S-ALKYLGLUTATHIONE, METHYLGLYOXAL METABOLISM AND CELL DIVISION

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SUMMARY

The effects of S-alkylglutathione on methylglyoxal metabolism in vitro and on cell division in vivo have been studied. S-hexylglutathione inhibited glyoxalase I, but had no effect on a-ketoaldehyde dehydrogenase from various sources including sheep, rat and mouse livers, and human malignant tumor cells. The alkylglutathione did not inhibit cell division in an in vivo mouse epidermis test system. It seems unlikely that S-alkylglutathiones will cause accumulation of ketoaldehydes in cells where both pathways for methylglyoxal degradation are in effective operation.

It has recently been shown that S-alkylglutathiones are competitive inhibitors of glyoxalase (1), the enzyme system which converts methylglyoxal into lactic acid. Methylglyoxal and other a-ketoaldehydes are known to possess carcinostatic activity (2). It has therefore been suggested that inhibitors of the glyoxalase system may be used to cause a build-up of methylglyoxal in cells and thus inhibit cellular growth (1). However, the glyoxalase system is not the only pathway by which methylglyoxal can be degraded. Mammalian cells (3) and certain microorganisms (4) contain an enzyme which catalyses the direct oxidation of methylglyoxal to pyruvic acid. This reaction requires NAD+ or NADP+ as cofactor, but is in contrast with glyoxalase, glutathione independent. The enzyme can also use other a-ketoaldehydes like glyoxal, phenylglyoxal, hydroxypyruvic aldehyde (3) and 2-keto-3-deoxyglucose (5) as substrate, and has therefore been called a-ketoaldehyde dehydrogenase (3).

Potential anticancer agents based on the principle of intracellular methylglyoxal accumulation should consequently possess the ability to inhibit not only the glyoxalase, but also the a-ketoaldehyde dehydrogenase.

The present investigation examines effects of S-alkylglutathiones on the a-ketoaldehyde dehydrogenase from various sources, including malignant tumors from man. Possible inhibitory effects of S-hexylglutathione, methylglyoxal and mixtures thereof on cell division in mouse epidermis in vivo were also tested.

MATERIALS AND METHODS

S-hexylglutathione was synthesised by reacting reduced glutathione (Sigma Chemical Co., USA) with hexyl iodide (Fluka AG., Switzerland) in aqueous ethanol containing a slight excess of sodium hydroxide. The product was isolated as described by Vince and Wadd (1) and recrystallized twice from water. The product gave correct analysis for N (Kjeldahl analysis). All experiments were carried out with S-hexylglutathione, since this is almost as potent an inhibitor of glyoxalase I as S-heptyl and S-octyl glutathione, but considerably more soluble than the latter compounds (1). Solutions of methylglyoxal (Koch-Light Ltd., England) free from acidic contaminants were prepared as described earlier (5). Glyoxalase I (EC 4.4.1.5) was obtained from Boehringer and Sohne, Germany, and was diluted before use with phosphate buffer (0.01M, pH 6.6).

Crude solutions of a-ketoaldehyde dehydrogenase were prepared from rat, mouse and sheep livers and from two malignant tumors (adenocarcinoma of the stomach from a 60 year old male patient, and retroperitoneal neurogeneous tumor from a 45 year old female patient). The tissues were homogenized in 5 volumes of 0.15 M sodium chloride in a Waring blendor and sonicated for 30 seconds

(20,000 cycles/sec and 6A) with a Branson S-75 sonifier. After centrifugation at 105,000 x g for 30 minutes the clear supernatants were passed through a column (30 x 1½ cm) of Sephadex G-25, equilibrated with 0.1 M phosphate buffer (pH 7.9). This removed all endogeneous cofactors including glutathione and pyridine nucleotides. The gelfiltered protein fraction was used as enzyme. In some experiments a partially purified a-ketoaldehyde dehydrogenase was used. The purification involved preparation of acetone powder from sheep liver, followed by extraction and fractionation with ammonium sulphate as previously described (5) following the method of Monder (3).

The activity of glyoxalase I was measured as increase in the absorbance at 240 mμ and the activity of the a-ketoaldehyde dehydrogenase was followed as increase in absorbance at 340 mμ in a Zeiss recording spectrophotometer (RPQ 20A), as previously described (5). The enzyme activities were also measured as disappearance of the substrate (methylglyoxal), which was determined using the 2,4-dinitrophenylhydrazine method previously described (5).

The effect of S-hexylglutathione on dividing cells in vivo was tested with the hairless mouse epidermis as assay system. The rate of cell proliferation was estimated by means of the Colcemid method (for discussion of this procedure see reference 6). In all experiments 0.15 mg of Colcemid was injected intraperitoneally together with the substance to be tested. The animals were killed by neck fracture exactly four hours after injection of Colcemid. After routine dehydration and paraffin embedding the sections were stained with Celestine-Blue/haemalum and mitoses were counted in 8 mm interfollicular epidermis. The number of Colcemid arrested mitoses represents the number of cells that has entered mitosis during the 4 hour period. This method has been extensively used

for testing the activity of various substances on the epidermal mitotic rate (7). The cytostaticum methotrexate was used as a control in the present experiments since this agent is known to produce a mitotic depression in the interfollicular epidermis (8).

RESULTS

Fig. 1a confirms the finding of Vince and Wadd that S-hexylglutathione inhibits glyoxalase I. Our data show that approximately

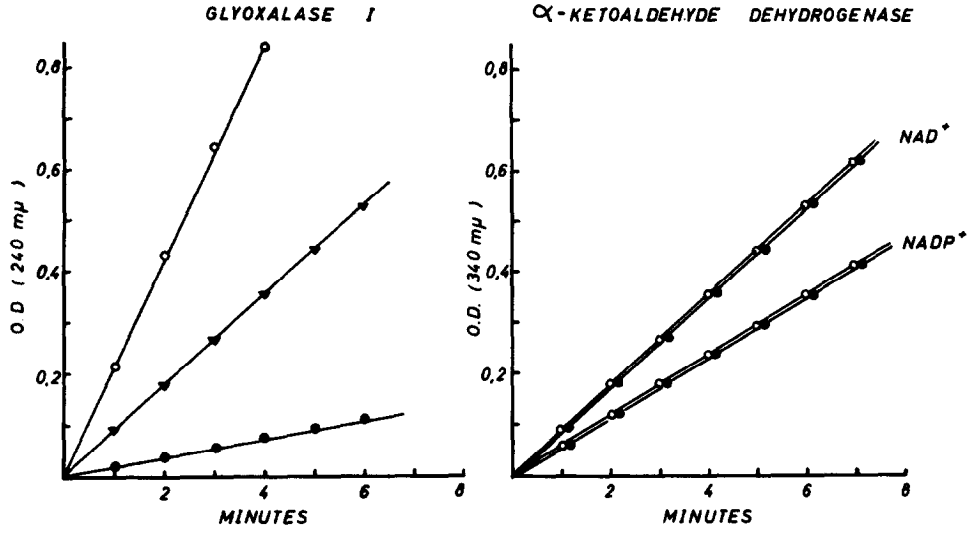

Fig. 1a. Effect of S-hexylglutathione on glyoxalase I. The assay mixture contained: phosphate buffer (pH 6.6) (67mM), glutathione (1.3mM), methylglyoxal (1.3mM), glyoxalase I (5 μg of protein) and S-hexylglutathione in concentrations as stated below, in a final volume of 1.5 ml at 22°.

—○—○— = control without hexylglutathione
—▼—▼— = 0.08 mM hexylglutathione
—●—●— = 0.8 mM hexylglutathione

Fig. 1b. Effect of S-hexylglutathione on partially purified sheep liver a-ketoaldehyde dehydrogenase. The assay mixture contained: Tris buffer (pH 7.8) (100mM), methylglyoxal (5mM), sheep liver enzyme (40 μg of protein), NAD+ or NADP+ (0.6mM) and S-hexylglutathione (10mM) in a final volume of 1.6 ml at 22°.

—○—○— = control without hexylglutathione
—●—●— = with 10 mM hexylglutathione

0.04 mM final concentration of S-hexylglutathione was sufficient to cause 50% inhibition of the glyoxalase, in very good agreement with the results of Vince and Wadd (1). It is evident, however, that S-hexylglutathione has no inhibitory effect on the pyridine nucleotide dependent oxidation of methylglyoxal. Even in the presence of large amounts of S-hexylglutathione (10^{-2}M) the sheep liver a-ketoaldehyde dehydrogenase catalyzed the oxidation of methylglyoxal to pyruvate at the same rate as in the control (Fig. 1b). The same result was obtained when a-ketoaldehyde dehydrogenases from mouse livers and rat livers were used (not shown). The two tumors tested also contained a-ketoaldehyde dehydrogenase activity, measured as increase in optical density at 340 mμ as well as disappearance of substrate (methylglyoxal). S-hexylglutathione (0.01 M) had no inhibitory effect on the tumor dehydrogenase activity (Fig. 2).

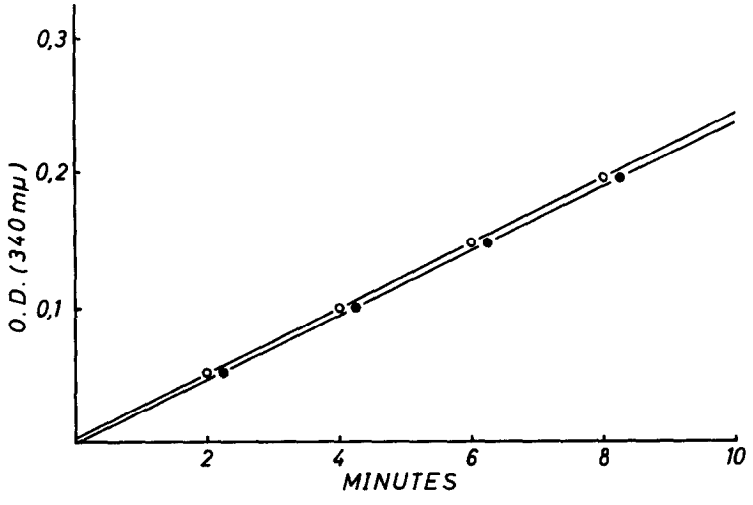

Fig. 2. Effect of S-hexylglutathione on a-ketoaldehyde dehydrogenase from adenocarcinoma cells. The assay mixture contained: sodium phosphate buffer (pH 7.9) (80mM), methylglyoxal (5mM), gelfiltered extract of adenocarcinoma cells (200 μg of protein), NAD+ (0.6mM) and S-hexylglutathione (10mM) in a final volume of 1.6 ml at 22°.

——○——○—— = control without hexylglutathione
——●——●—— = with 10 mM hexylglutathione

TABLE I

EFFECT OF S-HEXYLGLUTATHIONE AND METHYLGLYOXAL ON THE EPIDERMAL MITOTIC RATE

Treatment	Number of Animals	Number of arrested Mitoses
Methylglyoxal (25 µmoles i.p.)*	12	23.33 ± 1.12
S-hexylglutathione (5 µmoles i.p.)	8	24.62 ± 2.87
S-hexylglutathione (5 µmoles i.v.)*	7	23.00 ± 4.04
S-hexylglutathione (5 µmoles) + Methylglyoxal (25 µmoles) i.p.	8	23.50 ± 3.60
Controls (no treatment)	17	24.17 ± 1.98
Controls Methotrexate, i.p.)**	8	15.75 ± 2.45

* Abbreviations: i.p. = intraperitoneally
i.v. = intraveneously
Total volume injected into each animal: 0.5 ml.
** 5 mg/kg body weight

Table I shows that S-hexylglutathione had no effect on the cell division in the mouse epidermis. Even a mixture of the alkylated glutathione and methylglyoxal had no effect in our *in vivo* test system. It should be noted that the amount of S-hexylglutathione injected into the mice (5 µmoles per mouse weighing approximately 25 g) was sufficient to obtain a concentration in the animal of 0.6 - 0.7 mM which is about 15 - 20 times higher than required to inhibit the glyoxalase 50%.

DISCUSSION

The physiological role of glyoxalase is not known, although it has been suggested that this enzyme system might be involved in the regulation of cell division by breaking down growth-retarding a-ketoaldehydes (9).

The occurrance of a-ketoaldehydes in tissues is, however, doubtful, particularly after the finding that ketoaldehydes readily are produced as artifacts during recommended isolation procedures (10-12). The question whether ketoaldehydes are found in tissues therefore remains unsettled, but several reports suggest that methylglyoxal may be a metabolic intermediate (see reference 3). Inhibitors of methylglyoxal metabolism leading to a build-up of the ketoaldehyde in the cells might prove to be powerful growth-retarding substances, as suggested by Vince and Wadd (1). The present investigation shows that although being a potent inhibitor of the glyoxalase I, S-hexylglutathione has no effect on the a-ketoaldehyde dehydrogenase which converts methylglyoxal directly to pyruvate (3). It therefore appears unlikely that S-alkylglutathiones will cause an accumulation of ketoaldehydes in cells where both pathways for methylglyoxal degradation are in effective operation. This probably explains the failure of S-hexylglutathione to inhibit cell division in our <u>in vivo</u> test system. Another question is whether all cells, and in particular cancer cells, contain a-ketoaldehyde dehydrogenase. The results of Monder (3) indicate that several organs, e.g. kidney and lung from rat apparently are devoid of the enzyme, although kidney and lung from a different species (sheep) show a very high dehydrogenase activity (3). The present investigation shows that at least certain cancer cells, such as the cells of an adenocarcinoma, contain a-ketoaldehyde dehydrogenase activity. The possibility exists, however, that other types of cancer cells might be

devoid of the enzyme. It appears likely that only in such cases will S-alkylglutathiones show growth retarding activity.

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