Biochemical Pharmacology, Vol. 38, No. 11, pp. 1835–1840, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

THE EFFECT OF IFOSFAMIDE AND ITS METABOLITES ON INTRACELLULAR GLUTATHIONE LEVELS *IN VITRO* AND *IN VIVO*

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(*Received* 16 *November* 1988; *accepted* 16 *December* 1988)

Abstract—The effect of ifosfamide and its metabolites on intracellular levels of glutathione in P388 cells *in vitro* has been studied. It is demonstrated that glutathione depletion occurs only in the presence of 4-hydroperoxyifosfamide and chloroacetaldehyde. In contrast isophosphoramide mustard had no effect on glutathione levels in intact cells. The concentration of 4-hydroperoxyifosfamide required to reduce glutathione levels by 50% was approximately 1 mM and this represents a concentration far in excess of that achievable in patients receiving the drug. However the concentration of chloroacetaldehyde (approximately 100 μM) required to reduce intracellular levels of glutathione to a similar extent is attained in patients receiving ifosfamide. The glutathione levels in lymphocytes isolated from a patient undergoing an eight hour infusion of ifosfamide showed a marked decrease to about 30% of their original value. We conclude that ifosfamide causes glutathione depletion *in vivo* and the majority of this can be accounted for by the production of chloroacetaldehyde.

Ifosfamide, an isomer of cyclophosphamide, has been shown to be useful in the treatment of a number of solid tumours. It has distinct advantages over cyclophosphamide in its efficacy and toxicity profile [1]. Like cyclophosphamide, ifosfamide requires hepatic activation which results in the production of a number of active, potentially active and inactive metabolites [2]. A major difference in the metabolism of the two drugs is that ifosfamide can undergo *N*-dealkylation to liberate chloroacetaldehyde and monochloroethyl ifosfamide [3]. Chloroacetaldehyde has been shown to be highly toxic although its precise mechanism of action remains unknown [4].

Glutathione and other sulphydryl reagents have been implicated as a determinant of responsiveness to a variety of antitumour agents including cyclophosphamide [5] and other alkylating agents [6]. Deactivation of alkylating agents has been proposed to occur by conjugation of one of the nucleophilic groups to a sulphydryl group. This reaction may occur either spontaneously or in an enzyme mediated reaction involving the glutathione transferases [7].

In this study we describe the use of monochlorobimane and the technique of flow cytometry to measure intracellular glutathione levels in P388 lymphocytes and in lymphocytes isolated from a patient undergoing treatment with ifosfamide.

This method provides rapid quantification of glutathione levels in intact cells [8], and has been used to evaluate the effect of ifosfamide and its metabolites on *in vivo* and *in vitro* intracellular levels of glutathione.

MATERIALS AND METHODS

Ifosfamide, 4-hydroperoxyifosfamide (4HOO-IF), isophosphoramide mustard (IPM), carboxyifosfamide (CIF) and monochloroethyl ifosfamide (MCI) were kindly donated by Degussa (Bielefeld, F.D.R.). Chloroacetaldehyde, glutathione (GSH), rat and human placental glutathione transferase, and 1-chloro-2,4-dinitrobenzene (CDNB) were supplied by the Sigma Chemical Company (Poole, Dorset, U.K.).

Syn-monochlorobimane and *syn*-monobromobimane were synthesised from ethyl α-methylacetoacetate using a modification of the method of Kosower [9, 10]. Briefly, bromination of *syn*-tetramethylbimane afforded a mixture of *syn*-dibromobimane and *syn*-monobromobimane. These compounds were separated at this stage by flash chromatography in dim or red light using ethyl acetate as eluant [11]. *Syn*-monochlorobimane was prepared from the pure monobromobimane according to the published method [9, 10].

Cell culture. A P388 cell line was grown in RPMI medium supplemented with 10% horse serum (Gibco, U.K.). Cells are regularly screened and shown to be mycoplasma free.

Isolation of human lymphocytes. Lymphocytes were isolated by the use of a Ficoll–Paque (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Determination of intracellular glutathione levels. These were determined cytofluorimetrically on a Coulter EPICS V cytofluorimeter with a Spectrophysics 2025 Argon laser capable of producing up to 850 mW maximum in the ultraviolet. The laser power used in these experiments was 100 mW.

Glutathione levels were determined by quantification of the fluorescence from monochlorobimane treated cells. The fluorescence arises from the prod-

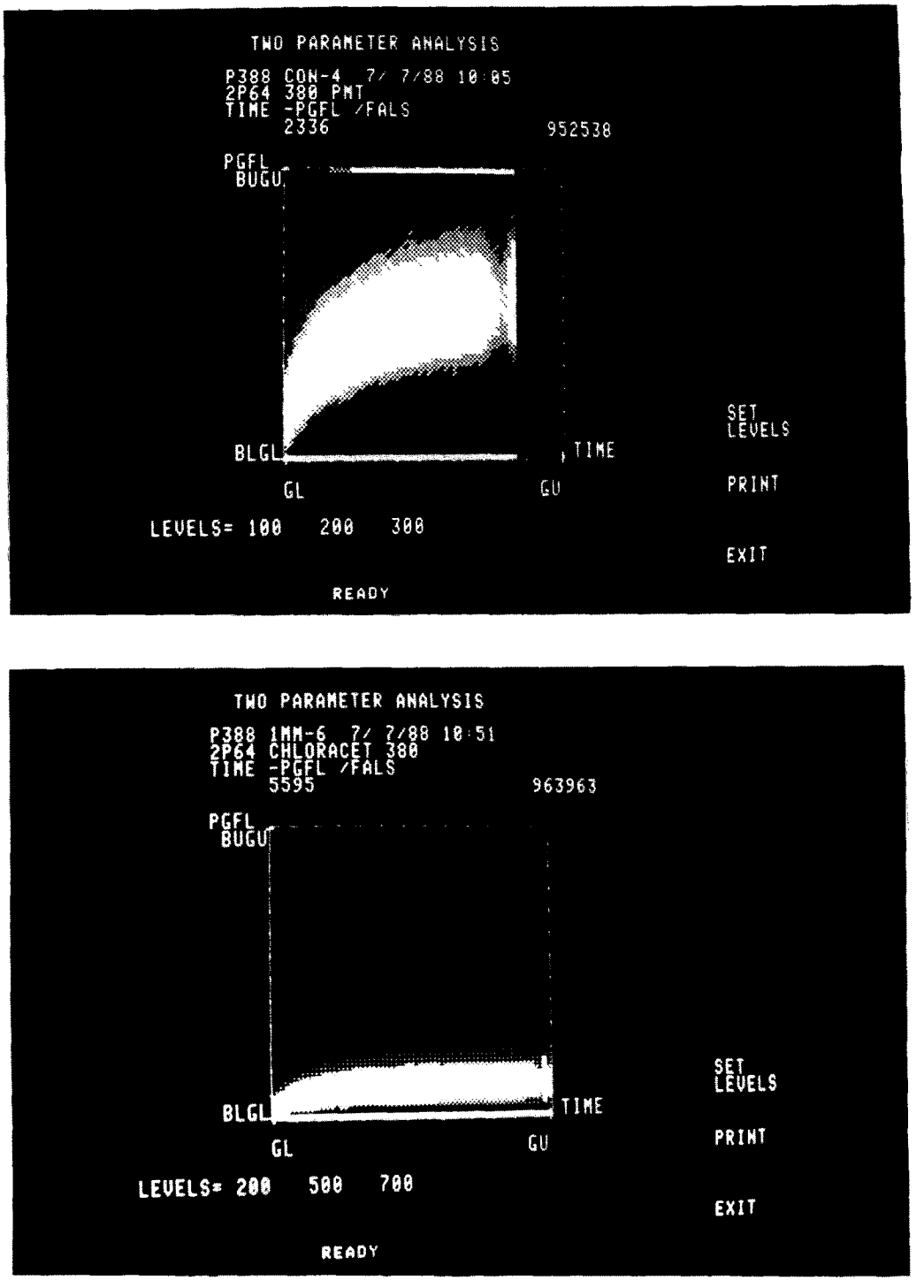

Fig. 1. Flow cytofluorimetric analysis of the rate of conversion of monochlorobimane to its fluorescent glutathione adduct. The abscissa (time) scale is 20 min, and the ordinate scale arbitrary fluorescent units. Figure 1(a) shows data from untreated P388 cells, while Fig. 1(b) shows the corresponding fluorescence-time distribution following treatment with chloroacetaldehyde (1 mM, 60 min, 37°).

uct of conjugation of glutathione with monochlorobimane (non-fluorescent) to yield a fluorescent bimane–glutathione adduct [12]. Fluorescence was measured using a 408 nm long pass filter. Cells (approximately 10^6/ml) were treated with monochlorobimane (20 μM final concentration) and the rate of development of cellular fluorescence was followed utilising the internal clock of the Coulter EPICS V. Mean fluorescence per cell was calculated from the fluorescence–time distribution.

Table 1. The effect of ifosfamide and ifosfamide metabolites on P388 glutathione levels and rat glutathione transferase

Metabolite	I	4HOO-I	IPM	CIF	MCI	CHA
I_{50}*	68.7	6.6	10.3	>100	>100	8.3
%GSH†	110	46	100	108	103	20

* I_{50} represents the concentration (mM) of drug or metabolite necessary to cause a 50% decrease in the rate of conjugation of glutathione (1 mM) to 1-chloro-2,4-dinitrobenzene by rat liver glutathione transferase.

† %GSH refers to the fluorescence from cells following treatment with ifosfamide or metabolites (1 mM) after incubation with monochlorobimane. All results are expressed as a percentage of control (untreated) cells.

I = ifosfamide; 4HOO-I = 4-hydroperoxyifosfamide; IPM = isophosphoramide mustard; CIF = carboxyifosfamide; MCI = monochloroethylifosfamide; CHA = chloroacetaldehyde.

Enzyme kinetic studies. Glutathione transferase activities were measured as described by Habig and Jakoby [13] using 1-chloro-2,4-dinitrobenzene (1 mM) and glutathione (1 mM) as substrates in potassium phosphate buffer (100 mM, pH 6.5, 25°). Absorption increases at 340 nm were measured on a Beckman DU8 spectrophotometer with a kinetics 11 accessory. The uncatalysed reaction was monitored in the absence of enzyme. Enzyme inhibition studies were carried out by addition of agents as described in the text.

Effect of chloroacetaldehyde on the sulphydryl group of glutathione. Free glutathione sulphydryl levels were determined using Ellman's reagent [di-(2-carboxy-3-nitrophenyl)disulphide] as described by Deakin *et al.* [14]. The concentration of sulphydryl was determined following incubation of glutathione (100 μM) with chloroacetaldehyde (1 mM) in the presence and absence of commercial glutathione *S*-transferase (1 unit/ml) for a period of up to 20 min. Free –SH levels were determined spectrophotometrically at 412 nm following addition of Ellman's reagent. The pseudo first order rate constants were calculated from log [–SH]/time plots under conditions where the concentration of chloroacetaldehyde (in excess) could be considered constant.

Patient treatment and blood collection. Serial blood samples were taken from a patient with epithelial ovarian cancer receiving an eight hour infusion of ifosfamide at a dose of 5 g/m^2 given with an equal dose of the uroprotector mesna.

RESULTS

The effects of chloroacetaldehyde on the rate and extent of development of fluorescence intensity in P388 cells following incubation with monochlorobimane are shown in Fig. 1. This shows the increase in fluorescence (ordinate) with time (abscissa). The intensity of shading is proportional to the number of cells. The average fluorescence per cell can be calculated from these distributions at each time point by the use of the computational facilities of the EPICS V.

Figure 1(a) shows the rapid rise in fluorescence for untreated (control) P388 cells. Figure 1(b) shows the effect of chloroacetaldehyde (1 mM) which results in a decrease in the fluorescence intensity observed following treatment with monochlorobimane.

Using similar techniques, the effect of ifosfamide and its metabolites on intracellular glutathione levels are shown in Table 1. It can be seen that ifosfamide, isophosphoramide mustard, carboxyifosfamide and monochloroethyl ifosfamide do not reduce glutathione levels in cultured P388 cells following treatment at 1 mM concentrations for 1 hr at 37°. However, 4-hydroperoxyifosfamide and chloroacetaldehyde can be seen to extensively deplete intracellular glutathione. The effect of altering the concentration of 4-hydroperoxyifosfamide on glutathione in P388 cells is shown in Fig. 2. This metabolite can be seen to deplete glutathione in a concentration-dependent manner.

The potency of 4-hydroperoxyifosfamide, however, is considerably less than that observed for chloroacetaldehyde (Fig. 3). These data show that the concentration required to reduce glutathione levels by 50% for 4-hydroperoxyifosfamide is approximately 1 mM whilst for chloroacetaldehyde this level is approximately 100 μM. This concentration of chloroacetaldehyde does not alter the ability of the cells to hydrolyse fluorescein diacetate and to retain the fluorescent product within the cell. These cells appear microscopically indistinguishable from untreated cells. The retention of this product is diagnostic of an intact cell membrane.

A comparison using the alternative fluorescent probe monobromobimane (Fig. 4) shows that there is little difference between it and monochlorobimane. The effect of 4-hydroperoxyifosfamide on rat glutathione transferase activity is shown in Fig. 5. It can be seen that the rate of conjugation of CDNB to glutathione is inhibited by 4-hydroperoxyifosfamide. The concentration required to reduce this rate by 50% is approximately 6.6 mM. Similar data for other agents is shown in Table 1.

From this table it is apparent that the most potent inhibitors of glutathione transferase action with regard to CDNB–glutathione conjugation are 4-hydroperoxyifosfamide, chloroacetaldehyde, and isophosphoramide mustard. In a separate experiment preincubation of enzyme with 4-hydroperoxyifosfamide for 30 min at 37° did not alter the rate at which glutathione was conjugated to CDNB. This

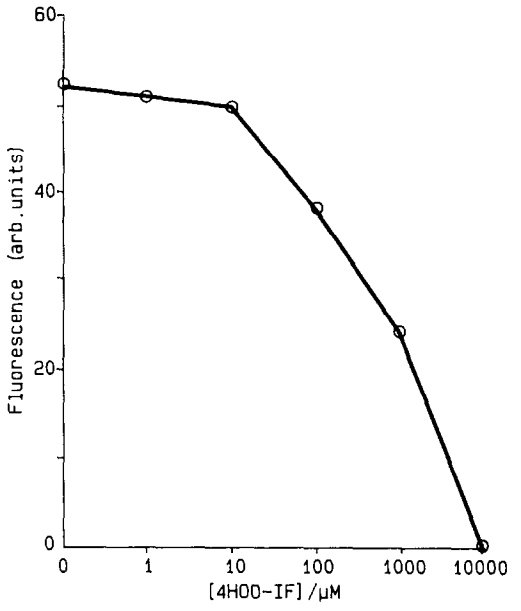

Fig. 2. Effect of concentration of 4-hydroperoxyifosfamide on intracellular glutathione levels in P388 cells following incubation for 1 hr at 37°. Intracellular glutathione is expressed as the mean intracellular fluorescence (arbitrary units) from bimane treated cells.

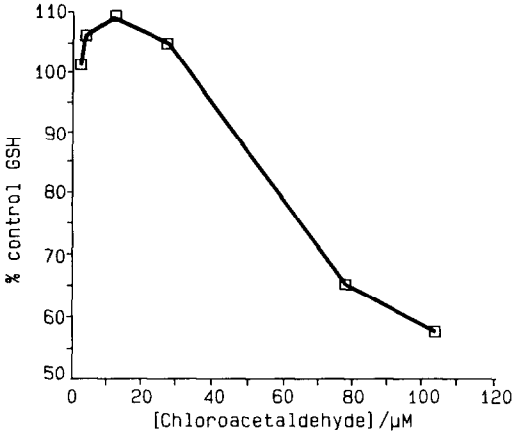

Fig. 3. Effect of chloroacetaldehyde on the glutathione levels in P388 cells following treatment for one hour at 37°. Glutathione levels are expressed as a percentage of control (untreated cells) and are obtained from the mean intracellular fluorescence values obtained by flow cytometry.

indicates that the enzyme is not deactivated by 4-hydroperoxyifosfamide. Further studies on the nature of chloroacetaldehyde inhibition of human placental glutathione transferase were carried out by keeping the concentration of glutathione in excess of 20 mM and varying the concentrations of chloroacetaldehyde and CDNB. A Dixon plot of the data is shown in Fig. 6. From this plot it can be deduced that chloroacetaldehyde inhibits human placental glutathione transferase in a competitive manner with a K_i value of approximately 1.5 mM. Rigorous

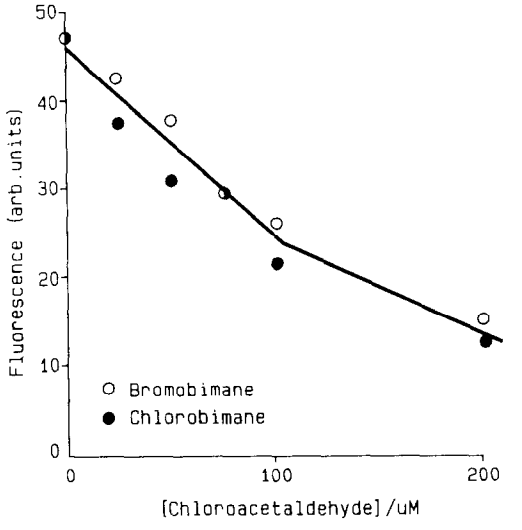

Fig. 4. Comparison of the intracellular glutathione probes monochlorobimane and monobromobimane, following treatment with chloroacetaldehyde.

enzyme inhibition studies could not be carried out on the other metabolites due to insufficient material.

The rate of conjugation of chloroacetaldehyde to glutathione was found to be independent of whether glutathione *S*-transferase was present in the incubation medium. The pseudo first order rate constant obtained in the presence of enzyme ($k = 6.5 \pm 0.3 \times 10^{-2}\,\text{min}^{-1}$) was identical to that obtained in the absence of enzyme ($k = 6.9 \pm 0.5 \times 10^{-2}\,\text{min}^{-1}$). Both rate constants were obtained from log[GSH] against time plots which gave excellent straight lines with correlation coefficients from linear regression calculations of > 0.99.

In order to determine whether such depletion of glutathione occurs *in vivo* following ifosfamide administration the glutathione levels in peripheral lymphocytes of a patient undergoing treatment with an eight hour infusion were followed for up to 27 hours following commencement of therapy (Fig. 7). It can be seen that during the infusion the glutathione level is drastically reduced to 30% of its initial value. This is followed by a slow recovery from the baseline level following cessation of the infusion, with a return to a glutathione level of 60% of its initial value approximately 20 hours after the start of the infusion.

DISCUSSION

Chloroacetaldehyde, which has been shown to be formed following the metabolism of ifosfamide but not cyclophosphamide [3], has been implicated in the neurotoxic syndrome associated with ifosfamide administration [15]. In this paper we describe the reduction of glutathione levels by this agent. The levels of chloroacetaldehyde used to accomplish this are comparable to those seen in patients receiving ifosfamide (50–100 μM, Ref. 15). However, the levels of 4-hydroperoxyifosfamide required to reduce intracellular glutathione are far in excess of those generally seen in patients (< 100 nM, Ref. 16).

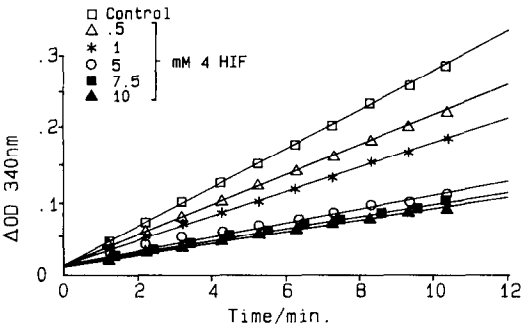

Fig. 5. Inhibition of commercial rat glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene by 4-hydroperoxyifosfamide (4 HIF) at the concentrations shown.

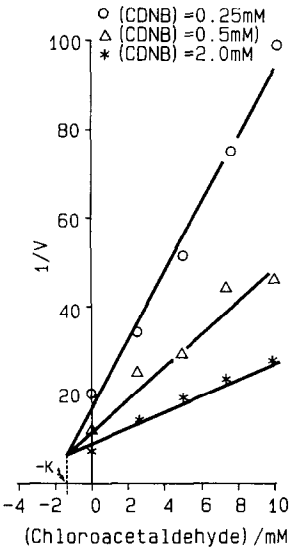

Fig. 6. Dixon plot of the inhibition of human placental glutathione *S*-transferase activity by chloroacetaldehyde against 1-chloro-2,4-dinitrobenzene.

It is therefore concluded that the majority of depletion of intracellular glutathione seen in the patient was due to chloroacetaldehyde liberation and not production of 4-hydroperoxyifosfamide or other metabolites.

Isophosphoramide mustard, which we have shown to be capable of inhibiting transferase-mediated conjugation of CDNB to glutathione (Table 1), did not reduce intracellular glutathione. It is proposed that this metabolite cannot enter cells due to its polar nature and hence has no effect on intracellular glutathione. This may account for the reduced cytotoxicity of the mustard as opposed to the 4-hydroxy metabolite [17].

A comparison of chlorobimane and bromobimane as probes for intracellular glutathione levels shows no difference between these agents. Bromobimane differs from chlorobimane in its ability to conjugate with glutathione in the absence of the enzyme glutathione transferase. In contrast, the rate of conjugation of chlorobimane with glutathione in the absence of the enzyme is insignificant [8] compared with the enzyme-mediated reaction. Following chloroacetaldehyde administration, the similarity of the results obtained using these two probes suggests that the enzyme is not deactivated by chloroacetaldehyde (Fig. 4). These data cannot, however, distinguish whether the conjugation of chloroacetaldehyde with glutathione is an enzymatic or chemical process. Evidence that the reaction of chloroacetaldehyde with glutathione is a chemical, and not an enzymatic, reaction is provided by the lack of enhancement of the rate of reaction when glutathione *S*-transferase is added. It may, therefore, be concluded that this reaction does not require the presence of the enzyme. However, the enzyme inhibition studies show chloroacetaldehyde to be a competitive inhibitor of human placental glutathione transferase (Fig. 6).

Data from the patient show that ifosfamide administration at a conventional dose of 5 g/m^2 produced marked depletion of intracellular glutathione. This decrease is attributed to the high level of chloroacetaldehyde production, compared to 4-hydroperoxyifosfamide and also its affinity for glutathione.

These observations have two important consequences. Firstly, ifosfamide is capable of depleting intracellular glutathione levels in peripheral lymphocytes *in vivo*. It has yet to be determined whether a similar effect occurs in either tumour or solid tissue. However, if this were to be the case this property could be used to sensitise tumours to other agents where resistance is known to be mediated by increased glutathione and glutathione transferases. Secondly, glutathione and glutathione transferase are responsible for the intracellular metabolism and detoxification of various ifosfamide metabolites, especially chloroacetaldehyde. Therefore intracellular glutathione levels may be important in determining the toxicity of ifosfamide. This may have particular importance with regard to the neurotoxicity syndrome which is thought to be caused by chloroacetaldehyde [15]. It has been noted by several investigators [18–20] that neurotoxicity from ifosfamide is commoner following oral than intravenous administration. Oral administration may result in

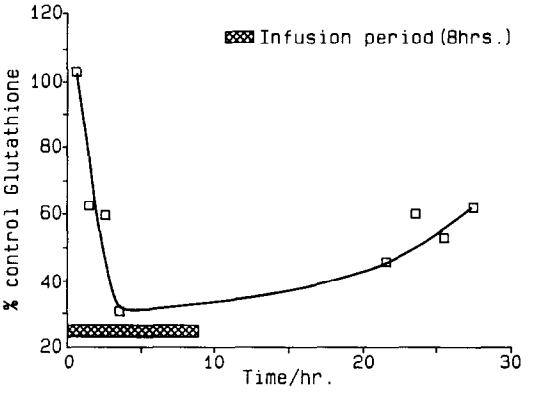

Fig. 7. Effect on lymphocyte glutathione levels following an eight hour infusion of ifosfamide (5 g/m^2).

higher concentrations of metabolites in the liver during the first pass. This would result in a more pronounced fall in hepatic glutathione levels than would be expected following intravenous administration where ifosfamide is distributed throughout the total body water leading to lower hepatic concentrations of glutathione-depleting metabolites. Thus there may be a failure of the liver to detoxify chloroacetaldehyde due to glutathione depletion when ifosfamide is given orally. Therefore chloroacetaldehyde levels in the blood would be elevated which could explain the higher frequency and severity of neurotoxicity with the oral route of administration. There is already some evidence that urinary metabolites of ifosfamide are found in higher quantities following oral administration [21].

In conclusion, ifosfamide is capable of depleting intracellular glutathione *in vivo* and *in vitro*. This property could be exploited in designing suitable combination chemotherapy. This method of reducing concentrations of intracellular glutathione may be an alternative to the use of agents such as buthionine *l*-sulphoximine as, in the case of ifosfamide, depletion is accomplished by an agent with proven antitumour activity.

Acknowledgements—This work was supported by the Cancer Research Campaign.

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