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Effective Quenching Processes for Physiologically Valid Metabolite Profiling of Suspension Cultured Mammalian Cells

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Global metabolite analysis approaches, coupled with sophisticated data analysis and modeling procedures (metabolomics), permit a dynamic read-out of how cellular proteins interact with cellular and environmental conditions to determine cell status. This type of approach has profound potential for understanding, and subsequently manipulating, the regulation of cell function. As part of our study to define the regulatory events that may be used to maximize production of commercially valuable recombinant proteins from cultured mammalian cells, we have optimized the quenching process to allow retention of physiologically relevant intracellular metabolite profiles in samples from recombinant Chinese hamster ovary (CHO) cells. In a comparison of a series of candidate quenching procedures, we have shown that quenching in 60% methanol supplemented with 0.85% ammonium bicarbonate (AMBIC) at −40 °C generates a profile of metabolites that is representative of a physiological status based upon examination of key labile cellular metabolites. This represents a key feature for any metabolomic study with suspension cultured mammalian cells and provides confidence in the validity of subsequent data analysis and modeling procedures.

Metabolomics offers a uniquely powerful interrogative approach for understanding the activity and regulation of cellular function in normal and aberrant conditions.[1−4] The profile of cellular metabolites presents a dynamic read-out of the interactions between cellular gene expression, protein function and environmental conditions, referred to as the phenotype. The metabolome is much more sensitive to perturbations in the environment than either the transcriptome or the proteome because the metabolites within a cell are an integrated reflection of the cellular phenotype.[5,6] Investigating the metabolome offers other advantages including high-throughput and lower cost per sample analysis.[7] Characterization of cellular metabolomes offers great potential in the technical armory available to investigate cell function, particularly when it is used in combination with other ‘omics approaches.

Although arguably a "re-invention" of the approaches of analytical biochemistry of the 1960s onward, metabolomics (a combination of sample collection/preparation, analytical and data interrogation techniques coupled in a workflow or pipeline[8]) has blossomed recently as a reflection of the success exhibited by other data-rich and holistic ‘omics approaches. Metabolomics approaches have been used extensively for understanding responses in yeast and bacteria to environment stimuli.[9−13] The knowledge gained from these approaches can have direct application in the areas of biotechnological processes, potentially leading to rational metabolic engineering,[14] as exemplified by the engineering of *Escherichia coli* for the production of fuel ethanol from lignocellulose.[15] Metabolomics approaches have also been applied in studies in plants and higher eukaryotic systems.[9,16,17] However,

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study of mammalian systems has predominantly focused on examination of metabolic profiling (biomarkers) from blood, serum, and other body fluids.[17−21] There are several compelling reasons for application of metabolomics to mammalian cell culture systems but this area has only recently started to be a focus of interest. Reports have started to appear of metabolomic studies of adherent cultured mammalian cells.[22−25] However, no simple technology has been developed to apply similar approaches to mammalian cells in suspension. One report describes the development of a microstructure heat exchanger for quenching the metabolism of suspension Chinese hamster ovary (CHO) cells; however, this requires specific equipment and the effectiveness of the quenching on the metabolite profile was not reported.[26] A major proportion of commercially valuable protein biopharmaceuticals are produced using CHO and other mammalian cells (e.g., mouse NS0 myeloma cells) in suspension culture as "factories" for large-scale bioprocessing. The environment (the culture medium composition and any feeding regimes) influences achievable cell biomass and biopharmaceutical specific productivity, yet medium design (and understanding the metabolic events that characterize "good" medium) remains non-predictive and is therefore a time-consuming and expensive process. The ability to apply a metabolomics approach to understanding the interaction of cell culture environment with the efficiency of mammalian cell culture growth and biopharmaceutical production offers the potential to rationalize and optimize bioprocess design.

For mammalian cell lines in culture, characterization of metabolites in the surrounding medium ("footprinting")[27] and within the cells (intracellular metabolites; "fingerprinting")[1] both have validity for assessment of cellular function. Metabolic footprinting offers technical simplicity, high-throughput, and automation as samples are simply centrifuged to separate medium and cells prior to analysis and is an appropriate tool for screening of large sample libraries. None of the difficulties associated with sampling intracellular metabolomes, including cell leakage during quenching, are present. However, the intracellular metabolite profile most accurately defines the metabolic status of the cell, and one of the most crucial technical features for characterization of intracellular metabolites is the quenching and extraction process. Many metabolites are extremely labile and, for example, ATP and glucose 6-phosphate have turnover rates of less than 1−2 s[28] and hence cellular metabolism must be stopped immediately (quenched) upon sampling of the cells to prevent/minimize metabolite turnover. It should be stressed that although changes may be profound for very labile metabolites, other "more stable" metabolites may also change to some degree. Hence, a fundamental requirement for intracellular metabolite measurement is to ensure that all enzymatic activity is stopped as quickly as possible so that metabolite data obtained represents accurate physiological concentrations. It is also crucial to ensure that procedures are reproducible. Current metabolite extraction protocols are generally two-step processes consisting of an initial quenching of the cells, to stop all metabolic activity, followed by extraction of the metabolites. Some extraction protocols seek to quench cell activity without removal of the medium (e.g., fast heating).[29−31] This approach presumes that the majority of cellular metabolites will not be present in the medium and thus can be presumed to have an intracellular localization. However, there are a number of metabolites that will be present in both the culture medium and within the cells (e.g., amino acids) or that may be secreted to the extracellular volume during growth. This is a particular restriction for mammalian cells in complex and "metabolite-rich" culture medium (which may be of proprietary composition). Hence, for mammalian cells in culture, extraction approaches for harvest of intracellular metabolites must remove (or, at the very least, minimize) contamination of intracellular metabolites by those in the surrounding culture environment. In addition, mammalian cells grow to relatively low density and, to obtain sufficient cell numbers for metabolite analysis, the harvest from large volumes has consequences for potential medium contamination as well as increasing the time taken to reach a quenched state (i.e., for recovery of labile intracellular metabolites).

One standard approach for quenching of cells involves placing cells in 60% methanol at −40 or −50 °C. This method stops metabolism by rapid cooling of the cells;[32] the lower the temperature the slower the turnover rate of all the enzymes within the cell and hence the more efficient the quenching process. Originally pioneered in *Saccharomyes cerevisiae*,[33] this method has subsequently been used for metabolite analysis of several bacterial strains.[34−36] However, it has been reported that cold methanol quenching in both Gram negative and Gram positive bacteria resulted in >60% loss of intracellular metabolites through leakage from the cells into the medium during harvest, and it was suggested that this leakage was caused by cell wall/membrane permeabilization created by cold-shock and/or contact with organic solvents.[36] Additional washes have also been used to remove the final traces of the medium but these can increase loss of metabolites because of leakage[37] or result in metabolite turnover and a change in the metabolite profile. To minimize

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leakage, several studies have examined the consequences of inclusion of additives to methanol (agents that act as buffers or that restrict osmotic shock), and there have been reports that such additives can decrease leakage with bacterial cells.[34,35,38,39] Minimizing the time of contact between organic solvent and cell may also decrease leakage.[37] Other methods which have examined the separation of medium from cells include fast filtering[36,40,41] and centrifugation,[41,42] and these techniques have proved to be effective with certain cell types, though the time between sampling and inhibition of metabolism can be longer than when applying quenching in cold solvents. Following quenching the metabolites are extracted using methods that lyse the cells, allowing release of metabolites under conditions in which the majority of the metabolites are soluble, stable, and in an enzymatically inert environment. Several different extraction methods have been reported including cold methanol (or methanol/water),[22,23,34,35,43] hot ethanol or methanol,[34,43−46] perchloric acid,[29,34,35,43,45] alkaline (KOH),[13,34,43,45] and chloroform (or chloroform/methanol/water)[34,45] extractions. The efficiency of each extraction method for specific metabolites varies, but two separate extensive studies have demonstrated that, at least in *E. coli*, cold methanol extraction is the best general method for metabolite profiling applications, although other methods may be more appropriate for specific metabolites or metabolite classes.[34,43] The methods for quenching and extraction are undoubtedly sample- and cell-dependent; differences in cellular composition (especially cell membrane and cell wall structure) and cell size may influence the efficiency of quenching and the rate of metabolite leakage.

To date there have been no reports of the effectiveness (and hence physiological relevance) of methods for quenching and extraction of intracellular metabolites from suspension cultured mammalian cells. In this paper, we report the effectiveness of four different quenching methods, followed by methanol extraction, applied to CHO cells. CHO cells are very important industrially for production of biopharmaceuticals, such as therapeutic antibodies, because of the requirement for mammalian-type post-translational processing and appropriate folding.[47,48] The application of metabolite profiling to CHO cell metabolism under defined culture conditions offers great potential for enhancement of industrial cell line selection and process development and, hence, overall yield and profitability. We have characterized and optimized quenching methods for the generation of physiologically relevant intracellular metabolite profiles from CHO cells and defined how quenching processes influence the recovery of a series of key intracellular metabolites (with their concentrations and relative ratios, e.g., energy charge, used as indicators of the robustness and physiological relevance).

MATERIAL AND METHODS

Media and Reagents. CD-CHO medium was obtained from Invitrogen (Carlsbad, U.S.); the chemical composition is defined but confidential. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

Cell Lines. The GS-CHO cell line LB01 was obtained from Lonza Biologics (Slough, U.K.), and was generated by transfection of CHOK1SV cells with a construct containing glutamine synthetase (GS) and a chimeric IgG4 antibody (cB72.3).

Cell Culture and Growth Assessment. Stocks of LB01 cells were revived in 20 mL of CD-CHO medium (Invitrogen), supplemented with 25 μM methionine sulfoximine (MSX). The cells were subcultured every 3−4 days with a seeding density of 0.2×10^6 cells mL^{-1} and were grown in 250 mL Erlenmeyer flasks in a volume of 50 mL medium. All cultures were grown at 37 °C with 100 rpm shaking. Growth was assessed by light microscopy using an improved Neubauer hemocytometer at 24 h intervals. Samples were appropriately diluted and mixed 1:1 with 0.5% Trypan Blue in PBS.

Metabolite Extraction. The cells were grown to mid-exponential phase (day 5) and then either quenched or harvested by centrifugation before extraction. The cells were rapidly quenched by addition of 1×10^7 cells to 5 volumes of quenching solution at −40 °C. Addition of the cells to the quenching solution increased the temperature by no more than 15 °C. The quenching solutions used were 60% methanol with (a) no additives, (b) 70 mM HEPES (pH 7.4), (c) 0.85% (w/v) ammonium bicarbonate (AMBIC) (pH 7.4) or (d) 0.85% (w/v) NaCl. The cells in the quenching solution were then centrifuged at $1000 \times g$ for 1 min, and the quenching solution was removed, with an aliquot being retained to assess intracellular metabolite leakage. Where specified, the cell pellet was washed with 5 mL of the appropriate quenching solution. The metabolites were extracted by resuspension of the cell pellet in 0.5 mL of 100% methanol followed by flash freezing in liquid nitrogen. After thawing on ice at 4 °C samples were vortexed for 30 s, centrifuged at $800 \times g$ and the supernatant removed. The pellet was resuspended in 0.5 mL of 100% methanol, and the extraction repeated. The methanol extracts were pooled, centrifuged at $15000 \times g$ for 1 min, and the supernatant removed and lyophilized. Cells collected without quenching in methanol solutions were harvested by centrifugation at $1000 \times g$ for 1 min and the medium was removed. The metabolites were extracted from the cell pellet using 100% methanol as described above for the quenched cells.

Metabolite Assays. Dried metabolite extracts were resuspended in 1 mL of water for use in each of the metabolite assays. ATP assays were performed using an ATP Bioluminescence Assay Kit CLS II (Roche). Each sample (50 μL) was mixed with an equal volume of luciferase reagent before measuring luminescence in a luminometer (Turner Designs). ADP and AMP assays were performed by converting ADP or AMP into ATP, followed by measurement using the ATP assay kit. Reaction mixtures (100 μL) contained 50 μL of sample, 100 mM triethanolamine (pH 7.8), 200 mM KCl, 30 mM $MgSO_4$, 1.5 mM phosphoenolpyruvate, and 5 units of pyruvate kinase (ADP assay) or 5 units of myokinase

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and 5 units of pyruvate kinase (AMP assay), and were incubated at 30 °C for 3 h. ATP assays were then performed on each reaction.

NAD^+/NADH assays were performed using an NAD^+/NADH Quantification Kit (BioVision) in 96 well plates. To detect combined NAD^+ and NADH concentrations, 25 μL of sample was mixed with 25 μL of extraction buffer (BioVision) and then assayed. NADH concentrations were determined by mixing 50 μL sample with 50 μL extraction buffer (BioVision) and decomposing the NAD^+ by incubation at 60 °C for 1 h after which 50 μL was assayed. The assay was performed by addition of 100 μL of NAD cycling mix (BioVision) followed by incubation at room temperature for 5 min and then addition of 10 μL of NADH developer (BioVision). Plates were incubated at room temperature for 3 h before the absorbance at 450 nm was measured using a Multiskan Ascent plate reader.

Glucose 6-phosphate assays were performed by converting glucose 6-phosphate in the samples to glucono-γ-lactone-6-phosphate with the concurrent conversion of NAD^+ to NADH using glucose 6-phosphate dehydrogenase and then measuring the increase in NADH. Reaction mixes (100 μL) containing 80 μL of sample, 200 mM Tris (pH 7.8), 10 mM $MgCl_2$, 10 μM NAD^+, and 5 units of glucose 6-phosphate dehydrogenase were incubated at 30 °C for 30 min. The NAD^+ was decomposed by addition of 50 μL of extraction buffer (BioVision) to 50 μL of reaction mix followed by incubation at 60 °C for 1 h, after which 50 μL was assayed using the NAD^+/NADH Quantification Kit (BioVision) as described above.

HPLC Analysis of Glucose Concentrations. Resuspended metabolite samples were analyzed using a Shimadzu SCL-10 HPLC system with autoinjector, and glucose was detected using a Waters 410 differential refractometer. Samples were separated on an HPX-78H column (Bio-Rad) using a flow rate of 0.5 mL min^{-1} and 5 mM sulfuric acid as the solvent. Class VP4.2 software (Shimadzu) was used for the HPLC setup, analyte calibration, and analysis of the data.

GC-TOF-MS Analysis. Intracellular metabolite extracts were prepared for analysis by lyophilization followed by a two-stage chemical derivatization procedure as described previously.[49] Samples were analyzed by gas chromatography (Agilent 6890N, Cheadle, U.K.) coupled to an electron impact time-of-flight mass spectrometer (Pegasus III, Leco, Stockport, U.K.) applying a method for the analysis of yeast metabolic footprints as previously described.[50]

RESULTS

Effect of Quenching Additives on Extraction and Recovery of Metabolites. Leakage of metabolites during the quenching step of metabolite extractions is a major problem particularly with cells lacking a cell wall. Metabolite extractions from bacterial cells using 60% methanol as the quenching solution have resulted in disruption of the cell membrane and therefore significant loss of metabolites.[36] One might consider that eukaryotic cells are even more delicate and, therefore, in an effort to protect the CHO cells against disruption of the cell membrane by the methanol in the quenching solution a number of additives were included, and these quenching solutions were compared to harvesting of the cells by centrifugation (without quenching) prior to extraction of the metabolites. The additives tested were 70 mM HEPES (pH 7.4), 0.85% ammonium bicarbonate (AMBIC) (pH 7.4), or 0.85% NaCl, since these additives have previously been found to protect bacterial cells.[35] To test the efficiency of each of the metabolite quenching solutions, the concentrations of six metabolites which are subject to rapid turnover were measured (viz., ATP, ADP, AMP, NAD^+, NADH, and glucose 6-phosphate). The concentrations of these metabolites are known to rapidly change upon environmental stress to the cells and the ratios of these (adenylate energy charge and catabolic reduction charge) give information on the relative "well-being" of the cells when metabolism was arrested. Initially ATP, NAD^+, and glucose 6-phosphate concentrations were measured (Figure 1). In each case quenching of the cells using 60% methanol + AMBIC resulted in the greatest recovery of the metabolites being measured. The addition of HEPES or NaCl did not give improved recovery of metabolites compared to 60% methanol alone. Harvesting of the cells by centrifugation resulted in >80% loss of ATP and glucose 6-phosphate; however, the concentrations of NAD^+ recovered were comparable to those from the quenched cells. This suggests that the cell membranes were not disrupted by centrifugation but that the process of centrifuging the cells perturbed the cells resulting in changes in the intracellular metabolite concentrations. The concentrations of these metabolites extracted from the CHO cells were comparable to those published for other mammalian cell types. ATP concentrations in rat hepatocyte extractions range from 4.52−10.89 mM[51−55] and are 2.11 mM in human erythrocytes[54,55] compared to 7.14 mM in CHO cells (Figure 1A and Table 1). ADP and AMP concentrations were also comparable to reference concentrations from rat hepatocytes (Table 1). NAD^+ concentrations in rat hepatocytes (1.14 mM[56]) were 6-fold higher than in CHO cells (0.19 mM); however, the concentrations compared well to human HEK293, U937, THP-1 cells (0.34−0.37 mM[57−59]) and human erythrocytes (0.06 mM[54,60]) (Figure 1B and Table 1). Glucose 6-phosphate concentrations in CHO cells were 0.06 mM compared to 0.09 mM in rat hepatocytes[56] and 0.04 mM in human erythrocytes[54,55] (Figure 1C and Table 1). These data indicate that the quenching methods rapidly stop cellular metabolism. Similar results were also observed when metabolites were extracted from mouse NS0 myeloma cells (data not shown). The

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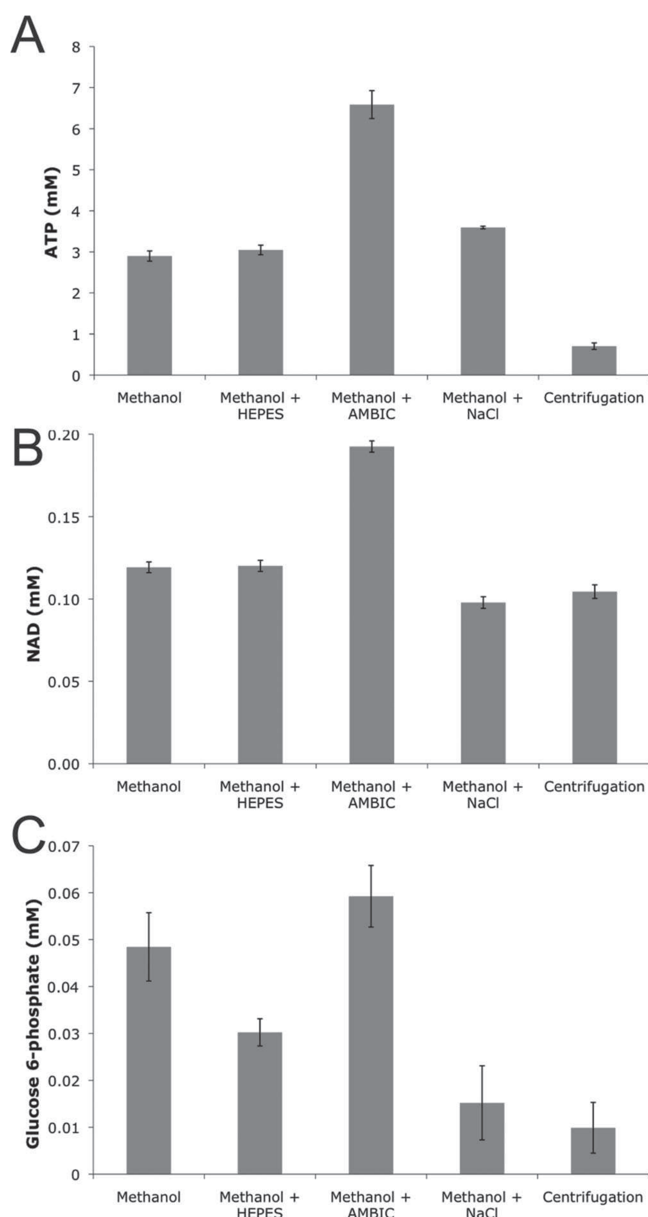

Figure 1. Recovery of rapidly turned over metabolites using different quenching methods. Cellular metabolism was quenched using each of the different quenching solutions or by harvesting of the cells by centrifugation, and then each preparation was extracted for analysis of intracellular concentrations of (A) ATP (B) NAD^+ and (C) glucose 6-phosphate. Values for each experiment represent the average of three biological replicates with two technical replicate readings of each, and standard deviation error bars are shown.

presence of AMBIC in the quenching solution improved the metabolite recovery from the cells. Therefore, extractions were also tested using double or half the initial concentrations of the additives [i.e., 1.7% or 0.4% AMBIC (pH 7.4) instead of 0.85%] to evaluate the effect of additive concentration on the recovery. Combinations of the additives (e.g., 0.85% AMBIC + 0.85% NaCl) were also tested to determine whether a cumulative effect could be seen. No improvements could be seen using any of these quenching solutions compared with the initial additives (data not shown). It was viewed as essential to define if leakage had occurred from cells during the quenching process and how this was affected by the quenching conditions. Quenching with methanol + AMBIC not only allowed greater recovery of ATP but also resulted in a greater retention (through both diminished leakage and enhanced recovery) of this metabolite (Figure 2). This contrasts markedly with all other methanol/additive combinations and verifies the recommendation that the chosen quenching method requires validation in terms of a combination of recovery and reduction of leakage.[34] Quenching of the CHO cells in 60% methanol + 0.85% AMBIC resulted in 20−60% greater recovery of the labile metabolites than any of the other methanol quenching solutions coupled with lower levels of ATP leakage.

Quenching the Cells Stops Metabolism Rapidly. The ratios of ATP, ADP, and AMP give insights into the state of the cell prior to metabolite extraction. This is in the form of the adenylate energy charge, which is defined as ([ATP] + [ADP]/2)/([ATP] + [ADP] + [AMP]), and was found to be in the range of 0.80 to 0.95 in bacteria[61] and 0.87 to 0.93 in rat hepatocytes and human erythrocytes.[51−55] The adenylate energy charge for metabolite extractions using methanol and methanol + AMBIC quenching solutions were 0.89 and 0.92, respectively, (Figure 3A) reflecting the sampling during mid-exponential phases of growth, and further demonstrates that metabolism must have been arrested rapidly enough to prevent enzymatic turnover of the ATP pool. Conversely, the adenylate energy charge for cells harvested by centrifugation was 0.55. One explanation for this is that the process of harvesting by centrifugation in the absence of methanol had perturbed the cells resulting in ATP catabolism. Alterations in the metabolite profile of cells because of centrifugation has previously been reported.[32] Another possible explanation is that the enzymes involved in nucleotide triphosphate decomposition are degrading the ATP during centrifugation of the cells (in the absence of quenching), which would explain the decrease in the total adenylate levels (Figure 3A). It has also been reported that in *E. coli* the enzymes involved in nucleotidetriphosphate decomposition are not deactivated even after methanol extraction of the metabolites,[62] which would also explain the decrease in the total adenylate levels; however, similar effects would be seen across all samples since the same extraction methods were used and this is not the case. Both explanations would reflect a consequence for the overall metabolite concentrations within cells and would also lead to under- or overestimation of metabolites. The ratio of NAD^+ and NADH also gives information on the state of a cell in the form of the catabolic reduction charge, which is defined as [NADH]/([NAD^+] + [NADH]). In growing cells the catabolic reduction charge is always low. This is because most NAD^+ in a cell is in the oxidized form to provide oxidizing power for catabolism. The catabolic reduction charge was found to be in the region of 0.03−0.07 in bacteria[63] and 0.08 in rat hepatocytes,[56] and this corresponds well with the values obtained from the methanol quenches (0.09 and 0.05 without or with AMBIC, respectively) but not with the cells harvested by centrifugation before extraction (0.16) (Figure 3B). This further supports the hypothesis that methanol + AMBIC quenching preserves metabolite concentrations at the physiological state.

Effect of Washing on Leakage of Metabolites. When the cells are quenched and harvested for extraction, some of the culture medium remains associated with the cells. The medium

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Table 1. Metabolite Concentrations in CHO Cells Compared to Other Mammalian Cells[a]

metabolite	CHO cells (this study, mM)	cell type	value (mM)	reference
ATP	7.14 ± 0.11	fed rat hepatocytes	5.08	51
		fed rat hepatocytes	10.89	52
		fed rat hepatocytes	4.52	53
		human erythrocytes	2.11	54,55
ADP	1.31 ± 0.10	fed rat hepatocytes	1.24	51
		fed rat hepatocytes	2.26	52
		fed rat hepatocytes	0.77	53
		human erythrocytes	0.304	54,55
AMP	0.04 ± 0.00	fed rat hepatocytes	0.22	51
		fed rat hepatocytes	0.55	52
		fed rat hepatocytes	0.10	53
		human erythrocytes	0.03	54,55
NAD^+	0.19 ± 0.00	fed rat hepatocytes	1.14	56
		human embryonic kidney (HEK293)	0.35	57
		human leukemic monocyte lymphoma (U937)	0.37	58
		human acute monocyte leukemia (THP-1)	0.34	59
		human erythrocytes	0.06	54,60
NADH	0.01 ± 0.00	fed rat hepatocytes	0.10	56
G6P	0.06 ± 0.01	fed rat hepatocytes	0.09	56
		human erythrocytes	0.04	54,55

[a] CHO concentrations are from this study. Metabolites from fed rat hepatocytes for ATP, ADP, and AMP measurements were extracted from the hepatic cells using 30% ice-cold perchloric acid.[51−53] Metabolites from HEK293, U937, and THP-1 cells for NAD^+ measurements were extracted using either 30%[57,58] or 10%[59] ice-cold perchloric acid. Metabolites for NAD^+, NADH and glucose 6-phosphate (G6P) from fed rat hepatocytes were extracted from whole liver samples.[56] In each case the liver was freeze-clamped, ground in liquid nitrogen, and metabolites were extracted with 30% ice-cold perchloric acid. Metabolites were extracted from erythrocytes using 10% ice cold perchloric acid.[55,60]

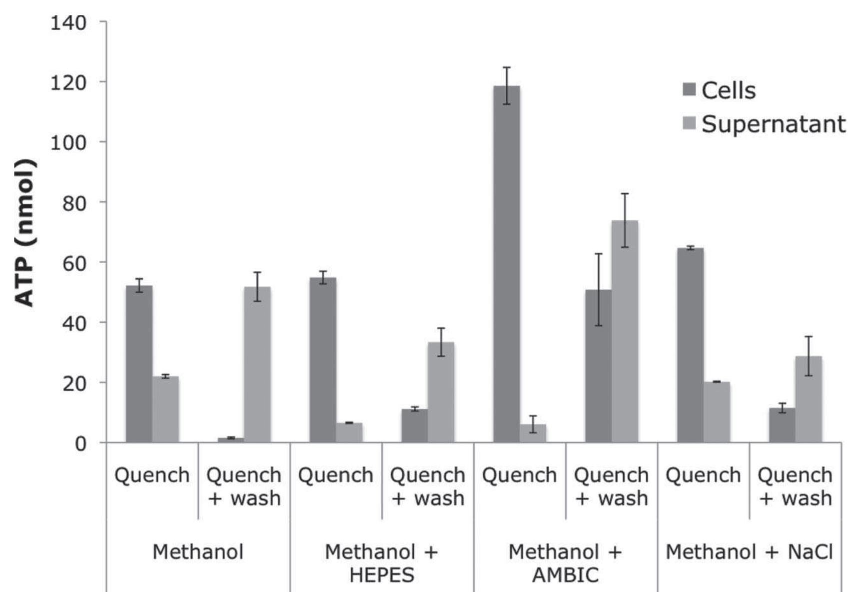

Figure 2. Metabolite leakage after methanol quenching. Leakage of metabolites into the quenching and wash solutions was assessed by measuring the ATP concentrations in the cells compared to the ATP amounts in the quench or wash supernatants. Values for each experiment represent the average of three biological replicates with two technical replicate readings of each, and standard deviation error bars are shown.

contains a number of nutrients which are also normal metabolic components of the cells. This means that the associated medium may cause the measured concentrations of metabolites to be artificially high. Quenching removes most of the medium, but it is possible that an additional washing step might improve the validity of the intracellular metabolite measurements. However, we also needed to check whether an additional washing step might cause further metabolite leakage. This is particularly important because it has been demonstrated that extension of the length of time of metabolite extractions resulted in increased loss of metabolites from bacterial cells.[37] Glucose concentrations were measured in the extracts from CHO cells to indicate the level of medium contamination in the metabolite sample. Glucose is a suitable indicator because free glucose is immediately converted to glucose-6-phosphate or other metabolites as soon as it enters the cell. Therefore, any glucose present in the cell extract must have originated from the culture medium. When unwashed methanol-quenched cells were analyzed, the data indicated that 0.8−1.3% of the medium had been carried down with, and contaminated, the cellular fraction (Figure 4A). However, the medium contamination decreased to 0.3−0.4% following an additional washing step with fresh quenching solution. Washing

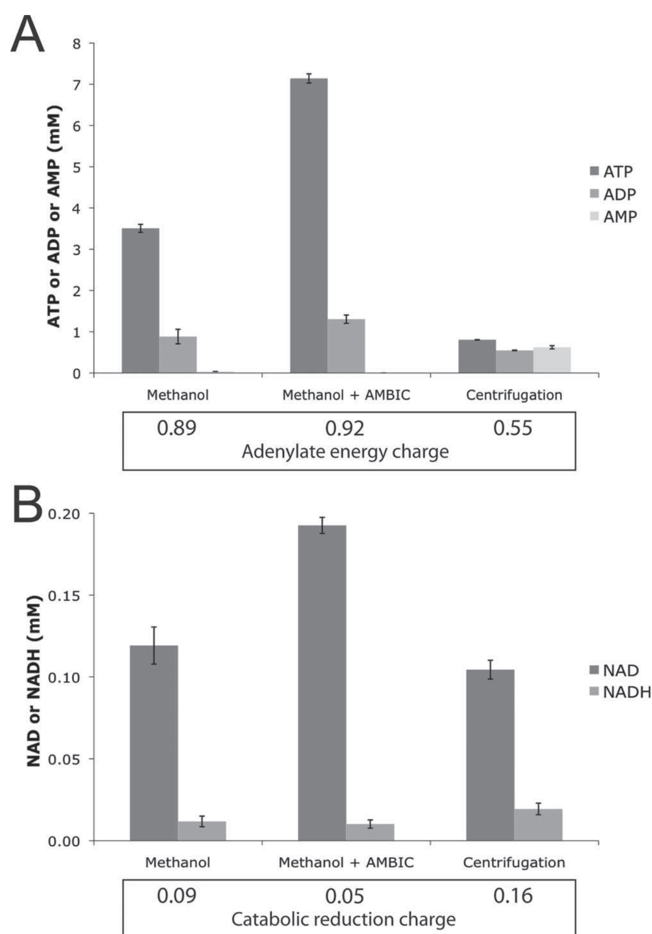

Figure 3. Comparison of energy charge and catabolic reduction charge after use of different quenching procedures. (A) Intracellular ATP, ADP, and AMP concentrations after quenching with methanol or methanol + AMBIC or harvesting of the cells by centrifugation. The adenylate energy charge is also shown and was calculated using the equation ([ATP] + [ADP]/2)/([ATP] + [ADP] + [AMP]). (B) Intracellular NAD^+ and NADH concentrations after quenching with methanol or methanol + AMBIC quenching or harvesting of the cells by centrifugation. The catabolic reduction charge was calculated using the equation [NADH]/([NAD^+] + [NADH]). Values for each experiment represent the average of three biological replicates with two technical replicate readings of each, and standard deviation error bars are shown.

decreased the contamination by the medium (an average of 60 ± 15%). However, we also measured the ATP concentrations in the cells with and without washing to determine the effects of washing on metabolite leakage. As observed previously, 60% methanol + AMBIC resulted in the recovery of the highest concentrations of ATP when the cells were quenched and extracted without an intermediate washing step. By contrast, 60% of the ATP was lost when the cells were quenched and then washed before extraction (Figure 4B). The loss of ATP was even greater when the cells were quenched and washed in 60% methanol alone (97% loss). Although not as effective as 60% methanol + AMBIC in retention of ATP, washing with 60% methanol + HEPES or NaCl did provide some protection to the cells compared to methanol alone (80% loss in both cases; Figure 4B). The loss of intracellular ATP was mirrored by an increasing leakage into the washing solutions (Figure 2). These data demonstrate once again that the addition of AMBIC results in the best recovery of metabolites. However, the drastic loss of metabolites following washing far outweighs the benefits derived from the wash; therefore, our standard procedure does not contain an extra washing step.

Presence of AMBIC Does Not Interfere with GC/MS Analysis of the Metabolite Samples. Although quenching of the cells with a methanol + AMBIC solution results in the best recovery of metabolites it is important that the presence of AMBIC does not interfere with downstream metabolomic analysis of the samples. Many metabolomic investigations employ chromatography−mass spectrometry platforms which can be salt intolerant because high levels of salts can reduce the efficiency of chemical derivatization procedures.[5,7] To investigate this, metabolite samples obtained using either methanol or methanol + AMBIC quenching were analyzed using GC/MS (Figure 5). GC/MS was chosen as it is one of the most frequently used MS-based analysis tools within the field of metabolomics.[5] The numbers and amounts of metabolites within each sample were compared and a *t*-test was performed to determine whether there were any significant differences between the two sample types. Both preparations exhibited approximately 100 well-defined major peaks, which did not include adenine or nicotinamide nucleotides, indicating that the presence of AMBIC does not interfere with the GC/MS analysis. A total of 41 unique metabolites were identified using each of the quenching methods (Table 2). There were no significant differences between the relative peak areas for all the identified peaks obtained in the presence and absence of AMBIC (unpaired *t*-test with 90% confidence limit for the peak areas for each individual peak; Average p-value = 0.557) (Figure 5). This indicates that quenching with methanol + AMBIC acts to prevent catabolism or decomposition of at least some of the most labile metabolites (i.e., ATP, NAD^+, and glucose 6-phosphate) but did not increase recovery of the more stable metabolites (e.g., amino acids). As suggested from the analysis of the ATP leakage into the quench and wash solutions (Figure 2), the GC/MS data also indicates that the increase in concentration of these metabolites in these rapid metabolite extractions is not through protection of the cell membrane from disruption by methanol but may instead be that the presence of AMBIC reduces any momentary stress/shock experienced by the cells when injected into the quenching solution.

DISCUSSION

In this study, we have addressed the quenching process for sample preparation of intracellular metabolites from mammalian cells in suspension culture, and we have shown, based on measurements of a group of labile metabolites, that quenching in −40 °C 60% methanol supplemented with 0.85% AMBIC generates a profile that is representative of a physiological status. Clearly, failure to prevent artifactual changes to metabolite profiles (with possible increases or decreases because of enzymatic activity) would prevent meaningful interpretation of metabolite changes in response to environment or cell line phenotype. For instance, inefficient quenching of cells has been demonstrated to result in reduced recovery of 6 carbon intermediates of glycolysis,[36] which will result in underestimation of these metabolites and consequently overestimation of the lower glycolysis metabolites. Several stages in the extraction process can result in artifactual changes to metabolite profiles. A key step is the separation (and hence concentration) of cells from a large volume of medium. For

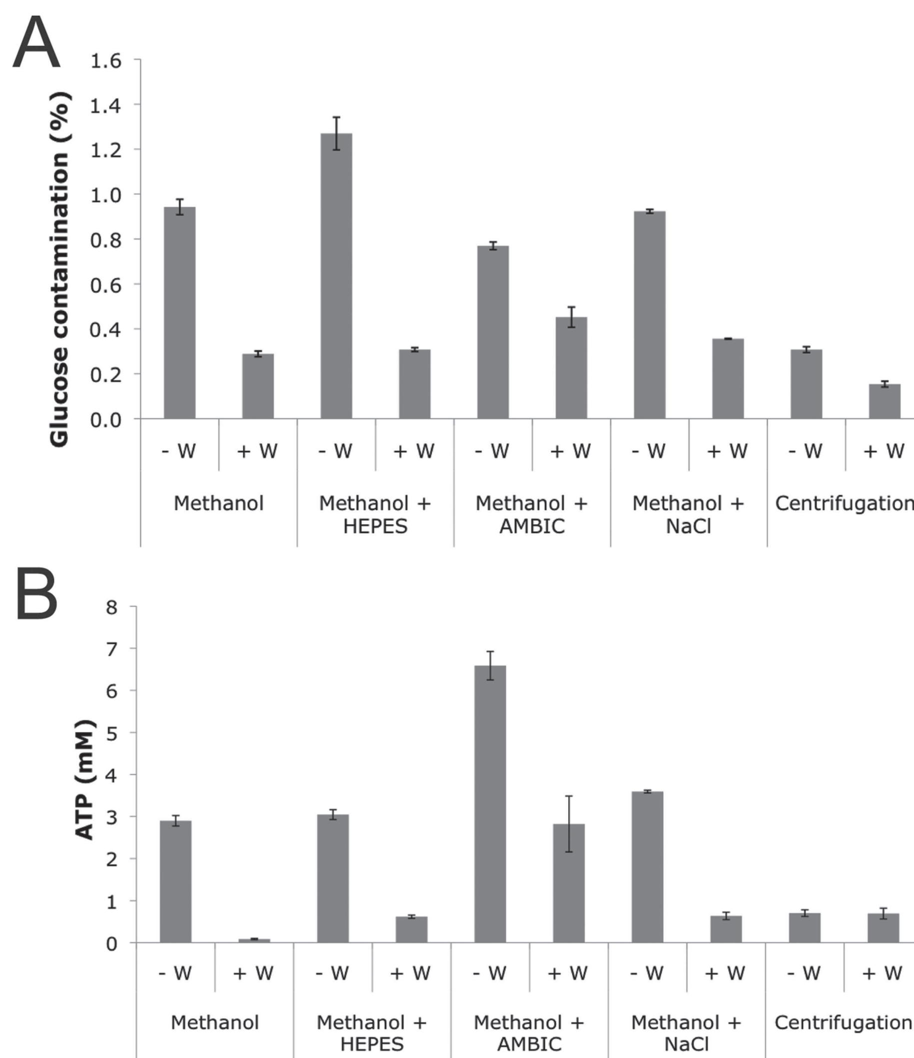

Figure 4. Effect of washing the cells after quenching. (A) Glucose levels in the metabolite extracts were assayed to determine the amount of medium contamination. Values represent percentage of the total glucose present in the original sample containing medium and cells. Glucose contamination from the medium is shown for extractions performed without (−W) or with (+W) washing of the cells (with appropriate quenching solution) following quenching. (B) ATP concentrations from extractions performed without (−W) or with (+W) washing of the cells (with appropriate quenching solution) following quenching. Values for each experiment represent the average of three biological replicates with two technical replicate readings of each, and standard deviation error bars are shown.

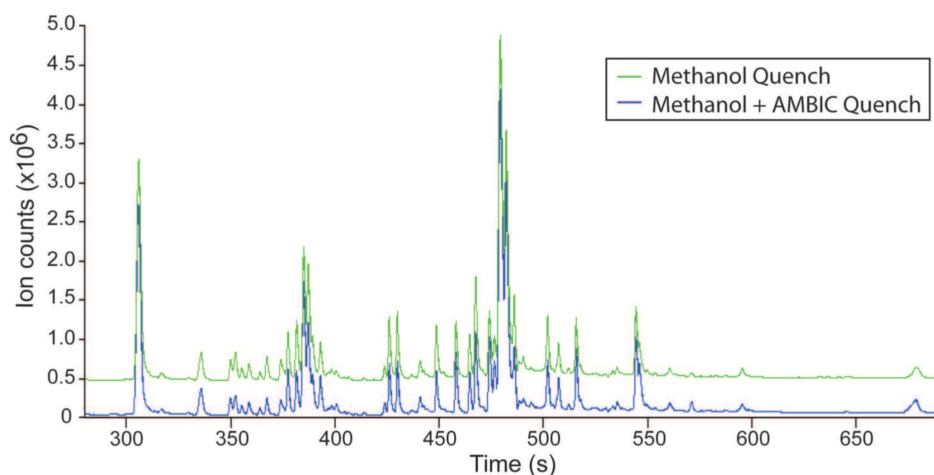

Figure 5. GC/MS analysis of intracellular metabolites extracted from CHO cells. Cells were either quenched with 60% methanol (green) or 60% methanol + 0.85% AMBIC (blue) before extraction of the metabolites using cold methanol. For clarity, the *y* axis for methanol alone has been offset.

Table 2. Unique Metabolites Identified in the Intracellular Metabolite Extractions by GC/MS Analysis[a]

3-Ureidopropionic acid
4-hydroxyproline
Alanine
Asparagine
Aspartic acid
Cholesterol
Citric acid
Cysteine
Glucose
Glutamic acid
Glutamine
Glycerol
Glycerol 3-phosphate
Glycine
Heptadecanoic acid
Hexadecanoic acid
Hypotaurine
Isoleucine
Lactic acid
Leucine
Lysine
Malic acid
Methionine
Monopalmitin
Monstearin
Myo-inositol
N-bromosuccinimide
Octadecanoic acid
Pantothenic acid
Pentadecanoic acid
Phenylalanine
Phosphate
Proline
Pyroglutamic acid
Pyruvic acid
Serine
Threitol or erythritol
Threonine
Tryptophan
Tyrosine
Valine

[a] Analysis of the data identified 96 individual peaks, of which 53 peaks were matched to specific metabolites by both retention time and mass spectrum.[49] A total of 41 unique metabolites were identified with no significant difference of recovery observed for methanol and methanol + AMBIC quenching.

effective harvest of cells from the quenching solution, centrifugation is required and this should be relatively gentle as high speed centrifugation may cause cellular damage leading to metabolite leakage or change. The relatively large size of mammalian cells provides an advantage as centrifugation times are significantly less (1−1.5 min) than those required for yeast and bacteria (typically 5+ minutes). This shortens the overall time required for harvest and reduces the exposure of the mammalian cells to the organic solvent and together these factors may explain the relatively low levels of leakage observed in contrast to bacterial cells.

To challenge the effectiveness of the quenching process to the greatest possible extent, we elected to use a series of labile intracellular metabolites as indicators of physiological validity of the approaches undertaken. Use of a −40 °C methanol + AMBIC extraction process generated metabolite extracts with ATP, NAD^+, and glucose 6-phosphate contents that are within physiological cellular concentrations.[51−60] The physiological profiling exhibited for these labile metabolites is reinforced by values obtained for the adenylate energy charge (reflecting ATP, ADP, and AMP concentrations) and the catabolic reduction charge (reflecting NAD^+ + NADH). Having shown the physiological validity of this approach at the level of labile metabolites, we extended our work to examine overall intracellular metabolite profiles by GC/MS and could show that the inclusion of (the salt) AMBIC in extracts was not detrimental to metabolite derivatization, separation, and detection.

Other methods can be used for harvesting/quenching of cells; however, the major limitation with each of these alternative methods is that in the absence of methanol the coldest that the solutions can be used at is 0–4 °C. At this temperature enzymatic activity is decreased but not arrested. This may not be a major problem for the more stable metabolites within a cell with a low turnover rate, such as amino acids, but the concentrations of the more labile metabolites, such as ATP, alter within seconds of a cell being stressed (e.g., by cold shock) in the absence of simultaneous quenching. This means that the amounts detected would be significantly different to those at the time of sampling. This was the case in the study of Bolten et al. who found that with bacterial cells the 6-carbon intermediates of glycolysis were obtained in higher concentrations from cold methanol-quenched extracts than from extracts produced by fast-filtering.[36]

We did not extensively test extraction methods following quenching because this aspect of metabolite extractions has been examined in a number of studies. Maharjan and Ferenci tested five different extraction methods (cold and hot methanol, hot ethanol, perchloric acid, and alkaline extractions) for extraction of metabolites from *E. coli* and concluded that the best overall method was cold methanol extraction.[43] A similar procedure was performed by Villas-Boas et al. for the yeast *S. cerevisiae*.[64] This method gave the best permeabilization of the membrane and was the simplest and most reproducible method. It also prevented the loss of heat-labile or pH-sensitive metabolites and had the advantage that the extraction was performed at −40 °C, which stops cellular metabolism. It was for these reasons that we used the cold methanol extraction and did not extensively test the other extraction methods. This was confirmed in the recent work by Winder et al. who used similar extraction methods followed by univariate and multivariate analysis to elucidate the best extraction method for metabolic profiling applications.[34] While cold methanol appears to be the best general method, it does not extract all metabolites equally and is positively biased toward polar metabolites;[34] therefore, it is likely that to get a complete metabolite profile each sample would require extraction of metabolites by a number of different extraction methods, most probably caused by differences in metabolite solubility, which has been shown for mammalian biofluids.[65]

One concern in profiling intracellular metabolites is the need for reassurance that the metabolite detected is from an intracellular pool rather than a contaminant from the culture medium. This may be of limited concern for specific metabolites and when cells are suspended in simple medium, but this is a major concern for mammalian cells grown in complex, rich medium (as in this case). In our studies a process has been developed which allows a suitable compromise between minimization of contamination of

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intracellular material with medium components and the need for speed to retain a physiological intracellular metabolite profile. In further studies knowledge of the (limited) extent of medium contamination together with determinations of concentrations from intracellular and medium samples will enable meaningful determination of intracellular metabolite concentrations.

CONCLUSIONS

Our study presents important information for metabolomics studies with mammalian cells in suspension culture. The method is now ready for deployment for analysis of the metabolome, and thus provides reassurance of the physiological validity of intracellular metabolite concentrations and a key starting point for any meaningful interpretation of relationships between cell physiology/ phenotype and critical metabolic events. For example, growth and production of exogenous recombinant proteins is sensitive to external conditions (e.g., cold shock[66,67]), and it is now possible to use metabolomics with confidence to analyze the metabolic events underlying the down regulation of protein synthesis. Now that appropriate validation has been completed, characterization of intracellular metabolites will offer a unique approach to interrogate the cellular events that generate concerted control of cell growth and recombinant protein production. Our quenching and extraction process has general validity for mammalian cells growing in suspension cultures. We have also carried out extensive analysis with mouse NS0 myeloma cells (data not shown), a second suspension mammalian cell type that is widely used in the biopharmaceutical/bioprocessing industry for the generation of recombinant proteins.[68] Data from NS0 myeloma cells are in close agreement with the findings from the experiments with CHO cells described here and indicate the wide effectiveness of the methanol + AMBIC process described in this manuscript. The widely applicable and technically simple method described here presents one initial significant step in the application of metabolomics approaches to mammalian cell lines in suspension culture.

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