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STUDIES ON THE LOW MOLECULAR WEIGHT THIOLS OF MOUSE EHRLICH ASCITES TUMOUR CELLS: GLUTATHIONE CONTENT

Michael Gronow[1]

Cambridge Cancer Research Fund, The Irwin Centre, Scotland Rd. Dry Drayton, Cambridge CB23 8AR, UK

Abstract: The total thiol contents of mouse Ehrlich ascites tumour cells and that of the acid soluble fraction (ASF), released on deproteinization of these cells, were measured using the Ellman reagent, 5,5' dithio (2-nitrobenzoic acid)(ESSE). It was shown that the ASF constituted 13.2% of the total cellular thiol at an average value of 3.20 ± 0.29 femtomoles of RSH per cell and this was confirmed using three different methods of thiol estimation. This extract was shown to contain practically negligible amounts of reducing inorganic sulphur ions, disulphide or vicinal disulphide material. The aromatic mixed disulphides (RSSE) formed by direct addition of the ASF thiols to a three fold excess of Ellman reagent (ESSE) were analysed by ion exchange chromatography and capillary electrophoresis. These analyses revealed only two major components, the glutathione adduct (GSSE) and excess ESSE. Some minor RSSE components were also present and this was confirmed by ^{35}S labelling of these cells. The GSH content, as calculated from the GSSE formed, was 1.28±0.04 femtomoles/cell which accounts for only 42±3% of the thiol content of the ASF and 5.3% of the total cellular thiol. Furthermore, the G^{35}S SE isolated from the radioisotope labelled cells contained only13.6% of the ^{35}S incorporated into the ASF. Apart from glutathione, a large proportion, ca 60%, of the thiols present in the ASF of these cells, although reacting with the Ellman reagent do not appear to retain the aromatic thionitrobenzoic acid residue, indicating that they do not form stable aromatic mixed disulphides. These results indicate that caution should be employed in interpreting Ellman values of cellular ASF thiols as attributable solely to the presence of glutathione.

Keywords: Mouse ascites tumour, non-protein thiols, radio-isotope labelling, Ellman reagent, Ellman thiol adducts, glutathione content, chromatography, capillary electrophoresis

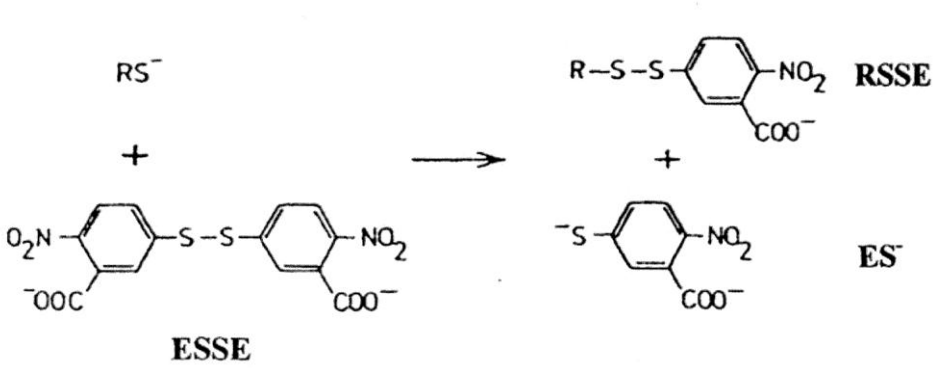
Introduction

Thiol compounds occupy a pivotal role in cellular metabolism. Many papers have been published over the past millennium stressing their important role in cell metabolism, particularly with regard to their essential function in the maintenance of cellular redox balance (e.g. see Packer, 1995) and their role in controlling oxidative stress (Sies, 1986]. They also have vital roles in protection against radiation damage (e.g. Eldjarn & Pihl,1960 and Bridges, 1969) and the control of cell division and gene activity (Blackburn et al.,1999).

Of the known cellular lower molecular weight thiols, apart from metallothioneins that are only present in quantity when specifically induced (see Krezoski et al.,1991) the cysteine containing tripeptide glutathione (GSH) stands alone as the most ubiquitous and is widely accepted as the major non protein thiol present in eukaryote cells and tissues; thought to comprise >90% of the acid soluble fraction (Larsson et al.,1993 and Meister and Anderson,1983). Apart from playing an important role in maintaining the cellular redox balance, perhaps its most important role in cellular metabolism is in detoxification by removing toxic, xenobiotic and carcinogenic reagents via the glutathione transferases (Ketterer,1988 and Hayes and Pulford 1995).

Michael Gronow (Correspondence)
cam.cancer@btconnect.com **or** michael@gronow-cambridge.co.uk

The large number of analytical methods that have evolved to measure the GSH content of cells and tissues mirrors its importance in these respects; however none of these analytical methods is entirely satisfactory. One of the most popular reagents employed for GSH and general thiol estimation has been that developed by Ellman (1959), utilizing 5'5 dithiobis (-2-nitrobenzoic acid)(DTNB or ESSE). This reagent, in conjunction with added enzyme glutathione reductase has formed the basis of the widely used Teitze assay (Teitze,1969) for the estimation of GSH. ESSE rapidly reacts with thiols in a quantitative fashion at physiological range between pH 7 to 8 as shown below:-



Using thiol standards it has been shown that, provided the thiol is added to a 2-3 fold excess of ESSE, there is a quantitative formation the aromatic mixed disulphide (RSSE) and yellow anion (often written as ES^-) (Russell et al. 1997). Generation of the latter can be followed spectrophotometrically; the λmax is 412nm; the extinction coefficient reported for this anion ranges from 13,600 to 14,145 $M^{-1}cm^{-1}$. Various groups have reported the preparation of various RSSE derivatives (Faulstick and Heintz 1995); while others have developed analytical systems to quantify these adducts using isoelectrofocussing and ion exchange (Gronow and Lewis,1975), HPLC (Beales et al.,1981, Kuwata, 1982 and Russell et al.,1997) and capillary electrophoresis(CE) (Russell and Rabenstein,1966).

Of the many investigators that have used the Ellman assay to determine the thiol content of deproteinized or acid soluble extracts of cells or tissues many now assume that nearly all the thiol present is glutathione (e.g. see Russo and Bump,1998). However, over the years there have been a number of reports indicating the presence of other low molecular weight components in cells, particularly in tumours (e.g.Calcutt and Doxey, 1962) but, apart from some very minor components the presence of significant quantities of other low molecular weight thiols has not been confirmed, perhaps because of their extreme reactivity. To investigate the veracity of these findings a method has been developed to investigate cellular thiol composition utilizing the selective extraction of ESSE and RSSE in bulk from the acid soluble fraction of cells and tissues after reaction with the Ellman reagent. The quantitative recovery of RSSE achieved facilitates further analytical study using ion exchange and capillary electrophoresis. From an analytical point of view these compounds have a convenient UV absorption peak at 308-358nm, the molar extinction coefficient depending on the nature of R. In the case of ESSE it is 17,780 $M^{-1}cm^{-1}$ at 324nm (Riddles et al.,1979), whereas the non-aromatic or aliphatic residue (RSSE) gives ca 8,900 $M^{-1}cm^{-1}$ at 330nm (Kuwata et al.,1982).

Materials and Methods

2.1 Cells and Cell Culture

In earlier experiments mouse Erhlich-Lettre ascites tumour (EAT) was propagated in CD-1 mice in up to 9-day transplants. The original cell line, strain E, was a kind gift from the Patterson Institute of Cancer Research (PICR).

The cells were removed from the mice and freed from blood contamination by harvesting into ice-cold phosphate buffered saline followed by gentle centrifugation. Haemolysed harvests were

discarded. Suitable, essentially erythrocyte free preparations were sedimented by centrifugation and the cell pellet stored at -70°C until use.
Cell counts were carried out, after suitable dilutions in Trypan blue solutions, in a Neubauer chamber.

In later experiments, ostensibly to carry out ^{35}S labelling, the EAT were propagated in cell culture, either in monolayers or suspension using RPMI 1640 medium containing 18mM MOPS (4 morpholinopropane sulphonic acid) buffer pH 7.6 supplemented with 10% FBS, 100µg/ml streptomycin,100 IU/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37°

2.2 Reagents, Chemicals and Chromatographic Materials

Unless otherwise stated all chemicals or biochemicals were supplied be Sigma-Aldrich, Poole, Dorset or Merck UK. Only "Analar" or higher grades of purity were used. 4,4'-bis-dimethylaminodiphenylcarbinol (BDC-OH) was supplied by the Nutritional Biochemicals Corp.,Cleveland, Ohio
Whatman anion exchange cellulose DE52 was supplied by The Lab Sales Co.,Over, Cambs. and XAD-2 from Supelco.
The ^{35}S labelling radio-isotope mixture, Redivue™/Promix™ was obtained from Amersham Pharmacia Biotech, UK Ltd..
A "glutathione assay" kit was obtained from Calbiochem Novabiochem Ltd.Nottingham UK

2.3 Measurement of total cellular thiol content and extraction of acid soluble thiols

A suspension was made up of EAT in water at 4°C containing 10^7 to 10^8 cells per ml using a 3 thou gap all-glass homogenizer. Aliquots of 50 and 100 µl were taken for estimation of total cellular thiol content. To measure the "apparent" thiol content 100mM phosphate buffer pH 7.6 was used containing excess ESSE (100µg/ml) and for "total" thiol content the cells were dissolved in 8M urea, 2M sodium chloride, 0.01MEDTA, 0.1 M sodium phosphate pH7.6 also containing an excess of Ellman's reagent (this fully opens up the secondary and tertiary structure of the cellular proteins). The OD of the yellow anion (ES^-) was recorded at 412 nm against suitable blanks made up in these solutions (without ESSE) and the thiol content calculated using a molar absorption coefficient of 14,100 $M^{-1}cm^{-1}$ (Various values have been quoted in the literature since the original value of 13,600 $M^{-1}cm^{-1}$ given by Ellman but in this laboratory with the buffers employed a standard value of 14,100 $M^{-1}cm^{-1}$ has been routinely obtained)
For the preparation of the acid soluble fraction an equal volume of 16% w/v trichloracetic acid (TCA) at 4°C was added and the mixture homogenized in a large gap Teflon glass homogenizer followed by short bursts in an "Ultraturrex" type stainless steel homogeniser. The homogenate was left at 4°C about 30 mins before centrifuging at 3000g to yield a clear supernatant. The pellet was re-extracted with half the original volume of 8% w/v TCA and the supernatants combined and filtered. This removed 98% of the "soluble" cellular thiols; a further extraction with 8% TCA removed the remaining 2% of non-protein thiol material.

2.4 Estimation of the thiol content of the ASF

Four different methods were employed each utilizing different chemistries.
1) Small aliquots were added to 0.6M sodium phosphate pH 7.6 containing an excess of Ellman's reagent (ESSE) and the concentration of thiol calculated from the yellow anion generated as measured by the absorbency at 412nm.
2) The thiol content was measured directly on the 8% TCA extract using the Saville method which is based on the formation of S-nitroso derivatives (R-S-NO) from thiols (Saville,1958, Gronow,1965).
3) A third method (Rohrbach et al.,1973) was also used utilizing another different chemistry at pH 5: the end point being the formation of a coloured thio-ether compound by the change in absorbancy at 610nm.
4) A fourth method was carried out on a metaphosphoric acid cell extract. This method also depends on the formation of a thio-ether derivative that is converted into a chromophoric thione (λmax 400nm). Supplied by Calbiochem as a specific glutathione assay kit.

The TCA extract was stored at 4°C and processed as soon as possible (approx 30% of the thiol is lost per month of storage at this temperature).

Vicinal dithiols were determined using the basic method of Zahler and Cleland (1968) by adding arsenite prior to the Ellman reagent.

The disulphide content was determined by modifications of the borohydride method (in borate buffer pH 9.5) followed by estimation of the thiol produced by the Ellman reagent (Modig, 1968)

2.5 Preparation and extraction of mixed disulphides for analysis

The thiol content of the ASF was found to decrease rapidly if the pH of the solution was raised above the 3-4 level attained after removal of the TCA by solvent extraction. To prevent this from occurring the following procedure was devised to minimize the loss of thiol by utilizing their very rapid reaction with Ellman's reagent to form mixed disulphide derivatives RSSE) suitable for further analysis. All procedures were carried out at room temperature: -

The bulk of the TCA was removed by two extractions of equal volume of ethyl acetate and the thiol content of the aqueous layer checked by the methods previously described. From this value the amount of ESSE required to react with the EAT thiols based on a stoichiometric ratio of approximately 3:1 was calculated and this amount of ESSE was dissolved on equal volume 0.6M sodium phosphate pH 7.8 containing 50mM EDTA. The ASF was added to this dropwise with rapid stirring to give the mixed disulphide (RSSE) and yellow anion ES^-. The A_{412} and A_{325} (absorption maximum of the yellow anion and aromatic disulphide bond respectively) were checked on completion.

The excess ethyl acetate was removed by rotary evaporation (or in stream of nitrogen) and solid NaCl added until saturated. The A_{325} of the solution was checked again and a specially prepared and optically clean hydrophobic resin, a non-ionic polymeric adsorbent, XAD-2, was added (1g per 15 A_{325} units). The mixture was stirred until very little A_{325} (ca 5%) was left in the supernatant, together with the bulk of the yellow anion. When greater than 95% absorption of the RSSE had occurred, as measured by the release of excess yellow anion from an aliquot of the supernatent after the addition of the thiol sodium 2-mercaptoethane sulphonate, the resin was filtered off on a sintered glass filter. Usually after 1 to 3 hours absorption was complete. The XAD-2 was then washed with 2M NaCl until the eluate contained very little UV absorbing material (in the range 200-400 nanometres) and finally with water until the conductivity had dropped significantly and A_{325} started appearing in the eluate.

The adsorbed A_{325} was removed with 50% methanol (20 volumes) overnight to give >90% recovery of the A_{325}. The mixture was reduced to low volume in a rotary evaporator prior to prior to fractionation of the mixed disulphides on the cellulose anion exchanger Whatman DE52.

2.6 Chromatography on Anion exchanger - Whatman DE52.

Generally a 30 x 2.0 cms column was found to be satisfactory but care was taken to adequately buffer this material as the Ellman mixed disulphides are unstable above pH 8.5 resulting in the release of the yellow anion (ES^-)
Pharmacia HiLoad™ system chromatography equipment was used for the fractionation with a GP-10 gradient programmer and a Uvicord S11 filter with a 313nm interference filter for monitoring the elution of the disulphides.
After application of the sample the column was washed with one bed volume of the starting buffer 20mM Tris-HCl pH 7.2 containing 5% v/v methanol. The aromatic mixed disulphides were eluted in a gradient system employing 20mM tris-HCl pH 7.2 to 0.35M guanidinium chloride 20mM Tris-HCl pH 7.2 containing 5% v/v methanol. 10ml fractions were collected. Usually 95% + recovery of the A_{325} was obtained.
The A_{325} of the fractions was determined using a sipette device on a Cecil 3000 series spectrophotometer and the conductivity measured using a conductivity probe and meter (Hanna HI8820).

Those mixed disulphides of the pooled fractions could be recovered and concentrated for capillary electrophoresis by adsorbing them on XAD2 resin as before.

2.7 Capillary electrophoresis analysis

A Crystal CE 310 electrophoresis equipment fitted with a Unicam 4225 UV detector (ATI Unicam, Cambridge) and a 65cms length capillary, ID 75µ was used for these investigations. The temperature of the sample carousel and capillary loop were set and controlled at 25°C. The most suitable running buffer was found to be 30mM sodium phosphate

pH 7.6 containing 5% isopropanol.

The capillary pre-wash regimen found to give the best results consisted of : 3min water, 3min ethanol:acetone (1:1) and 2min buffer - all at 2000mB pressure; a 20kV prerun in buffer for 2min. The sample was applied in 0.2min at 20mB pressure. After sample electrophoresis at 20kV for up to 40min the capillary was washed with 0.5M sodium hydroxide for 1min followed by water for 1min (all at 2000mB).

Each sample run of up to eight samples contained at least two runs of a mixed standard containing ESSE and GSSE (ca. 5-10 A_{325} units/ml).

2.8 ^{35}S labelling of cellular thiols

Cells for short-term ^{35}S labelling studies were propagated either in monolayer or cell suspension and harvested when cultures were about 80% confluent. A known number of cells, usually in the region of 10^8, were resuspended in 50 ml of RPMI medium deficient in methionine and cysteine containing 2mM L-glutamine, 2%w/v D-glucose and 5% FBS. The mixture, in a tightly capped tube, was then incubated at 37^{o}C for 15mins to deplete the sulphur pool. The cells were then gently centrifuged down and resuspended in 45 ml of the same medium containing 2.5mCi (92.5 MBq) of Redivue^{TM} (Promix^{TM}) ^{35}S in vitro cell labelling mixture (Amersham Pharmacia Biotech UK Ltd., (now GE Healthcare UK Ltd.) specific activity 800-1200 Ci/mmole) which contains [^{35}S] methionine and [^{35}S] cysteine in a ratio of approximately 70:30. The tightly capped tube was incubated in a water bath at 37^{o}C for 3 hours, resuspending the settled cells occasionally by gentle inversion. The tube was opened in a designated fume hood equipped with an activated charcoal filter; the cells were centrifuged down and drained as completely as possible from the radioactive medium. After a wash with 50 ml of the cold deficient medium the cells were resuspended in a 2ml of water and aliquots taken for ^{35}S and total thiol determination before preparing the ASF etc as described above.

Results and Discussion

3.1 Thiol determinations and values

The thiol values obtained for this strain of EAT are given in the table below.

Table 1

Thiol content of Ehrlich ascites tumour measured by Ellman reagent

"Apparent" femtomoles/cell	Total femtomoles/cell	ASF(8%TCA) femtomoles/cell	nanomoles–SH/mg protein*
12.2 ± 2.5 (8)	24.0 ± 3.0 (10)	3.20 ± 0.29 (10)	117 ± 8.0 (8)

* estimated by either Buiret or BCA (Sigma) methods

It is difficult to compare these values with other studies since many investigators do not always report the total thiol cell values obtained in strong denaturing solutions. They do show that a significant portion of the cellular thiol is buried in the protein matrix and this can be released by various denaturing agents.

The cellular total thiol values (femtomoles/cell) given in table 1 broadly agree with thiol values reported for EAT in the literature over the years as illustrated below; although there is some variability in the values obtained, possibly due to the incomplete denaturation of the cellular proteins attained or cell strain differences.

Table 2

	Thiol values in femtomoles per cell	
Author	Whole cells (not denatured)	Acid soluble fraction
Revesz & Bergstrand (1963)		1.91, 3.46, 2.56 (different clones of EAT)
Scaife (1964)	18.3 ± 1.2	2.7 ± 0.3
Gronow (1965)	10.6 – 20.4 (incubated ESSE)	3.37 – 4.04
Modig (1968)	16.71 ± 3.9	2.46
Harris (1968)	3.69-10.8	1.48

In the current investigations the acid soluble fraction of the EAT cells used constitutes 13.12 ± 0.94% of the total cellular thiol. Very little difference was found in the values when different acid extractants were employed, in contrast to older reports of ASF thiol analysis; but this is possibly due to the higher purity of these chemicals available nowadays. TCA was preferred in this study as it is easily removed with organic solvents. It was also found that there was very little difference in the overall thiol content of the ASF when determined by three totally different chemistries; very similar values were obtained, as shown in table 2.

Properties of the ASF thiols of EAT

1) Thiol content by different methods/chemistries of 8%TCA extract -femtomoles/cell

a) Ellman (ES^-)	3.20 ± 0.29 (10)
b) Saville (RS-NO)	3.34 ± 0.20 (7)
c) Rohrbach et al. (RS-C-BDC)	3.15 ± 0.31(4)
d) Calbiochem assay (RS-CR/)	3.6 (av.3 readings)

(as GSH content on metaphosphoric acid extract)

2) Vicinal dithiol content by reaction with $NaAsO_2$ 0.50± 0.25 %(4) of total thiol

3) Increase in thiol after reduction with $NaBH_4$ (disulphide) 1% ± 0.7% (4)

4) Loss of –SH :-

i) on removing TCA with ethyl acetate to pH 4-5 (after 40mins)	2 -10%
ii) raising pH to 7-8 (after 40mins)	40 -50%
iii) raising pH to 9-9.5 (after 40mins)	90%+
iv) reduction with $NaBH_4$ after iii), percentage of original thiol content restored	40%
v) storage in 8% TCA for 60 days at 4^0C	65%

Values given as means (± SEM) of the number of determinations given in parentheses.

Furthermore the ASF contained insignificant amounts of vicinal dithiols, as there was no difference in the A_{412} generated by the Ellman reagent when arsenite was added prior to this reagent. Arsenite complexes with vicinal dithiols temporarily preventing reaction with the Ellman reagent (Zahler and Cleland,1968). This result is important because on adding vicinal dithiols to ESSE the first mixed disulphide formed, being in a local concentration of 6-10M, could react with the second, favouring the formation of an internal disulphide and liberating a second molecule of ES^- (Gilbert,1990).

ESSE will, in addition to thiols, react with certain inorganic molecules such as sulphide, sulphite, bisulphite and thiosulphate to release yellow anion but these molecules will not form mixed disulphides. However, none of these inorganic ions produces the coloured end product in the Saville method, which gives virtually the same thiol value as the Ellman reagent, showing that these ions are not present in significant quantities in the ASF of these cells.

The third method employed, little used but highly convenient, was the Rohrbach method which depends on the formation of a thioether derivative. The reaction is almost instantaneous and can be followed at 600nm giving virtually the same thiol content with either whole cells or ASF as the other methods.

The 2 step Calbiochem assay, carried out on a metaphosphoric acid extract, works on a similar chemical basis for the first step followed by a second which it is claimed is specific for GSH. This gives a slightly higher thiol value than the other three methods.

Conditions were optimised for the estimation of the disulphide content of the ASF after reduction with sodium borohydride ($NaBH_4$ – a potent reducing agent) revealing that, in agreement with other investigators (see Russo and Bump, 1998)

very little disulphide is present. It is interesting, however, that after loss of virtually all thiol by raising the pH to 9 -10 in the presence of air, only 40% of the original thiol could be restored by $NaBH_4$ reduction, indicating that the bulk of the thiol is not converted to disulphide, the usual end-product of mild thiol oxidation. This finding clearly indicates that raising the pH of the ASF prior to thiol estimation, a step often carried by investigators in this field, clearly leads to erroneous, low thiol values.

3.2 Chromatography and Capillary Electrophoresis Analysis of Aromatic Mixed Disulphides.

The results of analysis of the RSSE products of the Ellman reaction by anion exchange chromatography on DE52 cellulose are illustrated in Figure 1.

Peaks were identified by their conductivity in milli-siemens (mS) using a conductivity meter

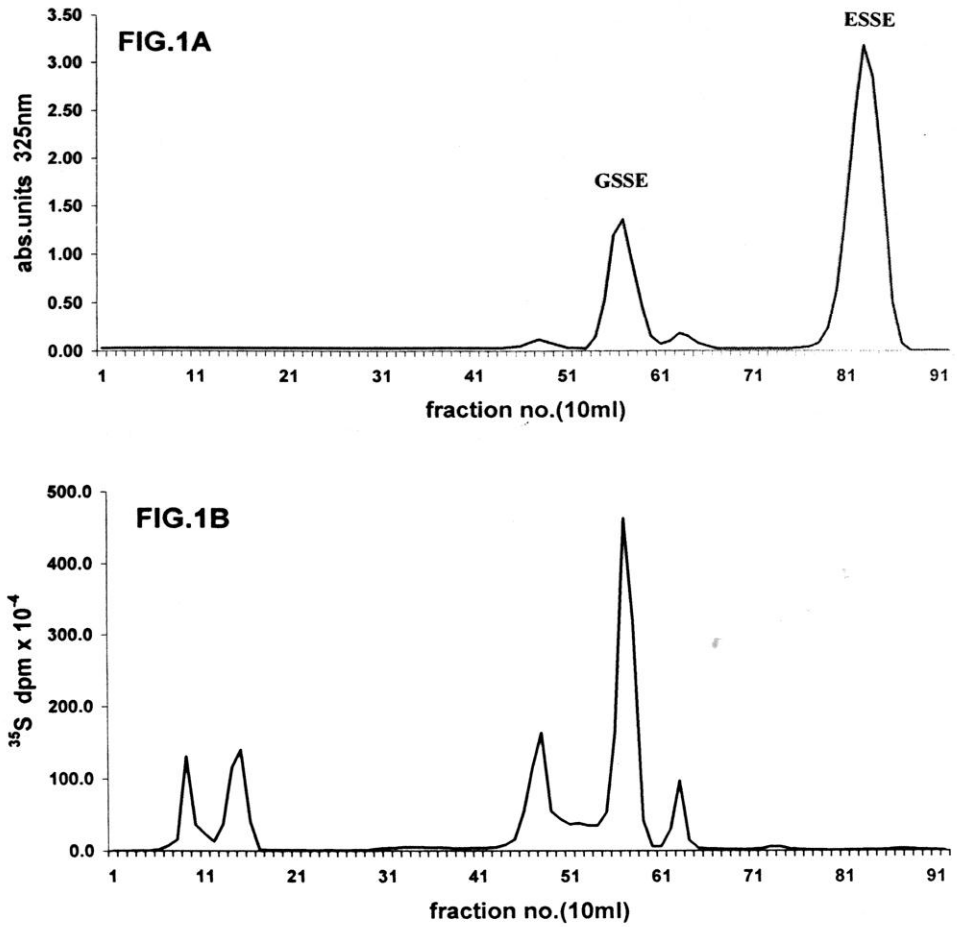


Figure 1: Ion-exchange chromatography of acid soluble thiols labelled with Ellman's reagent isolated on XAD-2 Column: 30 x 2cms DE52 cellulose

System: Pharmacia HiLoad™ system chromatography equipment: fraction collector with a GP-10 gradient programmer and a Unicord S11 filter with a 313nm interference filter

Sample A 100 to 300 A_{325} units of RSSE (bulk preparation)

Sample B 2.5×10^7 dpm $R^{35}SSE$ labelled material

Elution: 120mls wash 20mM Tris-HCl pH 7.2 containing 5% v/v methanol; then 800ml linear gradient to 0.35M guanidinium chloride 20mM Tris-HCl pH 7.2 containing 5% v/v methanol

Flow rate: 1ml/min

Recoveries of A_{325} and ^{35}S were generally greater than 95%. Figure 1A reveals that nearly all the A_{325} is present in two peaks; the first is GSSE which elutes at 15.1±0.5 mS conductivity while the largest is the excess ESSE eluting at 25.2±0.6mS; identification of which was confirmed by CE analyses as shown in Figure 2.

Figure 1B shows the DE52 pattern obtained from the $R^{35}SSE$ mixture isolated from the ASF by the XAD-2 resin clearly confirming that $G^{35}SSE$ is one of the major components of the $R^{35}SSE$ mixture representing 40 ± 2.5% of the ^{35}S in the form of $R^{35}SSE$ (table3) The presence of the two minor peaks on either side of the GSSE peak was confirmed by the ^{35}S labelling studies. Two additional $R^{35}SSE$ peaks, not detected in the A_{325} pattern shown in figure 1A, emerged in the column wash, ca 2mS . These components, the presence of which has been demonstrated in a previous study using ^{35}S labelled ESSE (used to prepare $RS^{35}SE$ see 15) are obviously trace thiols of high specific activity. These components could represent important metabolic intermediates but were present in too small a quantity to permit analysis in the present study, as were the two A_{325} components on either side of the $G^{35}SSE$ peak even though they were detected by their A_{325}. Clearly no ^{35}S labelled components were found to be present in the fractions where ESSE is eluted (25.2mS)

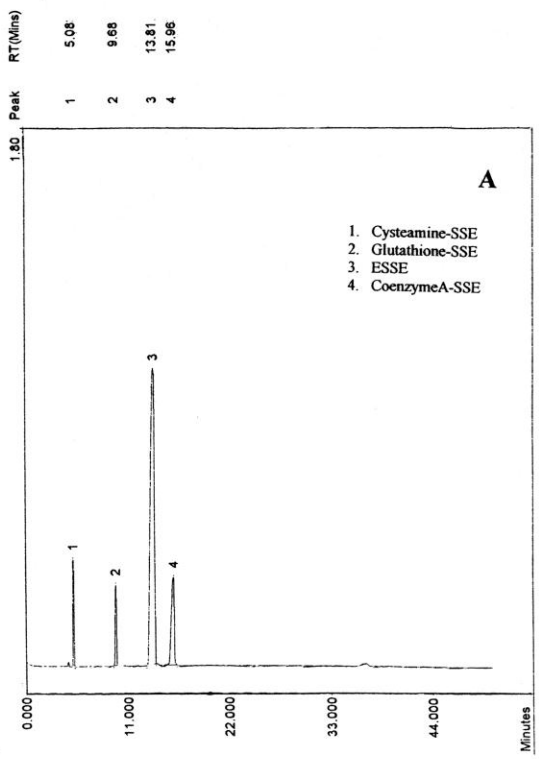


Figure 2

Capillary electrophoresis of aromatic mixed disulphides

Instrument: Crystal CE310 (ATI Unicam)

capillary; length 65cms, ID 75µ

UV detector; Unicam 4225 set at 325nm

Buffer; 30mM sodium phosphate pH 7.6 containing 5% isopropanol

Applied voltage 20kV

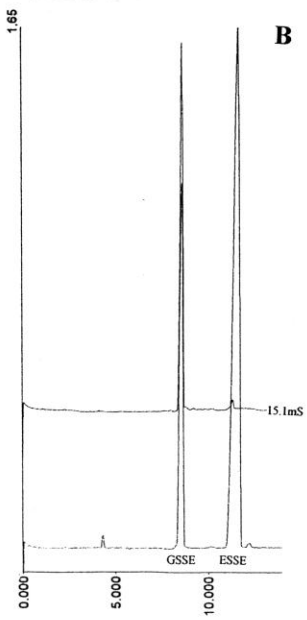
Temperature 25°C

Injection of sample; 0.2 min at 20mB pressure

A – Four RSSE standards;

B -DE52 15.1mS peak(top), GSSE & ESSE standards (bottom)

C –DE52 25.2mS peak



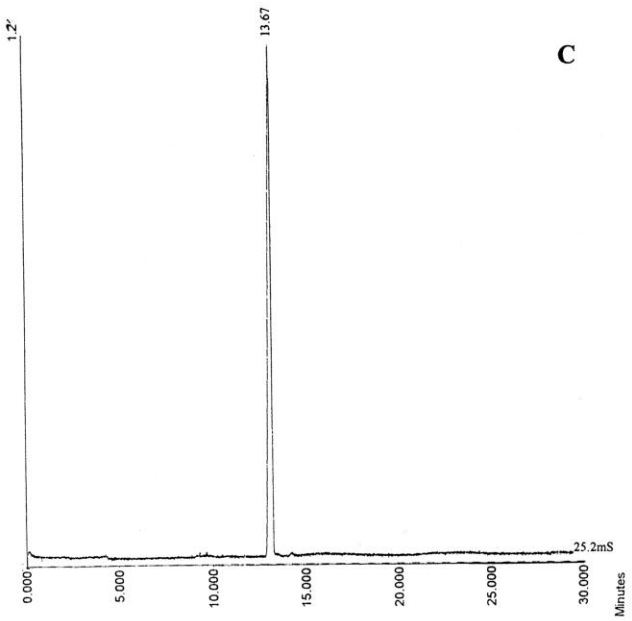


Figure 2A shows the pattern obtained in this CE system with various RSSE standards. The **relative mobility** of these standards, as measured by the time of emergence divided by the time of emergence of ESSE were as follows: (all results were the mean of 6 to 8 runs) CysteamineSSE 0.38±0.01(6), CysteineSSE 0.67±0.01, glutathioneSSE 0.74±0.02 (ESSE 1.00), coenzymeA-SSE 1.18±0.04.

Of the DE52 fractions, the 15.1mS peak (fig. 2B upper trace) contained mainly GSSE and one very minor component, probably ESSE, while the 25.2mS peak (fig. 2C) contained exclusively ESSE. The values obtained for the DE52 15.1 and 25.2mS peaks were 0.73±0.03 and 1.00±0.02 respectively confirming the identity of these components. (It was found that any attempt at storage or acidification of ESSE containing ES^- leads to the formation of multiple CE peaks due to the attack of the highly nucleophilic ES^- on the ESSE excess to give $ESSE(ES)_x$ thioether adducts – unpublished data)

From the DE52 separation the amount of GSSE recovered from the ASF was calculated either from the A_{325} in the 15.1mS peak **or** by the yellow anion released from this component after addition of excess sodium 2-mercaptoethane sulphonate. The result obtained is given in table 3; the value of 1.28 ± 0.04 femtomoles of GSH per cell obtained represents only 42 ± 3% of the total thiol material present in the ASF. If GSH was dissolved in 8% TCA at similar concentrations to that found in the ASF and the GSSE prepared as described above the 15.1mS peak contained greater than 85% of the theoretical GSSE yield.

Table 3

Glutathione content and ^{35}S labelling in EAT cells using the Ellman Reagent

GSH content of EAT cells by DE52/15.1mS analysis of RSSE	a) Whole cells	1.28±0.04 femtomoles/cell
	b) GSH content as percentage of ASF thiols	42±3%

^{35}S Promix (Redivue) cell labelling of EAT 3 hours at 37°	a) Percentage of ^{35}S label taken up by cells	30±2%
	b) Percentage of incorporated ^{35}S present in ASF	11.1±1%
	c) ^{35}S dpm x 10^{-8} / µmole –SH in whole cells	3.35±0.15
	d) ^{35}S dpm x 10^{-8} / µmole –SH in ASF	2.91±1.1

Analysis of ^{35}S labelled ASF	a) Percentage adsorbed by XAD-2 (=$R^{35}SSE$)	32.5±1.5%
	b) Percentage $R^{35}SSE$ in form of $G^{35}S$ SH (= DE52/15.1mS)	40.0±2.5%
	c) Percentage of total ASF – ^{35}S in form of $G^{35}S$ SE	13.6%
	d) Percentage of ASF-^{35}S **not** in form of $G^{35}S$ SE	86.4%

Results given as means (± SEM) of 5-6 determinations

Table 3 also illustrates that in the ^{35}S labelling experiments the bulk of the ^{35}S (>60%) was left in the supernatent after XAD-2 extraction and that this sulphur containing material is clearly not in the form of $R^{35}SSE$. Analysis of this mixture is difficult due to the high salt content generated and necessary for the hydrophobic adsorption of the RSSE onto the XAD-2. Preliminary analysis by gel filtration on Biogel P2 showed at least three to five ^{35}S labelled components are present, some of which may not have arisen from thiol components. Further investigations are required to determine the nature of these materials.

3.3 Discussion and conclusions

Many publications over the past decades have concluded that GSH is the principal low molecular weight thiol in eukaryote cells but this has been difficult to prove definitively due to the limitations of the analytical methods available. The Tietze enzyme assay, commonly used for GSH estimation using ESSE, has been shown to be subject to interference by unknown cellular constituents (Eady et al.,1995), possibly related to the unknown thiols reported here. Chemical and chromatographic methods for the analysis of cell and tissue thiols abound, but most give variable results due to the ease of autoxidation of the thiol group and the possible instability of labelling adducts. The current study seems to indicate that the latter could be true but one would not expect a mixed disulphide, such as RSSE, to exchange in the absence of free thiol. In these investigations it is possible that an unknown chemical reaction is taking place involving a spontaneous rearrangement of the RSSE after formation. Some preliminary mass spectrographic studies on the minor RSSE's have indicated this may be the case. The presence of such a reactive thiol species might also explain why it could not be measured or detected by the commonly employed thiol reagents which react at pH 7 and above (e.g. the formation of fluorescence adducts with bimane reagents (see Part A, section II in Packer, 1995)). However, it is also possible that these unknown, very reactive thiols found in the present study could be artefacts released after acid treatment of the cells.

These results indicate strongly that it must not be assumed that glutathione constitutes over 90% of the cellular ASF thiols and that caution must be employed in interpreting Ellman values of ASF thiols as attributable solely to the presence of this tripeptide.

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Abreviations Used: ASF acid soluble fraction obtained after deproteinization of cells; BDC-OH; 4,4'-bis-dimethylaminodiphenylcarbinol; DTNB or ESSE Ellman's reagent (1959), utilizing 5'5 dithiobis (-2-nitrobenzoic acid); DE 52 Whatman cellulose anion exchanger; EAT Erhlich-Lettre ascites tumour (mouse); ES^- yellow anion 5 thio- 2-nitrobenzoic acid; GSH glutathione; HPLC ; high pressure liquid chromatography; mS milli-Siemens (conductivity); OD optical density; RSSE ; aromatic mixed disulphide (s) formed on reaction of cellular thiols with Ellman's reagent; TCA trichloracetic acid: XAD-2 non-ionic polymeric absorbent, hydrophobic resin