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# Modification by simetryn sulphoxide of a specific thiol group in rat haemoglobin

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Native rat haemoglobins were found to bind simetryn sulphoxide to an extent 40-fold greater than human haemoglobin. This specific behaviour was studied by using only high-pressure ('performance') liquid chromatography for the preparative separation of globin chains and the isolation of peptides resulting from chemical and enzymic degradation. High recoveries (>80%) of peptides throughout the procedures in combination with microsequence techniques, allow a definitive assignment of the residue undergoing modification. The haemoglobin β-chain cystine-125 residue, with a stoichiometry of one per tetramer of rat haemoglobin, was found to be modified. Stereochemical implications of this finding are discussed. Simetryn sulphoxide would appear to be useful as a specific reagent for the mapping of exposed thiol residues in proteins.

Haemoglobin of rat is known to be heterogeneous (Garrick *et al.*, 1975), three α- and four β-chains have been isolated (Garrick *et al.*, 1978), and sequence data for two main α-chains (Garrick *et al.*, 1975; Chua *et al.*, 1975) and for the major β-chain (Garrick *et al.*, 1978) are available. The thiol content is relatively high in the major haemoglobin component; cysteine occupies positions 13, 104 and 111 in the α- (Chua & Carrell, 1974) and positions 93 and 125 in the β-chain (Garrick *et al.*, 1978). By assuming that the model of Perutz *et al.* (1968) for horse haemoglobin can be applied, then residues Cys-β-93 (β-chain cysteine-93) and Cys-β-125 would be expected to be relatively reactive. Cys-α-13 (α-chain cysteine-13) is at least partially shielded by Asn-α-12, and both Cys-α-104 and Cys-α-111 are in the $\alpha_1\beta_1$-dimer-contact region and therefore not accessible for solvent.

Rat erythrocytes, in contrast with those of the human and most other mammals, bind acetaldehyde (Eriksson *et al.*, 1977) and show unique behaviour towards thiol-oxidizing diazenes (Kosower *et al.*, 1977). These properties have been attributed to the presence of unusually reactive cysteine residues within the haemoglobin. However, considering that the reactive Cys-β-93 residue is common to all mammals, and, in addition, that cat haemoglobin, which contains a larger number of reactive thiol groups, has no marked reactivity with diazenes (Kosower *et al.*, 1977), then the reason for this peculiar reactivity is unclear.

In our study on the toxic effects of simetryn [*NN'*-diethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine]; see I, Scheme 1, a widely used herbicide, it was found that rats, fed with radio-labelled material retained the label for several weeks within the erythrocytes. *In vitro*, simetryn itself does not react with rat haemoglobin. However, the sulphoxide derivative [*NN'*-diethyl-6-(methylsulphinyl)-1,3,5-triazine-2,4-diamine; see II, Fig. 1], formed *in vivo*, as a result of oxidation by microsomal fractions (Hamboeck *et al.*, 1981), can form stable derivatives. On incubation *in vitro* of several mammalian haemoglobins with simetryn sulphoxide, stable derivatives were formed only with that from rat and guinea pig. Modification of cysteine, according to the reaction shown in Scheme 1, can be expected. Specific group modification can be important in protein structure–function studies, and accordingly a study was undertaken to determine the specificity and nature of this derivatization of rat hameoglobin.

In a previous paper (Hughes *et al.*, 1979*a*) and in the preceding papers (Honegger *et al.*, 1981; Wilson *et al.*, 1981*b*), we have shown that amino acids and peptides are conveniently analysed and isolated by

Abbreviations used: DABITC, 4-*NN*-dimethylaminoazobenzene 4'-isothiocyanate; DABTH, 4-*NN*-dimethylaminoazobenzene 4'-thiohydantoin; DITC, phenylene di-isothiocyanate; h.p.l.c., high-pressure ('performance') liquid chromatography.



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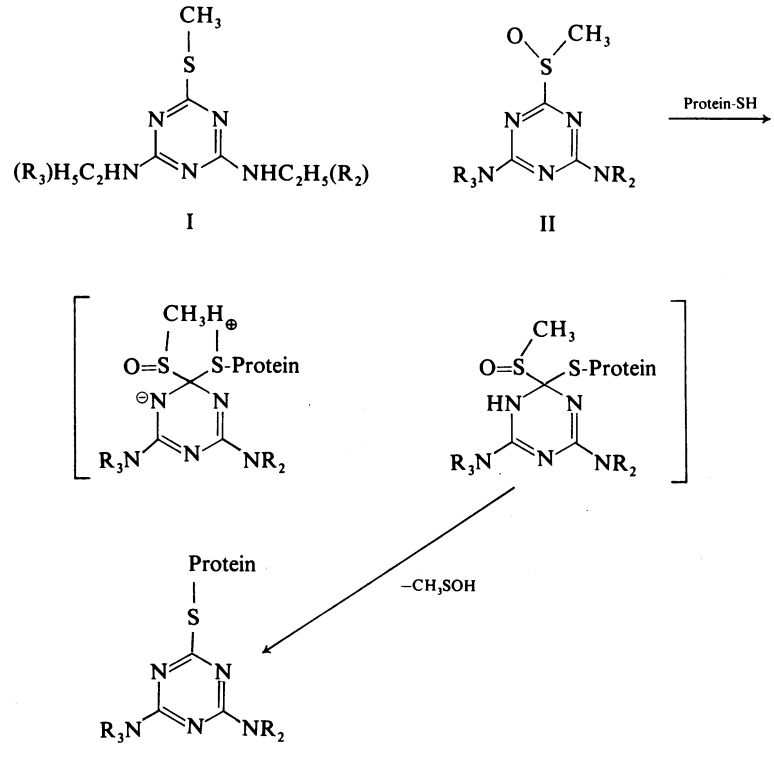


Scheme 1. *Proposed reaction of simetryn sulphoxide with cysteine*

using h.p.l.c. techniques. Here we show such technology can be applied in the study of post-translational protein modifications and report that Cys-$\beta$-125 in rat haemoglobin is specifically modified by simetryn sulphoxide.

## Materials and methods

### *Reagents*

All chemicals were of analytical grade and were obtained from either Merck or Fluka unless otherwise stated.

[$^{14}$C]Simetryn sulphoxide (sp. radioactivity 3.6 Ci·mol$^{-1}$) was kindly given by Ciba–Geigy. Iodo[$^{14}$C]acetamide (sp. radioactivity 24.9 Ci·mol$^{-1}$) was from New England Nuclear. Liquid-scintillation counting was performed with an Isocap 300 (Searle) instrument, with Lumagel SB (Lumac Systems) as scintillator.

### *Protein modification*

Rat haemoglobins were isolated as described by Garrick *et al.* (1975). Incubation with simetryn sulphoxide (2–8 molar excess over Hb tetramer) was at 4°C in 1 M-Tris/HCl buffer, pH 8.4, at a concentration of 2.5% (w/w) carboxyhaemoglobin. Protein (2–15 mg) and unchanged sulphoxide were separated by gel filtration through Sephadex G-15 equilibrated in 0.5 M-Tris/HCl, pH 8.5. In experiments where subsequent blockage with iodoacetamide was not performed, the fraction (typically <1 ml) containing haemoglobin (2–5 mg) was made to 3% (v/v) in formic acid. Globin was then precipitated by the usual acetone/HCl procedure. Residual acetone was removed by evaporation, and the globin was dissolved in 10% (v/v) formic acid. Portions (containing 3–10 nmol per globin chain) were diluted to 500 $\mu$l with 30% (v/v) formic acid and directly analysed by h.p.l.c.

Fragmentation and subsequent peptide isolation was performed on samples of the simetryn sulphoxide-modified haemoglobin in which the remaining cysteine residues had been blocked by iodoacetamide. Here, the haemoglobin fraction from gel filtration was made 6.0 M in guanidinium chloride, 10 mg of dithiothreitol was added and the mixture was incubated at room temperature. After 2 h, 24 mg (in 100 $\mu$l water) of unlabelled iodoacetamide was added and incubation, under $N_2$ and in the dark, was continued for 30 min. The protein was desalted by

chromatography on Sephadex G-15 in 5% formic acid. Samples were analysed by h.p.l.c. in 500$\mu$l of 30% (v/v) formic acid, either directly or after vortex evaporation.

Parallel experiments were performed on globin from unmodified rat haemoglobin V (Garrick *et al.*, 1975), which was kept at low pH in order to minimize oxidation of thiol groups. Here cysteine modification was performed by simultaneous addition of 500$\mu$l of 0.5 M-Tris base, containing 6.0 M-guanidinium chloride, and iodoacetamide (4$\mu$mol in 100$\mu$l of ethanol), to 2mg of globin in 1% formic acid (80$\mu$l). After 30min, 10mg of dithiothreitol was added to reduce any disulphides that could have been formed during the procedure. After 2h, excess cold iodoacetamide was added and the sample processed as for the sulphoxide-modified sample.

*Peptide fragmentation and characterization*

Fragmentation with CNBr was performed by standard techniques (Gross, 1967). Cleavage with *Staphylococcus aureus* V8 proteinase (Miles Laboratories) was performed in 0.1 M-sodium acetate, pH4.0, containing 2mM-EDTA at 37°C for 16h. Sequencing was with a solid-phase sequencer (Sequemat) modified for use with DABITC as described by Hughes *et al.* (1979*b*) and derivatives were identified by reverse-phase h.p.l.c. (Wilson *et al.*, 1979). Amino acid analysis was performed after hydrolysis with 6 M-HCl as described in an accompanying paper (Wilson *et al.*, 1981*a*). Native carboxyhaemoglobin concentrations were measured by absorbance at 540nm; other peptide/protein determinations were by amino acid analysis.

*H.p.l.c. of peptides*

The h.p.l.c. apparatus for analytical and preparative peptide isolation was described previously (Hughes *et al.*, 1979*a*). Monitoring was done by reaction of *o*-phthalaldehyde or fluorescamine; for globin chains either fluorogen was used, and for smaller peptides, only fluorescamine was utilized. Columns (4.6mm × 250mm) of LiChrosorb (10$\mu$m particle size) RP-8 or RP-18 were developed at room temperature with a flow rate of 0.65ml/min. Buffer compositions were: (i) 0.26 M-trifluoroacetic acid (20ml/litre) and 0.21 M-pyridine (17ml/litre), in water for buffer *A* (pH about 1.5) and in 60% (v/v) propan-1-ol for buffer *B*; (ii) 0.53 M-formic acid (20ml/litre) and 0.125 M-pyridine (9.5ml/litre) in water for *A* (pH3.0) and in 60% (v/v) propan-1-ol for *B*. Formic acid and propan-1-ol ('purum' grade) were each distilled over ninhydrin; trifluoroacetic acid was sequencer grade.

Pooled fractions after chromatography were dried by vortex evaporation (Buchler). Residual salt from the trifluoroacetic acid/pyridine buffers was removed by application of the sample in 10% (v/v) formic acid (500$\mu$l) to an RP-8 column equilibrated with buffer *A* from (ii) above. After 5ml of equilibrating buffer had passed through the column,

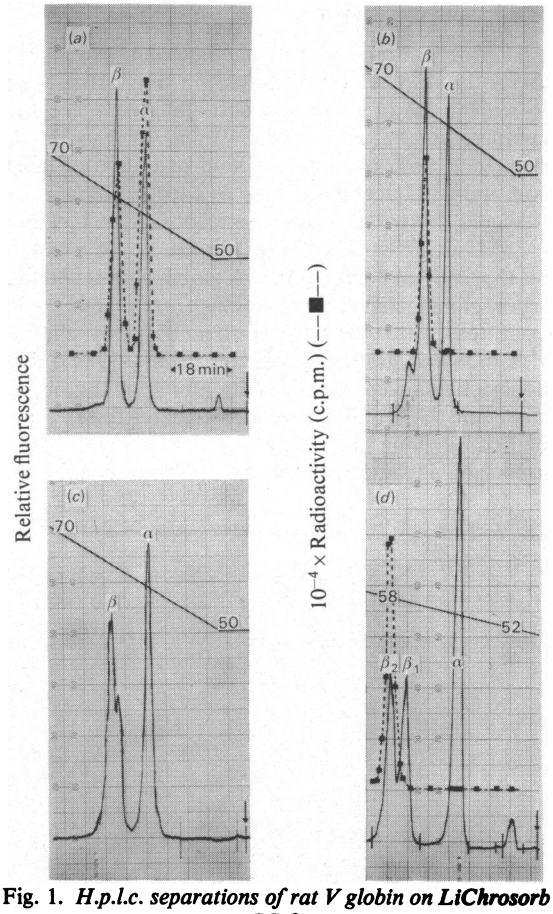


Fig. 1. *H.p.l.c. separations of rat V globin on LiChrosorb RP-8*

(*a*) Iodo[$^{14}$C]acetamide derivative, 3nmol per component; (*b*) [$^{14}$C]simetryn derivative, 30nmol per component; the $\beta$-chain fraction had 0.48mol of label/mol of protein; (*c*) simetryn modification followed by iodoacetamide-derivatization of chains, 3nmol per component; (*d*) [$^{14}$C]simetryn modification followed by iodoacetamide-derivatization of chains; 28nmol of $\alpha$- and $\beta$-chains; the yield of radioactivity (—■—) was quantitative; 92% was present in the $\beta_2$ fraction, which had 0.98mol of label per mol of protein. Elution was with trifluoroacetic acid/pyridine buffers; the gradients of increasing propan-1-ol are shown, marked with the percentages of buffer *B* in *A* + *B* mixture (see the Materials and methods section) at either end. Detection on 2% of the effluent was with *o*-phthalaldehyde in (*a*), (*b*), and (*c*) and with fluram in (*d*). This Figure and similar succeeding ones are shown with the time scale from left to right because a large percentage of recorders work in that manner. Injection points are marked by arrows and the continuous straight line represents the developed gradient.

elution of the peptide was performed with the buffer *B* from (ii) above.

## Results and discussion

The separation of rat haemoglobin V globin chains by h.p.l.c. is illustrated in Fig. 1(*a*). The prominent features of this chromatogram are the complete separation (within 40 min) and yields of more than 95% of protein. The distribution of label from iodo[$^{14}$C]acetamide was 3:2 for $\alpha/\beta$ chains as predicted from the known sequence (Chua *et al.*, 1978; Garrick *et al.*, 1975). It needs to be pointed out that human haemoglobin A globin chains and the major globin chains from goldfish are equally well separated, with yields better than 85%, by elution with modified gradients (Hughes *et al.*, 1981). Rat globin chains were identified by amino acid composition and *N*-terminal sequence (ten residues). This method, having in respect to time and yield important advantages over the classical techniques, which have involved ion-exchange chromatography in 8 M-urea, would appear to be of general use for preparative globin-chain separation in many species.

Similar results were obtained for separation of rat V globin chains from incubations of the native protein with $^{14}$C-labelled sulphoxide. A typical chromatogram is shown in Fig. 1(*b*). Virtually all radioactivity was associated with the $\beta$-chain, and in this particular experiment the yield was >95%. The other rat haemoglobins gave analogous results. However, calculation of the stoichiometry of the reaction from the specific radioactivity gave only 0.5 of a molecule per $\beta$-chain. This value was achieved within 3 h of incubation and could not be increased by quadrupling the molar excess of sulphoxide over Hb or by increasing incubation time to 15 h. Under identical incubation conditions human haemoglobin incorporated only minimal (40 times less) amounts of sulphoxide. This observation was completely unexpected, since incubation of [$^{14}$C]sulphoxide with glutathione indicated binding to thiol groups. An

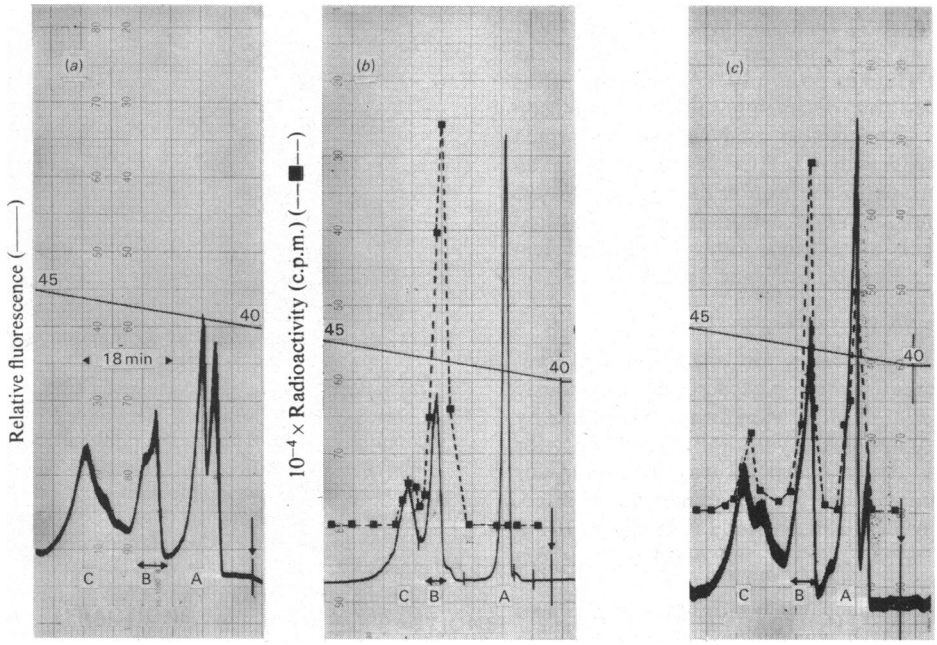


Fig. 2. *Separation of CNBr peptides from rat haemoglobin β-chains on LiChrosorb RP-18*
(*a*) $\beta_1$-Peptides, 9 nmol of sample. The $\beta_1$ fraction from the chromatography shown in Fig. 1(*d*) was dried by vortex evaporation and then desalted by reverse-phase h.p.l.c. 70% (v/v) Formic acid (500 $\mu$l) containing 1 mg of CNBr was added to the dried residue and incubation was at room temperature overnight. After removal of CNBr by vortex evaporation, the sample (90%) was applied in 500 $\mu$l of 30% formic acid; (*b*) $\beta_2$-peptides, 23 nmol from a similar separation (50 nmol per $\alpha$, $\beta$-chain) to that shown in Fig. 1(*d*). The sample was treated as in (*a*). Of the radioactivity applied to the column, 80% was found in pool B; *N*-terminal sequence (see Fig. 3) and amino acid analysis identified B as the *C*-terminal peptide (residues 110–146) with 0.94 mol of label/mol of peptide. (*c*) $\beta$-Chain peptides modified with iodo[$^{14}$C]acetamide (see Fig. 1*a*). Formic acid/pyridine buffers were eluants; the gradients of increasing propan-1-ol are shown, with the percentage of buffer *B* in *A* + *B* at either end. Of the effluent, 2.5% was removed for detection with fluram.

acid hydrolysis of the tripeptide yielded glycine and glutamic acid in stoichiometric amounts, plus two radioactively labelled peaks, one eluting immediately before carboxymethylated cysteine and the other co-eluting with glutamic acid. Hydrolysis of the labelled $\beta$-chain (Fig. 1*b*) gave peaks of radioactivity on amino acid analysis similar to the ones obtained in the glutathione experiment. This highly specific binding to rat haemoglobin but not to human haemoglobin and the indication that a cysteine residue was involved, prompted us to characterize this reaction more fully. In view of the fact that rat and human haemoglobins have the 'reactive' Cys-$\beta$-93 residue in common, comparable binding to the proteins of both species would have been expected.

Fig. 1(*c*) illustrates the chain separation of rat haemoglobin incubated with non-radioactive sulphoxide and subsequent modification of the remaining thiol groups by iodoacetamide. This indicates $\beta$-chain heterogeneity. In a second experiment, where the elution gradient was modified, the chains now carrying the [$^{14}$C]sulphoxide, henceforth designated as '$\beta_2$' were quantitatively separated from the ones ($\beta_1$) that had not bound the label (Fig. 1*d*).

Fig. 2(*a*) shows the chromatogram of CNBr peptides obtained from $\beta_1$ chains (Fig. 1*d*), whereas Fig. 3(*b*) shows the peptides from $\beta_2$ (Fig. 1*d*). Here all radioactivity (yield 80%) was associated with the fragment in pool B. As expected from the results in Fig. 1(*d*), this peptide containing the simetryn derivative elutes after the same peptide from the $\beta_1$-chain (Fig. 2*a*), which carries a carboxymethylated cysteine residue instead. Peptides were identified by *N*-terminal sequence as: A, internal peptide (residues 56–109); B, the *C*-terminal peptide (110–146); and C, the *N*-terminal peptide (1–55). Composition data on the B-peptide from Fig. 2(*b*) was in perfect agreement. Finally, Fig. 2(*c*) illustrates the distribution of $^{14}$C-labelled iodoacetamide in peptides A and B, as is predicted from the known sequence.

Fig. 3 illustrates the results from solid-phase sequencing of peptide B from Fig. 2(*b*). Radioactivity was only found in cycle 16, with an overlap into cycle 17. This corresponds to residue Cys-125

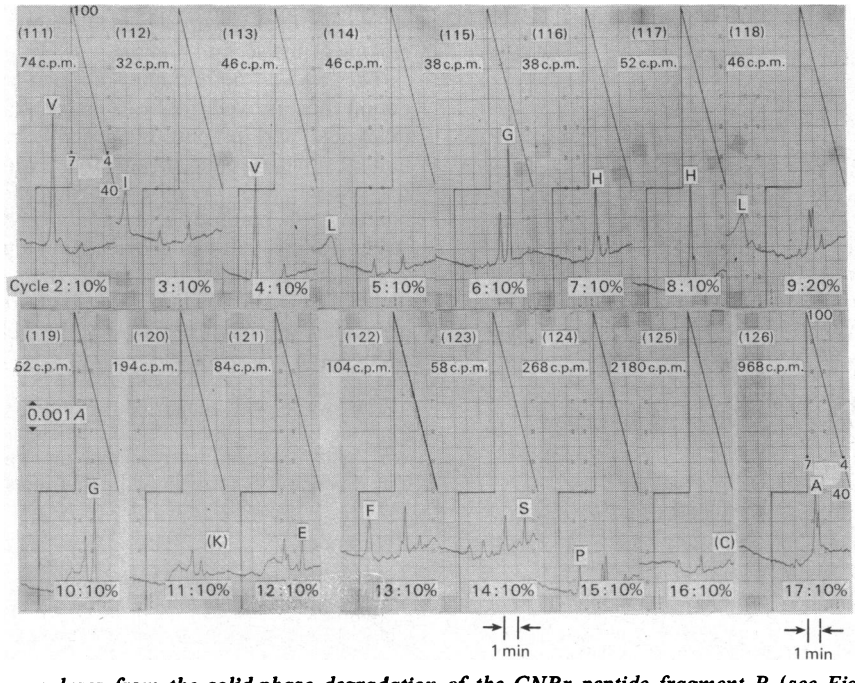


Fig. 3. *H.p.l.c. analyses from the solid-phase degradation of the CNBr peptide fragment B (see Fig. 2) carrying the [$^{14}$C]simetryn label*

Radioactive fragment from Fig. 2(*b*) (1.6 nmol, about 10%) was coupled in Quadrol buffer to aminopropyl-glass, via the DITC derivative. Peptide (1.2 nmol, 75% coupling yield) (containing 9000 d.p.m.) was degraded with DABITC as described for the Sequemat instrument by Hughes *et al.* (1979*b*). DABTH derivatives were identified by reverse-phase h.p.l.c. (Wilson *et al.*, 1979). Detection was by absorbance at 440 nm. Half of the fraction from each cycle was taken for liquid-scintillation counting of radioactivity. The total c.p.m. are shown (top middle) for each cycle; the counting efficiency was 80%. The radioactivity in cycle 16, namely 2700 d.p.m. (2200 c.p.m.), represents a 93% repetitive yield. The percentage of each fraction injected for identification (bottom centre) and position (in parentheses, top centre) in the rat $\beta$-chain are shown.

of the β-chain. As additional confirmation we obtained an *S. aureus* proteinase digest of peptide B (Fig. 2*b*) and separated the resulting peptides (Fig. 4). The radioactivity was then found in the fourth cycle of sequence of the radioactive peptide from Fig. 4, in keeping with the fact that enzymic cleavage occurred at residue 121, glutamic acid.

Position 123, in close contact with the binding site has been reported to have allelic variation of either serine or threonine; however, we found no differences in sequence between B-peptides. Serine was found exclusively in this position.

Analysis of the stereochemical positions of cysteine residues β-93 and β-125 yielded the following information: β-93 is buried in a cleft, the access to which is hindered by the carboxy group of Asp-β-94 and His-β-146. Whereas this apparently does not impede the access of *p*-chloromercuribenzoate, it does so for the sulphoxide derivative of *s*-triazines. Cys-β-125, on the other hand, is entirely on the surface of the haemoglobin molecule and is surrounded by hydrophobic residues. The access for the large sulphoxide derivative is not hindered. Two other cysteine residues, α-104 and α-111 in the rat haemoglobin tetramer, are located in the interchain contact surfaces and are therefore not to be considered. Additionally there would appear to be an effective blockage of Cys-α-13 by the residue Asn-α-12. These peculiar properties of the reaction described here appear to make the sulphoxide derivative a useful reagent for mapping certain thiol groups.

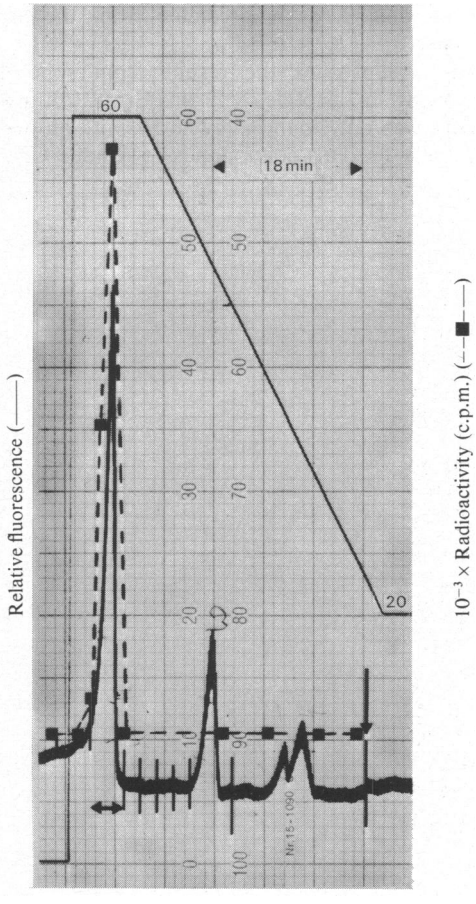


Fig. 4. *Peptides from the S. aureus proteinase digest of the [¹⁴C]simetryn-labelled CNBr peptide*

Half of the fraction B from Fig. 2(*b*) was dried, dissolved in 50μl of sodium aceate, pH4.0, and incubated overnight with 5μg of *S. aureus* proteinase. Of this material, 6nmol (70%) was injected in 500μl of 10% formic acid and chromatographed as described in Fig. 2. The yield of radioactivity was 87%.

Why only one of the two Cys-β-125 residues in the tetramer reacted has no apparent explanation. Whether the formation of higher aggregates than tetramers protects some β-chains requires further analysis. It is, however, noteworthy that rat haemoglobins are quite insoluble at neutral pH. Alternatively, incorporation of one molecule of simetryn within the tetramer may cause a change in the stereochemical environment around the Cys-125 residue of the second β-chain.

The h.p.l.c. methodology applied to this investigation has proved to be a rapid technique, both analytical and preparative, capable of high chromatographic resolution, and from which yields are respectably high. We believe that no other combination of methods commonly used in protein structural analysis can provide such a performance.

Finally, it must be stated that the common practice of the testing of pharmaceuticals and pesticides in rodents may not be relevant to the effects on other species.

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