Isolation and Characterization of Adenochrome, a Unique Iron(III)-binding Peptide from *Octopus vulgaris*

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Adenochrome, the iron(III)-containing pigment from the branchial heart of *Octopus vulgaris*, has been isolated as colourless desferri-adenochrome (DFA) and shown to consist of a mixture of closely related peptides derived from glycine and three isomeric amino-acids, adenochromines A, B, and C, which were formulated as the novel dopa derivatives (1a), (1b), and (1c), respectively, by spectral and chemical evidence.

AMONG the products of metabolism of dopa in animals, the catecholamines and the widespread dark pigments, the eumelanins, are indeed the best known. Another pathway, described more recently, is exemplified by the formation of the phaeomelanins,[1] which proceeds by way of the cystein-*S*-ylodopas derived by conjugation of dopa with cysteine through a sulphide bond.[2] Although this pathway has so far been established only in mammals and birds, it is remarkable that a related compound, 2,5-dicystein-*SS*-yldopa,[3] is found in the *tapetum lucidum* of the fish *Lepisosteus spatula*. It has been known for a long time[4] that the branchial hearts of some *Octopus* species possess a red iron-binding pigment, adenochrome,[5] containing nitrogen and sulphur. Because of its intractable nature, chemical investigations of this unusual metabolite have proceeded very slowly and only recently has it been isolated as the colourless desferri-adenochrome (DFA) from the branchial hearts of *Octopus vulgaris*.[6]

In this paper evidence is reported that DFA is an unusual type of peptide consisting of glycine and three novel iron-binding amino-acids, adenochromines A, B, and C, for which we propose the isomeric structures (1a), (1b), and (1c), respectively. These are biogenetically related to the cystein-*S*-yldopas, being derived by conjugation of dopa with the hitherto unknown 5-thiolhistidine.

Isolation and Properties of Desferri-adenochrome (DFA).—Branchial hearts contain, in addition to varying amounts of the violet ferri-adenochrome (FA), large amounts of the colourless desferri-adenochrome (DFA). Since the latter displayed more favourable solubility and chromatographic properties, an isolation procedure was developed which involved first the complete conversion of the material to DFA by removal of iron under reducing conditions. Thus, branchial hearts were extracted with 0.5M-$HClO_4$ containing 2% thioglycolic acid; Fe^{3+} was reduced to Fe^{2+} which was easily separated from DFA on Sephadex G-10. Successive chromatographies on Sephadex LH-20 and on Dowex 50W-X8 afforded an almost colourless preparation of DFA in an average yield of 10 mg per g wet weight.

DFA is an amorphous powder readily soluble in water as the hydrochloride and insoluble in common organic solvents. Its elemental analysis was consistent with a nitrogen : sulphur ratio of 9 : 2. Molecular weight determinations of the material by osmometry or Sephadex gel filtration gave an approximate value of 1 300—1 500. When examined by t.l.c. (cellulose) and paper electrophoresis, DFA gave a single spot, detected with both ninhydrin (red-purple) and ferric chloride (green) reagents. However in the course of degradative experiments it became evident that DFA was in fact a mixture of several compounds of similar structures.

The u.v. spectrum of DFA at pH 1 showed a well-defined peak at 306 nm which shifted to 320 nm at pH 10, suggesting the presence of an *ortho*-dihydric phenol chromophore. On account of the complex nature of the structure(s), the ^1H n.m.r. spectrum of DFA in 2M-DCl was not very informative, showing broadened signals between δ 3 and 5 and at *ca.* 6.9 and 8.8, the latter two being attributable to aromatic and heteroaromatic protons, respectively.

As expected, when an acid solution of DFA containing an excess of $FeCl_3$ was brought to pH 4.5, a blue, insoluble iron complex precipitated immediately which contained approximately one atom of iron per DFA unit [formed from one adenochromine and two glycine residues (hereafter referred to as the monomer)].

Acid Hydrolysis of DFA.—Hydrolysis of DFA under standard conditions (6M-HCl for 24 h at 110 °C) gave a complex mixture of unusual amino-acids. T.l.c. of the hydrolysate revealed four main ninhydrin-positive spots, only one of which was due to a known amino-acid, glycine. Two of the other components, named adenochromines (1) and secoadenochromines (2), were both positive to $FeCl_3$ and Pauly reagent (yellow). On the other hand, the remaining spot [the disulphide (4)] was negative to $FeCl_3$ and gave an intense brown-violet colour with Pauly reagent. Later, we found it convenient to carry out the hydrolysis with 6M-HCl containing 4% thioglycolic acid,‡ as it appeared to give higher yields of (1) and (2) with less by-products; also the thiol (3) that was formed instead of (4) was much easier to separate. Although the heterogeneity due to partial *N*-methylation (described later) hampered our efforts to separate (1) and (2) into their isomers, some separation was achieved by careful column chromatography on Dowex 50W resin coupled with paper chromatography in n-propyl alcohol–1M-HCl (3 : 2). Thus, for example, fractionation of a hydrolysate of 250 mg

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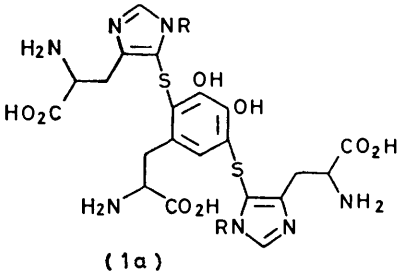
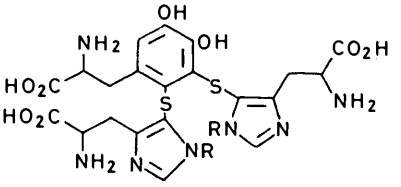
‡ Thioglycolic acid has been used [7] to prevent oxidation of certain amino-acids during acid hydrolysis of proteins.

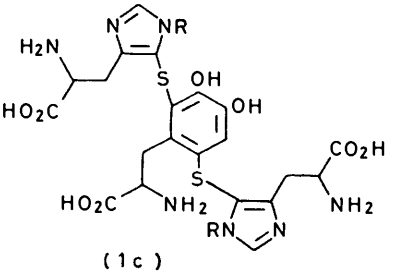
of DFA afforded 101 mg of (1a and c), 12.4 mg of (1b), 32.4 mg of (2a), 6.5 mg of (2b and c) [*ca.* 4 : 1 ratio, (n.m.r.)], 24.3 mg of (3), and glycine. Furthermore, compound (2c) was obtained practically pure as described later.



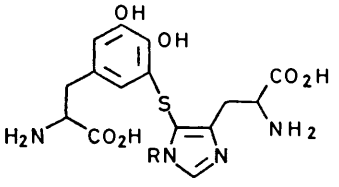
(1a)



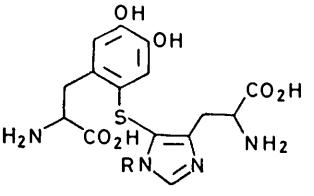
(1b)



(1c)



(2a)



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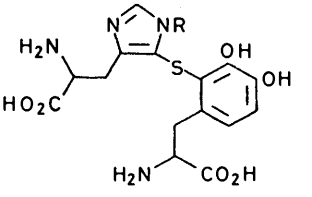
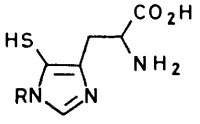


(2c)

R = H or Me



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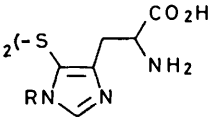

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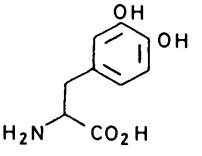
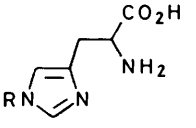


(5)



(6)

a; R = H

b; R = Me

Among the products of hydrolysis, the adenochromines (1) exhibited u.v. spectra which closely paralleled that of DFA, suggesting that the secoadenochromines (2) as well as the thiol (3) might be artifacts resulting from acid cleavage of (1) at the thioether bonds. In order to clarify this aspect, the hydrolysis of DFA with HCl–4% thioglycolic acid was examined under both mild and drastic conditions. The results (Table 1) indicated that (2) and (3) were indeed formed from the adenochromines (1), especially from isomer (1b), and that the ratio of (1a and c) : (1b) in DFA was approximately 2 : 1. Thus, it appeared likely that the actual amino-acid constituents of DFA were only glycine and the adenochromines A (1a), B(1b), and C (1c).

TABLE 1

Hydrolysis of DFA under mild and drastic conditions [a]

Product	Yield (mg) 3M-HCl, 20 h [b]	6M-HCl, 120 h
(1a and c)	22.0	20.6
(1b)	8.3 [c]	1.6 [c]
(2a)	0.8 [c]	9.7 [c]
(2b and c)	*d*	1.7 [c]
(3)	2.1 [e]	8.6 [e]
(5)	0	0.7

[a] For both experiments 63 mg of DFA were hydrolysed. [b] Hydrolysis of peptide bonds was incomplete. [c] Purified by paper chromatography (*ca.* 80% recovery). [d] Not determined. [e] Purified as the disulphide (4).

The acid cleavage of the adenochromines (1) at the thioether bonds may be explained in terms of electrophilic elimination of a sulphenyl ion His–S^+ which is then reduced to the thiol His–SH (3) by excess of thioglycolic acid (Scheme 1).

As the hydrolysis of DFA with 6M-HCl was somewhat misleading, an alternative reaction was needed to determine the ratio of glycine to adenochromines (1) in DFA. This was found in reductive hydrolysis with 57% HI in the presence of red phosphorus at 110 °C for 48 h. This proved suitable for the quantitative cleavage of the thioether bond to give glycine, 5-thiolhistidine (3), and L-dopa (L-3,4-dihydroxyphenylalanine) (5) in a molar

ratio 2 : 2 : 1. Hence the ratio of glycine to adenochromines (1) in DFA was determined as 2 : 1.

Structure of the Thiol (3).—The thiol (3), a colourless, amorphous amino-acid, was readily available from hydrolysis of DFA with HI. In acid solution it underwent aerial oxidation in the absence of oxidant, or more smoothly, in the presence of catalytic amounts of I_2, to give the crystalline disulphide (4), $C_{12}H_{16}N_6O_4S_2 \cdot 4HCl \cdot 2H_2O$,* $\lambda_{max.}$ 257 nm, which could be reconverted to the parent thiol by catalytic hydrogenolysis over 10% Pd–C in 0.1M-HCl. Desulphuration of (4) with Raney nickel yielded a product whose ^1H n.m.r. spectrum was practically identical to that of histidine, except that it exhibited a sharp singlet at δ 3.96 (*ca.* 0.8 H), characteristic of a heteroaromatic NMe group. Preparative paper electrophoresis at pH 6.5 separated the product into L-histidine (6a) and L-1-methylhistidine (6b) in *ca.* 3 : 1 ratio, reflecting the degree of methylation. The position of the thiol moiety in (3) was determined by its n.m.r. spectrum which exhibited a singlet at δ 8.87 (1 H) attributed to a proton at C-2 of an imidazole ring. Moreover, the thiol (3) was not identical to an authentic sample of 2-thiolhistidine. The thiol (3) was therefore adduced to be the hitherto unkown 5-thiolhistidine, partially methylated at position 1.

Structures of Adenochromines A, B, and C.—These characteristic constituents of adenochrome gave intense green colours with $FeCl_3$ and exhibited very similar u.v. spectra with an absorption maximum at *ca.* 305 nm, shifted to *ca.* 320 nm on basification. Common features of the ^1H n.m.r. spectra (in 2M-DCl) of the adenochromines were signals from three $-CH_2CH(NH_3^+)CO_2H$ residues, one aromatic proton (at δ *ca.* 7.0), and two heteroaromatic protons (at δ *ca.* 8.8, typical for H-2 of an imidazole ring). The spectra also exhibited two NMe signals at *ca.* δ 4.0 (each less than 1 H), indicating that the adenochromines were also partially methylated. Moreover, on reductive hydrolysis with 57% aqueous HI in the presence of red phosphorus they gave, as expected, dopa (5) and the thiol (3) in the molar ratio 1 : 2. Furthermore, the elemental analysis of adenochromines A + C agreed with the formula $C_{21}H_{25}N_7O_8S_2 \cdot 5HCl$. Accordingly, the adenochromines were regarded as isomers of the general structure (1) differing in the positions of the two 5-thiolhistidine residues on the dopa moiety.

* The analyses were in agreement with the values calculated by taking into account the 3 : 1 ratio of NH and NMe homologues. However, for the sake of clarity the molecular formulae of (4) as well as those of the related compounds described later refer only to the NH homologues.

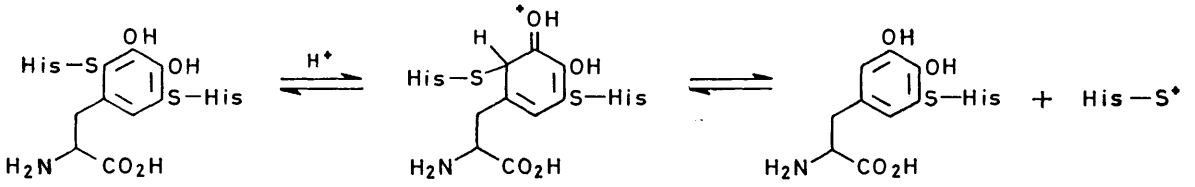
TABLE 2

Degradative reactions of adenochromines (1)

Adenochromine	Conditions	Products [a] [% yield]
(1a and c)	57% HI–red P, 110 °C, 2 days	(3) [86], (5) [81]
(1b)	57% HI–red P, 110 °C, 2 days	(3) [96], (5) [88]
(1a and c)	6M-HCl–4% TGA,[b] 110 °C, 7 days	(3), (5), [3 [c]], (2a) [28], (2b and c) [8], (1a and c) [45]
(1b)	6M-HCl–4% TGA, 110 °C, 4 days	(3) (5), (2a and b) [76], (1b)
(1a and c)	48% HBr–4% TGA, 110 °C, 1 day	(3), (5) [45], (2a) [37], (1a and c) (10)
(1b)	48% HBr–4% TGA, 110 °C, 1 day	(3), (5) [25 [c]], (2a) [73]
(1a and c)	H_2–Pt_2, room temp., 1 day	(6) [60 [d]], (5) [9], (2c) [25], (1a and c) (24)
(1b)	H_2–Pt_2, room temp., 1 day	(6), (5), (2b) [4 [c]], (1b) [37]

[a] Yields are not corrected for the starting material except for those marked *d*. [b] Thioglycolic acid. [c] Determined on the basis of u.v. absorbance. [d] Corrected for yields of (1) and (2c).

Table 2 summarizes the results of degradation experiments carried out on the adenochromines, upon which the structures were eventually established. On prolonged hydrolysis with 6M-HCl containing 4% thioglycolic acid, adenochromines A + C gave, besides recovered (1a) + (1c) (45%), the thiol (3), dopa (3%), secoadenochromine A (2a) (28%), and the secoadenochromines B + C (4 : 1 ratio) (8%). The formation of the three isomeric secoadenochromines can only be explained by assuming that the adenochromine submitted to the hydrolysis was a mixture of two isomers. On the other hand, the hydrolysis of adenochromine B resulted in the formation of two isomers of secoadenochromines, A and B, in the ratio *ca.* 6 : 1. In contrast to the hydrolysis with HCl, heating adenochromines A + C with 48% HBr containing 4% thioglycolic acid gave the thiol (3), dopa (45%), the sole secoadenochromine A(2a), (37%), and a 10% recovery of the adenochromines.



SCHEME 1

The remaining isomer, adenochromine B, also yielded selectively secoadenochromine A in 73% yield, along with dopa and the thiol (3). The fact that (2b) and (2c) were not obtained from the HBr hydrolysate may be due to their faster conversion to dopa than (2a). This was supported by model experiments which showed that 2- and 6-cystein-*S*-yldopa (7b and c) [2] were several times more rapidly converted to dopa with 40% HBr than 5-cystein-*S*-yldopa (7a) [2] (see Table 3).

Secoadenochromine A, analysed for $C_{15}H_{18}N_4O_6S \cdot 3HCl$, gave a well-defined ^1H n.m.r. spectrum which exhibited signals assigned to two $-CH_2CH(NH_3^+)CO_2H$

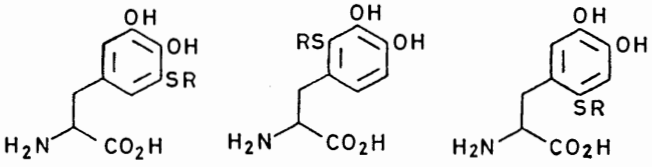
TABLE 3

Acid cleavage of cystein-*S*-yldopas (7)

Compound [a]	Conditions	Products [% yield [d]]
(7a)	HBr–TGA,[b] 14 h [c]	(7a) [74], dopa [13], cysteine
(7b)	HBr–TGA, 14 h	dopa [90], (7b) [7], cysteine
(7c)	HBr–TGA, 7 h	dopa [83], (7c) [trace], cysteine

[a] SR = $-SCH_2CH(NH_2)CO_2H$. [b] 40% HBr containing 4% thioglycolic acid under reflux. [c] Some (7a) still remained after 70 h. [d] Determined by amino-acid analyser.

residues, an NMe group (0.8 H), *meta*-oriented aromatic protons at δ 6.70 and 6.89 (ABq, *J* 1.8 Hz), and an imidazole C-2 proton at δ 8.82. Secoadenochromine A was therefore assigned the structure (2a). The assignment of the aromatic substitution pattern as in (2a) was substantiated by the ^{1}H n.m.r. spectra of the two related compounds, 5-cystein-*S*-yldopa (7a) (ABq at δ 6.93 and 7.01, *J* 2.0 Hz) and 6-cystein-*S*-yldopa (7c) [2]



(singlets at δ 6.94 and 7.20). Furthermore, a similarity of the u.v. spectra of the secoadenochromines with those of the cysteinyldopas ($\lambda_{max.}$ 292—295 nm) [2] was also in accord with the structures (2).

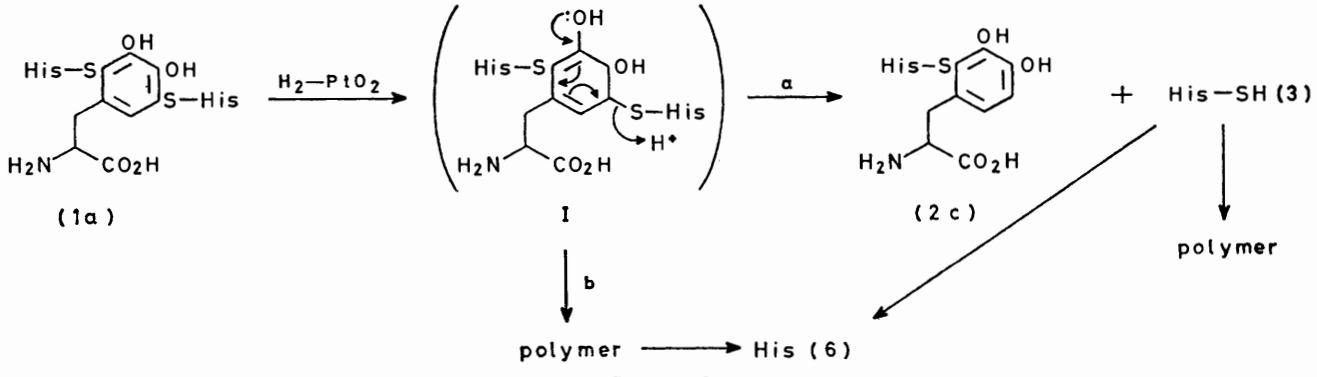
A further characterization of the adenochromines (1) was provided by hydrogenolysis. Catalytic hydrogenolysis of adenochromines A + C in 0.1M-HCl over large amounts of PtO_2 afforded, in addition to dopa (9%) and histidine (60%; partly methylated at N-1), secoadenochromine C in 25% yield and a 24% recovery of the starting material (1a) + (1c).

Secoadenochromine C was assigned structure (2c) on the basis of its ^{1}H n.m.r. spectrum which showed signals from *ortho*-oriented aromatic protons at δ 6.91 and 7.03 (ABq, *J* 8 Hz). The selective formation of (2c) from (1a) may be accounted for by preferential hydrogenation across the 4,5-position, presumably for steric reasons. The subsequent selective removal of the thiol residue instead of the hydroxy group may result from electron donation from the dienol moiety in the intermediate I (pathway a in Scheme 2). The 5-thiolhistidine (3) once formed would undergo further reduction to give histidine or a polymeric material. In fact, the disulphide (4) gave a 25% yield of (6) in addition to a brownish polymer under similar conditions. The fairly good yield of histidine from (1) compared with that from (4) suggested an alternative pathway, *i.e.* pathway b. The relatively low yield of dopa and (2c) may also be attributable to the formation of considerable amounts of a brownish polymeric material (pathway b). Hydrogenolysis of adenochromine B gave, besides the starting material (37%), low yields of (6) and (5), together with secoadenochromine B (4%), identified by amino-acid analysis.

The formation of (2a) by hydrolysis and (2b) by hydrogenolysis from adenochromine B indicated structure (1b) for the adenochromine. The adenochromines A and C were therefore assigned structures (1a) and (1c), consistent with the results of the degradative reactions summarized in Table 2.

Biogenetically, adenochromines may be formed by addition of 5-thiolhistidine to dopaquinone arising from tyrosine by oxidation with tyrosinase. Support for this pathway is provided by a recent study [8] which showed that under biomimetic conditions the reaction of 5-thiolhistidine with dopaquinone, generated by tyrosinase oxidation of dopa, proceeds smoothly and cleanly to give, albeit in small yields, the three adenochromines by way of the parent monoadducts, secoadenochromines A, B, and C.

In conclusion, our results provide information on the structure and origin of the adenochromines which are the essential amino-acid constitutents of adenochrome, the first example of a non-porphyrin iron-chelating metabolite from the animal kingdom. All the other ironophoric substances that are known, *e.g.* the siderochromes,[9] have been isolated from micro-organisms. Further work to define the full structure of adenochrome, *i.e.* the sequence of the peptide chain, has been so far unrewarding because the presence of the adenochromines prevents a straightforward peptide analysis by conventional methods.



SCHEME 2

EXPERIMENTAL

M.p.s were determined in capillaries. U.v. spectra were recorded with a Beckman model 25 spectrophotometer and optical rotations with a Perkin-Elmer model 141 polarimeter. ^{1}H N.m.r. spectra were recorded for solutions in 2M-DCl with a Varian EM-360 60 MHz spectrometer and chemical shifts are expressed in δ values using as internal reference the methyl signal of t-butyl alcohol at δ 1.28. Signals split due to the presence of *N*-methyl homologues are indicated by the mark $^+$. Analyses of ordinary amino-acids were performed with a Beckman model 120 B amino-acid analyser.

The adenochromines (1) and secoadenochromines (2) were examined on a Liquimat III (Kontron, Switzerland) amino-acid analyser. The chromatography was run with pico E lithium buffer (Durrum) at 79 °C for (1) or with pico E lithium buffer–H_2O (4 : 1 v/v) at 79° for (2). Unless otherwise stated, the resin used for ion-exchange chromatography was Dowex 50W-X2, 200—400 mesh. Columns used were: column 1, 1.8 × 22 cm, equilibrated and eluted with 3M-HCl at 55 ml h^{-1}, fractions of 20 ml being collected; column 2, 0.9 × 26 cm, equilibrated and eluted with 3M-HCl (9 ml h^{-1}, 5 ml fractions); column 3, 0.9 × 20 cm, equilibrated and eluted with 3M-HCl (12 ml h^{-1}, 4 ml fractions); column 4, 0.9 × 6 cm (3M-HCl), ×7 cm (2M-HCl), or ×8 cm (1M-HCl). For analytical t.l.c. glass plates of cellulose (Merck) were used and for preparative paper chromatography and electrophoresis (10—17 V cm^{-1}) Whatman No. 3MM paper was used. Solvent systems used for t.l.c. and paper chromatography were: solvent 1, n-propyl alcohol–1M-HCl, 3 : 2 v/v; solvent 2, n-propyl alcohol–1M-HCl, 2 : 1 v/v. Spots were located by u.v. lamp (254 nm), ninhydrin reagent, 1% $FeCl_3$ in 95% EtOH alone or followed by NH_3 vapour, or Pauly reagent. Unless otherwise stated, the amino-acids obtained by degradative reactions were isolated as the hydrochlorides by evaporation of the HCl solution and subsequent drying at room temperature with a mechanical pump, and therefore contained varying amounts of H_2O. Drying (80 °C under high vacuum) and elemental analyses [of (1), (2), and (4)] showed that the adenochromines (1) formed the $5HCl{\cdot}3H_2O$ salt (m.wt. 811), the secoadenochromines (2) the $3HCl{\cdot}2H_2O$ salt (m.wt.531), the thiol (3) the $2HCl{\cdot}H_2O$ salt (m.wt. 284), the disulphide (4) the $4HCl{\cdot}2H_2O$ salt (m.wt. 566), dopa (5) the $HCl{\cdot}H_2O$ salt (m.wt. 252), and histidine (+1-methylhistidine) (6) the $HCl{\cdot}H_2O$ salt (m.wt. 213).

Isolation of Desferri-adenochrome (DFA).—Branchial hearts (10 g) were homogenized in 0.5M-$HClO_4$ (100 ml) containing 80% thioglycolic acid (2 ml) and extracted for 1 h with vigorous stirring at room temperature. After centrifugation at 17 300*g* for 15 min, the precipitate was discarded and the supernatant was adjusted to pH 1.5 by addition of $KHCO_3$. After removal of the precipitated $KClO_4$ by centrifugation, the supernatant was concentrated to 10 ml under reduced pressure and clarified by a further centrifugation. The concentrate was then chromatographed on a column (3.5 × 85 cm) of Sephadex G-10 with 0.02M-HCl as the eluant at a flow rate of 120 ml h^{-1}. The fractions containing DFA, detected spectrophotometrically, were combined and evaporated to dryness at 40 °C. The DFA, taken up in the minimum amount of 0.02M-HCl, was centrifuged to remove insoluble material, chromatographed again on a column (2.5 × 93 cm) of Sephadex LH-20 using 0.02M HCl as the eluant at a flow rate of 60 ml h^{-1}, and fractions containing DFA were evaporated to dryness at 40 °C. The DFA thus obtained (264 mg) was further purified by ion-exchange chromatography on a column (2 × 46 cm) of Dowex 50W-X8 (200—400 mesh) at a flow rate of 20 ml h^{-1} with 2M-pyridine–acetate buffer, pH 4.8 containing 0.2% ethanethiol to minimize oxidation. The 'oxidized' DFA is characterized by a yellow-brown colour and by disappearance of the peak at 305 nm characteristic of DFA. Fractions of *ca.* 5 ml were collected and on the basis of their u.v. spectra were divided into (1) 'oxidized' DFA (20 mg), (2) a mixture of DFA and 'oxidized' DFA (57 mg), and (3) DFA (112.5 mg). The material in (3) was precipitated by addition of an equal volume of acetone. After two washings with acetone to remove the buffer, the DFA was dried in a desiccator (Found: C, 44.7, 43.2; H, 5.4, 4.9; N, 15.9, 16.9; S, 8.9, 8.9. Calc. for $C_{25}H_{31}N_9O_{10}S_2{\cdot}CH_3CO_2H$: C, 44.1; H, 4.8; N, 16.8; S, 8.6%).

Preparation of Ferri-adenochrome (FA).—To a solution of DFA (52 mg, 63 mmol as the monomer) in 0.1M-HCl was added $FeCl_3{\cdot}6H_2O$ (56 mg, 207 mmol) dissolved in a few ml of H_2O; the solution turned immediately an intense dark blue. Within 1 min the pH of the solution was raised to 4.5 with concentrated NaOAc solution and the mixture kept overnight in a freezer. After thawing, the dark blue precipitate was collected by centrifugation, washed three times with H_2O, and kept in a freezer overnight. The precipitate was filtered off, washed well with H_2O, and dried in a desiccator. The yield of FA was 42.4 mg (Found: C, 37.7; H, 4.2; N, 14.8; S, 7.1; Fe, 8.8. Calc. for $C_{25}H_{28}FeN_9O_{10}S_2{\cdot}5H_2O$: C, 36.8; H, 4.7; N, 15.2; S, 7.7; Fe, 6.7%).

Hydrolysis of DFA with Hydrochloric Acid.—(a) *Analytical.* DFA (3.58 mg) was hydrolysed with 6M-HCl (1 ml) containing 80% thioglycolic acid (0.04 ml) at 110 °C for 48 h. The hydrolysate was evaporated to dryness at 40 °C and taken up in 0.1M-HCl. A quantitative amino-acid analysis gave 1.62 μmol of glycine per μmol of DFA (as the monomer). A trace amount of aspartic acid (<2% of glycine) was also found.

(b) *Preparative.* A solution of DFA (250 mg) and 80% thioglycolic acid (0.5 ml) in 6M-HCl (12.5 ml) was heated at 110 °C for 24 h. The hydrolysate was evaporated to dryness under reduced pressure and the residue, taken up in 3M-HCl, was chromatographed on Dowex column 1: fraction 4 contained glycine (and thioglycolic acid); fractions 7—8, crude (3) (42 mg); 18—21, a mixture of (2b) and (2c) (7 mg); 22—31, a mixture of (2a) and (1b) (54 mg); and 36—45, a mixture of (1a) and (1c) (107 mg). The thiol (3) was purified as the disulphide (4) (described later) and (2a) and (1b) were separated by preparative paper chromatography on solvent 1. (2a) was again chromatographed on Dowex column 4 to remove trace amounts of impurities. (1b) was further purified on a column (1.8 × 115 cm) of Bio-Gel P-2, 200—400 mesh, using 10% CH_3CO_2H as the eluant at a flow rate of 13 ml h^{-1}. The yields were: 101 mg of adenochromine A + C (1a and c), 12.4 mg of adenochromine B (1b), 32.4 mg of secoadenochromine A (2a), 6.5 mg of secoadenochromine B + C (2b and c), and 24.3 mg of the disulphide (4).

Properties of the Adenochromines (1) and Secoadenochromines (2).—On a Liquimat III amino-acid analyser the adenochromines A + C [(1a and c)] gave a characteristic elution pattern of three peaks, at 64, 72 and 83 min, which were not identified. Adenochromine B (1b) gave three peaks emerging at 50, 59, and 71 min. Secoadenochromine

A (2a) gave two peaks at 42 and 50 min, secoadenochromines B + C (2b and c) three peaks at 32, 37, and 45 min and secoadenochromine C [(2c); described later] two peaks at 37 and 45 min. R_F Values on t.l.c. in solvent 1 were: (1), 0.14; (2), 0.33; and (4), 0.24 (glycine, 0.64).

Adenochromines A + C was an almost colourless, very hygroscopic powder, δ 3.2—4.2 (6 H, m centred at 3.7, β-CH_2 × 3), 3.97 (≤1 H, s, NMe), 4.03 (≤1 H, s, NMe), 4.2—4.8 (3 H, m centred at 4.5, α-CH × 3), 6.82 [+] [from (1c)] and 6.85[+] [from (1a)] [1 H, H-5 of (1c) and H-6 of (1a)], 8.79 (1 H, s, imidazole H-2), and 8.96[+] (1 H, s, imidazole H-2) (Found: C, 33.5; H, 4.4; N, 13.0; S, 8.8; Cl, 23.5. Calc. for $C_{21}H_{25}N_7O_8S_2$·5HCl: C, 34.1; H, 4.1; N, 13.0; S, 8.5; Cl, 23.4%). Adenochromine B had δ 3.2—4.0 (6 H, m centred at 3.6, β-CH_2 × 3), 3.92 (≤2 H, s, NMe × 2), 4.2—4.7 (3 H, m centred at 4.4, α-CH × 3), 7.08 (1 H, s, H-2), and 8.73 (2 H, s, imidazole H-2 × 2). Secoadenochromine A was a hygroscopic powder, δ 3.17 (2 H, d, *J* 6.5 Hz, $ArCH_2$), 3.60 (2 H, d, *J* 7.5 Hz, SCH_2) 3.96 (0.8 H, s, NMe), 4.37 (1 H, t, *J* 6.5 Hz, α-CH), 4.49 (1 H, t, *J* 7.5, α-CH), 6.70 and 6.89 (2 H, ABq, *J* 1.8, H-6 and -2), and 8.82[+] (1 H, s, imidazole H-2) (Found: C, 37.6; H, 4.3; N, 11.1; S, 6.5; Cl, 21.0. Calc. for $C_{15}H_{18}N_4O_6S$·3HCl: C, 37.0; H, 4.4; N, 11.3; S, 6.5; Cl, 21.0%). The ^1H n.m.r. spectrum of secoadenochromines B + C was complex, showing in the aromatic and hetero-aromatic region signals from (2b) at δ 6.89[+] (s, H-5), 6.96 (s, H-2), and 8.83 (s, imidazole H-2), and signals from (2c) at δ 6.91 and 7.03 (AB q, *J* 8 Hz, H-5 and -6), and 8.73[+] (s, imidazole H-2). The ratio of (2b) to (2c) was determined as *ca.* 4 : 1 by the integration of the imidazole protons. Secoadenochromine C showed δ 3.2—3.9 (4 H, m centred at 3.6, β-CH_2 × 2), 3.90 (0.8 H, s, NMe), 4.2—4.6 (2 H, m centred at 4.4, α-CH × 2), 6.91 and 7.03 (2 H, ABq, *J* 8 Hz, H-5 and -6), and 8.73[+] (1 H, s, imidazole H-2).

Hydrolysis of DFA with Hydrochloric Acid under Two Different Conditions.—(a) *Mild conditions.* A solution of DFA (63 mg) in 3M-HCl (3 ml) containing 80% thioglycolic acid (0.12 ml) was heated at 110 °C for 20 h. The hydrolysate was evaporated at 40 °C to dryness and the residue was chromatographed on Dowex column 2: fractions 4 and 5 contained glycine; 6—8, compound (3); 16—19, compounds (2b and c); 20—28, compounds (2a) and (1b); and 29—38, compounds (1a and c). The thiol (3) was purified as the disulphide (4). Fractions 20—28 were subjected to preparative paper chromatography and the components purified by rechromatography on Dowex column 4 (3M-HCl as eluant). Product yields are shown in Table 1.

(b) *Drastic conditions.* Conditions were as for (a) except for use of 6M-HCl containing thioglycolic acid for 120 h. Results are summarized in Table 1.

Hydrolysis of DFA with Hydriodic Acid.—(a) *Analytical.* A mixture of DFA (3.0 mg) and red phosphorus (5 mg) in 57% aqueous HI (1 ml) was heated at 110 °C for 48 h. The hydrolysate was evaporated at 60 °C under reduced pressure and the residue was taken up in 0.1M-HCl. An amino-acid analysis showed peaks of glycine and dopa in the ratio 2.00 : 0.98. Also found by amino acid analysis were trace amounts of aspartic acid, *m*- and *p*-tyrosine, (arising from dopa), and histidine [arising presumably from (3)]. Neither (3) nor (4) emerged from the columns.

(b) *Preparative.* DFA (200 mg) was heated at 110 °C for 48 h with 57% aqueous HI (10 ml) in the presence of red phosphorus (150 mg). The hydrolysate was evaporated to dryness at 60 °C under reduced pressure and the residue, taken up in 3M-HCl, was chronatographed on Dowex column 1: fractions 4 and 5 contained 53 mg of crude glycine, 6—8, 127 mg of the crude thiol (3); and 9—12, 56 mg of crude dopa (5). Rechromatography of the crude dopa on Dowex column 2 afforded 50 mg (0.2 mmol) of pure dopa. The crude thiol (3) was oxidised to the disulphide (4) by stirring it in 0.1M-HCl (10 ml) in the presence of a few crystals of I_2 for 24 h. Chromatography on Dowex column 4 (2M-HCl) gave 120 mg [0.42 mmol as (3)] of pure disulphide (4). The dopa was crystallized from H_2O containing $NaHSO_3$ at pH 4.5 and had m.p. 271—273° (decomp.), R_F 0.75 in solvent 1, $[\alpha]_D^{20}$ −9.7° (*c* 2.04, 1M-HCl). An authentic sample of L-dopa had m.p. 272—274 (decomp), $[\alpha]_D^{20}$ −9.5° (*c* 2.17, 1M-HCl) The disulphide (4) was crystallized from 6M-HCl–acetone to give prisms, m.p. 158° (softens) and >200° (decomp.); $\lambda_{max.}$ (0.1M-HCl) 257 nm (ε 9 200); δ 3.47[+] (2 H × 2, d, *J* 7 Hz, β-CH_2), 4.00 (1.2 H, s, NMe), 4.47 (1 H × 2, t, *J* 7, α-CH), and 8.97[+] (1 H × 2, s, H-2) (Found: C, 26.3; H, 4.9; N, 14.4; S, 11.2; Cl, 25.5. Calc. for $C_{12}H_{16}N_6O_4S_2$·4HCl·2H_2O: C, 26.6; H, 4.5; N, 15.0; S, 11.5; Cl, 25.3%).

Reduction of 5-Thiolhistidine Disulphide (4) *to 5-Thiolhistidine* (3).—A solution of (4) (42.0 mg) in 0.1M-HCl (3 ml) was hydrogenated over 10% Pd–C (14 mg). After 3 h the catalyst was filtered off and washed with 0.1M-HCl. The combined filtrates were evaporated to give a reddish residue (41 mg) which was then chromatographed on Dowex column 4. Elution with 2M-HCl gave 35.2 mg (83%) of the pure thiol (3), δ 3.57 (2 H, d, *J* 7 Hz, β-CH_2), 3.97 (0.8 H, s, NMe), 4.57 (1 H, t, *J* 7, α-CH), and 8.87 (1 H, s, H-2). T.l.c. gave elongated, multiple spots due to the rapid oxidation to (4) during development.

Raney Nickel Desulphuration of 5-Thiolhistidine Disulphide (4): *Formation of* L-*Histidine* (6a) *and* L-1-*Methylhistidine* (6b).—To a solution of (4) (118 mg) in H_2O (4 ml) and 95% EtOH (15 ml) was added a suspension of Raney nickel (1 ml) and the mixture was heated under reflux for 10 min with stirring. To the hot reaction mixture was added 6M-HCl (3 ml); the pH of the mixture dropped to *ca.* 1. After the evolution of H_2 gas had ceased, the mixture was centrifuged to remove solid metal and the supernatant was evaporated to dryness. The residue was successively chromatographed on Dowex 50W (0.9 × 13.5 cm in 1M-HCl), on paper in solvent 2, and then again on Dowex 50W (0.9 × 10.5 cm in 2M-HCl) to give 74 mg (83%) of a mixture of (6a) and (6b) in the ratio 3 : 1 (^1H n.m.r.). Separation of the mixture (65 mg) was effected by paper electrophoresis at pH 6.5, giving 33 mg of (6a) and 12 mg of (6b). The mobilities towards the cathode at pH 6.5 of histidine, 1-methylhistidine, and 3-methylhistidine were 2.4, 3.2, and 2.1 cm h^{-1}, respectively. The histidine was recrystallized as histidine·HCl·H_2O from H_2O–EtOH, m.p. 158° (softens) and 247° (decomp.), $[\alpha]_D^{20}$ +4.3° (*c* 1.1, 1M-HCl). An authentic sample of L-histidine·HCl·H_2O had m.p. 160° (softens) and 247° (decomp.), $[\alpha]_D^{20}$ +5.3° (*c* 2, 1M-HCl). The 1-methylhistidine from (4) had $[\alpha]_D^{20}$ +7.1° (*c* 0.49, 1M-HCl), while an authentic L-1-methylhistidine had $[\alpha]_D^{20}$ +9 (*c* 0.6, 1M-HCl).

Hydrolysis of the Adenochromines (1) *with Hydriodic Acid.*—(a) *Adenochromines A + C.* A mixture of (1a and c) (34.0 mg) and red phosphorus (35 mg) in 57% HI (2 ml) was heated at 110 °C for 48 h. The hydrolysate was evaporated to dryness at 60 °C, taken up in 3M-HCl, and chromatographed on Dowex column 3: fractions 6—9 contained 20.5 mg (86%) of crude (3) and 10—12, 8.5 mg

(81%) of crude (5). Rechromatography on the same column of (3) after conversion into (4) gave 7.0 mg of dopa (5) and 18.0 mg of the disulphide (4).

(b) *Adenochromine B.* (1b) (9.5 mg) was hydrolysed with HI under similar conditions as above. Chromatography on Dowex column 2 gave 2.6 mg (88%) of crude (5) and 6.4 mg (96%) of crude (3) [+ some (4)].

Hydrolysis of the Adenochromines (1) *with Hydrochloric Acid.*—(a) *Adenochromines A + C.* (1a and c) (72.0 mg) was heated at 110 °C for 168 h with 6M-HCl (3.8 ml) containing 80% thioglycolic acid (0.15 ml). The hydrolysate was evaporated to dryness at 40 °C and the residue chromatographed on Dowex column 2: fractions 6—13 gave (3) and (5) (3% by u.v.); 15—19, 3.8 mg (8%) of (2b and c) [*ca.* 4 : 1 ratio (n.m.r.)]; 22—28, 13.1 mg (28%) of (2a); and 31—39, 32.3 mg (45%) of recovered (1a and c).

(b) *Adenochromine B.* (1b) (2.2 mg) was hydrolysed with 6M-HCl (1 ml)–thioglycolic acid (0.04 ml) at 110 °C for 96 h. The hydrolysate was evaporated to dryness at 40 °C and chromatographed on a cellulose preparative t.l.c. plate using n-propyl alcohol–1M-HCl, 3 : 2 as eluant. The band corresponding to (2) was scraped off the glass and eluted with 0.1M-HCl. The fraction (2) (1.1 mg, 76%), was identified as (2a and b) (*ca.* 6 : 1) by amino-acid analysis under the conditions described above.

Hydrolysis of the Adenochromines (1) *with Hydrobromic Acid.*—(a) *Adenochromines A + C.* (1a and c) (31.8 mg) was heated at 110 °C for 24 h with 48% HBr (1.5 ml) containing 80% thioglycolic acid (0.06 ml). The hydrolysate was evaporated to dryness and the residue was chromatographed on Dowex column 2: fractions 6—12 contained (3) [+ some (4)] and (5); 21—28, 7.8 mg (37%) of pure secoadenochromine A (2a); 30—36, 3.2 mg (10%) of a mixture of recovered (1a and c). After converting (3) to (4), the fractions 6—12 were rechromatographed on the same column: fractions 10—11 gave 4.1 mg (45%) of dopa (5) and 13—16 gave 8.1 mg of the disulphide (4).

(b) *Adenochromine B*, (1b) (9.5 mg) was hydrolysed with 48% HBr–thioglycolic acid under similar conditions as for (a). Chromatography on Dowex column 2 gave (3) [+ some (4)], (5) [25% (u.v.)], and 4.5 mg (73%) of (2a).

Hydrolysis of the Cystein-S-yldopas (7) *with Hydrobromic Acid.*—A solution of (7a), (7b), or (7c) (5 mg) in 40% HBr (1.5 ml) containing 80% thioglycolic acid (0.06 ml) was heated under reflux as in Table 3. The hydrolysate was evaporated at 60 °C under reduced pressure and the residue, taken up in 0.1M-HCl, was analysed on the long column (Beckman type M 72 resin) of a Beckman model 120 B amino-acid analyser using as eluant the pH 4.25 buffer. Product yields are reported in Table 3.

Hydrogenolysis of the Adenochromines (1).—(a) *Adenochromines A + C: preparation of secoadenochromine C* (2c). (1a and c) (48.0 mg) was hydrogenated over PtO_2 (100 mg) in 0.1M-HCl (6 ml) at room temperature. After 24 h the catalyst was removed by centrifugation and washed with 0.1M-HCl. The brown supernatants were combined and evaporated to dryness under reduced pressure. The residue, taken up in 3M-HCl, was chromatographed on Dowex column 4 (3M-HCl as eluant); the brown polymeric material remained on the resin. The first 50 ml of eluate was evaporated and the residue was again chromatographed on Dowex column 2: fractions 6—8 gave 9.5 mg [60% of the calculated yield, corrected for (1) and (2c)] of histidine (6); 11—12, 1.4 mg (9%) of (5); 16—21, 8.1 mg (25%) of pure secoadenochromine C (2c); 30—40, 11.4 mg (24%) of recovered (1a and c).

(b) *Adenochromine B.* (1b) (12.4 mg) was hydrogenated over PtO_2 (30 mg) in 0.1M-HCl (6 ml) at room temperature for 24 h. Work-up as above yielded (6), (5), 4% (u.v.) of (2b) (identified by amino-acid analysis), and 4.6 mg (37%) of the starting material (1b).

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