METHYLGLYOXAL, GLYOXALASES AND CELL PROLIFERATION

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SUMMARY

The metabolic function of the glyoxalase system was investigated in (a) the differentiation and proliferation of human tumour cells <u>in vitro</u>, (b) the cell-free assembly of microtubules and (c) in the red blood cells during hyperglycaemia associated with Diabetes Mellitus.

Chemically-induced differentiation of human promyelocytic HL60 leukaemia cells to neutrophils, and K562 erythroleukaemia cells, was accompanied by a decrease and an increase in the activity of glyoxalase I, respectively. Growth-arrest of Burkitt's lymphoma Raji cells and GM892 lymphoblastoid cells was accompanied by an increase and a decrease in the activity of glyoxalase I respectively. However, differentiation and growth arrest generally proceeded with an increase in the activity of glyoxalase II. Glyoxalase I activity did not consistently correlate with cell differentiation or proliferation status; hence, it is unlikely that glyoxalase I activity is either an indicator or a regulator of cell differentiation or proliferation. Conversely, glyoxalase II activity consistently increased during cell differentiation and growth-arrest and may be both an indicator and regulator of cell differentiation or proliferation. This may be related to the control of cellular microtubule assembly.

S-D-Lactoylglutathione potentiated the cell-free, GTP-promoted assembly of microtubules. The effect was dose-related and was inhibited by glyoxalase II. During assembly, S-D-lactoylglutathione was consumed. This suggests that the glyoxalase system, through the influence of S-D-lactoylglutathione, may regulate the assembly of microtubules in cellular systems

The whole blood concentrations of methylglyoxal and S-D-lactoylglutathione were increased in Diabetes Mellitus. There was no significant difference between red blood cell glyoxalase activities in diabetics, compared to healthy controls. However, insulin-dependent diabetic patients with retinopathy had a significantly higher glyoxalase I activity and a lower glyoxalase II activity, than patients without retinopathy. Diabetic retinopathy correlated with high glyoxalase I activity and low glyoxalase II activity and suggests the glyoxalase system may be involved in the development of diabetic complications.

<u>Keywords</u> : Glyoxalase, S-D-lactoylglutathione, microtubules, differentiation, proliferation.

TO TERRY.

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ACKNOWLEDGEMENTS

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ABBREVIATIONS

CCRG 81045 : 8-carbamoyl-3-methylimidazo-[5,1-d]-
1,2,3,5-tetrazin-4(3H)-one

BL : Burkitt's Lymphoma

DMSO : Dimethyl Sulphoxide

EGTA : 1,2-Di(2-aminoethoxy)ethane-NNN'N'-
tetra-acetic acid

GSH : Reduced Glutathione

GTP : Guanosine 5'- Triphosphate

HEPES : N-2-Hydroxyethylpiperazine-N'-2-
ethanesulfonic acid

HL60 : Human Promyelocytic Leukaemia - 60 Cell
Line

HPLC : High Pressure Liquid Chromatography

MES : 2-(N'-Morpholino)ethanesulfonic acid

NMF : N-Methylformamide

RPMI 1640 : Roswell Park Memorial Institute 1640

TRIS : Tris(hydroxymethyl)aminomethane

1. INTRODUCTION

1.1 The Glyoxalase System - A Definition

The glyoxalase system catalyses the conversion of methylglyoxal to D-lactic acid, via the intermediate S-D-lactoylglutathione. It comprises two enzymes, glyoxalase I and glyoxalase II, plus a catalytic amount of reduced glutathione (1) - Scheme 1.

MeCOCHO

Glyoxalase I

GSH   MeCH(OH)CO-SG

Glyoxalase II

MeCH(OH)CO$_2$H
D-Lactic acid
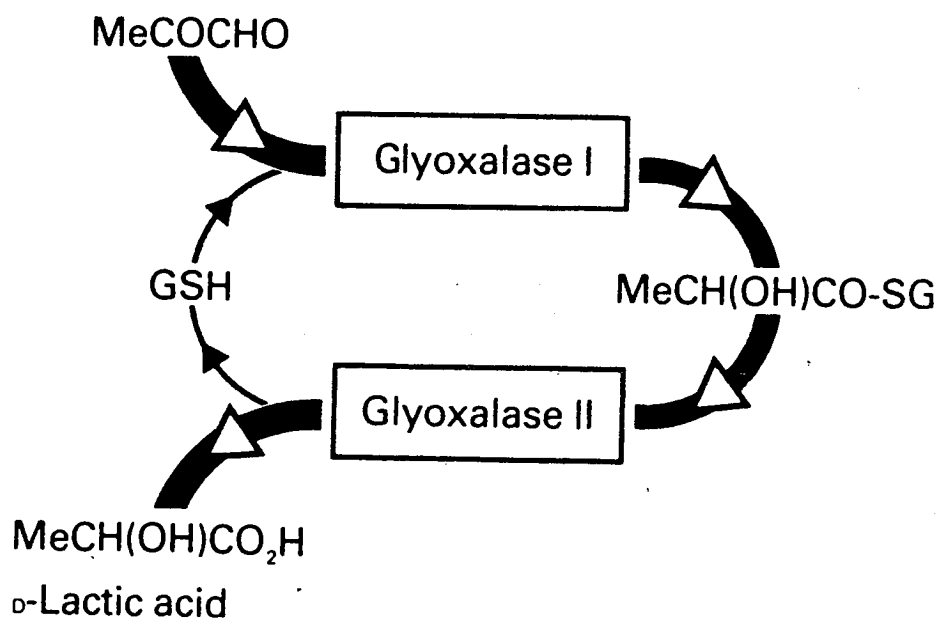
SCHEME 1   THE GLYOXALASE PATHWAY.

Glyoxalase I [E.C.4.4.1.5, lactoylglutathione lyase] catalyses the formation of S-D-lactoylglutathione from the hemimercaptal formed non-enzymatically between methylglyoxal and reduced glutathione (2).

MeCOCHO
+ $\rightleftharpoons$ MeCOCH(OH)-SG $\xrightarrow{\text{GLYOXALASE I}}$ MeCH(OH)CO-SG
GSH
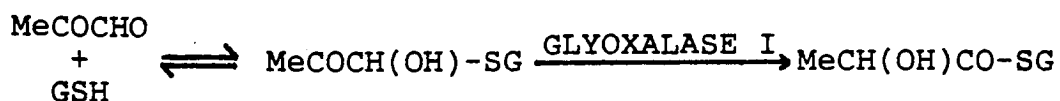
Glyoxalase II [E.C.3.1.2.6, hydroxyacylglutathione
hydrolase] catalyses the hydrolysis of
S-D-lactoylglutathione to D-lactic acid and regenerates
the reduced glutathione consumed in the glyoxalase
I-catalysed reaction (2).

$$\text{MeCH(OH)CO-SG} \xrightarrow{\text{GLYOXALASE II}} \text{MeCH(OH)COOH + GSH}$$
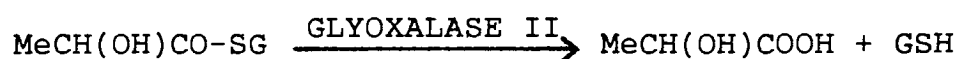
The glyoxalase II-catalysed reaction is generally
found to be the rate-limiting step of the glyoxalase
metabolic pathway. Glyoxalase II is the pacemaking
enzyme (2). The glyoxalase system is present in the
cytosolic fraction of cells and cellular organelles (3).
It is found throughout biological life and is thought to
be ubiquitous : Hopkins and Morgan considered the
glyoxalase system to be present in the cells of all life
forms (4).

1.1.1 Historical.

The glyoxalase system was discovered in 1913 by
Dakin and Dudley (5) and by Neuburg (6). It was
initially thought to consist of only one enzyme
catalysing the conversion of methylglyoxal to lactic
acid and phenylglyoxal to mandelic acid. "Glyoxalase
activity" was assayed as the rate of production of
lactic acid upon the addition of methylglyoxal to cell
extracts. At this time, the formation of lactic acid
was measured either by the manometric determination of
carbon dioxide released by acidification of a

bicarbonate buffer (1,4) or by optical rotation measurements (5,7).

In 1932 Lohmann (1) found that reduced glutathione was an essential cofactor required for "glyoxalase activity". This disclosure stimulated interest in the chemical interaction between methylglyoxal and reduced glutathione. In 1933 Jowett and Quastel (8) reported that reduced glutathione and methylglyoxal combine non-enzymatically to form a complex which, they hypothesised, was converted to lactic acid by "glyoxalase". By 1936 Yamazoye (9) was able to demonstrate that methylglyoxal was converted to an intermediate in liver extracts which was distinct from the non-enzymatic adduct of methylglyoxal and reduced glutathione. The structure of this intermediate remained a mystery for a further 15 years.

In 1951 Racker (2) recognised that the glyoxalase system was comprised of two discrete and consecutive reaction steps, catalysed by two separable enzymes which he called glyoxalase I and glyoxalase II - as defined above. The product of the first enzymatic step was the substrate of the second step; with methylglyoxal, this glyoxalase intermediate was found to be S-D-lactoylglutathione. He developed a direct spectrophotometric method for the assay of S-D-lactoylglutathione and, thereby, measured the activity of glyoxalase I by determining the rate of formation of S-D-lactoylglutathione from methylglyoxal

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and reduced glutathione, and the activity of glyoxalase II by determining the rate of disappearance of added synthetic S-D-lactoylglutathione; in the presence of cell extract. This formed the basis of the protocol for the spectrophotometric assay of the activities of the glyoxalase enzymes which is in use today.

In 1954 Racker (10) reported the crucial observation that D-lactic acid is the product of metabolism of methylglyoxal by the glyoxalase system. This fundamentally divorced the glyoxalase system from mainstream glycolytic metabolism which produces L-lactic acid. It was then concluded that the glyoxalase pathway is a minor pathway of non-glycolytic glucose metabolism.

In 1966 it was reported that glyoxalase I is a metalloprotein (11). Initial investigations, studying the loss of enzymatic activity with metal ion chelators and re-constitution of the apoenzyme with restoration of enzymatic activity, suggested the prosthetic metal ion was magnesium (11). However, later studies (12,13) analysing metal content of the purified enzyme defined zinc (II) ions as the physiological metal component of glyoxalase I.

The Glyoxalase Enzymes

1.2.Glyoxalase I.

1.2.1 Distribution and Molecular Characteristics.

Glyoxalase I activity appears to be present in all tissues of prokaryotic and eukaryotic organisms (4). It has been detected in : mammals, birds, fish, amphibians, plants, bacteria, fungi, and invertebrates (4,14-16). In mammals, the highest activities are found in the liver, kidney and brain (4,14,17). Typical activities of glyoxalase I are presented in Table 1. Recent comparison of glyoxalase I activities with radioimmunoassay of the enzyme protein suggest that there may be active and inactive forms of the enzyme in cells (18). Glyoxalase I is present at a concentration of approximately 0.2 μg/mg protein in most human tissues (18).

Mammalian glyoxalase I is a dimer with two identical subunits of molecular weight 20-23,000 daltons (19) whereas, from yeast and some strains of bacteria, glyoxalase I is a monomer with molecular weight 31-34,000 daltons (17). For most species, glyoxalase I is present in only one genetically determined form. However, the mammalian enzyme is found as a mixture of three separable forms (19,20). No differences in the catalytic and binding properties of these isozymes has been detected, indicating that the three subunit combinations (aa, ab, bb) are probably very similar.

In naturally occurring glyoxalase I, each subunit

Table 1.   The Activity of the Glyoxalase Enzymes in
Different Tissues

Source	Glyoxalase I mU/mg protein	Glyoxalase II mU/mg protein	Ref
Human Brain	138	245	3
Human Liver	26	108	3
Human Heart	71	80	3
Human Muscle	120	108	3
Pig Brain	274	80	3
Ox Brain	268	69	3
Rabbit Brain	117	86	3
Rat Brain	328	77	3
Yeast (Saccharomyces cerevisiae)	2040	nd	152

U = μmoles of S-D-lactoylglutathione produced
    (glyoxalase I) or decomposed (glyoxalase II) per
    minute at 25°C.

nd = Activity not determined

contains one equivalent of prosthetic zinc (II) ions -
the apoenzyme is catalytically inactive. Metal ion
substitution experiments have demonstrated that similar
specific enzymatic activities can be maintained with
magnesium Mg^{2+}, manganese Mn^{2+}, cobalt Co^{2+} and nickel
Ni^{2+} ions (21,22).

Mammalian glyoxalase I has an isoelectric point of
4.7 - 4.8, an acidic protein (23) whereas the yeast
enzyme is neutral with an isoelectric point of 7.0 (17).
These diverse properties are reflected in a dissimilar
amino acid composition for glyoxalase I from mammalian
and yeast origin (17). There are two free thiol groups
per molecule for all of the enzymes except the human
one, which contains four, and 5 - 12 disulphide bridges
(17). The molecular properties of glyoxalase I from
bacterial, yeast and mammalian sources are summarised in
Table 2.

Antibodies raised against glyoxalase I from one
mammalian species will cross-react with other mammalian
glyoxalase I enzymes but not with yeast glyoxalase I
(18). The substantial differences in the physical,
molecular and immunological properties of glyoxalase I
from mammalian and yeast sources suggests that the two
enzymes may have originated from different ancestral
proteins (17).

1.2.2. Substrate Specificity

The natural cofactor for glyoxalase I is reduced
glutathione [γ -L-glutamyl-L-cysteinylglycine] (1). A

Table 2   Molecular Properties of Glyoxalase I

Number of subunits	1 (Yeast) (17) 1 (Bacteria) (196) 2 (Mammalian) (23)
Molecular Weight (daltons)	30,000 (Yeast) (17) 20,000 (Bacteria) (196) 46,000 (Mammalian) (17)
Isoelectric Point at 4°C	7.0 (Yeast) (17) 4.7 - 4.8 (Mammalian) (23)
Metal Content	1 zinc atom per subunit (Yeast (17) and Mammalian (12))
Molecular Activity (k_{cat}) for Methylglyoxal	109,000 min^{-1} (Yeast) (17) 68,000 min^{-1} (Mammalian) (17)
Km for Methylglyoxal (at 2mM free glutathione)	0.53 mM (Yeast) (17) 0.12 - 0.14 mM (Mammalian) (17) 3.50 mM (Bacteria) (196)*

* Free Glutathione Concentration Not Known

number of glutathione analogues have been investigated as cofactors (24). L-Cysteine, oxidised glutathione [GSSG], L-cysteinylglycine and γ-L-glutamyl-L-cysteine have all been shown to be inactive as cofactors. L-cysteinylglycine and L-cysteine were inhibitory. Homoglutathione [γ-L-glutamyl-L-cysteinyl-β-alanine] (25), isoglutathione [α-L-glutamyl-L-cysteinylglycine] and some N-acyl derivatives of glutathione (26) have been found to be active as cofactors. The apparent Km values for these glutathione analogues are significantly higher than that of glutathione (the Km for glutathione is 7.4×10^{-4} M; for isoglutathione, it is 1.2×10^{-2} M) (27), while the maximal velocity is changed very little, indicating a lower binding affinity for the analogues than for reduced glutathione.

Glyoxalase I has a broad substrate specificity for α-oxoaldehydes. Substrates of glyoxalase I include methylglyoxal (7), hydroxypyruvaldehyde (28), phosphohydroxypyruvaldehyde (29), and several aromatic derivatives of glyoxal (30,31). The Km for the α-oxoaldehyde substrate decreases as the polarity of the side chain of the α-oxoaldehyde decreases. The trend in the V_{max} data parallels that of the Km for a series of alkyl α-oxoaldehyde homologues. Overall, methylglyoxal has the lowest Km and highest V_{max} for glyoxalase I, for enzymes from all known sources, and thus methylglyoxal is considered to be the physiological substrate.

1.2.3. The Rate of the Glyoxalase I Reaction.

From the kinetic analysis of the glyoxalase I reaction, it is generally accepted that the hemimercaptal adduct of reduced glutathione and methylglyoxal is the substrate of glyoxalase I (32,33,34). The hemimercaptal is formed in a non-enzymatic pre-equilibrium.

A neutral aqueous solution of methylglyoxal contains a complex equilibrium mixture of hydrates and oligomers of the α-oxoaldehyde with only a minor component existing in the free α-oxoaldehyde form (32). In the presence of reduced glutathione, the α-oxoaldehyde component reacts rapidly with the cysteinyl thiol of glutathione to form a hemimercaptal - residual methylglyoxal hydrate undergoes dehydration as hemimercaptal formation continues.

$$MeCOCH(OH)_2 \underset{+ H_2O}{\overset{- H_2O}{\rightleftharpoons}} MeCOCHO \underset{- GSH}{\overset{+ GSH}{\rightleftharpoons}} MeCOCH(OH)-SG$$
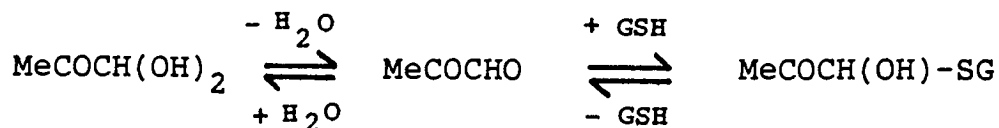
When the reduced glutathione concentration is equal to or greater than the concentration of methylglyoxal, the initial rate of formation of the hemimercaptal is dependent on the concentration of methylglyoxal and is independent of the concentration of reduced glutathione (32). The rate expression for the formation of hemimercaptal, HM, is therefore :

$$\frac{d[HM]}{dt} = k \, [methylglyoxal]$$

where k = 0.01 s^{-1} at pH 6.6 and 20°C (32). Eventually equilibrium is attained,

$$MeCOCHO + GSH \rightleftharpoons MeCOCH(OH)-SG$$

The equilibrium constant, K_{HM}, is 333 M^{-1} in the pH range 5-9 (34). The hemimercaptal is the molecular species which binds to the active site of glyoxalase I (34). The rate of S-D-lactoylglutathione formation from methylglyoxal and reduced glutathione cannot be increased above the rate of hemimercaptal formation by addition of glyoxalase I, indicating that there is no significant contribution from other (two substrate) reaction pathways (35).

The reaction kinetics of the conversion of hemimercaptal to S-D-lactoylglutathione catalysed by glyoxalase I approximate to Michaelis-Menten kinetics over a restricted substrate concentration range below saturation conditions. The initial-velocity saturation behaviour is non-Michaelian with apparent inhibition by reduced glutathione. With purified enzymes, Mannervik found the true kinetic behaviour was best described by the relationship :

$$v = \frac{Vmax. \quad [HM]}{Km(1+[GSH]/K_i) + [HM]}$$

where v was the reaction velocity, [HM] was the

concentration of hemimercaptal and K_i was the constant describing the competitive inhibition by reduced glutathione, GSH (36). The Michaelis constant, Km, for the hemimercaptal with purified enzyme is 120-140 μM from mammalian sources and 530 μM from yeast (17). The molecular activity (turnover number), $k_{cat.}$, is 59-71,000 min^{-1} for mammalian enzymes and 109,000 min^{-1} for the yeast enzyme (17). The molecular activity per subunit for mammalian enzymes is only ca. 30% of the activity of the yeast enzyme. The values of $k_{cat.}/Km$ are usually of the order $10^6 - 10^7$ $M^{-1}s^{-1}$ which is close to the theoretical diffusion limit maximum, indicating that glyoxalase I is a very efficient enzyme (17).

The glyoxalase I-catalysed conversion of hemimercaptal to S-D-lactoylglutathione may be reversed under conditions where liberated reduced glutathione is trapped and removed from the equilibrium. For the human red blood cell enzyme, kinetic parameters for this reverse reaction are k_{cat} = 3.6 s^{-1} and Km = 1.9 mM, which combined with parameters for the forward reaction, give an equilibrium constant of 1.1 x 10^4 for the isomerisation of hemimercaptal to S-D-lactoylglutathione (37).

1.2.4 Probing the Active Site

Glyoxalase I from yeast is inactivated by sulphydryl antagonists (38) whereas mammalian enzymes are resistant to a variety of oxidising, alkylating and mercaptide-forming sulphydryl-blocking agents (36,39).

Glyoxalase I from all sources is inhibited by the amino reagents 1-fluoro-2,4-dinitrobenzene, 5-dimethylaminonaphthalene-1-sulphonyl chloride and 2,4,6-trinitrobenzenesulphonate (36,39), and by arginine-specific reagents, 2,3-butanedione and camphorquinone-10-sulfonic acid (40). Modification of only two of the 10 - 17 arginine residues in the enzyme causes complete inactivation. One arginine residue is protected by glutathione; the hemimercaptal and S-D-lactoylglutathione may also provide substantial protection (40).

Nitration of the tyrosine residues of yeast glyoxalase I with tetranitromethane causes complete inactivation of the enzyme (41). The competitive inhibitor S-(p-bromobenzyl)glutathione provides very effective protection against inactivation, indicating that at least one residue must be in or near the active site of the enzyme.

Circular dichroism studies on yeast glyoxalase I show that the binding of S-(p-bromobenzyl)glutathione changes the electronic environment of the tyrosine residue(s) (42). This indicates that there is at least one tyrosine residue in close proximity to the active site. Although tyrosine residues may have an important structural role, they may also be acting as ligands for the zinc atom; it has been proposed that the ligands involved in the binding of the metal ion are probably nitrogen and oxygen (43).

Modification of the three tryptophan residues per subunit of human glyoxalase I with 2-hydroxy-5-nitrobenzyl bromide produces an almost total loss of activity (13). Studies with the competitive inhibitor S-p-bromobenzylglutathione have shown that one tryptophan residue is located in the active site of each subunit of glyoxalase I and may be involved in the binding of the substrate. Binding of the zinc ions to the apoenzyme changes the intrinsic fluoresence of the tryptophan residues, indicating a conformational change and, possibly, the close proximity of at least one tryptophan to the metal ion (13).

The activity of mammalian glyoxalase I is not markedly changed following the replacement of zinc ions with some other divalent cations (21,22), although the presence of zinc or other metal ion is essential for activity. This indicates that the prosthetic metal ion is not directly interacting with the substrate and may, rather, have an important structural role within the locus of the active site. The structural role for the metal ion is supported by the conformational changes occurring during metal ion binding by the apoenzyme, as judged by changes in tryptophan fluorescence (13).

The chemical properties of the metal ion binding site in yeast and mammalian glyoxalase I are very different. When the yeast apoenzyme is formed by removal of the zinc ions with the chelator 1-hydroxy-2,3-propanedithiol, the enzymatic activity

cannot be restored with zinc or other metal ions (44);
whereas, with the human red blood cell enzyme,
reconstitution of the enzyme can occur with a decrease
in activity of only a few percent. This may be related
to the particular sensitivity of the yeast enzyme to
sulphydryl agents.

Metal ion replacement studies of glyoxalase I have
been used to provide a paramagnetic metal ion derivative
which is amenable to electron paramagnetic resonance
spectroscopy (EPR). These studies have indicated that
the metal ion environment in cobaltous (Co^{2+})-glyoxalase
I is octahedral (21). Direct extended X-ray absorption
fine structure (EXAFS) measurements on native human
glyoxalase I suggest a distorted octahedral, or perhaps
heptahedral coordination to the zinc ion - see Figure 1
(45). Both EXAFS and nuclear magnetic resonance (NMR)
studies confirm that there is no coordination of the
substrate sulphur atom to the metal ion. NMR studies
have indicated that there are one or more water
molecules coordinated with the zinc ion, and the
carbonyl groups of the substrate and product reside in
the second coordination sphere of the zinc ion (46,47) -
probably held there by hydrogen bonding to the
associated water. These structural characteristics
proximate to the prosthetic metal ion may facilitate the
isomerisation of the substrate.

1.2.5. Catalytic Mechanism of Glyoxalase I.

The reaction catalysed by glyoxalase I is an
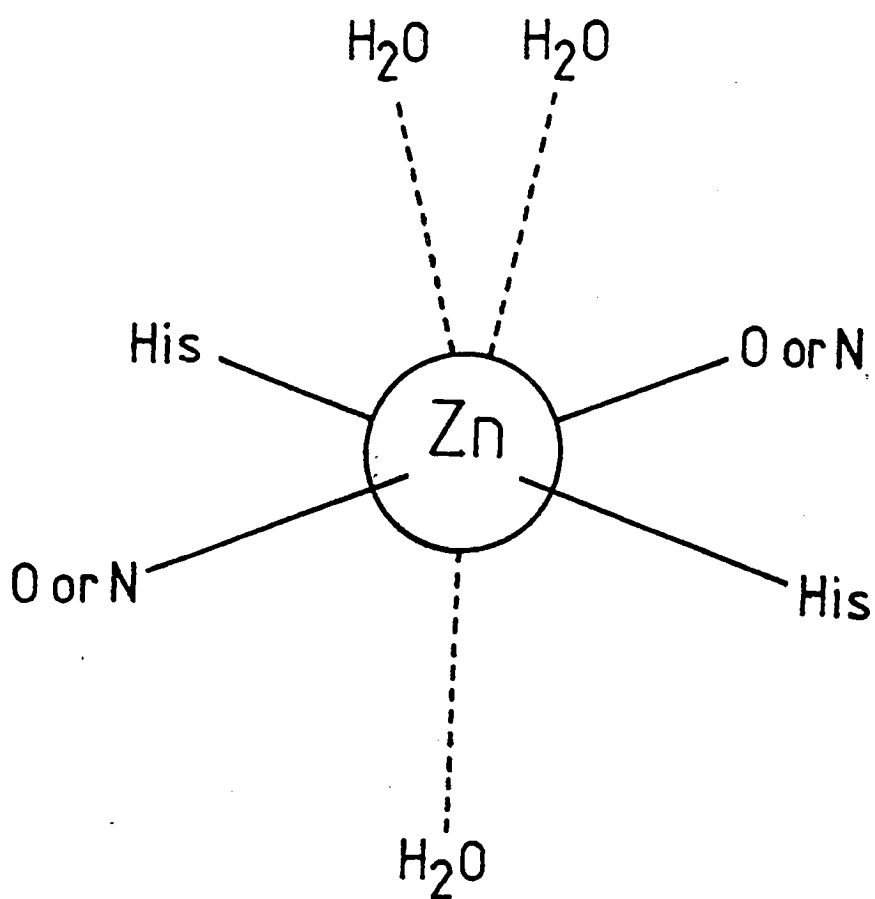
Figure 1. The Environment of the Metal Ion in the Active Site of Glyoxalase I.

isomerisation of the methylglyoxal-glutathione
hemimercaptal adduct to S-D-lactoylglutathione.
Experimental observations suggest the role of the
glutathione in this process is : (a) to provide specific
binding to the active site, and (b) to activate the C1
proton of the aldehydic group of the methylglyoxal by
hemimercaptal formation. The zinc ion is thought to be
important in the maintenance of optimum structure of the
active site for catalysis.

The mechanism proposed for the isomerisation
involves a shielded-proton transfer from C1 to C2 of the
hemimercaptal bound in the active site of the enzyme -
see Figure 2, (I). The resulting bound ene-diolate
species (II) ketonises to form S-D-lactoylglutathione.
Further substantiation of this mechanism has been
provided by evidence of the formation of the ene-diolate
species. This intermediate has been trapped by
oxidative reaction with flavinoid compounds (48,49).

The existence of a cis-dihydroxy intermediate has
been detected by NMR studies. As the temperature of the
reaction mixture is increased, the incorporation of
deuterium atoms into S-D-lactoylglutathione increases
from ca. 15% at $25^{O}C$ to ca. 22% at $35^{O}C$ (50). This is
consistent with a reaction mechanism involving an
ene-diol proton transfer. Glutathiohydroxyacetone
($GS-CH_2COCHO$), which is a substrate for glyoxalase I,
can be synthesised as two monodeuterated diastereomers
which differ very slightly in their NMR signals (51).
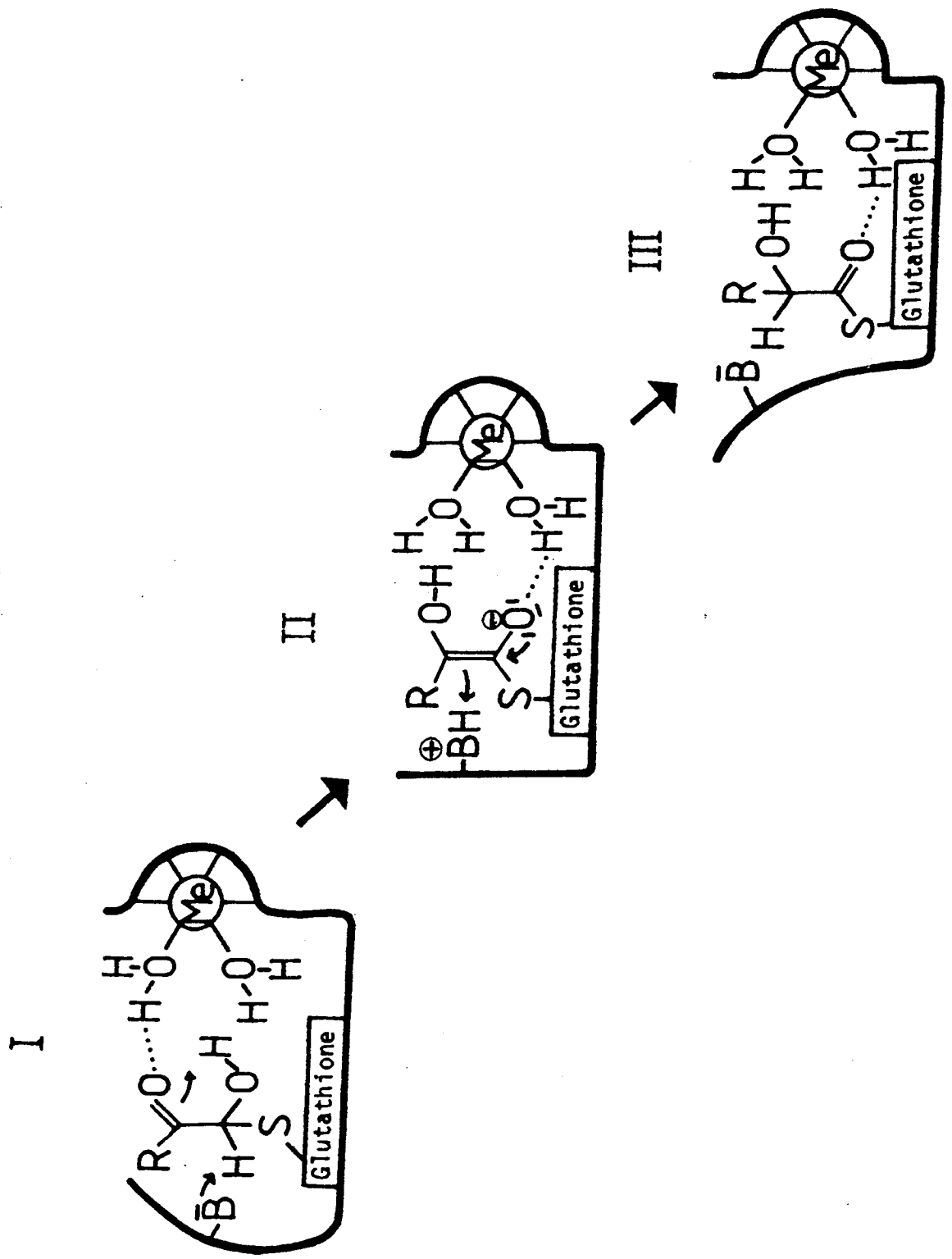
Figure 2. The Proposed Reaction Mechanism of Glyoxalase I.

Investigation using the thiohemiacetals derived from the reaction of the diastereomers with glutathione strongly suggest that only one diastereomer reacts with glyoxalase I and that the reaction proceeds via a cis-ene-diol intermediate. Ene-diol analogues have also proven to be good inhibitors of glyoxalase I - see below.

The role of the enzyme protein in the isomerisation is important for specificity of substrate binding and stereo-selectivity, and also for the provision of the basic group which is the putative initiator of the enolisation. The identity of this crucial basic component is not known.

1.2.6 Inhibition.

A large number of compounds have been investigated as potential inhibitors of glyoxalase I, some of them with a view to their prospective use as anti-cancer and anti-inflammatory agents.

Competitive Inhibitors.

S-Alkylglutathiones are competitive inhibitors of glyoxalase I. The relative inhibitory power increases with increase in alkyl chain length (52). This indicates that there is a non-polar region on glyoxalase I which plays an important part in the formation of the enzyme-inhibitor complex (52,53). The most potent reversible inhibitor of glyoxalase I known is S-p-bromobenzylglutathione [K_i = 9 μM] (53). However, this inhibition decreases 10-20 fold if the

zinc atom is replaced by other divalent metal ions (13).

Glutathione derivatives formed by modification of the N- and S-terminals are competitive inhibitors of glyoxalase I (54,55). The K_i values of these compounds, with glyoxalase I from human erythrocytes, indicate that there is no specific binding of the N-site of glutathione to the enzyme. This is in contrast with the yeast enzyme where a weak interaction between the N-site of glutathione and glyoxalase I is observed.

Yeast glyoxalase I is competitively inhibited by a number of porphyrin derivatives (56,57). The K_i value for these compounds is pH dependent, the inhibition being stronger at lower pH.

Mechanism-based Inhibitors.

The proposal that the glyoxalase I reaction proceeds via an ene-diol intermediate has prompted several investigators to test ene-diol analogues for inhibitory activity. Examples are : lapachol (the K_i is dependent on the source of the enzyme - yeast = 37.5 µM, rat liver = 0.16 mM and human erythrocyte = 9.5 µM) (58), flavones (myricetin, taxifolin, quercetin) (59) and substituted coumarins (4-methylesculetin, isoesculetin) (60). Modification of the ene-diol moiety greatly reduces the extent to which these compounds bind to glyoxalase I (61).

Miscellaneous inhibitors.

An inhibitor has been isolated from _Streptomyces griseoporeus_ -

2-Crotonyloxymethyl-4(R),5(R),6(R)-trihydroxycyclohex-2-enone. Its inhibitory action is potentiated by pre-incubation with glutathione (62). Some nucleic acid bases, nucleosides and nucleotides, and tryptophan have been proposed as potential inhibitors of glyoxalase I (63,64). The I_{50} for tryptophan with mouse liver glyoxalase I is 0.9 mM and for GTP is 0.15 mM (63).

1.2.7 Genetics and Polymorphism.

The mammalian glyoxalase I gene is located on chromosome 6 and exists in the form of two alleles, GLO*1 and GLO*2. These alleles are autosomally inherited in a simple 2-allele codominant manner. The alleles code for two slightly different monomers, GLO^1 and GLO^2. These can be seperated by their electrophoretic mobility : GLO^1 is electrophoretically "slow" and GLO^2 is electrophoretically "fast". Glyoxalase I can exist in the form of three isozymes - GLO^{11} and GLO^{22} homodimers and a GLO^{12} heterodimer. These three isozymes appear to be functionally identical but can be seperated by means of their different chromatographic and electrophoretic properties (19).

In lymphocytes stimulated with phytohemagglutinin and in lymphoblastoid cell lines, a third glyoxalase I allele (GLO*3) has been found (65). This allele codes for a GLO^3 monomer that is electrophoretically similar to GLO^1, but is catalytically inactive. Enzyme dimers that are homozygous for this monomer are inactive, while

heterozygous dimers have decreased enzymatic activity
(approx 50% or less of normal activity). The GLO^3
monomer has not been detected in red blood cells or
circulating lymphocytes either because the cell is
unable to resynthesize it after it has been broken down
by natural processes or because it is more unstable in
these cells than it is in the stimulated lymphocytes and
lymphoblastoid cell lines. A glyoxalase I monomer with
very low activity has been detected in some species of
chicken (66).

The glyoxalase I gene has been shown to exist in
the form of two alleles in the human (67), rat (68) and
mouse (69) genome. Experiments in the rat indicate that
the glyoxalase I gene is located on the same chromosome
as the genes for the Major Histocompatability Complex
(MHC) (68). It has also been linked to the H-2 gene
complex of the mouse (69). Both the MHC of the rat and
the H-2 gene complex of the mouse are equivalent to the
Human Leukocyte System A (HLA) gene complex in man. The
mammalian glyoxalase I gene is linked to the HLA gene
complex (70). The proposed order of the genes on
chromosome 6 is shown below :

HLA GENE COMPLEX

DR DR

GLO   D   Bf B C   A
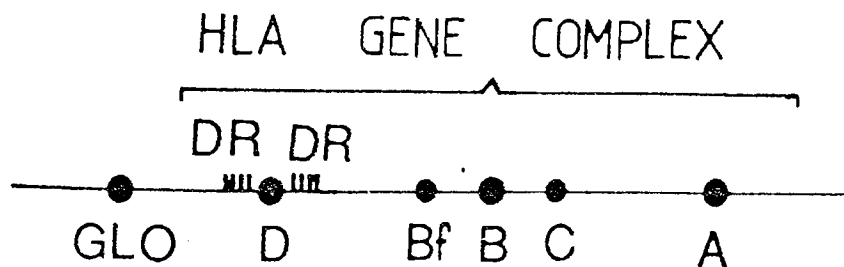
The glyoxalase I gene has been assigned to chromosome 1 of the Chinese hamster. It is not known where the HLA complex is situated in this animal (71).

Tissue typing of the HLA system in man has shown that the different glyoxalase I alleles are associated with different HLA haplotypes. In a French survey (72), GLO[1] was found to be mainly associated with the BW6 allele, while GLO[2] was mainly associated with the BW4 allele. A Spanish survey (73) has shown a significant association between the DR4 allele and GLO[2]. The DR3 and DR4 alleles were also found to be associated with GLO[2] in Insulin-Dependent Diabetes Mellitus patients (73). Investigation of one family indicated the possible existence of a glyoxalase I "null" allele (GLO*0) that was associated with the A(10)-B16-DW7 haplotype (74). In a different family, this GLO*0 allele was associated with the AW30-CW4-BW35 haplotype (75).

Glyoxalase I polymorphism has been suggested as a possibly useful tool in the study of human population genetics. The GLO[1] allele frequency has been measured in a large number of different populations. The frequency is highest in Caucasoid populations (0.42-0.45) (76), lower in Negroid populations (0.25) (77) and it is rare or non-existent in Asiatic populations (78,79).

Glyoxalase I polymorphism has been used forensically (80). The blood glyoxalase I phenotype in

a human individual is identical with the glyoxalase I
phenotype in as many as 23 different tissue sites (81),
including semen, muscle, skin and hair root tissue. If
the existence of a silent allele is taken into account,
the heredity pattern of glyoxalase I, and the frequency
with which it deviates from this pattern, is such that
the glyoxalase system can be used to exclude suspects in
paternity cases (82).

1.3 Glyoxalase II

1.3.1 Distribution and Molecular Characteristics

Glyoxalase II activity is found throughout
biological life in association with the activity of
glyoxalase I. Typical glyoxalase II activities in
biological tissues are presented in Table 1. Glyoxalase
II has been isolated from several different vertebrates
(83). The molecular weight of the enzyme varies between
18,000 and 29,000 daltons. The protein is normally
isolated as a monomer but a recent report (84) suggests
that it may exist in dimeric, trimeric and tetrameric
forms in rat hepatocytes. Glyoxalase II is a basic
protein, with an isoelectric point above pH 7.0. The
typical amino acid composition, primary sequence and
crystal structure of glyoxalase II have not yet been
reported. The molecular properties of glyoxalase II
from yeast and mammalian sources are summarised in Table
3.

Table 3   Molecular Properties of Glyoxalase II

Number of Subunits	1
Molecular Weight (daltons)	21,900 (Rat Erythrocyte) (197)
	22,900 (Human Liver) (85)
	23,000 (Calf Brain) (198)
	24,000 (Human Brain) (86)
	29,500 (Mouse Liver) (89)
Isoelectric Point	7.63 (Calf Brain] (198)
	8.10 (Mouse Liver) (89)
	8.35 (Human Liver) (85)
	8.50 (Human Brain) (86)
	9.25 (Python Liver) (83)
Km for S-D-Lactoylglutathione	0.190 mM (Human Liver) (85)
	0.270 mM (Mouse Liver) (89)
	0.304 mM (Human Brain) (86)
	0.230 mM (Python Liver) (83)
Molecular Activity (k_{cat}) for S-D-Lactoylglutathione	$16,000 \ \text{min}^{-1}$ (Mammalian) (198)

1.3.2 Substrate Specificity

Glyoxalase II can catalyse the hydrolysis of a wide range of S-2-hydroxyacylglutathiones. With S-lactoyl-, S-glycoloyl-, S-mandeloyl-, S-glyceroyl- and S-acetoacetoyl glutathiones, the relative kinetic activity of glyoxalase II is maintained during purification (85). In contrast, the activity of glyoxalase II with S-formyl-, S-succinoyl-, S-acetyl- and S-propionoyl glutathione is lost during purification, suggesting that there may be other S-acylglutathione hydrolases (85). Purified glyoxalase II is inactive with S-formyl-, S-acetyl-, S-propionyl-, S-succinoyl- and S-acetoacetyl thioglycolate, acetyl-CoA, succinyl-CoA and carboxyl esters, indicating a high specificity for the glutathione moiety of the substrate (85).

1.3.3 The Rate of the Glyoxalase II Reaction.

The hydrolysis of S-acylglutathiones to carboxylic acid and reduced glutathione, catalysed by glyoxalase II, follows Michaelis-Menten kinetics in the substrate range 5-500 μM, except under some experimental conditions. Competitive inhibition by the substrate may be observed at high substrate concentrations (85). For the S-acylglutathione derivatives studied, S-D-lactoylglutathione has the highest relative V_{max} and a relatively low Km (200-300 μM) in the glyoxalase II-catalysed reaction (85,86). There is also a well-defined biological source of S-D-lactoylglutathione

- the glyoxalase I-catalysed reaction. Therefore, S-D-lactoylglutathione is considered to be the physiological substrate of glyoxalase II.

It has recently been reported that glyoxalase II from liver and brain of Wistar rats exists in two forms, designated α and β. There was no significant difference in the kinetic activity of these two forms (87).

1.3.4 Probing the Active Site

Glyoxalase II does not require an active site metal ion for its activity (85). Several studies have shown that the activity of glyoxalase II is unaffected by serine modifying reagents (phenylmethanesulphonic acid, diisopropyl phosphofluoridate), indicating that there are no serine residues in the active site and glyoxalase II is not a serine-type thiolesterase (85,86,88). The activity of glyoxalase II is also not affected by treatment with 2-hydroxy-5-nitrobenzylbromide, indicating that there are no tryptophan residues in the active site (86). The effect of thiol antagonists is equivocal : N-ethylmaleimide is a weak inhibitor (86), mercuric chloride and p-hydroxymercuribenzoate produce strong inhibition only at high concentrations which is not reversible with dithiothreitol (85), and 5,5'-dithiobis(2-nitrobenzoic acid) is without effect (85). These observations are not typical of a enzyme with an active site cysteinyl residue, although there may be a cysteine group adjacent to the active site.

The arginine reagent phenylglyoxal produces a partial inhibition (86), the histidine reagents diethylpyrocarbonate and photo-oxidation with methylene blue produce a strong inhibition (86), and the amine reagent 2,4,6-trinitrobenzenesulphonic acid produces a strong inhibition (85), of glyoxalase II. This indicates that there are arginine and histidine residues, and possibly lysyl residues, in the active site which are essential for the catalytic function of the enzyme. The arginine residue is probably located in the active site and appears to be involved in binding the substrate's carboxyl group (86). The Km and V_{max} data suggests that the active site contains a hydrophobic pocket on the side which binds the acyl moiety of the substrate (86).

1.3.5 Inhibition

Mouse liver glyoxalase II is inhibited by nucleosides, nucleotides and tryptophan (63). The I_{50} for tryptophan is 0.8 mM and for GTP is 0.16 mM (63). Both methylgloxal and reduced glutathione are weak competitive inhibitors of glyoxalase II (for reduced glutathione with human liver glyoxalase II, K_i = 4 mM) (85). Their effects are increased in combination, possibly due to the formation of the hemimercaptal. Studies on the hemimercaptal show that it is a competitive inhibitor of glyoxalase II, but the K_i depends on the source of the enzyme (K_i = 0.3 mM (mouse liver glyoxalase II) (89) and 0.12 mM (human liver

glyoxalase II) (85)).

S-(Nitrocarbobenzoxy)glutathiones are the most
potent competitive inhibitors of glyoxalase II known (K_i
of the para isomer = 6.5 μM) but are very weak
inhibitors of glyoxalase I. This selective inhibition
of glyoxalase II facilitates their use in experiments to
investigate the function of glyoxalase II (90).

1.3.6 Genetics and Polymorphism

Experiments involving the fusion of human and
rodent cells have shown the location of the human
glyoxalase II gene to be on chromosome 16 (91). In
man, there is normally only one phenotype for the
glyoxalase II gene. However, in one Micronesian
population, a second phenotype of glyoxalase II has been
identified (92). When the blood glyoxalase enzymes from
this population were separated by electrophoresis, they
showed a band of activity at the normally expected
position for glyoxalase II and a second,
electrophoretically faster band of activity, suggesting
the presence of an allele variant at the glyoxalase II
locus. Investigation of family data showed that the
allele variant was genetically transmitted in an
autosomal codominant manner. This variant was
restricted to only one Micronesian population,
suggesting that it is a relatively recent mutation.
Dimorphic forms of glyoxalase II have also been isolated
from human erythrocytes (93) and the liver and brain of

Wistar rats (87). In each case, the two forms of glyoxalase II differed in their isoelectic points, suggesting that they were structurally distinct. Although glyoxalase II polymorphism is rare in man, it has been found to be common in anthropoid primates (94).

An heredity deficiency of glyoxalase II activity has been demonstated in a patient suffering from heredity elliptocytosis (95). The occurance of the two conditions was not related. The glyoxalase II deficiency was inherited by an autosomal recessive mechanism. The activity was deficient in the erythrocytes but not leukocytes of the affected subjects, suggesting that there may be a varying pattern of isozymes in different tissues. This deficiency of glyoxalase II activity was not associated with any clinical or haematological abnormalities.

1.4. Methylglyoxal

1.4.1 Formation

The formation of methylglyoxal in biological tissues occurs by several different routes. Dihydroxyacetone phosphate is converted to methylglyoxal by the action of methylglyoxal synthetase (E.C. 4.2.99.11). This enzyme has been isolated from goat liver (96) and bacteria (97,98). An alternative source of methylglyoxal in some bacteria is 1-hydroxyacetone (acetol), an intermediate in the catabolism of isopropanol and acetone (99). The enzyme involved in

the conversion of 1-hydroxyacetone to methylglyoxal is acetol dehydrogenase (E.C. 1.1.1.-), which uses NAD^+ as a co-factor.

In mammals, methylglyoxal is also formed from aminoacetone catalysed by amine oxidase, which has been isolated from goat plasma (100). Aminoacetone can be formed from L-threonine catalysed by L-threonine dehydrogenase (E.C. 1.1.1.103.) or from glycine and acetyl coenzyme A catalysed by aminoacetone synthase. Methylglyoxal exerts feedback inhibition on L-threonine dehydrogenase (101), binding to a site other than the active site, thus providing a route by which methylglyoxal can regulate it's own rate of production.

1.4.2 Metabolism

Glyoxalase I and three other enzymes may catalyse the metabolism of methylglyoxal; 2-oxoaldehyde dehydrogenase, S-formylglutathione hydrolase, and methylglyoxal reductase.

2-Oxoaldehyde dehydrogenase (E.C. 1.2.1.23.) has been isolated from mammalian liver (102,103) and micro-organisms (104). This enzyme catalyses the direct oxidation of methylglyoxal to pyruvate. It requires NAD^+ or $NADP^+$ as a cofactor, but does not require glutathione. The Km of the mammalian enzyme for methylglyoxal is 4.5 mM with NAD^+ and 0.4 mM with $NADP^+$ (105).

Formaldehyde dehydrogenase (E.C. 1.2.1.1) is present in yeast, several other micro-organisms and

mammalian liver (105,106). This enzyme requires glutathione and NAD$^+$ for activity. Its normal function is to convert the adduct formed between formaldehyde and glutathione (107) to S-formylglutathione (105). A second enzyme, S-formylglutathione hydrolase (E.C. 3.1.2.12), metabolises S-formylglutathione to formate and glutathione (108). Although formaldehyde dehydrogenase will readily convert the methylglyoxal-glutathione adduct to S-pyruvoylglutathione (109) (the Km of methylglyoxal is 1.2 mM; Km for glutathione = 0.13 mM) (105), pyruvoylglutathione is not a substrate acceptable to S-formylglutathione hydrolase (110). The role of formaldehyde dehydrogenase in the metabolism of methylglyoxal is therefore unclear.

The third enzyme involved in methylglyoxal metabolism is methylglyoxal reductase. This enzyme, isolated from goat liver (111), converts methylglyoxal to lactaldehyde. It uses NADH as a co-substrate and has a Km value for methylglyoxal of 0.4 mM

1.4.3 The Concentration of Methylglyoxal in Biological Tissues.

Methylglyoxal is generally assayed by determination of a chromophoric adduct of methylglyoxal with an amine (112,113). The major interference associated with this procedure is the prospect of the chromophoric amine catalysing the formation of methylglyoxal or other α-oxoaldehydes from monosaccharides (114) in the cell

extract during the analytical extraction procedure. This artifact, if unrecognised, greatly over-estimates the concentration of α-oxoaldehyde. The recent development of a rapid high pressure liquid chromatography (HPLC) method for the adduct of methylglyoxal with o-phenylene diamine, 2-methylquinoxaline, has improved the accuracy and precision of the assay of methylglyoxal in biological material (115).

The concentration of methylglyoxal in the liver and muscle of young Wistar rats is 0.88 and 0.90 μg/g wet weight respectively. The concentration in the heart is higher (1.10 μg/g wet weight) (115). The concentration of methylglyoxal in the liver is higher in mice than the rat (3.69 μg/g) (116).

1.4.4 Other Reactions

Methylglyoxal binds to DNA and RNA undergoing replication and transcription, through adduct formation with guanine residues (117,118).

It may express mutagenic or carcinogenic activity through this interaction with DNA (119,120) e.g. acting as a direct mutagen in Salmonella typhimurium TA100. Cells treated with methylglyoxal may develop DNA crosslinks but these are usually repaired within 24 hours of removal of the α-oxoaldehyde (121).

Methylglyoxal also binds and crosslinks proteins through adduct formation with arginyl residues. These reactions may mediate the inactivation of enzymes,

particularly those with arginine residues essential for enzymatic activity in the active site, and the denaturation of proteins. However, relatively high concentrations of methylglyoxal are required to exert this inhibitory effect. For example, the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglyceromutase, phosphoglycerate kinase and phosphofructokinase are inhibited by 50% within 50 minutes of incubation at 30^OC and pH 7.6 with 2.5 mM methylglyoxal (122), and the assembly of microtubules from purified tubulin at pH 6.6 and 37^OC is inhibited by 1 - 5 mM methylglyoxal (123).

Methylglyoxal inhibits the cellular synthesis of proteins (124,125). The inhibition is most marked during the S and G_2 phases of the cell cycle (126) . During these phases, RNA and DNA synthesis is inhibited much less than protein synthesis. The inhibition of protein synthesis does not involve an effect on mRNA (127) but may involve an inactivation of ribosomal enzymes which contain arginine residues essential for their function (128). Alternatively, this inhibitory effect may be due to methylglyoxal binding to tRNA and preventing its participation in protein synthesis.

All of these biological effects of methylglyoxal are observed under conditions which are extremely favourable for their occurrence; 1 - 5 mM methylglyoxal treatment, for example. It is unlikely that such concentrations of methylglyoxal (and biological

consequences) are ever maintained in normal
physiological circumstances because of the efficiency
of the glyoxalase system.

1.5. S-D-Lactoylglutathione.

S-D-Lactoylglutathione is considered to be the
physiological intermediate of the glyoxalase system. It
is stable in neutral or acid solution, but undergoes a
slow decomposition in alkali solution to give D-lactate
and reduced glutathione (9). S-D-Lactoylglutathione
expresses some remarkable bioactivities : (i) it
potentiates the GTP-promoted assembly of microtubules
from purified tubulin and microtubule-associated
proteins in vitro (129); (ii) it potentiates the
secretion of histamine from human leukocytes (130); and
it inhibits the proliferation of tumour cells in culture
(131). The mechanism of expression of these effects and
the normal cellular concentration of
S-D-lactoylglutathione is not known.

1.6. D-Lactate.

D-Lactate is the end product of the glyoxalase
system. It is very poorly metabolised by mammals (132)
and is normally excreted in the urine (133,134). It
may, however, be used by bacteria, such as Escherichia
coli, as part of the amino acid and β-galactoside
transport mechanism (135,136).

In man, the normal concentration of D-lactate in

plasma is 0.023 mM (137). This is only a small fraction
(1.5%) of the concentration of L-lactate. The
excretion, and presumably the production, of D-lactate
in man appears to be unaffected by the presence or
absence of disease - although this may deserve further
and more thorough investigation (134). In vitro,
D-lactate formation has been demonstrated in blood
following inhibition of glycolysis (138), presumably due
to an increased flux of glycolytic intermediates through
the glyoxalase system.

1.7.Physiological Role of the Glyoxalase System.

Although the existence of the glyoxalase system has
been known for over 70 years and the enzyme activity
found to be ubiquitous, the role of this system in
normal cellular metabolism still remains to be fully
elucidated. Several theories for the function of the
glyoxalase system have been proposed.

1) Glycolytic bypass in micro-organisms. Cooper and
Anderson have demonstrated that the micro-organism E.
coli can convert dihydroxyacetone phosphate to pyruvate,
via methylglyoxal and D-lactate (139). This reaction
sequence bypasses the normal glycolytic route for the
formation of pyruvate from dihydroxyacetone phosphate.
They proposed this may be a regulatory mechanism to
prevent the accumulation of a high concentration of
phosphorylated glycolytic intermediates, which are known
to inhibit bacterial growth (140). Alternatively, it

may be a means of producing D-lactate, via the
glyoxalase system, which may be important in E. coli for
amino acid and β-galactoside transport (135,136)

2) Detoxification of α-oxoaldehydes. Methylglyoxal is
a potentially noxious compound, if permitted to
accumulate to high concentrations in biological tissue.
The formation of methylglyoxal by methylglyoxal synthase
has been found in several strains of bacteria, including
enterobacteria (98). Mannervik and Aronsson have
proposed that methylglyoxal, released by enterobacteria,
enters the body and is transported to the liver, where
it is metabolised by glyoxalase I (23). It has also
been suggested that the major role of this system in
micro-organisms may be the removal of methylglyoxal
formed during metabolism (97)

3) Control of Cell Growth. In the 1960's,
Szent-Györgyi, Együd and others proposed that there was
a universal mechanism in multi-cellular organisms that
regulated growth (cell division). This mechanism would
involve a factor which existed in all living systems
which could permanently promote growth and would be
controlled in its action by an antagonist. The growth
status would depend on the equilibrium between the
growth factor and the antagonist. They also proposed
there was a common receptor which regulated all the
changes occurring in the cell during division. The
receptor would involve sulphydryl groups, since their
indispensibility for cell division had been known for

some time (141).

The inhibitory factor, called "retine" was considered to be a glyoxal derivative (142,143), as partial purification of tissue extracts capable of inhibiting tumour growth had revealed the presence of α-oxoaldehydes. It was already known that methylglyoxal (and all α-oxoaldehydes) would react readily with thiol groups (144), supporting the assumption that the growth inhibiting action of "retine" depended on blocking sulphydryl groups important for growth.

The accelerating factor, called "promine", was thought to involve the glyoxalase system, which converts the α-oxoaldehydes to the biologically much less reactive α-hydroxycarboxylic acids.

4) Control of microtubule assembly. Gillespie made the seminal observation that S-D-lactoylglutathione potentiates (and glyoxalase II inhibits) the cell-free GTP-promoted assembly of microtubules in vitro (129), although the data presented was equivocal - S-D-lactoylglutathione was shown to both potentiate and inhibit microtubule assembly. She later observed that the activity of glyoxalase I increased and the activity of glyoxalase II decreased during the functional activition (phagocytosis and degranulation) of neutrophils (145), and S-D-lactoylglutathione potentiated anti-IgE-induced secretion of histamine from basophils (130) - which is known to be a

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microtubule-mediated process. Gillespie proposed that
the glyoxalase system may regulate the assembly of
microtubules through the influence of
S-D-lactoylglutathione.

2. BACKGROUND TO THIS WORK

2.1 The Modification of the Glyoxalase System in Cell Proliferation and Maturation

2.1.1. The Activity of Glyoxalase I and Glyoxalase II.

The Szent-Györgyi hypothesis predicted that methylglyoxal was a growth-inhibiting (or anti-proliferative) substance and glyoxalase I, the physiological antagonist of methylglyoxal, was a growth-promoting substance; the conflict between these two species was the putative mechanism of growth control (142,143). This hypothesis was substantiated by a wealth of literature demonstrating the carcinostatic properties of relatively high doses of methylglyoxal (146,147,148,149). Therefore, rapidly proliferating tissue was expected to have a high activity of glyoxalase I and a low cellular concentration of methylglyoxal.

In 1978, Jerzykowski et al (3) reported the results of a survey of the glyoxalase activities in murine, rat and human tumours. In the majority of tumours, they found the activity of glyoxalase I was lower than in normal tissues and the activity of glyoxalase II was very small or undetectable. Despite this, the tumours exhibited rapid tissue growth kinetics. These observations contradicted the Szent-Györgyi hypothesis and stymied advance in this area of glyoxalase research.

In 1982, Principato et al published the first
report in a series of investigations into glyoxalase
activities in proliferating and maturing tissues. They
studied glyoxalase activities in the maturing chick
embryo, before and after hatching (150). They found
that the activity of glyoxalase I decreased and the
activity of glyoxalase II increased markedly from five
days before to fifteen days after hatching. This
demonstrated a major change in glyoxalase activities
during embryo maturation. A further investigation by
this group found that regenerating rat liver (after
hepatectomy) had a higher activity of glyoxalase I and a
lower activity of glyoxalase II than the sham control
liver (151). From these data, it appears that during
cell maturation (differentiation), there was a decrease
in the activity of glyoxalase I and an increase in the
activity of glyoxalase II.

Tissue maturation reflects both changes in the
differentiation status and the proliferation status of
cells. Therefore, it is still not clear if the changes
observed in glyoxalase activities during maturation
reflect changes in proliferation status or
differentiation status.

Studies on non-differentiating, growth-arrested
cells show no consistent relationship between
proliferation status and glyoxalase activities. In a
study with exponentially growing and G_1 arrested
temperature-sensitive cell division cycle mutants of

Saccharomyces cereviciae, the activity of glyoxalase I
was found to be substantially (35 - 75%) decreased in
growth-arrested cells (152). The activity of glyoxalase
I in mouse spleen was monitored during regeneration
following radiation-induced damage : the activity of
glyoxalase I was found to correlate with the increased
rate of DNA synthesis and cell proliferation during
regeneration (153). However, in a study of quiescent
and proliferating human fibroblasts, Bruschelli et al
(154) found no significant change in the activity of
glyoxalase I or glyoxalase II between growth-arrested
and rapidly proliferating cells. Further studies are
necessary before any firm conclusions can be made.

The glyoxalase system is a primary and important
feature of mammalian foetal development. The glyoxalase
I phenotype is one of the first to be developed and is
present, fully formed by the sixth-seventh week of
foetal life (155). The activity of both enzymes
increases as gestation proceeds. Overall, the cell
proliferation and maturation studies to date indicate
that the activity of the glyoxalase enzymes are linked
to the mitotic activity of the cell. As the mitotic
activity increases, the activity of glyoxalase I
increases and the activity of glyoxalase II decreases.

The regulatory mechanism by which these changes in
glyoxalase activity occur and why these changes occur
during proliferation and maturation is not known.
Similar changes in glyoxalase activities in

functionally-activated neutrophils are associated with the control of the cellular concentration of S-D-lactoylglutathione and the putative regulation of microtubule assembly (129,145,156). The changes in glyoxalase activities during proliferation and maturation may also be related to the control of S-D-lactoylglutathione for the regulation of the microtubular cytoskeleton.

2.1.2. The Concentration of Methylglyoxal and S-D-Lactoylglutathione.

The concentration of methylglyoxal and S-D-lactoylglutathione in tumour cells is not known.

2.1.3. Research Objectives and Plan of Action.

Objective 1) To study the modification of the glyoxalase system during the differentiation of human leukaemia cells in vitro.

Two well-established human leukaemia cell lines were used for this study : the HL60 human promyelocytic leukaemia line and the K562 human erythroleukaemia line.

A. Human Leukaemia - 60 Cells.

Human leukaemia - 60 (HL60) cells are a human promyelocytic leukaemia cell line initially established from peripheral blood leukocytes from a patient with acute promyelocytic leukaemia (157). HL60 cells can be induced to differentiate into two different types of mature cells. Exposure to dimethylsulphoxide (158) and N-methylformamide (159) induces differentiation to cells

which are morphologically and functionally similar to human neutrophils, although HL60-derived neutrophils have been shown to be deficient in some characteristic neutrophilic enzyme activities e.g. myeloperoxidase (160). However, the characteristic NADPH oxidase activity of neutrophils is present (161) and is a convenient marker of differentiation of HL60 cells.

Alternatively, exposure of HL60 cells to 12-O-tetradecanoylphorbol-13-acetate induces their differentiation into macrophages or monocyte-like cells (162). After treatment with dimethylsulphoxide, the cells are committed to differentiation within 8 - 18 hours (163). The initiation of the maturation of HL60 cells to neutrophils is not a cell-cycle related event (164).

B. K562 - Erythroleukaemia Cell Line.

The K562 human erythroleukaemia cell line was originally established in 1975. It was derived from the pleural effusion of a patient with chronic myelogenous leukaemia in terminal blast crisis (165). The K562 cell is thought to be an early differentiated stage of the granulocyte lineage (166). However, it shows clear differences from benign and malignant cells representing various stages of the myeloblast to granulocyte differentiation sequence : it lacks the normal B-cell markers of immunoglobin and T-cell antigens, and does not phagocytose or mediate antibody-dependent cytolysis or phagocytosis (166). Moreover, the K562 cell exhibits

several features common to human erythrocytes : the cell surface antigens are very similar to those of normal erythrocytes with the major red blood cell sialoglycoprotein, glycophorin, on its surface (167); K562 cells contain spectrin (a red blood cell protein) and may synthesise haemoglobin which is electrophorectically similar to haemoglobin from embryonic erythroblasts (168).

Objective 2) To study the modification of the glyoxalase system during changes in the proliferation status of the cell, in the absence of differentiation.

Two cell lines were used in this study : the GM892 transformed human lymphoblast and the Raji (Mer +) Burkitt's lymphoma cell lines.

A. The Raji (Mer +) Cell Line.

The Raji cell line was initially established in 1964 (169). It was derived from material obtained from a Burkitt's lymphoma (BL) tumour, and has many characteristics in common with other BL-derived tumour cell lines. Burkitt's lymphoma is characterised by the presence of a rapidly growing solid tumour (170) and is defined as a B-cell tumour of mucosa-associated lymphoid tissue (171). The origin of BL is unknown, but all BL-derived cell lines, including Raji, contain at least one copy of an Epstein-Barr virial (EBV) genome and express EBV membrane and nuclear antigens (170).

B. The GM892 Cell line

The GM892 cell line is a human lymphoblastoid cell line, which has been shown to be O^6-alkylguanine repair deficient (131).

Objective 3) To study the effect of S-D-lactoylglutathione on the cell-free assembly of microtubules

Following the pioneering work of Weisenberg (172), the GTP-dependent assembly of microtubules from purified tubulin (with microtubule-associated proteins) can be studied in cell-free systems _in vitro_ using a calcium-deficient medium with sulphonic acid buffers at pH 6.6. This was exploited to study the effect of S-D-lactoylglutathione on the kinetics of microtubule assembly and disassembly.

2.2. The Glyoxalase System in Hyperglycaemia : Possible Involvement in Diabetic Pathogenesis.

2.2.1 Diabetic Pathogenesis : Defining the Pathology.

Diabetic pathogenesis is the development of complications arising form chronic periodic hyperglycaemia, associated with Diabetes Mellitus. Diabetes Mellitus is a metabolic disorder in which there is a relative, or absolute deficiency, of insulin. The relative or absolute lack of insulin action produces a substantial change in carbohydrate, lipid and protein metabolism (173). Tissues with insulin-dependent glucose uptake (e.g. skeletal muscle) cannot utilise

glucose and the plasma glucose level rises from 5 mM to 10 - 50 mM. Tissues with insulin-independent glucose uptake (red blood cell, endothelial cells of the small capillaries - especially in the retina and kidney glomeruli, lens fibre cells, red blood cells, peripheral neurones) become hyperglycaemic. With insulin injections, the diabetic individual experiences periodic hyperglycaemia, particularly in the hours immediately prior to the insulin supplement. The toxicity arising from this managed, periodic hyperglycaemia produces a characteristic profile of clinical pathology : cataract, retinopathy, neuropathy, nephropathy and renal failure, generalised microangiopathy and arteriosclerosis (174).

2.2.2 Classification of Diabetes Mellitus

There are two main types of Diabetes Mellitus :

Type 1 Diabetes Mellitus (Insulin-Dependent Diabetes).

This form of diabetes normally occurs in children or young adults. The classification is based on a dependence on insulin and a disposition towards ketoacidosis. These patients usually have a short history of the symptoms of hyperglycaemia (polydipsia, polyuria, lethargy and weight loss) and, if not recognised quickly, may develop ketoacidosis. The amount of insulin remaining in the pancreas is extremely small and circulating levels are very low. Carbohydrate metabolism is extemely abnormal, producing a fasting hyperglycaemia and a marked abnormality of glucose tolerance (173).

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<u>Type 2 Diabetes Mellitus (Non-Insulin-Dependent</u>
<u>Diabetes).</u>

This is the normal "clinical" type of diabetes that occurs in middle or late life. Insulin is not usually required and ketoacidosis rarely arises (173). The plasma insulin levels are normal or elevated and the subjects are often obese (175,176).

The diagnosis of Diabetes Mellitus is made on the criterion of plasma glucose level. A plasma glucose level greater than 200mgs/100mls is usually indicative of diabetes, irrespective of other factors. A basal plasma glucose level of 115 - 129mgs/100mls is the boarderline for the diagnosis of diabetes. Values greater than 129mgs/100mls require the diagnosis of diabetes to be confirmed by other tests.

2.2.3 The Biochemical Basis for Diabetic Pathogenesis.

It is generally considered that the biochemical basis of the development of diabetic complications is expressed through pathways of non-glycolytic glucose metabolism which are enhanced during hyperglycaemia. Particularly pertinent examples are : (i) the characteristic formation of sorbitol from glucose (177,178), catalysed by aldose reductase (alditol : $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.21), and (ii) the non-enzymatic glycosylation of protein (179). Recent reports (114,180) proposed that the formation of α-oxoaldehydes may be enhanced during hyperglycaemia

and α-oxoaldehyde metabolism may be an aetiological factor in the development of diabetic microangiopathy.

To test and substantiate this hypothesis, a human cell type is required which has an active glyoxalase system and suffers hyperglycaemic metabolism in Diabetes Mellitus, and is readily isolable   for carefully-controlled in vitro experiments.

2.2.4 The Red Blood Cell as a Model For Hyperglycaemic Metabolism in Diabetic Pathogenesis.

The red blood cell has an insulin-independent glucose uptake mechanism and operates only the Embden-Meyerhof glycolytic pathway (181). In periods of hyperglycaemia, the intracellular concentration of glucose, normally ca. 5 mM, may rise to 20 - 30 mM, depending on the quality of glycaemic control (182,183). In the red blood cell suffering periodic hyperglycaemia : sorbitol is produced from glucose, catalysed by red blood cell aldose reductase (184); haemoglobin (185) and other proteins (179) are glycosylated, forming fructosamine adducts by Amadori rearrangement of the initial Schiff's base adduct, and chromophoric and fluorophoric pigments by further rearrangement and oxidation (186,187); the concentration of reduced glutathione is significantly decreased (188,189); and there are decreases in Ca^{2+}-ATP-ase activity (190) and membrane deformability (191,192). Although these metabolic changes do not produce measurable haemolysis, the circulatory lifetime of the red blood cell is

significantly decreased in Diabetes Mellitus (193,194).
The red blood cell is typical of many other
hyperglycaemic tissues in producing these characteristic
metabolic changes in hyperglycaemia.

The red blood cell has an active glyoxalase system.
Both glyoxalase I and glyoxalase II have been purified
from human red blood cells (93,195). The cellular
concentrations of methylglyoxal and
S-D-lactoylglutathione are not known.

Objective 4. To study the effect of hyperglycaemia on
the red blood cell glyoxalase system in diabetic
patients in vivo.

Glyoxalase activities and the concentrations of
glyoxalase metabolites may be assayed in clinical blood
specimens taken routinely from diabetic patients.

3. MATERIALS AND METHODS

3.1 Materials.

3.1.1 Cell Culture

Human promyelocytic leukaemia HL60, erythroleukaemia K562, Burkitt's lymphoma Raji, and GM892 lymphoblastoid cells were incubated at 37^OC in RPMI 1640 media containing 10% foetal calf serum under an atmosphere of 10% CO_2 in air, 100% humidity for the times stated. Tissue culture medium RPMI 1640 , foetal calf serum, and newborn calf serum were purchased from Gibco Europe Ltd. (Paisley, Scotland).

3.1.2 Enzymes, Co-factors and Substrates

Glyoxalase I (Grade X, from yeast, specific activity 1645 units/mg protein), glyoxalase II (from beef liver, specific activity 10.3 units/mg protein) and glutathione S-transferase (from equine liver, specific activity 65 units/mg protein) were purchased from Sigma Chem. Co. Ltd, (Poole, Dorset, U.K). Methylglyoxal, reduced glutathione GSH, S-D-lactoylglutathione (free acid), 1-chloro-2,4-dinitrobenzene, guanosine 5-triphosphate (GTP, Type II-S) and nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate) were purchased from Sigma. 5-(^{3}H-Methyl)thymidine (specific activity 5.0 Ci/mmcl) was purchased from Amersham International plc (Buckinghamshire, U.K).

3.1.3 Buffers and Miscellaneous Agents

2-(N'-Morpholino)ethanesulfonic acid (MES),

tris(hydroxymethyl)aminomethane (TRIS),

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and

1,2-di(2-aminoethoxy)ethane-NNN'N'-tetra-acetic acid (EGTA) were purchased from Sigma. Dimethyl sulphoxide (DMSO), N-methylformamide and N-ethylmaleimide were obtained from Aldrich Chem. Co. Ltd. (Poole, Dorset). The imidazotetrazinone, 8-carbamoyl-3-methylimidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045), was kindly donated by May and Baker Ltd. (Dagenham, Essex, U.K). OPTIPHASE scintillation fluid was purchased from LKB Instruments Ltd, (Croydon, Surry, U.K).

3.1.4 Miscellaneous

All other chemicals were purchased from BDH Ltd (Poole, Dorset) and were the highest grade available.

3.2 The Characterisation of the Glyoxalase System.

3.2.1 The Assay of the Activity of Glyoxalase I in Cell Extracts.

The activity of glyoxalase I was determined by measuring the initial rate of formation of S-D-lactoylglutathione from the hemimercaptal formed non-enzymatically from methylglyoxal and reduced glutathione. The formation of S-D-lactoylglutathione was followed spectrophotometrically at 240 nm, $\Delta \, \varepsilon_{240} = 2.86 \, mM^{-1}cm^{-1}$ (34). Nominal concentrations of hemimercaptal were calculated assuming, for the

equilibrium, MeCOCHO + GSH $\rightleftharpoons$ MeCOCH(OH)-SG,

K_{eq} = 333 M^{-1} (34). For routine estimates of tissue activities of glyoxalase I, the initial hemimercaptal concentration was nominally 1.33 mM. For investigation of the substrate - reaction velocity relationship, initial hemimercaptal concentrations were 0.2, 0.285, 0.5, and 2.0 mM. The hemimercaptal was prepared by incubating equimolar mixtures of reduced glutathione and methylglyoxal in 100 mM sodium phosphate, pH 6.6 , at 37°C for 30 minutes.

It has been reported by Hall et al that the hemimercaptal formed between methylglyoxal and reduced glutathione undergoes a non-enzymatic conversion to S-lactoylglutathione (199). For this study, the activity of glyoxalase I was determined under standard conditions. A temperature of 37°C was used for physiological relevance, and an incubation time, for hemimercaptal formation, of 30 minutes. The incubation time of 30 minutes was chosen to allow effective thermal equilibrium of the incubation mixture to 37°C and because the absorbance change in the blank was found to be sufficiently linear and accurately reproducable at this time. Some of the hemimercaptal may be converted to S-lactoylglutathione during the incubation period and, therefore, the hemimercaptal concentrations are only nominal values, but the conditions used gave accurately reproducible determinations for glyoxalase I activity. It is

critical that the preincubation times are kept exactly the same throughout the study.

After incubation of the hemimercaptal and reference (buffer only) solutions, an aliquot of cell extract was added to both reference and sample cuvettes and the initial rate of change of absorbance at 240 nm was measured. Blanks were assayed by the addition of an aliquot of sonication buffer only. The activity of glyoxalase I is given in units per 10^6 cells (or per ml of packed red blood cells) where one unit of activity is defined as the amount of enzyme required to catalyse the formation of one μmol of S-D-lactoylglutathione per minute under assay conditions.

Stock solutions of methylglyoxal were prepared by diluting commercial methylglyoxal solutions with water to ca. 20 mM. The concentration of methylglyoxal was calibrated by end-point assay with commercial glyoxalase I (grade X). Sufficient of the methylglyoxal solution was added to 2 mM reduced glutathione in 100 mM sodium phosphate, pH 6.6, in a quartz cuvette to make the final methylglyoxal concentration of ca. 0.3 mM. Glyoxalase I (5 units) was added to sample and reference cuvettes. The absorbance at 240 nm was followed until a steady maximum was reached. The mean increase in absorbance was used to calculate the concentration of S-D-lactoylglutathione at the end-point assuming $\Delta \varepsilon_{SLG} = 3.10 \text{ mM}^{-1}\text{cm}^{-1}$, and hence the concentration of methylglyoxal in the stock solution.

3.2.2 The Assay of the Activity of Glyoxalase II in Cell Extracts

The activity of glyoxalase II was determined by measuring the initial rate of hydrolysis of S-D-lactoylglutathione in 50 mM Tris/HCL buffer, pH 7.4, at 37°C. The decrease in the concentration of S-D-lactoylglutathione was followed spectrophotometrically at 240 nm; $\Delta \varepsilon_{240} = 3.10 \text{ mM}^{-1}\text{cm}^{-1}$ (34). The initial concentration of S-D-lactoylglutathione was 300 µM for routine estimates of tissue activities of glyoxalase II, and 25, 33, 50, 100 and 300 µM where the substrate - reaction velocity relationship was investigated. The blank was the spontaneous rate of hydrolysis of S-D-lactoylglutathione after addition of an aliquot of sonication buffer only. The activity of glyoxalase II is given in units per 10^6 cells (or per ml of packed red blood cells) where one unit of activity is defined as the amount of enzyme required to catalyse the hydrolysis of one µmol of S-D-lactoylglutathione per minute under assay conditions.

The concentration of S-D-lactoylglutathione in stock solutions was calibrated by assaying the S-D-lactoylglutathione using commercial glyoxalase II in an end-point spectrophotometric assay. A stock solution of ca. 3 mM S-D-lactoylglutathione (free acid) in water was prepared and a portion was diluted 10 fold in a quartz cuvette containing 50 mM Tris/HCl, pH 7.4, with

buffer only in the reference cuvette. One unit of glyoxalase II (Sigma) was added to both sample and reference cuvettes and the absorbance at 240 nm was followed until a steady minimum absorbance was reached. The concentration of S-D-lactoylglutathione was calculated from the mean decrease in absorbance using the extinction coefficient given above.

3.2.3 The Assay of Reduced Glutathione and S-D-Lactoylglutathione in Whole Human Blood

The concentration of S-D-lactoylglutathione in whole blood was assayed by a modification of the method of Thornalley et al (156). S-D-Lactoylglutathione was determined as the reduced glutathione released on the addition of glyoxalase II to de-proteinised extracts of whole blood samples; reduced glutathione was measured by the method of Davies et al (200), which determines glutathione as the chromophore S-2,4-dinitrophenyl-glutathione, formed from 1-chloro-2,4-dinitrobenzene in the presence of glutathione S-transferase.

2 mls of whole blood were treated with 1 mM N-ethylmaleimide for 10 minutes at room temperature to remove native reduced glutathione (201). 1 ml of ice-cold 20% trichloroacetic acid was added to the sample and mixed throughly. After keeping the samples on ice for 20 minutes, the precipitate was sedimented by centrifugation (500g, 10 minutes). The supernatant was removed and neutralised by extraction with 5 x 4

of water-saturated diethyl/ether. Residual ether was dispersed with nitrogen gas. This procedure produced a de-proteinised extract depleted of native reduced glutathione.

An aliquot of de-proteinised extract was incubated with 100 uM 1-chloro-2,4-dinitrobenzene and 10 units of glutathione S-transferase for 1 hour in 50 mM Tris/HCl, pH 7.4 and 37°C. The absorbance at 340 nm was recorded. 1 Unit/ml of glyoxalase II was added to the assay and reference samples and incubated for a further hour at 37°C. The final absorbance at 340 nm was recorded. The concentration of S-D-lactoylglutathione was calculated from the glyoxalase II-dependent increase in absorbance. Assays were calibrated and controlled by assaying standard solutions of S-D-lactoylglutathione and blanks (phosphate-buffered saline) respectively.

A typical calibration curve for reduced glutathione and S-D-lactoylglutathione is presented in Figure 3.

3.2.4 The Assay of Methylglyoxal in Whole Human Blood

The concentration of methylglyoxal in whole blood was assayed by a modification of the high pressure liquid chromatography (HPLC) method of Ohmori et al (115), which determines ∝-oxoaldehydes as quinoxalines formed by the incubation of ∝-oxoaldehydes with o-phenylene diamine. Whole blood samples were treated with two volumes of absolute ethanol, mixed thoroughly and incubated on ice for 10 minutes. The precipitate was sedimented by centrifugation (500g, 10 minutes).
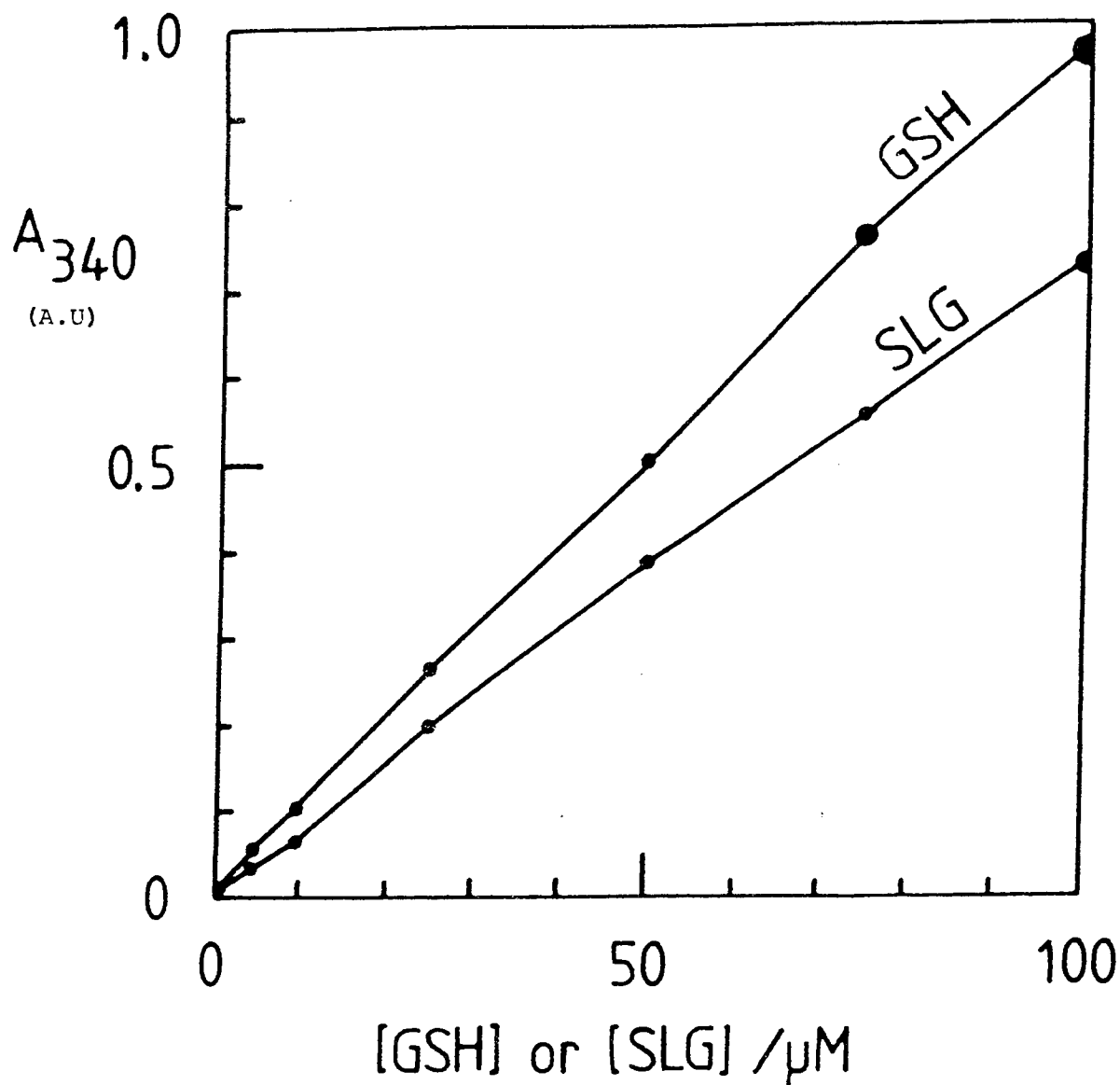
Figure 3. A Typical Calibration Curve for the Assay of
Reduced Glutathione and S-D-Lactoylglutathione.
Standard solutions of reduced glutathione and
S-D-lactoylglutathione were assayed as described in the
Materials and Methods section.

The clear aqueous ethanol supernatant was incubated with 100 µM o-phenylene diamine for two hours in the dark at room temperature, and then extracted 5 times with 4 volumes of chloroform. The chloroform extracts were pooled and evaporated to dryness under vacuum at room temperature. The residue was dissolved in 0.5 ml HPLC mobile phase and analysed for quinoxaline content.

HPLC analysis was performed on a Radial-PAK µBondapak C_{18} reverse-phase column (8 mm internal diameter x 10 cm) with a mobile phase of 10 mM potassium dihydrogen phosphate pH 2.1 : acetonitrile (80 : 20). Quinoxalines were detected by flow spectrophotometry at 312 nm. The internal standard was 2,3-dimethylquinoxaline. The assay was calibrated by assaying solutions of known methylglyoxal concentration. 200 µl of sample was injected per assay. The recovery of methylglyoxal was approximately 60%. A typical HPLC trace for the methylglyoxal assay is shown in Figure 4.

3.3 Modification of the Glyoxalase System During the Differentiation of Human Leukaemia Cells In Vitro.

HL60 and K562 cell cultures were grown in the absence and presence of 130, 150 and 185 mM N-methylformamide, and 13, 26, 51.5, 77 and 103 µM CCRG 81045 dissolved in DMSO such that the final concentration of DMSO in the culture medium did not exceed 0.001% - this concentration does not induce differentiation of HL60 or K562 cells (202). Cells were
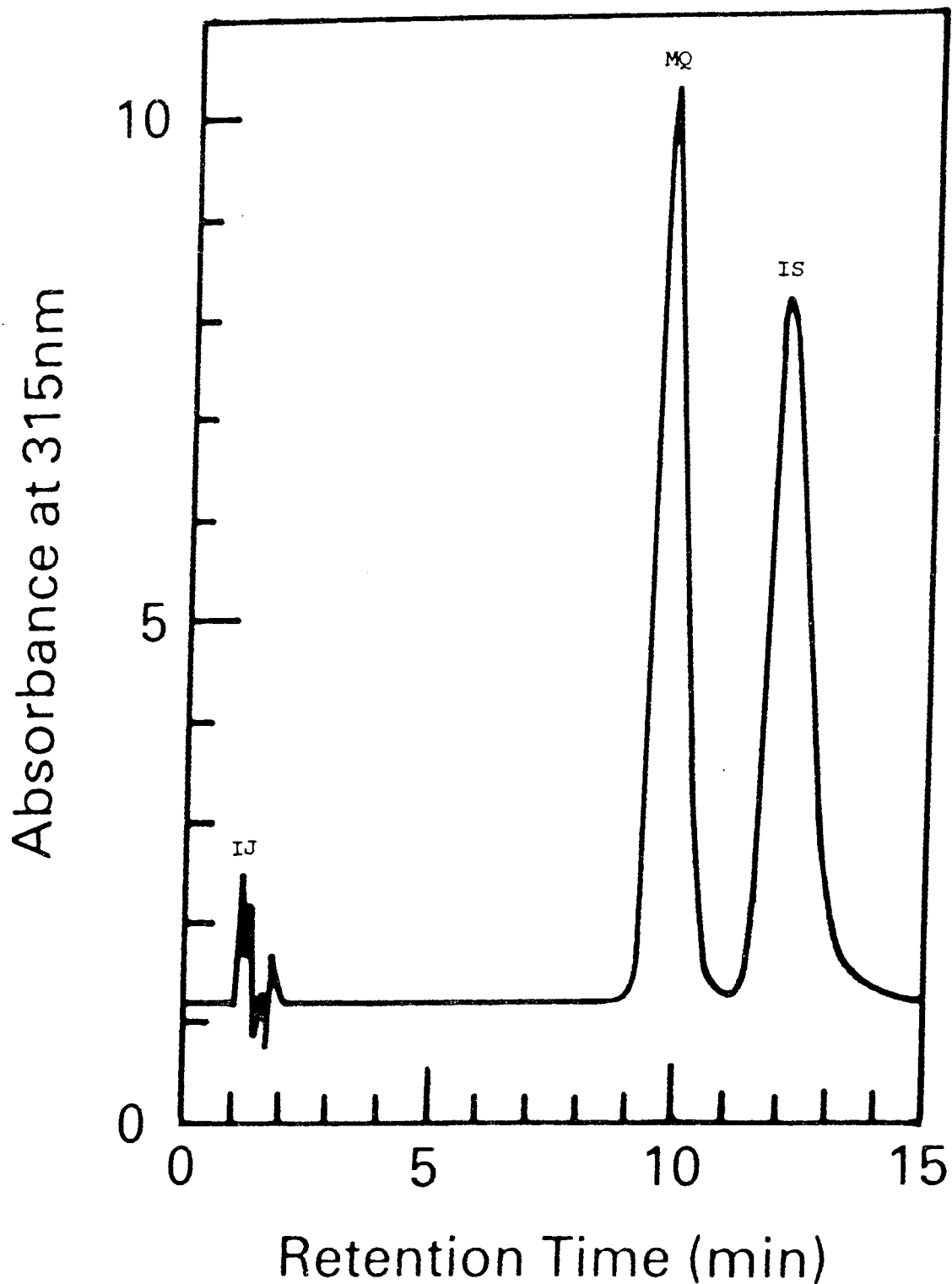
Figure 4. A Typical HPLC Trace for the Assay of
Methylglyoxal. A standard methylglyoxal solution was
prepared and assayed as described in the Materials and
Methods section. Key : IJ - Injection point, MQ -
Methylquinoxaline peak (10 μM), IS - Internal Standard
peak (10 μM).

seeded at an initial density of 5×10^4 cells/ml and incubated for 3 - 6 days. The development of HL60 maturation-dependent superoxide-forming NADPH oxidase activity was measured by the reduction of nitro blue tetrazolium (NBT) to formazan by the method of Mendelsohn et al (203). Erythroid differentiation of K562 cells was scored by benzidine staining (204).

For the assay of glyoxalase activities, the cells were sedimented by centrifugation (300g, 5 minutes), re-suspended in sonication buffer (10 mM sodium phosphate, pH 7.4) at 4^OC and ruptured by sonication (100 W, 20 seconds). The membranes were sedimented by centrifugation (50,000g, 30 minutes) and the supernatant assayed for glyoxalase I and glyoxalase II activity.

The intrinsic effect of NMF and CCRG 81045 on the glyoxalase enzyme activities, defined as the effect of these compounds on the activity of glyoxalase I and glyoxalase II in a cell-free system, was investigated by studying the effect of adding NMF and CCRG 81045 to sonicated cell extracts from HL60 and K562 cells. An aliquot of cell extract was incubated, in the presence or absence of 185 mM NMF or 103 µM CCRG 81045 (the highest concentration of each drug used in the differentiation study), for 10 minutes at 37^OC. After incubation, the residual activity of glyoxalase I and glyoxalase II in the sample was determined at a high and a low substrate concentration (0.2 and 2.0 mM hemimercaptal, 25 and 300 µM S-D-lactoylglutathione).

The effect of NMF and CCRG 81045 on the stability
of the glyoxalase substrates, hemimercaptal and
S-D-lactoylglutathione, was also investigated.
Solutions of substrate were incubated with or without
185 mM NMF or 103 µM CCRG 81045 and the rate of decrease
in substrate concentration was monitered at 240 nm, as
described above.

The stability of glyoxalase I and glyoxalase II in
the cell-free extract was also determined. The activity
of both enzymes was measured when the cell extract was
prepared. The cell extract was then stored overnight at
$4^{o}C$ and the activity of glyoxalase I and glyoxalase II
re-measured, as described above. The stability of both
enzymes was determined from the change in activity of
glyoxalase I and glyoxalase II following overnight
storage.

3.4 Changes in Glyoxalase Activities During Growth Arrest Of Raji and GM892 Cells.

GM892 and Raji cell cultures were incubated as
described. Growth arrest was achieved by decreasing the
concentrations of foetal calf serum to 0.1% , 0.5% ,
2.0% and 5.0% from the normal 10%. Cells were seeded at
an initial density of 2×10^{5} cells/ml and incubated for
2 days. For assay of glyoxalase activities, the cells
were sedimented by centrifugation (300g, 5 minutes),
re-suspended in sonication buffer (10 mM HEPES with
0.02% Triton X-100, pH 7.4) at $4^{o}C$ and ruptured by

sonication (100 W, 20 seconds). The membranes were sedimented by centrifugation (50,000g, 30 minutes) and the supernatant assayed for glyoxalase activity.

The rate of cell proliferation was assessed by the rate of incorporation of tritiated thymidine (131). An aliquot (1 ml) of cell suspension was added to 2.5 µCi of tritiated thymidine and gassed with 5% carbon dioxide. The tubes were capped and incubated at $37^{O}C$ for 1 hour. The solution was filtered, under vacuum, through a WHATMAN GF/C filter and washed once with physiological saline (0.9% sodium chloride), followed by three washes with ice cold 5% trichloroacetic acid, and a final wash with absolute ethanol. The filter was dried in air and placed in a scintillation vial containing 5 mls of OPTIPHASE scintillation fluid for counting. The amount of tritiated thymidine in the cell extract was measured with a Tricarb 2000CA liquid scintillation counter.

3.5 The Potentiation of Cell-Free Microtubule Assembly by S-D-Lactoylglutathione.

3.5.1 The Purification of Microtubular Protein from Porcine Brain

Microtubular protein (tubulin + microtubule-associated proteins, MAPS) was prepared by a modification of the method of Gaskin et al (205). Pig brains from 4 - 6 pigs, freshly killed at a local slaughter house, were chilled on ice and the meninges

and superficial blood vessels removed. The brains were homogenized at 0°C in assembly buffer (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 ml of buffer per gram wet weight of tissue). Homogenization was performed with a Waring blender (4 seconds at low speed) and then in a PotterElvejhem Teflon pestle homogenizer. Cellular debris was sedimented by centrifugation (90 minutes, 12,000g, 0°C). Tubulin was purified from the supernatant by three assembly - disassembly cycles. One volume of 8 M glycerol in assembly buffer was added to the supernatant and the solution was made 1 mM with respect to GTP and incubated at 37°C for 45 minutes. Assembled microtubules were sedimented by centrifugation (60 minutes, 39,000g, 37°C) and re-suspended in assembly buffer by homogenization. The re-suspended microtubules were disassembled by incubation at 0°C for 30 minutes, the solution was then centrifuged (60 minutes, 39,000g, 0°C) to produce a tubulin-rich supernatant. The supernatant was purified by a further two assembly - disassembly cycles, the last purification cycle being performed immediately prior to the microtubule assembly experiments, as shown in the purification scheme - Figure 5. Purified tubulin solution was stored at -20°C. Electrophorectic analysis has shown that protein purified by this method contains greater than 80% tubulin, the remainder being microtubule-associated proteins (205).
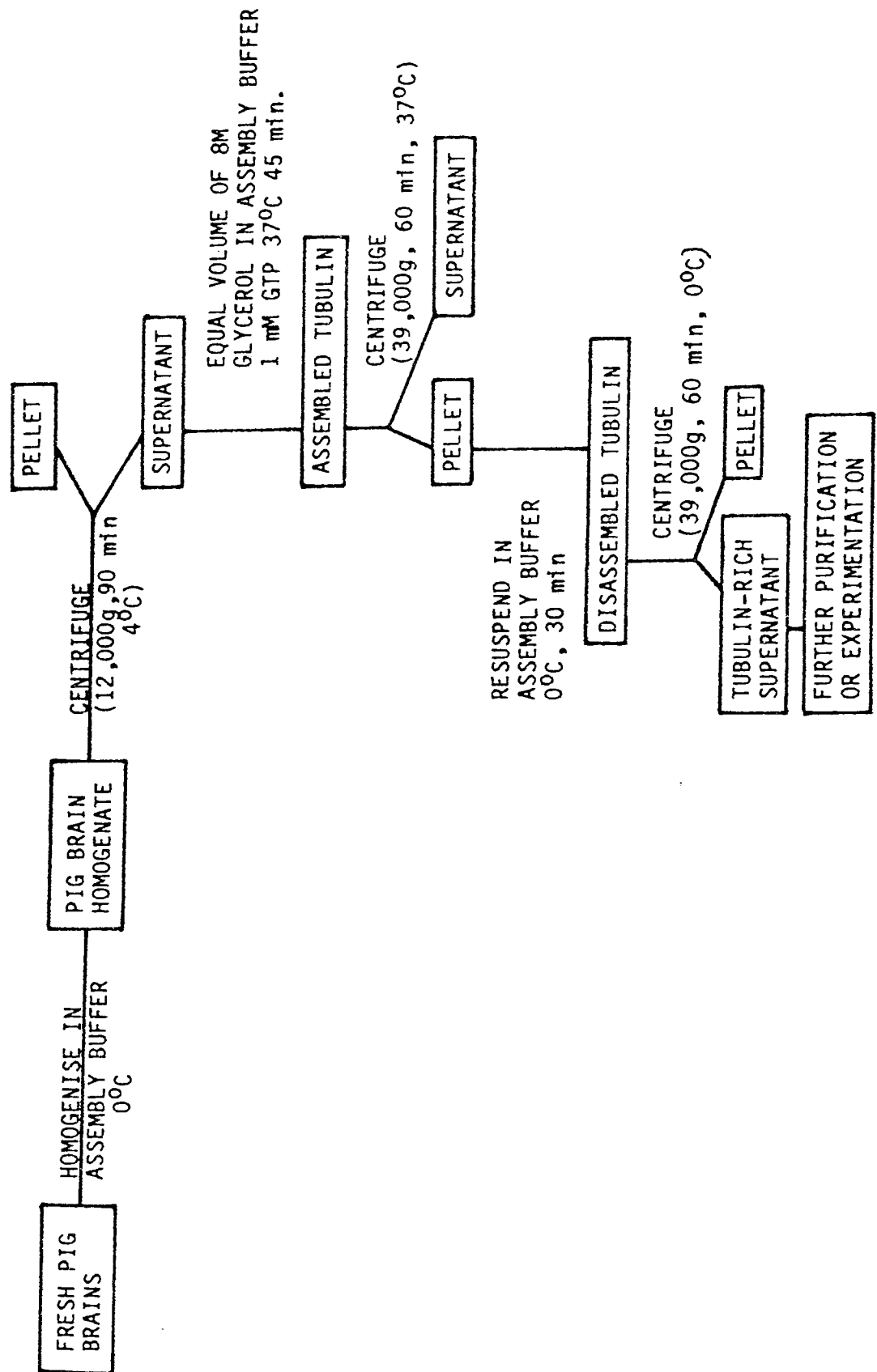
Figure 5. Flow Diagram for the Purification of Microtubular Protein from Porcine Brain. Microtubular protein was purified by the method of Gaskin et al (205).

3.5.2 The Measurement of Microtubular Assembly by Turbidity.

Microtubular formation was measured turbidometrically by following the absorbance of assembly incubations at 350 nm (205). The incubation mixture contained 1 mg/ml microtubular protein in assembly buffer, with and without 0.02 - 1.00 mM S-D-lactoylglutathione, at $37^{\circ}C$. The assembly of microtubules was initiated by the addition of 20 μM GTP. After incubation for one hour, the disassembly properties of the microtubules were investigated by : (i) the addition of calcium chloride (final concentration 1.05 mM), (ii) the addition of 0.1 mg/ml of the anti-tubulin nocodazole, and (iii) incubation of assembled microtubules in suspension at $0^{\circ}C$. The decrease in absorbance at 350 nm was followed for a further 30 minutes.

3.5.3 The Consumption of S-D-Lactoylglutathione on Assembly

Microtubular protein (1 mg/ml) was assembled at $37^{\circ}C$ for 30 minutes in the presence of 1 mM S-D-lactoylglutathione. The incubation was centrifuged (30 minutes, $37^{\circ}C$, 20,000g) and the supernatant removed. The concentration of S-D-lactoylglutathione in the supernatant was estimated from the decrease in absorbance after addition of glyoxalase II - as described in section 3.2.2. Controls were S-D-lactoylglutathione incubated with assembly buffer

and GTP, and assembly buffer with microtubular protein without GTP.

3.6. The Red Blood Cell Glyoxalase System in Diabetic Patients.

3.6.1. Patients.

Eighty-five diabetic patients, 40 with retinopathy and 45 clinically uncomplicated (no retinopathy by fundoscopy of the dilated eye and no "Albustix" positive proteinuria) were studied together with 45 healthy non-diabetic controls attending the same outpatients clinic with minor complaints. Venous blood samples taken by venupuncture were drawn into tubes containing lithium heparide at the West Midland Regional Health Authority Diabetic Clinic, East Birmingham Hospital, by Drs P. Jennings and C. Florkowski. Samples were transported to the laboratory, arriving within 1 hour of taking the blood sample. Retinopathy examinations and blood glucose measurements were performed by the staff of East Birmingham Hospital.

3.6.2. The Assay of Red Blood Cell Glyoxalase Activities

Red blood cells were sedimented from 4 mls blood by centrifugation (10 minutes, 2,000g). The plasma and white cells were removed and the red cells washed four times with 5 volumes of phosphate-buffered saline (0.9% sodium chloride : 0.1 M potassium phosphate buffer pH 7.4; 9 : 1). 1 ml of washed packed red blood cells was

lysed in four volumes of distilled water and vortexed to ensure complete lysis of the cells. The membrane fragments were sedimented by centrifugation (10 minutes, 2,000g). The supernatant was assayed for glyoxalase activity as described above.

In the glyoxalase I assay, the coefficient of variance for the intrabatch assay was 2.7% (n = 6) and 6.7% for the interbatch assay (n = 6). In the glyoxalase II assay, the coefficient of variance for the intrabatch assay was 1.5% (n = 6) and 3.2% for the interbatch assay (n = 6).

3.6.3. The Assay of Methylglyoxal and S-D-Lactoylglutathione in Whole Human Blood.

The concentration of methylglyoxal in whole blood was determined in 32 diabetic patients with and without complications and in 17 healthy control subjects by the methods described above. The concentration of S-D-lactoylglutathione in whole blood was determined in 28 diabetic patients with and without complications and in 24 healthy control subjects by the methods described above.

In the methylglyoxal assay, the coefficient of variance for the controls was 5.8% for the intrabatch assay (n = 4) and 14.7% for the interbatch assay (n = 6). In the S-D-lactoylglutathione assay, the coefficient of variance for the controls was 11.4% for the intrabatch assay (n = 4) and 12.1% for the interbatch assay (n = 6).

3.7 Instrumentation.

3.7.1 Spectrophotometry.

All spectrophotometric assays were performed on a CECIL CE5095 High Performance Scanning spectrophotometer equipped with a CECIL CE500 Control-record module (Series II) and a Gallenkamp 85 thermostirrer.

3.7.2 High Pressure Liquid Chromatography.

The HPLC system employed comprised an ALTEX 420 microprocessor controller/programmer, two ALTEX 100A pumps, a WATERS Lambda-Max model 480 LC spectrophotometer, a WATERS RCM-100 Radial Compression Module and an LKB 1-Channel recorder. The HPLC column (Type 8MBC 1801μ) was purchased from Waters Associates (Harrow, U.K.)

4. RESULTS.

4.1 Changes in Glyoxalase Activities During the Differentiation of Human Leukaemia Cells In Vitro.

4.1.1 Human Promyelocytic Leukaemia HL60 Cells.

Incubating HL60 cells with N-methylformamide (NMF) for 3 to 6 days results in a dose- and time- dependent increase in the formation of mature granulocytes, as determined by an increase in the percentage of cells reducing nitroblue tetrazolium (NBT) (159). During the incubation period, there were marked changes in the HL60 glyoxalase activities - the activity of glyoxalase I decreased and the activity of glyoxalase II increased (Figure 6). The activities of the glyoxalase enzymes in untreated HL60 cells was relatively constant throughout the 6-day period (Table 4). There was no reduction in the viability of NMF-treated cells, as judged by trypan blue exclusion (159). However, there was a dose-dependent decrease in cell growth, as indicated by the decrease in the final cell number.

The decrease in the activity of glyoxalase I was dependent on the concentration of NMF in the incubation media. Maximal inhibition of 40, 58 and 64% of control values occurred after incubation for 4 days with 130, 150 and 185 mM NMF, respectively. In contrast, the activity of glyoxalase I in the untreated cells varied by only 10% of the mean value throughout the 6 day incubation period (Table 4). The number of
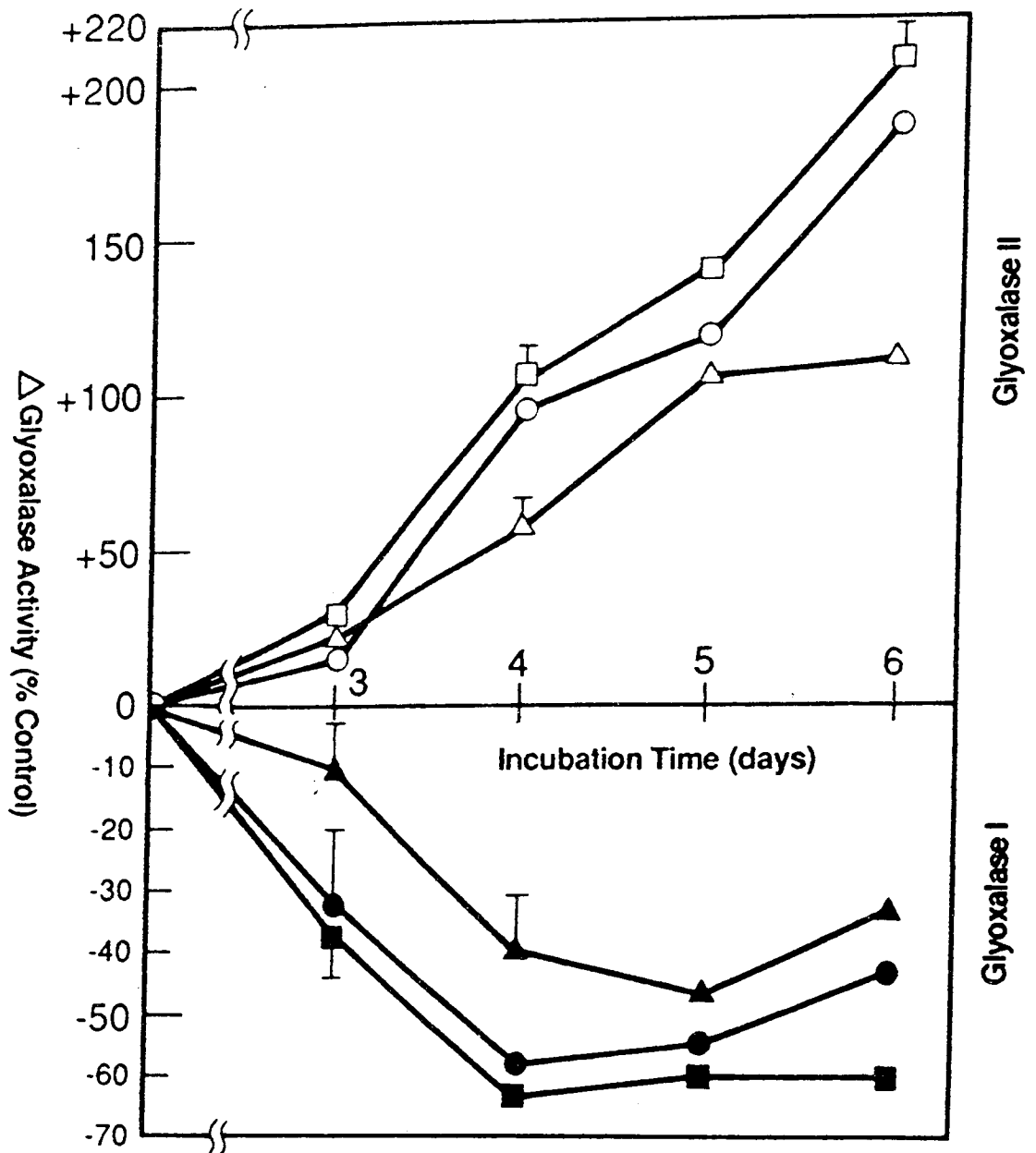
Figure 6. The Glyoxalase I and Glyoxalase II Activities in HL60 Cells Treated with N-Methylformamide (NMF). Drug treatment : (△ ▲) 130 mM NMF, (O,●) 150 mM NMF and (□ ■) 185 mM NMF. Closed symbols - glyoxalase I, open symbols - glyoxalase II. Data is the mean ± standard deviation of three independent experiments. The activities of the glyoxalase enzymes were determined as described in the Materials and Methods section.

Table 4. The Activity of Glyoxalase I and Glyoxalase II in Untreated HL60 Leukaemia Cells in Culture.

Culture Time (Days)	Glyoxalase I (mU/10^6 cells)	Glyoxalase II (mU/10^6 cells)
3	37.9 ± 6.9	1.96 ± 0.33
4	34.1 ± 1.2	2.24 ± 0.05
5	29.4 ± 0.7	1.96 ± 0.08
6	31.5 ± 1.6	1.92 ± 0.03

Glyoxalase activities were determined as described in the Materials and Methods section. Data is the mean ± standard deviation of a minimum of 7 experiments.

differentiated cells reached a maximum at 4 days after drug administration and remained relatively constant during days 5 and 6 (159). After the fourth day of incubation, the decrease in the activity of glyoxalase I showed a direct correlation with the percentage of cells reducing NBT (Figure 7). On continued incubation of the cells with 185 mM NMF, there was no further decrease in the activity of glyoxalase I, although by day 6 the activity of glyoxalase I in the cells treated with 130 and 150 mM NMF had increased by 10-15% . The data suggests that the general features of the change in glyoxalase I activity in NMF treated HL60 cells are : (a) a decrease in activity of approximately 50-60% within the first 4 days of treatment, and (b) the approximate maintenance of this decreased glyoxalase I activity thereafter.

During the 6-day incubation period, the activity of glyoxalase II in NMF-treated HL60 cells increased by between 110 and 210% of the control levels (Figure 6). The activity of glyoxalase II in untreated HL60 cells changed by only about 8% of the mean value during this period (Table 4). The activity of glyoxalase II in the NMF-treated cells showed a time- and dose-dependent increase throughout the 6 day incubation period, as was observed with glyoxalase I. In general, incubation of the cells with 130 mM NMF produced a smaller response than was seen with the higher concentrations. There is a smaller difference between the curves for 150 and 185
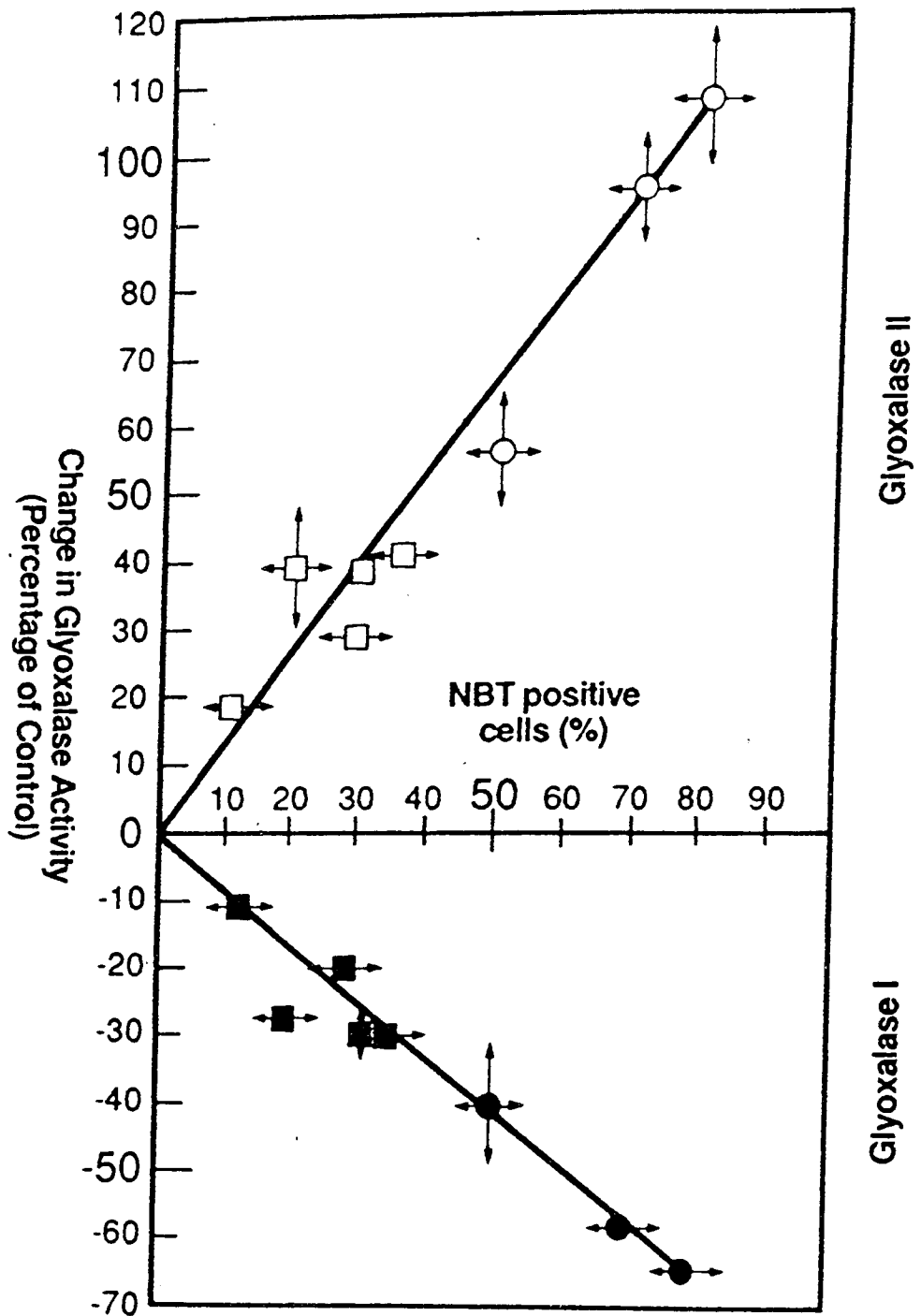
Figure 7. The Relationship Between Changes in the Activities of the Glyoxalase Enzymes and the Percentage of Differentiated HL60 Cells after Treatment with NMF and CCRG 81045 for 4 Days. Key : ○ ● - NMF, □ ■ - CCRG 81045. Closed symbols - glyoxalase I, open symbols - glyoxalase II.

mM NMF than between the curves for 130 and 150 mM NMF. This difference in the effect of NMF is reflected in the percentage of cells which could reduce NBT. On day 4 of treatment, there was a linear relationship between the increase in the activity of glyoxalase II and the number of differentiated cells (Figure 7). To compare the relative activities of the glyoxalase enzymes, the unit ratio of the activity of glyoxalase I to the activity of glyoxalase II (^{A}GI/^{A}GII) was calculated. This unit ratio decreased during the first 4 days of incubation and then remained relatively constant (Table 5).

When HL60 cells were incubated with CCRG 81045, the changes in the activities of glyoxalase I and glyoxalase II were similar to those seen with NMF. After a 6 day incubation, when the percentage of differentiated cells was maximal (203), there was a dose-dependent decrease in the activity of glyoxalase I and a dose-dependent increase in the activity of glyoxalase II (Figure 8). The decrease in glyoxalase I activity and the increase in glyoxalase II activity was maximal at CCRG 81045 concentrations greater than 70 µM. Moreover, there was a linear relationship between the changes in glyoxalase activities and the percentage of cells reducing NBT (Figure 7). The data for the differentiation of HL60 cells by CCRG 81045 has the same linear relationship as that obtained for the differentiation of HL60 cells by NMF.

When a crude preparation of glyoxalase enzymes,

Table 5. The Glyoxalase I/Glyoxalase II Activity Ratio (A_{GI}/A_{GII}) in N-Methylformamide Treated HL60 Leukaemia Cells in Culture

Culture Time (Days)	[NMF] (mM)	A_{GI}/A_{GII} (unit ratio)	$\Delta\, A_{GI}/A_{GII}$ (% Control)
3	Control	19.3 ± 6.7	---------
	130	10.2 ± 1.6^a	-47 ± 51
	150	7.8 ± 1.6^a	-60 ± 56
	185	9.4 ± 1.7^a	-51 ± 53
4	Control	15.2 ± 0.8	---------
	130	5.9 ± 0.9^b	-61 ± 23
	150	3.3 ± 0.5^c	-78 ± 21
	185	2.7 ± 0.5^c	-82 ± 24
5	Control	15.0 ± 1.0	---------
	130	5.7 ± 0.5^b	-68 ± 14
	150	4.2 ± 0.4^c	-77 ± 15
	185	4.1 ± 0.3^c	-77 ± 14
6	Control	16.4 ± 1.7	---------
	130	5.6 ± 0.3^b	-72 ± 15
	150	3.2 ± 0.2^b	-84 ± 16
	185	2.6 ± 0.2^b	-87 ± 18

The glyoxalase activity ratio, A_{GI}/A_{GII}, was calculated as the ratio of the activity of glyoxalase I/glyoxalase II, where the glyoxalase activities are expressed in units/10^6 cells as defined in the Materials and Methods section. Significance was determined using the Student's two-tailed "t" test : ^{a} - $P > 0.10$; ^{b} - $0.01 > P > 0.002$; ^{c} - $P < 0.002$.
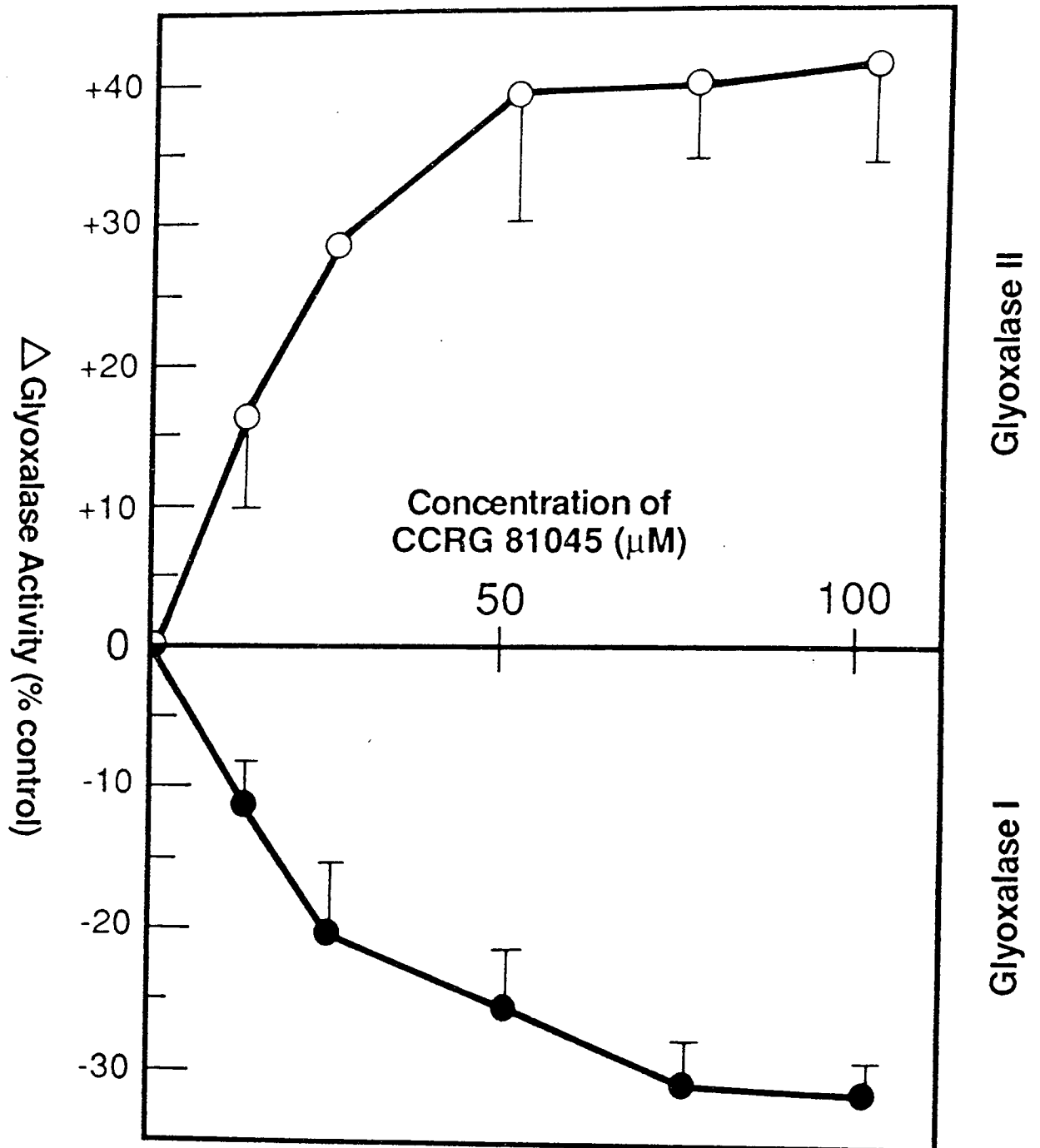
Figure 8. The Activity of Glyoxalase I and Glyoxalase II in HL60 Cells Treated with CCRG 81045 for 6 Days. Closed symbols - glyoxalase I, open symbols - glyoxalase II. Data is the mean ± standard deviation of three independent experiments. The activities of the glyoxalase enzymes were determined as described in the Materials and Methods.

prepared from the cytosol of HL60 cells, was incubated
with either 185 mM NMF or 103 µM CCRG 81045 for 10
minutes, there was no significant change in the
activities of glyoxalase I and glyoxalase II (Tables 6
and 7). This excludes the possibility that the presence
of NMF or CCRG 81045 in the assay systems for glyoxalase
activities produced the observed inhibitory and
stimulatory effects. Therefore, the changes in
glyoxalase activities observed during the 6 day
incubations with both NMF and CCRG 81045 may be due to
changes in enzyme synthesis, metabolic regulatory
modification, or to a longer term interaction between
NMF and CCRG 81045 with the glyoxalase enzymes.

When the glyoxalase substrates, 1.33 mM
hemimercaptal and 0.3 mM S-D-lactoylglutathione, were
incubated with 185 mM NMF and 103 µM CCRG 81045, there
was no significant change in the stability of
S-D-lactoylglutathione in the presence of NMF or CCRG
81045, or hemimercaptal in the presence of CCRG 81045.
However, NMF caused a significant increase in the rate
of change of absorbance of the hemimercaptal (Table 8).
This did not affect the activity of glyoxalase I. In
all cases, the rate of change of the blank was less than
10% of the rate of change of the enzyme catalysed
reaction.

When the HL60 cell extract was stored overnight at
$4^{O}C$, there was no significant decrease in the activity
of glyoxalase I or glyoxalase II (Table 9). This

Table 6. **The Effect of N-Methylformamide and CCRG 81045 on Glyoxalase I Activity in Cell-Free Systems**

	[Hemimercaptal]	
	0.2 mM	1.33 mM
HL60 Cell Extract		
Control	18.1 ± 0.1	32.8 ± 2.3
+ 185 mM NMF	18.5 ± 0.1	32.4 ± 3.7
+ 103 μM CCRG 81045	20.3 ± 2.3	35.0 ± 0.8
K562 Cell Extract		
Control	18.6 ± 1.0	86.8 ± 4.8
+ 185 mM NMF	14.2 ± 8.6	78.8 ± 4.0
+ 103 μM CCRG 81045	19.5 ± 2.9	85.3 ± 1.4

Glyoxalase I activity is expressed as $mU/10^6$ cells as defined in the Materials and Methods section. Data is the mean ± standard deviation from two independent experiments. All data gives $P > 0.10$ in the Student's two-tailed "t" test.

Table 7. The Effect of N-Methylformamide and CCRG 81045 on Glyoxalase II Activity in Cell-Free Systems

	[S-D-Lactoylglutathione]	
	25 µM	300 µM
HL60 Cell Extract		
Control	0.285 ± 0.025	2.02 ± 0.13
+ 185 mM NMF	0.235 ± 0.023	1.72 ± 0.04
+ 103 µM CCRG 81045	0.250 ± 0.028	1.98 ± 0.02
K562 Cell Extract		
Control	0.510 ± 0.074	2.80 ± 0.13
+ 185 mM NMF	0.420 ± 0.049	2.11 ± 0.33
+ 103 µM CCRG 81045	0.502 ± 0.60	2.57 ± 0.12

Glyoxalase II activity is expressed as $mU/10^6$ cells as defined in the Materials and Methods section. Data is the mean ± standard deviation from two independent experiments. All data gives $P > 0.10$ in the Student's two-tailed "t" test.

Table 8.   The Stability of Hemimercaptal and
                  S-D-Lactoylglutathione in the Presence
                  of 185 mM NMF and 103 µM CCRG 81045.

                                           Change in Absorbance
                                                (mAU/min)

0.3 mM S-D-Lactoylglutathione

Control $0.38 \pm 0.02_a$
+ 185 mM NMF 0.41 ± 0.03^a
+ 103 µM CCRG 81045 0.31 ± 0.04^a

1.33 mM Hemimercaptal

Control $3.36 \pm 0.26_b$
+ 185 mM NMF 11.73 ± 0.19^b
+ 103 µM CCRG 81045 3.39 ± 0.31^a

The stability of S-D-lactoylglutathione and
hemimercaptal was determined as described in the
Materials and Methods section. Statistical analysis was
performed using a one-tailed Student's "t" test –
[a] = $P > 0.05$, [b] = $P < 0.001$.

Table 9. The Stability of Glyoxalase I and Glyoxalase II from HL60 and K562 Cells.

| | Activity (% of Control) | |
	Day 0	Day 1
HL60 Cells		
Glyoxalase I	100 ± 4	97 ± 5
Glyoxalase II	100 ± 1	99 ± 1
K562 Cells		
Glyoxalase I	100 ± 7	99 ± 3
Glyoxalase II	100 ± 4	108 ± 1

The activity of glyoxalase I and glyoxalase II was determined as described in the Materials and Methods section. The activity of both enzymes on the day the samples were prepared (Day 0) is taken as the control activity. The activity of glyoxalase I and glyoxalase II was remeasured after storage for 24 hours (Day 1). Data is the mean ± standard deviation of two determinations.

indicates that both glyoxalase enzymes are stable for the time-period over which the experiment was conducted.

4.1.2 Human Erythroleukaemia K562 Cells

Unlike HL60 cells, K562 cells are not induced to differentiate by NMF at any of the concentrations used (202). Although NMF has no effect on either the proliferative activity or the expression of haemoglobin in these cells, it did induce an increase in the activity of both glyoxalase I and glyoxalase II over the 6 day period (Figure 9). The activities of the glyoxalase enzymes in untreated K562 cells showed a small fluctuation throughout this period (less than 5% of the mean value - Table 10). There was an inverse relationship between the increase in the activity of glyoxalase I in NMF-treated K562 cells and the concentration of NMF - after five days of treatment with 130 mM NMF, the glyoxalase I activity was increased by 70% of the control value, whereas, after treatment for five days with 185 mM NMF, the activity of glyoxalase I had increased by only 25% of the control value. After incubation for six days, the glyoxalase I activity, at all the NMF concentrations studied, was increased by 80 - 90% of the control value.

The activity of glyoxalase II in NMF-treated K562 cells increased during the 5-6 day incubation period (Figure 9), except for an anomalous decrease with 130 mM NMF between days 5 and 6. In contrast to the glyoxalase I data, NMF had a dose-dependent effect on glyoxalase II
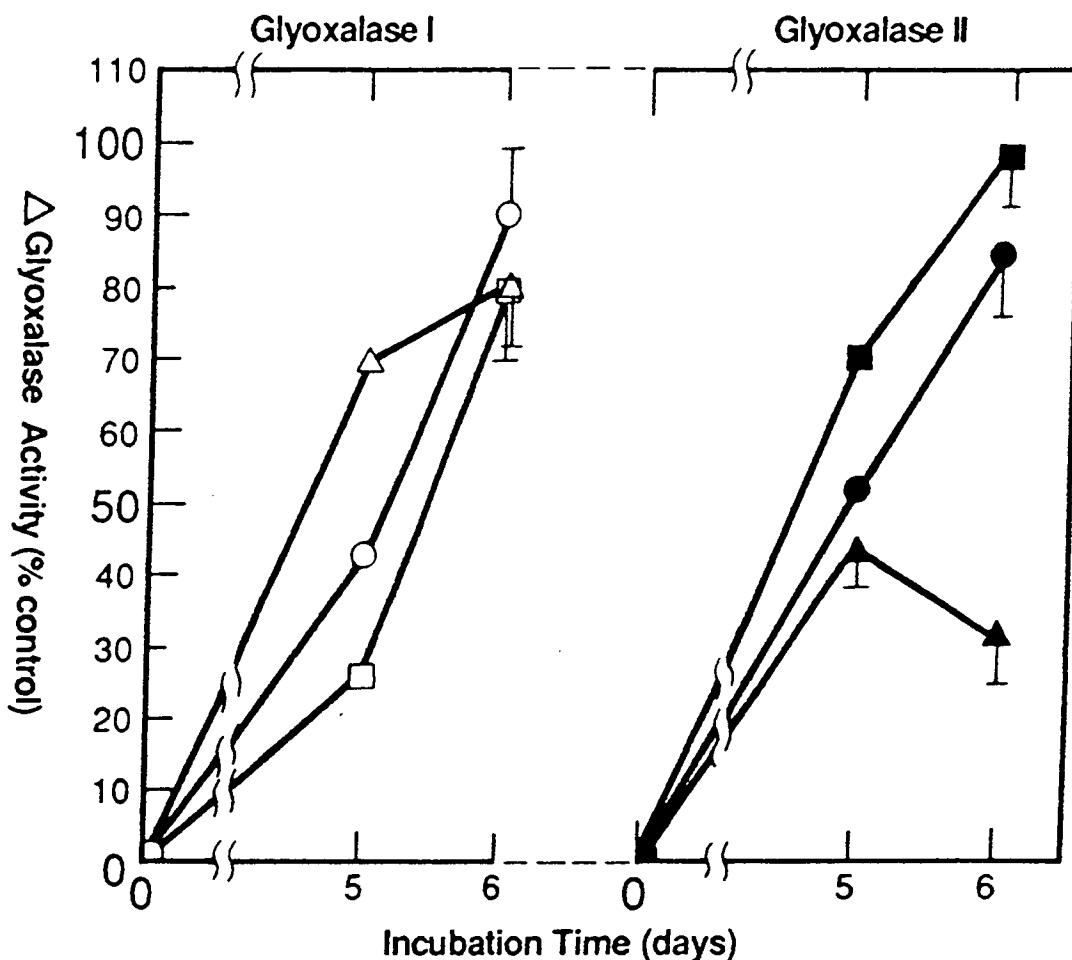
Figure 9. The Glyoxalase I and Glyoxalase II Activities
in K562 Cells Treated with N-Methylformamide. Key :
(△ ▲) 130 mM NMF, (○ ●) 150 mM NMF and
(□ ■) 185 mM NMF. Closed symbols - glyoxalase I, open
symbols - glyoxalase II. Data is the mean ± standard
deviation of three independent experiments. The
activities of the glyoxalase enzymes was determined as
described in the Materials and Methods.

Table 10.   The Activity of Glyoxalase I and Glyoxalase
             II in Untreated K562 Erythroleukaemia
                      Cells in Culture.

Culture Time (Days)	Glyoxalase I $(mU/10^6 cells)$	Glyoxalase II $(mU/10^6 cells)$
3	82.2 ± 5.5	2.83 ± 0.08
5	90.3 ± 4.3	2.78 ± 0.06
6	88.0 ± 6.9	2.93 ± 0.18

Glyoxalase activities were determined as described in
the Materials and Methods section. Data is the mean $\pm$
standard deviation of a minimum of 8 experiments.

activity. The maximum increase in the activity of
glyoxalase II observed was about 100% of the control
value. The unit ratio of the activity of glyoxalase I
to the activity of glyoxalase II showed only a minor
change after 6 days of incubation of K562 cells with NMF
(Table 11).

Unlike NMF, incubating K562 cells with CCRG 81045
produces a time- and dose-dependent increase in the
number of haemoglobin-producing cells, although no
functional alteration is apparent until after 3 days of
treatment (204). After treatment with CCRG 81045, there
was no significant change in K562 glyoxalase activities
for the first three days, but thereafter the activity of
both glyoxalase I and glyoxalase II increased (Figure
10). The glyoxalase activities in untreated cells
showed only a relatively small fluctuation throughout
this period (Table 10). The increases in the activities
of glyoxalase I and glyoxalase II were not markedly
dependent on the dose of CCRG 81045. Except for the
curve for glyoxalase I activity in cells treated with 26
μM CCRG 81045, most of the glyoxalase activity curves
are extraordinarily similar. There was a marked
increase in the activity of both glyoxalase I and
glyoxalase II during the sixth day of incubation. The
percentage increase in the activity of glyoxalase II is
approximately twice the percentage increase in the
activity of glyoxalase I. Therefore, the unit ratio of
the activity of glyoxalase I to the activity of

Table 11. The Glyoxalase I/Glyoxalase II Activity Ratio
(A_{GI}/A_{GII}) in N-Methylformamide
Treated K562 Erythroleukaemia Cells in
Culture

Culture Time (Days)	[NMF] (mM)	A_{GI}/A_{GII} (unit ratio)	$\Delta\ A_{GI}/A_{GII}$ (% Control)
3		NOT DETERMINED	
	Control	30.8 ± 1.5	----------
	130	36.3 ± 1.7^a	$+18 \pm 10$
5	150	28.9 ± 0.8^a	$- 7 \pm 8$
	185	22.7 ± 0.4^b	-26 ± 7
	Control	29.6 ± 0.8	----------
	130	41.0 ± 1.1^b	$+39 \pm 6$
6	150	30.5 ± 0.8^a	$+ 3 \pm 5$
	185	27.4 ± 0.4^b	$- 7 \pm 4$

The glyoxalase activity ratio, A_{GI}/A_{GII}, was calculated
as the ratio of the activity of glyoxalase I/glyoxalase
II, where the glyoxalase activities are expressed in
units/10^6 cells as defined in the Materials and Methods
section. Significance was determined using the
Student's two-tailed "t" test : [a] - P > 0.10;
[b] - P < 0.05.
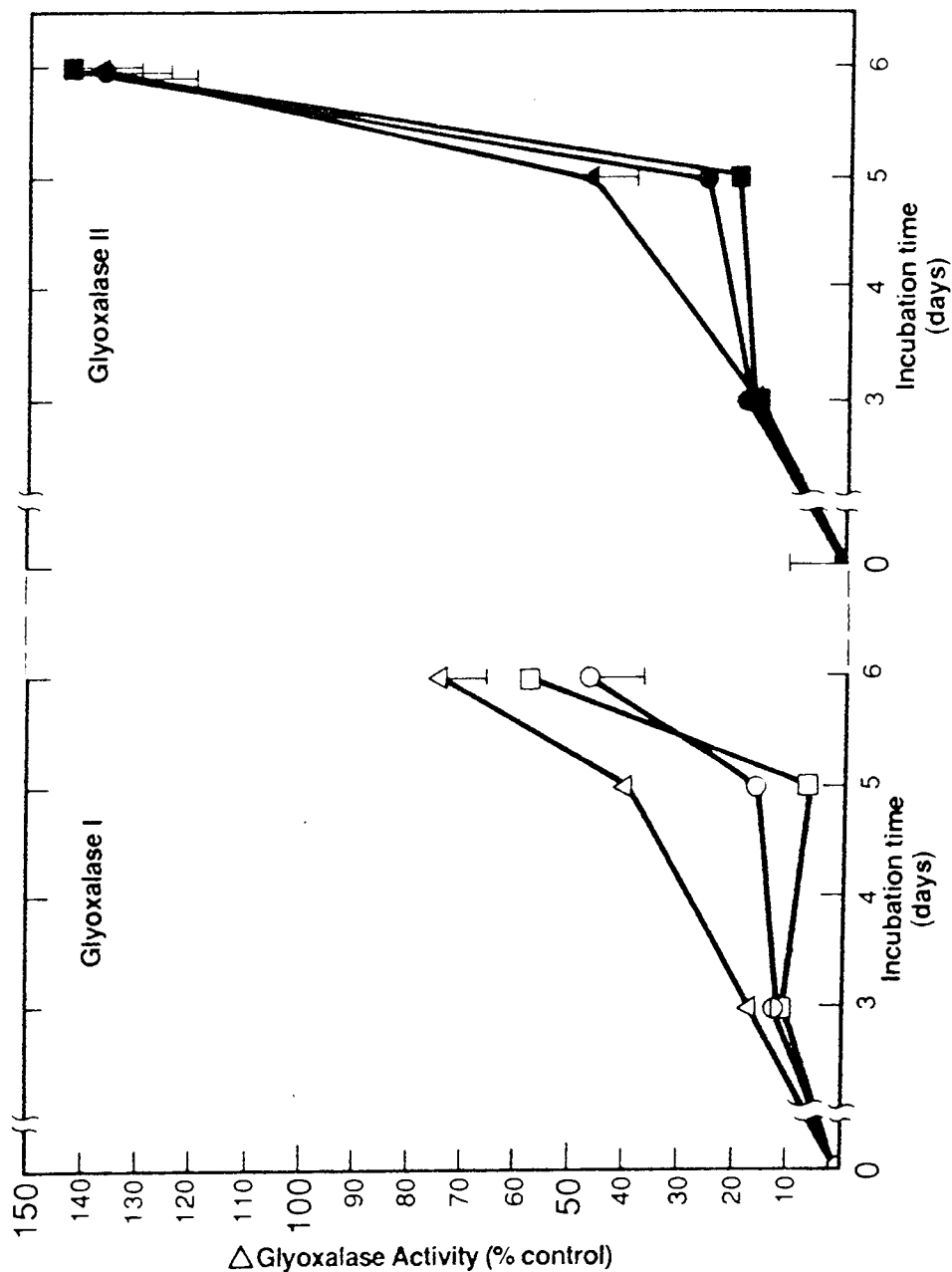
Figure 10. The Glyoxalase I and Glyoxalase II Activities in K562 Cells Treated with CCRG 81045. Key : (△ ▲) 26.0 μM CCRG 81045, (○ ●) 51.5 μM CCRG 81045 and (□ ■) 77.0 μM CCRG 81045. Closed symbols - glyoxalase I, open symbols - glyoxalase II. Data is the mean ± standard deviation of three independent experiments. The activities of the glyoxalase enzymes was determined as described in the Materials and Methods.

glyoxalase II falls sharply as differentiation ensues
(Table 12).

When a crude preparation of glyoxalase I and
glyoxalase II, prepared from the cytosol of K562 cells,
was incubated with either 185 mM NMF or 103 μM CCRG
81045 for 10 minutes, there was no significant change in
the activities of either enzyme (Tables 6 and 7). This
excludes the possibility that the presence of NMF or
CCRG 81045 in the assay systems for glyoxalase
activities produced the observed inhibitory and
stimulatory effects. The changes in glyoxalase
activities during the 6 day incubation period with both
NMF and CCRG 81045 may be due to changes in
enzyme synthesis, metabolic regulatory modification, or
a longer term interaction between NMF and CCRG 81045
with the glyoxalase enzymes in K562 cells.

The effect of overnight storage on the activity of
glyoxalase I and glyoxalase II in the K562 cell extract
is shown in Table 9. There was no significant decrease
in the activity of either enzyme. Therefore, glyoxalase
I and glyoxalase II can be regarded as stable over the
time period used for the experiment.

4.1.3 The Substrate - Reaction Velocity Relationship for Glyoxalase Enzymes During the Differentiation of Human Promyelocytic Leukaemia Cells to Neutrophils.

Incubation of HL60 promyelocytic leukaemia cells
with NMF induced their differentiation to
neutrophil-like cells with an accompanying decrease in

Table 12. The Glyoxalase I/Glyoxalase II Activity Ratio (A_{GI}/A_{GII}) in CCRG 81045 Treated K562 Erythroleukaemia Cells in Culture

Culture Time (Days)	[CCRG81045] (µM)	A_{GI}/A_{GII} (unit ratio)	$\Delta\, A_{GI}/A_{GII}$ (% Control)
3	Control	29.0 ± 2.8	----------
	26.0	29.0 ± 1.3^{a}	0 ± 14
	51.5	27.5 ± 2.1^{a}	$- 5 \pm 17$
	77.0	27.5 ± 0.8^{a}	$- 5 \pm 12$
5	Control	34.2 ± 1.3	----------
	26.0	41.0 ± 3.6^{a}	$+20 \pm 13$
	51.5	31.6 ± 1.0^{a}	$- 8 \pm 7$
	77.0	30.9 ± 1.6^{a}	-10 ± 9
6	Control	30.4 ± 2.7	----------
	26.0	22.3 ± 0.8^{b}	-27 ± 12
	51.5	18.8 ± 0.8^{c}	-38 ± 12
	77.0	19.7 ± 1.7^{c}	-35 ± 15

The glyoxalase activity ratio, A_{GI}/A_{GII}, was calculated as the ratio of the activity of glyoxalase I/glyoxalase II, where the glyoxalase activities are expressed in units/10^{6} cells as defined in the Materials and Methods section. Significance was determined using the Student's two-tailed "t" test : [a] - P > 0.10; [b] - 0.10 > P < 0.05; [c] - P < 0.05

the activity of glyoxalase I and an increase in the activity of glyoxalase II (Section 4.1.1). When the experimental activity data was analysed by the Lineweaver - Burke method, the substrate concentration (S) – reaction velocity (V) profiles for both glyoxalase I (Figure 11) and glyoxalase II (Figure 12) indicate that the changes in glyoxalase activity, during differentiation, were reflected by changes in the parameters of the apparent Michaelis-Menten kinetics. The apparent Michaelis constant, Km, and the relative maximum velocities, V_{max}, calculated from the experimental data are presented in Table 13. During differentiation, the apparent Km of both glyoxalase I and glyoxalase II remained constant, within the limits of experimental error. However, there was a 60% decrease in the apparent V_{max} of the glyoxalase I-catalysed reaction and an approximately 140% increase in the apparent V_{max} for the glyoxalase II-catalysed reaction, relative to the control HL60 promyelocyte values. These changes in the kinetic parameters of glyoxalase I and glyoxalase II indicate that the activity of glyoxalase I was non-competitively decreased during differentiation and the activity of glyoxalase II was non-competitively increased during differentiation.
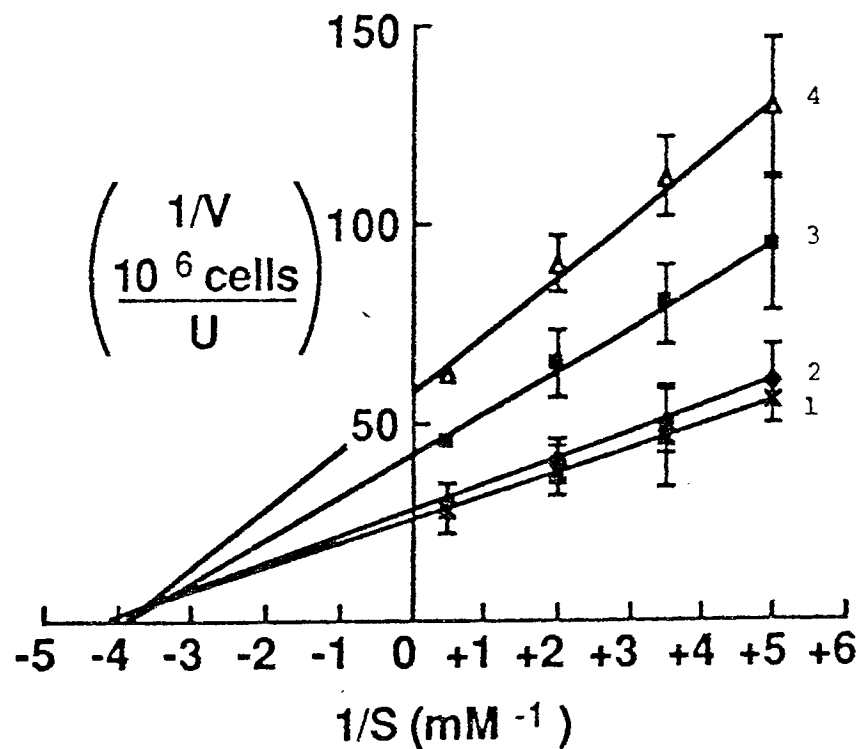
Figure 11. **The Substrate Concentration - Reaction Velocity Relationship for the Glyoxalase I-Catalysed Reaction During the Differentiation of HL60 Cells to Neutrophils.** Symbols : 1 - 0% (Control), 2 - 35%, 3 - 50% and 4 - 80% differentiation. The activity of glyoxalase I was determined as described in the Materials and Methods section. Data is the mean ± standard deviation for six independent experiments.
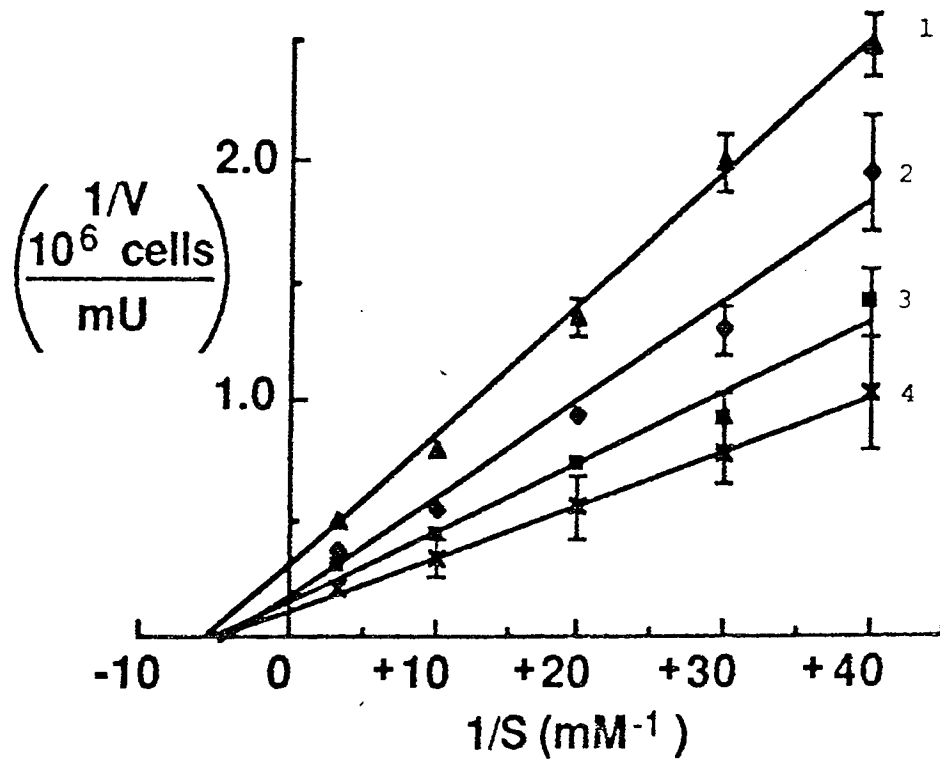
Figure 12. The Substrate Concentration - Reaction
Velocity Relationship for the Glyoxalase II-Catalysed
Reaction During the Differentiation of HL60 Cells to
Neutrophils. Symbols : 1 - 0% (Control), 2 - 35%,
3 - 50% and 4 - 80% differentiation. The activity of
glyoxalase II was determined as described in the
Materials and Methods section. Data is the mean ±
standard deviation for six independent experiments.

Table 13. The Apparent Michaelis-Menten Kinetic Constants for the Glyoxalase Enzymes During the Differentiation of HL60 Promyelocytes to Neutrophils by N-Methylformamide

NMF Treatment (mM)	Percent Different. (%)	Glyoxalase I		Glyoxalase II	
		App. Km (µM)	Rel. Vmax (%)	App. Km (µM)	Rel. Vmax (%)
0	0	241± 9	100± 9	211±55	100±11
130	34±5	250±12	91±18	205±32	137±17
150	50±5	256±22	60±14	208±54	185±33
185	80±5	260±34	43±12	216±34	236±50

4.2 Changes in Glyoxalase Activity During the Growth Arrest of GM892 Lymphoblastoid and Raji Burkitt's Lymphoma Cells In Vitro.

4.2.1 Growth Arrest of GM892 Lymphoblastoid Cells.

When cultures of GM892 cells, grown in media containing 10% foetal calf serum, were incubated in media containing decreased concentrations (5 - 0.1%) of foetal calf serum, there was a decrease in the rate of cell proliferation, as judged by a decrease in the rate of uptake of tritiated thymidine. The growth arrest of GM892 cells was accompanied by changes in the cellular glyoxalase activities : there was a decrease in the activity of glyoxalase I and an increase in the activity of glyoxalase II with progressive growth arrest (Figure 13). There was an approximately linear correlation between the activity of both enzymes and thymidine incorporation (correlation coefficients : glyoxalase I = 0.91 ; glyoxalase II = - 0.98). An approximately 100% increase in the rate of cell proliferation produced an approximately 200% increase in the activity of glyoxalase I and an approximately 20% decrease in the activity of glyoxalase II. Growth-arrested GM892 cells had a relatively low activity of glyoxalase I and a relatively high activity of glyoxalase II, whereas rapidly proliferating GM892 cells had a relatively high activity of glyoxalase I and a relatively low glyoxalase II activity.
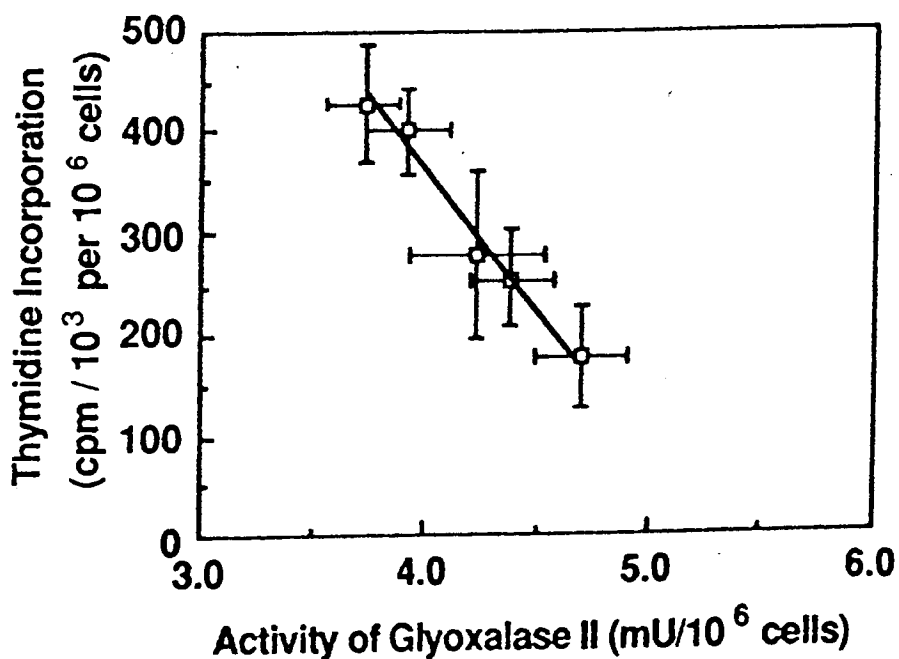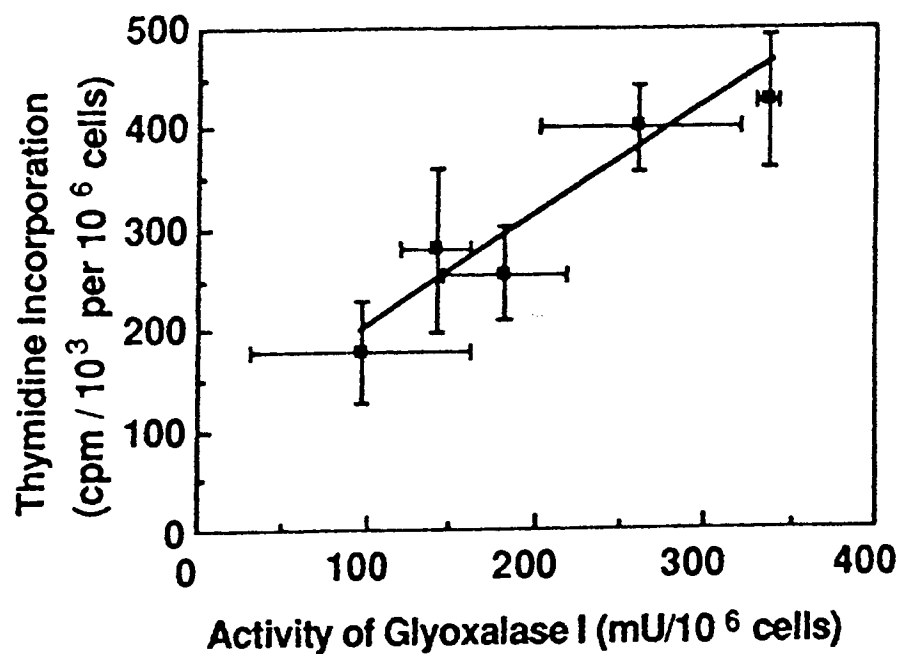
Figure 13. The Effect of Proliferation Rate on the Activity of Glyoxalase Enzymes in GM892 Cells. GM892 cells were incubated for two days in media containing 5%, 2%, 0.5% and 0.1% foetal calf serum. ^{3}H-Thymidine incorporation and the activity of glyoxalase I and glyoxalase II were measured as described in the Materials and Methods section.

4.2.2 Growth Arrest of Raji Burkitt's Lymphoma Cells.

Serum-deprivation of Raji cells produced a decrease in the cell proliferation status, as judged by the decrease in the rate of tritiated thymidine uptake. The activities of both glyoxalase I and glyoxalase II increased when Raji cells were growth-arrested (Figure 14). Therefore, growth-arrested Raji cells had relatively high activities of glyoxalase I and glyoxalase II, whereas, rapidly proliferating Raji cells had relatively low activities of glyoxalase I and glyoxalase II. Unlike GM892 cells, there was no linear correlation between the rate of proliferation and the glyoxalase activities in Raji cells. There does, however, appear to be a reciprocal (hyperbolic) relationship between glyoxalase activity and rate of cell proliferation in growth arrested Raji cells (Figure 14).

4.3 S-D-Lactoylglutathione and the Cell-Free Assembly of Microtubules.

4.3.1 The Potentiation of Microtubule Assembly by S-D-Lactoylglutathione.

When microtubular protein (1 mg/ml) was incubated in microtubule assembly buffer with 20 μM GTP at 37°C, the assembly of microtubules was initiated. The resulting increase in turbidity, due to the formation of microtubular fibres of greater than 1 μm in length in suspension, may be conveniently assessed by following
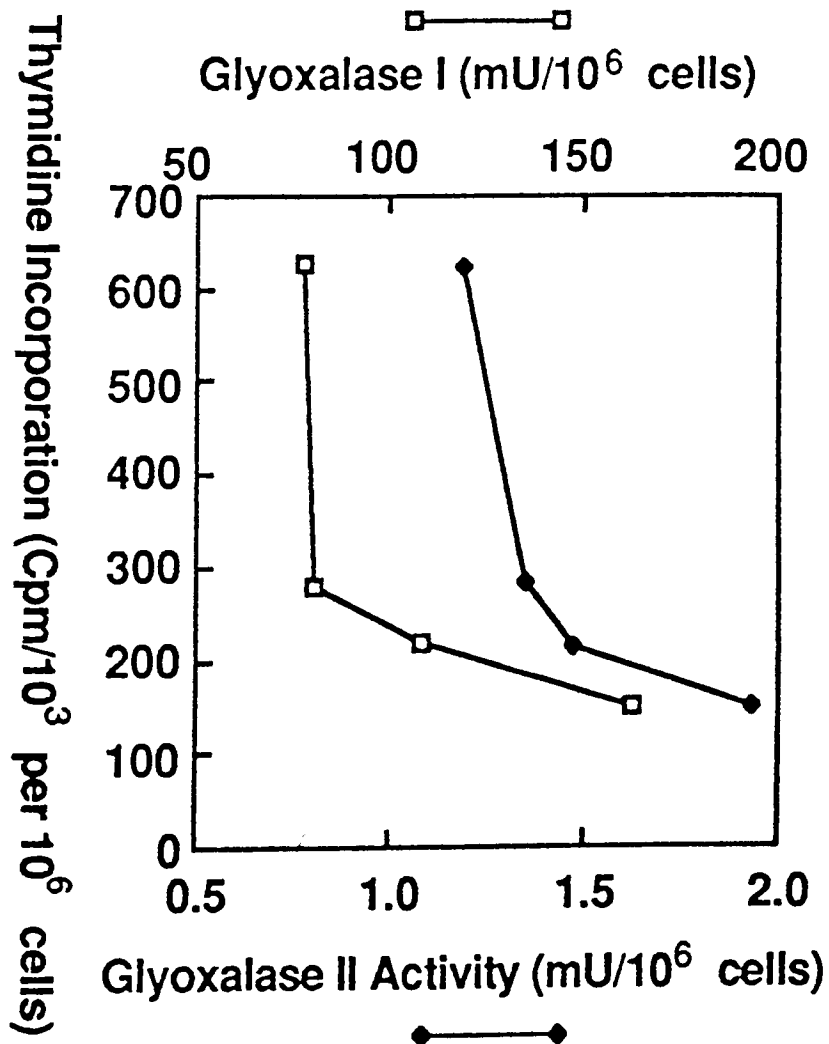
Figure 14. The Effect of Proliferation Rate on the
Activity of Glyoxalase Enzymes in Raji Cells. Raji
cells were incubated for two days in media containing
5%, 2%, 0.5% and 0.1% foetal calf serum. ^{3}H-Thymidine
incorporation and the activity of glyoxalase I and
glyoxalase II were measured as described in the
Materials and Methods section.

the absorbance at 350 nm. For the initial 1 - 2 minutes of the incubation, there was no increase in absorbance (Figure 15, curve d). This reflects the lag period during which nucleation centres for microtubule assembly were formed and microtubular assembly begins (205). After the nucleation lag phase, there was a rapid increase in absorbance over the next 10 minutes when the microtubules grow out from the nucleation centres. The light scattering measurement of the assembly of microtubules is responsive to microtubules greater than 1 μm long increasing in length (205). Therefore, this rapid change in absorbance reflects nucleating microtubules growing from 1 μm up to a typical length of 4 - 5 μm (206). During the following 50 minutes, there was a slow decrease in absorbance at 350 nm, representing a slow disassembly of the microtubules formed. This is thought to be due to phosphorylation of microtubule protein (207,208). The GTP-promoted cell-free assembled microtubules may be rapidly disassembled by treatment with 1.05 mM calcium chloride, 1 mg/ml of the anti-tubulin nocodazole, or by incubation at 0°C on ice. All these features of cell-free microtubule assembly have been well-documented (172,209).

When 20 - 1000 μM S-D-lactoylglutathione was included in the incubation, there was a dose-related decrease in the nucleation lag phase and an increase in the maximum absorbance (Figure 15, curves a, b and c).
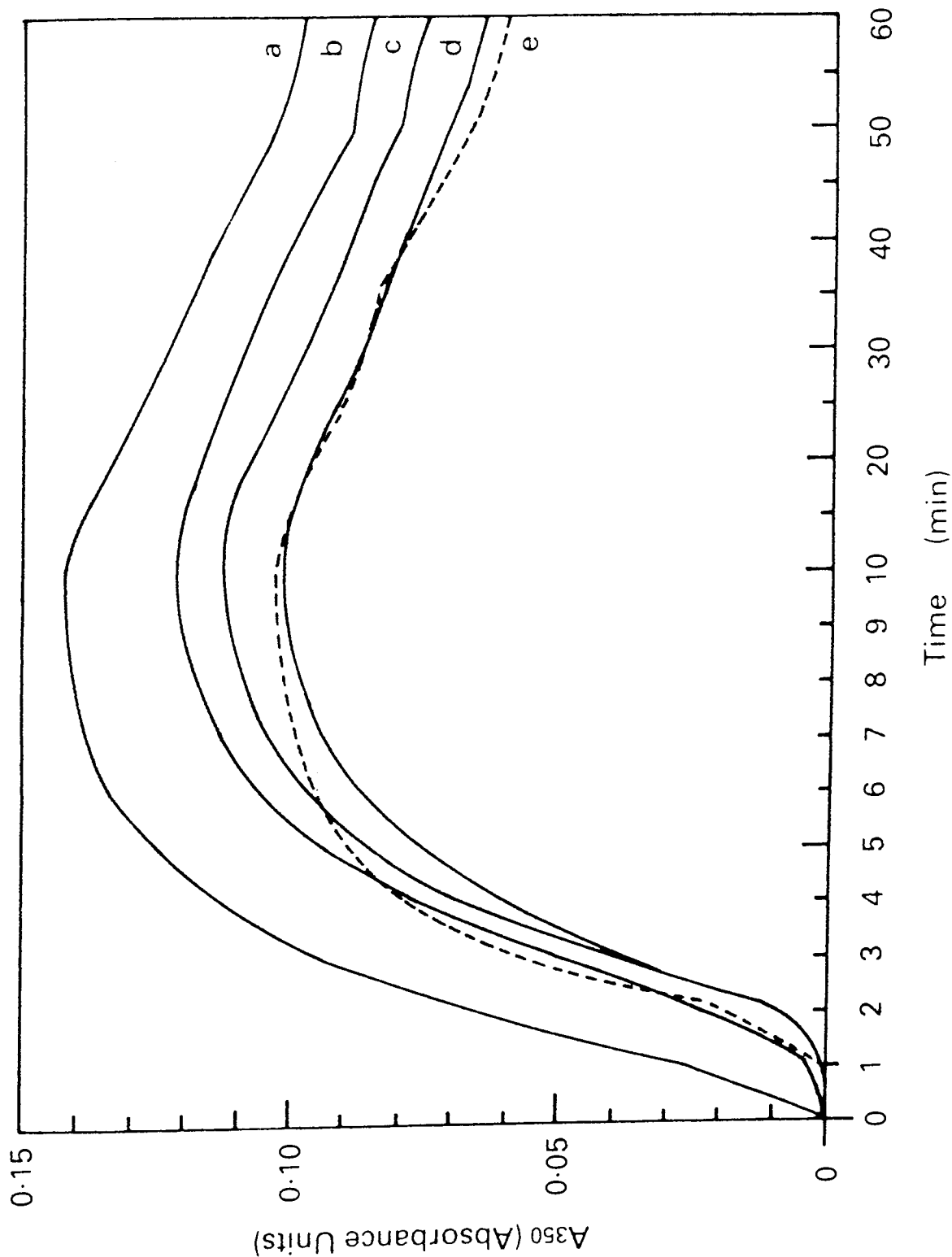
Figure 15. The Effect of S-D-Lactoylglutathione on the Assembly of Microtubules. Microtubules were assembled in the presence of increasing concentrations of S-D-lactoylglutathione as described in the Materials and Methods section. Key : d - 0 μM (control), c - 20μM, b - 100 μM, a - 1000 μM S-D-lactoylglutathione. Dashed line - 500 μM S-D-lactoylglutathione and 1 unit glyoxalase II

These effects were inhibited by glyoxalase II (Figure 15, dashed line). S-D-Lactoylglutathione did not stimulate the assembly of microtubules in the absence of GTP. Therefore, S-D-lactoylglutathione potentiated the GTP-promoted assembly of microtubules. The slow phosphorylation-associated disassembly of microtubules was unaffected by S-D-lactoylglutathione. A plot of maximum absorbance at 350 nm versus the concentration of S-D-lactoylglutathione suggests that, as a result of the potentiation of microtubule assembly by S-D-lactoylglutathione, the assembly of tubulin approaches a maximum, saturation level at high S-D-lactoylglutathione concentrations (Figure 16).

Microtubules assembled in the presence of S-D-lactoylglutathione were disassembled by treatment with 1.05 mM calcium chloride, 0.1 mg/ml nocodazole and by incubation on ice, at an identical rate to microtubules assembled in the absence of S-D-lactoylglutathione.

4.3.2 Consumption of S-D-Lactoylglutathione on Assembly of Microtubules.

When microtubular protein was assembled in the presence of 1 mM S-D-lactoylglutathione, there was a significant decrease in the S-D-lactoylglutathione concentration in the solution after assembly ($P < 0.001$ - Table 14). During assembly, 0.39 μmoles of S-D-lactoylglutathione were consumed. This indicates that approximately 20 nmoles of S-D-lactoylglutathione
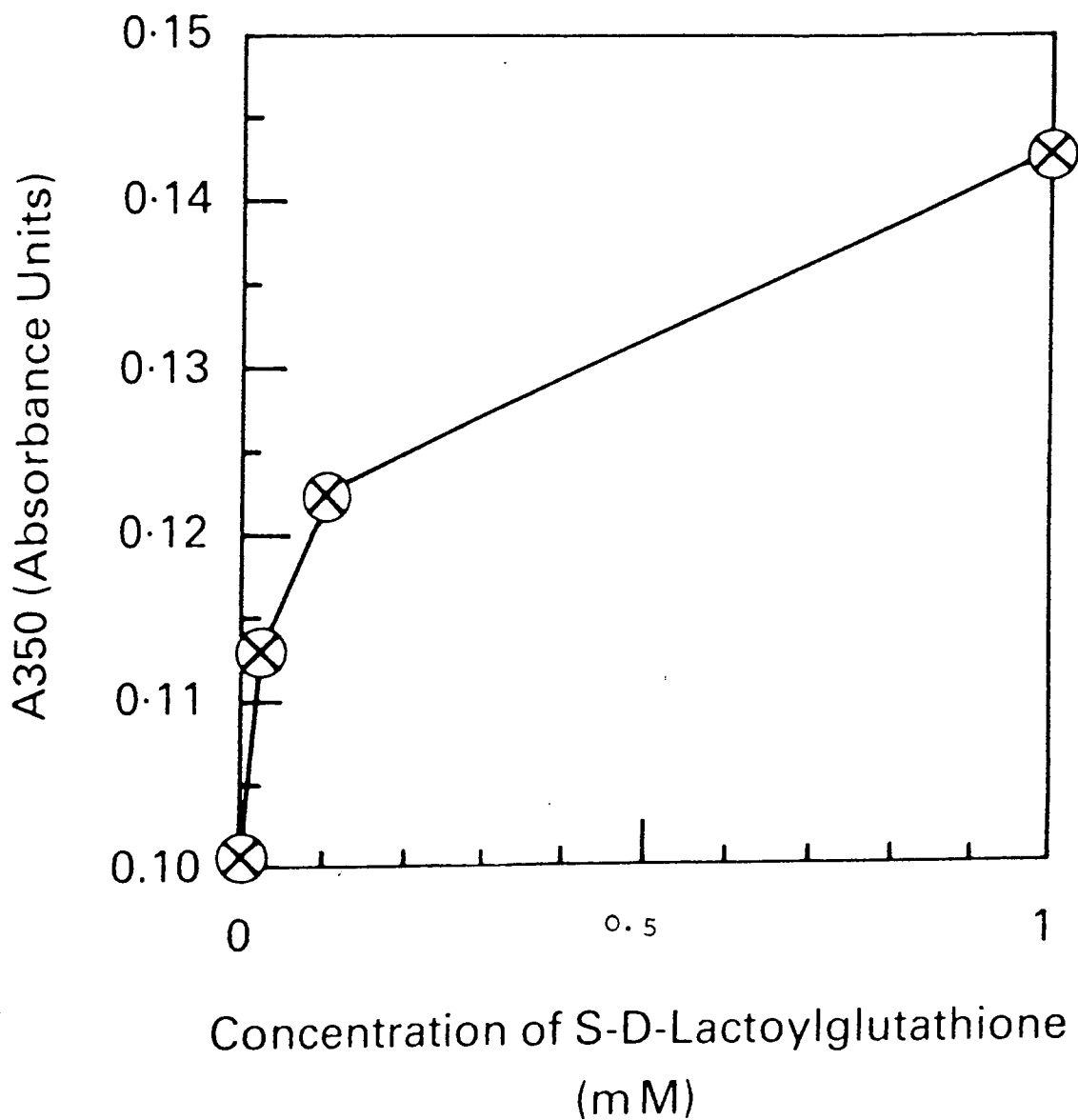
Figure 16. The Effect of the Concentration of
S-D-Lactoylglutathione on Microtubule Assembly.
Microtubule assembly was measured as described in the
Materials and Methods section. The concentrations of
S-D-lactoylglutathione in the incubation mixture were 0,
20, 100 and 1000 µM.

Table 14. The Consumption of S-D-Lactoylglutathione During Tubulin Assembly

Incubation	S-D-lactoylglutathione consumed (μmoles)	Microtubule Assembly
Tubulin, GTP S-D-lactoylglutathione	0.39 ± 0.01	+
Tubulin, S-D-lactoylglutathione	0.00	−
GTP, S-D-lactoylglutathione	0.02 ± 0.01	−

The initial concentration of S-D-lactoylglutathione was 1 mM and the initial tubulin concentration was 20 μM. Samples were incubated for 1 hour at 37°C. S-D-Lactoylglutathione concentration was determined by the decrease in absorbance on the addition of glyoxalase II. Data is the mean $\pm$ standard deviation of 3 determinations.

were consumed per nmole of tubulin during assembly. If
either microtubular protein or GTP was absent, there was
no loss of S-D-lactoylglutathione from the solution.
This indicates that a significant decrease in the
concentration of S-D-lactoylglutathione only occurs
under conditions necessary for the assembly of
microtubules.

4.4. The Red Blood Cell Glyoxalase System During Hyperglycaemia In Vivo.

4.4.1 Glyoxalase Activities.

The activities of glyoxalase I and glyoxalase II
were examined in red blood cells from 45 normal healthy
control individuals and from 85 patients with Diabetes
Mellitus (40 with retinopathy and 45 uncomplicated).
The diabetic patients were classified as either
insulin-dependent (Type I) or non-insulin-dependent
(Type II) diabetics. The results are presented as
frequency diagrams in Figures 17 and 18 (the thickened
envelope in each diagram represents the distribution of
glyoxalase activities in the controls). The statistical
analysis of the data is presented in Tables 15 and 16.

The distribution of the activity of glyoxalase I in
the red blood cells from diabetic patients and from
normal healthy controls was very similar (Figure 17).
Inspection of Figure 17 shows that, although the
frequency distributions for glyoxalase I in the red
blood cells from diabetics with and without
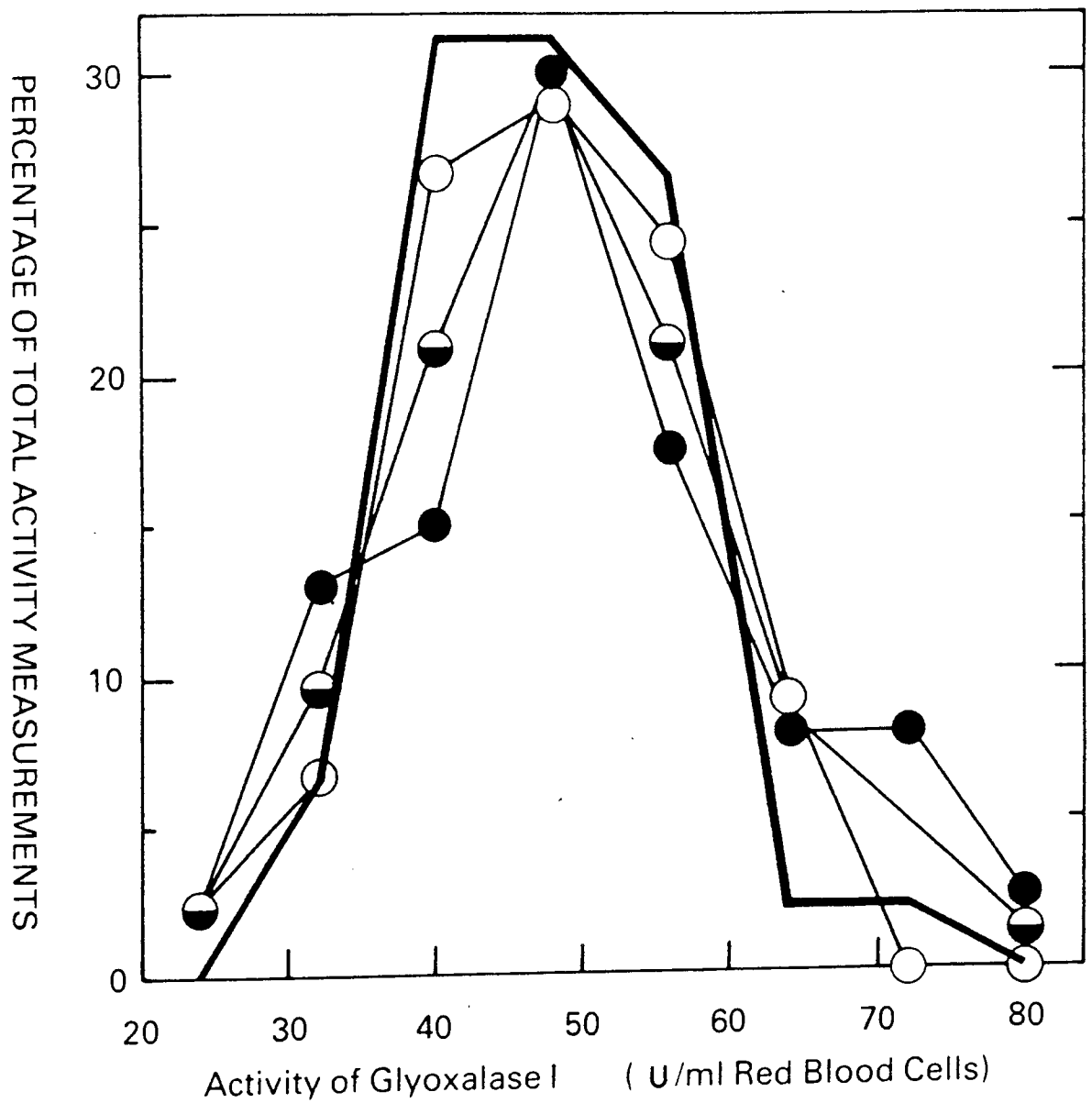
Figure 17. Frequency Distribution Curves for the
Glyoxalase I Activity of Red Blood Cells. Key :
thickened line envelope - normal healthy controls,
(-O-O-) - diabetic patients without complications,
(-●-●-) - diabetic patients with complications, (-◐-◐-)
- mean of all diabetic patients.

Figure 18. Frequency Distribution Curves for the Glyoxalase II Activity of Red Blood Cells. Key : thickened line envelope - normal healthy controls, (-O-O-) - diabetic patients without complications, (-●-●-) - diabetic patients with complications, (-◐-◐-) - mean of all diabetic patients. The mean activity for diabetic patients without complications > the mean activity for patients with complications (P < 0.05).

Table 15 . The Activity of Glyoxalase I and Glyoxalase
             II in Control and Diabetic Patients

Subjects (n)	Complications (+ or -)	Glyoxalase I (U/mlRBC)	Glyoxalase II (U/mlRBC)
Control (45)		47.8 ± 8.2	19.1 ± 4.0
All Diabetics (85)	±	48.7 ± 11.3	19.5 ± 4.0
Type I Diabetics (36)	±	48.0 ± 11.2	20.6 ± 4.0
Type II Diabetics (44)	±	49.3 ± 11.6	18.8 ± 4.1
All Diabetics (45)	-	47.6 ± 9.3	20.2 ± 3.7
All Diabetics (40)	+	49.9 ± 13.4	18.7 ± 4.5
Type I Diabetics (19)	-	45.0 ± 10.8	21.9 ± 3.2
Type I Diabetics (17)	+	51.4 ± 11.0	19.2 ± 4.4
Type II Diabetics (21)	-	48.9 ± 7.3	19.2 ± 3.7
Type II Diabetics (23)	+	49.7 ± 14.7	18.5 ± 4.6

Glyoxalase I and Glyoxalase II activity was measured as
described in the Materials and Methods section and
expressed as units/ml packed red blood cells. Data is
the mean ± standard deviation from n individuals.

Table 16.   Statistical Analysis of the Data from the
                        Diabetic Study

Comparison Groups	Data	P Value
Controls : All Diabetics	Glyoxalase I Activity Glyoxalase II Activity	>0.1 >0.1

There is no significant difference in the red blood cell
glyoxalase activities in control and diabetic subjects

All Diabetics(+)	All :Diabetics(-)	Glyoxalase II Activity Duration of Diabetes	<0.05 <0.001

Red blood cell glyoxalase II activity was significantly
lower in diabetic patients with complications.

Type I Diabetics(-)	Type I :Diabetics(+)	Glyoxalase I Activity Glyoxalase II Activity Duration of Diabetes	<0.05 <0.05 <0.001

Red blood cell glyoxalase I activity was significantly
higher and glyoxalase II activity lower in Type I
diabetics with complications.

Type II Diabetics(-)	Type II :Diabetics(+)	Glyoxalase I Activity Glyoxalase II Activity Duration of Diabetes	>0.1 >0.1 <0.01

There was no significant difference in the red blood
cell glyoxalase I or glyoxalase II activities in
diabetics with or without complications.

Significance was assessed using the Student's one-tailed
"t" test. Actual values for the different groups are
given in Table 17 (age of subjects and duration of
diabetes) and Table 15 (activity of glyoxalase I and
glyoxalase II). The presence or absence of
complications is indicated by + or -.

complications diverged at several points across the glyoxalase activity range, they converged and crossed at the next data point. This indicates that there was no consistent difference between the activity of glyoxalase I in red cells from diabetic patients with and without complications.

The statistical analysis of the glyoxalase I activity data (Table 16) confirms the observations from Figure 17. The activity of glyoxalase I in the red blood cells from diabetic patients was not significantly different from the glyoxalase I activity in the red cells from normal healthy controls. In addition, there was no significant difference between the glyoxalase I activity in the red blood cells of diabetic patients with complications, and the glyoxalase I activity in the red blood cells of diabetic patients without complications.

The frequency distributions for the glyoxalase II activities in red blood cells from diabetic patients and normal controls are similar (Figure 18). The curve for diabetic patients has a higher maximum frequency than the curve for the controls. There was a consistent difference in the frequency distribution curves of glyoxalase II activity in red blood cells from diabetics with and without complications. At relatively low glyoxalase II activities, the frequency distribution curve for patients without complications was below the curve for patients with complications. At relatively

high glyoxalase II activities, the reverse was true -
the frequency distribution curve for patients without
complications was higher than the curve for patients
with complications. This data suggests that the
frequency distribution curve for diabetic patients
without complications is skewed to relatively high
activities of glyoxalase II, and the frequency
distribution curve for diabetic patients with
complications is skewed to relatively low activities of
glyoxalase II.

The statistical analysis of the glyoxalase II
activity data (Tables 15 and 16) confirms : (i) that
there was no significant difference in the activity of
glyoxalase II in the red blood cells from diabetic
patients and controls and (ii)´ that the activity of
glyoxalase II in the red blood cells from diabetic
patients with complications was significantly lower than
the glyoxalase II activity in the red blood cells from
patients without complications (P < 0.05).

Taking the non-insulin-dependent diabetic patients
as a group, the statistical analysis revealed that there
was no significant difference between the red blood cell
glyoxalase activities in diabetic patients with and
without complications (Tables 15 and 16). The
significant difference that exists between the
glyoxalase activities in the red blood cells from
patients with and without complications was located in
the insulin-dependent diabetic subgroup. If the

insulin-dependent diabetic patients are considered alone, the patients without complications had a significantly lower red blood cell glyoxalase I activity and a significantly higher red blood cell glyoxalase II activity than did the patients with complications (Tables 15 and 16).

The frequency distributions for the activities of glyoxalase I and glyoxalase II in the red blood cells from insulin-dependent diabetic patients are presented in Figures 19 and 20 respectively. In the patients with complications (closed circles), the frequency distributions for the red blood cell glyoxalase activities are skewed to relatively high activities of glyoxalase I and relatively low activities of glyoxalase II. In contrast, in patients without complications (open circles), the frequency curves for the red blood cell glyoxalase activities are skewed to relatively low activities of glyoxalase I and relatively high activities of glyoxalase II.

Examination of Table 17 shows that, with both insulin-dependent and non-insulin-dependent patients, there was a significant bias in the age and duration of diabetes between the subgroups of patients with and without complications. To remove this bias, 14 pairs of patients from the non-insulin-dependent diabetes subgroup, one of each pair with and one without complications, were matched for age (criterion : age difference less than 10 years) and duration of diabetes
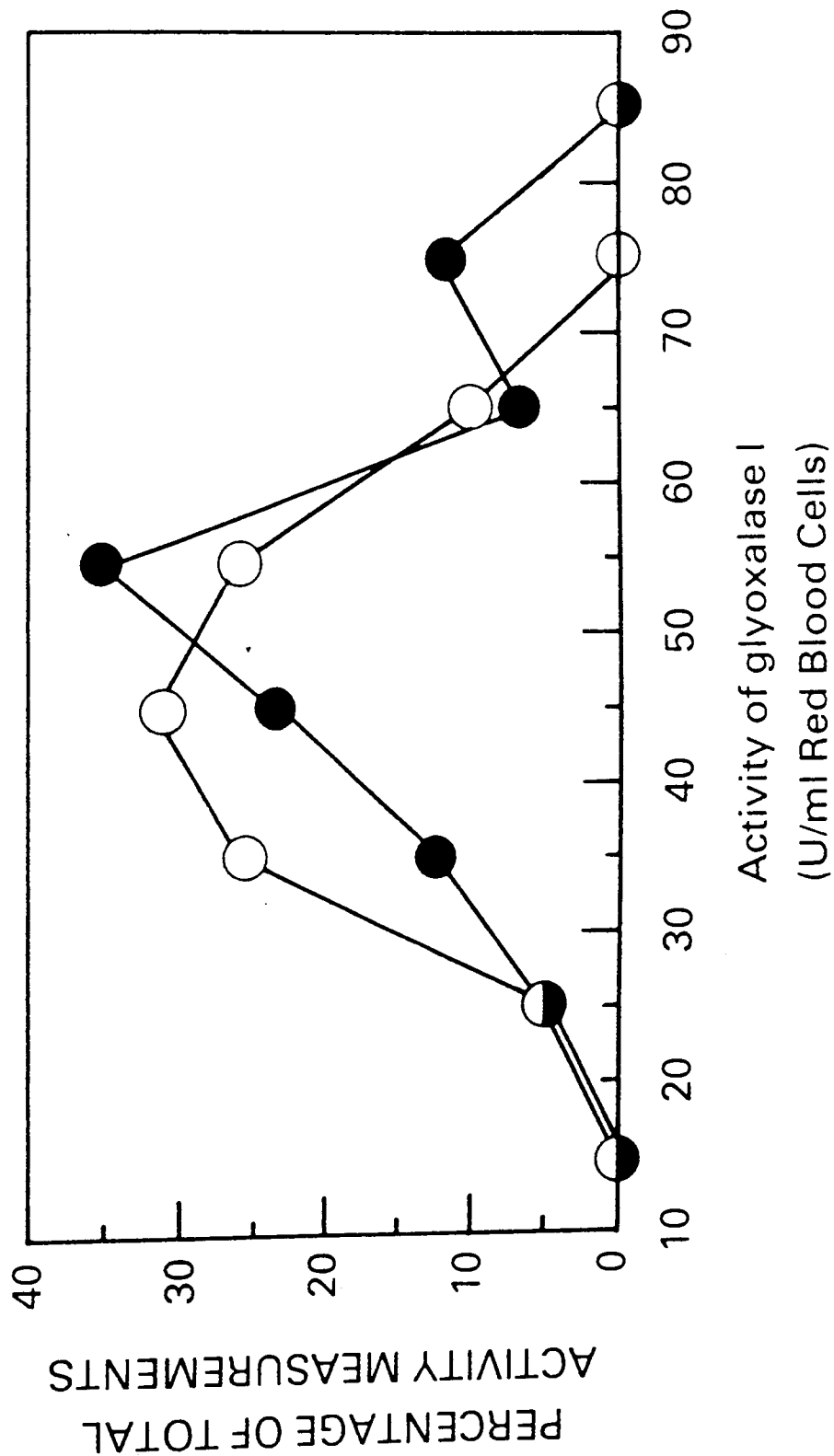
Figure 19. Frequency Distribution Curves of Glyoxalase I Activity in Red Blood Cells from Insulin-Dependent Diabetic Patients. Key : (-O-O-) - diabetic patients without complications, (-●-●-) - diabetic patients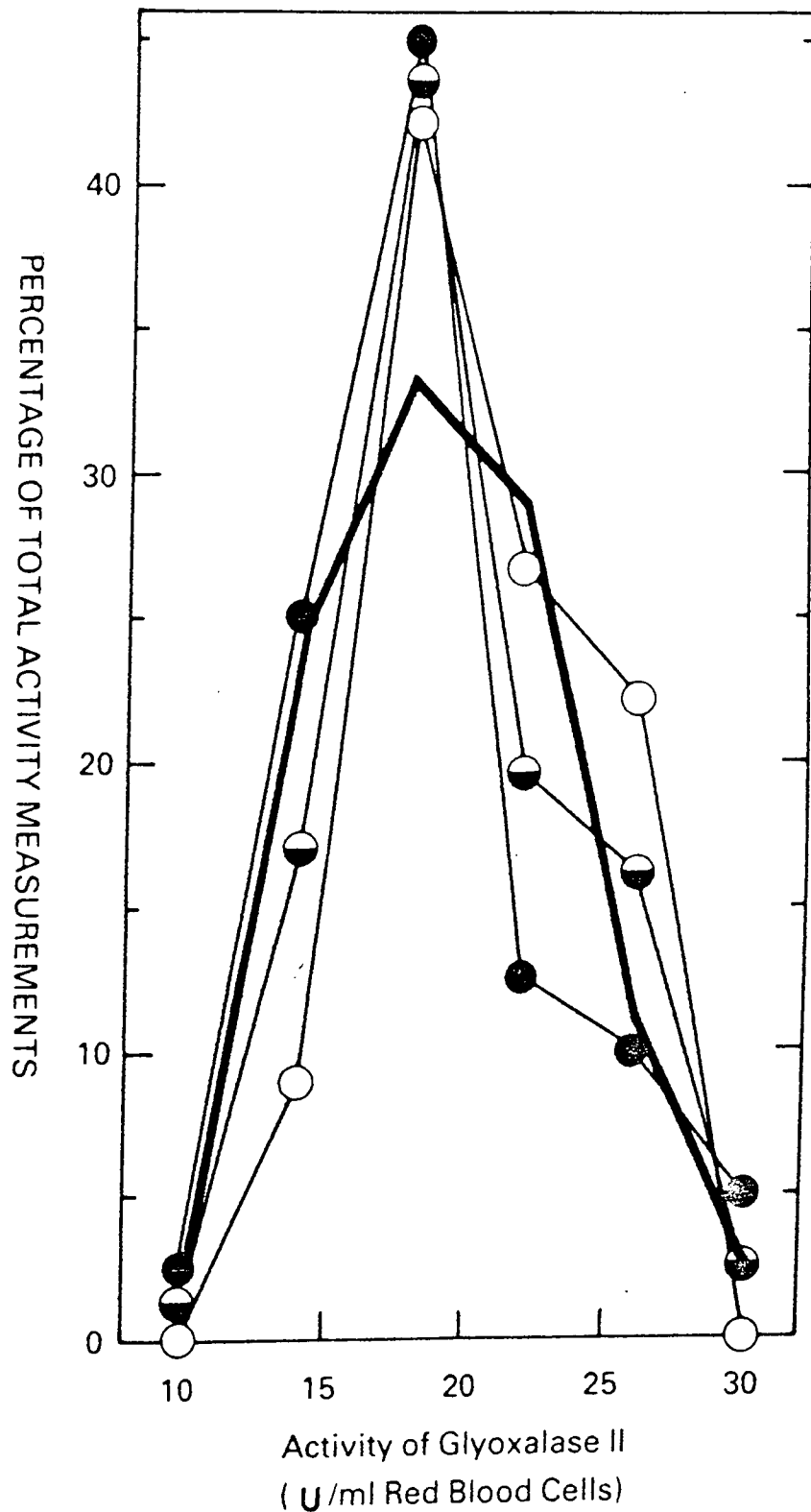 with complications. The mean activity for diabetic patients without complications < the mean activity for patients with complications (P < 0.05).
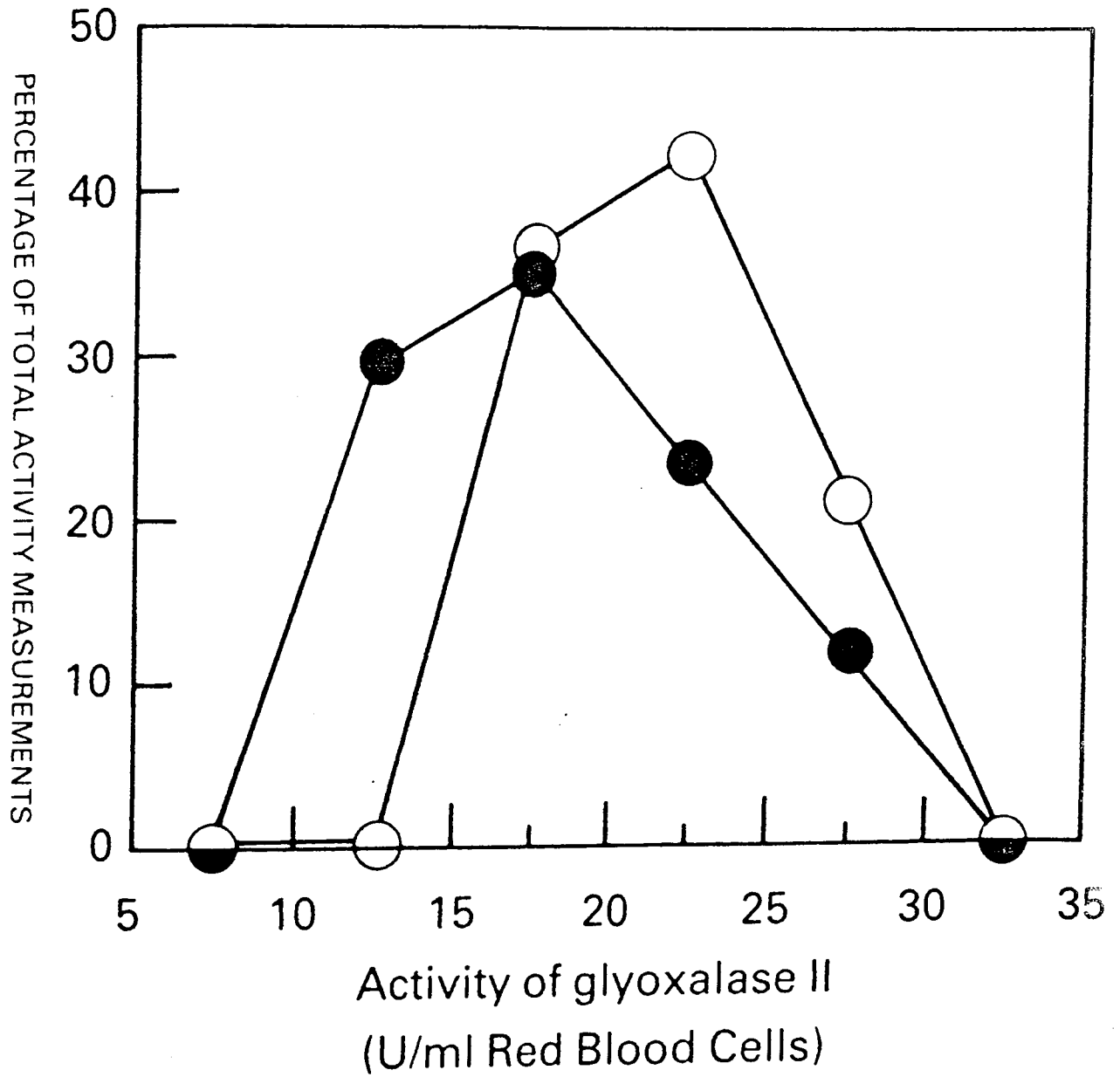
Figure 20. Frequency Distribution Curves of Glyoxalase
II Activity in Red Blood Cells from Insulin-Dependent
Diabetic Patients. Key : (-0-0-) - diabetic patients
without complications, (-●-●-) - diabetic patients with
complications. The mean activity for diabetic patients
without complications > the mean activity for patients
with complications (P < 0.05).

Table 17. The Age and Duration of Diabetes for Subjects in the Diabetes Study

Subjects (n)	Complications (+ or -)	Age (yrs)	Duration (yrs)
All Diabetics (85)	±	52.6 ± 17.5	10.8 ± 9.4
Type I Diabetics (36)	±	43.1 ± 17.4	15.2 ± 11.4
Type II Diabetics (44)	±	60.3 ± 13.7	7.3 ± 5.3
All Diabetics (45)	−	45.5 ± 19.4	6.9 ± 4.7
All Diabetics (40)	+	59.6 ± 12.2	14.8 ± 11.2
Type I Diabetics (19)	−	34.0 ± 16.9	8.8 ± 5.4
Type I Diabetics (17)	+	53.4 ± 11.6	22.4 ± 12.0
Type II Diabetics (21)	−	56.1 ± 15.1	5.2 ± 3.1
Type II Diabetics (23)	+	64.3 ± 10.6	9.2 ± 6.2

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(criterion : duration difference less than 2 years). The results are presented in Table 18. Even after age matching, there was still no significant difference between the activities of glyoxalase I and glyoxalase II in the patients with or without complications. It was more difficult to match the insulin-dependent diabetic patients for age and duration of diabetes. Using the criteria of age difference less than 10 years (except for one pair with a difference of 25 years) and duration difference less than 3 years, there were only 7 suitable pairs of patients with and without complications (the duration of diabetes was considered to be the most important criterion, but large age differences over 60 years were omitted). The results are presented in Table 19. The statistical analysis showed that the patients without complications had a significantly higher glyoxalase II activity ($P < 0.01$) and a significantly lower glyoxalase I activity ($P < 0.05$) than the patients with complications. Matching the insulin-dependent diabetics for age and duration of diabetes maintained and improved the significance of the differences between the red blood cell glyoxalase activities in patients with and without complications.

4.4.2 The Concentration of Methylglyoxal in Whole Human Blood.

The concentration of methylglyoxal in whole blood was determined in 17 normal healthy control individuals and 32 patients with insulin-dependent and

Table 18 . The Activity of the Glyoxalase Enzymes in
          Age-Matched Non-Insulin-Dependent Type II
          Diabetics With and Without Complications

	UNCOMPLICATED			COMPLICATED	
A(d)	Glyoxalase I	Glyoxalase II	A(d)	Glyoxalase I	Glyoxalase II
(yrs)	(U/mlRBC)	(U/mlRBC)	(yrs)	(U/mlRBC)	(U/mlRBC)
59(4)	63.7	21.1	57(4)	60.6	9.4
78(5)	45.6	26.8	72(5)	40.2	18.1
42(6)	58.1	16.6	70(7)	43.0	14.7
52(10)	41.2	25.1	56(10)	46.9	17.5
72(2)	44.9	18.2	78(3)	28.4	15.7
61(7)	50.3	19.0	57(7)	73.6	15.4
61(6)	56.4	23.7	58(6)	55.7	16.2
60(2)	44.1	18.2	61(2)	34.8	24.6
38(10	43.3	17.0	44(1)	65.9	25.7
74(10)	40.2	19.7	79(9)	54.5	17.1
65(1)	46.6	17.1	63(2)	68.5	16.3
74(10)	40.2	19.7	68(10)	53.6	19.1
59(3)	46.8	12.3	60(2)	47.8	21.2
59(8)	42.6	13.1	59(8)	33.2	18.9

Mean Activity ± S.D   Mean Activity ± S.D

Glyoxalase I : 47.40 ± 6.90   Glyoxalase I : 50.40 ± 13.3
Glyoxalase II: 19.10 ± 4.00   Glyoxalase II: 17.85 ± 3.90

Activity of glyoxalase I and glyoxalase II is expressed
as units/ml packed red blood cells as described in the
Materials and Methods section. A(d) - age of patient
(duration of diabetes). Statistical analysis performed
using a Students one-tailed "t" test : P > 0.1 for both
enzymes.

Table 19. The Activity of the Glyoxalase Enzymes in
         Age-Matched Insulin-Dependent Type I
         Diabetes With and Without Complications

	UNCOMPLICATED			COMPLICATED	
A(d)	Glyoxalase I	Glyoxalase II	A(d)	Glyoxalase I	Glyoxalase II
(yrs)	(U/mlRBC)	(U/mlRBC)	(yrs)	(U/mlRBC)	(U/mlRBC)
65(12)	52.8	21.4	58(10)	71.6	14.1
31(16)	50.7	23.4	56(16)	49.3	14.3
69(14)	45.1	19.3	51(13)	45.9	17.5
22(13)	40.1	18.1	24(12)	51.4	18.7
21(13)	32.2	26.3	57(14)	39.9	19.6
31(15)	37.1	21.4	38(13)	54.2	22.1
34(19)	39.6	24.0	54(22)	48.8	13.7

Mean Activity ± S.D   Mean Activity ± S.D

Glyoxalase I :42.51 ± 6.86   Glyoxalase I :51.58 ± 9.17
Glyoxalase II:21.99 ± 2.61   Glyoxalase II:17.14 ± 2.98

Activity of glyoxalase I and glyoxalase II is expressed
as units/ml packed red blood cells as described in the
Materials and Methods section. A(d) - age of patients
(duration of diabetes). Statistical analysis performed
using a Students one-tailed "t" test : Glyoxalase 1 - P
< 0.05; Glyoxalase II - P < 0.01.

non-insulin-dependent diabetes, with and without complications. The data is presented as frequency distribution diagrams in Figure 21, with a statistical analysis in Table 20. In diabetic patients, the distribution of whole blood methylglyoxal concentrations was skewed to the high concentration range, when compared with the concentrations in controls. The increase in the concentration of methylglyoxal in the blood of diabetic patients was statistically significant (P < 0.001). The control subjects had a mean whole blood concentration of methylglyoxal of 1.9 μM, while the concentration approximately doubled in the diabetic patients to 3.5 μM. This indicates that the red blood cell glyoxalase system experienced a substantial elevation in the concentration of methylglyoxal during hyperglycaemia.

4.4.3 The Concentration of S-D-Lactoylglutathione in Whole Human Blood.

The whole blood concentration of S-D-lactoylglutathione was assayed in 24 normal healthy control individuals and in 28 diabetic patients with insulin-dependent and non-insulin-dependent diabetes, with and without complications. The frequency distribution curves of the data are presented in Figure 22, with a statistical analysis in Table 20. In diabetic patients, the frequency distribution was skewed over to the high concentration range of S-D-lactoylglutathione, compared to the frequency
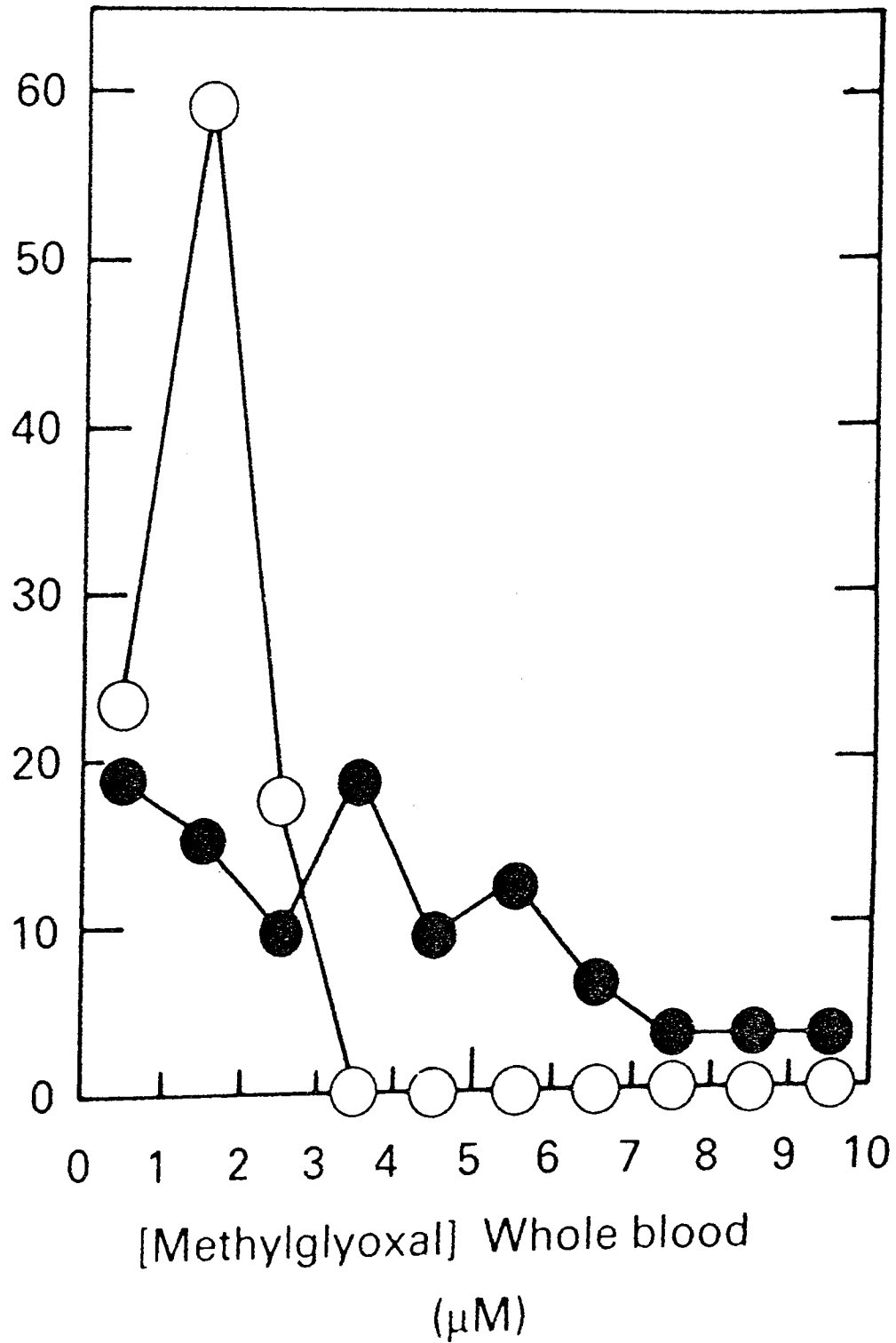
Figure 21. Frequency Distribution Curves of the
Concentration of Methylglyoxal in Whole Human Blood.
Key : (-●-●-) - diabetic patients, (-O-O-) - normal
healthy controls. The mean concentration in diabetic
patients > the mean concentration in controls
(P < 0.001).

Table 20. The Concentration of Glyoxalase Metabolites
in Whole Venous Human Blood

	Concentration (μM) (n)	Blood Glucose (mM) (n)
S-D-lactoylglutathione		
Controls[1]	12.4 ± 4.8(24)[a]	---------
Diabetics[1]	21.4 ± 9.3(28)[a]	9.9 ± 4.0(27)
Methylglyoxal		
Controls[2]	1.44 ± 0.51(17)[a]	---------
Diabetics[2]	3.59 ± 2.33(32)[a]	10.9 ± 4.3(30)

The concentration of S-D-lactoylglutathione and
methylglyoxal was determined as described in the
Materials and Methods section. Data is the mean ±
standard deviation of determinations from n individuals.
[1] - Diabetic group comprised 13 Type I and 15 Type II
patients. [2] - Diabetic group comprised 16 Type I and 16
Type II patients. Significance was determined using the
Student's two-tailed "t" test : [a] - P < 0.001
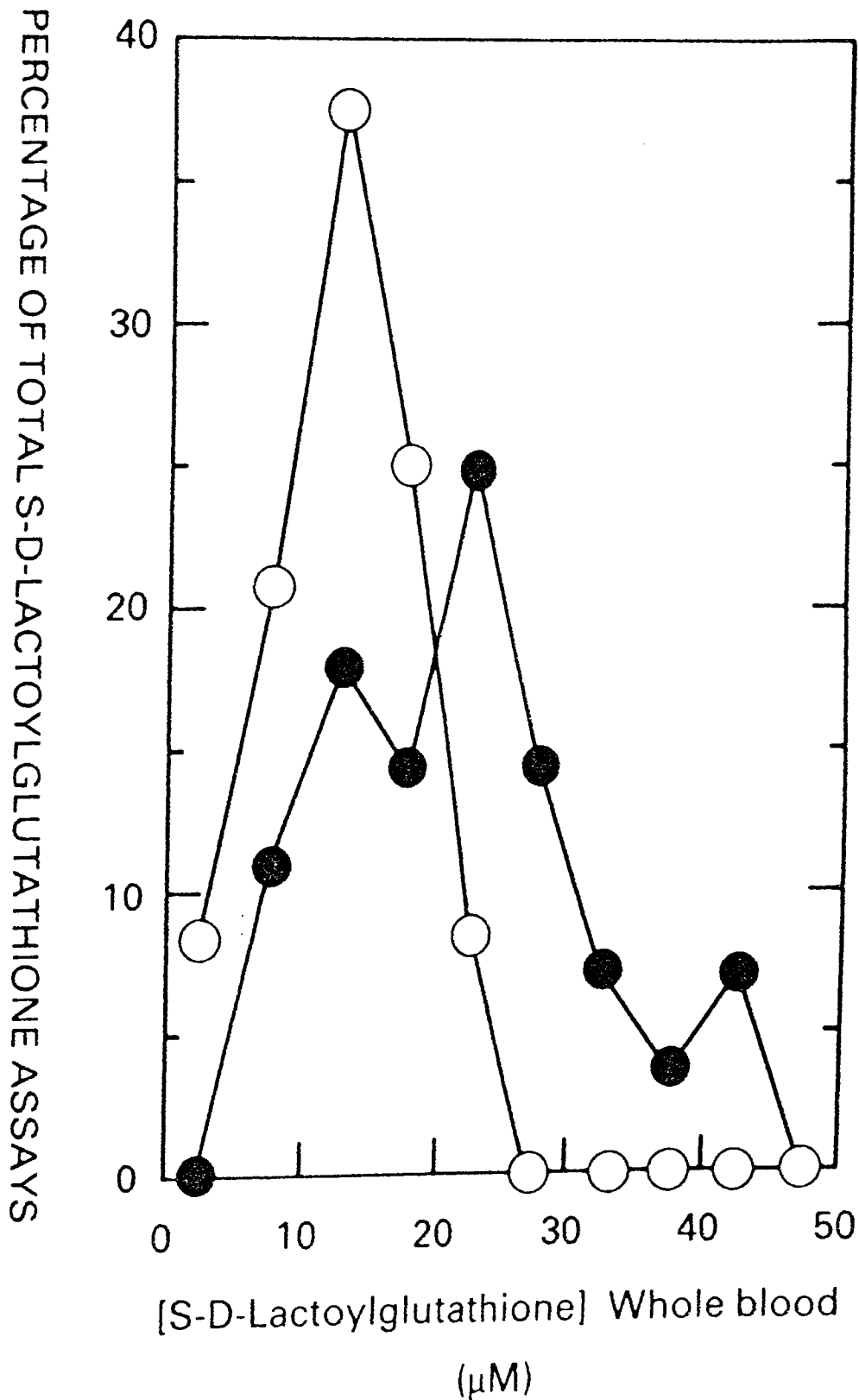
Figure 22. Frequency Distribution Curves of the
Concentration of S-D-Lactoylglutathione in Whole Human
Blood. Key : (-●-●-) - diabetic patients, (-O-O-) -
normal healthy controls. The mean concentration in
diabetic patients > the mean concentration in controls
(P < 0.001).

distribution of S-D-lactoylglutathione concentrations in the controls. The statistical analysis shows that the concentration of S-D-lactoylglutathione in the blood of diabetic patients was significantly increased when conpared to the controls ($P < 0.001$). The mean whole blood concentration of S-D-lactoylglutathione approximately doubled from 12.4 μM in the controls to 21.4 μM in the diabetic patients. This indicates that the red blood cell glyoxalase system experienced a substantial elevation in the concentration of S-D-lactoylglutathione during diabetic hyperglycaemia.

5. DISCUSSION.

5.1 The Glyoxalase Enigma.

The glyoxalase system appears to be present in the cytosol of cells of all life forms. It metabolises the α-oxoaldehyde methylglyoxal to D-lactic acid, via the intermediate S-D-lactoylglutathione. The widespread distribution of the glyoxalase system may suggest a fundamental role in biological metabolism; yet, the major product of the glyoxalase system is D-lactic acid which, although it may be incorporated into the glycolytic metabolism of bacteria, finds very little metabolic use in animals and is largely excreted in the urine (133,134). The ubiquitous occurrence of the glyoxalase system catalysing the formation of a product of minor metabolic importance is the biochemical paradox confronting those who seek to functionally characterise the glyoxalase system. This is the enigma of the glyoxalase system.

The metabolic significance of the glyoxalase metabolic pathway may be assessed by studying tissues which are deficient in one or more components of the glyoxalase system. Rapidly proliferating tumours and immature tissues are characteristically deficient in glyoxalase II activity (3,150,210). Studies of the modification of the glyoxalase system during the differentiation and growth arrest of human tumour cells in culture were major objectives of this work.

The clinical significance of the glyoxalase pathway may be assessed where a disease process gives rise to a modification of the glyoxalase system. A study of the red blood cell glyoxalase system in Diabetes Mellitus was undertaken since it was suspected (114,180) that there is an increase in the metabolic flux through the glyoxalase system during the hyperglycaemia associated with the development of clinical complications.

The results presented here provide further evidence towards the characterisation of the functional role of the glyoxalase system in biological metabolism.

5.2 Modification of the Glyoxalase System During the Differentiation of Human Leukaemia Cells In Vitro.

The human promyelocytic leukaemia HL60 and the human erythroleukaemia K562 cell lines in culture are convenient models for studying in vitro differentiation. The differentiation of the HL60 cell line to neutrophil-like cells can be induced by treatment with several compounds, including NMF (159) and the tetrazinone CCRG 81045. The K562 cell line undergoes transformation to haemoglobin-producing cells after treatment with CCRG 81045 (204), but not with NMF (202). This combination of cell lines and drugs therefore provides a practical test to see if an increase in the activity of glyoxalase II occurs concomitantly with the chemically-induced differentiation process, as is observed during physiological differentiation in embryo

maturation (150).

Optimum differentiation of HL60 cells to neutrophil-like cells occurs after a 4 day incubation with 180 mM NMF. The degree of differentiation is reduced if the cells are treated with 130 mM and 150 mM NMF, or with 180 mM NMF for shorter incubation periods (159). The activity of glyoxalase II increased steadily during the 6 day incubations with 130, 150 and 185 mM NMF (Figure 6). This is consistent with the observed increase in glyoxalase II activity during physiological differentiation. Treatment with CCRG 81045 produced a significant increase in the number of differentiated cells, accompanied by a marked increase in the activity of glyoxalase II (Figure 8). In HL60 cells, the increase in glyoxalase II activity was directly proportional to the number of differentiated cells present (Figure 7). This suggests that there is a direct correlation between differentiation and the increase in glyoxalase II activity.

The differentiation of K562 cells to erythrocyte-like cells, induced by CCRG 81045, is dose-dependent and does not occur until 3 days after drug administration (204). The activity of glyoxalase II in K562 cells treated with CCRG 81045 also increased after 3 days of incubation. Although NMF does not induce differentiation in K562 cells, there was an increase in the activity of glyoxalase II after the incubation of K562 cells with NMF (Figure 9).

Therefore, the glyoxalase II activity in K562 cells increased in both differentiation and during non-differentiation conditions.

The significance of the individual changes in the activity of glyoxalase I are more difficult to interpret. Since glyoxalase II is apparently the rate-determing enzyme in the glyoxalase system, changes in the activity of glyoxalase I may be of less metabolic significance than the changes in the activity of glyoxalase II. There is, however, one important feature of the activity changes of glyoxalase I. In HL60 cells, the activity of glyoxalase I decreased and the activity of glyoxalase II increased after treatment with both NMF and CCRG 81045 (Figures 6 and 8). In K562 cells, the activity of both glyoxalase enzymes increased following treatment with these drugs (Figures 9 and 10). This demonstrates that differentiation proceeded with an increase or decrease in the activity of glyoxalase I, and may indicate that changes in the activity of glyoxalase I are indeed of less functional significance than the changes in the activity of glyoxalase II.

The activity of glyoxalase II in K562 cells increased during both differentiating and non-differentiating conditions (Figures 9 and 10). However, the ratio of glyoxalase I to glyoxalase II activity decreased only during differentiation (Tables 11 and 12). The glyoxalase I/glyoxalase II activity ratio also decreased during the differentiation of HL60

cells by NMF and CCRG 81045 (Figure 23). This ratio may be more important than the absolute changes in enzyme activity during differentiation, due to the expected effects of cell volume changes on the absolute glyoxalase activity per cell number. The relative increase in the activity of glyoxalase II during differentiation suggests that mature, differentiated cells have a greater capacity to metabolise S-D-lactoylglutathione via glyoxalase II than immature, non-differentiated cells.

The differentiation of HL60 cells by 130-185 mM NMF was accompanied by a decrease in the activity of glyoxalase I and an increase in the activity of glyoxalase II (Figure 6). During the differentiation process, the apparent Km of both the glyoxalase I- and glyoxalase II-catalysed reactions was unchanged. However, differentiation of the cells decreased the relative V_{max} for the glyoxalase I-catalysed reaction and increased the relative V_{max} for the glyoxalase II-catalysed reaction (Table 13). This indicates that during the differentiation of HL60 promyelocytes to neutrophils, there was a non-competitive inhibition and/or inactivation of glyoxalase I, and a non-competitive activation and/or de novo synthesis of glyoxalase II. The non-competitive modification of the glyoxalase enzymes has also been reported for the functional activation of neutrophils (211).

These results indicate that there is a major
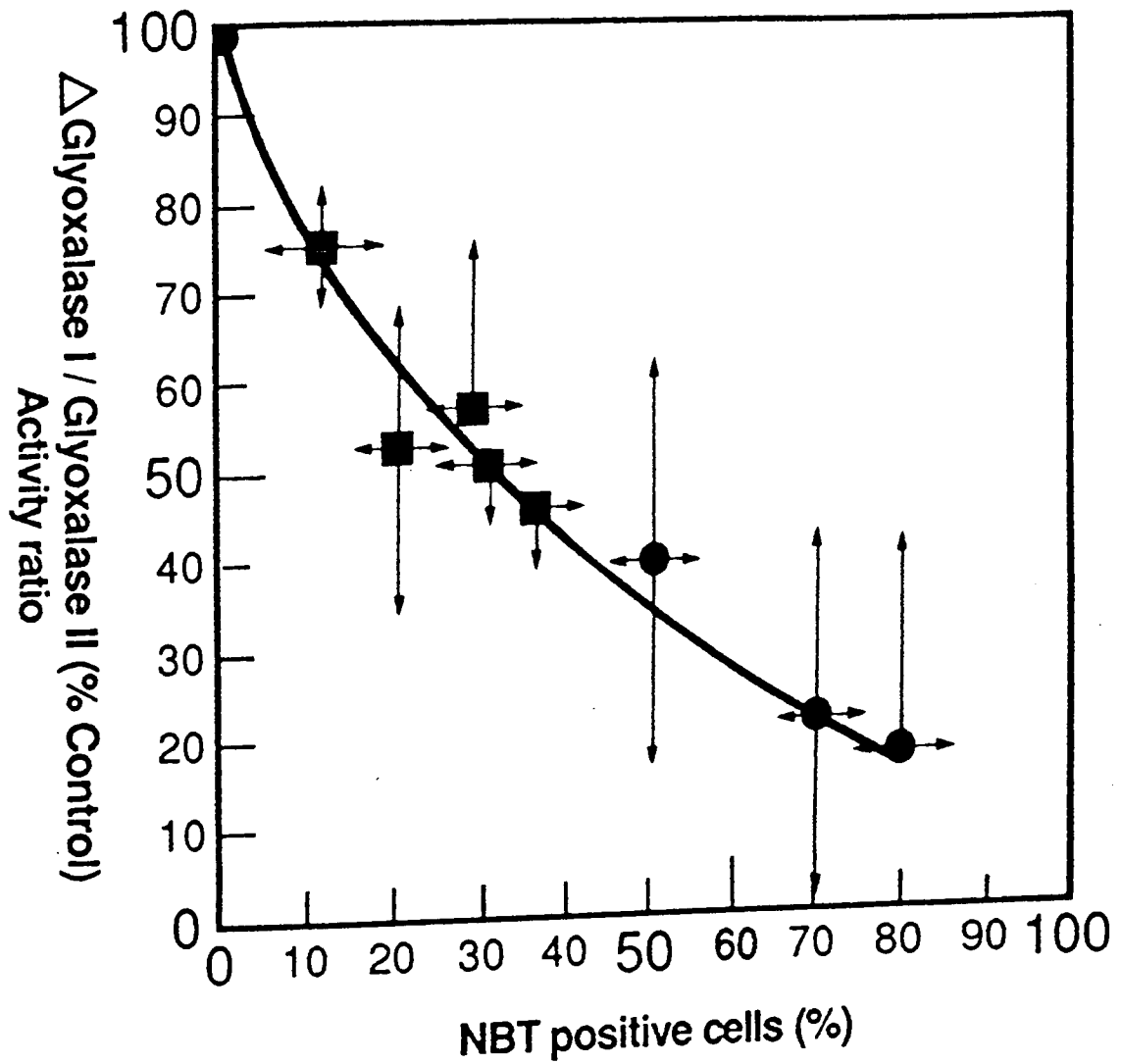
Figure 23. The Relationship Between the Degree of
Differentiation of HL60 Cells and the Decrease in the
Glyoxalase I/Glyoxalase II Activity Ratio. Data is for
day 4 of treatment with NMF (●) and CCRG 81045 (■).

modification of cellular glyoxalase activity during differentiation. If the flux of metabolites is unchanged, this would be expected to change the cellular concentrations of the glyoxalase metabolites, methylglyoxal and S-D-lactoylglutathione.

Inspection of the Michaelis-Menten kinetics of the glyoxalases in HL60 cell extracts (Figures 11 and 12) reveals that, at similar substrate concentrations, the activity of glyoxalase II is lower than the activity of glyoxalase I. Glyoxalase II is therefore expected to be the rate-determining enzyme of the glyoxalase pathway in HL60 promyelocytes.

Principato et al have also investigated the changes in the glyoxalase system during differentiation, using chick embryo liver as a model (150). They reported that the glyoxalase I activity, which was initially high, paralleled the mitotic activity of the tissue and decreased as the liver differentiated. The glyoxalase II activity showed the reverse effect - it was initially low and increased as the liver differentiated. Given a constant supply of methylglyoxal, the overall effect of these changes in the activity of the glyoxalase enzymes may be the production of an initially high concentration of S-D-lactoylglutathione, which then decreases as the liver becomes differentiated. These results are very similar to those obtained with the chemically-induced differentiation model used in this study.

5.3 Modification of the Glyoxalase System During Changes in Cell Proliferation Status.

Raji and GM892 cells may be growth-arrested in culture by decreasing the concentration of foetal calf serum in the culture medium. This decreases the proliferation status of the cells without inducing differentiation. This provides a convenient model to study the effect of cell proliferation status on the glyoxalase system.

In GM892 cells, there was a decrease in glyoxalase I activity and an increase in the activity of glyoxalase II during growth arrest (Figure 13). This indicates that the activity of glyoxalase I increases and the activity of glyoxalase II decreases with increasing proliferation of GM892 cells. In Raji cells, there was an increase in the activity of both glyoxalase I and glyoxalase II during growth-arrest (Figure 14). This indicates that the activity of both glyoxalase I and glyoxalase II decrease with increasing proliferation of RAJI cells. Therefore, in both GM892 and Raji cells there was a decrease in the activity of glyoxalase II with increasing cell proliferation. This suggests a consistent inverse relationship between the activity of glyoxalase II and the rate of cell proliferation. However, there was no similar consistent relationship between the activity of glyoxalase I and cell proliferation : the activity of glyoxalase I increased or decreased with increasing cell proliferation. This

suggests that the changes in glyoxalase I activity during growth arrest may not be metabolically significant and the activity of glyoxalase I is not a critical factor in the control of cell proliferation (cf. the Szent-Györgyi hypothesis, Section 1.7).

Removal or depletion of an essential nutrient (e.g. foetal calf serum) from cell culture media is a method routinely employed for growth-arresting cells in culture. During growth arrest, the rate of cell proliferation decreases and the distribution of cells around the cell cycle changes with an accumulation of cells in the non-proliferating G_0 phase of the cycle. The observed changes in the activities of glyoxalase I and glyoxalase II in both GM892 and Raji cells suggest that the activities of glyoxalase I and glyoxalase II change round the cell cycle.

If the flux of methylglyoxal metabolised via the glyoxalase system is maintained during growth arrest and round the cell cycle, the observed changes in glyoxalase activities would be expected to produce an increase in the concentration of the glyoxalase intermediate S-D-lactoylglutathione with increasing rate of cell proliferation, although this was not determined. An increased cellular proliferation rate will be reflected by an increased proportion of cells undergoing mitosis. The putative increase in S-D-lactoylglutathione concentration with increased rate of cell proliferation would correlate well with the observed effect of

S-D-lactoylglutathione on microtubular assembly.

Several other studies have investigated the role of the glyoxalase system in cellular proliferation. Experiments with <u>Saccharomyes cereviciae</u> (152,212) and <u>Datura callus</u> cells in culture (213) have shown that the activity of glyoxalase I increased with increasing rate of cell proliferation. This is consistent with the changes observed in the GM892 cell study described above. Using a <u>Datura callus</u> cell culture, Ramaswamy <u>et al</u> investigated the relationship between the activity of glyoxalase I and the cell proliferation rate (214). Incubation of the cells with a specific mitotic inhibitor (vinblastine) inhibited both cell number (a measure of the proliferation rate) and glyoxalase I activity. Vinblastine acts by binding to the growing end of the microtubule and preventing the formation of the mitotic spindle (215). When the cells were incubated with a specific glyoxalase I inhibitor (squaric acid), both the glyoxalase I activity and the cell number decreased. These results, therefore, indicate that glyoxalase I activity is related to cell proliferation rate, probably via a mechanism linked to the formation of the mitotic spindle.

In contrast, there was found to be no significant difference in the activities of glyoxalase I and glyoxalase II in growth-arrested and proliferating human fibroblasts (154). This conflicts with both the GM892 and Raji studies. Although the reason for this is not

understood, there are two possible explanations. It has been reported that the cell density at which fibroblasts are cultured affects the amount of polymerized tubulin (microtubules) in the cell (216). If the glyoxalase system is involved in the control of microtubule assembly, the activity of glyoxalase I may be dependent on the density at which the cells were cultured, as well as their actual proliferation rate. This may explain why there was no change in the activity of glyoxalase I, even though the proliferation rate of the cell was markedly increased. Alternatively, it is possible that glyoxalase activities in human fibroblasts may be different in individual phases of the cell cycle and yet the mean glyoxalase activities still be similar under proliferating and growth-arrested conditions. Further studies of the glyoxalase activities in cell cycle synchronised fibroblasts are required to resolve this problem.

5.4 The Effect of S-D-Lactoylglutathione on Cell-Free Assembly of Microtubules.

5.4.1 The Cell-Free Assembly of Microtubules.

Microtubules are hollow cylindrical structures, 24 nm in diameter, composed of longitudinally arranged tubulin protofilaments with small amounts of microtubular associated proteins (217). The cytosolic network of microtubules, the microtubular cytoskeleton, provides structural organisation for the cytosol and cell contents. It is particularly important where a

major re-organistion of the cytosol occurs. For example, the microtubules form the structural basis for the mitotic spindle, and, as such, are essential for cell division. They also form a cytoplasmic network that may be involved in the control of cell shape, cell growth and intracellular transport.

The cell-free assembly of purified microtubular protein (tubulin + microtubular associated proteins) in this study was pioneered by Weisenberg (172) and further developed by Shelanski _et al_ (218) and Borisy _et al_ (219). The assembly of microtubules is a dynamic process. It can be split into several different phases :

(i) The Nucleation Phase - This is the initial series of reactions between tubulin dimers that lead to the formation of polymerization nuclei. _In vivo_, the centrosomes act as the nucleation sites for cytoplasmic microtubules, but the exact identity of the nucleation species _in vitro_ is unknown (217).

(ii) The Microtubule Elongation Phase - The microtubule nuclei promote the further assembly of tubulin into microtubules. Elongation involves the sequential net addition of tubulin dimers onto the growing points at the ends of the microtubules. The presence of GTP or ATP is essential for this process. Tubulin and GTP form a complex which actually binds to the end of the microtubule, during which the GTP is hydrolysed to GDP.

As microtubular assembly is a dynamic process, there will be constant addition and loss of tubulin

dimers from the ends of the microtubules. If the
concentration of tubulin is below a certain value,
termed the "critial concentration", there will be no
spontaneous net assembly of tubulin into microtubules
(220).

In this study, the assembly of microtubular protein
to microtubules was followed by measuring the increase
in absorbance of the solution at 350 nm. The increase in
absorbance reflects an increase in the turbidity of the
solution, caused by the presence of long rods of
microtubules (205). Under these conditions, the
turbidity of the solution is primarily a measure of the
weight of the assembled microtubules and is reasonably
insensitive to their length. This method was,
therefore, considered as being suitable for simple
kinetic analysis of microtubule assembly.

5.4.2 The Effect of S-D-Lactoylglutathione on the
Kinetics of Cell-Free Assembly of Microtubules.

The cell-free assembly of purified brain
microtubular protein was promoted by GTP and was
characterised by an initial 1 - 2 minute lag phase when
no increase in absorbance at 350 nm occurred, a 10
minute period of rapid microtubular assembly when there
was a rapid increase in absorbance at 350 nm, and
followed finally by a slow disassembly when there was a
slow decrease in absorbance at 350 nm over a period of
several hours (Figure 15, curve d).
S-D-Lactoylglutathione decreased the nucleation lag-time

(particulary at high concentration) and increased the extent of maximum microtubule assembly - as judged by the maximum aborbance at 350 nm. However, S-D-lactoylglutathione had no significant effect on the rate of microtubule assembly during the rapid assembly phase (Figure 15). The decrease in the nucleation lag-time suggests that S-D-lactoylglutathione either increased the rate of formation of nucleation sites or enhanced the addition of tubulin to the pre-existing nucleation sites. From this experiment, it is impossible to distinguish between these two possibilities. The increase in the maximum absorbance obtained suggests that S-D-lactoylglutathione increased the percentage of total microtubular protein assembled at the steady state where the rates of microtubule assembly and disassembly are equal. In terms of the Weisenberg microtubule assembly notation, the concentration of unassembled tubulin under these conditions is termed the critical subunit concentration. The potentiation of GTP-promoted cell-free assembly of microtubules by S-D-lactoylglutathione may reflect a decrease in the critical subunit concentration, or an increase in the concentration of microtubular protein competent to assemble in the sample. Further experimentation is required to discriminate between these two possibilities.

The slow disassembly of microtubules has been assigned to the phosphorylation of tubulin by an

ATP-dependent kinase which co-purifies with microtubular protein (221). S-D-Lactoylglutathione did not affect the rate of this slow disassembly. This shows that the effect of S-D-lactoylglutathione is independent of the effect of this ATP-dependent phosphorylation.

5.4.3 The Consumption of S-D-Lactoylglutathione During the Cell-Free Assembly of Microtubules.

The assembly of microtubular protein in the presence of S-D-lactoylglutathione lead to a loss of S-D-lactoylglutathione from the solution. This occurred only under conditions necessary for microtubule assembly. This suggests that the potentiation of microtubule assembly leads to a consumption of S-D-lactoylglutathione. The mechanism of this effect is not known. The microtubules produced in the absence and presence of S-D-lactoylglutathione were identical in their instability in the presence of calcium ions, nocodazole and cold - normal features of cell-free assembled microtubules (172,205,209).

Although pig brain microtubules were purified on several occasions, the effect of S-D-lactoylglutathione on the assembly of the microtubules was not consistent. This lack of a consistent effect of S-D-lactoylglutathione has been noted by other workers (129,222). The reason for this inconsistency is currently unknown. However, a recent report has stated that the concentration of magnesium ions in the assembly buffer has an effect on the morphology of the early

intermediates in the microtubular assembly process (206). At low Mg^{2+} ion concentrations, a large number of polymerisation nuclei are formed, assembly is rapid and the assembled microtubules are short. Conversely, at high magnesium ion concentrations (greater than 1 mM), a small number of polymerisation nuclei are formed and the assembled microtubules are long. Magnesium ions are chelated by GTP, so the concentration of GTP in the solution can affect the final length of the assembled microtubules, by its action on magnesium ion concentration.

The increase in the final absorbance of the assembly solution in the presence of S-D-lactoylglutathione indicates that S-D-lactoylglutathione increases the amount of tubulin assembled into microtubules. It probably exerts this effect by increasing the mean length of microtubules after assembly (217). This increase in mean length will occur to a greater extent if there are a large number of short microtubules in the solution, than if there are a small number of long microtubules (206). The effect of S-D-lactoylglutathione on microtubular assembly would, therefore, be expected to be enhanced when there are a large number of nucleation sites present in the solution. The observed lack of consistency of S-D-lactoylglutathione on microtubule assembly may reflect variations in the number of nucleation sites present in the solution.

5.5 The Red Blood Cell Glyoxalase System During Hyperglycaemia In Vitro

5.5.1 Diabetes Mellitus and Retinopathy

Diabetes Mellitus is a metabolic condition that arises from a deficiency in the action of insulin. The lack of insulin action results in a chronic periodic hyperglycaemia, particularly if the condition is being treated with supplementary insulin injections. Diabetic complications, such as cataract, neuropathy and nephropathy, occur as a direct result of this periodic hyperglycaemia. The development of diabetic complications is frequently correlated with metabolic indicators, such as the formation of glycosylated haemoglobin (185), that are measures of the cumulative exposure of the individual to periodic hyperglycaemia.

The diabetic patients in this study were examined for the presence or absence of retinopathy. Retinopathy is the most common complication of diabetes. Diabetic retinopathy is the major cause of blindness between the ages of 30 and 64 in England and Wales (223). The age of the patient and the duration of diabetes have a marked effect on its time of appearence (224,225,226). Few diabetics have detectable retinopathy at the time of diagnosis (224,226), but most patients have some degree of retinopathy within 25 years. In young diabetics, the mean interval of appearence is approximately 13 years. If diabetes occurs after the age of 60, the mean interval of appearence drops to about 5 years.

5.5.2 Age and Duration of Diabetes

The mean age and duration of diabetes data (Table 17) for the patient groups used in this study shows several significant differences between the groups. In general, the patients with diabetic complications, irrespective of the type of diabetes from which they suffered, were older and had had diabetes for longer than those patients without complications. This is in agreement with other reports that the duration of diabetes is important in the development of diabetic retinopathy (224,226). The difference in the age of the patients with and without complications is important, as older people are prone to develop retinopathy due to old age and not simply due to the occurrence of diabetes.

5.5.3 Glyoxalase Activities

Glyoxalase activity has been detected in neutrophils (145,156) and platelets (227) as well as red blood cells, but the red blood cells are the major source of glyoxalase activity per ml of circulating human blood (Table 21). The results show that the glyoxalase activities in red blood cells from normal healthy control subjects were not significantly different from the glyoxalase activites in red blood cell from a mixed group of diabetic patients, which contained approximately equal numbers of insulin-dependent and non-insulin-dependent diabetics, with and without complications. However, if the diabetic subjects were split into those with

Table 21.   The Distribution of Glyoxalase I and
               Glyoxalase II Activity in 1 ml of Whole
                                Blood

Cell Type	Cells/ml Whole Blood	Glyoxalase I (units)	Glyoxalase II (units)
Red cells	$4-6 \times 10^9$	23.9	9.55
Neutrophils	$2.5-7.5 \times 10^6$	0.07	0.007
Platelets	$2-5 \times 10^8$	0.11	0.01

The activity of glyoxalase I and glyoxalase II is
expressed as units as defined in the Materials and
Methods section. The red cell glyoxalase activity was
calculated using the control data from the diabetic
study and adjusted for a 50% haematocrit. The
glyoxalase activity for the neutrophils and platelets
was calculated using the maximum value of the normal
range for the number of cells per ml.

complications (irrespective of Type) and those without

complications (irrespective of Type), it was found that

the glyoxalase II activity in the red blood cells from

those subjects with complications was significantly

lower than the red blood cell glyoxalase II activity in

diabetics without complications.

The most significant differences in

glyoxalase activities in red blood cells occurred in

patients with insulin-dependent diabetes. In these

patients, the glyoxalase activities were

correlated as follows :

Glyoxalase I : Non-complicated < Complicated (P < 0.05)

Glyoxalase II: Non-complicated > Complicated (P < 0.05)

Investigation of the glyoxalase activities in

insulin-dependent diabetics matched for age and duration

of diabetes improved the statistical significance of

these results (Table 19).

The distribution of the red blood cell glyoxalase

activities was not significantly affected by diabetes

(Figures 17 and 18). The correlation of a high

glyoxalase I and a low glyoxalase II activity in the red

blood cells of insulin-dependent diabetics suggests that

these patients may be genetically disposed to develop

diabetic complications. Insulin-dependent diabetes has

already been linked to the occurrence of certain alleles

of the HLA system (228,229). The most significant

linkage is with alleles on the HLA-D and HLA-DR (D Related) loci. As described in Section 1.2.7, the glyoxalase I locus is located very close to the HLA-D locus on chromosome 6, and has been shown to be linked to the HLA complex as a whole (70). The mechanism by which a genetic disposition to diabetic complications could occur is easy to see for glyoxalase I. The corresponding mechanism for glyoxalase II is harder to envisage, as glyoxalase II does not occur in the form of isozymes, unlike glyoxalase I, and the gene locus is situated on a different chromosome.

If the correlation between glyoxalase activity and insulin-dependent diabetes is repeated in the other tissues where the clinically important complications occur (lens, retina, kidney glomeruli, peripheral neurones), it suggests that the glyoxalase system may be an important factor in the development of diabetic complications.

The non-insulin-dependent diabetic patients did not show any significant correlations between the occurrence of diabetic complications and the red blood cell glyoxalase activities. The reason for this may be two-fold : (a) non-insulin-dependent diabetics do not generally suffer severe hyperglycaemia, and (b) the duration of the disease may not be accurately known (the potentially mild symptoms may prevent immediate diagnosis). In the insulin-dependent diabetic patients, the age of the patient and the duration of diabetes were

important factors in the correlation of glyoxalase activities in the red blood cell with the occurrence of diabetic complications . This is in accord with the correlation of diabetic complications with chronic periodic hyperglycaemia (174,178) and with the view that diabetic complications are an acceleration of the diseases of old age (230).

5.5.4 The Concentration of Methylglyoxal and S-D-Lactoylglutathione in Whole Human Blood

The concentrations of methylglyoxal and S-D-lactoylglutathione in whole blood were significantly increased in diabetic patients, relative to the controls (Table 20). These metabolites are mainly found in the cellular fraction of blood - in plasma, the concentration of methylglyoxal is less than 50 nM and S-D-lactoylglutathione less than 1 µM. In terms of their concentration per ml of whole blood, most of the methylglyoxal and S-D-lactoylglutathione is present in the red cell; the neutrophils (156) and presumably the platelets contain only relatively small amounts.

As there was no significant difference between the red blood cell glyoxalase activities in the control and diabetic groups, the increase in the concentration of the glyoxalase substrates that was observed in the diabetic patients is probably due to an increased flux of intermediates metabolised by the glyoxalase system during hyperglycaemia. The correlation of high glyoxalase I activities and low glyoxalase II activities

with the occurrence of diabetic complications suggests that S-D-lactoylglutathione may play an important role in the development of these complications.

Methylglyoxal may also have a role in the development of diabetic complications. Cataract formation is associated with high molecular weight cross-linked proteins in the eye (231). α-Oxoaldehydes are capable of cross-linking proteins (232). If the changes demonstrated in blood are replicated in other tissues, such as the eye, methylglyoxal may be envisaged as a factor in the formation of diabetic cataract.

6. CONCLUSION

The aims of this work were to investigate the metabolic functions of the glyoxalase system, and the glyoxalase metabolites methylglyoxal and S-D-lactoylglutathione, in normal and abnormal cellular metabolism. Three specific areas were investigated : (a) the differentiation and proliferation of human tumour cells in vitro; (b) the cell-free assembly of microtubules and (c) the effect of Diabetes Mellitus on the glyoxalase system in vivo.

During the chemically-induced differentiation of human leukaemia cells in vitro, there was a substantial increase in the activity of glyoxalase II, whereas differentiation proceeded with a decrease or an increase in the activity of glyoxalase I. A decrease in the proliferation rate of the GM892 and Raji cell lines, without differentiation, produced an increase in the activity of glyoxalase II, whereas a change in the proliferation status of the cells occurred with an increase or decrease in the activity of glyoxalase I. Therefore, during differentiation and changes in the proliferation status of the cell, there was no consistent change in the activity of glyoxalase I. This indicates that glyoxalase I was not a critical factor in the control of cell proliferation or differentiation. This is contrary to the Szent-Györgyi hypothesis (142,143). The activity of glyoxalase II changed

consistently with changes in cell differentiation and proliferation, and may have a critical role in some regulatory factor in cell differentiation and proliferation. The changes in the glyoxalase activities during differentiation may reflect the removal of cells from progress round the cell cycle.

The cell-free GTP-promoted assembly of microtubules in vitro was potentiated by S-D-lactoylglutathione. The potentiation of assembly was accompanied by the loss of S-D-lactoylglutathione from the solution, which indicates that it may be consumed during the assembly process. The glyoxalase system may have a physiological role in the regulation of microtubular assembly through its control of S-D-lactoylglutathione levels. This suggested role is consistent with the observed changes in the activity of the glyoxalase enzymes during maturation and differentiation. Immature tissues have a high mean microtubular flux for their increased mitotic spindle formation. They also have a decreased capacity to metabolise S-D-lactoylglutathione, via glyoxalase II, and hence may have an increased cellular concentration of S-D-lactoylglutathione.

In diabetes, there was an increase in the whole blood concentration of both S-D-lactoylglutathione and methylglyoxal, but there was no significant change in the activity of the red blood cell glyoxalase enzymes. This may reflect an increased flux of methylglyoxal metabolised via the glyoxalase system during

hyperglycaemia. The occurrence of complications in insulin-dependent Type I diabetics was correlated with a high glyoxalase I activity and a low glyoxalase II activity in the red blood cells. The glyoxalase system may be involved in the development of diabetic complications in red blood cells and possibly in other tissues.

The results presented in this thesis must now be considered in the light of the roles that have been suggested for the glyoxalase system (Section 1.7).

Cooper and Anderson have suggested that the glyoxalase mediated conversion of methylglyoxal to D-lactate may form a glycolytic by-pass route in micro-organisms (139). However, the changes that have been observed in the glyoxalase system during the differentiation and maturation of tissue (150), the functional activation of neutrophils (211) and during diabetes strongly suggest that the glyoxalase system has a different, and more complex role in the cell.

Aronsson and Mannervik proposed that the glyoxalase system was a mammalian detoxification route for methylglyoxal produced by enterobacteria (23). This putative role for the glyoxalase system was supported by the observed high glyoxalase activity in blood and liver. It did not, however, explain the fact that glyoxalase activity has also been detected in plants and in the bacteria themselves. As with the Cooper and

Anderson suggestion, it also fails to explain how tissues, such as immature embryo (150), tumours (3) and regenerating liver (151), can thrive and proliferate, with a deficiency in the activity of glyoxalase II - the pacemaking enzyme of the glyoxalase pathway.

Szent-Györgyi and his co-workers (142,143) suggested that cell growth was controlled by methylglyoxal (the growth inhibitor) and glyoxalase I (the growth promoter). The results presented here demonstrate that the activity of glyoxalase I increased or decreased during cell proliferation and differentiation. Glyoxalase I was not a critical marker or regulatory factor in either of these processes. This is inconsistant with the Szent-Györgyi hypothesis of a methylglyoxal/glyoxalase I growth controlling system.

Gillespie proposed that the glyoxalase system regulated microtubule assembly, by controlling the concentration of S-D-lactoylglutathione (129). This putative role for the glyoxalase system has received support, directly and indirectly, from a number of different investigations. Under conditions in which the assembly of microtubules is elevated, and given a constant supply of methylglyoxal, the activity of glyoxalase II is expected to be relatively low, thus producing a relatively high cellular concentration of S-D-lactoylglutathione. This has been demonstrated in such diverse tissues as differentiating chick liver (150), functionally activated human neutrophils (211),

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and regenerating rat liver (151). The two studies on differentiating and proliferating human tumour cells presented in this thesis support this role for the glyoxalase enzymes, assuming that the glyoxalase system controls the microtubular assembly that is required for mitotic spindle formation.

The effect of hyperglycaemia on the glyoxalase system was first proposed by Thornalley (114,180). The results presented in this thesis may represent the first discovery of an involvement of the glyoxalase system with a recognised clinical condition. The modification of the glyoxalase system during diabetes may be an aetiological factor in the development of diabetic complications, and as such, offers a new insight into the prevention of complications.

In the light of the results presented here, it is apposite to reappraise the cellular sites for the metabolism of S-D-lactoylglutathione. From the modifications to the glyoxalase system during the differentiation and proliferation of human tumour cells, and during Diabetes Mellitus, it is possible that there are sites, other than the active site of glyoxalase II, where S-D-lactoylglutathione can be metabolised by the cell, as suggested in the cell-free microtubule assembly experiments. The reaction of S-D-lactoylglutathione at sites other than the active site of glyoxalase II may be a crucial factor in the functional characterisation of

the glyoxalase system.

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