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HOST PARASITE RELATIONSHIP IN HYDATIDOSIS : COMPARATIVE ANALYSIS OF HYDATID CYST FLUID AND SHEEP SERUM

by

DIRK JANSSEN (1), MARICRUZ RUEDA RUBIO (2), PAUL H. DE RYCKE (1) and ANTONIO OSUNA (3)

(1) Universiteit Gent, Laboratorium voor Zoöfysiologie, K. L. Ledeganckstraat 35, B-9000 Gent (Belgium)
(2) Hospital Comarcal La Inmaculada, Avda Guillermo Reina, 04600 Huercal Overa (Spain)
(3) Universidad de Granada, Laboratorio de Parasitologia Molecular, c/ Severo Ochoa s/n, 18001 Granada (Spain)

SUMMARY

Various biochemical and physiological characteristics of fresh sheep serum were estimated and compared with those of pooled hydatid cyst fluid (HCF), isolated from *Echinococcus granulosus* cysts of ovine origin. Concentrations of the following compounds were determined : total protein, total albumin, total lipids, triglycerides, cholesterol, high density, low density and very low density lipoproteins, glucose, bilirubin, creatinine, uric acid, urea, calcium, inorganic phosphate, iron, sodium and potassium. Total activities of the following enzymes were measured : glutaminate-oxaloacetate transaminase, glutaminate-pyruvate transaminase, L-gamma-glutamyl transferase, alkaline phosphatase and α-amylase. Osmolarity and pH were also determined and total proteins were subjected to acetate cellulose electrophoresis. It was shown that compounds that are related to the carbohydrate and lipid metabolism, as well as nitrogenous catabolism, were predominantly present in HCF when compared with sheep serum. In contrast, the HCF contained few proteins and showed a low activity of enzymes related to protein metabolism. HCF, although osmotically comparable to serum of a putative host, is characterized by high levels of sodium and potassium. Microscopically, ammonium urate, amorphous phosphate and calcium carbonate crystals are found. The results are discussed in view of cestode physiology and metabolical (in-)dependence of hydatid cysts.

Key words : Echinococcus granulosus, hydatid cyst fluid, analysis, proteins, enzymes, lipids, carbohydrates, bilirubin, creatinine, uric acid, urea, calcium, phosphate, iron, sodium, potassium.

INTRODUCTION

Cystic echinococcosis/hydatidosis is part of a zoonosis, caused by the tapeworm *Echinococcus granulosus* (BATSCH, 1786). The adult parasite lives in the intestine of dogs and other canids. Shed proglottids and eggs infect intermediate hosts like sheep, cattle, horses, grazing on pasture contaminated with faecal material of infected dogs. Man, who is a dead-end host, is infected through close contact with infected dogs. The ingested eggs hatch and the oncospheres pass through the intestinal mucosa ; they are transported to the liver (or other organs) where they develop into hydatid cysts. Some of these cause severe complications such as intrabiliary and intraperitoneal ruptures, whereby liberated protoscoleces will form new cysts. Also, due to the presence of foreign substances in the freed hydatid cyst fluid, severe anaphylactic shocks may occur. The estimated global incidence of hydatidosis in man is more than 100,000 per year. In endemic areas, the surgical rate is more than 10/million population/year. Notwithstanding current experimental trials of chemotherapy (ECKERT, 1986), surgical treatment still remains the inevitable way of intervention. In the course of this type of treatment many technical difficulties often occur, sometimes demanding drastic procedures such as liver transplantation (LANDA GARCIA, 1989). Furthermore, many treated patients suffer from recurrent infections of hydatidosis, so that there is still a need for efficient chemotherapy and vaccines. It is believed that comparative biochemical studies on *E. granulosus* and on host tissues may eventually result in the development of adequate chemotherapeutic measures (VESSAL *et al.*, 1972). In the present study, we look into some biochemical features of hydatid cyst fluid (HCF), isolated from infected sheep. Values are compared with those found in the serum of sheep. Results are discussed in view of the physiological relationship between hydatid cysts and their hosts.

MATERIAL AND METHODS

A pooled sample (900 ml) of HCF, isolated from sheep was obtained through Leti Laboratorios, S.A. (Barcelona, Spain). The fluid was centrifuged at 4,000 rpm for 10 min and the supernatant was used for further analyses. The pellet was microscopically examined for organic and inorganic crystals as well as for the presence of protoscoleces to assess fertility of the hydatid cysts. Sheep blood was obtained from healthy sheep, stored at 37° C for 1 h, and left overnight at 4° C. Subsequently the serum was collected after centrifuging the blood at 900 rpm for 10 min. HCF and sheep serum were analyzed on an Hitachi System 704 unless indicated otherwise.

Protein analysis

1) The Biuret method (WEICHSELBAUM, 1946) was applied to determine the total protein content, using the Boehringer Mannheim HiCo Total Protein test. Additionally, an ATOM cellogel electrophoresis system was used to separate the

proteins on cellulose-acetate strips. For sheep serum, 5 µl serum was applied. HCF was previously incubated with 5 % TCA (trichloroacetic acid) overnight at 4° C and then centrifuged at 4000 rpm/10 min. The pellet was resuspended in 100 µl bidistilled water, and 5 µl of this was applied for electrophoresis. The strips carrying the separated proteins were stained with amido black (10 min for serum, 30 min for HCF). Proteinograms were scanned with an ATOM DIGISCAN (atom-430/429) and the amount of protein in the different fractions was calculated with respect to the amount of total protein that was measured with the Biuret method.

2) The total amount of albumin was analyzed according to DOUMAS *et al.*, (1971). Source of reagents : Boehringer Mannheim Albumin test.

3) Glutaminate-oxaloacetate transaminase (GOT) and glutaminate-pyruvate transaminase (GPT) were analyzed, using a Boehringer Mannheim Twin GOT/GPT test [GOT : BERGMEYER *et al.* (1986a) ; GPT : BERGMEYER *et al.* (1986b)].

4) L-gamma-Glutamyl transferase (EC 2.3.2.2) was analyzed, using a Boehringer Mannheim HiCo gamma-GT New test, following the method of PERSIJN and VAN DER SILK (1976).

5) Alkaline phosphatase (EC 3.1.3.1) was analyzed according to the DEUTSCHEN GESELLSCHAFT FÜR KLINISCHE CHEMIE (1972). Source of reagents : Boehringer Mannheim HiCo Alcalic Phosphatase Opt. test.

6) α-Amylase was measured colorimetrically using the Boehringer Mannheim α-Amylase PNP test. The method was according to RAUSCHER *et al.* (1985).

Lipid analysis

1) The total lipid content was determined with a Merckotest Total Lipids following ZÖLLNER and KIRSCH (1962).

2) Triglycerides : analysis was done using a Boehringer Mannheim Twin TG/CHO test according to the method of KERSCHER *et al.*, (1985).

3) Cholesterol : analysis was done according to KERSCHER *et al.* (1985) using a Boehringer Mannheim Twin TG/CHO test.

4) Lipoproteins : VLDL (Very Low Density Lipoproteins), LDL (Low Density Lipoproteins) and HDL (High Density Lipoproteins) were calculated and measured as follows : VLDL in serum were estimated as TG/5 ; since HCF cannot be expected to be entirely comparable to vertebrate serum, care should be taken for the interpretation of VLDL values for HCF. HDL was measured manually using a Boehringer Mannheim HDL-Cholesterol test. The LDL-cholesterol was calculated as : LDL-Cho = Total Cho - [HDL-Cho + VLDL-Cho] (FRIEDEWALD *et al.*, 1972). Since calculation of LDL-cholesterol involves VLDL-cholesterol, equal care is required in the interpretation of the values found for HCF.

Glucose analysis

Total monomeric glucose was determined according to ZIEGENHORN *et al.* (1977), using a Boehringer Mannheim Twin BUN/GLU test.

Analysis of nitrogen-containing metabolites

1) Total bilirubin was measured following the DPD-method (with 2,5-dichlorphenyldiazoniumsalt) of WAHLEFELD *et al.*, (1972). Source of reagents : Boehringer Mannheim HiCo Bilirubin test.

2) Creatinine was measured using a modification of the Jaffé method, according to BARTELS *et al.* (1972). Reagents were from a Boehringer Mannheim Creatinine test.

3) Uric acid was measured according to TOWN *et al.* (1985). Source of reagents : Boehringer Mannheim HiCo Uric Acid PAP test.

4) Urea was enzymatically determined according to PRENCIPE *et al.* (1983). Source of reagents : Boehringer Mannheim Twin BUN/GLU test.

Physiological analysis

1) Total calcium was measured automatically as well as by flame photometry (Eppendorf). In the automatic analysis we used the method of RAY SARKAR and CHAUHAN (1967). Source of reagents : Boehringer Mannheim Calcium test.

2) Inorganic phosphate was measured according to HENRY (1974). Source of reagents : Boehringer Mannheim Inorganic Phosphate test.

3) Iron was measured using the method of SIEDEL *et al.* (1984). Source of reagents : Boehringer Mannheim Iron test.

4) Sodium in HCF was measured on by flame spectrometry (Eppendorf) ; in sheep serum it was done with the CORNING 614 Na^+/K^+ ANALYSER. Preliminary measurements for HCF on the CORNING 614 Na^+/K^+ ANALYSER showed values that impeded appropriate quantification using this technique for HCF, so that this was confined to sheep serum only.

5) Potassium in HCF was measured by flame spectrometry (Eppendorf) ; in sheep serum it was done with a CORNING 614 Na^+/K^+ ANALYSER. Analyzing HCF potassium using the latter technique showed the same problems as for sodium and likewise two different techniques were applied for hydatid cyst fluid and sheep serum.

6) Osmolarity was measured using the freezing point method. For reference a standard dilution curve with NaCl was used.

7) pH was determined with a Beckman M3500.

8) Crystals were examined under a microscope and determined, taking into account the pH of the fluid and the solubility of the crystals.

RESULTS

The results of most assays are shown in Table 1. Microscopical examination of the sediments of HCF revealed the presence of ammonium urate, amorphous phosphate and calcium carbonate crystals. The 900 ml of fluid yielded 1.5 ml of packed protoscoleces. The cellulose acetate gel electrophoreses of serum and HCF proteins are shown in Figs. 1. and 2. ; albumin predominates in the proteinogram of sheep serum, in which also many bands can be distinguished that probably repre-

TABLE 1

Summary of analyses of E. granulosus *hydatid cyst fluid (HCF) and sheep serum.*
ND = not done.

TEST	HCF	SHEEP SERUM	UNITS
TOTAL PROTEIN CONTENT	0.06	7.74	g/dl
ALBUMIN	0.03	3.18	g/dl
GOT/ASAT	6	79	U/l
GPT/ALAT	0	17	U/l
g-GT	2	34	U/l
ALK. PHOSPHATASE	30	253	U/l
α-AMYLASE	21	48	U/l
TOTAL LIPIDS	135	370	mg/dl
TRIGLYCERIDES	60	19	mg/dl
TOTAL CHOLESTEROL	3	73	mg/dl
VLDL	[12]	3.8	mg/dl
LDL	[0.5]	26.2	mg/dl
HDL	11	43	mg/dl
GLUCOSE	105	57	mg/dl
TOTAL BILIRUBIN	0.01	0.08	mg/dl
CREATININE	0.63	0.96	mg/dl
URIC ACID	0.41	0.11	mg/dl
UREA	41	36	mg/dl
CALCIUM (HS 704)	12.52	9.60	mg/dl
CALCIUM (flame photometry)	14	ND	mg/dl
INORGANIC PHOSPHATE	1.70	4.65	mg/dl
IRON	27	104	µg/dl
SODIUM (CORNING 614)	ND	111	mg/dl
SODIUM (flame photometry)	340	ND	mg/dl
POTASSIUM (CORNING 614)	ND	18.8	mg/dl
POTASSIUM (flame photometry)	67.2	ND	mg/dl
Osmolarity	258	ND	mOsmol/l
pH	8.12	ND	6

sent the alpha 1,2, beta -and gamma globulin fraction as they can be found in human serum. The mean ratio albumin/globulin based on 3 gel electrophoresis scannings was found to be 1.47. The sheep proteinogram shows striking differences with respect to that of HCF proteins : albumin and a band that probably represents gamma-globulins predominate in the latter, giving a mean albumin/globulin ratio of 0.59.

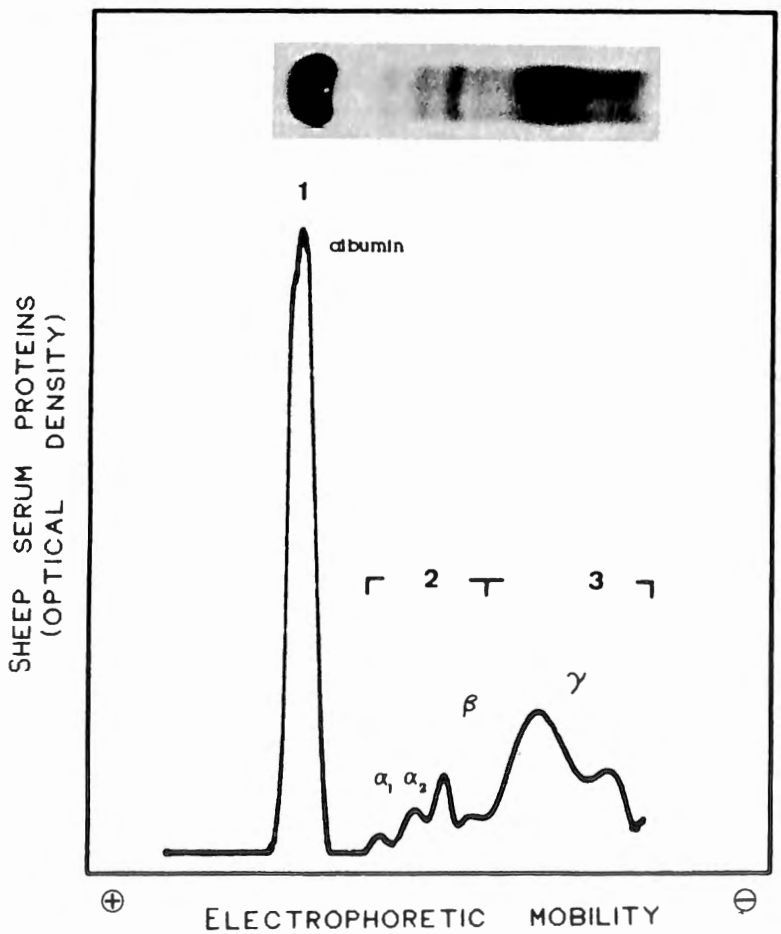

Fig. 1. — Cellulose acetate electrophoresis of sheep serum proteins. *Top :* stained gel ; *Bottom :* scanned gel. The amount of proteins per stained band (obtained after digitized scanning and considering the mean value of 3 gels) is calculated, based on a total protein content of 7.74 g/dl according to the Biuret method : b1 (albumine) : 4.60 g/dl ; b2.(alpha/beta globulines) : 0.50 g/dl ; b3.(gamma globulines) : 3.33 g/dl ; ratio albumin/globulin : 1.47.

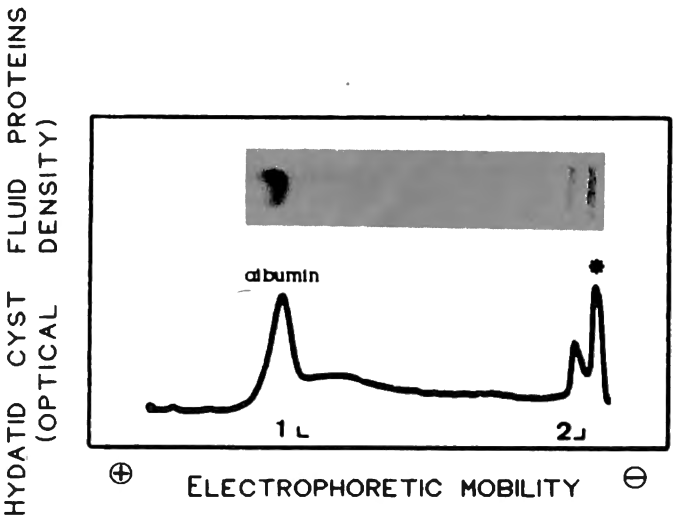

Fig. 2. — Cellulose acetate electrophoresis of HCF proteins. *Top :* stained gel ; *Bottom :* scanned gel. The amount of proteins per stained band (obtained after digitized scanning and considering the mean value of 3 gels) is calculated, based on a total protein content of 0.06 g/dl according to the Biuret method : b1.(alb) : 0.022 g/dl ; b2.(total globulines with a peak in the gamma globulin region) : 0.038 g/dl ; * = application situs of the sample ; ratio albumin/globulin : 0.59.

DISCUSSION

Protein metabolism

The HCF sample that we studied was found to contain 0.06 % (w/v) protein : this is about 130 times less than in sheep serum. Determinations of albumin and globulin indicated that these protein classes account for most of the total protein. Based on acetate cellulose electrophoresis, we found an albumin/globulin ratio of 0.59 in HCF and 1.47 in sheep serum. The striking differences in the distribution of the protein classes (see Figs. 1 and 2.), showing hardly any alpha or beta globulins, could be due to a selective accumulation mechanism in the cyst involving a higher rate of transport of the host immunoglobulins to the cyst tissue and fluid (cf. KASSIS and TANNER, 1977). How these proteins enter the cysts is not known but it may be by diffusion, through fissures in the cyst membranes (PAPPAS, 1978), or by endocytosis, which has been shown in the bladder of *Taenia crassiceps* (THREADGOLD and DUNN, 1983). In this respect, it is suggested that the ionic nature of proteins may also play an important role in their absorption (HUSTEAD and WILLIAMS, 1977). However, in view of the different distribution of predominant protein classes, and of the low amount of total protein found in HCF, it could be argued that the acquisition of host derived proteins is very low suggesting a signifi-

cant degree of biochemical independence on the part of the hydatid cyst residing inside an intermediate host. As an example of amino acid metabolism, we tested HCF and sheep serum for GOT, GPT and gamma-GT. gamma-GT is a cytosolic enzyme that acts within the so-called Meister cycle (the gamma-glutamyl cycle) that transports amino acids and several small peptides across the cell membrane. GOT and GPT are enzymes in the synthesis of amino acids (respectively L-aspartate and L-alanine). As such, GOT represents an important mechanism of transferring amino acids into useful compounds that can enter the Krebs cycle. Transaminations in general have been demonstrated in various cestodes (cf. SMYTH and MCMANUS, 1989). It is believed that, when compared to vertebrates, cestodes may have an extremely limited capacity for performing transaminations. This may reflect the fact that many cestodes live in an environment rich in amino acids, in which case synthesis may play a minor role in satisfying essential amino acid requirements. However, taking into account the very low amount of total proteins found in HCF, the parasitic fluid exhibited a considerable GOT activity (6 U/l). Sheep serum GOT on the other hand was much higher (79 U/l). No GPT activity was found in the HCF (17 U/l in sheep serum) and gamma-GT in HCF was 2 U/l (34 U/l in sheep serum). It should be noted that based on the ratio of enzymatic activity [sheep serum/HCF] (13.2 for GOT and 17 for gamma-GT) it cannot be decided whether these enzymes are host derived or represent metabolic activity of the parasite. The answer to this question could be positive as far as alkaline phosphatase activity in HCF is concerned : acid and alkaline phosphatases have already been demonstrated on the surface of protoscoleces of *E. granulosus* (MCMANUS and BARRETT, 1985). This finding supports previous evidence from in vitro studies that the scolex of *E. granulosus* can digest proteins at the host/parasite interface by membrane (contact) digestion (SMYTH, 1972).

Carbohydrates metabolism

α-Amylase is a hydrolytic enzyme that hydrolyses polymers of glucose containing α(1 → 4) glycosidic bonds (e.g. starch and glycogen). Several species of cestodes (incl. *Hymenolepis diminuta*) have been shown to absorb α-amylase, and adsorption appears to lead to an increase in amylolytic activity (BARRETT, 1981). In the case of adult cestodes this is a possible manifestation of the phenomenon of membrane (= contact) digestion. Our demonstration of α-amylase activity in the HCF could seem dubious : this particular enzymatic activity has hitherto never been detected in *E. granulosus* HCF. However due to the presence of glycogen in HCF (0.03 mg/ml) and in protoscoleces (218.76 mg/g lyophilized protoscoleces) (FRAYHA and HADDAD, 1980) this enzyme could provide an important mechanism for the parasite's carbohydrate metabolism. Earlier attempts to demonstrate α-amylase in larval *E. granulosus* have been unsuccessful (FRAYHA and HADDAD, 1980). To our knowledge this is the first demonstration of α-amylase activity in HCF. In conclusion : our results show that considerable quantities of sheep serum glucose could be taken up by hydatid cysts for consumption ; taking into account the earlier reports on the presence of endogenous glycogen, together with our demonstration

of α-amylase activity in HCF, our results also confirm the importance of carbohydrate metabolism in the biochemistry of *E. granulosus* hydatid cysts.

Nitrogenous compounds

Production of urea by cestodes in general suggests the existence of the urea cycle. One of the key enzymes, arginase, has been widely reported in cestodes (see SMYTH and MCMANUS, 1989). However, some of the other enzymes, notably carbamoyl phosphate synthetase and ornithine transcarbamoylase, are either absent, or present in only low quantities (BARRETT, 1981), and it is doubtful if a complete cycle operates in cestodes. It is likely that the urea excreted by tapeworms comes from the activity of arginase alone. We found the amount of urea in HCF to be 41 mg/dl. Since we determined the concentration of urea present in sheep serum to be 36 mg/dl it can be argued that the total urea present in the HCF is not entirely derived from host serum by diffusion or any other mechanism, but may be mainly of parasitic origin. In this respect it can be understood that HCF contains truly excretory products of protoscoleces, and the proliferative, germinal layer of the hydatid cyst which maintains a low urea concentration in the parasitic « tissue » could be a prophylactic measure to avoid deleterious effects on some vital processes like, for example, the inhibition of the phosphoenolpyruvate-pyruvate interconversion in the glycolytic scheme, (the blocking of this pathway by urea has been reported in certain ectoparasites (FRAYHA *et al.*, 1972). In our study we found 0.41 mg/dl uric acid in HCF, as compared to 0.11 mg/dl in sheep serum. These data confirm what has been said above in the case of urea, namely that HCF urea and uric acid represent proper parasitic catabolism. In addition, we refer to the abundant occurrence of ammonium urate crystals, present in the HCF. Measurements of bilirubin display a different pattern : the HCF contained 0.01 mg/dl and sheep serum 0.08 mg/dl. Since no data are available concerning the production or use of haemoglobin in cestodes, the present results suggest that the bilirubin found in HCF might indeed be host derived. In this respect bilirubin could represent a molecule of reference, due to its size, charge and relative quantity as compared to the host serum, to indicate which molecules present in the HCF could be parasitic and which derived from the host. In contrast, creatinine shows a ratio sheep serum/HCF of 1.52. With regard to the values found in HCF (0.63 mg/dl), creatinine has long been considered one of the end products of metabolism in *E. granulosus* (CODOUNIS and POLYDORIDES, 1936) despite the fact that FRAYHA and HADDAD (1980) found no creatinine in the protoscoleces. As is the case with other nitrogenous metabolites (such as urea), the pathways whereby these components are produced are almost unknown.

Lipid metabolism

Unfortunately, information on lipid-, phospholipid- and glycolipid metabolism in hydatid cysts is still scarce. Studies in this respect have been confined mainly to quantitative and qualitative examination of the lipid content and its distribution in

the larval stage of *E. granulosus*. Some data also exist on the total lipid content of *E. granulosus* adults and *E. multilocularis* protoscoleces. Recently, much of the available information has been comprehensively reviewed by FRAYHA and SMYTH (1983). Since it is generally accepted that lipid metabolism of cestodes is limited, it is likewise believed that these parasites largely depend on the acquisition of host lipids (*e.g.* for cholesterol see BAHR *et al.*, 1979). Our results, on the other hand, suggest that the lipid content of HCF is fundamentally different from that of sheep serum. The ratio [triglycerides/cholesterol] in the former is 20 whereas in sheep serum it was found to be 0.26. Furthermore, the measured ratio [total lipids/total proteins] is 2.25 in HCF and 0.05 in sheep serum. This suggests again the importance of lipid metabolism in the case of hydatid cysts and is evidence of their biochemical independence within the host. In addition it should be remembered that for instance the insect hormone ecdysone has been demonstrated in HCF (MERCER *et al.*, 1987). In the course of the present study we also compared lipids of different densities. Comparison of the ratio [high-density lipoproteins (HDL) : low-density lipoproteins (LDL)] between parasitic and host fluid reveals that HCF has considerably more HDL-bound lipids (22 as compared to 1.6 in sheep serum). This suggest that a relevant large portion of lipids in HCF is bound to proteins (about 50 % of vertebrate HDL weight consists of proteins).

Physiological parameters

Iron is required for the synthesis of the heme portion of hemoglobin and myoglobin. There are no data on the occurrence of hemoglobulin or myoglobulin in larval *E. granulosus*. However, the need for iron in cytochromes in the respiratory chain of the parasite suggests that a mechanism of iron absorption exists. In view of the relatively high amount of iron in HCF, this absorption (*e.g.* in the form of transferrin) could be a specific process. Sodium, potassium and chloride represent major ions of body fluids. Both sodium and potassium prove to be 3 to 3.5 times as concentrated in HCF, when compared with sheep serum. This indicates that these ions play an essential role in the physiology of larval *E. granulosus*. Calcium was found as $CaCO_3$ crystals in HCF. Soluble calcium was measured and showed to be higher in HCF than in sheep serum (ratio 1.3 to 1.4 depending on the method of assay). In view of the lower amount of inorganic phosphate in HCF as compared to sheep serum (ratio 0.36) it could be concluded that hydroxylapatite does not represent a major form of calcium storage in hydatid cysts. However, both ions are found as amorphous phosphate and calcium carbonate crystals in the HCF.

The finding of high levels of sodium and potassium in HCF raises the question of the osmotic and ionic relationships in hydatid cysts. We found the osmolarity of the HCF to be 258 mOsmol/l. Normal human serum has osmotic values of 285 to 295 mOsmol/l. Studies of water and electrolyte balance in protoscoleces of *E. granulosus* from sheep have been carried out *in vitro* by REISIN and ROTUNNO (1981) and REISIN *et al.*, (1981). Their data imply the existence of an active transport mechanism for Na^+ and K^+ and they suggest that the energy required to

maintain the Na-K balance within protoscoleces is largely provided by anaerobic pathways, with oxidative metabolism being only accessory to the energy balance.

CONCLUSION

A comparative biochemical examination of HCF and serum of a putative host suggests the following conclusion. In view of the relatively predominant amount of compounds related to carbohydrate and lipid metabolisms and nitrogenous catabolism, together with the low amount of total protein found in HCF, it is questioned whether much effort should be invested in the study of protein anabolism. Until now, attempts to produce commercially available vaccines based on larval *E. granulosus* material have proved unsuccessful. Recent strategies have been developed using genetic technology. However, we suggest more investigation of the carbohydrate and lipid metabolism of hydatid cysts, since in this respect too many gaps in our knowledge still exist. As mentioned above, ecdysone has been demonstrated in HCF. In addition, we are currently investigating HCF-derived lipid compounds with immunomodulatory activity. This is in line with the trend that treatment methods should be based on investigations of essentially different steps in parasite and host metabolism.

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