Biochemical Pharmacology, Vol. 40, No. 8, pp. 1893–1900, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

SPERMINE TOXICITY AND GLUTATHIONE DEPLETION IN BHK-21/C13 CELLS

VALERIE G. BRUNTON,*† M. HELEN GRANT*‡ and HEATHER M. WALLACE*†
Clinical Pharmacology Unit, Departments of *Medicine and Therapeutics and †Pharmacology, Polwarth Building, University of Aberdeen, Foresterhill, Aberdeen AB9 2ZD, U.K.

(*Received* 25 *April* 1989; *accepted* 21 *June* 1990)

Abstract—Spermine, a polycationic amine, produced a dose-dependent inhibition of BHK-21/C13 cell growth. This response was not due to the extracellular metabolism of spermine by an amine oxidase found in bovine serum, as the toxicity was observed when the cells were grown in medium supplemented with horse serum. Three indices were used to monitor cell growth, cell number, protein content and [^{3}H]thymidine incorporation into DNA. Spermine (2 mM) caused significant reductions in all three measurements after a 6–8 hr exposure. The amine was rapidly taken up into the cells reaching levels 15–16-fold greater than in control cells within 2 hr. There was a rapid loss of intracellular reduced glutathione following exposure to toxic concentrations of spermine, which occurred before any effect on cell growth. Three methods for the determination of intracellular glutathione content were compared in this system. The effect on both cell growth and glutathione was reversible following removal of spermine from the extracellular medium. The possible mechanisms involved in this toxic response are discussed with particular reference to the depletion in intracellular reduced glutathione.

The polyamines, spermidine [$NH_2(CH_2)_3NH(CH_2)_4NH_2$] and spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] and their precursor putrescine [$NH_2(CH_2)_4NH_2$] occur in all mammalian cells and are essential for normal cell growth and differentiation [1, 2]. Their intracellular levels are highly regulated and are found to be elevated in rapidly proliferating tissues. However, at high concentrations these amines have been shown to inhibit growth in a number of different cell lines [3–5]. These groups reported that the inhibition of cell growth was dependent on the activity of an amine oxidase which is present in ruminant serum. Replacement of calf serum by horse serum, which does not contain this enzyme, abolished the toxicity of the polyamines. The serum amine oxidase acts on spermidine and spermine to produce an aminomonoaldehyde [N^1-(4-aminobutyl)-amino propionaldehyde] or a dialdehyde [NN^1-bis-(3-propionaldehyde)-1,4-diaminobutane] respectively plus NH_3 and H_2O_2 [6]. These oxidized polyamines have been postulated to be responsible for the toxicity of the polyamines.

More recent reports have shown that polyamines are also toxic in the absence of ruminant serum [7, 8] and suggest that oxidation of the polyamines is still responsible, but by an enzyme produced by the cells themselves. Cells contain an FAD-dependent polyamine oxidase which will act on spermidine and spermine to produce 3-aminopropionaldehyde and H_2O_2, although the preferred substrates of this enzyme are the acetylated polyamines.

The tripeptide GSH§ is the most abundant intracellular non-protein thiol and is involved in many important cellular functions [9, 10]. One of its most widely recognized roles is that of conjugation with electrophilic compounds or metabolites. These reactions can occur spontaneously or more usually are catalysed by a group of isoenzymes called the glutathione-*S*-transferases. The conjugates are typically excreted in the urine as mercapturic acids [11]. Thus, conjugation with GSH can both detoxify and enhance excretion of reactive compounds.

GSH also plays a vital role in the maintenance of the intracellular redox state, by removal of H_2O_2, formed either as a normal cellular metabolite or, in excess, by the redox cycling of exogenous compounds, e.g. quinones [12]. Both these processes can lead to a depletion of intracellular GSH stores which is extremely detrimental to cellular integrity and is seen as a prerequisite for toxicity following exposure to certain compounds such as paracetamol [13].

In this paper we have examined the toxicity of spermine in a baby hamster kidney fibroblast cell line (BHK-21/C13) grown in the absence of ruminant serum, to investigate in more detail the intracellular events involved in the toxic response and, in particular, we have examined the role of intracellular GSH.

MATERIALS AND METHODS

Chemicals. [^{3}H]Thymidine was obtained from Amersham International (Amersham, U.K.). Spermine tetrahydrochloride, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, NADH, NADPH, 2,4-dinitrofluorobenzene, iodoacetic acid, pyruvic acid, Triton X-100 and Trypan Blue were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). *o*-Phthaldialdehyde was obtained from Fluka BioChemika (Buchs, Switzerland).

Cell culture. BHK-21/C13 cells were routinely

‡ Present address: Department of Bioscience and Biotechnology, Immunology Division, University of Strathclyde, The Todd Centre, 31 Taylor Street, Glasgow G4 0NR, U.K.

§ Abbreviations: HPLC, high performance liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione.

grown in monolayer culture in Dulbecco's medium supplemented with 10% (v/v) horse serum (DH_{10}) in a humidified atmosphere of 5% CO_2–95% air at 37°. For experiments, the cells were seeded at a density of $1.6 \times 10^4/cm^2$ on 9 cm diameter plastic plates and grown for 16 hr before exposure to spermine for up to 24 hr. At the required times the cells were harvested mechanically, using a 'rubber policeman', and polyamines and glutathione were extracted into 0.2 M $HClO_4$ at 4° for 10 min and stored at −20° prior to analysis. The cell pellet was resuspended in 0.3 M NaOH for protein determination. Polyamines were extracted from aliquots of medium in the same way. In the recovery experiments, the cells were exposed to spermine as before for a 12 hr period. The medium was then removed and the cell sheet washed twice with warmed medium containing no serum before addition of spermine-free DH_{10}. The control cells were treated in the same way.

Measurement of cell growth. Cell growth was determined by (a) change in cell number, (b) change in protein content and (c) [^{3}H]thymidine incorporation into acid-insoluble material. Cells were counted with a Neubauer improved haemocytometer in 0.1% Trypan Blue. Viability was assessed by both dye exclusion and lactate dehydrogenase leakage into the medium [14]. Protein content was determined by the method of Lowry *et al.* [15]. [^{3}H]Thymidine incorporation was measured by addition of the isotope (2.5 μCi/plate) to the cultures at 16 hr and harvesting the cells as before. The radioactivity in the acid-insoluble fraction was determined and the results expressed per mg protein.

Measurement of polyamines. Polyamines were measured by a modification of the high performance liquid chromatography (HPLC) method of Seiler and Knodgen [16] as described previously [17].

Measurement of glutathione. Glutathione levels were determined by three methods. (i) GSH was measured by derivatization with *o*-phthaldialdehyde [18]; (ii) GSH and GSSG contents were distinguished using the HPLC method of Reed *et al.* [19]; (iii) the enzymatic cycling method of Tietze [20], which measures total glutathione content, was also employed.

Statistical analysis. Non-paired Student's *t*-test and ANOVA with Dunnett's test were carried out to compare treatment groups with control values as indicated.

RESULTS

Measurement of BHK-21/C13 cell growth in spermine-treated cells

Spermine produced a dose-dependent inhibition of cell growth following a 24 hr exposure, with concentrations of 0.5 mM or less having no effect on cell growth (Fig. 1). High concentrations of spermine caused a loss in cell viability as determined by both dye exclusion and lactate dehydrogenase leakage (Table 1). The use of enzyme leakage into the medium as a measure of cell viability was a more sensitive index than dye exclusion. Concentrations of spermine which reduced cell growth by as much as 70% had no effect on cell viability.

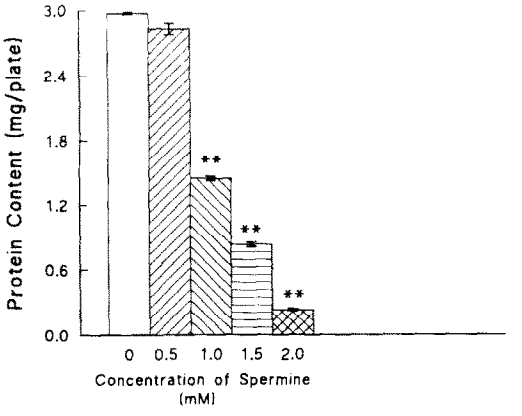

Fig. 1. Effect of a range of spermine concentrations on BHK-21/C13 cell growth. Cells were grown as described in Materials and Methods in the presence of a range of spermine concentrations and harvested after a 24 hr exposure. Values are mean ± SD (N = 6). **P < 0.01 were significantly different from untreated cells as determined by ANOVA.

Table 1. Effect of spermine on BHK-21/C13 cell viability

[Spermine] added (mM)	Viable cells (% of total)	
	Trypan Blue exclusion	LDH leakage
0	99.90 ± 1.04	89.59 ± 7.54
0.5	99.40 ± 3.15	87.37 ± 3.33
1.0	99.13 ± 5.41	85.75 ± 5.71
1.5	97.96 ± 5.36	80.48 ± 4.80
2.0	87.50 ± 4.81	53.22 ± 3.00

BHK cells were grown in the presence of a range of spermine concentrations for a period of 24 hr. The viability was then determined using Trypan Blue exclusion and lactate dehydrogenase leakage and the results expressed as the number of viable cells as a percentage of the total. Values are mean ± SD (N = 6).

From these initial findings two concentrations of spermine were chosen for a more detailed analysis of this response. Figure 2 shows the effect of spermine on BHK cell growth over a 24 hr exposure, using three indices of cell growth: cell number (Fig. 2a), protein content (Fig. 2b) and DNA synthesis (Fig. 2c). Spermine (2 mM) caused a rapid reduction in cell growth after an 8 hr exposure, as determined by all three methods. When cell number was used as an index of cell growth, this effect became significant 2 hr earlier than with the other two indices. Spermine (1 mM) caused about a 50% reduction in cell number and protein content after a 24 hr exposure, but had no significant effect on DNA synthesis over this period.

When cells were exposed to 2 mM spermine for 12 hr and then transferred to spermine-free medium, their growth rate increased (Fig. 3). Sixty hours after the removal of spermine the cell number and protein content had reached approximately 60% of control values, whereas there was a greater than 95% inhibition of cell growth in the presence of spermine at this time. Control cells had by this time reached

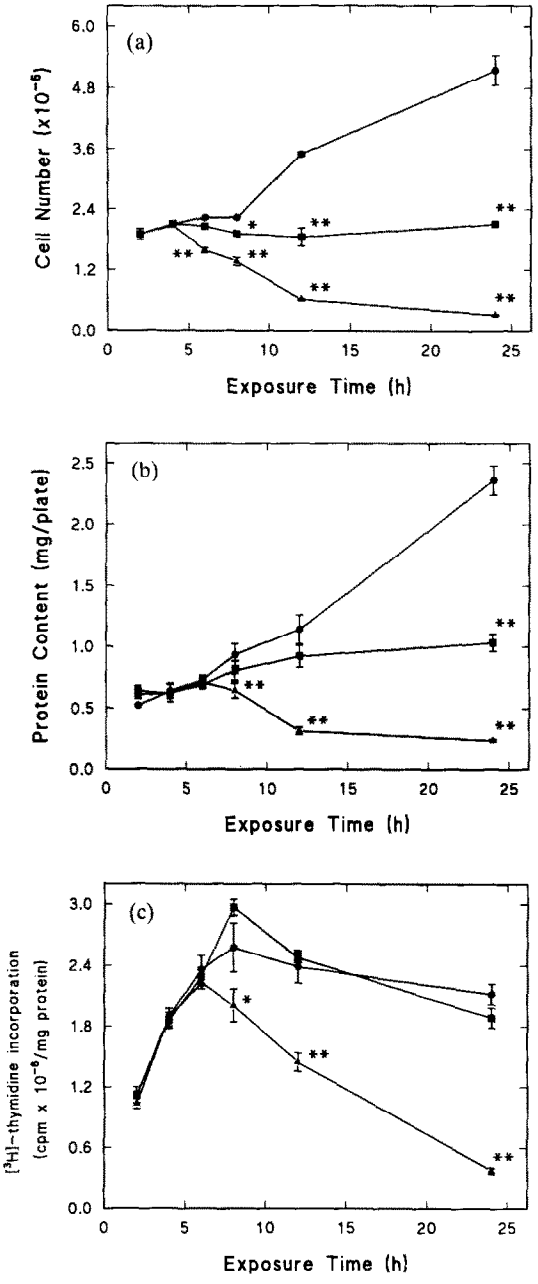

Fig. 2. Effect of spermine on BHK-21/C13 cell growth expressed as cell number (a), protein content (b) and DNA synthesis (c). (●) Control; (■) 1 mM spermine; (▲) 2 mM spermine. Cells were grown as described in Materials and Methods. Values are mean ± SD (N = 3). *P < 0.05, **P < 0.01 values were significantly different from control using ANOVA.

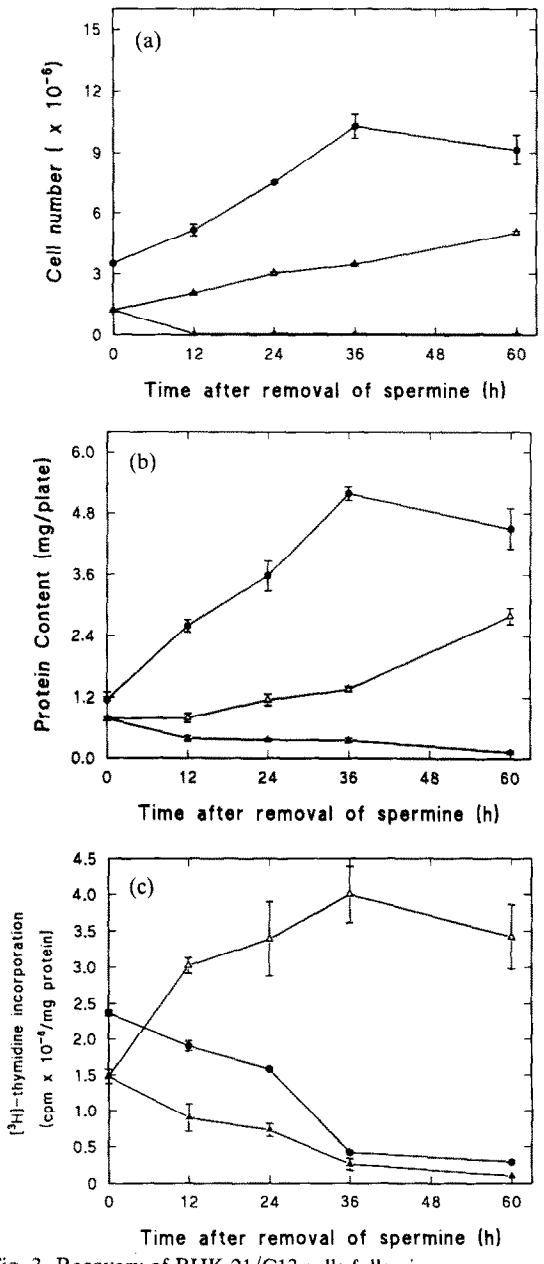

Fig. 3. Recovery of BHK-21/C13 cells following exposure to spermine. BHK cells were exposed to 2 mM spermine for 12 hr after which the spermine was removed from the extracellular medium. Cell growth was monitored by cell number (a), protein content (b) and DNA synthesis (c). (●) Control; (▲) continuous exposure to 2 mM spermine; (△) 12 hr exposure to 2 mM spermine. Values are mean ± SD (N = 3).

confluence and their growth rate was therefore low. The pattern of DNA synthesis in these cells was different. Following the removal of spermine there was a marked increase in DNA synthesis (Fig. 3c). In control cells approaching confluence the incorporation of [^{3}H]thymidine fell to levels which were similar to spermine-treated cells.

Measurement of polyamines in spermine-treated cells

Treatment of BHK cells with spermine caused a rapid uptake of the amine into the cells which resulted in marked changes in the intracellular polyamine profile (Table 2). There were no detectable levels of putrescine in spermine-treated cells and the spermidine levels were also significantly reduced. However, the total polyamine content of these cells was markedly increased due to the high levels of spermine accumulated. After a 24 hr exposure the total polyamine content of 2 mM spermine-treated cells was 4.3-fold greater than

Table 2. Effect of spermine on the polyamine profile of BHK-21/C13 cells

Exposure time (hr)	[Spermine] added (mM)	Intracellular polyamine content (nmol/mg protein)			
		Putrescine	Spermidine	Spermine	Total
2	—	0.73 ± 0.01	7.46 ± 0.61	3.23 ± 0.31	11.42 ± 0.92
	1	ND	0.96 ± 0.21**	26.19 ± 4.62**	27.16 ± 4.83**
	2	ND	3.77 ± 0.58*	50.77 ± 7.33**	54.54 ± 7.85**
4	—	0.64 ± 0.02	6.37 ± 0.54	2.90 ± 0.28	9.90 ± 0.81
	1	ND	3.56 ± 0.29	37.06 ± 0.77**	40.62 ± 1.00**
	2	ND	4.96 ± 0.09	62.57 ± 8.07**	69.51 ± 9.05**
6	—	0.73 ± 0.03	9.31 ± 0.89	4.43 ± 0.81	14.47 ± 1.69
	1	ND	2.61 ± 1.03**	44.94 ± 7.38**	47.55 ± 8.41**
	2	ND	2.06 ± 0.29**	65.20 ± 9.81**	67.27 ± 9.90**
8	—	0.99 ± 0.01	9.30 ± 1.13	6.21 ± 0.53	16.50 ± 2.46
	1	ND	1.64 ± 0.33**	34.39 ± 6.05**	36.03 ± 6.38**
	2	ND	2.53 ± 0.35**	69.81 ± 7.12**	72.34 ± 7.47**
12	—	0.73 ± 0.09	11.39 ± 0.71	5.99 ± 0.43	18.10 ± 1.22
	1	ND	1.46 ± 0.08**	38.68 ± 1.61**	40.14 ± 1.58**
	2	ND	2.17 ± 0.50**	74.09 ± 5.06**	76.26 ± 5.55**
24	—	0.77 ± 0.08	12.03 ± 0.83	7.43 ± 0.54	20.24 ± 1.34
	1	ND	2.33 ± 0.18**	44.36 ± 3.58**	46.68 ± 3.76**
	2	ND	4.05 ± 0.57**	83.97 ± 8.12**	88.02 ± 8.65**

BHK cells were grown in the presence of spermine for a period of 24 hr. The intracellular polyamine content of the cells was determined at various intervals in this period. Values are mean ± SD (N = 3).
* $P < 0.05$, ** $P < 0.01$ were significantly different from control values using ANOVA.
ND, not determined.

Table 3. Changes in intracellular polyamines following removal of spermine from the extracellular medium

Time after removal of spermine (hr)	[Spermine] added (mM)	Intracellular polyamine content (nmol/mg protein)			
		Putrescine	Spermidine	Spermine	Total
0	—	1.50 ± 0.22	10.06 ± 1.19	4.87 ± 0.39	16.42 ± 1.77
	2	ND	1.08 ± 0.09**	75.16 ± 4.89**	79.25 ± 1.98**
12	—	2.21 ± 0.05	12.33 ± 0.46	4.15 ± 1.59	18.39 ± 2.44
	2	ND	1.33 ± 0.20**	15.84 ± 1.76**	17.17 ± 1.82
24	—	1.56 ± 0.19	9.07 ± 0.16	3.55 ± 1.02	14.18 ± 0.88
	2	ND	1.34 ± 0.13**	6.85 ± 0.49*	8.19 ± 0.62*
36	—	0.67 ± 0.06	10.87 ± 0.83	7.80 ± 1.55	19.39 ± 1.99
	2	0.41 ± 0.02	2.96 ± 0.43**	13.48 ± 0.88	16.85 ± 1.22
60	—	ND	3.30 ± 0.46	8.42 ± 1.17	11.72 ± 1.37
	2	0.55 ± 0.17	5.26 ± 1.13*	19.46 ± 2.31**	25.27 ± 2.92**

BHK cells were exposed to 2 mM spermine for 12 hr after which it was removed from the extracellular medium. Thereafter the intracellular polyamine content of these cells was determined for a further 60 hr. Values are mean ± SD (N = 3).
* $P < 0.05$, ** $P < 0.01$ were significantly different from control values using a non-paired Student's *t*-test.
ND, not determined.

control cells and 2.3-fold greater in 1 mM spermine-treated cells. No acetyl polyamine derivatives were detected in the cells.

When spermine was removed from the extracellular medium, there was a rapid loss of spermine from the cells, with the intracellular content falling from 75.16 ± 4.89 to 15.84 ± 1.76 nmol/mg protein in the first 12 hr (Table 3). After a further 24 hr period putrescine was detected in these cells and there was a significant secondary increase in the spermine content. In control cells, as the cells approached confluence, their intracellular polyamine content decreased and putrescine levels fell below the limit of detection.

The excretion of polyamines into the extracellular medium following removal of spermine is shown in Table 4. In control cells the major excretory product was putrescine, with low levels of spermidine and N^1-acetylspermidine also being excreted. As these cells reached confluence, they also excreted spermine, with the total excretion of polyamines being 17-fold greater than from exponentially growing cells.

In spermine-treated cells we saw a rapid loss in the intracellular spermine content when the amine was removed from the extracellular medium (Table 3). The main excretory product from these cells was initially spermine but significant amounts of

Table 4. Excretion of polyamines from BHK-21/C13 cells following removal of spermine from the extracellular medium

Time after removal of spermine (hr)	[Spermine] added (mM)	Extracellular polyamine content (nmol/mL medium)				
		Putrescine	Spermidine	N^1-Acetyl spermidine	Spermine	Total
12	—	0.12 ± 0.01	ND	0.01 ± 0.00	ND	0.13 ± 0.01
	2	ND	0.20 ± 0.04	0.01 ± 0.00	0.50 ± 0.13	0.71 ± 0.13*
24	—	0.60 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	ND	0.64 ± 0.04
	2	0.05 ± 0.01**	0.43 ± 0.09*	0.01 ± 0.00	0.43 ± 0.06	0.92 ± 0.07
36	—	1.21 ± 0.09	0.15 ± 0.03	0.02 ± 0.01	ND	1.38 ± 0.12
	2	0.13 ± 0.01**	0.63 ± 0.08**	ND	0.15 ± 0.04	0.90 ± 0.12*
60	—	1.78 ± 0.33	0.03 ± 0.01	0.08 ± 0.01	0.33 ± 0.07	2.22 ± 0.56
	2	0.80 ± 0.22*	0.77 ± 0.03*	0.02 ± 0.01	0.14 ± 0.09	1.73 ± 0.19*

BHK cells were exposed to 2 mM spermine for 12 hr after which it was removed from the extracellular medium. Thereafter the polyamine content of the extracellular medium was determined for a further 60 hr. Values are mean ± SD (N = 3).

* $P < 0.05$, ** $P < 0.01$ were significantly different from control values using a non-paired Student's *t*-test.

ND, not determined.

spermidine were also detected in the medium (Table 4). The total polyamine excretion from treated cells in the first 36 hr was greater than from control cells, but as the growth of the control cells decreased so their excretion of polyamines increased.

Measurement of glutathione in spermine-treated cells

Three methods were used to measure the glutathione content of BHK cells (Fig. 4). The fluorometric method of Hissin and Hilf [18], which uses *o*-phthaldialdehyde as a fluorophore, gave an underestimate of the GSH content of the spermine-treated cells as the intracellular levels of spermine increased. The separation of the dinitrophenol derivatives of the thiols by HPLC was used to distinguish between the reduced and oxidized forms of glutathione. There were only low levels of the disulphide in the cells, accounting for less than 10% of the total glutathione pool (0.27 ± 0.03 and 0.67 ± 0.07 nmol/mg protein in 2 mM spermine-treated and control cells respectively). Spermine treatment did not alter the ratio of reduced to oxidized glutathione.

The enzymatic cycling method of Tietze was used to confirm the results obtained by HPLC. This method measures total glutathione. Spermine (up to 2 mM) did not interfere with the activity of the enzyme glutathione reductase used in this assay. Spermine (2 mM) caused a decrease in intracellular GSH levels, which was significant after a 2 hr exposure. This preceded any effect on cell growth. Spermine (1 mM) caused a small but significant fall in GSH initially, but by 24 hr it had risen significantly above control values. To enable us to distinguish between GSH and GSSG we chose the HPLC method for further studies.

Figure 5 shows the GSH levels in cells that are recovering after a 12 hr exposure to 2 mM spermine. There was a rapid recovery of GSH to control values in the first 12 hr. These intracellular levels were maintained, while in control cells the levels of GSH fell as the cells approached confluence. The GSH content remained low in the continuous presence of spermine.

DISCUSSION

We have shown that spermine-induced toxicity in BHK cells does not require the presence of ruminant serum but may result from the loss of intracellular GSH. Since this loss occurred before any significant effect on cell growth it may be that the toxicity is dependent on the prior loss of the cytoprotective role of GSH. Henle *et al.* [21] have demonstrated that when CHO cells were depleted of their glutathione stores using buthionine sulphoximine, they were more susceptible to spermidine-induced DNA strand breaks. This suggests that glutathione plays a definite role in the mechanisms involved in polyamine toxicity and that the depletion of glutathione does not occur only as a consequence of the toxic response.

There are two possible explanations for the depletion of intracellular GSH in spermine-treated cells: (i) conjugation or (ii) spermine-induced oxidative stress.

(i) The aminoaldehydes produced in the oxidation reactions of polyamines described earlier may conjugate with GSH resulting in their detoxification and enhanced excretion. Formation of such glutathione adducts in spermine-treated cells would result in a loss of glutathione from the cells. Polyamine–glutathione conjugates have been isolated from both *E. coli* [22] and trypanosomes [23], but as yet no such compounds have been identified in mammalian cells.

(ii) Some workers favour the theory that polyamine toxicity is due to oxidative stress within the cells. This would lead to a loss of glutathione in its oxidized form as the reducing equivalents in the glutathione redox cycle become exhausted. However, Gaugas and Dewey [24] showed that catalase, the enzyme involved in the removal of H_2O_2, was unable to prevent spermine-induced arrest of lymphocyte proliferation and that there was no evidence for the involvement of oxygen radicals in this response. We have also shown that inhibition of catalase or glutathione reductase had no effect on the toxicity of spermine in BHK cells (Brunton VG, Grant

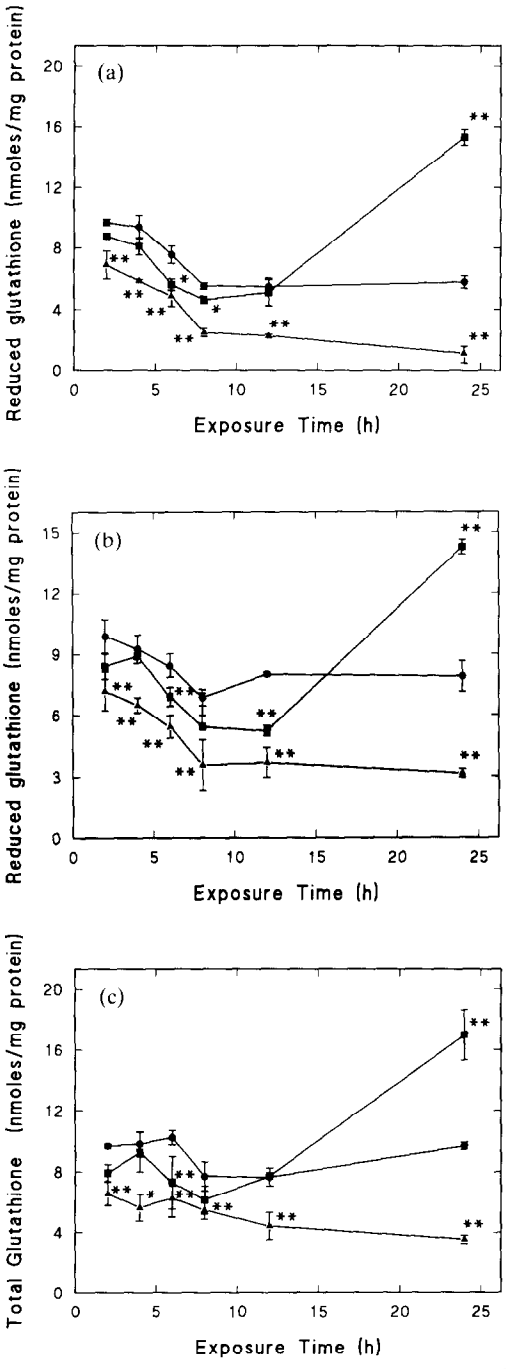

Fig. 4. Effect of spermine on the glutathione content of BHK-21/C13 cells. Reduced glutathione was measured by the method of Hissin and Hilf [18] (a) and by HPLC [19] (b). Total glutathione was determined by the method of Tietze [20] (c). (●) Control; (■) 1 mM spermine; (▲) 2 mM spermine. Values are mean ± SD (N = 3). *P < 0.05, **P < 0.01 values were significantly different from control using ANOVA.

MH and Wallace HM, unpublished observation) suggesting that over-production of H_2O_2 does not play a significant role in spermine toxicity in this model.

When measuring GSH by derivatization with *o*-phthaldialdehyde, the high intracellular spermine

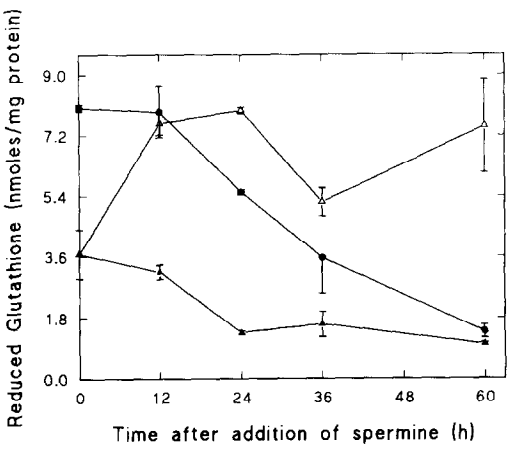

Fig. 5. Recovery of glutathione levels in BHK-21/C13 cells following exposure to spermine. BHK cells were exposed to 2 mM spermine for 12 hr after which the spermine was removed from the extracellular medium. The GSH was measured by HPLC [19]. (●) Control; (▲) continuous exposure to 2 mM spermine; (△) 12 hr exposure to 2 mM spermine. Values are mean ± SD (N = 3).

levels were found to interfere with the assay. Spermine competed with the thiol for the binding sites on the *o*-phthaldialdehyde molecule and therefore gave an underestimate of the GSH content of spermine-treated cells. Glutathione can also be quantified by the HPLC method of Reed *et al.* [19], which measures the dinitrophenol derivatives of glutathione. Dinitrofluorobenzene is used in the derivatization process. Amines are known to bind to this compound; however, dinitrofluorobenzene is present in such excess that we found that there was no interference with the derivatization of the thiol groups.

Using all three indices of cell growth, we showed that the inhibition of cell growth was reversible (Fig. 3). Longer exposure of BHK cells to polyamines causes the cells to round up and detach from the culture dishes. This is an irreversible process [25]. The reversibility of polyamine-induced growth inhibition has been reported previously [21, 26, 27], although again the length of exposure to the polyamines appears to be critical, with longer exposure times and high polyamine concentrations resulting in an irreversible effect. After removal of spermine from the extracellular medium, there was a rapid recovery of GSH (Fig. 5) in the first 12 hr, presumably due to its resynthesis. GSH exerts a negative feedback inhibition on its own synthesis [28]. In spermine-treated cells this inhibition will be absent. A rapid resynthesis of GSH in these cells would therefore be expected when the spermine was removed. In control cells the GSH content fell as the cells reached confluence. This has been demonstrated in other cell lines and may render the cells more susceptible to toxic insult [29]. In BHK cells, however, confluent cultures are more resistant to spermine than cells in exponential growth [30].

Addition of spermine to the extracellular medium resulted in its rapid accumulation within the cells (Table 2). Such high intracellular levels of spermine had marked effects on the polyamine profile of the

cells. Ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, two of the polyamine biosynthetic enzymes, are subject to negative feedback regulation by high levels of spermine [31]. This would lead to a decrease in intracellular putrescine and spermidine and would explain the fall in these two amines seen in spermine-treated cells.

The excretion of polyamines from cells is closely regulated by the growth status of the cells [32]. In rapidly growing cells excretion is low and the rate of polyamine biosynthesis high, whereas in cells approaching confluence the converse is true. This explains the changes in polyamine levels seen in cells which are recovering after exposure to spermine. After the removal of spermine from the extracellular medium polyamine excretion was high as the excess spermine was removed from the cell. As the growth of these cells increased so their rate of excretion fell. Conversely, in control cultures approaching confluence the excretion was high.

Further evidence that polyamine biosynthesis and excretion are highly regulated by the growth status of the cells was the detection of putrescine in cells recovering from prior exposure to spermine (Table 3). As the intracellular spermine levels fall so the negative feedback inhibition of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, will be removed, allowing the resynthesis of putrescine. N^1-Acetylspermidine was one of the excretory products of both control cells and those which were previously exposed to spermine. This has been identified previously as one of the excretory products of BHK cells [33]. Acetyl polyamines were not detected in the cells so their presence in the medium supports the link between acetylation and polyamine excretion [34], and acetylation may enhance or indeed be a prerequisite for the removal of excess polyamines from cells.

The levels of polyamines measured in the extracellular medium do not account entirely for the fall in intracellular polyamine content. However, some loss of spermine would be expected during the two wash periods before excretion measurements began.

The moiety directly responsible for polyamine-induced toxicity has not been identified. As indicated earlier it appears unlikely that the toxicity is due to the over-production of reactive oxygen species. Another possibility is that acrolein, which is formed by the spontaneous breakdown of the aminoaldehydes, may be responsible for the toxicity observed. However, we have shown that only very high concentrations of spermine cause any loss in cell viability. This is in agreement with a previous paper which reports that there is no loss in viability following spermine treatment, whereas it is known that low concentrations of acrolein cause marked reductions in cell viability [27], suggesting that acrolein production does not account for the toxicity seen on exposure to polyamines. However, a study of the oxidative metabolism of spermine in BHK cells must be carried out before this possibility can be disregarded.

Our work is currently focusing on the role of the aminoaldehydes produced in cells grown in medium containing horse serum on exposure to spermine and the possibility of the formation of a dioxidized spermine–glutathione conjugate.

Acknowledgement—We wish to thank the Wellcome Trust for its financial support of this work.

REFERENCES

1. Heby O, Role of polyamines in the control of cell proliferation and differentiation. *Differentiation* **19**: 1–20, 1981.
2. Pegg AE, Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem J* **234**: 249–262, 1986.
3. Alarcon RA, Foley GF and Modest EJ, Effects of spermine on mammalian cells. *Arch Biochem Biophys* **94**: 540–541, 1961.
4. Bachrach U, Abzug S and Bekierkunst A, Cytotoxic effect of oxidised spermine on Ehrlich ascites cells. *Biochim Biophys Acta* **134**: 174–181, 1967.
5. Allen JC, Smith CJ and Hussain JI, Thomas JM and Gaugas JM, Inhibition of lymphocyte proliferation by polyamines requires ruminant-plasma polyamine oxidase. *Eur J Biochem* **102**: 153–158, 1979.
6. Tabor CW, Tabor H and Bachrach U, Identification of the aminoaldehydes produced by the oxidation of spermine and spermidine with purified plasma amine oxidase. *J Biol Chem* **239**: 2194–2203, 1964.
7. Smith CJ, Maschler R, Maurer HR and Allen JC, Inhibition of cells in culture by polyamines does not depend on the presence of ruminant serum. *Cell Tiss Kinet* **16**: 269–276, 1983.
8. Brunton VG, Grant MH and Wallace HM, Toxicity of spermine in BHK-21/C13 cells. *Biochem Soc Trans* **17**: 223–224, 1989.
9. Reed DJ and Beatty PW, Biosynthesis and regulation of glutathione: toxicological implications. *Rev Biochem Toxicol* **2**: 213–241, 1980.
10. Meister A and Anderson ME, Glutathione. *Annu Rev Biochem* **52**: 711–760, 1983.
11. Habig WH, Pabst MJ and Jakoby WB, Glutathione-*S*-transferases. The first step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
12. Reed DJ, Regulation of reductive processes by glutathione. *Biochem Pharmacol* **35**: 7–13, 1986.
13. Mitchell JR, Thorgeirsson SS, Potter WZ, Jollow DJ and Keiser H, Acetaminophen-induced hepatic injury: protective role of glutathione in man and rationale for therapy. *Clin Pharmacol Ther* **16**: 676–684, 1974.
14. Lindstrom TD, Anders MW and Remmer H, Effect of phenobarbital and diethyl maleate on carbon tetrachloride toxicity in isolated rat hepatocytes. *Exp Mol Pathol* **28**: 48–57, 1978.
15. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
16. Seiler N and Knodgen B, High performance liquid chromatographic procedure for the simultaneous determination of the natural polyamines and their monoacetyl derivatives. *J Chromatogr* **221**: 227–235, 1980.
17. Wallace HM, Nuttall ME and Robinson FC, Acetylation of spermidine and methylglyoxalbis(guanylhydrazone) in baby-hamster kidney cells. *Biochem J* **253**: 223–227, 1988.
18. Hissin PJ and Hilf R, A fluorometric method for determination of oxidised and reduced glutathione in tissues. *Anal Biochem* **74**: 214–226, 1976.
19. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW and Potter DW, High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. *Anal Biochem* **106**: 55–62, 1980.

20. Tietze F, Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: application to mammalian blood and other tissues. *Anal Biochem* **27**; 502–522, 1969.
21. Henle KJ, Moss AJ and Nagle WA, Mechanism of spermidine cytotoxicity at 37°C and 43°C in Chinese hamster ovary cells. *Cancer Res* **46**: 175–182, 1986.
22. Tabor H and Tabor CW, Isolation, characterisation and turnover of glutathionylspermidine from *Escherichia coli*. *J Biol Chem* **250**: 2648–2654, 1975.
23. Fairlamb AH and Cerami A, Identification of a novel, thiol-containing co-factor essential for glutathione reductase enzyme activity in trypanosomatids. *Mol Biochem Parasitol* **14**: 187–198, 1985.
24. Gaugas JM and Dewey DL, Oxygen-dependent free radicals in spermine oxidation cytostasis and chemiluminescence and the role of superoxide dismutase. *Br J Cancer* **41**: 946–955, 1980.
25. Otsuka H, The toxic effect of spermidine on normal and transformed cells. *J Cell Sci* **9**: 71–84, 1971.
26. Byrd WJ, Jacobs DM and Amoss MS, Synthetic polyamines added to cultures containing bovine sera reversibly inhibit *in vitro* parameters of immunity. *Nature* **267**: 621–623, 1977.
27. Hussain JI, Smith CJ and Allen JC, Polyamine-mediated inhibition of *in vitro* cell proliferation is not due to acrolein. *Cell Tiss Kinet* **16**: 583–591, 1983.
28. Richman PG and Meister A, Regulation of γ-glutamylcysteine synthetase by nonallosteric feedback inhibition by glutathione. *J Biol Chem* **250**: 1422–1426, 1975.
29. Duthie SJ, Coleman CS and Grant MH, Status of reduced glutathione in the human hepatoma cell line, HEP G2. *Biochem Pharmacol* **37**: 3365–3368, 1988.
30. Brunton VG, Grant MH and Wallace HM, Spermine-induced toxicity in BHK-21/C13 cells: dependence on growth status. *Med Sci Res* **16**: 951–952, 1988.
31. Holtta E and Pohjanpelto P, Control of ornithine decarboxylase in Chinese hamster ovary cells by polyamines. *J Biol Chem* **261**: 9502–9508, 1986.
32. Wallace HM and Keir HM, Uptake and excretion of polyamines from BHK-21/C13 cells. The effect of serum on confluent cell cultures. *Biochim Biophys Acta* **676**: 25–30, 1981.
33. Wallace HM, MacGowan SH and Keir HM, Polyamine metabolism in mammalian cells in culture. *Biochem Soc Trans* **13**: 329–330, 1985.
34. Wallace HM, Polyamine catabolism in mammalian cells: excretion and acetylation. *Med Sci Res* **15**: 1437–1440, 1987.