# *myo*-Inositol hexakisphosphate is a major component of an extracellular structure in the parasitic cestode *Echinococcus granulosus*

Florencia IRIGOÍN*, Fernando FERREIRA†, Cecilia FERNÁNDEZ*, Robert B. SIM‡ and Alvaro DÍAZ*‡[1]

*Cátedra de Inmunología, Facultad de Química/Ciencias, Universidad de la República, Avenida Alfredo Navarro 3051, piso 2, CP 11600, Montevideo, Uruguay, †Laboratorio de Carbohidratos y Glicoconjugados, Departamento de Química Orgánica, Departamento de Desarrollo Biotecnológico, Facultad de Química/Medicina, Universidad de la República, Avenida Alfredo Navarro 3051, piso 1, CP 11600, Montevideo, Uruguay, and ‡MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

*myo*-Inositol hexakisphosphate (IP$_6$) is an abundant intracellular component of animal cells. In this study we describe the presence of extracellular IP$_6$ in the hydatid cyst wall (HCW) of the larval stage of the cestode parasite *Echinococcus granulosus*. The HCW comprises an inner cellular layer and an outer, acellular (laminated) layer up to 2 mm in thickness that protects the parasite from host immune cells. A compound, subsequently identified as IP$_6$, was detected in and purified from an HCW extract on the basis of its capacity to inhibit complement activation. The identification of the isolated compound was carried out by a combination of NMR, MS and TLC. The majority of IP$_6$ in the HCW was found in the acellular layer, with only a small fraction of the compound being extracted from cells. In the laminated layer, IP$_6$ was present in association with calcium, and accounted for up to 15 % of the total dry mass of the HCW. IP$_6$ was not detected in any other structures or stages of the parasite. Our results imply that IP$_6$ is secreted by the larval stage of the parasite in a polarized fashion towards the interface with the host. This is the first report of the secretion of IP$_6$, and the possible implications beyond the biology of *E. granulosus* are discussed.

Key words: calcium, cestode, hydatidosis, inositol polyphosphates, phytic acid.

## INTRODUCTION

Higher *myo*-inositol polyphosphates are ubiquitous components of animal cells. Of these, *myo*-inositol hexakisphosphate (IP$_6$) is the most abundant, with its concentration ranging from 10–100 $\mu$M in mammalian cells depending on cell type and developmental stage [1–4]. However, there are still major gaps in the understanding of this molecule. One of the important questions still to be answered is the complete elucidation of the pathways for IP$_6$ synthesis and degradation in animal cells. This has impaired the generation of knockout animals deficient in IP$_6$, thus making the physiological role of this molecule still a matter of debate [5,6]. The interaction of IP$_6$ with several intracellular proteins has been investigated by means of *in vitro* experiments [7–10]. In some cases, these interactions result in the inhibition [8,11] or potentiation [10] of the physiological activities of the proteins involved. The most compelling evidence arising from these studies suggests a role for IP$_6$ as a cofactor in DNA repair by non-homologous end-joining [10]. Recent studies in yeasts, using mutants deficient in IP$_6$ synthesis, have shown that the molecule is involved in mRNA export from the nucleus to the cytosol [12].

Another aspect of IP$_6$ biology that is not completely understood involves its cellular localization and the determination of whether the molecule is found in a free soluble form or in association with membranes or other cellular components. Cell permeabilization studies indicate that the majority of IP$_6$ in mammalian cells is present in, or in rapid exchange with, the cytosol, although the existence of a minor IP$_6$ pool in other membrane-bound compartments has not been excluded [13]. The unusual behaviour of IP$_6$ in solution [10,14,15] does not allow the prediction of whether the molecule can pass freely through nuclear pores. However, the suggestion that IP$_6$ has a role in the nucleus would imply that the molecule is present in this compartment. A further factor raising questions about the location of IP$_6$ arises from the finding that the only enzyme known to dephosphorylate IP$_6$, the multiple inositol polyphosphate phosphatase ('MIPP'), is present exclusively in the lumen of the endoplasmic reticulum (ER) [14]; the enzyme is nonetheless able to regulate the levels of cytosolic IP$_6$ to some extent [16]. These results suggest the existence of a low-affinity IP$_6$ translocator in the membranes of the ER. Finally, endogenously synthesized IP$_6$ has never been reported to be secreted from cells or to be present at high levels in extracellular compartments [5].

The current study demonstrates the presence of abundant extracellular IP$_6$ in the cyst wall of *Echinococcus granulosus*. This is a cestode parasite whose larval stage dwells within internal organs of livestock animals and humans. The larva has the form of a fluid-filled cyst (hydatid cyst) (Figure 1), which can reach up to 25 cm in diameter. The part of the cyst that is exposed to the host is the hydatid cyst wall (HCW). This structure is composed of two layers: the germinal and laminated layers. The innermost layer (germinal layer), which is only two or three cells thick, is the live parasite tissue and synthesizes the outer laminated layer. This layer is an acellular structure that resembles an exaggerated glycocalyx. It is a carbohydrate-rich meshwork that precludes host cells from direct contact with the parasite cells, but is permeable to host macromolecules. The germinal layer also gives
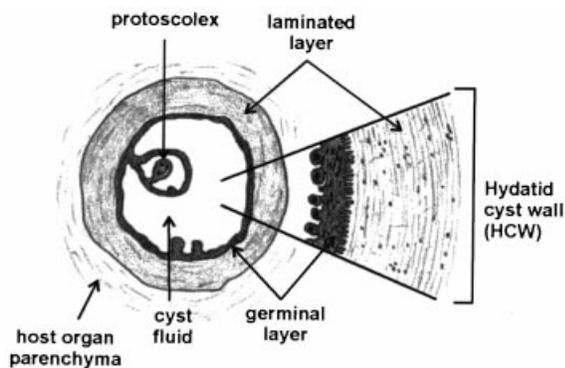
**Figure 1    The hydatid cyst of *E. granulosus***

The larval stage of the parasite *E. granulosus* is a fluid-filled cyst bounded by a HCW. The HCW consists of two layers: the germinal and laminated layers. The innermost layer (germinal layer) is the live parasite tissue that synthesizes and secretes an acellular, carbohydrate-rich layer (laminated layer) towards the host. The germinal layer also gives rise to the protoscoleces by budding towards the interior of the cyst.

rise, through budding towards the interior of the cyst, to the protoscoleces, which are the infective stage for the host in which the adult worm develops. Recently, during a study to identify molecular species from the hydatid cyst that interact with host immunity, we have isolated from the HCW a low-molecular-mass, heat-stable molecule with the capacity to inhibit complement activation [17]. In the present study, we describe the identification of this molecule as $IP_6$ and its localization to the laminated layer of the hydatid cyst.

## MATERIALS AND METHODS

### Materials

$IP_6$ was purchased from either Sigma (St Louis, MO, U.S.A.) or Calbiochem (La Jolla, CA, U.S.A.) and *myo*-inositol pentakis-phosphate ($IP_5$) was from Matreya Inc. (Pleasant Gap, PA, U.S.A.). Polyethyleneimine (PEI)-cellulose plates for TLC were obtained from Macherey–Nagel (Düren, Germany). The protease inhibitors PMSF, *trans*-epoxysuccinyl-L-leucylamido-(4-guani-dino)butane (E-64), and benzamidine hydrochloride, as well as general chemicals, were purchased from Sigma.

### *E. granulosus* extracts

#### 'Mother' bovine cysts

Large cysts (several cm in diameter) were obtained from natural infections in cattle and are referred to as 'mother' bovine hydatid cysts. After puncture and aspiration of the cyst fluid, the HCW collapse, detaching from host tissue. HCW were generally used as the whole entity. In specified cases, the germinal and laminated layers were separated from each other with forceps. A soluble extract from whole bovine HCW was obtained by homogenization with a pestle and mortar under liquid $N_2$, followed by extraction for 2 h at 25 °C with PBS containing 5 mM EDTA, 1 mM PMSF, 1 mM benzamidine and 10 $\mu$M E-64 (PBS/EDTA). This extract was used for the initial purification of the component under study. In addition, isolated germinal and laminated layers were washed extensively with PBS and then extracted for 4 h at 25 °C with PBS/EDTA containing 0.5 % (w/v) Tween 20 (PBS/EDTA/Tween) or PBS/EDTA respect-

ively. Extracts were clarified by centrifugation and kept for analysis.

#### Small cysts

We use the term small cysts to refer collectively to: (i) 'daughter' bovine cysts, which are hydatids that grow within larger cysts; and (ii) murine cysts, which were obtained from the peritoneal cavities of mice 8 months after intraperitoneal infection with protoscoleces [18]. Intact cysts (diameters ranging between 0.3–1.5 cm) were washed with PBS and then extracted with either PBS/EDTA for 3 h ('daughter' bovine cysts) or two separate PBS/EDTA washes for 1 h and 2 h respectively (murine cysts). Cyst fluid was removed by aspiration with a syringe, the HCW was opened with a scalpel blade and extracted at 4 °C with PBS/EDTA/Tween for 1 h ('daughter' bovine cysts) or for 30 min twice (murine cysts). Thus extracts were obtained by external washing of intact cysts and by detergent treatment of cysts opened to expose the cellular structures. Other murine cysts were extracted 'from the inside' by injecting EDTA solution so as to attain a final concentration equal to that used for extracting the cysts 'from the outside'. After incubation at 25 °C for 3 h, the liquid inside the cyst was retrieved with a syringe.

#### Protoscoleces

Protoscoleces from bovine cysts were washed with PBS, homogenized under liquid $N_2$ and extracted with PBS/EDTA for 2 h at 25 °C. The residue was incubated under conditions (200 mM EDTA for two weeks at 4 °C) known to dissolve the 'calcareous corpuscles', a conspicuous feature of this stage of the parasite (M. Marín, unpublished work). The soluble extracts obtained after the two steps were kept for analysis.

#### Adult worms

Adult worms were recovered from intestines of dogs 32 days after oral infection with protoscoleces, as described previously [19]. The extract from these worms was a gift from Silvia González (Cátedra de Inmunología, Montevideo, Uruguay), and was obtained by extensive sonication in PBS containing 5 mM EDTA and 2 mM PMSF.

### *E. multilocularis* material

Cysts from *E. multilocularis* were generously given by Dr Klaus Brehm (Institut für Hygiene und Mikrobiologie der Universität Würzburg, Germany). *E. multilocularis* larvae, obtained from infected patients and maintained in Mongolian jirds (*Meriones unguiculatus*), were cultivated *in vitro* for 6 weeks as described previously [20]. The cysts were washed with PBS and a soluble extract from the cyst walls was obtained as described above for the 'mother' bovine HCW.

### *In vitro* complement-activation assay

The human alternative complement pathway C3 convertase was formed from its purified components [45 ng of amidated C3, 70 ng of factor B and excess factor D] in the presence or absence of the samples to be tested. Incubations were performed at 37 °C for 1 h in 5 mM $NaH_2PO_4$/2.5 mM barbituric acid (adjusted to pH 7

with NaOH) containing 75 mM NaCl, 0.25 mM EDTA, 0.01 % (w/v) Triton X-100 and 1 mM $NiCl_2$. Complement activation was evaluated in terms of factor B (93 kDa) conversion into its activation products Bb (63 kDa) and Ba (30 kDa), as visualized by Western blotting. Samples were run on SDS/PAGE [8 % (w/v) acrylamide gels] and electrotransferred on to nitrocellulose membranes. For detection of factor B, blots were probed with a rabbit anti-(human factor B) polyclonal antibody (Dako, Carpinteria, CA, U.S.A.) and peroxidase-conjugated goat anti-(rabbit IgG) (Calbiochem). Blots were developed using enhanced chemiluminescence (ECL®; Amersham Biosciences, Piscataway, NJ, U.S.A.).

## NMR analyses

NMR spectra were recorded for solutions in $^2H_2O$ at 30 °C using a Brücker Avance DPX 400 instrument (Acton, MA, U.S.A.) at 400, 100.6 and 162 MHz for $^1H$, $^{13}C$ and $^{31}P$ respectively. $^1H$, $^{13}C$ and $^{31}P$ chemical shifts were reported in p.p.m. using 3-trimethyl-silyl propionate ($\delta_H$ 0.00) and acetone ($\delta_C$ 31.07) as internal references and phosphoric acid ($\delta_P$ 0.00) as the external reference. Two-dimensional [COSY and heteronuclear multiplet quantum coherence spectroscopy ('HMQC')] experiments were performed according to standard pulse sequences available in the Brücker software.

## MS analyses

Electrospray ionization MS was performed by Dr Robin Aplin (Oxford Centre for Molecular Sciences, University of Oxford, U.K.) employing a Micromass BioQ II-ZS mass spectrometer (Micromass U.K. Ltd., Altrincham, Cheshire, U.K.). Both positive- and negative-ion spectra were recorded over a mass range of 200–1200 Da, and were calibrated relative to a mixed polyethylene glycol standard. Matrix-assisted laser-desorption ionization–time-of-flight MS was carried out by Dr Rosario Durán and Dr Carlos Cerveñansky (Lab. de Bioquímica Analítica, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay) on a Voyager DE-PRO spectrometer (Applied Biosystems, Foster City, CA, U.S.A.) using 2,5-di-hydroxybenzoic acid as the matrix and a 337 nm nitrogen laser.

## Dephosphorylation of the HCW component and analysis of the resultant product

The purified component was freeze-dried and treated with 48 % (w/v) hydrofluoric acid overnight at 4 °C. Excess hydrofluoric acid was evaporated using $N_2$. The product was applied on to a P2 gel-filtration column (1 cm × 60 cm; Bio-Rad, Hercules, CA, U.S.A.) in 1 % (v/v) pyridine/0.4 % (v/v) acetic acid buffer (pH 6.0). Fractions corresponding to the refractive index peaks were analysed by $^1H$-NMR. The fraction containing the dephosphorylated molecule was acetylated as described previously [21] and analysed by GLC on a Hewlett Packard 6890 gas chromatograph (HP-5-capillary column; 0.25 $\mu$m × 30 m) with a gradient of 5 °C/min from 190–250 °C, and by GLC–MS on a Shimadzu QP 1100 instrument (PTE-5 column; 0.25 $\mu$m × 30 m; Supelco, Bellafonte, PA, U.S.A.) with a gradient of 5 °C/min from 80–260 °C. The identity of the dephosphorylated molecule was confirmed by GLC using co-injection of hexa-acetylated *myo*-inositol prepared from commercial *myo*-inositol as described previously [21].

## TLC for high inositol polyphosphates

TLC was performed on PEI-cellulose plates, with the detection of phosphate by ammonium molybdate as described previously

[22]. The detection limit of the system was 6 nmol of phosphate. Crude extracts were dialysed against water and treated with activated charcoal prior to TLC to minimize interference with the detection of inositol polyphosphates. For quantitation of $IP_6$, the plates were scanned and the intensity of the spots was quantitated by densitometry (integration of density over area) using Quantity One software (Bio-Rad). Different amounts (3–15 nmol) of an $IP_6$ standard (Sigma) were run in parallel with the samples and employed to construct linear regression curves ($r^2 > 0.98$).

## Detection of *E. granulosus* intracellular proteins

The intracellular parasite proteins, P-29 and cyclophilin A (CyPA), were detected by Western blotting. P-29 was detected as described previously [23]. For the detection of CyPA, samples were separated by SDS/PAGE [10 % (w/v) acrylamide gel] and electrotransfered on to nitrocellulose. Blots were probed with a rabbit polyclonal antiserum raised against CyPA from *Meso-cestoides corti*, a related cestode; the antiserum cross-reacts strongly with the homologous protein from *E. granulosus*. The blots were probed with peroxidase-conjugated goat anti-(rabbit IgG) (Calbiochem) and developed using ECL®. *M. corti* CyPA, known to migrate similarly to the *E. granulosus* protein (M. Margenat, M. Marín and C. Fernández, unpublished work) was included as a positive control.

## Quantitation of calcium and magnesium in the extracts

The quantitation of calcium and magnesium in extracts from murine cysts was carried out by Lourdes Salvarrey and D. Moisés Knochen (Cátedra de Análisis Instrumental, Montevideo, Uruguay) by using a PerkinElmer 380 atomic absorption spectrophotometer (Indianapolis, IN, U.S.A.). Samples were diluted in 0.5 % (w/v) lanthanum in order to avoid interference of phosphates in the determination of calcium and were subsequently burnt in an acetylene/air flame. The measurements were made using a hollow cathode lamp for calcium/magnesium/aluminium (PerkinElmer) at 20 mA, and wavelengths of 285.2 and 422.7 nm for magnesium and calcium respectively.

## Quantitation of total phosphorus in the HCW

'Daughter' bovine hydatid cysts were washed extensively with Milli Q water, cut into small pieces, and the content of phosphorus was determined as described previously [24]. Results were expressed as the means of four individual determinations.

## RESULTS

### Purification and identification of $IP_6$ from the HCW of *E. granulosus*

A component, subsequently identified in the present study as $IP_6$, was detected in and purified from the HCW of *E. granulosus* as part of a search for host complement-inhibitory activities from the parasite [17]. Thus detection of the component, both in crude extracts from the HCW and during its purification, was carried out by means of an assay for inhibition of complement activation. Details of the analysis of the interaction of $IP_6$ with the complement system will be reported elsewhere (F. Irigoìn, A. Laich, A. M. Ferreira, C. Fernández, R. B. Sim and A. Díaz, unpublished work). The complement-inhibitory activity was not solubilized from the HCW in the absence of EDTA, suggesting that the active components were immobilized in the
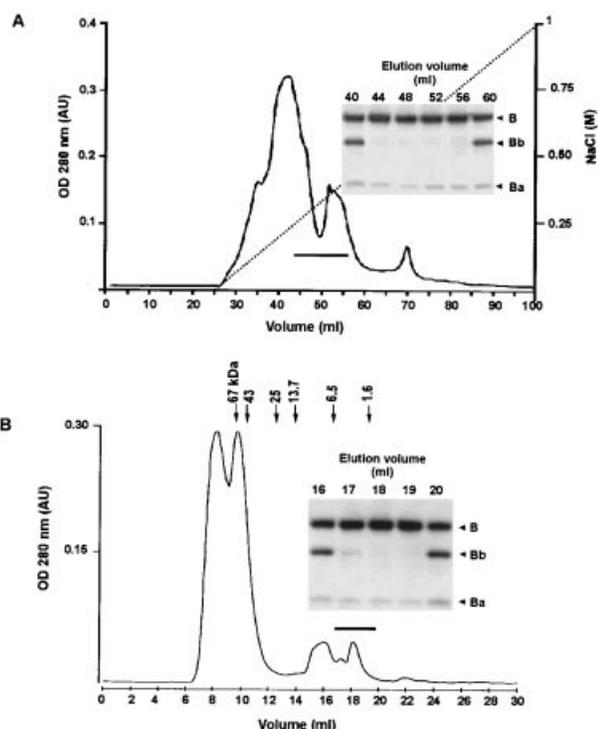
**Figure 2   Purification of a component of the HCW of *E. granulosus* with the capacity to inhibit complement activation**

A molecule, subsequently identified as $IP_6$, was purified from a soluble extract of bovine HCW on the basis of its ability to interfere with complement activation. Complement activation was measured in terms of the conversion of factor B (93 kDa) into its activation fragments, Bb (63 kDa) and Ba (30 kDa), as detected by Western blotting. Hence, inhibitory activity was visualized as the absence of the bands corresponding to Bb and Ba. The results of the complement activation assay for the selected fractions are shown in the Figure insets. (**A**) Anion exchange on a 25 ml Source 15Q column (BioPilot; Amersham Biosciences) at pH 8.0. The elution was performed with increasing concentrations of NaCl and the absorbance (shown as optical density, O.D.) was measured at 280 nm. No absorbance at 280 nm was detected with the material that did not bind to the column. The inhibitory activity eluted at 0.25–0.4 M NaCl. (**B**) Active fractions from the anion-exchange step were pooled, dialysed against a solution containing 10 mM sodium phosphate buffer (pH 7.0), 250 mM NaCl and 0.5 mM EDTA and refractionated by FPLC-gel filtration on Superdex 75 (Amersham Biosciences). Absorbance (O.D.) of the eluted proteins was measured at 280 nm. Elution volumes of protein standards (in kDa) are indicated above the panel. Solid bars in both (**A**) and (**B**) represent the fractions pooled for further purification or characterization. The concentration of $IP_6$ in the active fractions from the second step was 3–5 mg/ml. AU, absorbance units.

cyst wall through the interaction with bivalent cations. Purification of the active molecule was achieved by two successive chromatographic steps: the activity was eluted from an anion-exchange column between 0.25–0.4 M NaCl (Figure 2A), and on refractionation by gel filtration on Superdex 75, it was eluted with a molecular mass of approx. 3 kDa (Figure 2B) when compared with protein standards. In spite of this apparent low molecular mass, the inhibitory activity did not dialyse through conventional 12 kDa cut-off membranes.

The purified fraction obtained was analysed by NMR, MS and TLC. The one-dimensional $^1H$- (Figure 3A), $^{13}C$- (four signals at 77.8, 76.9, 76.5 and 73.8 p.p.m., results not shown) and $^{31}P$-NMR spectra (four signals at 2.95, 1.95, 0.95 and 0.05 p.p.m. with an intensity ratio of 1:2:2:1 respectively, results not shown), as well as the two-dimensional $^1H$-$^1H$-, $^1H$-$^{13}C$- and $^1H$-$^{31}P$-NMR spectra (results not shown), were compatible with a pure small molecule containing six carbon atoms, none of them anomeric in nature, and each bound to a phosphate group. The
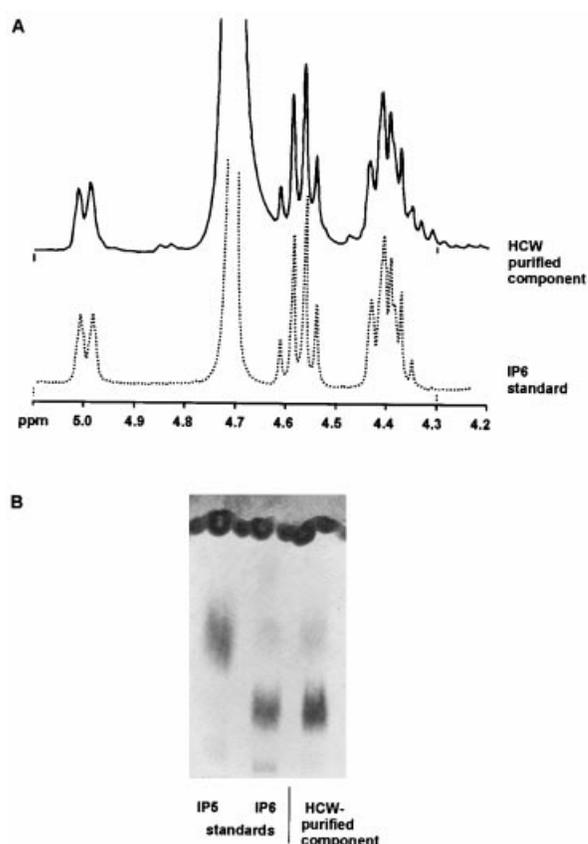


**Figure 3   Identification of the purified component of the cyst wall as $IP_6$**

The purified component of the HCW was analysed in parallel with $IP_5$ and $IP_6$ standards. (**A**) Commercial $IP_6$ (broken line) and the purified component (solid line) were passed through a Dowex 50 resin (H$^+$ form), freeze-dried and redissolved in $^2H_2O$ for $^1H$-NMR spectroscopy as described in the Materials and methods section. Chemical shifts are expressed in p.p.m. using 3-trimethylsilyl propionate ($\delta_H$ 0.00) as an internal reference. (**B**) The purified component and the standards were run on PEI-cellulose TLC.

molecule was subjected to hydrolysis in the presence of hydrofluoric acid, with the aim of removing the phosphate groups, and the resulting product was analysed by NMR (one-dimensional $^1H$- and $^{13}C$-NMR and two-dimensional $^1H$-$^1H$- and $^1H$-$^{13}C$-NMR). The results suggested that the dephosphorylated molecule was *myo*-inositol; this was confirmed by GLC with co-injection of the acetylated derivative of commercial *myo*-inositol (results not shown). Electrospray ionization and matrix-assisted laser-desorption ionization–time-of-flight MS of the purified untreated molecule indicated a molecular mass of 660 Da (results not shown), which was compatible with an inositol ring with six phosphomonoester groups attached. Confirmation of the identity of the purified molecule was achieved by NMR. The $^1H$- (Figure 3A) and $^{13}C$- (results not shown) NMR spectra of the molecule were identical with those of commercial $IP_6$. Finally, analysis of the purified fraction by TLC on PEI-cellulose, under conditions usually employed for separating inositol polyphosphates, revealed the presence of more than 95 % pure $IP_6$, with a minor contaminant migrating consistently with $IP_5$ (Figure 3B).

## $IP_6$ is an intrinsic component of the HCW

The parasite material from which $IP_6$ was isolated, namely HCW from bovine lung cysts, exists *in vivo* in intimate contact with

host inflammatory cells. Host-derived molecules can find their way *in vivo* into the meshwork constituted by the laminated layer and be retained within it. For example, this takes place for the usually cytosolic protein annexin II [25]. Hence, it was possible that the $IP_6$ isolated from bovine HCW originated from host cells. Metabolic labelling studies are not straightforward to carry out with the cyst stage of the parasite. Thus we used an approach consisting of assessing the presence of $IP_6$ in cysts from other origins in which it is known that the contact with host inflammatory cells is minimal or nil.

One type of material analysed was cysts obtained from experimental infection in mice. These cysts grow free in the peritoneal cavity with little inflammatory reaction around them (M. Breijo, G. Anesetti, L. Martínez, R. B. Sim and A. M. Ferreira, unpublished work). The second, even 'cleaner', type of material used was constituted by small ('daughter') cysts, which arise inside larger ('mother') bovine ones. As host cells cannot penetrate the HCW of the 'mother' cyst, 'daughter' cysts are free of contact with host cells. Moreover, the chemical nature of $IP_6$ (charge, insolubility in the presence of calcium) is expected to preclude it from diffusing across the membrane barriers of the germinal layer and the calcium-containing cyst fluid of the 'mother' cyst. Extracts were made from murine and 'daughter' bovine cysts, and $IP_6$ was detected and quantitated in them by TLC. Of the total dry masses of HCW from murine and 'daughter' bovine cysts, $IP_6$ accounted for 8 and 15 % respectively (expressed in terms of its free acid form), whereas the corresponding value for 'mother' bovine cysts was 3 %. This latter value is probably an underestimation of the actual levels of $IP_6$ in the 'mother' bovine HCW, as it was observed that is more difficult to solubilize $IP_6$ from these thicker cyst walls than it is from the HCW of small cysts (results not shown). Taken together, these observations confirmed that $IP_6$ is an intrinsic component of the HCW, and is therefore, in all likelihood, produced by the parasite.

$IP_6$ was not detected by TLC in other *E. granulosus* materials, other than the HCW. Taking into account the amounts of material loaded and the detection limit of the method, $IP_6$ in protoscoleces and adult worms constituted less than 0.09 % and 0.43 % of the total dry mass respectively. Similarly, the maximum possible $IP_6$ concentration in hydatid cyst fluid would be 9 $\mu$g/ml. We also analysed the presence of $IP_6$ in the cysts from another species of *Echinococcus*, namely *E. multilocularis*. $IP_6$ was not detected in the cyst wall from this species (less than 0.7 % of the starting material dry mass).

## $IP_6$ is a component of an extracellular structure: the laminated layer of the HCW

The extracts analysed above originated from whole HCWs, with no attempt made to separate physically the germinal and laminated layers. Since $IP_6$ has been described as an intracellular cytosolic molecule [13], it could be assumed that the $IP_6$ extracted from the HCW was contained within the cells of the germinal layer. However, some observations were not consistent with this possibility, suggesting that $IP_6$ was a constituent of the laminated layer.

One of the first observations that caught our attention was the very high amounts of $IP_6$ extracted from the HCW. A calculation on the basis of the $IP_6$ content of small cysts, their diameters and an average germinal layer thickness of 10 $\mu$m, as reported for cysts in this size range [26], yielded a hypothetical intracellular $IP_6$ concentration of approx. 300 mM. This is hundreds of times higher than the concentration of $IP_6$ in *Dictyostelium discoideum*, one of the highest reported [27]. If present at high concentrations

**Table 1  Quantitation of $IP_6$, calcium and magnesium in different extracts from murine hydatid cysts**

$IP_6$ was quantitated in different extracts from murine cysts by TLC on PEI-cellulose plates followed by densitometry. Calcium and magnesium were quantitated in the same extracts by atomic absorption. $IP_6$, calcium and magnesium contents were expressed as the total amounts per g of HCW dry mass ($\mu$mol/g of HCW dry mass) extracted by washing the intact cysts externally (washes from outside) or by treatment of the resultant cyst walls with Tween 20 and EDTA (opened-up cysts). Values in parentheses indicate the molar ratios between each atom and $IP_6$.

| Extract | $IP_6$ | Calcium | Magnesium |
|---|---|---|---|
| Washes from outside | 101 | 804 (8) | 61 (0.6) |
| Opened-up cysts | 22 | 200 (9) | 22 (1.0) |
| Total | 123 | 1004 (8) | 83 (0.7) |

in the cytosol, $IP_6$ would be present (mostly in insoluble form) as its magnesium salt [6]. In contrast, extracellular $IP_6$, even at submicromolar concentrations, would be present as its calcium salt, which is even more insoluble than the magnesium one. This reasoning led us to quantitate the amounts of calcium and magnesium co-extracted with $IP_6$. As shown in Table 1, calcium and magnesium were extracted at molar ratios with respect to $IP_6$ of 8 and 0.7 respectively. These results suggested that most of the $IP_6$ is present as the $Ca^{2+}$ salt; assuming this is the hexacalcium salt, association with $IP_6$ would account for approx. 74 % of the total calcium extracted from the HCW. These data also suggested that the majority of the $IP_6$ extracted was not present in the cytosol of cells.

We then attempted the selective stepwise extraction of $IP_6$ from the cellular and acellular layers of the structure. The pattern of extraction was then compared with those of two intracellular markers: the parasite proteins P-29 [23] and CyPA [28]. Both proteins are known to be expressed by the germinal layer cells and have relatively low molecular masses (29 and 16 kDa for P-29 and CyPA respectively). CyPAs are exclusively cytosolic proteins [29]; the subcellular location of P-29, which is absent from parasite secretions, has not been determined experimentally, but the lack of sorting signals in its coding sequence suggests that the protein is also cytosolic.

Intact murine or 'daughter' bovine cysts were washed with PBS and then PBS/EDTA in an attempt to extract any $IP_6$ present in the laminated layer without disrupting the integrity of the germinal layer cells. Cyst fluid was then removed by aspiration with a syringe, and the resultant HCW was opened up and extracted with PBS/EDTA/Tween in order to solubilize cellular components. Washing the intact cysts in the presence of EDTA allowed the solubilization of the majority of the total extractable $IP_6$ (Figure 4A and Table 2). No $IP_6$ was recovered from the cyst fluid, but the treatment of the opened-up cyst walls with PBS/EDTA/Tween allowed the extraction of some $IP_6$ (Figure 4A). In contrast with $IP_6$, the two intracellular proteins P-29 and CyPA were only extracted when cells were disrupted with the use of detergent. No contamination with these intracellular markers was observed in the external washes of intact cysts with the EDTA-containing buffer (Figures 4B and 4C). In a parallel experiment, EDTA was injected into intact murine cysts, so as to attain the same final concentration of the chelating agent in the cyst fluid as that used in the external washings. This extraction of the HCW 'from the inside', did not solubilize any $IP_6$ (Figure 4A). This indicated that $IP_6$, solubilized during the external washings of the cysts, could not have arisen from the disruption of cells as a result of the chelation of bivalent cations by EDTA.
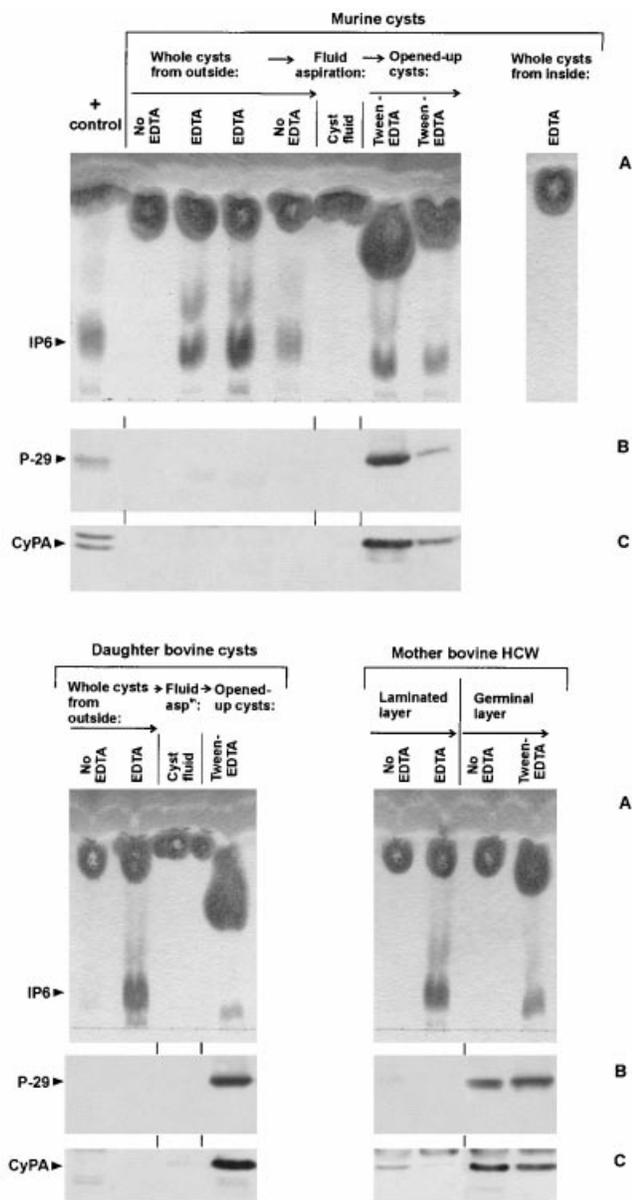
**Figure 4      Demonstration of the extracellular presence of IP$_6$ in the cyst wall**

Sequential extracts from cysts were analysed for the presence of IP$_6$ and the *E. granulosus* intracellular proteins P-29 and CyPA. Intact murine (upper panel) and 'daughter' bovine (lower, left panel) cysts (11 and 9 cysts respectively) were successively washed with PBS (no EDTA) and PBS/EDTA (EDTA). An extra PBS wash was then performed on the murine cysts. All of these extracts are grouped under 'whole cysts from outside'. After cyst fluid removal, HCW were opened up (opened-up cysts) and extracted with PBS/EDTA/Tween (Tween-EDTA). In a parallel experiment, murine cysts (6 cysts) were extracted with EDTA 'from the inside' (whole cysts from inside) by injecting the chelating agent, so as to attain an intracystic concentration equal to that used for washing the intact cysts 'from the outside'. After incubation, the cyst fluid was aspirated with a syringe and analysed. A 'mother' bovine HCW (lower, right panel) was subjected to mechanical separation of its constituent layers using forceps. After washing with PBS, the germinal and laminated layers were extracted with PBS/EDTA/Tween (Tween-EDTA) or PBS/EDTA (EDTA) respectively. (**A**) Extracts were analysed for the presence of IP$_6$ by TLC on PEI-cellulose plates. (**B**) P-29 and (**C**) CyPA were detected in the extracts by Western blotting. In the case of CyPA detection, *M. corti* CyPA, known to migrate similarly to the *E. granulosus* protein, was included as a positive control. When sequential extracts from the same starting material were analysed, an identical fraction of the total of each extract was loaded in each lane. In this way, band intensities for each type of molecule analysed reflect the relative total amounts of the component solubilized at each step.

**Table 2      Quantitation of IP$_6$ in the laminated layer of hydatid cyst walls from different origins**

IP$_6$ was quantitated in crude extracts as described in Table 1. IP$_6$ present in the laminated layer was considered to be that solubilized by washing intact murine and 'daughter' bovine cysts externally. In the case of 'mother' bovine cyst walls, it was the IP$_6$ extracted from the laminated layer, which had been mechanically separated from the germinal layer. IP$_6$ concentrations in the laminated layers of murine and daughter bovine cysts were estimated by assuming that cysts were perfectly spherical, using the measured diameters of the cysts, and assuming a laminated layer thickness of 80 $\mu$m, which is the average reported for the size range of cysts analysed [26]. n.d., not determined.

| Cyst origin | IP$_6$ in laminated layer/total IP$_6$ (%) | IP$_6$ in laminated layer (mM) |
|---|---|---|
| Murine | 82 | 29 |
| 'Daughter' bovine | 99 | 36 |
| 'Mother' bovine | 85 | n.d. |

Working with intact cysts is not feasible for 'mother' bovine cysts, which cannot be easily separated from host tissue without disruption. Hence, in a slightly different approach from that described above, the germinal and laminated layers of a 'mother' bovine HCW were separated mechanically and extracts were prepared from both materials. When the germinal layer was treated with PBS/EDTA/Tween and the laminated layer washed with PBS/EDTA, IP$_6$ was solubilized from both materials, with the majority being recovered from the acellular structure (Figure 4A and Table 2). In contrast, P-29 and CyPA were detected only in extracts from the germinal layer (Figures 4B and 4C).

Taken together, these observations indicate that IP$_6$ is a component of the laminated layer, i.e. it is found *in vivo* in the extracellular space. By estimating the volume of the laminated layer, taken to be a perfectly spherical shell, values of approx. 30 mM were obtained for the IP$_6$ concentration in this acellular structure (Table 2). Measurement of total phosphorus content in 'daughter' bovine HCW yielded a value of 1.5 mmol/g of dry mass. As this material contained 0.23 mmol of IP$_6$/g of dry mass, IP$_6$ accounted for approx. 92 % of the total phosphorus in the HCW.

## DISCUSSION

In the current study, we report the presence of high amounts of IP$_6$ in the laminated layer of the HCW of the parasitic cestode *E. granulosus*. IP$_6$ was purified from a soluble extract of the HCW and was shown to account for up to 15 % of the total dry mass of this structure. However, the actual amounts of IP$_6$ in the HCW could be higher than those estimated in the present study. Although IP$_6$ behaves in dialysis as if it had a higher molecular mass than predicted from its formula [10,15], it is expected that a minor percentage of the IP$_6$ in the samples is lost during dialysis against water prior to quantitation. In any case, this underestimation is unlikely to have been large, as the value for total phosphorus in the HCW was only slightly higher than that calculated to derive from IP$_6$. A previous study [30] has demonstrated strikingly high amounts of IP$_6$ in animal species: IP$_6$ insolubilized as its Mg$^{2+}$ salt accounted for 50 % of the total dry mass of the dispersal larvae of the mesozoan *Dicyema typus*. Even in that particular case, IP$_6$ is present intracellularly. In contrast, the majority of the IP$_6$ in the HCW of *E. granulosus* occurs in the acellular laminated layer. The secretion of endogenously synthesized IP$_6$ by cells has not been reported to date, causing the various extracellular activities observed for IP$_6$

to be regarded as unlikely to be of physiological importance [5]. Our findings constitute the first observation of $IP_6$ being an intrinsic component of an extracellular structure.

The building blocks of the laminated layer are synthesized and secreted by the cells of the germinal layer. The possibility of $IP_6$ being accumulated in the laminated layer by other means, i.e. as a result of necrosis of germinal layer cells, seems remote. Firstly, there is no evidence of extensive necrosis of germinal layer cells. Secondly, if we assume that the $IP_6$ concentration in the cells of the germinal layer were as high as in *D. discoideum*, i.e. about 1 mM [27], the whole germinal layer would have to release its contents 200 times over for the amount of $IP_6$ found in the laminated layer to be attained. Thus $IP_6$ has to be synthesized by the cells of the germinal layer and subsequently secreted, in a polarized fashion, into the extracellular environment. If the synthesis of $IP_6$ in *E. granulosus* cells takes place in the cytosol, as it is the case in unicellular eukaryotes for which the synthetic pathway of $IP_6$ has been elucidated [31,32], $IP_6$ would have to cross a membrane barrier at some point along its secretion pathway. This would imply the presence of a membrane translocator for $IP_6$. The existence of such a transporter in the membranes of the ER of mammalian cells has been suggested to explain the observation that multiple inositol polyphosphate phosphatase, a phosphatase present in the lumen of the ER, affects the cytosolic levels of $IP_6$ [16]. As the *E. granulosus* germinal layer must have a very active machinery to synthesize and secrete $IP_6$, this constitutes an interesting system for studying the metabolism and possible membrane translocation of this molecule.

$IP_6$ in the laminated layer is immobilized by the interaction with $Ca^{2+}$, as it was solubilized only in the presence of EDTA, co-solubilizing with $Ca^{2+}$ and not with $Mg^{2+}$. Chemical analyses of the laminated layer have shown that the structure is much richer in $Ca^{2+}$ than in $Mg^{2+}$ [33]. It is likely that $IP_6$ is involved in this selective retention of $Ca^{2+}$. $Ca^{2+}$ deposits in the laminated layer have been ascribed by X-ray microanalysis to the naturally electron-dense aggregates, which are one of the main ultra-structural features of the structure [34]. The aggregates are found irregularly dispersed within the microfibrillar matrix, the major ultrastructural element of the laminated layer. This organization has been observed consistently in *E. granulosus* HCWs from various origins [26]. The microfibrillar matrix is a common feature of the laminated layers of different *Echinococcus* species [35,36]. In contrast, the naturally electron-dense aggregates have only been described in *E. granulosus* [37]. The structures of these aggregates have been described in detail: they are composed of granules that are homogeneous in size ($41 \pm 1$ nm) and possess 8 nm electron-lucent spheres within. The granules are also present within vesicles in cells of the germinal layer, which appear to arise from the Golgi apparatus and to be involved in exocytosis towards the laminated layer. It seems likely that these $Ca^{2+}$-containing granules contain the $IP_6$ present in the HCW. We are currently testing this hypothesis.

The biological function of $IP_6$ in the *E. granulosus* laminated layer remains to be answered. Although a strictly structural role is plausible, the large extracellular amounts of the molecule found specifically at the interface with the host suggest a function related to the interaction with host molecules. $IP_6$ would provide the laminated layer, which is a structure composed mainly of neutral sugars [38], with charge. It is well established that certain extracellular cascades of the mammalian host, namely the alternative and classical pathways of complement and the intrinsic pathway of coagulation, are sensitive to distributions of charge present or absent on surfaces [39,40]. There have been several reports on the selective adsorption of host proteins in the

*E. granulosus* HCW. These proteins, namely factor H [41], annexin II [25], cathepsin K [42] and the extracellular superoxide dismutase (G. Salinas, unpublished work), share the capacity to bind to negatively charged species, often glycosaminoglycans (GAGs). GAGs could not be detected in the HCW by histo-chemical techniques (A. Díaz and R. B. Sim, unpublished work). The possibility that $IP_6$ is responsible for the retention of GAG binding host proteins in the HCW deserves further study. Finally, the fact that $IP_6$ does not appear to be a major component of the cyst wall of *E. multilocularis* needs to be taken into account when considering the function of the compound in *E. granulosus*. Although in both species the laminated layer protects the para-site from host immune cells, several other features of the host–parasite relationships established by the two are very different. Whereas the unilocular, non-invasive cyst of *E. gran-ulosus* manages not to trigger inflammation in most host species [43], *E. multilocularis* displays a tumour-like growth, with external cyst budding and invasion of the host organ parenchyma, leading to necrosis, chronic granulomatous inflammation and extensive fibrosis [44].

## REFERENCES

1 Szwergold, B. S., Graham, R. A. and Brown, T. R. (1987) Observation of inositol pentakis- and hexakis-phosphates in mammalian tissues by $^{31}$P NMR. Biochem. Biophys. Res. Commun. **149**, 874–881

2 Pittet, D., Lew, D. P., Mayr, G. W., Monod, A. and Schlegel, W. (1989) Chemoattractant receptor promotion of $Ca^{2+}$ influx across the plasma membrane of HL-60 cells. A role for cytosolic free calcium elevations and inositol 1,3,4,5-tetrakisphosphate production. J. Biol. Chem. **264**, 7251–7261

3 Bunce, C. M., French, P. J., Allen, P., Mountford, J. C., Moor, B., Greaves, M. F., Michell, R. H. and Brown, G. (1993) Comparison of the levels of inositol metabolites in transformed haemopoietic cells and their normal counterparts. Biochem. J. **289**, 667–673

4 Guse, A. H., Greiner, E., Emmrich, F. and Brand, K. (1993) Mass changes of inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate during cell cycle progression in rat thymocytes. J. Biol. Chem. **268**, 7129–7133

5 Shears, S. B. (2001) Assessing the omnipotence of inositol hexakisphosphate. Cell. Signalling **13**, 151–158

6 Irvine, R. F. and Schell, M. J. (2001) Back in the water: the return of the inositol phosphates. Nat. Rev. Mol. Cell. Biol. **2**, 327–338

7 Voglmaier, S. M., Keen, J. H., Murphy, J. E., Ferris, C. D., Prestwich, G. D., Snyder, S. H. and Theibert, A. B. (1992) Inositol hexakisphosphate receptor identified as the clathrin assembly protein AP-2. Biochem. Biophys. Res. Commun. **187**, 158–163

8 Norris, F. A., Ungewickell, E. and Majerus, P. W. (1995) Inositol hexakisphosphate binds to clathrin assembly protein 3 (AP-3/AP180) and inhibits clathrin cage assembly *in vitro*. J. Biol. Chem. **270**, 214–217

9 Mehrotra, B., Myszka, D. G. and Prestwich, G. D. (2000) Binding kinetics and ligand specificity for the interactions of the C2B domain of synaptogmin II with inositol polyphosphates and phosphoinositides. Biochemistry **39**, 9679–9686

10 Hanakahi, L. A., Bartlet-Jones, M., Chappell, C., Pappin, D. and West, S. C. (2000) Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. Cell (Cambridge, Mass.) **102**, 721–729

11 Ye, W., Ali, N., Bembenek, M. E., Shears, S. B. and Lafer, E. M. (1995) Inhibition of clathrin assembly by high affinity binding of specific inositol polyphosphates to the synapse-specific clathrin assembly protein AP-3. J. Biol. Chem. **270**, 1564–1568

12 York, J. D., Odom, A. R., Murphy, R., Ives, E. B. and Wente, S. R. (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science (Washington, D.C.) **285**, 96–100

13  Stuart, J. A., Anderson, K. L., French, P. J., Kirk, C. J. and Michell, R. H. (1994) The intracellular distribution of inositol polyphosphates in HL60 promyeloid cells. Biochem. J. **303**, 517–525

14  Ali, N., Craxton, A. and Shears, S. B. (1993) Hepatic Ins(1,3,4,5)P$_4$ 3-phosphatase is compartmentalized inside endoplasmic reticulum. J. Biol. Chem. **268**, 6161–6167

15  Van der Kaay, J. and Van Haastert, P. J. (1995) Desalting inositolpolyphosphates by dialysis. Anal. Biochem. **225**, 183–185

16  Chi, H., Yang, X., Kingsley, P. D., O'Keefe, R. J., Puzas, J. E., Rosier, R. N., Shears, S. B. and Reynolds, P. R. (2000) Targeted deletion of Minpp1 provides new insight into the activity of multiple inositol polyphosphate phosphatase *in vivo*. Mol. Cell. Biol. **20**, 6496–6507

17  Díaz, A., Irigoín, F., Ferreira, F. and Sim, R. B. (1999) Control of host complement activation by the *Echinococcus granulosus* hydatid cyst. Immunopharmacology **42**, 91–98

18  Araj, G. F., Matossian, R. M. and Frayha, G. J. (1977) The host response in secondary hydatidosis of mice. I. Circulating antibodies. Z. Parasitenkd. **52**, 23–30

19  Smyth, J. D. and Howkins, A. B. (1966) An *in vitro* technique for the production of eggs of *Echinococcus granulosus* by maturation of partly developed strobila. Parasitology **56**, 763–766

20  Jura, H., Bader, A., Hartmann, M., Maschek, H. and Frosch, M. (1996) Hepatic tissue culture model for study of host–parasite interactions in alveolar echinococcosis. Infect. Immun. **64**, 3484–3490

21  Jansson, P. E., Kenne, L., Liedgren, H., Lindberg, B. and Lunngren, J. (1976) A practical guide to the methylation analysis of carbohydrates. Chem. Commun. (Univ. Stockholm) **8**, 1–74

22  Spencer, C. L., Stephens, L. R. and Irvine, R. F. (1990) Separation of higher inositol phosphates by polyethyleneimine-cellulose thin layer chromatography and by Dowex chloride column chromatography. In Methods in Inositide Research (Irvine, R. F., ed.), pp. 39–43, Raven Press, New York

23  González, G., Spinelli, P., Lorenzo, C., Hellman, U., Nieto, A., Willis, A. and Salinas, G. (2000) Molecular characterization of P-29, a metacestode-specific component of *Echinococcus granulosus* which is immunologically related to, but distinct from, antigen 5. Mol. Biochem. Parasitol. **105**, 177–184

24  Hancock, I. and Poxton, I. (1988) Estimation of organic phosphorus. In Bacterial Cell Surface Techniques (Hancock, I. and Poxton, I., eds.), pp. 274–275, John Wiley and Sons, Chichester

25  Díaz, A., Ibarguren, S., Breijo, M., Willis, A. C. and Sim, R. B. (2000) Host-derived annexin II at the host-parasite interface of the *Echinococcus granulosus* hydatid cyst. Mol. Biochem. Parasitol. **110**, 171–176

26  Bortoletti, G. and Ferretti, G. (1978) Ultrastructural aspects of fertile and sterile cysts of *Echinococcus granulosus* developed in hosts of different species. Int. J. Parasitol. **8**, 421–431

27  Martin, J. B., Foray, M. F., Klein, G. and Satre, M. (1987) Identification of inositol hexaphosphate in $^{31}$P-NMR spectra of *Dictyostelium discoideum* amoebae. Relevance to intracellular pH determination. Biochim. Biophys. Acta **931**, 16–25

28  Lightowlers, M. W., Haralambous, A. and Rickard, M. D. (1989) Amino acid sequence homology between cyclophilin and a cDNA-cloned antigen of *Echinococcus granulosus*. Mol. Biochem. Parasitol. **36**, 287–289

29  Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J. and Speicher, D. W. (1984) Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science (Washington, D.C.) **226**, 544–547

30  Lapan, E. A. (1975) Magnesium inositol hexaphosphate deposits in mesozoan dispersal larvae. Exp. Cell Res. **94**, 277–282

31  Stephens, L. R. and Irvine, R. F. (1990) Stepwise phosphorylation of *myo*-inositol leading to *myo*-inositol hexakisphosphate in *Dictyostelium*. Nature (London) **346**, 580–583

32  Ongusaha, P. P., Hughes, P. J., Davey, J. and Michell, R. H. (1998) Inositol hexakisphosphate in *Schizosaccharomyces pombe*: synthesis from Ins(1,4,5)$P_3$ and osmotic regulation. Biochem. J. **335**, 671–679

33  Sadanand, A. V. (1971) Biochemical analyses of the cyst wall of *Echinococcus granulosus* Batsch. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. **40**, 797–805

34  Rogan, M. T. and Richards, K. S. (1989) Development of the tegument of *Echinococcus granulosus* (Cestoda) protoscoleces during cystic differentiation *in vivo*. Parasitol. Res. **75**, 299–306

35  Ingold, K., Gottstein, B. and Hemphill, A. (2000) High molecular mass glycans are major structural elements associated with the laminated layer of *in vitro* cultivated *Echinococcus multilocularis* metacestodes. Int. J. Parasitol. **30**, 207–214

36  Ingold, K., Dai, W., Rausch, R. L., Gottstein, B. and Hemphill, A. (2001) Characterization of the laminated layer of *in vitro* cultivated *Echinococcus vogeli* metacestodes. J. Parasitol. **87**, 55–64

37  Richards, K. S., Arme, C. and Bridges, J. F. (1983) *Echinococcus granulosus equinus*: an ultrastructural study of the laminated layer, including changes on incubating cysts in various media. Parasitology **86**, 399–405

38  Kilejian, A., Sauer, K. and Schwabe, C. (1962) Host-parasite relationship in Echinococcosis. VIII. Infrared spectra and chemical composition of the hydatid cyst. Exp. Parasitol. **12**, 377–392

39  Sim, R. B. and Malhotra, R. (1994) Interactions of carbohydrates and lectins with complement. Biochem. Soc. Trans. **22**, 106–111

40  Conrad, E. (1998) Heparin-binding proteins in hemostasis. In Heparin-Binding Proteins pp. 239–300, Academic Press, New York

41  Díaz, A., Ferreira, A. and Sim, R. B. (1997) Complement evasion *by Echinococcus granulosus*: sequestration of host factor H in the hydatid cyst wall. J. Immunol. **158**, 3779–3786

42  Díaz, A., Willis, A. C. and Sim, R. B. (2000) Expression of the proteinase specialized in bone resorption, cathepsin K, in granulomatous inflammation. Mol. Med. (N.Y.) **6**, 648–659

43  Ferreira, A. M., Irigoín, F., Breijo, M., Sim, R. B. and Díaz, A. (2000) How *Echinococcus granulosus* deals with complement. Parasitol. Today **16**, 168–172

44  Ammann, R. and Eckert, J. (1995) Clinical diagnosis and treatment of Echinococcosis in humans. In *Echinococcus* and Hydatid Disease (Thompson, R. C. A. and Lymbery, A. J., eds.), pp. 411–463, CAB International, Wallingford