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THE EFFECT OF PARTITION OF FATTY ACID BETWEEN OIL AND MICELLES ON ITS UPTAKE BY EVERTED INTESTINAL SACS

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SUMMARY

1. Everted sacs of rat jejunum were used to measure the effect on uptake of labelled oleic acid, 1 mM, when an unabsorbable oil (glyceryl-trioleyl ether) was present. The incubation medium also contained monoolein 1 mM, and pure bile salts (sodium taurocholate–sodium taurodeoxycholate, 4:1 on a molar basis).

2. Increasing concentrations of triether had no effect on the low uptake of oleic acid in the absence of a micellar phase, bile salts 1 mM, when virtually all the lipid was in emulsified particles.

3. When most of the oleic acid (and monoolein) was present in a micellar phase, bile salts 4 mM or 5 mM, uptake was greatly accelerated. Addition of increasing amounts of triether now led to partition of an increasing proportion of oleic acid into the emulsified oil particles with a corresponding reduction in concentration of oleic acid in the micellar or isotropic phase. Uptake of oleic acid was reduced in linear proportion to reduction in micellar concentration.

4. The regression of oleic acid uptake on isotropic aqueous concentration was the same in these experiments, in which bile salt concentration remained constant, as in previous experiments in which the isotropic concentration was varied by altering the bile salt concentration.

5. These experiments provide further evidence that uptake *in vitro* depends on the concentration of fatty acid in the isotropic aqueous phase, and not on the concentration of bile salts *per se*.

INTRODUCTION

The uptake of fatty acid by everted intestinal sacs is accelerated by bile salts above the critical micellar concentration[1,2]. This could be explained by solubilization which increases the concentration in the aqueous phase and so increases transport by diffusion through an unstirred layer of fluid immediately external to the brush border. Other mechanisms which may play a part are (i) increase of mucosal (brush border) permeability by bile salts[3,4] and (ii) an intracellular effect of bile salts promoting incorporation of fatty acid into triglyceride[5].

Previous experiments from this laboratory have shown a linear relation of fatty acid uptake to concentration in the isotropic phase[1]. In these experiments while the total fatty acid concentration remained constant, the aqueous concentration of fatty acid was increased by increasing bile salt concentrations above the critical micellar concentration. Since bile salt concentrations varied, a membrane or metabolic effect was not excluded. In the present experiments both total fatty acid and bile salt concentrations were held constant while the concentration of aqueous (micellar) fatty acid was altered by partition between the aqueous phase and increasing concentrations of emulsified unabsorbable oil, glyceryl triether.

MATERIALS AND METHODS

Tissue

Male rats, 200–220 g of a Wistar strain locally inbred for 12 years were fasted overnight. The small intestine was removed under ether anaesthesia, rinsed with saline at room temperature, everted over a stainless steel rod, and segments weighing approx. 0.4 g cut from below the ligament of Treitz. After weighing, the segments were distended with 0.2 ml of normal saline. In each experiment, paired sacs from each animal were used to compare variables.

Materials

Sodium taurocholate and sodium taurodeoxycholate were prepared by the method of Norman as modified by HOFMANN[6]. A 2-mg sample of each bile salt ran as one spot on thin-layer chromatography when developed in the system ethyl acetate–methanol–glacial acetic acid (70:20:10, by vol.).

[1-^{14}C]Oleic acid was purchased from the Radiochemical Centre, Amersham. The label was in excess of 98 % class pure when run on thin-layer chromatography in the system hexane–diethyl ether–glacial acetic acid (80:20:2, by vol.). It was used as supplied. Unlabelled oleic acid was purchased from May and Baker, England. It was 99 % pure and was used as supplied. Monoolein was purchased from Calbiochem. It was 90 % pure and was further purified by solvent partition. The final product ran as one spot on thin-layer chromatography in a system of hexane–diethyl ether–glacial acetic acid (30:70:2, by vol.). 1,3-Dodecyl-2-hexadecyl-glyceryl ether (triether) was prepared by the method of BAUMANN AND MANGOLD[7,8]. It was purified by elution from a column of silicic acid using a hexane–benzene solvent system. The final product ran as one spot on thin-layer chromatography using a hexane–diethyl ether–glacial acetic acid system (90:10:2, by vol.).

All reagents used were of analytical grade. Ethanol and water were redistilled.

Solutions

Solutions were made up in a phosphate buffer (pH 6.4) of the following composition: HPO_4^{2-}, 7.5 mM; $H_2PO_4^-$, 15 mM; Cl^-, 137 mM; Ca^{2+}, 1 mM; K^+, 7.5 mM; Na^+, 157 mM; glucose, 10 mM. The buffer was oxygenated with O_2–CO_2 (95:5, by vol.), for 15 min prior to use. A mixture of bile salts was used, sodium taurocholate–sodium taurodeoxycholate (4:1, on a molar basis).

Two stock solutions of 100 mM monoolein and [1-^{14}C]oleic acid plus unlabelled carrier to make oleic acid 100 mM were made up in chloroform. The specific activity of

oleic acid was measured. Appropriate volumes of the stock solutions were evaporated to dryness under N_2. Appropriate amounts of bile salts were added in 5 ml of aerated buffer and the mixture insonated with a Branson Sonifier until all the oil was emulsified. Buffer was added in approx. 5-ml aliquots, with insonation between additions, until the final volume was reached. In the final solution, oleic acid and monoolein concentrations were 1 mM respectively. Triether concentration varied from 0 to 7 mM. Bile salt concentration was either 1, 4 or 5 mM.

Uptake

5 ml of the solution containing emulsified lipid and bile salts was pipetted into a 10-ml round bottomed stoppered flask and shaken in a water bath at 35° at a rate of 140 cycles/sec. After allowing time for temperature equilibration, freshly prepared sacs were added and incubated for 30 min.

Extraction and counting

At the end of an uptake experiment, the sac was washed out with 0.15 M NaCl and drained of serosal fluid. The mucosa was scraped off and homogenized in a glass homogenizer (Kontes Glassware), using chloroform–methanol (2:1, by vol.) as solvent.

Thin layer chromatography (Silica gel G, 0.25 mm thickness) was performed on an aliquot of extract from each sac, in the system hexane–diethyl ether–glacial acetic acid (80:20:2, by vol.). The lipids were divided in 3 fractions (i) triglycerides plus cholesteryl esters; (ii) free fatty acid; (iii) partial glycerides, cholesterol and phospholipids. Silica gel was scraped from the glass plates and lipid eluted with chloroform–methanol.

The scintillant used was 10 ml of the mixture 4 g PPO and 0.05 g 1,4-bis-(2-(4-methyl-5 phenyloxazoyl)- benzene in 1 l toluene. Quench correction was made by the channels ratio method[9].

Ultracentrifugation

To determine the isotropic concentration of fatty acid, 9-ml aliquots of solutions of lipid were pipetted into cellulose nitrate tubes. The tubes were placed in a type 30.2 rotor and spun at $7 \cdot 10^7 \times g$ min in a Beckmann L2-65 preparative ultracentrifuge The temperature during spins was $30 \pm 2°$.

After centrifugation 5–6 ml of the clear lower phase was removed from each centrifuge tube. An aliquot was taken and extracted by solvent partition in ethanol–petroleum ether–diethyl ether (1:1:1, by vol.)[10]. Solvents were evaporated under N_2, scintillant was added and the sample was counted. Solubilization was calculated by expressing the amount of radioactivity in an aliquot of spun solution as a percentage of that in a similar aliquot of unspun solution. Isotropic concentration of oleic acid was calculated by multiplying percentage solubilization by molar concentration in the unspun solution. The isotropic concentration of bile salts was unaffected by varying the volume of unabsorbable oil.

RESULTS

Increasing oil phase in the absence of micellar phase, bile salts 1 mM

The concentration of labelled fatty acid in the isotropic phase and the rate of uptake did not alter significantly when the oil phase was increased from 0.5 to 1.5 mM

triether, Table I. Hydrolysis of monoolein, liberating unlabelled fatty acid into the incubate and so altering the specific activity, was not a significant factor. Although Table I might suggest decreasing incorporation of fatty acid label into mucosal triglyceride when the volume of oil phase was increased, the mass of label incorporated was small and did not vary significantly between groups. Optical sizing of emulsion particles with a phase contrast microscope and image-splitting eyepiece indicated that the emulsion was finer with 0.5 mM triether than with 1.0 or 1.5 mM triether. In no emulsions were particles greater than 3 μm apparent diameter. For 0.5 mM triether there were fewer particles of greater than 0.5 μm diameter than for either 1.0 or 1.5 mM triether.

TABLE I

Effect of increasing oil phase on uptake of [1-^{14}C]oleic acid in the absence of a micellar phase. All emulsions contained 1 mM [1-^{14}C]oleic acid, 1 mM monoolein, 1.0 mM bile salt and triether concentration as indicated. Concentration of oleic acid in the isotropic phase was determined by ultracentrifugation at $7 \cdot 10^7 \times g$ min. Values represent means $\pm$ S.E. $N = 12$ for uptake values. For thin-layer chromatography $N = 8$, 8 and 6 for emulsions containing 0.5, 1.0 and 1.5 mM triether, respectively. PG, percentage of recovered ^{14}C present in thin-layer chromatography fraction corresponding to partial glycerides and phospholipid. TG, percentage of recovered ^{14}C present in thin-layer chromatography fraction corresponding to triglyceride and cholesteryl ester

Triether oil concn. (mM)	*Total fatty acid uptake (μmoles/g wet wt. tissue per 30 min)*	*Isotropic oleic acid concn. ($\cdot 10^{-5}$ M)*	*% total recovered radioactivity*	
			PG	*TG*
0.5	0.198 $\pm$ 0.088	0.49 $\pm$ 0.05	15.4 $\pm$ 0.9	33.1 $\pm$ 3.2
1.0	0.229 $\pm$ 0.047	0.44 $\pm$ 0.01	13.6 $\pm$ 0.9	22.7 $\pm$ 3.0
1.5	0.141 $\pm$ 0.016	0.49 $\pm$ 0.07	15.1 $\pm$ 0.8	16.1 $\pm$ 2.0

Thus below the critical micellar concentration, which was about 2.5 mM for the bile salt mixture used, uptake of fatty acid was not affected by volume of oil phase or size of oil emulsion particles which contained the bulk of the fatty acid. The concentration of fatty acid in the isotropic aqueous phase was very low and of the order expected for monomolecular solution (10^{-5} M).

Increasing oil phase in the presence of micellar phase

All the lipid mixtures in this group of experiments contained fatty acid and monoolein in micellar solution. For both bile salt concentrations it can be seen, Fig. 1, A and B, that the concentration of labelled fatty acid in the micellar phase decreased as the volume of inert oil phase, triether, was increased. A close correlation between uptake and solubilization curve is apparent. When uptake was plotted against isotropic concentration of labelled fatty acid the relationship was linear, as shown in Fig. 2, lines B and C.

Comparison with previous data

In previous experiments from this laboratory[1] the concentration of fatty acid in the aqueous phase was varied by varying the bile salt concentration above the critical micellar concentration, from 3 mM to 10 mM. In those experiments the tissue

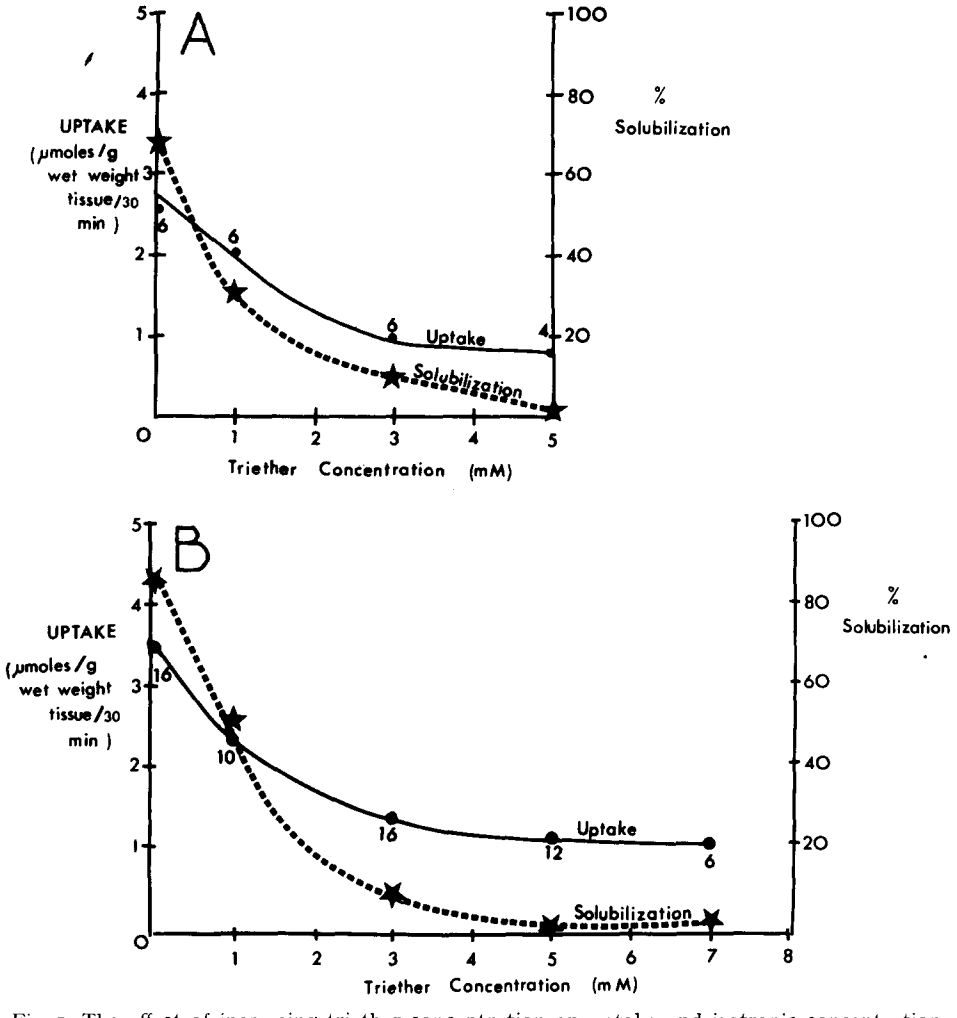

Fig. 1. The effect of increasing triether concentration on uptake and isotropic concentration of [1-^{14}C]oleic acid. A. 4 mM bile salt. B. 5 mM bile salt. Lipid compositions as in Table I. Figures in graphs represent number of experiments. Points represent means. S.E. too small to be plotted clearly.

was weighed after incubation and since incubation altered tissue weight in a predictable manner[11] a correction factor was applied to uptake figures. There was no significant difference ($P > 0.05$) between the regression line under these conditions (line A, Fig. 2) and the lines from the present experiments in which bile salt concentration was held constant at either 4 mM (line B, Fig. 2) or 5 mM (line C, Fig. 2).

Metabolism

The total incorporation of labelled fatty acid into triglyceride increased with rate of fatty acid uptake (Fig. 3). The increase was somewhat slower for uptake from bile salts, 4 mM than for bile salts, 5 mM. The difference was statistically significant ($P < 0.001$).

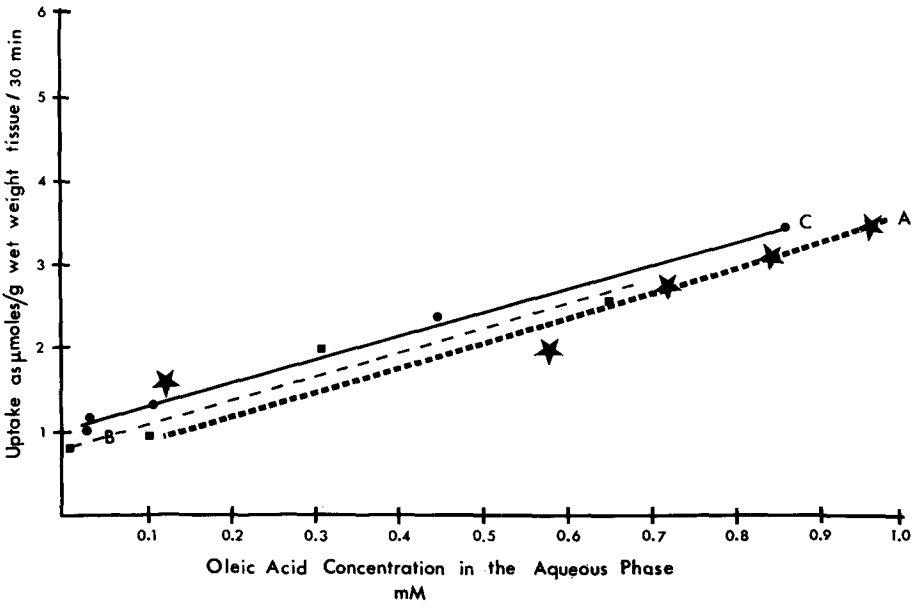

Fig. 2. The effect of increasing isotropic concentration of [1-^{14}C]oleic acid on uptake by everted sacs. Isotropic oleic acid varied in: A. by varying bile salt concentration from 3 to 10 mM. B. and C. by keeping bile salt concentration constant at 4 and 5 mM respectively, and varying triether concentration. The equations of the lines, with correlation coefficient in brackets, are: A, $Y = 1.1216 + 0.0230\,X$ (0.90); B, $Y = 0.8349 + 0.0279\,X$ (0.92); C, $Y = 1.0435 + 0.0286\,X$ (0.90).

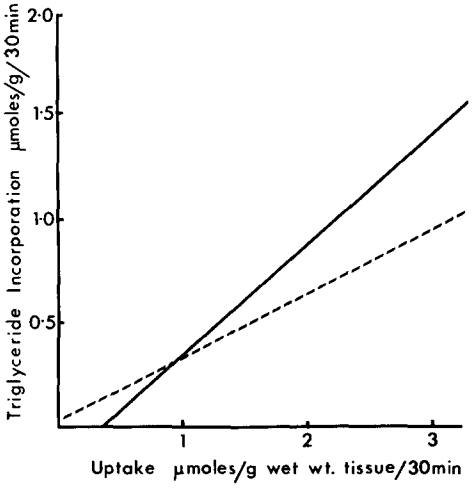

Fig. 3. The effect of increasing rates of uptake of [1-^{14}C]oleic acid on rate of incorporation into triglyceride. Two bile salt concentrations were used: 4 mM (– – – –) and 5 mM (———). The equations of the lines, with correlation coefficients in brackets, are: 4 mM, $Y = -0.0076 + 0.3521\,X$ (0.85); 5 mM, $Y = -0.1985 + 0.5952\,X$ (0.91).

DISCUSSION

The rate-limiting steps in uptake of fatty acid by everted sacs *in vitro* have not been identified. Three obvious possibilities are (i) diffusion through an unstirred

aqueous layer immediately preceding entry into the brush border; (ii) penetration of the brush border; (iii) maintenance of a low intracellular concentration of absorbed free fatty acid by incorporation into triglyceride and other esters. Bile salts could mediate an increased rate of uptake by influencing one or more of these steps. The present experiment and others from this laboratory suggest that the major and possibly the only influence of bile salts is on the diffusion-limited step.

If fatty acid uptake were diffusion-limited the following predictions could be made:

(a) Uptake should be linearly related to aqueous (isotropic) concentration in the incubation medium. This has been demonstrated under 3 sets of conditions (i) total fatty acid constant, solubilized fatty acid varied by altering bile salt concentration[1]; (ii) total fatty acid and bile salt concentrations both constant, aqueous fatty acid concentration varied by partition between micellar phase and unabsorbable oil phase (present experiments); (iii) bile salt concentration constant fatty acid concentration varied but always completely solubilized[12]. Moreover, as emphasized below, the linear relation of uptake to aqueous fatty acid concentration was unaffected by bile salt concentration.

(b) The gradient relating uptake to isotropic fatty acid concentration should be a function of the diffusion coefficient for fatty acid in the medium. Such a relationship was found when uptake gradient and diffusion coefficient were compared for fatty acid solubilized in a high molecular weight, non-ionic detergent (Pluronic F68) and in bile salts[13].

(c) The effect of temperature on uptake should be small, consistent with the low activation energy of aqueous diffusion, and metabolic inhibitors should not affect uptake. Independence of uptake from temperature or metabolic inhibition has been reported by others[11,14,15]. Experiments in this laboratory (HOFFMAN, unpublished) gave a temperature coefficient indicating an activation energy of -5.2 and -4.4 kcal/mole respectively for uptake from bile salt and non-ionic micelles, which is consistent with a diffusion-limited process.

Evidence such as that above suggests that any specific role of bile salts in uptake can only be accepted after taking into account the possibility of increased flux by micellar diffusion. It has been found that the relationship of uptake to aqueous concentration of fatty acid was independent of bile salt concentration. If bile salts specifically affected some other rate-limiting process such as permeability of brush border or intracellular metabolism, it would be expected that the line relating uptake and aqueous fatty acid concentration would be displaced upwards by increased bile salt concentration or that its slope would be altered. As previously noted, this was not the case. It is true that the slope of the line was less for uptake from a non-ionic detergent, but this was accounted for by a decrease in the overall diffusion coefficient of fatty acid in the medium[13]. Obviously little useful information on uptake mechanisms can be obtained when concentrations of fatty acid in the isotropic phase are not measured or when comparisons are made for a single isotropic concentration.

The above considerations minimize the possibility that bile salts increase uptake by increasing membrane permeability or by intracellular stimulation of esterification—at least if such effects are dependent on extracellular bile salt concentrations. They do not exclude the possibility that bile salts have an effect on intracellular metabolic processes which are not rate limiting for uptake. Observations suggest that

metabolic events in the cell may be related to the rate of uptake of free fatty acid. If so, this must be taken into account when evaluating a specific intracellular role of bile salts. In the present experiments it was shown that the rate of incorporation of labelled fatty acid into triglyceride varied with the rate of uptake when bile salt concentration remained constant. With bile salts 5 mM incorporation increased significantly more steeply with uptake than when bile salt concentration was 4 mM. This might suggest a specific effect of bile salts. On the other hand, however, previous work from this laboratory has shown that, for a given rate of uptake, esterification was the same from non-ionic detergent as from bile salts and that the maximum rate of incorporation into triglyceride was the same for the two detergents[13]. Such observations argue against a major specific role of bile salts apart from solubilization.

REFERENCES

1 N. E. HOFFMAN, *Biochim. Biophys. Acta*, 196 (1970) 193.
2 A. F. HOFMANN AND B. BORGSTROM, *Fed. Proc.*, 21 (1962) 43.
3 S. FELDMAN AND M. GIBALDI, *J. Pharm. Sci.*, 58 (1969) 425.
4 S. FELDMAN AND M. GIBALDI, *J. Pharm. Sci.*, 59 (1970) 579.
5 A. M. DAWSON AND K. J. ISSELBACHER, *J. Clin. Invest.*, 39 (1960) 730.
6 A. F. HOFMANN, *Acta Chem. Scand.*, 17 (1963) 173.
7 W. J. BAUMANN AND H. K. MANGOLD, *J. Org. Chem.*, 29 (1964) 3055.
8 W. J. BAUMANN AND H. K. MANGOLD, *J. Org. Chem.*, 31 (1966) 498.
9 R. W. HENDLER, *Anal. Biochem.*, 7 (1964) 110.
10 D. H. BLANKENHORN AND E. H. AHRENS, *J. Biol. Chem.*, 212 (1955) 69.
11 N. E. HOFFMAN, Doctoral Thesis, University of Western Australia, 1971.
12 N. E. HOFFMAN AND V. J. YEOH, *Biochim. Biophys. Acta*, in the press.
13 N. E. HOFFMAN AND W. J. SIMMONDS, *Biochim. Biophys. Acta*, in the press.
14 D. PORTE AND C. ENTENMAN, *Am. J. Physiol.*, 208 (1965) 607.
15 J. M. JOHNSTON AND B. BORGSTROM, *Biochim. Biophys. Acta*, 84 (1964) 412.