Chemical Studies on Hemocyanins. I. Amino Acid Composition*

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ABSTRACT: Hemocyanins prepared from the blood of *Octopus vulgaris*, *O. macropus*, *Eledone moschata* (Cephalopoda); *Murex trunculus*, *M. brandaris* (Gastropoda); *Eriphia spinifrons*, *Callinectes sapidus*, *Homarus vulgaris*, *Palinurus vulgaris* (Crustacea); and *Limulus polyphemus* (Xiphosura) have been analyzed, after acid hydrolysis, by column chromatography. Tryptophan has been determined after enzymatic hydrolysis as well as by titrimetric and spectrophotometric methods. The data obtained are reported as g of amino acid residues/100 g of protein and as numbers of residues/mole of protein containing two copper atoms. The amino acid composition was determined as ratios to 2 g-atoms of bound copper. Half-cystine and sulfhydryl groups were determined by several techniques. Hemocyanins were oxidized with performic acid or alkylated with iodoacetic acid after reduction, and the resulting cysteic acid and *S*-carboxymethylcysteine were determined chromatographically. The data thus obtained have been compared with the results from amperometric titration with silver ions and from determinations with *p*-mercuribenzoate on reduced hemocyanins. The number of half-cystine residues per molecule of protein was found to vary from 3, 4, 6, and 8 to as many as 10 in hemocyanins from different animal species. Free sulfhydryl groups in both native and copper-free hemocyanins have been determined by amperometric titration in the presence or in absence of urea. Most of the hemocyanins examined (*Octopus*, *Homarus*, *Palinurus*) do not reveal any free SH groups; after removal of copper, however, a single sulfhydryl group can be demonstrated for each one (*Homarus*) or two (*Octopus*, *Eriphia*) functional units, *i.e.*, for two or four atoms of copper removed.

Hemocyanins are copper proteins found in the hemolymph of several marine and terrestrial invertebrates belonging to Mollusca and Arthropoda. The presence of copper confers to the protein the ability to combine reversibly with molecular oxygen and to function as an oxygen-carrying pigment. When deoxygenated, hemocyanins are colorless; during the oxygenation reaction one molecule of oxygen combines stoichiometrically with two atoms of copper and the protein becomes blue. The spectrum of oxygenated hemocyanins shows bands (the "copper bands") in the near ultraviolet and in the visible region; they disappear in the absence of oxygen.

Hemocyanins are proteins of high molecular weight, which can be easily dissociated into subunits; the dissociation–association reaction depends on protein concentration, ionic environment, pH, and other factors. This has been established for all hemocyanin species (for review, see Redfield, 1950; Manwell, 1960; Bielig and Bayer, 1960; Ghiretti, 1962). There are, however, several marked differences among hemocyanins of different origin. On the basis of the elemental composition, minimal molecular weight, electrophoretic behavior, electron micrographs of the "molecular aggregates," and position of the "copper bands," these respiratory proteins have been classified into two groups: molluscan hemocyanins and arthropod hemocyanins.

In contrast to the large body of physical data, information on the chemical composition of hemocyanins is limited. Apart from old determinations of some amino acid components (Roche, 1935; Mazur, 1937), the only complete analyses recently reported are those on the hemocyanin of *Helix pomatia* by Moring-Claesson (1956) and of *Octopus vulgaris* by Bayer and Fiedler (1962). Accurate determinations of the sulfhydryl groups are also scanty; this is more surprising in view of the current interest in the role of SH groups in proteins in general and in their possible participation in the structure of the "active site" of hemocyanins in particular, *i.e.*, in the linkage between the metal and the protein (Klotz and Klotz, 1955).

The main objective of this study was the determination of the amino acid composition of hemocyanins prepared from various species of Mollusca and Arthropoda. Before detailed analyses were undertaken, procedures had to be developed for purification of the protein from each animal species considered, for the determination of the copper/protein ratio, and for the preparation of apohemocyanins. The experimental conditions for the acid hydrolysis were first studied herein with the purpose of evaluating in quantitative terms the effect of copper and of acid concentration upon the digestion of hemocyanin. Tryptophan, cysteine, and cystine were determined by special methods;

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the number of sulfhydryl groups was evaluated in both native and copper-free protein.

Materials and Methods

Preparation of Native and Copper-free Hemocyanins. The hemocyanins of the following invertebrate species were used: *Octopus vulgaris*, *O. macropus*, *Eledone moschata*, *Murex trunculus*, *M. brandaris* (Mollusca); *Eriphia spinifrons*, *Callinectes sapidus*, *Palinurus vulgaris*, *Homarus vulgaris* (Crustacea); *Limulus polyphemus* (Xiphosura).

The blood was taken directly from the vessels or from the heart or hemocoel according to the species used. Lymphocytes and particulate impurities were removed by centrifugation at 600*g*, and hemocyanin was sedimented from the supernatant in a Spinco preparative ultracentrifuge (Model L, rotor 50) for 5 hr at 180,000*g*. The protein was dissolved in a small amount of water and dialyzed overnight in the cold. The small precipitate occasionally formed was centrifuged off at 3000*g*, and the hemocyanin solution was then lyophilized in the presence of sucrose (two parts to one part of protein, according to Heirweg *et al.*, 1959).

Apohemocyanin was prepared by incubating the native protein with cyanide (10 moles of KCN/mole of copper) at 4° in air for 18–20 hr; the copper–cyanide complex was removed by passage through a Sephadex G-25 or an Amberlite MB-1 deionizing column (Ghiretti-Magaldi and Nardi, 1963). Copper-free hemocyanins were also lyophilized in the presence of sucrose, as described above. In several experiments, fresh preparations of native hemocyanin or apohemocyanin were used. In all other cases, the frozen dried proteins were dissolved in water before use and dialyzed *vs.* distilled water.

Amino Acid Analysis. Hydrolysis was carried out at 110 ± 2° for 22, 45, or 70 hr with redistilled hydrochloric acid (Mallinckrodt) in evacuated Pyrex ampoules. Hemocyanin solution (0.5 ml) (*ca.* 4 mg of protein) and 0.5 ml of HCl were frozen in a Dry Ice–acetone bath; the ampoule was evacuated twice to <50 μ and sealed under vacuum. The colorless or light yellow hydrolysates were taken to dryness; the amino acid hydrochlorides were then dissolved in 6 ml of 0.2 M sodium citrate buffer at pH 2.2, and the amino acid composition was determined in duplicate (1 mg of protein/run) by ion-exchange chromatography in a Beckman Spinco Model 120 automatic amino acid analyzer (Spackman *et al.*, 1958).

Determination of Tryptophan. Hemocyanin samples were analyzed for tryptophan content either spectrophotometrically (Goodwin and Morton, 1946; Beaven and Holiday, 1952), or by titration with *N*-bromosuccinimide (NBS)[1] (Patchornick *et al.*, 1958), or after enzymatic digestion of the protein with Pronase (Nomoto *et al.*, 1960). For the NBS titration the reaction mixture contained: 9 M urea, 0.1 M acetate buffer, pH 4.2, and variable amounts (0.2–0.6 mg/ml) of protein. Aliquots (10 μl) of 5×10^{-3} M NBS solution were added at intervals. Readings were taken at 280 mμ *vs.* a blank containing the same mixture except NBS and were corrected for dilution. The digestion with Pronase (Panprotease, Worthington Biochemical Corp.) was carried out for 7 days at pH 8 and 39° in evacuated Thunberg tubes containing 10 mg of protein and 1 mg of enzyme (final volume, 1 ml). In some experiments 0.1 ml of 10^{-3} M $CaCl_2$ and 0.05 ml of 98% ethanol were added to the reaction mixture. Controls without hemocyanin were always included. Aliquots (0.3 ml) of the enzymatic digest (both of the samples and of the controls) were dialyzed *vs.* distilled water; the dialysates were dried under vacuum and the residues redissolved in 0.2 M citrate buffer at pH 2.2 and analyzed by ion-exchange chromatography. Additional samples of the enzymatic digest were further hydrolyzed with hydrochloric acid for 22 hr at 110° and their amino acid content, determined as above, was used to calculate the extent of the enzymatic hydrolysis.

Determination of Total Cysteine. Cysteine was determined by the following procedures: (1) cleavage of the disulfide bonds by oxidation with performic acid (Schram *et al.*, 1954) and determination of the cysteic acid formed by column chromatography; (2) reduction of disulfides with sodium borohydride (Moore *et al.*, 1958) followed by amperometric titration of SH groups (Benesch *et al.*, 1955), or spectrophotometric titration with *p*-mercuribenzoate (PCMB) (Boyer, 1954), or alkylation with iodoacetic acid and determination of *S*-carboxymethylcysteine after acid hydrolysis (Cole *et al.*, 1958). The reaction mixture contained equal amounts (100 mg) of both hemocyanin and of sodium borohydride in the presence of 8 M urea; reduction was complete after 1 hr at 50°.

Determination of Free Sulfhydryl Groups. Fresh, nonlyophilized native and copper-free hemocyanin preparations were used as soon as possible to avoid changes due to aging of the protein (Ghiretti-Magaldi *et al.*, 1962). The amperometric titration was carried out in the presence or absence of urea as denaturing agent, and the results obtained were checked by titration with PCMB.

Other Analytical Methods. PROTEIN concentration was estimated by conventional titrimetric, colorimetric, and spectrophotometric methods. On the basis of the dry weight of the protein and of the analytical values obtained, the conversion factor from nitrogen to protein and the extinction coefficients at 278 mμ were calculated for each hemocyanin preparation. COPPER was determined with 2,2′-biquinoline (Felsenfed, 1960) after reduction of the Cu^{2+} with hydroxylamine. Control analyses were carried out with the diethyldithiocarbamate method after wet digestion (Eden and Green, 1940). Spectra were obtained in an automatic recording Beckman DK-2 spectrophotometer. Performic acid was prepared by adding 1 volume of 30% hydrogen peroxide to 9 volumes of 88% formic acid; commercial

[1] Abbreviations used: NBS, *N*-bromosuccinimide; PCMB, *p*-mercuribenzoate; Hc, hemocyanin.

TABLE I: Properties of Native and Apohemocyanins.

Animal Species	Native Hc Cu/ Protein (%)	Apo-Hc Cu/ Protein (%)	Cu Removed (%)	Max (mμ)	E_{278}/ mg of Protein	N (%)	S (%)	Ref
Mollusca								
O. vulgaris	0.250	0.044	82					Kubowitz (1938)
O. vulgaris	0.245	0.075	69.4			15.9		Bayer and Fiedler (1962)
O. vulgaris	0.250	0.036	85.6	348	1.35	16.3	1.26	This work
O. macropus	0.254	0.041	84	346	1.66	16.4		This work
E. moschata	0.252	0.036	86	347	1.49	15.0		This work
M. brandaris	0.246	0.080	67.5	345	1.81	14.6		This work
M. trunculus	0.257	0.050	80.5	345	1.89	14.7		This work
Loligo pealei	0.260			345	1.58			Van Holde and Cohen (1964)
Arthropoda								
E. spinifrons	0.167	0.006	97	338	1.61	16.2	1.29	This work
P. vulgaris	0.170	0.020	88	337	1.38	15.4		This work
H. vulgaris	0.169	0.002	99	335	1.44	15.5		This work
L. polyphemus	0.170	0.005	97	341	1.12			This work
C. sapidus	0.132	0.012	90	336	1.24			This work
Cancer magister	0.166	0.025	84.3[a]	338				Thomson *et al.* (1959)

[a] Calculated from the data reported in Table II (Thomson *et al.*, 1959).

urea was purified on Amberlite "monobed" MB-1 column and recrystallized.

Results

Properties of the Hemocyanin Preparations. It was observed (Ghiretti-Magaldi *et al.*, 1962) that in hemocyanins kept frozen at −20°, up to 80% of the copper (as determined in the absence of oxygen) is converted to Cu^{2+}. In lyophilized preparations, however, no oxidation of the metal occurs and all the chemical, physical, and immunological properties of the protein remain unchanged.

For most of the arthropod hemocyanins, *ca.* 100% removal of copper by cyanide was obtained; for molluscan hemocyanins, however, no more than 85% of the metal was removable even when the experiments were carried out in the absence of air or the presence of hydrogen, with or without thioglycolate as reducing agent. Numerous attempts using chelating agents other than cyanide, as Versene, *o*-phenanthroline, α,α′-dipyridyl, and sequestering resins such as Dowex A-1 or Chelex, failed to remove the metal from the protein.

Copper-free hemocyanin shows the same electrophoretic behavior and the same immunological properties as the native protein. When apohemocyanin was incubated with Cu_2O, 80% of the original hemocyanin was reconstituted (Ghiretti-Magaldi and Nardi, 1963). In the 278-mμ region, the ultraviolet spectrum of apohemocyanin appears to be identical with that of the native protein. As shown in Table I, in crustacean hemocyanins the first "copper band" is displaced toward shorter wavelengths when compared with that of molluscan hemocyanins. The per cent nitrogen and the extinction coefficient values are also reported.

Amino Acid Composition. The recovery of amino acids from hemocyanins, for the three time periods of acid hydrolysis, and the average values, were calculated as g of anhydroamino acid/100 g of protein. The values obtained show deviations of <3% from the average values. For those amino acids which undergo incomplete liberation during acid hydrolysis, the results of the 22-hr hydrolysis period were excluded from the average calculations. A few erratic results which showed higher deviations from the average were also excluded. Threonine and serine, which are subject to extensive destruction during the acid hydrolysis, were calculated by extrapolation to zero time. In all experiments, the HCl concentration was maintained at *ca.* 6 N, and 1 ml of the acid was used to hydrolyze *ca.* 4 mg of protein.

The minimal molecular weights of molluscan and arthropod hemocyanins, as calculated from the copper/protein ratio,[2] are 25,400 and 37,450, respectively. Being the copper/oxygen ratio equal to 2 (Redfield, 1950), the minimal molecular weight of the functional unit is 50,800 for the hemocyanin of Mollusca and 74,900 for that of Arthropoda. The minimal molecular

[2] The copper content of hemocyanins ranges from 0.24 to 0.26% for Mollusca and from 0.16 to 0.18% for Arthropoda. For calculations we used the mean values of 0.25 and 0.17%.

TABLE II: Amino Acid Composition of the Hemocyanin from *O. vulgaris*.

Amino Acid	Amino Acid Residues (g)/100 g of Protein, Time of Hydrolysis (hr) 22	45	70	Av[a]	Min Mol Wt[b]	Assumed *n* of Residues	Calcd Mol Wt[c]	Calcd *N* of Residues for Mol Wt 50,800	*N* of Residues to Near Integer
Lysine	(4.15)	5.02	5.04	5.03	2,554	20	51,080	19.89	20
Histidine	5.91	5.99	6.32	6.07	2,259	22	49,698	22.49	22
Ammonia	1.05	1.03	1.19	1.09	...	...	...	...	...
Arginine	4.95	4.93	5.15	5.01	3,178	16	50,848	15.98	16
Tryptophan[d]	...	...	...	2.47	7,538	7	(52,766)	6.74	7
Aspartic acid	(10.28)	11.10	11.06	11.08	1,039	49	50,911	48.89	49
Threonine[e]	4.42	4.41	4.36	4.45	2,272	22	49,984	22.36	22
Serine[e]	3.40	3.25	3.01	3.60	2,419	21	50,799	21.00	21
Glutamic acid	(10.03)	10.23	10.26	10.25	1,269	40	50,760	40.03	40
Proline	(4.21)	4.48	4.67	4.58	2,120	24	50,880	23.96	24
Glycine	2.46	2.56	2.48	2.50	2,284	22	50,248	22.24	22
Alanine	(3.83)	3.89	4.07	3.98	1,786	28	50,008	28.44	28
Half-cystine[f]	...	...	...	2.02	5,105	10	(51,050)	9.95	10
Valine	(4.41)	4.95	5.25	5.10	1,943	26	50,518	26.15	26
Methionine[g]	...	...	...	2.84	4,620	11	50,820	11.00	11
Isoleucine[h]	(4.16)	4.78	4.94	4.86	2,329	22	51,238	21.81	22
Leucine	(7.84)	8.45	8.59	8.52	1,329	38	50,502	38.22	38
Tyrosine	(5.58)	5.92	5.80	5.86	2,785	18	50,130	18.24	18
Phenylalanine	(6.58)	6.84	7.22	7.03	2,094	24	50,256	24.26	24
Total	86.6	90.3	91.9	96.3		420		421.65	420
Av[i]							50,543 ± 375		

[a] Calculated from the analytical data at different times of hydrolysis. The numbers in brackets were excluded from the average calculation. [b] Calculated from the relationship: mol wt of amino acid residue × 100/per cent amino acid residue. [c] Calculated on the basis of two copper atoms/mole of protein. See text for details. [d] Determined chromatographically after enzymatic hydrolysis. [e] Calculated by linear extrapolation to zero time. [f] Calculated by independent determinations. See text. [g] Determined after performic acid oxidation. [h] Corrected for the formation of alloisoleucine. [i] This is the average molecular weight for all amino acids except tryptophan and half-cystine.

weight of the protein containing one residue of each individual amino acid was derived from the relationship mol wt of amino acid residue × 100/per cent amino acid residue, and the nearest integral numbers of amino acid residues for 50,800 and 74,900 were computed. Most of these numbers range between 20 and 50 for the protein of Mollusca and exceed 50 for that of Arthropoda. With the precision of the method (3% average deviation from the mean), a good estimation of the molecular weight could be made from the weight percentage of those amino acids present to the extent of 10 residues or less/mole of protein. Only tryptophan and half-cystine, for which the analyses are least reliable, fall in this category. A new molecular weight, therefore, was calculated, which is the average value of all the molecular weights obtained for each amino acid with the exception of cysteine and tryptophan. For the hemocyanins of *O. vulgaris* and of *E. spinifrons* the values of 50,543 ± 375 and 74,770 ± 769 were obtained, respectively; they are in good agreement with those calculated from the copper/protein ratio. An example of this calculation is reported in Table II. The average per cent recovery and the number of amino acid residues/mole of protein for all hemocyanins studied are listed in Table III.

Tryptophan was determined chiefly spectrophotometrically from the ratio moles of tryosine/mole of tryptophan (Beaven and Holiday, 1952) and by using the values of tyrosine obtained by the amino acid analyses of the acid hydrolysates. All determinations were carried out with copper-free hemocyanin; the native protein absorbs *ca.* 340 mμ and a large correction factor should, therefore, have been introduced into the calculations.

The spectrophotometric method was controlled by titration with NBS and by ion-exchange chromatography after enzymatic digestion. As shown in Table IV good agreement was found by the three methods. The NBS reaction which occurs between pH 4.2 and 7.0 is maximal at 4.2 (Patchornick *et al.*, 1958). Since

TABLE III: Amino Acid Composition of Molluscan and Arthropod Hemocyanins.

	Mollusca										Crustacea								Xiphosura	
	O. vulgaris		*O. macropus*		*E. moschata*		*M. brandaris*		*M. trunculus*		*E. spinifrons*		*H. vulgaris*		*P. vulgaris*		*C. sapidus*		*L. polyphemus*	
Amino Acid[a]	%	N[b]	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N
Lysine	5.03	20	5.28	21	4.69	19	4.50	18	4.93	20	4.32	25	4.93	29	4.75	28	5.14	30	6.14	36
Histidine	6.07	22	6.54	24	6.70	25	7.95	29	7.48	28	7.93	43	7.37	40	7.62	42	6.90	38	8.36	46
Ammonia	1.09		0.92		1.47		1.04		0.94		1.19		1.31		1.34		1.35		1.08	
Arginine	5.01	16	5.72	19	5.01	16	6.16	20	6.07	20	5.97	29	6.11	29	6.48	31	6.39	31	6.47	31
Tryptophan[c]	2.47	7	2.07	6	2.61	7	2.63	7	2.63	7	2.42	10	1.74	7	2.04	8	1.98	8		
Aspartic acid	11.08	49	11.04	49	11.31	50	11.53	51	11.81	52	12.48	81	12.19	79	14.41	94	13.51	88	10.98	71
Threonine[d]	4.45	22	5.16	26	4.20	21	4.40	22	4.30	22	4.44	33	4.95	37	4.20	31	4.50	33	4.27	31
Serine[d]	3.60	21	3.92	23	3.96	23	3.45	20	3.67	21	3.90	34	3.05	26	2.86	25	3.48	30	3.50	30
Glutamic acid	10.25	40	10.92	43	10.16	40	12.21	48	12.41	49	10.61	62	11.77	68	12.12	70	12.36	72	12.14	70
Proline	4.58	24	4.47	23	4.29	22	4.22	22	4.42	22	3.63	28	3.57	28	3.89	30	4.06	31	3.42	26
Glycine	2.50	22	2.76	25	2.73	24	2.87	26	3.04	27	2.71	36	2.73	36	2.95	39	3.29	43	2.71	36
Alanine	3.98	28	3.97	28	3.80	27	3.95	28	4.08	29	3.56	38	3.44	36	3.34	35	4.27	45	2.83	30
Half-cystine[e]	2.02	10	1.69	8	1.63	9	1.33	7	1.29	6	0.45	3	0.64	5	0.70	5	0.85	6	1.40	10
Valine	5.10	26	4.75	24	4.58	24	4.73	24	4.92	25	5.46	41	5.27	40	5.25	40	5.78	44	5.44	41
Methionine[f]	2.84	11	2.70	10	2.55	10	2.33	9	2.48	10	2.86	16	2.45	14	3.24	19	2.80	16	2.63	15
Isoleucine[g]	4.86	22	5.29	24	4.89	22	3.83	17	4.02	18	4.02	27	4.53	30	4.76	32	4.84	32	4.97	33
Leucine	8.52	38	8.97	40	8.25	37	8.24	37	8.51	38	6.87	45	6.79	45	6.78	45	7.20	48	7.87	52
Tyrosine	5.86	18	6.01	19	5.40	17	5.20	16	5.47	17	4.98	23	4.60	21	4.66	21	5.73	26	4.87	22
Phenylalanine	7.03	24	6.89	24	6.77	23	7.75	27	7.73	27	7.21	37	6.80	34	6.89	35	7.04	36	6.34	32
Total	96.3	420	99.1	436	95.3	415	98.3	428	100.1	438	95.0	611	94.3	604	98.3	630	101.5	657	95.4	612

[a] Calculated from the analytical data at different times of hydrolysis. Amino acid residues (g)/100 g of protein (average value). [b] Calculated for a molecular weight of 50,800 (Mollusca) and 74,900 (Arthropoda). Number of residues to nearest integer. [c] In *O. vulgaris* and *E. spinifrons* obtained by chromatography after enzymatic hydrolysis; in all other hemocyanins by spectrophotometric determination. [d] Calculated by linear extrapolation to zero time. [e] In *C. sapidus* and *L. polyphemus* calculated by linear extrapolations to zero time; all other values obtained by independent determinations. See text. [f] In *O. vulgaris* and *E. spinifrons* determined after performic acid oxidation. All other values corrected for methionine sulfoxides. [g] Corrected for alloisoleucine.

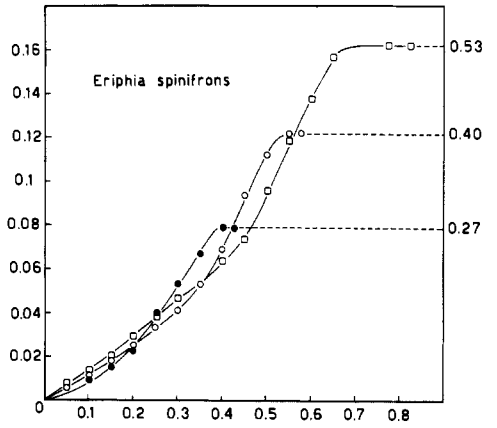

FIGURE 1: NBS titration of tryptophan in different amounts of hemocyanin from *E. spinifrons*. Abscissa: μmoles of NBS; ordinate left: optical density at 280 mμ; right: milligrams of protein used.

hemocyanin is insoluble at this pH, determinations were carried out at pH 7.0. Under these conditions, low recovery of tryptophan was obtained as compared with the results of the two other methods. It was found, however, that the end point of the reaction between the amino acid and NBS was reached when the protein was titrated at pH 4.2 in the presence of 9 M urea. A good proportionality was also observed between the end-point reaction and the amount of protein used (Figure 1).

TABLE IV: Tryptophan Content of Hemocyanins from *O. vulgaris* and *E. spinifrons* as Determined by Independent Methods.[a]

	Spectrophotometric Detn	Titration with NBS	Anal. after Enzymatic Hydrolysis
O. vulgaris	2.44	2.44	2.47
E. spinifrons	2.47	2.04	2.42

[a] G/100 g of protein.

Digestion with Pronase (Nomoto *et al.*, 1960) also gives satisfactory values. The extent of hydrolysis of the *Octopus* and *Eriphia* hemocyanins was equal to 74–80% of the total protein. Native and copper-free hemocyanins gave exactly the same results, either in the presence or absence of calcium chloride and of ethanol.

Distribution of Sulfur. Table V shows the total number of cysteine residues as determined by various methods. Several difficulties were encountered with the oxidation procedure. As is known, the yield of cysteic acid residues from the protein is not quantitative; under the conditions used, an over-all recovery of 90% (Schram *et al.*, 1954) is obtained. However, cysteine values determined as cysteic acid after performic acid oxidation were higher than those obtained with other analyses.

Among the several reagents that can be used for reductive cleavage of disulfide bonds, sodium borohydride was found to give the most reproducible results. Thioglycolic acid and β-mercaptoethylamine were also tried (Bailey, 1962). These reagents, however, when applied to hemocyanin, presented technical difficulties and gave inconsistent results. The estimation of cysteine residues in hemocyanin as *S*-carboxymethylcysteine was not quantitative. A partial inhibition of the methylation reaction by traces of copper still present in the apohemocyanin preparations could account for the lower recoveries obtained, as compared with the values given by the amperometric titration and by the interaction with PCMB. The methylation reaction, however, has to be considered complete, since no residual half-cystine was found among the amino acids of the acid hydrolysate after carboxymethylation.

All the estimations of cysteine by determination of sulfhydryl groups (amperometric and titrimetric with PCMB) were carried out on reduced hemocyanins in the presence of urea as a denaturing agent. It was found that, under our experimental conditions, native hemocyanin undergoes oxidative changes, primarily due to the presence of copper, which restricted the estimation to apohemocyanin only. Both silver and PCMB titrations gave somewhat similar results. The number of cysteine residues per molecule of protein was found to vary from 3, 4, 6, and 8 to as many as 10. The sulfur content of the hemocyanin of *O. vulgaris* amounts to 1.26%; by adding the sulfur of cysteine and methionine residues, a value of 1.30% was obtained.

The determination of sulfhydryl groups was repeated using native and apohemocyanins, freshly prepared, not lyophilized and not treated with reducing agent, with the aim of establishing whether free SH groups are present in the protein molecule. The results obtained by amperometric titrations are reported in Table VI.

Native hemocyanin of *O. vulgaris* did not take up silver ion, indicating the absence of reactive thiol groups. However, after the removal of copper, the presence of 0.56 mole of SH/mole of protein was revealed, corresponding to one new sulfhydryl for each four atoms of copper removed. Silver ion uptake was reduced either when air was bubbled into the mixture or after incubation with PCMB. Similar results were obtained with the hemocyanin of *E. spinifrons*, which was found to bind 0.5 mole of Ag^+ in the native state and 1 mole after copper was removed. Also in the hemocyanin of *H. vulgaris*, sulfhydryl groups could be titrated only after the removal of copper. In this case, however, one new SH is revealed for each two atoms of copper removed.

TABLE V: "Total Cysteine" Content as Determined by Different Methods.[a]

Species	As Cysteic Acid[b]	As *S*-Carboxymethylcysteine[c]	Amperometric Silver Titration[d]	Reaction with PCMB[e]
Mollusca				
O. vulgaris	10.49 (6)	7.65 (6)	9.84 (2)	9.75 (13)
O. macropus	...	...	...	8.34 (6)
E. moschata	...	7.08 (1)	...	8.02 (6)
M. trunculus	...	...	...	6.36 (5)
M. brandaris	...	...	...	6.55 (4)
Crustacea				
E. spinifrons	3.90 (6)	1.47 (6)	2.73 (3)	2.90 (6)
H. vulgaris	...	...	4.61 (1)	4.61 (4)
P. vulgaris	...	...	...	5.04 (6)

[a] Moles/mole of protein containing two copper atoms. In parentheses number of experiments. [b] Amounts of hemocyanin containing 1–2 mg of cystine were added to 25 ml of performic acid and left at 0° for 4 hr. Excess reagent was removed under vacuum at 40° and the oxidized protein was hydrolyzed in an evacuated sealed tube with 6 N HCl at 110° for 22 hr. Cysteic acid was determined on the long column of the amino acid analyzer. [c] Iodoacetic acid (12 mg) was added to 40 mg of protein. Incubation was for 3–4 hr at 25° and pH 9. After 20 hr of dialysis *vs.* water, the carboxymethylated protein was hydrolyzed and chromatographed. Pure *S*-carboxymethylcysteine (Michaelis and Schubert, 1934) was used as standard. [d] Reduced hemocyanin was brought to pH 5.0 with HNO_3, diluted with water, and kept for 20–30 min at room temperature under a stream of argon. The reaction mixture consisted of 10 μl of hemocyanin solution (50 mg of protein), 3 ml of 1 M Tris–nitric acid buffer at pH 7.4 made up in 8 M urea, and 0.3 ml of 1 M KCl. Titration was carried out at 4° in ice bath with 5×10^{-3} M $AgNO_3$. A known amount of reduced glutathione was titrated before and after each determination. [e] The test Beckman cuvet contained 1 mg of reduced hemocyanin in 3 ml of 0.1 M phosphate buffer pH 7 containing 8 M urea, and mercaptide formation was followed after addition of 10-μl aliquots of 1×10^{-3} M PCMB. The reference cuvet contains no hemocyanin. Protein concentration had been previously calculated spectrophotometrically on the basis of the specific absorption coefficient determined under the same experimental conditions.

TABLE VI: Free Sulfhydryl Groups of Native and Apohemocyanins as Determined by Amperometric Titration.[a]

Species	Native	Cu Free
Mollusca		
O. vulgaris	0.04 ± 0.04 (12)	0.56 ± 0.09 (12)
O. macropus	0.08 ± 0.08 (7)	0.39 ± 0.04 (7)
M. trunculus	0.46 ± 0.09 (7)	0.54 ± 0.09 (8)
M. brandaris	0.54 ± 0.09 (10)	0.54 ± 0.06 (7)
Crustacea		
E. spinifrons	0.59 ± 0.02 (11)	0.93 ± 0.12 (8)
H. vulgaris	0.08 ± 0.08 (5)	0.80 ± 0.06 (10)
P. vulgaris	0.00 (6)	0.00 (6)

[a] Moles/mole of protein containing two copper atoms. Number of experiments in parentheses.

When the amperometric titration was carried out on the hemocyanins of the gastropod *M. trunculus* and *M. brandaris* and of the lobster *P. vulgaris*, no difference was found before and after the removal of copper. *Palinurus* hemocyanin seems to lack free SH groups; however, native hemocyanin and apohemocyanin of *Murex* show 0.5 mole of SH/protein molecule. The nonstoichiometric values obtained and the different results given by hemocyanins from different animal species called for repeated estimations in a number of preparations and under a variety of experimental conditions.

Spectrophotometric titration (Boyer, 1954) on 0.1 M phosphate buffer at pH 7 indicated no reaction between PCMB and either native or copper-free hemocyanins. It is known that the nature of buffers and salts in the titration mixture is critical for accurate quantitative determination of thiol groups with PCMB. It has also been reported recently (Cooms *et al.*, 1964) that the reaction of PCMB with carboxypeptidase is instantaneous and complete in Tris and sodium perchlorate. Since the solubility of apohemocyanin also increases in concentrated Tris buffer, native and copper-free hemocyanins were titrated with PCMB in the presence of 1.0 M Tris and 1.0 M sodium perchlorate. The results obtained with the hemocyanin of *O. vulgaris* under these conditions were exactly the same as those of the amperometric titration, *i.e.*, 0.5 mole of mercaptan was titrated/mole of protein only after the removal of copper. With other hemocyanins, however, the results of the spectrophotometric titration under the same conditions were entirely

different and unreliable. It was observed that when dissolved in a mixture of 1.0 M Tris–$NaClO_4$, hemocyanin shows spectral changes, mainly due to the gradual disappearance of the "copper band" in the near-ultraviolet and the increase of absorption at 250 mμ. Whereas in the hemocyanin of *O. vulgaris* these spectral modifications are practically negligible, in the other hemocyanins, especially that of *E. spinifrons*, the rapid increase of the 250-mμ absorbance is responsible for the higher values obtained when compared with the results of amperometric titration.

Attempts to determine free SH groups by PCMB in the presence of high concentrations (2 M) of Tris buffer alone gave values close to those obtained with silver titration. However, increase in optical density at 250 mμ due to mercaptide formation was always low, sometimes below the limits of reliability of the instrument used.

Discussion and Conclusions

The number of analyses reported here is not large enough to allow a comparative study of the amino acid composition of hemocyanins. Among the variety of invertebrate animals which possess this protein in the blood, only 10 marine species from Mollusca and Arthropoda have been considered. A classification of hemocyanins on the basis of their amino acid composition can be attempted when other species, aquatic and terrestrial, are studied. This work is in progress.

The hemocyanins so far examined do not present striking dissimilarities in their amino acid content. All the most common amino acids, cysteine and tryptophan included, are present. In general, hemocyanins from closely related species, when not identical, resemble each other more than they do those from species belonging to distant groups. Previous experiments (Parisi *et al.*, 1962) have shown that the immunological response is the same for the protein of *O. vulgaris* and *O. macropus* and of *M. trunculus* and *M. brandaris*. On the contrary, Cephalopoda and Gastropoda do not show cross reactions with Crustacea. The hemocyanin of *L. polyphemus* does not give an immunological cross reaction with that of any other species.

Peptide composition analyzed by the "fingerprint" method after tryptic digestion also indicated a great dissimilarity between hemocyanins from different sources, the peptides present in the digest being typical for each species examined (A. Ghiretti-Magaldi, C. Nuzzolo, and F. Ghiretti, unpublished data).

No difference was found in the amino acid composition of native and copper-free hemocyanins, neither with respect to total recovery of per cent of amino acid residues, nor to the number of residues of each amino acid. This is a further indication that the removal of copper does not modify the composition of the protein. As mentioned, also the electrophoretic behavior of apohemocyanin is the same as that of the native protein. In this connection, Cohen and Van Holde (1964) could find no detectable configurational changes in hemocyanin of *Loligo pealei* after the removal of copper, and Van Bruggen *et al.* (1962) could detect no difference in electron micrographs of the native hemocyanin and the apohemocyanin of *H. pomatia*.

The high percentage of aspartic and glutamic acids is a noteworthy aspect of the amino acid composition of hemocyanin, dicarboxylic acids accounting for *ca.* 25% of the total residues. High figures were also found for histidine, leucine, and phenylalanine. As for tryptophan, with the exception of *H. vulgaris*, the content is fairly similar in all the species studied and the values obtained are of the same order as those found in other proteins, such as hemoglobins, globulins, and cytochromes (Tristram and Smith, 1963).

The number of cysteine residues appears to be characteristic for each species and is different even in molluscan hemocyanins which present many similarities in their amino acid composition. From three to six cysteine residues can be titrated in Crustacea, in a total of >600 amino acid residues; higher values were found in Mollusca, as many as 10 in a total of 423 amino acid residues (*O. vulgaris*). They represent all the SH groups which can be estimated after reduction of the protein with borohydride, in the presence of urea as denaturing agent.

The results of titration in nonreduced protein, before and after the removal of copper, seem to indicate that most of the hemocyanins examined do not have any free SH group available for titration; in some of them, however, after the removal of copper, one SH group is revealed per each two functional units, *i.e.*, per four atoms of copper removed. Similar results have been reported by Thomson *et al.* (1959). This could indicate either that no bonds of the Cu–S type are present in the hemocyanin molecule, or that sulfhydryl groups may participate in the composition of the "active center" through the linkage of only one atom of copper. Our finding (A. Ghiretti-Magaldi, C. Nuzzolo, and F. Ghiretti, unpublished data) that molluscan hemocyanin consists of two polypeptide chains, one of which has a COOH-terminal cysteine, would support the latter hypothesis.

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References

Bailey, J. L. (1962), Techniques in Protein Chemistry, Amsterdam, Elsevier, p 304.

Bayer, E., and Fiedler, H. (1962), *Ann. Chem. 653*, 149.
Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Benesch, R. E., Lardy, H. A., and Benesch, R. (1955), *J. Biol. Chem. 216*, 663.
Bielig, H. J., and Bayer, E. (1960), Handbook Physiology and Pathological Chemical Analysis, Vol. 4, Germany, Hoppe-Seyler Thierfelder, 714.
Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.
Cohen, L. B., and Van Holde, K. E. (1964), *Biochemistry 3*, 1809.
Cole, R. D., Stein, W. H., and Moore, S. (1958), *J. Biol. Chem. 233*, 1359.
Cooms, T. L., Omote, Y., and Vallee, B. L. (1964), *Biochemistry 3*, 653.
Eden, A., and Green, H. H. (1940), *Biochem. J. 34*, 1202.
Felsenfeld, G. (1960), *Arch. Biochem. Biophys. 86*, 247.
Ghiretti, F. (1962), *in* The Oxygenases, Hayaishi, O., Ed., 517.
Ghiretti-Magaldi, A., Ghiretti, F., Nardi, G., Parisi, V., and Zito, R. (1962), *Boll. Soc. Ital. Biol. Sper. 38*, 1851.
Ghiretti-Magaldi, A., and Nardi, G. (1963), *in* Protides of Biological Fluids, Vol. 11, Peeters, H., Ed., p 507.
Ghiretti-Magaldi, A., and Nuzzolo, C. (1965), *Comp. Biochem. Physiol. 16*, 249.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Heirweg, K., Borginon, H., and Lontie, R. (1959), *Arch. Intern. Physiol. Biochim. 67*, 514.
Klotz, I. M., and Klotz, T. A. (1955), *Science 121*, 477.
Kubowitz, F. (1938), *Biochem. Z. 32*, 299.
Manwell, C. (1960), *Ann. Rev. Physiol. 22*, 191.
Mazur, A. (1937), *J. Biol. Chem. 118*, 631.
Michaelis, L., and Schubert, M. P. (1934), *J. Biol. Chem. 106*, 331.
Moore, S., Cole, R. D., Gundlach, H. G., and Stein, W. H. (1958), *Symp. Proteins, 4th Intern. Congr. Biochem., Vienna.*
Moring-Claesson, J. (1956), *Arkiv Kemi 10*, 1.
Nomoto, M., Narahashi, Y., and Murakami, M. (1960), *J. Biochem. (Tokyo) 48*, 593.
Parisi, V., Nardi, G., Ghiretti, F., and Ghiretti-Magaldi, A. (1962), *Boll. Soc. Ital. Biol. Sper. 38*, 1848.
Patchornick, A., Lawson, W. B., and Witkop, B. (1958), *J. Am. Chem. Soc. 80*, 4747.
Redfield, A. C. (1950), *in* Copper Metabolism, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., John Hopkins, p 174.
Roche, J. (1935), Essay sur la Biochemie Générale et Comparée des Pigments Respiratoires, Paris, Masson, p 170.
Schram, E., Moore, S., and Bigwood, E. J. (1954), *Biochem. J. 57*, 33.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Thomson, L. C. G., Hines, M., and Mason, H. S. (1959), *Arch. Biochem. Biophys. 83*, 88.
Tristram, G. R., and Smith, R. H. (1963), *Advan. Protein Chem. 18*, 227.
Van Bruggen, E. F. J., Wiebenga, F. H., and Gruber, M. (1962), *J. Mol. Biol. 4*, 1.
Van Holde, K. E., and Cohen, L. B. (1964), *Biochemistry 3*, 1803.