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INHIBITORY EFFECT OF CHOLEPHILIC ANIONS ON FATTY ACID UPTAKE BY THE PERFUSED RAT LIVER

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Abstract—The mechanisms of uptake and intracellular transport of plasma free fatty acids by the liver cell are poorly known. A cytosolic protein, termed *Z* protein, has been identified in the liver, intestine and other tissues; *in vitro* studies show a high affinity of fatty acid and organic anions for this protein and suggest that it could be involved in the cellular transport of fatty acids *in vivo*. The present experiments demonstrate an inhibitory effect of various organic cholephilic anions (B.S.P., D.B.S.P., flavaspidic acid, bilirubin, etc) on fatty acid uptake by the isolated perfused rat liver. This inhibition is rapidly reversible when the liver is perfused with a medium lacking cholephilic anions, which together with the absence of any functional or ultrastructural damage to the organ, excludes any hepatotoxic effect. Such an inhibition of fatty acid uptake could be due to a competition for binding either to membrane sites and/or to intracellular carriers common to both fatty acids and cholephilic anions. These data are consistent with the involvement of carrier-mediated processes in fatty acid transport in the liver. Finally, simultaneous uptake of radioactive fatty acid and cholephilic anions does not interfere with further fatty acid utilization by the cell.

Long chain fatty acids are water-insoluble compounds, and as a result, they must be transported in aqueous biological fluids (e.g. blood plasma, cytosol) either bound to soluble protein or in the form of more polar water-soluble derivatives. The role of albumin as a plasma free fatty acid (F.F.A.) carrier is firmly established. Moreover, at least three water-soluble fatty acyl derivatives, namely fatty acyl CoA, acylcarnitine and lysophosphatides, are known to fulfil an important function in the transfer of fatty acids among different subcellular compartments.

A cytosolic protein (F.A.B.P.), which binds fatty acids *in vivo* and *in vitro*, and which is probably identical to *Z* protein, has been recently isolated by Ockner *et al.* [1, 2] from the supernatant of intestinal mucosal homogenates and from some other tissues, including liver. These authors have suggested that this protein could play a role in fatty acid transport in intestinal cells and perhaps in other tissues.

In vitro studies showed that after incubation of radioactive fatty acids with a hepatic or intestinal cytosolic fractions, (105,000 *g* supernatant of a tissue homogenate), followed by chromatography on Sephadex columns, fatty acids were eluted in association with contaminating albumin as well as *Z* protein peaks [2, 3].

Flavaspidic acid and α-bromopalmitate compete with fatty acids for binding to F.A.B.P. *in vitro*, though they do not interfere with fatty acid uptake by everted gut sacs [4]. Competition for binding to hepatic *Z* protein between fatty acid and B.S.P. and other cholephilic anions *in vitro* has been described [3]. These results, suggest that F.A.B.P. or *Z* protein could play a specific role in fatty acid transport in the liver cell.

The present experiments were performed in order to investigate the influence of organic cholephilic anions on fatty acid uptake *in vivo* using the isolated perfused rat liver.

The results reveal an inhibitory effect of several organic anions on fatty acid uptake by the liver, and suggest the existence of some common acceptor or carrier mechanism for fatty acids and cholephilic anions.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats of about 350 g body wt, which were fed a standard laboratory diet, were used. Animals were fasted 18 hr before perfusion, with free access to a 20%, w/v glucose solution.

Liver perfusion. The liver perfusion technique has been described elsewhere [5]. The standard perfusate consisted of heparinized rat blood diluted (1:2, v/v) with a Krebs-bicarbonate buffer (pH 7.38) containing 4 g/100 ml of human albumin. The F.F.A./albumin molar ratio ($\bar{v}$) in the perfusate was adjusted to 0.45 by binding appropriated amounts of palmitic acid to delipidated albumin [6].

Perfusion was performed for 20 min in order to allow blood flow and bile secretion to become steady.

Measurement of fatty acid uptake was commenced after the portal cannula had been connected to a reservoir containing the standard perfusate to which 10 μCi [^{3}H]palmitic acid ($\bar{v}$ = 0.45) has been added (specific activity: 45 Ci/m-mole).

The hepatic venous effluent was collected in plastic tubes at 30 sec intervals for 10 min. A 10-min-recirculating perfusion with standard medium lacking labelled fatty acid was subsequently performed in order to wash the previous perfusate out of the liver. Thereafter the liver was perfused with the standard perfusate supplemented with 10 μCi-[^{14}C]palmitic acid (specific activity: 49.2 mCi/m-mole) and one of the following anions: tetrabromosulfonephthalein (B.S.P.),

dibromosulfonephthalein (D.B.S.P.), Flavaspidic acid, Bilirubin complexed to albumin, Indocyanin Green (I.C.G.), Taurocholic acid or Rifampicin in amounts sufficient for an anion/F.F.A. molar ratio of 1.

In some experiments (see Results), the alternative perfusion with perfusate to which a cholephilic anion had either been added or omitted was repeated twice and fatty acid uptake by the same liver was measured on each occasion.

Chemicals. All the chemicals for routine use were obtained from Prolabo, B.S.P. and D.B.S.P. were from S.E.R.B. Laboratories, Rifampicin from Lepetit Laboratories, Indocyanine Green from Hynson, bilirubin from Sigma and taurocholic acid from Calbiochem. Flavaspidic acid *N*-methyl glucaminate was a gift from Prof. I. Arias (Albert Einstein College of Medicine, Bronx, NY) and Dr Ahö (Turku, Finland). Iso-Octan was purchased from Merck and Nile Blue from Edward Gurr Ltd. Radioactive fatty acids were purchased from C.E.A. (Saclay, France) and their radio-chemical purity, as checked by thin layer chromatography (t.l.c.) and radioactive counting was higher than 98 per cent for [^{14}C] and 95 per cent for ^{3}H-labelled palmitic acid.

Determination of the F.F.A. uptake. A perfusion flow rate through the liver of 1 ml/g liver/min was obtained by adjusting the hydrostatic pressure in the portal vein; usually blood flow remained steady throughout the experiment.

The net uptake of F.F.A. was calculated every 30 sec from the difference between the F.F.A. concentration in the portal blood and that in the hepatic veinous blood samples [7]. The concentration of F.F.A. was determined according to Trout's modification of the Dole method [8]. Since more than 95 per cent of perfusate (inflow and outflow) radioactivity was recovered as free fatty acid by t.l.c., plasma samples (0.1 ml) were routinely dissolved in 10 ml of Bray's scintillation mixture and counted in a β-spectrometer (Intertechnique, France) [9]. Fatty acid uptake was determined both as the porto-caval difference in its concentration and in its radioactivity.

Statistical methods. The significance of differences in F.F.A. uptake between perfusions in which cholephilic anions had been either added or omitted were determined by the Student's '*t*' test.

Dissolution of bilirubin. Immediately before use, bilirubin was dissolved in 0.1 N NaOH and subsequently complexed to albumin by slow addition to albumin-containing Krebs buffer with constant stirring in the dark.

RESULTS

Validation of the uptake measurements. Indeed the determination of the liver's uptake of circulating substances by porto-caval differences in their concentration or radioactivity has been widely used in studies of a number of compounds, including free fatty acids [7, 10]. This method has been validated in the present experiments since cholephilic anions might modify the clearance of fatty acids from the circulation without having any effect on cellular uptake. In a typical experiment with an non-recycling perfused liver, 2 μCi of [^{14}C]palmitic acid complexed to albumin were injected into the portal vein catheter. After 0.5 min the liver circulation was washed out for 4.5 min with a standard non-radioactive perfusate. Duplicate liver samples were immediately homogenized in cold methanol and the lipids extracted by the method of Folch *et al.* [11]. Lipids were separated by t.l.c. on silica gel and material in the different spots quantitatively transferred into counting vials containing 10 ml Instagel.

The distribution of the label between the fractions was: free fatty acids, 5 per cent; triglycerides, 29 per cent; phospholipids, 58 per cent; mono- and diglycerides, 5 per cent; and cholesterol esters, 2 per cent.

Conversion of [^{14}C]palmitate to $^{14}CO_2$ and water-soluble metabolites was not directly measured but it could be estimated to be lower than 0.3 per cent, since recovery of radioactivity in the lipid extract of the total liver was 99.7 per cent of the ^{14}C radioactivity cleared from the perfusate under these conditions.

Indeed when cholephilic anions were present in the perfusate, the recovery of radioactivity in the liver lipids and in non-metabolised ([^{14}C]-free fatty acid) and metabolised palmitate ([^{14}C]-lipids) was unchanged. Accordingly, in the subsequent experiments we considered fatty acid clearance from the perfusate to be an expression of cellular uptake.

Fatty acid uptake under standard conditions. As in the competition experiments, fatty acid uptake was measured using two perfusates, the first containing [^{3}H]palmitic acid as a tracer and the second, the cholephilic anion and [^{14}C]palmitic acid. This technique was validated by preliminary studies: two samples of the standard perfusate containing [^{3}H] or ^{14}C-labelled palmitic acid respectively were sequentially perfused (without recirculation) with intermediary washout as described in Methods; fatty acid uptake was estimated by measurement of radioactivity in the portal and hepatic vein perfusates. The results clearly showed both the validity of the measurements of the rate of uptake by both labels and the constancy in the rate of fatty acid uptake by the perfused liver (Table 1).

Fatty acid uptake in competition experiments. Seven cholephilic anions were tested. These anions differ in their chemical structure and hepatic metabolism but all are rapidly removed by the liver and excreted in bile. All of them significantly decreased fatty acid uptake (mass and radioactivity determinations) by the liver in a range from 28% (bilirubin) to 43% (B.S.P.) (Table 2).

Representative data on the inhibition of F.F.A. uptake by Indocyanin Green are shown in Table 3. Although as previously described, a higher uptake rate was obtained by radioactivity measurement, the effect of I.C.G. was evident with either method [12].

In order to test whether the effects of cholephilic anions on the rate of fatty acid uptake are reversible, some isolated livers were perfused with standard perfusate for 10 min, then with the B.S.P.-containing perfusate for the same period and finally again with the standard perfusate. Between each of these two periods the perfusate which had been previously used was washed out of the liver as described in Methods. As shown in Table 4, B.S.P. did not permanently modify the rate of fatty acid uptake, since it returned to control values when a standard medium lacking B.S.P. was perfused.

In the inhibition experiments, fatty acid and the organic anion under examination were present in the perfusate at equimolar concentrations. The influence of the organic anion concentration was further investigated. In these experiments, the perfusate fatty acid concentration was adjusted to a physiological level (0.45 μEq/ml) in four perfusate samples to which adequate amounts of B.S.P. were added, in order to obtain a B.S.P./F.F.A. molar ratio in the range 0–1.0 Although, an absolutely linear dose-response effect was not observed, increase in the B.S.P./F.F.A. ratio resulted in a proportionate decrease in the uptake of F.F.A. by the liver (Table 5).

In other experiments, B.S.P. concentration was kept constant (0.130 μmole/ml) and F.F.A. concentration was adjusted to two different levels. Thus, inhibition

Table 1. Uptake of radioactive palmitic acid in perfused rat liver under standard conditions

Period of perfusion 0–10 min Percentage of 3H Palmitic acid uptake*	20–30 Min Percentage of 14C Palmitic acid uptake*
26.8 ± 0.7	26.6 ± 0.8 N.S.

* Uptake is expressed as the percentage difference in the level of radioactive palmitate in the portal and caval perfusates (see Methods). Values are the mean ± S.E.M. of six perfusions in each group; a series of eighteen determinations were made in the course of each perfusion. F.F.A. concentration = 0.460 μEq/ml; F.F.A./albumin molar ratio: 0.70. The transhepatic flow rate of the perfusate was 0.77 ml/g of liver/min.

Table 2. Effect of various organic anions on F.F.A. uptake by isolated perfused rat liver

Organic anion added into perfusate	% Of [^{3}H]palmitic acid uptake in the absence of an organic anion	% Of [^{14}C]palmitic acid acid uptake* in the presence of an organic anion	% Inhibition of uptake	P†
Bilirubin	28.9 ± 0.6	20.8 ± 0.4	28	0.001
Rifampicin	25.1 ± 0.5	16.8 ± 0.1	33	0.001
D.B.S.P.	32.3 ± 0.9	21.8 ± 0.8	33	0.001
Taurocholic acid	33.1 ± 0.6	21.8 ± 0.8	34	0.001
Indocyanin green	30.1 ± 0.8	19.0 ± 0.7	37	0.001
Flavaspidic acid	26.5 ± 0.5	15.5 ± 0.4	42	0.001
B.S.P.	27.8 ± 0.7	15.9 ± 0.4	43	0.001

Experimental conditions were as described in Methods. For each organic anion added to the perfusate, the anion/F.F.A. molar ratio was equal to 1. Values are the mean ± S.E.M. of six perfusions in each group.

* F.F.A. uptake was determined as described in Methods.

† Student's '*t*' test.

Table 3. Effect of Indocyanin Green on F.F.A. uptake by perfused rat liver. Comparison of two methods of estimation of uptake: mass and radioactive porto-caval difference

	% Of F.F.A. uptake under standard conditions	% Of F.F.A. uptake in presence of I.C.G. I.C.G./F.F.A. molar ratio = 1	%*	P†
Radioactivity	30.10 ± 0.75	19.00 ± 0.71	37.0	0.001
Mass (Dole)	22.54 ± 1.41	15.51 ± 0.68	32.0	0.001

Rat liver perfusion was performed as described in Methods. The transhepatic flow rate of the perfusate was 0.77 ml/g of liver/min. Values are means ± S.E.M. from six perfusions.

* % Of inhibition.

† Student's '*t*' test.

Table 4. Reversibility of the inhibition of F.F.A. uptake after perfusion with medium containing B.S.P.

Time, min		% Of F.F.A. uptake (porto-caval difference in radioactivity)	
0–10	A: Standard perfusate	24.25 ± 0.92	P*
20–30	B: Perfusate with B.S.P. 40 μM/100 ml	19.00 ± 0.80	0.001†
40–50	C: Standard perfusate	23.43 ± 2.26	N.S.‡

Mean ± S.E.M.

* Student's '*t*' test ($n = 4$).

Perfusions were performed as described in Methods.

† B vs A.

‡ C vs A.

Table 5. Molar ratio of B.S.P./F.F.A. and its relationship to F.F.A. uptake

B.S.P./F.F.A. molar ratio	% Of F.F.A. uptake (porto-caval difference in radioactivity)	% Inhibition	P*
No B.S.P.	38.5 ± 0.6	—	
0.25	33.7 ± 0.3	13	0.001
0.50	31.3 ± 0.5	18	0.001
1.00	21.8 ± 0.8	43	0.001

Perfusion and F.F.A. determination as in Methods. F.F.A. concentration = 0.45 μEq/ml; transhepatic flow rate of perfusate = 0.67 ml/g of liver/min. F.F.A./albumin molar ratio = 0.77. Mean ± S.E.M. from four perfusions.
* Student's '*t*' test.

Table 6. Effect of F.F.A. concentration on the inhibition of F.F.A. uptake by B.S.P.

	F.F.A. concentration μEq/ml	B.S.P.	B.S.P./F.F.A. molar ratio	% FFA uptake	% Inhibition	P*
A	0.260	no B.S.P. added	0	14.4 ± 1.0	—	
B	0.260	0.130 μM/ml	0.50	7.4 ± 0.4	50	B versus A: 0.001
C	0.485	no B.S.P. added	0	30.8 ± 0.6	—	
D	0.485	0.130 μM/ml	0.25	27.0 ± 0.8	13	D versus C: N.S.

The perfusion technique was as described in Methods.
* Student's '*t*' test.
Mean ± S.E.M. from four perfusions.

of fatty acid uptake was observed at the low F.F.A. concentration (0.260 μmole/ml), but at higher fatty acid concentrations (0.485 μmole/ml), the inhibitory effect fell to a mere 13 per cent (Table 6). These observations suggest a competition for hepatic uptake between B.S.P. and F.F.A.

Metabolic utilization of fatty acids. The metabolism of radioactive fatty acid was studied in liver samples after perfusion for 10 min with control medium or with the same medium to which cholephilic anions had been added.

Though the amount of fatty acids cleared by the liver differed in both groups, their overall metabolic utilisation was not affected by the cholephilic anions. Thus for all the tested anions most of the label was found in triglycerides (54.8 ± 2.3 per cent) and phospholipids (34.7 ± 2.9 per cent); less than 10 per cent of the radioactivity was recovered in the form of fatty acids, mono- and diglycerides or cholesterol esters. Since, fatty acids oxidation was not directly measured, differences in the amount of fatty acids oxidized by individual anions cannot be excluded.

DISCUSSION

Although many tissues can extract and utilize plasma F.F.A., in man and other animals, the liver extracts very efficiently non-esterified fatty acids and bilirubin from the blood in addition to organic anions (dyes) which may have been infused into the circulation for diagnostic or experimental purposes. Other tissues can also utilize plasma F.F.A. but do not possess such a high capacity for uptake of these compounds; then it appears reasonable to assume that the hepatic cell might have selective membrane and cytosolic carriers which do not exist in other cells. According to current views, fatty acid uptake is a non-energy-dependent process, probably involving a simple diffusion of fatty acid chains through the lipoprotein membrane bilayers. Even if the existence of membrane specific acceptors is not required fatty acids have to be transported through the aqueous cytosolic phase bound to some carrier protein, since the enzymatic reactions necessary to transform them into water-soluble compounds (acylCoA and acylcarnitine) do not occur in the plasma membrane.

Experiments *in vitro* have established the affinity of fatty acids for F.A.B.P. and for the *Z* protein isolated from the cytosolic fraction of liver, intestine and other tissues. Other organic anions exhibit the same affinity for these proteins. Furthermore, such cholephilic anions compete with fatty acids for binding to *Z* protein *in vitro* [3]. These data suggest that F.A.B.P. or *Z* protein could play a role in the cellular transport of a variety of organic anions, including fatty acids.

The present *in vivo* experiments represent an attempt to examine the hypothesis that a part of the fatty acids taken up by the liver cell are carried by *Z* protein. It has been postulated that in this case, an inhibition of fatty acid uptake by organic anions would occur *in vivo*.

Uptake has been estimated by differences in the radioactivity or mass of fatty acids between the portal and hepatic vein perfusates. In these studies, fatty acid clearance was shown in preliminary experiments to be an expression of cellular uptake. Fatty acids which disappeared from the circulation were very rapidly taken up by the cell and subsequently metabolised into different lipid classes, water-soluble compounds (cetonic acids) and CO_2. A minor proportion of radioactivity was found as F.F.A. in the extract of liver lipid. In order to minimize any specific effect which might be due to a particular organic anion, a variety of them differing in chemical structure, hepatic metabolism and relative affinity for *Z* protein *in vitro*, were chosen for study. Any effect of mass could

be neglected, since in the competition experiments, fatty acids and the other organic anions were present at identical molarity.

Their concentrations were adjusted so as to be well below that at which albumin binding sites were saturated and also below the maximal capacity of uptake by the liver for each anion.

An inhibition of F.F.A. uptake was observed with each of the cholephilic anions examined and this ranged from 28 per cent for bilirubin to 43 per cent for B.S.P. (Table 2). This effect was reversible when the organic anion was absent from the perfusate and could be lessened by increasing the molar ratio of F.F.A./anion (Tables 4 and 6). Though a perfect linearity was not observed, when the molarity of F.F.A. was kept constant the percentage of inhibition of uptake was roughly proportional to the B.S.P. concentration in the perfusate (Table 5). Conversely, the inhibitory effect of B.S.P. was diminished when the concentration of F.F.A. in the perfusate increased (Table 6). These results suggest that organic anions inhibit hepatic fatty acid uptake by a competitive mechanism, although the exact location of inhibition in the process of fatty acid transport remains to be established. Yet, it cannot be excluded that individual organic anions could compete with fatty acid transport at different locations. Mishkin *et al.* [13] have recently shown that in the whole rat and in perfused liver preparations, flavaspidic acid and B.S.P. do not interfere with or even increase fatty acid uptake. These results conflict with those previously reported in competition experiments *in vitro* [3], with our own results and with a recent report by Wu-Rideout *et al.* [14] using hepatocytes. A possible explanation for this discrepancy may arise from differences in perfusion techniques and especially from the fact that these authors used very high concentrations of fatty acids (4 μmoles/ml, F.F.A./albumin molar ratio 9.3), such levels are in contrast with the fatty acid concentration (0.5 μmole/ml) and fatty acid/albumin molar ratio ($\bar{v} = 0.5$–0.8) used in our experiments, which are in the physiological range for normal rat plasma. Indeed, it is possible that those experiments demonstrate a different phenomenon than that shown by Wu-Rideout [14] and ourselves.

Since fatty acids in the perfusate are bound to albumin it is possible that they could be displaced by other anions. Such a possibility can be excluded because all the anions tested, with the exception of bilirubin, are water-soluble and do not have to be transported bound to albumin. Each molecule of albumin can accept up to 8–10 fatty acid molecules and six of bilirubin; these molar ratios are much higher than those obtained in our perfusates. Moreover, a displacement of fatty acid from albumin should result in an increased hepatic uptake, and in fact the converse was observed in our competition experiments. This point has been further substantiated by phase-partition experiments (unpublished observations) showing that albumin-bound fatty acids (0.3–5.0 mM, $\bar{v} = 0.5$–8.0) are not displaced by equimolar or higher concentrations (0.3–10.0 mM) of bilirubin, B.S.P., I.C.G. or cholyltaurine. An inhibition of fatty acid transport could occur at the membrane level, although membrane receptors for fatty acids probably do not exist [15–17].

On the basis of measurements of kinetic uptake, it has been postulated that membrane receptors and carriers should be involved in the transport of cholephilic anions [18].

The higher *in vitro* affinity of bilirubin for albumin than for cytosolic protein seems indicative of a carrier-mediated process for bilirubin uptake *in vivo* [19]. In this respect, the existence of membrane receptors common to some organic anions could be supported by the observations of Arias *et al.* [20] regarding a competitive effect between B.S.P. and flavaspidic acid for binding to plasma membrane preparations from rat liver; however, no competition between B.S.P. and cholyltaurine or B.S.P. and bilirubin occurred in these studies.

Further experiments are currently in progress in order to evaluate the possible competition for binding sites between fatty acids and organic anions in purified plasma membrane suspensions. Cholephilic anions are not hepatotoxic at concentrations much higher than those used in our experiments. Transformation of radioactive fatty acid into liver lipids was not significantly affected by such anions. Moreover, their inhibitory effect on F.F.A. uptake was rapidly reversible, (at least for B.S.P., other anions were not tested) suggesting a direct effect rather than an indirect one through some modification of fatty acid metabolism. The present data and the above consideration suggest that organic anions compete with fatty acids for binding to *Z* protein. Such anions are less efficiently transported in the cell and therefore a decreased uptake results. Though extrapolation of *in vitro* binding experiments to uptake of F.F.A. *in vivo* must be made with caution, there is some agreement between previously published data and our present observations that *Z* protein may fulfil a physiological role in fatty acid transport.Thus,

(a) The F.A.B.P. or *Z* protein is particularly abundant in organs (liver and intestine) which are involved in the transport and metabolism of large amounts of fatty acids, (b) Administration of fat-rich diets increases the F.A.B.P. concentration in the intestine, [2] (c) Organic anions that compete more strongly with fatty acid for *Z* binding *in vitro* are the stronger inhibitors of F.F.A. uptake in the perfused liver, (d) Our results in the perfused liver are in agreement with those of Wu-Rideout *et al.* [14], using isolated hepatic cell suspensions, and by Ockner *et al.* [4, 1] which suggest that F.A.B.P. or *Z* protein operates as a fatty acid carrier in the cytoplasm of hepatic and intestinal cells.

Finally in recent experiments, we have shown that clofibrate administration to rats increases the *Z* protein concentration by 98 per cent and the B.S.P. binding capacity by 160 per cent [21]. Clofibrate-treated livers took up more F.F.A. (+75 per cent) from the perfusate than controls. Measurement of plasma membrane surface, lipid and protein content, did not show any significant modification under such conditions. These findings suggest a relationship between *Z* protein and fatty acid transport in the liver cell.

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