Urea denaturation and thermal denaturation studies throw light on the importance and organization of the helical structure of collagen fibrils on its affinity for bilirubin. These observations may be of physiological significance in view of the fact that skin provides a semi-solid matrix for the connective tissue. The importance of the role of collagen fibrils is further strengthened by the observation on the lack of affinity of gelatin for bilirubin under identical conditions.

The liver conjugate being still in a developing state may not be able to cope up with all the bilirubin encountered in neonatal jaundice. In such a situation it is presumable that the skin takes the main load of free bilirubin employing native collagen as the binding agent. Collagen thus provides a matrix for the photooxidation of bilirubin to relatively polar degradation products which are then eliminated by the kidney.

The author wishes to record his gratefulness to Dr. C. R. Krishna Murti for many thoughtful suggestions and guidance.

1. Kapoor, C. L., Krishna Murti, C. R. and Bajpai, P. C., *Biochem. J.*, 1973, 136, 35.
2. —, — and —, *New Eng. J. Med.*, 1973, 288, 583.
3. — and —, *Biochem. J., Proceedings of 540th Biochemical Society*, Oxford, (In press), 1973.
4. Glimcher, M. J. and Krane, S. M., *Biochemistry*, 1964, 3, 195.
5. Woessner, J. F., *Arch. Biochem. Biophys.*, 1961, 93, 440.
6. Gross, J. and Krich, D., *J. Biol. Chem.*, 1958. 233, 355.
7. Van Roy, F. P., Meuwissen, J. A. T. P., De Meuter, F. and Heirwegh, K. P. M., *Clin. Chem. Acta*, 1971, 31, 109.
8. Mustafa, M. G. and King, T. E., *J. Biol. Chem.*, 1970, 245, 1084.
9. Odell, G. B., *Pediatrics*, 1970, 46, 16.
10. Silverman, W. A. M., Adersen, D. H., Blanc, W. A. and Crozier, D. N., *Ibid.*, 1956, 18, 614.

ECOLOGICAL IMPLICATIONS OF HAEMOLYMPH PROTEIN PATTERNS IN SOME AMPHIPOD AND ISOPOD SPECIES

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AMONG the numerous proteins that are to be found in the crustacean haemolymph, two particular components have been easily recognized, when present, owing to their obvious properties—the respiratory pigments and the coagulable proteins. The chemical nature and physical properties of both these proteins have been recently reviewed by Jenuiaux[6], Redmond[9] and by Grégoire[5].

The remaining protein fraction has been resolved into a series of components with different electrophoretic properties[1,2,4,7,11,12]. Although the nature of these proteins is poorly understood, their electrophoretic mobilities in some cases have been used tentatively in taxonomic studies[7].

The aim of the present investigation is to define some of the characteristics of haemocyanin and other blood proteins in a few marine, freshwater and terrestrial isopods, and to determine if the haemolymph protein electrophoretic pattern in these

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species has any taxonomic or ecological implications.

Material and Methods

Animals

AMPHIPODA :

1. *Gammarus fossarum* Koch : ♀♀ 8–11 mm, ♂♂ 10–14 mm, collected from a stream originating from the Niebieskie Źródla, an artificial lake formed by the Pilica river near Tomaszów Mazowiecki (Poland).
2. *Gammarus lacustris* G.O. Sars : ♀♀ 13–15 mm, ♂ 15–18 mm, from the peat-bog near Blonie, Lenczyca District (Poland), and
3. *Gammarus roeseli* Gerv. : ♀♀ 7–10 mm, ♂♂ 9–13 mm, from the uppermost reach of the river Notec, near Izbica Kujawska, Kolo District (Poland).

ISOPODA :

1. *Asellus aquaticus* (L.): ♀♀ 7–11 mm, ♂♂ 9–13 mm, from ponds and streams in various localities near the city of Lódz (Poland),
2. *Oniscus asellus* L. : ♀♀ 11–14 mm, ♂♂ 10–13 mm, from a stock colony maintained at the Institute of Botany and Zoology (The University of Lódz, Poland) at 21° C and 94–100% relative humidity, on pieces of potatoes and carrots,
3. *Porcellio laevis* Latr. : ♀♀ 15–18 mm, ♂♂ 14–20 mm, collected from the various parks in the city of Prague (Czechoslovakia), and
4. *Ligia oceanica* (L.): ♀♀ 20–25 mm, ♂♂ 16–20 mm, procured from under the stones at the beach near Bologne-sûr-mer (France).

The crustaceans were dried on filter-papers for about one minute, and were bled by the method of Alikhan[1].

Cellulose acetate electrophoresis was performed by a slight modification of the method described by Alikhan[1]. The following details are of importance : cellulose acetate strips, 11 × 2·5 cm ; buffer, veronal, pH 8·6 ; μ, 0·05 ; current, 2·5 mA per strip ; usually four to five strips were run simultaneously ; stain, amido black ; washing in acetic acid : methanol : water ; clearing in bromonaphthalene : paraffin oil ; scanning, with Densitometer ERJ-65. Protein fractions were identified by the criteria outlined by Decleir and Vercauteren[3], and by Alikhan and Lysenko[2].

Whenever required, peaks of individual protein bands were measured from densitometric tracings of the cellulose acetate strips by means of a polar planimeter. These units were converted to percentage of the total number of units for the entire protein pattern, and expressed as such.

Results

As is obvious from Fig. 1, the haemolymph from almost all species showed four distinct bands of various mobilities. Fraction 1, a dense, fast moving band, when denatured by heat, displayed distinct peroxidase activity. This was considered to be the respiratory pigment, haemocyanin. This haemocyanin fraction was always followed by a relatively smaller band (fraction 4, in Fig. 1), which gave a negative lipoprotein, but a positive glycoprotein reaction.

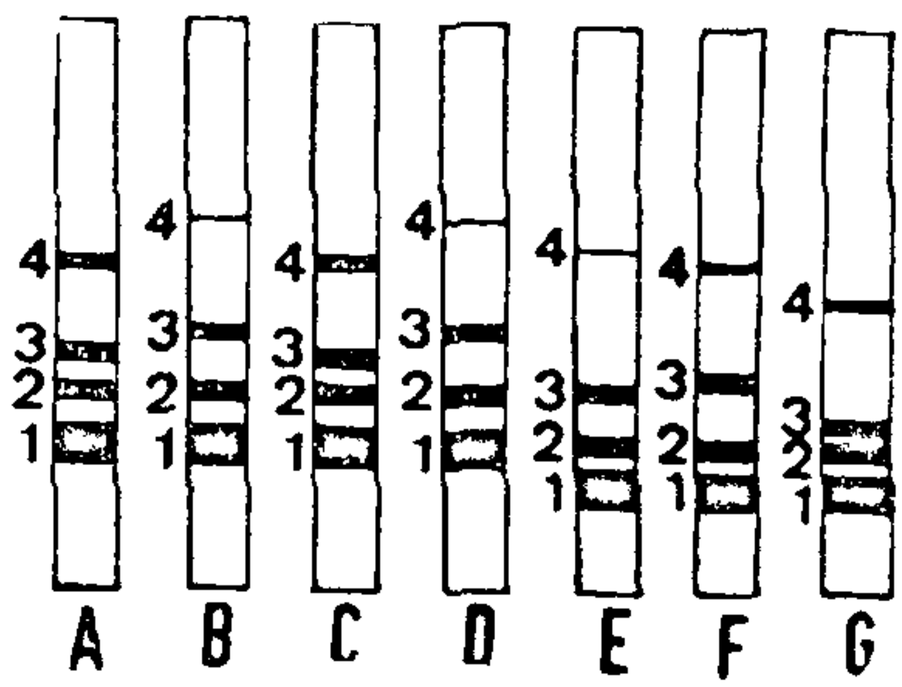

Fig. 1. Cellulose acetate electrophoretic pattern of the haemolymph proteins in various amphipod and isopod species. 1, haemocyanin ; 2 and 3, apo subunits of haemocyanin ; 4, glycoprotein ; A, *Gammarus fossarum* ; B, *Gammarus lacustris* ; C, *Gammarus roeseli* ; D, *Asellus aquaticus* ; E, *Oniscus asellus* ; F, *Porcellio laevis* ; and G. *Ligia oceanica*.

The remaining two bands (fractions 2 & 3) could not be identified with certainty. However, on the basis of their molecular weights (Alikhan & Lysenko, *unpublished data*), they have been regarded as apo subunits of haemocyanin.

The data on the haemolymph protein measurements in various species (Table II) revealed that major part (about 82–96%) of the plasma proteins in these species is formed by haemocyanin. According to their plasma haemocyanin contents, these seven species fall roughly into two categories : category 1, comprising of *G. fossarum*, *Oniscus asellus* and *P. laevis*, and category 2, formed by *G. lacustris*, *G. roeseli*, *A. aquaticus* and by *L. oceanica*.

Females in all species, in general, contained relatively more haemocyanin than did the males.

TABLE I

Haemolymph coloration in various species

Species	Coloration scale	Usual colour
Gammarus fossarum	bluish-yellow, turning blue when exposed to air	blue
Gammarus lacustris	Yellowish-green, dirty green, dirty blue	brownish-green
Gammarus roeseli	yellow, yellowish green, green	green
A. aquaticus	colourless, pale, yellow	pale yellow
O. asellus	colourless, pale yellow	pale yellow
P. laevis	light yellow, dark yellow	light yellow
L. oceanica	colourless, yellow	yellow

TABLE II

Relative concentration (in percentage) of haemolymph proteins in various species

Species	Haemocyanin	Glycoprotein
Gammarus fossarum ..	95·60±7·8	4·4 ±1·2
Gammarus lacustris ..	83·52±4·4	16·4 ±0·5
Gammarus roeseli ..	83·39±6·9	16·6 ±0·9
Asellus aquaticus ..	81·30±3·1	18·7 ±0·7
Oniscus asellus ..	93·5 ±1·7	6·5 ±0·2
Porcellio laevis ..	94·2 ±1·9	5·8 ±0·2
Ligia oceanica ..	85·76±5·1	14·2 ±0·9

Average of 30–36 samples in each case ± S.E.

DISCUSSION

The blood in members of the class Crustacea is an important medium for the transportation of ions and molecules involved in energy metabolism. As a consequence, the composition of blood supply an important information on the physiological and pathological state of these animals.

The haemolymph proteins in several amphipod and isopod species have previously been analyzed using cellulose acetate[1,11]. The result of these studies demonstrated the presence of a maximum number of five protein fractions: a glycoprotein, a fibrinogen, a heteroagglutinin, an apohaemocyanin and the haemocyanin. In the present studies, the presence of fibrinogen and heteroagglutinin could not be demonstrated. Nevertheless, the present studies did show certain intraspecific differences, in spite of the fact that there were large variations among the relative contents of the various proteins within the same species. These differences have been related to the physiological state of the animal[1–2 10].

The result obtained in the present study clearly shows that in its haemolymph protein composition, *G. lacustris* is somewhat identical to *G. roeseli* on the one hand, and *A. aquaticus* and *L. oceanica* on the other. Morphologically, *G. fossarum* and *G. lacustris* have been considered to belong to the same so-called "pulex-group"[8], while *G. roeseli*, as well as *A. aquaticus* and *L. oceanica*, fall into morphological categories far removed from *G. lacustris*. The implication here is that the similarity in the protein compositions in *G. lacustris* and *G. roeseli* could be due to their ecological affinity. Both of these species, along with *A. aquaticus* and *L. oceanica*, have been considered as euryoxybionts[8] in relation to other *Gammarus* species, since they can withstand variations in the oxygen tension of their environments. *G. fossarum* (like *Oniscus* and *Porcellio*), on the other hand, lives only in well aerated medium, and as a consequence is regarded as polyoxybiont[8]. This situation is also reflected by the "saprobiont system", defined by Breitig[8]. Under this system, *G. roeseli* has been defined as a β-mesosaprobiont, and *G. fossarum* as a poligosaprobiont, implying again that in their oxygen and other life requirements, these two species differ from each other.

Similarly, the protein composition similarity between *O. asellus*, *P. laevis* and *G. fossarum* imply similarity in their habitat and oxygen requirements. Both *O. asellus* and *Porcellio laevis* are terrestrial species, and as such are unable to tolerate any variation in the oxygen tension of their environments. *A. asellus*, a closely related species to *P. laevis*, is an α-mesosaprobiont, which again places it in the category of *G. lacustris*.

Wieser[11] also contends that the haemolymph protein compositions in a given species are affected by its ecological position. According to him, the relative haemocyanin content of the haemolymph tends to become more stable as the species acquires more terrestrial habitat. However, this hypothesis needs further verification.

ACKNOWLEDGEMENTS

The work was supported by a Senior Travel Fellowship (1973–74) to the senior author from the National Research Council of Canada. One of us (M. A. A.) thanks RNDr. Oleg Lysenko (Czechoslovak Academy of Sciences, Prague), Prof. Dr. R. Vercauteren (Laboratorium voor Fysiologische scheikunde, Rijksuniversiteit-Gent, Belgium) and the Institut de Biologie Maritime et Regionale, Wimereux (Université de Lille), France, for laboratory facilities.

1. Alikhan, M. A., *Comp. Biochem. Physiol.*, 1971, **39 A**, 735.
2. Alikhan, M. A., *Comp. Biochem. Physiol.*, 1973, **45 B**, 539.
3. Decleir, W. and Vercauteren, R., *Cah. Biol. mar.*, 1965, **6**, 163.
4. Frentz, R., *C.r. Acad. Sci. Paris*, 1958, **247**, 2205.
5. Grégoire C., In : *Chemical Zoology* (Florkin, M. and Scheer, B. T., Eds.), Academic Press, New Ytrk, 1971, **6**, 145.
6. Jeuniaux, C. In : *Chemical Zoology* (Florkin, M. and Scheer, B. T., Eds.), Academic Press, New York, 1971, **6**, 63.
7. Manwell, C. and Baker, C. M. A., *Comp. Biochem. Physiol.*, 1963, **8**, 193.
8. Pinkster, S., *Bijdr. Dierk.*, 1972, **42**, 164.
9. Redmond, J. R. In : *Chemical Zoology* (Florkin, M. and Scheer, B. T., Eds.), Academic Press, New York, 1971, **6**, 119.
10. Tyler, A. and Metz. C. B., *J. exp. Zool.*, 1945, **100**, 387.
11. Wieser, W., *J. mar. Ass. biol. U.K.*, 1965, **45**, 507.
12. Zuckerkandl, E., *Ann. Inst. Océanogr. Monaco*, 1960, **38**, 1.

MORPHOGENESIS IN STEM-CALLUS TISSUE OF *CITRUS GRANDIS* IN LONG-TERM CULTURES—A BIOCHEMICAL ANALYSIS

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ABSTRACT

Stem-callus tissue of *C. grandis,* in long-term cultures, differentiated into two types of tissues : type-A, which was compact-nodular and slow growing and type-B, which was friable-spongy and fast growing. In a medium containing 0·25 mg/l BAP + 0·1 mg/l NAA + 500 mg/l ME, the type-A tissue produced numerous shoot-buds and shoots, whereas the type-B tissue did not. The two types of tissues also differed in respect of their nitrogen, protein, free amino acid and sugar contents.

INTRODUCTION

THERE are several reports[1–3] of the gradual loss of the regenerative capacity of plant callus tissues grown *in vitro* for a long time. Some workers[4–7] have studied the changing cytological conditions in the tissue during its prolonged culture and the correlated loss of its regenerative capacity. However, since callus tissue of varied ploidy including haploidy are known to differentiate organs and plantlets[8–10], and those with abnormal polyploid chromosome numbers, to form abnormal shoots[6], it appears that besides cytological alterations, some biochemical changes in the tissue during its prolonged culture may also be involved in the phenomenon of the loss of its morphogenetic potentiality. It seems that the latter aspect has not been studied so far, though some analyses of free amino acids in *in vitro*-grown tissues have been made[11–12]. In the present investigation, certain biochemical changes in long-term culture have been studied with a view to find out any correlation between the metabolic changes in the tissue and the loss of its regenerative capacity.

EXPERIMENTAL PROCEDURE

Composition (in mg/l) of MS medium, a variant of Murashige and Skoog's medium[13], where it differed from the litter, was : 150 NH_4NO_3, 1500 KNO_3, 400 $CaCl_2$, 150 KH_2PO_4, 360 $MgSO_4$. $7H_2O$, 10 thiamine-HCl, 2·5 pyridoxine-HCl, 2·5 nicotinic acid, 0·1 folic acid, 0·1 riboflavin, 0·1 biotin, 5 ascorbic acid, 50,000 sucrose and 7000 agar. Sterilization procedure and other cultural conditions were as reported earlier[14]. Stem-callus tissue of *C. grandis* was maintained in MS medium