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Premeditated enzyme inactivation : the development of mechanism-based irreversible inhibitors of glyoxalase I as potential anti-cancer agents

Stuart Neal Isaacs
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PREMEDITATED ENZYME INACTIVATION: THE DEVELOPMENT OF MECHANISM-BASED IRREVERSIBLE INHIBITORS OF GLYOXALASE I AS POTENTIAL ANTI-CANCER AGENTS

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March 6, 1985

(Date)

PREMEDITATED ENZYME INACTIVATION:

THE DEVELOPMENT OF MECHANISM-BASED IRREVERSIBLE

INHIBITORS OF GLYOXALASE I AS POTENTIAL ANTI-CANCER AGENTS

A Thesis Submitted to:
The Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

BY

STUART NEAL ISAACS

1985

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This thesis is dedicated to my Parents, whose sacrifice and dedication have inspired me; and to David, Steven and Mary Beth, for the things we have done and for the things we will do.

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ABSTRACT

Premeditated Enzyme Inactivation: The Development of Mechanism-Based Irreversible Inhibitors of Glyoxalase I as Potential Anti-Cancer Agents

Stuart Neal Isaacs
1985

The development of a mechanism-based irreversible inhibitor of an enzyme is the ultimate proof that a postulated enzymatic mechanism is operative. Halogenated methylphenylglyoxals (XV) were synthesized based on the theory that glyoxalase I proceeds via an enediol intermediate. The newly synthesized compounds could divert the enediol intermediate to a highly reactive species which would then covalently modify the enzyme and inactivate it. The *in vitro* findings of halogen ion release and the demonstration of irreversible enzyme inhibition by p-bromomethylphenylglyoxal provides strong support for the premise that glyoxalase I acts via an enediol intermediate. While p-fluoromethylphenylglyoxal resulted in no enzyme inhibition, the enzyme inhibition by p-bromomethylphenylglyoxal might represent the first mechanism-based inhibitor of glyoxalase I.

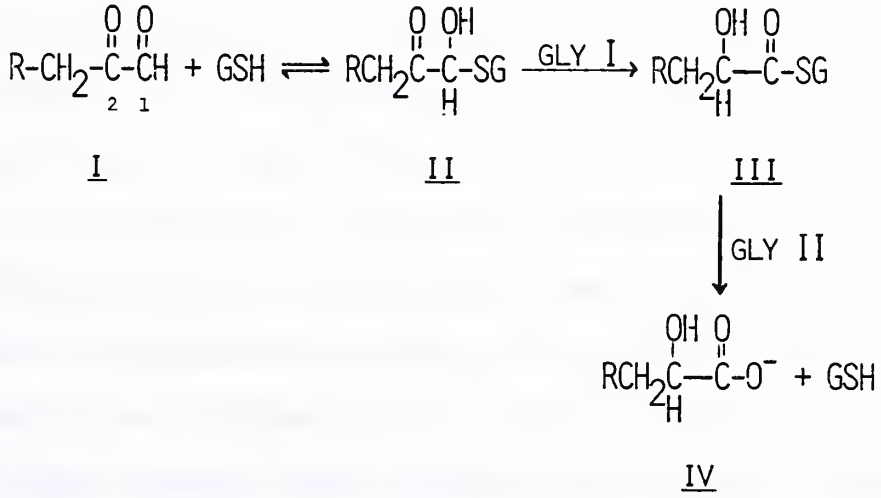
Based on the theory that the glyoxalase enzyme system constitutes a fundamental regulatory mechanism of cell growth and division the halomethylphenylglyoxals were tested as anti-cancer agents in tissue culture and in tumor-bearing mice. Significant cytotoxic effects of fluoro- and bromomethylphenylglyoxal against L1210 and P388 cell lines were observed. In tumor-bearing mice p-bromomethylphenylglyoxal revealed extreme toxicity, and early death to the mice. This is consistent with the potent effect of an irreversible enzyme inhibitor which causes the overwhelming accumulation of substances which inhibit cell growth and division.

INTRODUCTION

> It is ...difficult to exaggerate the importance to biology...of extended studies of enzymes and their action.
>
> — F.G. Hopkins (Presidential address to the Royal Society - 1932) (1)

The glyoxalase enzyme system is now known to convert the thiohemiacetal (II) of an α-ketoaldehyde (I) and glutathione (GSH) to the corresponding α-hydroxythioester (III) which is then hydrolyzed by glyoxalase II to the corresponding D-hydroxyacid (IV) and free GSH (Scheme I). Since its independent discovery in 1913 by Dakin & Dudley (2,3) and

SCHEME I:



I II III IV

Neuberg (4), the glyoxalase system has been an area of considerable biological and mechanistic controversy. Although it has been found widely distributed (3,5) in animals, plants, and microorganisms, the actual physiological function of the system remains obscure to this day. There are many theories, most with tenuous evidence, of the definitive function of the glyoxalase system.

> The theories serve to satisfy the mind, prepare it for 'an accident' and keep one going. I must admit that most of the new observations I made were based on wrong theories. My theories collapsed, but something was left afterward.
> -A. Szent-Györgyi (6)

Probably the most controversial theory of the function of the glyoxalase enzyme system is that proposed by Albert Szent-Györgyi, the 1937 Nobel Prize winner in Physiology and Medicine for his work on ascorbic acid and his studies of cellular respiration. Szent-Györgyi has proposed that methylglyoxal and the glyoxalase system constitute a fundamental regulatory mechanism of cell division and growth (7-10). His theory is based on his observations that a tissue extract, thought to be a ketoaldehyde (9), and later identified as methylglyoxal (11), reversibly inhibited cell growth (8). Szent-Gyorgyi, therefore, designated methylglyoxal as a retarder of division, the cell's internal "brake". The glyoxalase enzyme system is thought to be the promoter of division by the metabolism of methylglyoxal to D-lactate (10). A disturbance in this equilibria may be connected with a cause of cancer. Szent-Györgyi has extended this theory further into an even more controversial realm of "bioelectronics" (12-19). In this submolecular theory, he hypothesized that methylglyoxal is inherently important in maintaining the energy state of normal living matter, and that the energy state of living matter is altered in cancer. His studies using electron spin resonance have revealed that molecules of major biological importance, with low chemical reactivity, can give off a whole electron, thus forming a free radical. This suggests that charge transfer may be a common and fundamental biological reaction and he believes that the presence of methylglyoxal allows such a charge transfer. He calls methylglyoxal the "acceptor impurity of protein" whose action is to desaturate the otherwise electron filled ground state of proteins (15-18). "If there is a shortage of methylglyoxal or and excess of glyoxalase, there

is no charge transfer, and cells revert to the simple, non-stoppable proliferative state, which is cancer." (16) Therefore, according to Szent-Györgyi's theory, electronic desaturation of protein is a central event of cell life and a low level of desaturation is seen in cancer (14,17).

Other researchers have tried to assign different functions to the glyoxalase system. Kun (19), who found that methylglyoxal inhibited succinic dehydrogenase and other similar enzymes suggested that the glyoxalase enzyme system eliminated spontaneously formed methylglyoxal to prevent the inactivation of enzymes containing sulfhydryl-groups. Salem (20) supported this idea that spontaneously formed methylglyoxal was detoxified by glyoxalase and that the wide distribution of the enzyme in all types of tissues from species of all phyla is in keeping with a role of this kind. A Belgium group (21), gave strong evidence that in yeast, the glyoxalase pathway is a defense mechanism against methylglyoxal produced by the spontaneous decay of accumulating triose phosphates during glycerol catabolism.

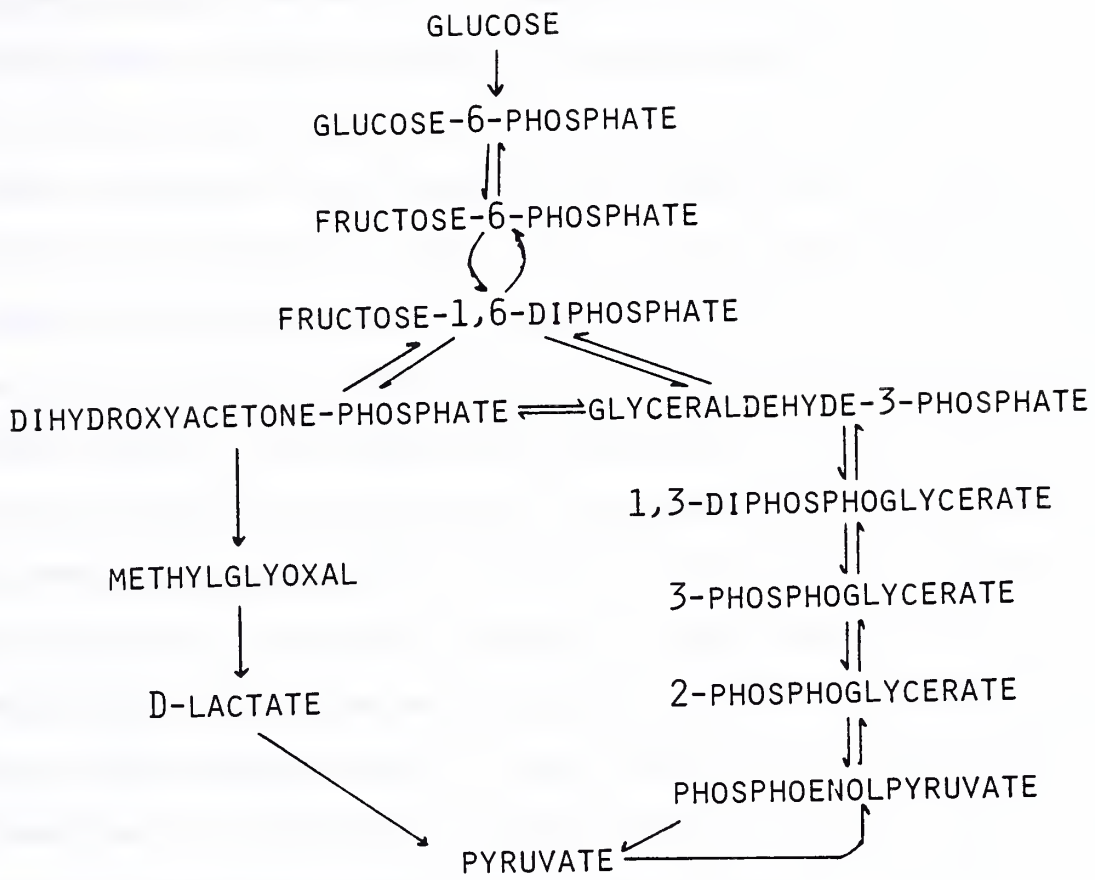
Recent studies by Gillespie (22-25) suggest that methylglyoxal and the glyoxalase enzyme system may be involved in the mediation of the immune and inflamatory responses. She has shown that the product of the glyoxalase I reaction, S-lactoylglutathione (III, R=CH_3; Scheme I), is a physiologically important species regulated by the glyoxalase system. Concanavalin A is known to increase centriole-associated microtubules in polymorphonuclear leukocytes (23). In vitro studies revealed that this assembly occurred in the same time frame of glyoxalase I activation, resulting in the buildup of S-lactoylglutatione. This thioester was also shown to be a potentiator of histamine release in human leukocytes (24). Her work with a phorbol ester, a known inflamatory agent and potent carcinogen, revealed that it caused elevated glyoxalase I activity and decreased glyoxalase II activity

in human leukocytes (25). This is compatible with the hypothesis that increased levels of S-lactoylglutathione are a concomitant of inflamation. It is also possible that the increased levels of this thioester is related to the tumor promoting properties of phorbol esters. This is all consistent with Szent-Györgyi's theory that cancer cells have increased glyoxalase activity which cause a decrease in endogenous methylglyoxal.

Others doubted the existence of intracellular methylglyoxal and postulated that the glyoxalase system was involved in the reactions of other intracellular ketoaldehydes. Thus, some naturally occuring ketoaldehydes, phosphohydroxypyruvic aldehyde (26), hydroxypyruvic aldehyde (27), and γ,δ-dioxovalerate (28), were shown to be substrates of the glyoxalase enzyme system.

The debate over the existence of methylglyoxal as a natural constituent of cells was finally concluded in 1970 when Cooper and co-workers (29-31) made the important discovery of the enzyme methylglyoxal synthase. This enzyme originally found in E. coli, catalyzes the conversion of dihydroxyacetonephosphate (DHAP) to methylglyoxal and phosphate. Previous to this discovery, some thought that the methylglyoxal found in cells was formed non-enzymatically (32,33). Methylglyoxal synthase serves as a bypass of triose-phosphate isomerase. Methylglyoxal synthase, in conjunction with glyoxalase and D-lactate dehydrogenase, constitutes an alternative to the Embden-Meyerhof glycolysis pathway (29). (See Scheme II). This bypass does not result in ATP formation because it bypasses both substrate-linked phosphorylations of glycolysis. At first, Cooper (29) thought that it might play a role by preventing the accumulation of high concentrations of phosphorylated glycolytic intermediates which inhibit growth. Later, his data (30,31) suggested that the pathway would be important when glycolysis was restricted by reduced availability of inorganic phosphate (P_i). With decreased P_i,

SCHEME II:[29]



glyceraldehyde-3-phosphate dehydrogenase would be inhibited and as a consequence the triose phosphate concentration would rise. The bypass scheme would then function by methylglyoxal synthase liberating P_i, thus replenishing intracellular P_i, and then glyoxalase would generate lactate which is an energy source under aerobic conditions. Methylglyoxal synthase was initially found in bacteria and has more recently been found in rat liver (34) and isolated from goat liver (35). Thus, the existence of an enzyme to make methylglyoxal suggests that this compound may indeed have a significant biological role.

Even though Szent-Györgyi's theory that the glyoxalase system constitutes a fundamental regulatory mechanism for cell division and cell growth remains controversial, it has, none-the-less, stimulated much thought and research on the possible connections of this enzyme system and

cancer. Work which lends some support to Szent-Györgyi's theory are the studies of glyoxalase activity in cancerous tissues and tissues undergoing rapid growth; as well as studies of the keto-aldehyde's inhibitory effects on cell growth and division. Early studies (36) generally showed no evident differences between cancerous and normal animals in the glyoxalase content of corresponding organs. More recent studies by Norton and co-workers (37) have shown that for the first ten days after tumor implant, there is an increase in tissue glyoxalase enzyme activity versus tissues of normal animals. The increased glyoxalase activity during the early stages of tumor formation goes along with Szent-Györgyi's theory that glyoxalase, by metabolizing methylglyoxal, is a cell growth promoter. However, after ten days there is an overall decrease in glyoxalase activity in tumor-bearing mice, but this decreased activity paralleled the decreased activity seen in other enzymes studied in tumor bearing mice. Norton's group also showed that the glyoxalase I enzymes isolated from the liver of tumor-bearing mice and normal mice were different protein molecules, but, with the same pH optima and Km's. They concluded that the glyoxalase I isolated from tumor-bearing mice was modified due to the presence of tumor (38). Brandt (39) studied the possible changes in the glyoxalase system with rapid growth. He found that the final product of the glyoxalase reaction, D-lactate, was increased in the blood of rats at the end of their rapid growth phase. He concluded that if D-lactate concentration reflects cellular methylglyoxal levels, then the hypothesis that methylglyoxal is a natural growth inhibitor is supported because, as observed, its level would increase as the animals reach the end of their rapid growth phase. An Italian group (40) studied glyoxalase I and II activity in the regenerating rat liver. Rat liver regeneration peaks at 28 hours and then falls rapidly. They found significantly increased glyoxalase I activity at 24 to 48 hours after partial hepatectomy. The high glyoxalase I activity during

the regeneration period support Szent-Györgyi's theory that glyoxalase I activity reduces the intracellular content of growth inhibitors. Increased glyoxalase II activity at 16, 48, and 72 hours with a transient decrease in activity at 24 hours, was correlated with an interval when proliferative activity is nearly stopped. Therefore, they proposed that it was the changes in concentration of the thioester, the intermediate compound between the glyoxalase I and glyoxalase II reaction, that is also important in growth regulation. That is, a decrease in this substance, by increased glyoxalase II activity, causes cellular proliferation to be shut down. In support of this, a Polish group (41) found that glyoxalase II activity was absent in cancerous tissues and cells. They claimed that this is a characteristic feature that distinguishes cancerous tissues from normal tissues. Thus, they agree with Szent-Györgyi that a fundamental factor in the cancer process is a disruption in the glyoxalase enzyme system.

The effects of ketoaldehydes on growth and division have been known for a long time. They have been tested as viricidal agents (42-46), and are known inhibitors of bacterial and cellular growth and division (8,47-50). Együd & Szent-Györgyi (8) found that low levels of methylglyoxal inhibited cell division of E. coli, fertilized sea urchin eggs, flagellates and cells in tissue culture. Methylglyoxal has also been found to inhibit adult kidney cell growth (50). Ketoaldehydes have also been extensively studied as possible carcinostatic agents (51-55), and, in fact, the ketoaldehyde, 3-ethoxy-2-ketobutyraldehyde (Kethoxal) was at one time marketed as a carcinostatic drug. In experimental set-ups, the glyoxal compounds have been found to be most active against mice ascites carcinoma, mammary carcinoma, leukemia, adenocarcinoma, lymphosarcoma, and sarcoma (53,54). Együd & Szent-Györgyi (54) noted that cancer cells in tissue culture were more sensitive to the inhibitory effects of methylglyoxal than normal cells in culture. However, the chemotherapeutic value of α-

ketoaldehydes are limited by their rapid metabolism by the glyoxalase system.

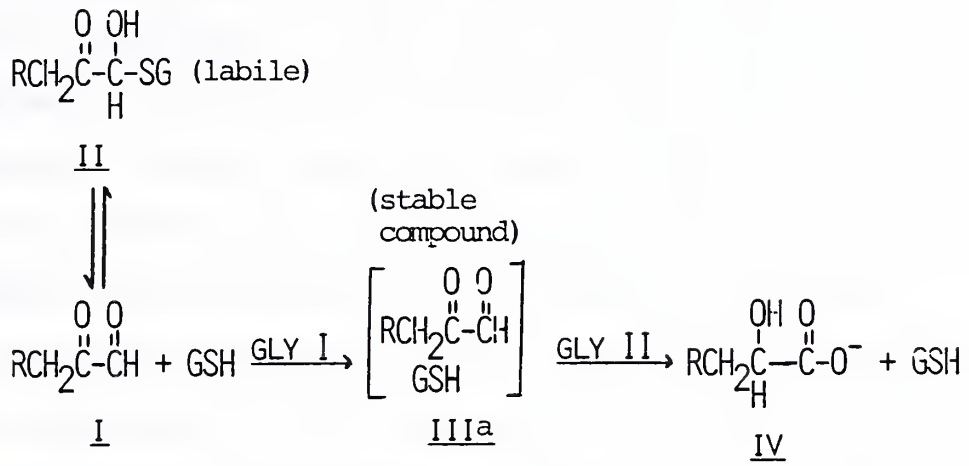
The observations of the potent inhibitory effects of the ketoaldehydes led to the studies of how this inhibition occurred. Early on, Együd & Szent-Györgyi (8) showed that the inhibition was not due to the cessation of oxidative metabolism. Their biological studies on E. coli (56) using radioactive compounds revealed that methylglyoxal inhibited protein synthesis and to a lesser degree, DNA and RNA synthesis. This has been confirmed (57,58) and similar results have been obtained using malignant ascites cells (59), mouse lymphoma cells (49), and cultured guinea pig keratocytes (GPK) (60). The effect of the inhibition is to stop cells from continuing through their life cycle, regardless of where the cell is in the cell cycle when the ketoaldehyde is added. One study on E. coli (58) showed that methylglyoxal, by interfering with protein synthesis, inhibited DNA synthesis by interacting with an initiation complex. They also found that methylglyoxal disrupted cell division by preventing the synthesis of a termination protein required for cell division. Another study on GPK cells (60) showed that methylglyoxal directly inhibited protein and RNA synthesis individually. The inhibition of protein synthesis resulted from an inhibition of the initiation of translation. RNA synthesis was inhibited by a block in the maturation of the 32S rRNA precursor. Methylglyoxal has also been shown to react with the 7-methylguanosine cap structure of eukaryotic mRNA to cause an inhibition of protein synthesis (61). Recently, Riley (62) determined that a methylglyoxal-ascorbate adduct (NFCR 278021) isolated by Fodar, effectively inhibited protein synthesis. The inhibitory effect of the adduct on protein synthesis was found to be decreased in the presence of GSH and glyoxalase.

> To [attempt] cure...without understanding it is similar to trying to repair a machine without knowing how it works.
>
> -A. Szent-Györgyi (63)

Throughout the years of controversy of the biological role of the glyoxalase enzyme system, a detailed understanding of the catalytic mechanism was accumulated. The first theory of the mechanism by which glyoxalase functions was put forward by Dakin & Dudley (3) who observed that the reaction was similar to the *in vitro* reaction of ketoaldehydes in alkaline solution (i.e. what is now called a Cannizarro Reaction). Lohmann (64) discovered that the tripeptide, -glutamylcysteineglycine or "glutathione" (GSH) was a coenzyme of glyoxalase. Jowett & Quastel (65) proposed the formation of a condensation product between methylglyoxal and glutathione (GSH) as an intermediate to lactic acid production. Yamazoye (66) was first to show the presence of an intermediate distinct from the addition complex between methylglyoxal and GSH in crude tissue extracts. It was not until 1950 when Hopkins & Morgan's (67) "factor" which accelerated the glyoxalase reaction was identified by Racker (68,69) and Crook & Law (70,71) as another enzyme which was present in the original crude glyoxalase preparations. Thus, two individual enzymes, glyoxalase I and II, were firmly identified. Of equal importance, Racker introduced a spectophotometric method of following the glyoxalase reactions, thus replacing the cumbersome manometric techniqes used in the past. Both groups also determined a difference between the chemical condensation product and Yamazoye's biological product. The chemical intermediate (proposed as *II*, Scheme III) was unstable in acid and alkali, decomposing to methylglyoxal and GSH. The biological product (proposed as *IIIa*, Scheme III) formed by the action of glyoxalase I was stable on the acid side of neutrality and decomposed to lactic acid and GSH on the alkaline side of

neutrality. Both groups proposed reaction pathways similar to Scheme III.

SCHEME III: (adapted from 70)



At that time, Crook & Law (71) also noted the low specificity of the enzymes for the glyoxal substrate but the very high specificity of glyoxalase for the GSH coenzyme. In 1961, Cliffe & Waley (72) gave evidence that the thiohemiacetal adduct II was the substrate for glyoxalase I. This was shown in a reaction at high glyoxalase I concentration and low methylglyoxal and GSH levels. The rate of the reaction was revealed to be determined by the non-enzymatic formation of the thiohemiacetal (II). This was confirmed by Davis & Williams (73) who concluded that glyoxalase I acts as an oxidoreductive isomerase. Thus, Scheme I is the accepted pathway of the glyoxalase enzyme system. Further confirmation of a one substrate pathway have been offered by Vander Jagt (74) and Marmstål & Mannervik (75). It is known that the product of glyoxalase I reaction is stereospecific and an initial study (76) using high-resolution ^{1}H-NMR revealed that glyoxalase I selected the proper thiohemiacetal diastereomer for processing. The investigators concluded that the most likely origin of the product stereochemistry was a selective binding site on glyoxalase I for the proper diastereomer. However, this was not confirmed by Creighton

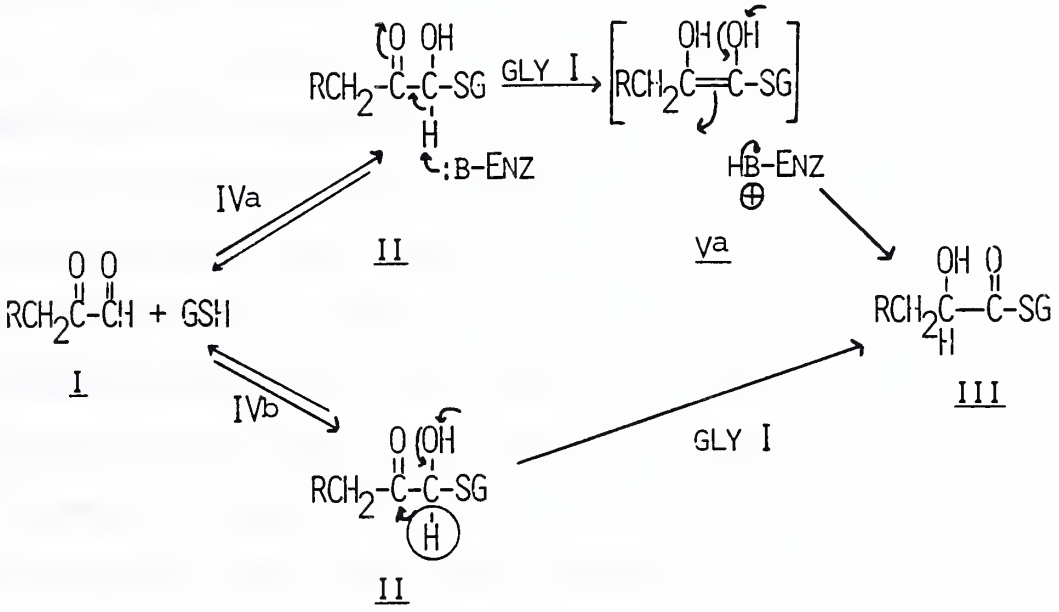
(77) who found through a series of isotope-trapping experiments that glyoxalase I operated on the mixture of thiohemiacetal diastereomers. Chari & Kozarich (78) have recently deduced the absolute stereochemical course of glyoxalase I. Their work supported the finding of a single stereochemical outcome of the two thiohemiacetal isomers when processed by glyoxalase I. However, this most likely occurred by an epimerization of the wrong isomer prior to the stereospecific proton abstraction by glyoxalase I which results in a single stereospecific product.

As stated previously (71,74,79), glyoxalase I was found to have a broad specificity for both aliphatic and aromatic α-ketoaldehydes (reflected by nearly identical V_{max}). However, the V_{max} dropped abruptly if the side chain of the α-ketoaldehyde was sterically crowded or polar in nature. Thus, the active site has a non-polar region and has a space constraint. Mannervik (80,81) has shown that sulfhydryl groups on the enzyme are not involved in glyoxalase's catalytic activity and that a tryptophan near the active site is involved in the binding of the substrate. Reed (82) using a similar experimental technique found an arginine residue near the active site that is involved in substrate binding via the GSH.

A metal ion at the active site was suggested by Davis & Williams (83) when they found that during purification a metal-chelator totally inactivated the enzyme. The activity was restored most effectively when Mg^{++} ions were added. They theorized that the divalent cation played a direct part in the catalysis of the thioester formation. Later, Mannervik and co-workers (84) found that it was actually Zn^{+2} that was tightly bound to glyoxalase I. They felt that the metal ion acted in the catalytic reaction or that it functioned to maintain the enzyme structure. Later work (85,86) by this group proved that the zinc ion was at the active site of the glyoxalase I enzyme.

The mechanism of glyoxalase I has been an area of as much controversy as that centered on the biological role of the glyoxalase enzyme system. Racker (69) proposed that the mechanism of glyoxalase I was through an enediol intermediate (Va, See Scheme IVa) which would proceed via a proton

SCHEME IV:



transfer. However, when Rose (87) ran the glyoxalase I reaction in tritiated water, he failed to observe the incorporation of tritium into the product and this contradicted the possibility of an enediol intermediate. Because the conversion of methylglyoxal to lactic acid resembled the Cannizzaro reaction, (an alkaline catalyzed reaction known to convert methylglyoxal to lactic acid by a direct 1,2-hydride shift) he proposed that the glyoxalase I reaction was analogous to the Cannizzaro reaction and therefore the mechanism was a direct 1,2-hydride shift (See Scheme IVb). These results were confirmed when Franzen (88) failed to detect the incorporation of deuterium in the product of the glyoxalase I reaction run in deuterated solvent.

Davis & Williams (83) supported a hydride shift mechanism which would proceed via a cyclic transition state of the thiohemiacetal (II) and the

newly discovered metal cation. Such a mechanism was a variation of the Meerwein-Ponndorf-Oppenauer reaction. Vander Jagt (79,89,90) favored the 1,2-hydride shift mechanism of glyoxalase I, however, some of his results made him question this conclusion. For instance, in his work on the broad specificity of glyoxalase I for α-ketoaldehydes, he found no substituent effect of the substituted α-ketoaldehydes on the rate of the reaction (79). If the mechanism was a hydride shift, one would expect to see a substituent effect depending on the polarity of the α-ketone carbonyl (due to the transition state stabilization by electron withdrawing groups). This substituent effect existed in the alkaline catalyzed Cannizzaro reaction (89). He felt that the above observations suggested that either the hydride shift was not the rate determining step or that the hydride migration was facilitated through the polarization of the α-ketone group by the enzyme. In the latter case the effects of substituents may be small and the hydride shift could still be rate determining (79). However, experiments (90) measuring deuterium isotope effects revealed that the hydride shift was rate determining, and he therefore concluded that the side chains of substituted α-ketoaldehydes do not make a significant contribution to the polarity of the α-ketone group because the enzyme polarizes the α-ketone group in some way.

Hall (91) studied the rearrangement of α-ketoaldehydes (I) and thiols to α-hydroxyesters (III) in the presence of a "general base" (e.g. a tertiary amine) and deuterated solvent and found deuterium incorporation in the α-hydroxyester. But it was not until 1976 when Hall, et al. (92) detected the incorporation of solvent protons into the product of the glyoxalase I reaction. The percentage of incorporation increased with temperature. His group proposed a mechanism whereby a fast proton transfer via an enediol was taking place in a highly protected active site. Experiments measuring deuterium isotope effects (93) indicated that the

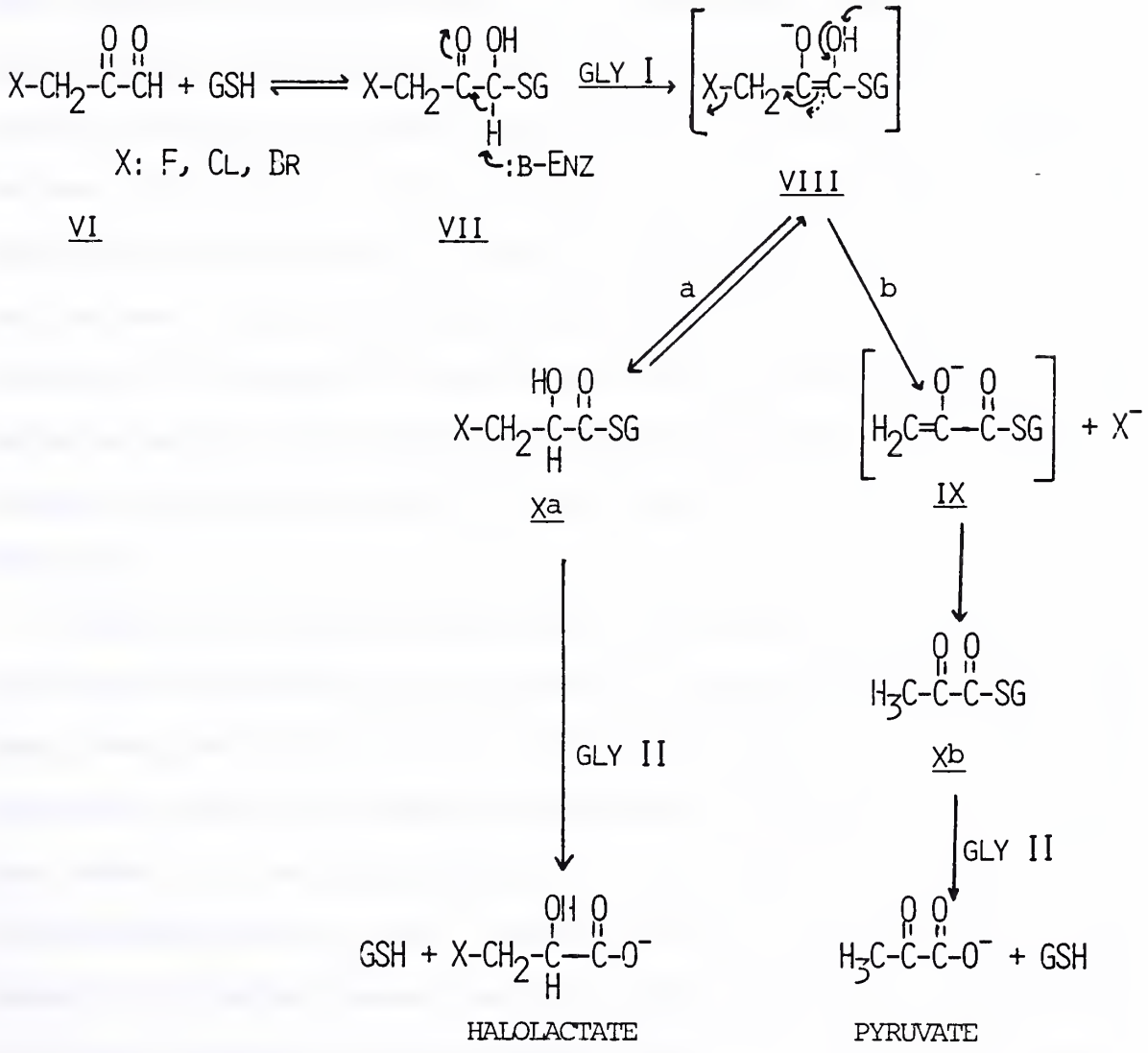
hydrogen transfer was rate limiting, but did not distinguish the nature of the hydrogen transfer. Another group (94) provided further evidence for a proton transfer mechanism when they detected the slow exchange of the hydroxymethylene proton of the product, S-(D-lactoyl)glutathione (III) with solvent.

A problem with solvent incorporation as a means of determining the reaction mechanism is the possibility of proton exchange occurring by a fortuitous side reaction after the catalytic step and prior to product release. This had led researchers to continue to investigate the reaction mechanism of glyoxalase I. Shinkai (95) using a "flavin-trapping" technique provided evidence that the chemical reaction occurred via an enediol intermediate. Complete reaction inhibition was seen in a test model system of the general base catalyzed rearrangement of thiohemiacetals to the corresponding α-ketoacids in the presence of a flavin compound. Flavin compounds react with transient carbanion intermediates, but do not serve as efficient hydride acceptors.

Recently, Kozarich (96-98) designed experiments which definitively proved the glyoxalase I mechanism to be that of a proton transfer. The design (98) centered around the expected differences in the charge densities on the C-2 position of the ketoaldehyde. In the enediol mechanism (Scheme IVa) one would expect the buildup of carbanionic character at this carbon in order to enhance proton transfer from the active site base. In the hydride shift pathway (Scheme IVb) one would expect an increase in carbonium ion character at this carbon in order to facilitate the transfer of the hydride from the C-1 position of the thiohemiacetal. With the proper substrate, one could distinguish between the two mechanisms. The group synthesized halomethylglyoxals (VI) which would be sensitive to the charge density on the C-2 carbon. Thus, if the enediol mechanism was correct one would expect the possibility of halide

elimination (Scheme V). However, one would expect no halide elimination if

SCHEME V: (adapted from 96)



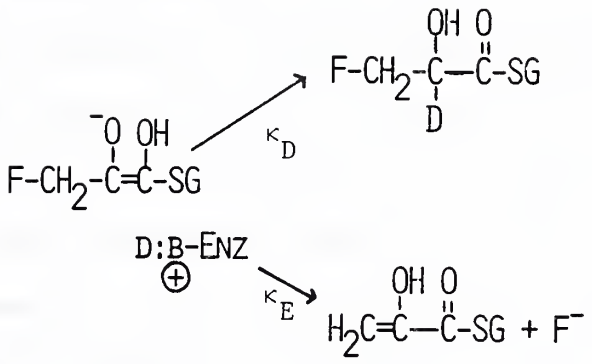
the hydride transfer mechanism was operating. And, in fact, under typical Cannizzaro reaction conditions, no halogen was released. The above experimental design eliminated the possible ambiquity of solvent incorporation.

Fluoromethylglyoxal (VI; X=F) was studied (96) and the results were consistent with a rapid proton-transfer mechanism. As hypothesized, the enzymatic reaction with glyoxalase I yielded total fluoride release which could best be accounted for by a carbanionic character at C-2. If the

same reaction was performed in the presence of both glyoxalase I and II, then three products were obtained (See Scheme V). ^{19}F-NMR determined that the only fluorine containing products were fluorolactate and fluoride. The other product was pyruvate (determined to be present enzymatically). The above results could best be accounted for by Scheme V, which shows the unique glyoxalase I catalyzed partitioning of the enediol intermediate between protonation to yield S-fluorolactoylglutathione (Xa, path a) and elimination of fluoride to first form an enol (IXb, path b) which then tautomerizes to form S-pyruvylglutathione (Xb, path b). Hydrolysis of the thioesters by glyoxalase II yields the products fluorolactate (path a) and pyruvate (path b) which undergo no further reactions. This example of product partitioning provided strong support for the proton transfer mechanism.

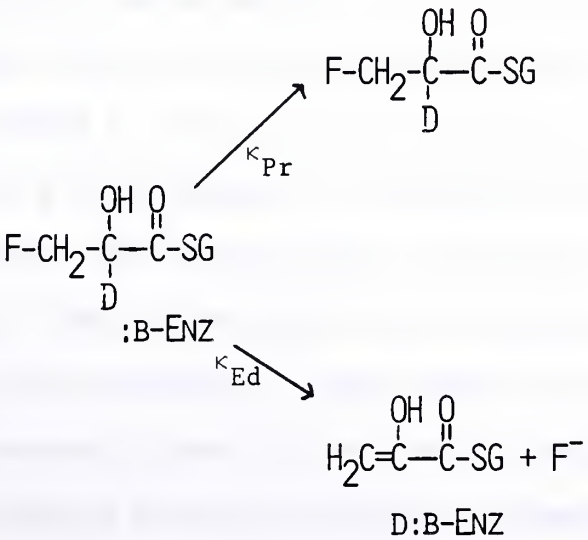
However, the results outlined above could also be accounted for by a fortuitous side reaction by an active site base after the catalytic step. Thus, the possibility of a 1,2-hydride shift was not ruled out. The definitive proof of the enediol intermediate of the glyoxalase I reaction was shown by isotope effects on the product partitioning of fluoromethylglyoxal-d_1, (97). Isotope effects in the past (90,93) were unable to distinguish between the two possible mechanisms. The difference in Kozarich's design was that an isotope effect on the unique partitioning of products of the glyoxalase system reaction would allow one to distinguish between proton transfer and hydride shift with β-elimination. Since the observed partitioning measures the competition of two kinetic processes (i.e. fluoride elimination versus protonation) the selective disruption of one of these rates would have resulted in a change in the observed partitioning. If the enediol mechanism was correct (Scheme VI), abstraction of the deuterium from C-1 by the active base would result in

SCHEME VI:[97]



the formation of the enediol and deuterated base. Now a primary isotope effect on the reprotonation of the enediol at C-2 would result in a delay in the formation of fluorolactoylglutathione and the overall effect would have been to <u>increase</u> fluoride elimination (k_D is selectively decreased by the isotope effect on the dueterated base, Scheme VI). The opposite result was expected if the hydride shift mechanism was operative. A 1,2-deuteride shift from the thiohemiacetal would have yielded deuterated fluorolactoylglutathione directly (Scheme VII). The primary isotope effect

SCHEME VII:[97]



would result in a decrease in the rate of elimination and the overall effect would have been to decrease fluoride elimination (k_{Ed} is selectively decreased by the isotope effect on deuterated C-2, Scheme VII). Chari & Kozarich's results (97) clearly demonstrated an increase in the amount of fluoride released when the deuterated substrate was compared to the non-deuterated compound. This finding was only consistent with a primary isotope effect on the partitioning reaction involving a proton transfer to an enediol intermediate. Thus, this established a compelling argument for the proton transfer mechanism of glyoxalase I first proposed over thirty years ago (69).

During Kozarich & Chari's studies with halomethylglyoxals, they discovered a unique example of "inverse" substrate processing by glyoxalase I. This work (99) has re-emphasized the need for glutathione for substrate binding, as well as the requirement of thiohemiacetal formation for catalysis. It also gives further information about the "size" of the enzymatic active site, which in the past (74) was found to have a poor fit with sterically hindered side chains of substituted α-ketoaldehydes. Scheme VIII depicts the method by which Kozarich & Chari were able to form β-(alkylthio)-α-ketoaldehydes (XI) and specifically the glutathione adduct (XI, R=GSH). When β-(glutathiomethyl)glyoxal was processed through Scheme VIII, pathway a, the majority of product formed was L-lactate. Thus, one observed a total reversal of the expected stereochemistry of the glyoxalase enzyme. The investigators, therefore, proposed that the substrate binds to the active site in an inverted manner allowing the stereochemistry to be reversed. They termed this process "mirror-image" catalysis and proposed Figure 1 as a possible explanation. Figure 1A depicts normal binding in which glyoxalase processing would form the D-isomer. Figure 2B illustrates the inverted binding of the "inverse"

SCHEME VIII: (adapted from 99)

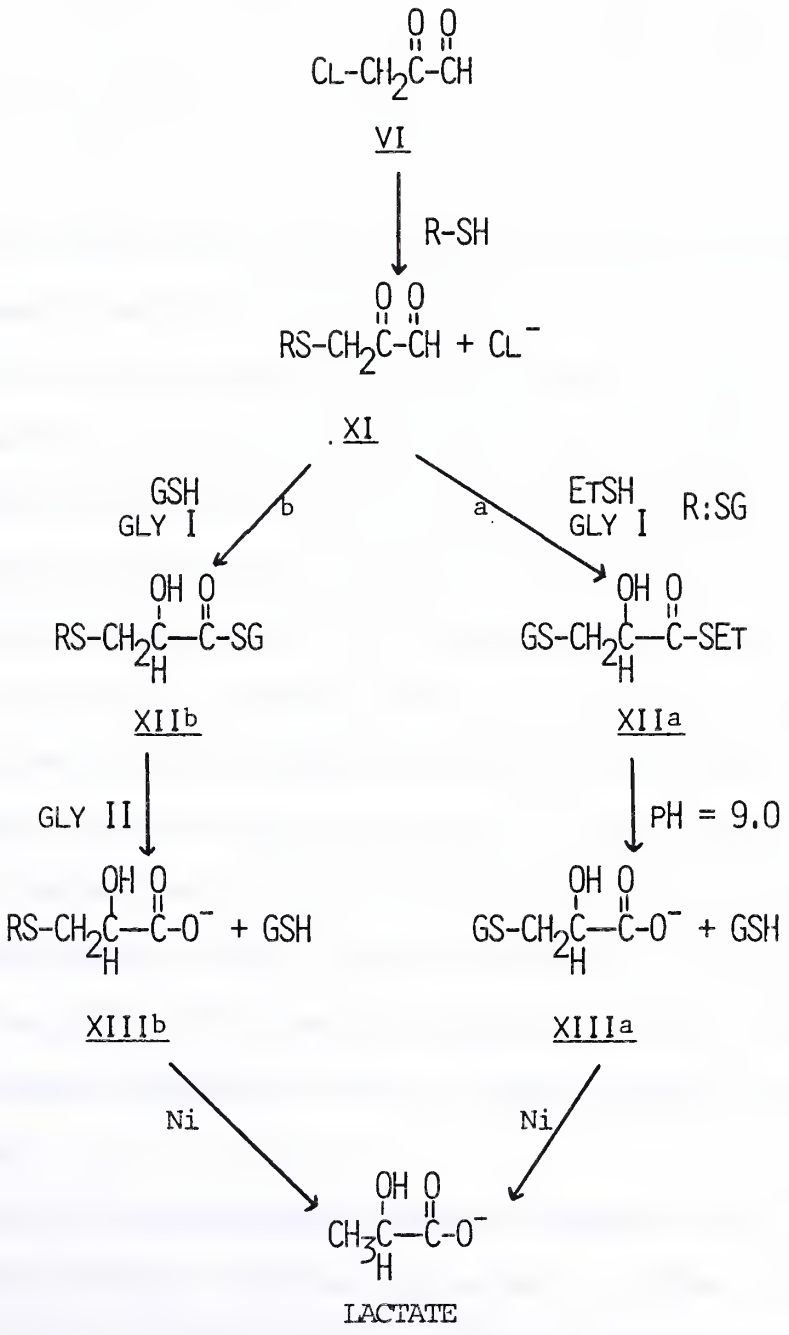
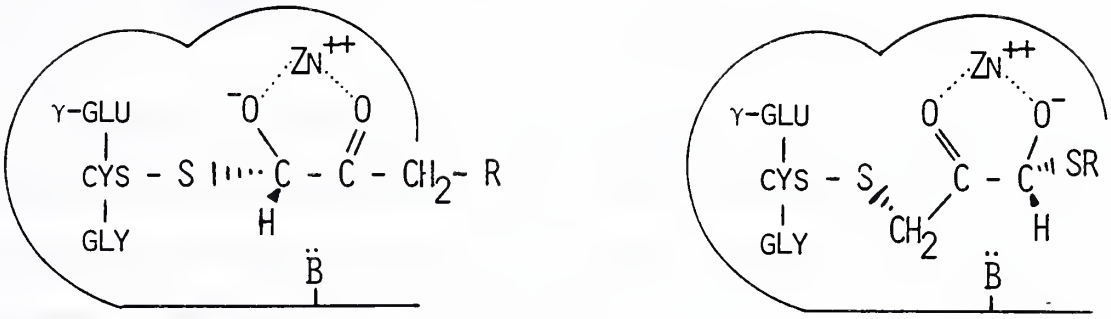


FIGURE 1: (99)



A. B.

substrate which due to the constraints of the system necessitates the opposite processing resulting in the L-isomer.

Glyoxalase I [S-lactoylglutathione methylglyoxal-lyase (isomerizing) EC 4.4.1.5] has been isolated in high purity from a number of different sources ranging from yeast (90), mouse liver (38,100,101), sheep liver (102), and various tissues from rat (103). The molecular weight of the mammalian enzyme has been found to be between 45,000-52,000. In contrast to the yeast enzyme, Oray & Norton (100) were the first to discover that mammalian glyoxalase I consisted of two identical subunits. It appears that each subunit has an active site (80,81). Vander Jagt (103) showed that within a species there is only a single form of the enzyme. However, between species, the activities, properties and amino acid composition of glyoxalase I vary (104). This has been supported by Chari & Kozarich (97) who found different partitioning ratios of products depending on the origin of enzyme used.

Glyoxalase II [S-2-Hydroxyacylglutathione hydrolase, EC 3.1.2.6) which catalyzes the hydrolysis of the thioester to the corresponding α-hydroxyacid has also been highly purified. Like glyoxalase I it is highly specific for GSH, but unspecific for the acylgroup of the thioester (105). Its molecular weight is about 30,000 (106) and its activity is normally less than than of glyoxalase I (107).

> ...I believe...[methylglyoxal] is the universal brake of proliferation...and glyoxalase is its switch.
>
> -A. Szent-Györgyi (14)

Because one of the major roles suggested for the glyoxalase enzyme system is that of regulation of cell growth and division, the possibility exists that cancer cells have lost their ability to maintain a proper balance of methylglyoxal. Attempts have been made to try and influence the complex equilibria between methylglyoxal, glyoxalase, and lactic acid. α-Ketoaldehydes are known growth inhibitors. The problems of achieving suitable doses of methylglyoxal or other reactive ketoaldehydes for systemic control of cell growth and division are due to their inherent toxicities, as well as the widely distributed glyoxalase system which causes their inactivation. Another approach to disturb the regulatory system has been the development of inhibitors of glyoxalase I, the enzyme which converts α-ketoaldehydes and glutathione to thiohemiacetals. In the past, this enzyme has been shown to have increased activity in cancerous and regenerating tissues. Therefore, by inhibiting this enzyme one could possibly disrupt growth by allowing the intracellular buildup of growth inhibiting substances. Another goal in the development of enzyme inhibitors is their usefulness as probes in elucidating the mechanism and possible biological significance of an enzyme system.

Research first centered around the design of competitive inhibitors of glyoxalase I. Kermack & Matheson (108) found that various analogues of GSH, the most active having a large alkyl group substitution, to be pure competitive inhibitors of glyoxalase I. Similar inhibitors were developed by Vince and co-workers (109,110) whose compound, S-p-bromobenzylglutathione, is presently the best reversible inhibitor known, with 50% enzyme inhibition K_i of 0.009 mM. They found these compounds to be cytotoxic versus L1210 leukemia and KB cells in tissue culture. Vince

(110) stated that by increasing the intracellular methylglyoxal, one inhibited protein synthesis. Protein inhibition occurred by methylglyoxal binding to RNA guanine residues. This adduct was stable at acid pH (111) and therefore Vince hypothesized that one might be able to have selectivity in methylglyoxal's effect on cancer versus normal tissue. Norton (112), using the fact that S-substituted glutathiones strongly interacted with glyoxalase I, developed S-substituted glutathiones for the use as ligands for the affinity chromatography purification of glyoxalase I.

Vince (113,114), noted that the S-substituted competitive inhibitors were rapidly metabolized in vivo by glutathionase, rendering the inhibitors inactive. He then developed glyoxalase I inhibitors which could resist degradation. However, these S- and N- substituted cysteinylglycines exhibited non-competitive inhibition of glyoxalase I and Vince therefore concluded that they were binding to a site other than the active site. Recently (115) esters of several glutathione analogues were synthesized in which the glycine was replaced by a straight-chain fatty acid and the mercapto group was benzylated. This type of compound, which inhibited glyoxalase I, was not hydrolyzed by glutathionase or cysteinylglycinase and has high lipohilicity. However, they did not inhibit P388 lymphocytic leukemia in mice.

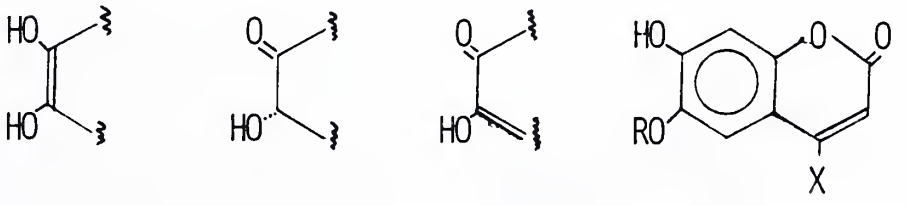
Hall (116) synthesized α-hydroxythiol esters and found them to be competitive inhibitors of glyoxalase I. However, when tested against L1210 leukemia cells, he found no antitumor activity and thought this might be due to the ease of hydrolysis of the compound in vivo.

Oray & Norton (117) found that both glyoxalase I and II from different tissues were equally inhibited by nucleotides and nucleosides in an apparently cooperative manner. They raised the point that intracellular levels of the tested nucleotides are at concentrations higher than what was needed to inhibit both enzymes. The physiological significance of this has

not been shown. Another interesting competitive inhibitor of glyoxalase I was studied by Douglas (118). He showed that yeast glyoxalase I was inhibited by porphyrin derivatives, possibly by active site occlusion. As referred to earlier (28), γ, δ-dioxovalerate, the transamination product of the first committed intermediate of porphyrin biosynthesis, is a known substrate of glyoxalase I. This possible complex interrelationship of glyoxalase with the heme biosynthetic pathway has not been worked out.

From the above data, it appears that some competitive inhibitors are excellent glyoxalase I inhibitors *in vitro*. However, mostly because of their own metabolism and inactivation, competitive inhibitors are not likely to be useful inhibitors of glyoxalase I *in vivo*. Therefore, attention turned to the development of mechanism-based, transition-state (119) inhibitors where tighter binding of the transition state analog occurs. The mechanism of glyoxalase I is known to proceed through an enediol intermediate. Therefore, Douglas & Nadvi (120) synthesized compounds designed on transition state analogy. The compounds all resembled the enediol intermediate, (See Figure 2) and showed excellent

FIGURE 2: (119)



a. b. c. d.

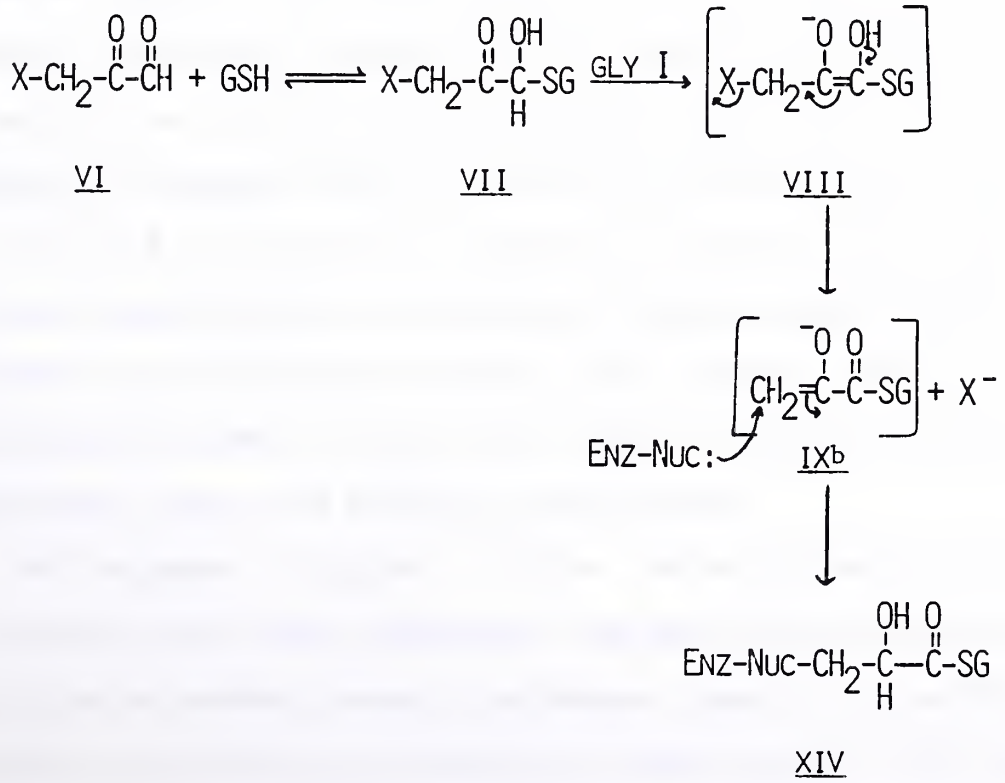
inhibition, but had inhibition constants no better than the competitive inhibitor p-bromobenzylglutathione and are therefore excluded as tansition state analogs. Similarly, Brandt, et al. (121) selected compounds to test on their similarity to the enediol intermediate of the glyoxalase I

reaction. They found that some derivatives of coumarin (See Figure 2d) to be the most effective inhibitors of this type with a K_i of 0.03 mM. But this still is not as good as inhibition by p-bromobenzylglutathione. Therefore, no effective transition state based inhibitor has been found for glyoxalase I.

> Suicide inactivators...depend upon the specific catalytic capabilities of the active site.
> -Abeles & Maycock (122)

The main goal of my project was to synthesize a potential mechanism-based, suicide inhibitor of glyoxalase I. Kozarich and co-workers (96) were aware of the possibility that their halomethylglyoxals could function as mechanism-based inhibitors of glyoxalase I. They proposed a mechanism (see Scheme IX) whereby the transient enol (IXb) of S-pyruvylglutathione

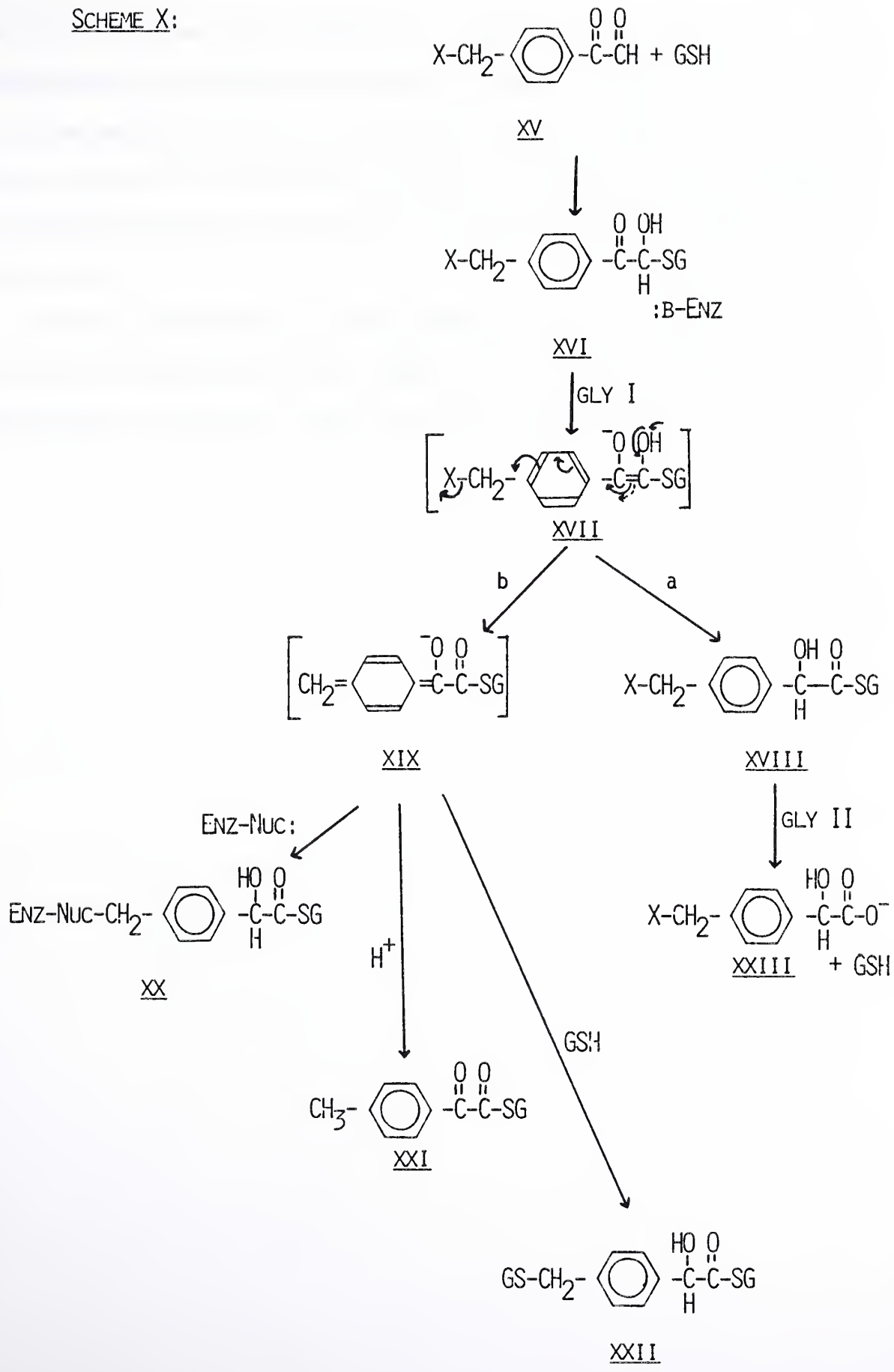
SCHEME IX:



generated by halide elimination, might be susceptible to Michael addition by an active site nucleophile. This would result in covalent modification and inactivation of the enzyme (XIV). Kuo, et al. (123) have shown that the enol of pyruvate is sufficiently long lived for Kozarich's proposed Michael addition to occur. However, no inactivation was detected (96). Trapping experiments (98) suggested the presence of (IXb) in solution. Therefore, inactivation might not have been seen because an active site base was not accessible to the enol (IXb) for attack, or that IXb was not sufficiently active to undergo Michael addition. In order to take advantage of the enediol intermediate, p-halomethylphenylglyoxals were synthesized. These compounds could divert the enediol to a highly reactive species which could covalently modify glyoxalase I and inactivate it (See Scheme X). When the thiohemiacetal of XV and GSH react with glyoxalase I, the corresponding enediol (XVII) is generated. The intermediate could follow path a, Scheme X, and undergo fast-shielded protonation to yield the thioester (XVIII). Or the enediol could follow path b, Scheme X, and eliminate the halogen to generate the very reactive quinoid-like structure XIX which is an electron deficient species. XIX would then be highly susceptible to Michael addition by an active nucleophile which could attack it and form a covalently modified enzyme, XX. Thus, the halomethylphenylglyoxals are potential mechanism-based inhibitors of the glyoxalase enzyme system and therefore might ultimately prove to be useful antineoplastic agents, as well as be useful in further elucidating the physiological role of the glyoxalase enzyme system.

The compounds I prepared, p-bromo- and p-fluoromethylphenylglyoxal had previously never been synthesized. They were studied with glyoxalase I *in vitro* using standard enzymatic techniques. Some of the products of the enzymatic reactions were identified by NMR analysis. The reaction mechanism of an intermediate enediol was studied by employing techniques of

SCHEME X:


X-CH2-
-C-CH + GSH
XV
X-CH2-
-C-C-SG
H
:B-ENZ
XVI
GLY I
X-CH2-
-C≡C-SG
XVII
b
a
CH2=
=C-C-SG
X-CH2-
-C—C-SG
H
XIX
XVIII
ENZ-NUC:
GLY II
ENZ-NUC-CH2-
-C-C-SG
H
XX
X-CH2-
-C-C-O
H
XXIII
+ GSH
H+
GSH
CH3-
-C-C-SG
XXI
GS-CH2-
-C-C-SG
H
XXII

specific ion electrode analysis. Inhibition of glyoxalase I was investigated using standard techniques of kinetic analysis. The compounds were also tested in cell culture against L1210 and P388 cell lines as an initial screen for antitumor activity. Finally, the halogenated methylphenylglyoxal's anti-cancer activity were tested in vivo in tumor-bearing mice.

The newly synthesized halomethylphenylglyoxals were also used to form p-glutathiomethylphenylglyoxal (XXIV) which was then studied as both a substrate and a potential inverse substrate of glyoxalase I.

MATERIALS AND METHODS

A. SYNTHETIC METHODS

Starting materials were of analytical grade from either Aldrich or Fisher Scientific, unless otherwise noted. Solvents were distilled and stored over molecular sieves. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H-NMR spectra were routinely recorded in the noted solvent on a Varian T-60A Analytical NMR Spectrometer. The chemical shifts are expressed in δ values (parts per million) relative to Me_4Si internal standard. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta. Analyses are indicated by symbols of the elements and represent analytical values which were within $\pm$ 0.1 % of theoretical values. Ultraviolet spectra were determined in a potassium phosphate buffer with a Beckman Model 35 Spectrophotometer. Mass spectra data was obtained on a Hewlett-Packard 5985 GC Mass Spectrometer.

Purification of N-bromosuccinimide (NBS) -- Following a literature procedure (124), NBS (25g) was added to boiling distilled water (250 ml) and swirled for about two minutes until all the crystals were dissolved. The resulting yellow solution was poured through a fluted filter into a flask immersed in an ice-water bath and allowed to stand in the ice bath for two hours. The resulting mixture was then filtered through a Buchner funnel with suction. The white crystals were washed with two portions of ice-cold water (50ml) and then dried first under water vacuum and then in a lyophilyzer for 24 hrs. Yield: 58%.

Synthesis of p-bromomethylacetophenone -- Following a previously reported procedure (125), NBS (14.0 grams; 0.079 moles), benzoylperoxide (10 mg),

and p-methylacetophenone (10.6 g; 0.079 moles) were mixed in carbon tetrachloride (90 ml). This solution was held at reflux and irradiated with a 275-W sun lamp for about one hour (until the originally floating NBS appeared to be converted entirely to succinimide which sinks in CCl_4). The solution was filtered and the solvent removed by rotary evaporator. The liquid was distilled under reduced pressure to yield 10.6g of p-bromomethylacetophenone (63% yield), bp 99-100° (0.05 mmHg), NMR ($CDCl_3$): δ =7.2-7.8 (q,4H), 4.4 (s,2H), 2.2 (s,3H).

p-Bromomethylphenylglyoxal [The proper name is 4-bromomethyl-α-oxo-benzeneacetaldehyde, XV] — This compound was synthesized by oxidation of p-bromomethylacetophenone with selenous acid following a modified literature procedure (126). A solution of selenous acid (2.24 g; 0.017 moles) and dioxane (15 ml) was heated at ~55°C for 15 minutes until all the solid had gone into solution. p-Bromomethylacetophenone (3.89 g; 0.018 moles) was added in one lot and the reaction mixture, under a constant stream of argon gas, was slowly brought to reflux and held at 98-100°C for 2 hours. The warm solution was decanted off the precipitated black selenium metal and concentrated on a rotary evaporator. Column chromatography was performed to isolate the desired reaction product. A glass column (inner diameter = 3.5 cm; length = 80 cm) was filled with silica gel (175 g). A total of ~2 liters of solvent starting with 20% ethylacetate in hexanes and ending with 100% ethylacetate was used for product elution. A Buchler Automatic Fraction collector was used to collect ~20 ml fractions. The isolated products within each fraction were identified by thin layer chromatography (TLC eluting solution: 50% hexanes, 50% ethylacetate). The desired product, p-bromomethylphenylglyoxal eluting at 20% hexanes and 80% ethylacetate solution, was concentrated under reduced pressure and determined to be >95%

pure by nmr. (Column yield: 37%) mp. 101-103°C; NMR (acetone-d_6): δ=9.2 (s,0.2H), 6.9-7.6 (q,4H), 5.4 (s,0.6H), 4.2 (s,2H); NMR (D_2O): δ =7.1-7.8 (q,4H), 5.6 (s,1H), 4.3 (s,2H), 4.0 (H_2O); λ_{max}=262nm, ε_{262}=~15,800 $M^{-1}cm^{-1}$. A sample was recrystalized twice from ethylacetate and determined to be the monohydrate by elemental analysis. Anal. ($C_9H_9BrO_3$) C,H,Br. EI mass spectrum, m/e (relative intensity): 197,199(M^+-CHO, 100), 169,171 (M^+-COCHO,5), 147(M^+-Br,10), 118(M^+-Br-CHO, 100), 90(M^+-Br-COCHO, 100).

p-Fluoromethylacetophenone -- p-Fluoromethylacetophenone was synthesized from p-bromomethylacetophenone using a fluorinated resin (127). The anion exchange resin (1 kg; Sigma, Amberlite IRA-900; Chloride) was first converted to the hydroxy-form by washing it with 1N NaOH (3 liters) followed by warm distilled H_2O (~10 liters) until the wash solution pH turned ~7.0. In a completely glass-free aparatus, the resin was then converted to the fluoride-form by washing it with 1 M hydrofluoric acid (2.5 liters) followed by washings with H_2O (~15 liters) until washings reached near neutral pH. The resin was then washed with 95% EtOH (~1 liter) and then with benzene (~250 ml). The resin was dried overnight in a dessicator under vacuum, then rotovapped. A portion of this partially dried resin (250 g) was suspended in dry benzene (previously dried over sodium metal, distilled, and then stored over molecular seives) for additional dehydration which was performed by refluxing it for 24 hrs in a Soxhlet extractor containing molecule sieves. The benzene was then removed by vacuum and low heat. The fluorinated resin (64 g; 3.4 mequiv. F^-/gram) was suspended in 375 ml of pentane. p-Bromomethylacetophenone (5.7 g; 0.027 moles) was added and the vigorously mixed solution was slowly heated to reflux and left refluxing for ~2 days. The liquid was decanted off the resin and concentrated under reduced pressure to yield 0.35 g (9%) of product. Ether washings of the resin resulted in the collection of 0.5 g

(12%) of product. Methylene chloride washings (total of 400 ml) yielded 2.4 g (59%) of desired product, NMR ($CDCl_3$): δ=7.2-8.0 (q,4H), 5.8 (s,0.7H), 5.0 (s,0.7H), 2.5 (S,2.9H). The combined samples were distilled under reduced pressure to yield 1.8 g (overall yield 45%) of >95% pure p-fluoromethylacetophenone, bp 45°C (0.1 mmHg), NMR ($CDCl_3$): δ=7.2 - 7.9 (q, 4H), 5.8 (s,1H), 4.9 (s,1H), 2.5 (s,3H) Anal. (C_9H_9FO) C,H.

p-Fluoromethylphenylglyoxal - (The proper name is 4-fluoromethyl-α-oxo-benzeneacetaldehyde). This compound was synthesized in the same manner as the bromo-analog. Thus, fluoromethylacetophenone (0.95 g; 0.0063 moles), selenous acid (0.84 g; 0.0065 moles), and dioxane (5 ml) were reacted under argon gas for 2 hrs. The crude reaction product was then purified on a 55 gram silica gel column (inner diameter = 2.2 cm; length = 50 cm). The desired product, p-fluoromethylphenylglyoxal-monohydrate, eluted with a 35% hexanes and 65% ethylacetate solution in 60% yield. mp. 120-122°C, NMR (acetone-d_6, poorly soluble): δ=6.8-7.7 (q,4H), 5.3 (s,0.7H), 5.0 (1.6,H), 4.5 (s,0.7H). λ_{max}=253, ε_{253}=~12,400 $M^{-1}cm^{-1}$. Anal. ($C_9H_9FO_3$) C,H.

B. ENZYMATIC METHODS

All pH measurements were determined with an Orion Research model 601A Digital Ionalyzer equipped with an Orion Research Semi-micro Combination pH Electrode. Enzymatic reactions were usually carried out in a 0.05 to 0.2M potassium phosphate buffer at a pH between 6.0 to 7.5. Ketoaldehyde and GSH concentrations ranged between 0.1 to 2.0 mM. Glyoxalase I [S-lactoylglutathione methylglyoxallyase (isomerizing) EC 4.4.1.5 (Sigma, Yeast, Grade X)] was commonly used at 1 to 3 units per ml of reaction

mixture where 1 unit is defined as the amount of enzyme which will convert 1.0 umole of substrate to product per minute at pH 6.6 at 25°C. Glyoxalase II [S-2-hydroxyacylglutathione hydrolase EC 3.1.2.6 (Sigma, Beef liver)] was used at 0.5 to 1.5 units/ml of reaction mixture where 1 unit is defined as the amount of enzyme that would hydrolyze 1.0 umole of S-lactoylglutatione per minute at pH 7.4 at 25°C. Reactions were followed by observing the decrease in absorbance of the halogenated methylphenylglyoxals at the noted wavelengths on a Beckman Model 35 Spectrophotometer. Conditions of individual enzymatic experiments, including inhibition studies are reported in detail in the Results Section. Attempts were made to identify the products of the enzymatic reactions by both purification of the reaction mixture by passage through a Dowex-1 formate column and by high pressure liquid chromatography. These samples, as well as actual enzymatic reaction mixtures were analyzed on a Bruker WM 500-MHz NMR Spectrometer. Fluoride ion and bromide ion release were followed with an Orion Research Digital Ionalyzer (Model 601A) equipped with either an Orion Fluoride Electrode (Model 96-09) or an Orion Bromide Electrode (Model 94-35).

Ketoaldehyde Stock Solution - Example of preparation: A stock 50 mM bromomethylphenylglyoxal-monohydrate solution would be prepared by dissolving 5.1 mg (0.021 mmoles) of compound in 420 ul of methanol or ethanol. Typical enzymatic reactions were run at a ketoaldehyde concentration of 0.2 mM. Therefore, 4 ul of stock solution would be added to a 1 ml aqueous reaction mixture.

GSH Stock Solution - Because glutathione is easily oxidized, GSH solutions were kept cold and prepared fresh daily. A stock 50 mM GSH solution was prepared by dissolving 15.4 mg (0.05 mmoles) glutathione

(Sigma) in 1000 ul water. Typical enzymatic reactions had an equimolar to a ten times excess of GSH based on the ketoaldehyde concentration.

1. Enzyme-Substrate Studies

Spectrophotometric Studies - The halogenated methylphenylglyoxals were studied as substrates of the glyoxalase enzyme system. Reactants were mixed in a spectrophotometer cuvette (either 10 mm or 3 mm UV path length) in order to follow the course of the reaction spectrophotometrically. Thus, to a 3 mm path cuvette containing 974 ul of pH 6.0, 0.1 M potassium phosphate buffer, 8 ul of 46 mM stock substrate solution (final reaction concentration = 0.37 mM) and 16 ul of 51 mM stock solution (final reaction concentration = 0.8 mM) were added. The reaction was then followed spectrophotometrically with no enzyme present, with glyoxalase I (1 unit/ml of reaction mixture), or with glyoxalase I (1 unit/ml) and glyoxalase II (0.5 unit/ml of reaction mixture). Reactions containing p-fluoromethylphenylglyoxal were followed at λ=256nm. Reactions containing p-bromomethylphenylglyoxal were followed at λ=262nm.

High Resolution NMR Studies - The reactions of p-bromomethylphenylglyoxal and GSH with the glyoxalase enzyme system were also followed by high resolution NMR. Reactions were carried out in an NMR tube containing buffer made with deuterated water and analyzed with a 500 MHz NMR spectrometer. Bromomethylphenylglyoxal (3.2 mg, 0.013 mmoles) was disolved in 100 ul of deuterated methanol (CD_3CD_2OD). A 200 mM GSH solution was made by dissolving GSH (30.8 mg, 0.1 mmoles) in 500 ul of deuterated water. To 564 ul of a pD 6.1, 0.05 M potassium phosphate solution, 20 ul of the p-bromomethylphenylglyoxal solution (final concentration in reaction mixture = 4.4 mM) and 14 ul of the GSH solution

(final concentration in reaction mixture = 4.8 mM) was added and an NMR spectrum of the formation of the thiohemiacetal was recorded. Addition of 2 ul of a glyoxalase I solution containing 5 units/ul of solution (final concentration of enzyme in reaction mixture =~17 units/ml) resulted in the conversion of the thiohemiacetal to its corresponding thioester.

Isolation of Products for High Resolution NMR -- Attempts were made to isolate the products of the reaction of p-bromomethylphenylglyoxal and GSH in the presence of glyoxalase I and II. Thus, to a pH 6.0, 0.1 M potassium phosphate buffer (30 ml), GSH (61.1 mg, 0.2 mmoles) was added. Because the pH fell to 4.7, 1 N NaOH was added to raise the pH to 6.0. Glyoxalase I (120 ul, 60 units), glyoxalase II (120 ul, 12 units), and a methanol solution of p-bromomethylphenylglyoxal (24.2 mg; 0.1 mmoles in 200 ul MeOH) was then added and the reaction mixture was reacted for 1 hr under an argon atmosphere. The reaction mixture was then loaded onto a Dowex-1 formate column (outer diameter = 1.3 cm; length = 50 cm) at 3KC. The product was eluted off the column with a 0 to 6 M formic acid linear gradient (400 ml + 400 ml). Fractions of ~5 ml were collected by an automatic fraction collector. The presence of a product in a fraction was identified by measuring the absorbance of each fraction on a Beckman DU Spectrophotometer (Model 2400) at λ=266 nm. The fractions which contained product were combined and concentrated under reduced pressure. Before NMR analysis was performed, the sample containing product was washed numerous times with D_2O.

High pressure liquid chromatography (HPLC) was used to further purify the major product found by Dowex-1 formate column chromatography. The concentrated solution was placed on a Alltech HPLC Column (Cat. #8250; Frit size: 2 u; inner diameter = 4.6 mm; length = 25 cm; packing material:

Partisil 10 ODS2, size 10 u) in 50 ul alliquots and eluted with distilled water. The major peak detected by a Gilson UV Spectrophotometer at λ=266 nm was collected by numerous injections of the concentrated material from the Dowex-1 formate column onto the HPLC column. The combined samples were concentrated under reduced pressure, washed numerous times with deuterated water, and then analyzed by high resolution NMR.

2. "Inverse" Substrate Studies

Spectrophotometric Studies — p-Bromomethylphenylglyoxal was tested as an inverse substrate of glyoxalase I. Thus, 0.3 mM p-bromomethylphenylglyoxal-monohydrate was incubated with an equimolar amount of GSH at pH 7.5, 0.1 M potassium phosphate buffer for 3 to 6 hours under an argon atmosphere. The pH of this solution was then adjusted to a pH 7.2 by adding to it an equal volume of pH 7.0, 0.1 M potassium phosphate buffer. Glyoxalase I (1 unit/ml of reaction mixture) was added to this solution. (Any thiohemiacetal of p-bromomethylphenylglyoxal and GSH which had inadvertently formed was converted to its corresponding thioester and would undergo no further reactions.) Then, a ten times molar excess of either GSH (334 mM) or ethanethiol (334 mM; EtSH) was added. Reactions were followed by measuring the change in absorbance at λ=260nm. After observing any reaction for half an hour, an additional ten times excess of ethanethiol was added to the mixture already containing ethanethiol, followed by the addition of an excess of GSH. Reactions were continuously followed spectraphotometrically as noted above.

Isolation of Products for High Resolution NMR - The product of the "inverse" substrate reaction of p-(glutathio-methyl)phenylglyoxal and excess GSH with glyoxalase I and II was isolated and identified by high

resolution NMR. To a pH 7.5, 0.1 M potassium phosphate buffer (30 ml), GSH (120.9 mg, 0.39 mmoles) and p-bromomethylphenylglyoxal-monohydrate (22.8 mg, 0.09 mmoles in 200 ul of MeOH) was added and allowed to react at room temperature for ~2 hours. Glyoxalase I (60 units) and glyoxalase II (12 units) was then added and allowed to react for ~1 hr. This reaction mixture was then pumped onto a Dowex-1 formate column (outer diameter = 1.3 cm; length = 50 cm) at 3°C. A 0 to 6 M formic acid linear gradient (400 ml + 400 ml) was used to elute the products off the column. Fractions of ~5 ml were collected by an automatic fraction collector. The presence of a product in a fraction was identified by measuring the absorbance of each fraction on a Beckman DU Spectrophotometer (Model 2400) at λ=266 nm. The fractions that revealed the presence of a product were combined, concentrated under reduced pressure, and then washed numerous times with deuterated water.

3. Enzyme Inhibition Studies

An initial simple screening study was first carried out to determine if p-bromomethylphenylglyoxal would inhibit glyoxalase I activity. The initial velocity of the enzymatic reaction of 0.12 mM bromomethylphenylglyoxal and 2 mM GSH was observed with 0.5 units glyoxalase I/ml of reaction mixture. With the addition of more bromomethylphenylglyoxal, a new initial velocity was observed. As a control, an identical procedure was carried out with 0.12 mM phenylglyoxal.

A more detailed and rigorous study of the irreversible inhibitory effect of p-bromomethylphenylglyoxal on glyoxalase I was performed by incubating p-bromomethylphenylglyoxal (at 0.02, 0.2, and 2.0 mM) with two equivalents of GSH in the presence of 25 units glyoxalase I/ml of reaction

mixture in a pH 5.5, 0.1 M acetate buffer. At various time points an aliquot from this inhibition reaction mixture was added to an assay solution containing 0.4 mM methylglyoxal, 2 mM GSH in a pH 6.6, 0.1 M potassium phosphate buffer. The calculated enzyme concentration in this assay mixture after the transfer was ~0.1 units glyoxalase I/ml of solution. An initial velocity of the conversion of the thiohemiacetal of methylglyoxal to its corresponding thioester was measured by following the change in absorbance at λ =240nm. Control experiments included a run in which the "inhibitor reaction mixture" contained no p-bromomethylphenylglyoxal, but contained the proper amount of ethanol (the solvent p-bromomethylphenylglyoxal was dissolved in). A second type of control study, which contained no GSH in the inhibitor reaction mixture, was run at each inhibitor concentration. This would reveal non-enzymatic inhibition. A third type of "control" allowed the formation of the inverse substrate, p-(glutathiomethyl)phenylglyoxal. This involved the pre-incubation of p-bromomethylphenylglyoxal and GSH for 45 minutes in a pH 7.5, 0.1 M potassium phosphate buffer. To this solution, glyoxalase I was added and inhibition was followed as above.

4. Methods of Measuring Ion Release

Fluoride Ion Release - Fluoride release was determined following procedures outlined in the fluoride electrode Instruction Manual (128). A glass free apparatus was used. A calibration curve was made by mixing commercial standard samples (Orion) of known fluoride ion concentration with an equal volume of potassium phosphate buffer. Fluoride release during the enzymatic reactions (2 mM GSH, 0.5 mM fluoride substrate, glyoxalase I, 3 units/ml reaction mixture, glyoxalase II, 0.6 units/ml reaction mixture in 1000 ul of pH 6.0, 0.1 M potassium phosphate buffer)

was followed continuously using the fluoride ion electrode. Side by side controls were run using fluoromethylglyoxal, a known fluoride eliminating compound (96).

Bromide Ion Release - Bromide ion release was followed using modified procedures outlined in the bromide electrode Instruction Manual (129). Because of the bromide electrode's marked sensitivity to free thiols in solution (i.e. GSH), it was necessary to eliminate any free thiols before a bromide ion determination was performed. This involved stopping the enzymatic reaction by adding an excess of N-ethylmaleimide (NEM) which is known to react with free GSH (130,131) and thus protected the electrode from poisoning by free sulfhydryls. Calibration curves were made by mixing commerical standard samples (Orion) of known bromide ion concentration with an equal volume of potassium phosphate buffer containing GSH (1mM) and NEM (5mM). Bromide release during enzymatic reactions, run at the usual compound concentrations (i.e. 2 mM GSH, 0.5 mM bromide substrate, glyoxalase I, 3 units/ml, glyoxalase II, 0.6 units/ml in pH 6.0, 0.1 M potassium phosphate buffer), was measured with the bromide electrode by taking alliquots from the reaction mixture then adding it to a solution containing a five times excess of NEM. Bromomethylglyoxal (132) was used as a control. Because one was limited by the time it took for each bromide ion determination, a further modification of the procedure allowed the rapid time course of the reaction to be followed. This was done by removing 500 ul of the reaction mixture at the desired time points and adding it to 5 ul of 30% nitric acid. This lowered the solution's pH to 2.5, a level that inactivated both glyoxalase I and II. Bromide ion release was then measured with the electrode by taking this solution and adding it to an equal volume of a potassium phosphate buffer at pH 8.0, thus making the pH of the resulting solution 6.8. The GSH in solution was

then immediately "inactivated" by adding a five-times molar excess of NEM before a bromide reading was taken. Calibration curves for this type of experiment were generated by adding a buffer solution which had undergone identical modifications to the standard samples of known bromide ion concentration. Five types of bromide elimination studies were performed. Three experiments were controls in which bromide ion measurements were taken on bromomethylphenylglyoxal alone, bromomethylphenylglyoxal with GSH, and bromomethylphenylglyoxal with glyoxalase I. The two other experiments performed were with bromomethylphenylglyoxal and GSH in the presence of glyoxalase I and in the presence of glyoxalase I and II.

C. TISSUE CULTURE METHODS

Standard tissue culture experiments were run on P388 and L1210 mouse leukemia cell lines. The cells were maintained in suspension culture at 37°C in a 5% CO_2 humidified atmosphere. Three times per week, 10^5 cells/ml were passed in PRMI 1640 medium supplemented with 10% beef inactivated fetal calf serum (GIBCO), streptomycin (100 ug/ml), penicillin (100 units/ml), and 1% glutamine (100 mM). Agents were added 24 hours after seeding (10^5 cells/ml). Cell counts were taken every 24 hours for three days. Agents tested were run at approximately, 10^{-4}, 10^{-5}, and 10^{-6} M. The compounds used were of highest purity available. Standard solutions for this set of experiments were made by first dissolving the desired compounds in methanol to make a 50 mM solution. The appropriate volume was then added to 5 ml H_2O to make a 10^{-3} M solution. The compounds tested were: methylacetophenone, p-bromomethylacetophenone, p-fluoromethylacetophenone, phenylglyoxal, p-bromophenylyglyoxal, p-bromomethylphenylglyoxal and p-fluoromethylphenylglyoxal.

D. BIOLOGICAL TEST METHODS

The newly synthesized halogenated methylphenylglyoxals were evaluated for their anti-cancer activity against L1210 tumor bearing mice following previously described procedures (133). L1210 ascites cells were withdrawn from the peritoneal fluid of donor CDF_1 mice bearing 7-day growths. The suspension was centrifuged for 2 minutes, the supernatant decanted, and a 10 fold dilution with isotonic saline was made. The cell number was determined with a Coulter particle counter, and the cell population was adjusted to 10^6 cells/ml. Transplantation was then performed by injecting 0.1 ml (containing approximately 10^5 cells) of this solution intraperitoneally into each animal. The test compounds were administered by intraperitoneal injection, beginning 24 hours after tumor implantation followed by once daily injections for a total of 6 consecutive days. The test compounds were injected as fine suspensions following homogenization in 2 to 3 drops of 20% aqueous Tween 80 and then made up to the proper volume with isotonic saline. All test compounds were administered intraperitoneally in a volume of 0.5 ml. Anti-cancer activity of the test compounds were carried out at various concentrations. Bromomethylphenylglyoxal was tested at 1, 2.5, 5, 10, 25 and 50 mg/kg. Fluoromethylphenylglyoxal was tested at 10 and 50 mg/kg. For any one experiment, the mice were distributed into groups of 5 of comparable weight. Throughout the experiment, the mice had unlimited access to Purina Laboratory Chow pellets and water. Control tumor-bearing animals were given injections of comparable volumes of the vehicle solution not containing any test compound.

RESULTS

A. Synthesis of Halogenated Methylphenylglyoxals

Bromo- and fluoromethylphenylglyoxal were successfully synthesized from p-methylacetophenone. Methylacetophenone was selectively brominated with N-bromosuccinimide to yield p-bromomethylacetophenone. This compound was either directly oxidized with selenous acid to p-bromomethylphenylglyoxal or fluorinated with an ion-exchange resin to yield p-fluoromethylacetophenone. The fluorinated compound was then oxidized with selenous acid to p-fluoromethylphenylglyoxal. Both compounds were positively identified by NMR analysis and elemental analysis. The bromo-compound was also identified by mass spectroscopy. For details of syntheses and analyses see the Materials and Methods section.

B. Halomethylphenylglyoxals: A Substrate of Glyoxalase I and II

Bromo- and fluoromethylphenylglyoxal were found to be substrates of glyoxalase I and II. At enzymatic reaction conditions of 0.37 mM bromomethylphenylglyoxal and two equivalents of GSH at pH 6.0 one could follow the rate of the reaction spectrophotometrically by observing the decrease in absorbance at λ=260 nm. The conversion of the thiohemiacetal (λ_{max}=262 nm; ε_{262}=~14,300 $M^{-1}cm^{-1}$) of bromomethylphenylglyoxal and GSH to its corresponding thioester (λ_{max}=234; ε_{262}=~3,100 $M^{-1}cm^{-1}$) was enzyme dependent and could be accelerated by the presence of glyoxalase II. (See Table 1). This was most likely due to the regeneration of GSH which results in a higher concentration of the thiohemiacetal which is the substrate of glyoxalase I. At identical enzymatic reaction conditions the rate of the enzymatic conversion of the thiohemiacetal (λ_{max}=252 nm) of fluoromethylphenylglyoxal and GSH to its corresponding thioester

TABLE 1:

GLYOXALASE I (Unit/ml)	GLYOXALASE II (Unit/ml)	VELOCITY (OD/min) BrMeφG[1]	FMeφG[2]
1	-	0.81	0.44
1	0.5	0.77	0.47
1	1.5	1.00	
3	-	0.95	
3	1.5	1.13	

[1] BrMeφG: Bromomethylphenylglyoxal (0.37mM)
[2] FMeφG: Fluoromethylphenylglyoxal (0.33mM)

(ε_{256}=~2,500 $M^{-1}cm^{-1}$) appeared slower than that of the bromo-compound. At λ=256 nm, the initial velocity of the glyoxalase I (1 unit/ml of reaction mixture) catalyzed conversion of fluoromethylphenylglyoxal to its thioester was 0.44 O.D./minute. In the presence of glyoxalase I (1 unit/ml) and glyoxalase II (0.5 unit/ml of reaction mixture) the velocity was only slightly higher at 0.47 O.D./minute.

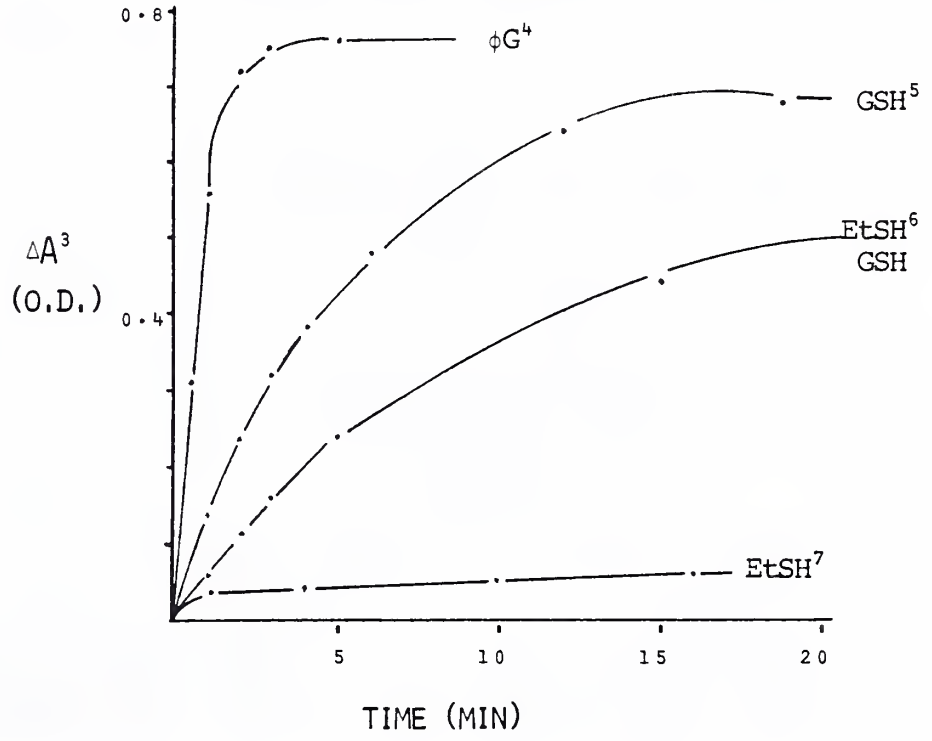
C. p-Bromomethylphenylglyoxal as a Precursor of an "Inverse" Substrate

It was noted that p-bromomethylphenylglyoxal and GSH underwent a time dependent change in absorbance at λ= 262 nm when mixed in a pH 7.2 to 7.5 potassium phosphate buffer. After about 80 minutes, no further significant drop in absorbance was noted. This suggested that at high pH, GSH was attacking bromomethylphenylglyoxal at the reactive brominated benzylic carbon forming glutathiomethylphenylglyoxal (XXIV) [λ_{max}=258]. This was first shown enzymatically by adding glyoxalase I to a previously incubated mixture of approximately equimolar bromomethylphenylglyoxal and GSH. The change in absorbance noted at λ = 260 nm was about less than a third of

the expected change in absorbance of enzymatic reactions at lower pH. This revealed that much of the GSH was in a form which prevented the formation of the thiohemiacetal. It appeared that the GSH was covalently attached to the benzylic carbon. To test if p-glutathiomethylphenylglyoxal could act as an "inverse" substrate of glyoxalase I, excess ethanethiol (EtSH) was added to the mixture to form the thiohemiacetal (XXVb). This activated the C-1 hydrogen making it acidic and allowed for potential proton abstraction by the active site base via inverse chemistry . In the presence of glyoxalase I, no change in absorbance was noted. However, when excess GSH was added, a large change in absorbance was noted, consistent with a typical glyoxalase I catalyzed reaction forming the corresponding thioester (ε_{262}=~3,500 $M^{-1}cm^{-1}$). At roughly 0.11 mM glutathiomethylphenylglyoxal and 10 equivalents of GSH the initial velocity of the glyoxalase I (1 unit/ml) catalyzed reaction was 0.14 O.D./min. When excess GSH was added to the reaction mixture containing ethanethiol, there was a typical change in absorbance noted, however, the initial velocity of the conversion of the thiohemiacetal to the thioester was about half the rate noted above. This finding was consistent with the competition of GSH and EtSH to form the thiohemiacetal of glutathiomethylphenylglyoxal. In this case, GSH must form the thiohemiacetal for processing by glyoxalase I. (See Figure 3). Thus while p-bromomethylphenylglyoxal forms glutathiomethylphenylglyoxal, it does not undergo "inverse" substrate processing by glyoxalase I. However, the glutathione substituted methylphenylglyoxal does undergo enzymatic processing in the presence of excess glutathione. As further proof of the above, the reaction product (XXVI) of glutathiomethylphenylglyoxal, excess GSH, and glyoxalase I and II was isolated by Dowex-1 formate column chromatography (first of two peaks; eluting at 1M formic acid) and identified as the proper product by high resolution NMR (See Figure 4).

FIGURE 3:

GLUTATHIOMETHYLPHENYLGLYOXAL[1] AS A SUBSTRATE OF GLYOXALASE I[2]



[1]Glutathiomethylphenylglyoxal was formed by incubating bromomethylphenylglyoxal (0.29mM) and GSH (0.3mM) for greater than 3 hours in a 0.1M potassium phosphate buffer at pH 7.5 under argon gas.

[2]After formation of the glutathiomethylphenylglyoxal, the solution was added to an equal volume of 0.1M potassium phosphate buffer at pH 7.0 (the final solution's pH = 7.2). Reactions were followed at λ = 260nm by first adding glyoxalase I (1 Unit/ml reaction mixture) to react with any excess GSH, then by adding the proper amount of thiol.

[3]ΔA = Change in Absorbance = Absorbance - Initial Absorbance.

[4]Control experiment containing approximately an identical concentration of phenylglyoxal in a 0.1M potassium phosphate buffer at pH 7.2, along with GSH (1.5mM) and Glyoxalase I (1 Unit/ml).

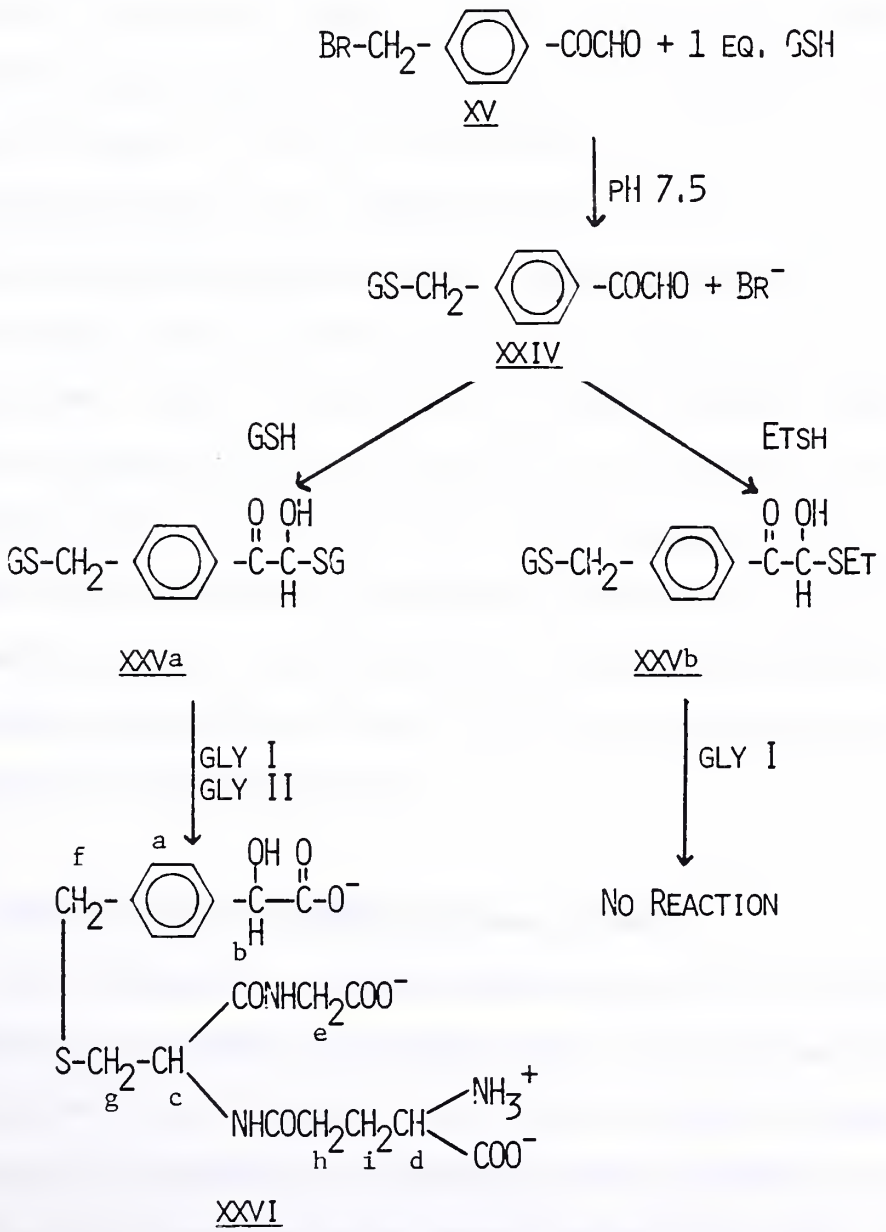
[5]Reaction mixture with excess GSH (1.5mM).

[6]Reaction mixture initially with excess ethanethiol (EtSH) (1.5mM), then with excess GSH (1.5mM).

[7]Reaction mixture with excess EtSH (1.5mM).

FIGURE 4:

FORMATION OF THE "INVERSE" SUBSTRATE AND IDENTIFICATION OF THE ENZYMATIC PRODUCT BY ^1H - NMR



a: 7.2(q,4H), b: 5.0(s,1H), c: 4.2(q,1H), d,e: 3.7-3.9(3H), f: 3.5(s,2H), g: 2.7(q,1H), g: 2.5(q,1H), h: 2.1(m,2H), i: 1.9-2.0(m,2H).

D. Fluoride Release by Fluoromethylphenylglyoxal?

Under typical enzymatic reaction conditions with glyoxalase I alone or with both enzymes present, absolutely no fluoride ion elimination was observed for fluoromethylphenylglyoxal. (However, as shown previously, this compound was determined to be a substrate for glyoxalase I). No fluoride ion release was noted at a pH range of 6.0 to 7.3 or after extended reaction times of greater than 60 hours. Thus, p-fluoromethylphenylglyoxal, while acting as a substrate of the glyoxalase enzyme system, does not eliminate fluoride ion. Nor does p-fluoromethylphenylglyoxal undergo attack by GSH at the benzylic carbon to form glutathiomethylphenylglyoxal. Control experiments studying fluoride ion release using fluoromethylglyoxal confirmed previously published results (96). Thus, at pH 6.0 and 7.3, total fluoride release was observed when fluoromethylglyoxal and GSH were incubated at standard reaction conditions with glyoxalase I only. When glyoxalase I and II were present a single burst of fluoride release was detected. The ratio of fluoride released to total fluoride was 0.35 which agrees with the previously published partitioning ratio of 0.32 for fluoromethylglyoxal - yeast glyoxalase enzyme system (96).

E. Bromide Release by Bromomethylphenylglyoxal

The bromide ion release experiments were fraught with many methodological problems, some which remain unresolved and therefore prevent quantification of results. The first problem of the bromide ion electrode's sensitivity to GSH was nicely overcome by quenching the reaction with N-ethylmaleimide before reading were taken. This procedure reproducibly eliminated the GSH from causing incorrect Br^- release measurements. The unresolved problem which prevented the quantification of data was the apparent instability of the tested compounds in the presence

of the bromide electrode. Thus, when one of the bromide containing compounds was present with the bromide electrode, a reading could be obtained showing bromide release. A second measurement of the same sample would show further bromide release. However, what can be preliminarily reported is the finding the p-bromomethylphenylglyoxal affords a substantial amount of bromide ion released which is enzyme catalyzed (See Figure 5). The expected trends are seen. The glyoxalase I catalyzed reaction appears to be on a path which would lead to total Br^- elimination. The glyoxalase I and II catalyzed reaction exhibited a burst of bromide release, but then no further release is measured. And, at pH 6.0 and a two times excess of GSH, there is a slow non-enzymatic release of bromide due to "inverse" substrate formation. The important control studies demonstrated little to no sponteneous bromide ion release from bromomethylphenylglyoxal or from this compound in the presence of glyoxalase I.

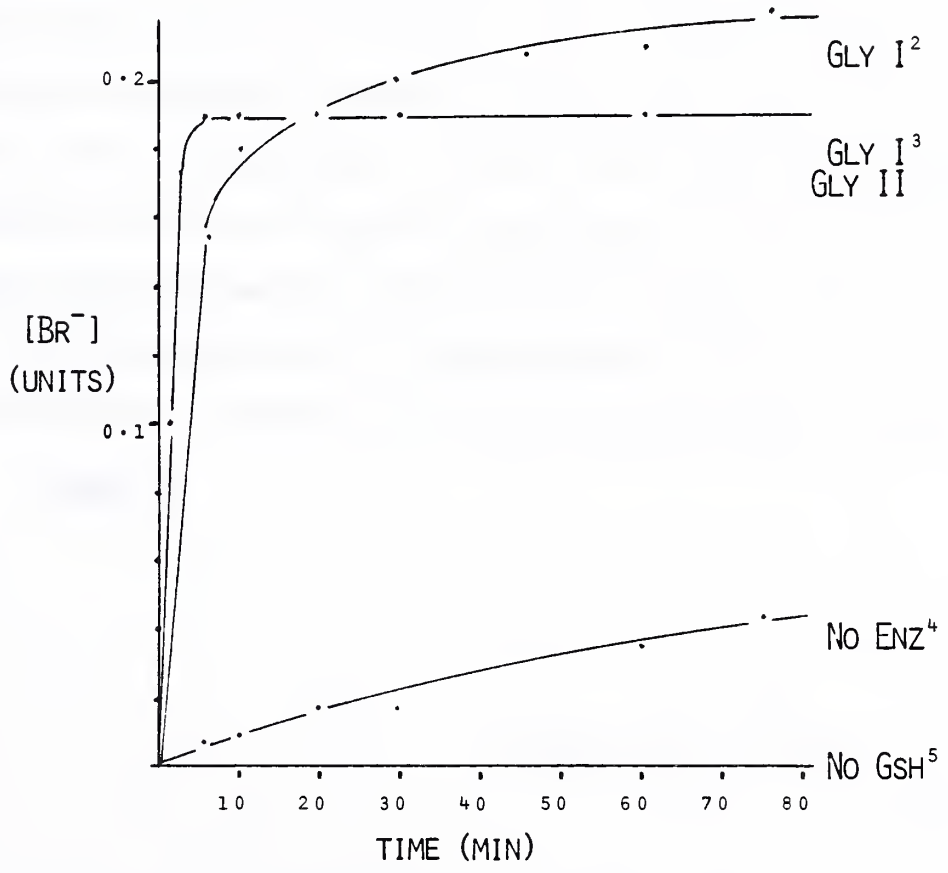
F. Isolation of Enzymatic Products for High Resolution NMR

Many attempts were made to isolate the products of the enzymatic reaction of bromomethylphenylglyoxal and two equivalents of GSH at pH 6.0 with glyoxalase I (2 units/ml) and glyoxalase II (0.4 units/ml). Most attempts at isolating the enzymatic products by Dowex-1 formate column separation and by HPLC were unsuccessful and resulted in NMR spectra of unsatisfactory quality. A product which had undergone bromide elimination could not be clearly identified. Definitive identification of enzymatic products such as p-bromomethylmandelic acid were unsuccessful due to the interference of a large water signal on NMR tracings.

Subsequent work in our laboratory (135) has identified the product of p-fluoromethylphenylglyoxal and GSH in the presence of glyoxalase I and II as p-fluoromethylmandelic acid.

FIGURE 5:

BROMOMETHYLPHENYLGLYOXAL -- BROMIDE ION ELIMINATION[1]



[1]Reactions were carried out in a 0.1M potassium phosphate buffer at pH 6.0 with bromomethylphenylglyoxal at 0.4mM and other reactants at the concentrations noted below. Analyses were performed as described in the METHODS section.

[2]GSH (0.8mM); Glyoxalase I (1 Unit/ml of reaction mixture).

[3]GSH (0.8mM); Glyoxalase I (1 Unit/ml); Glyoxalase II (0.5 Unit/ml).

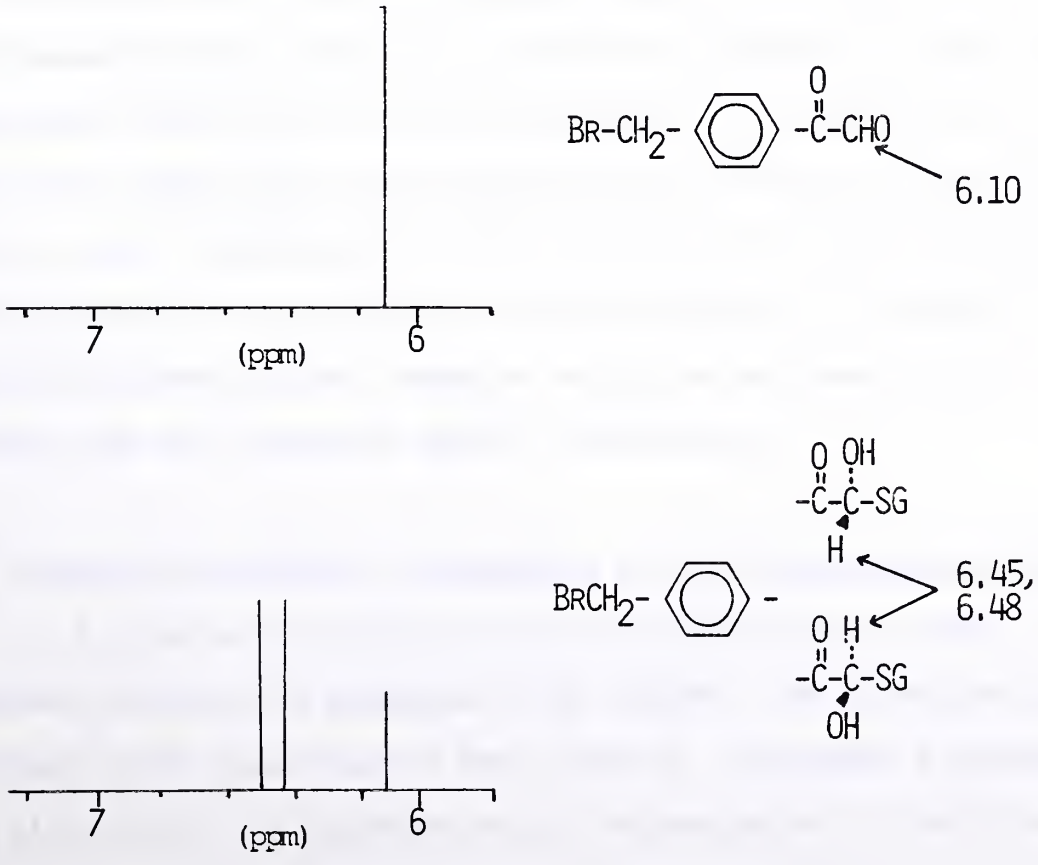
[4]GSH (0.8mM); No enzymes present.

[5]Glyoxalase I (1 Unit/ml); No GSH present.

G. Identification of Enzymatic Products by High Resolution NMR

Identification of products by NMR by direct analysis of reaction mixtures of bromomethylphenylglyoxal and GSH in the presence of glyoxalase I yielded more definitive results. At equimolar substrate and GSH concentrations, the formation of the thiohemiacetal could be observed by following the C-1 hydrogen [6.1 ppm (s, 1H)]. With the addition of GSH, this singlet decreased in intensity as new peaks formed, indicating the formation of the thiohemiacetal. In the newly formed thiohemiacetal the diastereotopic protons of the C-1 hydrogen are clearly resolved. In agreement with the previously reported (76) chemical shifts of similar protons of the thiohemiacetal of phenylglyoxal and GSH, two singlets of equal intensity were observed at 6.45 and 6.48 ppm as seen in Figure 6. In

FIGURE 6:



the presence of glyoxalase I there is total disappearance of these protons of the thiohemiacetal, thus suggesting glyoxalase I processing of the thiohemiacetal of bromomethylphenylglyoxal and GSH to a thioester. The formation of a methyl-group after protonation of the bromide elimination product (XXI) was not detected by high resolution NMR. It was also impossible to detect accurately the decrease in the intensity of the bromomethylene peak which would suggest bromide elimination. This was unachievable because the nmr signal of the brominated methyl-group of the starting material fortuitously fell at the same ppm of the H_2O signal. However, if one attempted to ignore the large interference by the water peak, one could determine a slight decrease in the brominated methylene signal which might have revealed that bromide elimination had occurred to some extent.

H. Bromomethylphenylglyoxal as a Glyoxalase Inhibitor

The initial test screen for enzyme inhibition by the serial addition of bromomethylphenylglyoxal to a reaction containing greater than 15 equivalents of GSH and 0.5 units of glyoxalase I/ml reaction mixture gave the first evidence that the compound was an inhibitor of glyoxalase I. That is, when compared to the initial velocity of the serial additions of phenylglyoxal to an identical reaction mixture, the addition of bromomethylphenylglyoxal caused a continuous decrease in the initial velocity with each subsequent additon (see Figure 7).

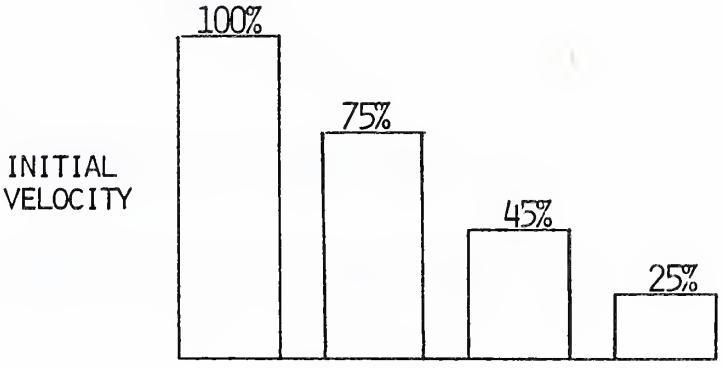
I. Irreversible Inhibition of Glyoxalase I by p-Bromomethylphenylglyoxal

At a given concentration of p-bromomethylphenylglyoxal, a time dependent inhibition of glyoxalase I was observed. The inhibition appeared to follow first order kinetics (see Figure 8). Glyoxalase I inactivation was also found to be dependent on the concentration of inhibitor (See

FIGURE 7:

INHIBITION OF GLYOXALASE I BY SERIAL ADDITION OF BROMOMETHYLPHENYLGLYOXAL (BrMEφG)

ADDITION (#)	1	2	3	4
BrMEφG (mM)	0.12	0.12	0.06	0.03
GSH (mM)	2.0			
GLY I (U/ML)	0.5			



CONTROL EXPERIMENT WITH PHENYLGLYOXAL (φG)

ADDITION (#)	1	2	3
φG (mM)	0.12	0.12	0.06
GSH (mM)	2.0		
GLY I (U/ML)	0.5		

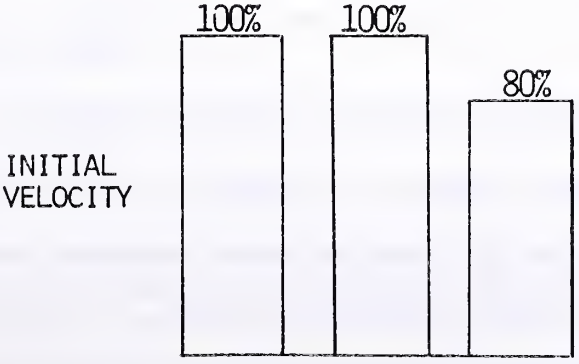
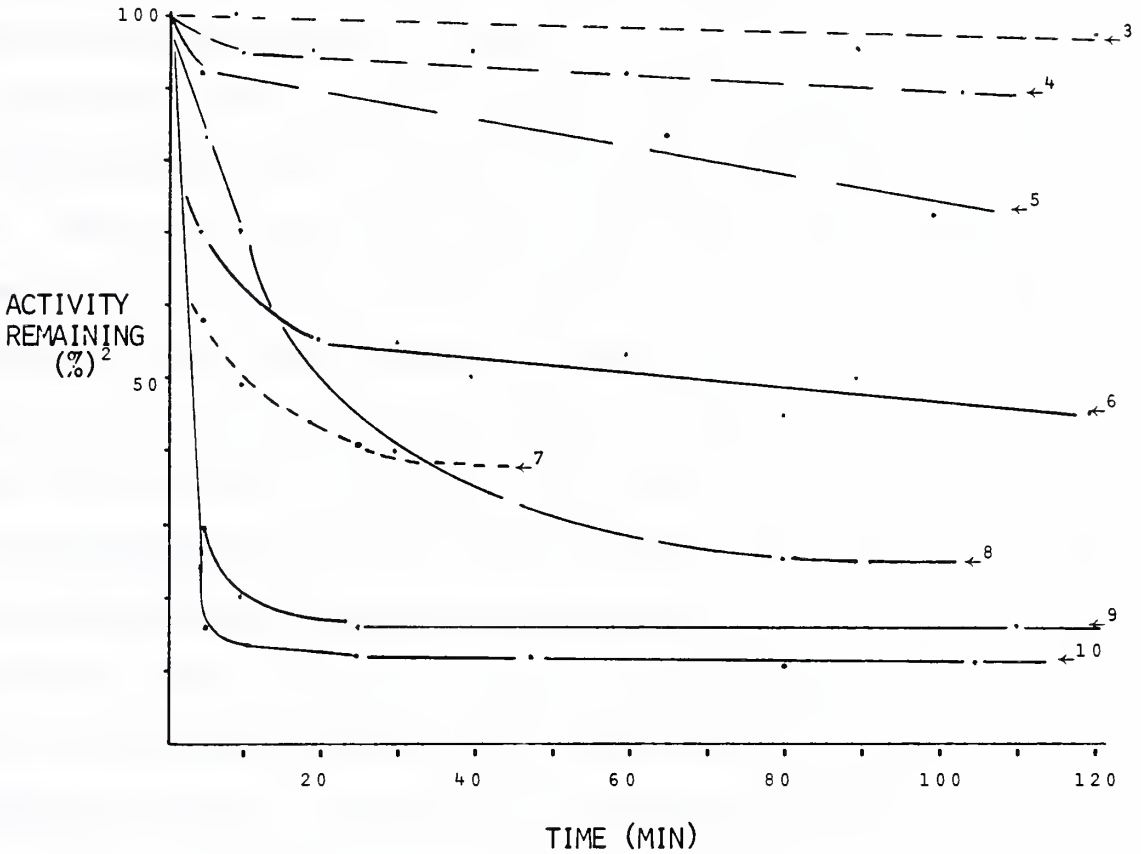


FIGURE 8:

BROMOMETHYLPHENYLGLYOXAL: IRREVERSIBLE INHIBITION OF GLYOXALASE I

PLOT OF TIME VERSUS ACTIVITY REMAINING[1]



[1]Activity Remaining was a measure of the initial velocity of the glyoxalase I catalyzed reaction of methylglyoxal (0.4mM) and GSH (2.0mM) as described in the METHODS section.

[2]% Activity Remaining is defined as the change in absorbance per min. divided by the initial measurement of the change in absorbance per min. at time zero for each of the different reaction conditions.

[3]Over Control: Inhibition solution contained glyoxalase I (25 units/ml of reaction mixture) and GSH (4mM). No inhibitor, BrMeϕG (bromomethylphenylglyoxal) added.

[4][0.02mM] Control: Inhibitor solution contained glyoxalase I (25 U/ml); BrMeϕG (0.02mM). No GSH.

[5][0.2mM] Control: Inhibitor solution contained glyoxalase I (25 U/ml); BrMeϕG (0.2mM). No GSH.

[6]Inhibition Study: BrMeϕG (0.02mM), GSH (0.04mM), Glyoxalase I (25 U/ml).

[7]Inverse Substrate Formation: BrMeϕG (0.2mM), GSH (0.4mM) were incubated for 45 mins. at pH 7.5 to form glutathiomethylphenylglyoxal. Then glyoxalase I (25 U/ml) was added and inhibition was followed with time.

[8][2.0mM] Control: Inhibitor solution contained glyoxalase I (25 U/ml); BrMeϕG (2.0mM). No GSH.

[9]Inhibition Study: BrMeϕG (0.2mM); GSH (0.4mM); glyoxalase I (25 U/ml).

[10]Inhibition Study: BrMeϕG (2.0mM); GSH (4.0mM); glyoxalase I (25 U/ml).

Figure 8). The graph of time of incubation versus the semi-log of the % activity remaining (See Figure 9) revealed the $t_{1/2}$ (inactivation) to be approximately 9, 2.8, and 1.7 minutes at 2×10^{-5}, 2×10^{-4}, and 2×10^{-3} M respectively. The K_I, extrapolated from the graph of (1/inhibitor concentration) versus ($1/k_{inact.}$) was equal to 0.078 mM (See Figure 10). The first control study which contained no inhibitor demonstrated >90% of enzyme activity present throughout the time period studied. The controls run at varying inhibitor concentrations, but without GSH present revealed slight enzyme inhibition, but at a much slower rate, and following non-first order kineticcs. The inhibition study in which glutathiomethylphenylglyoxal (the "inverse" substrate) was formed by incubating 0.2 mM p-bromomethylphenylglyoxal with 0.4 mM GSH at high pH resulted in enzyme inhibition, but at a lower level than when compared with 0.2 mM p-bromomethylphenylglyoxal which had not undergone "inverse" substrate formation. The inhibition observed was probably due to unreacted p-bromomethylphenylglyoxal which had not undergone "inverse" substrate formation at high pH.

As expected, further work in our laboratory (135) has demonstrated that fluoromethylphenylglyoxal does not cause irreversible inhibition of glyoxalase I.

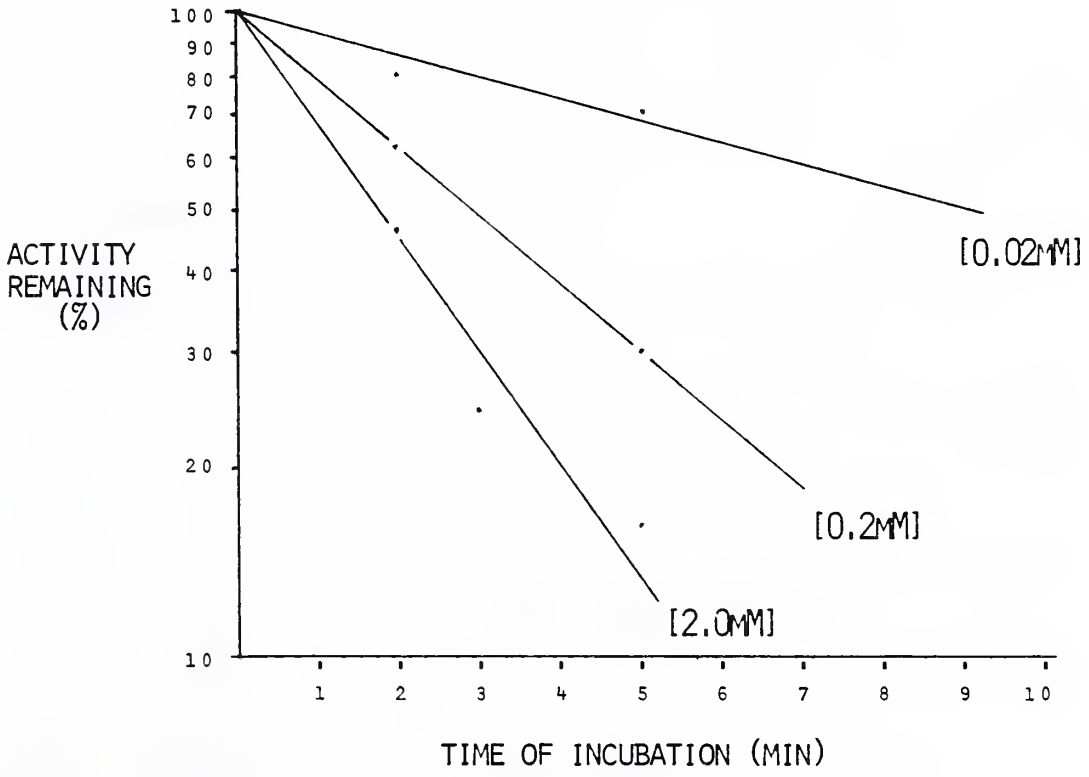
J. Tissue Culture Experiments

Bromo- and fluoromethylglyoxal were tested as growth inhibitors of L1210 and P388 cell lines in tissue culture as a preliminary test screen for the compound's anti-cancer activity. The results are summarized in Figure 11 which graphs the ratio of the cytotoxic effects on tumor growth of the test compounds to controls versus time. Both compounds at 10^{-4} M showed greater that 50% inhibition of growth of the L1210 cell line at 24 hours when compared to controls. Growth was shown to be further inhibited

FIGURE 9:

BROMOMETHYLPHENYLGLYOXAL: IRREVERSIBLE INHIBITION OF GLYOXALASE I

PLOT OF TIME VERSUS THE SEMI-LOG OF ACTIVITY REMAINING



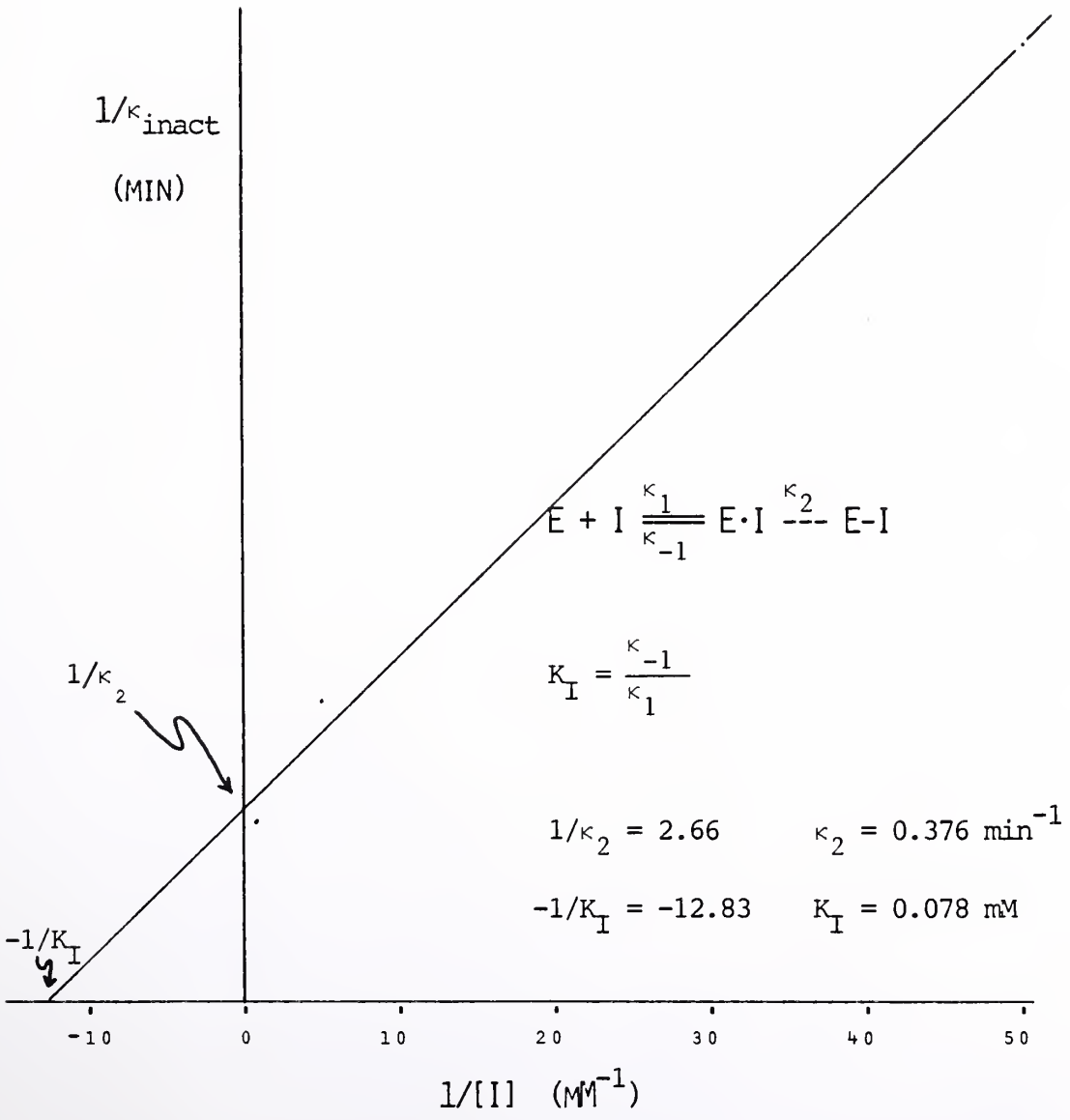
[I] (mM)	1/[I] (mM^{-1})	$T_{1/2}$ (min)	κ_{inact}[a] (min^{-1})	$1/\kappa_{inact}$ (min)
0.02	50	9.0	0.077	13.0
0.2	5	2.8	0.248	4.0
2.0	0.5	1.7	0.408	2.6

[a] $\kappa_{inact} = 0.693/T_{1/2}$

FIGURE 10:

BROMOMETHYLPHENYLGLYOXAL: IRREVERSIBLE INHIBITION OF GLYOXALASE I

PLOT[a] OF $1/\kappa_{inact}$ VERSUS $1/[I]$

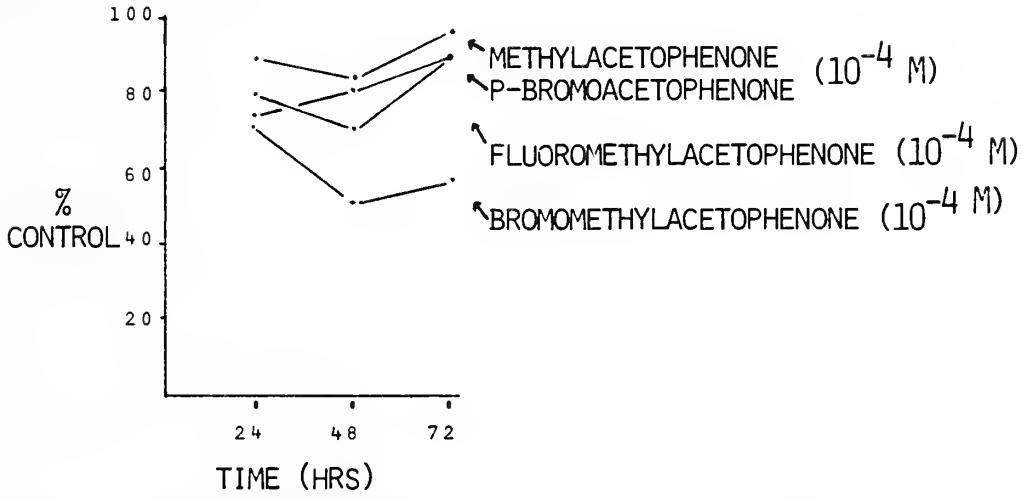


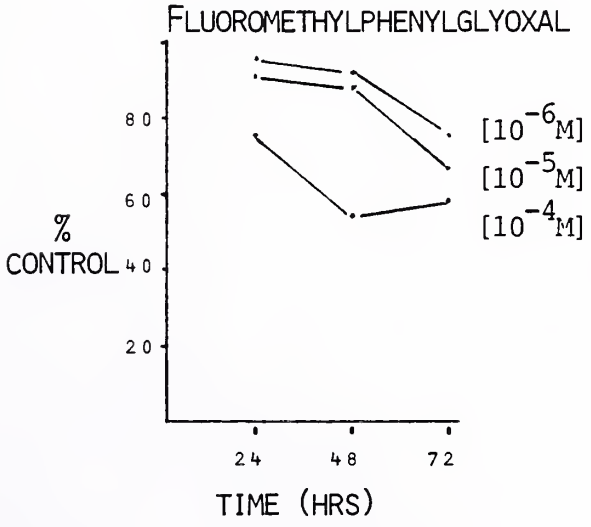
[a]Plot of best line by linear regression.

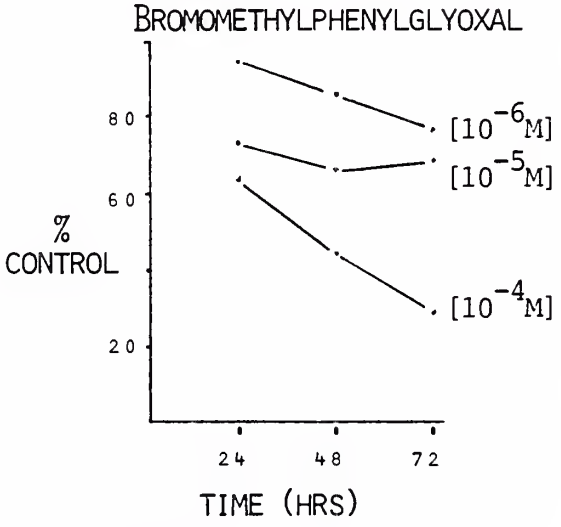
FIGURE 11:

TISSUE CULTURE EXPERIMENTS

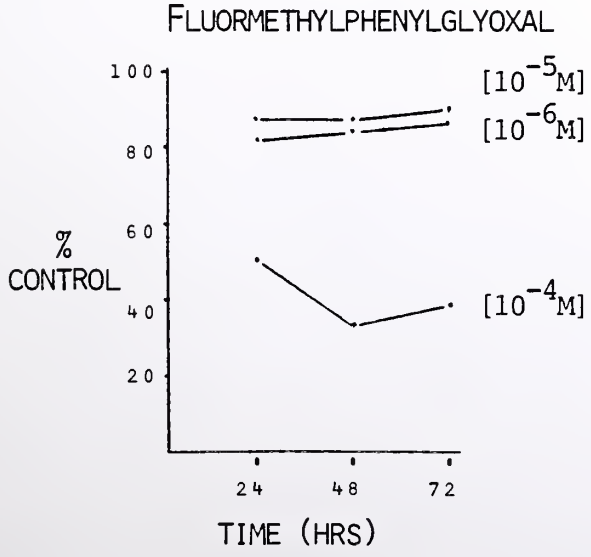
P388

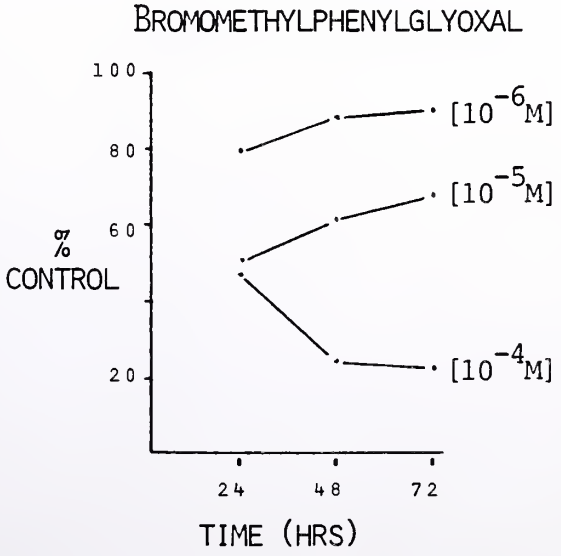






L1210





by 72 hours to levels of 60% for fluoromethylphenylglyoxal and ~80% for bromomethylphenylglyoxal. Against the P388 cell line, fluoromethylphenylglyoxal at 10^{-4} M showed no more than a 40% growth inhibition. Bromomethylphenylglyoxal at 10^{-4} M resulted in ~70% cell growth inhibition at 72 hours. The experiments which tested cell growth inhibition of p-methylacetophenone, p-bromoacetophenone, and p-fluoromethylacetophenone resulted in no significant inhibition. Bromomethylacetophenone resulted in ~40% cell growth inhibition at 72 hours when compared to controls. This was most likely due to impurities present in the p-bromomethylacetophenone solution.

K. Biological Evaluation in Tumor-Bearing Mice

The halogenated methylphenylglyoxals were further evaluated for anti-cancer activity against L1210 tumor bearing mice. The compounds were given on 6 consecutive days at the same dose beginning 24 hours after intraperitoneal tumor implantation. The results are summarized in Table 2. Fluoromethylphenylglyoxal, at a dose of 10 mg/kg and 50 mg/kg showed no significant antineoplastic activity in this system and showed no toxicity at the doses tested. However, bromomethylphenylglyoxal was very potent and showed extreme toxicity at 25 mg/kg and 50 mg/kg with all mice dying within 24 hours after the first dose was given. Lesser toxicity was seen at doses of 5 mg/kg and 10 mg/kg, with all mice surviving the 6 consecutive treatments, but then all dying before the untreated controls [(T/C)% less than 100%]. There was no significant anti-cancer activity observed at doses of bromomethylphenylglyoxal at 1.0 mg/kg and 2.5 mg/kg.

TABLE 2:

BIOLOGICAL TEST OF HALOGENATED METHYLPHENYLGLYOXALS IN TUMOR-BEARING MICE[1]

	DOSE (mg/kg)	NO. OF INJ.[2]	TUMOR EVALN.[3] (test/control)	L_{1210}[4] (T/C) %
BrMEφG[5]	50	1	2.0/8.8	23
	25	1	2.0/8.8	23
	10	6	6.8/8.8	77
	5	6	8.4/8.8	95
	2.5	6	9.8/8.8	110
	1.0	6	9.2/8.8	105
FMEφG[6]	50	6	8.8/8.8	100
	10	6	9.4/8.8	107

[1]Compounds were injected intraperitoneally as suspensions 24 hours after a standard inoculum of 10^5 L1210 lymphoid leukemia cells were implanted in CDF_1 mice. Five mice were in each test group.

[2]Number of injections given once daily at the same dosage level.

[3]Mean survival time of animals in days.

[4](T/C)% is the ratio of the mean survival time of treated animals to control animals expressed as percent. In general, a decrease in survival time of treated animals as compared to control animals resulting in a (T/C)% of less then 100% reflected drug toxicity.

[5]BrMeφG: Bromomethylphenylglyoxal

[6]FMeφG: Fluoromethylphenylglyoxal

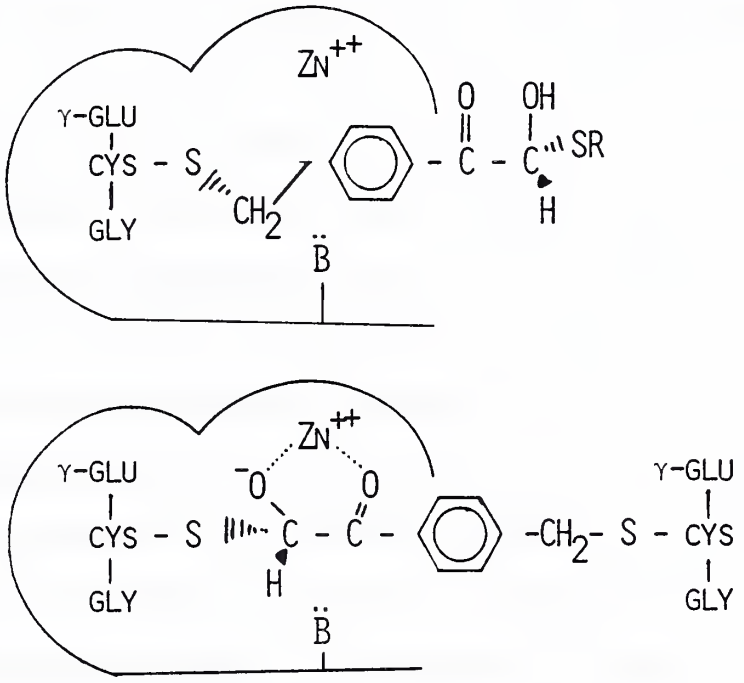
DISCUSSION

Novel halogenated keto-aldehydes were successfully synthesized in high yield. The basis for their design was to synthesize a substrate for glyoxalase I that might, when activated by the enzyme, form a highly reactive compound that would be susceptible to Michael addition by a nucleophile at the active site. Such a strategy would create a covalently linked compound in the active site, thus inactivating the enzyme. This scheme, which proceeds via halogen elimination, provides further evidence that the glyoxalase I mechanism proceedes through a fast-shielded proton transfer and not by a hydride shift. The newly synthesized compounds were also tested as possible "inverse" substrates of glyoxalase I.

Like other halogenated methylglyoxals described by Kozarich (99) which form "inverse" substrates of glyoxalase I, bromomethylphenylglyoxal was found to react non-enzymatically with GSH at high pH forming p-glutathiomethylphenylglyoxal. This could be observed by the slow change in UV absorbance, as well as by the slow elimination of bromide ion detected by the bromide electrode. This type of reaction was not observed when fluoromethylphenylglyoxal was incubated under identical conditions with GSH. This might be expected because the fluoride ion is not as a sufficiently good leaving group when compared to the bromide ion. However, when the uniquely formed glutathiomethylphenylglyoxal was incubated with glyoxalase I and ethanethiol, which renders the former aldehydic proton acidic and thus potentially susceptible to proton abstraction by the active site base, no enzymatic reaction was observed. This is in marked contrast with glutathiomethylglyoxal which Kozarich & Chari (99) found to undergo inverse substrate processing. Because glutathiomethylphenylglyoxal was unreactive in the inverse reaction, this suggests, as we recently reported (134), that there is a constraint on the distance and geometry between the

glutathione moiety and the α-keto-aldehyde in the inverse reaction (See Figure 12). However, when glutathiomethylphenylglyoxal was incubated with

FIGURE 12:



excess GSH, glyoxalase I successfully converted it to its corresponding α-hydroxythioester which, in the presence of glyoxalase II, was hydrolyzed to the hydroxy acid. This product was successfully isolated and identified by high resolution NMR. The processing of the thiohemiacetal of glutathiomethylphenylglyoxal and GSH by glyoxalase I provides an additional example of the requirement of glyoxalase I for GSH for binding and the enzyme's nonspecificity for the α-keto-aldehyde. In this case, a methylphenylglyoxal with a tripeptide side chain was successfully processed by glyoxalase I and II. This might indicate the ability of the glyoxalase system to process large macromolecules containing keto-aldehyde side chains. Such a proposition could be significant in yet to be discovered important intracellular substrates which might be key to the definitive physiological function of glyoxalase.

The findings that the thiohemiacetals of bromo- and fluoromethylphenylglyoxal and GSH are substrates of glyoxalase I are similar examples of the non-specific nature of glyoxalase for the α-keto-aldehyde and the highly specific nature of its binding to GSH. As can be expected, the thiohemiacetal of bromomethylphenylglyoxal and GSH was found, by high resolution NMR, to form two stereoisomers (76,77). These two isomers are presumed to undergo equal processing by glyoxalase I (in the presence of enzyme, both disappear). The newly formed thioester was not successfully identified. Of equal disappointment was our inability to identify, by NMR, the product of the enzymatic elimination of bromide. The possibility, however, remains that our attempts to identify the protonated intermediate, XXI, were unsuccessful because the quinoid-like intermediate, XIX, diffused out of the active site and was immediately attacked by a nucleophile (e.g. GSH) forming XXII. No attempts were made to isolate this product. And, because of the presence of GSH in the reaction mixture, such a product could not be easily identified by direct analysis by NMR of reaction mixtures.

Nevertheless, enzyme dependent bromide elimination was observed using the techniques of bromide ion electrode analysis. These experiments strongly indicated the mechanism of glyoxalase I proceeds through a fast-shielded proton transfer with an enediol intermediate. Of significance, the elimination of the halide generating the quinoid-like structure XIX, requires a disruption of the aromatic ring. This loss of resonance energy might be expected to constitute a formidable energy barrier to halide release. It appears that bromide ion is a sufficiently reactive leaving group to over-come this energy barrier. Fluoride ion electrode studies demonstrated no fluoride ion release. Therefore, it appears that fluoride ion is not a sufficiently reactive leaving group to overcome the energy barrier of disrupting the aromatic reasonance structure. Consequently, if

bromide ion elination could be successfully quantified, one might be able to measure partitioning ratios as Kozarich & Chari did with fluoromethylglyoxal (96). The existence of various products is supported by the tentative identification of the non-elimination product, p-bromomethylmandelic acid (XXIII) and the observation that bromide ion release is an enzyme dependent process. In the presence of glyoxalase I, the trend appears to be toward total bromide elimination due to the continuous re-entry of the thiohemiacetal and thioester product into the active site for further processing. While in the presence of glyoxalase I and II, there appears to be a trend toward no further bromide release after the final hydroxy-acid product is formed.

The demonstration of an enzyme dependent bromide ion release supports the hypothesis that the quinoid-like intermediate, XIX, is formed at the active site. This has important implications. The quinoid-like intermediate formed by bromide elimination takes on a planar configuration. Thus, it might be inferred that the active pocket is sufficiently nonspecific to permit the formation of the planar quinoid-like intermediate. Of greater significance is that this compound is highly susceptible to Michael addition by a nucleophile at the active site. This would then cause the covalent modification of the enzyme and thus irreversibly inhibit it. Such a possibility would represent the first mechanism-based irreversible inhibitor of glyoxalase I.

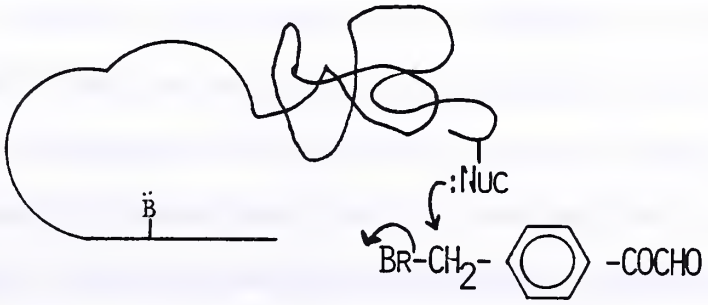
Initial experiments studying the inhibition of glyoxalase I by bromomethylphenylglyoxal provide strong evidence that the compound is acting as a mechanism-based, irreversible inhibitor of glyoxalase I. The studies showed that the novel new haloketoaldehyde fulfills the initial criteria (122) for identifying suicide inactivation. The experiments demonstrated the loss of enzyme activity was time dependent, following first-order kinetics. The rate of inactivation was found to be

proportional to the inhibitor concentration. Saturation kinetics are observed at high concentrations of inhibitor (135). Further experiments that would allow us to definitively state that bromomethylphenylglyoxal is an irreversible inhibitor include substrate protection studies, where the rate of inactivation is decreased as one increases the concentration of substrate (e.g. methylglyoxal). Other important studies include extensive dialysis and column separation to try and reactivate the enzyme. For further proof of the covalent modification of the enzyme, one could radioactively label the inactivator and show that the radioactivity becomes irreversibly associated with the protein.

The control experiments of the enzyme inhibition studies indicated an alternative process by which enzyme inactivation could occur. This was demonstrated in experiments which contained bromomethylphenylglyoxal at various concentrations incubated with glyoxalase I without GSH present. This result suggests that a non-mechanism-based enzyme inactivation is operative in the absence of GSH.

At low concentrations there was a slow inactivation of the enzyme probably due to random alkylation of the enzyme by bromomethylphenylglyoxal (See Fig. 13). While at a high concentration of inhibitor, this non-

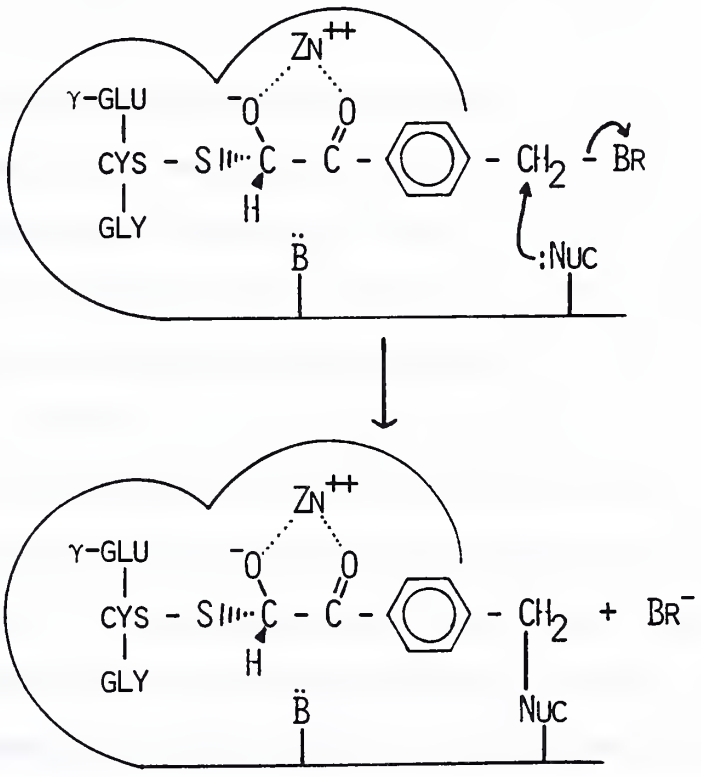
FIGURE 13:



enzymatic inactivation appeared more rapid, the latter was later shown to represent no more than 50% inactivation after 2 hrs. (135). The above

observations raise an interesting dilemma. In the presence of GSH, which "carries" the ketoaldehyde to the active site, is the inactivation of the enzyme due to an active site nucleophile simply displacing the bromide and forming a covalent link (See Fig. 14)? Or is the compound truly processed

FIGURE 14:



through the quinoid-like intermediate followed by Michael addition? The former mechanism, which is due to an intrinsically reactive α - halomethylene reacting with an active site nucleophile, would represent an "affinity label" inactivation and not the hypothesized mechanism-based inactivation. In support of the former was our inability to identify bromide elimination products which had not been covalently attached to the protein. However, there is also data that strongly supports the hypothesis that bromomethylphenylglyoxal is being processed through the highly reactive quinoid-like intermediate which then inactivates the enzyme. If enzymatic inactivation occurred by an active site nucleophile attacking the

reactive α-halomethylene, for every bromide ion released, one would expect the result of an inactive enzyme. Thus, for "affinity label" inactivation, there would be a very small, almost nonmeasureable amount of bromide ion released. This was certainly not observed. One observed a significant amount of bromide ion release that was enzyme dependent. On the other hand, this finding is possible if the inhibitor proceeds through the proposed quinoid-like intermediate. In such a scheme it is possible to have bromide elimination without requiring enzyme inactivation. Further proof of the probability of mechanism-based enzyme inhibition over affinity label type of inhibition is seen in the bromide ion electrode studies. The control experiment containing just inhibitor and glyoxalase I (no added GSH) revealed that an unmeasurably small amount of bromine was eliminated. This observation indicates that the enzyme inactivation that was seen when the inhibitor and glyoxalase I were incubated occurred by a very small number of alkylations. If it had occurred via numerous alkylations, than a measureable quantity of bromide ion should have been present. It also gives strong evidence that bromomethylphenylglyoxal is being processed via the proposed intermediate which allows for the observed enzyme dependent bromide elimination. This is the only explanation for the findings that indicate that for inactivation to occur, bromide ion must be eliminated, but bromide ion elimination does not necessarily need to cause enzymatic inactivation.

Further proof is needed to definitively assign bromomethylphenylglyoxal as a mechanism based irreversible inactivator of glyoxalase I. Besides the studies mentioned earlier which are required to conclude that a compound is a suicide inhibitor, other useful studies could be designed. One could prepare a compound like $BrCH_2-C_6H_4-CH_2CHO$ which would undergo thiohemiacetal formation with GSH. The thiohemiacetal would be able to specifically bind to the active site. One could then compare the

rate of enzymatic inactivation by this type of compound, which could only cause enzyme inactivation by an "affinity label" type mechanism, versus enzyme inactivation by bromomethylphenylglyoxal, which causes enzyme inactivation via a mechanism-based process. Such an experiment could give further evidence toward solving the dilemma of mechanism-based inhibition versus affinity label inhibition.

The studies of the halogenated methylphenylglyoxal's cytotoxic effect on tumor cells in tissue culture and their anti-cancer activity in tumor-bearing mice strongly support the finding that p-bromomethylphenylglyoxal is an inhibitor of glyoxalase I and can potentially act as a useful anti-neoplastic agent. As expected, bromomethylphenylglyoxal inhibited the growth of L1210 and P388 cell lines is tissue culture. As an inhibitor of glyoxalase I, the compound could cause the build-up of growth inhibiting substances in cells in tissue culture and thus dramatically decrease tumor cell growth. When p-fluoromethylphenylglyoxal was incubated with the P388 and L1210 cell lines, some growth inhibition was noted. The findings that fluoromethylphenylglyoxal inhibited tumor growth, while it was not an *in vitro* mechanism-based irreversible inhibitor of yeast glyoxalase I, can be explained by one of two mechanism. Because fluoromethylphenlyglyoxal was shown to be a substrate of glyoxalase I, it can act as a competitive inhibitor. As a competitive inhibitor, *in vivo*, it can cause an initial accumulation of growth inhibiting substances that result in tumor growth inhibition. Another possible explanation of this compound's growth inhibiting effect *in vivo* is that the mammalian glyoxalase I enzyme may differ from the yeast enzyme and in contrast to the yeast enzyme the mammalian enzyme may cause fluoride elimination and formation of the reactive quinoid-like intermediate that inactivates the enzyme.

In support of the discovery that bromo- and fluoromethylphenylglyoxal cause tumor growth inhibition in tissue culture by specifically interacting

with the glyoxalase enzyme system is the finding that their synthetic precursors, methylacetophenone, p-fluoromethylacetophenone, and p-bromomethylacetophenone result in little to no inhibition of tumor growth in tissue culture.

Bromo- and fluoromethylphenylglyoxal were also tested in L1210 tumor-bearing mice by the intraperitoneal injection of the compounds for six consecutive days. As expected, at the doses tested, fluoromethylphenylglyoxal showed no increased survival in the treated tumor-bearing mice. This is an agreement with the *in vitro* enzymatic studies which demonstrated that the fluoro- compound did not act as an irreversible inhibitor of glyoxalase I. However, bromomethylphenylglyoxal at 50 mg/kg and 25 mg/kg resulted in extreme toxicity. The L1210 tumor-bearing mice only survived through one dose of the compound. The mice treated with this compound at 10 mg/kg and 5 mg/kg survived the complete course of six treatments, but died before the non-treated controls. At lower doses, no significant increase in survival was noted. The extreme toxicity of bromomethylphenylglyoxal in tumor-bearing mice is in keeping with the potent effect of an irreversible, suicide inhibitor which completely inactivates an enzyme. In this case, the inhibition of glyoxalase I leads to the overwhelming accumulation of substances which inhibit cell growth and division and cause the death of the animal. Thus, the treatment schedule of therapy beginning 24 hours after tumor implantation followed by treatment for six consecutive days was not the optimum regimen for evaluating the anti-cancer effects of p-bromomethylphenylglyoxal. Perhaps a different evaluation program specified by the Drug Evaluation Branch of the National Cancer Institute will yield more definitive results. One such evaluation involves the treatment of tumor-bearing mice beginning 24 to 48 hours after tumor implantation followed by treatment intervals of every fourth day (133). Such an

evaluation program could optimize the anti-tumor effect and diminish the toxic effects of bromomethylphenylglyoxal.

> .....the cancer cell is unable to arrest growth because it is unable to inactivate its glyoxalase, which destroys the ketone-aldehyde that keeps the cell at rest...
>
> -A. Szent-Györgyi (13)

The design and synthesis of inhibitors of specific key metabolic enzymes has been an important new approach in the development of potential anti-cancer agents. p-Bromomethylphenylglyoxal appears to be the first mechanism-based, irreversible inhibitor of glyoxalase I. This compound may be a potential anti-neoplastic agent and may help to further elucidate the definitive function of the glyoxalase enzyme system. And if Szent-Györgyi's theory is one day proven wrong, something will be left afterward.

REFERENCES

1. Hopkins, F.G. in Enzymes, Dixon, M., Webb, E.C., eds. (Academic Press, Inc., New York), 1958, p.v.

2. Dakin, H.D., Dudley, H.W. "An Enzyme Concerned with the Formation of Hydroxy Acids from Ketonic Aldehydes". J. Biol. Chem 14: 155 (1913).

3. Dakin, H.D., Dudley, H.W. "On Glyoxalase" J. Biol. Chem. 14: 423 (1913).

4. Neuberg, C. "The Destruction of Lactic Aldehyde and Methylglyoxal by Animal Organs" Biochem. Z. 49: 502 (1913).

5. Hopkins, F.G., Morgan, E.J. "On the Distribution of Glyoxalase and Glutathione" Biochem. J. 39: 320 (1945).

6. (Szent-Györgyi, A.) Bendiner, E. "The Art in Being Wrong" Hosp. Pract. May: 179 (1982).

7. Szent-Györgyi, A. "Cell Division and Cancer" Science 149: 34 (1965).

8. Együd, L.G., Szent-Györgyi, A. "Cell Division, SH, Ketoaldehydes and Cancer" Proc. Natl. Acad. Sci. USA 55: 388 (1966).

9. Szent-Györgyi, A. "On Retine" Proc. Natl. Acad. Sci. USA 57: 1642 (1967).

10. Szent-Györgyi, A., Együd, L.G., McLaughlin, J. "Keto-Aldehydes and Cell Division" Science 155: 539 (1967).

11. Fodor, G., Mujumdar, R., Szent-Györgyi, A. "Isolation of Methylglyoxal from Liver" Proc. Natl. Acad. Sci. USA 75: 4317 (1978).

12. Szent-Györgyi, A. "Bioelectronics" Science 161: 988 (1968).

13. Szent-Györgyi, A. "The Living State and Cancer" Proc. Natl. Acad. Sci. USA 74: 2844 (1977).

14. Szent-Györgyi, A. "The Living State and Cancer" (Marcel Dekker, Inc., New York), 1978.

15. Szent-Györgyi, A. "The Living State and Cancer" Ciba Found. Symp. 67: 3 (1978).

16. (Szent-Györgyi, A.) Holden, C. "Albert Szent-Györgyi: Electrons and Cancer" Science 203: 522 (1979).

17. Szent-Györgyi, A. "The Living State and Cancer" Physiol. Chem. Phys. 12: 99 (1980).

18. Pethig, R., Szent-Györgyi, A. "Electronic Properties of the Casein-Methylglyoxal Complex" Proc. Natl. Acad. Sci. USA 74: 226 (1977).

19. Kun, E. "Inhibitors of Succinic Dehydrogenase by Methylglyoxal" J. Biol. Chem. 187: 289 (1950).

20. Salem, H.M. "Glyoxalase in Thiamine-Deficient Rats" Biochem. J. 57: 227 (1954).

21. Penninckx, M.J., Jaspers, C.J., Legrain, M.J. "The Glutathione-Dependent Glyoxalase Pathway in the Yeast Saccharomyces cerevisae" J. Biol. Chem. 258: 6030 (1983).

22. Gillespie, E. "Cell-Free Microtubule Assembly: Evidence for Control by Glyoxalase" Fed. Proc. 34: 541 (1975).

23. Gillespie, E. "Concanavalin A Increases Glyoxalase Enzyme Activities in Polymorphonuclear Leukocytes and Lymphocytes" J. Immun. 121: 923 (1978).

24. Gillespie, E. "Effects of S-Lactoylglutathione and Inhibitors of Glyoxalase I on Histamine Release from Human Leukocytes" Nature 277: 135 (1979).

25. Gillespie, E. "The Tumor Promoting Phorbol Diester, 12-0-Tetradecanoylphorbol-13-Acetate (TPA) Increases Glyoxalase I and Decreases Glyoxalase II Activity in Human Polymorphonuclear Leukocytes" Biochem. Biophys. Res. Commun. 98: 463 (1981).

26. Weaver, R.H., Lardy, H.A. "Synthesis and Some Biochemical Properties of Phosphohydroxypyruvic Aldehyde and of 3-Phosphoglyceryl Glutathione Thiol Ester" J. Biol. Chem. 236: 313 (1961).

27. Reeves, H.C., Ajl, S.J. "Enzymatic Synthesis and Metabolism of Hydroxypyruvic Aldehyde" J. Biol. Chem. 240: 569 (1965).

28. Jerzykowski, T., Winter, R., Matuszewski, W. " γ , δ -Dioxovalerate as a Substrate for the Glyoxalase Enzyme System". Biochem J. 135: 713 (1973).

29. Cooper, R.A., Anderson, A. "The Formation and Catabolism of Methylglyoxal During Glycolysis in Escherichia coli" FEBS Lett. 11: 273 (1970).

30. Hooper, D.J., Cooper, R.A. "The Regulation of Escherichia coli Methylglyoxal Synthase; A New Control Site in Glycolysis?" FEBS Lett. 13: 213 (1971).

31. Hooper, D.J., Cooper, R.A. "The Purification and Properties of Escherichia coli Methylglyoxal Synthase" Biochem. J. 128: 321 (1972).

32. Salem, H.M., Crook, E.M. "Production and Utilization of Methylglyoxal by Tissue" Biochem. J. 46: xxxvii (1950).

33. Riddle, V., Lorenz, F.W. "Nonenzymic, Polyvalent Anion-Catalyzed Formation of Methylglyoxal as an Explanation of its Presence in Physiological Systems" J. Biol. Chem. 243: 2718 (1968).

34. Sato, J. Wang, Y., van Eys, J. "Methylglyoxal Formation in Rat Liver Cells" J. Biol. Chem. 255: 2046 (1980).

35. Ray, S., Ray, M. "Isolation of Methylglyoxal Synthase from Goat Liver" J. Biol. Chem. 256: 6230 (1981).

36. Platt, M.E., Schroeder, E.F. "Glyoxalase: The Distribution of Glyoxalase in Tissues of Normal and Cancerous Albino Rats" J. Biol. Chem. 106: 179 (1934).

37. Strzinek, R.A., Vela, G.R., Scholes, V.E., Norton, S.J. "Further Studies on Liver Glyoxalase Activity in Mice Bearing Lymphosarcoma" Cancer Res. 30: 334 (1970).

38. Strzinek, R.A., Scholes, V.E., Norton, S.J. "The Purification and Characterization of Liver Glyoxalase I from Normal Mice and from Mice Bearing a Lymphosarcoma" Cancer Res. 32: 2359 (1972).

39. Brandt, R.B., Waters, M.G., Muron, D.J., Bloch, M.H. "The Glyoxalase System in Rat Blood (41376)" Proc. Soc. Exp. Biol. Med. 169: 463 (1982).

40. Principato, G.B., Locci, P. Rosi, G. Talesa, V., Giovannini, E. "Activity Changes of Glyoxalase I-II and Glutathione Reductase in Regenerating Rat Liver" Biochem. International 6: 249 (1983).

41. Jerzykowski, T., Winter, R., Matuszewski, W., Szczurek, Z. "Glyoxalase II Activity in Tumours" Experientia. 31: 32 (1975).

42. Underwood, G.E., Sheldon, W.D. "Glyoxal and Related Compounds as Potential Blood Sterilizing Agents" Proc. Soc. Exper. Biol. Med. 93: 421 (1956).

43. Massarani, E., Nardi, D., Degen, L., Magistretti, M.J. "Antiviral Compounds. XIII. Aminoacethydrazones of Aromatic α -Ketoaldehydes" J. Med. Chem. 9: 617 (1966).

44. Baylor, M.B., Egyud, L.G. "The Effects of Methylglyoxal on Phage Infection of Escherichia coli" Virology 31: 380 (1967).

45. Kuchler, C., Kuchler, W., Shulze, W. "Antiviral Effect of Methylglyoxal-Bis-Guanylhydrazone" Acta. Virol. 12: 441 (1968).

46. Underwood, G.E. "Kethoxal for Treatment of Cutaneous Herpes Simplex (33294)" Proc. Soc. Exp. Biol. Med. 129: 235 (1968).

47. Sparkes, B.G., Kenny, C.P. "Identification of a Bacertial Growth Inhibitor from HeLa Cells: A Keto-aldehyde" Proc. Natl. Acad. Sci. USA 64: 920 (1969).

48. Freedberg, W.B., Kistler, W.S., Lin, E.C.C. "Lethal Synthesis of Methylglyoxal by Escherichia coli During Unregulated Glycerol Metabolism" J. Bacteriol. 108: 137 (1971).

49. Gregg, C.T. "Inhibition of Mammalian Cell Division by Glyoxals" Exp. Cell Res. 50: 65 (1968).

50. Scaife, J.F. "Mitotic Inhibition Induced in Human Kidney Cells by Methylglyoxal and Kethoxal" Experientia 25: 178 (1969).

51. Bahner, C.T., et al. "Compounds for Cancer Studies" J. Am. Chem. Soc. 75: 4838 (1953).

52. French, F.A., Freedlander, B.L. "Carcinostatic Action of Polycarbonyl Compounds and their Derivatives" Cancer Res. 18: 172 (1958).

53. Apple, M.A., Greenberg, D.M. "Arrest of Cancer in Mice by Therapy with Normal Metabolites. I. 2-Oxopropanal (NSC-79019)" Cancer Chemother. Rep. 51: 455 (1967).

54. Együd, L.G., Szent-Györgyi, A. "Cancerostatic Action of Methylglyoxal" Science 160: 1140 (1968).

55. Furst, A., Freedlander, B.L., French, F.A., Gross, H.K., Demsher, D. "B-Ethyoxy- α-ketobuteraldehyde (Kethoxal) as a Carcinostatic Agent in Mouse Tumors" Proc. Amer. Ass. Cancer Res. 2: 204 (1957).

56. Együd, L.G., Szent-Györgyi, A. "On the Regulation of Cell Division" Proc. Natl. Acad. Sci. USA 56: 203 (1966).

57. Krymkiewicz, N., Diéguez, E., Rekarte, U.D., Zwaig, N. "Properties and Mode of Action of a Bactericidal Compound (=Methylglyoxal) Produced by a Mutant of Escherichia coli" J. Bacteriol. 108: 1338 (1971).

58. Fraval, H.N.A., McBrien, D.C.H. "The Effect of Methylglyoxal on Cell Division and Synthesis of Protein and DNA in Synchronous and Asynchronous Cultures of Escherichia coli B/r" J. Gen. Microbiol. 117: 127 (1980).

59. Otsuka, H., Együd, L.G. "Nucleic Acid and Protein Synthesis of Malignant Ascites Cells in the Presence of Liver Extract and Methylglyoxal" Cancer Res. 27: 1498 (1967).

60. White, J.S., Rees, K.R. "Inhibitory Effects of Methylglyoxal on DNA, RNA, and Protein Synthesis in Cultured Guinea Pig Keratocytes" Chem-Biol. Interactions. 38: 339 (1982).

61. Kozarich, J.W., Deegen, J.L. "7-Methylguanosine-Dependent Inhibition of Globin mRNA Translation by Methylglyoxal" J. Biol. Chem. 254: 9345 (1979).

62. Riley, P.A. "Inhibition of Protein and DNA Synthesis in Tissue Culture Cells by a Derivative of Methylglyoxal and Ascorbate" Chem-Biol. Interactions 31: 73 (1980).

63. Szent-Györgyi, A. "Studies on Cellular Regulations" Perspectives Biol. Med. 11: 350 (1968).

64. Lohmann, K. "A Study of the Enzymic Transformation of Synthetic Methylglyoxal to Lactic Acid" Biochem. Z. 254: 332 (1932).

65. Jowett, M., Quastel, J.S. "LXX. The Glyoxalase Activity of the Red Blood Cell. The Function of Glutathione" Biochem. J. 27: 486 (1933).

66. Yamazoye, S. "Glyoxalase and its Coenzyme. III. Mechanism of the Action of Glutathione as the Coenzyme of Glyoxalase" J. Biochem., Japan 23: 319 (1936).

67. Hopkins, F.G., Morgan, E.J. "Studies on Glyoxalase: A New Factor" Biochem J. 42: 23 (1948).

68. Racker, E. "Glyoxalases" Fed. Proc. 9: 217 (1950).

69. Racker, E. "The Mechanism of Action of Glyoxalase" J. Biol. Chem 190: 685 (1951).

70. Crook, E.M, Law, K. "The Mode of Action of Glyoxalase" Biochem. J. 46: xxxvii (1950).

71. Crook, E.M., Law, K. "Glyoxalase: The Role of the Components" Biochem. J. 52: 492 (1952).

72. Cliffe, E.E., Waley, S.G. "The Mechanisms of the Glyoxalase I Reaction, and the Effect of Ophthalmic Acid as an Inhibitor" Biochem. J. 79: 475 (1961).

73. Davis, K.A., Williams, G.R. "Glyoxalase I, a Lyase or an Oxidoreductive Isomerase?" Can. J. Biochem. 47: 553 (1969).

74. Vander Jagt, D.L., Daub, E., Krohn, J.A., Han., L.B. "Effects of pH and Thiols on Kinetics of Yeast Glyoxalase I. An Evaluation of the Random Pathway Mechanism" Biochem. 14: 3669 (1975).

75. Marmstal, E., Mannervik, B. "Evaluation of the Two-Substrate Pathway of Glyoxalase I from Yeast by Use of Carbonic Anhydrase and Rapid-Kinetic Studies" FEBS Lett. 131: 301 (1981).

76. Brown, C., Douglas, K.T., Ghobt-Sharif, J. "Direct Demonstration of 'H NMR Spectrometry of the Stereoselectivity of Yeast Glyoxalase I Towards the Diastereomeric Forms of the α-Ketoaldehyde-Glutathione Hemithioacetal" J.C.S. Chem. Comm. 944 (1981).

77. Griffis, C.E.F., Ong, L.H., Buettner, L., Creighton, D.J. "Non Stereospecific Substrate Usage by Glyoxalase I" Biochem. 22: 2945 (1983).

78. Chari, R.V.J., Kozarich, J.W. "Glutathiohydroxyacetone: 'H NMR Determination of the Stereochemistry of Proton Exchange by Gloyoxalase I. Evidence for a cis-Enediol Intermediate Based on Mirror-Image Catalysis" J. Am. Chem. Soc. 105: 7169 (1983).

79. Vander Jagt, D.L., Han, L.B., Lehman, C.H. "Kinetic Evaluation of Substrate Specificity in the Glyoxalase I-Catalyzed Disproportionation of α-Ketoaldehydes" Biochem. 11: 3735 (1972).

80. Mannervik, B., Marmstål, E., Ekwall, K., Górna-Hall, B. "Inactivation of Glyoxalase I from Porcine Erythrocytes and Yeast by Amino-Group Reagents" Eur. J. Biochem. 53: 327 (1975).

81. Aronsson, A., Sellin, S., Tibbelin, G., Mannervik, B. "Probing the Active Site of Glyoxalase I from Human Erythrocytes by Use of the Strong Reversible Inhibitor S-p-bromobenzylglutathione and Metal Substitutions" Biochem. J. 197: 67 (1981).

82. Schasteen, C.S., Reed, D.J. "Involvement of Arginine Residues in Glutathione Binding to Yeast Glyoxalase I" Biochem. Biophys. Acta 742: 419 (1983).

83. Davis K.A., Williams, G.R. "Cation Activiation of Glyoxalase I" Biochem. Biophys. Acta. 113: 393 (1966).

84. Aronsson, A., Marmstål, E., Mannervik, B. "Glyoxalase I, a Zinc Metalloenzyme of Mammals and Yeast" Biochem. Biophys. Res. Commun. 81: 1235 (1978).

85. Sellin, S., Eriksson, L.E.G., Mannervik, B. "Fluorescence and Nuclear Relaxation Enhancement Studies of the Binding of Glutathione Derivatives to Mangenese-Reconstituted Glyoxalase I from Human Erythrocytes. A Model for the Catalytic Mechanism of the Enzyme Involving a Hydrated Metal Ion" Biochem. 21: 4850 (1982).

86. Garcia-Iniquez, L., Powers, L., Chance, B., Sellin, S., Mannervik, B., Mildvan, A.S. "X-ray Absorption Studies of the Zn^{2+} Site of Glyoxalase I" Biochem. 23: 685 (1984).

87. Rose, I.A. "Mechanism of the Action of Glyoxalase I" Biochem. Biophys. Acta 25: 214 (1957).

88. Franzen, V. "The Action and Mechanism of Glyoxalase I" Chem. Ber. 89: 1020 (1956).

89. Vander Jagt, D.L., Han, L.B., Lehman, C.H. "Effects of Substituents on the Rates of Disproportionation of Substituted Phenylglyoxals in Alkaline Solution" J. Org. Chem. 37: 4100 (1972).

90. Vander Jagt, D.L., Han, L.B. "Deuterium Isotope Effects and Chemically Modified Coenzymes as Mechanism Probes of Yeast Glyoxalase I" Biochem. 12: 5161 (1973).

91. Hall, S.S., Poet, A. "Metal Ion - Base Catalyzed Rearrangement of α-Ketohemimercaptals to α-Hydroxythiolesters" Tet. Lett., 2867 (1970).

92. Hall, S.S., Doweyko, A.M., Jordon, F. "Glyoxalase I Enzyme Studies. 2. Nuclear Magnetic Resonance. Evidence for an Enediol-Proton Transfer Mechanism" J. Am. Chem. Soc. 98: 7460 (1976).

93. Hall, S.S., Doweyko, A.M., Jordon, F. "Glyoxalase I Enzyme Studies. 4. Catalyzed Enediol Proton Transfer for Rearrangement of Methyl-and Phenylglyoxalglutathionyl-hemithiol Acetal to S-Lactoyl- and S-Mandeloylglutathione Followed by Hydrolysis. A Model for the Glyoxalase Enzyme System" J. Am. Chem. Soc. 100: 5934 (1978).

94. Sellin, S., Rosevear, P.R., Mannervik, B., Mildvan, A.S. "Nuclear Relaxation Studies of the Role of Essential Metal in Glyoxalase I" J. Biol. Chem. 257: 10023 (1982).

95. Shinkai, S., Yamashita, T., Kusano, Y., Manabe, O. "Coenzyme Models. 25. Facile Oxidation of Hemithiol Acetals by Flavin. Supporting Evidence for the Enediol Mechanism of Glyoxalase I" J. Am. Chem. Soc. 103: 2070 (1981).

96. Kozarich, J.W., Chari, R.V.J., Wu, J.C., Lawrence, T.L. "Fluoromethylglyoxal: Synthesis and Glyoxalase I Catalyzed Product Partitioning Via a Presumed Enediol Intermediate" J. Am. Chem. Soc. 103: 4593 (1981).

97. Chari, R.V.J., Kozarich, J.W. "Deuterium Isotope Effects on the Product Partitioning of Fluoromethylglyoxal by Glyoxalase I." J. Biol. Chem. 256: 9785 (1981).

98. Kozarich, J.W. "Enzyme Mechanisms in Three Carbon Metabolism" Department of Health and Human Services, Public Health Service, Grant Application. April, 1984.

99. Kozarich, J.W., Chari, R.V.J. "(Glutathiomethyl)glyoxal: Mirror-Image Catalysis by Glyoxalase I" J. Am. Chem. Soc. 104: 2655 (1982).

100. Oray, B., Norton, S.J. "A two-step Purification of Mouse Liver Glyoxalase I and Evidence of its Dimeric Constitution" Biochem. Biophys. Acta 483: 203 (1977).

101. Oray, B., Norton, S.J. "Glyoxalase I from Mouse Liver" Methods. Enzymol. 90: 542 (1982).

102. Uotila, L., Koivusalo, M. "Purification and Properties of Glyoxalase I from Sheep Liver" Eur. J. Biochem. 52: 493 (1975).

103. Han, L.B., Davison, L.M., Vander Jagt, D.L. "Purification and Kinetic Study of Glyoxalase I from Rat Liver, Erythrocytes, Brain and Kidney" Biochem. Biophys. Acta 445: 486 (1976).

104. Marmstål, E. Aronsson, A. Mannervik, B. "Comparison of Glyoxalase I Purified from Yeast (Saccharomyces cererisiae) with the Enzyme from Mammalian Sources" Biochem. J. 183: 23 (1979).

105. Uotila, L. "Purification and Characterization of S-2-Hydroxyaclyglutathione Hydrolase (Glyoxalase II) from Human Liver" Biochem. 12: 3944 (1973).

106. Oray, B., Norton, S.J. "Glyoxalase II from Mouse Liver" Methods Enzymol. 90: 547 (1982).

107. Oray, B., Norton, S.J. "Purification and Characterization of Mouse Liver Glyoxalase II" Biochem. Biophys. Acta 611: 168 (1980).

108. Kermack, W.O., Matheson, N.A. "The Effects of Some Analogues of Glutathione on the Glyoxalase System" Biochem. J. 65: 48 (1957).

109. Vince, R., Wadd, W.B. "Glyoxalase Inhibitors as Potential Anticancer Agents" Biochem. Biophys. Res. Comm. 35: 593 (1969).

110. Vince, R., Daluge, S. "Glyoxalase Inhibitors. A Possible Approach to Anticancer Agents" J. Med. Chem. 14: 35 (1971).

111. Litt, M. "Structural Studies on tRNA. I. Labeling of Exposed Guanine Sites in Yeast Phenylalanine - tRNA with Kethoxal" Biochemistry 8: 3249 (1969).

112. Kester, M.V., Reese, J.A., Norton, S.J. "Mouse Liver Glyoxalase I Inhibition by S-Substituted Glutathiones" J. Med. Chem. 17: 413 (1974).

113. Vince, R., Wolf, M., Sanford, C. "Glutaryl-S-(p-bromobenzyl)-L-Cysteinylglycine. A Metabolically Stable Inhibitor of Glyoxalase I" J. Med. Chem. 16: 951 (1973).

114. Lyon, P.A., Vince, R. "Synthesis and Kinetic Evaluation of S- and N-Substituted Cysteinylglycines as Inhibitors of Glyoxalase I" J. Med. Chem. 20: 77 (1977).

115. Foye, W.O., Jan, C. "Synthesis and Evaluation of N-Alkanoyl-S-Benzyl-L-Cysteinylglutamic Acid Esters as Glyoxalase I Inhibitors and Anticancer Agents." J. Pharm. Sci. 73: 559 (1984).

116. Hall, S.S., Doweyko, L.M., Doweyko, A.M., Zilenovski, S.R. "Synthesis and Evaluation of α-Hydroxythiol Esters as Antitumor Agents and Glyoxalase I Inhibitors" J. Med. Chem. 20: 1239 (1977).

117. Oray, B., Norton, S.J. "Inhibitors of Glyoxalase Enzymes" Biochem. Biophys. Res. Comm. 95: 624 (1980).

118. Douglas, K.T., Sharif, J.G. "pH Dependence of the Inhibition of Yeast Glyoxalase I by Porphyrins" Biochim. Biophys. Acta 748: 184 (1983).

119. Lienhard, G.E. "Enzymatic Catalysis and Transition-State Theory" Science 180: 149 (1973).

120. Douglas, K.T., Nadvi, I.N. "Inhibition of Glyoxalase I: A Possible Transition-State Analogue Inhibitor Approach to Potential Antineoplastic Agents?" FEBS Lett. 106: 393 (1979).

121. Brandt, R.B., Brandt, M.E., April, M.E., Thomson, C. "Inhibitors of Glyoxalase I in vitro" Biochem. Med. 29: 385 (1983).

122. Abeles, R.H., Maycock, A.L. "Suicide Enzyme Inactivators" Acc. Chem. Res. 9: 313 (1976).

123. Kuo, D.J., O'Connel, E.L. Rose, I.A. "Physical, Chemical, and Enzymological Characterization of Enolpyruvate" J. Am. Chem. Soc. 101: 5025 (1979).

124. Dauben, Jr., H.J., McCoy, L.L. "N-Bromosuccinimide. I. Allylic Bromination, a General Survey of Reaction Variables" J. Am. Chem. Soc. 81: 4863 (1959).

125. Jarvis, B.B., Saukaitis, J.C. "Nucleophilic Displacements on Halogen Atoms. II. Kinetic Study of the Reactions of α-Halo Sulfones with Triphenylphosphine" J. Am. Chem. Soc. 95: 7708 (1973).

126. Riley, H.A., Gray, A.R., in Organic Synthesis, Collective, Volume 2, Blatt, A.H., ed. (John Wiley & Sons, Inc., New York) 1943.

127. Colonna, S., Re, A., Gelbard, G., Cesarotti, E. "Anionic Activation in Polymer-Supported Reactions. Part 2. Stereochemical Studies on the Introduction of Fluorine at Chiral Centres and in Biologically Significant Molecules" J. Chem. Soc. Perkin I, 2248 (1979).

128. Orion Research: Instruction Manual. Fluoride Electrodes. Model 94-09, 96-09. Form IM94, 96-09/7721 (Orion Research Inc., USA) 1977.

129. Orion Ionalyzer: Instruction Manual. Halide Electrodes. Model 94-17, 94-35, 94,53, 96-17. Form 94H/776 (Orion Research Inc., USA) 1977.

130. Gregory, J.D. "The Stability of N-Ethylmaleimide and its Reaction with Sulfhydryl Groups" J. Am. Chem. Soc. 77: 3922 (1955).

131. Alexander, N.M. "Spectrophotometric Assay for Sulfhydryl Groups Using N-Ethylmaleimide" Analytical Chem. 30: 1292 (1958).

132. Chari, R.V.J., Kozarich, J.W. "Synthesis and Properties of Halohydroxyacetones and Halomethylglyoxals" J. Org. Chem. 47: 2355 (1982).

133. Lin, T., Antonini, I., Cosby, L.A., Sartorelli, A.C. "2,3-Dimethyl-1,4-naphthoquinone Derivatives as Bioreductive Alkylating Agents with Cross-Linking Potential". J. Med. Chem 27: 813 (1984)

134. Dirmaier, L.J., Chari, R.V.J., Isaacs, S.N., Kozarich, J.W. "Further Studies on Mirror-Image Catalysis by Glyoxalase I" Fed. Proc. 42: 1923 (1983).

135. Dirmaier, L.J. Private Communication.

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