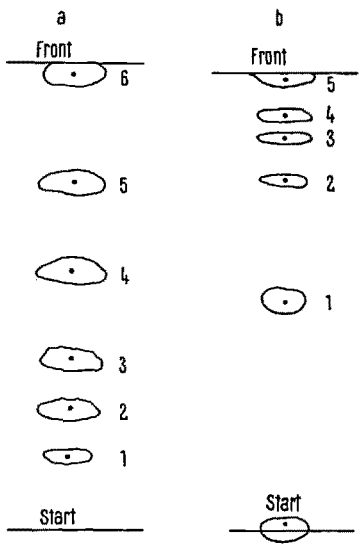
Specol spectrophotometer (Carl Zeiss, Jena). The results of the separation of the pigments by means of column and thin-layer chromatography in the specimens of Arachnoides mentioned above are given in Tables I and II and in the Figure. As the Rf values and the maximum absorption of the various fractions of the column and thin-layer chromatography show, the colour of the specimens of the Arachnoides investigated is due to the presence of a whole series of carotenoids.

From comparison of the absorption maxima of the fractions obtained by means of column and thin-layer chromatography with literature data[8–16], it is evident that such carotenoids as β-carotene, echinenone, cantaxanthin, lutein, astacene, and free astaxanthin are present. The absorption maxima of the fourth orange fraction from column chromatography indicate the presence of cryptoxanthin. A third yellowish-orange fraction of a carotenoid occurs which we have not been able to identify. Our results when compared with those obtained during investigations on other species of Hydracarina, show that[17] β-carotene, lutein, astaxanthin and keto-carotenoid are present in *Eylais extendens*. The authors of this paper have however noted in *Eylais hamata* the presence of the same carotenoids as those found in *Hydrachna geografica* and *Piona nodata*[18].



Thin-layer chromatograms of carotenoids from *Hydrachna geografica* and *Piona nodata* in systems of solvents A and B.

Résumé. Les auteurs à l'aide des méthodes de chromatographie sur colonne et sur couche fine, ont effectué la séparation des caroténoïdes chez *Hydrachna geografica* (Müller 1776) et *Piona nodata* (Müller 1776), qui appartiennent à Hydracarina (Arachnoidea). Ces recherches ont attesté la présence du β-carotène, de l'échinénone, de la cantaxanthine, de la lutéine, de l'astacène et de l'astaxanthine libre.

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Peroxidative Activity of Hemepeptides from Horse Heart Cytochrome *c*

Tuppy and Paléus[1] isolated hemepeptide by peptic digestion of cytochrome *c* and showed that it is an undecapeptide (HUP) with the sequence of

Val-Glu(NH_2)-Lys-Cys-Ala-Glu(NH_2)-Cys-His-Thr-Val-Glu
heme

By using pyrogallol as a hydrogen donor, Paléus et al.[2] reported that this compound exhibited a peroxidase-like activity 20 times higher than that of cytochrome *c*. Treatment of HUP with trypsin removed Val-Glu(NH_2)-Lys, giving a hemeoctapeptide (HOP). Although the peroxidative action of several hemepeptides has been reported, no extensive study has been made.

Horse heart cytochrome *c* (Type III, 99%) was purchased from Sigma Chemical Company and was further purified by means of an IRC-50 ion-exchange resin column. HUP was prepared by peptic digestion of cytochrome *c* according to the method of Tuppy and Paléus[1]. HOP was prepared by tryptic digestion of HUP following the method of Harbury and Loach[3]. However, a purification procedure involving Hyflo-super cel was repeated twice instead of once as in the procedure of Harbury and Loach. The HOP prepared by the Harbury and Loach method was further purified by using Sephadex G-25.

Peroxidative activities of the samples were measured spectrophotometrically at 460 nm in 0.01 *M* phosphate buffer pH 6.0, using *o*-dianisidine as a hydrogen donor[4,5]. The effect of substrate concentration on the rate of reaction was studied and all the enzyme activities were measured at the substrate concentration which gave the maximum velocity (Figure 1).

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[3] H. A. Harbury and P. A. Loach, J. biol. Chem. *235*, 3640 (1960).
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The amino acid compositions of HUP and HOP are shown in Table I. The results were comparable to those of MARGOLIASH et al.[6] and HARBURY and LOACH[3]. From amino acid analysis, it was shown that HUP contains 1 mole each of lysine, histidine, threonine, and alanine; 2 moles each of valine and cysteine; and 3 moles of glutamic acid residues. The analysis also indicated that HOP contains 1 mole each of histidine, threonine, alanine, valine, and 2 moles each of glutamic acid and cysteine residues.

Paper chromatograms of both HUP and HOP gave only 1 spot at their respective Rf values (0.30 for HUP and 0.54 for HOP). The solvent system, butanol-acetic acid-water (4:1:5), was used. The paper electrophoresis experiments also indicated that each hemepeptide was homogenous. Each hemepeptide also yielded only 1 band in Sephadex G-25 and hyflo-super cel columns.

Cytochrome *c*, HUP and HOP gave typical Soret bands at 407, 417, and 410 nm respectively in 0.1 *M* imidazole solution. On reduction of cytochrome *c*, HUP and HOP with sodium dithionite, all compounds gave typical absorption maxima at 550 and 520 nm. In the UV-region, cytochrome *c* gave a typical protein absorption maximum at 280 nm but both HUP and HOP gave no such absorption. This further confirmed the absence of tyrosine and tryptophane residues in HUP and HOP as found by the amino acid analysis shown before.

Simple inorganic ferric compounds such as ferric ammonium sulfate, ferric ammonium chloride, and ferric ammonium nitrate gave no peroxidative activity. Protohemin itself showed very little activity (Table II).

Unlike horseradish peroxidase, heat treatment of HUP and HOP did not alter the peroxidative activity. Heat-denatured cytochrome *c* gave slight increase in activity. Addition of nitrogenous compounds such as imidiazole, histidine, and ammonium chloride increased the peroxidative activity with the exception of cytochrome *c*.

Peroxidative activity of cytochrome *c* and horseradish peroxidase were readily inhibited at relatively low concentrations of cyanide ion. Both HUP and HOP were not inhibited by the cyanide concentration which inhibited the peroxidative activities of cytochrome *c* and horseradish peroxidase. However, at much higher concentrations of cyanide ion, the peroxidative activity of both HUP and HOP was inhibited (Figure 2).

Low fluoride ion concentration also inhibited the peroxidative activity of horseradish peroxidase. The peroxidative activity of cytochrome *c* was less sensitive to fluoride ion and that of HOP and HUP was not inhibited (Figure 3).

It is of interest to note that the pH optimum for each compound was different. Horseradish peroxidase gave the lowest pH value of 4.5 and cytochrome *c* gave the value of 6.0. Optimum pH of HUP and HOP were quite different (Figure 4).

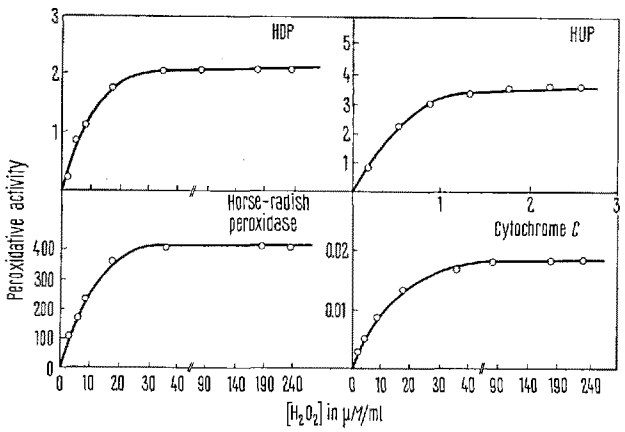

Fig. 1. Effect of substrate concentration on peroxidative activity of HOP, HUP, horseradish peroxidase, and cytochrome *c*.

Table I. Amino acid composition of hemepeptides

Amino acids	Hemeundecapeptide			Hemeoctapeptide		
	MARGOLIASH[6] et al.[6]	HARBURY and LOACH[3]	Present study	HARBURY and LOACH[3]	Present study [a]	Present study [b]
Lysine	1.6	1.02	1.12	0	0	0
Histidine	1.2	1.00	1.33	0.97	0.93	0.93
Arginine	–	0	0	0	0	0
Aspartic acid	–	0.06	0	0.14	0	0
Threonine	0.8	0.91	1.32	0.92	0.95	0.96
Serine	–	0.21	0.08	0	0	0
Glutamic acid	2.9	2.97	2.90	1.98	2.10	2.06
Proline	–	0	0	0	0	0
Glycine	–	0.06	0.09	0.20	0.12	0.10
Alanine	1.3	1.06	0.93	1.10	1.10	1.10
Valine	2.1	1.98	2.12	1.01	1.00	1.00
Methionine	–	0	0	0	0	0
Isoleucine	0.2	0	0	0.19	0.10	0.10
Leucine	0.2	0	0	0.09	0.15	0.06
Cysteine as cysteic acid	1.7	0.85	2.02	–	–	1.80

– The values were not reported. [a] HCl hydrolysis. [b] Performic acid treatment before HCl hydrolysis.

Table II. Peroxidative activity of horse cytochrome *c*, hemeundecapeptide (HUP), hemeoctapeptide (HOP), horseradish peroxidase, and ferric salt

Sample	Addition of nitrogenous compounds	Heat treatment	Enzyme activity[a]
Cytochrome *c*			0.019
		94 °C for 30 min	0.110
	0.1 *M* imidazole		0.018
Horseradish peroxidase			478
		94 °C for 30 min	0
	0.1 *M* imidazole		713
HUP			1.9
	0.1 *M* imidazole		3.4
		94 °C for 30 min	3.5
HOP			2.9
		94 °C for 30 min	5.5
	0.1 *M* imidazole		7.4
Protohemin			0
	0.1 *M* imidazole		0.09
Ferric ammonium sulfate			0
Ferric ammonium nitrate			0

[a] The enzyme activity was defined as μmole *o*-dianisidine oxidized/min/mg sample.

[6] E. MARGOLIASH, N. FROHWIRT and E. WIENER, Biochem. J. *71*, 559 (1959).

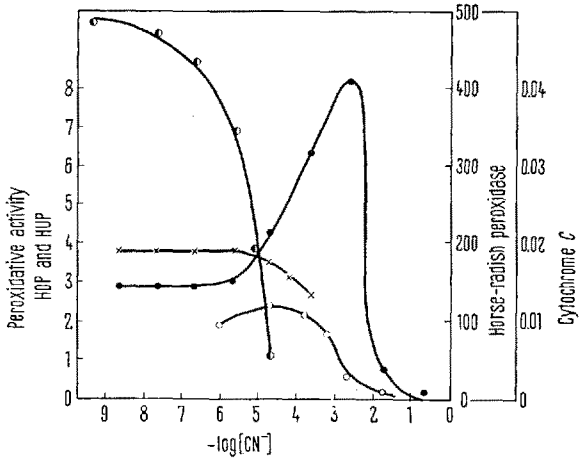

Fig. 2. Effect of cyanide concentration on peroxidative activity of •——•, HOP; ○——○, HUP; ◐——◐, horseradish peroxidase; x——x, cytochrome *c* in 0.01 *M* phosphate buffer at pH 6.0.

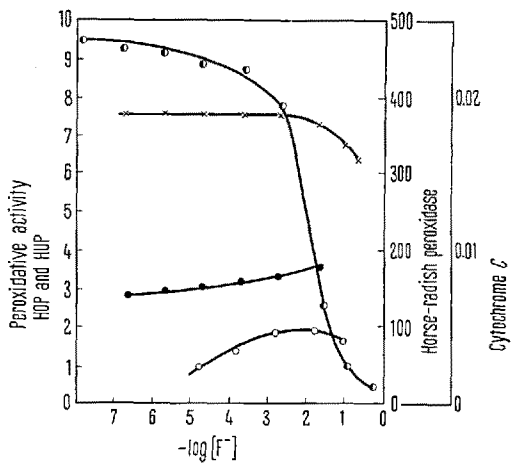

Fig. 3. Effect of fluoride ion concentration on peroxidative activity of •——•, HOP; ○——○, HUP; ◐——◐, horseradish peroxidase; x——x, cytochrome *c* in 0.01 *M* phosphate buffer at pH 6.0.

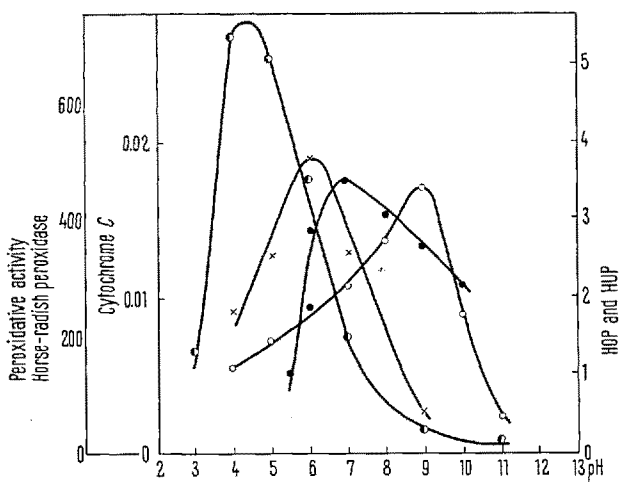

Fig. 4. Effect of pH on peroxidative activity of •——•, HOP; ○——○, HUP; ◐——◐, horseradish peroxidase; x——x, cytochrome *c*. Acetate buffer was used for pH 3–5 (0.01 *M*) and phosphate buffer (0.01 *M*) was used for pH above 5.5.

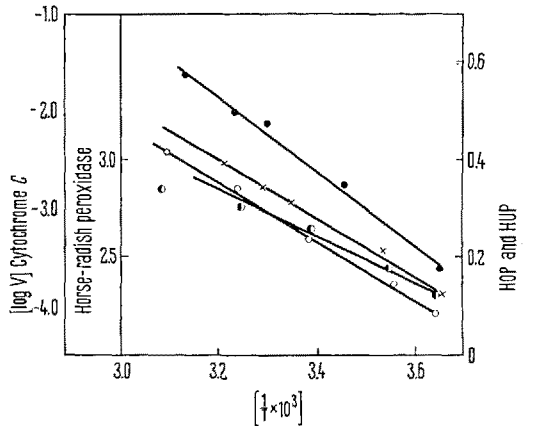

Fig. 5. Effect of temperature on peroxidative activity of •——•, HOP; ○——○, HUP; ◐——◐, horseradish peroxidase; x——x, cytochrome *c* in 0.01 *M* phosphate buffer pH at 6.0.

At higher temperatures, the enzyme activity of horseradish peroxidase decreased as expected because of partial denaturation of protein. However, peroxidative activity of cytochrome *c* did not decrease at higher temperatures. The activity of both HUP and HOP increased as the temperature became higher, following an Arrhenius plot (Figure 5).

No catalase activity was detected on HUP or HOP as shown by the fact that the same titers of $KMnO_4$ were used for the H_2O_2 blank.

Incubation of HUP or HOP with *o*-dianisidine without H_2O_2 did not yield any absorbance change. Therefore, the absorbance increase is not due to the oxidation of *o*-dianisidine by HUP or HOP. Elimination of hydrogen donor, *o*-dianisidine, from the system did not decompose the H_2O_2, indicating there was no catalase activity. Three compounds: H_2O_2, hydrogen donor, HUP or HOP are necessary for the absorbance change. Therefore, the absorbance change in a complete system is due to the peroxidative action of HUP or HOP.

Résumé. L'hémoundécapeptide (HUP) a été préparé par digestion pepsique du cytochrome *c* qui a été extrait et purifié à partir du cœur de cheval. L'hémooctapeptide (HOP) a été obtenu par digestion trypsique de l'HUP. L'un et l'autre ont donné des produits homogènes par chromatographie sur colonne et sur papier, par électrophorèse sur papier à différents pH et par analyse des acides aminés. On a effectué une analyse spectrométrique de HUP et HOP et déterminé leur activité peroxydasique dans diverses conditions. Ces 2 composés n'ont pas montré d'activité catalasique.

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[9] This paper is based on a thesis submitted by Y. Y. Hsiao in partial fulfillment for the degree of Master of Science at Utah State University.