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Studies On the Composition of The Low Molecular Weight Thiols Present in Tumour Cells– Is There "An Elephant in The Room"? An Autobiographical Review

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ABSTRACT

This is an account of the author's research investigations on the identification of the low molecular weight thiols (LMWT) present in a variety of eukaryote cells, particularly cancer cells. The work was carried out periodically over some 60 years in a number of university and private laboratories as, and when, resources permitted: as a result, this research can be roughly divided into 7 phases.

The first phase arose from initial research into ways to improve cancer treatment by radiation treatment. The second was involved with studies of the thiols present in isolated nuclei and nucleoli. The third consisted of attempts to develop new techniques to isolate and identify the various nuclear protein thiol containing moieties of a rat hepatoma tumour. The fourth phase involved the successful isolation of the nuclear nonhistone proteins which contain the bulk of the nuclear thiol material, leading to study nuclear thiol changes during rat liver carcinogenesis. The fifth phase consisted of the discovery and isolation of "hidden" low molecular weight thiols in isolated nuclei from rat tumours and other tissues. After a break of some 14 years, when bulk preparations of whole human cells became available, it became possible to continue work again on the low molecular weight thiols. Phase six consisted of studies on the thiols found in the ASF of various tumour cell lines using the new analytical technologies which were developing at that time. The seventh phase evolved into studies on human prostate cancer cell lines. Here a method was developed which clearly demonstrated that, among the low molecular weight thiols present in these cells, there was "an elephant in the room". This new thiol moiety, constituting 56.4% of the total cell thiols, was found to be concealed within the protein matrices of the cell, held in place, not covalently but possibly by ionic and hydrophobic bonding; deemed to be a "Conthiol". It does not contain any of the constituents of known cellular thiols and has proved difficult to analyse due to its reactive nature.

Keywords

Low molecular weight thiols, Cancer cells, Cell nuclei, Cellular thiol components.

Abbreviations

LMWT: Low Molecular Weight Thiols; ASF: Acid Soluble Fraction; NHP: Nonhistone Protein; NEM: N-Ethylmaleimide; IEF: Isoelectric Focusing; LC-MS: Liquid Chromatography-Mass Spectra; RSMNP: Thiol Adduct of 2-Mercuri-4-Nitrophenol; RSSE: Mixed Disulphide from Ellman Reagent; ROS: Reactive Oxygen Species.

Introduction

Over the last 100 years or so many papers, reviews and books have been published featuring research on one of the most ubiquitous and reactive chemical groupings found in cells and living tissues, namely the thiol or sulphydryl components which are widely found in proteins, lipids and other low molecular weight cellular compounds.

These studies have clearly established that thiols play **major** roles in cellular metabolism. These range from early research findings on:

- Cell division
- The control and maintenance of redox balance
- Energy metabolism and the regulation of respiration
- Defence against damage induced by ionizing radiation
- Reaction with xenophobic compounds, carcinogens and drugs

Also, to recent research studies involving:

- Gene activation, activity and signal transduction
- Defence against oxidative stress and damage, especially to the genetic material, especially with relation to cancer.
- The control of cell death by apoptosis.

All this research has clearly established the importance of thiols in cellular metabolism and other life processes.

Although much has been revealed regarding thiol metabolism in proteins and other cellular macromolecules interest has been mainly focussed on the ubiquitous tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) (GSH) as a major player in the control of thiol metabolism. Discovered by Hopkins in the 1920's it has been often quoted as accounting for over 90% of the low molecular weight thiols present in the cell. The universality of its presence in the intracellular fluids of living cells, whether in animal, plant or bacteria, is regarded as an indication that glutathione has general functions common to all cells. As one early thiol reviewer said "no substance of biological significance has had so many vicissitudes as glutathione"! It has been shown that this tripeptide has a major role in maintaining the redox balance of the cell and has recently been linked to what has become known as redox signaling, sometimes involved in gene activation. In many other studies GSH clearly been shown to also have a vital cellular function in the removal xenobiotic, carcinogenic and other toxic materials via the GSH transfer enzymes (GST). Additionally in the last 20-30 years it has been shown to be an important factor in the control of oxidative stress in some cancer cell studies.

Some cogent references to these facets of thiol metabolism and my own publications on this subject are given at the end of this review.

My own research into the biochemistry of thiols, a pursuit sometimes described as "thiology"(!), has been carried out in a number of different laboratories and can be divided into 7 phases as follows.

Phase 1 – Getting "hooked" on thiol metabolism

In the early 1960's I started my research career as a PhD student at the University Department of Radiotherapeutics based at Addenbrookes Hospital in Cambridge. This was in the field of radiobiology working on a project involving the synthesis of new drugs for cancer treatment.

Here my interest in the biochemistry of thiols developed while working on the role and use of radiosensitizers in the cancer treatment. It had been clearly established at this time that thiols, particularly aminothiols, were able to protect cells from the lethality of ionizing radiation. Conversely blocking reagents were able to sensitize cells to the killing effects of ionizing radiation.

I was able to show that treatment of mouse ascites tumour cells with a known radiosensitizing drug, 2-methyl-1,4 napthaquinonol bis disodium phosphate)(known as Synkavit), lead to a decrease in cellular thiol levels, in both protein and low molecular weight compounds. It was known that, after dephosphorylation of this compound within the cell, menadione was formed, which is able to react with thiols to give an adduct. It was speculated at the time that this reaction did in fact play an important role in the compound's radiosensitization properties.

A radioactive, tritiated, form of this drug which I helped to synthesize was shown to further decrease the cellular content of low molecular weight thiols in tumour cells. Later radioautographic studies using Synkavit, labelled with a radioactive labels ^{211}At or ^{261}I, showed that the drug localized in the nucleus and, in a some cases, unmistakeably in the nucleolus.

Later, attempts were made to identify the thiols which could be involved in the radiosensitization of these Ehrlich ascites tumour cells. At the time the major low molecular weight thiol component of eukaryote cells was thought to be the tripeptide, glutathione (GSH). However, during my analytical investigations at this time I found that this was **not** the case. Paper chromatograms of cellular acid extracts and simple detection agents revealed the presence of large amounts of thiols other than GSH (or cysteine) in these cells, as illustrated in the figure 1 below.

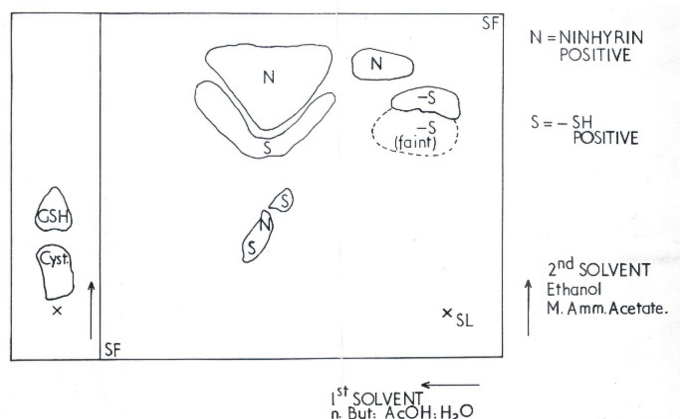


Figure 1: Two-dimensional paper chromatography analysis of mouse tumour low molecular weight thiols.

Only some fairly crude analytical work could be done which failed to identify the unknown thiols but did establish that they did not contain any amino acids. Lack of suitable equipment and time did not allow me to follow up this observation, but the finding has haunted me ever since!

Phase 2 – Thiols investigations on isolated nuclei and nucleoli

At this point I left Cambridge to do postdoctoral work with Professor Harris Busch in the Department of Pharmacology at

Baylor University in Houston, Texas. Here I learnt the valuable skills of the large-scale isolation of rat liver cell nuclei and nucleoli and where my fascination with "thiology" developed further. I reasoned that if thiols played a major role in cellular metabolism probably those that were most important would be those closest to the genetic material ie. in the nucleus. My work on isolated nuclei and nucleoli confirmed that they were indeed rich in thiol material, particularly in the nucleoli. I found out later that this had already been observed by a number of other workers using cytological stains.

At that time all that was known about the nuclear proteins was that there were two broad fractions, the histones and proteins rich in acidic amino acids or non-histone proteins (abbreviated as NHP); the latter being the most metabolically active based on radio-isotope turnover studies. Histones were just being sequenced and only thiol containing histone (cysteine in the peptide chain) was found to be the H3 (or F3). In contrast to the histones, the acidic proteins, which form the bulk of the nuclear proteins were rich in thiols. Frustratingly, at the time this group of ill-defined proteins were very difficult to analyse due to their insolubility in normal buffers and solvents. They were known to be very metabolically active, rich in thiols and thought to be important in the control of transcription so I developed a technique to solubilize them and separate them from the chromatin complex of DNA and histones (later called nucleosomes).

After a year this work was halted as, for personal reasons, I had to leave the States and return home to the UK.

Phase 3 – Method developed for the extraction and analysis of tumour cell nuclear thiols

In 1967 I obtained a two year postdoctoral grant enabling me to continue my thiol analysis studies in the laboratory of Dr.Lloyd Stocken at the Dept. of Biochemistry, University of Oxford who was studying thiol containing histones. Here, in addition to developing a new method for measuring the thiol content of histones, I was able to do further research on the NHP of a rat tumour (Hepatoma 223) confirming the work done in Houston; demonstrating the efficacy my technique in extracting these proteins. This is illustrated in the electron micrograph photos shown below (Figure 2).

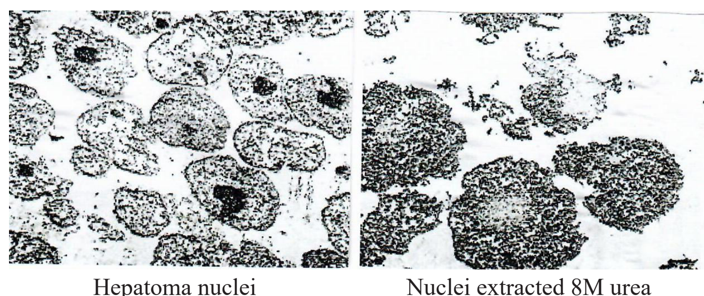


Figure 2: Extraction of rat tumour nuclear non-histone proteins.

Using this method nuclei were extracted with 8M urea 50mM phosphate pH 7.6 resulting in the complete dissolution of the NHP producing a hole where the nucleolus was situated and leaving the insoluble DNA and histone complexes; known as chromatin at the time but later found to be derived from nucleosomes.

The chromatin fraction containing the DNA:histone complex could be removed by centrifugation and the NHP in the supernatant crudely fractionated by anion exchange chromatography. The work was published in the European Journal of Cancer in 1969.

It was subsequently shown that this extraction removed up to 70% of the nuclear protein material and approximately 88% of the thiol present from the isolated nuclei. This technique worked well for many cell types. After a short while progress was made on the fractionation of the NHP from the nuclei of various cells and tissues. For these studies the NHP thiol groups where always blocked with N-ethylmaleimide (NEM, usually ^{14}C labelled,) prior to analysis to prevent the formation of oxidation artefacts. Thiols react with NEM as follows (Figure 3).

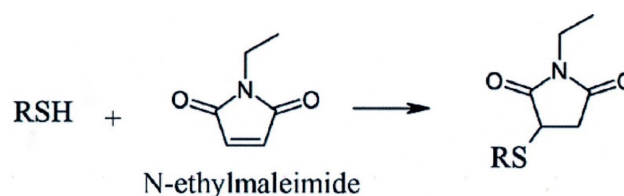


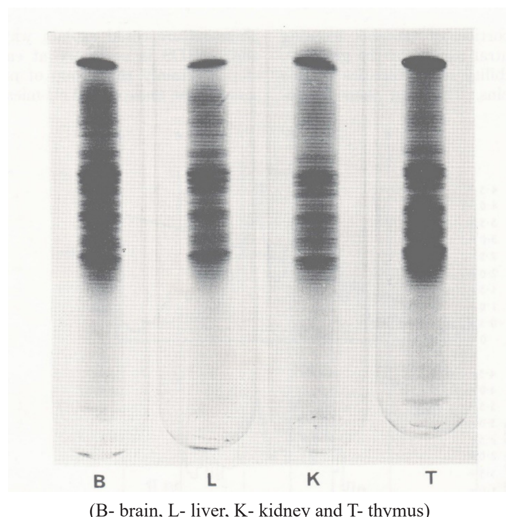
Figure 3: Reaction of thiols with N-ethylmaleimide.

Phase 4 – Analysis of thiol containing non-histone nuclear proteins

In 1970, after obtaining a research lectureship post at the Department of Experimental Pathology and Cancer Research at University of Leeds in Yorkshire, I was able to continue my attempts to develop suitable analytical techniques to resolve the complex mixture of thiol containing proteins present in the urea soluble NHP fraction of normal and tumour cells.

Here the first clear separation of the NHP components was achieved using a new technique of isoelectric focusing (IEF) in polyacrylamide gels revealing the true heterogeneity of this fraction. The photograph below is the first published example of the successful isolation and separation of the non-histone proteins. These were studied in the nuclei of different rat tissues, after the thiols had been blocked with N-ethylmaleimide. They were the first reported studies clearly demonstrating the heterogeneity of the NHP of cell nuclei. The isoelectric focusing gel patterns in the pH range 3-10 obtained from the 8Murea 50mM phosphate nuclear extracts of some rat tissues are illustrated below (Figure 4).

Further studies using ^{14}C labelled NEM labelled NHP in these IEF polyacrylamide gels showed the **apparent** variation in the composition and isoelectric points (pI's) of these proteins in various tissues and cells. The gel patterns obtained are shown below (Figure 5).



(B- brain, L- liver, K- kidney and T- thymus)

Figure 4: Isoelectric focusing gel patterns of NHP obtained from the nuclei of various rat organs.

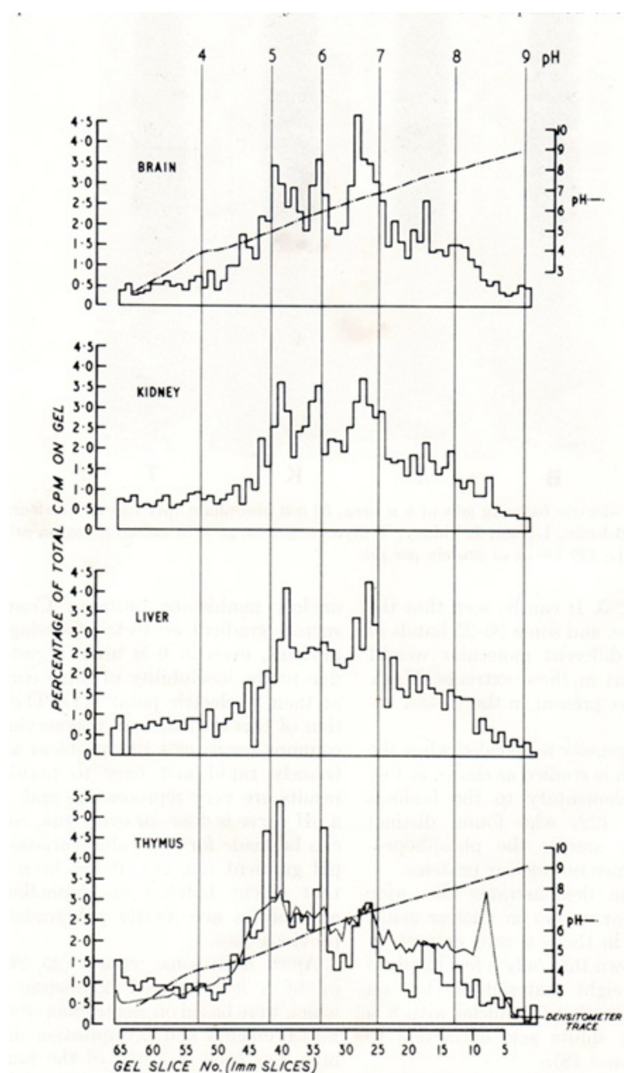


Figure 5: Distribution of ^{14}C NEM label in isoelectric focusing gels of NHP isolated from various rat organs.

Similar complex patterns were found in isolated rat tumour nuclei. On the face of this it seemed that fairly heterogeneous mixtures of proteins might be present in the nuclei from different tissues. Similar heterogeneous patterns were obtained later when the protein fractions were analysed by SDS polyacrylamide electrophoresis; a separation being based on molecular weight.

Phase 5 – low molecular weight thiols found to be non-covalently bound in nuclear non-histone protein structures.

In 1976, at the request of the funding charity, Yorkshire Cancer Research Campaign, I left Leeds to set up and establish a new carcinogenesis research unit at the University of York taking my invaluable technicians Fraser Lewis and Tony Thackrah. Here I was able to quickly continue my studies on the thiol containing proteins of cell nuclei.

In these new laboratories at York my team were able to continue our work to improve the separation of these proteins using isoelectric focusing using an alternative label to the ^{14}C labelled NEM used in the Leeds studies.

For these new studies a well-known and specific thiol reagent, 5,5' dithio-2 bis nitrobenzoic acid (Ellman's reagent, DTNB or ESSE) labelled with the radioisotope ^{35}S was synthesised. This was able to label thiols as shown below (Figure 6).

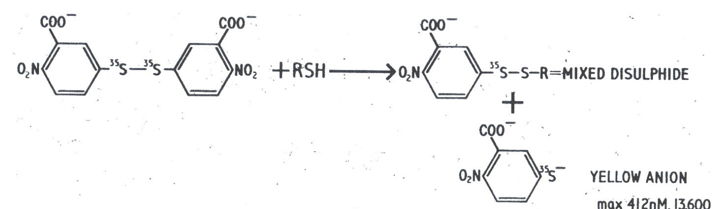


Figure 6: Reaction of ^{35}S labelled Ellman reagent with thiols.

The mixed disulphide formed from the thiol has an extra carboxyl group which it was thought should help in analytical separations, especially using isoelectric focusing where it was expected that the labelled proteins would have new isoelectric points due to the addition of an extra carboxyl group.

The isolated NHP labelled with ^{35}S labelled thio-nitrobenzoic acid (TNB) was shown to have a very similar specific activity to the ^{14}C-NEM labelled material as illustrated in table 1 below:

Table 1: Thiol contents calculated from specific activities of nuclear non-histone proteins.

Isotope label and specific activity	dpm/mg Protein	nanomoles -SH per mg Protein
^{35}S-TNB 17.5mCi/mM	2.65×10^6	68.5
^{14}C-NEM 2.4mCi/mM	3.96×10^5	75.0

However, when the distribution of ^{35}S label in the IEF gels was analysed a somewhat different pattern was obtained to the ^{14}C labelled gels. A comparison of the patterns obtained from rat liver nuclei NHP are shown below (Figure 7).

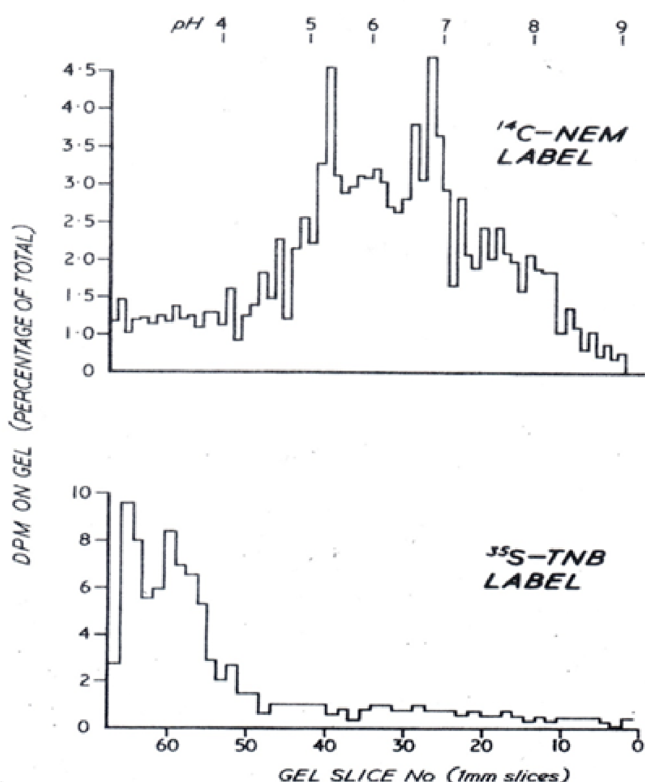


Figure 7: Distribution of radiolabels in isoelectric focusing gels.

Very little of the ^{35}S label applied was recovered from the gel. The table below illustrates this. Most of the ^{35}S was found to be in the anode electrode solution (10mM phosphoric acid) of the IEF equipment. No protein could be detected in this solution. Also, the stained gel pattern (Coomassie Brilliant Blue) was almost identical in both gels.

Approximate percentage recovery of ^{35}S applied to the large scale IEF gels of 8M urea extracts from rat liver nuclei is shown in table 2.

Table 2: Distribution of ^{35}S label obtained in large scale IEF gels.

	Gel slices extracted			
Bottom electrode solution, 10 mM H_3PO_4	(1) with 8 M urea 50 mM phosphate pH 7.6	(2) N sodium hydroxide	Top electrode solution, 20 mM NaOH	Percentage recovery
56–60	10–11	2–4	0.3–0.5	72–78

In addition, ~3% was present in the initial gel overlay.

From these experiments it was clear that low molecular weight thiol adducts, labelled with the ^{35}S reagent, could be released from the NHP mixture during isoelectric focussing.

Unfortunately, very little of this data was published at the time. The final paper on this research which was based mainly on tumour cell nuclei was submitted to an analytical biochemistry journal but it was rejected by two referees on very tenuous grounds; one referee said that, despite the fact that he (or she) hadn't received the copies of the 5 diagrams and 4 tables with the paper, it was rejected out of hand!!

Much of this data was lost but later some of it was recovered and submitted to an open access online journal and published in 2020. In this paper I also showed that the ^{35}S labelled adducts found in the IEF anode solution could be adsorbed onto XAD2 resin and 100% recovered in methanol. Following this, gel filtration on a 30x2 cms column of Bio-Gel P2, eluted with 50mM phosphate buffer pH 7.6, revealed the presence of adducts of molecular weight of less than 1500 (exclusion limit of this gel is 1500 daltons) (Figure 8).

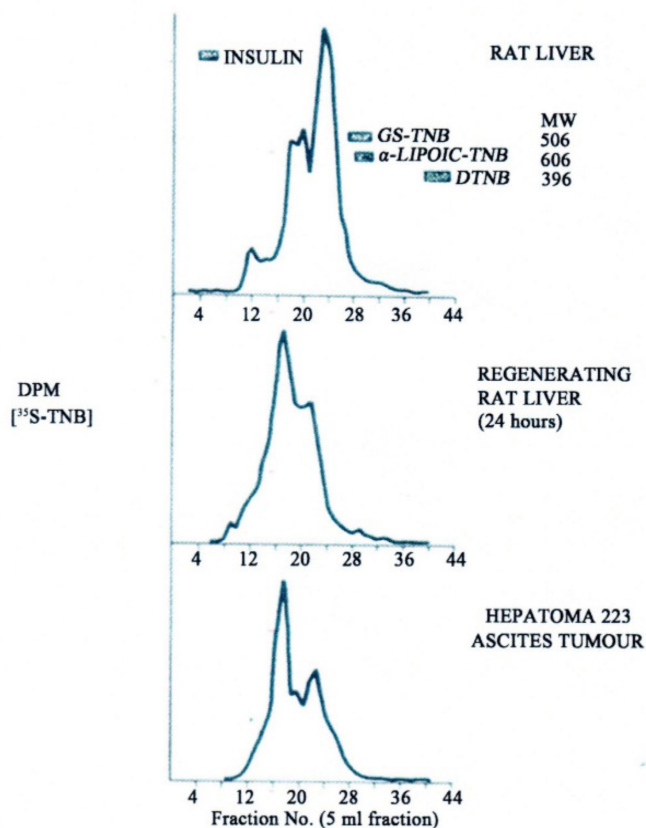


Figure 8: Gel Filtration chromatography (on Bio-Gel P2) of the ^{35}S labelled adducts isolated from the IEF anode solution.

The patterns obtained indicated that there were differences in the tumour, regenerating and normal cell nuclear patterns. Comparison of the elution times of the TNB adducts of glutathione (GSH) and reduced α-lipoic acid and DTNB itself indicated the unknowns to have molecular weights of 600 or more, but less than 1500, as shown by the Blue Dextran or insulin markers which are excluded from the P2 gel. Subtracting the 5-thio-nitrobenzoic acid mass indicated the thiols to have molecular weights of about 400-500.

Phase 6 Change of career followed by a fresh start and new approach to thiol studies.

At this stage I was also trying to isolate and analyse the bioactive fraction of normal and neoplastic cell nucleoproteins by two-

dimensional gel electrophoresis which was a formidable task. Although I had a tenured position University of York my funding started to dry up and the University showed little interest in my work so I decided to leave the research world and enter the commercial sphere.

I joined a large international "think tank" company, PA International, in Melbourne just south of Cambridge in 1979 to set up a Life Sciences consultancy service to industry. Two years later I left to help establish several new biotechnology companies in the Cambridge Science Park – but that is another story!

It was some 15 years later that I was able to "retire" and return to my research studies on thiols! During the time I had been out of the biological thiol research field (since 1979) the scene had changed greatly. One of the main buzzwords in "thiology" at the time involved the condition known as "Oxidative stress". Glutathione (GSH) was being moted as a major cell constituent involved in the cellular defense against oxidative stress and also in the regulation of redox signaling. Many papers had been published on this area of thiol research on cancer cell metabolism.

With some support from one of my commercial ventures I was able to help in setting up a cancer charity named Cambridge Cancer Research Fund. With their support laboratory space was obtained for me to continue my research on thiol biochemistry at the Strangeways Research Laboratories in Cambridge.

Unfortunately these laboratories did not have the expensive equipment or resources to prepare nuclei and so I was unable follow up my previous work, so I chose to go back to working on whole cell thiols. I began by looking at the acid soluble fraction (ASF) obtained after precipitation of the cellular proteins where I had originally spotted the presence of unknown LMWT. In contrast to my previous work on nuclei from rat tissues my work now focused solely on tumour cell lines.

My first paper on thiol cellular thiol components since leaving research at York University was published in the journal "Cancers" in 2010; an abstract of which is as follows:
"The low molecular weight thiol (-SH) content of the acid soluble fraction (8%TCA extract) of a human prostate carcinoma cell line (LNCaP) was investigated using three forms of thiol assay each utilizing different chemistries and a new technique developed to estimate the glutathione content.

Mixed aromatic disulphides, prepared by reaction of the ASF thiols with a three fold excess of the Ellman reagent, were isolated by chromatography on C_{18} reverse phase silica gel followed by DE52 anion exchange separation and then analysed by capillary electrophoresis. The glutathione adduct (GSSE) and an unknown disulphide (RSSE) were the major components isolated on DE52 together with two minor ones. However, the GSSE isolated could only account for $28.5 \pm 4.3\%$ of the total ASF thiols isolated from these cells.

From the absorbency at 325nm of the RSSE isolated it appeared that bulk of the ASF thiol had not formed a stable mixed disulphide with Ellman's reagent and this was confirmed by ^{35}S labelling of the cells. A large proportion of the ^{35}S labelled components, obtained after reaction of the ASF thiols with the Ellman reagent did not consist of the expected mixed aromatic disulphides and passed straight through C_{18} reverse phase silica gel unabsorbed. This mixture could be separated into at least six components by gel filtration on Biogel P2."

This research demonstrated that there were non-protein LMWT other than the ubiquitous glutathione present in tumour cells.

Later returning to some freshly refurbished laboratory facilities in my incubator unit at Cambridge Research Laboratories I was able to analyze the LMWT of several tumour cell lines by high pressure liquid chromatography (HPLC) using different chromogenic thiol labelling compounds. This work was published in two papers in 2012 and 2013. All of this research work clearly confirmed my previous findings that there were non-protein LMW thiols other than the ubiquitous glutathione present in tumour cells. ^{35}S labelling of the cells confirmed the presence of unknown thiols. However, later liquid chromatography and mass spectrographic analysis (LC-MS) analysis (by an external laboratory) of the unknown adducts I had isolated failed to identify any of these thiol components.

The results indicated that only 30-40% of the LMW non-protein thiols present in these cells were in fact glutathione. I suspected at the time that unknown, unidentified thiol components of the ASF could be leaching out from the precipitated protein mass.

Only much later did I identify some of them when I was able to collaborate with an analytical group at Sunderland who had the sophisticated equipment necessary for this analysis. Using LC-MS-MS and NMR technology insight was gained into the identity of the other thiols present in the ASF of LNCaP tumor cells. They turned out to be cysteine, cysteinylglycine (part of the GSH molecule) and traces of an unknown thiol identified as cysteinylglycerate. The ASF constituted about 20-23% of the total thiol measured in these cells. This work was published in 2017, an abstract of this paper is as follows:

"The low molecular weight thiols present in the deproteinized extract of a prostate cancer cell line (LNCaP-FGC) were analysed after derivatization with the Ellman reagent (ESSE). The mixed disulphides formed (RSSE) were fractionated, characterized and quantified by liquid chromatography on a C-18 column using UV detection. This revealed the presence, in femtomoles per cell, of glutathione (8.3 ± 0.73), cysteine (2.71 ± 0.04) and cysteinylglycine (0.83 ± 0.10), accounting for the bulk of the thiol present. Further analysis of the cell extracts using a novel and sensitive mass spectrometry technique allowed the detection, at a low level, of an additional RSSE derivative which was identified as cysteinylglycerate using NMR spectroscopy."

Phase 7: Further investigations into the total thiol content and **composition of whole cells**

While the Sunderland investigations on the acid soluble fraction were going on I was able to do some further research on the protein bound thiols, ie those precipitated by the protein denaturant (trichloacetic acid). Having removed the GSH and other LMWT it would be logical to assume that the remaining thiols were protein constituents in the cysteine residues of the polypeptide chains - but that proved not to be the case! On labelling the total cell thiols of two human prostate cell lines, supplied in bulk by the Public Health laboratories at Porton Down, I made a surprising discovery.

Using the Ellman reagent (ESSE) again to label all of the thiols of the human LNCaP tumour and a virally transformed human prostate cell line (PNT2) gave interesting results that tied up with the findings I had made on isolated cell nuclei at York University in the 1970's. An abstract of this paper, published in 2018 (open access), is as follows:

"The protein thiol contents of two human prostate cell lines, LNCaP (malignant cell line) and PNT2 (a virally transformed prostate cell line) were analysed after removal of the cellular glutathione by trichloroacetic acid precipitation. The protein precipitate was then dissolved in buffered 8M urea containing the Ellman reagent (ESSE); this reagent reacts with thiols (RSH) to give a yellow anion (ES) and a mixed disulfide (RSSE). Fractionation of the latter by gel filtration chromatography on Biogel P2 revealed that the excluded protein components eluted in the void volume (M_r > 1500) could only account for a fraction (18-19%) of the total thiol originally present. A low molecular weight fraction liberated from the protein matrix accounted for 56-61% of the total cellular thiol detected with the Ellman reagent. This fraction contained the excess ESSE, possible RSSE and the yellow anion liberated (ES). After removal of the ES a HPLC analysis revealed that only one major component was present. This was shown by MS analysis to be ESSE (m/z 395); traces of some other derivatives were found on the chromatogram of mass 592m/z and 791m/z but these were almost certainly artefacts formed by the addition of ES molecules to ESSE. No amino acids or cysteine could be detected in this low molecular weight ESSE/RSSE fraction.

It was concluded that a considerable amount of "labile" low molecular weight thiol had been released from the protein matrix by extraction with buffered 8M urea which did not form an RSSE adduct with the Ellman reagent. This may be a simple divalent sulfur moiety, possibly sulfide (S^{2-}), polysulfides or derivatives of sulfane sulfur (S^0) associated with vital metabolically active/ regulatory cellular proteins, such as those involved in respiratory functions."

The surprising result of these experiments was the finding that the isolated cellular protein only contained 18-19% of the total measurable protein thiol. It had always been assumed that, in addition to the LMWT thiols consisting mainly of GSH, the rest of the cellular thiols were in the form of cysteine residues in the various thiol containing proteins of the cell.

The method developed for this whole cell study is summarized in the following chart (Figure 9).

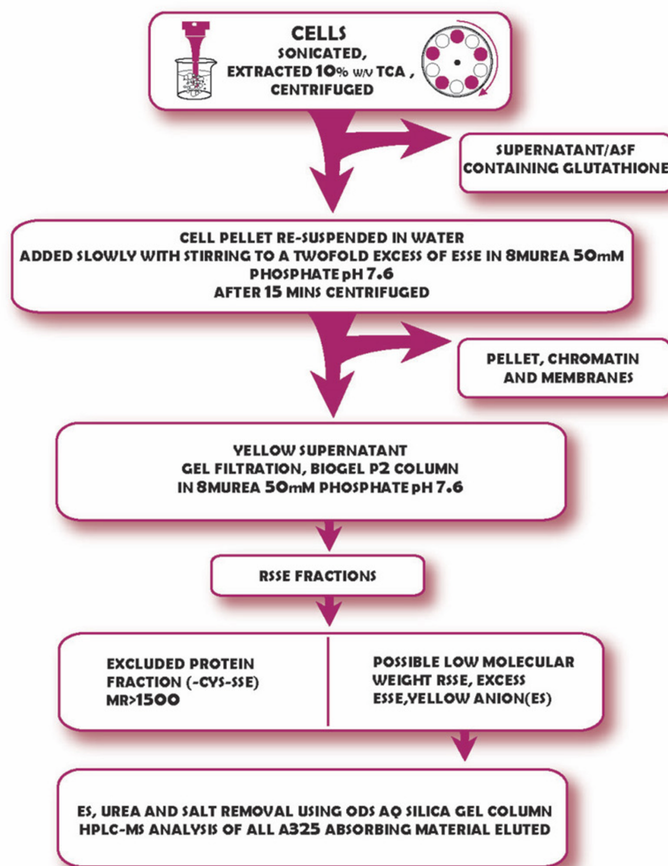


Figure 9: Schematic diagram of isolation and separation of the cellular thiols of LNCaP cells.

The calculated thiol contents of the fractions obtained by this procedure are shown in the table below (Table 3):

Table 3: Thiol contents of LNCaP and PNT2 cells.

Distribution of cellular thiols in LNCaP (red) and PNT2 (green) cells				
	femtoles thiol*/cell		% of total thiols	
Total thiol content	56.3 ± 3.6	28.5 ± 1.7	100.0	100.0
ASF 10%TCA soluble	13.1 ± 0.8	4.9 ± 0.3	23.3	17.2
Total protein pellet	43.2 ± 2.9	23.6 ± 1.4	76.7	82.8
Protein pellet after extraction with 8M urea containing ESSE				
Chromatin bound –SSE (insoluble fraction)	1.3 ± 0.2	0.7 ± 0.1	2.3	2.4
Biogel P2 Protein-SSE (soluble fraction)	10.1 ± 0.6	5.4 ± 0.4	17.9	19.0
Reduced P2 protein thiol content (includes protein disulphide)	12.2 ± 1.0 (+20.8%)	6.5 ± 0.4 (+20.3%)	------	------
Thiol released from protein by difference –"labile thiol"	31.8 ± 2.2	17.5 ± 1.6	56.5	61.4

*Average of 4 results

These results also showed clearly that the bulk of the thiol materials precipitated with the protein were of low molecular weight, accounting for some 56.5% to 61.4% of the total cellular thiol. Although the cellular thiol contents of the cells were different, similar distribution results were obtained from the tumour cell line LNCaP

and the virally transformed prostate cell line known as PNT2.

Unfortunately, unlike my previous studies on cell nuclei, at this time I was unable to isolate any Ellman mixed disulphides from this labile thiol fraction. Whatever they were, despite reacting with the Ellman reagent to give the expected yellow anion, which quantifies thiol present, they did not seem to form mixed disulphides with this reagent. I was therefore unable, despite LC-MS analysis, to get any clues as to the nature of these labile thiols using this reagent. However I now know that I made a mistake in the isolation procedures that probably lost the unknown Ellman adducts in these experiments.

The results obtained in these experiments encouraged me to find another suitable way to identify these low molecular weight thiols. If I had had the facilities to do radioisotope labelling work this would have been the chosen route but alas this was not available. After some thought and reading through the voluminous literature on thiols that I have accumulated over the years I came to the conclusion that in order to do this analysis with the facilities I had available I would have to use a thiol blocking agent containing a chromogenic group to give a visible adduct. After an extensive literature survey I chose an aromatic mercurial, 2-mercuri-4-nitrophenol, unavailable commercially but easy to synthesize. Having always enjoyed chemical synthesis I soon made it. This reagent reacts with thiols in a highly specific manner as follows (Figure 10).

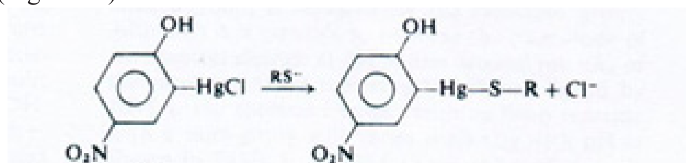


Figure 10: Formation of mercurial adduct from 2-chloromercuri-4-nitrophenol.

Using this reagent the yellow adduct(s) formed (λ_{max} at 410nm, neutral pH) enabled me to isolate and examine the adducts of low molecular weight thiols concealed in the protein matrix of human prostate cancer cells (LNCaP). These results were published in Bioscience Reports an online journal (open access) in 2020 and I feel that this is one of the most important papers that I have published.

A schematic of the method of analysis I developed is shown in Figure 11 below.

Due to the chemical nature of the mercuri-thiol adducts I had a great deal of difficulty in getting a suitable gel filtration column to work. However, I eventually managed to obtain a good chromatographic pattern on Sephadex G15 as shown in figure 12. Looking at the percentage of the yellow label (A 410) in the peaks, as shown in table 4 it was clear that, in agreement with the previous work using the Ellman reagent, the bulk of the labelled thiol is of low molecular weight. Over 90% of the applied A410 was achieved:

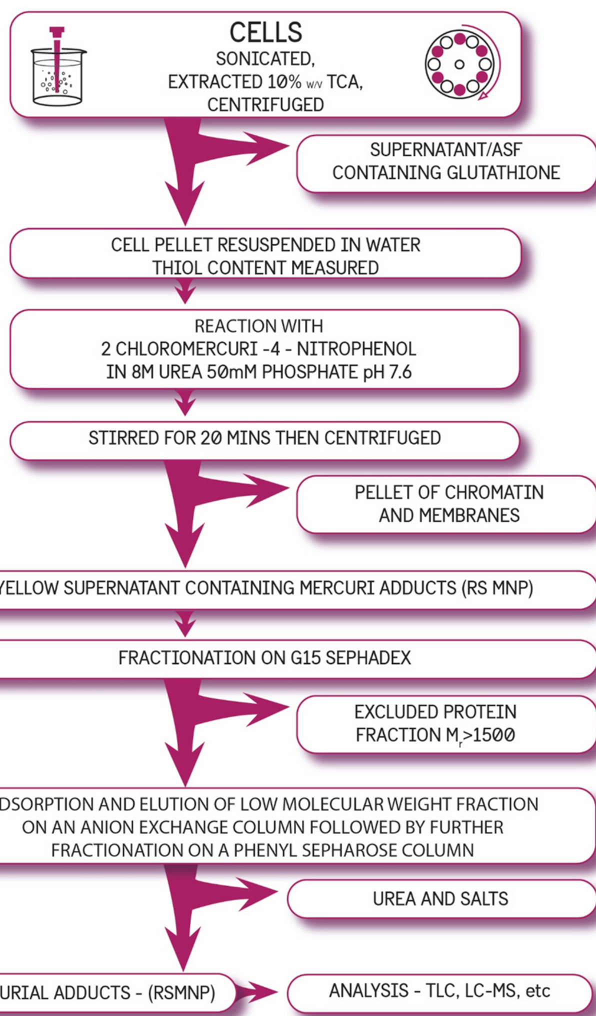


Figure 11: Flow chart of procedure to prepare thiol mercurial adducts.

The LMW mercurial adducts turned out to have some very peculiar properties and I had quite a few problems in isolating enough material for analysis. To cut a long story short I found that I could isolate the yellow adduct by hydrophobic interaction chromatography on a column of PhenylSepharose with the bonus of concentrating what started as a large band on the column into a single tight band on elution.

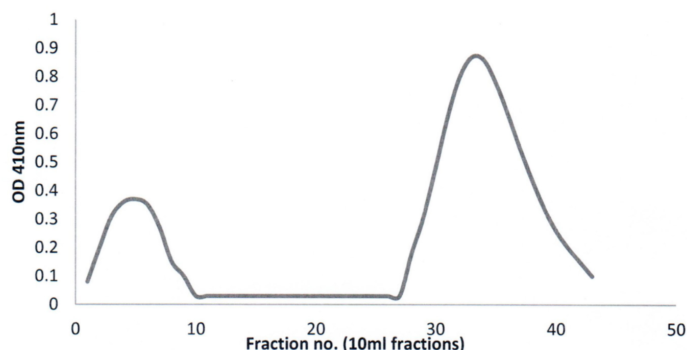


Figure 12: Chromatography of 2-thiomercuri-4-nitrophenol adducts on G15 Sephadex.

Table 4: Recovery of 2-thiomercuri-4-nitrophenol adducts on G15 Sephadex.

Percentage of the applied A_{410} recovered *		
Protein	"Intermediate"	LMW
16.0 ± 4.1 %	5.9 ± 1.2%	77.2 ± 3.7

*Average 4 runs (± SD)

After some high-pressure liquid chromatography development by a commercial analytical laboratory using a C18 column and an acidic solvent gradient it was eventually shown that the LMW peak consisted of only one major component was present as shown in figure 13.

The MS obtained from the ion trace obtained from this peak revealed a complex pattern consisting of a number of mercury clusters derived from the 7 stable isotopes of this element. The negative ion mass spectrum of this 3.3mins peak is illustrated in figure 14 below.

The formula weight of the label 2-mercuri-4-nitrophenol present in the adduct should be around 339 daltons but it can be seen that no ions of this size were found, nor those of the label plus a single sulphur atom (371daltons).

Taking The 588m/z peak and subtracting the MNP label fragment indicates a thiol of around 249 daltons or with the 706 m/z peak a thiol of 367 daltons. In the cases of the 822 and 941 m/z fragments it is possible that, depending on the ionization conditions employed in the MS, these could contain mercury dimers or trimers, making interpretation of the data difficult. However at this time it was felt that the data strongly indicated that this matrix thiol was not a simple sulphide, persulphide or some form of sulphane (S^0) sulphur.

Similar results to those obtained with the RSMNP adduct were obtained from preparing and isolating the similar adducts obtained from 4-chloromercuribenzoic and 4-chloromercurisulphonic acids.

As a result of the work done in this study and the previous work on the RSSE adducts the thiol contents of the LNCaP cellular fractions were shown to be as follows (Table 5):

Table 5: Thiol contents of LNCaP cells.

Thiol fraction	femtomoles of thiol/cell	Percentage of total thiol
Total cellular thiol	56.3 ± 3.6	100.0
Glutathione	8.3 ± 0.7	14.7
Minor ASF LMW components	3.5	6.4
Protein fraction-gel filtration	11.4 ± 0.3	20.2
Chromatin pellet	1.3 ± 0.2	2.3
Protein matrix release -LMW	31.8 ± 2.2	56.4

Thus the amount of the protein matrix released thiol, dubbed "Conthiol", was shown to be nearly four times more than the GSH present, making it the major low molecular weight thiol in these cells. Further studies with other non-malignant human prostate cell lines gave a similar result. At this stage I was convinced that there was a "thiol elephant" in the room!

Recent unpublished data on the isolated Conthiol adducts

Attempts have been made to isolate the Conthiol from its mercurial adduct by passing the RSMNP through a column of ThiopropylSepharose. This was expected to release the thiol, leaving the label on the column. The sample was applied at pH 7.5 but the pH of the eluate dropped to 5. It was found that, in addition to the thiol released, a precipitate slowly formed which did not contain thiol. On redissolving it was found to have a UV spectrum with a peak at 317nm which was not seen in the RSMNP adduct spectrum. Preliminary LC-MS analysis of the thiol column eluate indicated that that 2 components were present, a thiol component with low UV adsorption giving an MS ion fragment of 352m/z (negative mode) and the A_{317} nm absorbing compound producing a 427m/z ion. Further MS-MS studies are being carried out but it appears that a complex rearrangement or breakdown has occurred.

Summary of the findings made on analysis of Conthiols

Most analyses carried out on the various preparations of Conthiol adducts gave little indication as to the nature of the thiol. Some of the some of the findings are as follows:

- Full cysteine and other amino acid assays were carried out on an anion exchange auto analyser by the Protein & Nucleic acid chemistry facility of the department of Biochemistry at the University of Cambridge but no amino acids could be detected in the released thiol or any of the different thiol adducts prepared.
- Tests for ribose/pentoses using the classic Bial's Orcinol method and for deoxyribose (2'-deoxypentose sugars) using the Dische Diphenylamine reagent gave negative results showing these sugars are not present.
- Analyses for sulphur, selenium and phosphorus contents were carried out by Inductively Coupled Plasma Mass-Spectrometry (ICP-MS) using a Thermo Finnigan Element 2 Magnetic-Sector ICP-MS by Philip Holdship of the Oxford University Innovation Services at the Department of Earth Sciences, University of Oxford. The sulphur results correlated exactly with the thiol measured by the Ellman reagent. No selenium or phosphate was detected.
- As already pointed out from the LC-MS studies on the RSMNP adduct it appears that the Conthiol could have a molecular weight ranging from 249 to 367 daltons. From the fragments observed it does not seem to contain 1-N-methyl-4-mercaptohistidine, known as Ovothiol A (molar mass 201), found in high quantities in the ovary, eggs and biological fluids of sea urchins and marine cephalopods.
- Passing this adduct through a ThiopropylSepharose column released a thiol moiety giving negative ions at 213, 363, 395 and 427m/z which according to the MS nitrogen rule indicates that an odd number of nitrogen atoms are present. However, it is still unsure that these ions are not artefactual arising from a rearrangement of the thiol on release from the mercurial label.
- The UV spectra of the RSMNP and RSSE adducts is very

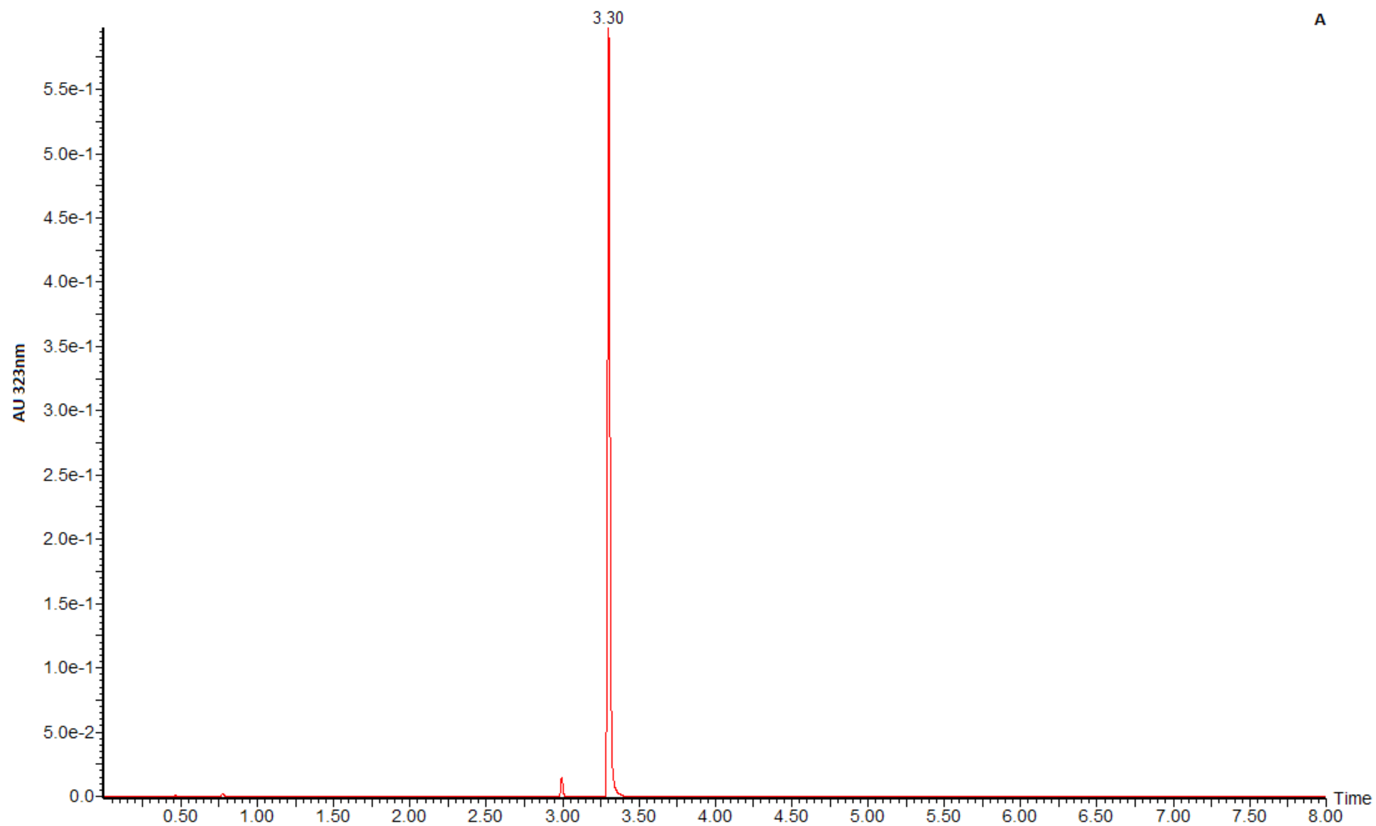


Figure 13: LC trace of LNCaP-RSMNP at 323nm; Ord. AU 323nm; Abs. Time of elution (mins).

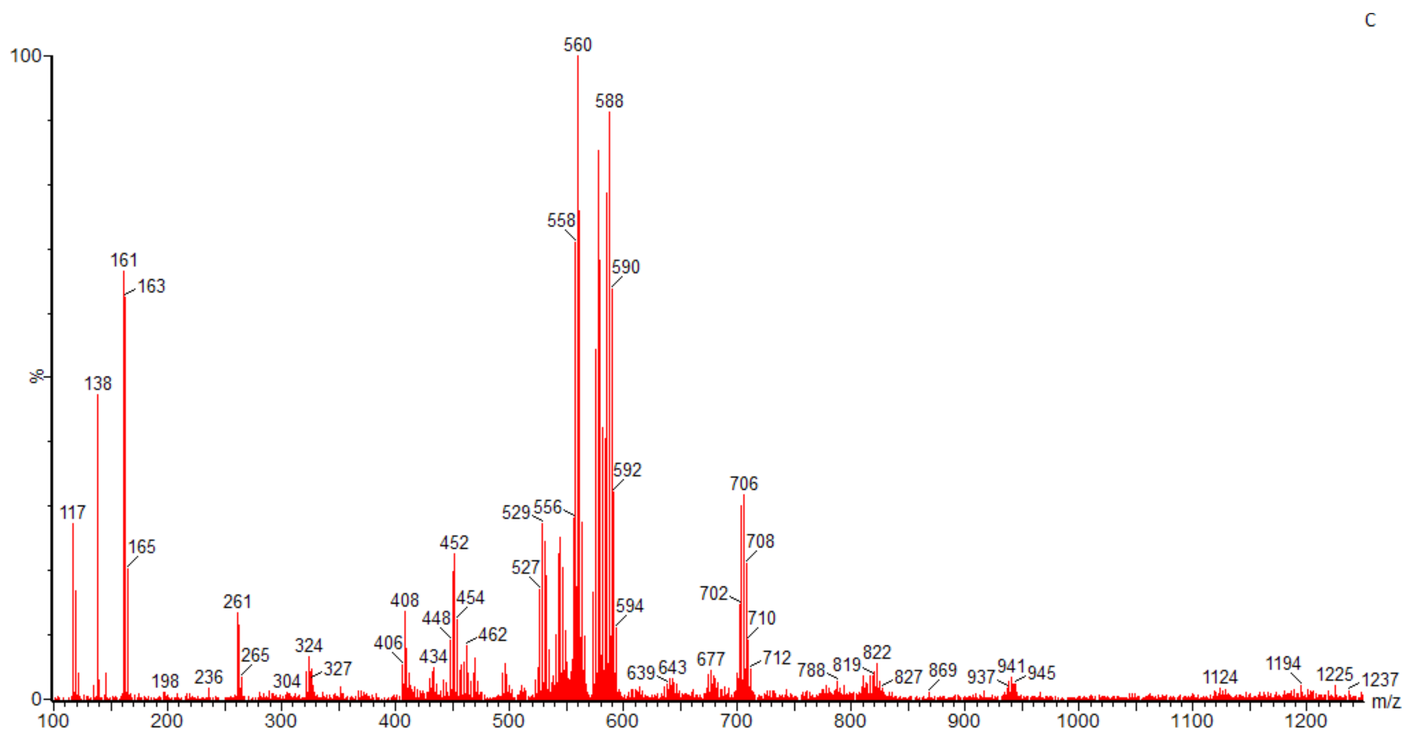


Figure 14: MS pattern of 323nm, 3.3mins peak.

similar to their labelling compounds with very little difference at 260nm ruling out the presence of any nucleic acid bases.
- The only clue on the nature of the adduct, which is very soluble in water, is that it has amphoteric properties in that it can be adsorbed on both anion and cation exchange columns. As phosphate is not present his indicates that a carboxyl group could be present. The basic grouping is not a primary or secondary amine but might be a tertiary or quaternary amine but further work needs to be done to identify the basic group present.
- FTIR analyses, as performed by a commercial company, Intertek, only seemed to show the possible presence of lactam ring structures.
- NMR analysis (in D_2O) of the RSMNP adduct by the Chemistry Department at the University of Cambridge revealed very little information except that they could not detect the presence of any unsaturated aliphatic carbons.

Further LC-MS^n investigations to identify these Conthiols are, of course, currently underway. In addition to LNCaP cell studies similar results (unpublished) were obtained when the thiols and RSMNP adducts were studied in PC3 and DU145 metastastic prostate cancer cell lines.

Discussion and Conclusion

The author's investigations over the years clearly show that GSH is not the major low molecular weight thiol present in the tissues and cell lines he has studied. Instead, an "elephant in the room" has emerged which is an unidentified thiol concealed within some of the cellular protein matrices, deemed to be a "Conthiol". It is present in the region of four times the concentration of the GSH present in the cells studied.

Initial analysis studies have not been able to fully identify this Conthiol structure except to establish that it does not contain glutathione or any of the thiol sulphur containing amino acids or any other well-known low molecular weight thiols.

Furthermore, it appears to be a highly reactive molecule. Its significance in cell metabolism remains to be elucidated but based on the considerable evidence in the literature indicating the importance of cellular thiol components, strongly suggests that it plays an important role in cellular metabolism.

So, what is the relationship of Conthiols with GSH?

Being buried in the protein matrices of the cell probably means that Conthiols are well shielded from ROS, toxic agents and other factors that give rise to oxidative stress problems, an area where GSH plays an important role. In the light of the opinion by a number of investigators (see Harris et al 2015) that combined inhibition of GSH and Thioredoxin (Trx) antioxidant pathways leads to a synergistic cancer cell death, both in vitro and in vivo, it would be interesting to determine if GSH is capable of maintaining the Conthiols in a reduced state. The unique tripeptide structure of GSH may well be the key which allows it to enter the protein matrices and interact with Conthiols.

Which cellular protein matrices could this Conthiol be mainly associated with?

From my work on cell nuclei and the fact that many histological studies have shown high concentrations of thiols to be present in cell nuclei, and in particular nucleoli, it seems highly likely that Conthiols could be part of the nucleic acid polymerase enzymes. Nearly all known types of these enzymes are dependent on intact thiols for their reactivity. This would fit in with the established role of thiols in radiosensitivity due to the indirect effects of radiation damage to cellular macromolecules as a result of free radical generation in conjunction with the direct damage of radiation known to affect to the cell's genetic material.

What will the be the impact of Conthiol discovery on cancer therapy?

At present chemotherapy has limited use in treating metastatic prostate cancer. As a result of my work on prostate and metastatic prostate cancer cells I feel sure that identification of the Conthiols I have discovered may provide vital pointers to the development of more effective chemotherapeutic agents for prostate tumours and other currently untreatable cancer growths.

The evolution of a new rational design approach to redox therapies as suggested by the work of Oberley and his team could be important in this respect.

In conclusion, elucidation of the structure and metabolic role of Conthiols in tumour metabolism promises to open up an exciting new era for therapeutic advances in the treatment of cancer.

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My thanks are due to the British Empire Cancer Campaign (now Cancer Research UK), the Yorkshire Cancer Research Campaign for their continuing financial support during my earlier research career, and finally to my fellow Trustees, Dr Robert Carpenter, Dr. Bill McCrae and Ruth Fermor of the Cambridge Cancer Research Fund for their generous support in recent years.

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