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Haemagglutinins and Haemolysins in Maritime Lichens and the Effects of Various Parameters on *Cladonia pyxidata* Anti-Rabbit Erythrocyte Agglutinin Activity

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Abstract

Extracts of 10 maritime lichen species contained relatively non-specific, naturally-occurring agglutinins (titre range neat to 2^{-9}) and lysins (2^{-1} to 2^{-7}) against 14 animal and 8 human red blood cell (RBC) types. Maximum agglutinin levels were detected in *Cladonia pyxidata* against rabbit, human ARh+ and ABRh+ RBC while maximum lysin titres were found in *Xanthoria parietina* against horse RBC. Specificity towards either goat, rabbit, rat, sheep, frog, chicken, human BRh+, ARh+ or ABRh+ RBC was displayed by at least one but no more than two of the lichen species. Both exposure and aspect of the sea-shore collection sites influenced haemagglutinin and haemolysin titres. The effects of simulated environmental parameters on *C. pyxidata* anti-rabbit RBC agglutination activity were investigated. The *C. pyxidata* haemagglutinin was thermolabile, unaffected by freezing and thawing treatments, displayed optimum activity at neutral to slightly alkaline pH and required the presence of Ca^{2+} ions for maximum haemagglutination. Furthermore artificial rain-water and simulated acid rain at low pH values, both used as diluent in the agglutination assays, buffer NaCl concentrations of 0.2 M or above and the inclusion of common heavy metal ion pollutants, especially Fe^{3+}, in the buffer all either significantly lowered or negated haemagglutination titres. However exposure to high doses of γ-radiation did not effect haemagglutinin levels. The possible effects of various ecological factors on *C. pyxidata* haemagglutinin activity is discussed.

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Keywords: Maritime lichens, *Cladonia pyxidata*, haemagglutinins, haemolysins, environmental factors

1. Introduction

Plant-derived agglutinins that display a high degree of specificity for certain RBC types of the human ABO(H) blood group were initially described by Renkonen (1948) and Boyd and Reguera (1949). Since that time agglutinins reactive towards RBC from other human blood groups and animals, and against both prokaryotic and eukaryotic cells, have been reported in and isolated from seed extracts of angiosperm species, mainly the Leguminoseae (Sandhu and Reen, 1982; Toms, 1971). Many of these substances are now regarded as lectins (Liener et al., 1986) or receptor-specific proteins (Balding, 1981).

Concurrently, attention has been directed towards lectins (agglutinins) and lectin-like molecules in lower plants predominantly algae (Ingram, 1985a, 1985b) and fungi (Nordbring-Hertz, 1986). In these groups, lectins appear to mediate cell-cell interactions during gamete fusion (Adair, 1985; Callow, 1985), colony formation (Maki and Mitchell, 1986), symbiotic associations (Bubrick et al., 1985; Galun et al., 1984), nutrition (Nordbring-Hertz, 1986) and parasite-plant (Vranken et al., 1987) and parasite-invertebrate interactions (Pendland and Boucias, 1986). Moreover, agglutinins and lectins have also been found mainly in terrestrial lichens (Barrett and Howe, 1968; Estola and Vartia, 1955; Fillho et al., 1980; Lockhart et al., 1978; Petit, 1982). Relatively few attempts have been made to isolate and determine the physico-chemical properties of lichen lectins (Howe and Barrett, 1970; Petit et al., 1983; Tassabehji, 1987) and only in two instances have sugar-binding specificities been investigated (Hardman et al., 1983; Ingram and Tassabehji, 1988).

In previous studies, Ingram (1982, 1984) reported the occurrence of agglutinins and lysins in maritime lichens whose activities were temperature-dependent. To the authors' knowledge there are no data available concerning the effects of ecological factors on lichen haemagglutinins and haemolysins. This paper presents the results of a study on the effects of various simulated environmental parameters on maritime lichen agglutinin and lytic activities, with particular reference to the anti-rabbit RBC agglutinin(s) from *Cladonia pyxidata*, a foliose lichen present in the upper supralittoral zone of a rocky sea shore.

Table 1. Haemagglutinin and Haemolysin Titres (log_2) of Maritime Lichen Extracts against Erythrocytes from Humans and Various Vertebrate Species.

Type	*Species At	Ct	Cm	Lac	Lh	La	Xp	Rs	Op	Cp
Human O Rh+	–	**(1)	***–	3(2)	4	4	4(3)	2(3)	3	5(1)
Human O Rh-	NT	NT	NT	NT	NT	NT	–	–	NT	–
Human A Rh+	–	–	(1)	–	–	–	0	3(1)	(1)	8
Human A Rh-	NT	NT	NT	NT	NT	NT	6	4	NT	1
Human B Rh+	4(1)	–	6(2)	–	–	–	–	–	–	3(1)
Human B Rh-	NT	NT	NT	NT	NT	NT	–	–	NT	2
Human AB Rh+	–	–	(1)	3(3)	–	6(2)	4	3(3)	4(2)	8(1)
Human AB Rh-	NT	NT	NT	NT	NT	NT	3	1	NT	3
Rabbit	–	7(1)	(2)	4(2)	4(3)	4(3)	–	3(4)	6(4)	9
Goat	6(6)	5(1)	1(2)	(2)	–	–	1	1	6(1)	6
Mouse	–	–	–	–	–	–	1(1)	–	–	3
Guinea Pig	–	0	1	–	–	–	2	1	–	–
Rat	–	–	–	–	–	–	–	–	–	6
Ox	–	0	–	–	–	–	–	2	–	2
Calf	–	1	–	–	–	–	–	1	–	3
Horse	2(3)	3	–	3(2)	4(1)	3(1)	(7)	(1)	2	2
Sheep	–	–	–	(1)	7(1)	2	2(1)	3(1)	(1)	–
Pig	–	–	–	–	–	–	1	1	–	2
Chicken	4(4)	5(3)	7(2)	(1)	–	4(2)	4(4)	4	7(3)	(1)
Goose	(5)	2	1	–	–	5	4(4)	3	1	4(2)
Frog	3(3)	–	–	–	–	7(4)	2	3(2)	–	–
Trout	–	4(2)	–	–	–	–	–	2(1)	1	–

* At, *Anaptychia fusca*; Ct, *Caloplaca thallincola*; Cm, *Caloplaca marina*; Lac, *Lecanora actophila*; Lh, *Lecanora helicopis*; La, *Lecanora atra*; Xp, *Xanthoria parietina*; Rs, *Ramalina siliquosa*; Op, *Ochrolechia parella*, Cp, *Cladonia pyxidata*

** Haemolysin values given in parentheses

*** –, negative for both haemagglutinins and haemolysins

NT,not tested

O, reaction in undiluted (neat) extract

2. Materials and Methods

Collection and identification of specimens

Lichens (see Table 1) were obtained from sandstone rocks in the mid-supralittoral zone of sea shore areas on the Dale Peninsula, Dyfed, South-west Wales, U.K. *Cladonia pyxidata* was collected from the surface of clumps of the moss *Grimmia maritima* on the upper supralittoral zone at the base of the cliff top. All specimens were speciated by both morphological and chemical criteria (Duncan, 1970; Hale, 1983).

Lichen extracts

Following collection, the lichens were stored overnight at 4°C and washed in phosphate buffered saline (PBS) pH 7.2 to remove soil particles and surface debris. The samples were dried slowly at 15°C for 36 hr to avoid possible denaturation of proteins. The dried thalli (2 g) were finely chopped up, ground and 10 ml chilled PBS were added. The material was homogenised for 6–7 min in an ice bath and then kept at 4°C for 15 hr to further extract any soluble material present. The mixture was centrifuged at 4000 rpm and the supernatant divided into small aliquots and stored at –20°C.

Erythrocyte preparation

RBC types from the human ABO(H) blood group, both Rhesus + and –, were obtained from a local blood bank whereas RBC from various vertebrate species were purchased commercially (goat, ox, calf, horse,sheep, pig, chicken and goose) or sampled from stock animals (rabbit, mouse, rat, guinea pig, frog and trout). The washing and adjustment of the RBC to 2% suspensions have been described elsewhere (Ingram, 1982, 1984).

Haemagglutination assay

Serial doubling dilutions of the initial 1:5 (w/v) lichen extract were made in PBS and to each dilution was added an equal volume of the appropriate 2% RBC suspension. The mixtures were incubated at 4, 21 or 37°C for 1.5 hr and the results read and scored subjectively from 0 (no agglutination) to 4+ (100% agglutination). Endpoint titres, expressed as $\log_2$ values, were taken as the highest dilutions that just showed visible agglutination. The plates were further incubated overnight at 4°C and rescored. Controls comprised RBC plus PBS, and a sheep RBC/rabbit anti-sheep RBC serum system. All assays were conducted in triplicate with fresh RBC and samples used on each occasion.

Effects of various environmental parameters

Exposure and aspect

The effects of both exposure and aspect on the levels of haemagglutinin and haemolysin against rabbit RBC (or chicken RBC in the cases of *C. marina, X. parietina* and *A. fusca*) in lichens present on north- and south-facing shores were investigated. These 2 RBC types were chosen because their usage gave the highest titres with the lichen species examined. The incubations were performed at either 4°C or 37°C because these temperatures gave optimum lysis and agglutination titres respectively. Comparisons

were made between sheltered and exposed shores with northern and southern aspects. Exposure ratings defined by Ballantine (1961) were used and designated III (exposed), IV (semi-exposed), V (semi-sheltered),VI (sheltered) and VII (very sheltered).

Rabbit RBC (RRBC) repeatedly gave the highest agglutination titres with the *C. pyxidata* extract and consequently RRBC were used along with this lichen species in all further experiments. On the basis of previously determined optimum conditions for agglutinin activity (Ingram and Tassabehji, 1988), all incubations were carried out in the dark at 21°C for 1.5 hr with RRBC and lichen extracts stored for no longer than 2 weeks and 15 months respectively.

Effect of temperature

Aliquots of fresh extract were either frozen at −20°C followed by rapid thawing at 21°C 4 consecutive times; frozen at 0°C for 12 hr and then thawed slowly at 5°C or stored at 4°C for 4 weeks. Samples of extract were cooled to 4°C or 15°C or heated at various temperatures from 25°C to 100°C for 1 hr. All treated extracts were examined for haemagglutination.

Effect of pH

The effect of buffer alkalinity and acidity on *C. pyxidata* agglutinin was examined. Buffer solutions, each of molar ionic strength 0.1, ranging in pH from 2.0 to 12.0 were prepared and checked to ensure that the solutions did not haemolyse RRBC. Dilutions of extract were made in the appropriate buffer and samples examined for agglutination.

Sodium chloride concentration

High levels of NaCl in the environment are usually either toxic or cause osmotic dehydration in non-halophytic plans. The effect of varying amounts of NaCl in the PBS, ranging from 0.1 to 0.9 M, on agglutination activity was therefore examined.

Requirement for divalent cations

Some higher plant agglutinins need metal ions, usually obtained from soil solution or rain water, as co-factors which are essential for their specific activities and structural integrity (Liener et al., 1986). Ethylene glycol-bis (β-amino ethyl ether) N,N, N′-tetra-acetic acid (EGTA) is a chelator of only Ca^{2+} ions whereas ethylenediaminetetraacetic acid (EDTA) chelates all divalent cations. EDTA and EGTA were used to examine whether the *C. pyxidata* agglutinin required the presence of two common environmental divalent

cations, calcium and magnesium. Solutions of EDTA and EGTA, 0.1 and 0.2 M respectively, were prepared in PBS. Equal volumes of lichen extract and either EDTA or EGTA were added together and incubated at 21°C for 1–2 hr. The mixtures were then centrifuged at 10,000 rpm for 10 min and a small portion of each was tested for haemagglutination activity following preparations of serial dilutions in PBS. In addition, untreated extracts were prepared in PBS or PBS containing $MgCl_2$ and $CaCl_2$, both salts at 0.1 mg/ml. The remaining EDTA-treated samples were dialysed against PBS overnight at 4°C and re-examined for haemagglutination after being serially diluted in PBS containing either $CaCl_2$, $MgCl_2$ or a mixture of both these salts. A similar doubling dilution series of the EGTA-treated sample was prepared with PBS containing $MgCl_2$ or $CaCl_2$, and similarly assayed.

Effect of pollutants

Acid rain

Lichens are sensitive to any changes in their surroundings caused by natural or man-made pollution which may directly affect metabolism and activity of lichen substances including agglutinins (Hawksworth and Rose, 1976; Johnsen, 1986).

The effects of artificial rainwater and the presence of SO_4^{2-} and NO_3^{2-} ions in a simulated acid rain solution on haemagglutination were investigated. The artificial rain solution was prepared according to Jacobson et al. (1985) and adjusted to different pH levels. The acid rain solution comprised the background inorganic ions present in simulated rain with a sulphate/nitrate mass ratio of 2:1 (v/v) and adjusted H^+ ions to pH 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0. Agglutination assays were performed as previous but using dilutions of extract prepared with the different rainwater and acid rain solution pH values.

Gamma radiation

Studies on the effect of ionising radiation on lichens revealed that growth rate decreased proportionally with increasing ionising radiation dose and that some *Cladonia* species are able to tolerate very high daily levels of 10 Gy for almost 2 years (Brodo, 1964) or 27 Gy for 1 year (Woodwell and Gannutz, 1967). The effect of γ-radiation on *C. pyxidata* agglutinin activity was investigated. Aliquots of lichen extract, each 0.1 ml, were placed in sterile vials. The samples were exposed to a Co^{60} γ-ray source and irradiated for 2, 8, 10 or 15 min at a dose of 220 grays (Gy)/min (i.e. 22 krad/min). The irradiated samples were tested for agglutination.

Table 2. Effects of exposure and aspect on the haemagglutinin (HMGN) and haemolysin (HLYN) titres (log_2) of maritime lichens

	NORTH			SOUTH			NORTH			SOUTH		
	*Exp	HMGN	HLYN	Exp	HMGN	HLYN	Exp	HMGN	HLYN	Exp	HMGN	HLYN
C. thallincola	6–7	4	3	5–6	7	1	4	3	4	3	5	1
C. marina	5–6	4	2	5–7	6	0	4	3	3	3	5	1
L. actophila	5–6	2	2	5–7	4	1	4	1	2	3	3	2
L. helicopis	5–6	2	2	5–6	4	0	4	1	3	3	3	1
L. atra	7	0	2	7	4	1	4	1	2	3	4	0
X. parietina	6–7	1	4	7	4	2	4	0	4	3	3	3
R. siliquosa	7	2	2	7	3	2	4	0	4	3	1	1
O. parella	7	5	4	7	6	2	4	3	3	3	4	3
A. fusca	7	2	2	7	2	4	4	2	4	3	3	3
C. pyxidata	7	7	–	7	9	–	4	6	2	4	8	0

* Exp, exposure rating designated as per Materials and Methods under sub-heading Exposure and Aspect

–, negative; 0, reaction in undiluted extract

Heavy metals

The effect of the presence of common heavy metal pollutants on haemagglutination was determined. Different concentrations of heavy metal ions were made in PBS from the appropriate salts and the resultant solutions readjusted to pH 7.2 if necessary (Fig. 3). The amounts of each divalent cation used were within the ranges previously determined for the open Atlantic Ocean (Preston, 1973) and sea shore fucoid seaweeds (Preston et al., 1972) found for South-west Wales near the Dale area or the highest levels detected in maritime lichen thalli (Fletcher, 1976, 1980). Dilutions of extract in PBS containing the appropriate metal ion were prepared and haemagglutination was carried out as described above.

Statistics

The results were analysed by either the chi-squared test or Student's t-test.

3. Results

The extracts gave variable and in most cases relatively non-specific haemagglutination and/or haemolysis titres which depended upon the lichen species and RBC types used (Table 1). Overall the levels of agglutinin(s) were higher than those of the lysin(s). The least number of lichen species causing lysis and/or agglutination or neither, occurred when rat, ox, calf, guinea pig, mouse, pig, trout, human ARh+ and BRh+ RBC were used whereas the highest number was found against horse, rabbit, chicken, human ORh+ and ABRh+ RBC. The lichens examined displayed agglutination of more of the RBC types than lytic activity. Agglutinin titres varied from neat (undiluted) to 2^{-9} and those of the lysins from 2^{-1} to 2^{-7}. Relative agglutinin specificity was demonstrated in *C. marina* towards human BRh+ and chicken RBC, *C. thallincola* (rabbit), *L. actophila* (sheep), *L. atra* (human ARh+ and frog), *X. parietina* (rabbit, humanARh+ and ABRh+). In the cases of *A. fusca* and *X. parietina*, specific lysin activity was directed against goat and horse RBC respectively.

One to 16-fold lower haemagglutinin and one to 8-fold greater haemolysin titres were detected in lichens collected from exposed and sheltered north-facing shores compared to those sampled from south-facing areas of similar exposure conditions (Table 2). However the differences in titres were not significant ($P>0.05$). In addition, lysin and agglutinin titres were greater for exposed and sheltered shores respectively irrespective of the aspect (Table 2).

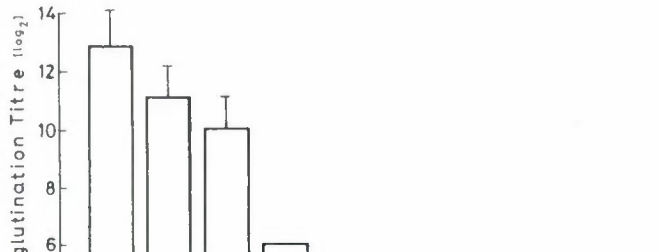

Figure 1. Mean titre of *C. pyxidata* anti-rabbit erythrocyte agglutinin at various sodium chloride concentrations in PBS. The bars indicate titre range where applicable.

Table 3. Effect of varying pH on *C. pyxidata* anti-rabbit agglutinin erythrocyte titres (log_2)

Diluent	pH 2	2.5	3	3.5	4	4.5	5	5.5	6	7.2	8	8.3	9	10	11	12
PBS alone	–	NT	–	NT	–	NT	[a]1	[c]3–4	[d]6	9	8–9	7–8	[d]5–6	[b]2	–	–
Simulated rain soln.	–	NT	–	NT	–	NT	0	[b]2–3	[d]5–6	8	7–8	NT	[c]4–5	[a]1	–	–
Simulated acid rain	NT	–	–	–	–	[a]0–1	[a]1–2									

NT, not tested; –,negative; 0, undiluted (neat) extract

[a]$p<0.001$; [b]$p<0.002$; [c]$p<0.01$; [d]$p<0.05$, when pH values are compared to the maximum titre of 2^{-9} at pH 7.2

The following results, Table 3 and all the figures refer exclusively to the investigations into the effects of various parameters on the activity of the *C. pyxidata* anti-RRBC agglutinin(s).

Both the slow and fast freezing and thawing treatments, storage at 4°C, cooling to 4°C or 15°C, and heating at 37°C caused no change in the initial *C. pyxidata* agglutinin endpoint titre against RRBC of 2^{-9}. However titres progressively declined when samples were heated at 45°C (2^{8}), 55°C (2^{-7}), 60°C ($2^{-5}-2^{-6}$; $P<0.02$), 70°C ($2^{-3}-2^{-4}$; $P<0.005$), or 80°C (2^{-1}; $P<0.001$) and were negated at 90°C and 100°C.

The pH range for optimum haemagglutination activity was 7.2 to 8.0 with significant decreases or negation of titres as acidity or alkalinity increased (Table 3). At pH 8.3 (normal sea-water value), high titres were still detected and under artificial rainfall conditions (normal rainwater pH range of 5.5–6.0), 4- to 8-fold reductions in titre were found when compared to levels at neutral pH 7.2. When samples were subjected to simulated acid rain solutions of pH 2.5 to 5.0, titres were either significantly lowered or negated (Table 3).

A maximum titre of 2^{-14} was obtained using NaCl-free phosphate buffer (PB) and as the amount of NaCl increased in the PB from 0.1 to 0.9 M, the mean agglutinin titre decreased from 2^{-13} to 2^{-2} (Fig. 1). Normal PBS containing 0.145 M NaCl and buffer containing 0.6 M NaCl (sea-water approximately 0.58 M NaCl) gave titres of 2^{-9} and 2^{-8} respectively. When the agglutinin titres found for each NaCl concentration were compared to the value obtained with NaCl-free PB, the decreases were significant for 0.1 M ($P<0.05$), 0.2 M ($P<0.01$), 0.3 M ($P<0.002$) and 0.4–0.9 M ($P<0.001$).

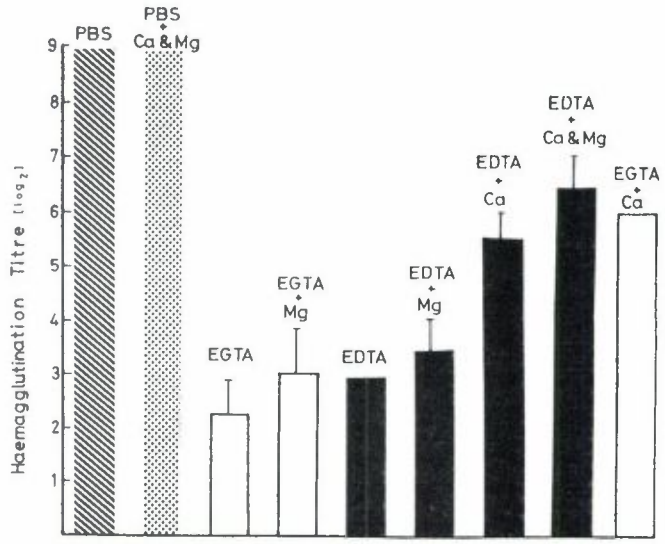

Figure 2. Mean haemagglutinin titre, together with range where appropriate, of the *C. pyxidata* extract after treatment with EDTA or EGTA alone or followed by addition of either Ca^{2+} and/or Mg^{2+} ions. Mean titre for divalent cation-free or inclusion in the phosphate buffered saline (PBS) diluent is also shown.

Removal of Ca^{2+} from the extract by EGTA and all divalent cations by EDTA resulted in 64- to 128-fold reductions ($P<0.002$) in mean agglutinin levels compared to untreated samples (Fig. 2). The addition of Mg^{2+}, Ca^{2+} or both these ions to EDTA-treated and dialysed samples resulted in titre increases ($P<0.01$) by approximately 2-fold, 8-fold and 16-fold respectively. However when Mg^{2+} ions were added to EGTA-treated and dialysed samples, no difference in titre was observed but the addition of Ca^{2+} ions resulted in an 8-fold increase in agglutinin levels (Fig. 2).

Exposure of the *C. pyxidata* samples to an overall dose of 3.3×10^2 Gy γ-radiation resulted in no change in the pre-exposure haemagglutinin value of 2^{-9}.

The inclusion in the PBS of various heavy metals within normal sea-water concentration ranges caused a 2- to 4-fold but not significant, except for Pb^{2+} and Cd^{2+} ($P<0.05$), decline in mean haemagglutinin titres (Fig. 3). When sea shore amounts of metal were used significant 6-fold to 24-fold decreases were found with Cd^{2+} and Mn^{2+} ($P<0.05$), Ni^{2+} and Zn^{2+} ($P<0.02$), Pb^{2+} and Cu^{2+} ($P<0.01$) and Fe^{2+} ($P<0.001$). In the case of high thallus concentrations, the titres were almost negated ($P<0.001$) with the exception of Cd^{2+} where levels declined 32-fold ($P<0.01$).

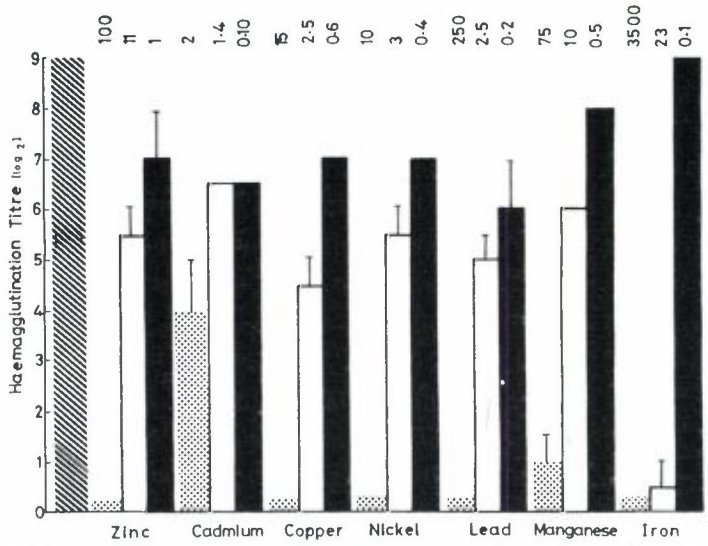

Figure 3. Mean titre and range where applicable of *C. pyxidata* anti-rabbit erythrocyte agglutinin subjected to various heavy metals in PBS at normal sea-water (μgl^{-1}) ■; normal sea shore seaweed (μgl^{-1}) □; or maximum lichen thallus accumulated (mgl^{-1}) ▒ concentrations and the amounts of each cation are displayed at the top of the Figure. The haemagglutinin titre of heavy metal-free PBS is also shown ▨.

4. Discussion

The presence of relatively non-specific, anti-human and anti-animal RBC agglutinins and titre ranges in maritime lichens are consistent with the findings for other woodland and maritime species especially against chicken, rabbit, sheep and horse RBC (Barrett and Howe, 1968; Ingram, 1982, 1984). However guinea pig RBC gave lower titres in maritime lichens compared to woodland species and specificity against some of the animal RBC types used has been observed in certain species of *Parmelia* (Barrett and Howe, 1968). Nevertheless, as is the situation with *C. pyxidata*, the use of RRBC gave maximum titres in several woodland species including *Ramalina* and *Cladonia* (Barrett and Howe, 1968). The capacity to agglutinate RBC of human ABO(H) blood group appears to vary between different genera, species and strains of the same species. Lack of selective reactivity for human RBC has been found by several workers (Dubovoy et al., 1966: Estola and Vartia, 1955; Fillho et al., 1980). However, relative specificity for 'A' (*C. pyxidata* and *X. parietina*), 'B' (*C. marina*) and 'ABC' (*L. atra* and *C. pyxidata*) RBC were found in the present study. Similar anti-A, anti-B and anti-AB specifications have been demonstrated in some foliose (Barrett and Howe, 1968;

Lockhart et al., 1978; Petit, 1982), fruticose and crustose species (Barrett and Howe, 1968; Fillho et al., 1980). However, the occurrence of specific anti-O agglutinins does not seem to be widespread and only in 2 foliose species have anti-H specificities been detected (Barrett and Howe, 1968; Hardman et al., 1983). Therefore it is also feasible that sampling habitat, season of collection, extraction protocol and lichen morphology may influence both titres obtained and degrees of haemagglutination.

Maximum *C. pyxidata* agglutinin titres were found at temperatures within the annual range recorded for the Dale Peninsula of minimum –1.20°C and maximum 17.73°C (D.C. Emerson, pers. comm). Furthermore, freezing and thawing treatments and storage of extract for 1 month at 4°C did not affect agglutinin activity. Similar findings have been documented for basidiolichens in which agglutination of animal RBC was not affected by storage at 5°C for 20 days (Fillho et al., 1980). However heating the *C. pyxidata* extracts at temperatures of over 40°C lowered titres which were negated at 90°C or above. By comparison, some woodland foliose lichen lectins are heat-stable at 55°C (Fillho et al., 1980) or 100°C (Howe and Barrett, 1970; Petit, 1982; Petit et al., 1983) suggesting possible differences in the structural configuration of the maritime *C. pyxidata* agglutinin(s). In this context, short-term exposure to a high dose of γ-radiation under laboratory conditions failed to reduce *C. pyxidata* agglutinin levels. Under normal conditions this lichen would only be subjected to ultraviolet rays during sunshine and an approximately 900 μGy low background radiation dosage per year calculated for the U.K. (Mellanby, 1980). However, caution must be observed in the interpretation of results for comparisons between exposure of intact lichen thalli to either annual natural environmental radiation or daily artificial doses of γ-radiation (see Brodo, 1964; Woodwell and Gannutz, 1967) and the artificial treatment of lichen extracts with γ-rays under laboratory conditions for a relatively short period of time.

Interestingly the optimum pH for haemagglutination occurred in neutral to alkaline conditions which implies that *C. pyxidata* retains the ability to synthesize and maintain agglutinin activity under conditions of sea spray (pH 8.3) striking the cliff top flora. Furthermore, the pH of the moss and soil on which this species was growing was determined as pH 6.3. However, in the simulated acid rain and normal rainwater experiments, pH values less than 5.0 severely reduced or abolished agglutination. The rainwater pH for south-west Wales varies between 4.0 and 4.75 (Watch, 1986). Therefore acid rain would tend to influence soil pH while freshwater runoff downwards

from the cliff top and seawater splash upwards may establish a pH gradient (Fletcher, 1980) and lead to local environmental pH variations that may regulate haemagglutinin activity. Nevertheless some investigators have found foliose lichen lectins to be active over a pH range of 5.0 to 11.0 (Petit, 1982) and others obtained optimum agglutination at pH 5 to 6 (Howe and Barrett, 1970; Fillho et al., 1980). These findings correlate with those reported in the current work.

High saline concentrations drastically affect haemagglutination and this finding suggests that *C. pyxidata*, obtained from a sheltered shore cliff-top environment, would be susceptible to wind-blown seaspray, splash or deposition of salt crystals on the thalli due to evaporation in adverse conditions. Lack of agglutination at high salt concentrations have also been reported for woodland foliose lichens (Howe and Barrett, 1970).

The inclusion of various heavy metals in the buffer at concentrations determined for seaweeds and in amounts accumulated in sea shore lichen thalli caused either significant reductions in or no agglutination. However comparisons with the amounts of heavy metals found by other workers is difficult due to the use of different units, usually w/w dry weight (Nieboer et al., 1978; Pakarinen, 1985). In the present study the concentrations of metal ions used for sea-water fall below the background levels reported for cliff-top (Fletcher, 1976)and non-maritime species (Brown, 1976; Nieboer et al., 1978). Lichens obtain essential divalent cation nutrients (Mg, Ca, Mn, Fe, Cu, Zn) from chemical weathering of substratum minerals usually under acid (pH 3.5–5) conditions, the seashore ecosystem, air-borne fall-out from pollution (Pb) or rainfall (Fe) by chelation reactions involving lichenic, oxalic or carbonic acids (Jones and Wilson, 1985; Nieboer et al., 1978). In *Cladonia* species the presence of excess metals causes toxicity and leads to morphological distortion (Zn), phycobiont breakdown and photosynthetic inhibition (Zn, Cd, Cu, Pb, Ni) or membrane damage (Cu, Pb) (Brown and Beckett, 1984). Moreover, toxic Fe^{3+} ions are strongly complexed by fulvic and humic acids produced by decomposition of organic matter (Jones and Wilson, 1985) and efficiently retained while both Pb and Fe are significantly increased in dead thalli (Parkarinen, 1985). On this basis it is feasible that the *C. pyxidata* agglutinin may be precipitated by the excessive uptake of certain heavy metals, especially Fe, with loss of activity. Alternatively heavy metal pollutants may indirectly interfere with agglutinin synthesis by the symbionts.

Agglutinin activity also depends partly on the presence of Ca^{2+} ions in the buffer (Fig. 3). Calcium ions are of importance to some higher plant lectins

for structural stabilization and activity (Liener et al., 1986). Nevertheless, the addition of Ca^{2+} and/or Mg^{2+} ions to EDTA- and EGTA-treated and dialysed samples did not fully restore the initial agglutinin titres. This may be caused by dilution factors during treatment or the possibility that another divalent cation is required for activity. On the other hand, it may be due to partial agglutinin denaturation caused by temporal irreversible chelation. Petit (1982) suggested that lichen lectins may be stimulated by divalent cations (Mg, Mn, Ca, Cu, Cd) although he did not present details of magnitude of activity or amount of divalent cation used.

Both exposure and aspect affect agglutinin and lysin titres determined for the various lichens and Ferry and Sheard (1969) have shown that varying degrees of exposure influence the occurrence of certain rocky sea shore lichen species and hence lectin presence. Exposure covers a wide range of parameters including rock type, climate, topography, wind and wave action, tidal influence, temperature and amount of sea spray (Fletcher, 1980). The maritime *Cladonia* species used in the current study was collected from a south/south south west-facing site,reasonably sheltered from prevailing winds but exposed to sunlight and subjected to fine sea spray in inclement weather. It is possible that as a result of the findings of the present study, a combination of different environmental factors may influence *C. pyxidata* haemagglutinin activity.

Algal-binding proteins (Bubrick et al., 1985; Galun et al., 1984) and lectins (Petit, 1982) originate from the fungal symbiont of lichens but their function(s) remains unclear. It has been suggested that these molecules may participate in mycobiont-phycobiont interactions by recognition, contact and/or adhesion processes between compatible symbionts during lichen thallus initiation or resynthesis (Bubrick et al., 1985; Galun et al., 1984). Furthermore, the natural occurrence of relatively non-specific lichen haemagglutinins appears to be more widespread than expected and these substances may play several as yet unidentified roles. Ingram and Tassabehji (1988) demonstrated the *C. pyxidata* anti-RRBC agglutinin(s) to be glycoprotein in nature with specificities directed towards glucose, methyl glucoside, mannitol, mannosamine and N-acetyl derivatives of glucose, mannose and galactose.

It is likely that lichens contain heteroagglutinins, with multispecificity for carbohydrate or glycoprotein moieties on the erythrocyte surface, which exhibit antibody-like properties based on the results of the current work on the effect of various factors on *C. pyxidata* lectin activity. Moreover, the cell walls of various micro-organisms share common surface antigens (Mirelman, 1986), which could cross-react with natural agglutinins or similar lectin-like

molecules (Pistole, 1981). Therefore such heterogeneity of lichen lectins could enable a protective benefit, if one assumes a survival advantage or potential defence function, by interaction with and counteraction of the vast array of environmental pathogens.

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