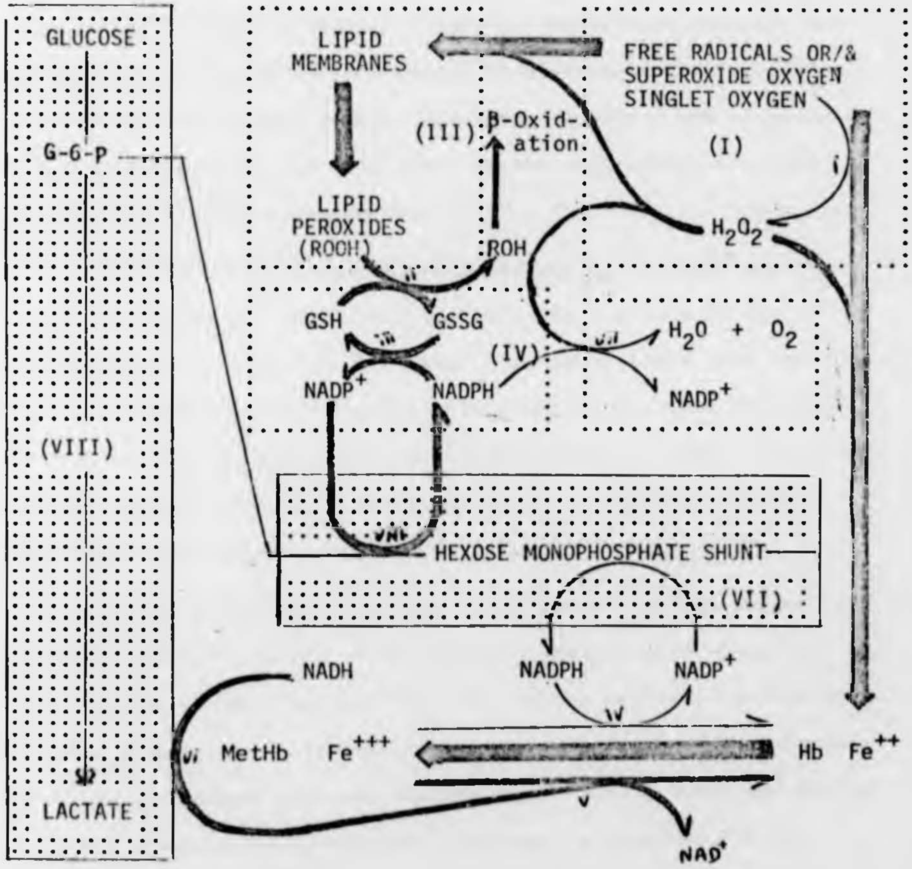
s (I) on haemoglobin (II)
ythrocyte defense
athione reductase system
tase systems (VI) supported
olysis (VIII).

ductase.

luctase.

hydrogenase.

Defence mechanism against oxidative stress in the red cell



The red cell requires a source of energy for synthetic processes for the maintenance of ion concentration gradients and other essential functions. Under physiological conditions the chief source of energy comes from the breakdown of glucose through anaerobic glycolytic pathway (Embden-Meyerhoff) which generates NADH and ATP. Secondarily, there is a direct oxidative pathway of glucose metabolism, the phosphogluconate oxidative pathway or the hexose monophosphate shunt. Under normal conditions the latter pathway accounts for only 5-10% of glucose utilized by the red cell, but this shunt is the only source of NADPH (Murphy, 1960) available to erythrocytes.

The flavin-dependent enzymes, methaemoglobin reductase and glutathione reductase are absolutely dependent on a supply of the reduced coenzymes. There are two forms of methaemoglobin reductase one of which is FMN-dependent, requires NADH and is the more important. In addition, another form exists which is dependent on NADPH, does not appear to be as physiologically important and also requires a flavin prothetic group for its activity (Matsuki, Yubisui,Tomado, 1978).

Glutathione reductase is primarily dependent on NADPH (Visser and Veeger, 1969; Carlberg, and Mannervik,1974) while in-vitro studies have shown that it will also metabolise NADH but with only 20% of the activity (Waller,1968). In fact, the glutathione reductase system may well be the main metabolic pathway which uses NADPH (Schrier, Kellermeyer, Carson, Ickes and Elving, 1958). GSH produced by glutathione reductase is required for the detoxification of peroxide compounds by glutathione peroxidase (Mills, and Randall, 1958; Cohen and Hochstein, 1961). In the absence of glucose in-vitro, red cell GSH concentrations fall relatively rapidly when cells are exposed to oxygen (Fegler, 1952; Klebanoff, 1957) being converted at least initially to GSSG.

Catalase also utilizes NADPH in its function to remove H_2O_2 but the relative importance of the two erythrocyte enzymes, glutathione peroxidase and catalase is difficult to define.

The blood circulation of any mammal consists of red blood cells at all stages of ageing, and it has been found that the composition of the red cell is often dependent on its age (Bartos and Desforges, 1967; Bishop and Van-Gastel, 1969; Blume, Busch, Haffhaver, Arnold, and Löhr, 1970; Fischer and Waller, 1971). Changes in the levels of many metabolites and the activities of different enzymes within the cell have been reported to occur during cell ageing. It has been suggested that these changes may interfere with the ability of old cells to maintain functional stability (Löhr and Waller, 1962). Concentrations of reduced glutathione (Rigas and Koler, 1961), total sulfhydryl groups (Jacob and Jandle, 1962) and ATP (Löhr and Waller, 1962) are lower in the old cells, total haemoglobin is unchanged (Allison and Burn, 1955) while methaemoglobin, lipid peroxidation (Walls, Kumar and Hochstein, 1976) and density (Allison and Burn, 1955) are increased. In the case of enzyme activity, this was found in general to fall with age, e.g. glucose-6-phosphate dehydrogenase (G6PD) in man (marks, 1958) EGR in man and animals (Ganzuni, Barras, and Marti, 1976; Powers and Thurnham, 1976) methaemoglobin reductase in man (Berger, Zuber, and Miescher, 1960). Ganzuni *et al.*, (1976) reported that the age of red blood cells falls with age, while loss of potassium ions and susceptibility to haemoglobin by H_2O_2 increased (Walls *et al.*, 1976) as also did osmotic fragility (Danon, 1961). The ageing red cell appears to progressively lose the ability to maintain functional haemoglobin for at least two changes in haemoglobin structure. Firstly, there is an increase in the methaemoglobin concentrations with age (Keitt, Smith and Jandl, 1966; Walls *et al.*, 1976) and secondly, falling enzyme activities in the red cell may be responsible for increasing concentrations of haemoglobin A_3. This is a form of haemoglobin (Hb) in which oxidised glutathione is bound in the beta-Hb chain and the product becomes non-functional. Glutathione in this form becomes unavailable for further use and this factor may be relevant to the functioning of the glutathione peroxidase system.

b - Auto-oxidation and its control in the red cell

Early in 1954 Commoner and his colleagues reported that mammalian tissues contain two types of radicals:

a - free radicals associated with protein moieties.

b - A number of polymeric condensed ring substances which contained unpaired electrons in a highly stable configuration (Commoner, Townsend and Pake, 1954). Other authors have shown that whenever organisms live under aerobic conditions or gain their bio-energy by the reduction of molecular oxygen to water, the possible formation of highly reactive intermediates of oxygen reduction such as the free radical superoxide oxygen($O^{\bar{\cdot}}{}_2$) and singlet oxygen (O^1_2,the high energy form of molecular oxygen) or peroxides must be considered (Halliwell, 1974; Loschen, 1975; Boveris, Oshino and Chance, 1972; Boveris and Chance, 1973; Flohe and Zimmerman, 1974; Loschen, Azzi, Richter and Flohe, 1974;

In addition it was shown that H_2O_2 is produced by such enzymes as superoxide dismutase and xanthine oxidase not only within the peroxisomes, but also in microsomal, mitochondrial and soluble fractions of the cell (Loschen, 1975; Boveris et al., 1972). The amount of peroxide production in the tissues is not often realised, in rat liver, for example, up to 5% of the oxygen utilized is consumed in the production of hydrogen peroxide (Boveris et al., 1972; Boveris and Chance, 1973). Much of this hydrogen peroxide results from the action of dismutase which combines superoxide oxygen and hydrogen ions (Halliwell, 1974; Loschen, 1975; Loschen et al., 1974).

$$2H^{+} + 2O_2^{\dot{-}} \rightleftharpoons H_2O_2 + O_2^1$$

The latter implies the peroxide and superoxide anions may be present simultaneously in biological material. This condition favours the formation of hydroxyl ions (OH^-) and singlet oxygen (O_2^1)

$$H_2O_2 + O_2^{\dot{-}} \rightleftharpoons OH^{\cdot} + OH^{-} + O_2^1$$

which in turn may attack a variety of cellular constituents (Arneson, 1970; Flohe and Zimmerman, 1974; Cohen and Heikkila, 1974; Leipzig, Brewer and Kruckerberg, 1975).

It has been shown that the superoxide oxygen can also be generated by auto-oxidation of leucoflavins, thiols, (Misra, 1974), tetrahydropteridine (Fisher and Kaufman, 1973) ferrodoxins and haemoproteins (Misra and Fridovich, 1972; Weser, 1973). Auto-oxidation of such substances will release electrons which may be taken up by oxygen, forming the superoxide oxygen. This anion is also produced by intact granulocytes during phagocytosis (Root and Metcalf, 1977). However, it is still not possible to quantitate the importance of any of the different sources of superoxide oxygen in any tissue, it is only possible to say that significant amounts of superoxide oxygen are produced within all respiratory cells (Misra and Fridovich, 1972).

The pronounced reactivity of hydrogen peroxide, superoxide oxygen and singlet oxygen provide the basis for such important biochemical reactions as hydroxylation (Coon, Strobel, Heidema, Kaschnitz, Autar and Balton, 1972), synthesis of thyroxine (Serif and Kirkwood, 1958) and α-oxidation of fatty acids (Mead and Levis, 1963). On the other hand the reactivity of the oxygen species referred to above has been shown to be high enough to attack non-specifically a variety of biologial structures susceptible to oxidative damage (Halliwell, 1974; Loschen, 1975; Flohe and Zimmerman, 1974; Loschen et al., 1974; Cohen and Heikkila, 1974).

In addition to the effect of superoxide oxygen, the red cell is exposed to oxygen tensions of a magnitude experienced by few of the other tissues of the body. Because of this constant association with high oxygen pressure, the red cell may be more susceptible to oxidative damage than the other tissues, thus it is not surprising that evolution has ensured the presence of enzymes regulating the steady state levels of H_2O_2, other hydroperoxides and superoxide oxygen. The cellular device for meeting this demand consists of haem-containing enzyme catalase (Sies, Gerstenecker, Summer, Menzel and Flohe, 1974) and GSH-peroxidase (Flohe and Gunzler1974) to remove peroxides and hydroperoxides respectively and the copper-containing protein dismutase to remove superoxide oxygen (McCord, Beauchamp, Goscin, Misra and Fridovich, 1973) and possibly also singlet oxygen (Loschen et al., 1974; Richter, Wendell, Weser and Azzi, 1975). The pentose phosphate shunt represents the first line of defence in the first two protective mechanisms for they rely on an adequate supply of NADPH for full activity (Figure 2).

There is continuing argument concerning the relative importance of the glutathione peroxidase system and catalase in the detoxification of peroxide (Cohen and Hochstein, 1963; Jacob, Ingbar and Jandl, 1965). There is evidence in man that both systems are able to protect the red cell but the studies of Jacob et al. (1965) would seem to suggest that in acatalasia far greater activity of the hexose monophosphate shunt is required to enable the glutathione peroxidase system to cope with peroxide than in normal cells. This would suggest that the glutathione peroxidase system is less efficient and catalase is the first line of defence. However, more recently it has been suggested that the two systems react preferentially with different substrates; catalase with H_2O_2 and glutathione peroxidase with the lipid hydroperoxide (Chow, Reddy and Tappel, 1973; Cohen, 1975).

The functions of glutathione peroxidase/glutathione reductase system are established, but the relative importance of the FAD-dependent enzyme glutathione reductase has not been extensively studied as is outlined below.

c - Glutathione reductase and red cell stability

Gerald Cohen (1975) reported that erythrocytes deficient in glucose-6 phosphate dehydrogenase (G6PD) responded in-vitro to sustained low levels of H_2O_2 with the following changes:

1 - Disappearance of reduced glutathione.

2 - Accumulation of methaemoglobin.

3 - Depression of the glycolytic pathway.

4 - Depression of hexose monophosphate shunt.

The effects are probably caused by metabolic adjustments to, as well as, direct oxidative damage by H_2O_2. The interest in G6PD deficiency arose from the fact that the red blood cells from these subjects undergo oxidative damage and haemolysis in-vivo when exposed to a number of oxidant drugs (Beutler, 1966; Hochstein, 1971). The intracellular generation of H_2O_2 by some of these drugs has been observed both in-vitro and in-vivo (Cohen and Hochestein, 1963; 1964). The sensitivity to H_2O_2 is believed to derive primarily from the inability of G6PD-deficiency to maintain concentrations of reduced glutathione (Cohen and Hochstein 1962), thus causing the activity of GSH-peroxidase to fall and affecting the ability of red cells to handle lipid hydroperoxides or H_2O_2 within the cell. Desforges, Thayer and Dawson, (1959) suggested that glutathione reductase may be particularly important in the maintenance of red cell stability because of its role in the regeneration of GSH. The enzyme is mainly dependent on NADPH to reduce oxidised glutathione (Beutler, 1963) but in-vitro experiments have shown that enzyme activity is also reduced by deficiency of riboflavin.

Hill, Haut, Cartwright and Wintrobe (1964) suggested that GSH is the preferred metabolite to neutralise oxidative potential within the red cell thereby protecting haemoglobin and other important metabolites. Factors which interfere with the regeneration of GSH may affect the ability of the red cell to protect itself from oxidation denaturation. It has been suggested that there were four metabolic lesions which might affect the synthesis or utilization of GSH in the red blood cell (Hill et al., 1964).

1 - G6PD deficiency may affect the supply of NADPH.

2 - A deficiency of glutathione reductase may restrict the regeneration of GSH.

3 - Genetically low levels of GSH where G6PD and glutathione reductase were normal.

4 - A deficiency of glutathione peroxidase which might affect the ability of the cell to use GSH i.e. unable to protect itself against oxidant activity.

Of the four lesions the first three have been found to occur in different situations while the fourth has not been discovered.

With the discovery that glutathione reductase was FAD-dependent (Scott, Duncan and Ekstrand, 1963; Staal. et al., (1969) the possibility of a fifth lesion affecting GSH concentrations arose, namely a deficiency of riboflavin. Studies to investigate this possibility from the basis of the investigations reported in this thesis are outlined below.

PURPOSE OF STUDY

Carson, Schrierer and Kellermeyer (1959) and Carson, Brewer and Ickes, (1961) reported that reduced activity of red cell glutathione reductase caused red cell GSH concentrations to fall when exposed to oxidant drugs and a reduced erythrocyte life span and they argued that riboflavin-deficiency might have similar effects. Beutler and Srivistava (1970) however, reported that riboflavin deficiency in man and rats did not shorten the red cell life span in-vivo in the presence of oxidant drugs. In addition, Bamji and Sharada (1971) working with blood from severely riboflavin-deficient subjects, reported that concentrations of red cell GSH in-vitro were not reduced and the regeneration of GSH in-vitro was not affected by exposure to oxidant drugs. It was concluded therefore that riboflavin deficiency had little physiological effect on those mechanisms involved in protecting the red cell against drug-induced haemolysis. It was suggested, however, by Walls and Hochstein (1974) that drug-induced haemolytic anaemia in G6PD-deficient persons might be due not only to an accumulation of peroxides but also to the availability of circulating thyroid hormone. For example, they found that increasing amounts of thyroid hormone increased the in-vitro haemolysis produced by peroxide or G6PD-deficient red cells in man when incubated in the presence of glucose. Thyroid hormones are reduced in riboflavin-deficiency (Rivlin et al., 1968) and the possibility that thyroid hormones were involved in the physiological role of glutathione reductase was considered worthy of further investigation. Experiments were planned therefore in which red cells from control and riboflavin-deficient rats were exposed to peroxide with and without thyroxine. The effects of thyroxine on rat erythrocytes were found to be different from the effects reported in man, however, the in-vitro technique showed that the erythrocytes from riboflavin-deficient rats were more fragile than those from control animals. Subsequent studies on red cells separated by density gradient fractionation showed that greater fragility was present in the old cells (densest cells). The fractionation technique made it possible to investigate red cells which were particularly severely affected by riboflavin-deficiency and therefore assisted the investigation of the effects of riboflavin deficiency on the metabolism of the red cell.

Some of the results of these studies have already been reported in the medical press and copies are attached, (Hassan and Thurnham, 1977a; 1977b; 1977c).

CHAPTER TWO

MATERIAL AND METHODS

MATERIALS AND METHODS

1. GENERAL PROCEDURE

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

1 - Animals and Diets:

(a) Experimental animals

Weanling, Wister, specified-pathogen-free, albino male rats obtained from A.Tuck and Son, Breeding Station,Rayleigh, Essex, were used in all experiments. Their individual weights were 40-50g on arrival from the suppliers. The rats were housed individually in white plastic, wire-bottomed cages in a well-ventilated room at a temperature of 26°C. They were weighed every five days for the first two weeks and then daily throughout each experiment.

Rats were divided into three groups on arrival in such a way that the mean weight in each group was about the same. The groups were used as controls, pair-fed controls and rats fed a riboflavin-deficient diet. Pair-fed controls received an amount of diet based on the mean intake of animals receiving the riboflavin-deficient diet.

Precautions taken to avoid coprophagy in the experimental animals were, use of wire-bottomed cages, a low fibre diet, cleaning of cages at weekly intervals and removal of all faecal waste twice a day. If a riboflavin-deficient animal refected, as shown by increasing weight, animals were transferred to cages with extra wide gaps between the wires of the grid at the bottom of the cage. Food and water containers were cleaned regularly to minimise the growth of bacteria; a possible source of riboflavin.

b - Riboflavin deficient diets and food intake of rats

(i) Composition

Early experiments used the rat diet described by Carpenter, Harris and Kodicek (1948) modified by using corn oil instead of cotton seed oil and acid-extracted casein (Tables 1,2 and 3 diet A). The diet was not successful in producing clinical signs of riboflavin deficiency and only marginal biochemical evidence. Greater success was achieved with the diet marketed by the Nutritional Biochemical Corporation and it was prepared in this laboratory to the formula given by Hoppel and Tandler (1975). (Tables 1,2 and 3 diet B).

(ii) Food intake

The powdered food was given to the animals ad-libitum in ceramic pots usually between 4 p.m. and 5 p.m. and removed the following morning usually between 10 a.m. and 11 a.m. The diet was fed to the animals on the day following their arrival. Food consumption of the rats on the riboflavin-deficient diet was approximately 10g while control animals ate 18grams.

The criterion of a clinical riboflavin deficiency was a gain in weight of no more than 5grams during a 14-day interval and this meant that most of the animals became deficient within 3-4 weeks and also showed clinical symptons of deficiency.

(iii) Extraction of riboflavin from crude casein (Yagi,1951)

Crude casein (Casumen sodium caseinate, Unigate Foods, Limited) was heated with approximately 5 %(v/v) sulphuric acid up to 80°C.

Table 1:

Major Constituents of Diet A and Diet B

Component	Diet A (gram %)	Diet B (gram %)
Acid washed casein	10.50	18.00
Sucrose	81.35	68.00
Fat	3.00*	10.00**
Salt mixture	5.00	4.00
Cystine	0.15	—

* Corn oil was used instead of cotton seed oil

** Fat used was groundnut oil

Table 2:

Vitamin Mixtures per kg Diet

Component	Diet A	Diet B
Thiamine-HCl	3mg	22mg
Riboflavin*	3mg	22mg
Ascorbic acid	—	0.99g
Pyridoxine	3mg	22mg
Ca pantothenate	20mg	66mg
Nicotinamide	25mg	99mg
Biotin	0.1mg	0.44mg
B_{12}	50ug	30ug
Choline-HCl	2g	1.65g
Folic acid	—	1.98mg
Inositol	—	0.11g
p-Amino benzoic acid	—	0.11g
Vitamin A	1000 i.u.	5000 i.u.
D_2	200 i.u.	250 i.u.
Menadione	0.05mg	1mg
Tocopherol	2mg	2mg

* Riboflavin was omitted from the riboflavin-deficient mixtures. 44mg were used for the control diet B in experiments concerning iodino-depletion and that comparing different lipids in the diet.

Table 3:

Salt mixtures per kg

Component	Diet A	Diet B
Dipotassium hydrogen phosphate	—	333.1g
Potassium dihydrogen phosphate	310.8g	—
Sodium chloride	311.0g	173.0g
Aluminium potassium sulphate	—	0.2g
Calcium chloride	304.8g	309.9g
Calcium hydrogen phosphate	—	98.12g
Cobalt chloride	0.02g	0.26g
Copper sulphate	0.38g	0.21g
Ferric citrate	—	28.4g
Ferric sulphate	21.6g	—
Magnesium sulphate	45.8g	51.1g
Manganous sulphate	4.6g	4.13g
Potassium iodide*	0.6g	0.83g
Sodium flouride	—	0.26g
Sodium borate	—	0.26g
Zinc chloride	—	0.26g
Zinc carbonate	0.4g	—

* Potassium iodide was omitted from the iodine-deficient salt mixture.

The casein was washed several times with hot tap water through a wire sieve to remove riboflavin in the acidic washings. The riboflavin-free casein was then dried at $95-105^{\circ}C$ for at least 24 hours. The dried riboflavin-free casein was then crushed and powdered through a mixer (Moulinex, Model MC UIII). Riboflavin estimated in samples of riboflavin-extracted casein showed that this method removed 95% of riboflavin present in crude casein, leaving behind approximately 240ug/Kg.

c - Iodine-deficient diet

The rat diet described by Hoppel and Tandler (1975) diet B (Tables 2 and 3) was used with and without potassium iodide in the salt mixture. Rats were kept on an iodine-deficient diet with distilled water ad-libitum for five weeks. Distilled water supplemented with 0.1% propyl thiouracil was then substituted for the distilled water until the rats showed a regular reduction of body weight for two weeks.

d - Estimation of riboflavin in casein and stool (Arnold, 1945)

This method depends on extraction of the vitamin with dilute acid, filtration, treatment of the filtrate with permanganate and hydrogen peroxide to destroy the interfering pigments and measurements of the fluorescence. The vitamin content of the extract is evaluated by means of an internal standard.

A known weight (approx.0.5g) of dried, riboflavin-extracted casein or a day's collection of stool was finely ground in a glass homogenizer at $0-4^{\circ}C$ with 9ml 0.1N-H_2SO_4. It was heated in a boiling water bath for 45 minutes with shaking at intervals. The mixture was cooled and adjusted to pH4.3 with 2.5M-sodium acetate. The volume was made up to 10ml with distilled water and then centrifuged at 2500 g for ten minutes at room temperature. The supernatent was then treated with 0.2ml 4%(v/v)$KMnO_4$ solution and after three minutes the permanganate colour was discharged with freshly prepared 3%(v/v) hydrogen peroxide solution. The froth was broken with a few drops of acetone and the contents diluted to 6.5ml with distilled water. After mixing and filtering, two 1.5ml portions were pipetted out of the filtrate. To one was added 0.1ml distilled water and fluorescence measured (A). To the other was added 0.1ml riboflavin standard and fluorescence measured (B). The blank was obtained (C) after adding 2mg of sodium hydrosulphite to (A) or (B).

The riboflavin content of the sample (µg/g) was obtained using the following formula:

$$= \frac{A - C}{B - A} \times \frac{1}{15} \times \frac{65}{60} \times \frac{100}{wt} \times 10$$

where "wt" is the weight of sample taken in grams.

e - Blood sampling, killing disection of animals

Blood samples were obtained either from the tail vein or from the heart of the animals. In the first case, the tail was immersed in warm water for approximately 30 seconds to one minute, the tip was then removed, dried and 50ul blood collected in heparinized capillary tubes (Benjamin Haematocrit Tubes, Harshaw Chemicals,Ltd.) for Packed Cell Volume, whole blood glutathione reductase (BGR) and erythrocyte glutathione reductase (EGR).

In order to obtain blood from the heart the rat was anaesthetized with chloroform and blood removed by syringe from the heart. Liver, left kidney, spleen and a piece of intestine were then removed, weighed and stored in distilled water at -20°C for later analysis of iron, peroxides and peroxidase.

11. BIOCHEMICAL MEASUREMENTS

1 - Experiments with Whole Blood

a - Measurements of packed cell volume (PCV)

A heparinized capillary tube was filled with approximately 50ul blood, sealed with a small flame and centrifuged for five minutes at about 12000 g (using a Hawkesly haematocrit centrifuge). The height of cells in the tube was expressed as a percentage of the total height of the tube contents to give a PCV.

b - Red blood cell and white cell counts

Whole blood (50µl) were mixed with 20ml Isoton, (Coulter Electronics Ltd.) to give a dilution of 1:400. From this dilution 100 ul were added to an additional 20ml of Isoton to give a final dilution of 1:80,000. The latter was transferred to a 'Coulter Counter Model D1' and red cells were counted at room temperature. White cells were counted in the dilution (1:400) after haemolysing the red cells by adding one drop of saponin for three minutes at room temperature. White cells were counted during the next 10 minutes.

c - Reticulocyte count (Dacie and Lewis,1963)

Principle: Reticulocytes are juvenile red cells; they contain remnants of the basophilic ribonucleoprotein which was present in larger amounts in the cytoplasm of the nucleated precursors from which they were derived. This basophilic material has the property of reacting with certain dyes, such as brilliant cresyl blue, to form a blue precipitate of granules or filaments. This reaction takes place only in vitally stained unfixed preparations. The most immature reticulocytes are those with the largest amount of precipitable material; in the least immature only a few dots or strands are seen.

Staining solution: Brilliant cresyl blue (0.1g) was dissolved in 100ml of citrate-saline solution (1 part of 3%(w/v) hydrated sodium citrate to 4 parts 0.85%(w/v) sodium chloride). The mixture was filtered before use.

Method

Heparinized whole blood (50µl) was mixed with 20µl cresyl blue solution and the mixture was incubated for ten minutes at 37°C. The incubated red cells were resuspended by gentle mixing, films were made on glass slides. Slides were left to dry and then microscopically examined without being fixed or counterstained using 2-mm oil-immersion objective. The total erythrocytes and reticulocytes were counted in a field, the size of which was reduced to contain approximately 50 cells. Approximately 1000 erythrocytes were counted.

d - Preparation of haemolysates for glutathione reductase Assay (BGR)

The whole capillary tube containing 50µl heparinized blood was placed in 8ml of 0.1M-Potassium phosphate buffer at pH7.4 containing 20mM-EDTA at room temperature. The capillary tubes were later crushed with a glass rod, 20ul saponin added and haemolysates left to stand at 4°C for at least one hour. The haemolysates were then centrifuged at about 2500 g and 4°C for ten minutes to remove the stroma. The clear supernatent was used in estimating BGR activity as described in this chapter, section 2b.

e - Estimation of haemoglobin derivitives: Methaemoglobin Sulfhaemoglobin and Oxyhaemoglobin: (Evelyn and Malloy, 1938)

Principle: (i) Methaemoglobin (MetHb)

When sodium cyanide is added to a solution of MetHb the characteristic absorption band at 635nm is almost completely abolished by the conversion of MetHb into cyanmethaemoglobin (metHbCN). The resulting change in optical density may be measured colorimetrically at 635nm.

(ii) Sulfhaemoglobin (SHb)

The absorption of SHb at 620nm is unchanged by the addition of cyanide. Hence the concentration of SHb in a solution containing oxyhaemoglobin (HbO_2) and SHb is proportional to the residual optical density of the solution after the MetHb has been converted into MetHbCN by addition of cyanide. The measurement of SHb can be made on the same solution as used for the MetHb determination. A correction must of course, be made for the small, though not negligible absorption of HbO_2 and MetHbCN at 620nm.

(iii) Oxyhaemoglobin (HbO_2)

The concentration of HbO_2 is obtained by subtracting the values for MetHb and SHb from the concentration of the total haemoglobin determined by a modification of the methods of Austin and Drabkin (1935; 1936). All forms of haemoglobin are converted into MetHbCN and the absorption measured at 540nm.

Method

Fresh whole blood (30µl) was added to 3ml 16.7mM-potassium phosphate buffer at pH6.6 in a cuvette. The solution was allowed to stand for five minutes, and the absorption measured at 635nm against distilled water as a blank. The optical density was recorded as L_1 and 0.2ml 12%(w/v) sodium cyanide neutralised with ammonium hydroxide was then added to the solution to convert any MetHb into MetHbCN. After two minutes a second reading (L_2) is made at 635nm. The difference (L_1 minus L_2) is proportional to the concentration of MetHb. The solution, which up to that time was very slightly turbid, was then cleared for the SHb determination by adding 0.02ml of concentrated ammonium hydroxide and a reading (L_3) was made at 620nm and against a distilled water blank. Finally 0.6ml of the solution was pipetted into a second cuvette containing 2.4ml of 67mM potassium phosphate buffer pH6.6and 0.1ml of 20% (w/v) potassium ferricyanide and 0.01ml 10%(w/v) sodium cyanide and (L_4) was read at 540 nm. The concentration of the haemoglobin derivatives were calculated from the following equations in which MetHb, SHb and HbO_2 were calculated as g per dl.

$$T = \frac{100 \times L_4}{2.38} \qquad (1)$$

$$MetHb = \frac{100 \times (L_1 - L_2)}{2.77} \qquad (2)$$

$$SHb = \frac{100 \times L_3 - (8.5 \times MetHb + 4.4 \times T)}{100}$$

Where T. = the total Hb

Corrected T = T calculated from equation (1) + 0.22 x SHb

HbO_2 = Corrected T - (MetHb + SHb)

f - Determination of haemoglobin in haemolysate samples

(Wootton, 1964)

Principle: This method is based on the reaction of potassium cyanide with all forms of haemoglobin pigment to form cyanmethaemoglobin which in the presence of potassium ferricyanide solution absorbs at 540nm. The cyanmethaemoglobin in a known volume of the test haemolysate is measured by comparing the absorption with that of commercial standards.

Reagents: Drabkin's solution was prepared by disolving sodium bicarbonate (10g) potassium cyanide (0.5g) and potassium ferricyanide (2g) in 1000 ml distilled water. The solution was stored at 4°C.

Method:

Drabkin's solution (4.5ml) was mixed well with 0.5ml haemolysate. The optical density was measured after ten minutes using Drabkin's solution as a blank and the concentration of haemoglobin determined by comparison with cyanmethaemoglobin standards.

Haemoglobin concentration of test solution in g/ml

$$= \frac{t}{s} \times \text{Hb concentration of standard} \times 10$$

where t = optical density of test solution

s = optical density of the standard

10 = dilution of haemolysate (0.5ml) in Drabkin's solution (4.5ml).

g - Fluorometric determination of reduced glutathione (GSH)

(Hissin and Hilf, 1976)

Principle: GSH reacts specifically with O-phthalaldehyde (OPT) at pH8.0, yielding a highly fluorescent product that can be activated at 350nm with an emission peak at 420nm. The fluorescence is dependent on the final pH as fluorescence intensity is less when the reaction is done at a pH below 8.0, as originally reported by Cohn and Lyle (1966). Care must also be taken not to increase the pH above pH8.0 for this causes the conversion of GSH to GSSG (Hissin and Hilf, 1976).

Method:

Heparinized whole blood (0.1ml) was added to 3.9ml of 0.1M-sodium phosphate buffer (pH8.0) containing 0.005M-EDTA and 1ml of 25%(w/v) metaphosphoric acid to precipitate the protein. The total volume was then centifruged at 4°C and 2500 g for 15 minutes. The supernatent was then diluted 10 times with sodium phosphate-EDTA buffer and 100µl were added to 1.8ml with sodium phosphate-EDTA buffer and 100µl OPT solution (100µg OPT 100ml methyl alcohol) to form a final assay mixture of 2ml). After thorough mixing and incubation at room temperature for 15 minutes, the solution was transferred to a quartz cuvette and fluorescence determined at 420nm after activation at 350nm.

Standard curve: GSH was dissolved in sodium phosphate-EDTA buffer (pH8.0). Known amounts of GSH in 100ul solution were mixed with 100ul of the OPT solution. The final volume of this mixture was adjusted to 2ml with buffer and measured as described for the sample.

2. Experiments with Erythrocytes (RBCs)

a - Preparation of erythrocytes

Heparinized blood was centrifuged at 2500 g for ten minutes at room temperature. Plasma was separated and stored at -20°C to be used later for thyroxine estimations and plasma iron.

Red blood cells were washed three times with 0.85%(w/v) sodium chloride solution containing 0.1%(w/v)albumin. The washed red cells were then ready to be used in the following procedures listed below.

Measurement of glutathione reductase activity

Density gradient fractionation of red cells

Measurement of the red cell fragility

Measurement of endogenous formation of hydroperoxides

Measurement of glutathione peroxidase activity

Measurement of NADH — methaemoglobin reductase activity

Measurement of NADPH — methaemoglobin reductase activity

Measurement of aspartate aminotransferase activity

b - Estimation of glutathione reductase activity

Haemolysates for the measurement of glutathione reductase activity were prepared from washed red cells (section 2a) when using terminal blood (EGR activity) or unwashed packed red cells from a capillary tube when using tail blood (BGR activity). Capillary tubes were cut below the leucocyte layer and the red cell fraction (approximately 25µl) transferred to 8ml 0.1M-potassium phosphate buffer at pH7.4 containing 20mM-EDTA, crushed with a glass rod, 20ul saponin added and the haemolysates left to stand at $4^{\circ}C$ for at least one hour. The haemolysate was then centrifuged at 2500 g and $4^{\circ}C$ for ten minutes to remove glass and any stroma and the clear supernatent was stored at $-20^{\circ}C$.

Glutathione reductase activity was measured *in-vitro* in 3.0ml haemolysate (1:560 dilution in 0.1M-potassium phosphate-EDTA buffer,pH7.4) with 3.95mM-oxidised glutathione, with or without 18.5uM-FAD in a reaction initiated by 120uM-NADPH and followed at 334nm and $35^{\circ}C$ in a total volume of 3.3ml. Rate of activity was calculated from the steepest part of the slope recorded on the chart paper and expressed as the fall in absorbance over ten minutes. The measure of riboflavin status, the AC was calculated as follows:

$$AC = \frac{\text{Change in O.D. in 10 (with FAD)}}{\text{Change in O.D. in 10 (without FAD)}}$$

c - Density gradient fractionation of erythrocytes

(Turner, Fisher and Harris, 1974)

Principle: Fractionation of erythrocytes was based on the fact that the density of the red blood cell increases progressively with increasing cell age (Rigas and Koler, 1961; Prentice and Bishop, 1965). Red cells were layered onto solutions of Ficoll and Triosil set up to form a step-wise gradient and centrifuged under defined conditions to separate red cell population into fractions of different mean age.

Method

Solutions of Ficoll (Pharmacia, Uppsala, Sweden) were prepared by dissolving 4.40, 5.00, 5.60 and 7.00g of Ficoll in aliquots of 20ml distilled water by standing overnight at 4°C. Aliquots (12ml) of Triosil (Vestric Limited, Runcorn, Cheshire) solution, prepared by mixing the contents of one Triosil ampoule with 24ml distilled water, were added to each Ficoll solution and mixed thoroughly to form densities of 1.113, 1.119, 1.125 and 1.140g/ml respectively. 4ml of each solution were then layered into 19.5ml cellulose tubes (Beckman, California), starting with the heaviest at the bottom. The gradient was used to fractionate 0.5ml washed erythrocytes at 80,000 g and 4°C for two hours in an SW 27.1 swing-out rotor on a Servall OTD-2, Ultracentrifuge. Four fractions were carefully removed with Pasteur pipettes from above the separating layers and washed three times with isotonic saline solution containing 20mg/dl human albumin (Kabi Vitrum Limited, Ealing, London, W5 2TH). The number of red cells at the bottom of the tube was negligible.

The washed fractions were then haemolysed, after measuring the approximate volume of each fraction with a 1ml pipette, by diluting with 20 volumes of distilled water. Haemolysates were centrifuged for ten minutes at 2500 g and 4°C and the supernatent stored at -20°C prior to use.

d - Preparation of Krebs-Ringer phosphate buffer pH7.0

Krebs-Ringer phosphate buffer was prepared by mixing 100 parts of 0.154M-sodium chloride with 4 parts of 0.154M-potassium chloride, 3 parts of 0.1M-calcium chloride and 1 part of 0.154M-magnesium sulphate ($MgSO_4.7H_2O$). Twenty parts of 0.1M-sodium phosphate buffer pH7.4 were added slowly with mixing to avoid salt precipitation. The 0.1M-sodium phosphate buffer was prepared by dissolving 17.8g hydrated disodium monohydrogen phosphate in one litre distilled water containing 20ml 1N-HCl and adjusted to a pH7.4 by 0.1M-monosodium dihydrogen phosphate solution.

The pH of the Krebs-Ringer phosphate buffer was then adjusted by using 0.1N-NaOH or 0.1N-HCl after which the buffer was stored in a glass stoppered vessel at 4°C.

To oxygenate the buffer, oxygen was bubbled through 2.3 to 2.5ml aliquots of the buffer in 10ml flasks for 15 seconds and just prior to other experimental procedures.

e - Fragility experiments

(i) Exposure to peroxide

The conditions were based on those described by Walls and Hochstein (1974). Washed red blood cells (0.5ml 6%(v/v) haematocrit) were incubated in a hydrogen peroxide-generating system containing 1.6mM-hypoxanthine and 0.02 units xanthine oxidase (both from Boehringer) in 2.3ml oxygenated Ringer phosphate buffer (see above), pH7.0 for 45 minutes at 37°C in a shaking water-bath (70 r.p.m., Gallenkamp). Following incubation, the medium was centrifuged for ten minutes at 2500 g. Haemoglobin was estimated in the supernatent by the cyanmethaemoglobin method, as described in this chapter (section 1c).

(ii) Exposure to hypotonic saline

Washed red cells (0.5ml 6%v/v) haematocrit were incubated for 45 minutes at 37°C in 2.5ml 0.90%, 0.72%, 0.54% and 0.36%(w/v) solutions of sodium chloride respectively. Samples were shaken in a water-bath and haemoglobin assayed as described above.

f - Estimation of hydrogen peroxide (Vogel, 1953)

Principle: Estimation of hydrogen peroxide (H_2O_2) is based on the reduction of a solution of permanagate by H_2O_2 in acidic medium according to the following equation:

$$2KMnO_4 + 3H_2SO_4 + 5H_2O_2 \longrightarrow K_2SO_4 + 2MnO_4 + 8H_2O + 5\ O_2$$

Method

Dilute sulphuric acid (0.5ml, 0.2N) was added to the solution containing peroxide which was then titrated with a standard solution of 0.1N-$KMnO_4$. The amount of H_2O_2 present in the solution was calculated from the equation:

$$1ml \text{ of } N\text{-}KMnO_4 = 0.01701g\ H_2O_2$$

g - Estimation of malonyldialdehyde (Mengel, Kann and Meriwether, 1967)

Principle: Endogenously lipid reacts with hydrogen peroxide in vitro to form malonyldialdehyde. The latter reacts with 2-thiobarbituric acid (TBA) to form a chromogen which can be measured at 535nm.

Procedure - Red cells (0.5ml 6%(v/v) haematocrit) incubated in the H_2O_2-generating system (section 2ci) in oxygenated Ringer phosphate buffer (total volume 3ml) were added to 2ml 10%(w/v) trichloroacetic acid (TCA).

Solutions were mixed and then centrifuged at 2500 g and 4°C for 15 minutes. The clear supernatant (2ml) was added to

2.5ml 0.67%(w/v) thiobarbituric acid and heated in a boiling water-bath for 15 minutes, the mixture was left to cool at room temperature and then absorption read at 535nm.

h - Estimation of endogenous hydroperoxide (Heath and Tappel, 1976)

Principle: In this method a blood sample was reacted with glutathione peroxidase to reduce all endogenous hydroperoxides present. The GSSG formed was reduced by glutathione reductase and the fall in absorption at 340nm (that is the consumption of NADPH) was used as an estimate of the original concentration of total hydroperoxides.

Method

The assay comprised three stages:

1. Red cell haemolysates (0.1ml) as a test solution were added to 2.9ml of 0.124M-Tris-HCl pH7.6 and 0.2mM-EDTA tetra-sodium salt in a test tube and allowed to react at room temperature for five minutes prior to stage (2).

2. NADPH (50μl, 2mM), 10μl of purified glutathione peroxidase (Boehringer, enzyme activity 2500umol of NADPH oxydised/minute/1ml at 37°C) and 100μl of 4.25mM-GSH were added to a tube. Timing of reaction began at the point of adding the GSH and the solution was incubated at 33°C for ten minutes.

3. Absorbance of the solution was read, after which 5ul glutathione reductase (Boehringer, 100 units/ml) was added. The solution was then reacted for ten minutes. Absorbance was again read and the net amount of NADPH oxidised was calculated using the extinction coefficient of 6200/min./Cm at 340nm. Absorbance of the unreacted NADPH was 0.336 ±

0.005 when all the NADPH was oxidised the absorbance was about 0.015.

i - Estimation of glutathione peroxidase activity (Beutler, 1971)

Principle: Glutathione peroxidase catalyzes the oxidation of GSH by hydrogen peroxide (1)

$$2GSH + H_2O_2 \overset{\text{GSHPeroxidase}}{\rightleftharpoons} GSSG + 2H_2O \qquad (1)$$

and the rate of formation of GSSG is measured by linking GSSG formation with the oxidation of NADPH by means of glutathione reductase (reaction 2) and measuring the fall in absorbance at 340nm

$$GSSG + NADPH + H^+ \overset{\text{GR}}{\rightleftharpoons} 2GSH + NADP^+ \qquad (2)$$

Azide is added to the reaction mixture in order to inactivate catalase, which would otherwise decompose the hydrogen peroxide (Cohen and Hochstein, 1963).

Ferricyanide-cyanide solution is used in the preparation of haemolysate to minimise interaction between haemoglobin and NADPH and it improved reproducibility (Beutler, 1971).

Method: The reaction mixture (3ml) contained 2ml 0.1M-potassium phosphate buffer (pH7.0), 30ul 0.1M-GSH, 60ul 0.2M-EDTA, 300ul glutathione reductase (diluted to 10 i.u./ml with potassium phosphate buffer) 30ul 0.4M-sodium azide, 300ul 2mM-NADPH, 500ul distilled water and 100ul 1:20 ferricyanide-cyanide haemolysate. The haemoglobin was prepared by mixing one volume of red blood cells with 19 volumes of 0.9mM-ferricyanide +2.04mM-cyanide mixture. The reaction was incubated at 37°C for ten minutes after which 0.036M-hydrogen peroxide was added. No GSH, but all other components were added to a blank cuvette. Rate of reaction was measured at 37°C by following the decrease in

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j - Estimation of NADH-methaemoglobin reductase (NADH-MR)

(Hegesh, Calmanovici and Avron 1956)

Principle: In the conversion of methaemoglobin to haemoglobin by ferrihaemoglobin reductase, ferric ions are reduced to ferrous ions within the haemoglobin molecule. The formation of haemoglobin is followed by measuring the increase in absorption at 575nm. NADH-MR was measured in the presence of a 4:1 molar ratio of potassium ferrocyanide: ferrihaemoglobin since it was found that the methaemoglobin ferrocyanide complex is a better substrate *in vitro* than methaemoglobin.

Method

Tetrasodium EDTA (0.5ml) 0.27M was pipetted into a mixture comprising 1ml 5mM-sodium citrate buffer (pH4.7), 1.0ml 0.5mM-potassium ferricyanide and 1ml of haemoglobin substrate (see following subsection) in a test tube. The mixture was brought to a total volume of 10.0ml with distilled water, mixed thoroughly with 10ul of freshly obtained whole blood, and left to stand at room temperature for five minutes. The mixture was then centrifuged at 2500 g and 4°C for five minutes. Two aliquots (2.97ml) of the supernatent were transferred into cuvettes (1cm width) and incubated for ten minutes at 37°C. Distilled water (30μl) was added to the first (blank cuvette) and 30μl 10mM-NADH at zero time to the second cuvette (test cuvette). The increase in optical density was read at 575 at 37°C.

Calculation - Change in absorbance over ten minutes from the blank cuvette (OD_b) from that of the test cuvette (OD_r). Calculation of the enzyme activity is based on an optical density difference between ferro-and ferrihaemoglobin of 42 for 1mM-solution. The activity (A) in micromoles of Hb reduced in the 1ml system/minute is obtained as follows:

$$A = \frac{OD_r - OD_b}{42 \times 10}$$

The activity (E) in international units/gram Hb is as follows:

$$E = \frac{A}{Hb_F}$$

Where Hb_F is the number of grams of haemoglobin in the final assay system which is calculated as shown below.

$$Hb_F = Hb \times \frac{1}{100} \times \frac{0.01}{10.00} \times 2.97$$

Where Hb is the haemoglobin concentration of the blood sample in gram per decilitre.

1/100 converts the haemoglobin concentration to g/ml The other two factors take into account the dilution of the sample. Therefore, umol haemoglobin reduced per minute (E) is as follows:

$$E = \frac{OD_r - OD_b}{Hb} \times 80.17$$

k - Haemoglobin substrate

Principle: In order to estimate the activity of NADH-methaemoglobin reductase (NADH-MR) in a blood sample, a Hb substrate was required from which the enzyme was removed. Hb substrate was freed from NADH-MR by mixing with diethylaminoethyl (DEAE) cellulose and filtering. DEAE cullulose has a strong absorptive capacity for NADH-MR.

Method

Washed red cells (1 volume) was mixed with distilled water (6 volumes) and the haemoglobin was thoroughly mixed with 0.16g of dry DEAE cellulose (Sigma). The mixture was left for ten minutes at room temperature and then centrifuged at 2500g at 4°C. Absorption of the enzyme was repeated twice using fresh quantities of DEAE cellulose. Hb was then estimated in the clear supernatent and after checking the residual activity of NADH-MR (ΔOD 0.003/min.) the concentration of Hb was adjusted to (1.224g%).

l - Estimation of NADPH-methaemoglobin reductase

(NADPH-methaemoglobin reductase) (Huennekens,Caffrey,
Basford and Gabria, 1957)

Principle: NADPH-methaemoglobin activity is measured by its ability to transfer hydrogen ions from NADPH to methylene blue (MeBl), reducing it to leucomethylene blue (leuk MeBl).

The rate of reaction was followed by measuring the fall in absorption at 340nm.

Method

Two aliquots (50µl) of haemolysate (1:20 erythrocytes in distilled water) were transferred into cuvettes containing 3ml of 1M-Tris-HCl pH8.0. 60µl of 2mM-NADPH were added to the first cuvette and 60µl distilled water to the second cuvette (blank). The two cuvettes were incubated for ten minutes at $37^{\circ}C$ and then the contents of each cuvette were mixed with 30ul of 0.8mM-methylene blue at zero time. The change in optical density of the test cuvette was measured at 340nm against that of the blank.

Calculation - The activity of NADPH-methaemoglobin reductase can be calculated from the equation

$$A = \frac{\Delta OD}{6.22} \times \frac{V_c}{V_H}$$

where A is the enzyme activity in i.u., that is, µmoles NADPH oxidised/min./g Hb

V_c is the cuvette volume and V_H is the volume of haemolysate in milliliters in the reaction medium and ΔOD is the change in optical density per minute at 340 nm

m - Estimation of aspartate aminotransferase activity

Wilkinson, Baron, Moss and Walker (1972);
Rosalki, and Wilkinson (1959).

Principle: Aspartate aminotransferase (AST) requires

B_6 in the form of pyridoxal phosphate to function. Thus, the rate of the enzymic reaction in vitro is controlled by the amount of pyridoxal phosphate present in the haemolysate if all other requirements are added in excess. Under these conditions, the addition of an excess of exogenous pyridoxal phosphate (PP) stimulates the rate of reaction and the amount of stimulation produced is a measure of the initial deficiency.

The rate of transamination may be measured indirectly by coupling the reaction to the oxidation of NADH and measuring a decrease in absorbance at 340nm. Oxaloacetate, formed during the transaminase reaction, is reduced to malate by NADH in the presence of malate dehydrogenase (MDH).

$$\text{Aspartate} + \text{2 - Oxoglutarate} \underset{PP}{\overset{AST}{\rightleftharpoons}} \text{glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \overset{MDH}{\rightleftharpoons} \text{malate} + \text{NAD}$$

Method

Aspartate aminotransferase activity was measured in-vitro in 1.5ml haemolysate (1:315 dilution in 0.1M-Potassium phosphate buffer, pH7.4) with an 1.17mM-NADH in 0.1M-phosphate buffer, pH7.4, 0.127M-L.aspartate in distilled water and 0.5i.u. malate dehydrogenase in distilled water with or without 1.27mM-pyridoxal phosphate in 0.1M-phosphate buffer in a reaction initiated by 33.3-mM-2-Oxoglutarate in phosphate buffer and followed at 340nm and 35°C in a total volume of 3.0ml. Rate of activity was calculated from the steepest part of the slope recorded on the chart paper and expressed as the fall in absorbance over ten minutes.

3. Experiments with Plasma

(a) Estimation of total thyroxine by radioimmunoassay

Principle: The method requires a high affinity antiserum for plasma thyroxine. Prior to addition of the antiserum, plasma samples were diluted with a fixed amount of $^{125}I\text{-}T_4$ and the amount of label which attached to the antiserum is inversely proportional to the amount of thyroxine in plasma. Bound T_4 was then precipitated by polyethylene glycol and after careful removal of the supernatent the labelled material in the precipitate was counted in a gamma counter.

Method

The T_4 radioimmunoassay was carried out at room temperature and plastic tubes were set up as follows:

1. Two tubes containing 50ul of T_4-free serum (O-standard).

2. Two tubes containing 50ul of T_4-free serum and 250μl distilled water (diluent blank).

3. Two tubes containing 200ul of $^{125}I\text{-}T_4$ alone (total count).

4. Two tubes for each concentration of standard (30, 15, 8, 4, 2μg/100 ml), test sample and quality control, 50ul of appropriate sample were added to each tube followed by 200ul of $^{125}I\text{-}T_4$ and 250ul of antiserum.

All the tubes were incubated at room temperature for one hour. Polyethylene glycol was then added to each tube and mixed thoroughly to produce a homogenous opalescent solution and centrifuged at 2500 g and 4°C for 30 minutes. The supernatent was then decanted ensuring that no drops of fluid remained in the tubes. The precipitate in each tube containing antibody-bound hormones was counted for 30 seconds using a gamma counter (LKB Walla, 1280 Ultrogamma).

The concentration of T_4 in the samples was calculated by reference to a standard curve drawn from the standard results on 1cm graph paper. The equation for standard curve when counts (Y) were plotted against the logarithm of T_4 concentration (X) was:

$$Y = -12623(X) + 25266$$

b - Estimation of plasma iron (Reference method recommended, by International Committee for Standardization in Haemotology (1966).

Principle: Plasma proteins are precipitated by a solution composed of trichloroacetic acid, thioglycolic acid and redistilled hydrochloric acid. Iron in the supernatent is reacted with bathophenanthroline to produce a pink colour which can be measured at 535nm.

Method

Protein precipitant solution, 1ml, 0.43 M-thioglycollic acid in 2M-hydrochloric acid and 0.61M-trichloracetic acid was added to 1ml plasma in a dry Fe-free test tube. Plasma and protein precipitant were mixed thoroughly, allowed to stand for five minutes and then centrifuged at 2500 g for 15 minutes at 4°C. A reagent blank and standard were prepared for samples by substituting Fe-free distilled water or standard solution (2µg Fe/ml) for plasma. Supernatent solution (1ml) was taken from each tube and added to 1ml of chromogen solution (0.75M-bathophenanthroline sulphonate in 2M-sodium acetate). Each tube was mixed thoroughly and allowed to stand for five minutes.

Optical density of the chromogen treated solution was then measured at 535nm, against a blank of distilled water. The optical density of the reagent blank should not exceed 0.015 against distilled water in 1cm light path.

Plasma iron concentration was calculated from the formula:

Iron $\mu g/100$ ml plasma =

$$\frac{OD_t - OD_b}{OD_{St} - OD_b} \times 2 \times 100$$

Where OD_t = optical density of the test,

OD_b = optical density of the blank,

OD_{St} = optical density of the standard

2 = concentration of the standard $\mu g/ml$

100 = conversion factor for 100ml plasma

4. Experiments with Tissue

a - Preparation of the tissue for the different assays

A known weight of tissue (0.5-1g) was mixed with 10ml of distilled water and homogenized in an electrically powered glass homogenizer. The homogenized tissues were then centrifuged for one hour at 80,000 g at 4°C. The supernatent which contained only the soluble fraction from the cells was then used in the different assays reported below.

b - Estimation of total peroxide and peroxidase activity in tissue extracts.

Method

Tissue extract was diluted 1 part in 20 parts in ferricyanide-cyanide reagent. An aliquot ($100\mu l$) of this

solution was then reacted in the assay described previously for the estimates of erythrocyte peroxidase activity (p
The calculation was made according to the formula outlined below:

umol total peroxides/g tissue

$$= \frac{OD_{340} \quad \times \quad 6200 \times 100}{\text{Tissue weight}}$$

Where OD_{340} = OD of the incubation medium before adding glutathione reductase minus OD of the incubation medium after 10 minutes incubation with glutathione reductase

6200 = the molar extinction coefficient of NADPH/min./Cm at 340nm.

100 = the dilution factor

Tissue wt = weight of the liver tissue used

c - Estimates of non-haem iron in the liver's tissues

(Foy, William, Cortell and Conrad, 1967)

Principle: Non-haem iron of tissue homogenates was extracted by boiling the homogenates in a mixture of trichloroacetic acid and sodium pyrophosphate solution. The supernatent produced was then reacted with a chromogen (α,α' bipyridine) to produce a pink colour measured at 520nm.

Method

Aliquots (5ml) of tissue homogenates or extract of Ferritin-Fe (P.82) were transferred to iron-free test tubes and mixed with 4ml 25%(w/v) trichloroacetic acid and 4%(w/v) sodium pyrophosphate solution. The test tubes were placed in a boiling water-bath for 15 minutes and agitated several times during boiling to mix thoroughly. The mixture was then centrifuged at 2500 g for 20 minutes at 4°C, Aliquots

(3ml) of the supernatent were added to 2 iron-free cuvettes. To one (the test sample), 1ml 2%(w/v) sodium ascorbate solution was added, 1ml α,α bipyridine (0.4%(w/v) in redistilled 0.005N-HCl) and 2ml of 2M-sodium acetate buffer pH4.8. The same reagents were added to the other tube (the sample blank B_1) except the a,abipyridine solution, which was replaced with 1ml iron-free distilled water. A standard solution was prepared by substituting 1ml of an aqueous iron standard (5μg/ml), 1ml 4%(w/v) sodium phosphate and 1ml 25% redistilled triochloroacetic acid solution for the supernatant and using the same reagents for the test sample. A standard blank (B_2) was prepared in a manner similar to the standard, except that 1ml iron-free distilled water was substituted for the standard solution. A reagent blank (B_3) was made up with 1ml of 4% sodium pyrophosphate 1ml 25% trichloroacetic acid solution, 2ml iron-free distilled water, 1ml 2% sodium ascorbate and 2ml 2M-sodium acetate buffer pH4.8. Contents of the cuvettes were mixed thoroughly and allowed to stand at room temperature for 30 minutes before measuring the absorbance at 520nm.

The non-haem iron content of the tissue sample is calculated as below.

Iron content of tissue specimen (in μg)

$$= \frac{(O.D_{t} - O.D._{B_1}) - (O.D._{B_1} - O.D._{B_3})}{O.D._{St} - O.D._{B_2}} \times 5 \times 20$$

Where $O.D._{t}$ is the optical density of the test sample.

$O.D._{B_1}$ is the optical density of the sample blank

$O.D._{B_2}$ is the optical density of the standard blank.

$O.D._{B_3}$ is the optical density of the reagent blank

Where 5 is the iron standard concentration µg/ml

20 is the dilution factor of the tissue extract

Extract of ferritin-iron:

Aliquots (5ml) of tissue homogenate were heated for 30 minutes at 80°C in water-bath, centrifuged to remove the tissue residues and the supernatent was then mixed with saturated ammonium sulphate solution (1:1). The precipitate was separated by centrifugation at 2500 g and mixed with 5ml Fe-free distilled water.

CHAPTER THREE

RESULTS

CHAPTER THREE

RESULTS

Part A: ARIBOFLAVINOSIS IN GROWING RATS.

1. Effect of Different Diets on Weight Gain and Food Consumption

1 - Diet A

Tables 4 and 5 and Figure 3 show details of food consumption and weight gain over the initial period of 35 days of rats fed diet A with and without riboflavin. Over the period, 6 rats on the riboflavin-deficient diet consumed an average 77.5% by weight of the food consumed by the control animals i.e. the intake was 12g per control rat per day while rats on the riboflavin-deficient diet consumed 9g per rat per day. This resulted in a weight gain by rats on the riboflavin-deficient diet which was 43% of that obtained on the control diet. Altogether the experiment lasted for two months, during the last three weeks, rats on the deficient diet gained weight at an increased rate and almost caught up with the control animals. However, at two months when blood samples were taken, the difference in weight of control and deficient animals was still significant. In spite of the considerable depression of the growth rate of rats on the riboflavin-deficient diet, no clinical signs appeared during the two-month experiment. The only difference observed between the control and deficient rats was a gradual change in the appearance of the fur, which lost its lustre and silkiness in the latter.

Table 4:

Food Consumption and Weight Gain of Rats on Diets A or B over 35 Days of Feeding

	Diet	Control	Pair-fed	Deficient
Food consumption over 35 days in grams	A	425±14(6)	*	325±15(6)
	B	525±33(9)	*	342±32(14)
Weight gain over 35 days in grams	A	140±9 (6)	90±1(6)	60±9 (6)
	B	200±11(9)	133±2(6)	53±10(14)

* Food consumption of the pair-fed group is the same as that of the deficient group.

Table 5:

Mean Weight Gains (g) of Rats Fed
Diet B Measured every Five Days from the Beginning
of the Experiment

	Control (9)	Pair-fed (6)	Deficient (14)
Mean weight gain/5 days	17±3	14±2	9±2
" " " 10 "	37±4	35±4	13±1
" " " 15 "	63±4	49±3	27±4
" " " 20 "	105±8	89±5	39±2
" " " 25 "	140±7	105±7	46±4
" " " 30 "	167±6	112±5	49±3
" " " 35 "	200±11	133±2	50±2

Figures in parentheses indicated the number of animals used in each group. The data shown represented the mean±SD of the weight gain measured every five days over a period of 35 days.

Figure (3):

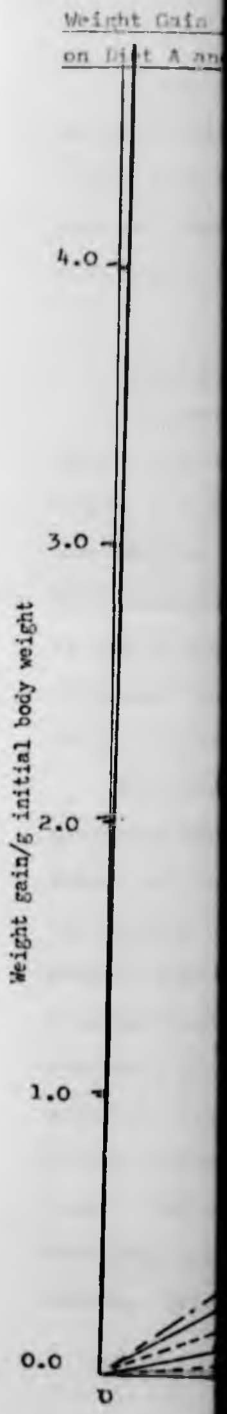
Weight Gain per Gram Initial Body Weight for Rats on Diet-A and Diet-B for 35 days

Figure 3:

Weight gain per gram initial body weight for rats on diet A and diet B for 35 days.

Points represent mean weight gain per gram initial body weight measured every 5 days for the above period for control (○——○), pair-fed (●- - - -●) and riboflavin-deficient (●- - - -●) groups on Diet B.

Points on the transparent overlay represent those for controls (○————○), pair-feds (○- - - ——○) and riboflavin-deficients (●— — —●) groups when diet A was used.



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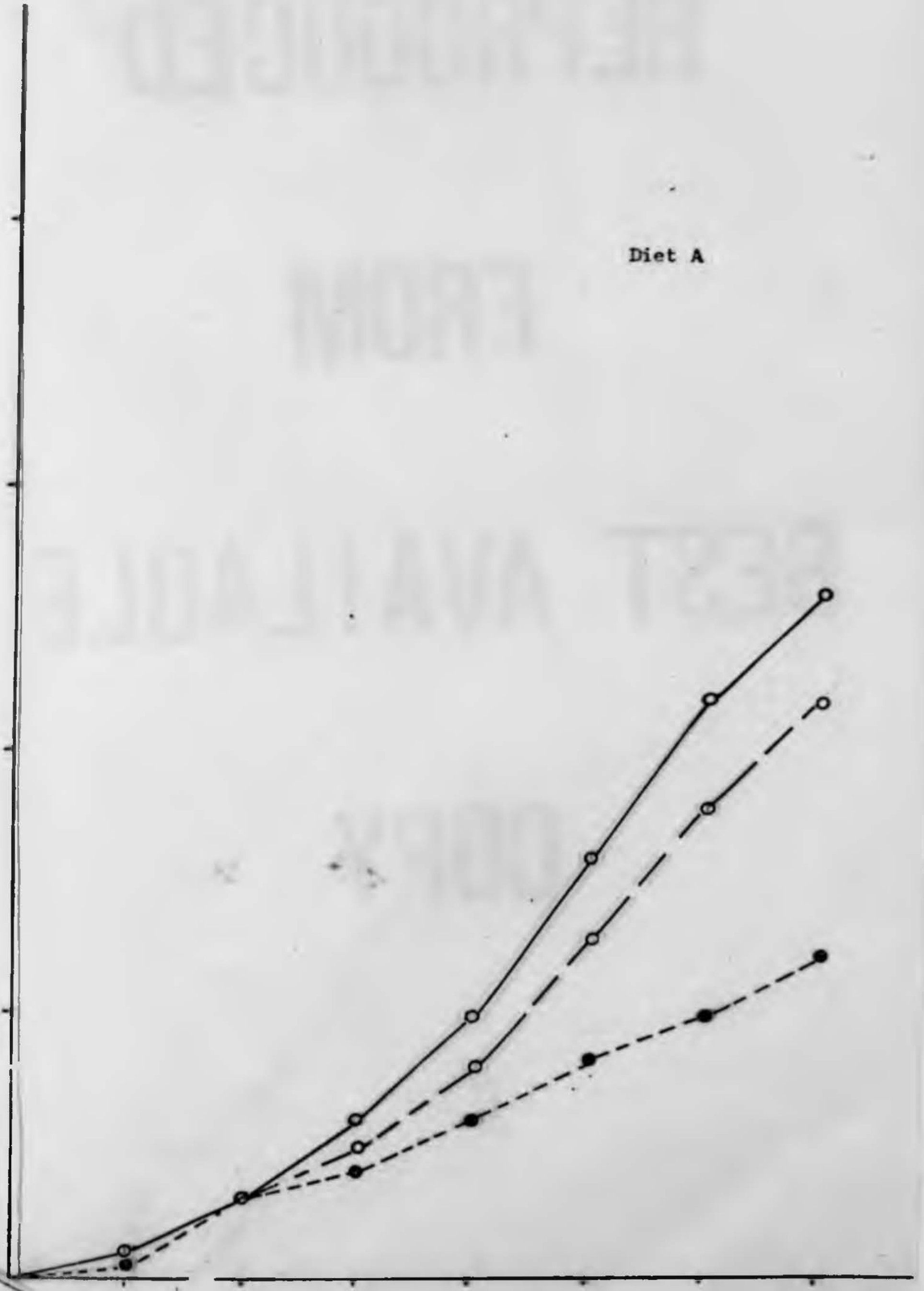

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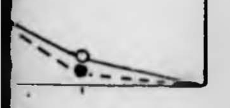
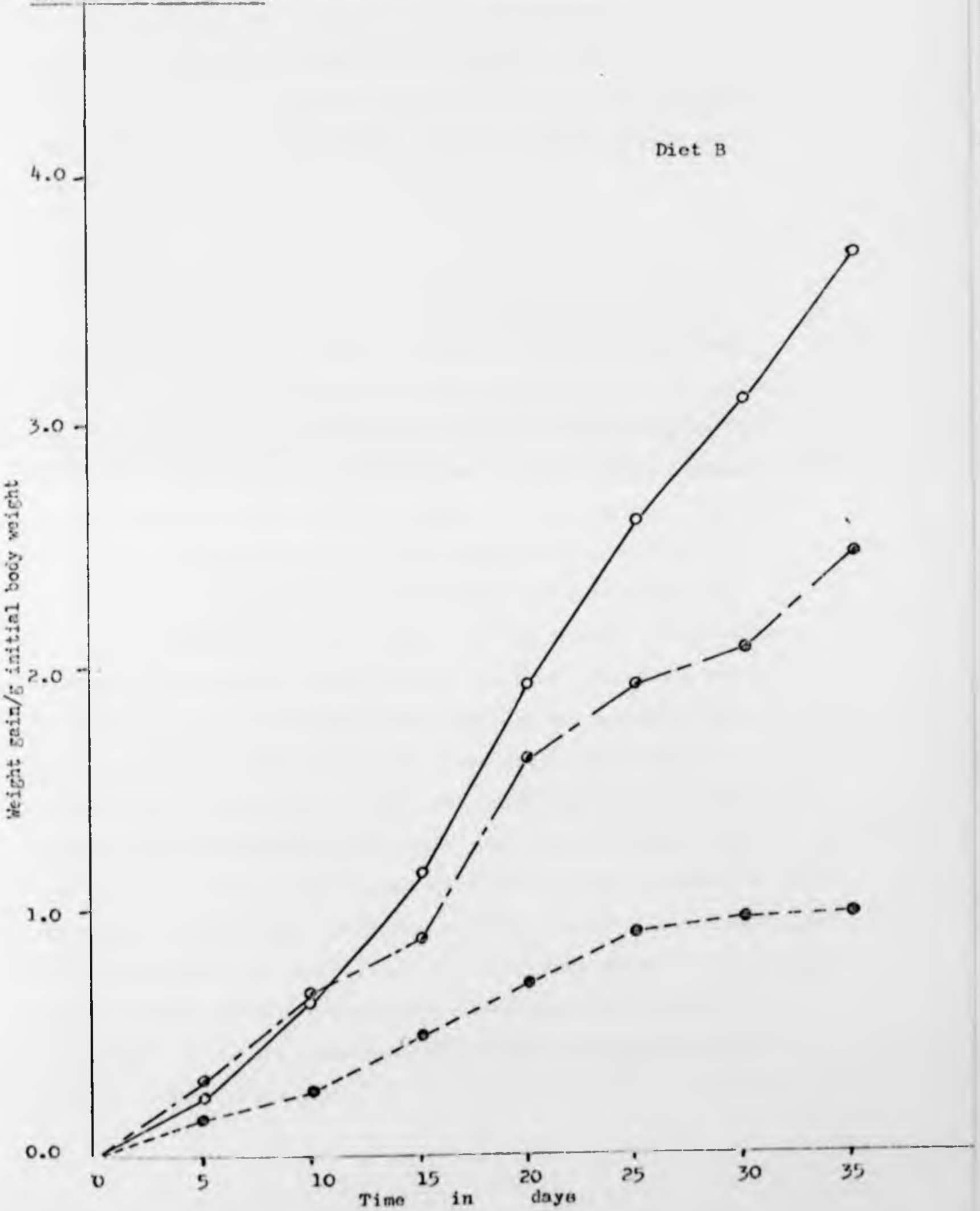


Weight Gain per Gram Initial Body Weight for Rats on Diet A and Diet B:



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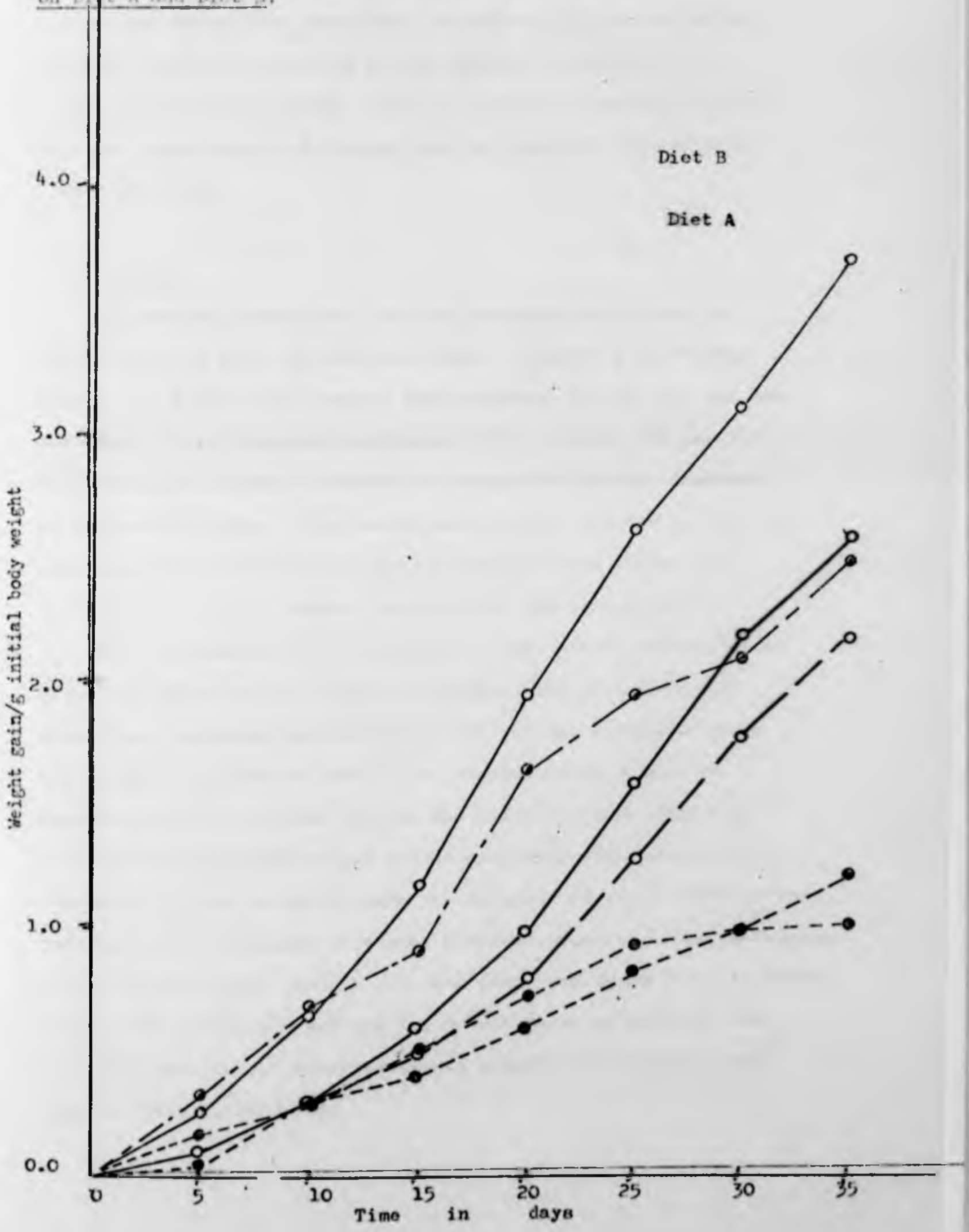
Weight Gain per Gram Initial Body Weight for Rats on Diet A and Diet B:



At the end of the experiment the mean (± SD) of the EGR-AC of blood collected from rats on the deficient diet A was 1.38 ± 0.21 (6) while those collected from pair-fed and controls were not significantly different and the combined results were 1.25 ± 0.23 (12).

2 - Diet B

The average weight gain and food consumption of rats on diet B over the first 35 days are shown in Tables 4 and 5 and Figure 3. Rats on the control diet consumed 15g per rat per day and those on the riboflavin-deficient diet, 10g per rat per day; i.e. deficient animals consumed an average 67% of that consumed by the control rats. The weight gain of rats fed the riboflavin-deficient diet expressed per gram of initial body weight was 26.5% of that of the control animals over the same period.

Food consumption of the animals on the control and deficient diets was approximately equal during the first five days, but weight gain per gram initial body weight of the deficient group was already only 62% of that of the control group (Table 4). Between days 5 and 20 the rats on the deficient diet showed decreasing food consumption and weight gain also decreased until it reached a plateau at which stage the majority of the animals on the deficient diet developed different clinical signs of ariboflavinosis. At the plateau stage, weight gain was less than 5g per 14 successive days. The difference between the growth rates of controls and deficient rats in all experiments was significant from day five onwards. (Fig.3, $P < 0.001$).

The difference between the total food consumed by control groups on diet A and diet B was 100g per rat over the 35 days. Animal growth was therefore much better on diet B and in all further experiments diet B was used particularly since symptoms of riboflavin deficiency were more reproduceably obtained. Riboflavin in the casein used for both diets A and B was 0.126μg ± 0.016 per g of casein based on 12 assays. Final concentration of riboflavin was about 0.013mg per Kg diet A and 0.023mg per Kg diet B.

Influence of different fats on production of ariboflavinosis by Diet B:

Fifty-three rats having a mean weight (± SD) of 53.04 ± 3.72g were used to investigate the effect of different dietary fats on the production of riboflavin deficiency using the diet B formula. The rats were divided into four main groups 13, 13, 15 and 13 and animals in each group were allocated as follows: 4 controls, four pair-fed and the rest on the riboflavin-deficient diet. The four groups were given diets prepared with the following fats; pure corn oil (Mazola; CPC United Kingdom Ltd.,Esher, Surrey, England), deodorised cotton seed oil (Loders and Mucoline Ltd., Cairn Mills, England), deodorised groundnut oil (Croda Premier Oils Ltd., Hull, England) and Seven Seas Cod Liver Oil (British Cod Liver Oils Ltd., Hull, England).

The percentage of food consumed over 35 days by riboflavin-deficient animals on corn oil, cotton seed oil, groundnut oil and cod liver oil diets were, 81, 56, 62.5 and 59% of their controls respectively. There were no significant differences between food consumed by control groups (range 550-570g over 35 days).

The change in weight of the different riboflavin-deficient groups expressed as a percentage of change in weights of the respective control groups is shown in (Fig.4). The figure shows that the lowest weight gain was made by rats on cotton seed oil followed by cod liver oil and groundnut oil. Rats on corn oil showed high weight gain and their growth was only slightly depressed by riboflavin deficiency.

Characteristic signs of severe riboflavin deficiency appeared on most animals fed the cotton seed oil, riboflavin-deficient diet during the second and third week of the experiment. The average weight gain of the riboflavin-deficient rats on cod liver oil was very similar to that of the cotton seed oil group, but the animals never showed any clinical signs of deficiency. Clinical signs of riboflavin deficiency were present in those receiving the groundnut oil diet, but were absent in those animals on the corn seed oil diet. The mean ± SD of the EGR-AC ratios of the riboflavin-deficient groups was 1.86 ± 0.31 (5), 1.84 ± 0.26(5), 1.62 ± 0.41 (6) and 1.67 ± 0.24 (5) for the cotton seed, cod liver oil, groundnut oil and corn oil diets respectively.

These biochemical measurements are not strictly comparable since they were done on blood samples taken over a number of days (day 35 to day 60) but they do indicate the approximate riboflavin status of the groups.

Figure (4):

Effect of Different Dietary Fats on the

Development of Riboflavin Deficiency in Rats

Figure 4:

Effect of different dietary fats on the development of riboflavin deficiency in rats

Curves show mean change in weight of groups of riboflavin-deficient rats calculated as a percentage of the change recorded in the respective control groups at 5-day intervals

(○——○) Diets containing corn oil
(■——■) Diets containind cotton seed oil
(●——●) Diets containing groundnut oil
(□——□) Diets containing cod liver oil

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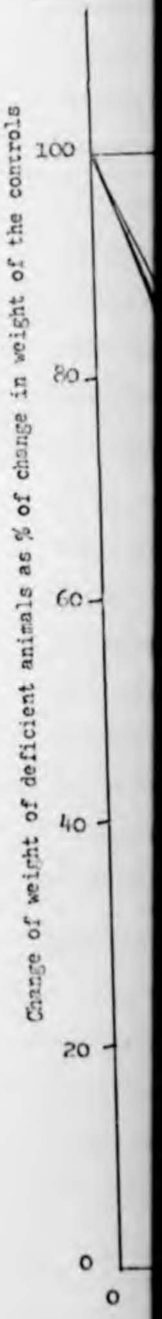

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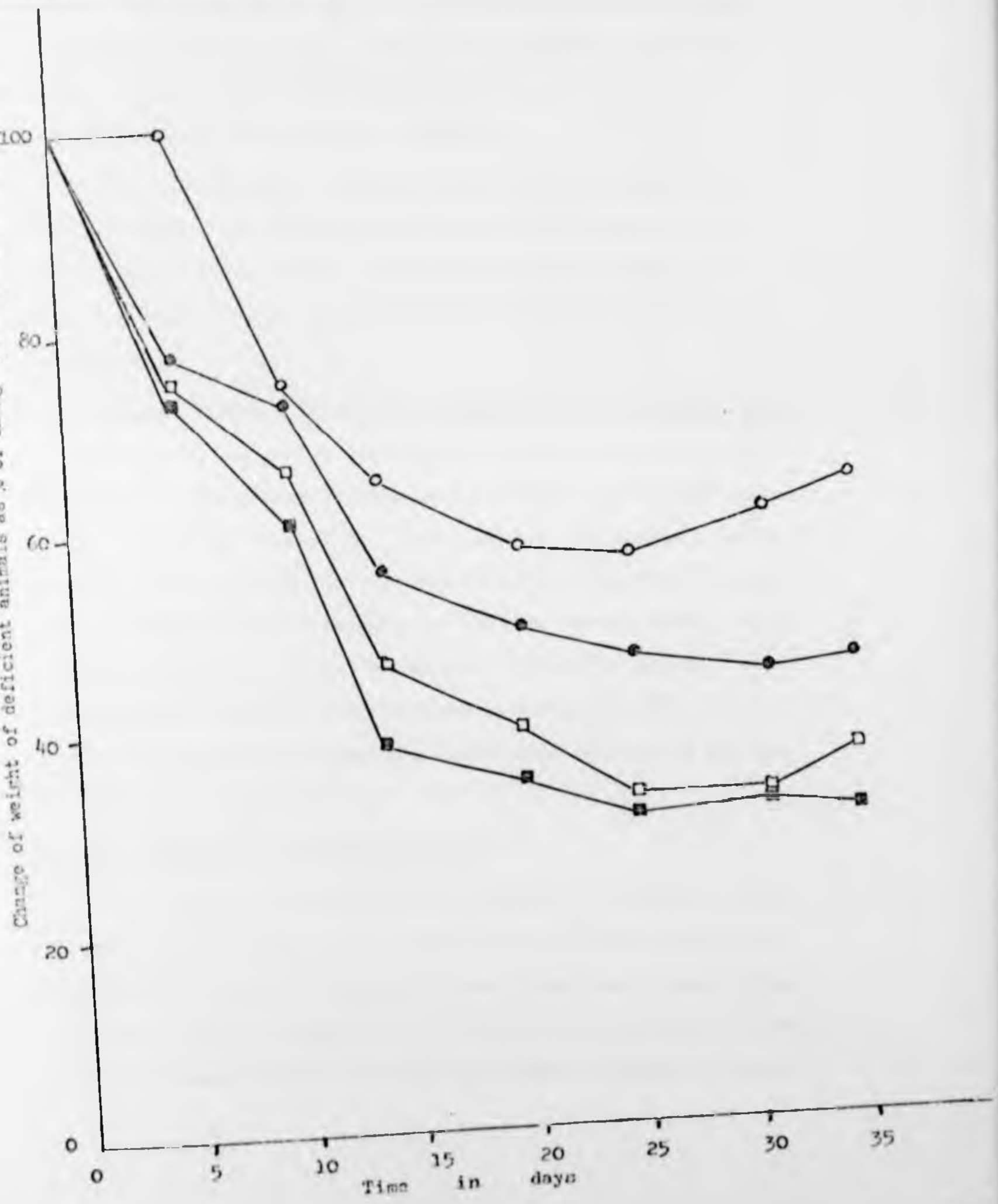
Effect of different Dietary Fats on Development

Riboflavin Deficiency in Rats



The results of this experiment show that cotton seed oil, groundnut oil, cod liver oil and corn oil diets produced very similar biochemical status. There was a complete absence of clinical signs from animals receiving the corn oil, and surprisingly also the cod liver oil diet.

It was noticed that corn oil and cod liver oil had one thing in common, they both contained added vitamin E, 84 and 100mg/100g oil respectively. The groundnut and cotton seed oils contained negligible amounts of vitamin E (Manufacturers information).

Vitamin E present in diet B was approximately 2.2mg/Kg diet, an amount which has proved satisfactory for many years in this Department. The amount of vitamin E recently recommended for rat diets (Clarke, Coates, Eva, Ford, Milner, O'Donoghue, Scott and Ward, 1977) was 12mg/Kg diet and it seemed possible that some of these signs studies attributed to riboflavin deficiency, may be exaggerated or influenced by marginal vitamin E status. The following experiment was done to examine the possibility that vitamin E deficiency was affecting riboflavin deficiency in rats on diet B.

Adequacy of vitamin E content in diet B:

In this experiment 44 rats (mean weight ± SD; 57.00 ± 2.71g) were used to investigate what effect vitamin E might have on the production of riboflavin deficiency when using diet B containing cotton seed oil or groundnut oil. Cod liver oil or corn oil were not used as these could not be obtained without vitamin E already added. The rats were divided into two equal groups according to which oil they received and further sub-divided according to whether extra vitamin E (100mg/Kg diet) was added or not, and into the controls, pair-fed and riboflavin-deficient groups.

Fresh samples of recently deodorised, vitamin E-free cotton seed and groundnut oils (Croda Premier Oils, Ltd.,Hull) were obtained and mixed with a solution of mixed tocopherols (tocopherol potency 52%; Molecular Distillation Ltd., Manchester) as required. Diet was prepared at weekly intervals and stored at room temperature.

Figure (5) shows the mean weight gain and food consumption of control and riboflavin-deficient rats on the four different diets used in this experiment. The results show that all four groups of control animals consumed almost the same amount of food (range 524-530g) and consequently the weight gain over 35 days was almost the same, approximately 180g/rat (Fig.5). Likewise food consumption of riboflavin-deficient rats in the four groups was not significantly different; those receiving cotton seed oil without vitamin E ate least, while those on groundnut oil plus vitamin E the most (10 and 11.5g/rat/day respectively).

The total weight gained by the four groups of riboflavin-deficient rats over the first 35 days was not significantly different from one another (Fig.5) but when change in weight of deficient animals was calculated as a percentage of that of their respective controls (Fig.6), weight gains of both groups on cotton seed oil was below those of the rats having groundnut oil.

Figure 5:

Food Consumption and Weight Gain Over 35 Days of Rats on Cotton seed oil ± Vitamin E, + Groundnut Oil ± Vitamin E

Figure 5:

Food Consumption and weight gain over 35 days of rats on cotton seed oil + vitamin E, Groundnut oil + vitamin E

Diagrams-represent mean food consumption and weight gain of rats (3 in each group) on diets containing cotton seed oil + vitamin E and groundnut oil + vitamin E over 35 days.

▭ Control (food consumption)
▥ Control (weight gain)
▨ Deficient (food consumption)
▤ Deficient (weight gain)

Food Consumption and Weight Gain of Rats on
Cotton Seed Oil + Vitamin E and Groundnut Oil + Vitamin E

Food Consumption and Weight Gain of Rats on

Cotton Seed Oil + Vitamin E and Groundnut Oil + Vitamin E

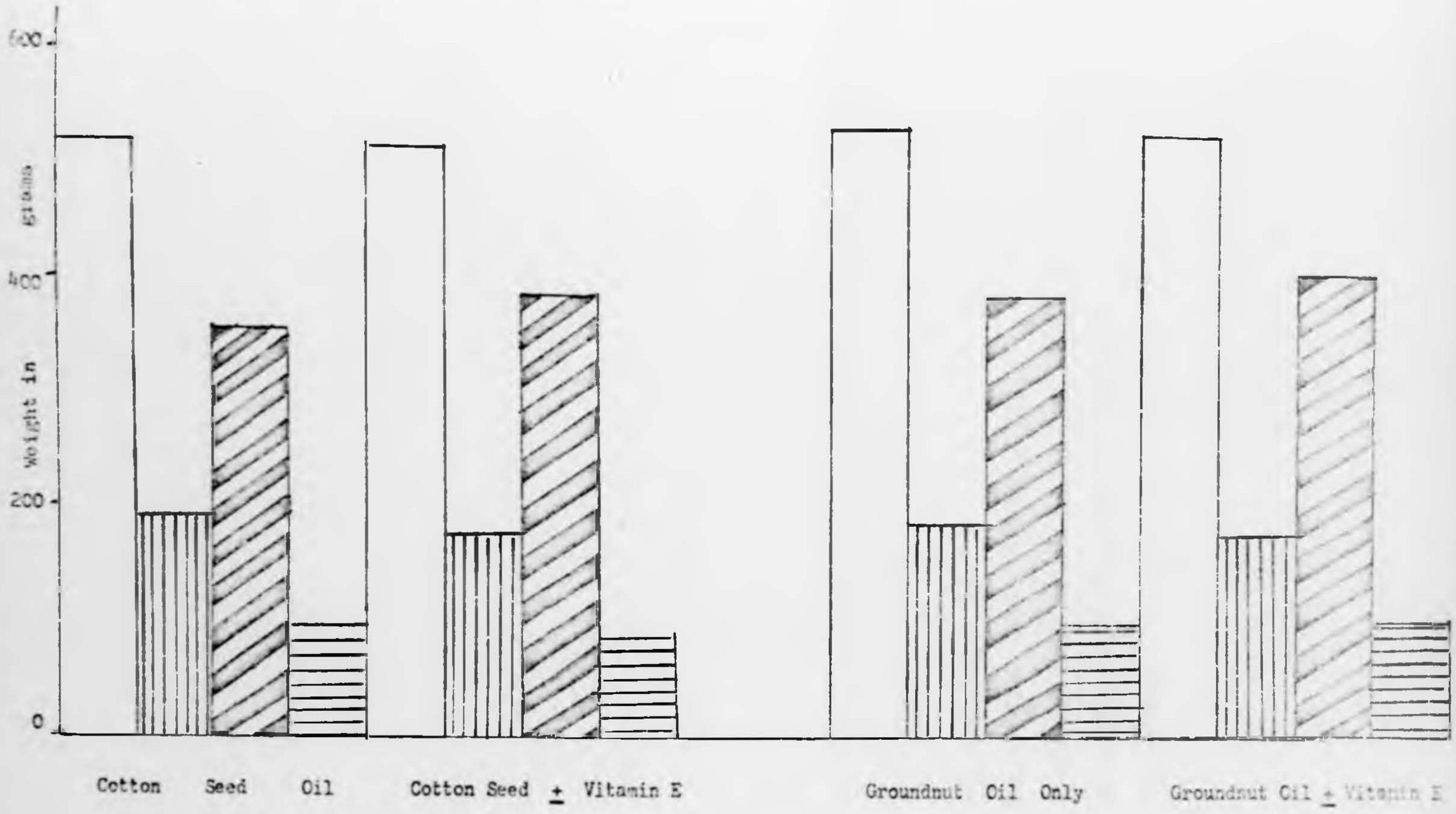


Figure (6):

Influence of Vitamin E on Development of Riboflavin Deficiency in Rats Receiving Diets Containing Cotton Seed Oil and Groundnut oil

Figure 6:

Influence of Vitamin E on development of riboflavin-deficiency in rats receiving diets containing cotton seed oil or groundnut oil.

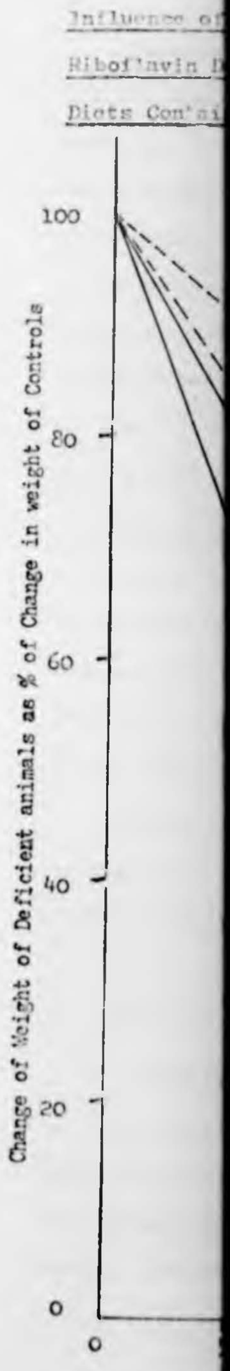
Change in mean weight of riboflavin-deficient rats expressed as percentage of change in mean weight of respective control animals at 5 day intervals.

■---■ Cotton seed oil

□—□ Cotton seed oil + Vitamin E

●—● Groundnut oil

○---○ Groundnut oil + Vitamin E



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Influence of Vitamin E on Development of
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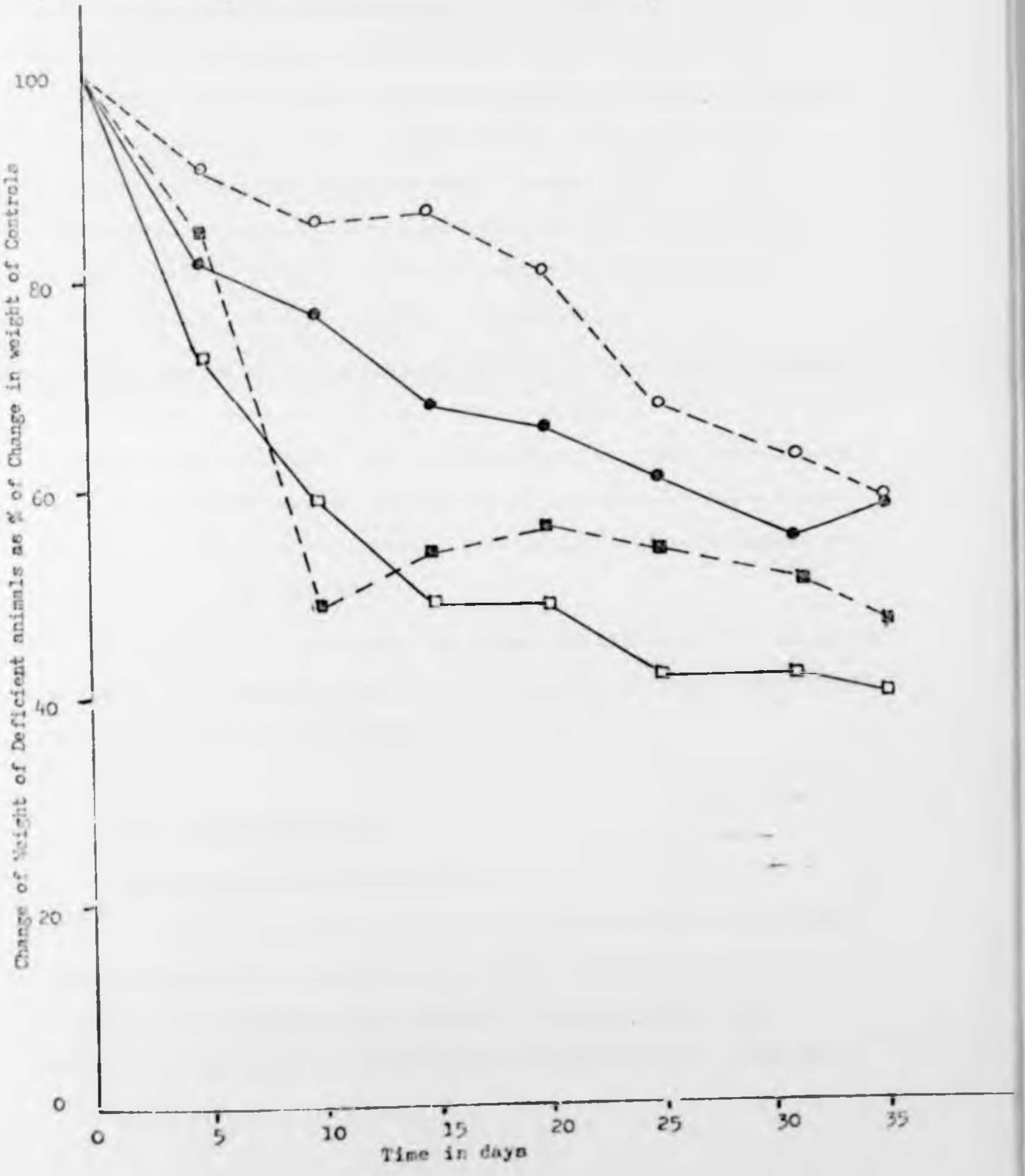

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Influence of Vitamin E on Development of
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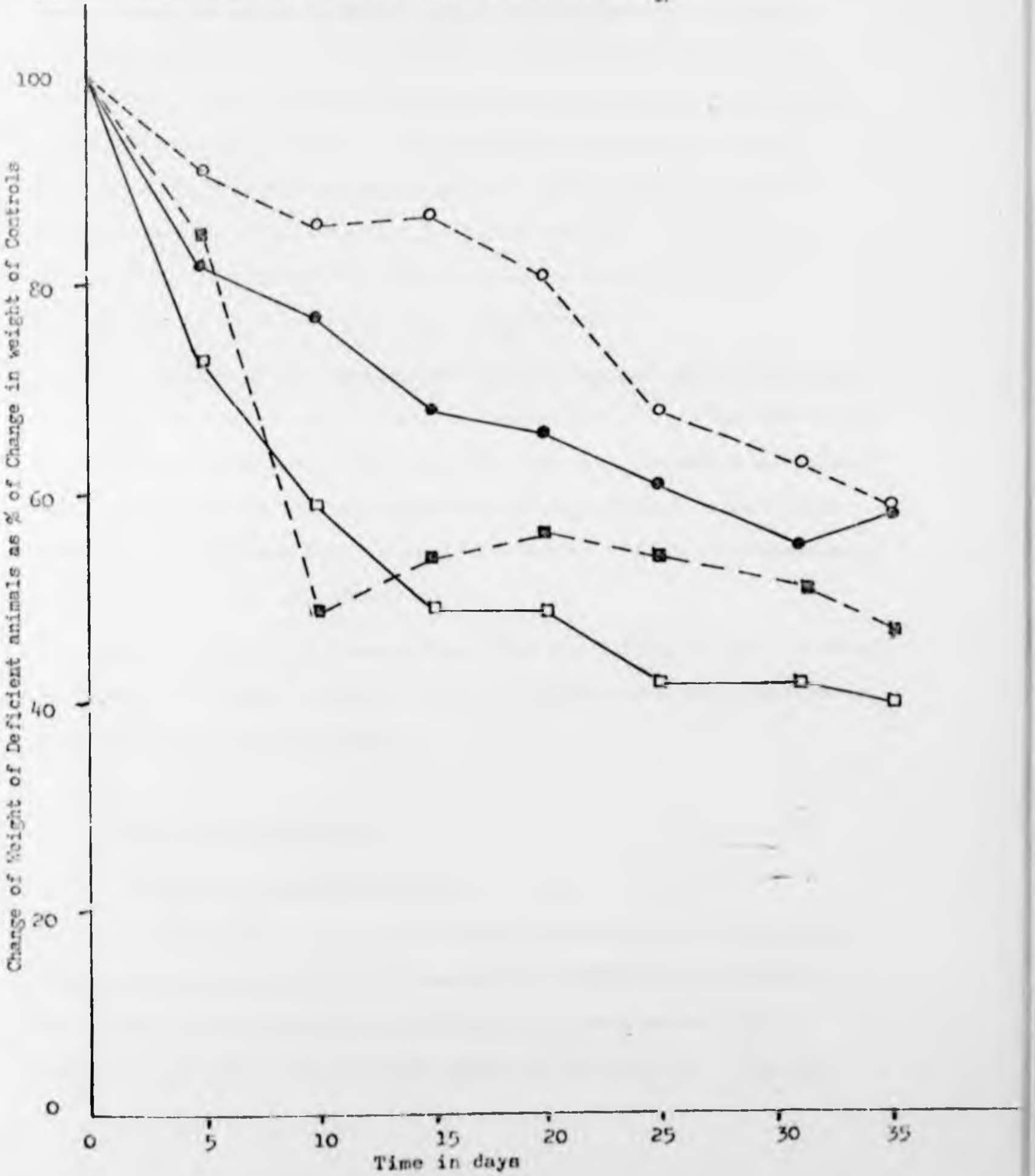


Clinical signs of riboflavin deficiency, appeared in almost all the animals on the deficient diets within the second and third week, except those on groundnut oil supplemented with vitamin E. The latter showed signs of deficiency in the fourth week of the experiment. Rats in three riboflavin-deficient groups, in receipt of cotton seed oil, vitamin E supplemented cotton seed oil and groundnut oil were all killed on day 40, while rats on vitamin E supplemented groundnut oil were killed on day 45. The EGR-ACs of the different groups were 2.44 ± 0.29(3), 2.22 ± 0.33 (3), 2.20 ± 0.07 (3) and 2.01 ± 0.17(3) respectively).

The results of this experiment did not explain why the 4 diets containing the different oils had different effects on the development of riboflavin deficiency, but they did show that vitamin E deficiency was not responsible for the appearance of the clinical signs associated with riboflavin deficiency. The latter will be discussed more fully elsewhere (p. 194-200).

Growth of pair-fed animals receiving the different oils is shown in figure 5. Growth rate and food consumption were not significantly different between the 4 groups.

II. Signs of Ariboflavinosis

1 - Biochemical Riboflavin status

Riboflavin status was established by measuring the erythrocyte glutathione reductase activity coefficient (EGR-AC: see Methods). The animals on the riboflavin-deficient diet were never killed before clinical signs of ariboflavinosis had appeared. Progress of deficient state was assessed by using the weight gain criterion. i.e. <5g/14 successive days, after which careful observations were made daily to avoid refection occuring if possible and maintain the deficient state.

Usually when clinical signs of riboflavin deficiency appeared, preparations were made to kill the rat within a few days together with pair-fed and control rats if possible.

In the first experiment using diet A because clinical signs never appeared, blood samples were not taken until the end of two months. Mean EGR-AC of deficient animals was slightly raised (see p 88) but the difference was not significant.

Results taken from the first eight experiments showed that the EGR-AC of all control animals ranged between 1.03 and 1.54 while that of the pair-fed animals from 1.08 - 2.20. The means and SD of the two groups respectively were 1.28 ± 0.17 (44) and 1.27 ± 0.24 (32). These values were taken as the normal range of the AC in this study.

The EGR-AC of the animals on the riboflavin-deficient diet varied according to the riboflavin status of the animals and the severity of its clinical condition. The highest EGR-AC values obtained in this study was 4.35 for an animal which showed skin lesions around its body in addition to brown pigmentation around its limbs and fur, and showed angular stomatitis early in its deficiency and alopecia. Clinical symptoms were apparent for two weeks before the animal was killed.

On the basis of the range of EGR-AC values obtained from control rats, EGR-AC values from the deficient rats were assigned arbitarily into three groups:

1 - Animals in which AC$>$2.0 were probably riboflavin deficient.

2 - Animals in which AC$<$2.0 but $>$1.6 and they exhibited clinical signs of riboflavin deficiency were probably marginally deficient or showing early signs of refection.

3 - Animals in which AC $<$ 1.6 were often free of clinical signs of deficiency and/or were undergoing obvious refection.

The mean basic activity for EGR in the first experiment on diet B where the AC $\geq$ 2.0 was 1.04$\pm$ 0.53 (13) and was significantly lower than that of control samples, 2.49$\pm$ 1.04 (9), P$<$0.001.

For the first 29 rats fed the riboflavin deficient diet B, glutathione reductase activity was measured in haemolysates prepared from both whole blood (BGR) and washed erythrocytes (EGR). This was done to determine whether the results were interchangeable. The values obtained by the two techniques were significantly correlated ($r = 0.89$, $P < 0.001$; Fig.7) and the equation of the regression line was calculated to be

$$AC_{EGR} = 1.45 \times AC_{BGR} - 0.24$$

In general the BGR-AC was 0.4 below that of EGR-AC. Likewise when basic activities of glutathione reductase of haemolysates for whole blood and washed erythrocytes were plotted against one another (Fig.8), the values were also significantly correlated ($r = 0.67, P < 0.001$). The equation of the regression line was calculated to be :

$$\text{Basic Activity of BGR} = 0.88 \times \text{Basic activity of EGR} + 0.95$$

Basic activity of the whole blood preparation was slightly more active than that from washed erythrocytes.

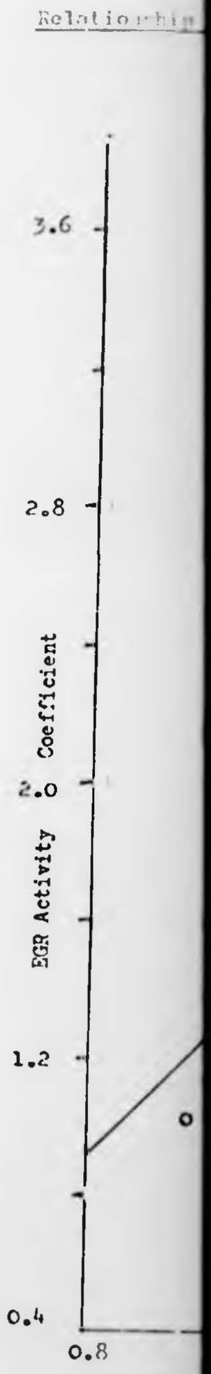
Figure (7):

Relationship between BGR-AC and EGR-AC

Figure 7:

Relationship between BGR-AC and EGR-AC

BGR-AC (whole blood) and EGR-AC (erythrocytes) were measured in samples from control (■), pair-fed (○) and riboflavin-deficient rats (●) as described in the Methods Chapter. AC ratios; the measures of riboflavin status, were calculated from measurements of glutathione reductase activity with and without FAD.



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Relationship between BGR-AC and EGR-AC

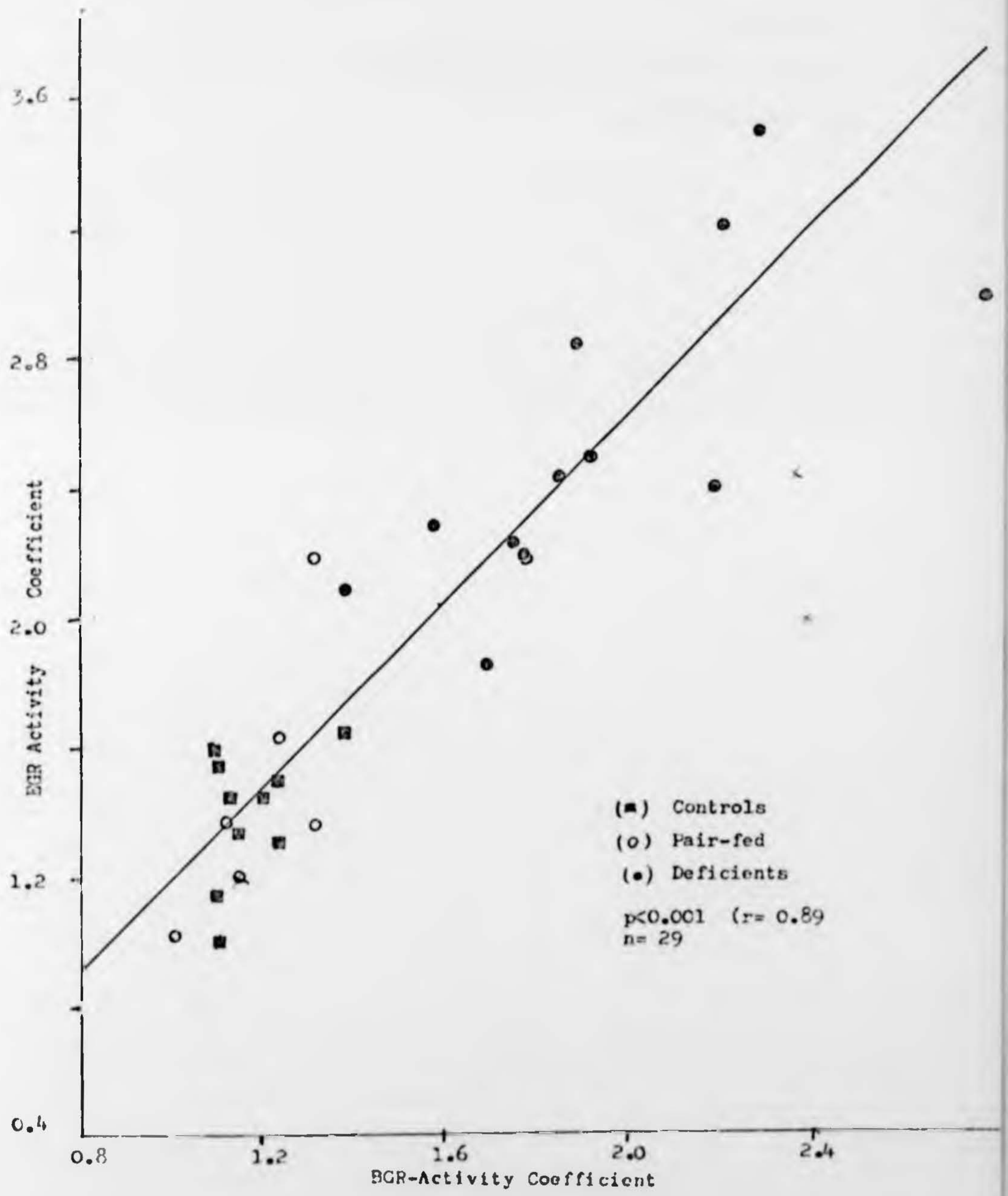


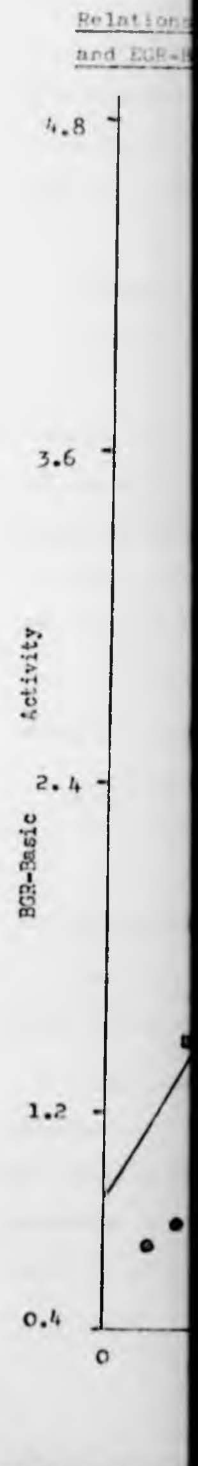
Figure (8):

Relationship between BGR-Basic Activity
and EGR-Basic Activity

Figure 8:

Relationship between BGR-Basic activity and EGR-Basic activity

Measurements of the fall in the optical density per 10 min. without FAD used in the determination of both BGR and EGR-ACs (Fig. 7) were used to calculate the basic activity of both BGR and EGR as i.u./g Hb.(Beutler, 1971). Hb concentration (g/100ml haemolysate) was measured in the haemolysate used in the assay as described in the Methods Chapter. Points represent the results obtained from Control (■), pair-fed (○) and riboflavin-deficient animals (●). Each point represents one sample on which duplicate assays were done.



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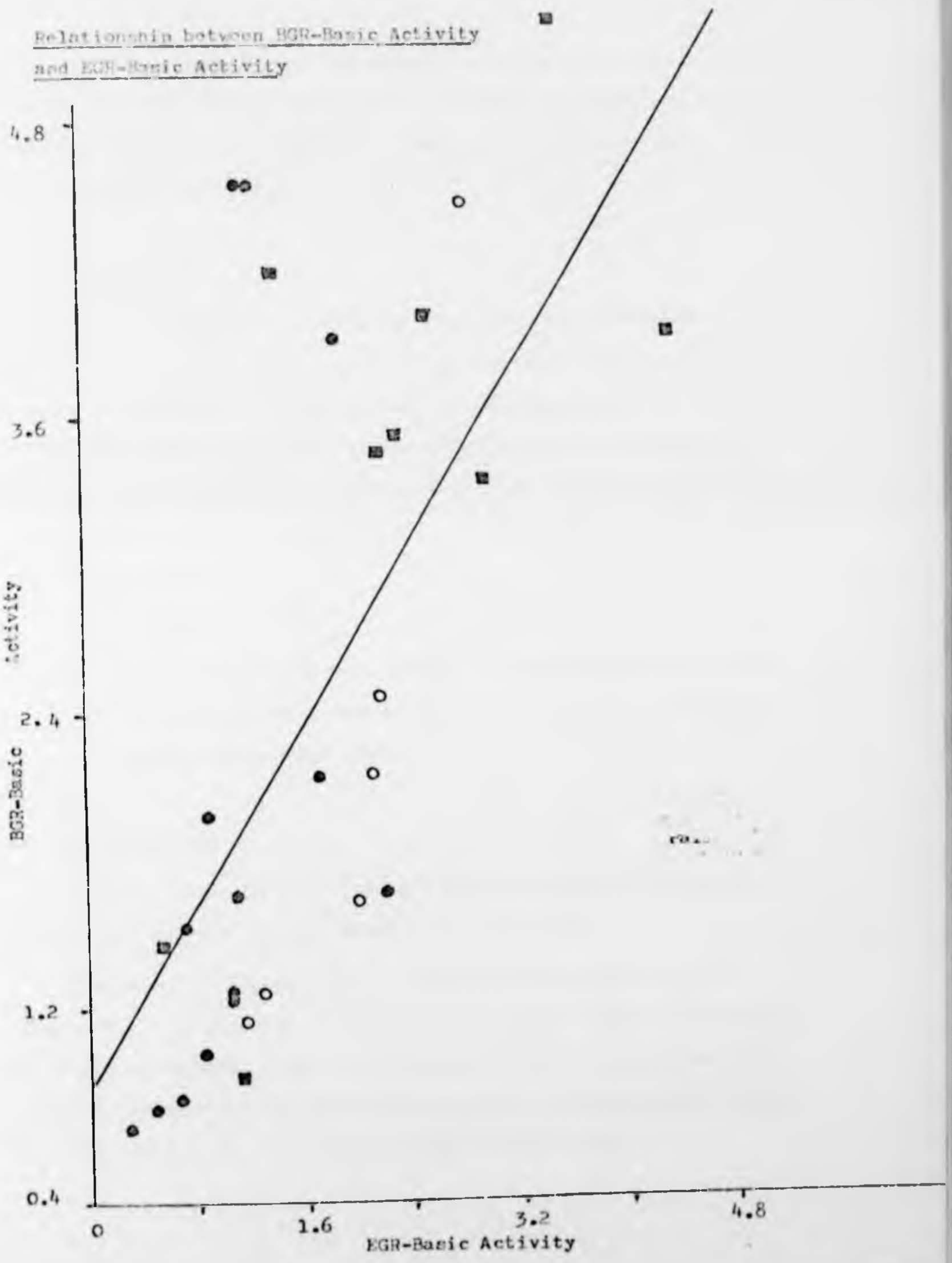
Relationship between BGR-Basic Activity and EGR-Basic Activity



It was decided to use EGR activity for the assay rather than BGR mainly since erythrocytes would be washed for other assays and also the fact that the EGR-AC was greater, i.e. possibly more sensitive than the BGR-AC.

2. Liver weight

The liver weight of riboflavin-deficient rats expressed as a percentage of the total body weight were greater than those of either pair-fed or ad-libitum-fed controls. It was also found that the increased liver weight of riboflavin-deficient rats was directly correlated to the EGR-AC ($P<0.001$) when the increased weight was expressed as a percentage (P) of the weight of the control (Fig.9)

$$P = \frac{D - C}{C} \times 100$$

where D = liver weight/100g body weight of riboflavin-deficient rat

C = liver weight/100g body weight of control rat of same age and killed at same time.

3. Clinical signs

Table 6. summarises the clinical signs recorded in ten experiments involving over 90 rats mostly receiving diet B.

Diarrhoea was a common sign in rats on diets deficient in riboflavin. It occurred at the end of the first week and continued for about seven days. During this period, fur on the venter, the posterior extremities and genitals was matted with dark green faeces. Alopecia of the groin commonly followed the diarrhoea.

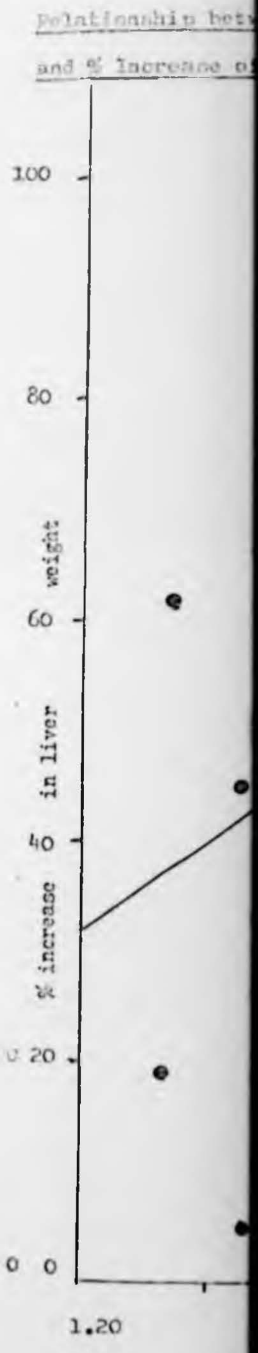
Figure (9)

Relationship between Riboflavin Deficiency and Percentage Increase in the Liver Weight

Figure 9:

Relationship between riboflavin deficiency and percentage increase in the liver weight

The plotted points in the figure represent the % increase of the liver weight from riboflavin-deficient rats of those of the control animals of the same age as a percentage of total body weight against their appropriate riboflavin status. Percentage increase in the liver weight of the riboflavin-deficient rats of those of the control was calculated as described in page 101. Riboflavin status was measured by EGR-AC assay described in figure 7.



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Relationship between Riboflavin Deficiency and % Increase of Liver Weight

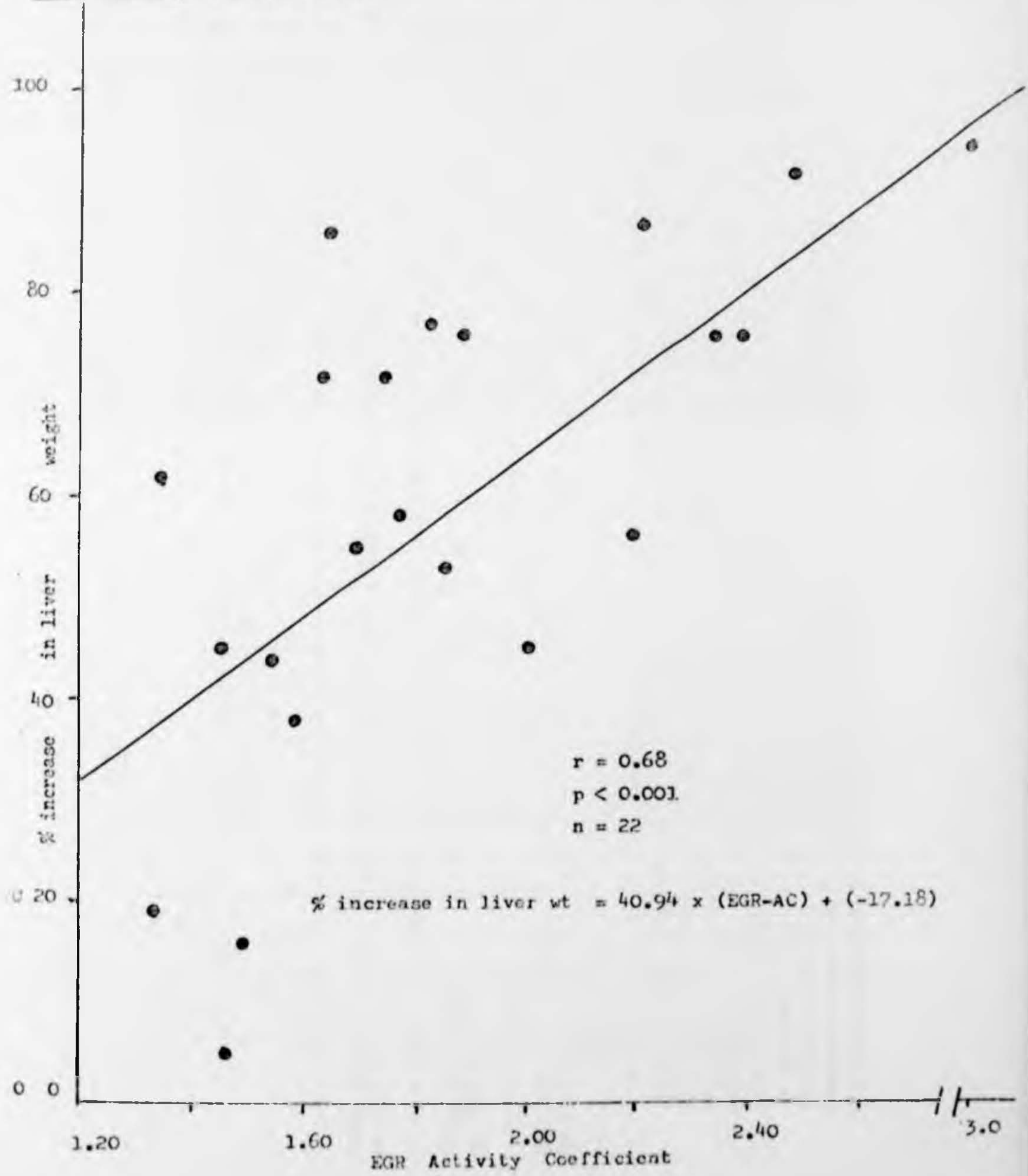


Table 6:

Summary of the Clinical Symptoms Appeared on the Rats on Riboflavin-Deficient Diets

	No. of rats (2)	Growth retardation (3)	Diarrhea	Skin Manifestation									Eye lid immflamn. (XX)	Cataract (++)	Paralysis	Weight loss (4)
				Change on fur	Reddish deposit on skin & fur	Alopecia	Alop. + lesion	Purple swollen lips (+)	Stomatitis	Skin lesion alone	Scaly skin	Kyphosis				
Young rats on diet A	6	35	None	50	None	None	None	50	None	None	50	None	17	None	None	None
Young rats on diet B	80	74	71	100	63	34	44	71	58	53	90	90	75	18	19	None
Old rats on diet B (1)	7	None	29	71	29	29	14	57	14	43	57	57	29	14	43	43

(1) Old rats on diet B fed a riboflavin-deficient diet supplemented with 22mg galactoflavin/kg diet for the first month raised to 44mg galctofalvin/kg diet later. (2) No of rats listed are the number of the rats on the riboflavin-deficient diet only. (3) Growth retardation was calculated by substracting the mean of the final weight at the end 35 days of the experiment from the mean of the final weight of the controls and divided by the mean of the initial weight of the controls multiplied by 100. (4) Weight loss calculated by substituting the mean final weight of the deficient animals from the mean initial weight divided by their mean initial weight multiplied by 100 (in the old rats only). (+) Normal lip colour is pale buff while in the deficient animals often become inflamed and swollen possibly oedematous. (++) Upper part of the eye appears watery and cloudy with some evidence of epithelial surface irregularities. (XX) Eye lids reddish, lids often remaining closed until handled, evidence of discharge around the eye and "spectacle" alopecia.
The percentages indicate the proportion of rats with the appropriate clinical signs except for (3) and (4).

Changes on the skin and fur: Many physical changes were observed in the skin and fur of animals on riboflavin-deficient diets from the third week onwards. Mild scaling unaccompanied by erythema occasionally occurred on the trunk, but more frequently, as mentioned above, around the venter the hair became loose and partial alopecia resulted, while the remaining hairs, which are normally short in this region, were bristled and matted together. The scales were small, white and dry. Scales on the tail appeared to increase in number and became larger and darker as the deficiency progressed. The fur on the trunk became moist, uneven and ragged. It was matted together in tufts. The rat then failed to groom its fur, displayed a lack of interest in the care of its body and became dirty.

As the condition progressed, the fur of many of the animals became coated and stained with a dark reddish brown or rust-coloured substance. The rims of the ears, the vibrissae, facial fur and the dorsal aspects of the paws were the sites most commonly involved, but sometimes the fur over the entire body was affected. The consistency of the stain was sticky and it was easily washed from the fur.

Characteristic "spectacle" alopecia occurred in the majority of the animals followed by adhesion of the eye lids to each other and inflammation. On the vertex of the scalp and on the shoulders of about 30% of the rats, there were symmetrical areas of alopecia sometimes accompanied by inflammation and lesions. The lips and nose were often erythematous, swollen and moist and in many animals there was a loss of hair over the anterior aspect of the labial regions.

Angular stomatitis which is a skin lesion around the angles of the mouth was sometimes an early sign of riboflavin deficiency. It was commonly observed in the majority of the rats between the third and sixth week (58% of all animals kept on diet B were affected).

Cataracts: These were seen in a small group of rats (18% of all animals on the deficient diet) especially those which were kept on deficient diet more than ten weeks. The upper part of the cornea of the eye became clear and watery, sometimes showing signs of epithelial scars, while the lower part of the cornea appeared cloudy and granular.

Partial paralysis: About 19% of rats on the deficient diet for more than ten weeks showed partial paralysis of lower limbs. This was observed while weighing the rats when stiffness of movement and tremor became evident on the balance, or were noticed when animals were endeavouring to climb onto their food pots while feeding.

Kyphosis: In addition to the above clinical symptoms,the majority of riboflavin-deficient rats assumed a chargcteristic humped-back posture, due possibly to stunted growth and possible distortion and malformation of the spine. Alternatively the enlarged liver may be causing the posture due to discomfort.

On killing riboflavin-deficient rats, loss of subcutaneous fat was evident and the absence of the fat from the serum was a common observation after centrifuging.

Part B: INFLUENCE OF RIBOFLAVIN DEFICIENCY ON THE STABILITY OF RED BLOOD CELLS

1. Development of the Peroxide - Generating System to Assess Fragility:

Walls and Hochstein (1974) described a method to assess fragility of human red cells in which the erythrocytes were exposed to a peroxide-generating system. The assay was modified to make it suitable for use with rat erythrocytes (Methods, section II2e) since the latter were much more easily haemolysed than human cells. Some of the pertinent experiments done in the development of this method are described below.

a - Concentration of xanthine oxidase:

Using either 0.08 or 0.04 units of xanthine oxidase in the incubation medium caused rapid oxidation and precipitation of the cells as a dark brown material during the first few minutes of oxidation. The use of 0.02 units of this enzyme caused approximately 40% red cell lysis after 45 minutes with slight discolouration but no detectable precipitate. Smaller amounts of xanthine oxidase (0.01 or 0.008 units) caused no detectable oxidation effect on haemoglobin of the lysed cells. It was decided that 0.02 units was probably the optimal concentration for greater sensitivity - but many subsequent experiments included xanthine oxidase at other concentrations (Fig. 12, 13 and 14) to ensure the conditions were sensitive enough for all blood examined. It was not found necessary to alter the concentration of hypoxanthine which remained at 1.6mM.

b - Time of Incubation

Walls and Hochstein (1974) incubated their red cells for many hours whereas effects of peroxide on rat erythrocytes were seen much more rapidly. Figure 10 shows the percentage haemolysis produced by 0.02 units of xanthine oxidase when incubated for different times with erythrocytes from well-nourished rats. The results suggested that during the first twenty minutes very little lysis occurred but that it progressed linearly until 45 minutes. After 45 minutes the rate of reaction fell and solution became increasingly discoloured presumably due to oxidative effects on haemoglobin. An incubation time of 45 minutes was therefore chosen as optimal for screening purposes.

c - Oxygenation of Krebs-Ringer Phosphate Buffer

It was found with human red cells that gassing the Krebs-Ringer phosphate buffer with oxygen before use caused slightly greater haemolysis (Fig. 11). In addition, when using rat blood and 0.04 units xanthine oxidase it appeared that gassing lessened the amount of haemoglobin which precipitated. The gassing was included therefore in the procedure with all erythrocytes to standardize the method.

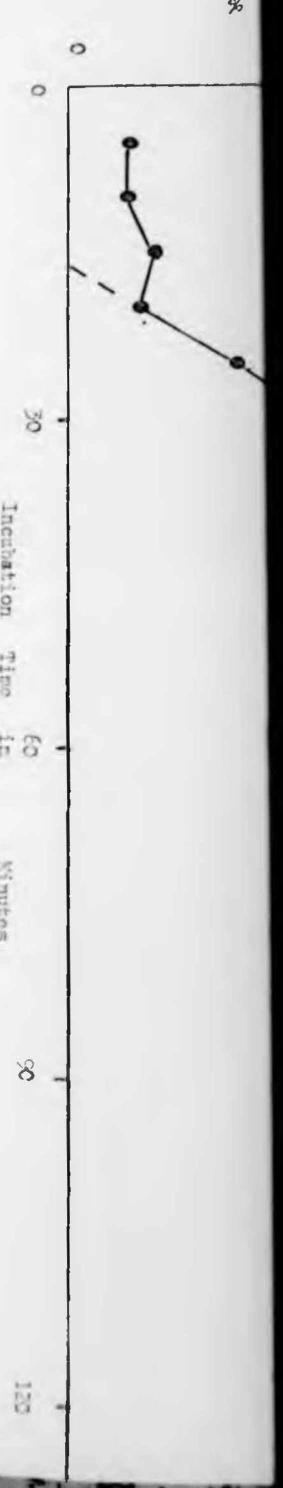
Figure (10):

Influence of Incubation Time on Peroxide-Induced Haemolysis of Rat Erythrocytes

Figure 10:

Influence of incubation time on peroxide-induced haemolysis of rat erythrocytes

To show the haemolysis produced by 0.02 units xanthine oxidase against time on 3ml rat erythrocytes (1%) haemotocrit for control animals in oxygen gassed Krebs-Ringer phosphate buffer (pH7.0) containing 1.6 mM hypoxanthine. Haemolysis in supernatent solution was expressed as a percentage of total haemolysis in tubes containing xanthine oxidase.



Effect of Incubation Time on the Haemolysis of Rat Erythrocytes

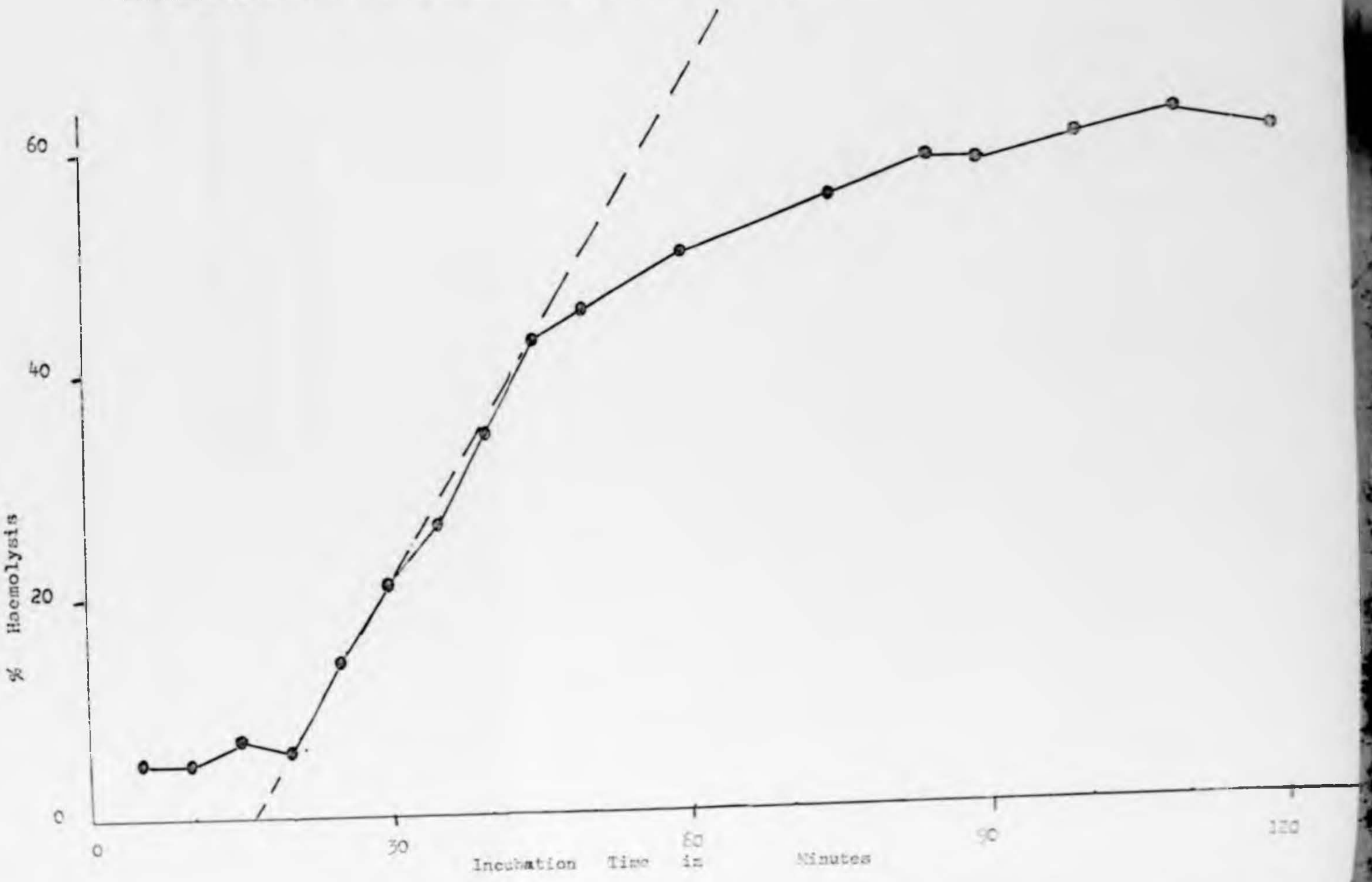


Figure (11):

Relation between Incubation Time and Haemolysis of Human Erythrocytes Produced by Peroxide-Generating System

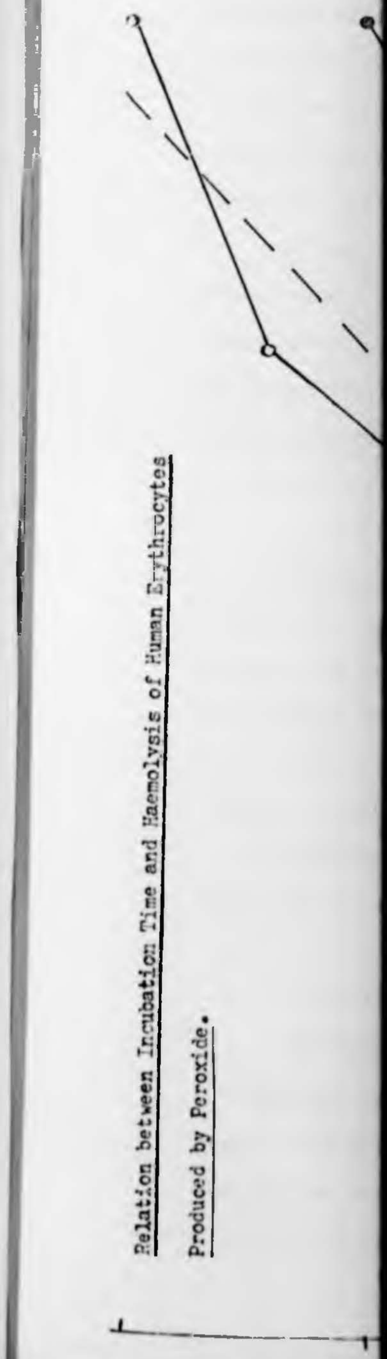
Figure 11:

Relation between incubation time and haemolysis of human erythrocytes produced by peroxide.

Conditions were those described in Fig.(9) except that human erythrocytes from a well-nourished subject were used and the flasks were shaken at 70rpm in a water bath at 37°C. ( ) Oxygenated

(•) Non oxygenated

(---) mean for oxygenated



Relation between Incubation Time and Haemolysis of Human Erythrocytes

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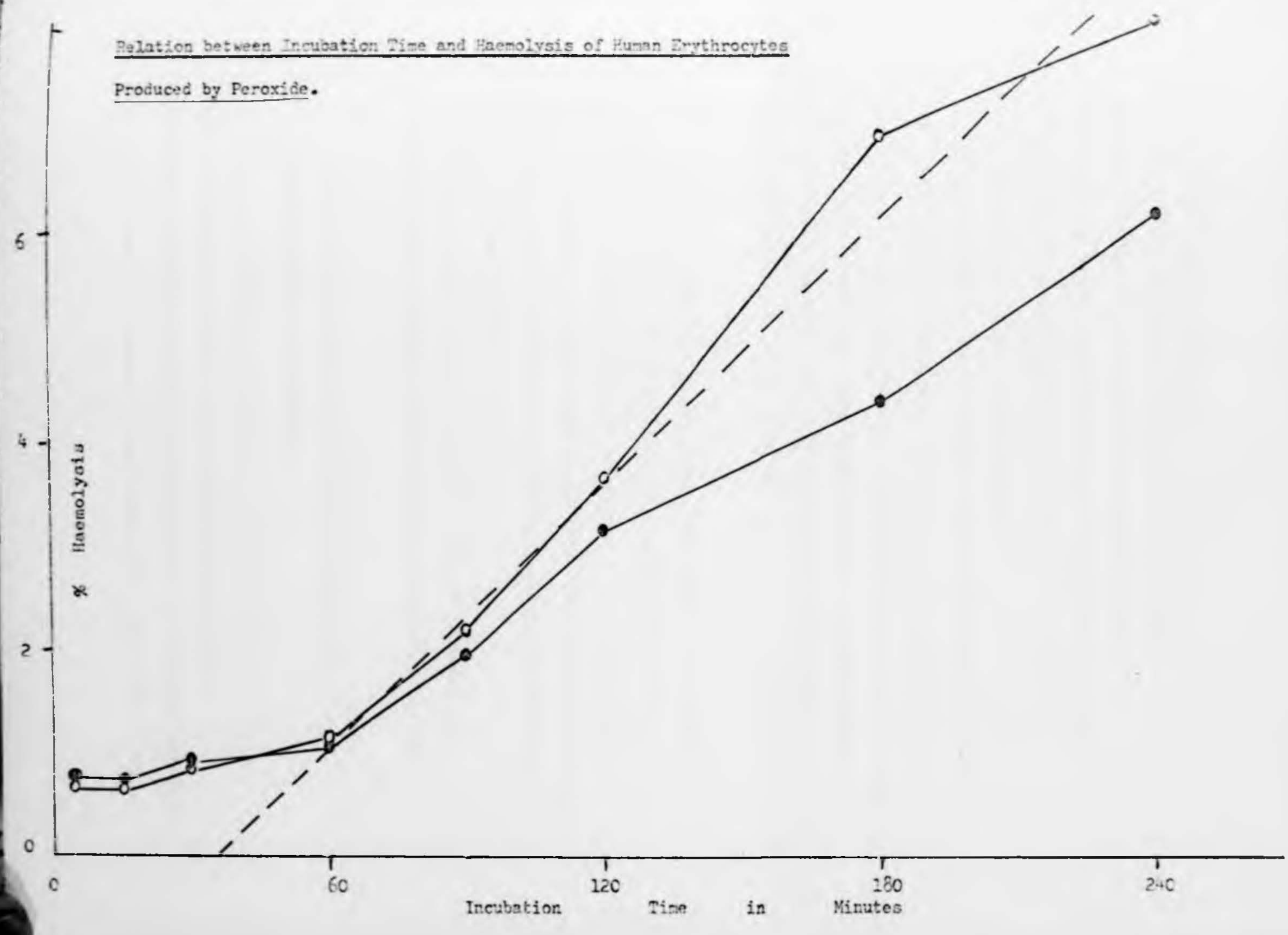

d- Shaking during incubation: Table 7 shows results obtained when the effect of shaking or not shaking was tested on the haemolytic action of the peroxide-generating system. No difference was found under a variety of conditions although more of the standard deviations were smaller in the shaken samples suggesting greater reproducibility.

It was decided to shake during incubation therefore, since this would keep the cells in suspension and the solution more homogenous. It was reasoned that future experiments might require more sensitive conditions than those necessary for experiments with cells from control rats, and shaking might give better reproducibility.

e- Storage and use of erythrocytes for assay: Walls and Hochstein (1974) stated that their experiments were done on human cells utilised immediately or stored at 4°C for periods up to three days. It was found with rat erythrocytes that cells older than 24 hours haemolysed on standing, and haemolysed more rapidly on exposure to peroxide.

All experiments therefore were done within 12 hours and most were set up to begin two hours after collection of blood.

2- In-vitro Susceptibility of Riboflavin-Deficient Erythrocytes to Haemolysis

One of the mechanisms present in the red cell to detoxify peroxide is the glutathione reductase/peroxidase system. Glutathione reductase requires riboflavin for its full activity therefore it was of interest to see whether red cells from ribo-

Table 7:

Effect of Shaking on the Susceptibility of Red Cells from Control, Pair-Fed and Riboflavin-Deficient Rats to Haemolysis in the Presence of Peroxidee-Generating System Containing 0.04 units Xanthine Oxidase in the Absence and Presence of Other Substrates

	Control		Pair-Fed		Riboflavin-Deficient	
	Shaking	No Shaking	Shaking	No Shaking	Shaking	No Shaking
H_2O_2-Generating system	38.19±7.76	37.19±4.66	41.21±3.09	41.20±2.17	73.21±8.56	76.186.44
H_2O_2-generating system + glucose	28.73±7.09	29.43±5.41	34.15±4.74	32.00±7.15	83.24±3.0	93.93±3.4
H_2O_2-generating system + 1.9×10^{-5}M-T_4	2.95±0.52	2.63±0.43	2.73±0.28	3.06±0.91	5.3±4.27	4.76±2.64
H_2O_2-generating system + 1.9×10^{-7}M-T_4	28.65±5.64	25.31±4.19	30.91±4.22	26.72±4.68	72.74±8.66	81.29±3.87
H_2O_2-generating system +18.5mM-FAD	37.76±4.13	35.82±4.16	38.11±2.92	34.86±3.50	61.45+6.58	58.42+5.60
EGR-AC	1.33±0.15		1.27±0.08		2.29±0.49	

Data show means(±SD) for results obtained from four animals for percentage haemolysis (see figure 9) produced using 0.04 unites xanthine oxidase alone or in the presence of glucose (0.01M-), thyroxine or FAD. Incubation temperature was 37°C and shaking was 70 excursions per minute.

flavin-deficient rats were more easily damaged by peroxides in-vitro. The experiments described below suggested that they were, and additional experiments in which the red cells were exposed to hypotonic saline were also done to see whether cells were equally sensitive to chemical and physical stresses.

a- Effect of peroxide-generating system on red cells from riboflavin-deficient rats

Erythrocytes from rats on control, pair-fed and riboflavin-deficient diets were exposed in-vitro to peroxide generated by 0.04, 0.02 and 0.008 units of xanthine oxidase.

The susceptibility of red cells to haemolysis as riboflavin status falls is shown in Figures 12, 13 and 14 in which the percentage haemolysis is plotted against riboflavin status (EGR-AC). Table.8 shows mean ± S D of the results of the individual groups of animals given in Figures 12, 13 and 14. A graded response to the increasing concentration of xanthine oxidase in all three groups of animals is shown in Table 8 as is also the increased susceptibility to haemolysis of red blood cells of riboflavin-deficient animals in the presence of both peroxide and hypotonicity.

The results in Table 9. showed that highly significant correlations were obtained between riboflavin status as measured by the activity coefficient (EGR-AC) and the degree of haemolysis produced by exposure to the H_2O_2-generating system irrespective of whether 0.008, 0.02 or 0.04 units of xanthine oxidase were used. Except for the controls, the correlations were statistically significant whether results for the groups were combined or examined alone.

Figure (12):

Relation between Riboflavin Status and Susceptibility to Haemolysis Of Red Cells in Presence of Peroxide-Generating System Containing 0.04 units Xanthine Oxidase

Figure 12:

Relation between riboflavin status and susceptibility to haemolysis or red cells in presence of peroxide-generating oxygen containing 0.04 units xanthine oxidase.

Red cells from control (■) pair-fed (○) and riboflavin-deficient rats (●) were incubated in the peroxide-generating system described in Fig 10 except for the use of 0.04 xanthine oxidase units. Riboflavin status was measured using the glutathione reductase assay. All points represent the mean duplicate assays.

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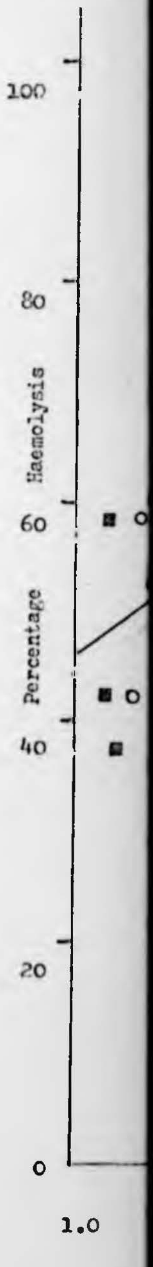

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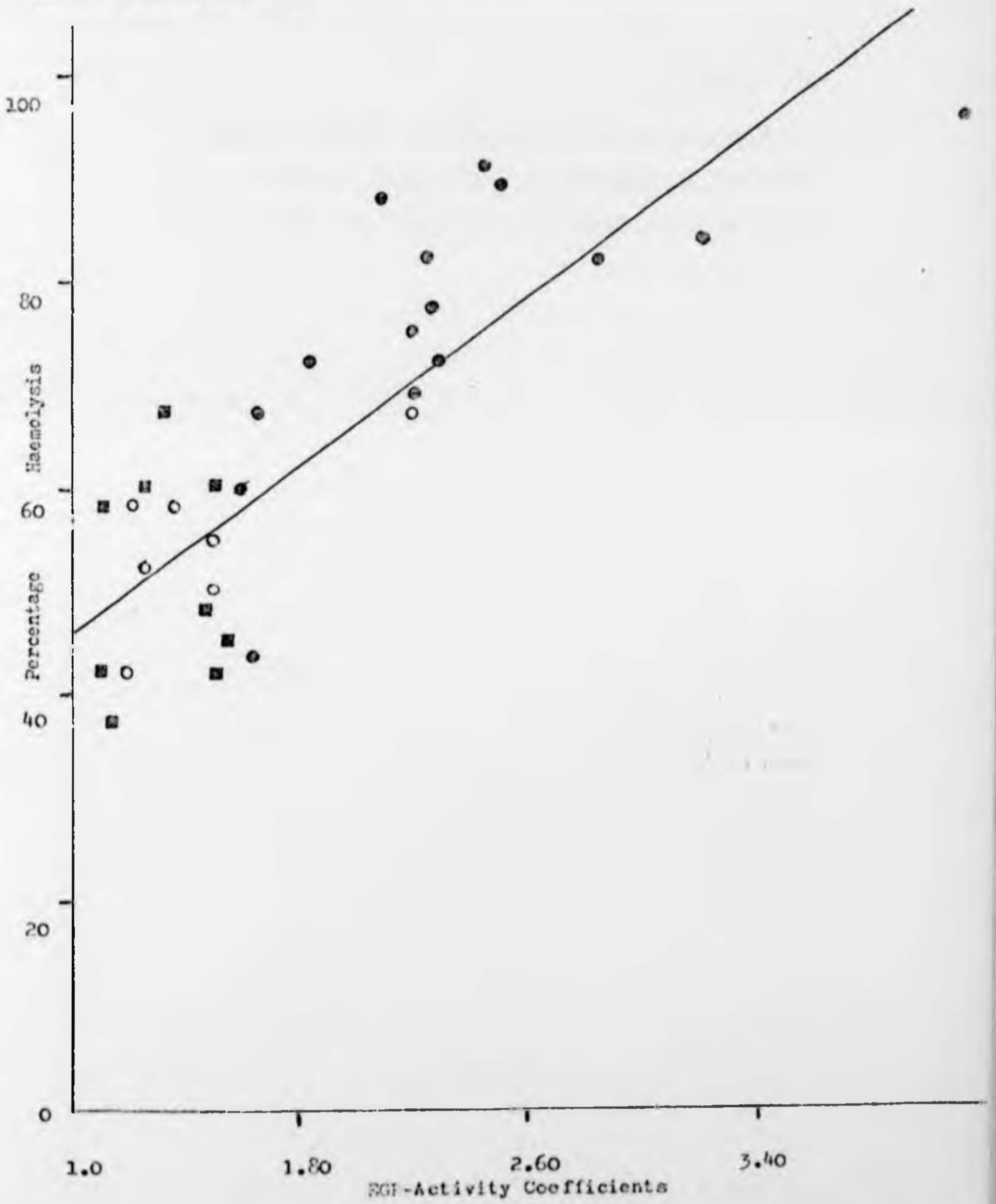


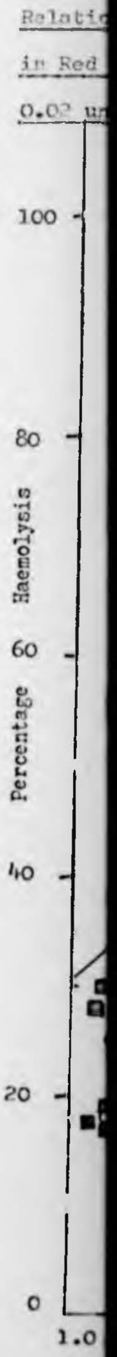
Figure (13):

Relation between Riboflavin Status and susceptibility to Haemolysis in Red Cells in Presence of Peroxide-Generating System Containing 0.02 Units Xanthine Oxidase

Figure 13:

Relation between riboflavin status and susceptibility to haemolysis in red cells in presence of peroxide-generating system containing 0.02 units xanthine oxidase

Red cells from control (■), pair-fed (○) and riboflavin-deficient rats (◐) were incubated in the peroxide-generating system described in Figure 10. Riboflavin status was measured using EGR-AC assay. Each point represents one blood sample on which duplicate assays were done.



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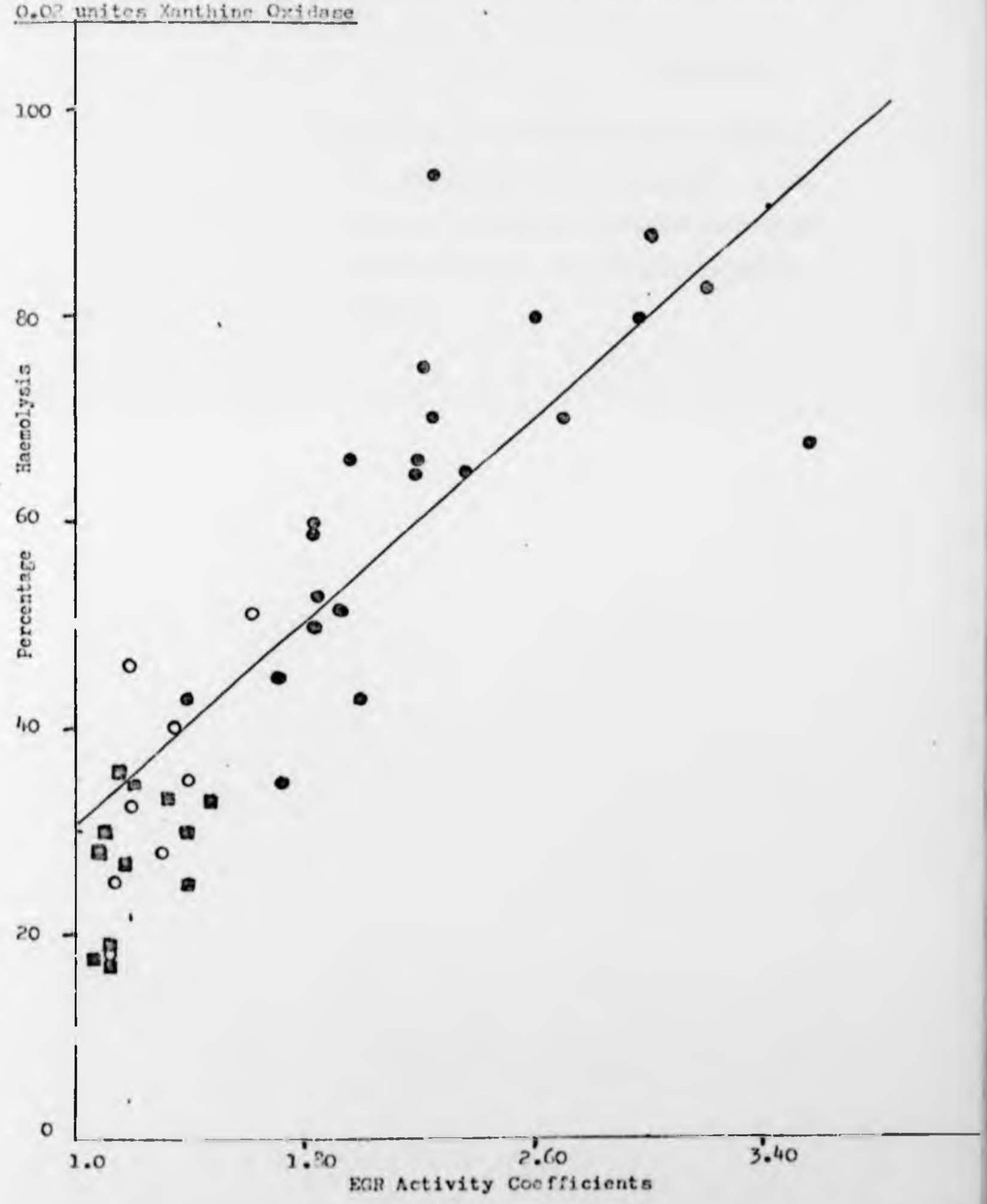

Figure (14):

Relationship between Riboflavin Status and Susceptibility to Haemolysis in Red Cells in Presence of Peroxide-Generating System Containing 0.008 Units Xanthine Oxidase

Figure 14:

Relationship between riboflavin status and susceptibility to haemolysis in red cells in presence of peroxide-generating system containing 0.008 xanthine oxidase

Red blood cells from control (■) pair fed (○), and riboflavin deficient rats (●) were incubated in the peroxide generating system described in Figure 10 except for the use of 0.008 units xanthine oxidase.

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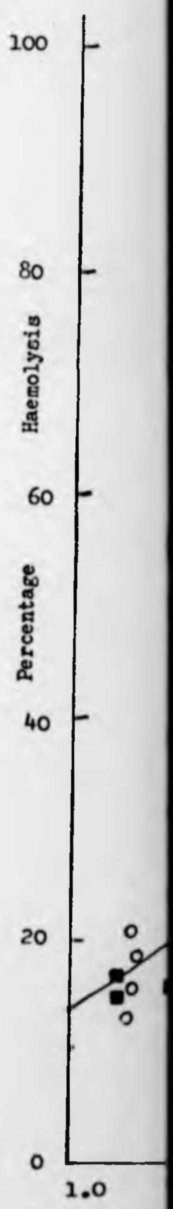

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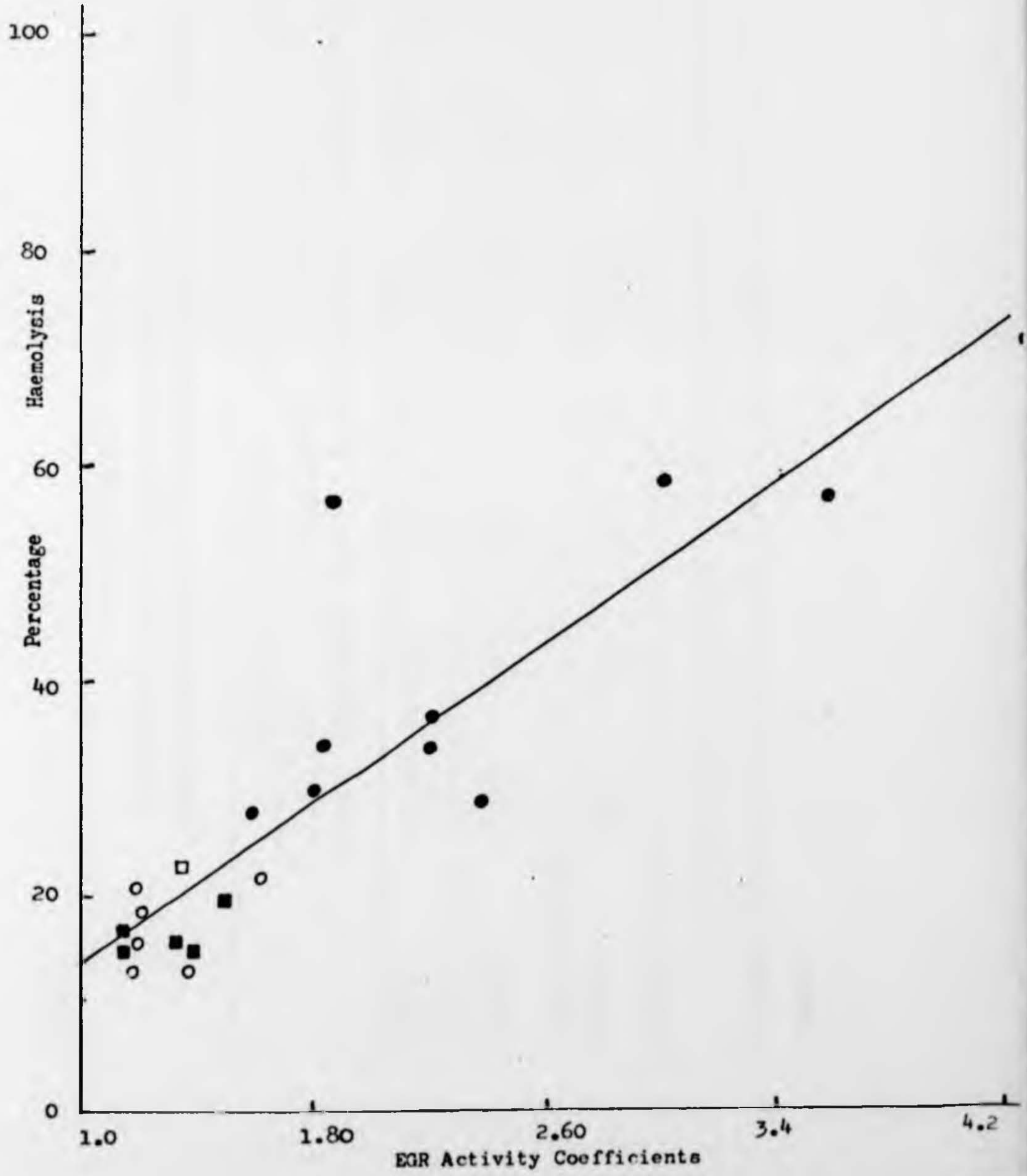

Table 8:

Mean and Standard Deviation ef EGR-AC of Percentage Haemolysis of Red Cells from Control, Pair-Fed and Riboflavin-Deficient Rats in the Different Assays of Red Cell Fragility

	Concentration of Xanthine Oxidase						0.36% Saline Solution	
	0.04 units		0.02 units		0.008 units			
	EGR-AC	%Haemolysis	EGR-AC	%Haemolysis	EGR-AC	%Haemolysis	EGR-AC	%Haemolysis
Control	1.31±0.18 (10)	49.99±10.12 (10)	1.27±0.17 (12)	24.05±6.54 (12)	1.31±0.14 (6)	13.43±3.13 (6)	1.29±0.21 (11)	15.06±6.85 (11)
Pair-fed	1.45±0.38 (6)	53.45±8.30 (6)	1.36±0.24 (9)	32.32±13.03 (9)	1.30±0.18 (6)	13.83±4.80 (6)	1.34±0.31 (12)	14.92±15.30 (12)
Deficient	2.35±0.69 (15)	74.60±13.21 (15)	2.37±).68 (22)	65.36±13.29 (22)	2.47±).87 (10)	38.45±15.80 (10)	2.28±0.71 (19)	58.04±18.13 (19)

All measurements for EGR-ACs and percentage haemolysis were done in duplicate on the same sample. The number of animals used in the different assays is shown in parentheses. For other details see legend to figure 11.

Table 9:

Correlation Coefficients (r) for Percentage Haemolysis Against EGR-Activity Coefficients

Experimental conditions	Controls			Pair-Feds			Deficients			Total		
	n	r	P<	n	r	P<	n	r	P<	n	r	P<
0.04 xanthine oxidase units	10	0.037	N.S	6	0.73	0.05	15	0.71	0.01	31	0.81	0.001
0.02 xanthine oxidase units	12	0.78	0·01	9	0.84	0.01	22	0.63	0.01	43	0.84	0.001
0.008 xanthine oxidase units	6	0.77	0·05	6	0.81	0.05	9	0.79	0.01	22	0.90	0.001
Osmotic fragility (0.36% saline)	11	0.59	0.05	12	0.93	0.001	19	0.70	0.001	42	0.85	0.001

The table summarises the information shown in figures 12, 13, and 14 and 17. Red blood cells from the experimental animals were prepared as described in the methods and exposed to the peroxide generating system or hypotonic saline. The resultant haemolysis was measured and expressed as a percentage of a totally haemolysed comparable sample. Glutathione reductase activity coefficients were also measured on each sample. and the two indices were correlated against one another. n = the number of animals, and P = the probability.

The assay using 0.008 units xanthine oxidase generated peroxide at the lowest rate and caused least haemolysis but correlation coefficient with the EGR-AC was marginally higher in experiments using this concentration.

b- Substances modifying the action of peroxide-generating system on red cell haemolysis

i- Thyroxine: Walls and Hochstein (1974) reported that thyroxine considerably increased haemolysis of human red cells in the presence of the peroxide-generating system. The action of thyroxine on rat red cells however, was completely the opposite. Table 7 shows the effect of the presence and absence of thyroxine (1.9×10^{-5}M-thyroxine and 1.9×10^{-7}M-thyroxine) on red cells from control, pair-fed and deficient rats when exposed to the peroxide-generating system. The concentration of thyroxine which prevented haemolysis completely was 1.9×10^{-5}M-thyroxine, smaller quantities inhibited to a lesser extent.

The influence of thyroxine on the in-vitro generation of H_2O_2 is shown in Table 10. The concentration of T_4 (1.9×10^{-5}M) which completely prevented haemolysis of rat erythrocytes was found to inhibit production of peroxide between 30 and 50% when incubated with 0.01 to 0.04 units of xanthine oxidase. The result obtained when this quantity of thyroxine was incubated with 0.008 units of xanthine oxidase was unexpected in that hydrogen peroxide production was increased by approximately 80%. When the lower concentration of thyroxine (1.9×10^{-7}M) was added to the peroxide-generating system

Table 10:

Amount of H_2O_2 in umols/Incubated Medium Produced by the H_2O_2-Generating System after 45 Minutes Incubation in Absence and Presence of Thyroxine

	Xanthine oxidase concentration			
	0.04 units	0.02 units	0.01 units	0.008 units
H_2O_2-generating system alone	587	218	62.5	41
H_2O_2-generating system + 1.9×10^{-5}M-T_4	303.5 * 48%	147.5 * 32%	32.5 * 48%	73.5 ** 79%
H_2O_2-generating system + 1.9×10^{-7}M-T_4	327 * 44%	201 * 8%	47 * 25%	30.8 * 25%

Data presented are the mean of duplicate assays in umols of H_2O_2 generated using different concentrations of xanthine oxidase.

* Data indicated percentage of reduction in the H_2O_2 produced levels in presence of thyroxine.

** Percentage increase in the H_2O_2 levels produced.

peroxide production was lowered at all concentrations of xanthine oxidase. The explanation is not known for the single pair of results which suggested that thyroxine stimulated peroxide production and it may have been an error. All the other results suggested that thyroxine inhibited peroxide production by xanthine oxidase and this was in agreement with the protective effect of thyroxine on red cell haemolysis.

It is also possible that thyroxine may have a direct protective effect itself on the red cell membrane in preventing haemolysis. The experiment shown in Table 11 illustrates the apparent protection by thyroxine of red cells from haemolysing in hypotonic saline (0.36g/100ml). This experiment was only done in 4 rats but two of these were riboflavin-deficient and a very similar effect was seen in both. This effect of thyroxine needs further investigation since thyroxine given <u>in vivo</u> appeared also to exert a small protective effect against osmotic lysis <u>in vitro</u> (Table 24;EIII,1 .)

ii-<u>Glucose</u>: Walls and Hochstein (1974) demonstrated the protective effect of 0.1M-glucose on the lysis of erythrocytes from normal and G6PD-deficient human subjects. Glucose in the primary metabolite which is catalysed in the hexose monophosphate shunt to provide the energy for the glutathione reductase/peroxidase and catalase systems (Murphy, 1960). It was considered of interest to investigate what influences glucose might have on the susceptibility of rat erythrocytes to haemolysis in the H_2O_2-generating system. The results in Table 7 show that the degree of haemolysis of erythrocytes from control animals was reduced by approximately 22% in both control and pair-fed amounts by 0.1M-glucose while eryth-

Table 11:

Effect of Thyroxine on the Susceptibility of Red Cells from Riboflavin-Deficient Rats to Osmotic Lysis

	% Haemolysis in 0.36% Saline		
Additions	0.36% saline only	+ 1.9×10^{-5}M-T_4	+ 1.9×10^{-7}M-T_4
Control (1)	17.15	5.15	13.19
Pair-fed (1)	19.36	6.76	11.47
Riboflavin deficients (2) Individual values	78.25 75.96	21.66 25.73	21.66 49.56

The data represent the results obtained from two experiments done separately. The first was done on erythrocytes from control (1), Pair-fed (1) and riboflavin-deficient (1) rats while in the second, one riboflavin deficient rat only was used. Percentage haemolysis was measured as described in the methods.

rocytes from riboflavin-deficient rats were slightly more haemolysed by the presence of glucose.

iii- Flavin adenine dinucleotide: The concentration of riboflavin in erythrocytes from riboflavin-deficient rats is low and it was wondered whether the addition of riboflavin or FAD to the incubated red cell suspensions would increase glutathione reductase activity, raise concentrations of GSH and protect against peroxide-induced haemolysis.

Absorption of riboflavin by red cells is poor (Mandela and Beutler, 1970) therefore only FAD was used. Results shown in Table 7 suggest that the degree of haemolysis was partly reduced by the presence of FAD, but, this reduction was only significant ($P<0.01$) in erythrocytes from all deficient animals.

c- Osmotic fragility: In view of the differing sensitivities shown by results from control and riboflavin-deficient rats to peroxide, the ability to withstand hypotonic saline was also measured.

Fig.15 shows the degree of haemolysis caused by 0.9, 0.72 and 0.36%(w/v) sodium chloride solution when incubated with red cells from control, pair-fed and riboflavin-deficient rats. The red cells from the riboflavin-deficient rats were more sensitive to haemolysis at all the concentrations tested below normal but the difference was considerably greater at 0.36%.

The lowest concentration of saline, 0.36%, was used more frequently therefore to measure erythrocyte fragility. Figure 16 shows the association between riboflavin status and the degree of haemolysis caused by this concentration of saline. The results show a steep rise in the fragility of red cells from riboflavin-deficient rats up to a plateau when about 80% of the red cells haemolyse at EGR-AC $\geqslant 2.40$.

Figure (15):

Effect of Varying Saline Concentrations on Red Cells from Control, Pair-Fed and Riboflavin-Deficient Rats.

Figure 15:

Effect of varying saline concentrations on red cells from control, pair-fed and riboflavin-deficient rats

Red cells from control (■), pair-fed (○) and riboflavin-deficient rats (●) incubated as a 1% haematocrit in 3ml normal or hypotonic saline at 37°C for 45 min. in a shaking water-bath (70 r.p.m.). After centrifuging the contents, haemoglobin in the supernatent was measured as a percentage of total haemoglobin following 100% haemolysis.

Each point represents one blood sample from one animal on which duplicate assays were done.

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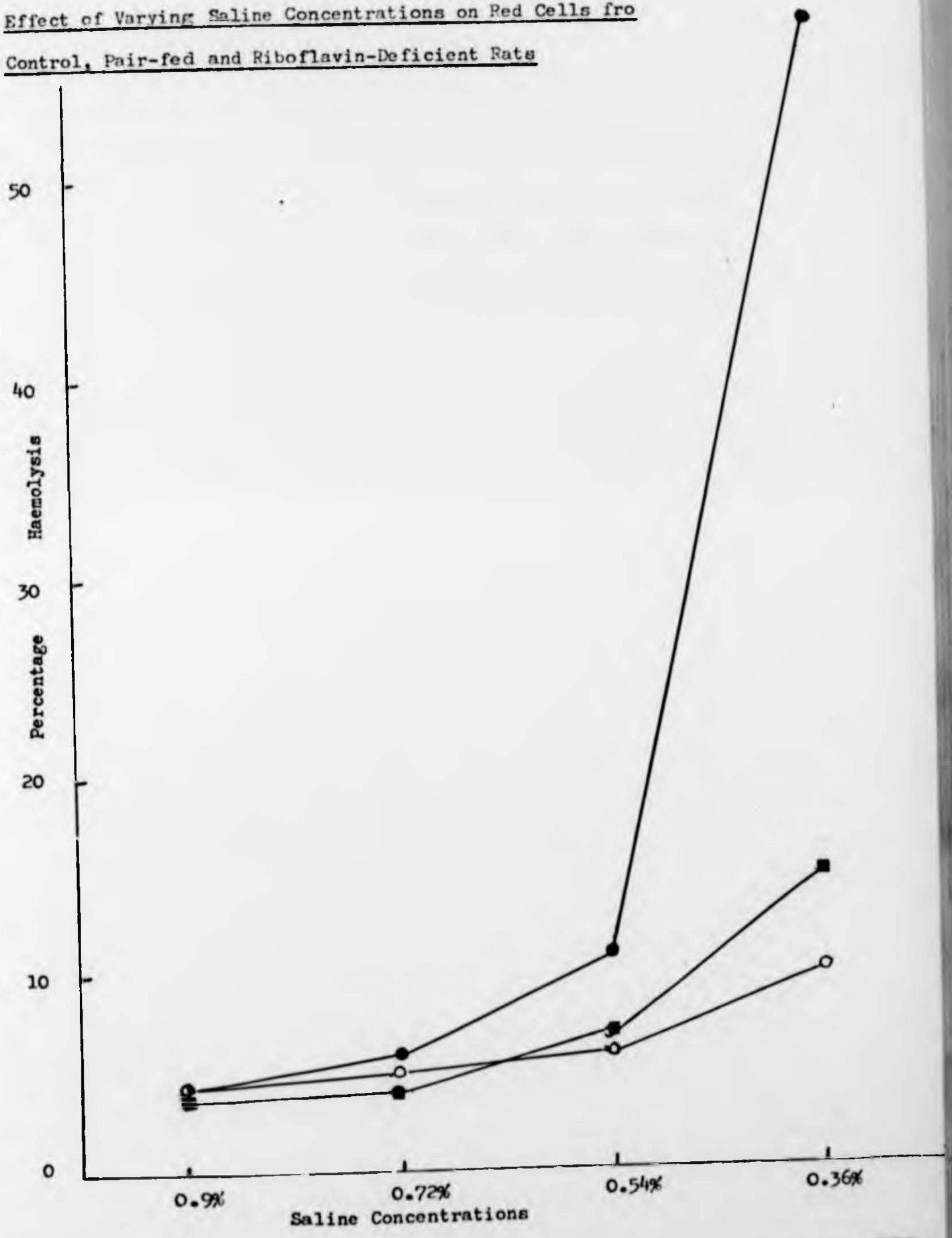

Figure (16):

Effect of 0.36% Saline on Red cells from Control, Pair-Fed and Riboflavin-Deficient rats.

Figure 16:

Effect of 0.36% saline solution on red cells from control pair-fed and riboflavin-deficient rats.

Red cells from contro (■), pair-fed (O) and riboflavin-deficient rats (●) incubated in 0.36% saline solution as previously described in Figure 15. Percentage haemolysis was measured as described in Figure 10.
Each point in the figure represents one blood sample on which duplicate assays were done.

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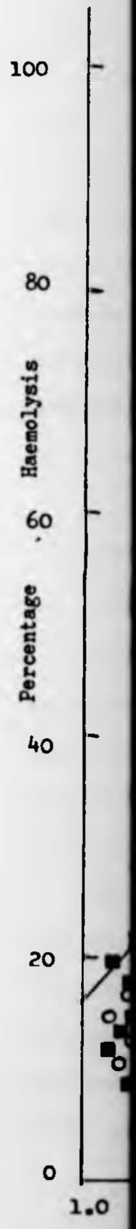

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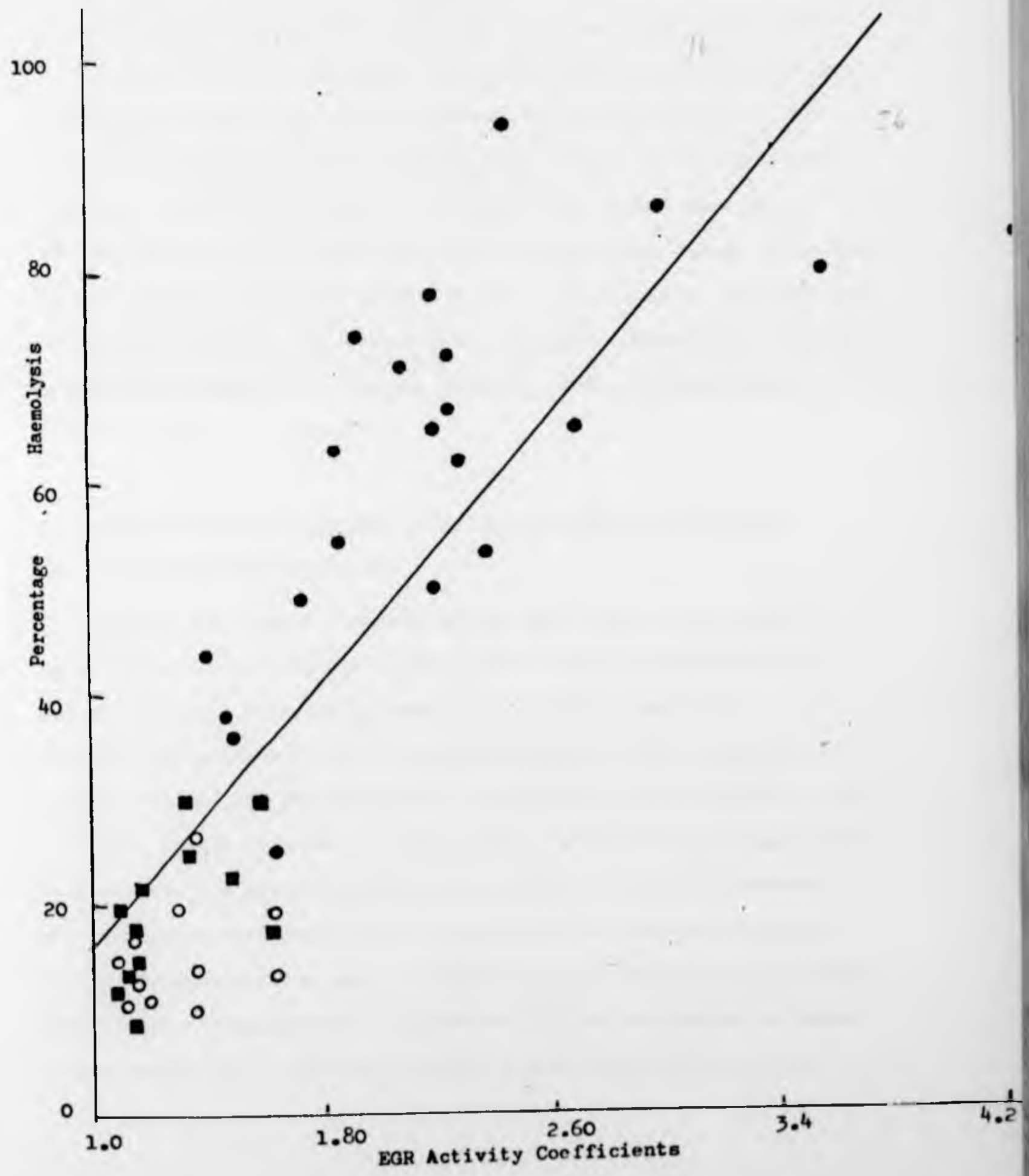
Effect of 0.36% Saline on Red Cells from Control, Pair-Fed and Riboflavin-Deficient Rats



The mean (± SD, n) figures for the percentage haemolysis occurring when red cells of the control, pair-fed and riboflavin-deficient groups of rats were incubated in 0.36% saline were, 15.06 (± 6.85,11), 14.92 (15.30, 12) and 58.04 (± 18.13, 19) respectively (Table 8).

Figure 17 shows regression lines for the data obtained for the four sets of conditions used to measure red cell fragility. Regression lines for percentage haemolysis produced by the peroxide-generating systems lie parallel with one another and show the increasing haemolysis resulting from the increasing amount of xanthine oxidase present in the incubation media. The slope of the line for percentage haemolysis caused by the 0.36% saline however, is steeper, suggesting possibly that the two types of system are measuring different types of fragility.

3- Concentrations of Reduced Glutathione (GSH) in Red Cells in Riboflavin-Deficiency:

Reduced glutathione is produced by the enzyme glutathione reductase. The results obtained showed that GSH concentrations decreased as the EGR-AC increased and a highly significant, negative correlation (Fig.18) was obtained when the concentration of red cell GSH was plotted against the EGR-AC ($r=0.80, P<0.001, n=64$).

The results obtained from the riboflavin-deficient animals were divided into two groups on the basis of their clinical appearance. The riboflavin-deficient group in which obvious clinical signs of deficiency were present and a 'recovered group' in which the clinical symptoms were disappearing. The means ± SD of the EGR-AC of these groups were 2.28 ± 0.48 (22) and 1.59 ± 0.20 (16) and the concen-

Figure (17):

Correlation between EGR-AC and Susceptibility of Red Cells when Exposed to Different Concentrations of Xanthine Oxidase and NaCl.

Figure 17:

Correlation between EGR-AC and susceptibility of red blood cells to haemolysis when exposed to different concentrations of xanthine oxidase and NaCl

The Figure shows the regression lines of the regression equations obtained from the data plotted in Figures 12, 13, 14 and 16. The regression equations were obtained when % of haemolysis of red cells from control, pair fed and riboflavin deficient rats were correlated with the respective EGR-AC ratios. % of haemolysis was measured as described in Figure 10.

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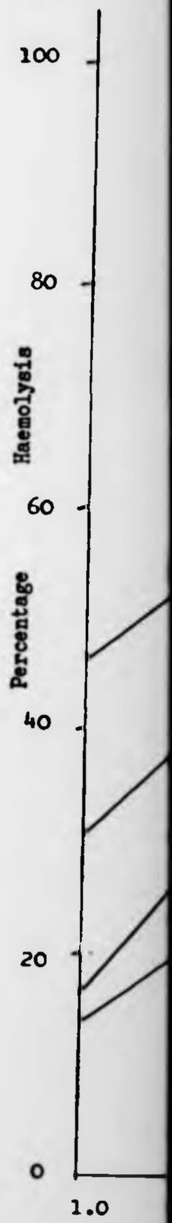

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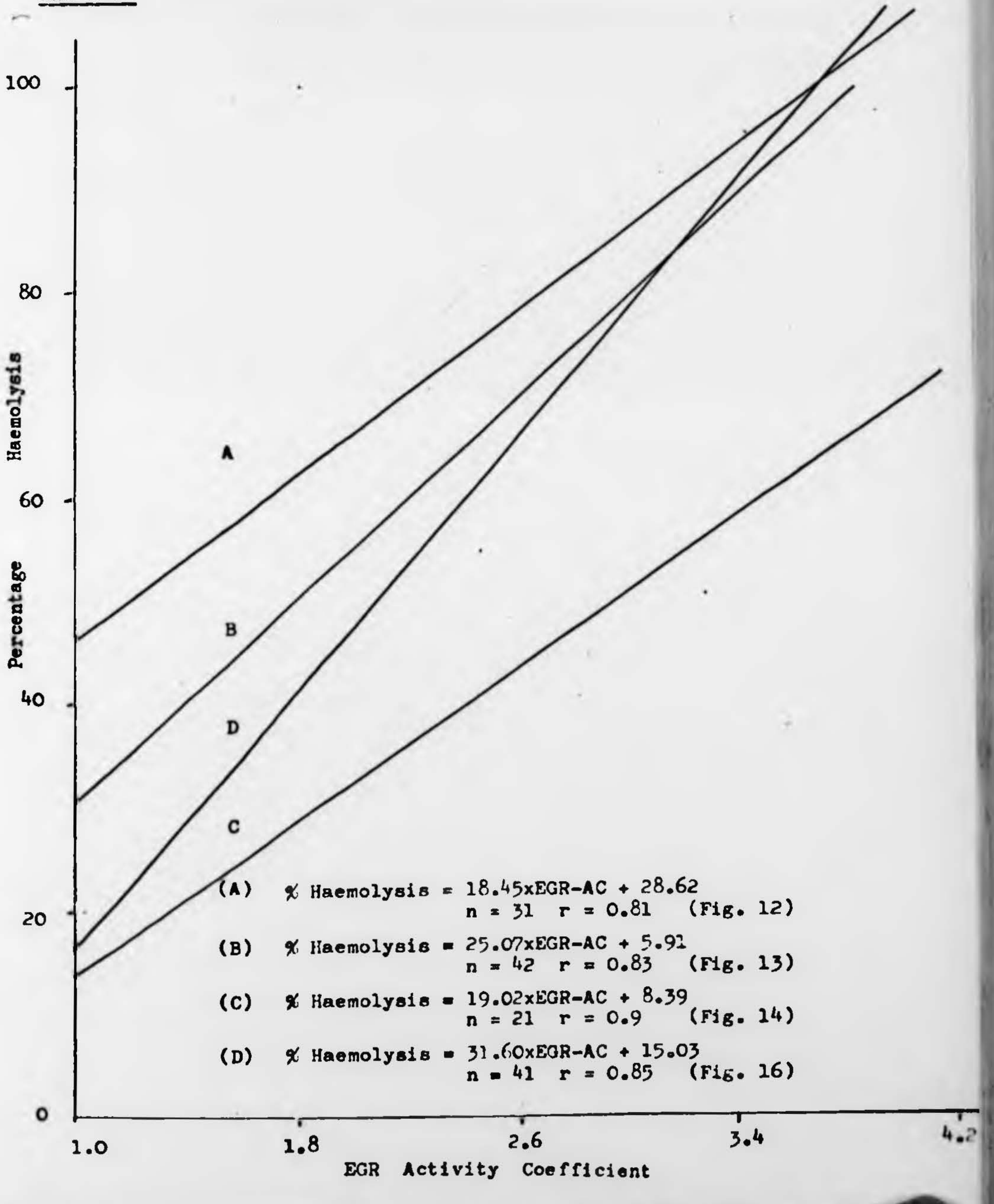


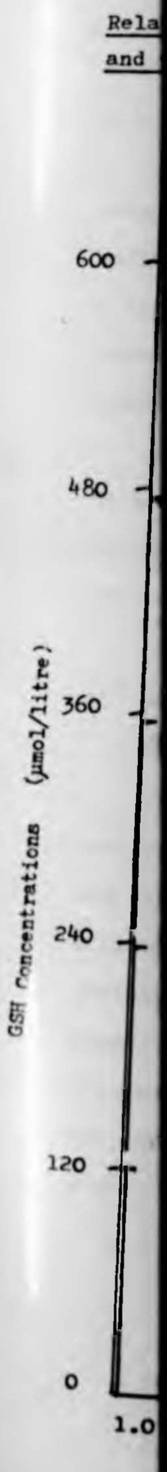
Figure (18):

Relationship between EGR-Activity Coefficients and Whole Blood Concentrations of Reduced Glutathione (GSH)

Figure 18:

Relationship between EGR-Activity coefficients and Whole blood concentrations of reduced glutathione (GSH).

GSH was measured fluorometrically (see Methods) on Trichloracetic acid extracts reacted with OPT reagents. Measurements of EGR-AC is described elsewhere. Points represent results obtained from blood samples from control (■), pair-fed (○) and riboflavin-deficient rats (●). Each point represents one sample on which duplicate assays were done. Points on the transparent overlay represent results fromriboflavin-deficient rats showed clinical evidence of recovery at the time of killing.



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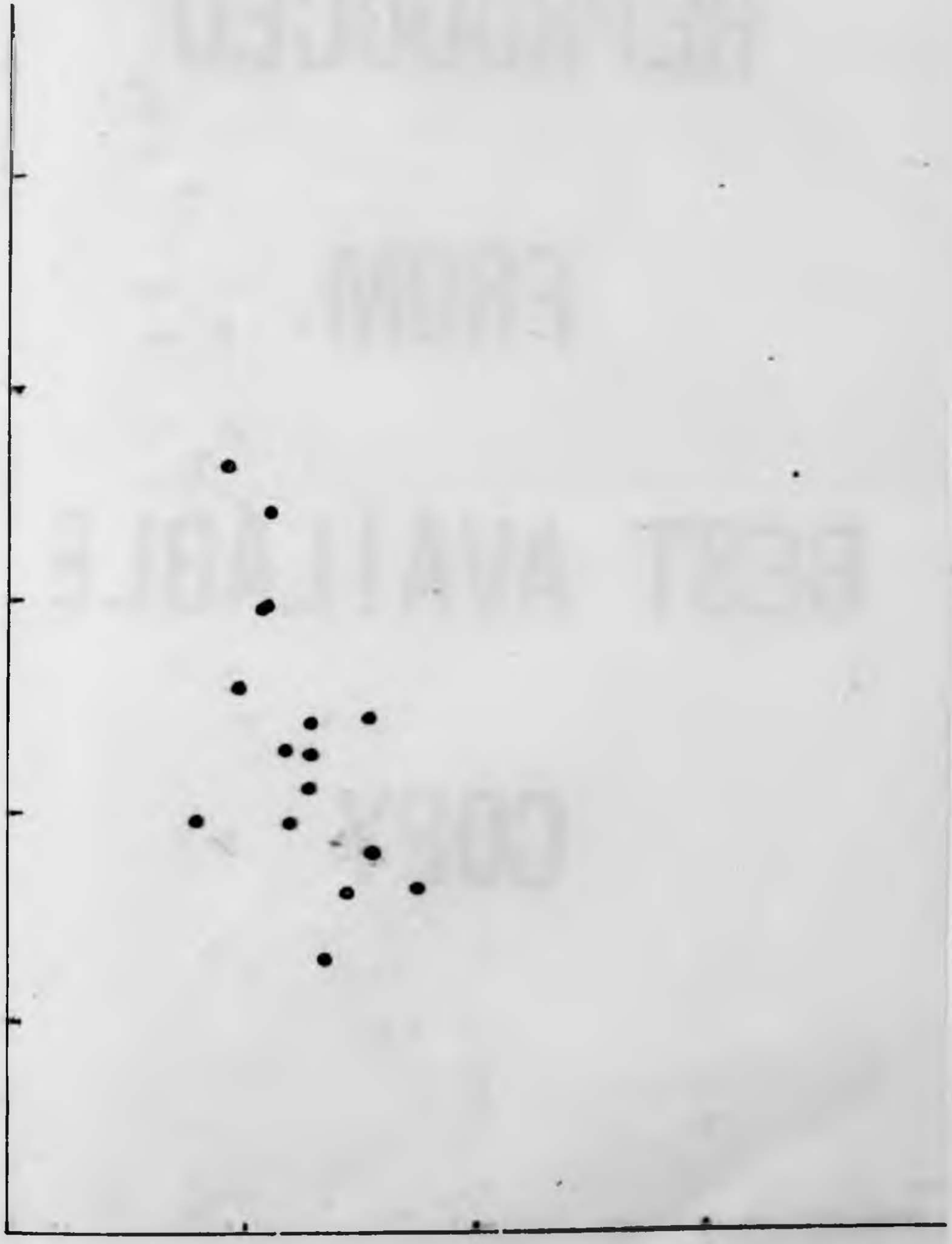

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Relationship between EGR-Activity Coefficients
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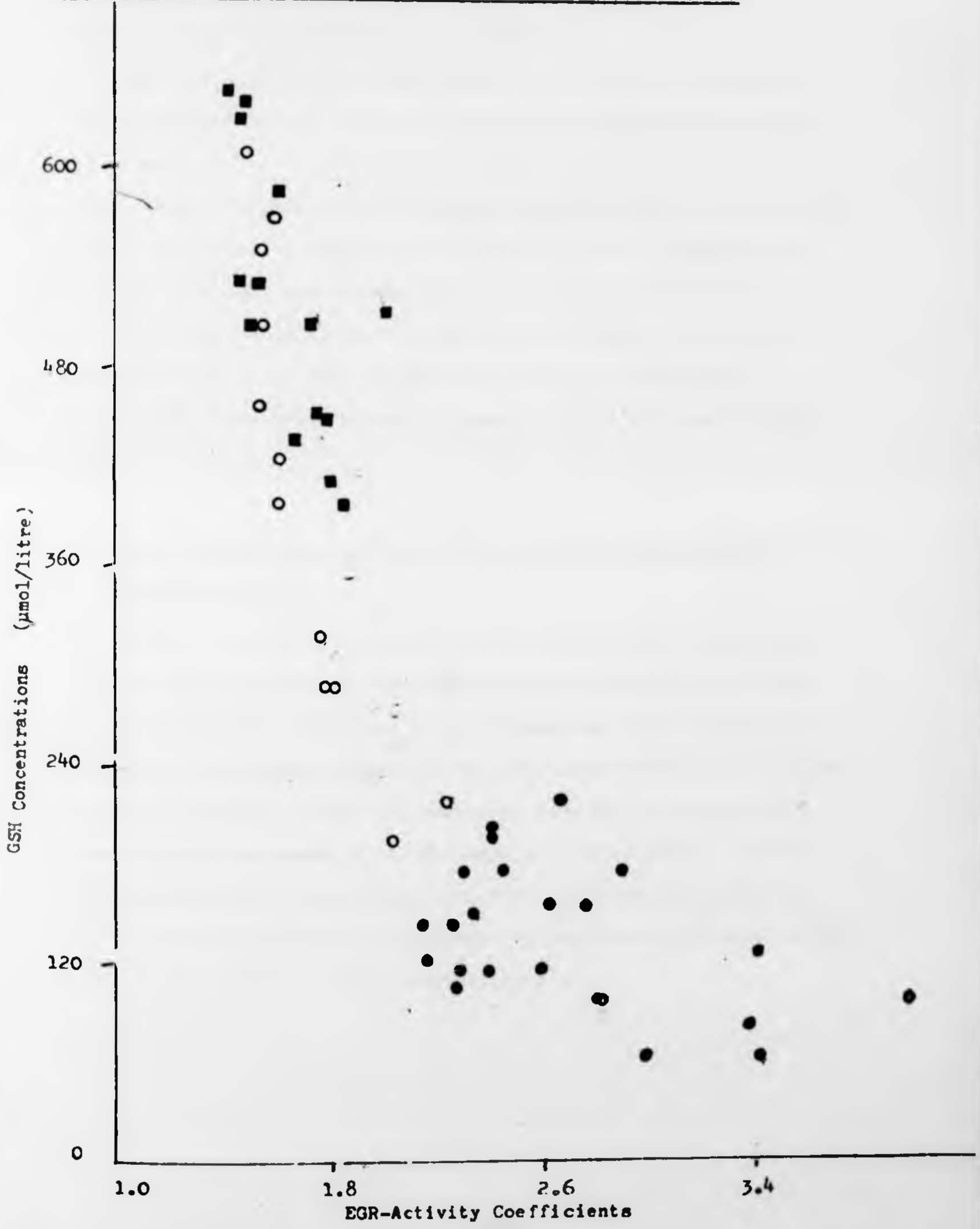

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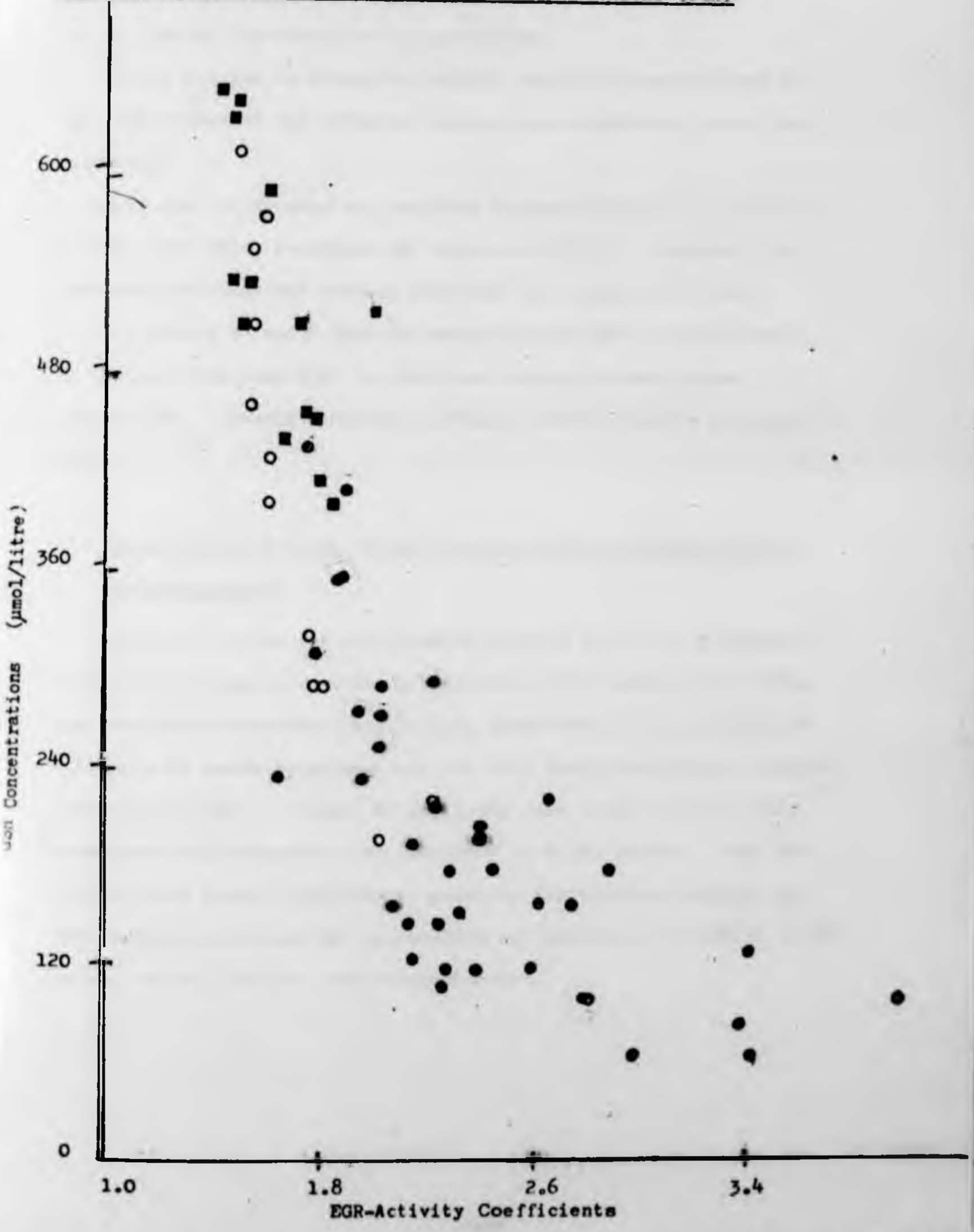

trations of reduced glutathione were 109.76 ± 35.03 (22) and 234.38 ± 65.63 (16)μmol/litre respectively.

Using t-tests to determine whether the difference between the mean GSH values of the different groups was significant showed the following:

A significant difference was obtained between deficient and pair-fed animals ($P<0.001$), recovered and pair-fed ($P<0.01$), deficient and controls ($P<0.001$) and between pair-fed and controls ($P<0.02$).

It should be noted that concentrations of GSH in whole blood of the pair-fed rats fell to quite low amounts in some cases (figure 18). Possible reason for these results will be discussed later.

IV- Relationship between "Fragility" of Red Blood Cells and GSH Concentrations:

Figure 19 shows the relationship between degree of haemolysis obtained from exposure of the erythrocytes from control, pair-fed, and riboflavin-deficient rats to H_2O_2 generated in the presence of 0.02units of xanthine oxidase and red cell concentrations of reduced glutathione (GSH). Figure 20 shows the same thing only red cell haemolysis was measured in the presence of 0.36% saline. The two figures show highly significant, negative correlations between the GSH concentrations and the measurement of haemolysis ($r=0.83$, P 0.001, $n=25$; $r=0.86$, $P<0.001$, $n=16$ respectively).

Figure (19):

Relationship between Erythrocyte Peroxide-Induced Fragility and Reduced Glutathione (GSH) Levels

Figure 19:

Relationship between erythrocyte peroxide-induced-fragility and reduced glutathione GSH levels

Erythrocyte peroxide-induced fragility was measured in red cells from Control (■), Pair-Fed (○) and (●) Riboflavin-deficient rats as described in Figure 13. GSH concentrations in whole blood was measured as described in Figure. 18.

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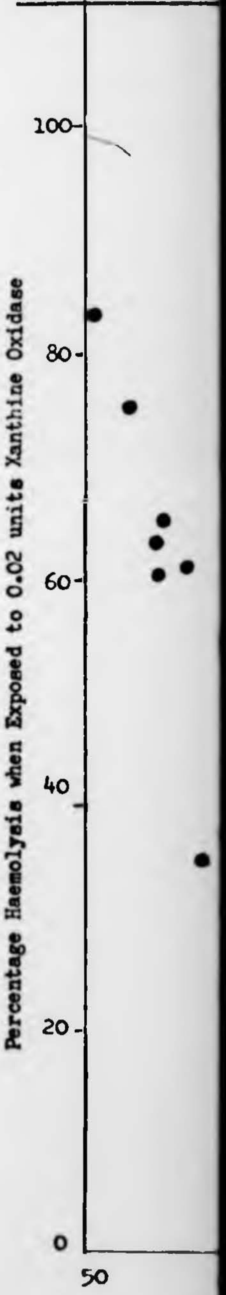

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Relationship between Erythrocyte Osmotic Fragility

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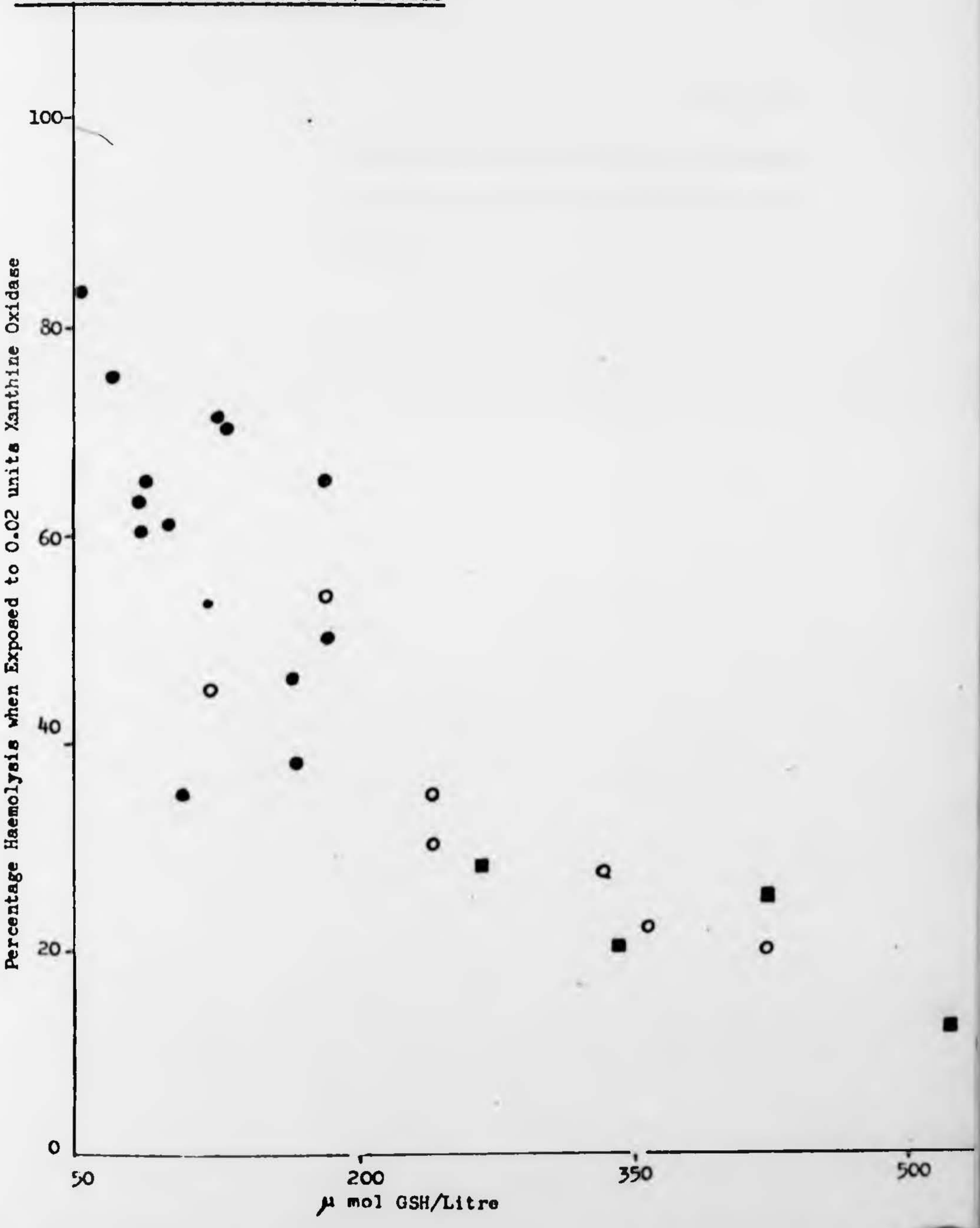

Figure (20):

Relationship between Erythrocyte Osmotic Fragility and Reduced Glutathione (GSH) Levels

Figure 20:

Relationship between erythrocyte osmotic fragility fragility and reduced glutathione (GSH) levels

Osmotic fragility of erythrocytes from Control (■), pair-fed (○) and riboflavin-deficient (●) were measured as % of haemolysis obtained when these cells were incubated in hypotonic solution Figure (16) GSH was measured in whole blood as described in Figure (18).

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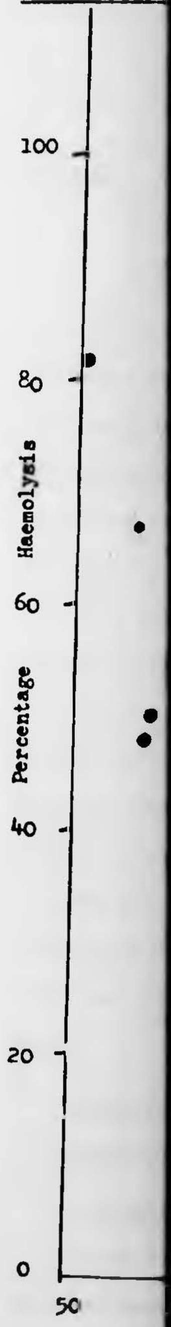

Figure 20:

Relationship between erythrocyte osmotic fragility fragility and reduced glutathione (GSH) levels

Osmotic fragility of erythrocytes from Control (■), pair-fed (○) and riboflavin-deficient (●) were measured as % of haemolysis obtained when these cells were incubated in hypotonic solution Figure (16) GSH was measured in whole blood as described in Figure (18).

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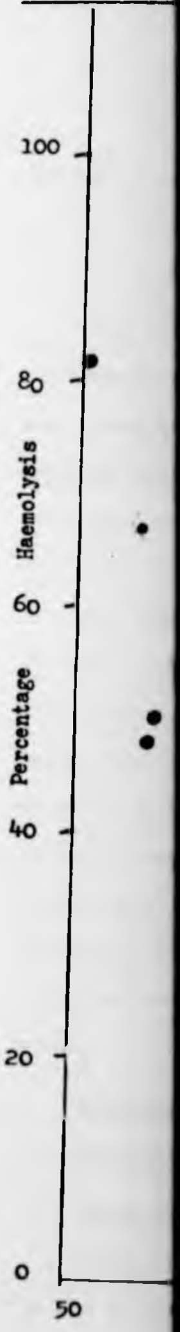

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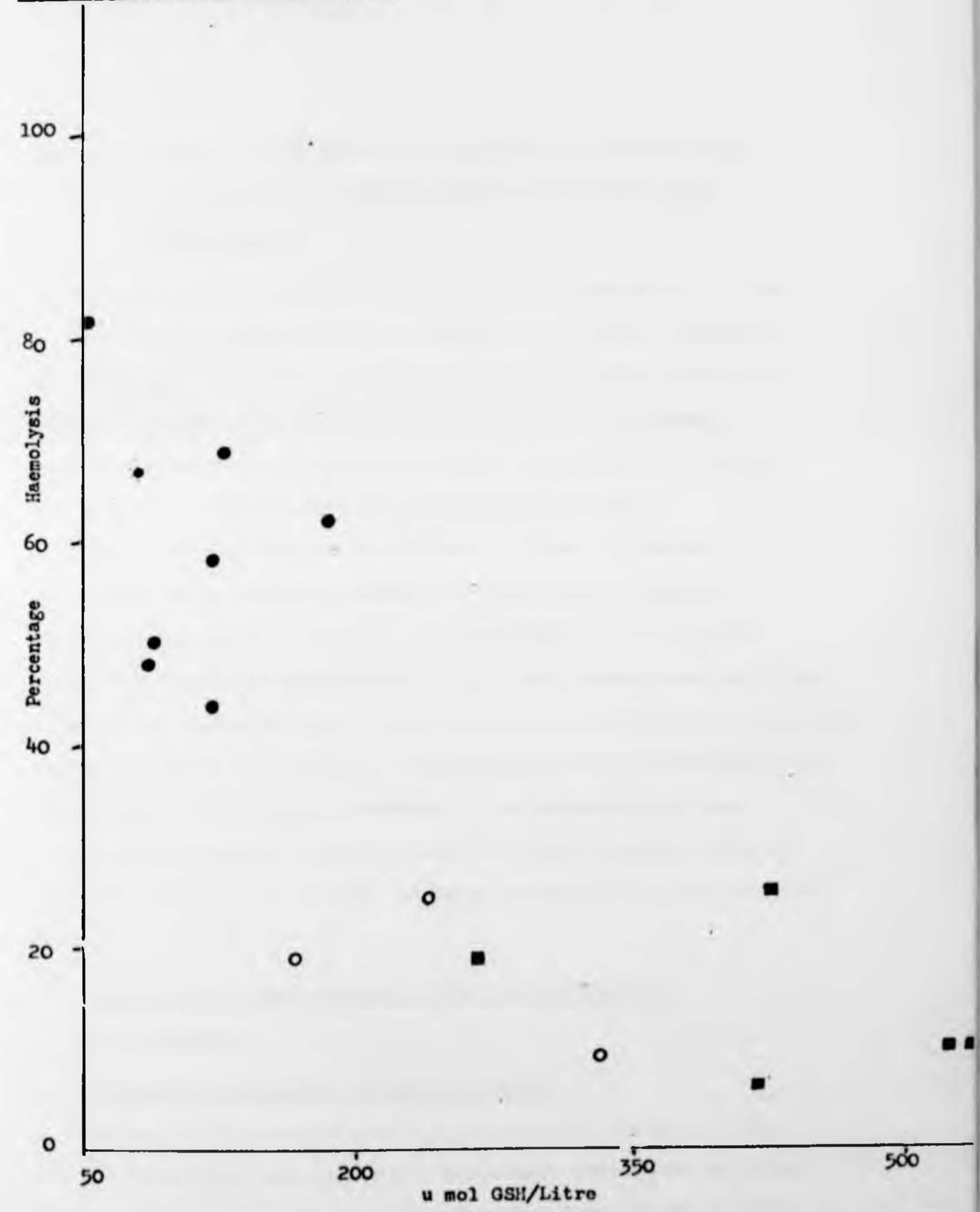
Relationship between Erythrocyte Osmotic Fragility and Reduced Glutathione (GSH) Levels



Part C: EFFECT OF RIBOFLAVIN DEFICIENCY ON THE FUNCTIONING OF GLUTATHIONE REDUCTASE/PEROXIDASE SYSTEM WITHIN ERYTHROCYTES

The results in Part (B) showed that the riboflavin deficient erythrocytes were susceptible to haemolysis and this haemolysis was found to be inversely correlated with the concentrations of reduced glutathione (GSH) in the erythrocytes. The results suggested that the reduction in the GSH concentration may reduce the ability of the red cell to maintain its stability.

The important role of glutathione in the reduced state in the red cells is achieved through the glutathione reductase-glutathione peroxidase system which functions as a detoxifier mainly for lipid hydroperoxides. Since both glutathione reductase activity and concentrations of GSH are lowered in riboflavin-deficient erythrocytes, it was decided to investigate what other pathological changes may be occurring in metabolites or enzyme activities indirectly or directly involved with the detoxification system in red blood cells. The results of these investigations are reported below.

I. Toxic Metabolites Produced in Riboflavin-Deficient Erythrocytes

1 - Endogenous formation of hydroperoxides

Cohen (1975) reported that H_2O_2 is normally produced within the red blood cell and glutathione peroxidase represents the first defence against its toxicity. Erythrocytes from G-6-PD subjects undergo oxidative damage and haemolysis either in-vivo when exposed to a number of drugs (Beutler, 1966; Hochstein, 1971) or in-vitro when exposed to a small amount of H_2O_2 (Walls and Hochstein, 1974). The sensitivity to H_2O_2 derives primarily from the disappearance of GSH. As the GSH concentrations fall, so does the activity of glutathione peroxidase, the enzyme which is primarily responsible for scavenging low levels of H_2O_2 (Cohen and Hochstein, 1963; Nicholls, 1972). Polyunsaturated fatty acids are particularly sensitive to peroxidation and an increase in concentrations of tissue peroxides leads to the formation of lipid hydroperoxides which in turn can give rise in-vitro to malonyldialdehyde.

In the following experiment erythrocytes from control, pair-fed and riboflavin-deficient animals were analysed for the in-vitro formation of malonaldehyde and the endogenous formation of total peroxides.

The results (Figures 21 and 22) show that both the concentration of red cell malonyldialdehyde and peroxides are directly correlated with the EGR-AC (i.e. riboflavin status). The correlation coefficients, significance and number of samples were r=0.83,P<0.001, n=43 and r=0.91, P<0.001 n=35 respectively. Mean and standard deviations of concentrations of malonyldialdehde and total peroxides of control groups were respectively 1.9 ± 0.1μmol/gHb (7); 126 ± 36 μmol/gHb (9) and for deficient groups 7.9 ± 4.0 (28); 293±88 μmol/gHb (19). The differences between the results from control and deficient animals were significant at P<0.001 and 0.01 for malonylaldehyde and total peroxides respectively (Table 12).

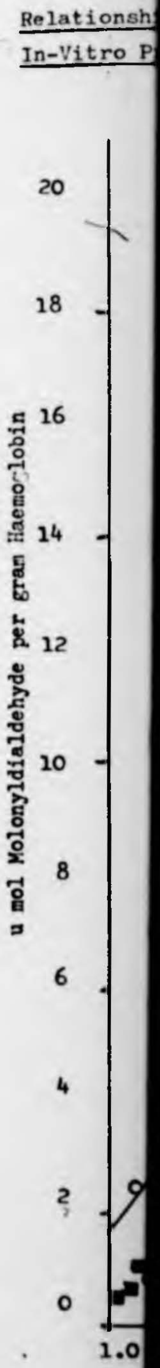
Figure (21):

Relationship between Riboflavin Status and and In-Vitro Production of Malonydialdehyde In the Red Cells

Figure 21:

Relationship between riboflavin status and in-vitro production of malonydialdehyde in the red cells

Glutathione reductase activity coefficients and the in-vitro production of malonyldialdehyde in the red cells were measured as described in the Methods Chapter in erythrocytes from control (■), pair-fed (○) and riboflavin-deficient rats (●) Each point represents a blood sample from one rat on which duplicate measurements were done.



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Relationship between Riboflavin Status and In-Vitro Production of Malonyldialdehyde in the Red Cells

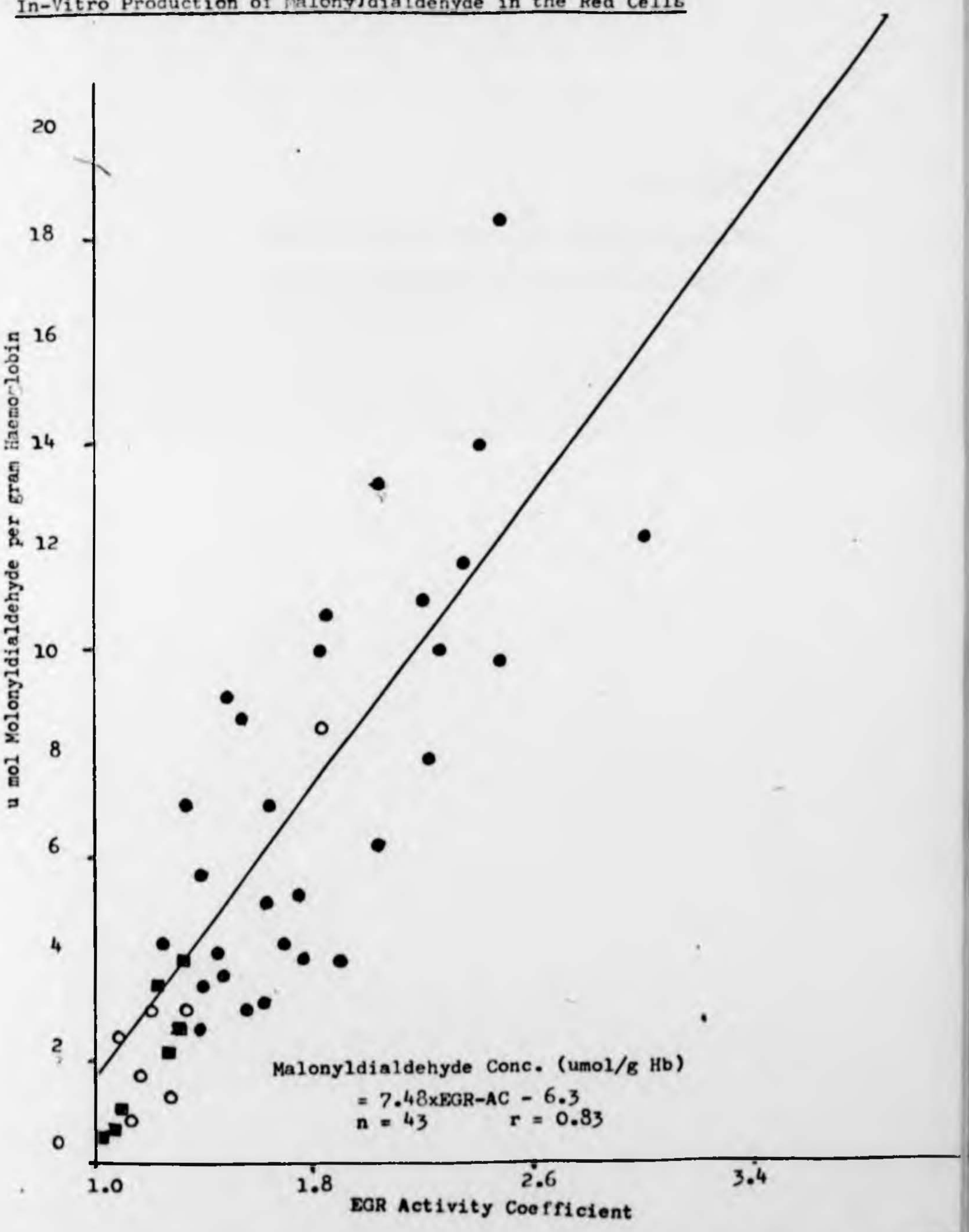


Figure (22):

Relationship between Riboflavin-Deficiency and the Concentration of Lipid Peroxides in the Erythrocytes

Figure 22:

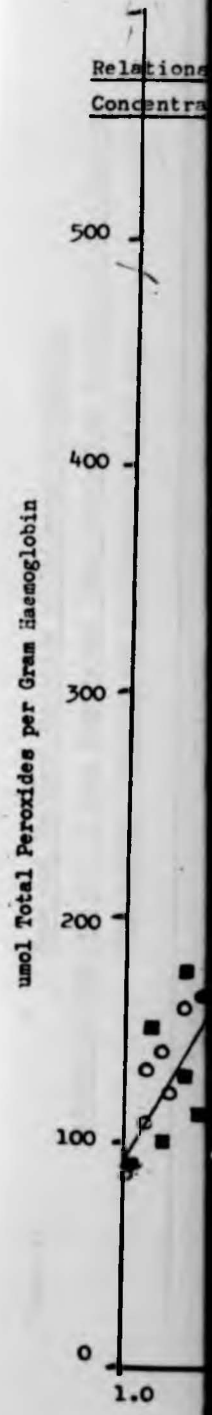
Relationship between riboflavin-deficiency and concentrations of lipid peroxides in the erythrocyte

Glutathione reductase activity coefficients and concentrations of total peroxides were measured as described in the Methods Chapter. Each point represents a blood sample from animal on which duplicate measurements were made. The points shown on the transparent overlay were from riboflavin-deficient animals which showed clinical evidence of recovery at the time of killing.

(■) Controls

(○) Pair-Feds

(●) Riboflavin-Deficients



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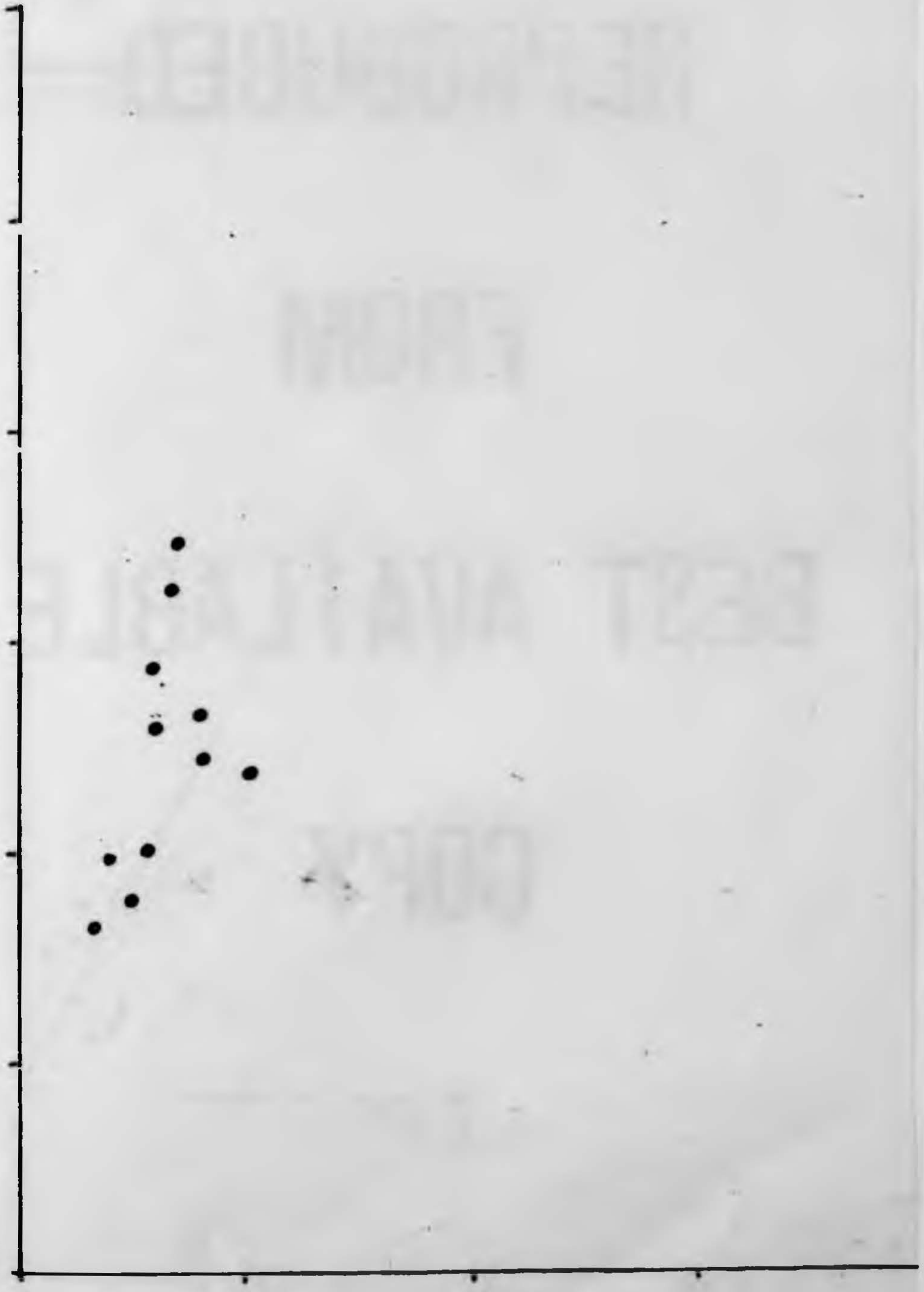
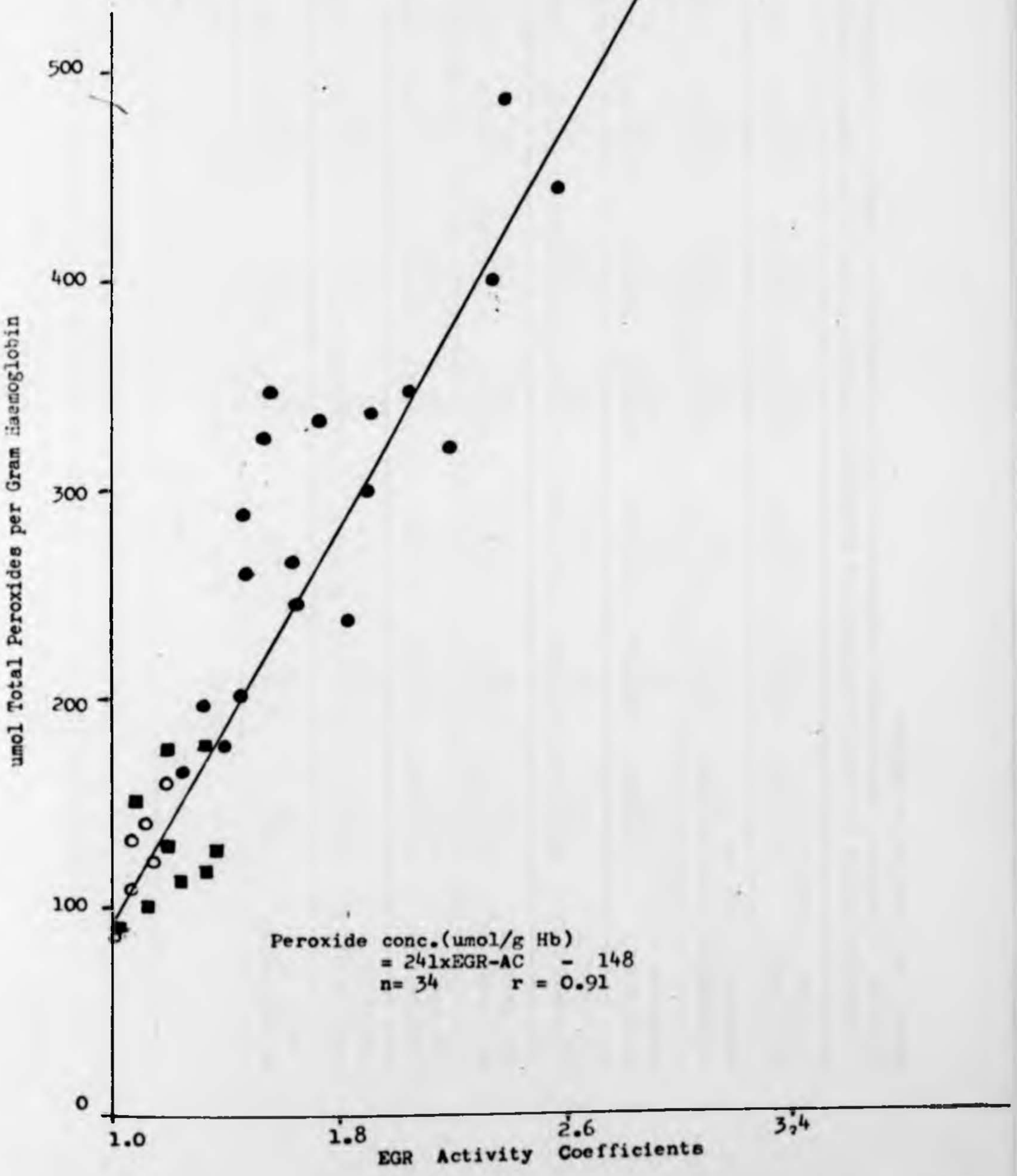


Relationship between Riboflavin Deficiency and Concentrations of Lipid Peroxides in the Erythrocytes



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Relationship between Riboflavin Deficiency and Concentrations of Lipid Peroxides in the Erythrocytes

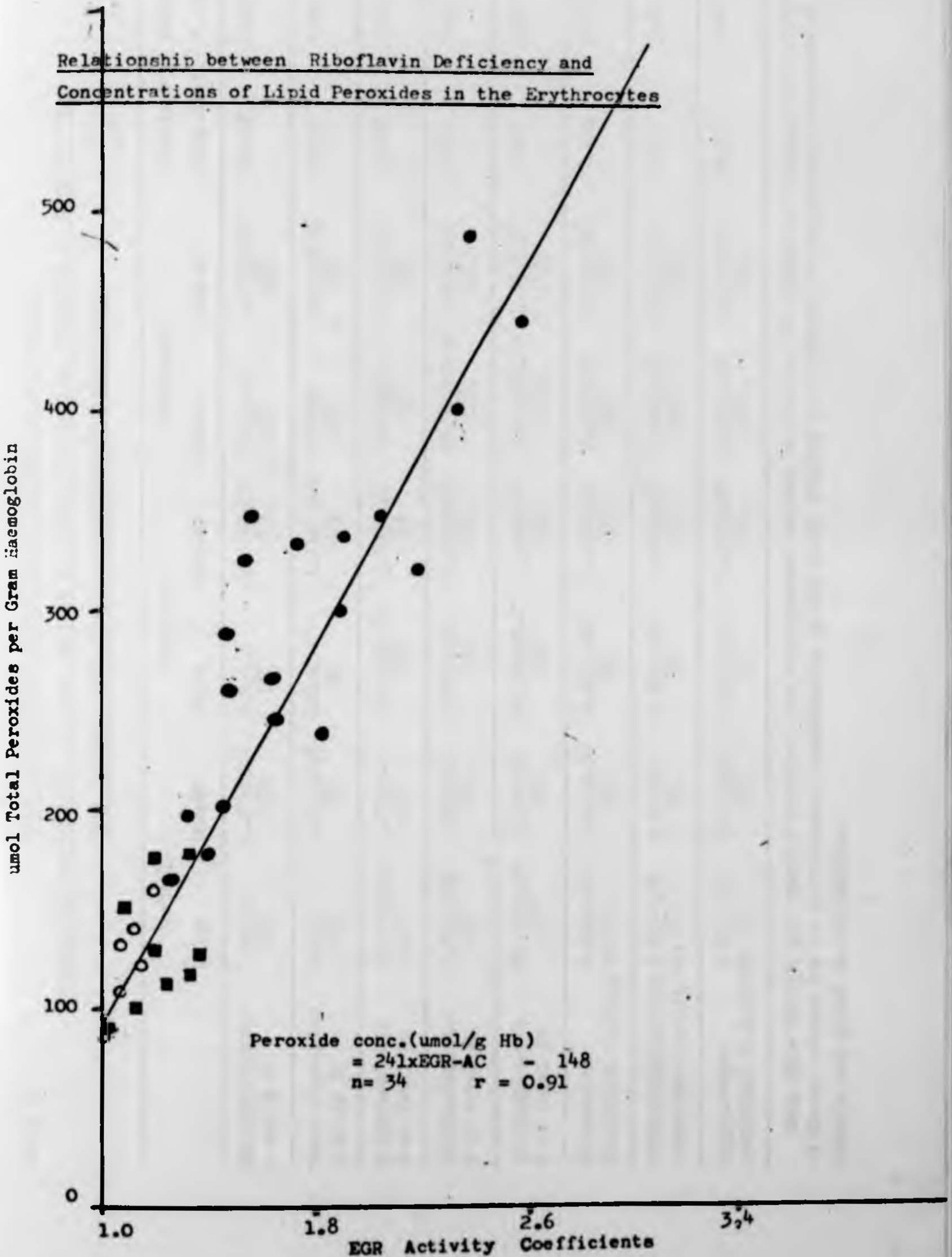

Table 12 Mean and Standard Deviation of Different Erythrocyte Metabolites and Activity of some Enzymes and Their Statistical Analysis in Erythrocytes from Control, Pair-fed and Riboflavin-Deficient Rats

	Control		Pair-Fed		Deficient		Statistical Analysis	
	Value	EGR-AC	Value	EGR-AC	Value	EGR-AC	Cont.×Def.	P.f.×Def.
Malonylaldehyde u mol/g Hb	1.9±1.1 (7)	1.21±0.14 (7)	3.0±2.6 (7)	1.31±0.25 (7)	7.9±4.0 (28)	1.85±0.43 (28)	0.001	0.01
Total Peroxides u mol/g Hb	126±36 (9)	1.23±0.13 (9)	116±15.8 (6)	1.11±0.004 (6)	293±88 (19)	2.96±0.88 (19)	0.01	0.01
Methaemoglobin % of Total Hb	10.75±5.53 (13)	1.21±0.2 (13)	11.01±8.18 (6)	1.29±0.27 (6)	30.04±15.3 (22)	1.99±0.56 (22)	0.001	0.01
Sulfhaemoglobin % of Total Hb	2.36±2.50 (9)	1.23±0.14 (9)	3.64±3.65 (6)	1.29±0.27 (6)	10.51±10.43 (22)	1.99±0.56 (22)	0.05	N.S.
Oxyhaemoglobin % of Total Hb	86.38±5.94 (9)	1.23±0.14 (9)	85.34±10.67 (6)	1.29±0.27 (6)	59.34±23.17 (22)	1.99±0.56 (22)	0.02	0.02
Glutathione Peroxidase (i.u./g Hb)	4.46±1.00 (9)	1.26±0.36 (9)	4.32±1.22 (6)	1.14±0.09 (6)	7.39±2.07 (27)	1.73±0.38 (27)	0.001	0.01
NADH-Methaemoglobin reductase (i.u./g Hb)	7.41±1.66 (5)	1.17±0.1 (5)	5.61±1.61 (5)	1.17±0.05 (5)	9.50±5.55 (20)	2.09±0.35 (20)	N.S.	N.S.
NADPH-MetHb reductase (i.u./g Hb)	3.73±1.53 (8)	1.18±0.18 (8)	3.60±1.49 (9)	1.17±0.08 (9)	6.92±3.26 (22)	1.97±0.41 (22)	0.05	0.01

Data show Mean±SD for results obtained from experimental animals on diet B. The number of animals used in the different assays are in parentheses. Measurements on all blood samples were assayed in duplicates. Experimental detaila are given in the methods.

Table 12

Mean and Standard Deviation of Different Erythrocyte Metabolites and Activity of some Enzymes and Their Statistical Analysis in Erythrocytes from Control, Pair-fed and Riboflavin-Deficient Rats

	Control		Pair-Fed		Deficient		Statistical Analysis	
	Value	EGR-AC	Value	EGR-AC	Value	EGR-AC	Cont.×Def.	P.f.×Def.
Malonylaldehyde u mol/g Hb	1.9±1.1 (7)	1.21±0.14 (7)	3.0±2.6 (7)	1.31±0.25 (7)	7.9±4.0 (28)	1.85±0.43 (28)	0.001	0.01
Total Peroxides u mol/g Hb	126±36 (9)	1.23±0.13 (9)	116±15.8 (6)	1.11±0.004 (6)	293±88 (19)	2.96±0.88 (19)	0.01	0.01
Methaemoglobin % of Total Hb	10.75±5.53 (13)	1.21±0.2 (13)	11.01±8.18 (6)	1.29±0.27 (6)	30.04±15.3 (22)	1.99±0.56 (22)	0.001	0.01
Sulfhaemoglobin % of Total Hb	2.36±2.50 (9)	1.23±0.14 (9)	3.64±3.65 (6)	1.29±0.27 (6)	10.51±10.43 (22)	1.99±0.56 (22)	0.05	N.S.
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NADH-Methaemoglobin reductase (i.u./g Hb)	7.41±1.66 (5)	1.17±0.1 (5)	5.61±1.61 (5)	1.17±0.05 (5)	9.50±5.55 (20)	2.09±0.35 (20)	N.S.	N.S.
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Data show Mean±SD for results obtained from experimental animals on diet B. The number of animals used in the different assays are in parentheses. Measurements on all blood samples were assayed in duplicates. Experimental detaila are given in the methods.

It was also found that when concentrations of GSH in samples of whole blood from control, pair-fed and riboflavin-deficient animals were plotted against the concentrations of peroxide in the same samples, a regression line was obtained (Fig.23) with the equation shown below:

$$\text{Total peroxides (umol/g Hb)} = -24 \times \text{GSH (mg/100ml bl.)} + 439$$

The correlation was highly significant ($r = 0.$, $P<0.001$, $n = 27$) suggesting that endogenous formation of total peroxides may be associated with a reduction in the level of GSH.

2 - Methaemoglobinemia:

The increased levels of peroxide in riboflavin-deficient erythrocytes led to an investigation of any possible pathological effect on the nature and function of haemoglobin under these circumstances.

Whole blood was collected at the time of killing under paraffin oil and stored at 4°C for analysis within the following 24 hours. Figures 24, 25 and 26 show the relationship between methaemoglobin (MetHb), sulfhaemoglobin (SHb) and oxyhaemoglobin (HbO_2) calculated as a percentage of total Hb and the EGR-AC. The rise in the concentrations of MetHb ($P<0.001$) and SHb ($P<0.001$ and reduction in HbO_2 ($P<0.001$) was significantly associated with increasing EGR-ACs i.e. with a fall in riboflavin status. As the figures suggest, MetHb and HbO_2 showed the greatest changes as riboflavin deficiency progressed. It is also apparent from the overlays to Figures 24, 25 and 26 that blood from animals

Figure (23):

Relationship between Peroxide Concentration and GSH concentration in Riboflavin-Deficiency

Figure 23:

Relationship between peroxide concentration and GSH concentration in riboflavin deficiency

Total peroxide concentration in erythrocyte haemolysate and GSH concentration in whole blood from control (■), pair-fed (○) and riboflavin-deficient (●) rats were measured as described in Figure 22 and 18 respectively.

The line represents that of the regression eqation which was calculated from the results obtained to be:

Peroxide concentration (umol/g Hb)

= -24 x GSH conc. (mg/100ml bl.) + 439

n = 23

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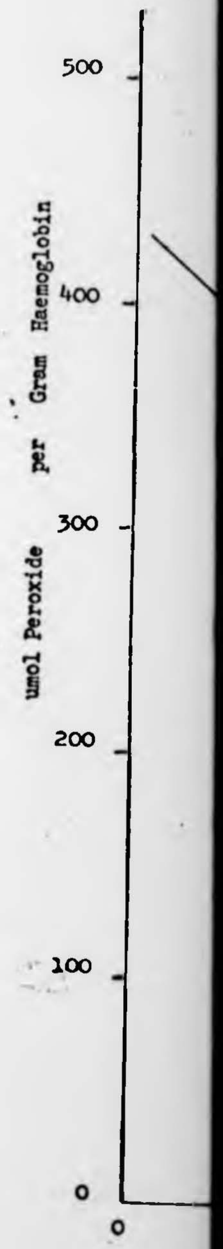

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Relation between peroxide Concentration and
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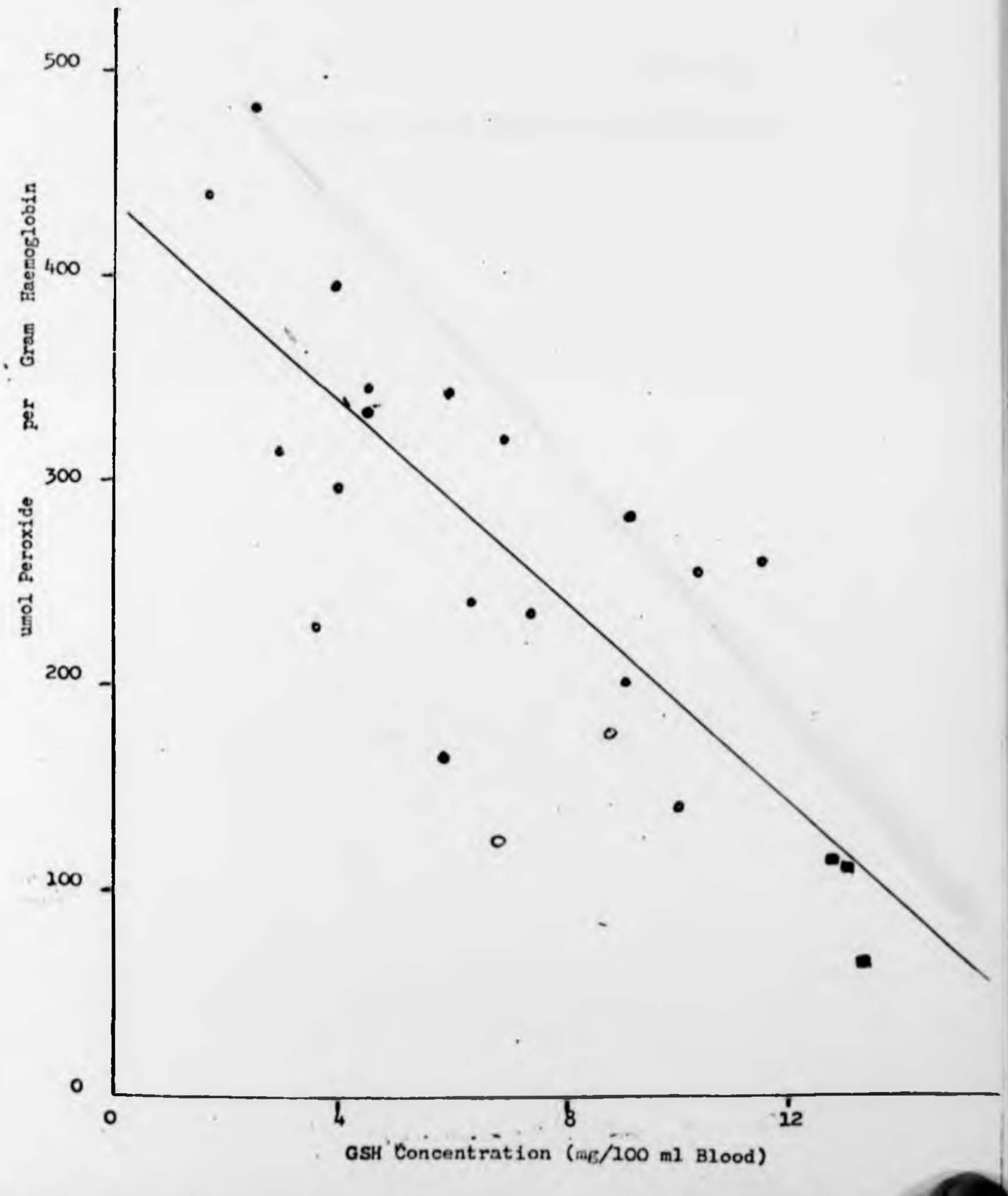


Figure (24):

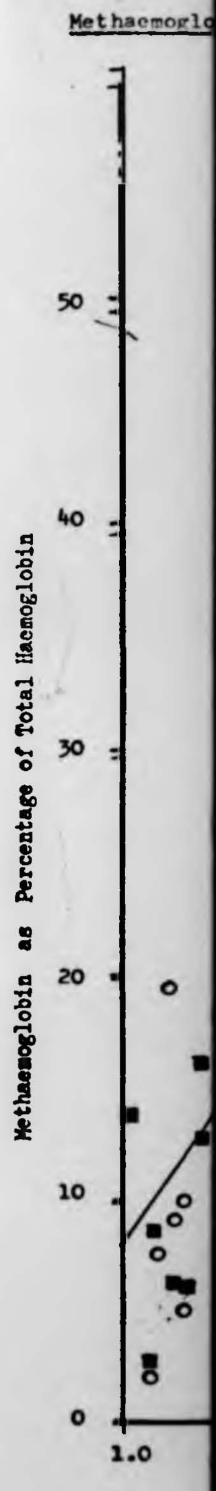
Methaemoglobin and Riboflavin-Deficiency

Figure 24:

Maethaemoglobin and riboflavin deficiency

Methods are described in Chapter two. Methaemoglobin concentrations as percentage of total haemoglobin in blood samples from control (■), pair-fed (○) and riboflavin-deficient animals (●) are plotted against the appropriate EGR-ACs. Each point represents the result obtained from one blood sample from one animal on which duplicate assays were done.

Points on the transparent overlay represents results from riboflavin-deficient rats which showed clinical evidence of recovery at the time of killing.



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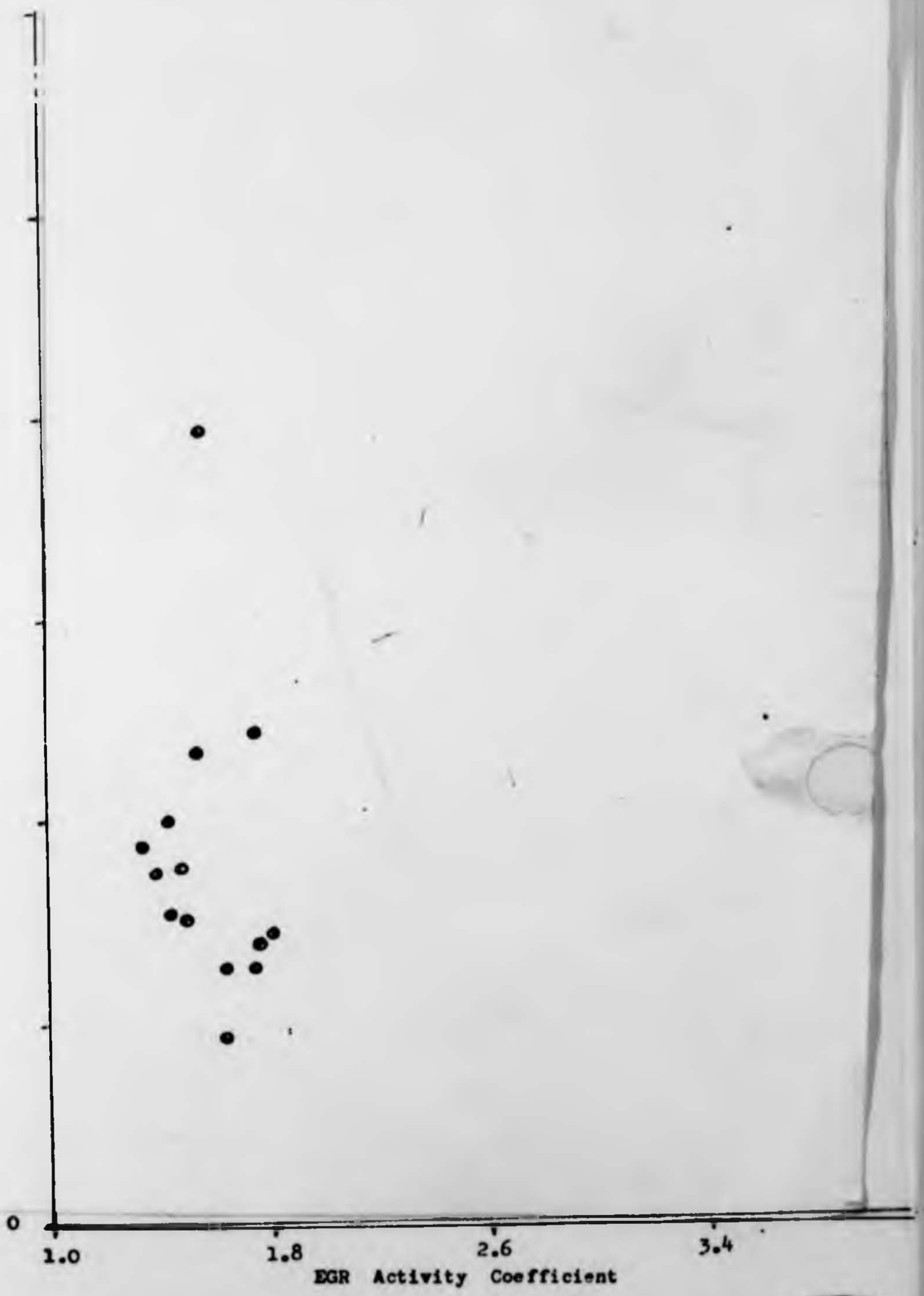

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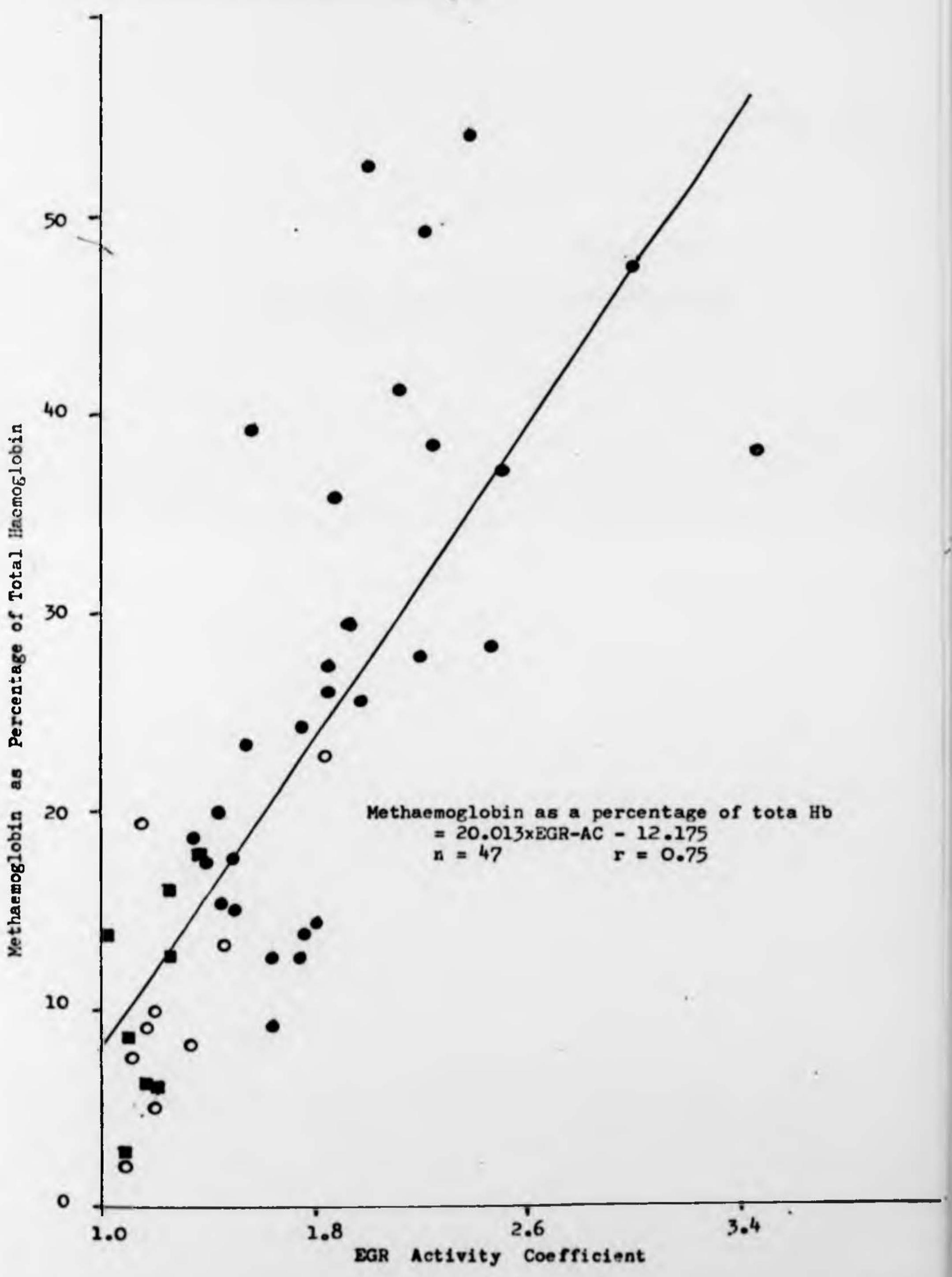

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Methaemoglobin and Riboflavin Deficiency

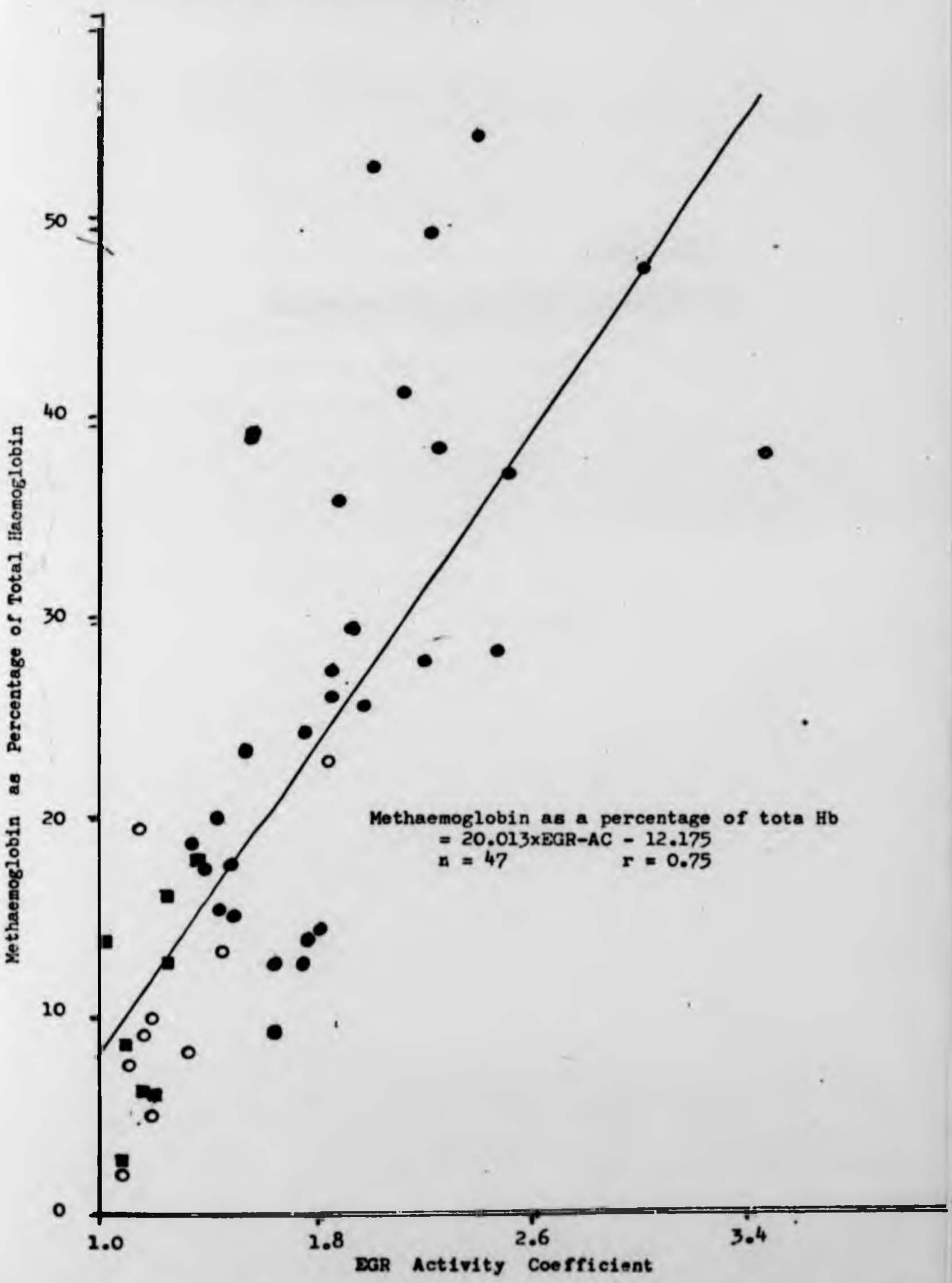


Figure (25):

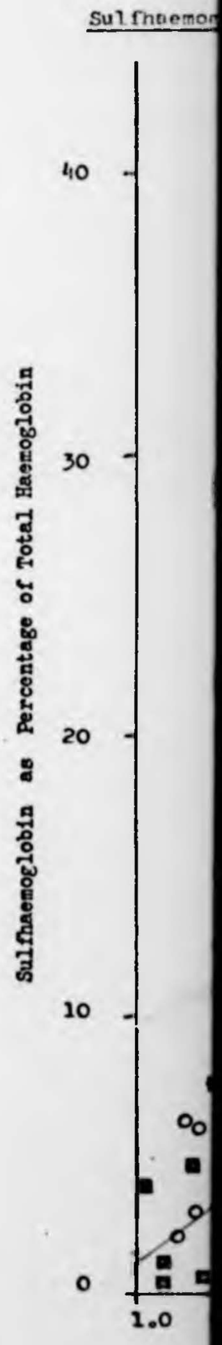
Sulfhaemoglobin and Riboflavin-Deficiency

Figure 25:

Sulfhaemoglobin and Riboflavin-deficiency

Sulfhaemoglobin was measured on whole blood from control (■), pair-fed (O) and riboflavin deficient rats (●) as described in the Methods section.

Each point represent results obtained from one animal on which duplicate measurements were made.



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Sulfhaemoglobin and Riboflavin Deficiency

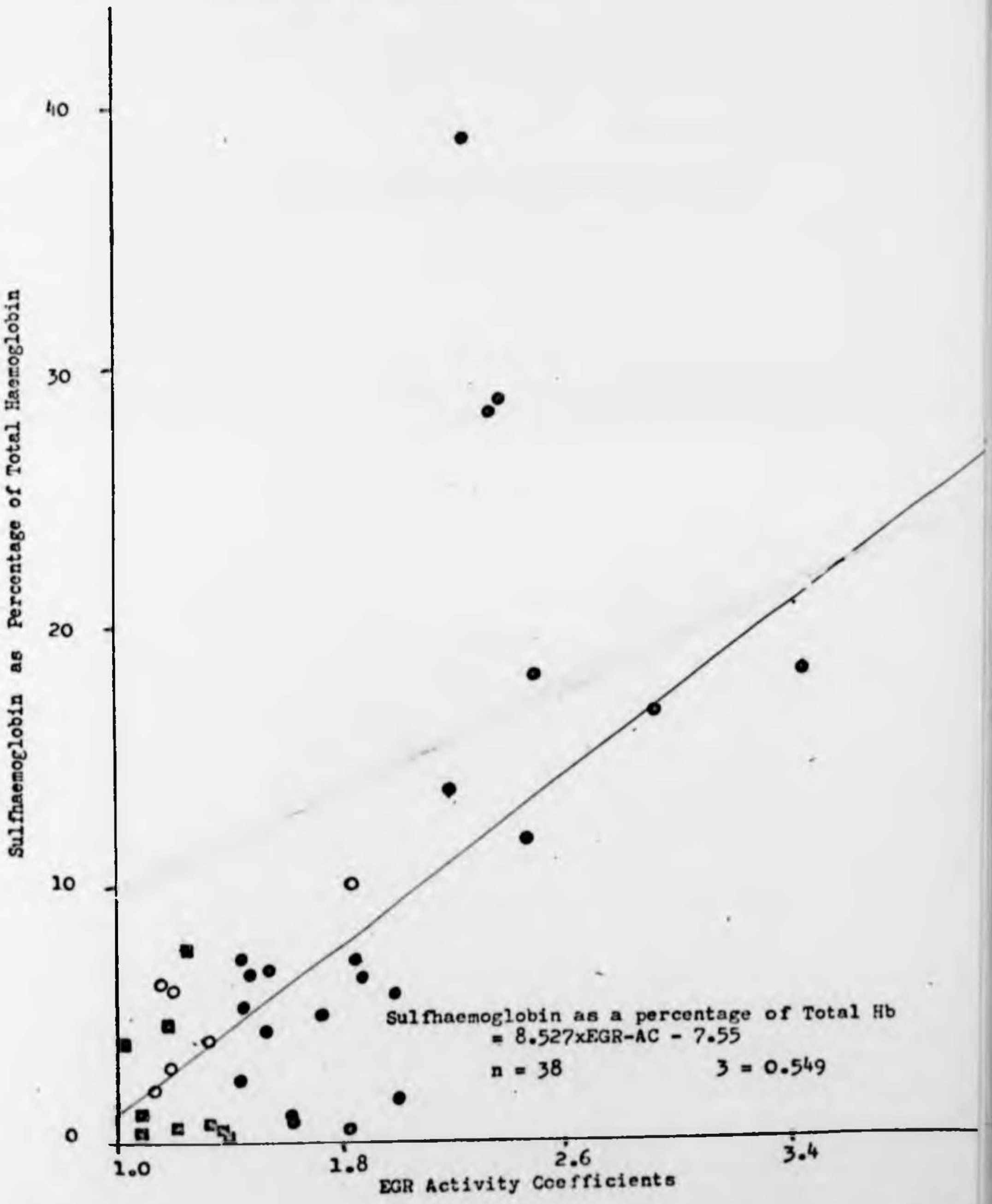


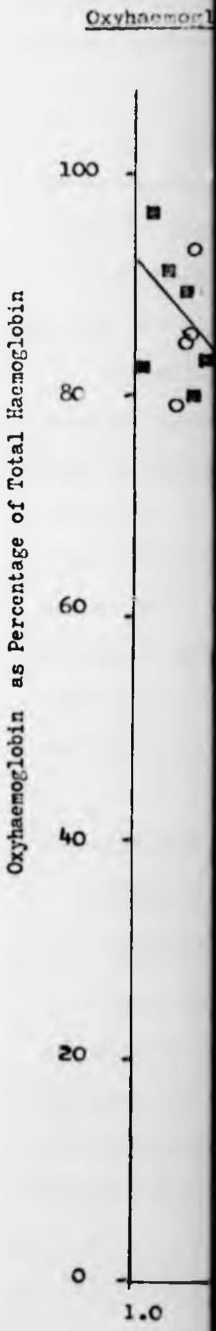
Figure (26):

Oxyhaemoglobin and Riboflavin-Deficiency

Figure 26:

Oxyhaemoglobin and riboflavin-deficiency

Oxyhaemoglobin was calculated by haemoglobin difference between total haemoglobin and the sum of methaemoglobin and sulfhaemoglobin as described in the Methods. The results obtained from control (■), pair-fed (○) and deficient (●) groups. Each point represents the results obtained from one animal on which duplicate assays were made.



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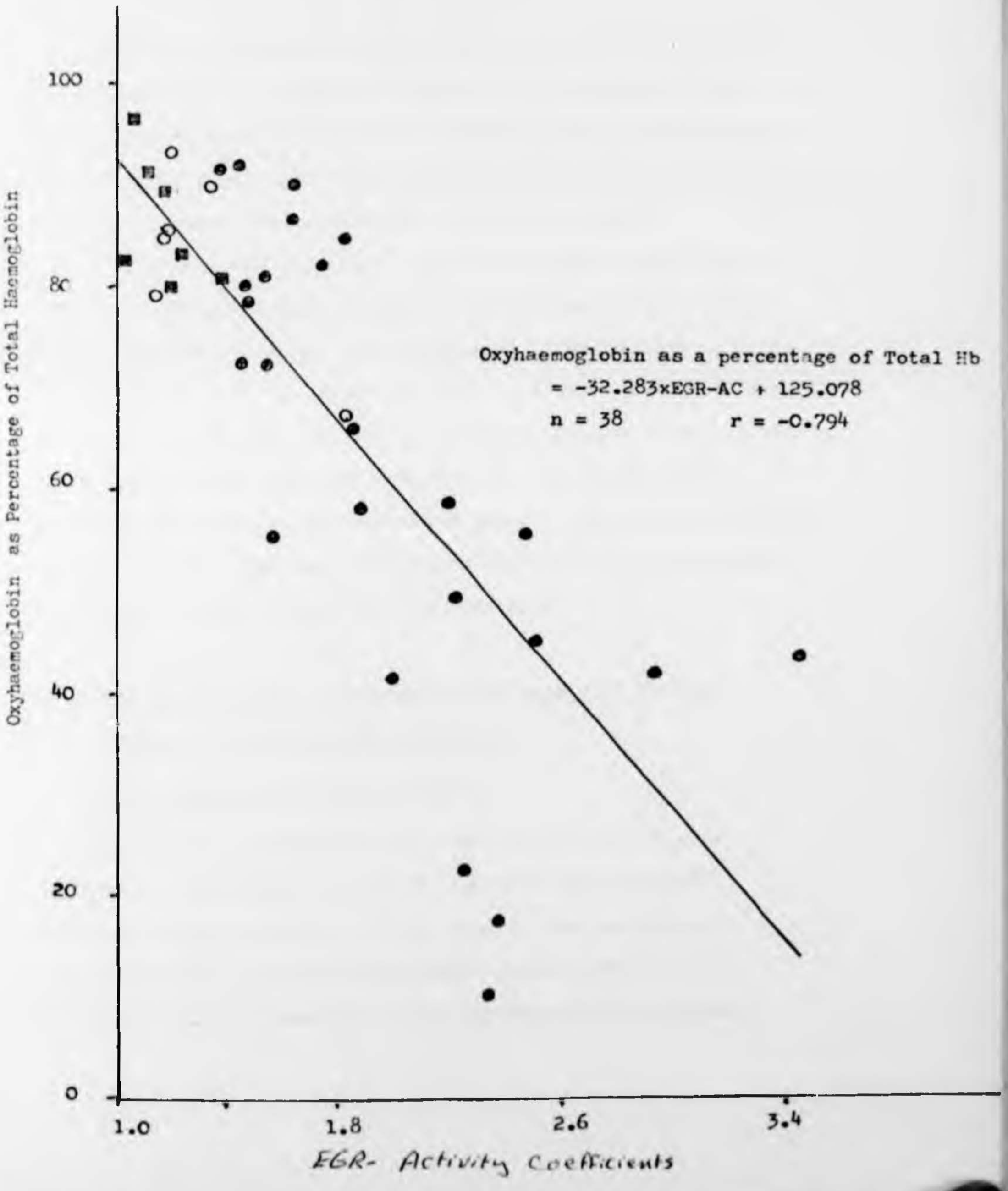
Oxyhaemoglobin and Riboflavin Deficiency



which showed evidence of clinical recovery at the time of killing showed changes which were less marked for the three haemoglobin derivities.

The mean and standard deviations for concentrations of the three haemoglobin derivatives in control, pair-fed and riboflavin-deficient rats are shown in Table 12.Differences between means of the different groups of animals for MetHb, SHb and HbO_2 values were tested for significance using the t test (Table 12).

All values were different at $P < 0.05$ between results from riboflavin-deficient animals and controls or pair-fed controls with the exception of SHb concentrations. SHb in control rats (2.36 ± 2.50) were significantly lower than that of the deficient group (10.51 ± 10.43), but SHb in pair-fed animals (3.69 ± 3.65) was slightly higher than the controls and not significantly different from that of the deficient group. Differences between means for control and pair-fed groups for any of the haemoglobin derivitives were not significantly different.

II. Changes in Activity of Enzyme Associated with Raised Concentrations of Toxic Metabolites

- Glutathione peroxidase activity

The results in Figure 28 show that when activities of glutathione peroxidase in control, pair-fed and deficient erythrocytes were combined and correlated with the EGR-AC, the correlation coefficient was highly significant ($r = 0.84$, P 0.001, n = 39). Mean values for erythrocyte glutathione

Figure (27):

Relationship between GSH Concentrations and Methaemoglobin

Figure 27:

Relationship between GSH concentration and methaemoglobin contration

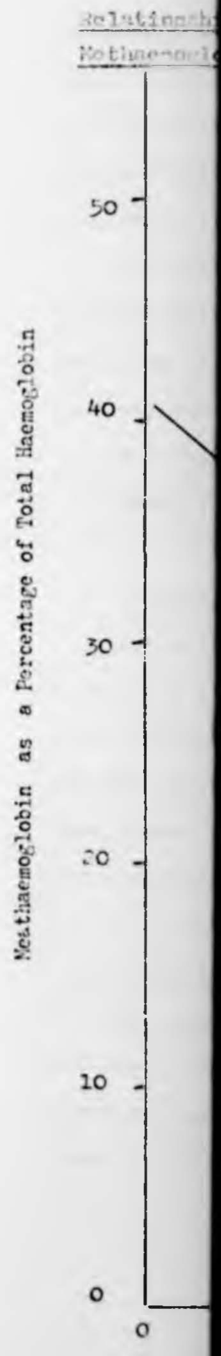
GSH and methaemoglobin as a percentage of total haemoglobin were assayed in whole blood as described in the Method. Regression line represents that of the regression equation which was calculated from the results obtainen to be:

Methaemoglobin concentration as a % of total Hb

= −2.25 x GSH Concn. (mg/100ml bl.) + 40.44

No of animals = 27

r = −0.65



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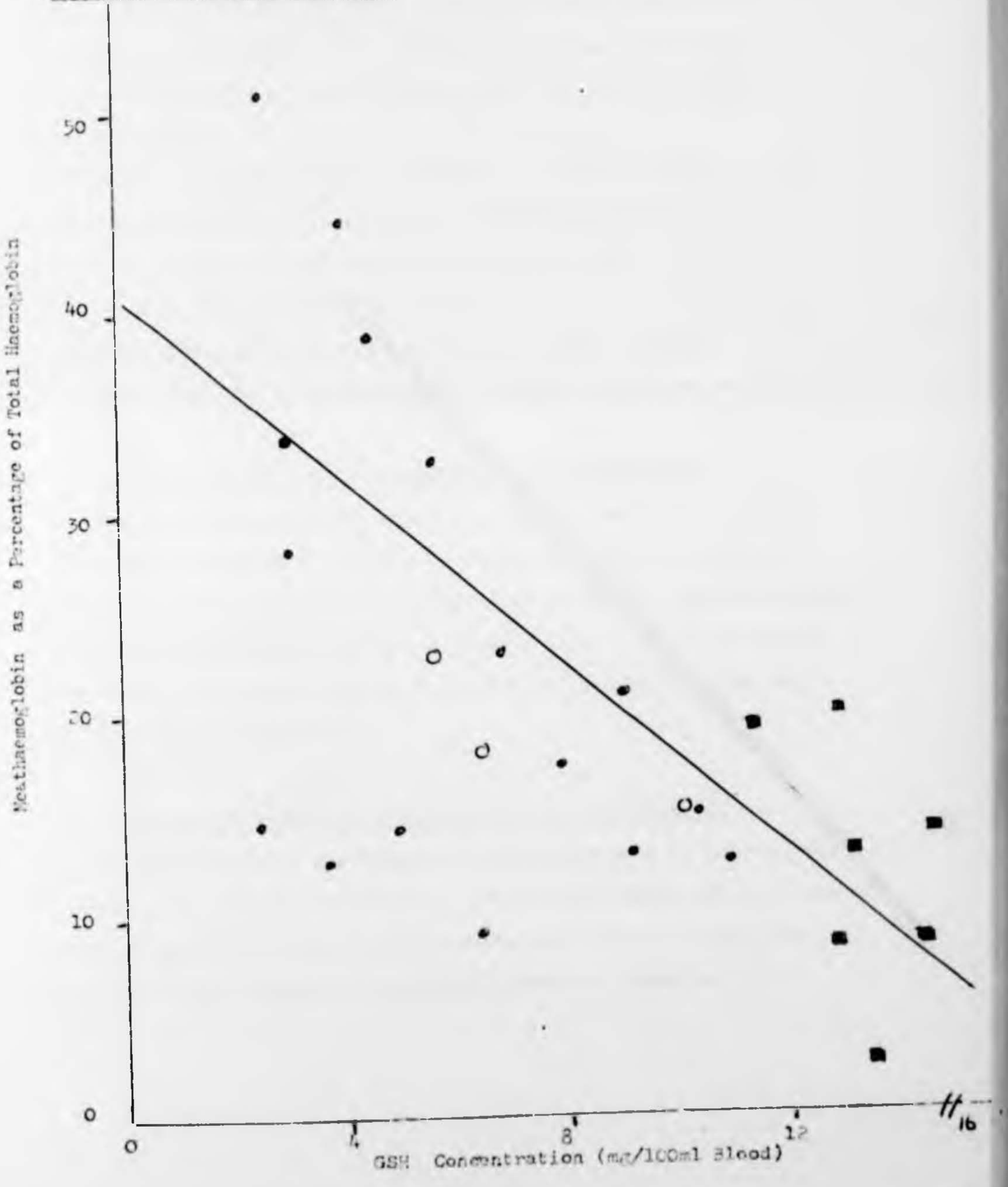
Relationship betwee GSH Concentrations and Methaemoglobin Concentrations



peroxidase activity of control, pair-fed and deficient groups were; 4.46 ± 1.00 (9), 4.32 ± 1.22 (6) and 7.39 ± 2.07 (27) i.u./mg Hb respectively. The differences between control and deficient ($P<0.001$) and pair-fed and deficient ($P<0.01$) were both significant.

Erythrocyte glutathione peroxidase activity was also significantly associated with the concentrations of total peroxides in the red cell ($r = 0.78$, $P<0.001$, $n = 27$). The equation for the regression line was

$$P = 0.388 \times \text{peroxidase activity (i.u./mgHb)} - 0.153$$

Where P is the concentration of peroxides in μmol/mg Hb (Fig.28)

2 - Activity of NADH-Methaemoglobin Reductase (NADH-MR)

Table 12 also shows the means and standard deviations for the activities of NADH-MR in i.u./mg Hb in erythrocytes from control, pair-fed controls and riboflavin-deficient animals. The mean value for the riboflavin-deficient group (9.50 ± 5.55; $n = 20$) was higher than either controls or pair-fed controls but the differences were not significant (Table 12).

3 - Activity of NADPH-Methaemoglobin reductase (NADPH-MR)

NADPH-MR activity was assayed in erythrocytes from control, pair-fed and riboflavin-deficient rats. The results showed that the enzyme activity was elevated in riboflavin-deficient rats above both the controls ($P<0.05$) and pair-fed controls ($P<0.01$) Table 12.

Figure (28):

Relationship between Glutathione Peroxidase Activity and Riboflavin Status

Figure 28:

relationship between glutathione peroxidase activity and riboflavin status.

Glutathione peroxidase activity in haemolysate and GSH in blood from control (■), pair-fed (○) and riboflavin-deficient rats (●) were measured described in the Methods chapter. All points represent one animal on which duplicate assays were made.

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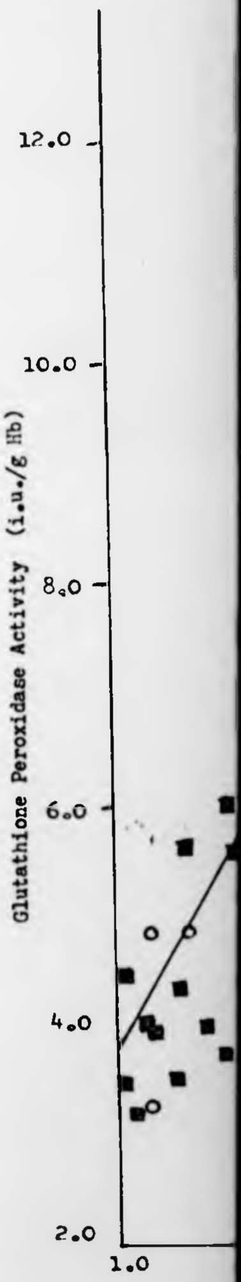

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Relationship between Glutathione Peroxidase and Riboflavin Status

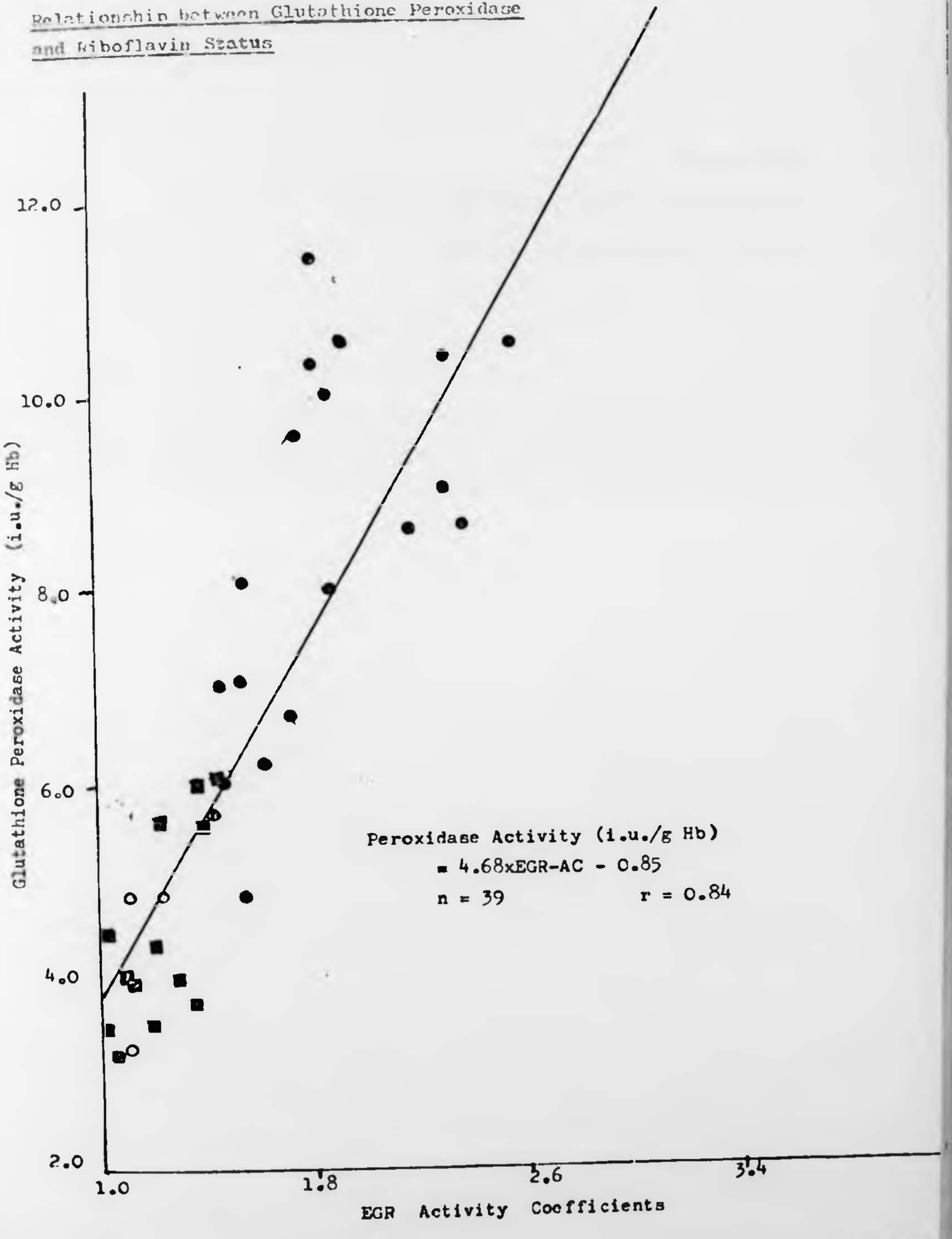

Figure (29):

Relationship between Glutathione Peroxidase Activity and Formation of Peroxides

Figure 29:

Relationship between Glutathione Peroxidase Activity and formation of peroxides

Glutathione reductase activity coefficients and activity of glutathione peroxidase are measured on haemolysates prepared from rat blood as described in the Methods Chapter. All points represent one animal on which duplicate assays were done from control (■), pair-fed (○) and riboflavin-deficient rats (●).

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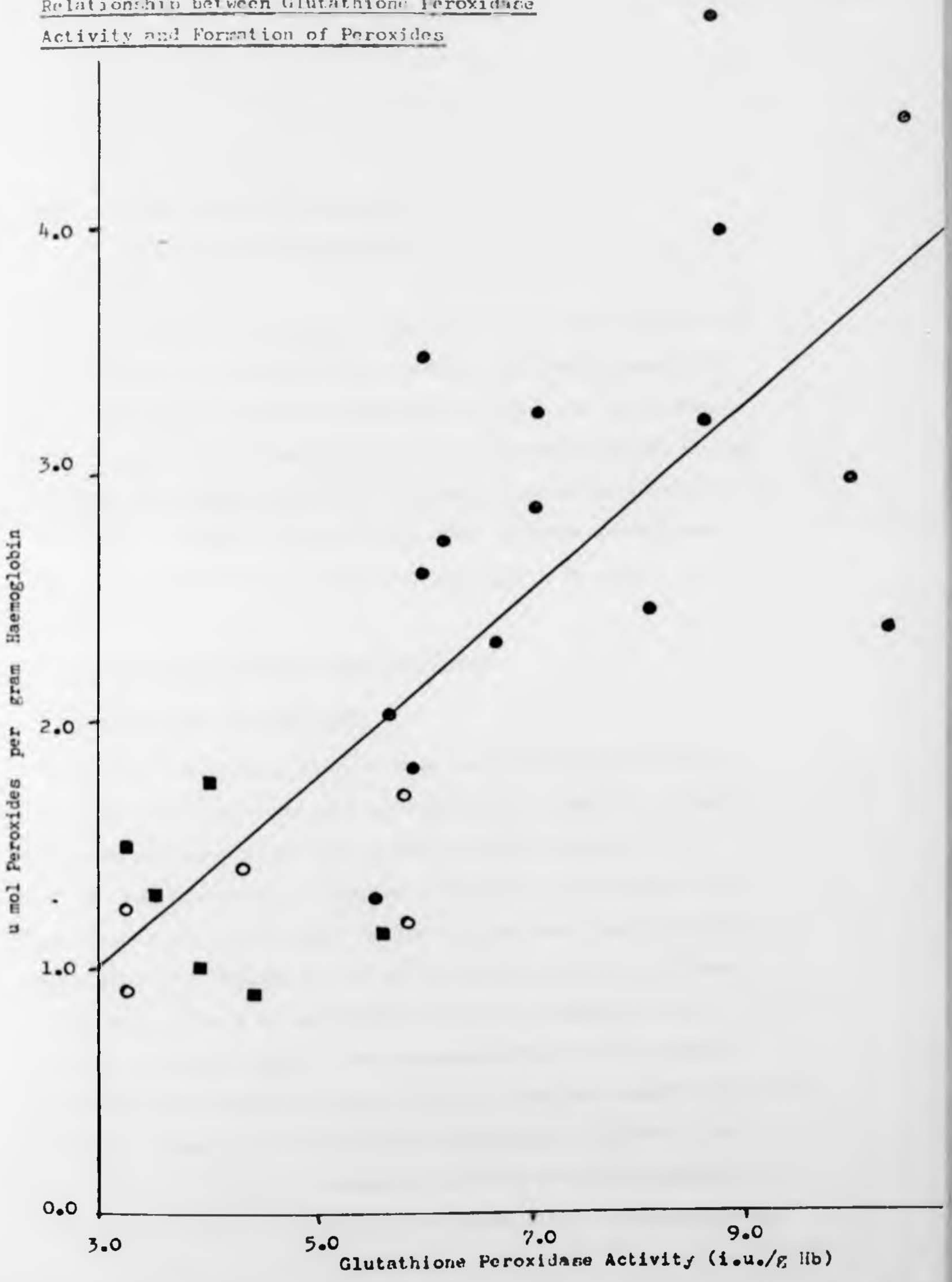
Relationship between Glutathione Peroxidase Activity and Formation of Peroxides



Part D: HAEMATOLOGICAL CHANGES IN RIBOFLAVIN-DEFICIENT RATS

In the current study, data reported in Part (B) demonstrated that red blood cells from riboflavin-deficient rats appeared to be more susceptible to haemolysis in-vitro than red cells from normal animals. The possible correlation between EGR-AC and red cell fragility suggested further studies to determine whether the increased fragility in red cells from riboflavin-deficient rats was associated with a shortened in-vitro life span.

1- Physical Characteristics of Red Cells

a - Observations on the blood

All blood samples collected from the riboflavin-deficient animals during this study were noticed to be brighter in colour than those obtained from control and pair-fed animals. The riboflavin-deficient blood samples appeared to clot faster therefore heparin was always added to the syringe when removing blood from the heart. Plasma in the deficient sample was very clear without any evidence of the turbidity which is normally associated with plasma lipids. The volumes of the blood collected from the deficient animals ranged between 2 and 4ml from 80-140g rats. After separating the blood cells by centrifuging at 2500 g, the plasma appeared reddish to brownish possibly due to the presence of haemolysed cells, and possibly the appearance of methaemoglobin.

The red blood cells from riboflavin-deficient rats appeared even brighter when washed with physiological saline solution containing 0.1% albumin in the washing proceedings.

b - Packed Cell Volume

Values for Packed Cell Volume (PCV) were measured on blood from rats on control, pair-fed and riboflavin-deficient diets. Figure 30. shows the relationship between the EGR-AC and PCV measurements made on blood samples of rats in many experiments. The PCV and EGR-AC were found to be significantly correlated, but PCV results from rats in the different groups were very scattered. For seventy animals of which 42 were riboflavin-deficient the correlation coefficient was 0.59 and the equation of the regression line is shown below:

$$\text{PCV} = (-6.39) \times (\text{EGR-AC}) + 54.88$$

The fall in PCV with riboflavin deficiency was not very large as is seen in Table (13) but nevertheless the mean was significantly different from that of both control and pair-fed animals ($P < 0.01$). The difference between mean PCV of controls and pair-fed rats was not significant.

3 - Red blood cell counts

Erythrocyte counts were done on a smaller number of animals than PCV according to whether blood and time was available. When the results were plotted against EGR-AC a negative correlation was obtained but the fall in erythrocytes counts as riboflavin status deteriorated was not very great (Fig. 31). The mean and standard

Figure (30):

Relationship between Packed Cell Volume (PCV) and Riboflavin Ststus

Figure 30:

Relationship between packed cell volume (PCV) and riboflavin status

Packed cell volume measured on heparinised whole blood samples from control (■), pair-fed (○) and riboflavin deficient rats (●) as described in the methods were plotted against their appropriate EGR-AC ratios determined on the haemolysate. Each point represents one sample from one rat on which duplicate measurements were done.

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Relationship between Packed Cell Volume and Riboflavin Status

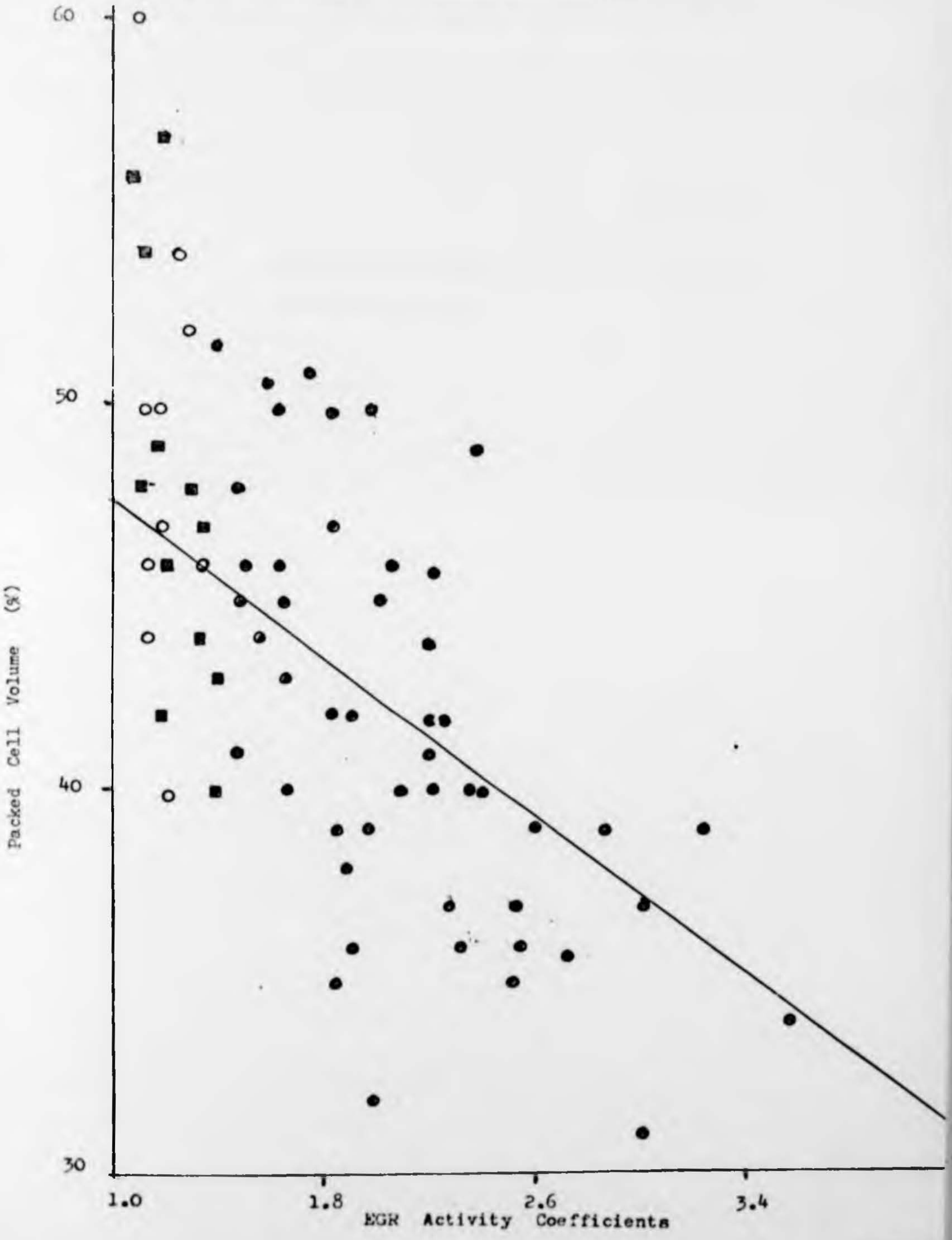


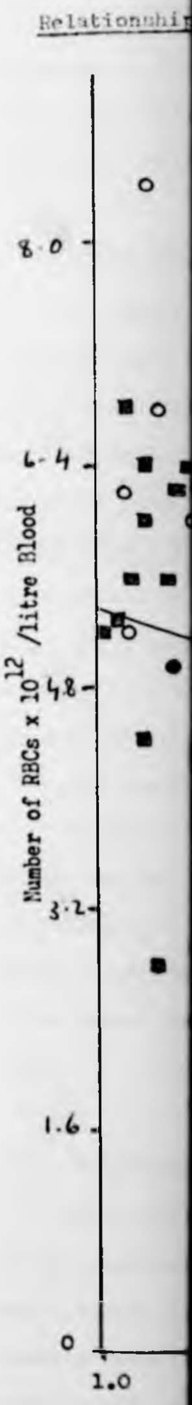
Figure (31):

Relationship between Erythrocyte Counts and Riboflavin Ststus

Figure 31:

Relation between erythrocyte counts and riboflavin status

Erythrocyte counts made on heparinised whole blood samples from control (■), pair-fed (○) and riboflavin-deficient rats (●) were plotted against their appropriate EGR-AC activity ratio determined on the haemlysate.



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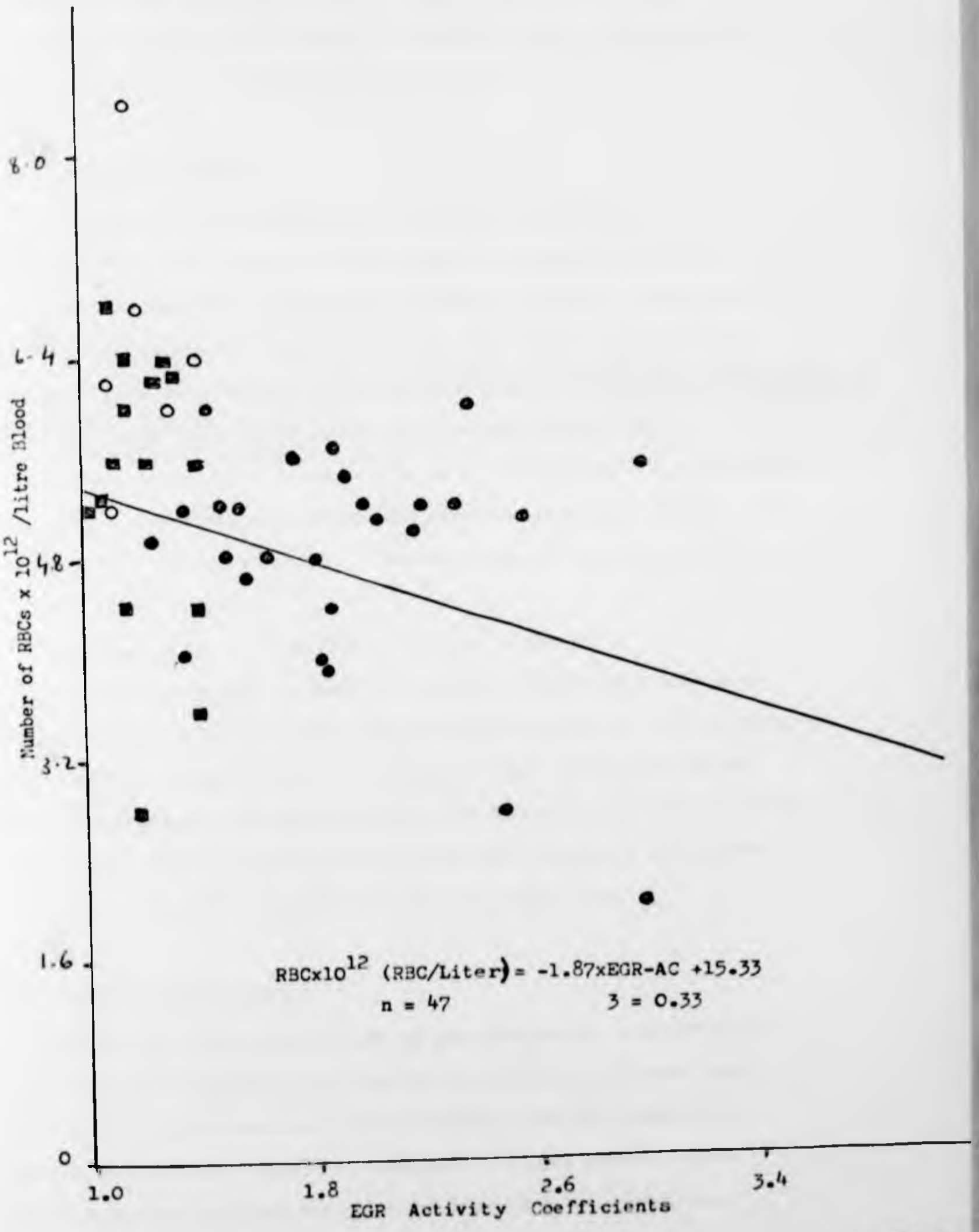
Relationship between Red Blood Cell Counts and Riboflavin Status



deviation of the erythrocyte counts from riboflavin-deficient animals was slightly lower than that of both control groups but the differences were not significant (Table 13).

4 - White cell counts

The number of leucocytes in the blood of riboflavin-deficient rats was found to be lower than the number found in both pair-fed ($P<0.01$) and control ($P<0.001$) animals. Means and standard deviations of leucocytes counts of control, pair-fed and deficient rats were $10.08 \times 10^9 \pm 2.26 \times 10^9$ (9), $9.10 \times 10^9 \pm 2.15 \times 10^9$ (6) and $4.29 \times 10^9 \pm 1.9 \times 10^9$ (30) per litre respectively (Table 13). In addition, when white blood counts were plotted against the EGR-ACs (Fig. 32) a negative correlation was obtained which was highly significant ($r = -0.70$, $P<0.001$). The equation of the regression line was calculated to be:

$$\mathrm{WBC(counts/l)} = (-32.78) \times (\mathrm{EGR\text{-}AC}) + 107.05$$

Attempts were made to make differential white cell counts on blood from the different groups by making blood smears and staining with 0.2%(w/v) Wright stain in methanol. The leucocytes in the blood of riboflavin-deficient animals did not stain properly and the faded colour made differentation between the different leucocytes difficult. Further attempts have not yet been made.

5 - Reticulocytes counts

Table (13) shows means ± SDs of percentages of reticulocytes in the blood of control, pair-fed and riboflavin-deficient rats. The differences between reticulocyte counts made on blood from riboflavin-deficient rats, and both pair-fed and control rats, were significant at ($P<0.001$), whilst there was no significant

Table 13:

Haematological Indices of Red Blood Cells from Rats on Control, Restricted Control and Riboflavin-Deficient Diets

	PCV	RBCs $\times 10^{12}/l$	WBCs $\times 10^{9}/l$	Reticulocytes % of total	Plasma Fe ug/100 ml	Ferritin-Fe ug/g liver	Total non-haem Fe ug /g liver
Controls	46.7±4.5 (13)	5.53±1.10 (11)	10.08±2.26 (9)	2.44±0.46 (6)	242.0±46.9 (8)	70.72±12.99 (8)	111.5±22.12 (8)
Pair-fed	48.3±5.8 (9)	5.36±1.45 (10)	9.10±1.15 (6)	2.44±0.32 (6)	220.6±50.7 (5)	64.05±15.46 (8)	108.05±18.56 (8)
Riboflavin-Deficient	40.9±5.4 (43)	4.84±0.94 (26)	4.29±1.90 (30)	1.36±0.60 (19)	190.69±24.0 (13)	120.8±27.6 (16)	191.83±50.90 (24)
p< Cont. x Def.	0.001	N.S.	0.001	0.001	0.01	0.001	0.001

Measurements were made on blood and liver tissues from rats on control and riboflavin-deficient diets.

The data shown are mean±SD for a number of animals in parentheses.

Figure (32):

Relationship between White Blood Cell Counts and Riboflavin Status

Figure 32:

Relationship between white blood cell counts and riboflavin status

White blood cell counts were made om heparinized whole blood samples from control (■), pair-fed (○) and riboflavin-deficient rats (●) and were plotted against their appropriate EGR-AC ratios determined on the haemolysates.

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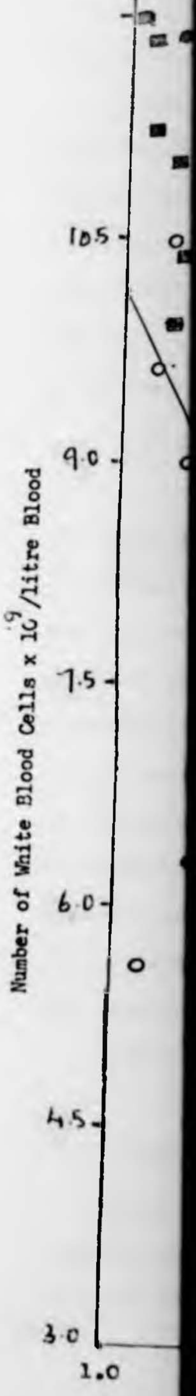

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Relationship between white blood cell counts and riboflavin status

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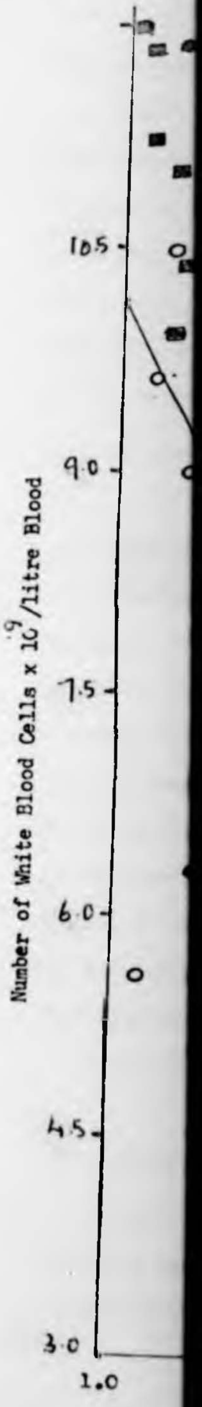

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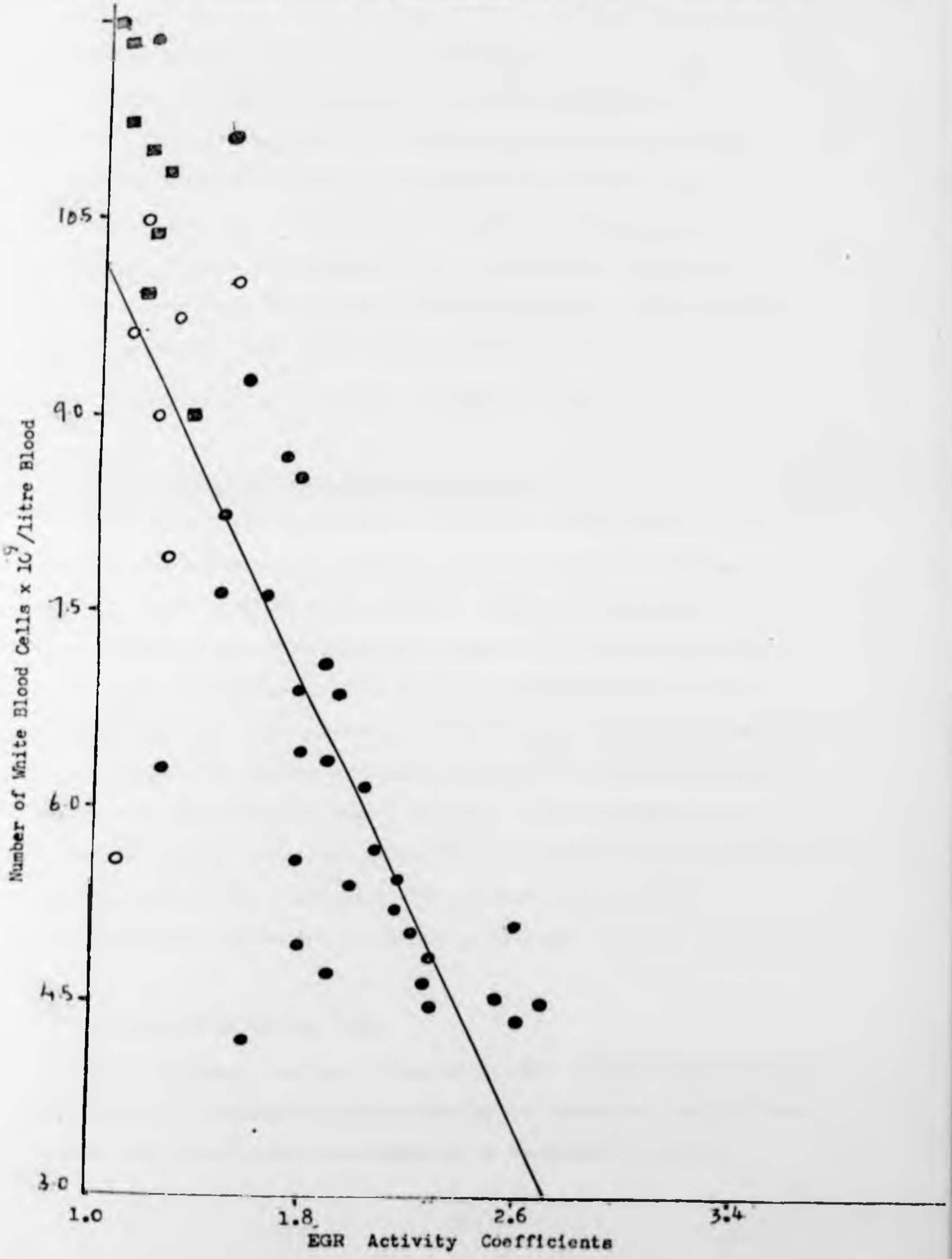
Relationship between White Blood Cell Counts and Riboflavin Status



difference between results from control and pair-fed animals.

Figure (33) shows the relation between percentage of reticulocytes circulating in the blood of control, pair-fed and riboflavin-deficient animals, and the EGR-AC of these rats. The results plotted in this figure suggested that riboflavin-deficiency in rats is associated with a significant reduction ($P < 0.001$, $r = 0.58$) in the number of reticulocytes. The equation of the regression line was calculated to be as follows:

$$\% \text{ reticulocytes} = (-0.89) \times (\text{EGR-AC}) + 3.34$$

2- Iron Metabolism in Riboflavin-Deficiency

Riboflavin-deficiency appears to increase the fragility of the erythrocytes and be associated with a small decrease in PCV and possibly also of blood cell counts. However, there was no reticulocytosis and if anything the number of circulating reticulocytes appeared reduced. The following experiments were done to investigate the role of riboflavin-deficiency in erythropoiesis, in particular the effects of iron metabolism and the concentrations of plasma iron as riboflavin status worsened. That is plasma iron concentration was inversely correlated with EGR-AC ($r = -0.53$, P 0.01, $n = 25$ The equation for the regression line was calculated to be:

$$\text{plasma iron } \mu g/100ml) = (-46.11) \times (\text{EGR-AC}) + 281.25 \quad \text{(Fig. 34)}$$

a - Iron storage in the liver

Total non-haem iron was estimated in liver tissue from control, pair-fed and riboflavin-deficient rats by the method of Foy Williams, Cortell and Conrad (1967) and found to be increased in animals

Figure (33)

Relationship between Riboflavin-Deficiency and Reticulocyte Counts

Figure 33:

Relationship between riboflavin-deficiency and reticulocyte counts

Reticulocyte counts made on heparinized whole blood samples from control (■), pair-fed (●) and riboflavin-deficient rats (○) as a percentage of total erythrocytes were plotted against their appropriate EGR-AC ratios determined on the haemolysis.

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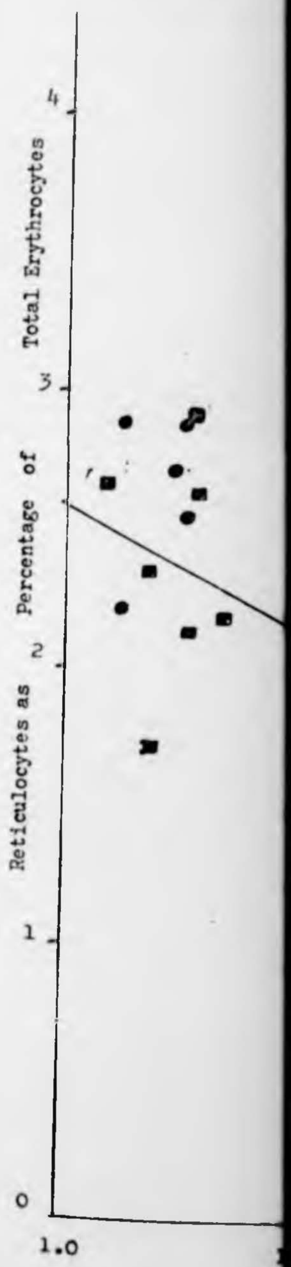

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Relationship between Riboflavin Deficiency and Reticulocyte Counts

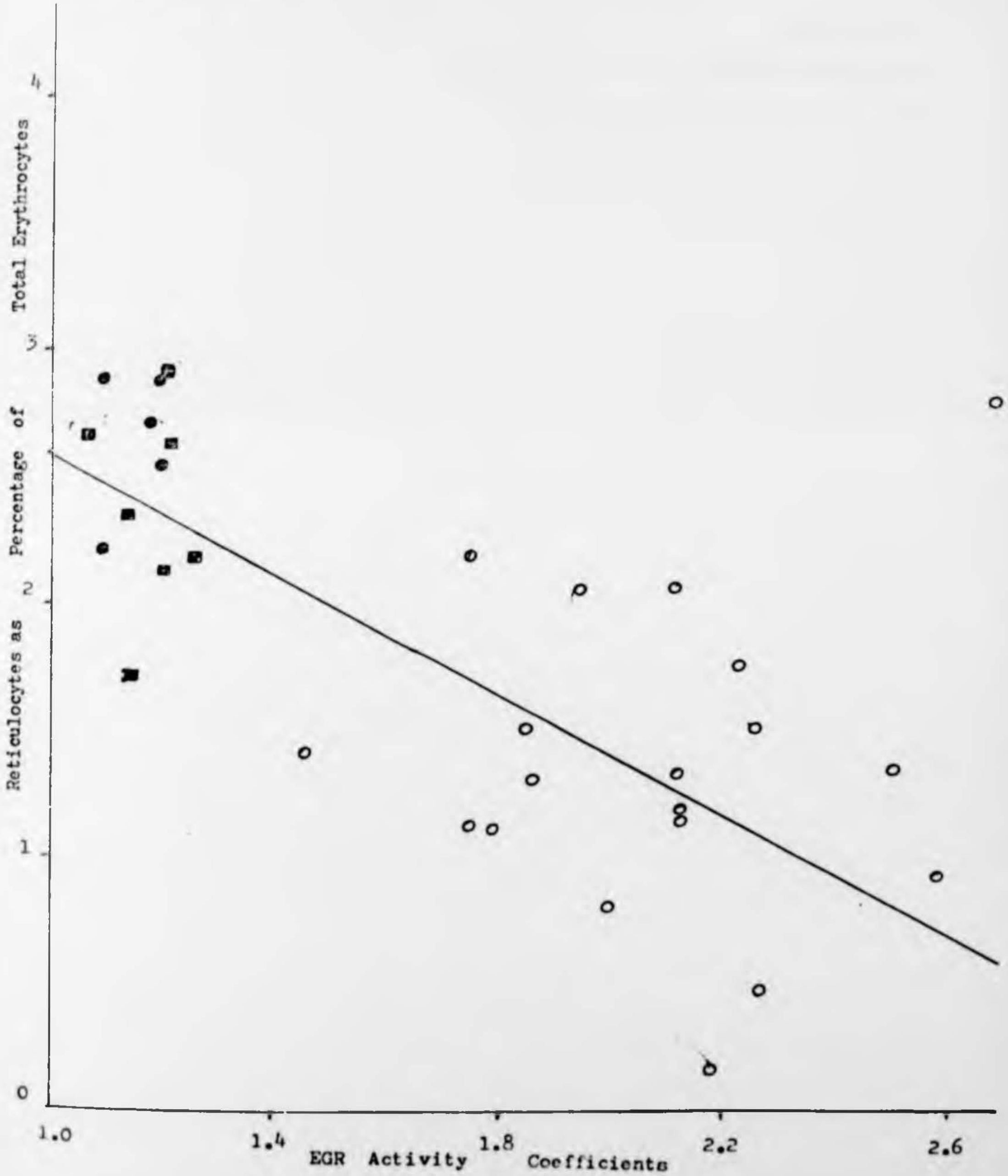


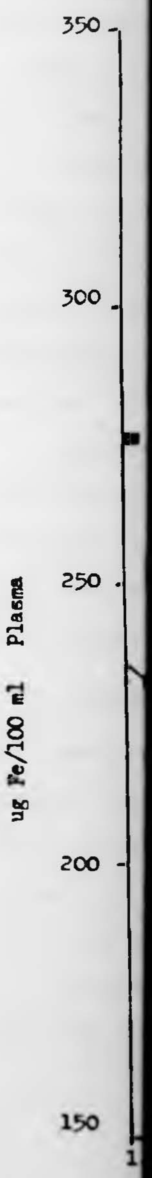
Figure (34):

Relationship between Riboflavin-Deficiency and Plasma Fe Concentration in Rats

Figure 34:

Relationship between riboflavin deficiency and plasma Fe concentration in rats

Plasma Fe and EGR-AC were estimated on heart blood collected from control (■), pair-fed (○) and riboflavin-deficient (●) rats when killed for analysis.



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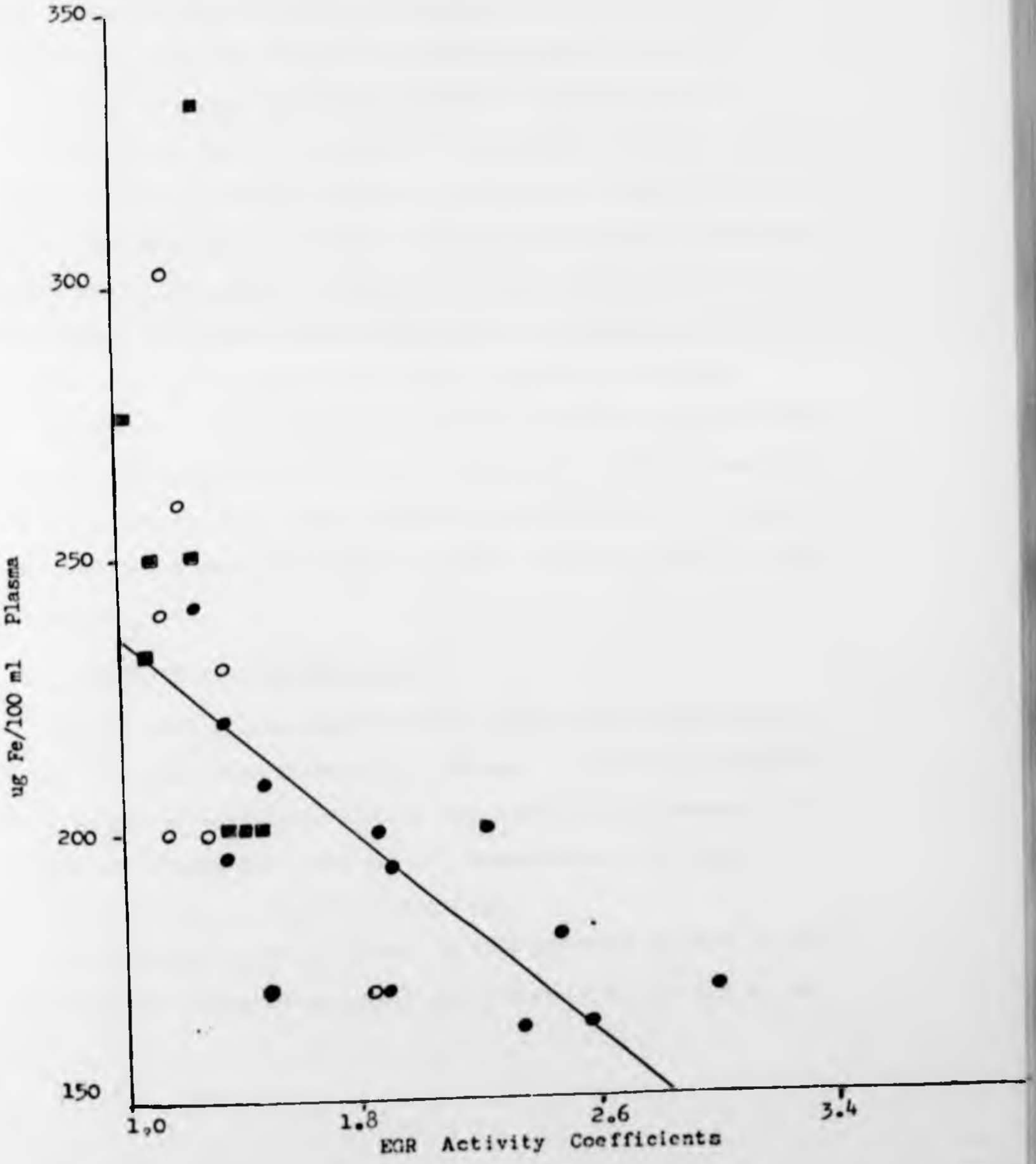
Relationship between Riboflavin Deficiency and Plasma Fe Concentrations in Rats



receiving the riboflavin-deficient diets. The increased levels of the total non-haem iron in the liver tissues was found to be positively correlated ($r = 0.69$, P 0.001) with EGR-AC in the different groups i.e. the riboflavin deficiency is associated with increased levels of total non-haem iron stored in the liver tissues (Fig. 35). The equation of the regression line was:

Non-haem Fe μg/gram tissue = (83.84) x (EGR-AC) +19.22

Means and standard deviation for the concentrations of total non-haem iron in livers of different groups are shown in Table (13). The differences between non-haem iron concentrations of riboflavin-deficient and both control and pair-fed groups were significant ($P<0.001$). No significant difference in the concentrations of non-haem iron in the livers of control and pair-fed groups.

Means and standard deviations of liver ferritin concentrations (Fig. 36) of control, pair-fed and riboflavin-deficient groups are reported in Table 13. Liver ferritin concentrations in riboflavin-deficient group were significantly higher ($P<0.001$) than the other two groups.

b - Measurement of absorbed iron

Measurement of absorbed iron was calculated by subtracting faecal iron loss from dietary iron intake. Diet (B) contained 4% by weight of salt mixture which was calculated to supply ferric citrate as 189.4μg iron/gram diet. Measurements were made over 3 x 5 day periods as shown in Table (14).

In absolute terms the amount of iron absorbed by rats on both pair-fed and riboflavin-deficient diets fell by 68 and 80% in the receiving the riboflavin-deficient diets. The increased levels of the total non-haem iron in the liver tissues was found to be positively correlated ($r = 0.69$, P 0.001) with EGR-AC in the different groups i.e. the riboflavin deficiency is associated with increased levels of total non-haem iron stored in the liver tissues (Fig. 35). The equation of the regression line was:

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Figure (35):

Relationship between Non-Haem-Fe Storage in the Liver Tissue and Riboflavin Status

Figure 35:

Relationship between non-haem iron storage in the liver tissue and riboflavin status

Estimated non-haem iron in liver tissue from control (■), pair-fed (○) and riboflavin-deficient rats (●) were plotted against the appropriate EGR-AC ratios. Each point represent a liver tissue sample from one animal on which duplicate estimates were done.

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Relationship between Non-Haem Iron Storage in the Liver Tissue and Riboflavin Deficiency

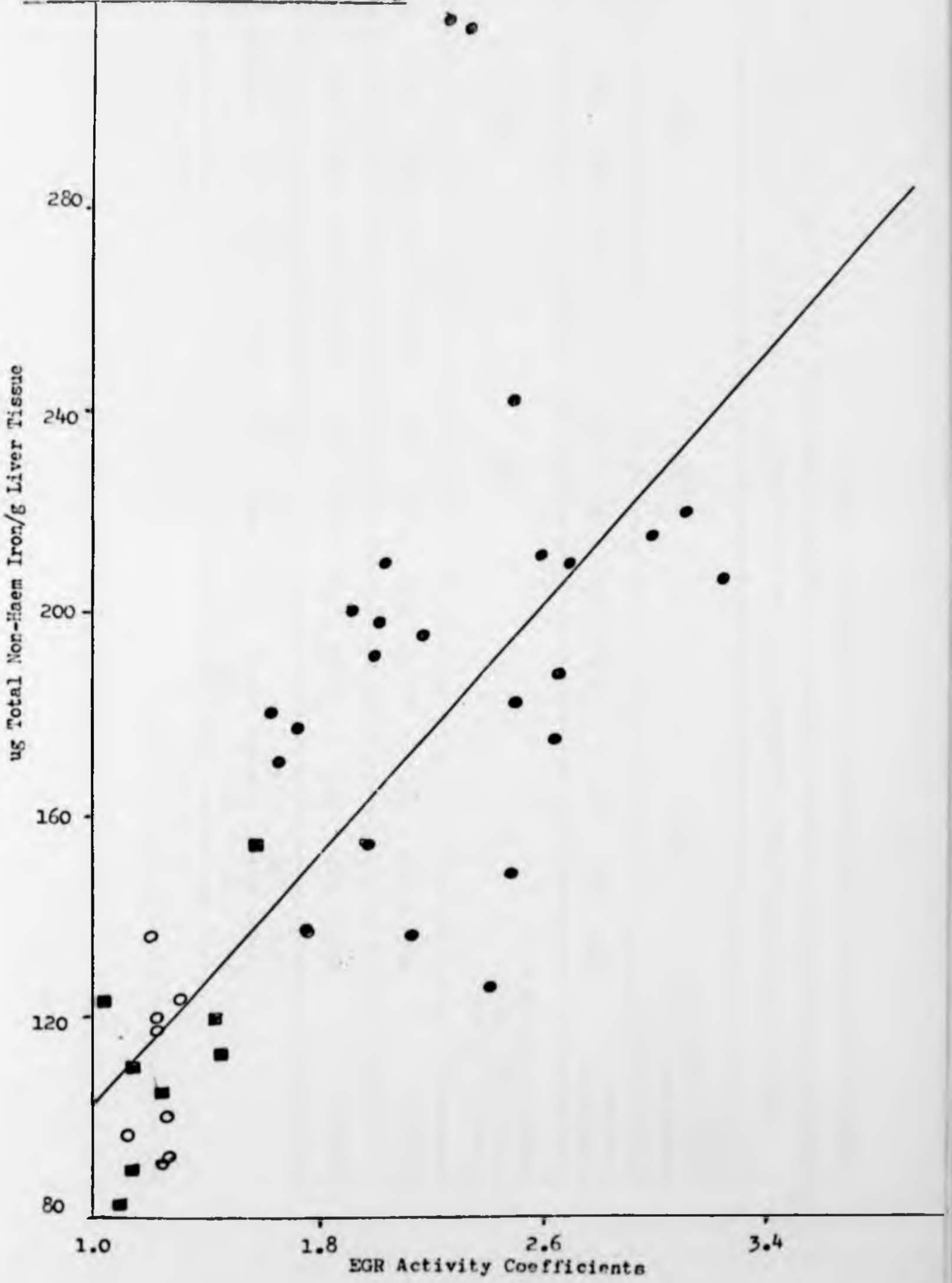

Table 14:

Iron Absorption in Riboflavin Deficiency in Rats

	0 - 5 days				20 - 25 days				40 - 45 days			
	Fe intake ug/d	Fe passed ug/d	Fe absorbed ug/d	% Fe absn.	Fe intake ug/d	Fe passed ug/d	Fe absorbed ug/d	% Fe absn.	Fe intake ug/d	Fe passed ug/d	Fe absorbed ug/d	% Fe absn.
Control	2320	1967	353	15.22	3220	2295	925	28.73	3504	2608	896	25.57
Pair-fed	1894	1559	335	17.68	1989	1689	300	15.08	2320	2053	267	11.51
% reduction in Fe absn. by pair-fed compared to controls	5%				68%				70%			
Riboflavin-deficients	1894	1667	227	11.99	1989	1808	181	9.1	2320	2170	150	6.47
% reduction in Fe absorption by def-itients compared to controls	36%				80%				83%			

Average of diet eaten and stool produced over 5 days were measured in 4 animals on control, pair-fed and riboflavin-deficient diets. Iron intake (ug/day) is calculated by multiplying 189.4 (ug Fe/g diet) x food intake. Faecal loss was analysed and Fe absorption was calculated by difference.

Figure (36):

Relationship between Stored Ferritin Fe in the Liver Tissue and Riboflavin Status

Figure 36:

Relationship between Ferritin-Fe storage in the liver tissue and riboflavin status

Estimated ferritin iron in liver tissue from control (■) pair-fed (○) and riboflavin-deficient rats (●) as described in the Methods chapter were plotted against the appropriate EGR-AC ratios. Each point represents a liver tissue sample from one animal on which duplicate estimates were done.

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Relationship between Ferritin-Fe Storage and

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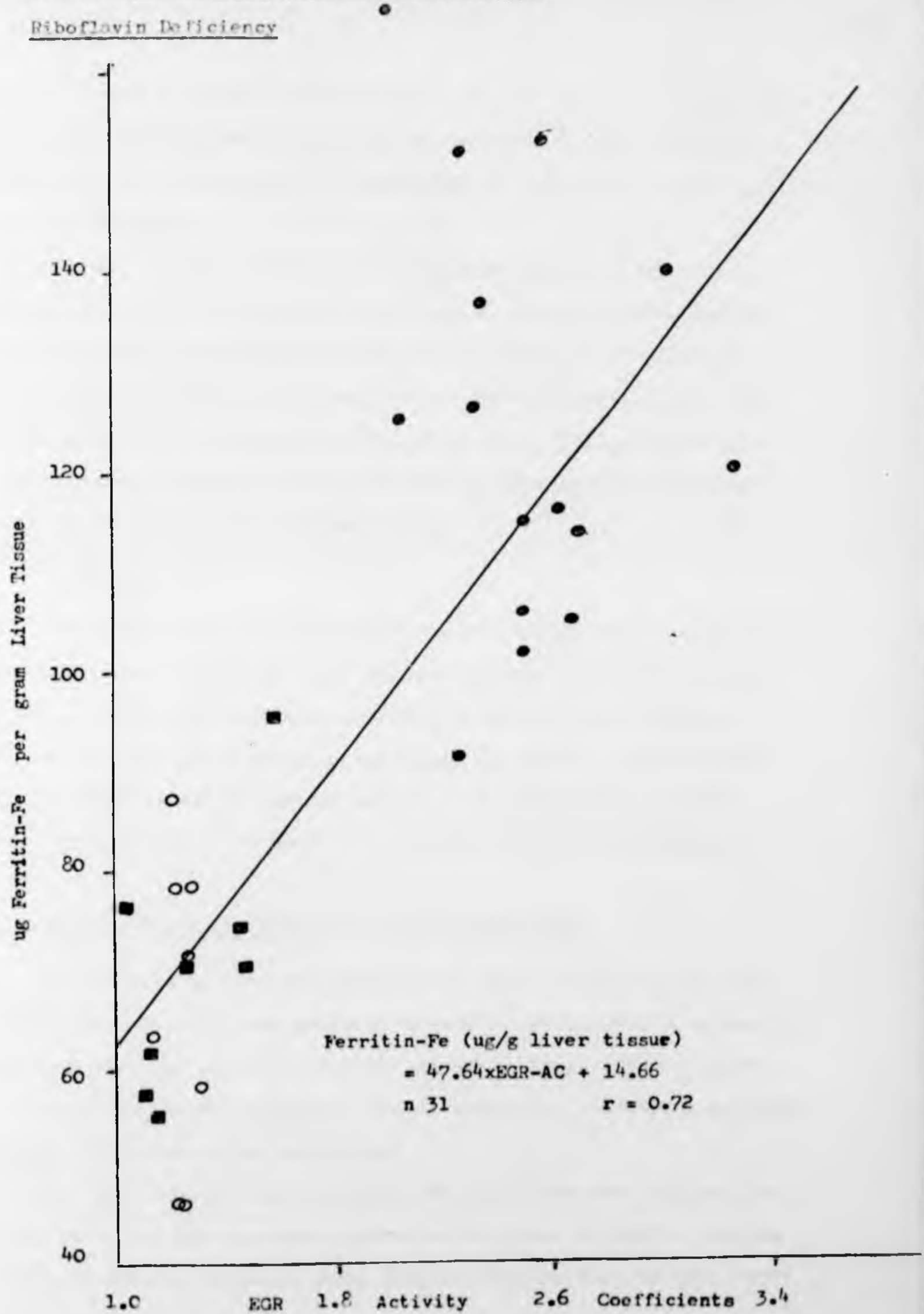

first 25 days and by 70 and 83% between days 40-45. Rats on the pair-fed and riboflavin-deficient diets are smaller, however, and when absorption was calculated as a percentage of intake the results were more interesting.

In the first five days all rats absorbed approximately 15% of the iron in the diet but even at this stage control animals absorbed slightly more than the riboflavin-deficient group. By 20-25 days, control and riboflavin-deficient animals absorbed about 29% and 9% while the pair-fed animals absorbed about 15%. Between 40-45 days control animals absorbed 26%, pair-fed 12% and riboflavin-deficient animals had fallen still further to 6%.

c- Plasma iron

Table (13) shows the means and standard deviations of plasma iron concentrations of control, pair-fed and riboflavin deficient groups. Plasma iron in the riboflavin-deficient group was lower than that found in both control and pair-fed groups ($P<0.001$). Mean plasma iron concentrations of control and pair-fed groups were not significantly different. Figure 34 shows the fall in relationship.

3- Density Gradient Fractionation of Erythrocytes

The results in Part (B) demonstrated that red blood cells from riboflavin-deficient rats appeared to be more susceptible to haemolysis *in-vitro* than red cells from normal animals. These findings posed the question, whether *in-vitro* red cell fragility indicated a shortened *in-vivo* life span of the red cells.

Red cell density increases with the age of the cell (Allison and Burn, 1955) and fractionation studies on the blood of the two biochemically riboflavin-deficient human subjects (Powers and Thurnham, 1976) suggested that there were fewer old cells as would be expected if red cell life span was shortened. Fractionation studies on blood from riboflavin-deficient rats were therefore done to determine whether the vitamin deficiency was increasing the fragility of red cells with age *in vivo* and as a consequence shortening their life span.

a- Influence of riboflavin-deficiency on distribution of red cells within the fractions:

Tables (15) and (16) show haemoglobin concentrations obtained when washed red cells from riboflavin-deficient and control rats were fractionated on a gradient using discrete separations. Red cells get denser as they age but the concentration of haemoglobin is not believed to change as the cell gets older (Allison and Burn,1955). Haemoglobin concentration was therefore assumed to be proportional to the number of red cells in each fraction. Fractionations of red cells from control (9) and pair-fed (10) animals produced very similar red cell distributions and t-tests on the mean haemoglobin concentrations of corresponding fractions in the two groups showed no significant differences (Table 15).

There were significant differences between the haemoglobin concentrations in corresponding fractions when the red cell distributions from riboflavin-deficient animals was compared with that of the control groups (Table 15). However, the distribution of red cells from riboflavin-deficient rats appears to be influenced by the degree of riboflavin-deficiency, therefore a straightforward comparison between the riboflavin-deficient and control groups was not justified as the degree of deficiency was not the same in all the riboflavin-deficient rats. Instead, changes in red cell distribution associated

Table 15:

Density Gradient Fractionation of Red Cells from Control, Pair-Fed, and Riboflavin-Deficient Rats

Density gradient (g/ml)		<1.113	1.113-1.119		>1.119-1.125			>1.125-1.140		
	EGR-AC	F_1	F_2	p< $F_1 \times F_2$	F_3	p< $F_1 \times F_3$	p< $F_2 \times F_3$	F_4	p< $F_1 \times F_4$	p< $F_3 \times F_4$
Controls (9) Mean±SD	1.19±0.07	30.1±12.5	36.1±8.3	N.S.	20.0±8.2	N.S.	0.001	12.0±5.2	0.01	0.05
Pair-fed (10) Mean±SD	1.23±0.23	40.6±13.9	26.1±8.6	0.02	21.0±11.2	0.01	N.S.	10.5±2.2	0.001	0.001
Deficient (20) Mean±SD	1.78±0.35	16.9±14.7	28.5±6.7	0.01	34.7±11.6	0.001	0.05	20.8±8.1	N.S.	0.001
p< Cont×Def.	N.S.	N.S.	0.02		N.S.			N.S.		

Haemoglobin concentration in each of the four fractions is expressed as a percentage of the total recovered The EGR-ACs were measured on unfractionated blood. All data shown are mean±SD and numbers of animals are shown in parentheses. F_1 to F_4 indicate fraction numbers.

Table 16:

Density Gradient Fractionation of Red Cells from Individual Riboflavin-Deficient Rats

Density of cell fractions (g/ml)	<1.113	1.113-1.119	>1.119-1.125	>1.125-1.140	EGR-AC
Deficients (20) Individual Values	51.22	26.00	15.30	6.48	1.28
	42.0"	28.80	12.46	16.71	1.34
	34.49	31.35	25.55	8.59	1.40
	33.92	28.78	23.81	13.49	1.44
	25.10	40.13	20.67	14.09	1.49
	31.43	40.77	16.19	13.69	1.53
	17.50	33.12	31.45	17.93	1.60
	14.54	28.19	35.65	20.90	1.64
	13.08	27.13	38.19	22.17	1.67
	11.25	32.41	42.35	15.32	1.78
	10.15	27.73	42.97	19.15	1.80
	6.94	32.55	37.63	22.88	1.80
	9.54	24.42	49.36	16.68	1.86
	9.08	30.39	38.95	23.54	1.88
	9.41	25.28	40.25	26.22	1.90
	5.93	16.86	48.06	29.12	1.95
	1.90	16.80	45.50	35.80	1.97
	2.59	28.66	41.19	27.56	2.13
	5.24	22.89	42.84	29.03	2.47
	1.20	18.18	44.82	35.80	2.61

Table shows haemoglobin concentrations in individual fractions of red cells following fractionation of washed red cells on a Ficoll/Triosil gradient. Results are listed in order of increasing EGR-AC with riboflavin-deficiency were investigated by determinating the correlation between the EGR-AC of the unfractionated erythrocytes and the proportion of haemoglobin in the respective fractions (Table 16). The correlation between the haemoglobin concentrations in the two lighter fractions and the EGR-AC ratios showed an inverse relationship ($P<0.001$ for both). The equation for the regression lines of fraction 1 (F_1) and fraction 2 (F_2) are:

Haemoglobin % of total (F_1) = (-34.95) x (EGR-AC) + 79.02

where $r = -0.83$; $P<0.001$, $n = 20$ (Fig. 37).

Haemoglobin % of total (F_2) = (-12.71) x (EGR-AC) + 51.11

where $r = -0.66$; $P<0.01$, $n = 20$

That is as riboflavin-deficiency increased, the number of red cells in the less dense fractions decreased. This trend was particularly significant ($P<0.001$) in the fractions containing erythrocytes with densities <1.113g/ml and also for erythrocytes with densities 1.113 - 1.119g/ml. On the other hand, in those fractions containing the heavier red cells, the proportion of haemoglobin i.e. the number of red cells increased as the degree of deficiency increased. Haemoglobin concentrations in both fractions 3 and 4 were directly correlated with the EGR-AC of the unfractionated blood ($P<0.001$). The equations of the regression lines for haemoglobin concentrations in fractions 3 and 4 against EGR-AC were calculated and are shown below:

Haemoglobin % of total (F_3) = 25.46 x (EGR-AC) -10.57

where $r = 0.77$, $P<0.001$ $n = 20$

Haemoglobin % of total (F_4) = 19.79 x (EGR-AC) -14.39

where $r = 0.85$, $P<0.001$, $n = 20$ (Fig . 38).

Figure (37):

Relationship between the circulated young Cells with Densities 1.113g/ml from Rats from Different Groups and Riboflavin Status

Figure 37:

Relationship between the circulated young cells with densities 1.113g/ml from riboflavin-deficient rats and riboflavin status

Measured haemoglobin concentration of erythrocytes with densities 1.113 following fractionation of washed red cells from riboflavin-deficient rats on a Ficoll/Triosil gradient were plotted against the appropriate EGR-AC ratios measured on non fractionated erythrocytes. Each point represent a blood sample from one animal.

* Represent Mean+SD of EGR-AC (horisontal) and Hb concentration (vertical) of the control group.

** As above for the pair-fed group.

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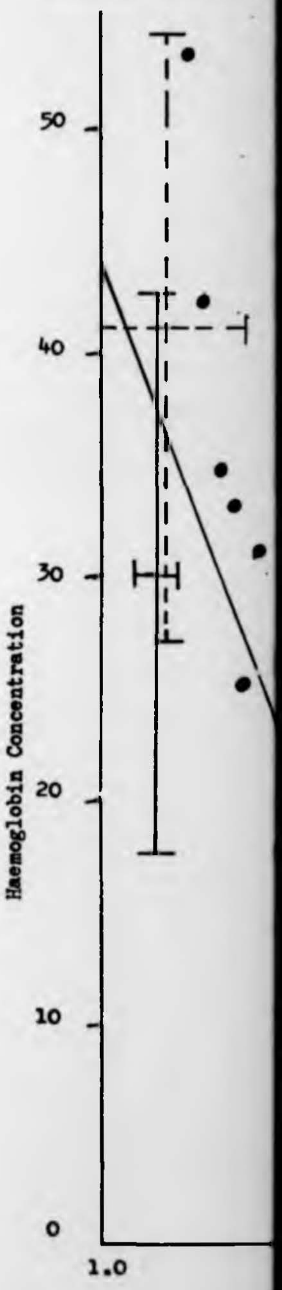

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Relationship between the Circulated Young Cells with Densities <1.113g/ml from Riboflavin-Deficient Rats and Riboflavin Status

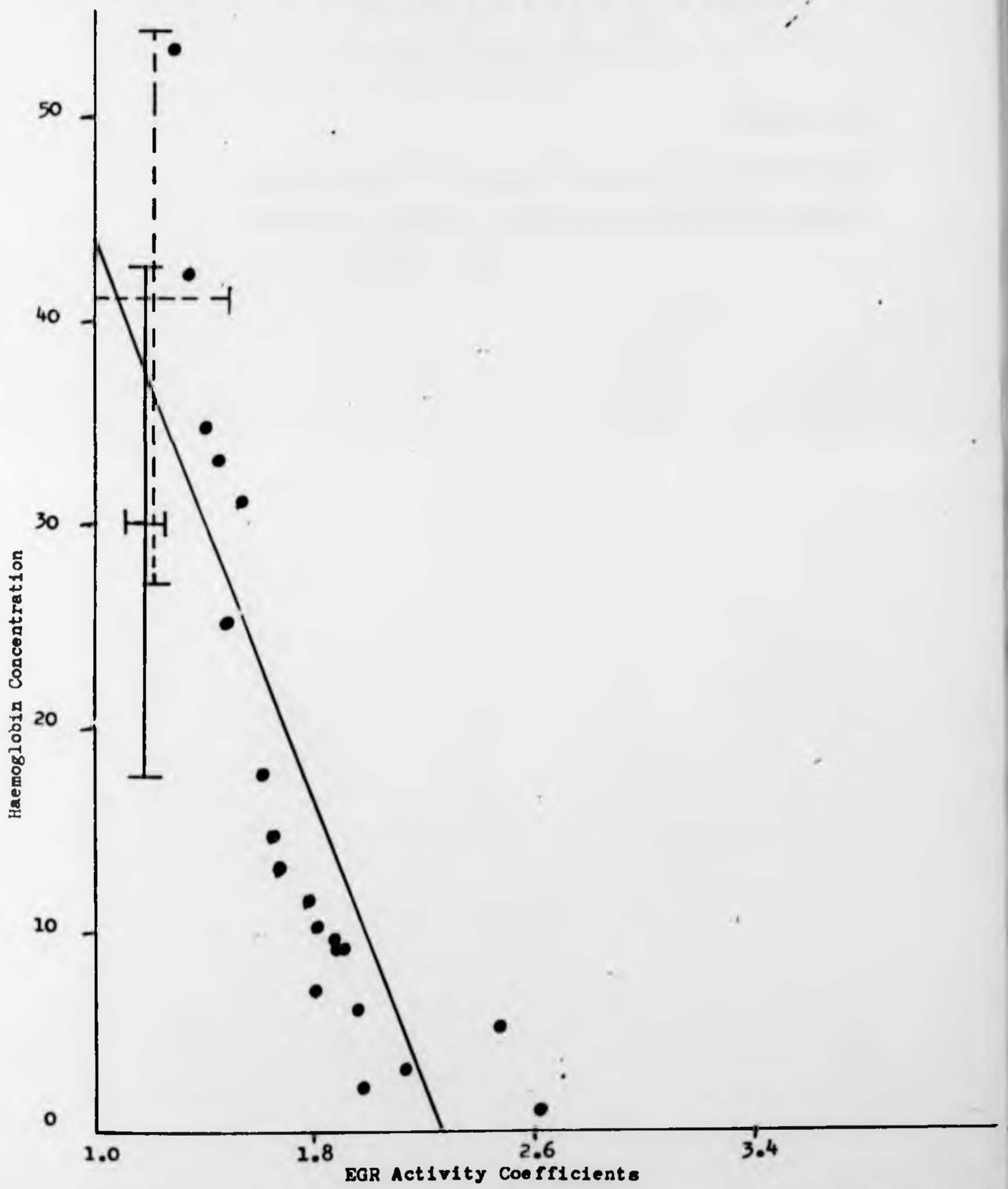

Figure (38):

Relationship between the Circulated Old Red Cells with Densities 1.125g/ml from Rats from Different Groups and Riboflavin Status.

Figure 38:

Relationship between the circulated old red cells with densities densities 1.125g/ml from riboflavin-deficient rats and riboflavin status

Haemoglobin concentration of erythrocytes with densities 1.125g/ml following fractionation of washed red cells from riboflavin-deficient rats on a Ficoll/Triosil gradient were plotted against the appropriate EGR-AC ratios measured on non fractionated erythrocytes. Each point represent a blood sample from one animal.

* Represent Mea±SD of EGR-AC (horisontal) and Hb concentration (vertical) of the control group.

** As above for the pair-fed group.

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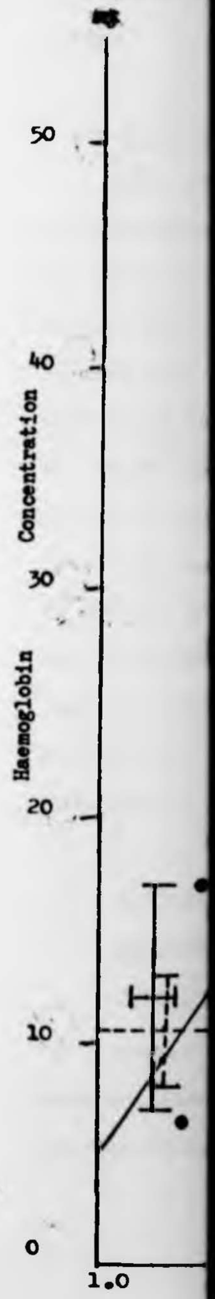

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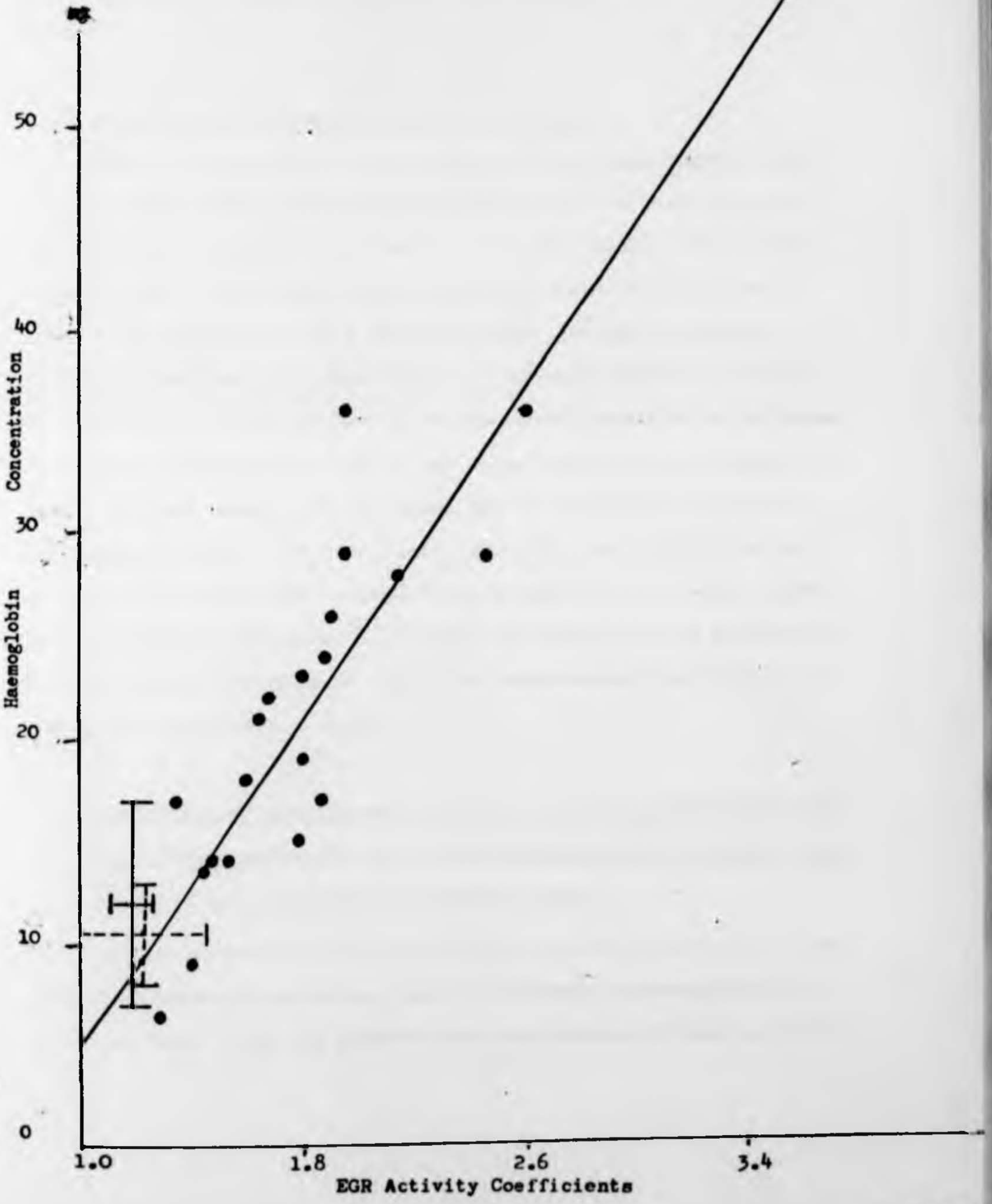
Relationship between the Circulated Old Cells with Densities >1.125g/ml from Riboflavin-Deficient Rats and Riboflavin Status



The data reported here suggested that riboflavin deficiency in rats is associated with fewer young cells and an increased number of old cells.

b- Fragility of red cells within the fraction:

Table (17) shows the effect of both the H_2O_2-generating system and hypotonic saline on the stability of washed red blood cells in the different fractions from control (6) and riboflavin-deficient animals (10). Red blood cells in all four fractions from control animals were haemolysed to a similar extent (19-29%) suggesting that there were no large differences in red-cell fragility between the fractions. Mean values for the haemolysis produced in the three fractions containing the heavier red cells from riboflavin-deficient rats were significantly greater than that in comparable fractions in control animals. The fractions containing the lightest cells from riboflavin-deficient animals were haemolysed to a lesser extent than red cells in the other 3 fractions ($P<0.001$) and the haemolysis produced was very similar in amount to that produced in the same fractions of the control animals.

c - Activities of glutathione reductase, aspartate aminotransferase, glutathione peroxidase and NADPH-methaemoglobin reductase in the four red cell fractions of different ages:

Washed erythrocytes from control and riboflavin-deficient rats were fractionated by stepwise, density-gradient centrifugation into four fractions. EGR was measured with and without FAD and aspartate-

Table 17:

Effect of Riboflavin Deficiency on Susceptibility of Red Blood Cells of Different Densities to Haemolysis in Presence of a Peroxide-Generating System and Hypotonic Saline System

	EGR-AC	H_2O_2-Generating System									Hypotonic Saline System								
		< 1.113 (1)	1.113-1.119 (2)	P< (1)×(2)	>1.119-1.125 (3)	P< (1)×(3)	P< (2)×(3)	>1.125-1.140 (4)	P< (1)×(4)	P< (3)×(4)	< 1.113 (1)	1.113-1.119 (2)	P< (1)×(2)	>1.119-1.125 (3)	P< (1)×(3)	P< (2)×(3)	>1.125-1.140 (4)	P< (1)×(4)	P< (3)×(4)
Control	1.20± 0.0'	18.6± 4.0	25.2± 3.7	N.S.	22.3± 3.3	N.S.	N.S.	22.2± 4.7	N.S.	N.S.	18.4± 6.0	21.4± 11.2	N.S.	20.9± 9.7	N.S.	N.S.	29.1± 14.2	N.S.	N.S.
Riboflavin Deficient	1.99± 0.33	21.2± 4.4	65.5± 12.8	0.001	65.1± 13.0	.001	N.S.	72.2± 11.8	.001	N.S.	18.7± 3.3	65.0± 10.4	.001	63.8± 11.0	.001	N.S.	69.)± 8.3	.001	N.S.
P< between Con.&Def.	.001	N.S.	.001		.001			.001			.001	.001		.001			.001		

Washed red blood cells were prepared as described in tables 15 and 16 from 6 controls and 10 riboflavin-deficient rats. Aliquots from fractions (1), (2), (3), and (4) respectively were exposed to either peroxide-generating system and/or hypotonic saline for 45 min. before centrifuging and measuring haemoglobin in the supernatent. Total haemolysis was determined by exposing the red cells to distilled water and haemoglobin in the supernatent is expressed as a percentage of the total. All results are shown as mean±SD.

aminotransferase (AST) was measured with and without pyridoxal phosphate (PP).

The fall in activity of EGR both with and without FAD, was quite marked between the two lightest fractions, but, thereafter, the fall was more gradual. The mean EGR-AC in the control animals increased from 1.11 in the lightest cells to 1.31 in the heaviest cells, while comparable values in red cells from deficient animals were 1.26 and 1.79. The results suggest therefore that there was both a fall in EGR-activity as well as an increasing loss of FAD as the cells become heavier.

In contrast to EGR, AST activity falls more gradually as the cells increase in density. In the control animals, the overall fall in activity between the lightest and heaviest cells was approximately 50% while in cells from the riboflavin-deficient groups; it fell by 60-70%. There was no significant increase in the AST-ACs with increasing cell density in either the control or riboflavin-deficient groups (Table 18).

Glutathione peroxidase activity in cells from control animals fell in a similar manner to AST activity as cell density increased. By contrast, red cells from riboflavin-deficient animals showed no fall in activity. In a similar manner the activity of NADPH-methhaemoglobin reductase fell approximately 5-fold between the lightest and heaviest fractions of control animals, but in red cells from the riboflavin-deficient group the drop was only 2-fold. In addition only NADPH-methaemoglobin reductase activity in the heaviest cells in the riboflavin-deficient group was significantly lower than that in the lightest fraction ($P < 0.05$).

Table 18:

Activity of Defense Mechanism Enzymes in Erythrocytes from Control and Riboflavin-Deficient Rats Separated into Fractions of Different Mean Ages

Density gradients g/ml			<1.113	1.113-1.119	>1.119-1.125	>1.125-1.140
Controls	EGR (5) Activity	B	8.44±2.00	2.86±1.74	1.51±0.39	1.72±0.39
		S	9.39±2.19	2.78±0.99	2.04±0.[illegible]	2.24±0.98
	AST (4) Activity	B	5.90±0.35	3.75±0.80	2.58±1.13	2.28±0.96
		S	6.04±1.64	4.23±1.50	3.25±1.58	2.75±1.24
	EGP (3) Activity		7.25±0.44	4.35±0.38	3.44±0.43	2.90±0.21
	NADPH-MR Activity (3)		3.35±0.65	3.19±0.06	2.05±0.29	0.73±0.05
Deficients	EGR (8) Activity	B	6.07±1.81	1.63±6.7	0.98±0.39	0.76±0.28
		S	7.06±1.60	2.72±1.31	1.80±0.78	1.36±0.39
	AST (8) Activity	B	5.96±1.80	3.05±0.93	2.38±0.64	1.70±0.73
		S	7.65±2.13	3.31±0.95	2.65±0.65	1.76±0.77
	EGP (5) Activity		7.62±1.05	8.92±2.24	7.74±2.18	7.16±1.65
	NADPH-MR Activity (5)		3.68±0.82	3.43±1.16	3.05±1.44	1.89±1.12

Data shown are mean±SD of different enzyme activities (i.u./g Hb) measured in haemolysate from each of the four fractions from control and riboflavin-deficient rats. Figures in parentheses indicate the number of a animals used. B = Basic activity and S = Stimulated activity.

d - Miscellaneous experiments

i - Weekly estimation of EGR-AC and glutathione peroxidase activity:

EGR-AC and peroxidase activity were measured at weekly intervals over four weeks in tail blood taken from two control, two pair-fed and two riboflavin-deficient rats. The first samples were collected at seven days. The results in Table (19) showed that EGR-AC and peroxidase activity in riboflavin-deficient rats increased gradually over the first fourteen days and more rapidly in the second period. There were no changes in either measurement in the control or pair-fed rats over the same period. The relationship between EGR-AC and peroxidase activity is shown in Figure 39 where results from all three groups of animals are combined.

ii - Peroxidase activity and endogenous formation of total peroxides in liver tissue:

Both peroxidase activity and total peroxides were assayed in liver tissue from control, pair-fed and riboflavin-deficient rats stored at -20°C prior to analysis. Means and standard deviations of the data obtained in these two assays are reported in Table 20 together with their EGR-AC of the animals used. Glutathione peroxidase activity was found to be three times higher in livers from riboflavin-deficient rats than those of control or pair-fed animals ($P<0.001$). Total peroxides in livers from deficient rats was higher than in control ($P<0.01$) and pair-fed ($P<0.02$) animals. Glutathione peroxidase activity was directly correlated with EGR-AC and the equation for the regression lines was calculated to be:

Table 19:

Effect of increasing riboflavin Deficiency on Erythrocyte Peroxidase Activity

		First Week		Second Week		Third Week		Fourth Week	
	Rat No	1	2	1	2	1	2	1	2
Control	EGR-AC	1.06	1.16	1.12	1.18	1.10	1.12	1.20	1.12
	Peroxidase *	45.2	52.2	52.9	52.2	44.7	51.5	57.2	50.0
Pair-fed	EGR-AC	1.38	1.21	1.31	1.20	1.30	1.20	1.31	1.26
	Peroxidase *	56.3	49.4	52.9	48.4	52.2	43.5	52.4	57.4
Deficient	EGR-AC	1.42	1.34	1.50	1.78	2.06	2.26	2.64	2.48
	Peroxidase *	57.7	54.7	61.2	75.0	87.9	98.4	115.2	107.4

Measurement of EGR-AC and peroxidase activity were done on whole blood collected from the rats' tail at 7 days and weekly intervals for 4 weeks. Data presented are means of duplicate measurements on 2 animals.

* Data for peroxidase activity shown as i.u./g Hb.

Relationship between EGR-AC and Glutathione Peroxidase Activity

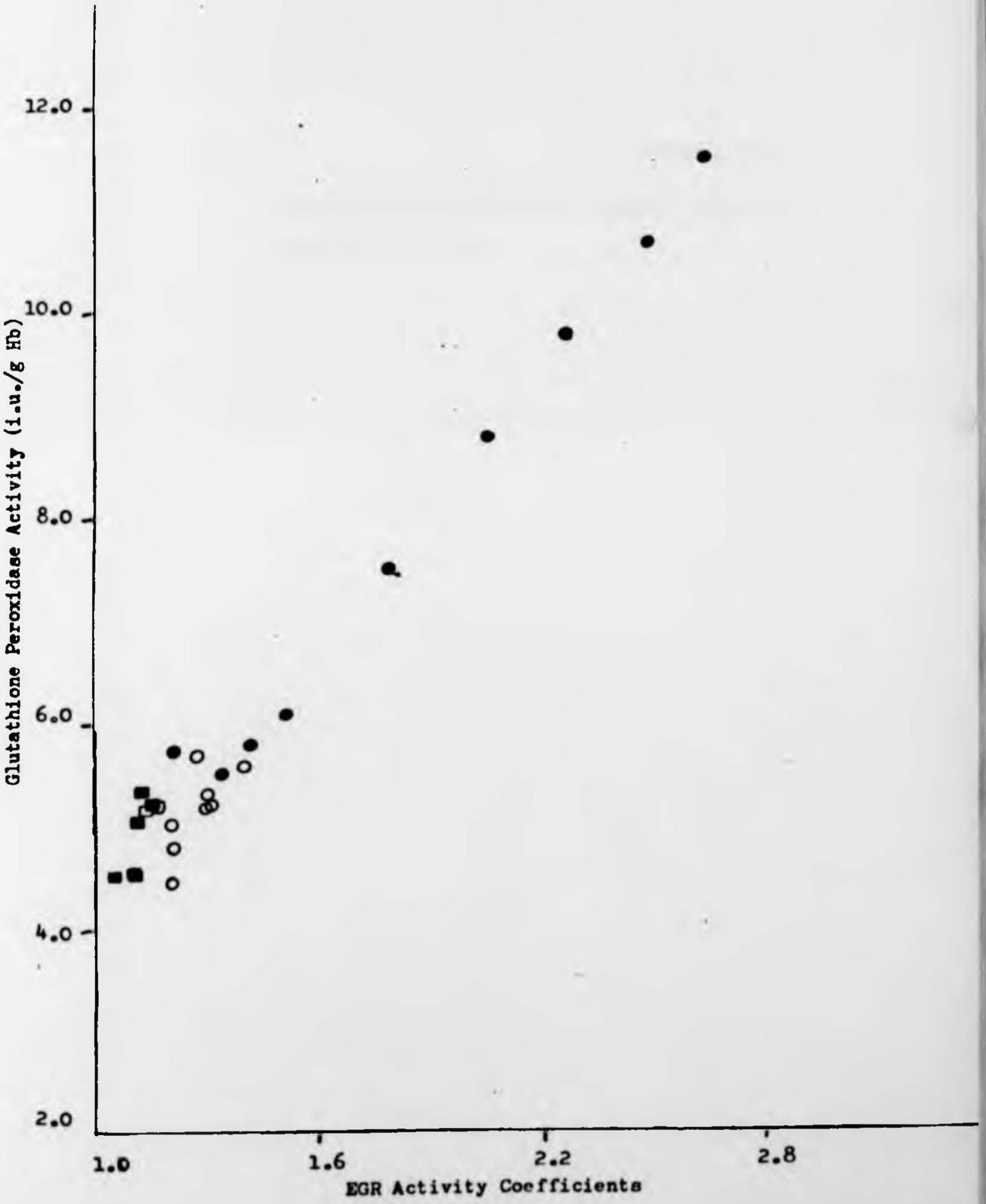


Figure (39):

Relationship between EGR-AC and Glutathione Peroxidase Activity

Figure 39:

Relationship between EGR-AC and glutathione peroxidase (EGP) activity

EGR-AC and EGP activity were measured on haemolysates from 2 controls (■), 2 pair-feds (○) and 2 riboflavin-deficient rats (●) prepared from tail blood samples collected weekly over a period of 4 weeks during which riboflavin deficiency was increasing.

Table 20:

Erythrocyte Glutathione Reductase Activity Coefficients and Glutathione Peroxidase (GP) Activity and Total Peroxides in Liver Tissue from Control, Pair-Fed and Riboflavin-Deficient Rats

Table 20:

Erythrocyte Glutathione Reductase Activity Coefficients and
Glutathione Peroxidase (GP) Activity and Total Peroxides in Liver Tissue from
Control, Pair-Fed and Riboflavin-Deficient Rats

	Controls (3)	Pair-feds (3)	Riboflavin-Deficients (7)	p< Cont×Def.	p< Pair-fed×Def.
Glutathione peroxidase activity (i.u./g)	33.43±5.34	36.56±12.11	98.23±10.00	0.001	0.001
Total peroxides m mol/g	17.36±1.71	20.31±0.80	42.68±10.87	0.01	0.02
EGR-AC	1.09±0.01	1.19±0.02	2.27±0.19	0.001	0.001

Livers were stored at -20°C prior to analysis for 1 to 2 weeks. Data presented are mean±SD. Number of animals used are shown in parentheses.

Glutathione peroxidase activity (i.u./g.tissue) = 53.13x(EGR-AC)-23.85

where $r = 0.94$, $P<0.001$, $n = 13$

The equation for regression line for total peroxides and EGR-ACs was:

Total peroxides (mmol/g tissue) = 21.24 x (EGR-AC) - 5.46

where $r = 0.88$, $P<0.001$ $n = 13$

These associations suggest that poor riboflavin status in rats is associated with an increased activity of glutathione peroxidase and an accumulation of total peroxides in the liver tissues.

c - Galactoflavin-induced riboflavin-deficiency in rats

It is well known that galactoflavin induces riboflavin-deficiency by competitively displacing riboflavin from the tissues (Lane, Alfery, Mengel, and Doherty, 1964, Prosky, Burch, Berjrablayas Lowry and Combs, 1964) /. A few experiments were done on adult rats in which galactoflavin was given to see whether the riboflavin deficiency produced was similar to the one obtained in the young animals by witholding only riboflavin.

Twelve old rats (398-476g range weight) divided into three groups; 3 controls (437 ± 55g), 3 pair-fed (445 ± 36g) and 6 on a galactoflavin-supplemented riboflavin-deficient diet (478 ± 44g). After eight weeks the rats receiving the galactoflavin-supplemented riboflavin-deficient diet showed a reduction in their weight of about 26%. Four of these rats showed severe riboflavin-deficiency associated with severe anaemia. One of these animals died before killing. The results in Table (21) show that the following measurements were significantly lower in blood from these riboflavin-deficient rats, PCV, erythrocyte counts, plasma Fe, thyroxine, reduced glutathione and methaemoglobin (percentage of total haemoglobin) than in control and pair-fed animals. Also in these

Table (21):

Effect of Galactoflavin-Induced Riboflavin Deficiency on Various Measurements in Erythrocytes and Plasma

		Controls (3)	Pair-fed (3)	Deficients (5)*
Initial Body Weight (g)		437±55	445±36	474±44
Body weight on killing (g)		511±28	469±36	351±88
Packed Cell Volume (PCV)		48.3±3.5	50.7±3.1	38.4±17.3
Red Blood Cells (RBCs) RBCsx10^{12}/litre Blood		5.23 ± 1.02	6.18 ± 0.39	4.63 ± 2.15
EGR-AC		1.16±0.02	1.25±0.08	2.27±0.45
Plasma Fe (ug Fe/100ml plasma)		242.3±38.7	210.7±25.7	158.8±81.8
Thyroxine (ug/100ml plasma)		5.46±1.29	5.53±0.61	2.24±0.79
GSH (umol GSH/litre Blood)		256.6±44.8	257.3±39.0	87.7±20.8
Methaemoglobin (% of total Hb)		5.60±0.50	4.79±0.54	36.16±6.45
Fractionation (Hb concentration)	<1.113	28.12±7.80	36.47±3.31	34.43±13.26
	1.113-1.119	42.30±7.80	34.62±4.96	32.09±6.99
	>1.119-1.125	17.01±1.81	12.47±0.99	16.60±4.23
	>1.125-1.140	12.36±9.45	16.45±3.92	16.95±7.92

Means±SDs are shown for various measurements. No of rats in each group are in parentheses. Galactoflavin was adminstered mixed with the riboflavin deficient diet (22mg/kg diet). Red cell fractions were prepared using Ficoll/Triosil gradient and the densities shown indicate the position of the red cells in the fractionation tube.

riboflavin-deficient animals, concentrations of methaemoglobin were raised but sulfahaemoglobin concentrations were unaltered by comparison with the controls.

When red blood cells from control (3), pair-fed (3) and galactoflavin-induced, riboflavin-deficient rats (5) were fractionated into cells of different densities, an interesting result was obtained. The proportion of young cells density (<1.113g/ml) was significantly higher than that of the old cells (densest). This was the opposite finding to the one obtained when red blood cells from young riboflavin-deficient rats were fractionated.

In many cases the effect of the galactoflavin-containing diet on the haematological measurements made in the old rats was much greater than that of the riboflavin-deficient diet done in young animals.

Part E: THYROID HORMONES AND RIBOFLAVIN DEFICIENCY

The results in Table 10 in Part B suggest that riboflavin has either a direct inhibitory effect on the activity of the xanthine oxidase or may inactivate the superoxide oxygen which is initially generated by the H_2O_2-generating system in-vitro. The net result of the action of thyroxine on xanthine oxidase in-vitro was a reduction in the amount of H_2O_2 produced by the H_2O_2-generating system and may account for the less erythrocyte haemolysis in the presence of thyroxine. In addition, however, the haemolysis produced by hypotonic saline was reduced (Table 11) when thyroxine was present with the red cells from riboflavin-deficient rats. Thus thyroxine may directly protect red cells from riboflavin-deficient rats against hypo-osmolarity.

Riboflavin-deficiency is associated with hypothyroidism Rivlin et al. (1968) therefore it was decided to investigate whether thyroxine given in-vivo could in any way influence the subsequent in-vitro susceptibility to haemolysis of erythrocytes from riboflavin-deficient rats.

Weanling Wistar rats (54) were divided into five groups fed the following diets: control (9) riboflavin-deficient (15) iodine-deficient (12) and pair-fed groups for the two deficient diets respectively (9,9).

1-Effect of Iodine Deficiency on the Weight Gain, Food Consumption and Riboflavin Status:

The average weight gain of rats on the iodine-deficient diet over the first 12 weeks is shown in Figure 40. Weight gain increased until it reached 167g (SD 13.9) in about nine weeks, after which a gradual loss in weight was noted. Weight gain of rats on the iodine-deficient diet was approximately three times that of riboflavin-deficient rats over the first 8-9 weeks and half that of control animals. The weight gain of rats fed the riboflavin-deficient diet behaved normally. Rats on the iodine-deficient diet were killed at least three weeks after the observed reduction in weight gain. Rats on the control diet showed abnormal growth in the first four weeks and their condition was diagnosed by the Animal House Superintendant as pneumonia. They recovered without any treatment and weight gain subsequently took place as usual. Since their initial weight gains were unusual, growth data were not used in Fig.39 for comparison with the experimental groups, but when blood was analysed later the composition did not differ from control results obtained in other experiments, therefore this data is used in Table (22).

Food consumption for rats on the iodine-deficient diet was 13 - 16g/rat/day over the first eight weeks, which then gradually decreased during the third month until they were killed. Food consumption during the last four weeks was; 16.2 ± 2.7, 13.8 ± 2.2, 11.2 ± 1.15 and 10.4 ± 2.7 grams per rat per day for weeks 9, 10, 11 and 12 respectively.

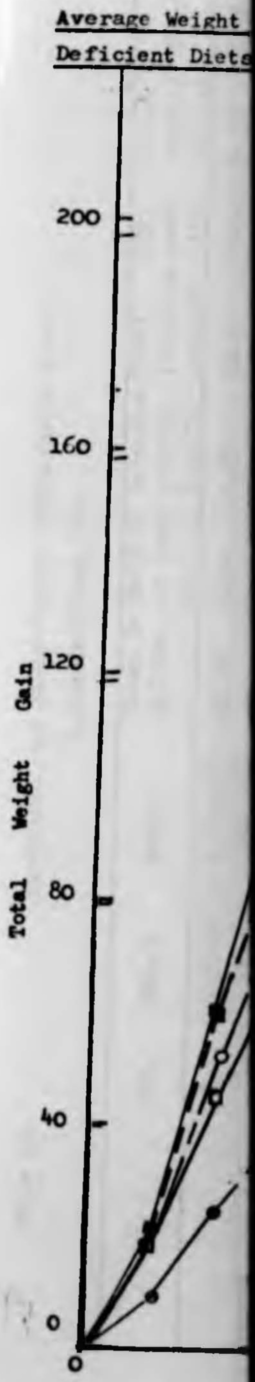
Figure (40):

Average Weight Gain of rats on Restricted and Iodine-Deficient Diets over a Period of 12 Weeks

Figure 40:

Average weight gain of rats on restricted and iodine-deficient diets over a period of 12 weeks

Points represent mean weight gain (g) measured over 12 weeks for iodine-deficient rats (□——□) and their pair-feds (■- - - -■). Points on the transparent overlay represent average weight gain of rats on control (■——■), pair-fed (○- - -○) and riboflavin-deficient rats (●——●) over a period of 35 days for comparison.



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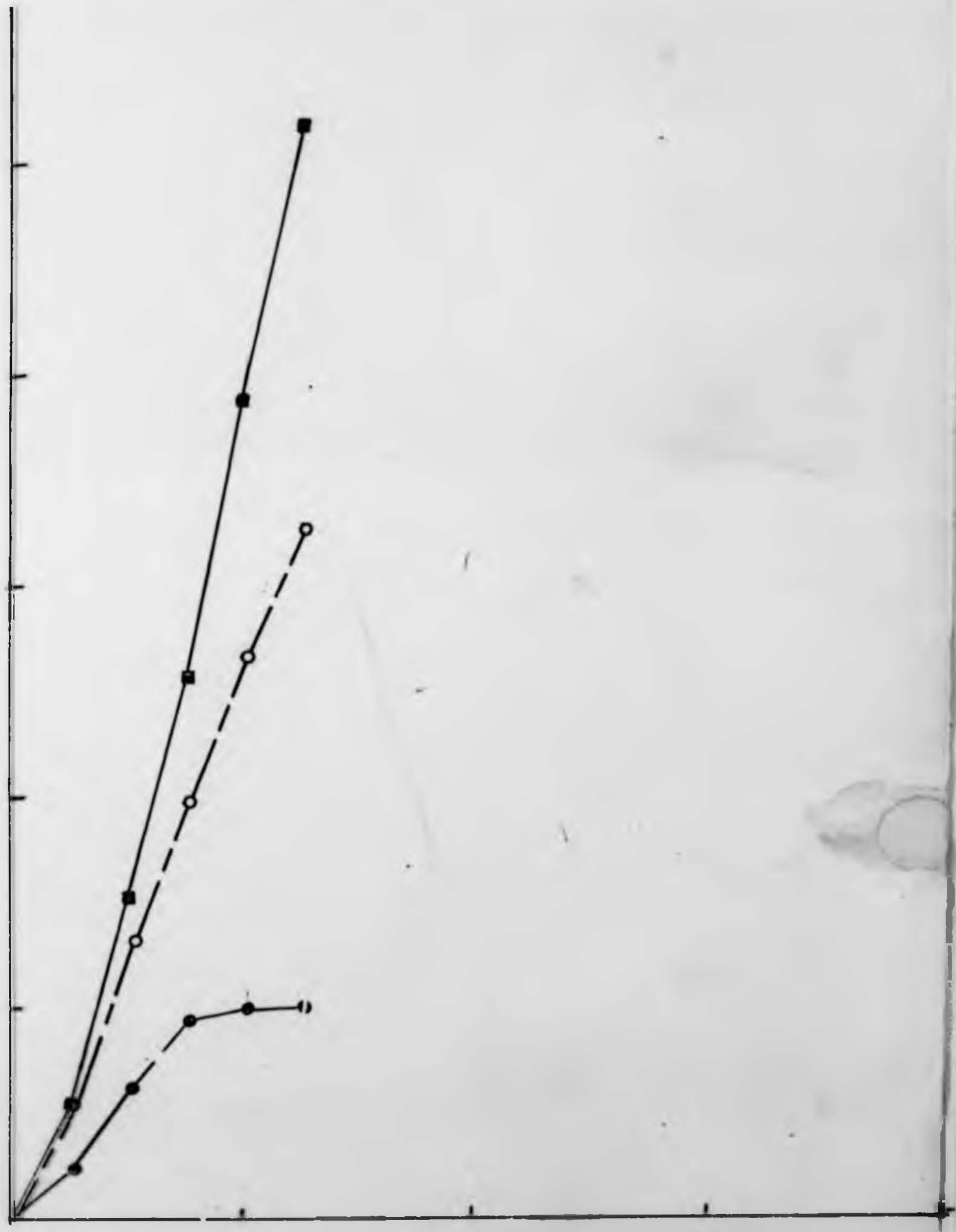
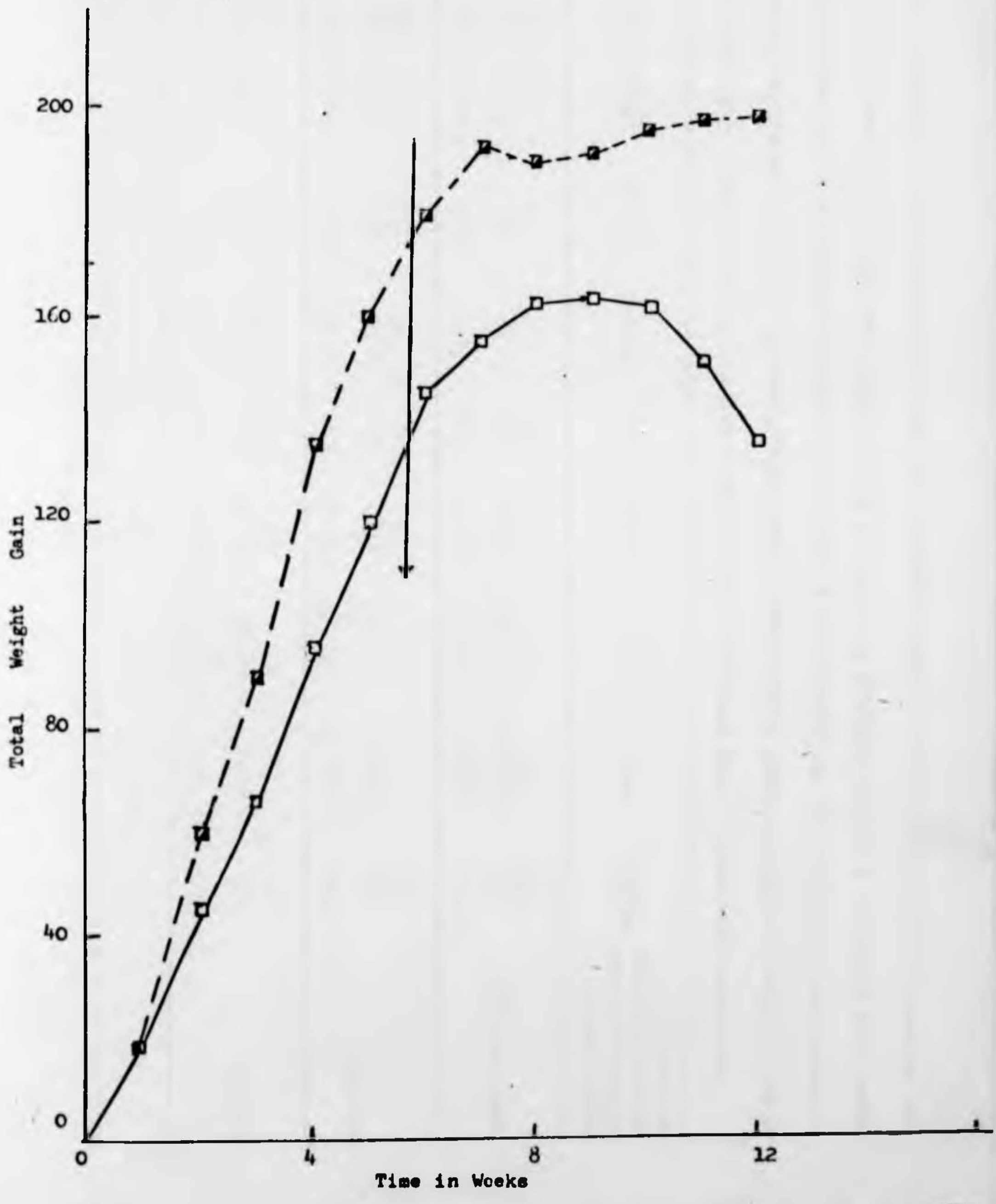


Average Weight Gain of Rats on Restricted and Iodine-Deficient Diets over a Period of 12 Weeks



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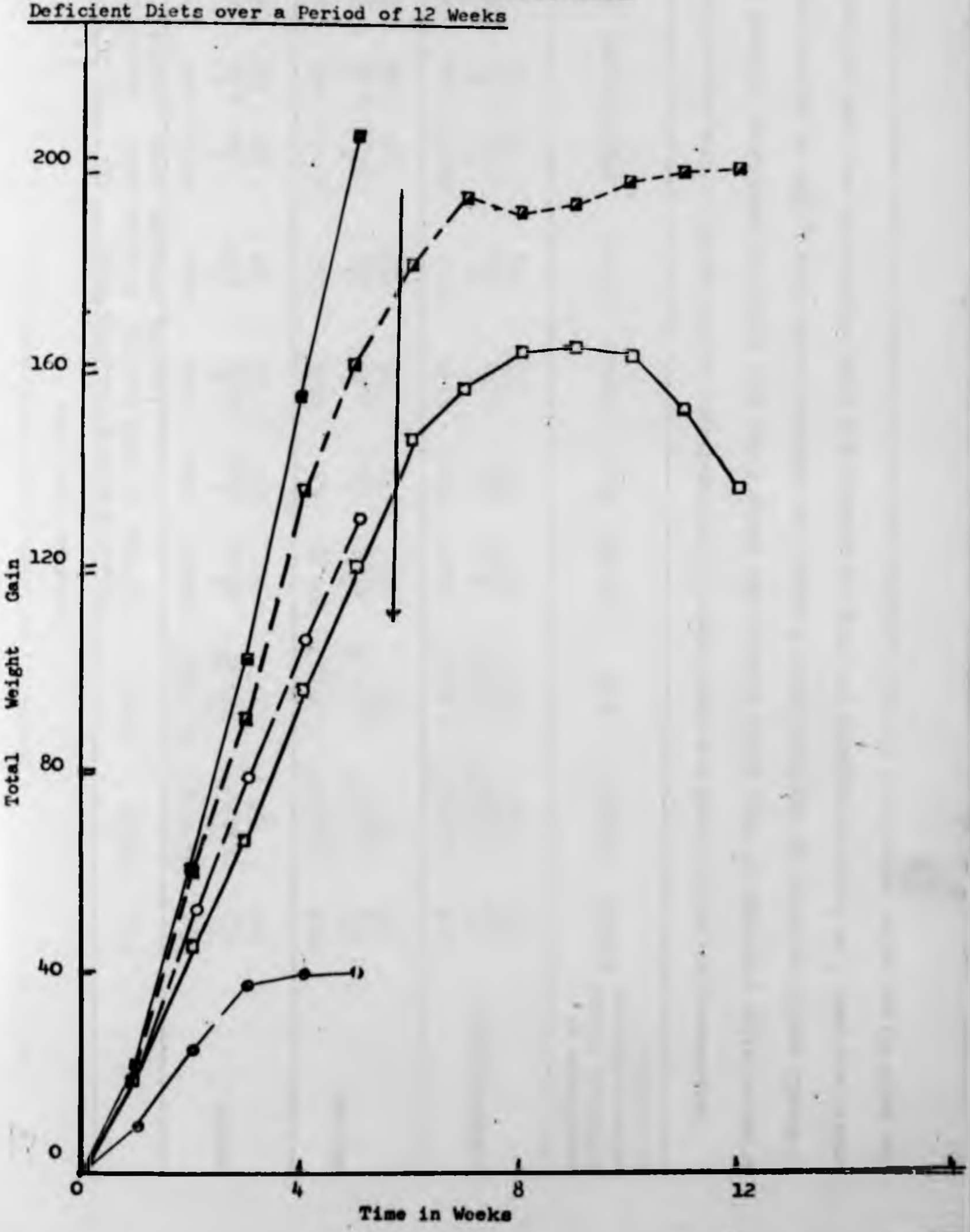

Table 22: Biochemical and Haematological Measurements Done on Iodine-Deficient Rats

	PCV.	RBCs/1	WBCs/1	Plasma Fe ug/100ml	MetHb % of total Hb	Peroxidase Activity i.u./gHb	Total Peroxides umol/gHb	GSH umol/ litre	Liver Weight/ 100gbwt	EGR-AC..
Control	49.5 ± 5.0 (6)	5.76×10^{12} ± 0.55×10^{12} (3)	10.73×10^{9} ± 1.58×10^{9} (3)	252.67 ± 22.12 (3)	6.14 ± 2.25 (3)	4.95 ± 0.30 (3)	10.6 ± 1.4 (3)	432.12 ± 9.84 (3)	4.70 ± 0.30 (3)	1.18 ± 0.10 (9)
Pair-fed	49.8 ± 4.5 (6)	6.00×10^{12} ± 0.69×10^{12} (3)	10.79×10^{9} ± 2.52×10^{9} (3)	244.33 ± 51.05 (3)	8.51 ± 1.86 (3)	4.88 ± 2.54 (3)	12.9 ± 0.3 (3)	353.79 ± 20.68 (3)	4.85 ± 0.25 (3)	1.27 ± 0.10 (9)
Iodine-deficient	37.4 ± 4.3 (9)	4.88×10^{12} ± 0.47×10^{12} (9)	8.18×10^{9} ± 3.27×10^{9} (9)	169.37 ± 32.38 (9)	18.99 ± 9.87 (6)	8.40 ± 1.30 (3)	32.9 ± 6.2 (3)	289.57 ± 57.61 (3)	3.10 ± 0.10 (5)	1.53 ± 0.19 (9)
Significance of difference between iodine-deficients and controls	P<0.001	P<0.05	N.S.	P<0.001	N.S.	P<0.05	P<0.01	P<0.05	P<0.001	P<0.001

Measurements were made on blood from those animals in the experiment in which control animals received thyroxine or FAD or saline injections 24 hours before killing, (see tables 24 and 25). Figures in parentheses indicated the number of animals used to calculate the statistic. Where 9 animals are indicated results from T_4, FAD and saline-injected animals were used; 5 or 6 animals indicates that data was obtained from blood samples which were used for fractionation (see table 26) and saline controls and 3 animals indicates that data was obtained from bloods which were fractionated

The mean and standard deviation of the EGR-AC of rats on the iodine-deficient diet was 1.53 ± 0.19 (9) which was significantly higher than that of the control animals ($P<0.001$) at the time of killing. Iodine deficiency was also found to increase the activity of erythrocyte peroxidase (Table 22) which showed a significant increase above that of control animals ($P<0.05$). In addition there was also an increase in total erythrocyte peroxides ($P<0.01$). This increased peroxidase activity in the iodine-deficient erythrocyte was not influenced by either 100ug T_4 or 1mg FAD both given (i.p.) in 0.1ml physiological saline solution 24 hours previously.

Unfortunately plasma thyroxine concentrations were not estimated in the blood from any of the rats in the experiment as the method kits used to assay thyroxine became unobtainable when Precision Assays ceased trading. It was therefore an assumption that keeping rats on the iodine-deficient diet and including 0.1% propyl-thiouracil in the drinking water from day 40 onwards would produce hypothyroidism.

In general the clinical condition of the iodine-deficient rats when killed was not as bad as that of the riboflavin-deficient rats. Rats on the iodine-deficient diet showed very little brown colouration of the fur, or changes in the appearance of the nose area, lips and limbs. Some brown pigmentation was present on the abdomen and the fur was yellow, moist and matted. Stools of iodine-deficient rats were small and surrounded by a viscous, deep yellow material. Iodine-deficient rats were generally unwilling to move, they lost their appetite and appeared sleepy all the time.. The weight of livers of iodine-deficient rats was about 34% smaller than those of controls. The mean weight of the livers was 3.1 ± 0.1g (5) which compared with a weight in the control animals of 4.7 ± 0.3g (5). The kidneys were also smaller by about 27%. The surface of the kidneys were rough in appearance and darker in colour than the controls.

2- Concentrations of Thyroxine in Plasma from Riboflavin-Deficient Rats:

Figures (~~40 and~~ 41) and Table (23) show the concentrations of the plasma thyroxine in young and old, control, pair-fed and riboflavin-deficient animals. The young rats used for these assays were the ones routinely used for studies on riboflavin-deficiency. The old rats were mature animals which had refected and galactoflavin was subsequently added to the riboflavin-deficient diet (22mg/kg) when the animals were about 450g. In both groups, young and old rats, thyroxine concentrations were found to be inversely correlated with the EGR-AC ($P<0.001$) the regression equations of both young and old rats respectively were as follows:

Young Rats:

$$\text{Plasma thyroxine } (\mu g/100ml) = (-6.57)\,(\text{EGR-AC}) + 22.57$$

$$r = -0.73,\ P<0.001,\ n = 27$$

Old Rats:

$$\text{Plasma thyroxine } (\mu g/100ml) = (-2.61) \times (\text{EGR-AC}) + 8.28$$

$$r = -0.83;\ P<0.001\ ;\ n = 13$$

The mean and standard deviation of plasma thyroxine concentrations in the different groups are reported in Table 23. Plasma thyroxine concentrations in old rats on control diets were significantly less than those in young animals. The effect of riboflavin-deficiency on concentrations of thyroxine in the plasma of the

Figure (41):

Relationship between Thyroxine Concentrations and Riboflavin-Deficiency in Young and Old Rats

Figure 41:

Relationship between thyroxine concentration and riboflavin-deficiency in young and old rats

Thyroxine concentrations (ug/100ml plasma) measured by the radioimmunoassay method described in the Methods section in control (■), pair-fed (○) and riboflavin-deficient rats (●) were plotted against the appropriate EGR-AC ratios.

(A) represents the regression line of the regression equation from results on young rats.

(B) represents the regression line of the regression equation obtained from results on old rats.

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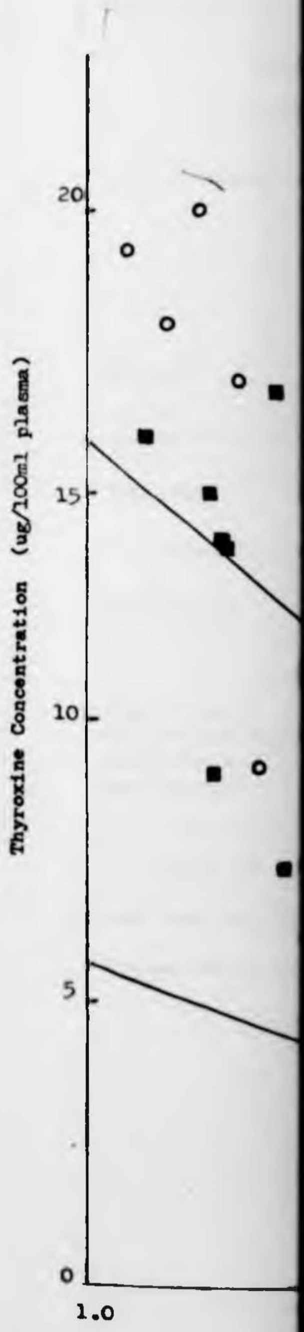

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Relationship between Thyroxine Concentration and Riboflavin Deficiency in Young and Old Rats

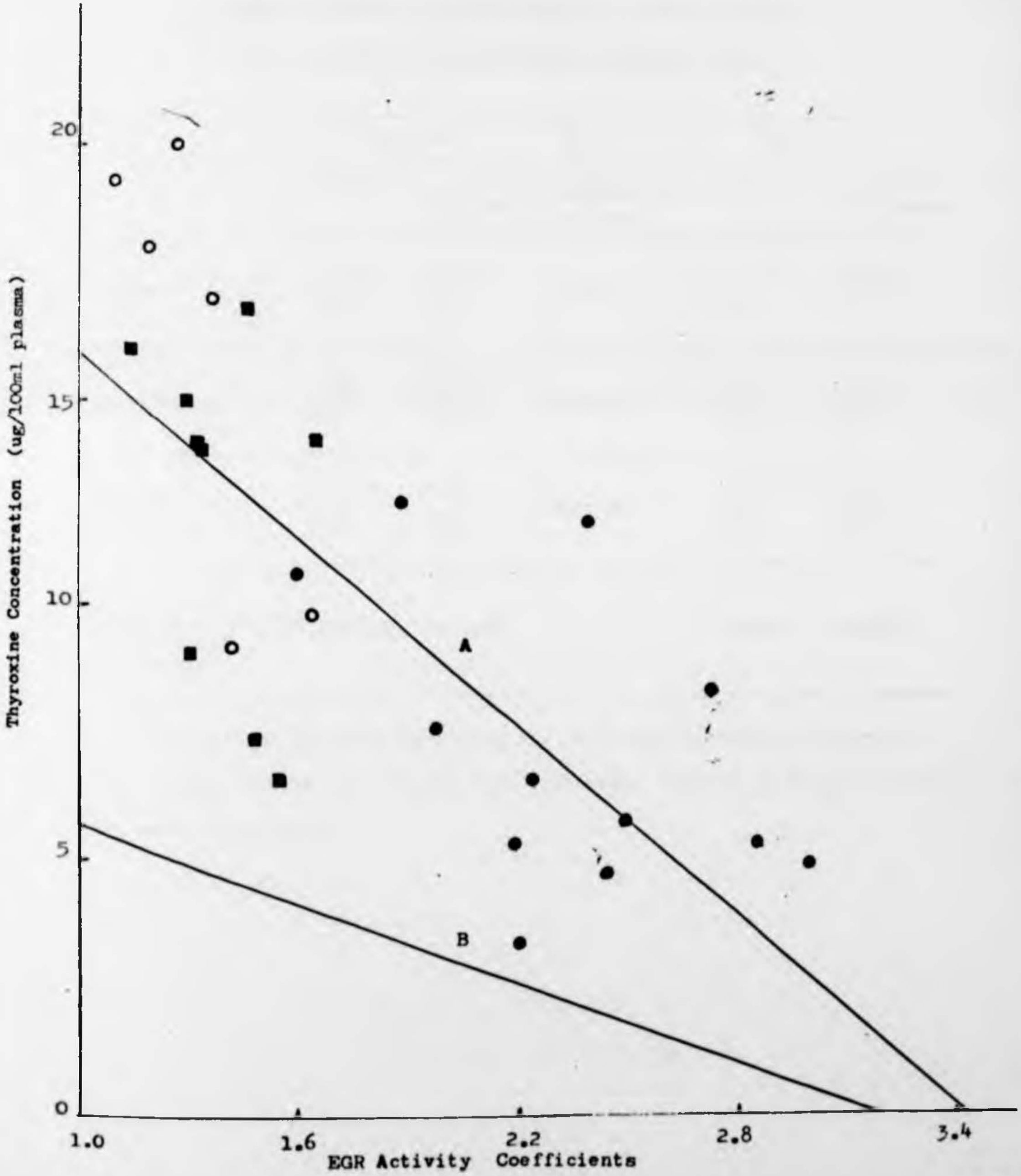

Table 23:

Plasma Thyroxine Concentrations in Young and Old Rats on Control and Riboflavin-Deficient Diets.

	Young Rats		T_4 significance difference between old and young	Old Rats	
	EGR-AC	T_4 (ug/100 mlplasma		EGR-AC	T_4(ug/100 ml plasma
Control	1.40 ± 0.15 (9)	12.58 ± 3.84 (9)	$P<0.02$	1.15 ± 0.01 (3)	5.80 ± 1.59 (3)
Pair-fed	1.33 ± 0.18 (6)	15.55 ± 4.80 (6)	$P<0.02$	1.17 ± 0.08 (3)	5.60 ± 0.86 (3)
Riboflavin-Deficients	2.33 ± 0.41 (12)	7.22 ± 0.85 (12)	$P<0.001$	2.06 ± 0.52 (7)	2.51 ± 0.83 (7)
Significance difference between controls and deficients	$P<0.001$	$P<0.001$		$P<0.001$	$P<0.001$

Five of the old rats receiving the riboflavin-deficient diet also received galactoflavin (22mg/kg diet). Separate details on those 5 animals are shown in table 21.

two groups of rats was therefore examined separately and was found in both cases to be significantly different from the respective controls.

3- In-vivo Effect of Thyroxine and FAD on Red Blood Cells in Riboflavin-Deficient and Iodine-Deficient Rats

a-Erythrocyte fragility

Table 24 shows measurements of erythrocyte fragility in iodine-deficient and riboflavin-deficient rats following an i.p. injection 24 hours earlier of FAD (1 mg) or thyroxine (100 µg) or saline (0.1ml). Both the H_2O_2-generating systems and hypotonic saline were used to measure fragility but results were essentially the same in that the H_2O_2 system always caused 5-20% greater haemolysis. Riboflavin status was measured before giving the injection and at the time of death. The decision concerning which animals were to be given which drug was made on the basis of clinical condition by attempting as far as possible to make all groups similar to one another on clinical grounds.

There were very few differences between the controls and both groups of pair-fed animals. Those that did occur were within the control range and were probably due to experimental error.

The results obtained from the saline-injected control, pair-fed (riboflavin) and riboflavin-deficient rats were no different from those obtained in earlier experiments (p. 118). It seems unlikely therefore that the saline injection would have affected the response of red cells from iodine-deficient or pair-fed (iodine) rats to the haemolysis tests used. The haemolysis produced by the tests on red cells from saline-injected, iodine-deficient

Table 24:

In-Vivo Effect of T_4 and FAD on the Susceptibility of Red Blood Cells from the Control, Pair-Fed, Iodine-Deficient and Riboflavin-Deficient Rats to haemolysis

	24 hrs FAD injection *				24 hrs T_4 injection *				24 hrs saline injection *			
	H_2O_2 GS'	O. F."	EGR-AC**		H_2O_2 GS	O.F.	EGR-AC **		H_2O_2 GS	O.F.	EGR-AC **	
	% Haemolysis		Before	After	% Haemolysis		Before	After	% Haemolysis		Before	After
Control	23.95 ± 1.80	15.37 ± 3.25	1.16 ± 0.12	1.16 ± 0.11	36.80 ± 1.33	27.59 ± 3.54	1.16 ± 0.08	1.20 ± 0.08	30.57 ± 4.38	18.00 2.52	1.23 ± 0.10	1.22 ± 0.09
Pair-fed for riboflavin-deficients	25.57 ± 5.06	17.67 ± 3.89	1.22 ± 0.09	1.19 ± 0.08	38.89 ± 3.19	32.40 ± 3.05	1.24 ± 0.18	1.45 ± 0.33	37.93 ± 2.64	31.00 ± 4.40	1.27 ± 0.07	1.27 ± 0.07
Pair-fed for Iodine-Deficient	26.34 ± 2.48	18.32 ± 6.00	1.26 ± 0.03	1.20 ± 0.05	35.62 ± 4.78	33.12 ± 4.24	1.29 ± 0.03	1.40 ± 0.03	30.55 ± 2.52	27.96 ± 5.24	1.21 ± 0.01	1.22 ± 0.01
Riboflavin-Deficients	32.18 ± 4.27	19.61 ± 6.16	2.15 ± 0.24	1.40 ± 0.04	82.00 ± 4.48	56.24 ± 6.72	1.81 ± 0.16	2.14 ± 0.25	89.83 ± 4.94	68.05 ± 4.19	2.08 ± 0.21	2.07 ± 0.22
Iodine-Deficients	56.33 ± 7.75	36.56 ± 3.30	1.54 ± 0.23	1.52 ± 0.24	54.09 ± 7.17	50.81 ± 2.80	1.57 ± 0.21	1.55 ± 0.22	55.27 ± 6.13	34.50 ± 8.24	1.63 ± 0.23	1.67 ± 0.11

* FAD and T_4 were injected (1mg and 100ug respectively) in 0.1ml saline i.p. 24 hours before killing.

** EGR-AC was measured before and after injection. ' H_2O_2-generating system (H_2O_2GS) contained 0.02 units xanthine oxidase. " Osmotic fragility (O.F.) was measured in 0.36% saline.
Data shown are the mean and standard deviation of measurements from three animals in each group.

rats was intermediate between the effect on the controls and riboflavin-deficient results and appeared to be unaffected by injections of either FAD or thyroxine. Likewise the riboflavin deficiency i.e. the mean EGR-ACs in the iodine-deficient groups were also intermediate between the control and the riboflavin-deficient groups and also appeared to be unaffected by either FAD or thyroxine.

The injection of FAD had the expected effect on the EGR-AC of riboflavin-deficient rats i.e. was significantly reduced ($P<0.001$). The effect on red cell fragility was similar when the results from the FAD-injected rats were compared with those injected with saline. Haemolysis in red cells from FAD-injected riboflavin-deficient rats was no different from the FAD-injected control rats whereas red cells from saline-injected, riboflavin-deficient rats were extensively haemolysed by both systems.

The injection of thyroxine appeared to reduce the fragility of red cells from riboflavin-deficient animals. Haemolysis was less when results from the thyroxine-injected, riboflavin-deficient rats were compared with those from the saline-injected, riboflavin-deficient animals. Percentage haemolysis produced by the H_2O_2-generating system was reduced by 8%($P<0.2$) while in the osmotic system the reduction was 12%($P<0.1$). These reductions are small, not statistically significant, and may simply be due to experimental variability, but the degree of riboflavin-deficiency in the two groups was very similar, so they may be due to the effect of the thyroxine injection.

b - Erythrocyte peroxidase activity and EGR-ACs

The results in Table (25) show the in-vivo effect of an i.p. injection of thyroxine or FAD on the activities of erythrocyte glutathione reductase (EGR) and peroxidase (EGP) in riboflavin-deficient and iodine-deficient rats. Blood samples were collected from the tails before injection of thyroxine (100µg in 0.1ml saline) or FAD (1mg in 0.1ml saline). The results show that EGP-activity was higher in red cells from both riboflavin-deficient and iodine-deficient rats ($P<0.05$ and $P<0.01$ respectively); about twice the activity in control animals. There was no difference between EGP-activity of erythrocytes from iodine-deficient and riboflavin-deficient rats. The increased activity of EGP and the EGR-ACs fell to within the normal range after injecting the riboflavin-deficient rats with FAD but did not affect results in the iodine-deficient animals.

3-Miscellaneous Measurements on Blood of Iodine-Deficient Rats including Liver Weights

The measurements made are shown in Table (22) and are principally haematological indices and measurements concerning the glutathione reductase/peroxidase system. In addition, Table (26) shows density gradient fractionation on red cells from iodine-deficient rats. Iodine-deficiency in the rats was associated with significant falls in PCV, erythrocyte and plasma Fe. The mean leucocyte count fell, but this was not significant. The changes are similar to those recorded for riboflavin-deficient rats Table(13).

Table 25:

Effect of T_4 and FAD In-vivo on EGR-ACs and the Activity of Peroxidase in Control, Pair-fed, Riboflavin and Iodine Deficient Rats

	1mg FAD				100 ug T_4				Saline			
	EGR-AC		Peroxidase		EGR-AC		Peroxidase		EGR-AC		Peroxidase	
			Before	After	Before	After	Before	After	Before	After	Before	After
Control	1.16 ±0.12	1.16 ±0.11	4.95 ±0.30	5.62 ±0.66	1.16 ±0.08	1.20 ±0.08	4.69 ±0.81	5.87 ±0.77	1.23 ±0.10	1.22 ±0.09	4.47 ±1.41	4.07 ±0.79
Pair-fed for Riboflavin-Deficients	1.22 ±0.09	1.19 ±0.08	5.79 ±1.28	5.65 ±0.76	1.24 ±0.18	1.45 ±0.33	4.68 ±1.92	6.71 ±0.88	1.27 ±0.07	1.27 ±0.07	4.55 ±2.17	4.86 ±2.57
Pair-fed for Iodine-Deficients	1.26 ±0.03	1.20 ±0.05	4.99 ±0.32	5.04 ±0.77	1.29 ±0.03	1.40 ±0.03	4.41 ±0.88	6.01 ±0.75	1.21 ±0.01	1.22 ±0.01	4.54 ±1.21	4.60 ±1.19
Riboflavin-Deficient	2.15* ±0.24	1.40 ±0.04	9.59** ±1.38	5.64 ±0.10	1.81 ±0.16	2.14 ±0.25	8.68 ±1.51	10.47 ±0.08	2.08 ±0.21	2.07 ±0.22	7.86 ±0.93	7.90 ±1.05
Iodine-Deficients	1.54+ ±0.23	1.52 ±0.24	8.68++ ±0.60	9.63 ±0.49	1.57 ±0.21	1.55 ±0.22	6.16 ±0.64	7.25 ±0.13	1.63 ±0.23	1.67 ±0.11	9.57 ±0.51	9.68 ±0.58

Measurements were made on tail blood collected before i.p. injection of T_4 (100ug), FAD (1mg) or saline and on heart blood taken on killing 24 hours later. Mean and standard deviations are shown for three animals.

Table 26:

Distribution of Haemoglobin after Separation of Red Cells from Control, Pair-Fed and Iodine-Deficient Rats into Fractions of Different Mean age

	EGR-AC	<1.113	1.113-1.119	>1.119-1.125	1.125-1.14
Controls	1.09 ± 0.03 (3)	25.93 ± 5.33 (3)	32.04 ± 6.38 (3)	29.71 ± 4.14 (3)	12.19 ± 3.11 (3)
Pair-Feds	1.18 ± 0.09 (3)	21.52 ± 5.15 (3)	30.98 ± 8.77 (3)	28.03 ± 10.88 (3)	19.40 ± 6.07 (3)
Iodine-Deficients	1.46 ± 0.15 (5)	7.52 ± 3.22 (5)	32.8 ± 2.26 (5)	30.52 ± 3.09 (5)	27.77 ± 4.25 (5)
Significance difference between def. and controls	$P<0.01$	$P<0.01$	N.S.	N.S.	$P<0.01$

Number of rats used in the fractionation are shown in parentheses.

EGR-AC were measured on unfractionated blood. All data shown are mean±SD

The results of the measurements made on the glutathione reductase/peroxidase system in the red cells from iodine-deficient rats showed similar trends to those obtained from riboflavin-deficient rats (Tables 12 and 13). The falls occurring in methaemoglobin and reduced glutathione, although significantly different from the control results were not as great in the iodine-deficient rats as those found in riboflavin-deficiency.

Liver weights (g/100g body weight) were the only measurements made where a difference was found from the behaviour in riboflavin-deficiency. In iodine-deficiency, liver weights fell to more than 30%, whereas in riboflavin-deficiency they were almost doubled Table (22).

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

DISCUSSION

1- Diertary Development of Ariboflavinosis

Early in the 1940's it had been demonstrated by several investigators that riboflavin has a profound effect on food utilization both for growth and maintenance. The primary effect of riboflavin reduction is cessation of growth because riboflavin is a fundamental constituent of animal tissues, and new tissues cannot be formed unless a minimum amount of riboflavin is available (Horwitt, 1954). It is necessary for both growth and tissue repair.

The work reported here was done to determine whether riboflavin deficiency had any effect on the metabolism of the erythrocyte.

In the current study only two basically different diets were used; a low-protein low-fat diet (Diet A) and a high-protein high-fat diet (Diet B) (Tables 1,2 and 3). Table 27 shows that diet A contained 10% of the calories as protein while diet B contained 16.6%. The fat contents of diets A and B was 6.5 and 20.7% of the calories respectively.

Rats fed the control diet B grew faster than those on diet A. Rats on riboflavin-deficient diet A gained weight slowly and rarely showed clinical or biochemical signs of deficiency. By contrast, rats on riboflavin-deficient diet B reached a growth plateau and showed clinical signs of deficiency within the first three weeks (Fig.3). The failure of rats to thrive on control diet A as well as those on control diet B suggested that diet A was inadequate for

Table 27:

Comparison of Diets A and B as Calories Expressed as Percentage of Total Calories

	Type of oil used	Protein	Carbohydrate	Fat					Total Calories per 100g Diet
				Sat. F.A.	Monounsat. F.A.	Diunsat. F.A.	Polyunsat. F.A.	Total	
Diet A	Corn oil	10.12	83.37	0.78%	2.93%	2.73%	Trace-0.023%	6.51	415
Diet B	Corn oil	16.59	62.67	2.49%	9.33%	8.71%	Trace-0.21%	20.74	434
	Cotton seed oil	16.59	62.67	5.18%	6.22%	9.12%	Trace-0.21%	20.74	
	Ground-nut oil	16.59	62.67	3.32%	3.11%	14.10%	Trace	20.74	
	Cod liver oil	16.59	62.67	4.15%	10.78%	1.04%	4.77%	20.74	

Data was calculated from dietary composition (see tables 1, 2, and 3) and from data supplied by the manufacturers in the case of the fatty acid composition.

optimal growth of weanling rats. The weight gain of control animals on diet A was about 33% of those on diet B after 35 days. Czazkes and Guggenheim (1946) used a diet containing 11% protein calories as a low protein diet and the weight gain in their rats was very similar to rats on diet A in these experiments. Therefore, the reason for the poor growth by rats on diet A may have been protein insufficiency. Rats on the riboflavin-deficient diet A never reached a growth plateau (Fig.3) and did not show any clinical signs of deficiency during the two-month experiment. In addition, biochemical evidence of riboflavin deficiency did not appear in 6 rats on diet A. The mean EGR-AC of these rats was 1.38 ± 0.23. It has been reported that there is a decreased excretion of riboflavin on diets low in protein (Sarett, Klein and Perlz weig, 1942) and this might explain the failure to produce riboflavin deficiency with diet A.

On the other hand, Table 1 shows that diet B had a high-fat content. Mannering _et al._,(1941) and Reiser and Pearson (1949) have suggested that fat increases the riboflavin requirement of rats. Not only has an increased level of fat an influence on the development of riboflavin deficiency but also the type of fat used in the diet. Reiser and Pearson (1949), in experiments with chicks when they used cotton seed oil, lard or hydrogenated vegetable oil in different riboflavin-deficient diets, found that it was the diet containing cotton seed oil as 20% of the calories which produced the lowest weight gain and also the weight gain plateau after four days on this deficient diet. One difference between cotton seed oil, lard and hydrogenated vegetable oil in their experiment is the higher concentration of unsaturated fatty acids in the cotton seed oil. By contrast, the experiment described in Figure 4 also illustrates a different response of rats to diets containing different fats. However, the degree of biochemical riboflavin deficiency which developed in these rats was directly proportional to the proportion of saturated fat present and there was very little difference in the total concentrations of unsaturated fatty acids present.

In the experiment using the four oils, namely, corn oil, cotton seed oil, ground nut oil and cod liver oil as sources of fat for diet B, the weight gain and clinical signs of deficiency in each group of rats receiving no riboflavin varied considerably. Rats on diets containing corn oil and cod liver oil showed no clinical signs of riboflavin deficiency, while rats on diets containing the other two oils did.

Rats on the riboflavin-deficient, cotton seed oil diet showed the poorest weight gain, reaching a growth plateau in three weeks, and rats on the cod liver oil diet showed very similar behaviour. Rats on the riboflavin-deficient groundnut oil-based diet showed the next poorest growth response, while the corn oil-based diet had the least effect on growth. The effect of the riboflavin-deficient diets on the biochemical response, i.e. EGR-AC, was similar to the growth response (Fig.4).

The amount used of the different types of fat was the same in each case (i.e. 20.7% total calories) but there were differences in the fatty acid composition. Table 27 shows that cotton seed oil contained the highest level of saturated fatty acid, followed by cod liver oil, groundnut oil and corn oil (5.18, 4.15, 3.32 and 2.49 calories % respectively).

Biochemical response (as mentioned above) and growth response were in direct proportion to the concentration of saturated fatty acids. The lack of clinical evidence of riboflavin deficiency in rats on the cod liver oil-based diet was surprising.

Closer examination of the composition of the oils showed that two of them, corn oil and cod liver oil were fortified by the manufacturer with 84 and 100mg vitamin E/100g oil respectively. It was not expected that this would have produced the clinical response to the riboflavin-deficient diets since the amount of vitamin E already added to the rat's diet (2mg/kg diet) was considered adequate. The A.R.C. (Clarke _et al_, 1977) have recently recommended that rat diet should contain 60mg/kg diet. In view of this the experiment described on p. 92 was done to exclude any possibility that the cotton seed oil and groundnut oil-based diets were marginally deficient in vitamin E. As the results showed, addition of vitamin E at the level of 100mg/100g oil (i.e. 100mg/Kg diet) did not alter the development of riboflavin deficiency in any way in the rats fed those two diets.

The explanation for the lack of clinical signs of riboflavin deficiency in rats on the cod liver oil-based diet could not be resolved on the basis of vitamin E concentrations. The only remaining difference between the four fats which is known, but which has not yet been examined for its effect on the development of clinical signs, is the concentration of polyunsaturated fatty acids, i.e. those with three or more double bonds, but predominantly linolenic acid. Only the cod-liver oil contains a significant amount of linolenic acid while all the oils contain significant amounts of linoleic acid.

Bhat and Belavady (1970) have suggested that follicular hyperkeratosis in man is associated with deficiences of essential fatty acids and the B-group of vitamins. It is possible that the clinical signs attributed to riboflavin-deficiencies in these studies may have been additionally influenced by the lack of linolenic acid in the diets. This will have to be examined at a later stage.

The association between riboflavin deficiency and those diets high in saturated fat is interesting. It has never been reported that saturated fats increase riboflavin requirement more than unsaturated fats, but the presence of double bonds in the fats undergoing B-oxidation precludes the necessity for FAD at the acyl CoA-dehydrogenase step. That is, the subsequent reaction does not occur with unsaturated fat. It is possible, therefore, that saturated fat in the diet increases riboflavin requirements and is the reason for the results obtained in the experiment involving the different fats.

Although outward signs of riboflavin deficiency were not evident in the rats fed the riboflavin-deficient, cod-liver oil-based diet, the weight of their livers (8g/100g body weight) was approproiate to their biochemical status (see overlay Fig.9). The liver weights of riboflavin-deficient rats, when expressed as a percentage of the total body weight, were considerably greater than those of either pair-fed or ad-lib. controls (Fig.9) in all experiments. This was first reported by Burch et al., (1956) who suggested that the increase in liver weight occurred in order to compensate for a loss of oxidating efficiency. The increase in the size may be associated with increased tenderness and may account for the curving body which is characteristic of riboflavin deficiency in rats.

Various workers have reported disturbances in fat metabolism. The concentrations in serum and livers of linoleic, linolenic and arachidonic acids were below those of normal rats (Mookerjia and Hawkins, 1960 ; Koyanagi and Oikawa, 1965; Williams, McIntosh, Hincenbergs, et al., 1967). It has been suggested that the dehydrogenation of fatty acids is reduced in riboflavin deficiency (Chatterjee and Ghosh, 1967), but this could only affect the concentrations of arachidonic acid which can be synthesized in the body.

The essential fatty acids are an important constituent of phospholipids in membranes. An increased turnover of phospholipids in the absence of an increased intake might explain the findings of Mookerjia and Hawkins (1960) and some of the results described in this thesis might be evidence of increased phospholipid turnover in riboflavin deficiency. For instance, there is increased activity of erythrocyte glutathione peroxidase in riboflavin deficiency but the concentration of red cell GSH is reduced. The increased peroxidase activity may be evidence of increased oxidation of membrane lipids and the increased red cell fragility may be due to oxidative damage of the membrane which results.

2- Riboflavin Status

In the present study erythrocyte glutathione reductase activity coefficient (EGR-AC) was used as a measure of riboflavin status to monitor biochemical and physiological functions of riboflavin in the rat's body. This index has been used by many investigators in man (Glatzle et al., 1968; Bamji, 1969) and in rats (Glatzle et al., 1973; Tillotson and Sauberlich, 1971; Prentice and Bates, 1978). The mean and standard deviation of the EGR-AC in 44 rats fed the control diet B was 1.28 ± 0.17 while that of the 32 pair-fed animals was 1.27 ± 0.24. If one takes the upper limit of the 'normal' range as the mean ± 2 SD, the figure would be approximately 1.60. However, an examination of many of the Figures 12 to 41 which show associations between EGR-AC and various measurements in the red cell, suggest that changes occur when the EGR-AC rises above 1.40. That is, 1.40 may be a better threshold of the normal range in rats than 1.60. Even 1.40, however, is Similar to that of 1.45 suggested by Prentice (1978) but not as high as the upper limit of the normal range of Glatzle et al.,(1973) whose figure was calculated to be 1.80. Tillotson and Sauberlich (1971) used whole blood in their studies but did not provide a regression equation to compare their results (BGR-AC) with those from washed erythrocytes (EGR-AC). Using the regression equation for these variables obtained in this thesis, the upper limit of the EGR-AC obtained in their control animals was 1.65. By contrast, using the regression equation of Glatzle et al. (1973), an EGR-AC of 1.72 was obtained for the upper limit of the control range of the Tillotson and Sauberlich (1971) data. These results suggest that data on EGR-ACs of control animals reported in this thesis were very similar to those previously reported by Tillotson and Sauberlich (1971) and Glatzle et al., (1973). Regression lines for BGR-AC versus EGR-AC reported by Glatzle et al. (1973) and in these studies were also very similar in spite of the alterations in methodology discussed below.

The technique used in this study to measure riboflavin status was modified from that of Glatzle et al., (1973), in which they used 223.5μM GSSG to start the reaction after adding 117.6 μM-NADPH with a concentrate in a total volume of 1.7ml. Beutler (1969) has shown that NADPH slightly inhibited EGR activity in the absence of GSSG and decreased the activity coefficient. This observation was confirmed by Tillotson and Sauberlich (1971) and Thurnham *et al.* (1972) who reversed the order of addition of GSSG and NADPH. It was also found that FAD has an inhibitory effect at concentrations above 2.5µm on the EGR enzyme activity in human blood which leads to a decreased EGR-AC (Schorah and Messenger, 1975). No such effect at this concentration was found in rats' blood and 18.5µM-FAD was found to stimulate maximally (Hassan, 1975). In the present study 3.95 mM-GSSG and 120µM-NADPH in a total volume of 3.3 ml gave maximal activity and activation coefficient.

Clinical symptoms were found in association with high values of EGR-AC ($>$2.0). Some of the animals sometimes showed some evidence of recovery before they were killed in that clinical signs were less severe, for example the re-appearance of hair where skin lesions had previously existed. EGR-ACs in such animals were usually less than 2. In addition, a few animals never showed any clinical signs and the probable reason for both failure to produce a deficient state and recovery was believed to be coprophagy. Barnes *et al.* (1957) estimated that 50-65% of faeces are ingested by rats even when dietary composition was adequate for all nutrients, while in the presence of a nutritional deficiency, faecal consumption may be even greater. The amount of riboflavin in the 12 lots of riboflavin-extracted casein was 0.126 ± 0.014 µg/g, that is 0.023mg riboflavin/kg diet. This was about one-fourth the amount used by Tillotson and Sauberlich (1971) whose diet contained 0.5µg riboflavin/g casein, that is 0.1mg/kg diet.

With the disappearance of clinical signs of ariboflavinosis, it was commonly observed that coprophagy was taking place e.g. nibbled stools at the sides of the cages and on the collecting trays. Analysis of faeces (Hassan, 1975) showed there was approximately 18µg riboflavin/g faeces. The amount of stool passed per rat per day on diet B was about 0.5-0.7g which could be a source of at least half the minimum requirement of the rat as estimated by Mannering _et al_. (1941).

The rapid recovery on obtaining riboflavin was seen in the experiment in which 1mg FAD was injected into 3 riboflavin-deficient rats (Tables 24 and 25). The EGR-AC fell from 2.15 ± 0.24 to 1.40 ± 0.04 in 24 hours. In man too, a rapid response to either riboflavin or riboflavin tetrabutyrate was seen within four hours (Ramakrishnan and Sheth , 1977).

The EGR-AC values obtained throughout the present study can be divided into three categories:

1- EGR-AC values of the control and pair-fed rats on riboflavin-supplemented diet B ranged from 1.03 to 1.40. This was in agreement with the other physiological indices studied in association with riboflavin-deficiency and showing normal values (Figures 12 - 41).

2 - EGR-AC values of the animals recovering from riboflavin-deficiency ranged from 1.40 to 2.0. Results from these rats are shown on the overlays attached to the different figures in Chapter 3. These values were associated with small changes in the concentrations of the other indices studied such as erythrocyte fragility, GSH, peroxides, ... etc.

3 - EGR-ACs of rats on the riboflavin-deficient diet B showing clinical signs ranged from 2.0 to 4.25. High EGR-ACs were also associated with large changes in the concentrations of several red cell constituents to be discussed below.

Measurements of the EGR-AC are not influenced by sex, age or dietary protein in man (Glatzle et al., 1970; Tillotson and Baker, 1972) and no reports to the contrary have been reported in rats. However, it is widely recognised that a great number of clinical disorders have been reported in association with altered EGR activities (Carson et al., 1959; 1961; Beutler, 1969; Rivlin et al., 1968). Flatz (1970) suggested that there was enhanced binding of the FAD by the EGR in red cells in glucose-6-phosphate dehydrogenase deficiency but did not pursue the involvement of other enzymes associated with riboflavin metabolism.

In man, EGR-activity may be increased 20 to 30% or more in patients with G-6-PD deficiency (Schrier et al., 1958) gout (Long, 1962), diabetes mellitis (Long and Carson, 1961) and following the administration of 4 grams of nicotinic acid (McNamara, Frischer, Rieckmann, Stockert, Powell and Carson 1967). Also it was found that EGR-activity is influenced in-vivo and in-vitro by certain drugs and chemicals e.g. nitrofurantoin (Furadantin) and hexavalent chromium which cause a reduction in GR activity (Buzard, Kapko and Paul, 1960; Koutras, Schneider, Hattari and Valentine, 1965).

3- Role of EGR Activity in the Red Blood Cell Stability

Erythrocyte glutathione reductase (EGR) is necessary to generate reduced glutathione (GSH) and the latter is required by glutathione peroxidase to detoxify peroxides which reduce cell integrity. It has been suggested, however, that EGR is not rate-limiting (Beutler and Srivistava, 1970), therefore, riboflavin-deficiency alone would be unlikely to affect the generation of GSH to any significant extent such that it would reduce cell integrity.

When red blood cells from control, pair-fed and riboflavin-deficient rats were exposed to H_2O_2-generating systems, red blood cells from riboflavin-deficient rats were found to be highly susceptible to haemolysis. This susceptibility to haemolysis increased as the amount of hydrogen peroxide generated was increased (Fig.12, 13 and 14). The H_2O_2-generating system was based on the following equation:

$$\text{Hypoxanthine} + \text{xanthine oxidase} \rightarrow \text{Uric acid} + 2H_2O_2$$

When 0.04 units of xanthine oxidase were used, approximately 600µmols of peroxide were generated in the incubation medium during 45 minutes (Table 10). This concentration of H_2O_2 had a deleterious effect on the stability of the red cell and the haemoglobin. Almost all cells from the riboflavin-deficient rats were lysed and the haemolysates were turned dark brown by a precipitated material, possibly Heinz bodies. Less and less dark brown material appeared as the concentration of xanthine oxidase was reduced. That is, when erythrocytes were exposed to H_2O_2-generating system containing 0.008 units xanthine oxidase, haemolysates were not turned dark brown and there was only a slight change from the bright reddish colouration of haemoglobin to a slight brown reddish colouration. The H_2O_2 produced by 0.008 units was 41µmols H_2O_2 per incubation medium per 45 minutes (Table 10).

Erythrocytes from control and pair-fed rats exposed to the different concentrations of H_2O_2 exhibited the least haemolysis (Figures 12, 13 and 14) even in the presence of 0.04 units xanthine oxidase. In these cells, reduced glutathione (GSH) concentrations (Fig.12) are maintained and peroxide is probably detoxified via glutathione reductase/peroxidase system in an adequate manner. In red cells from riboflavin-deficient rats concentrations of GSH were lower(Fig. 15) and this was coupled with lower activities of glutathione reductase (Fig.18). These GSH-depleted cells when exposed to peroxide may have been unable to generate sufficient GSH to maintain cell integrity. The same effects were seen when red cells from riboflavin-deficient rats were suspended in a hypotonic saline (0.36% NaCl) solution alone. Endogenous GSH again may have been not enough to maintain cell integrity and thus cause lysis of the cells. The haemolysis obtained from the lysed red cells after incubation in hypotonic saline solution was bright red in colour, i.e. there was no change in the physical characteristic of haemoglobin on lysis of the red cell. These results suggested that riboflavin-deficiency, through its effect on the activity of EGR, reduces the concentration of GSH and affects the stability of the red blood cell in-vitro when incubated in a H_2O_2-generating system (chemical stress) or even when incubated in hypotonic saline (0.36% NaCl) solution (physical stress). Even red cells from riboflavin-deficient rats, when centrifuged to remove plasma or washed in isotonic saline, showed some haemolysis.

Two systems in the red cells are responsible for detoxifying H_2O_2 in the surrounding medium, catalase and the glutathione reductase/ glutathione peroxidase systems (Fig.2). Catalase was reported to be inefficient in decomposing low concentrations of H_2O_2 (Keilin and Hartree, 1945; Cohen and Hochstein, 1961). Cohen and Hochstein (1961) demonstrated that continuous low levels (less than 10^{-6}M, i.e. 34 µmols in the incubation medium used in these experiments) of H_2O_2 will cause oxidative damage to erythrocytes regardless of normal levels of catalase.

As previously stated 0.008 units xanthine oxidase (Fig.14) produces 41 µmols H_2O_2 in the incubation medium/45 minutes (Table 10). Cohen and Hochstein (1963) concluded that low levels of H_2O_2 are detoxified almost entirely by glutathione peroxidase with resultant stimulation of the hexose monophosphate shunt mechanism to supply energy in the form of NADPH to maintain the erythrocyte concentration of GSH.

On the basis of the central position of glutathione reductase between the hexose monophosphate shunt and glutathione peroxidase, Desforges *et al.*(1959) suggested that glutathione reductase might control the activity of glutathione peroxidase via the concentration of GSH. The possibility, however, was discounted by Beutler and Srivistava (1970). They did experiments to determine *in-vivo* ^{51}Cr red cell survival in rats and man with low and high EGR-activity coefficients and in the presence and absence of oxidative drugs. They failed to reveal any differences which might provide evidence of limiting EGR activity having a controlling influence on the red cell stability. However, experiments reported in this thesis have shown that red cells from rats with high EGR-ACs i.e. low EGR activity showed a greater tendency to lysis in the presence of peroxide or hypotonic saline than those from control rats.

The fragility experiments were *in-vitro* tests and are not necessarily indicative of *in-vivo* fragility. The haemolysis test may be more stressful than the conditions experienced *in-vivo.* The ^{51}Cr survival test may not detect small reductions in red cell life span. Such experiments usually only follow the disappearance of ^{51}Cr during its first half life. Riboflavin deficiency is a progressive state and effects on red cell integrity may only become apparent later in the experiment. However, there may indeed be no alteration in *in-vivo* red cell survival.

4- Oxidative Stresses on Red Blood Cells in Riboflavin Deficiency

In the present studies, two important oxidative products accumulated within red blood cells of riboflavin-deficient rats; total peroxides and methaemoglobin. The increased concentration of methaemoglobin was associated with an increased concentration of sulfhaemoglobin and a decrease in oxyhaemoglobin (Figures 24, 25, 26). These changes were associated with a depletion in red cell GSH (Figures 27 and 28).

a- Lipid Peroxidation:

The increase in total peroxides measured in red cells from riboflavin-deficient rats is probably due to the lower activity of glutathione reductase in the presence of reduced amounts of FAD. Reduced activity of glutathione reductase appears to reduce the supply of GSH (Fig.18) and may in turn reduce the activity of glutathione peroxidase (GP) *in-vivo*. Evidence for a reduced utilisation of GP may be obtained from Fig.28 and Table 18. As red cells in control animals get older, GP activity falls and one presumes the loss in activity is due to loss of functional enzyme brought about by conformational changes occasioned by normal metabolic function. If the enzyme is inactivated in-vivo, conformational changes would be less likely to occur and more functional enzyme would be retained. Table 18 illustrates that this situation may exist in red cells from riboflavin-deficient rats since the oldest red cells still contained GP activity similar to the youngest cells in both control and deficient rats.

If the activity of glutathione peroxidase in-vivo is reduced in red cells from riboflavin-deficient rats, this would explain why concentrations of total peroxides are higher in both tissues where these were measured i.e. red cells and liver. The formation of free radicals of oxygen occurs in all organisms living in aerobic conditions (Flohé and Gunzler. 1976) and these free radicals can produce peroxide through the action of superoxide dismutase (Leipzig *et al.*, 1975). Some of the possible mechanisms concerning the formation of free radicals in the red cells are described in the introduction (p 44-47).

In normal cells, two systems detoxify free radicals produced inside the red cells; catalase and the glutathione reductase/peroxidase system. Catalase can detoxify high concentrations of peroxides and the mechanism needs a continuous supply of NADPH. Catalase may therefore prevent peroxides accumulating in toxic amounts but evidence from Fig.22 suggests that the peroxide continued to accumulate as the deficiency progressed i.e. there was no evidence of a plateau. In addition, evidence for increasing damage to lipids as the deficiency progressed was also evident (Fig.21). Malonylaldehyde is a breakdown product of lipid hydroperoxides and this too was increased in the red cells from riboflavin-deficient rats and showed no evidence of a plateau when the deficiency was severe.

The glutathione reductase/glutathione peroxidase system can act on low concentrations of peroxide. In the reduction of H_2O_2 two molecules of GSH are oxidised to one molecule of GSSG. Glutathione reductase regenerates GSH and this requires the presence of NADPH. That is, in normal conditions EGR functions to give a continuous supply of GSH. This function is mainly dependent on the availability of FAD and NADPH in the erythrocyte. Ten percent of the glucose consumed by the red cells is metabolised by the way of hexose monophosphate pathway to supply NADPH (Brin and Yonemoto, 1958; Murphy, 1960). Since glutathione reductase specifically requires, in addition to FAD, NADPH as its cofactor (Rall and Lehninger, 1952), and since glutathione is present in relatively large amounts in red cells, the reduction of oxidised glutathione to GSH presumably represents an important determinant of hexose monophosphate shunt activity. Glutathione reductase is believed to be of crucial importance to the cell, since GSH is believed to maintain other thiol groups of the cell in the reduced state through an oxidation-reduction interaction (Allen and Jandl, 1961; Jacob and Jandl, 1962).

In erythrocytes from riboflavin-deficient rats in the present study, it was found that both the activity of EGR and the concentration of GSH were considerably reduced.

The fall in the concentrations of GSH coupled with a reduced *in-vivo* activity of glutathione peroxidase (p.) may well have disturbed the GSH/GSSG ratio i.e. increased the concentration of GSSG. and reduce the activity of the pentose phosphate shunt (Jacob and Jandl 1965)

It has been reported that an increased concentration of GSSG inhibits the activity of the enzyme hexokinase (Eldjarn and Bremer, 1962; Beutler and Teeple, 1969) which is used to phosphorylate glucose to glucose-6-phosphate, the first step in the hexose monophosphate shunt and glycolysis. Increased concentrations of GSSG therefore may reduce available energy in the red cell by reducing the formation of both NADH and NADPH. It is also reported that a depletion of GSH reduces the activity of the sulfbydryl-requiring enzyme glycaraldelyde-3-phosphate dehydrogenase (Mengel, Kann, Lewis and Harton, 1964) which might also decrease concentrations of NADH in the red cell.

Concentrations of NADH and NADPH have not yet been estimated in this study and neither were the activities of hexokinase of glyceraldehyde-3-phosphate dehydrogenase. The need to do these assays only became apparent as other investigations were completed. If the supply of NADPH is reduced, this might also explain why catalase does not act to reduce the increased amounts of peroxide but this too needs more investigation than has yet been possible.

The failure to detoxify free radicals and H_2O_2 leads to accumulation of these oxidant agents which form deleterious stresses on both lipid membranes and haemoglobin. Both free radicals, singlet oxygen (O_2^1) and hydroxyl (OH) attack unsaturated fatty acids in lipid membranes. Mengel (1968) reported that increased lipid peroxidation might occur in circumstances of: (1) increased availability of oxygen or other prooxidants; (2) alterations of lipid composition, especially increases in unsaturated fatty acids or reductions in antioxidant capacity, such as in tocopherol deficiency. However, experiments showed that the riboflavin-deficient rats were not tocopherol deficient (Fig.5).

The mechanism of lipid peroxidation as suggested by Mengel (1968) is that a hydrogen atom escapes from the carbon in the alpha position to the double bond of an unsaturated fatty acid followed by non-enzymatic reaction of molecular oxygen with that carbon atom. This results in the sequential formation of unstable lipid-free radicals, (H-C-), and free peroxidic radicals, (H-C-O-O-). Peroxidic radicals capture hydrogen atoms from other unsaturated fatty acid molecules to form relatively stable lipid hydroperoxides and create other lipid-free radicals. This chain reaction proceeds autocatalytically with the continuing formation of free radicals, lipid hydroperoxides, and a number of intermediate breakdown products including dienes, trienes, and malonylaldehyde and its precursors. This leads to anatomical defects in the cell membrane with subsequent alterations of permeability characterisitics and eventual lysis. Experiments showed that red cells from riboflavin-deficient rats, when incubated with hypotonic saline solution were more easily haemolysed than those from control rats (Figs. 15 and 16).

It should also be mentioned that spleen size was increased in the riboflavin-deficient rats by about 30%. Weights of spleens in 7 control animals (± SD) were 0.257 ± 0.036 while those in 17 riboflavin-deficient rats were 0.338 ± 0.020. An increase in the size of the spleen often accompanies haemolytic disease and may be an indication of the increased <u>in-vivo</u> fragility of the red cells in the riboflavin-deficient rat. On the other hand, it has been suggested that splenomegaly itself may cause an increased red cell fragility (Sagawa and Shiraki, 1978). The latter authors suggested that stasis of red cells in enlarged sinuses where plasma glucose concentrations may fall, may lead to increased fragility in the red cells. Concentrations of plasma glucose in 6 riboflavin-deficient rats (5.20 ± 0.70 μmol/l) was in fact lower than that in 6 control rats (8.46 ± 0.85 μmol/l P<0.001) and was inversely associated with the degree of deficiency (r= -0.8 P<0.001). The importance of this observation is not known.

The lipid content of the rat diets was very high in unsaturated fatty acids. The composition of the body fat will be considerably influenced by the dietary intake. This by itself does not increase red cell fragility as susceptibility of red cells from control rats to haemolysis by hypotonic saline was not increased. The high content of unsaturated fatty acids in the rat diets might, however, have increased the susceptibility of the tissues of riboflavin-deficient rats to the effects of riboflavin deficiency. That is, increased fragility in the red cells may be an artefact caused by the diet but this point has not yet been investigated.

One final point: when riboflavin-deficient rats were injected with 1mg FAD (Table 24) the susceptibility of their red cells to haemolysis was reduced within 24 hours, but splenomegaly was still evident. This would suggest that increased fragility was previously caused by blockage of the glutathione reductase/glutathione peroxidase system and that splenomegaly was a consequence of increased red cell fragility.

b - <u>Methaemoglobinemias</u>

When methaemoglobin and sulfhaemoglobin were measured in red cells from riboflavin-deficient rats the concentrations were significantly increased by comparison with those in control and pair-fed rats. The concentration of methaemoglobin was also directly correlated with the

EGR-AC, that is, as riboflavin deficiency increased the concentrations of methaemoglobin in the red cells also increased. By contrast oxyhaemoglobin levels fell as riboflavin deficiency increased.

Methaemoglobin is formed from haemoglobin when the ferrous iron is oxidised to ferric by acquiring an electron (Lehmann, and Huntsman, 1966) Oxidation of the ferrous to ferric atom occurs in the non-oxygenated state to produce methaemoglobin, rendering that haem unit incapable of carrying oxygen. Since the main function of the haemoglobin is to carry oxygen it is useless until it is reduced either enzymatically by NADH-methaemoglobin reductase and NADPH-methaemoglobin reductase or non-enzymatically by GSH and ascorbic acid (Harris and Kellermeyer, 1974). It is well known that the prime function of the erythrocyte is to feed oxygen to all tissues. For this to occur, very precise and delicate mechanisms are set up and maintained in the red cell, so that (a) the formation of methaemoglobin is kept to a minimum and (b) any that is formed is reduced to the functional state required for reversible oxygenation (Rossi-Fanelli , Antonini and Mondovi, 1957). Oxidant compounds capable of changing haemoglobin to the oxidised or methaemoglobin form (especially H_2O_2) may be destroyed by (a) peroxidase and catalase (Fig.2), (b) various reducing substances such as ascorbic acid, GSH and other sulfhydryl compounds that are present in the red cell for direct action on oxidants, but the latter probably only function with failure of the enzyme systems (Mills, 1957; Mills and Randall, 1958).

In view of the increase in methaemoglobin concentrations in red cells of riboflavin-deficient rats, the activities of both NADH and NADPH-methaemoglobin reductases were assayed. In both cases the activity of the enzymes was higher in red cells from riboflavin-deficient rats than that found in the controls (Table 12). Measurements were also made on the activity of NADPH-methaemoglobin reductase in fractions of red cells of different ages. The activity was found to behave in a similar manner to that of peroxidase in that although there was some fall in activity as red cells from riboflavin-deficient rats increased in age (2-fold) it was smaller than that occurring in control erythrocytes (5-fold; Table 18).

It can be postulated that the maintenance of higher NADPH-methaemoglobin reductase in red cells from riboflavin-deficient rats may be due to its not being used in-vivo by the red cells possibly because of failure in the supply of the nicotinamide co-enzymes for the reasons discussed above. In addition, it was recently reported that NADPH-methaemoglobin reductase is also a riboflavin-dependent enzyme (Matsuki, et al., 1978) which is an additional factor in support of the idea that the enzyme has reduced activity in-vivo.

NADH-methaemoglobin reductase has not yet been measured in cells of different ages but the activity of the enzyme too was higher in erythrocytes from riboflavin-deficient rats than controls, it is riboflavin-dependent (Rivlin, 1975) and its activity too would be affected by the supply of nicotinamide co-enzymes. It seems reasonable to suggest, therefore, that the activity of this enzyme too may well be reduced in-vivo and account for the increased concentrations of methaemoglobin in red cells.

The mechanism of the formation of sulfhaemoglobin in red cells from riboflavin-deficient rats is probably similar to that occurring in-vitro when normal red cells are exposed to oxidant drugs or conditions in which autoxidation occurs. In such conditions, Allen and Jandl (1961) demonstrated the following sequence of events, first, GSH is oxidised i.e. 2 GSH → GSSG and second, GSSG interacts with 'reactive' sulfhydryl (β93 cysteine) of haemoglobin, forming a mixed disulfide. This haemoglobin remains in solution but is functionally abnormal with increased oxygen affinity, decreased cooperativity and reduced Bohr effect (Huisman and Dozy, 1962). In the presence of NADPH, glutathione reductase permits its reconversion to native haemoglobin (Srivastava and Beutler, 1967). Third, with increased oxidant stress, methaemoglobin is formed, followed by increasing amounts of brown to green haemoglobin proteins (sulfhaemoglobin). Lastly, following the oxidation of other globin thiols (β112 Cysteine and α104 Cysteine), haemoglobin molecules aggregate irreversibly to form colloid granules, corresponding to the intracellular Heinz bodies.

It is likely that a similar progression of events occurs in the intact cell following the oxidant injury of red cells *in-vivo*. In addition, oxidant compounds may attack membrane thiols resulting in accelerated splenic sequestration - probably on the basis of increased cation permeability and osmotic swelling (Jacob and Jandl, 1962 a and b).

In the present study the concentration of sulfhaemoglobin in erythrocytes from rats on riboflavin-deficient diets was only significantly increased above that of the controls in rats which were severely riboflavin-deficient (Fig.25).

This is possibly due to the fact that sulfhaemoglobin is just one intermediate product in a series of oxidative reactions of haemoglobin.

In summary, the increased concentrations of methaemoglobin and sulfhaemoglobin in red cells from riboflavin-deficient rats probably arises as a result of increased oxidant stresses. Riboflavin deficiency may directly reduce the *in-vivo* activity of both methaemoglobin reductases. In addition, riboflavin deficiency reduces the activity of glutathione reductase, lowering the concentrations of GSH in the red cell and probably increasing GSSG concentrations. The latter may partially inhibit hexokinase activity and then reduce available energy for the methaemoglobin reductase enzymes.

5- Inhibition of Erythropoiesis in Riboflavin Deficiency

The results in Tables 15 and 16 show the percentage of haemoglobin in the four red cell fractions for control, pair-fed and riboflavin-deficient animals. There was no difference between the results of the pair-fed and control rats, but the results do suggest that as riboflavin deficiency progressed there was a reduction in the number of young cells circulating in the blood stream and an increase in the number of old cells. The data in (Fig. 37 and 38) also support the idea that as riboflavin deficiency became more severe young cells were not released (Fig.37) into the circulation and the relative number of old cells increased (Fig.38). Evidence of anaemia in these rats is reported in Tables 13 and 14 and Figures 30, 31, 32, 33, 34, 35, 36, 37 and 38. This evidence suggested that riboflavin deficiency in rats was associated with decreased levels of PCV, RBCs, WBCs reticulocytes and plasma iron. The reduced levels of the above haematological indices were inversely related with increased BGR-ACs. In an attempt to explain the decreased haematological indices riboflavin-deficient rats, iron absorption from the diet and stored iron as non-haem and ferritin-iron in the livers were measured. Control and pair-fed rats behaved similarly and there were no significant differences between the two groups in the data obtained in the above measurements. The amount of iron absorbed by pair-fed rats was substantially less than that of control rats but this reductase, lowering the concentrations of GSH in the red cell and probably increasing GSSG concentrations. The latter may partially inhibit hexokinase activity and then reduce available energy for the methaemoglobin reductase enzymes.

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The results reported in Table 14 suggest therefore that the absorption of iron is decreased in riboflavin-deficient groups by about 80%. On the contrary, non-haem iron and ferritin-iron were found to be significantly correlated with the increased EGR-AC ratios. This indicated that although there is a reduction in the absorption of iron, this iron accumulated in the liver in its ferric state as non-haem and ferritin iron. In order to be released after its reduction from the ferric state to its ferrous state, it needs a flavoprotein enzyme: NADH-FMN oxidoreductase, in the liver to operate. If the latter enzyme is inhibited this would explain the lower concentrations of plasma iron in riboflavin-deficient rats. These findings were in agreement with the suggestions of Zaman and Verwilghen (1977) who reported that riboflavin deficiency decreased hepatic iron mobilization and probably also the absorption of iron from food. The data obtained from the control group in the present study were similar to that of the latter authors, but that of the deficient group were completely different. Zaman and Verwilghen (1977) reported that non-haem iron was decreased in liver tissues of riboflavin-deficient rats while Table 13 shows that non-haem iron was doubled in this group. The reason for this difference may be due to age differences between these animals. Zaman and Verwilghen (1977) used animals which were 27 weeks old for their measurements of non-haem iron, which in the experience of the writer would only produce mild riboflavin deficiency without the use of antimetabolites. No antimetabolite was used and neither was any evidence given of the biochemical riboflavin status of their animals . In the current study the animals used for these measurements were not more than 7 weeks old which is a critical period for rapidly growing animals to be given a deficient diet; the deficiency is then produced fairly consistently.

All the evidence discussed above supported the idea that riboflavin deficiency in rats was associated with a mild anaemia and inhibition of erythropoiesis. A direct estimation of the inhibition of erythropoiesis in riboflavin deficiency was attempted by fluorometric measurement of accumulated free erythrocyte porphyrin (FEP), a test suggested by Piomelli (1973). Unfortunately the volume of blood taken was too small to obtain satisfactory readings from the three groups. However, those from riboflavin-deficient rats were double the readings from either the control or the pair-fed groups. The method used measured FEP in 20µl human blood. It was hoped to repeat the measurements using a larger volume (0.5ml) but this was not possible.

In the experiment on old rats given a supplement of galactoflavin the changes in haematological indices were greater than those occurring in young rats, possibly due to the competitive effects of galactoflavin, at sites not usually affected or not affected so soon by the simple deficiency. When erythrocytes were fractionated into different age groups the results showed that the proportion of young cells was higher than that found in the fraction containing the oldest cells (i.e.densest). However, if the haemoglobin distribution on the four fractions from the galactoflavin-treated rats are compared with those from the control or pair-fed rats there are no significant differences in the distribution (Table 21). The mean EGR-AC (2.27 ± 0.45) of the galactoflavin-treated rats indicates that the riboflavin deficiency was quite severe but the haemoglobin distribution in the fractions is more like that of mild deficiency or animals recovering from riboflavin deficiency (Table 16). It can only be concluded that these differences between the two sets of results from rats in different age groups may be a consequence of the different ages (the greater resistance on the part of the old rats to deficiency) or the fact that galactoflavin will act differently by comparison with simple riboflavin deficiency.

In summary, the results reported in Part D suggested that riboflavin deficiency in rats was associated with a mild anaemia which was possibly partly due to a fall in the iron absorption and partly to a block in the release of ferrous iron from the stored ferric iron in the liver tissue.

6- Riboflavin Deficiency and Altered Thyroid Function

Tables 22 and 26 demonstrate the similarities and differences between riboflavin deficiency and altered thyroid status induced by iodine deficiency in rats. The similarities between riboflavin deficiency and iodine deficiency were an increase in the EGR-ACs and erythrocyte fragility (Table 24), higher glutathione peroxidase activity, higher concentrations of peroxides and methaemoglobin (Table 22) and an increase in the number of old cells (Table 26). There were also some haematological similarities: PCV, red blood cell count, plasma iron (Table 22) and the number of young cells (Table 26) were reduced in both riboflavin and iodine deficiency. However, all changes associated with iodine deficiency were not as great as those associated with riboflavin deficiency.

There were some differences between riboflavin-deficient and iodine-deficient rats. Their appearance was somewhat different, liver weights declined in iodine deficiency and some biochemical responses to T_4 and FAD injections were different. Injection of FAD into riboflavin-deficient rats was followed by falls in EGR-ACs and peroxidase activity (Table 25) and reductions in the percentage haemolysis produced by both peroxide or hypotonicity. T_4 injection to riboflavin-deficient rats was associated with a slight increase in the EGR-AC (Table 26) but none of these effects were recorded in iodine-deficient rats.

Thyroxine was also found to have the unexpected property of protecting both control and riboflavin-deficient red cells from haemolysis _in-vitro_ in both the peroxide-generating system (Table 7) and hypotonic saline (Table 12). An experiment was also done to see whether thyroxine would have the same effects if injected _in-vivo_, 24 hours prior to collecting the blood for study (Table 24). Some reduction in the susceptibility of red cells from riboflavin-deficient rats to haemolysis in the presence of peroxide and hypotonic saline was observed following injection of thyroxine. The amount of thyroxine injected (100µg) was considerably smaller than the amount incubated with red cells (Tables 7 and 12). If one assumes that a riboflavin-deficient rat contains 4ml blood, one should have injected 2.8mg. The small reductions in the susceptibility to the peroxide and hypotonic saline may therefore be a real effect in response to thyroxine. Further experiments with different doses of thyroxine need to be done, however, to confirm the observations. The results demonstrated in Table 10 suggest that thyroxine appears to interfere with the generation of H_2O_2 possibly by inhibition of xanthine oxidase. Hydrogen peroxide is also known to cause deiodination of thyroxine. (Reinwein, Rall and Durrer, 1968). These workers investigated the deiodination reaction and showed that it is mediated by H_2O_2 itself, that the reaction could be detected when the H_2O_2 concentration was as low as 5×10^{-5}M and that it takes place rapidly over a pH range of 6 - 9. However, the H_2O_2 produced in the peroxide-generating system used in this study was very high. When 0.04 units xanthine oxidase were used the H_2O_2 produced in 45 minutes was 115 times higher than the minimum required for the reaction (Reinwein et al., 1968). When the lowest concentration of xanthine oxidase (0.008 units) was used, the H_2O_2 produced was 8 times that concentration.

The protective effect of thyroxine may be due to inhibition of xanthine oxidase or a direct inactivation of peroxide but these would seem unlikely in view of the protective effects of haemolysis against hypotonic saline. A direct action of thyroxine on the erythrocyte membrane seems a more likely explanation.

Adenosine triphosphatase (ATPase) functions to control the Na^+ and K^+ transport across the cell membrane. In riboflavin deficiency, there is a possibility that glycolysis is reduced by the inhibitory action of GSSG on hexokinase activity and there may be a fall in available ATP. Activity of adenosine triphosphatase is also reduced in hypothyroidism (Goolden, Bateman and Torr, 1971), a state which has many similarities to riboflavin deficiency, and it is possible that ATPase activity may be directly linked to the presence of thyroxine in the blood. It was confirmed in this study that plasma concentrations of thyroid hormone were reduced in riboflavin-deficient rats (Nolte, Brdiezka, and Staudte, 1972). A fall in ATPase activity interferes with the cation gradient across cell membranes allowing a loss of K^+ and gain of Na^+ and water that ultimately leads to swelling of the cells and haemolysis. It can be postulated that thyroxine may act on erythrocyte membranes stabilising or stimulating ATPase activity and protecting the cell from haemolysis. There is no evidence at present for this hypothesis and it needs to be investigated.

In the current study, riboflavin deficiency was associated with low levels of plasma thyroxine i.e.hypothyroidism (Fig.41 and Table 23). Iodine-deficient rats also had high EGR-ACs which were significantly higher than those of the controls but lower than those of the riboflavin-deficient rats (Table 25). In both deficiencies therefore, there may be limiting concentrations of FMN and FAD. In riboflavin deficiency the limitation in FMN and FAD is due to riboflavin depletion; while in hypothyroidism it is probably due to a decreased rate of conversion of riboflavin to FMN and FAD (Domjan and Kokai, 1966; Rivlin and Langdon, 1969). Those two groups of authors have shown that the hepatic concentration of FAD was reduced to nearly two-thirds of normal in hypothyroid rats and could be restored to normal by treatment with thyroid hormones.

Treatment of iodine-deficient rats with 100µg of thyroxine had no effect on the measurements made in these studies (Tables 24 and 25) but the dose may have been too small or 24 hours too short a time to see any effects. A small increase in the EGR-AC and peroxidase activity were apparent however in the riboflavin-deficient rats which received thyroxine (Table 24). The differences were not significant however and were probably due to experimental variation.

Although there are many similarities in the pathological changes which occur in the two deficiencies, the response to the administration of FAD illustrates very clearly the different mechanism occurring in the two groups. Administration of FAD to riboflavin-deficient rats was followed by a rapid decrease in EGR-AC and an equally rapid fall in the peroxidase activity. Likewise in red cells from riboflavin-deficient rats, FAD administration reduced the susceptibility to haemolysis to that of control rats within 24 hours. No effects were seen in iodine-deficient rats, (Table 25). This would suggest that the red cells in iodine-deficient rats were unable to utilise the FAD. Rivlin and Langdon, (1966) suggested that the thyroid deficiency reduces available riboflavin co-enzymes by depressing their enzyme synthesis. In addition, it would seem that riboflavin-dependent apoenzymes in thyroid deficiency are unable to utilise the administered FAD.

Iodine-deficient rats showed some signs of anaemia and the fractionation technique demonstrated the presence of fewer young cells in the blood stream. Recently, it was shown by Golde, Bersch, Chopra and Cline (1977) that thyroid hormones have important effects on erythropoiesis in man and animals. They suggested that thyroid hormones have a direct effect on the proliferative capacity of erythroid-precursors, a finding which they suggested may have relevance to the mechanism of erythropoietic dysfunction in human thyroid disease.

Red cell life span has been found to be normal or slightly prolonged in hypothyroidism (Kiely, Purnell and Owen, 1967; Tudhope and Wilson, 1960; McClellan, Donegan, Thorup and Leavell, 1958). The decreased concentrations of plasma thyroxine in riboflavin deficiency may be responsible for the effects on erythrocyte production discussed earlier. The fall in plasma thyroxine may depress erythropoiesis in riboflavin deficiency in the same way that it does in iodine deficiency. The effect may be secondary to a depressant effect of riboflavin deficiency on the synthesis of thyroid hormone. Examination of Tables 15 and 16 suggests that early in riboflavin deficiency (EGR-AC <1.60) there is an increased production of young red cells. This may be the response to a marginal riboflavin-deficiency and is in fact similar to the findings reported by Powers and Thurnham (1977) in man. These authors suggested that marginal riboflavin deficiency stimulates erythropoiesis to replace erythrocytes lost as a result of the increased *in-vitro* fragility. As the riboflavin deficiency becomes more severe thyroid-hormone synthesis may become depressed and responsible for depression of erythropoiesis. These ideas are somewhat speculative for it is not known yet at what stage in riboflavin deficiency thyroid concentrations fall and whether this does in fact coincide with a depression of erythropoiesis.

SUMMARY AND CONCLUSION

The study examined erythrocyte pathophysiology in riboflavin deficiency in rats. Rats fed the experimental diet showed a gradual increase in EGR-AC, which was used as a measure of riboflavin deficiency, with the appearance after about three weeks of the characteristic signs of severe deficiency in most animals. Most of the experimental work was done with diets which contained groundnut oil but deficiency was more quickly obtained when rats were fed a riboflavin-deficient diet containing 10% cotton seed oil. It was suggested that this might be due to the fact that cotton seed oil contained more saturated fatty acids than the other oils. The possibility that the diets may have also been deficient in vitamin E was not confirmed.

Erythrocytes from riboflavin-deficient rats in this study were found to be more fragile than red cells from control rats and less capable of maintaining cell integrity when exposed to hydrogen peroxide or hypotonic saline in-vitro. Susceptibility to these stresses was considerably reduced by including thyroxine in the in-vitro test system and injection of much smaller quantities in-vivo may have had a similar effect though the differences were not significant.

The activity of erythrocyte glutathione reductase in riboflavin deficiency was lower than that of control rats and the concentration of reduced glutathione in the red cell was also lower. Other changes in the red cells accompanying riboflavin deficiency were higher activities of glutathione peroxidase, NADH-methaemoglobin reductase and NADPH-methaemoglobin reductase, increased concentrations of total peroxides and proportions of methaemoglobin and sulfhaemoglobin.

Evidence of anaemia accompanied riboflavin deficiency in the later stages. Packed cell volume, reticulocyte count, total blood count and also the number of white cells, all fell. Iron absorption was also reduced and concentrations of iron ferritin and non-haem iron increased in deficiency. Whether the anaemia is directly due to riboflavin deficiency reducing iron absorption or blocking the release of storage iron, is discussed, as is also the role of thyroxine in regulating erythropoiesis.

Fractionation of red cells from riboflavin-deficient rats showed that as the deficiency progressed the proportions of young cells decreased and old cells rose. The activities of glutathione peroxidase and NADPH-methaemoglobin reductase did not fall in cells from riboflavin-deficient rats as much as those found in control red cells. These observations may mean that these enzymes are not being used as much _in-vivo_ in erythrocytes from riboflavin-deficient rats as in controls and may explain why total peroxides and methaemoglobin are increased in red cells in deficiency. Shortage of GSH, FAD and nicotinamide co-enzymes may account for the lack of activity in these enzymes _in-vivo._ The activity of aspartate aminotransferase fell with age and, if anything, even more in the red cells from the riboflavin-deficient rats than in the controls. The metabolism of this enzyme is not involved in any direct way with glutathione reductase or associated metabolites and the similar decline in activities in riboflavin-deficient and control rats may mean that red cell survival is very similar also. If anything, the lower activity in the oldest red cells from riboflavin-deficient rats may mean that survival of cells in severe riboflavin deficiency is increased. There is certainly no evidence to suggest that erythrocyte survival is reduced as a result of the increased fragility in the red cells in severe riboflavin deficiency although it is possible that in the early stages the apparent increase in young cells may be stimulated by increased fragility.

The relationship between riboflavin deficiency and thyroid hormone metabolism was studied in this report. The results obtained showed that red cell metabolism in iodine and riboflavin deficiencies show many similar disturbances: high EGR-ACs, increased red cell fragility, methaemoglobinemia, increased peroxides and decreases in the number of young cells and in the concentration of plasma iron. Low plasma thyroxine was confirmed in riboflavin deficiency. The interactions between thyroid hormone metabolism and riboflavin metabolism in the genesis of the biochemical disturbances seen in riboflavin deficiency are discussed.

In summary, renewed interest in riboflavin metabolism has resulted from the improved methods for measuring functional riboflavin status, the erythrocyte glutathione reductase is of vital importance for the integrity of the red cell and that riboflavin deficiency renders the cell less able to combat oxidative damage.

PLANS FOR FUTURE WORK

There are three areas where this work should be expanded. Certain aspects of the pathophysiology of riboflavin-deficient red cells need more investigation. In this thesis it is postulated that hexokinase may be inhibited by increasing concentrations of intracellular GSSG which might depress glucose oxidation and reduce available NADH and NADPH. Methaemoglobin increases in red cells in riboflavin deficiency, but the reason is not clear. Both methaemoglobin reductases are riboflavin-dependent enzymes but attempts to stimulate NADH-methaemoglobin reductase from red cells of riboflavin-deficient subjects in-vitro with FMN were unsuccessful. (Beutler, 1969). The latter would suggest that NADH-methaemoglobin reductase in riboflavin deficiency is saturated in-vivo. These studies have shown that enzyme activity of the methaemoglobin reductases in riboflavin deficiency is high when measured in-vitro and that there appears to be a block in their utilisation in-vivo. Unavailability of nicotinamide co-enzymes might explain these observations and should be investigated.

These studies have shown that erythropoiesis is reduced in riboflavin deficiency and the rats became mildly anaemic. The fall in activity of aspartate aminotransferase activity with age indicates a normal or slightly increased red cell survival time while the increased fragility of riboflavin-deficient red cells would argue for the reverse. Beutler and Srivastava (1970) using red cells labelled with ^{51}Cr suggested that red cell survival was not affected by riboflavin deficiency and nor was it influenced by the presence of oxidant drugs. There was little indication of the degree of riboflavin deficiency in their animals and in view of the new evidence concerning red cell integrity, it would seem worthwhile repeating their experiments. In addition the relative importance of thyroid hormone deficiency and riboflavin deficiency in reducing erythropoiesis needs clarifying.

Lastly, the experiments in which rat diets were modified by using different fats raises some interesting possibilities. Using ground nut oil or cotton seed oil, riboflavin-deficient rats experienced skin lesions, while those rats receiving cod liver oil showed no skin lesions. Biochemical evidence of riboflavin deficiency was similar in all three groups of rats and it is possible that cod liver oil contains some metabolite not produced in-vivo in riboflavin-deficient rats but which is necessary for skin integrity. It is planned to fractionate cod liver oil and use the fractions in diets usually causing skin lesions in the hope of isolating and later identifying the active material.

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Influence of riboflavin status on the red blood cell fragility in rats. By

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Erythrocytes deficient in glucose-6-phosphate dehydrogenase are known to be susceptible to haemolysis upon exposure to various drugs possibly due to a diminished generation of NADPH (Beutler, 1960). It has been suggested that the oxidative changes observed during haemolysis, e.g. loss of reduced glutathione (GSH) and oxidation of haemoglobin, are manifestations of the presence of hydrogen peroxide (Mills & Randall, 1958). Glutathione reductase, a riboflavin dependent enzyme, is important for the maintenance of GSH and possibly for controlling the redox state of $NADP^+$ in tissues (Rall & Lehninger, 1952).

Experiments have been done to determine whether a marginal deficiency of riboflavin in riboflavin-deficient rats impairs the physiological function of red blood cells. Washed red blood cells (0·5 ml of 6% haematocrit in Krebs–Ringer phosphate buffer, KRP) from 6 normal, 6 pair-fed and 8 riboflavin-deficient rats and an H_2O_2-generating system (hypoxanthine, 1·6 mM; xanthine oxidase, various amounts) were incubated with 2·3 ml oxygenated KRP, pH 7·0, in a total volume of 3·0 ml in 10 ml conical flasks. In addition, red blood cell fragility was measured by incubating the washed red cells (as above) with 2·5 ml sodium chloride solutions (0·9, 0·72, 0·54 and 0·36%). All incubations were done at 37° for 45 min in a shaking waterbath. Erythrocyte glutathione reductase activity coefficients were estimated in every blood sample as a measure of riboflavin status by a modification of the method of Glatzle, Weiser, Weber & Wiss (1973).

The results showed on using 0·02 units xanthine oxidase a highly significant correlation between riboflavin status (as measured by the AC) and the degree of haemolysis produced by exposure to the H_2O_2-generating system (r 0·756, $P<0·01$). The slower production of peroxide produced by 0·008 units of xanthine oxidase caused less haemolysis but an even closer correlation (r 0·829, $P<0·01$). Measurements of red cell haemolysis made with decreasing concentrations of saline showed that erythrocytes from riboflavin-deficient rats were more easily haemolysed than those from control and pair-fed animals but this was apparent only when 0·36% saline was used. Under these conditions the degree of haemolysis was correlated with the riboflavin AC (r 0·531, $P<0·02$).

It has been suggested that an increasing susceptibility to haemolysis may be due to a lower GR activity being unable to generate sufficient GSH (Kaplan, 1971). The results presented suggest that riboflavin-deficiency through its effect on EGR activity may also reduce GSH levels and affect the stability of the red blood cell, for the H_2O_2 and salinity experiments show that a high EGR AC is associated with increased red cell fragility.

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Effects of riboflavin deficiency on the metabolism of the red blood cell

F.M. Hassan and D.I. Thurnham

Hassan and Thurnham (1977) demonstrated that red blood cells from riboflavin-deficient rats appear to be more susceptible to haemolysis *in vitro* than red cells from normal animals. We reported a positive correlation between the erythrocyte glutathione reductase (EGR) activity coefficient (AC) - a measure of riboflavin status - and red cell fragility. This paper reports further studies to determine whether the increased fragility in red cells from riboflavin-deficient rats was associated with a shortened *in vivo* life span.

Washed red blood cells (0.5 ml) from normal and deficient animals were layered onto density gradients (1.113, 1.119, 1.125 and 1.140 g/ml) of Ficall/Triosil mixture in cellulose nitrate tubes. Ultra centrifugation at 80,000 g for 2 hours at 4°C, produced four bands of red cells of differing mean ages. Fractions were separated, washed with isotonic saline solution containing 20 mg/dl human albumin. EGR - AC (Glatzle et al, 1973), haemoglobin (a measure of cell numbers; Wootton 1964) and a fragility test using hydrogen peroxide (Hassan and Thurnham 1977) were done in each fraction while reduced glutathione (GSH; Hissin and Hilf 1976) and haemoglobin derivatives (methaemoglobin, sulfhaemoglobin and oxyhaemoglobin (Evelyn and Malloy 1938) were measured in the unfractionated blood samples.

Haemoglobin measurements on the red cell fractions showed that in riboflavin-deficient animals there were fewer young cells circulating in the blood stream and that the majority of the red blood cell population were old cells. The results from thirteen riboflavin-deficient rats showed a highly significant negative correlation ($r = 0.832$, $P < 0.001$) between the AC of the unfractionated blood and the Hb content of the fraction containing the youngest cells. On the other hand, in the two fractions containing the oldest cells there were positive correlations ($r = 0.766, 0.702$, $P < 0.001$) between EGR - AC and haemoglobin.

These results suggest that as riboflavin deficiency becomes more severe young red cells are not being released into the circulation and the relative number of old cells is increasing. In young riboflavin-deficient rats there appears to be no evidence of anaemia as the deficiency progresses which would suggest, if erythropoiesis is being inhibited, that the life span of the cells is increasing.

Red cells from riboflavin-deficient animals (6) were significantly more fragile in all four fractions than their comparable fractions in three control animals. There was no significant difference in fragility of the four fractions from the control animals and no difference in the three oldest fractions from the blood of the riboflavin-deficient animals. The youngest fractions, however, from the riboflavin-deficient animals were significantly less fragile ($P < 0.01$) than the three older fractions.

GSH levels measured in red cells from nine riboflavin-deficient rats were significantly ($P < 0.001$; $\bar{x}$ ±SD, 2.78 ± 0.73) below those found in two control animals (15.56 ± 0.79) while levels of methaemoglobin in seven deficient animals were significantly ($P < 0.01$, 31.6 ± 7.1) higher than in three controls (4.3 ± 2.3).

We suggest that riboflavin deficiency through its effect on EGR activity may reduce the levels of GSH in the red cell. GSH is necessary for the stability of the membrane of the red cell, to convert methaemoglobin to haemoglobin and as source of reducing potential in the detoxifying function of glutathione peroxidase. The increased levels of methaemoglobin may therefore also be a consequence of reduced EGR activity and contribute to the increased fragility of the red cells. An impaired glutathione peroxidase activity may account for the increased susceptibility of the red cell from riboflavin-deficient animals to peroxide-induced haemolysis.

The increased red cell fragility in riboflavin deficiency appears to be due firstly to the reduced activity of EGR and secondly to the increased number of older cells. Unexpectedly, the increased fragility of the red cells does not appear to be of physiological importance in the severe deficiency to which the rat is exposed. This is contrary to results of human studies where the deficiencies are less severe and a reduction of old cells appears to occur (Powers, personal communication).

The tolerance shown in the rats to the more fragile cells may therefore be an adaptive mechanism which operates when erythropoiesis is blocked.

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– Erythropoiesis
– Red blood cells
– Fragility
– Fractionation
– Methaemoglobin
– Reduced glutathione

Effect of Riboflavin Deficiency on the Metabolism of the Red Blood Cell

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Summary: *Red blood cells from control and riboflavin-deficient rats were separated into fractions of different mean age. Measurement of haemoglobin in the red cell fractions showed a progressive reduction in the number of young cells as the severity of deficiency increased and a corresponding increase in the old cells. The red cells from the deficient animals were significantly more fragile in all fractions than their comparable fractions in the control animals when exposed to a peroxide generating system. The increased fragility of the red cell in the riboflavin deficient rat may be due to the reduced level of red cell reduced glutathione since this substance is a necessary substrate for glutathione peroxidase which functions to destroy peroxides. Reduced glutathione is also necessary to convert methaemoglobin to haemoglobin and methaemoglobin levels were increased in red blood cells from deficient animals.*

Thus riboflavin deficiency through its control of the activity of glutathione reductase appears to influence red cell fragility in the rat but there is no evidence yet to suggest that the life span of the erythrocyte is reduced.

Introduction

HASSAN and THURHAM [7] demonstrated that red blood cells from riboflavin-deficient rats appear to be more susceptible to haemolysis *in vitro* than red cells from normal animals. These findings posed the question of whether *in vitro* red cell fragility shortened the *in vivo* life span of red cells.

Red cell density increases with age of the cell [1] and fractionation studies on the blood of the two riboflavin-deficient humans [12] suggested that there were fewer old and more young red cells as would be expected if red cell life span was shortened. Fractionation studies on blood of riboflavin deficient rats were therefore done to determine whether the vitamin deficiency was increasing the fragility of red cells *in vivo* with age and as a consequence shortening their life span.

Material and Methods

Animals

Riboflavin-deficiency was produced by feeding a riboflavin-deficient diet [9] *ad libitum* and in individual pots to weanling male Wistar rats (50–60 grams) housed individually in wire-bottomed cages. Characteristic signs of severe deficiency were produced in most animals including weight loss, hair discolouration and skin lesions. Control animals were fed the riboflavin-deficient diet plus 22 mg of riboflavin/kg of diet. Pair-fed animals were given the average amount of food which was eaten by the deficient rats the previous day.

Fractionation experiments

Mixtures (4 ml) of Ficoll and Triosil [13] with densities of 1.113, 1.119, 1.125 and 1.140 g/ml were discretely layered into 19.5 ml-cellulose nitrate tubes. The gradient was used to fractionate 0.5 ml washed erythrocytes at 80 000 g and +4° C for 2 hours in an SW 27.1 swing-out rotor on a Beckman Ultracentrifuge. Four fractions were carefully separated with Pasteur pipettes and washed three times with isotonic saline solution containing 20 mg/dl human albumin (Kabi Vitrum Ltd., Ealing, London W5 2TH). The number of red blood cells at the bottom of the tube was negligible.

Biochemical measurements

1. **Erythrocyte glutathione reductase activity coefficient (EGR-AC):** Erythrocyte glutathione reductase (EGR) was measured spectrophotometrically using 3.0 ml haemolysate (1:560 dilution, 0.1 M phosphate buffer, pH 7.4) with 3.95 mM GSSG, 20 mM EDTA and with or without 18.5 µM FAD in a reaction initiated by 120 µM NADPH and followed at 334 mµ and 35° C in a total volume of 3.3 ml. The measure of riboflavin status, the AC, was calculated as follows:

$$AC = \frac{\text{Change in O. D. in 10 minutes (with FAD)}}{\text{Change in O. D. in 10 minutes (without FAD)}}$$

2. Reduced glutathione (GSH): GSH was measured in the unfractionated blood by a fluorometric method [8].

3. Methaemoglobin (MetHb) was measured by the spectrophotometric method o. Evelyn and Malloy [5] which is based on reading the absorption band at 635 mµ of MetHb before and after its conversion into cyanmethaemoglobin by sodium cyanide.

4. Haemoglobin was measured by the Cyanmethaemoglobin method as described by Wootton [15].

5. Fragility test: Washed red cells (0.5 ml, 6% haematocrit) were incubated in a hydrogen peroxide-generating system containing 1.6 mM hypoxanthine and 0.02 units xanthine oxidase (both from Boehringer) in 2.3 ml oxygenated Ringer phosphate buffer, pH 7.0 [14] for 45 minutes in a shaking water-bath.

Results

Table 1 shows the haemoglobin concentrations obtained in the four fractions of red cells of different densities. The concentration of haemoglobin in the red cell is not believed to change as the cell gets older and denser [1] therefore haemoglobin concentration indicates the proportion of red cells in each fraction. Frac-

Tab. I: Distribution of haemoglobin after separation of red cells from control and riboflavin-deficient rats into fractions of different mean ages

Red cell density g/ml	Haemoglobin (% of Total)				Activity coefficient (AC)
	< 1.113	1.113–1.119	> 1.119–1.125	> 1.125–1.14	
Control (9) Mean ± SD	30.1 ± 12.5	36.1 ± 8.3	20.0 ± 8.2	12.0 ± 5.2	1.19 ± 0.07
Pair-fed control (5) Mean ± SD	37.8 ± 6.2	29.3 ± 5.3	17.6 ± 6.0	11.0 ± 2.6	1.28 ± 0.31
Deficient (13) Individual Values	53.2	25.0	15.3	6.5	1.28
	42.0	28.8	12.5	16.7	1.34
	29.5	36.4	25.6	8.6	1.40
	25.1	45.1	20.7	9.1	1.48
	31.4	40.8	11.2	18.7	1.53
	17.5	33.1	31.5	17.9	1.60
	13.1	27.1	38.2	22.2	1.67
	11.3	32.4	42.4	15.3	1.78
	10.2	27.7	43.0	19.2	1.80
	9.1	30.4	39.0	23.5	1.88
	9.4	50.3	30.3	11.2	1.90
	1.9	16.8	45.5	35.8	1.97
	5.2	22.9	42.8	29.0	2.47

Red cells were separated into fractions of densities shown using gradient centrifugation. After washing, haemoglobin in the fractions was measured and expressed as a percentage of the total recovered for the individual rat or group of rats as indicated. AC is the measurement of riboflavin status obtained from glutathione reductase measurements on the unfractionated blood haemolysate. Number of rats shown in parentheses.

tionations of red cells from control and pair-fed animals produced very similar red cell distributions and t-tests on the mean haemoglobin concentrations of corresponding fractions showed no significant differences.

Fractionation of blood from riboflavin-deficient rats showed a different distribution of red cells by comparison with the control animals but a straightforward comparison between the two groups was not justified as the degree of deficiency was not the same in all the group. Instead, changes in haemoglobin distribution associated with riboflavin deficiency were investigated by determining the correlation between the AC ratio of the unfractionated blood and the proportion of haemoglobin in the respective fractions (table II). The correlation between the haemoglobin concentrations in the two lighter fractions and the AC ratios showed an inverse relationship. That is, as riboflavin deficiency increased, the haemoglobin concentration in the lighter fractions decreased. This trend was particularly significant ($P < 0.001$) in the fraction containing

Tab. II: Correlation between activity coefficient (AC) of unfractionated blood of riboflavin-deficient rats and haemoglobin (% of total) in the four red cell fractions of different densities

Red cell density g/ml	< 1.113	1.113–1.119	> 1.119–1.125	> 1.125–1.140
Correlation coefficient	– 0.832	– 0.410	0.766	0.702
P <	0.001	N. S.	0.001	0.01

Experimental procedure is described in table I.

Tab. III: Effect of riboflavin deficiency on susceptibility of red cells to haemolysis in presence of a peroxide-generating system

Density g/ml	*Control* AC = 1.17 ± 0.01 (n = 3) % Haemolysis *	P <	*Deficient* AC = 1.96 ± 0.11 (n = 6) % Haemolysis *
< 1.113	14.9 ± 4.7	0.01	22.1 ± 4.7
1.113–1.119	(N. S.) 19.7 ± 10.7	0.01	64.3 ± 15.4 (P < 0.001)
> 1.119–1.125	(N. S.) 20.6 ± 10.6	0.02	59.1 ± 19.6 (P < 0.01)
> 1.125–1.140	(N. S.) 21.1 ± 9.7	0.05	63.6 ± 23.7 (P < 0.01)

Red cells were prepared as described in table I. Aliquots were exposed to the peroxide-generating system for 45 minutes, centrifuged and the haemoglobin *, measured in the supernatant, was expressed as a percentage of the total haemoglobin in saponin-haemolysed, identicle but non-incubated tubes. All results are shown as mean ± standard deviation. Parentheses indicate significance of the difference between erythrocytes in the lightest fraction and the three other fractions (t-tests). (n) equals the number of animals used.

erythrocytes with densities < 1.113 g/ml. On the other hand, in those fractions containing the heavier red cells, the proportion of haemoglobin increased as the degree of deficiency increased.

When red blood cells from six riboflavin-deficient rats were incubated in a H_2O_2-generating system, the red blood cells in all four fractions were haemolysed to a significantly greater extent than those from comparable fractions in the three control animals (table III). There was no significant difference in the percentage haemolysis produced within the four fractions from the control rats. However the fraction containing the lightest cells from riboflavin-deficient animals was haemolysed to a lesser extent than red cells in the other three fractions.

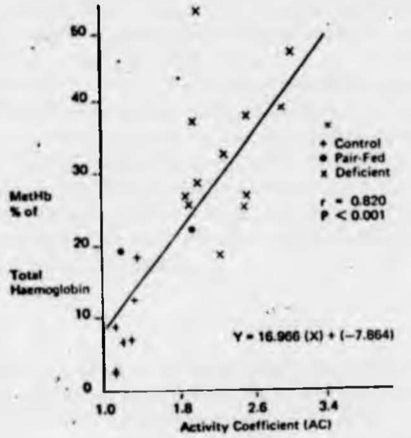

Fig. 1: Methaemoglobin was measured in red blood cells from control (7), pair-fed (2) and riboflavin-deficient (13) rats and expressed as a percentage of total haemoglobin. AC ratios, the measure of riboflavin status, were calculated from measurements of erythrocyte glutathione reductase activity with and without flavin adenine dinucleotide.

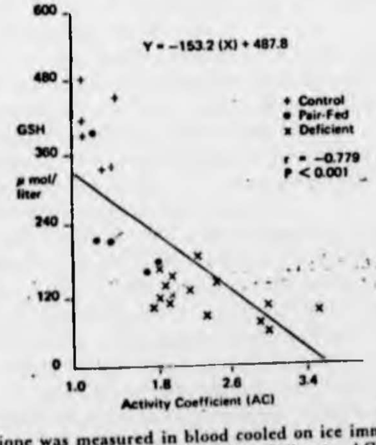

Fig. 2: Reduced glutathione was measured in blood cooled on ice immediately after collection from control (6), pair-fed (5) and riboflavin-deficient (15) rats. AC ratios are a measure of riboflavin status (see figure 1).

Figures 1 and 2 show results obtained for red cell methaemoglobin and red cell reduced glutathione respectively plotted against the AC ratios of the same samples. The results suggest that as the AC ratio increases there is a decrease in GSH and an increase of MetHb levels.

Using the t-test to determine whether the differences between the mean GSH values of the different groups was significant showed that there was a significant difference between deficient and pair-fed animals ($P < 0.02$), deficient and control ($P < 0.001$) and between pair-fed and control ($P < 0.001$) groups. Likewise, analysis of the MetHb results showed that there was no significant difference between the pair-fed and deficient groups while there was a significant difference between deficient and control ($P < 0.001$) and between the pair-fed and control ($P < 0.01$) groups.

Discussion

Glutathione reductase is necessary for the synthesis of GSH and for this function it is dependent on a supply of FAD, one of the metabolically active forms of riboflavin. Riboflavin deficiency has been shown to reduce the enzyme activity of glutathione reductase in rats [6] and man [2] although a fall in red cell GSH has not previously been demonstrated.

The data in figure 2 suggest that riboflavin deficiency in rats is associated with low red cell GSH levels. It must be noticed however that the red cell GSH in the pair-fed controls is also reduced and that AC ratios in these animals are slightly raised. Thus low GSH levels may be associated with reduced food intake and not riboflavin deficiency.

The reason for the slightly elevated AC ratios in some of the pairfed rats is not known. Daily riboflavin requirements of the rat have been variously estimated at 18–60 μg [3] but food intake in the pair-fed group did not fall below 10 g/rat/day which would supply at least 220 μg.

GSH is necessary for the stability of the red cell membrane [10] to convert MetHb to haemoglobin [11] and as a source of reducing potential in the detoxifying function of glutathione peroxide [4].

The increased level of methaemoglobin (fig. 1) may therefore be a consequence of reduced red cell GSH levels. Likewise the increased tendency to haemolyse in the peroxide-generating system (table III) probably represents increased red cell fragility due to lowered GSH levels. Low red cell GSH and reduced EGR activity may impair the detoxifying function of glutathione peroxidase and make the red cell more susceptible to red cell damage.

The results in table I show the percentage of haemoglobin in the four red cell fractions for control, pair-fed control and riboflavin-deficient animals. There was no difference between the results of the pair-fed and control rats but the results do suggest that as riboflavin deficiency progresses there is a reduction in the number of young cells circulating in the blood stream and an increase in the number of old cells. Likewise results in table II also support the idea that as riboflavin deficiency becomes more severe, young cells are not being released into the circulation and the relative number of old cells is increasing. In these riboflavin-deficient rats there was no evidence of anaemia as the deficiency progressed which suggests that the mean life span of the red cells is increasing if erythropoiesis is being inhibited.

Unexpectedly the increased fragility of the red cells does not appear to be physiologically important in the severe deficiency to which these rats were exposed since the animals did not become anaemic and the relative number of old cells appears to increase. This is contrary to results of human studies where the deficiencies are less severe and a reduction of old cells appears to occur (H. Powers, personal communication). The tolerance shown in riboflavin deficient rats to the more fragile cells may therefore be an adaptive mechanism which operates when erythropoiesis is blocked.

An alternative hypothesis to explain the apparant increase in the number of old cells in the riboflavin-deficient animals may be that the vitamin deficiency alters the metabolism of the red cell in such a way as to increase its density. We feel that this hypothesis merits more attention as some preliminary experiments have suggested that the enzyme activity of aspartate amino-transferase appears higher than would be expected from the density (*i. e.* age) of the cells obtained from riboflavin-deficient animals.

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