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COMPOSITION AND METABOLISM OF PHOSPHOLIPIDS OF *FASCIOLA HEPATICA*, THE COMMON LIVER FLUKE

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Summary

1. The phospholipid composition of *Fasciola hepatica*, the common liver fluke, was compared to that of the liver of the host animals (rats and cattle). Considerable differences were found: monoacyl-*sn*-glycero-3-phosphorylcholine, hardly detectable in the liver, was found in significant amounts in the parasite. On the other hand, sphingomyelin, a normal constituent in the liver, appears to be absent in the liver fluke. *Fasciola hepatica* isolated from rat and cow liver had a strikingly similar phospholipid composition.

2. Qualitative and quantitative differences were also found between the fatty acyl constituents of the phospholipids of the parasite and the liver. The major difference was the presence of eicosaenoic and eicosadienoic acids in the parasite, whereas these acids were not detected in the liver.

3. In vitro incubations of *Fasciola hepatica* in the presence of [^{32}P]phosphate and [2-^{3}H]glycerol resulted in the labelling of all phospholipids of the parasite, except that the ^{3}H label did not incorporate into ethanolamine plasmalogen. This is in agreement with the concept that in animals, glycerol is introduced into plasmalogens via dihydroxyacetonephosphate.

4. Homogenates of liver flukes were found to catalyze the synthesis of phosphatidylcholine from 1,2-diacyl-*sn*-glycerols and CDPcholine.

5. These results strongly suggest that *Fasciola hepatica* is capable of synthesizing at least part of its fatty acids and phospholipids.

Introduction

Fasciola hepatica, the common liver fluke, is a parasite belonging to the class of trematodes. In the adult phase of its life cycle, *F. hepatica* inhabits the liver of mammals, particularly cattle and sheep. It causes considerable damage to the liver parenchyma and bile ducts and, therefore, represents a major problem in veterinary medicine. During the course of an investigation on the inter-

mediary metabolism of *F. hepatica*, we became interested in the capability of this parasite to synthesize its phospholipids. At present, our knowledge of the composition and origin of the phospholipids of parasites in general and those of *F. hepatica* in particular, is very scarce [1,2].

In the present paper we report the phospholipid composition of *F. hepatica* in comparison to that of the infected liver tissues. In addition, the fatty acid patterns of the various phospholipids isolated from *F. hepatica* and from the liver of the host animals were compared. In conjunction with this analytical data, the results obtained with in vitro studies on the incorporation of $^{32}P_i$ and of [2-^{3}H]glycerol into the phospholipids of *F. hepatica* support the conclusion that this parasite has the capacity to synthesize its phospholipids and/or to induce alterations in lipids obtained from the liver.

Materials and Methods

Growth and isolation of F. hepatica

Adult liver flukes from rats were obtained by the following procedures [3]: Eggs from *F. hepatica* were collected at the local slaughterhouse from the bile of naturally infected sheep. The miracidiae from the eggs were used to infect laboratory-reared *Limnea trunculata* in order to obtain metacercariae. Young male Wistar rats, weighing 40—50 g, were subsequently inoculated orally with 30—35 metacercariae. From 10 weeks post-infection, the rats were killed by stunning and bleeding and the liver flukes were immediately collected from the bile ducts and immersed in standard medium kept at 38° C. This medium was refreshed three times at intervals of 1 h. Care was taken not to expose the flukes to temperatures below or above 38° C. Subsequently, the flukes were incubated in fresh standard medium (usually 20 flukes per l) at 38° C to free them from bile components. After 16 h of incubation the flukes had excreted their intestinal load into the medium and could be used for experiments. The standard medium which was used in all experiments was modified after Van Noordwijk and De Wolf [4] and contained the following components (mM): NaCl, 120; KCl, 4; $CaCl_2$, 0.9; $MgSO_4$, 2.4; $NaHCO_3$, 18; glucose, 5.5; sodium phosphate buffer (pH 7.5), 4.0. To 1 l of medium 10^6 units of benzylpenicilline and 100 mg streptomycine sulphate were added to prevent bacterial growth in the medium.

Liver flukes from cattle were collected at the local slaughterhouse. As soon as possible after the death of an animal the bile ducts were examined for the presence of liver flukes. The flukes were immediately transferred into the standard medium which was kept at 38° C. Subsequently, the flukes were prepared for the experiments exactly as described for the flukes obtained from rat liver.

Analysis of phospholipids and their plasmalogen analogues

After removal of the bile components by incubation of the liver flukes overnight, the lipids were extracted via the following procedure: 10 flukes were homogenized in 5 ml of methanol with the aid of an Ultraturrax (Janke and Kunkel KG, Germany). Subsequently, 10 ml of chloroform were added and the mixture stirred for 15 min at room temperature. After centrifugation for 10

min at room temperature in a clinical centrifuge at maximal speed, the chloroform/methanol supernatant was carefully aspirated and the pellet reextracted twice with 15 ml of chloroform/methanol (2 : 1, v/v). The chloroform/methanol extracts were combined and evaporated in vacuo until dryness. The lipid residue was taken up in 5 ml of chloroform. After two washings with 5 ml of 2 M KCl and one washing with 5 ml of water, the lipids were subjected to two-dimensional thin-layer chromatography on silica plates impregnated with 2% magnesium silicate. The thin-layer plates were prepared and activated exactly as described by Broekhuyse [5]. Chloroform/methanol/conc. NH_3/water (90 : 54 : 5.5 : 5.5, by vol.) was used as solvent for development in the first direction. After removal of the solvent under a stream of N_2, the plate was developed in chloroform/methanol/acetic acid/water (90 : 40 : 12 : 2, v/v) in the second direction. Detection of the phospholipids was accomplished by exposure to iodine vapour or by spraying with a 0.05% aqueous solution of Rhodamine 6G. Subsequently, the various phospholipids were scraped from the plate for phosphorus determination according to the procedure of Bartlett [6]. The plasmalogen contents of phosphatidylcholine and phosphatidylethanolamine were determined by subjecting the lipids to methanol/conc. HCl (88 : 12, v/v) prior to development of the plate into the second direction. Phosphorus determination was used to assay the amount of monoacyl-phosphoglycerides derived from the plasmalogens [7]. The various phospholipids were identified by cochromatography with defined standard phospholipids and after mild alkaline hydrolysis by the method of Dawson [8] as modified by Chang and Kennedy [9]. The deacylated phospholipids were separated on Whatman-1 paper chromatograms which were run in ammonium acetate (1 M, pH 7.4)/alcohol (1 : 2, v/v). *sn*-Glycero-3-phosphocholine, *sn*-glycero-3-phosphoethanolamine, *sn*-glycero-3-phosphoserine and *sn*-glycero-3-phosphoinositol were used as reference compounds. Detection was carried out by staining with ammonium molybdate [10], ninhydrin and by radioactivity measurements.

Gas-liquid chromatography of the fatty acyl constituents of the phospholipids of rat liver flukes and rat liver

After two-dimensional thin-layer chromatography the phosphatidylcholine and -ethanolamine fractions were scraped from the plate and transferred into tubes containing 4.5 ml of methanol/conc. H_2SO_4 (95 : 5, w/w). After flushing with N_2, the tubes were closed and heated for 2 h at 70°C. After cooling, 5.5 ml of water were added and the methylesters were then extracted with three 12.5-ml portions of hexane. The combined hexane layers were washed once with 12.5 ml of water and dried over anhydrous Na_2SO_4. Gas-liquid chromatography was carried out with a Beckers Research Chromatograph, model 3810 (Beckers, Delft, The Netherlands), equipped with flame-ionization detection and a 10% EGSS-X column (Applied Science, U.S.A.) which was operated at 160°C. The identification was based on comparison with known standard methylesters, supplied by Applied Science and by a semi-logarithmic plot of the retention time versus carbon number of the fatty acids [11]. In addition, hydrogenation of the methylesters in hexane using Adams catalyst (Fluka, Switzerland) was used to further identify the unsaturated methylesters.

In vitro uptake of $^{32}P_i$ and [2-^{3}H]glycerol by F. hepatica

After removal of the bile components by incubation of the liver flukes in standard medium as described above, 20 flukes were transferred to 100 ml of fresh medium to which 1 mCi of $^{32}P_i$ had been added. At the indicated times four flukes were removed from the incubation medium. The lipids were extracted immediately and applied on two-dimensional thin-layer plates, as described above. The detection of the various phospholipids was accomplished by staining with iodine followed by autoradiography. The thin-layer plates were covered with Agfa Osray T4 X-ray plates for the appropriate period of time. The plates were then developed with reagents supplied by Agfa. The radioactive phospholipids were scraped from the plate and transferred into scintillation vials containing 10 ml of Instagel (Packard, U.S.A.). The radioactivity was assayed in a Packard Tricarb model 2425 B. In some experiments, the flukes were incubated in the presence of [2-^{3}H]glycerol instead of $^{32}P_i$. In this case the radioactive phospholipids were detected by a radiochromatogram scanner (Panax Equipment, Great Britain) followed by quantitative assay of the radioactivity by means of liquid scintillation counting.

Synthesis of phosphatidylcholine by homogenates of liver flukes

(a) From 1,2-diacyl-sn-glycerols and CDPcholine. Six flukes were homogenized in 4 ml of 0.125 M KCl/0.1 M Tris, pH 7.4, with the aid of a Potter-Elvehjem tube and a teflon pestle. The protein content of the homogenate was estimated by the method of Lowry et al. [12]. The formation of phosphatidylcholine from 1,2-diacyl-*sn*-glycerols and CDP-[*Me*-^{14}C]choline was assayed in a medium of the following composition: 1,2-diacyl-*sn*-glycerols (4 mM, pipetted from a sonicated 20 mM emulsion in 0.1 M Tris, pH 7.4, 0.03% Tween 20) CDP-[*Me*-^{14}C]choline (4 mM, spec. act. 0.25 Ci/mol), $MgCl_2$ (10 mM), glutathione (4 mM), Tris · HCl (100 mM, pH 7.4) and the appropriate amounts of liver fluke homogenate. The formation of radioactive phosphatidylcholine was measured after 0, 3, 6, 12 and 20 min of incubation by the filter-disc method introduced by Goldfine [13].

(b) By methylation of phosphatidyl-N, N-dimethylethanolamine. The formation of phosphatidylcholine by methylation of phosphatidyl-*N*, *N*-dimethylethanolamine was studied as follows: 10 μmol of phosphatidyl-*N*, *N*-dimethylethanolamine were suspended by sonication using a MSE-sonifier in 5 ml of a 0.2 M solution of Tris (pH 8.2) containing 20 mg of sodium deoxycholate. 50 μl of this suspension, containing 0.1 μmol of the substrate, were added to an incubation mixture containing: 1 mM *S*-adenosyl-L-[*Me*-^{14}C]methionine (spec. act. $2.2 \cdot 10^5$ dpm/μmol), 50 mM Tris (pH 8.4) and the required amount of liver fluke homogenate. The formation of radioactive phosphatidylcholine was determined at 0, 10 and 20 min using the filter-disc method [13].

Radiochemicals

Sodium [^{32}P]phosphate (spec. act. 165 Ci/mol) was purchased from Philips-Duphar, Petten, The Netherlands. [2-^{3}H]Glycerol (spec. act. 496 Ci/mol) and *S*-adenosyl-L-[*Me*-^{14}C]methionine (spec. act. 53.1 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Great Britain. CDP-[*Me*-^{14}C]choline (spec. act. 40 Ci/mol) was bought from N.E.N.-chemicals, Dreieichenhain, Germany.

Results and Discussion

Phospholipid composition of F. hepatica compared to that of the host liver

Table I shows a comparison of the phospholipid composition of *F. hepatica* with that of the livers of rats and cattle infected with this parasite. The phospholipid pattern of the infected liver tissues is very similar to that reported by Rouser et al. [14] for the normal liver. Evidently, the liver flukes do not cause significant alterations in the phospholipid composition of the liver during their passage through this tissue of the host. Only the percentage of ethanolamine plasmalogen in the infected tissue (6.8%) is higher than that reported for the normal liver [15]. Striking differences can be noted between the lipid patterns of the liver flukes and of the liver of the host: (1) Whereas monoacyl-*sn*-glycero-3-phosphocholine is hardly detectable in the liver, it occurs in significant quantities in the liver flukes. (2) Sphingomyelin, a significant constituent in the liver, is completely absent in the parasite. (3) Phosphatidylethanolamine is more abundant in the liver whereas phosphatidylserine is more enriched in the parasite. This data strongly indicates that the liver fluke does not simply copy the lipid pattern of the liver. Apparently, this parasite has the capacity either to take up lipids from the liver in a very selective way and/or to synthesize its own phopholipids. In this respect, it is very interesting to notice the striking similarity of the lipid compositions of the liver flukes isolated from rat and cattle, respectively.

Fatty acid composition of the major phospholipids of F. hepatica

Table II presents the fatty acid patterns of phosphatidylcholine and phosphatidylethanolamine of the liver fluke isolated from infected rats. For comparison, the fatty acyl constituents of the corresponding phospholipids in the liver of the infected rats have also been included. It is known that dietary changes profoundly change the fatty acid patterns of liver lipids [16]. Therefore, we limited our studies on the fatty acid composition to *F. hepatica*

TABLE I

PHOSPHOLIPID COMPOSITION (%) OF *F. HEPATICA* COMPARED TO THAT OF THE LIVER OF THE HOST

	*F. hepatica**		Liver**	
	From cattle	From rat	Cattle	Rat
Monoacyl-*sn*-glycero-3-phosphocholine	5.6	4.2	0.2	0.2
Phosphatidylcholine	46.8	49.2	52.5	52.0
Choline plasmalogen	3.4	3.2	3.0	0.9
Sphingomyelin	—	—	4.2	3.4
Phosphatidylserine	13.6	13.6	4.6	4.8
Phosphatidylinositol	6.9	6.9	9.6	8.8
Phosphatidylethanolamine	18.2	16.5	19.2	20.5
Ethanolamine plasmalogen	3.9	3.6	4.8	6.8
Cardiolipin	1.7	2.8	1.8	2.7

* Average value of ten experiments.
** Average value of four experiments.

TABLE II

FATTY ACID COMPOSITION (%) OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE FROM *F. HEPATICA* AND FROM RAT LIVER

Averages of three experiments are presented. +, trace amounts present

Fatty acids	Phosphatidylcholine		Phosphatidylethanolamine	
	Liver	*F. hepatica*	Liver	*F. hepatica*
16 : 0	26.8	39.3	28.2	9.9
18 : 0	20.1	19.6	30.3	38.5
18 : 1	8.6	9.5	9.0	7.4
18 : 2	20.4	5.8	9.6	4.7
20 : 1	—	7.1	—	4.9
20 : 2	—	4.6	—	0.7
20 : 4	23.9	13.6	10.3	22.0
22 : 6	+	+	12.8	11.7

isolated from rats which had been fed with a regular laboratory chow-pellet diet. The fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine from the liver are in good agreement with data reported for these phospholipids by other investigators [17]: phosphatidylethanolamine is richer than phosphatidylcholine in stearic and docosahexaenoic acid whereas phosphatidylcholine contains higher amounts of linoleic and arachidonic acid. The fatty acids of the corresponding phospholipids in the liver fluke are quite different: in phosphatidylcholine there is an increase of palmitic acid and a decrease of linoleic and arachidonic acid. Also qualitative differences can be noted; eicosaenoic ($C_{20:1}$) and eicosadienoic ($C_{20:2}$) acid occur in significant amounts in the parasite, whereas these acids cannot be detected in the liver. It is of interest that another trematode, *Schistosoma mansoni*, also contains significant amounts of $C_{20:1}$ and $C_{20:2}$ [18,19]. Presumably, these acids are formed by the parasite by elongation of octadecenoic ($C_{18:1}$) and octadecadienoic ($C_{18:2}$) acid, respectively. In this light it is relevant to mention that another trematode, *S. mansoni*, and a cestode, *Hymenolepis diminuta*, have been shown to possess the capacity to elongate fatty acids [19,20]. Further experiments are in progress to investigate the elongation system of *F. hepatica*. Also for phosphatidylethanolamine, the major qualitative difference is the occurrence of $C_{20:1}$ and $C_{20:2}$ in the parasite and the absence of these fatty acids in the liver. Similarly as found for the polar head group of the phospholipids, the fatty acid pattern of the phospholipids of the parasite is not merely a reflection of that of the phospholipids of the liver. Evidently, *F. hepatica* has the capacity to synthesize at least part of its fatty acids de novo or to introduce modifications in fatty acids obtained from the liver.

In vitro uptake of $^{32}P_i$ *and [2-*^{3}H*]glycerol by F. hepatica*

Incubation of *F. hepatica* isolated from rats in standard medium to which $^{32}P_i$ had been added resulted in the labelling of all phospholipids of this parasite. In Fig. 1 the rate of incorporation of $^{32}P_i$ into the various phospholipids is studied. Phosphatidylinositol was found to have the highest specific activity over the whole incubation period. Probably, the specific activity of

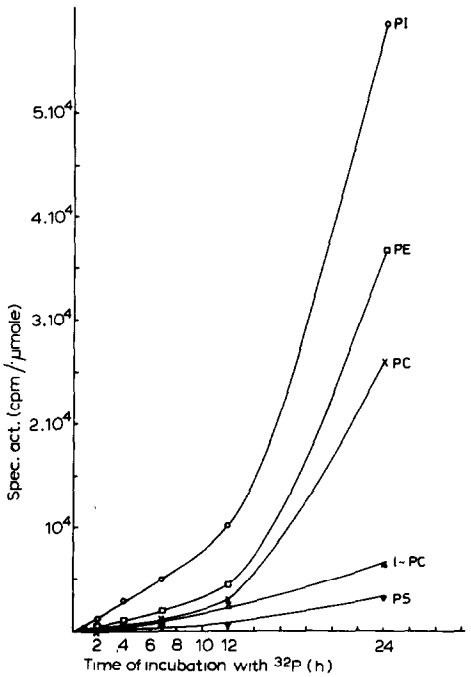

Fig. 1. In vitro uptake of $^{32}P_i$ by *F. hepatica* isolated from rats. o———o, phosphatidylinositol (PI); □———□, phosphatidylethanolamine (PE); X———X, phosphatidylcholine (PC); △———△, monoacyl-*sn*-glycero-3-phosphocholine (1-PC); ▲———▲, phosphatidylserine (PS).

phosphatidic acid is even higher than that of phosphatidylinositol, but its specific activity could not be measured accurately due to the extreme small amounts of this phospholipid in *F. hepatica*. Phosphatidylethanolamine and phosphatidylcholine were labelled at an almost constant rate during the first 7 h of incubation, after which time a strong increase in the rate of incorporation was noted. Similar observations were noted by Webb and Mettrick [21] who studied the uptake of $^{32}P_i$ and by Buteau and Fairbairn [22] who followed the incorporation of [^{14}C]glycerol into the phospholipids of the rat tape-worm, *H. diminuta*.

Monoacyl-*sn*-glycero-phosphocholine and phosphatidylserine were labelled at a much slower rate than the other phospholipids of *F. hepatica*, probably because of a much slower turn-over. After 24 h of incubation the percentage of label incorporated into the choline-containing lipids, phosphatidylserine and cardiolipin was smaller than the weight percentage of these lipids (Table III). On the other hand, the percentage of label incorporated into phosphatidylinositol and phosphatidylethanolamine exceeded the weight percentage of these phospholipids. The higher labelling of phosphatidylinositol and phosphatidylethanolamine may be related to a preliminary observation that these phospholipids are enriched in the eggs produced by the liver fluke. Incubation of liver flukes in the presence of [2-^{3}H]glycerol resulted in a similar labelling pattern of the various phospholipids, with the following exceptions: (1) Phosphatidylserine is labelled more strongly by glycerol than by phosphate. (2) Ethanol-

TABLE III

DISTRIBUTION (%) OF RADIOACTIVITY AMONG THE PHOSPHOLIPIDS OF *F. HEPATICA* AFTER 24 h INCUBATION IN THE PRESENCE OF $^{32}P_i$ OR [2-^{3}H]GLYCEROL
+, trace amounts present.

	$^{32}P_i$	[2-^{3}H] Glycerol	Weight distribution
Monoacyl-*sn*-glycero-3-phosphocholine	2.6	1.0	4.2
Phosphatidylcholine	37.2	37.0	49.2
Phosphatidylserine	1.9	7.8	13.6
Phosphatidylinositol	22.4	23.9	6.9
Phosphatidylethanolamine	29.0	29.4	16.5
Ethanolamine plasmalogen	7.1	0.8	3.6
Cardiolipin	+	+	2.8

amine plasmalogen is labelled by $^{32}P_i$ but hardly by [2-^{3}H]glycerol. These observations are in excellent agreement with the concept [23,24] that in animals glycerol is introduced into alkyl lipids via dihydroxyacetonephosphate. Since it is known that ethanolamine plasmalogen is derived from 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine [25], [2-^{3}H]glycerol should indeed loose its ^{3}H label upon incorporation into ethanolamine plasmalogen. It cannot be excluded, however, that the biosynthesis of plasmalogens in *F. hepatica* proceeds via another mechanism as has been described for mammalian tissues [25], since the metabolism of the fluke is essentially anaerobic and because there is evidence [20] that helminths might lack the capacity to desaturate fatty acids. These results obtained in experiments on the uptake of $^{32}P_i$ and [2-^{3}H]glycerol into the phospholipids of *F. hepatica* during in vitro incubations clearly demonstrate that this parasite is equipped with the machinery to synthesize and renew its own phospholipids de novo.

Synthesis of phosphatidylcholine by homogenates of F. hepatica

The synthesis of phosphatidylcholine in mammalian cells proceeds via the CDPcholine pathway, established by Kennedy [26]. An additional pathway for

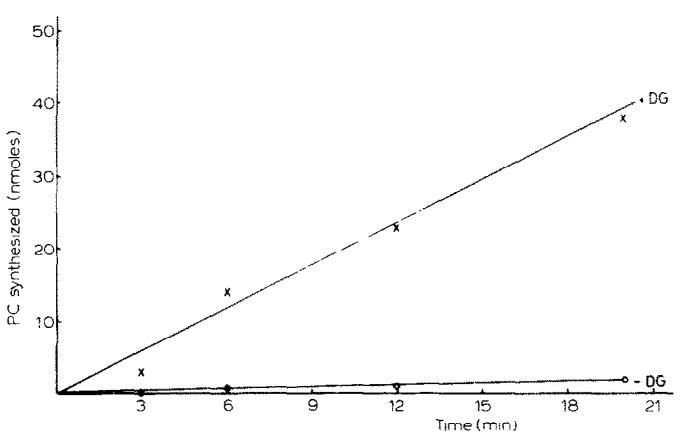

Fig. 2. Synthesis of phosphatidylcholine from 1,2-diacyl-*sn*-glycerols and CDP-[*Me*-^{14}C]choline by homogenates of *F. hepatica*. Details of the incubation mixture have been mentioned in Materials and Methods. DG, 1,2 diacyl-*sn*-glycerols.

the synthesis of phosphatidylcholine, viz. the N-methylation of phosphatidylethanolamine [27—29], has been shown to be almost exclusively confined to the liver [30], where it is believed to be operative mainly in the production of hexaenoic lecithins [31,32]. It was thought of interest to investigate whether the liver fluke synthesizes phosphatidylcholine via CDPcholine or via N-methylation. Fig. 2 shows that homogenates of *F. hepatica* contain choline phosphotransferase (EC 2.7.8.2): the synthesis of phosphatidylcholine from added 1,2-diacyl-*sn*-glycerols and CDP-[*Me*-^{14}C]choline was found to be proportional to time and amount of protein. However, in strong contrast to rat liver microsomes, homogenates of *F. hepatica* were not able to convert dimethyl phosphatidylethanolamine into phosphatidylcholine by N-methylation. Apparently, the liver fluke only uses the de novo synthesis via CDPcholine for the formation of phosphatidylcholine. In future studies it will be of interest to investigate the presence of a deacylation-reacylation cycle [33] in *F. hepatica*, especially in view of the exceptionally high level of monoacyl-*sn*-glycero-3-phosphocholine in this parasite.

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