Table II. Inhibitory effects of soluble fractions of *Nycticebus coucang* skin areas on mushroom tyrosinase

Skin (10 mg fresh tissue)	Activity[a] of mushroom tyrosinase (% of control[b])			
	Fresh preparations		3 week preparations	
	H	S	H	S
Forepaw, palm skin	35.2 ± 1.2	33.5 ± 1.0	98.8 ± 1.4	99.1 ± 1.0
Interorbital light streak	13.5 ± 0.7	10.7 ± 0.4	97.3 ± 0.8	101.0 ± 1.2
Forehead	18.9 ± 0.8	15.4 ± 0.7	99.3 ± 1.0	98.8 ± 1.2

[a] 10 µg mushroom tyrosinase was incubated with 40 µg L-tyrosine-^{14}C (sp. act. 0.329 mCi/mmole) in phosphate buffer, 0.1 *M*, pH 6.8, for 1 h at 30 °C. H and S, see footnotes in Table I. [b] Control: activity of mushroom tyrosinase in the absence of homogenates and soluble fractions or in the presence of the corresponding particulate fractions.

Table III. Substrate concentration in the reaction mixture at the beginning and end of incubation with mushroom tyrosinase and with or without soluble fractions of the skin from *Nycticebus coucang*

Origin of soluble fractions (10 mg fresh skin)	Tyrosine concentration (µg/ml)			
	Initial		Terminal	
	No soluble fraction	Soluble fraction	No soluble fraction	Soluble fraction
Forepaw, palm	39.9 ± 0.7	39.5 ± 0.3	36.8 ± 1.4	40.5 ± 1.0
Interorbital light streak	39.9 ± 0.7	40.5 ± 0.5	36.8 ± 1.4	39.4 ± 0.8
Forehead	39.9 ± 0.7	39.9 ± 0.7	36.8 ± 1.4	41.2 ± 0.8

the fraction was derived. Such an increase in tyrosinase activity of the particulate fraction may result from the separation of inhibitors present in the soluble fraction of the homogenate. The in vitro effects of these 3 skin homogenates and their soluble fractions on mushroom tyrosinase supported this thesis (Table II). The inhibitor factor(s) may be reducing agent(s) or substances susceptible to oxidation since skin preparations maintained at 0°–4 °C for 3 weeks lost the ability to inhibit mushroom tyrosinase (Table II). As mushroom tyrosinase incubated in the absence of these skin preparations (Control activity, 100%) was unchanged in the presence of the corresponding particulate fractions, only the soluble fraction appears responsible for the reduction of the tyrosinase activity in the homogenate. To study the 3 soluble fraction preparations which reduce tyrosinase activity, the substrate concentration of mushroom tyrosinase at the beginning and end of incubation was examined in the presence or absence of these fractions (Table III). At the beginning of incubation, the L-tyrosine concentration in the various groups were not different, thereby indicating that little or no free tyrosine was present in the soluble fractions under study. At the end of the incubation period, the L-tyrosine concentration in the absence of the soluble fraction decreased but that in the presence of the soluble fraction was unchanged. Since the tyrosine concentration was determined in the supernates of the TCA precipitated pre- and post-incubation mixtures, the uptake of L-tyrosine by non-tyrosinase reactions in the soluble fraction is eliminated[5].

Résumé. L'analyse radiométrique de la tyrosinase tégumentaire a montré que les régions de la peau de *Nycticebus coucang* diffèrent enzymatiquement. Cette peau renferme un inhibiteur enzymatique de tyrosinase ainsi que des champignons.

Y. M. CHEN and W. CHAVIN

Department of Biology, Wayne State University, and Michigan Cancer Foundation, Detroit (Michigan 48202, USA), 21 January 1973.

[5] Contribution No. 301, Department of Biology, Wayne State University.

The Carotenoid Pigments of Six Species of Adult Acanthocephala

Adult acanthocephalans are all intestinal parasites of vertebrates and many are coloured orange or red, although the degree of pigmentation varies widely from individual to individual. The orange pigment of *Polymorphus minutus* has been identified as a carotenoid, esterified astaxanthin[1] and carotenoids have also been described from another acanthocephalan *Pallisentis nagpurensis*[2]. In this report the nature of the pigments in 6 species of adult Acanthocephala has been investigated.

Materials and methods. Filicollis anatis were obtained from the common Eider (*Somateria mollissima mollissima*) *Macracanthorhynchus hirudinaceus* from the small intestines of pigs (*Sus scrofa*), *Neoechinorhynchus pseud-*

[1] J. BARRETT and P. E. BUTTERWORTH, Comp. Biochem. Physiol. *27*, 575 (1968).

[2] R. RAVINDRANATHAN and A. M. NADAKAL, Jap. J. Parasit. *20*, 1 (1971).

emydis were recovered from the Map turtle (*Pseudemys scripta elegans*), *Nipporhynchus ornatus* were obtained from the Skipjack tuna (*Katsuwonis pelamis*), *Pomphorhynchus laevis* were recovered from the intestines of chub (*Squalius cephalus*) and *Pseudoacanthocephaloides galaxis* from the intestines of small galaxid fish from New Zealand.

The parasites were homogenized and the pigments extracted with a 1:1 (v/v) mixture of hexane: acetone, in the dark at 4°C for 12 h. The extract was filtered and washed with water to remove the acetone and any water soluble compounds.

The carotenoids were analyzed as previously described[1]. A silica gel/methanol column[3] was used to separate the carotenoids into: carotenes and carotenoid esters, monohydroxycarotenoids and polyhydroxycarotenoids. The different fractions were then further separated on a magnesium oxide column[1]. The total number of components in the different pigment fractions was monitored by thin layer chromatography, the carotenoids being separated on 8.25 × 12 cm plates coated with silica gel G (250 μm thick) developed with hexane/benzene (1:1 v/v) and by reverse phase partition chromatography on silica gel plates impregnated with liquid paraffin[4].

Pigments were saponified in 5% KOH in 70% methanol at room temperature for 12 h in the dark. Partition coefficients were determined by the method of PETRACEK and ZECHMEISTER[5]; the presence of acidic groups was tested for by partition under acidic and alkaline conditions.

The presence of allylic hydroxyl groups (4-hydroxyl groups) was demonstrated by dehydration in acid chloroform[6,7] and carotenoid epoxides were detected by the appearance of a blue colour when the pigment, dissolved in diethyl ether/methanol (1:1 v/v), was treated with concentrated HCl[8,9]. The absorption spectra of the pigments were examined with a Beckman D-B recording spectrophotometer or a Zeiss P.M.Q. 11 spectrophotometer. The carotenoids were identified from their spectra and elution characteristics by comparison with published results[9–11].

Results and discussion. The results are summarized in the Table; none of the pigments examined gave a positive reaction when tested for acidic groups, 4-hydroxyl groups or epoxides. All of the species of Acanthocephala investigated contained a single major carotenoid. *M. hirudinaceus*, however, did contain a very minor unidentified component which eluted from the silica gel/methanol column with methanol and from the magnesium oxide column with 50% acetone in hexane (v/v). This pigment showed maximum absorption in hexane at 476,445 and 420 nm and was unchanged by mild saponification.

The pigment of *N. ornatus* showed a symmetrical absorption spectrum, the absorption maximum in acetone and chloroform being 474 and 485 nm respectively Following saponification this pigment had a single broad

The carotenoids of adult Acanthocephala

	Elution from silica gel/methanol column	Elution from magnesium oxide column	Maximum absorption in hexane (nm)	Maximum absorption after saponification	Partition coefficient hexane/95% methanol	Pigment
Class Archiacanthocephala						
Macracanthorhynchus hirudinaceus	methanol	15% acetone in hexane (v/v)	473, 445, 420	unchanged	0:100	Lutein
Pseudoacanthocephaloides galaxis	methanol	10% acetone in hexane (v/v)	475, 445, 420	unchanged	0:100	Lutein
Palaeacanthocephala						
Pomphorhynchus laevis	hexane	hexane	475, 450 shoulder at 425	unchanged	95:5	β-carotene
Filicollis anatis	hexane	1% acetone in hexane (v/v)	475, 450 shoulder at 425	unchanged	94:6	β-carotene
Nipporhynchus ornatus	hexane	10% acetone in hexane (v/v)	468	472–475	80:20	Esterified astaxanthin
Eoacanthocephala						
Neoechinorhynchus pseudemydis	methanol	15% acetone in hexane (v/v)	472, 445, 420	unchanged	0:100	Lutein

[3] A. E. PURCELL, Analyt. Chem. *30*, 1049 (1958).
[4] K. RANDERATH, *Thin Layer Chromatography*, 1st edn. (Academic Press, New York 1963).
[5] F. J. PETRACEK and L. ZECHMEISTER, Analyt. Chem. *28*, 1484 (1956).
[6] P. KARRER and E. LEUMANN, Helv. chim. Acta *34*, 445 (1951).
[7] B. C. L. WEEDON, in *Chemistry and Biochemistry of Plant Pigments* (Ed. T. W. GOODWIN; Academic Press, New York 1965), p. 75.
[8] A. L. CURL and G. F. BAILEY, J. agric. Fd Chem. *9*, 403 (1961).
[9] B. M. GILCHRIST, Comp. Biochem. Physiol. *24*, 123 (1968).
[10] T. W. GOODWIN, in *Modern Methods of Plant Analysis*, 1st edn. (Eds. K. PAECH and M. V. TRACEY; Springer-Verlag, Berlin 1955) vol. 3, p. 272.
[11] P. J. HERRING, Comp. Biochem. Physiol. *24*, 187 (1968).

peak in hexane (472–475 nm) and was entirely hypophasic when partitioned between hexane and 95% methanol. The carotenoid from *N. ornatus* was, therefore, identified as esterified astaxanthin which after saponification yielded astacene.

Although all the different species of Acanthocephala so far investigated do not possess the same carotenoids, each species appears to contain a single major carotenoid and not a mixture of pigments. There are, with the possible exception of the mammalian corpus luteum[12] no authenticated cases of de novo carotenoid synthesis in animals, so the parasites must obtain their carotenoids from their hosts. However, the carotenoids found in adult acanthocephalans do not necessarily appear to reflect the spectrum of carotenoids available to the parasite from their hosts diet.

There seems to be no correlation between the type of intermediate host and the carotenoid found in the adult parasite, although the majority of coloured Acanthocephala have Crustacea as their intermediate host. In a secondary host, such as *Gammarus pulex*, a wide range of carotenoids are present[1] although astaxanthin forms the major carotenoid available in the haemolymph[13]. Yet the adults of the 2 species of Acanthocephala which utilize this intermediate host, namely *P. laevis* and *P. minutus* contain different carotenoids. Possibly there is a correlation between the diet of the final host and the carotenoid present in the adult acanthocephalan.

The uptake of carotenoids by adult acanthocephalans would appear to be selective, since they only concentrate one of the available carotenoids. Selective uptake of carotenoids has been reported in certain other invertebrate groups[14]; some polychaetes contain only β-carotene and do not appear to absorb hydroxycarotenoids[15], whilst certain molluscs show a preferential uptake of xanthophylls[16]. The uptake of carotenoids by adult Acanthocephala may be associated with the uptake of other compounds, such as lipids and/or proteins in the form of lipocarotenoid complexes or carotenoproteins. In *P. minutus* cystacanths the esterified astaxanthin is associated with wax esters which constitute nearly 90% of the total cystacanth lipids[17].

The role of carotenoids in adult Acanthocephala is obscure, carotenoids may have an anti-oxidant function[14] or they may be involved in oxidative metabolism[18]. Alternatively the vitamin A function of carotenoids could be important in Acanthocephala, or carotenoids may be involved in reproduction[14]. However, carotenoids do not appear to be essential to the adult acanthocephalan parasite[1] and the concentration of carotenoids can vary widely from individual to individual and is completely absent in many species.

Whether the distribution of α- and β-carotene and their derivatives in the different classes of Acanthocephala has any phylogenetic significance is not known. β-Carotene and its derivative astaxanthin occur only in Palaeacanthocephala, whilst lutein (an α-carotene derivative) is found in the Archiacanthocephala and Eoacanthocephala. This may represent a biochemical difference between the 3 classes of Acanthocephala or merely reflect the ecological differences of their hosts.

Zusammenfassung. Die Carotinoide in 6 Spezies erwachsener Acanthocephalen wurden identifiziert. Lutein wurde als einziges Carotinoid in *P. galaxis* und *N. pseudemydis* gefunden und als wichtigstes Carotinoid in *M. hirudinaceus* festgestellt. *F. anatis* und *P. laevis* enthielten nur β-Carotin, während das veresterte Astexanthin als einziges Pigment in *N. ornatus* auftrat.

J. Barrett and P. E. Butterworth[19]

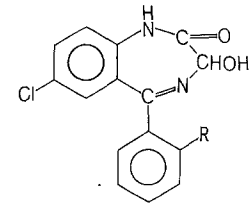
Dept. of Zoology, University of Oxford, South Parks Road, Oxford OX1 3 PS, and The Molteno Institute, Downing Street, Cambridge (England), 17 November 1972.

[12] B. M. Austern and A. M. Gawienowski, Lipids *4*, 227 (1969).
[13] D. F. Cheesman, W. L. Lee and P. F. Zagalsky, Biol. Rev. *42*, 132 (1967).
[14] T. W. Goodwin, *The Comparative Biochemistry of Carotenoids* (Chapman and Hall, London 1952).
[15] G. Y. Kennedy, in *Chemical Zoology* (Eds. M. Florkin and B. T. Scheer; Academic Press, New York 1969), vol. 4, p. 311.
[16] B. T. Scheer, J. biol. Chem. *136*, 275 (1940).
[17] J. Barrett and P. E. Butterworth, Lipids *6*, 763 (1971).
[18] V. N. Karnaukhov, Expl Cell Res. *64*, 301 (1971).
[19] We thank Dr. M. Hine and Dr. A. Pike for sending us material which was used in this study. Part of the work was carried out in the Dept. of Zoology, University of Massachusetts, Amherst (Mass., USA).

Identification of Oxazepam and Lorazepam Glucuronides by Chemical Ionization Mass Spectrometry

Oxazepam (7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one) and lorazepam (7-chloro-5-(*o*-chlorophenyl)-3-hydroxy-1, 3-dihydro-2H-1, 4-benzodiazepin-2-one) are benzodiazepines in clinical use by virtue of their antianxiety, tranquilizing and sedative properties. Their respective glucuronide conjugates are the major urinary metabolites in man and in several other species[1–3]; they also circulate together with the free drugs in human blood[4,5]. Furthermore, oxazepam glucuronide is a metabolite of diazepam[6,7] prazepam[8] temazepam[9] and medazepam[10,11]. In view of their presence in blood and their importance as final detoxification products it is highly desirable to obtain conclusive evidence for the structure of these conjugates which so far have been characterized only by enzymic cleavage. This communication describes the isolation, chemical analysis and identification by chemical ionization (CI) mass spectrometry[12] of the glucuronides of oxazepam and lorazepam.

Isolation of the metabolites was accomplished by first processing on XAD-2 resin urines from miniature swine orally dosed with 50 mg/kg oxazepam or lorazepam. Elution from the resin was accomplished with 80% aqueous acetone. After evaporation of acetone it was further purified by adsorption on charcoal and subsequent



Oxazepam R=H
Lorazepam R=Cl