Incidence and potential implications of the toxic metabolite methylglyoxal in cell culture: A review

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Abstract

Methylglyoxal is a toxic metabolite unavoidably produced in mammalian systems as a by-product of glycolysis. Detoxification of this compound occurs principally through the glyoxalase pathway, which consists of glyoxalase I and glyoxalase II, and requires reduced glutathione as a co-enzyme. Recently, it has been demonstrated that variations in glucose, glutamine and fetal bovine serum levels can cause significant changes in the intracellular concentration of methylglyoxal. More importantly, comparative studies involving wild-type Chinese hamster ovary cells and clones overexpressing glyoxalase I indicate that glucose and glutamine, within the range normally found in cell culture media, can cause decreased cell viability mediated solely through increased production of methylglyoxal. In addition, endogenously produced methylglyoxal has been shown to cause apoptosis in cultured HL60 cells. While the exact mechanism of the impact of methylglyoxal on cultured cells is unknown, methylglyoxal is a potent protein and nucleic acid modifying agent at physiological concentrations and under physiological conditions. Protein modification occurs mainly at arginine, lysine and cysteine residues and is believed to be an important signal for the degradation of senescent proteins. Modification of arginine and lysine results in the irreversible formation of advanced glycation endproducts, whereas modification of cysteine results in the formation of a highly reversible hemithioacetal. Methylglyoxal also forms adducts with nucleic acids, principally with guanyl residues. At high extracellular concentrations, it is genotoxic to cells grown in culture. Even at physiological concentrations (100 nM free methylglyoxal), methylglyoxal can modify unprotected plasmid DNA and cause gene mutation and abnormal gene expression.

Abbreviations: AGE, Advanced Glycation Endproduct; BSA, Bovine Serum Albumin; CFA, Colony Forming Ability; CHO, Chinese Hamster Ovary

Introduction

A major goal of process development in industrial cell culture is to achieve high titers of consistent product while operating with high volumetric productivities for long periods of time. Mammalian cells grown in culture produce a number of inhibitory compounds that can negatively affect cell growth and function resulting in decreased specific and volumetric productivities and poorer product quality. An important consideration during process optimization, therefore, has been the impact of bioreactor operating conditions, in particular medium composition, on the production and subsequent accumulation of toxic metabolites in the

cell growth environment. Most interest has focused on lactate and ammonium ion, generally considered to be the major inhibitory waste products in mammalian cell culture systems (Miller, 1991; Anderson and Goochee, 1995). Such research has resulted in significant improvements in culture productivity through the development of optimal reactor operating strategies and the selection or engineering of cell-lines that produce lower levels of toxic compounds or are more resistant to their accumulation (Glacken *et al.*, 1986; Inlow *et al.*, 1992; Schumpp and Schlaeger, 1992; Bebbington *et al.*, 1990; Brown *et al.*, 1992). In addition to lactate and ammonium ion, there are other inhibitory compounds secreted by cells grown in culture that are

much less well-studied and may also have a significant impact (Siwiora *et al.*, 1994). Identification and characterization of these compounds is also important if culture productivities are to be maximized (Bibila and Robinson, 1995).

Recently, it has been demonstrated that certain conditions in industrial cell culture cause higher levels of the toxic metabolite methylglyoxal with a resulting decrease in Chinese hamster ovary (CHO) cell viability (Chaplen *et al.*, 1996a). In addition, increased intracellular methylglyoxal induces apoptosis in HL60 cells grown in culture (Thornalley *et al.*, 1996). Methylglyoxal is unavoidably produced in mammalian systems as a by-product of glycolysis (Thornalley, 1993). While the exact mechanism of its negative impact on CHO cell viability has yet to be elucidated, methylglyoxal is a potent protein and nucleic acid modifying agent (Thornalley, 1996). Protein modification occurs readily under physiological conditions and is believed to be a mediator in Advanced Glycation Endproduct (AGE) formation and by extension an important signal for degradation of senescent proteins (Lo *et al.*, 1994; Westwood and Thornalley, 1996). Furthermore, physiological concentrations (100 nM free methylglyoxal) of methylglyoxal can modify unprotected plasmid DNA and cause gene mutation and abnormal gene expression (Papsoulis *et al.*, 1995). As a result of these various interactions, methylglyoxal has the potential and capacity for wide-ranging negative impacts on the productivity of cells grown in culture. These include: 1) alteration of the product gene, message or protein leading to protein leading to protein microheterogeneity; 2) alteration of critical housekeeping or other genes, messages or proteins causing a decrease in cellular efficiency and possible apoptotic events; 3) general interference with protein processing resulting in a decrease in specific productivity; and 4) modification of recombinant or genomic DNA in long-term culture leading to genetic drift.

First reported in separate studies by Darkin and Dudley (1913) and Neuberg (1913), the history of methylglyoxal and its associated catabolic activities in the glyoxalase pathway is a long one. Initially, it was thought that the glyoxalase enzymes were a major missing link in glycolysis. Although this hypothesis was later disproved, these activities were widespread in nature (Hopkins and Morgan, 1945) and this hinted at some important, but as of yet undiscovered physiological function for methylglyoxal metabolism. In ensuing years, many other theories have been suggested for the existence of methylglyoxal and the glyoxalase activi-

ties including the control of cell proliferation (Együd and Szent-Györgyi, 1966) and to provide a glycolytic by-pass in microorganisms (Cooper, 1984). Currently, there is much interest in the role of methylglyoxal as a mediator in the long-term pathophysiology of diabetes (Phillips and Thornalley, 1993; Wells-Knecht, 1995). In addition, the use of glyoxalase inhibitors to cause toxic levels of intracellular methylglyoxal holds some promise in the development of novel cancer therapies (Thornalley, 1995). These and other aspects of methylglyoxal metabolism in normal systems are presented in excellent reviews by Thornalley (1993, 1995 and 1996). The purpose of the current review is to provide an overview of the incidence and potential effects of methylglyoxal in animal cell culture.

Metabolism

Methylglyoxal is a naturally occurring α -ketoaldehyde formed as a by-product of glycolysis and found in most living organisms (Carrington and Douglas, 1986). Figure 1 shows methylglyoxal metabolism in mammalian systems. Intracellular methylglyoxal exists in an equilibrium mixture of free and bound forms (Lo *et al.*, 1994). As a result, the steady-state concentration of free methylglyoxal is a complicated function of anabolism, catabolism and interactions with cellular structures. The concentration of free methylglyoxal in rat tissues ranges from 0.16 μ M in whole blood to 2.40 μ M in sciatic nerves (Phillips and Thornalley, 1993), that in CHO cells ranges from 0.3 to 1.8 μ M under normal growth conditions (Chaplen *et al.*, 1996a). By contrast the level of reversibly bound methylglyoxal can be 100 μ M or higher (Chaplen *et al.*, 1996a; Chaplen, 1996), a level 2–3 orders of magnitude greater than for free methylglyoxal.

The rate of formation of methylglyoxal in normal systems is on the order of 120 μ M/day or 0.1–0.4% of glycolytic flux (Thornalley, 1988; Phillips and Thornalley, 1993). While this represents only a small proportion of the total flux, it is, however, important because of the high-reactivity of methylglyoxal with nucleic acids and proteins (Thornalley, 1996). The rate of formation of methylglyoxal is unknown in cells grown in culture, but is quite possibly higher than that of normal systems because tumor-lines generally lose the ability to control glycolytic flux on the basis of energy needs (Eigenbrodt *et al.*, 1985). As a result, glycolysis is controlled by the concentration of glucose in the extracellular growth medium; in cell

Figure 1. Methylglyoxal metabolism in mammalian systems. DHAP, dihydroxyacetone phosphate; G-3-P, glyceraldehyde-3-phosphate; P_i, inorganic phosphate.

culture, glucose is often present at significantly higher concentrations (up to 50 mM) than found in the blood stream in normal systems (5 mM or less), especially in the high-depth culture medium used in many culture systems.

The primary source of methylglyoxal in mammalian cells is phosphate elimination from triose 1,2-enediol-3-phosphate, the enediol intermediate in the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Richard, 1991; Richard, 1984). Phosphate elimination occurs spontaneously but is also accelerated in the presence of triose phosphate isomerase, possibly as a result of leakage of triose 1,2-enediol-3-phosphate from the active site of the enzyme (Richard, 1991). A methylglyoxal synthase activity has been reported for goat liver tissue

(Ray and Ray, 1981), but it is unclear at this time under what conditions, or in which specific animal tissues, this enzymatic activity becomes an important source of methylglyoxal (Thornalley, 1993). Secondary sources of methylglyoxal are from the enzymatic conversion of intermediates generated during fatty acid metabolism and amino acid catabolism (Argiles, 1986; Ray and Ray, 1987). In amino acid catabolism, aminoacetone can be formed during both L-threonine and L-glycine breakdown. Aminoacetone is then converted to methylglyoxal by monamine oxidase. Under normal physiological conditions, this pathway is reported to be a minor source of methylglyoxal (Ohmori *et al.*, 1989); however, under certain conditions where these amino acids are available as carbon sources, methylglyoxal levels are elevated (Ohmori *et al.*, 1989). In fatty

acid metabolism, fatty acids are converted to acetoacetate and then to methylglyoxal via acetone and acetol. This pathway is probably triggered as a starvation response in diabetics to allow gluconeogenesis from fats (Argiles, 1986).

In mammalian cells, the principal route for methylglyoxal detoxification is believed to be the glyoxalase pathway. This pathway consists of two enzymes, glyoxalase I (EC 4.4.1.5 lactoylglutathione lyase) and glyoxalase II (EC 3.1.2.6 hydroxyacylglutathione hydrolase). The physiological substrate for glyoxalase I is the hemithioacetal that forms non-enzymatically between reduced glutathione and methylglyoxal. Glyoxalase I converts this addition complex to *S*-D-lactoylglutathione. *S*-D-Lactoylglutathione is biologically active when added to the growth media of cells grown in culture, causing toxicity and growth arrest, but does not appear to impact the cells when produced as a result of methylglyoxal metabolism (Thornalley, 1995). Glyoxalase II then hydrolyzes *S*-D-lactoylglutathione to D-lactic acid, regenerating reduced glutathione in the process (Thornalley, 1990). In some tissues, the D-lactic acid is excreted, in others it is oxidized to pyruvate and respired to CO₂ (Baum, 1967). Another reported enzymatic route for methylglyoxal removal is aldose reductase (EC 1.1.1.21). Aldose reductase first reduces methylglyoxal to acetol and then further reduces it to L-1,2-propanediol. Two equivalents of NADPH are required for every equivalent of methylglyoxal reduced to L-1,2-propanediol. While the relative importance of these two enzymatic routes for methylglyoxal removal is not definitively known in animal cells grown in culture, order-of-magnitude estimates indicate that *ca.* 10-40-fold more methylglyoxal is detoxified by the glyoxalase system than by aldose reductase in most human tissues (Thornalley, 1996). In addition, it is probably energetically more advantageous for an organism to use the "redox neutral" glyoxalase system (Vander Jagt *et al.*, 1992).

Protein modification

Methylglyoxal is a potent protein modification agent (Thornalley, 1996). Indeed, the majority of intracellular methylglyoxal (> 99%) is associated with cellular macromolecules, particularly proteins (Lo *et al.*, 1994; Chaplen, 1996). Protein modification occurs as a result of nucleophilic addition reactions at arginine, lysine and cysteine residues, and at the *N*-terminus α -amino group. Interactions with arginine are essen-

tially irreversible, with several reaction products being possible. Initial formation of *N* δ -(4,5-dihydroxy-4-methylimidazolidin-2-yl) ornithine (Reaction 1 in the scheme in Table 1) is followed by formation of *N* δ -(5-hydro-5-methylimidazol-4-on-2-yl) ornithine (Reaction 2) and then further reactions to AGE-type compounds (Lo *et al.*, 1994). Interactions with lysine may also result in the formation of novel AGE (Reactions 1A and 2 in the scheme depicted in Table 1) and also in the formation of lysine-lysine crosslinks (Reactions 1B and 3). Lysine-lysine cross-linking *in vivo* is putative but has been demonstrated in a reaction system containing methylglyoxal and bovine serum albumin at physiological conditions (Lo *et al.*, 1994). The isolation of an AGE-type compound (imidizoline) resulting from methylglyoxal interactions with lysine residues in proteins has recently been reported (Nagaraj *et al.*, 1996). Interactions with cysteine are highly reversible and result in the formation of hemithioacetals under physiological conditions (Table 2; Lo *et al.*, 1994). Modification of the *N*-terminus α -amino group of proteins and peptides is rapid generally results in irreversible deamination (Table 2; Takahashi, 1968).

A strong indication of the role that methylglyoxal plays in AGE formation in cells grown in culture was recently demonstrated (Shinohara *et al.*, 1996). Addition of the diethyl ester of *N*-[Hydroxy-*N*-(4-chlorophenyl) carbonyl] glutathione, a glyoxalase I inhibitor, to GM7373 endothelial cells grown in culture resulted in 2-4-fold increase in the level of AGE measured. Conversely, a 2.8 fold increase in glyoxalase I activity was sufficient to reduce AGE formation by a factor of 10. While AGE are formed with both arginine and lysine, arginine believed to be the principle irreversible modification target in proteins at physiological concentrations of free methylglyoxal (< 5 μ M; Westwood and Thornalley, 1995). Low levels of methylglyoxal modification (1-2 AGE/protein molecule) makes proteins an acceptable substrate for AGE-receptor binding in monocytes and macrophages, with subsequent endocytosis and lysosomal degradation (Westwood and Thornalley, 1996). Moreover, methylglyoxal-modified proteins out-compete proteins modified by glucose to similar levels, indicating that methylglyoxal-modified proteins are an important physiological substrate for this receptor, and by extension an important signal for the degradation of senescent macromolecules (Lo *et al.*, 1994; Westwood *et al.*, 1994; Westwood and Thornalley, 1995.)

Methylglyoxal modification of proteins could affect cell physiology in several ways. Many enzymes

Table 1. Methylglyoxal reactions with arginine and lysine (Adapted from Lo *et al.*, 1994; Thornalley, 1996)

have cysteine and arginine in their active sites – for example, glyceraldehyde-3-phosphate dehydrogenase (Jocelyn, 1972) and the cysteine proteases, cathepsin and calpain (Polgár, 1989) contain sulfhydryl groups, while all but one of the fourteen glycolytic enzymes contain an active-site arginine (Riordan *et al.*, 1977). As a result, modification of active-site amino acid residues by methylglyoxal and related compounds can significantly decrease enzyme activity (Takahashi, 1968; Takahashi, 1977a). Moreover, the recognition sequences for many proteolytic cleavage reactions are often paired basic amino acids, such as arginine-arginine or arginine-lysine (Rehemtulla, 1992). Trypsin, for instance, only cleaves as the carboxyl side of lysine and arginine residues, and thrombin hydrolyses arginine-glycine bonds on specific peptide chains. While the effect of methylglyoxal on these cleavage reactions is unknown, the presence of phenylglyoxal-arginine adducts in RNase-A has been

reported to inhibit proteolytic cleavage by trypsin and chymotrypsin (Takahashi, 1968). Phenylglyoxal has a similar biological activity to methylglyoxal but is significantly less reactive (Cheung and Fonda, 1979; Takahashi, 1977a, 1977b). A reduction in proteolytic cleavage reactions could have several effects on the physiological state of the cell. For instance, it would prevent the final processing of many pro-proteins, such as neuropeptides, polypeptide hormones, growth factors and plasma proteins, resulting in their eventual degradation (Dorner *et al.*, 1992). Furthermore, many of the signal sequences that dictate the final intracellular location of the protein usually contain lysine and arginine (Alberts *et al.*, 1994). Modification of these sequences could prevent the correct transport of proteins in the cell. Finally, methylglyoxal is membrane permeable (Thornalley, 1988) and most probably is present in the endoplasmic reticulum and other non-cytosolic compartments in the cell. Steric hindrance

Table 2. Methylglyoxal reactions with lysine, N-Terminal α -amine groups and the guanyl bases in nucleic acids (Adapted from Lo *et al.*, 1994; Thornalley, 1995; Takahashi, 1977a; Takahashi, 1968; Shapiro *et al.*, 1969; Vaca *et al.*, 1994)

Species	Reaction
Free cysteine and cysteine residues	
N-Terminal α -amine groups	
Guanyl bases of nucleic acids	

resulting from the formation of methylglyoxal adducts could prevent proteins from folding or constituent subunits from combining properly.

Nucleic acid modification

Methylglyoxal is both a mutagen and genotoxic agent. At high extracellular concentrations, methylglyoxal causes increased sister chromatid exchanges and endoreduplication in CHO-AUXBI cells (Tucker *et al.*, 1989). It also induces DNA strand breaks in human lymphocytes and increases point mutations in *Salmonella typhimurium* (Miglore *et al.*, 1990). In addition, methylglyoxal causes single strand breaks in DNA and DNA-protein cross-links in CHO.K1 cells (Brambilla *et al.*, 1985), and an increased mutational frequency at the hypoxanthine-guanine phosphoribosyl transferase locus of V79 cells (Cajelli *et al.*, 1987). In

the presence of hydrogen peroxide, a byproduct of oxidative stress, the mutagenic effects of methylglyoxal are increased by an order of magnitude beyond the sum of the mutagenicities of each compound alone (Fujita *et al.*, 1985).

Methylglyoxal favors interactions with guanyl nucleotides and nucleosides (Shapiro *et al.*, 1969); the relative binding of methylglyoxal to poly-guanosine, adenine, cytosine and uracil is 100:7:3:0 (Krymkiewicz *et al.*, 1973). Guanyl modification is believed to involve nucleophilic attack by the aldehyde moiety of methylglyoxal at the amine side-group of the purine ring (Reaction 1 in the scheme depicted in Table 2) followed by cyclization between the less reactive ketone group and the guanine ring (Reaction 2; Vaca *et al.*, 1994; Shapiro *et al.*, 1969). This reaction proceeds rapidly with RNA or denatured DNA, but not with duplex DNA (Krymkiewicz, 1973). At physiological concentrations (100 nM free methylglyoxal modifies

unprotected plasmid DNA and causes gene mutation and abnormal gene expression (Papsoulis *et al.*, 1995). No *in vivo* reaction products of endogenously produced methylglyoxal have yet been directly identified. However, reaction products of malondialdehyde, another endogenously produced aldehyde, have been detected (Chaudhary *et al.*, 1994) and studies are underway to isolate methylglyoxal-DNA adducts from diabetic and aged subjects (Al-Abed *et al.*, 1996). Finally, it should be noted many of the studies establishing the link between intracellular methylglyoxal and nucleic acid modification utilized commercially methylglyoxal, which is relatively impure. As a consequence of this, these results await confirmation under more controlled conditions (Thornally, 1996).

Quantification

Historically, difficulties associated with the accurate quantification of methylglyoxal have impeded the study of methylglyoxal metabolism in biological systems (Chaplen *et al.*, 1996b; Thornalley, 1993). Some of these difficulties were a result of the assay procedures utilized. Others were a result of the state in which intracellular methylglyoxal exists, a complex mixture of free, reversible and irreversibly bound forms.

Early assay procedures for free methylglyoxal involved derivatization with dinitrophenylhydrazine followed by thin-layer chromatography (Sawicki and Sawicki, 1977). These were subject to interference from glycolytic pathway intermediates that reacted with the derivatizing agent to form the same as methylglyoxal (Ohmori *et al.*, 1989). Assay specificity was improved through the use of 1,2-diaminobenzene compounds, such as *o*-phenylenediamine, as alternative derivatizing agents. The resulting quinoxalines are strong chromophores or fluorophores or both and can easily be measured with high-performance liquid or gas chromatography (Ohmori *et al.*, 1987a; Ohmori *et al.*, 1987b; Hara *et al.*, 1988; Yamaguchi *et al.*, 1989). Other potential sources of assay error was methylglyoxal formation during the assay procedure as a result of phosphate elimination from glycolytic pathway intermediates (McLellan *et al.*, 1992) and breakdown of the carbohydrate backbone of nucleic acids (Chaplen *et al.*, 1996b). Phosphate elimination can be minimized through the use of acidic sample processing conditions and by minimizing sample preparation temperatures (McLellan *et al.*, 1992). Ironically, acidic processing conditions are also conducive to the formation of

methylglyoxal from nucleic acid degradation. This, however, can be accounted for through the use of control experiments with calf thymus DNA (Chaplen *et al.*, 1996a; Chaplen *et al.*, 1996c). Finally, an additional and indeterminate source of assay error in earlier studies may have resulted from the use of the quinoxaline derivative of 2,3-butanedione as an internal standard, as this compound is also produced as a result of nucleic acid degradation in perchloric acid. This source of error can be avoided by utilizing other internal standards, such as 5-methylquinoxaline (Chaplen *et al.*, 1996b).

Currently, the most effective assays for free methylglyoxal still utilize a 1,2-diaminobenzene derivatizing agent (McLellan *et al.*, 1992; Chaplen *et al.*, 1996a; Chaplen *et al.*, 1996c). Cells are lysed and deproteinized with perchloric acid. Free methylglyoxal in the resulting cell lysate is derivatized and quantified as a quinoxaline with reverse-phase HPLC. (McLellan *et al.*, 1992; Chaplen *et al.*, 1996c). With minor modifications, it is also possible to utilize this assay to quantify the free methylglyoxal present in cell growth media (Chaplen *et al.*, 1996c). Interestingly, when this is done for CHO cells grown in culture, there is 10-fold less free methylglyoxal in the extracellular environment than in cell extracts (Chaplen *et al.*, 1996c). Large disparities between intra- and extracellular measurements were unexpected since methylglyoxal freely translocates across cell membranes (Thornalley, 1988). One possibility is that the assay for intracellular free methylglyoxal is subject to interference from unavoidable recovery of reversibly bound methylglyoxal (Chaplen *et al.*, 1996c). This explanation is supported by differences between observed and predicted free methylglyoxal levels in a recent kinetic study of the glyoxalase pathway in red blood cells (Shih *et al.*, 1997). The steady-state, intracellular free methylglyoxal concentrations were predicted on the basis of measured fluxes to D-lactate, the unique end-product of the glyoxalase pathway in red blood cells. These predicted concentrations were orders of magnitude below those measured with the assay for free methylglyoxal indicating that there might be some unaccounted for interference with that assay (Shih *et al.*, 1997).

Much of the reversibly bound methylglyoxal is expected to associate with sulfhydryls *in vivo* (Leo *et al.*, 1994; Chaplen, 1996). In systems with low sulfhydryl concentrations it is relatively easy to recover the majority of the reversibly bound methylglyoxal. For example, when methylglyoxal is reacted with bovine serum albumin (BSA) under physiological con-

ditions, it is possible to recover methylglyoxal by rinsing the modified BSA. However, in systems with higher concentrations of sulfhydryls, such as cells, a more efficient approach is to utilize a thermodynamic trap, such as the 1,2-diaminobenzene derivatizing agent used in the free methylglyoxal assay. A thermodynamic trap sequesters the available free methylglyoxal forcing the reversibly bound methylglyoxal to return to solution as the system attempts to re-equilibrate. This general approach has been used to assay CHO cell samples for reversibly methylglyoxal (Table 3; Chaplen *et al.*, 1996a). In this assay, the derivatizing agent is added along with the perchloric acid and the entire sample is incubated for 24 hours before analyzing for quinoxaline content by reverse-phase HPLC. This contrasts with the free methylglyoxal assay where the derivatizing agent is added to the cell extract following the removal of the perchloric acid precipitate. Nucleic acid degradation can be accounted for through the use of control experiments (Chaplen, 1996). Levels of methylglyoxal measured in CHO cells with this new approach can exceed 100 μM as compared to 0.1–1.8 μM for free methylglyoxal (Table 3; Chaplen *et al.*, 1996a).

Impact on cultured cells

In mammalian systems, methylglyoxal is produced primarily as a by-product of glycolysis. As a result, methylglyoxal concentrations in cells are affected by changes in glycolytic flux. In red blood cells, for example, methylglyoxal increases in response to higher concentrations of glucose in the extracellular environment (Thornalley, 1988). This effect is also evident in CHO cells grown in culture where increasing glucose from 5 to 100 mM causes a 150% elevation in methylglyoxal. In addition, increasing glutamine from 2 to 5 mM causes a 30% increase in methylglyoxal (Chaplen *et al.*, 1996a). In absolute terms, increasing glucose from 5 to 25 mM and glutamine from 2 to 5 mM causes an increase in intracellular methylglyoxal from 0.3 ± 0.2 to $0.57 \pm 0.06 \mu\text{M}$. Glutamine probably had an effect because glutamine and glucose are partially substitutable as energy and carbon sources (Hu and Himes, 1989), with glutamine being the preferred energy source when present in excess of cellular needs for cell mass production. Methylglyoxal concentrations also increase as growth serum levels decreased (Chaplen *et al.*, 1996a). Low fetal bovine serum concentrations correspond to low levels of growth and oth-

er mitogenic factors. Cells under these conditions are effectively in a state of low proliferation, which would cause less carbon to be utilized in cell mass production via the pentose shunt and increase the carbon flux down the glycolytic pathway. Metabolic flux analysis indicates that such behavior occurs in hybridoma cells, at least, where, based on the study of Bonarius *et al.* (1996), the proportion of the glucose carbon being utilized by the pentose phosphate pathway is reduced by 17% in favor of glycolysis under conditions of low cell proliferation. Another contributing factor to the higher levels of methylglyoxal seen in CHO cells under conditions of high glucose and glutamine or low serum may have been that the cells were more highly stressed. Stressed cells require more energy for maintenance (Ozturk *et al.*, 1992) and obtain a greater fraction of that energy from glycolysis (Miller *et al.*, 1988), resulting in increased glycolytic flux.

In order to allow comparative studies to determine the possible impact of endogenously produced methylglyoxal on animal cells grown in culture, the gene for *Pseudomonas putida* glyoxalase I was expressed in CHO cells. This resulted in the isolation of cell populations with naturally lower levels of free and reversibly bound methylglyoxal (Table 3; Chaplen *et al.*, 1996a). While the magnitude of increased enzyme activity did not correspond exactly with the decrease in methylglyoxal seen, the relative free and reversibly bound methylglyoxal levels for each cell population were consistent. This indicates that the bacterial enzyme was functioning effectively and lowering methylglyoxal concentrations *in vivo*. Comparative studies indicate that increased levels of endogenous methylglyoxal can have a negative effect on cultured cells. In these studies, the survival ability of the CHO wild-type and *P. putida* glyoxalase I expressing cells were tested with colony-forming assays under growth conditions (high glucose, high L-glutamine) known to cause increased production of methylglyoxal by the cells. When the colony-forming ability (CFA) in medium containing higher levels of glucose and L-glutamine (25 mM and 5 mM, respectively) is compared to CFA in the basal medium (5.6 mM glucose and 2 mM L-glutamine), the wild-type cells show a significant decrease in CFA (Table 3; Chaplen *et al.*, 1996a), whereas the clones do not with fair to good significance. Thus, in this particular test, the clones are able to grow equally well in the basal and supplemented media. More importantly, the clones are better able than the wild-type cells to survive under conditions previously shown to increase methylglyoxal levels. The CFA result indicates that

Table 3. Characterization of various CHO cell populations and the effect of an enhanced methylglyoxal detoxification ability on CHO cell survival under conditions that cause an increase in endogenous methylglyoxal production¹

Clone ²	Glyoxalase I Activity ³ (U/mg total protein)	Free methylglyoxal		Reversibly bound methylglyoxal		Change in CFA ⁷ (%)	Anova ⁸ (Medium Conditions)	ANOVA ⁹ (Relative CFA)
		[Methylglyoxal] ⁴ (μ M)	ANOVA ⁵	[Methylglyoxal] ⁶ (μ M)	ANOVA ⁵			
WT	310 $\pm$ 43	1.8 $\pm$ 0.7	–	100 $\pm$ 30	–	–10 $\pm$ 2	P > 0.99	–
Clone 1	510 $\pm$ 43	0.15 $\pm$ 0.07	P > 0.95	60 $\pm$ 14	P > 0.95	+1 $\pm$ 8	–	P > 0.75
Clone 2	780 $\pm$ 85	0.4 $\pm$ 0.2	P > 0.95	85 $\pm$ 5	P > 0.95	+3 $\pm$ 8	–	P > 0.95
Clone 3	910 $\pm$ 97	0.4 $\pm$ 0.4	P > 0.95	80 $\pm$ 13	P > 0.95	+4 $\pm$ 8	–	P > 0.90

¹ See Chaplen *et al.* (1996a) for full experimental protocols.

² Wild-type (WT) denotes cells that were not transfected with the gene for *P. putida* GloI.

³ 1 Unit is defined as 1 μ mol S-D-lactoylglutathione formed/min at 20 °C. Values are specific activity $\pm$ standard error.

⁴ Values are mean $\pm$ standard deviation; n = 6.

⁵ Analysis of variance test (ANOVA) (Box *et al.*, 1978). P value represents the probability that the methylglyoxal concentration in the clone is significantly different from that in the wild-type cells.

⁶ Methylglyoxal concentrations represent sum of free and reversibly bound methylglyoxal bound methylglyoxal. Mean $\pm$ standard deviation; n = 6.

⁷ Mean $\pm$ standard deviation; n = 6. Change in colony-forming ability (CFA) of the cells grown in supplemented medium (25 mM glucose, 5 mM L-glutamine) relative to those grown in basal medium (5.6 mM glucose, 2 mM L-glutamine).

⁸ P value represents the probability that the increase in CFA as a result of the change in medium conditions is significant.

⁹ P value represents the probability that the difference between the clonal and wild type CFA is significant.

endogenously produced methylglyoxal can have a negative effect on cell function. In addition, these results are interesting from a cell culture engineering perspective because the test conditions utilized are commonly found in cell culture media.

Finally, endogenously produced methylglyoxal is associated with apoptosis in populations of HL60 cells exposed to a glyoxalase I inhibitor diester (Thornalley *et al.*, 1996). Apoptosis was noted within six hours of exposing the cells to the glyoxalase I inhibitor. Control experiments indicate that the inhibitor does not in of itself cause apoptosis. The increase in free methylglyoxal necessary for induction of apoptosis is only *ca.* 0.07 μ M (Thornalley *et al.*, 1996), an increase easy to achieve in CHO cells grown within the normal envelope of growth conditions found in industrial cell culture.

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