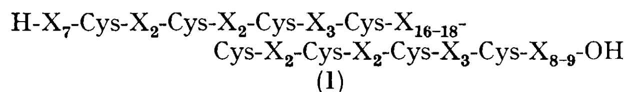
Studies relating to the Ferredoxins. Part 2.[1] Exchange Reactions of Some Cysteine–Glycine Peptides with the Iron–Sulphur Cluster Compound Bis(tetramethylammonium) Tetrakis(μ_3-sulphido-t-butylthioiron)

By **Roger J. Burt, Brian Ridge,** and **H. N. Rydon,*** Department of Chemistry, The University, Exeter EX4 4QD

Four peptides of the general structure Ac-Gly$_2$-(Cys-Gly$_2$)$_n$-Cys-Gly$_2$-NH$_2$ (n = 0—3) have been synthesised. They all exchange readily in dimethyl sulphoxide solution with the title iron–sulphur cluster compound to give peptide iron–sulphur cluster compounds. The exchange reactions have been followed quantitatively by ^{1}H n.m.r. spectroscopy. The four successive equilibrium constants have been evaluated in the case of the unidentate ligand (n = 0), but the other three cases are too complex for such a treatment.

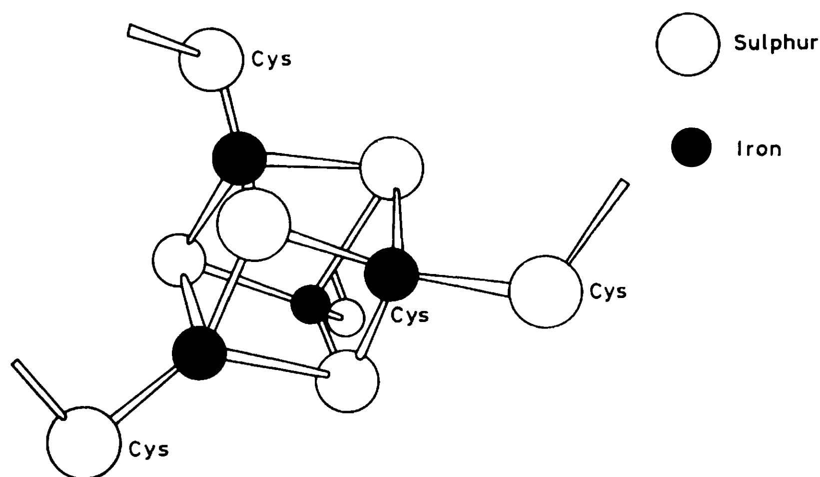
THE ferredoxins[2] are a widely distributed class of iron–sulphur proteins, present in bacteria, algae, higher plants, and mammals, which function as electron-transfer agents in many metabolic processes, ranging from photosynthesis and nitrogen fixation to steroid hydroxylation. Most bacterial ferredoxins contain eight iron and eight 'inorganic' sulphur atoms attached to very similar apoproteins with the general structure (1; X = an amino-acid residue other than cysteine). The electron-transfer



H-X$_7$-Cys-X$_2$-Cys-X$_2$-Cys-X$_3$-Cys-X$_{16-18}$-Cys-X$_2$-Cys-X$_2$-Cys-X$_3$-Cys-X$_{8-9}$-OH

(1)

properties are associated with two approximately cubic clusters (2) of four iron and four sulphur atoms attached by the iron atoms to cysteine (Cys) side chains in the apo-protein. The *X*-ray studies of Jensen and his co-workers[3] have shown that in *Peptococcus aerogenes* ferredoxin the four cysteine residues attached to each cubic cluster are not those closest together in the primary structure, but three from one such group and one from the other, as in Figure 1; it seems almost certain that a similar arrangement occurs in the other [8Fe–8S] bacterial ferredoxins. This unexpected arrangement seems both to provide stabilising cross linkages and to encase the cluster in a protective hydrophobic environment of non-polar amino-acid side chains.



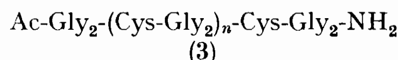
(2)

We have embarked on a programme of work with model cysteine-containing peptides designed to elucidate the influence of (a) the number and spacing of the cysteine residues and (b) the nature of the non-cysteine side chains on the formation, stability, and properties of ferredoxin-like cluster compounds derived from these. The present paper is concerned with the formation of such cluster compounds from cysteine–glycine peptides with an inter-cysteine spacing of two amino-acid residues. Since our work began, Holm and his co-workers[4,5] have published the results of similar studies.

RESULTS AND DISCUSSION

Model building showed that a tetracysteine peptide of the type H-Cys-X$_n$-Cys-X$_n$-Cys-X$_n$-Cys-OH could be wrapped around a cubic Fe$_4$S$_4$ cluster only if $n \geqslant 2$; when $n = 2$, β loops involving hydrogen bonding of the CO of the ith cysteine residue to the NH of the (i + 1)th cysteine residue can be formed throughout the sequence. It was for this reason that we adopted a two-glycine spacing in these initial studies. To reduce possible interference from the *N*-terminal amino- and *C*-terminal carboxyl groups we replaced these by

N-acetylglycylglycyl and glycylglycine amide respectively. The synthetic goal was therefore the four peptides represented by (3; $n = 0, 1, 2,$ or 3).*



$$\text{Ac-Gly}_2\text{-(Cys-Gly}_2)_n\text{-Cys-Gly}_2\text{-NH}_2$$
(3)

In order to obviate possible racemisation of the L-cysteinyl residues, and to economise in effort, we used the fragment-condensation strategy, based on cysteinyldiglycyl units, shown for the tetradecapeptide case in the Scheme. The key intermediates, prepared in

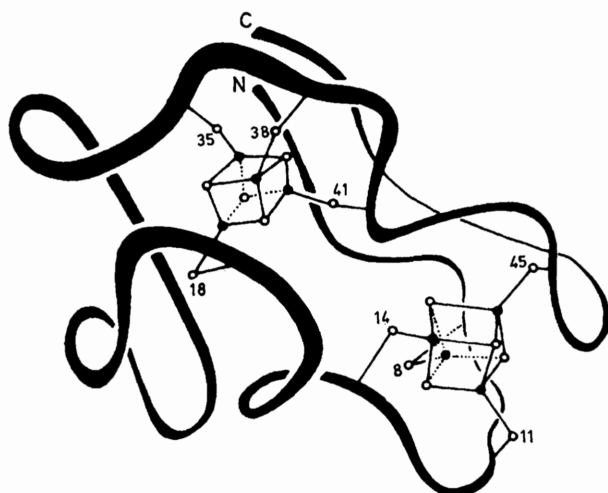
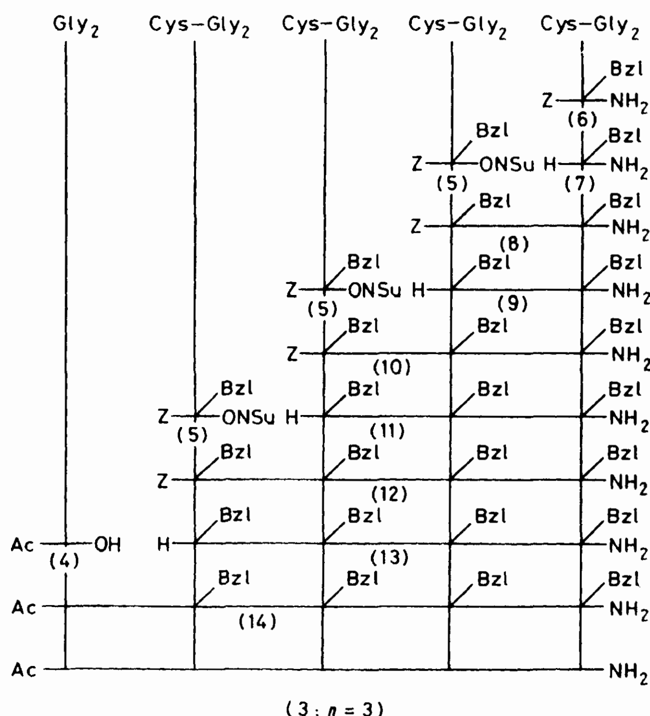


FIGURE 1 Structure of *P. aerogenes* ferredoxin

quantity, were *N*-acetylglycylglycine (4),[6] *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycine succinimido-ester (5), and the corresponding amide (6). *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycine was prepared in good yield, by coupling *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine succinimido-ester [7] with glycylglycine in aqueous dioxan in the presence of sodium hydrogencarbonate, and converted, also in good yield, into its succinimido-ester (5) by means of *N*-hydroxysuccinimide and *NN'*-dicyclohexylcarbodi-imide in dimethylformamide; the ester (5) decomposes on keeping and was therefore used at once for further coupling reactions. Glycylglycinamide was best prepared by removal of the *N*-protecting group from its *N*-benzyloxycarbonyl derivative [8] by catalytic hydrogenolysis or with hydrogen bromide in acetic acid; the former process was preferred owing to the inconvenient presence of additional hydrogen bromide in the hydrobromide prepared by the latter process. Condensation with *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine succinimido-ester in aqueous dioxan gave the required protected tripeptide amide (6) in good yield.

For the synthesis of the protected tetradecapeptide (14) (see Scheme), the *N*-protecting group was removed from (6) with hydrogen bromide in acetic acid and the resulting tripeptide amide (7) coupled with the succinimido-ester (5) in aqueous dioxan in the presence of sodium hydrogencarbonate. Since this coupling procedure gave the product (8) in only 70% yield, subsequent succinimido-ester couplings of (5), with (9) and (11), were carried out in anhydrous dimethylformamide, in the presence of a catalytic amount of acetic acid, which notably increased the yield. In all cases in the synthesis, *N*-benzyloxycarbonyl groups were removed, from (8), (10), and (12), by treatment with hydrogen bromide in acetic acid. Owing to the instability of *N*-acetylglycylglycine succinimido-ester, the terminal *N*-acetylglycylglycine residue was added to the partially deprotected dodecapeptide (13) by the mixed carbonic anhydride method, in dimethylformamide, to give the required *N*-acetyltetradecapeptide (14). The analogous protected *N*-acetyl-penta-, -octa-, and -undeca-peptides were obtained similarly from the partially deprotected peptides (7), (9), and (11), respectively.

Two methods were used to remove the *S*-benzyl groups from the protected peptides to give the free cysteine



SCHEME

peptides (3; $n = 0$—3). For complexing studies in aqueous solution, in which the presence of sodium chloride is of no consequence, the usual reductive procedure with sodium in liquid ammonia was used. For ligand-exchange reactions with pre-formed cluster compounds, however, the presence of sodium chloride is unacceptable and we therefore used cleavage with anhydrous hydrogen fluoride in the presence of anisole as carbonium-ion scavenger.[9] Both boron tris(trifluoroacetate) [10] and trifluoromethanesulphonic acid [11] were unsatisfactory for this purpose; the former reagent failed to remove the *S*-benzyl groups completely and the latter led to exten-

* Here and elsewhere abbreviations are those recommended by the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature (*Specialist Periodical Report*, 'Amino-acids, Peptides and Proteins,' The Chemical Society, London, 1972, vol. 4, ch. 5).

sive cleavage of peptide bonds. After work up of the hydrogen fluoride cleavage reactions, the products contained considerable amounts (up to 35%) of disulphide, as shown by estimation of free thiol with 4,4′-bis(dimethylamino)diphenylcarbinol;[12] reduction with 2-mercaptoethanol gave the pure cysteine peptides (3; $n = 0$—3).

Attempts to prepare iron–sulphur cluster compounds from the peptides (3) and ammonium iron(II) sulphate and sodium sulphide in aqueous solution [tris(hydroxymethyl)methylamine (tris) buffer, pH 7.4], with or without added dithiothreitol (*threo*-1,4-dimercaptobutane-2,3-diol), a procedure similar to that used to reconstitute ferredoxins from apoferredoxins,[13] were unsuccessful;* the electronic spectra of the resulting solutions showed no real resemblance to that of ferredoxin. So also were attempts to synthesise such compounds in methanolic solution from the peptides, iron(III) chloride, and sodium hydrogensulphide by the technique used successfully in the synthesis of cluster compounds from simple thiols (see below). The products were very dark brown powders, only one of which, that from (3; $n = 0$), was sufficiently soluble for n.m.r. spectroscopy; the ^1H n.m.r. spectrum of this was that of a diamagnetic species, no contact-shifted resonances being observed. Holm and his co-workers [16] have reported a similar lack of success with *N*-acetylcysteine methylamide.

At this stage in our work, Holm and his co-workers [4,16] showed that the cluster compound from *N*-acetylcysteine methylamide could readily be prepared by ligand exchange with the t-butyl cluster compound (15), used as its bis(tetramethylammonium) salt; subsequently,[5] they prepared similar compounds in this way from the tridentate nonapeptide Boc-Gly-Cys-Gly_2-Cys-Gly_2-Cys-Gly-NH_2 and the quadridentate dodecapeptide Boc-Gly-Cys-Gly_2-Cys-Gly_2-Cys-Gly_2-Cys-Gly-NH_2. We accordingly investigated the behaviour of our four peptides (3; $n = 0$—3) in the exchange reaction (i).

$$[Fe_4S_4(SBu^t)_4]^{2-} + 4\ RSH \rightleftharpoons [Fe_4S_4(SR)_4]^{2-} + 4Bu^tSH \quad (i)$$
(15)

Preliminary studies with the unidentate pentapeptide (3; $n = 0$) and the bis(tetramethylammonium) salt [17,18] of (15) and the bidentate octapeptide (3; $n = 1$) and the bis(tetraphenylarsonium) salt [17,19] of (15) showed that exchange was rapid in hexadeuteriodimethyl sulphoxide at 31 °C and that, with an excess of peptide, there was complete displacement of the SBu^t ligands. The reactions were followed by means of the ^1H n.m.r. spectra, in which the broad signal at $\delta = 2.6$ p.p.m. from hmd,† due to the protons of the co-ordinated SBu^t groups, was progressively replaced by a sharp signal at $\delta = 1.3$ p.p.m., due to the CH_3 protons of the liberated Bu^tSH. Of the two salts of (15), the bis(tetramethylammonium) salt seemed the more suitable for quantitative studies. Such studies were carried out with all four cysteine peptides (3; $n = 0$—3) in hexadeuteriodimethyl sulphoxide at 31 °C, aliquots of solutions of the

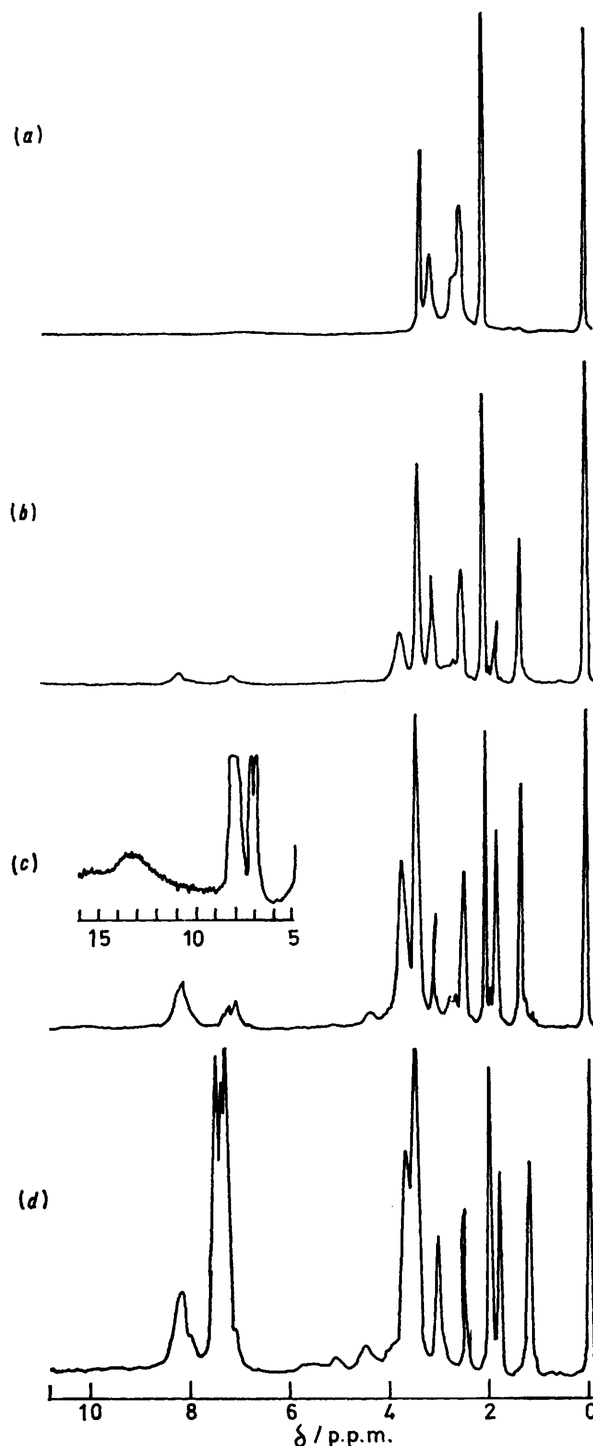
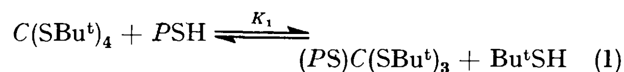


FIGURE 2 Proton n.m.r. spectra for exchange of the cluster compound (15) with the unidentate pentapeptide ligand (3; $n = 0$): (*a*), (15) plus Me_2CO and hmd; (*b*), after addition of 3.2 equivalents of peptide SH per mol (15); (*c*), after addition of 9.2 equivalents of peptide SH per mol (15); (*d*), after addition of excess of PhSH

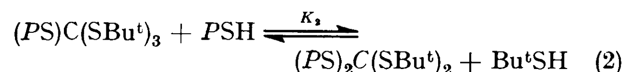
* We have since found [14] that the peptide cluster compounds can be prepared from their components in 8% aqueous dimethyl sulphoxide solution by the procedure of Christou *et al.*,[15] providing the iron(III) chloride is added last to prevent the precipitation of iron sulphide.

† hmd. = $Me_3SiOSiMe_3$.

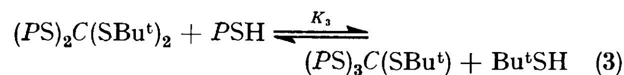
peptides being added to a solution of the cluster compound to which a known amount of acetone had been added; the solutions were made up and the exchange experiments carried out under strictly anaerobic conditions (nitrogen rigorously freed of oxygen). The 100 MHz spectra of the solutions were recorded after each addition of peptide. Figures 2 and 3 show some of the spectra obtained with the unidentate pentapeptide (3; $n = 0$) and the quadridentate tetradecapeptide (3; $n = 3$), respectively. The amount of Bu^tSH released during the course of the exchange reaction was determined by the integrated area of the t-butyl peak at $\delta = 1.3$ p.p.m. relative to that of the acetone peak at $\delta = 2.0$ p.p.m. The 100% exchange amount of Bu^tSH was determined by adding an excess of benzenethiol, to displace all aliphatic ligands,[19] and again measuring the areas of the peaks at $\delta = 1.3$ and 2.0 p.p.m. The persistence of the Fe_4S_4 cluster throughout the exchange was shown by an accumulated spectrum, run at the end of the reaction, but before adding benzenethiol. In all four cases a very broad peak, centred at $\delta = 13$ p.p.m., due to the co-ordinated cysteine CH_2 groups, was obtained;[5] the width of this band was *ca.* 300 Hz for the cluster compound from the pentapeptide (3; $n = 0$) and *ca.* 600 Hz for the other three. To confirm the persistence of the cluster, electronic spectra were recorded in dimethyl sulphoxide at room temperature; addition of the peptide changed the spectrum of the initial t-butyl cluster ($\lambda_{max.}$ 302, ε 21 900; $\lambda_{max.}$ 415 nm, ε 16 700 $dm^3\,mol^{-1}\,cm^{-1}$) [19] into that of the peptide cluster ($\lambda_{max.}$ 282—290, ε 22 600—25 500; $\lambda_{max.}$ 402—408 nm, ε



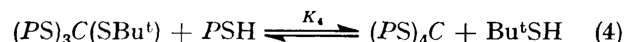
$$C(SBu^t)_4 + PSH \underset{}{\overset{K_1}{\rightleftharpoons}} (PS)C(SBu^t)_3 + Bu^tSH \quad (1)$$



$$(PS)C(SBu^t)_3 + PSH \underset{}{\overset{K_2}{\rightleftharpoons}} (PS)_2C(SBu^t)_2 + Bu^tSH \quad (2)$$



$$(PS)_2C(SBu^t)_2 + PSH \underset{}{\overset{K_3}{\rightleftharpoons}} (PS)_3C(SBu^t) + Bu^tSH \quad (3)$$



$$(PS)_3C(SBu^t) + PSH \underset{}{\overset{K_4}{\rightleftharpoons}} (PS)_4C + Bu^tSH \quad (4)$$

15 800—17 000 $dm^3\,mol^{-1}\,cm^{-1}$); addition of benzenethiol then gave the characteristic spectrum of the benzenethiol cluster ($\lambda_{max.}$ 458 nm, ε 17 600 $dm^3\,mol^{-1}\,cm^{-1}$).[5,19]

In the case of the unidentate ligand (3; $n = 0$) the experimental results can be very satisfactorily interpreted in terms of the four stages (1)—(4) of the exchange reaction [$C(SBu^t)_4 \equiv$ (15); $PSH \equiv$ (3; $n = 0$)]. The four equilibrium constants can readily be evaluated using the modified Bjerrum formation function (5),[20]

$$\bar{n} = \frac{K_1\alpha + 2K_1K_2\alpha^2 + 3K_1K_2K_3\alpha^3 + 4K_1K_2K_3K_4\alpha^4}{1 + K_1\alpha + K_1K_2\alpha^2 + K_1K_2K_3\alpha^3 + K_1K_2K_3K_4\alpha^4} \quad (5)$$

where $\bar{n}$ is the average degree of replacement of the *S*-t-butyl residues (*i.e.* mols Bu^tSH released per mol of cluster compound) and $\alpha = [PSH]/[Bu^tSH]$. In the curve-fitting process we imposed the further limitation that the four equilibrium constants should be as nearly

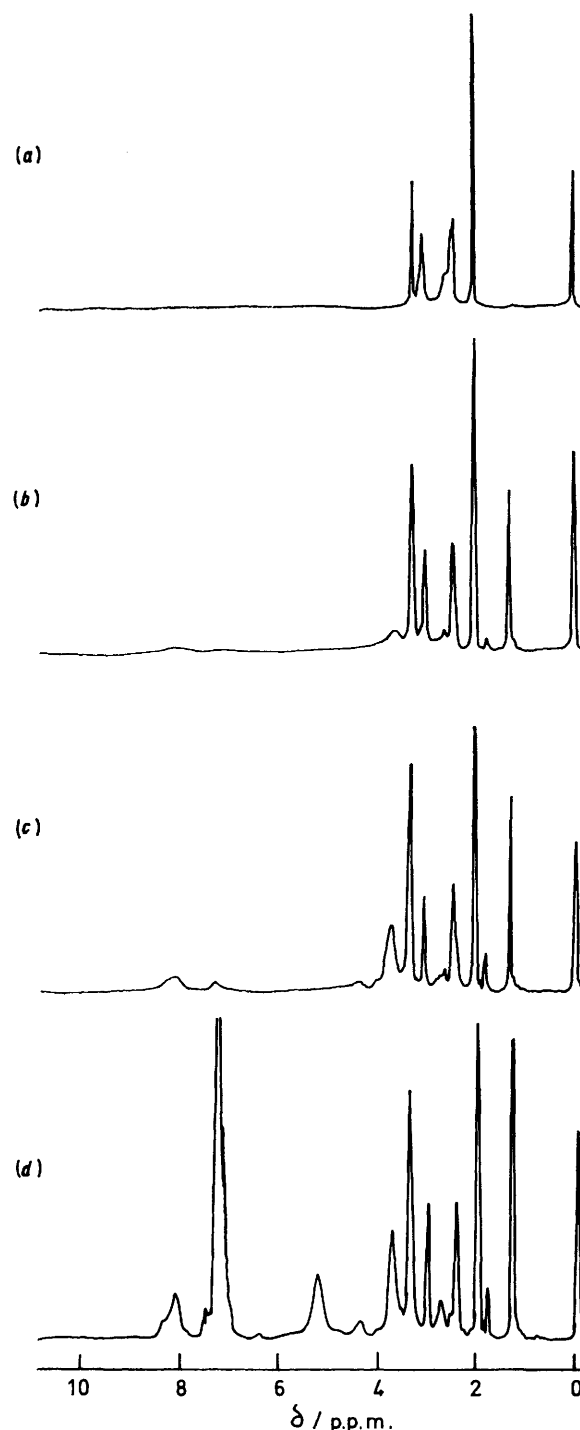


FIGURE 3 Proton n.m.r. spectra for exchange of the cluster compound (15) with the quadridentate tetradecapeptide ligand (3; $n = 3$): (*a*), (15) plus Me_2CO and hmd; (*b*), after addition of 3.6 equivalents peptide SH per mol (15); (*c*), after addition of 12.0 equivalents peptide SH per mol (15); (*d*), after addition of excess of PhSH

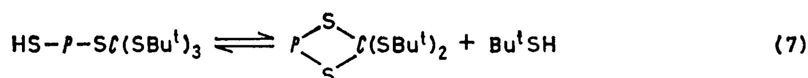
as possible statistically related ($K_1/K_2 = 8/3$; $K_2/K_3 = 9/4$; $K_3/K_4 = 8/3$).[20] The experimental results for the

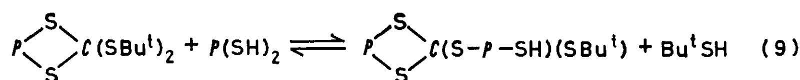
unidentate ligand (3; $n = 0$) are shown in Figure 4, together with the formation curve calculated for the following values of the equilibrium constants:

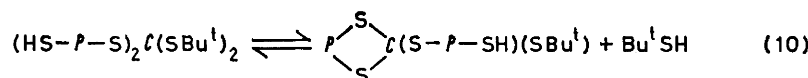
K_1	K_2	K_3	K_4	K_1/K_2	K_2/K_3	K_3/K_4
16	8	4	2	2	2	2

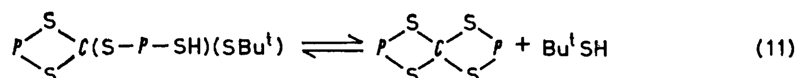
The fit is very satisfactory (standard deviation ±0.06) and the ratios of the equilibrium constants are at least as close to those expected statistically as in most cases in the literature. Although the deviation from the theoretical ratios is in the opposite sense to that usually encountered [21] it is noteworthy that Holm and his co-workers [19] found similar, lower, values ($K_1/K_2 = 2.0$, $K_2/K_3 = 1.7$, and $K_3/K_4 = 1.9$) for the ligand-exchange reaction of (15) with toluene-4-thiol.

This treatment cannot properly be applied in the case of the polydentate ligands (3; $n = 1$—3) owing to the complexity of the exchange reactions. Thus, even in the simplest case of the bidentate ligand (3; $n = 1$) at least six equilibria are involved, all of which have to be considered separately since, owing to the large distance between them, the thiol groups will react independently [equations (6)—(11)].[22] The results for the polydentate ligands are shown in Figures 5—7 with the calculated formation curve for the unidentate ligand for comparison. It will be seen that in all three cases complete exchange occurs with a smaller excess of peptide than is required in the unidentate case. The flattening out of the formation curve in the later stages is probably due to the presence of some disulphide in the peptides, as shown by the appearance in the n.m.r. spectra of a small separate peak due to t-butyl disulphide accompanying the main peak due to Bu^{t}SH.









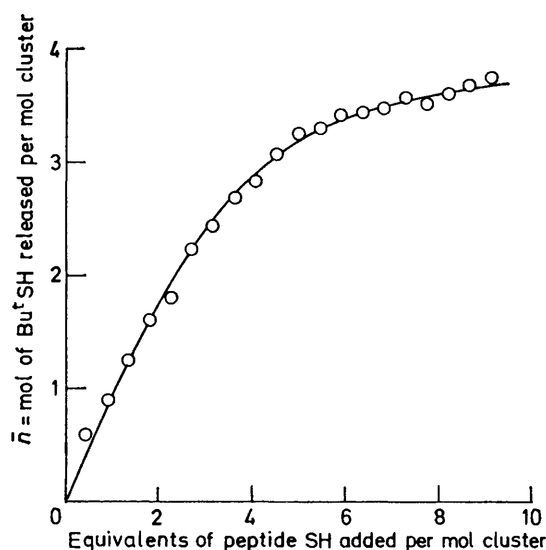


FIGURE 4 Ligand-exchange reaction between (15) and (3; $n = 0$). The line is calculated for $K_1 = 16$, $K_2 = 8$, $K_3 = 4$, and $K_4 = 2$

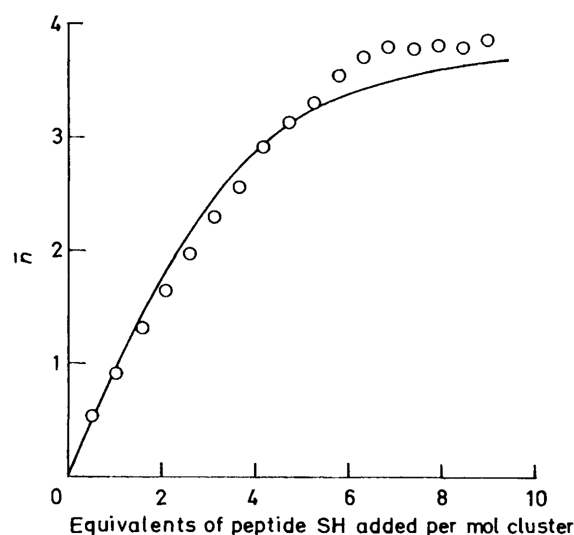


FIGURE 5 Ligand-exchange reaction between (15) and (3; $n = 1$). The line is calculated as for Figure 4

Although our results show that the number of thiol groups in the peptide ligands has no marked effect on their ability to form cluster compounds they throw no light on the question as to whether these are monomeric or polymeric with respect to the central Fe_4S_4 core.

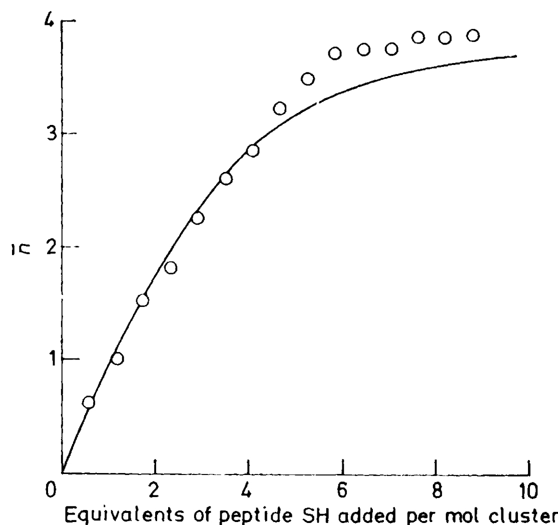


FIGURE 6 Ligand-exchange reaction between (15) and (3; $n = 2$). The line is calculated as for Figure 4

Holm and co-workers [5] assumed, for simplicity, that the peptide cluster compounds they prepared were monomeric. Although it seems most likely that this is the case for the cluster compounds from the uni- and bidentate peptide ligands (3; $n = 0$ and 1), stoicheiometry requires the cluster from the tridentate ligand (3; $n = 2$)

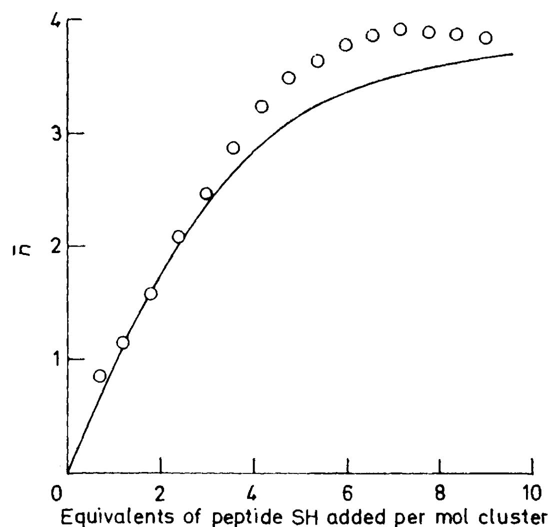


FIGURE 7 Ligand-exchange reaction between (15) and (3; $n = 3$). The line is calculated as for Figure 4

to be at least trimeric. The peptide cluster from the quadridentate ligand (3; $n = 3$) could equally well be monomeric, as in *Chromatium* high-potential iron protein,[23] or dimeric as in most bacterial ferredoxins. The ^{1}H n.m.r. and u.v. spectra of all four of our peptide cluster compounds are very similar and afford no evidence on this question, which remains an open one.

EXPERIMENTAL

All solvents were dried and purified by appropriate methods; organic solutions were dried over magnesium sulphate and evaporated or concentrated under reduced pressure at room temperature using a rotary evaporator. Reactions in liquid hydrogen fluoride were carried out in a Kel–F vacuum line (Protein Research Foundation, Osaka). The purity of all peptides and peptide derivatives was confirmed by t.l.c. in at least two solvent systems and their structures by ^{1}H n.m.r. and, where appropriate, i.r. spectroscopy; decomposition points are not recorded. Iron–sulphur cluster compounds were manipulated in an atmosphere of oxygen-free nitrogen, purified by passage over BASF Catalyst R3-11 at 145—150 °C, in a Schlenk-type apparatus [24] or a gas-tight Perspex glove-box (Mecaplex model G.B. 3011).

Optical rotations * were measured with a Bendix N.P.L. 143A polarimeter, i.r. spectra with a Perkin-Elmer 237 recording spectrophotometer, and u.v.–visible spectra with a Pye–Unicam SP 800B recording spectrophotometer. Proton n.m.r. spectra were recorded on either a Perkin-Elmer R10 60 MHz spectrometer at 33.5 °C or on a JEOL MH-100 100 MHz spectrometer at 31 °C, the latter instrument being used for the quantitative studies; chemical shifts (δ) are given in p.p.m. from tetramethylsilane.

(*A*) *Synthesis of Peptides.—General methods.* (*a*) *Couplings using succinimido-esters in aqueous dioxan.* An aqueous solution of the amino-component was added to a solution of the succinimido-ester of the carboxyl component (1 equivalent) in dioxan and the mixture stirred at room temperature for 24 h, after which an equal volume of water was added. In the case of peptide amides, the product precipitated; in the case of peptide sodium salts, the solution was cooled to 0 °C and the product precipitated by acidification (pH 2) with concentrated hydrochloric acid. In either case, the product was filtered off after some hours at 0 °C and recrystallised.

(*b*) *Couplings using succinimido-esters in anhydrous media.* Triethylamine (1 equivalent) was added to the hydrobromide of the amino-component in the anhydrous solvent. After stirring for 5 min at room temperature, the succinimido-ester of the carboxyl component (1 equivalent) was added, the mixture stirred at room temperature until the reaction was complete (t.l.c.), and the solvent removed by evaporation.

(*c*) *Mixed anhydride couplings.* Triethylamine (1 equivalent) was added to a solution of the carboxyl component in anhydrous dimethylformamide. After cooling to −15 °C, ethyl or isobutyl chloroformate (1 equivalent) was added and the mixture stirred for 10 min at −15 °C. A solution of the amino-component (1 equivalent; from the hydrobromide and triethylamine) in dimethylformamide was added, the mixture stirred for 1 h at −10 °C, left overnight at room temperature, and evaporated.

(*d*) *Selective removal of* N-*benzyloxycarbonyl groups.* A solution (45% w/v) of hydrogen bromide in acetic acid, with or without added trifluoroacetic acid, was added to the finely powdered *N*-benzyloxycarbonyl derivative and the mixture stirred vigorously at 20 °C. After 50 min the

* $\alpha_m(t,\lambda) = [\alpha]_\lambda^t$, the units being ^{0} cm^3 dm^{-1} g^{-1}; concentrations c are in g per 100 cm^3.

resulting hydrobromide was precipitated with anhydrous diethyl ether or with water, washed suitably (finally with anhydrous ether), and dried *in vacuo* over sodium hydroxide pellets.

N-*Benzyloxycarbonyl*-S-*benzyl*-L-*cysteinylglycylglycine succinimido-ester*, (5). Glycylglycine hydrochloride monohydrate [25] (14.3 g, 75 mmol), in aqueous 1 mol dm^{-3} sodium hydrogencarbonate (150 cm^3), was coupled by method (*a*) with *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine succinimido-ester [7] (33.3 g, 75 mmol) in dioxan (150 cm^3). The resulting *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycine (30.2 g, 87%) was recrystallised from aqueous methanol, m.p. 128—129 °C, α_m (22 °C, 589 nm) −20.3 (*c* 1.0 in MeOH) [lit.,[26] m.p. 129—130 °C, α_m (20 °C, 589 nm) −9.4]. *N*-Hydroxysuccinimide (2.88 g, 25 mmol) was added to this tripeptide (13.0 g, 25 mmol) in anhydrous dimethylformamide (100 cm^3); after cooling to 0 °C, *NN'*-dicyclohexylcarbodi-imide (5.15 g, 25 mmol) was added and the mixture stirred for 2 h at 0 °C. Next day the precipitated dicyclohexylurea was filtered off and washed with acetone. The combined filtrate and washings were evaporated and the residue dissolved in a little acetone. After 4 h at −10 °C a little more urea was filtered off. Evaporation of the filtrate and recrystallisation of the residue from ethyl acetate gave the *tripeptide ester*, (5) (11.2 g, 80%), m.p. 146—148 °C, α_m (22 °C, 589 nm) −24.8 (*c* 1.0 in dioxan) (Found: C, 56.3; H, 5.2; N, 9.9. $C_{26}H_{28}N_4O_8S$ requires C, 56.1; H, 5.1; N, 10.1%).

N-*Benzyloxycarbonyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*, (6). Glycylglycinamide acetate [8] (7.65 g, 40 mmol) in water (120 cm^3) was coupled by method (*a*) with *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine succinimido-ester [7] (17.75 g, 40 mmol) in dioxan (120 cm^3). Recrystallisation of the product from aqueous methanol gave the *tripeptide amide*, (6) (15.52 g, 85%), m.p. 149—150 °C, α_m (24 °C, 589 nm) −20.8 (*c* 1.0 in MeOH) (Found: C, 57.9; H, 5.7; N, 11.7. $C_{22}H_{26}N_4O_5S$ requires C, 57.6; H, 5.7; N, 12.2%).

N-*Acetyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*. *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycinamide, (6) (10.0 g, 22 mol), in acetic acid (20 cm^3) was treated with 45% hydrogen bromide in acetic acid (20 cm^3) by procedure (*d*). Precipitation with diethyl ether and reprecipitation with ether from ethanol gave the hygroscopic hydrobromide of *S*-benzyl-L-cysteinylglycylglycinamide, (7) (8.4 g, 91%). This salt (810 mg, 2 mmol) was coupled with *N*-acetylglycylglycine [6] (348 mg, 2 mmol) by method (*c*) using isobutyl chloroformate (0.262 ml, 2 mmol) in dimethylformamide (35 cm^3). Precipitation from methanol with ether gave the N-*acetylpentapeptide amide*, α_m (23 °C, 589 nm) −19.8 (*c* 1.0 in $MeCO_2H$), δ [100 MHz, $(CD_3)_2SO$] 8.4 (1 H, br, Cys-NH), 8.15 (4 H, br, Gly-NH), 7.3 (7 H, complex, C_6H_5 and $CONH_2$), 4.55 (1 H, dt, Cys-α-CH), 3.8 (10 H, complex, Gly-CH_2 and $PhCH_2$), 2.8 (2 H, br m, Cys-β-CH_2), and 1.9 (3 H, s, CH_3CO) (Found: C, 49.7; H, 5.9; N, 17.3. $C_{20}H_{28}N_6O_6S$ requires C, 50.0; H, 5.9; N, 17.5%).

N-*Acetyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*. *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycine succinimido-ester, (5) (4.45 g, 8 mmol), and *S*-benzyl-L-cysteinylglycylglycinamide hydrobromide (3.25 g, 8 mmol) were coupled by method (*a*) in 0.08 mol dm^{-3} sodium hydrogencarbonate (100 cm^3) and dioxan(100 cm^3). Recrystallisation from aqueous methanol gave N-*benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*, (8) (4.28 g, 70%), m.p. 166—168 °C, α_m (21 °C, 589 nm) −31.5 (*c* 1.0 in $HCONMe_2$) (Found: C, 56.3; H, 5.6; N, 12.5. $C_{36}H_{43}N_7O_8S_2$ requires C, 56.45; H, 5.65; N, 12.8%). Removal of the *N*-benzyloxycarbonyl group by method (*d*) gave the hydrobromide of the hexapeptide amide, (9), in 85% yield after reprecipitation from ethanol with diethyl ether. This salt (2.50 g, 3.5 mmol) was coupled with *N*-acetylglycylglycine (0.61 g, 3.5 mmol) in dimethylformamide (100 cm^3) by method (*c*); recrystallisation from aqueous acetic acid gave the N-*acetyloctapeptide amide* (1.93 g, 70%), α_m (23 °C, 589 nm) −26.9 (*c* 1.0 in Me_2SO), δ [100 MHz, $(CD_3)_2SO$] 8.4 (2 H, br, Cys-NH), 8.1 (6 H, br complex, Gly-NH), 7.3 (12 H, complex, C_6H_5 and $CONH_2$), 4.55 (2 H, dt, Cys-α-CH), 3.75 (16 H, complex, Gly-CH_2 and $PhCH_2$), 2.7 (4 H, m, Cys-β-CH_2), and 1.85 (3 H, s, CH_3CO) (Found: C, 51.7; H, 5.8; N, 15.6. $C_{34}H_{45}N_9O_9S_2$ requires C, 51.8; H, 5.75; N, 16.0%).

N-*Acetyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*. *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycine succinimido-ester (278 mg, 0.5 mmol) and the hydrobromide of (9) (356 mg, 0.5 mmol) were coupled by method (*b*) in dimethylformamide (20 cm^3) in the presence of acetic acid (30 mg). Crystallisation from aqueous acetic acid gave N-*benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*, (10) (470 mg, 87%), α_m (22 °C, 589 nm) −29.7 (*c* 1.0 in $HCONMe_2$) (Found: C, 56.1; H, 5.6; N, 12.6. $C_{50}H_{60}N_{10}O_{11}S_3$ requires C, 55.95; H, 5.6; N, 13.05%). Removal of the *N*-benzyloxycarbonyl group from (10) by method (*d*), followed by coupling of the resulting hydrobromide of (11) with *N*-acetylglycylglycine in dimethylformamide [method (*b*)] and recrystallisation from aqueous dimethyl sulphoxide, gave the N-*acetylundecapeptide amide* in 76% overall yield; α_m (22 °C, 589 nm) −28.5 (*c* 1.0 in Me_2SO), δ [100 MHz, $(CD_3)_2SO$] 8.3 (3 H, br, Cys-NH), 8.0 (8 H, br complex, Gly-NH), 7.2 (17 H, br complex, C_6H_5 and $CONH_2$), 4.5 (3 H, br d, Cys-α-CH), 3.7 (22 H, complex, Gly-CH_2 and $PhCH_2$), 2.65 (6 H, complex, Cys-β-CH_2), and 1.85 (3 H, s, CH_3CO) (Found: C, 51.9; H, 5.75; N, 14.3. $C_{48}H_{62}N_{12}O_{12}S_3 \cdot 0.5\ C_2H_6OS$ requires C, 51.9; H, 5.8; N, 14.8%).

N-*Acetyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*, (14). *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinylglycylglycine succinimido-ester (560 mg, 1 mmol) and the hydrobromide of (11) (500 mg, 0.5 mmol) were coupled by method (*b*) in dimethylformamide (10 cm^3) in the presence of acetic acid (30 mg). Recrystallisation from dimethylformamide–methanol gave N-*benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinyldiglycyl*-S-*benzyl*-L-*cysteinylglycylglycinamide*, (12) (600 mg, 87%), α_m (23 °C), 589 nm) −28.6 (*c* 1.0 in Me_2SO) (Found: C, 55.4; H, 6.0; N, 12.5. $C_{64}H_{77}N_{13}O_{14}S_4 \cdot CH_3OH$ requires C, 55.3; H, 5.8; N, 12.9%). Removal of the *N*-benzyloxycarbonyl group from (12) by method (*d*), followed by coupling of the resulting hydrobromide of (13) with *N*-acetylglycylglycine in dimethylformamide [method (*b*)] and recrystallisation from dimethyl sulphoxide–methanol, gave the N-*acetyltetradecapeptide amide*, (14), in 84% overall yield; α_m (22 °C, 589 nm) −28.5 (*c* 1.0 in Me_2SO), δ [100 MHz, $(CD_3)_2SO$] 8.45 (4 H, br, Cys-NH), 8.15 (10 H, br complex, Gly-NH), 7.35 (22 H, complex, C_6H_5 and $CONH_2$), 4.6 (4 H, br, Cys-α-CH), 3.8 (28 H, complex, Gly-CH_2 and $PhCH_2$), 2.7 (8 H, complex m, Cys-β-CH_2), and 1.85 (3 H, s, CH_3CO) (Found: C, 52.4; H, 5.7; N, 14.3. $C_{62}H_{79}N_{15}O_{15}S_4 \cdot 0.5\ C_2H_6OS$ requires C, 52.5; H, 5.7; N, 14.6%).

(*B*) *Ligand-exchange Reactions*.—Bis(tetramethylammonium) tetrakis(μ_3-sulphido-t-butylthioiron) (15) and the corresponding bis(tetraphenylarsonium) salt were prepared essentially as described by Holm and his co-workers,[17] except that lithium methoxide was used in place of sodium ethoxide and lithium sulphide in place of sodium hydrogensulphide.[27] Their spectroscopic properties (u.v., ^{1}H n.m.r., Mössbauer) were virtually identical with those reported by Holm and his co-workers.[16,17,28]

The *N*-acetylpeptide amides (3; n = 0—3) were prepared from their *S*-benzyl derivatives [(14) and analogues] by the following procedure developed on the basis of trial experiments with the *S*-benzyl derivative of (3; n = 0) and shown to result in the complete removal of the *S*-benzyl groups and the reduction of any disulphide bridges produced during the treatment with hydrogen fluoride.

The protected peptide (160 μequivalents of SCH_2Ph) and anisole (48 μl, 480 μmol) were dissolved, at −70 °C, in anhydrous hydrogen fluoride distilled into the reaction vessel from cobalt(III) fluoride. The vessel was sealed, the temperature allowed to rise to 20 °C, and the mixture stirred for the time necessary to remove all the *S*-benzyl groups [3 h for (3; n = 0); 4 h for (3; n = 1); 5 h for (3; n = 2 or 3)]. Hydrogen fluoride was then removed by pumping at 14 mmHg * for 3 h and the residue kept overnight over sodium hydroxide pellets at 0.01 mmHg. The residue was dissolved in water (20 cm^3) and extracted with diethyl ether (4 × 5 cm^3). The aqueous residue was concentrated to half its volume and 2-mercaptoethanol (2.24 g, 28 mmol) added. After 10 min the solution was evaporated and the residual oil triturated with 5% 2-mercaptoethanol in ethyl acetate (20 cm^3). The resulting white solid was filtered off, washed successively with 5% 2-mercaptoethanol in ethyl acetate (20 cm^3), 1% 2-mercaptoethanol in ethyl acetate (2 × 20 cm^3), and ethyl acetate (2 × 20 cm^3), and finally dried at 60 °C (0.01 mmHg).

A weighed amount of peptide was dissolved in deoxygenated hexadeuteriodimethyl sulphoxide (220 μl) under strictly anaerobic conditions. Aliquots (10 μl) of this solution were added, under strictly anaerobic conditions, by means of a Hamilton gas-tight syringe to a solution of the cluster compound (15) in deoxygenated hexadeuteriodimethyl sulphoxide, containing an accurately measured amount of acetone and hexamethyldisiloxane (1 μl), in an n.m.r. tube filled with oxygen-free nitrogen and closed with a serum cap. The contents of the tube were thoroughly mixed by shaking and the tube was placed in the n.m.r. spectrometer at 31 °C. After 15 min the ^{1}H n.m.r. spectrum was recorded and the area of the acetone (1.92—2.16 p.p.m. from hmd) and Bu^{t}SH peaks (1.16—1.24 p.p.m. from hmd) obtained by integration. From these areas the amount of Bu^{t}SH was calculated. After the addition of the last aliquot of peptide the mixture was kept for 24 h and another spectrum recorded; a large excess of benzenethiol was then added and the amount of released Bu^{t}SH again determined, thus giving the 100% point for the reaction.

In the case of the unidentate ligand (3; n = 0), values of $\bar{n}$ were calculated from the formation function (p. 1231) for a range of values of α, using different values of K_1—K_4, with the limitation on their ratios referred to on p. 1231; these calculated values of $\bar{n}$ were plotted against the corresponding calculated values of the amount of peptide added [= $\bar{n}(1+\alpha)$] to give a calculated formation curve. This process was repeated with modified values of K_1—K_4 until a satisfactory fit to the experimental points was obtained (Figure 4).

* Throughout this paper: 1 mmHg ≈ 13.6 × 9.8 Pa.

We thank the S.R.C. for the award of a Research Studentship (to R. J. B.).

[9/1222 *Received*, *2nd August*, 1979]

REFERENCES

[1] Part 1, M. J. Gunter, B. Ridge, H. N. Rydon, and R. Sharpe, *Bio-org. Chem.*, 1979, **8**, 371.
[2] For reviews see 'Iron–Sulphur Proteins,' ed. W. Lovenberg, Academic Press, New York, 1973—1976, vols. I—III.
[3] E. T. Adman, L. C. Sieker, and L. H. Jensen, *J. Biol. Chem.*, 1973, **248**, 2987.
[4] M. A. Bobrik, L. Que, and R. H. Holm, *J. Amer. Chem. Soc.*, 1974, **96**, 285.
[5] L. Que, J. R. Anglin, M. A. Bobrik, A. Davison, and R. H. Holm, *J. Amer. Chem. Soc.*, 1974, **96**, 6042.
[6] R. E. Steiger, *Helv. Chim. Acta*, 1934, **17**, 563.
[7] J. Kovacs, G. L. Mayer, R. H. Johnson, R. E. Cover, and U. R. Ghatak, *J. Org. Chem.*, 1970, **35**, 1810.
[8] J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, 1942, **145**, 253.
[9] S. Sakakibara in 'Chemistry and Biochemistry of Amino-acids, Peptides and Proteins,' ed. B. Weinstein, Marcel Dekker, New York, 1971, vol. I, p. 51.
[10] J. Pless and W. Baur, *Angew. Chem. Internat. Edn.*, 1973, **12**, 147.
[11] H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J.C.S. Chem. Comm.*, 1974, 107.
[12] M. S. Rohrbach, B. A. Humphries, F. J. Yost, W. G. Rhodes, S. Bratman, R. G. Hiskey, and J. H. Harrison, *Analyt. Biochem.*, 1973, **52**, 127.
[13] R. Malkin and J. C. Rabinowitz, *Biochem. Biophys. Res. Comm.*, 1966, **23**, 822; E. Bayer, H. Eckstein, H. Hagenmaier, D. Josef, J. Koch, P. Krauss, A. Röder, and P. Schretzmann, *European J. Biochem.*, 1969, **8**, 33.
[14] A. Balasubramaniam, B. Ridge, and H. N. Rydon, unpublished work.
[15] G. Christou, B. Ridge, and H. N. Rydon, *J.C.S. Chem. Comm.*, 1977, 908.
[16] B. V. De Pamphilis, B. A. Averill, T. Herskovitz, L. Que, and R. H. Holm, *J. Amer. Chem. Soc.*, 1974, **96**, 4159.
[17] B. A. Averill, T. Herskovitz, R. H. Holm, and J. A. Ibers, *J. Amer. Chem. Soc.*, 1973, **95**, 3523.
[18] R. H. Holm, W. D. Phillips, B. A. Averill, J. J. Mayerle, and T. Herskovitz, *J. Amer. Chem. Soc.*, 1974, **96**, 2109.
[19] L. Que, M. A. Bobrik, J. A. Ibers, and R. H. Holm, *J. Amer. Chem. Soc.*, 1974, **96**, 4168.
[20] J. Bjerrum, 'Metal Ammine Formation in Aqueous Solution,' Haase, Copenhagen, 1957.
[21] *Cf.* S. F. A. Kettle, 'Co-ordination Compounds,' Nelson, London, 1969, p. 51.
[22] G. Schwarzenbach, *Helv. Chim. Acta*, 1952, **35**, 2344.
[23] C. W. Carter, J. Kraut, S. T. Freer, N. H. Xuong, R. A. Alden, and R. G. Bartsch, *J. Biol. Chem.*, 1974, **249**, 4212.
[24] D. F. Shriver, 'The Manipulation of Air-Sensitive Compounds,' McGraw-Hill, New York and London, 1969, ch. 7, p. 145.
[25] H. F. Schott, J. B. Larkin, L. B. Rockland, and M. S. Dunn, *J. Org. Chem.*, 1947, **12**, 490.
[26] K. C. Hooper, H. N. Rydon, J. A. Schofield, and G. S. Heaton, *J. Chem. Soc.*, 1956, 3148.
[27] G. N. Schrauzer, G. W. Kiefer, K. Tano, and P. A. Doemeny, *J. Amer. Chem. Soc.*, 1974, **96**, 641.
[28] R. B. Frankel, B. A. Averill, and R. H. Holm, *J. Phys. (Paris)*, 1974, **35**, **C6**, 107.