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Bile acid activity in the presence of dietary fibres, casein, calcium, phospholipid, fatty acid and cholesterol: factorial experiments *in vitro*

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Binding of ^{14}C-deoxycholic acid (DCA) in the presence of all combinations of digested wheat bran, casein tryptic peptides, Ca^{2+}, lecithin, cholesterol and stearate, and of ^{14}C-glycocholic acid in the presence of all combinations of pectin, Ca^{2+} and casein tryptic peptides has been studied *in vitro* using membrane microseparation. The level of free DCA was significantly lowered by all components individually, except for cholesterol. Large first-order interactions involving DCA–bran were obtained with casein peptide and lecithin, and a number of higher-order interactions were significant. Greatest significant binding involving wheat bran was observed with DCA–bran–casein peptide–lecithin (51·7%), DCA–bran–Ca^{2+}–lecithin–stearate (45%), and DCA–bran–Ca^{2+}–lecithin (42·5%), compared with DCA–bran (27·5%). Glycocholic acid was significantly bound by casein peptide with a small significant interaction with Ca^{2+}, but was not bound by pectin. These results suggest that possible health benefits, which arise through binding of secondary bile acids by cereal fibre, may be enhanced by other dietary components.

INTRODUCTION

Dietary fibre is able to bind bile acids, a property which is thought to be important to the protection which high-fibre diets provide against both cancer of the colon (Eastwood, 1987) and heart disease (Khaw & Barrett-Conner, 1987). In the colon, deconjugated secondary bile acids, such as deoxycholic acid (DCA) are thought to promote cancer (Reddy, 1986). In the terminal ileum the availability of conjugated bile acids from enterohepatic recycling to the liver is important in setting the degree of utilisation of hepatic cholesterol for bile acid replacement, thus influencing the size of the plasma cholesterol pool and, hence, the risk of atherosclerosis (Anderson *et al.*, 1990). Several *in vitro* studies have shown that bile acids bind to a range of fibres (Eastwood & Mowbray, 1976; Vahouny *et al.* 1980, 1981) and interaction with fibre in digesta from the small intestine of rats has recently been demonstrated (Ebihara & Schneeman, 1989).

Bile acids are amphophilic steroids (Coleman, 1987); they have the potential to interact with a large number of species within the intestine, including proteins, lipids and cations (Hofmann & Roda, 1984; Coleman, 1987), and many are capable of interacting among themselves. The action of any particular component of the digesta on bile acid binding to fibre may therefore be complex when influences of other components of digesta are taken into account. However, as yet, there has been little systematic study of the interactive effects of digesta constituents on bile acid binding to fibre in a manner which would allow systematic identification of the interactions.

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Apart from previously studied components of mixed micelles—including bile acids, cholesterol, phospholipids and fatty acids—binding of calcium is of relevance because of its postulated role in protecting against colon cancer through neutralisation of free fatty acids and bile acids as their insoluble salts (Newmark *et al.*, 1984). Conversely, binding of Ca^{2+} by dietary phosphoproteins (such as casein) or their fragments may increase the bioavailability of bile acids in the terminal ileum, leading to hypercholesterolaemia in susceptible species (van der Meer *et al.*, 1985). An appreciable quantity of undigested protein has been shown to reach the terminal ileum and colon in humans (Chako & Cummings, 1988) so that it has the potential to influence conditions at both sites within the intestine. Furthermore, consumption of dietary fibre has been shown to reduce protein digestibility in a number of studies (Gallaher & Schneeman, 1986).

This paper reports results of two experiments on the *in vitro* binding of bile acids to various digesta components, including dietary fibre, using physiological levels of digesta and buffer components. First, measurements were made of the binding of DCA (the main deconjugated bile acid in humans) to wheat bran and to other constituents of digesta, including phospholipids, cholesterol, stearic acid, Ca^{2+} and casein peptide in all combinations. Second, binding of glycocholic acid (the main conjugated bile acid in humans) was measured in the presence of a soluble fibre (pectin), Ca^{2+}, and casein peptide, in all combinations. Factorial designs were used, so that interactive effects on the levels of free bile acids could be systematically identified—an approach not hitherto used in studies of bile acid binding to dietary fibre. The aim of the present research was to determine whether some components of digesta have the potential to modulate the health benefits of fibre consumption, which derive from its capacity to bind bile acids.

MATERIALS AND METHODS

Materials

Deoxycholic acid (D2510), glycocholic acid (G2878), cholesterol (C8667), stearic acid (S4751) and pectin (polygalacturonic acid: P1879) were obtained from Sigma Chemical Co., St. Louis, MO, USA. ^{14}C-deoxycholate (200 μCi/ml), ^{14}C-glycocholate (200 μCi/ml) and ^{14}C-cholesterol (100 μCi/ml) were from Amersham Laboratories, Bucks., UK. Phospholipid used was defatted 95% soybean phospholipid (Emulpur-N) from Lucas Meyer, Hamburg, Germany. Casein peptides from tryptic digestion and gel filtration of lactic casein, were provided by the New Zealand Dairy Research Institute, Palmerston North, New Zealand.

Wheat bran was purchased at a local supermarket. It was stirred in hot distilled water (5 g/50 ml, 100°C, 5 min) to gelatinise the starch, filtered over sintered glass, and suspended in 50 ml amyloglucosidase (Sigma, A7255) solution (0·2% w/v) in acetate buffer (pH 5·0, 0·2 M, 30 min) to hydrolyse the residual starch. The bran was then filtered again and thoroughly washed with 1% NaCl solution (100 ml, three times) and distilled water (100 ml, three times) by suspension and refiltering. It was freeze-dried and ground to powder in a coffee mill before use in the binding experiments.

The binding studies were carried out in Centrisart I membrane separation units (Sartorius, Gottingen, Germany) using 10 000 mol. wt. cut-off membranes.

Binding studies

All binding experiments were conducted in triplicate using simple balanced factorial designs. Preliminary experiments established that the membranes were permeable to the bile acids but not to ^{14}C-cholesterol. The experiments were carried out using digesta and buffer components at levels approximately those of the colon (experiment 1) and ileum (experiment 2), respectively. Bile acid activity was measured as DPM/ml in aliquots of liquid recovered through the membrane, by scintillation counting in toluene–triton (2:1) containing omnifluor (6 g/litre).

Experiment 1: deoxycholate binding

This experiment involved six factors (cholesterol, lecithin, stearic acid, calcium chloride, casein peptide and digested wheat bran) at two levels (absent and present), giving a 2^6 factorial design in three replicated blocks.

DCA binding was measured in buffer pH 7·4 (Na_2HPO_3, 50 mM; acetate, 100 mM; NaCl, 100 mM). In tubes that were to contain cholesterol and/or stearic acid, the component was dissolved in ethanol before transfer to the tube, the solvent was removed under vacuum and 0·35 ml buffer was added per dried component to compensate, at the time of the binding study. Final concentrations used were DCA (spiked with ^{14}C-DCA to $76{\cdot}6 \times 10^3$ DPM/ml), 5 mM; cholesterol, 250 μM; lecithin, 625 μM; stearic acid, 500 μM; calcium chloride, 10 mM; casein peptide, 10 mg/ml; and digested wheat bran, 16 mg/ml; in a final volume of 2·5 ml of buffer. The tubes were incubated with gentle shaking for 4 h at 37°C, and then centrifuged at 1500*g* for 80 min before aliquots of filtrate were removed for scintillation counting.

Experiment 2: glycocholate binding

This experiment involved three factors (calcium chloride, casein peptide and polygalacturonate) at three levels, giving a 3×3 factorial design.

Binding studies were carried out as above, using the following components in all combinations in buffer

Table 1. Levels of significance for interactive effects of digesta constituents on deoxycholate activity, from analysis of variance

	Bran	Calcium	Casein peptide	Lecithin	Cholesterol	Stearate
Main effects	0·001	0·001	0·001	0·001	NS[a]	0·001
First-order interactions						
Bran	—	0·02	0·001	0·001	NS	0·01
Calcium			NS	0·002	NS	NS
Casein peptide				0·03	NS	NS
Lecithin					0·03	NS
Cholesterol						NS
Second-order interactions						
Bran–calcium			NS	0·005	NS	0·02
Bran–casein peptide				0·006	NS	NS
Calcium–lecithin						0·04

[a]NS = not significant ($p > 0·05$).

(pH 7·4; 50 mM Na_2HPO_4, 100 mM NaCl). Concentrations were glycocholic acid, 2·7 mM; $CaCl_2$, 5 and 10 mM; casein peptides, 5 and 10 mg/ml; and polygalacturonate, 10 and 20 mg/ml. The glycocholate was spiked with ^{14}C-glycocholate to $34·9 \times 10^3$ DPM/ml.

Statistical analysis

Results were subjected to analysis of variance to identify significant main effects and interaction, using the SAS statistical package.

RESULTS AND DISCUSSION

Significant effects of digesta constituents on DCA binding from Experiment 1 were identified by analysis of variance (Table 1). All individual components, except for cholesterol, caused a significant reduction in DCA activity, although the effects of Ca^{2+} and stearate were small. The results in Table 2—treatment means for significant effects—show that interactions involving wheat bran generally caused a greater decrease in DCA activity than other combinations of components, except for casein-lecithin. This finding supports earlier studies that showed bile acid adsorption to dietary fibre (Kritchevsky & Story, 1986), and the extent of binding was of the same order as that observed in a previous study of DCA binding to wheat bran (Story *et al.*, 1982).

The action of individual soluble components which limit DCA movement through the membrane can be explained as the result of formation of aggregates larger than the 10000 mol. wt. exclusion limit of the membranes used. Micelles that DCA forms with lecithin and stearic acid, which reduced filterable DCA, are larger than those which it forms with cholesterol (Coleman, 1987), which did not affect filterable DCA activity. The bile acids in the absence of other components were not excluded by the membrane, because they are relatively soluble in water, and on their own form small micelles (Carey, 1985).

Calcium–DCA interaction was evident, the most obvious explanation being a lower solubility product for the Ca^{2+} salt of DCA than for its sodium salt. How-

Table 2. Treatment means for significant effects of digesta constituents on deoxycholate activity

Treatment	Mean activity (DPM × 10^{-3}/ml)		% Decrease in activity
	(−)	(+)	
Main effects			
Bran	51·6	37·4	27·5
Calcium	45·2	43·7	3·3
Casein peptide	49·7	39·2	21·1
Lecithin	48·9	40·0	18·2
Stearate	45·0	43·9	2·4
First-order effects			
Bran–calcium	52·9	37·2	29·7
Bran–casein peptide	59·0	34·4	41·7
Bran–lecithin	57·6	34·7	39·8
Bran–stearate	52·7	37·3	29·2
Calcium–lecithin	50·3	39·8	20·9
Casein peptide–lecithin	54·6	35·1	35·7
Cholesterol–lecithin	49·6	40·0	19·2
Calcium–stearate	46·4	43·7	5·8
Second-order effects			
Bran–calcium–lecithin	60·2	34·6	42·5
Bran–calcium–stearate	55·2	37·1	32·8
Bran–casein peptide–lecithin	65·9	31·8	51·7
Calcium–lecithin–stearate	52·1	39·7	23·8
Third-order effect			
Bran–calcium–lecithin–stearate	63·4	34·9	45·0
Overall mean	44·4		
LSD	0·83 ($p < 0·05$)		

Table 3. Treatment means and levels of significance for effects of digesta constituents on glycocholate activity

Treatment		Mean activity (DPM × 10^{-3}/ml)		% Change	Significance
		(−)	(+)		
Main effects					
Pectin	5 mg/ml)	27·6	27·9	—	NS[a]
	(10 mg/ml)	27·6	28·0	1·5	$p < 0·05$
Calcium	(5 mM)	28·1	27·7	—	NS
	(10 mM)	28·1	27·9	—	NS
Casein peptide	(5 mg/ml)	35·7	24·3	31·9	$p < 0·001$
	(10 mg/ml)	35·7	23·7	33·6	$p < 0·001$
First-order interaction					
Casein peptide (mg/ml)	*Calcium (mM)*				
0	0	35·36		—	
	5	35·70		0·96	
	10	35·91		1·50	
5	0	24·48		30·8	
	5	24·03		32·0	$p < 0·05$
	10	24·28		31·3	
10	0	24·32		31·2	
	5	23·28		34·2	
	10	23·63		33·2	
Overall mean:	27·9				
LSD ($p < 0·05$):	0·39				

[a]NS = not significant ($p > 0·05$)

ever, as Ca^{2+} promotes micelle formation, the effect may also have been the result of aggregate formation, particularly since the colonic pH used was below that optimal for micelle formation by unconjugated bile acids in the absence of Ca^{2+} (Hofmann & Roda, 1984; Carey, 1985).

A significant effect of casein peptides on DCA binding was measured. Casein, like the bile acids, is amphophilic; about 35% of its amino acid residues, such as phenylalanine, valine, leucine and isoleucine, possess hydrophobic side chains, and a similar proportion, including aspartic acid, glutamic acid and lysine, are highly charged. Monomeric bile salts are known to have a strong affinity for some proteins, involving both the hydrophobic and hydrophilic domains (Coleman, 1987). A proportion of the peptides derived from partial digestion of casein are likely to retain the amphophilic character of the parent molecule and, consequently, the ability to interact with a range of amphophiles, including bile acids such as DCA.

Treatment means of significant interactions (Table 2) show that binding of DCA by wheat bran was enhanced by lecithin and casein. Significant interactions, those in which the binding of DCA by wheat bran increased substantially from 27·5% of DCA–bran, were DCA–bran–casein peptide (41·7%), DCA–bran–lecithin (39·8%), DCA–bran–Ca^{2+}–lecithin (42·5%), DCA–bran–casein peptide–lecithin (51·7%) and DCA–bran–Ca^{2+}–lecithin–stearate (45%). One can only speculate on the precise mechanisms behind the observed changes in binding, given the possible number of bonding permutations. However, the involvement of lecithin suggests that aggregation of micelles on the hydrophobic surfaces of bran preparation was occurring. Wheat bran is also amphophilic, the carbohydrate fraction being polar, but with a high proportion of lignin in which the condensed phenylpropanoid units confer hydrophobicity.

The *in vitro* data presented in Tables 1 and 2 suggest that, *in vivo*, a reduction in levels of unbound secondary bile acids in the colon due to dietary fibre may be enhanced by the presence of some other digesta components, including casein peptides. Because dietary protein reaches the colon (Chako & Cummings, 1988), it is possible that fibre-rich diets or products may be modified to increase the protection which they provide against colonic cancer, by the inclusion of components such as low-fat dairy products. Any such benefits will be limited by the susceptibility of peptides to bacterial degradation in the colon, which may in turn be affected by binding to resistant fibre.

Results of experiment 2, on the effects of pectin, casein peptide and Ca^{2+} on filterable glycocholate activity, are shown in Table 3. Casein had a much greater effect than any other component; this was enhanced to a small but significant extent by Ca^{2+}. The ileal digestibility of casein is about 0·9 (Maccoll & James, 1988) so that in the terminal ileum casein-derived peptides may be present at high enough levels to affect free bile acid concentrations *in vivo*, given the strong *in vitro* effect.

The lack of a main effect of Ca^{2+} indicates that precipitation of glycocholate as its insoluble calcium salt was not an influential factor under the conditions used here. The slight cooperative effect of Ca^{2+} and casein peptides is, however, of interest, as it is not consistent with the suggestion that phosphoserine residues (abundant in casein) lead to a decrease in free glycocholate levels because they bind casein to insoluble calcium phosphate, and thereby reduce availability of Ca^{2+} for precipitation of glycine-conjugated bile acids (van der Meer *et al.*, 1985). It is possible that the observed opposite effect—a decrease in glycocholate activity—occurred because calcium has a charge-neutralising effect, allowing stronger net attraction of glycocholate to casein peptides, or even the formation of phosphoserine–Ca^{2+}–glycocholate complexes, in much the same way that Ca^{2+} may stabilise associations between pectin polymer chains.

Binding of glycocholate to pectin was not observed, although binding of taurocholate to pectin in the presence of Ca^{2+} has been reported recently (Koseki *et al.*, 1987), and a reduced uptake of bile acids, as a result of binding to fibre, has been posited as a mechanism underlying the hypocholesterolaemic effects of pectin and other soluble fibres (Anderson *et al.*, 1990). The present experiment does not shed light on the role of viscosity in limiting the rate of bile acid reabsorption from the gut.

Health implications of the binding effects noted here are difficult to deduce from the *in vitro* situation, and will depend largely on their *in vivo* influence on availability of the various species for absorption by the intestinal epithelium. Formation of mixed lipid/bile acid micelles is part of normal lipid digestion, in which micellar components are dynamic and readily available. Effects on binding to non-digestible materials such as dietary fibre, which maintain their presence in the gut, are likely to be more important.

The simple experiments reported here have identified a number of significant effects of selected digesta constituents on binding of a conjugated primary and a deconjugated secondary bile acid. Further research could now be aimed at determining the extent to which the theoretically desirable binding effects may be enhanced by manipulation of constituent levels *in vitro*, and at prediction and testing for *in vivo* effects which may be of practical benefit to health.

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