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PROMOTION OF CYSTINE UPTAKE AND ITS UTILIZATION FOR GLUTATHIONE BIOSYNTHESIS INDUCED BY CYSTEAMINE AND *N*-ACETYLCYSTEINE

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Abstract—Chinese hamster ovary (CHO) cells obtain a high capacity to utilize cystine from the growth medium by exposure to cysteamine (2-mercaptoethylamine, MEA) or *N*-acetylcysteine (NAC). For uptake studies a modified McCoy's 5A medium supplemented with 0.1 mM [^{35}S]cystine was used. The uptake of cystine was dependent on the time of exposure (0–60 min) and the concentrations of MEA or NAC (0–8 mM). At high concentrations of MEA or NAC, the uptake of cystine became saturated. Half-maximal uptake of cysteine was observed at concentrations of 0.12 mM MEA and 0.66 mM NAC, respectively. Increase in temperature (37–44°) or pH (6.0–8.0) during MEA or NAC exposure further increased the cystine uptake. The increased uptake of cystine was not affected in the presence of glutamate or homocysteate which both inhibited the cystine uptake of control cells. Determination of both reduced (GSH) and oxidized (GSSG) cellular glutathione showed a twofold increase in MEA- or NAC-treated CHO cells. DL-buthionine-*S*,*R*-sulfoximine (BSO), an inhibitor of GSH biosynthesis completely blocked the promotion of cystine uptake by MEA and NAC. By further analysis using reversed-phase HPLC of cell extracts, more than 90% of the [^{35}S] radioactive cystine taken up by the cells could be recovered within the pool of GSH. The results demonstrate that exposure of CHO cells with MEA and NAC leads to a promoted uptake of cystine from the culture medium and its rapid utilization for cellular GSH biosynthesis.

The aminothiol cysteamine (2-mercaptoethylamine, MEA)‖ is a well-known radioprotector *in vivo* and *in vitro* (for review see Ref. 1). Several factors are discussed to explain this property which it shares with certain other thiol compounds. It is thought to be the result from scavenging free radicals, either directly by the SH-compounds [2] or by GSH released from protein-bound mixed disulfide [3]. It is also suggested that thiol-linked autoxidation may be responsible for radioprotection by producing hypoxic conditions and thus reducing O_2 consumption within cells [4]. The thiol derivative *N*-acetylcysteine¶ (NAC) is clinically used for its mucolytic effects [5]. More recently, NAC has been found to be a chemoprotective agent reducing the side-effects of a number of cytotoxic compounds [6].

Both MEA and NAC have been shown to increase the level of GSH in mammalian cells [7–9]. In our previous study we could demonstrate that the increase of GSH in Chinese hamster ovary (CHO) cells was dependent on the presence of cystine in the culture medium [9]. The rate-limiting step for the increase of GSH seems to be the intracellular supply of cysteine, which is the reduced form of cystine and can be utilized by mammalian cells for GSH synthesis [10]. It is well-known that cysteine routinely added to growth medium is rapidly autoxidized to its disulfide form (cystine) within a few hours after preparation. Therefore, utilization of cystine from the growth medium is critical for the cysteine supply of these cells.

In the present *in vitro* study we used MEA and NAC in order to investigate their potential to increase the capacity of CHO cells to utilize cystine from the growth medium for cellular biosynthesis of glutathione.

MATERIALS AND METHODS

Chemicals. L-[^{35}S] cystine (specific activity 50–120 mCi/mmol) was obtained from Amersham Buchler (Braunschweig, F.R.G.). Cystine-dihydrochloride, cysteamine, cystamine, *N*-acetyl-L-cysteine and glutathione-*S*-transferase were obtained from Sigma Chemical Co. (Taufkirchen, F.R.G.). DL-buthionine-*S*,*R*-sulfoximine (BSO) was kindly supplied by Dr John Biaglow of the Case Western University, Cleveland, OH, U.S.A. Trypsin (0.25%), newborn and fetal calf serum, McCoy's 5A medium and McCoy's 5A medium without cystine, glutathione and methionine were purchased from Gibco (Eggenstein, F.R.G.). NADPH and other enzymes

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‖ Abbreviations used: BSO, DL-buthionine-*S*,*R*-sulfoximine; CHO, Chinese hamster ovary; GSH, glutathione, reduced; GSSG, glutathione, oxidized; GS–DNP, *S*(2,4-dinitrophenyl)-glutathione; HPLC, high pressure liquid chromatography; MEA, 2-mercaptoethylamine; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline.

¶ Fluimucil®, Inpharzam, Munich.

were from Boehringer (Mannheim, F.R.G.). Reagents for liquid scintillation counting were obtained from New England Nuclear (NEN, Dreieich, F.R.G.) and from Roth (Karlsruhe, F.R.G.). All other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Cell culture. CHO cells were routinely grown and subcultured in McCoy's 5A medium supplemented with 10% newborn and 5% fetal calf serum at 37° and in an atmosphere with 5% CO_2. Colony-forming efficiency was 80–90% and the population doubling time was 12–15 hr. For uptake studies, CHO cells were trypsinized and plated in 35 mm plastic Petri dishes (0.5×10^6 cells) containing 2 ml complete medium per dish. At the time of the experiment (≈24 hr after plating) the cell number was 1.5–2×10^6 cells per dish and the cells had reached about 50% confluency.

[^{35}S] *Cystine-uptake.* Incubations at different temperatures (37–44°) were performed in a temperature-controlled water bath, the bottom of the Petri dishes being submersed in the water. For experiments at 5°, the cells were kept for 20 min in a refrigerator before the addition of the cold uptake medium and further incubated at this temperature. The growth medium was then removed, the layer of cells was rinsed with phosphate-buffer saline (PBS), pH 7.4 (Dulbecco's solution with 0.1% glucose) and 0.5 ml of the uptake medium was added for different lengths of time as indicated. The uptake medium was the same as McCoy's 5A medium, except that glutathione and methionine were omitted and cyst(e)ine replaced by 0.1 mM [^{35}S] cystine. For experiments studying the influence of various amino acids upon the uptake of [^{35}S] cystine, the uptake was measured in PBS supplemented with 0.1% glucose. Uptake of [^{35}S] cystine at different pH (6.0–8.0) was also measured in PBS where the medium was adjusted to the indicated pH value by variation of the Na_2HPO_4/KH_2PO_4 ratio. In all cases, using thiols (MEA, NAC), BSO or different amino acids, these reagents were dissolved in N_2-gassed PBS and added in small volumes (5–50 μl) to the medium. After drug exposure in the presence of [^{35}S] cystine, the cell layer was rinsed with ice-cold PBS and then cells were trypsinized (0.25% trypsin) for 2 min at room temperature. The cell suspension (1 ml) was transferred into Eppendorf cups, precipitated with 1 N perchloric acid and kept on ice. The acid-insoluble pellet was solubilized for scintillation counting by an 18 hr incubation with 5 vol. of Protosol at room temperature. The acid-soluble and the acid-insoluble radioactivity was counted in a xylene-based cocktail. Quench correction was achieved by the external standard channel ratio technique. If not otherwise indicated, the data for [^{35}S] cystine uptake are expressed as total uptake (acid-soluble and acid-insoluble fractions). Uptake rates are based on incubations of 4 min duration. The cell number was determined for each experiment from 5 replicate dishes by counting the trypsinized cell suspensions in a Coulter Counter. Protein was assayed by the Lowry method [11].

GSH-determination. Experiments designed to measure total intracellular GSH were initiated by inoculating ≈10^6 cells/T_{25} flask in two replicates containing 4.5 ml of fresh medium (total volume). After exposure with MEA or NAC, the medium was removed, and cells were washed twice with PBS and then trypsinized (0.25% for 2 min). At the time of GSH determination, more than 95% of cells were metabolically viable assessed by trypan blue exclusion. After dilution in cold PBS (5 ml), the cells were counted and centrifuged at 4°.

Total GSH

Total cellular glutathione (GSH + GSSG) was quantified following the method described by Tietze [12]. The pellets were resuspended in 0.3 ml of 0.04 M EDTA and 0.6 ml of 7.5% trichloroacetic acid and centrifuged at 4°. The cold supernatant was removed and total glutathione was determined by the glutathione reductase procedure. The total GSH content of control cells was (N = 9) 26.3 ± 5.6 (SD) nmol/mg protein or 2.6 ± 0.5 (SD) nmol/10^6 cells.

GSH and GSSG

The reduced (GSH) and oxidized (GSSG) form of glutathione was determined by using the HPLC method as described by Reed [13]. For this procedure, the cell pellet was extracted with 0.5 ml 1 N perchloric acid and γ-glutamylglutamate was added as an internal standard. The extract was incubated for 120 min with iodoacetic acid at pH 8.5 to form *S*-carboxymethyl derivatives of free thiol groups. Subsequently, the amino groups were derivatized with 1-fluoro-2,4-dinitrobenzene (Sanger's reagent). Aliquots (50 μl) of the reaction mixture were injected onto a μ-Bondapak amine column (4 × 250 mm, Waters, Eschborn, F.R.G.) and eluted with a sodium acetate gradient (flow rate: 2 ml/min) in a water–methanol–acetic acid solvent at pH 4.5. The dinitrophenyl derivatives were detected at 365 nm. GSH and GSSG derivatives were quantified in relation to the internal standard. The GSH content of control cells (N = 4) was 20.8 ± 2.1 (SD) nmol/mg protein or 3.3 ± 0.3 (SD) nmol/10^6 cells. The GSH content (N = 4) was 0.51 ± 0.08 (SD) nmol/mg protein or 0.08 ± 0.01 (SD) nmol/mg protein.

Measurement of [^{35}S] *cystine incorporated into glutathione.* Recovery of [^{35}S] cystine incorporated into GSH was determined by HPLC and scintillation counting as recently described [14]. The incubation of cells in [^{35}S] cystine containing McCoy's 5A medium was essentially the same as described for the uptake method (see above). The cell pellet was extracted with ice-cold 1 N perchloric acid containing 1 mM EDTA (50 μl). The supernatant was neutralized with 22 μl of 1.57 M K_3PO_4, vortexed, and the resulting precipitate of $KClO_4$ pelleted after 4 min on ice. GSSG was reduced by adding 6 μl of a mixture of 20 μl 100 mM glucose-6-phosphate (in water), 20 μl of 1 mM NADPH (in 1% $NaHCO_3$), 20 μl of 5 mg/ml glucose-6-phosphate-dehydrogenase (from yeast, 140 U/mg, in water), 20 μl of glutathione reductase (from yeast, suspension of 120 U/ml) and finally 40 μl of (1 mg/ml) glutathione-*S*-transferase. After 10 min at 37°, 5 μl of 1-chloro-2,4-dinitrobenzene (10 mM in methanol) was added and the reaction mixture further incubated for 30 min at 37°. Then the mixture was acidified with 5 μl of 1 M H_3PO_4, centrifuged, and injected onto a

μ-Bondapak C_{18} column (4 × 300 mm, Waters, Eschborn, F.R.G.). The *S*-(2,4-dinitrophenyl)-glutathione conjugate (GS–DNP) was eluted with 0.1 mM H_3PO_4/methanol (60/40, v/v, flow rate 1.5 ml/min, R_t = 4.65 min, detection at 340 nm) and the radioactivity in the collected GS–DNP fractions counted as described. It should be noted that the possible 2,4-dinitrophenyl derivatives of cysteine and cysteamine were eluted at R_t = 3.65 min.

RESULTS

Time course of cystine uptake induced by MEA and NAC

The influence of MEA and NAC upon the uptake of [^{35}S] cystine from McCoy's 5A medium by CHO cells is shown in Fig. 1. Both thiols promote an immediate and pronounced increase in [^{35}S] cystine uptake compared to untreated control cells. Under the experimental conditions, it became apparent that longer exposure with MEA or NAC can lead to "floating off" effects of CHO cells. Also a slight increase of the pH-value of the uptake medium was observed during extended thiol exposure. Therefore, in order to approximate the initial uptake rates of cystine, only incubation periods of 4 min were used. During such a short period of exposure with MEA or NAC, neither a change of pH nor a significant detachment of cells could be observed.

Dependence of cystine uptake upon concentrations of MEA or NAC

In four independent experiments the initial uptake rate of cystine from the medium was 0.45 ± 0.15 (SE) nmol half-cystine/min/mg protein for untreated control cells. About 35% of the total radioactivity was found in the acid-soluble pool with an uptake rate of 0.13 ± 0.03 (SE) nmol half-cystine/min/mg protein. It should be emphasized that this latter rate, which mainly reflects incorporation of [^{35}S] cystine into protein synthesis or unspecific binding to the pellet, was not substantially changed by either thiol treatment. Almost all of the increased radioactivity taken up after treatment with MEA or NAC was found in the acid-soluble fraction of the cells.

MEA or NAC promote cystine uptake in a concentration dependent manner (0.1–8 mM). For example, exposure with 1 mM MEA increases the uptake approximately by a factor of six. NAC is not as effective and higher concentrations are required to obtain comparable uptake rates of [^{35}S] cystine. In experiments using cystamine (MEA-S-S-MEA) or *N*-acetylcystine (NAC-S-S-NAC), the oxidized forms of MEA and NAC, the uptake rates were quantitatively similarly increased (data not shown). The relation between MEA or NAC concentrations and cystine uptake in four repeated experiments allowed us to determine half-saturation constants (K_u) and maximal velocities (V_u) of [^{35}S] cystine uptake based on a Michaelis–Menten type of kinetics. These parameters were calculated by a non-linear least-squares regression program with subtraction of the control uptake rate. The values obtained for MEA were K_u = 0.12 ± 0.04 (SE) mM and V_u = 2.0 ± 0.2 (SE) nmol half-cystine/min/mg protein. For NAC, we obtained K_u = 0.66 ± 0.28 (SE) mM and V_u = 2.2 ± 0.2 (SE) nmol half-cystine/min/mg protein, respectively. The excellent fit and the striking similarity of the V_u-values become most apparent when the data are plotted in a Lineweaver–Burk diagram (see Fig. 2).

Influence of temperature upon cystine uptake induced by MEA and NAC

The temperature dependence (37–44°) of the MEA and NAC induced increase of [^{35}S] cystine uptake is shown in Fig. 3. A further slight increase of the rate of cystine uptake in thiol-treated cells

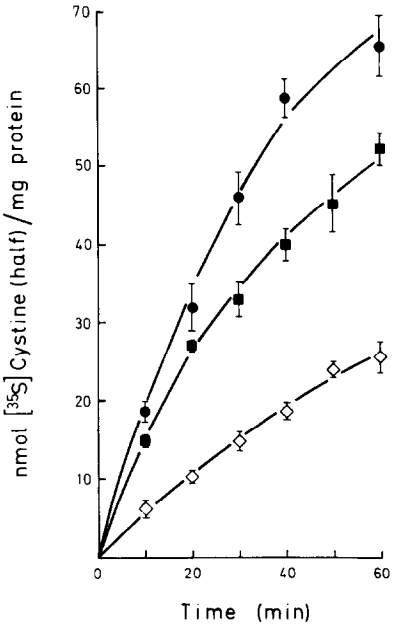

Fig. 1. Time course (0–60 min) of the uptake of 0.1 mM [^{35}S] cystine by CHO cells in McCoy's 5A medium at 37°. The medium contained: —●—, 0.4 mM MEA; —■—, 0.8 mM NAC; —◇—, no additional thiol (control). Means ±SD of three independent experiments.

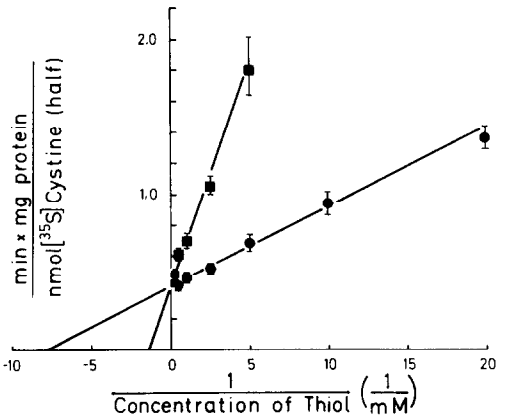

Fig. 2. Lineweaver–Burk plots of the thiol specific uptake of [^{35}S] cystine with: —●—, MEA; and —■—, NAC. The data were corrected for the control uptake Means ± SD of three replicates of one experiment.

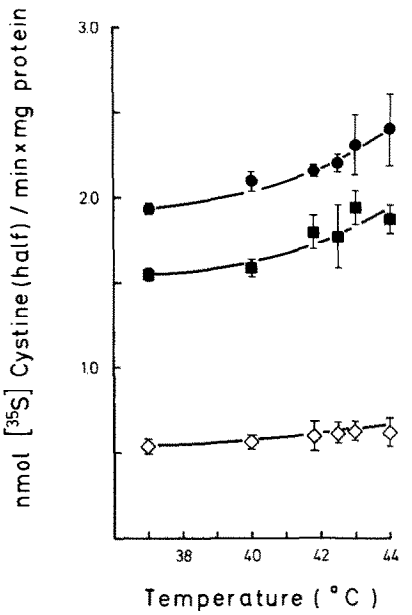

Fig. 3. Temperature dependence of the uptake rate of 0.1 mM [^{35}S] cystine by CHO cells in McCoy's 5A medium. —●—, 0.4 mM MEA, —■—, 0.8 mM NAC, or —◇—, no additional thiol (control). Means ± SD of three independent experiments.

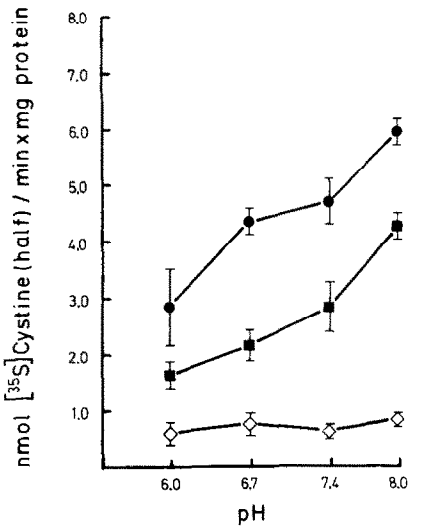

Fig. 4. Influence of pH of the growth medium (PBS supplemented with 0.1% glucose) upon the uptake of 0.1 mM [^{35}S] cystine by CHO cells at 37°. The medium was adjusted to the indicated pH value (6.0–8.0). —●—, 0.4 mM MEA; —■—, 0.8 mM NAC; —◇—, no additional thiol (control). Means ± SD of two independent experiments.

could be observed only at the higher temperatures (42–44°). In contrast, the control uptake of cystine in the absence of thiols was not significantly changed at temperatures from 37 to 44°. When cells were kept at 5° during thiol exposure (for details see Materials and Methods) no uptake of [^{35}S] cystine could be observed (data not shown).

Influence of pH upon cystine uptake induced by MEA and NAC

The effect of MEA and NAC upon the [^{35}S] cystine uptake in CHO cells revealed to be largely dependent from the pH of the uptake medium. As shown in Fig. 4, the corresponding uptake rate induced by either MEA or NAC significantly increased by a factor of two when the pH of the uptake medium was increased from 6.0 to 8.0, respectively. In contrast, the control uptake rate of [^{35}S] cystine in the absence of MEA or NAC was not modified under these conditions.

Inhibition of cystine uptake by other amino acids

The effects on the control and thiol promoted [^{35}S] cystine uptake in CHO cells by a number of different amino acids are shown in Table 1. In order to avoid the interference with other amino acids usually present in complete McCoy's 5A medium, the experiments were performed in PBS supplemented with 0.1% glucose. Compared to the uptake from complete McCoy's 5A medium, the initial uptake of [^{35}S] cystine in control cells from PBS was about twofold. The data given in Table 1 show the relative rates of cystine uptake in the presence of different amino acids (2.5 mM) with and without the addition of MEA or NAC. In the absence of either MEA or NAC, control uptake of cystine

Table 1. Effects of amino acids (2.5 mM) upon the uptake of 0.1 mM [^{35}S] cystine in CHO cells

Addition	Relative uptake		
	Control	+0.4 mM MEA	+0.8 mM NAC
None	1.00 ± 0.03*	1.00 ± 0.03	1.00 ± 0.04
L-Ala	0.81 ± 0.05	0.55 ± 0.03	0.66 ± 0.03
L-Ser	0.80 ± 0.09	0.52 ± 0.03	0.63 ± 0.03
L-Leu	0.84 ± 0.07	0.60 ± 0.04	0.78 ± 0.04
L-Lys	0.87 ± 0.04	1.19 ± 0.08	1.02 ± 0.08
L-Arg	0.84 ± 0.02	1.16 ± 0.06	1.33 ± 0.15
L-Glu	0.32 ± 0.09	0.94 ± 0.04	0.93 ± 0.09
DL-homocysteate	0.26 ± 0.03	0.89 ± 0.05	0.91 ± 0.07

The initial rate of [^{35}S] cystine uptake was measured in PBS supplemented with 0.1% glucose at 37° in the presence of different amino acids with and without the addition of MEA or NAC. The values are expressed as ratios of the individual uptake relative to control uptake.

The ±SE, N = 3 or more independent experiments.

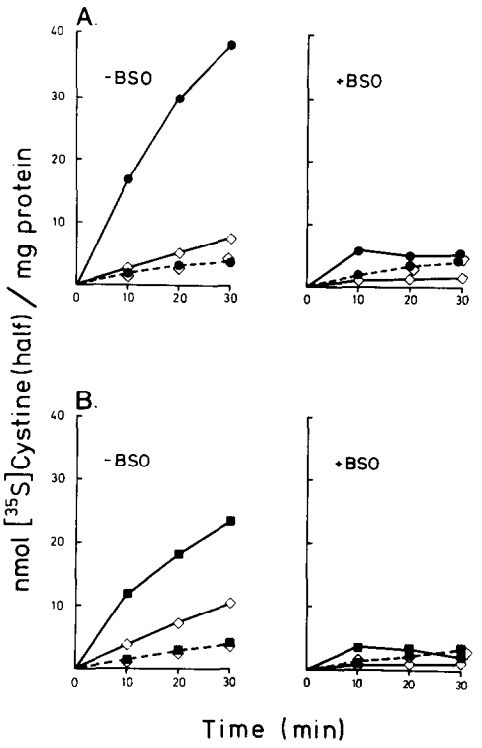

Fig. 5. Influence of DL-buthionine-*S*,*R*-sulfoximine (BSO) on the uptake of 0.1 mM [^{35}S] cystine by CHO cells in McCoy's 5A medium at 37°. 1 mM BSO was added to the cells 10 min before the addition of thiols. Full lines (———) represent the radioactivity of the acid-soluble supernatant, broken lines (– – – –) the radioactivity of the acid-insoluble pellet.

A. The uptake medium contained: —●—, 0.4 mM MEA; or —◇—, no additional thiol (control).

B. The uptake medium contained —■—, 0.8 mM NAC; or —◇—, no additional thiol (control).

was inhibited (≈70%) by L-glutamate and DL-homocysteate. In the presence of MEA or NAC, the increased uptake of [^{35}S] cystine was only inhibited (≈50%) by L-alanine and L-serine, but, interestingly, the inhibition by L-glutamate and DL-homocysteate could not be observed.

Effect of BSO upon the uptake of cystine induced by MEA and NAC

DL-buthionine-*S*,*R*-sulfoximine (BSO) is a potent and irreversible inhibitor of γ-glutamylcysteine synthetase, the first step of glutathione synthesis [10]. When 1 mM BSO was added to the uptake medium 10 min prior to the addition of MEA or NAC, the increased uptake of [^{35}S] cystine was completely blocked (see Fig. 5A and B). The uptake of [^{35}S] cystine into the acid-insoluble fraction either in the presence or absence of MEA and NAC was not influence by BSO. In order to determine whether the cellular level of GSH content at the time of MEA or NAC exposure could affect the [^{35}S] cystine uptake, we also repeated these experiments using CHO cells pretreated with 5 μM BSO for 17 hr at 37°. The BSO pretreatment of CHO cells leads to a reduction of total GSH to less than 20% of the control value [9]. Such BSO pretreated cells also showed no increase of [^{35}S] cystine uptake in the presence of MEA or NAC (data not shown).

Increase of GSH induced by MEA and NAC

The influence of MEA or NAC on cellular GSH content after exposure of CHO cells for 2 hr at 37° is shown in Table 2. Total cellular glutathione (GSH + GSSG) as quantitated by the glutathione reductase procedure [12] increased about twofold after both MEA (0.4 mM) or NAC (0.8 mM) treatment. The elevation of total GSH could be completely blocked by BSO (1 mM) added 10 min prior to the addition of MEA or NAC. The exposure with BSO alone for 2 hr only slightly reduced the total GSH content of cells. By determination of the reduced (GSH) and oxidized (GSSG) form of glutathione using the HPLC method described by Reed [13], a relatively similar increase of both forms after MEA or NAC exposure was observed. Although the GSH content of control cells measured by HPLC was slightly lower than the value obtained by the photometric assay, both methods led to comparable results in regard to the evaluation of GSH after MEA or NAC, respectively.

Recovery of [^{35}S] cystine incorporated into glutathione

We further determined the relative amount of [^{35}S] labelled cystine incorporated into glutathione after

Table 2. Glutathione content of CHO cells after exposure with MEA or NAC for 2 hr at 37°

Treatment	Photometric assay GSG + GSSG (nmol/mg protein)	HPLC method GSH (nmol/mg protein)	HPLC method GSSG (nmol/mg protein)
Controls	26.3 ± 5.6*	20.8 ± 2.1	0.51 ± 0.08
+BSO†	17.5 ± 0.9		
0.4 mM cysteamine	61.0 ± 14.0	54.8 ± 16.2	1.31 ± 0.26
+BSO†	16.7 ± 0.4		
0.8 mM *N*-acetylcysteine	41.4 ± 18.9	39.7 ± 9.1	0.80 ± 0.22
+BSO†	16.0 ± 1.4		

* Means ± SD, N = 3 or more independent experiments. †DL-buthionine-*S*,*R*-sulfoximine (1 mM) was added 10 min prior to the addition of MEA or NAC.

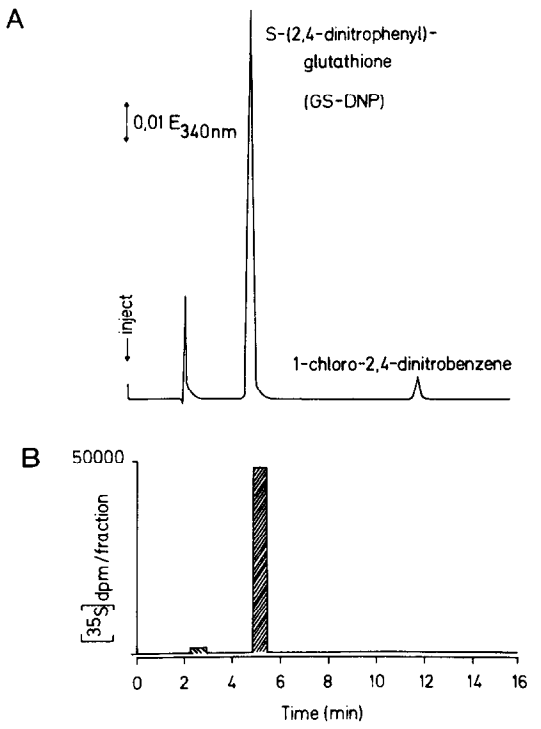

Fig. 6. Recovery of [^{35}S] radioactivity incorporated into *S*-(2,4-dinitrophenyl)-glutathione (GS–DNP).

A. Typical elution profile of GS–DNP at 340 nm after HPLC chromatography on μ-Bondapak C_{18} column; 0.1 M H_3PO_4/methanol 60/40 (v/v); flow rate 1.5 ml/min. After MEA exposure of CHO cells for 60 min at 37° in McCoy's 5A medium containing 0.1 mM [^{35}S] cystine, total GSH of the acid-soluble fraction was transferred to 1-chloro-2,4-dinitrobenzene by glutathione-*S*-transferase forming the GS–DNP conjugate. The identity of the radioactive peak with GS–DNP was checked by sample spiking with authentic GS–DNP [14].

B. Measurement of [^{35}S] radioactivity in fractions of the eluate after HPLC. More than 90% of the total [^{35}S] radioactivity applied to the column could be recovered in the peak of GS–DNP.

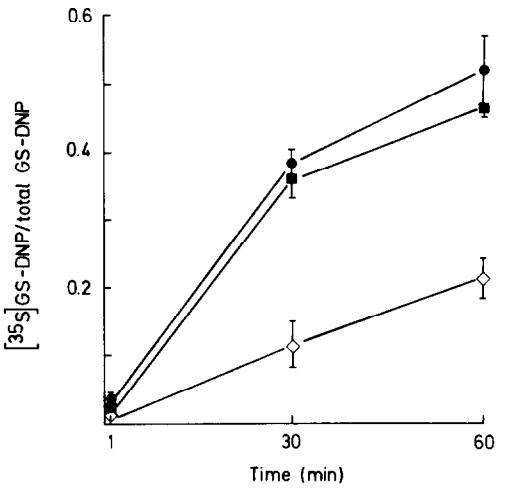

Fig. 7. Increase of the ratio of [^{35}S] GS–DNP/total GS–DNP during the exposure (0–60 min) of CHO cells with MEA or NAC at 37° in McCoy's 5A medium containing 0.1 mM [^{35}S] cystine. Total GSH of the acid-soluble fraction of cells was derivatized to GS–DNP and quantified by HPLC. The ratio represents the amount of radioactive [^{35}S] GS–DNP measured by scintillation counting relative to the amount of total GS–DNP: —●—, 0.4 mM MEA; —■—, 0.8 mM NAC; —◇—, no additional thiol (control). Means ± SD of four independent experiments.

exposure of cells with MEA or NAC. For this analysis, total glutathione was measured by HPLC after its reduction and derivatization to the GS–DNP conjugate. Figure 6A illustrates a typical elution profile with the peak of the GS–DNP conjugate at a retention time of ≈5.2 min. More than 90% of the total [^{35}S] radioactivity could be recovered within the GS–DNP conjugate of the acid-soluble fraction of cells treated with 0.4 mM MEA (Fig. 6B). Similar results were obtained after exposure of cells with 0.8 mM NAC (data not shown). The time dependent increase of [^{35}S] labelled GS–DNP conjugate after MEA (0.4 mM) or NAC (0.8 mM) exposure of cells at 37° is shown in Fig. 7. The ratio of [^{35}S] GS–DNP/total GS–DNP rapidly increased within 30 min after MEA or NAC treatment compared to the ratio of untreated control cells. For example, 35% of GS–DNP was found to be [^{35}S] labelled after 30 min MEA or NAC exposure, but only 10% of GS–DNP in untreated cells. The relative increase of this ratio did not differ significantly from each other by further incubation (30–60 min) with MEA or NAC compared to control cells, respectively.

DISCUSSION

Several studies have shown that exposure of cells with aminothiols or cyst(e)ine derivatives leads to an increase of cellular GSH content [7–9]. However, the mechanisms of GSH elevation by supplying these compounds to the growth medium can differ depending upon the potential of cells to utilize them as precursors for GSH biosynthesis. For example, L-2-oxothiazolidine-4-carboxylate (OTZ) acts as a potent intracellular delivery system for cysteine to which OTZ itself can be metabolized in mammalian cells [15]. Similarly, methionine can be utilized for intracellular supply of cysteine via the cystathionine pathway in rat hepatocytes but not in rat spleen lymphocytes or murine L5178Y and L1210 lymphoma cells (for review see [16]). According to this concept, promotion of GSH synthesis by *N*-acetylcysteine in hepatocytes was explained by the fact that NAC could be intracellularly deacetylated to yield cysteine [7, 17]. The cysteine formed in this manner could be then rapidly utilized for GSH synthesis.

In contrast, there is no evidence in the literature that MEA, which represents the decarboxylated form of cysteine, could act as a precusor for GSH synthesis. The previous explanation for the GSH elevation in MEA-treated cells by the release of protein-bound GSH [3] became insufficient because the increase of GSH was found to be blocked by BSO [8, 9], an inhibitor of γ-glutamyl synthetase

which is the first enzymatic step of GSH-biosynthesis [10].

The present study strongly suggests that SH-compounds such as MEA or NAC are not substrates for GSH synthesis, but that both act as delivery systems for cystine sulfur from the medium outside the cells into the cells. According to our previous report showing that the increase of cellular GSH by MEA and NAC in CHO cells was dependent on the presence of cystine in the medium [9], the results of this paper show that the cystine content of the growth medium apparently serves as an extracellular pool for the intracellular supply of cysteine for GSH synthesis promoted by MEA and NAC.

The data as plotted in Fig. 2 fit well in a Lineweaver–Burk diagram suggesting a saturation type of kinetics for the uptake in the presence of MEA or NAC. By comparison of the half-saturation constants (K_u-values) as calculated from the initial [^{35}S] cystine uptake from the medium, the potential to promote the uptake is greater for MEA than for NAC. The mechanism might be similar to the proposed growth-promoting action of 2-mercaptoethanol on mouse lymphoma L1210 cells, which are deficient in the capacity to take up cystine from the medium [18]. In contrast to L1210 cells, however, CHO cells take up cystine quite efficiently with a rate of 0.45 ± 0.15 (SE) nmol half cystine/min/mg protein which is comparable to human fibroblasts [19]. Also similar to human fibroblasts, we found that the control uptake of cystine in CHO cells is only inhibited (approx. 70%) by L-glutamate and DL-homocysteate (see Table 1). Therefore, the uptake of cystine is apparently mediated by the same transport system previously described as highly specific for cystine and glutamate [19] and designated as system x_c^- by Makowske and Christensen in fibroblasts [20]. It is interesting to note that the cystine uptake is also blocked by BSO in the absence of MEA or NAC (see control uptake in Fig. 5A and 5B). These results further support the idea of a likeness between substrates of system x_c^- and of γ-glutamylcysteine synthetase [21]. According to this concept, cystine, after entering cells in exchange for glutamate, is reduced to cysteine, which then reacts with glutamate to form γ-glutamylcysteine. Our results after blocking the latter enzymatic step with BSO might be explained with an inhibitory feedback effect on the x_c^- transport system for cystine. In CHO cells control uptake of cystine was not significantly affected by increasing temperature (37–44°) (Fig. 3) or by changing extracellular pH over the range of 6.0–8.0 (Fig. 4). The lack of increased cystine uptake at higher pH values as observed in isolated rat hepatocytes [22] can be explained by the difference in cell density used which largely affects cystine uptake. The promoted uptake of cystine sulfur in the presence of MEA or NAC significantly differs from the control uptake in the absence of these thiols. Neither L-glutamate nor DL-homocysteate could inhibit the uptake, but L-Ala, L-Ser, and L-Leu significantly reduced (approx. 50%) the uptake (see Table 1). Therefore, part of the sulfur uptake promoted by MEA or NAC occurs most likely in the form of cysteine which is mainly transported by the ASC system in a variety of cells. This system is especially reactive with neutral amino acids and serine [21]. The reported effect of different amino acids on the uptake of cystein in human fibroblast [19] is very similar to our results observed on the uptake promoted by MEA or NAC. The reaction of MEA or NAC with cystine in the medium produces an equimolar amount of a mixed disulfide and cysteine as shown for 2-mercaptoethanol [18]. Beside the uptake in the form of free cysteine via the ASC system, also the uptake of mixed disulfide (e.g. CYS-S-S-MEA or CYS-S-S-NAC) is most likely. For example, we found that the initial rate of [^{35}S] labelled cysteamine uptake in the presence of cystine was in the same order as the uptake of cystine at equimolar concentrations (data not shown).

From the analysis of the data, the observed increase of cystine uptake by the addition of MEA or NAC must include at least two different steps: firstly, the formation of the cysteine mixed disulfide with MEA or NAC and the simultaneous generation of equimolar amounts of cysteine in the medium; secondly, the uptake of cysteine and/or the mixed disulfide via different transport systems. The strong dependency of the promoted uptake (see Fig. 4) on extracellular pH should not be assigned to the nature of the transport system itself. The change of the ionic state of the reactants (e.g. MEA or NAC) at different pH values might influence the formation of the mixed disulfide (CYS-S-S-MEA or CYS-S-S-NAC) under the experimental conditions. The uptake of the mixed disulfide via the x_c^- transport system—as described above for the uptake of cystine—can be ruled out since L-glutamate or DL-homocysteate had no effect. The mixed disulfide is taken up presumably by the cells via the L-system as described previously by Ishii *et al.* [18]. They also found an inhibitory effect on this system by branched amino acids with apolar side chain, e.g. L-leucine.

The effect of higher temperatures (42–44°) on the cystine uptake is in accordance with our previous report on the effect of heat and MEA on cellular GSH content [9]. By exposure with MEA at 44°, GSH increased more rapidly to high levels as compared to exposure at 37°. Heat alone had no marked effect [9]. Similar to these results, the uptake promoted by MEA and NAC is increased by increasing temperature, while the cystine uptake of untreated cells is not significantly affected (see Fig. 3). This enhancement persists up to at least 30 min (data not shown). We conclude that despite the previously observed toxicity of MEA at higher temperatures [23], the transport mechanisms for the uptake of cystine are not markedly impaired and the cells are still able to maintain membrane integrity to an extent which allows intracellular accumulation of GSH promoted by MEA.

With regard to the further utilization of cystine sulfur taken up from CHO cells in the presence of MEA or NAC, our results clearly demonstrate that the cysteine moiety of the newly synthesized GSH derives from the extracellular pool of cystine. In addition to our previous observation that cystine uptake in CHO cells paralleled GSH elevation [24], it now becomes evident that MEA and NAC act as promoters for the cystine supply rather than being precursors for GSH synthesis. Promotion of cystine

uptake might be regulated by the amount of intracellular cysteine as shown by our results using BSO. In this experiment, inhibition of γ-glutamylcysteine synthetase by BSO completely blocked the utilization of cystine sulfur from the medium, independent of the actual GSH content of cells. By this regulatory mechanism cystine uptake promoted by thiols might be closely linked to GSH synthesis in order to avoid intracellular accumulation of cystine sulfur which has been shown to be toxic for cells [25]. The specificity of BSO as an inhibitor of γ-glutamylcysteine synthetase remains unclear in the literature. For example, there are two reports that describe an inhibition of transport phenomena by BSO treatment [26, 27]. Therefore, we cannot rule out other mechanisms for the effect of BSO upon the uptake of cystine promoted by MEA or NAC, but we feel that the reported transport inhibitions by BSO could be also related to the inhibition of GHS biosynthesis.

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