

The effect of *Gypsophila* saponins in the diet on mineral status and plasma cholesterol concentration in the rat

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1. Immature, male Wistar rats were allocated to one of six groups and caged individually. The first group was given a semi-synthetic diet containing 38 mg iron and 55 mg zinc/kg (basal group). The second and third groups were given a diet containing 10 mg Zn and 12 mg Fe/kg respectively (low-Zn and low-Fe groups). Groups four, five and six were given similar diets containing 20 g *Gypsophila* saponins/kg. After 21 d the Fe and Zn status of the rats was estimated and plasma cholesterol concentration determined.

2. Measurements of whole blood haemoglobin concentration, packed cell volume and liver Fe stores indicated that rats in the basal + saponin and low-Fe + saponin groups had a significantly reduced Fe status when compared with their controls. Rats in the low-Zn + saponin group also showed a trend toward reduced Fe stores.

3. Zn status, as judged by femur Zn concentration, was not adversely affected by the inclusion of *Gypsophila* saponins in the diet.

4. Consumption of the saponins resulted in a significant reduction in blood cholesterol concentration, with rats in both the low-Fe groups having significantly lower concentrations than their basal and low-Zn counterparts.

5. In view of suggestions that the consumption of saponins should be encouraged because of their ability to lower blood cholesterol, possible effects on Fe metabolism should be investigated further, particularly with respect to the levels and sources of saponin in the human diet.

Saponins are glycosides which occur widely in a range of plants and are present in many foods consumed by animals and man (Cheeke, 1971). In view of the emphasis being placed on increasing the consumption of plant foods, the intake of saponins is also expected to increase. It has been suggested that increased consumption of saponins might be beneficial to some sections of the population because of the ability of many saponins to lower serum cholesterol markedly; elevated plasma cholesterol levels being a significant risk factor in the aetiology of cardiovascular disease (Malinow *et al.* 1981; Oakenfull, 1981).

There is, however, extensive evidence that saponins can also have deleterious effects, in that the inclusion of some saponins into feeds leads to reduced growth in poultry and simple-stomached mammals (Petersen, 1950; Cheeke *et al.* 1978). The mechanism by which growth is retarded is uncertain but it has been suggested that the primary effect is due to reduced palatability of the food (Cheeke *et al.* 1983), and indeed the consumption of feeds containing saponin is often associated with reduced food intake (Gaunt *et al.* 1974).

An alternative explanation is that the presence of saponins at high concentrations may limit the availability, for absorption or metabolism, of essential micro-nutrients. It has been reported, for example, that certain saponins form insoluble complexes with iron and zinc *in vitro*, and so may reduce the proportion of dietary mineral which is available for absorption *in vivo* (West *et al.* 1978). Fe and Zn deficiencies, like many other mineral deficiencies, can result in poor growth and development and in disorders of appetite. The present study was therefore undertaken to determine whether there is any indication that dietary saponins reduce mineral utilization in the rat model. The source of saponin for the study was a *Gypsophila* root extract which consists of a mixture of acidic triterpenoid saponins similar in structure to those found in a variety of foods consumed by humans (Price *et al.* 1987a).

In view of evidence that some human diets are of low mineral concentration and poor

mineral availability (Southon *et al.* 1988), the investigation included diets which were low in Fe or Zn, in addition to a normal mineral-replete diet. Effects which may not be apparent in animals maintained on optimal mineral levels, may become important when the diet is marginal in this respect.

MATERIALS AND METHODS

Animals and diets

Sixty immature, male Wistar rats (approximately 100 g) were randomly divided into six groups of ten and caged individually. The first group was given a semi-synthetic diet containing 38 mg Fe and 55 mg Zn/kg; this was designated the basal group. For the second and third groups the mineral mix was adjusted to give final dietary concentrations of 10 mg Zn and 12 mg Fe respectively, while maintaining basal levels of all other minerals; these groups were designated the low-Zn and low-Fe groups respectively. For groups four, five and six similar diets were prepared containing 20 g *Gypsophila* saponins/kg, isolated from the roots of *Gypsophila* sp. (Sigma Chemical Co., Poole, Dorset); these groups were designated the basal+saponin, low-Zn+saponin, and low-Fe+saponin groups respectively. The composition of the diet was similar to that previously described (Southon *et al.* 1985) with the addition of 2.5 g methionine/kg diet. All rats received distilled water *ad lib*. Preliminary studies showed that rats found the diets containing saponin less palatable than the normal control diet, particularly over the first 4–5 d of feeding, after which intake rose to approximately 80% of the control value. To determine the effect of the saponin *per se* on mineral status and blood cholesterol concentration it was necessary to control food intake so that all animals consumed the same amount. Rats received 5 g food/d for the first 2 d, 10 g/d for the next 2 d, followed by 12 g/d rising to 20 g/d towards the end of the experiment. Allowance was made each day for any spillage of food. All animals were killed in random order on day 21 and Fe and Zn status estimated.

Experimental

Rats were deeply anaesthetized by intraperitoneal injection of sodium pentobarbital (160 mg/ml; 1 ml/kg body-weight) and killed by cervical dislocation. The abdomen was opened and a sample of blood (2.5 ml) obtained from the vena cava and transferred to a heparinized vial. The liver was excised, rinsed in ice-cold saline (9 g sodium chloride/l distilled water), blotted dry and weighed.

The whole liver and femur were dried at 80° for 24 h, reweighed and ground to a fine powder. Weighed samples were ashed at 470° for 48 h, the ash dissolved in a minimum volume of concentrated hydrochloric acid (11.5 M-HCl; Analar; BDH) and, after further dilution with distilled water, analysed for Zn and Fe by atomic absorption spectroscopy.

Haemoglobin (Hb) concentration was measured in samples (40 μ l) of whole blood by the cyanmethaemoglobin method (Cannon, 1958). Approximately 50 μ l blood was taken up into a heparinized heamatocrit tube for the determination of packed cell volume (PCV), which was expressed as a proportion of total blood volume.

Total cholesterol concentration was determined in plasma using the Boehringer CHOD-PAP method (Boehringer Corporation Ltd, Lewes, Sussex). Samples (20 μ l undiluted plasma or standard cholesterol solution, range 0–10.3 mmol/l in propan-2-ol) were incubated at 20–25° with Test System Reagent for 10 min, and the absorbance at 500 nm measured against a reagent blank within 1 h. Results were expressed as μ mol cholesterol/ml plasma.

Isolation and partial characterization of Gypsophila saponin

Crude saponin from Sigma Chemical Co. was purified by means of reversed-phase flash chromatography (Price *et al.* 1987*b*). Crude saponin (1 g) was dissolved in water (25 ml) and eluted through a column of octasilane bonded to silica gel (40 g) (J. T. Baker Chemical Co. Phillipsburg, N.J.) successively with water (375 ml) and redistilled Analar methanol (375 ml). The methanol fraction was collected and evaporated under reduced pressure which gave a saponin mixture representing 604 g/kg starting material. The aqueous fraction was freeze-dried. Portions of each were dissolved in aqueous methanol and subjected to reversed-phase high-performance thin layer chromatography (RP-HPTLC) using octadecyl silane-bonded silica (100 × 100 mm; BDH-Merck, Poole) and developed for 55 mm with a solvent mixture comprising methanol-water (3:2, v/v). Seven spots with R_f values ranging from 0.24 to 0.54 were observed for the methanol 'saponin' fraction, while the aqueous 'non-saponin' fraction contained only one spot running close to the solvent front.

Further analysis of these two fractions were carried out using the products of their acid hydrolysis. A portion of each fraction (25 ml) was hydrolysed using 50 ml HCl/1 methanol (4 ml) for 3 h under reflux conditions, neutralized with alkali (0.880 ammonia) and extracted with ethyl acetate (3 × 5 ml). The combined ethyl acetate layers were dried over sodium sulphate, evaporated to dryness and converted to their trimethylsilyl derivatives using bis-trimethylsilyltrifluoroacetamide (200 μ l) in pyridine (200 μ l) at 50° for 30 min. Each sample was analysed by gas-liquid chromatography using a Perkin-Elmer Sigma 3B chromatograph fitted with a 1 m × 2 mm i.d. column containing 3% OVI on diatomite CO (100–200 mesh) with a flow-rate of 35 ml/min and oven temperature of 280°. No derivatives of an acid-released aglycone were detected in the aqueous fraction while a major peak in the methanol fraction was identified from its retention time, and by subsequent coupled gas-liquid chromatography-mass spectrometry, as gypsogenic acid, the aglycone known to be present in the *Gypsophila* saponins (Luchanskaya *et al.* 1971). Due to the lack of knowledge on the composition of the individual *Gypsophila* saponins and hence the carbohydrate:gypsogenic acid ratios, no absolute value for saponin purity can be given from the information quoted. However, all the evidence suggests that the methanol fraction contains only saponin, while the aqueous phase contains compounds which do not exhibit chromatographic or chemical properties typical of saponins.

Assuming therefore that the commercially supplied saponin was 60.4% pure, the concentration of *Gypsophila* saponin in the diets consumed by the 'saponin' groups of rats was 12 g/kg.

Statistical analysis

The significance of difference between means was estimated by Student's *t* test for unpaired comparisons.

RESULTS

In a preliminary feeding trial it was found that the daily food intake of rats given diets containing 20 g crude *Gypsophila* saponins/kg diet was initially only 60% of the value for control animals, rising to 80–90% after approximately 5 d. In order to assess the effect of the saponin *per se* on mineral status and blood cholesterol levels, it was necessary to limit the food intake of the control rats. All rats were given the same amount of food each day, which was estimated from preliminary food intake values and the total food intake for each animal over the 21 d experimental period was 303 g.

The body-weight gain of the basal + saponin and low-Fe + saponin rats over this period

Table 1. *Body-weight gain, haemoglobin (Hb), liver iron and femur zinc concentrations, and packed cell volume (PCV) for rats given diets with and without crude Gypsophila saponins (20 g/kg diet)†*
(Values are means with their standard errors for ten rats)

Dietary treatment‡...	Basal		Basal + saponin		Low-Fe		Low-Fe + saponin		Low-Zn		Low-Zn + saponin	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body-wt gain (g)	134	2	131	2	133	2	131	2	134	3	126*	1
Hb (g/l)	125	3	119	3	76	2	70	3	127	2	122	2
PCV	0.401	0.008	0.363*	0.016	0.259	0.007	0.248	0.006	0.401	0.005	0.393	0.007
Liver fresh wt (g)	10.1	0.2	9.2*	0.2	9.1	0.2	8.9	0.3	9.8	0.2	9.3	0.3
Liver Fe (μ g/g dry wt)	282	20	246	18	124	3	113*	3	288	19	264	21
Total liver Fe (g)	849	55	672*	55	344	14	303*	10	825	48	753	56
Femur Zn (μ g/g dry wt)	220	10	211	5	228	7	242	10	155	6	158	9

* Mean values were significantly different ($P < 0.05$) from those for rats given a similar diet without saponin.

† Crude extract 60.4% pure.

‡ For details of dietary treatment, see p. 50.

Table 2. Total cholesterol concentration in plasma from rats given diets with and without *Gypsophila saponin*
(Values are means with their standard errors for ten rats)

Dietary treatment‡	Cholesterol ($\mu\text{mol/ml}$ plasma)	
	Mean	SE
Basal	2.76	0.11
Basal + saponin	2.28*	0.06
Low-Fe	2.44†	0.08
Low-Fe + saponin	1.89*†	0.04
Low-Zn	2.80	0.10
Low-Zn + saponin	2.49*	0.09

* Mean values were significantly different ($P < 0.05$) from those for rats given a similar diet without saponin.

† Mean values significantly different ($P < 0.05$) from basal and low-Zn counterparts.

‡ For details of dietary treatment, see p. 50.

was not significantly different from that of rats given a similar saponin-free diet, but the weight gain of the low-Zn + saponin animals was lower than that of their controls, despite the pair-feeding regimen (Table 1).

Measurements of whole-blood Hb concentration, PCV and liver Fe stores indicated that rats in the basal + saponin and low-Fe + saponin groups had a reduced Fe status when compared with their controls. The basal + saponin group, which received an adequate level of dietary Fe, had significant reductions in PCV and liver Fe stores, compared with rats given a similar diet without saponin. The decrease in total liver Fe was partly related to a reduction in liver fresh weight, but mean liver Fe concentration was also slightly lower (Table 1). Rats consuming the low-Fe diet with added saponin had significant reductions in both liver Fe concentration and total liver Fe, compared with their controls (Table 1). There was no significant change in PCV or liver Fe stores in the low-Zn + saponin animals, but mean values for liver Fe concentration and total liver Fe were lower than those for the low-Zn group (Table 1).

Zn status, as judged by femur Zn concentration, was not adversely affected by the inclusion of 12 g *Gypsophila* saponins/kg diet. It was noted, however, that the Zn concentration in femurs taken from the low-Fe + saponin rats was significantly higher ($P < 0.05$) than that for rats given a mineral-replete diet containing the saponin (Table 1).

Inclusion of *Gypsophila* saponins in the diet resulted in a significant reduction in the blood cholesterol level in each of the three dietary groups. This hypocholesterolaemic effect appeared to be greater when the saponin was incorporated into the low-Fe diet; reductions in cholesterol concentration being 11, 17 and 23% in the low-Zn + saponin, basal + saponin and low-Fe + saponin groups respectively. Cholesterol concentration was also influenced by dietary Fe concentration, since rats in the low-Fe and low-Fe + saponin groups had significantly lower concentrations than their basal and low-Zn counterparts (Table 2).

DISCUSSION

Recently, it has been strongly suggested that consumption of foods rich in saponin, or supplements of the purified component, could be beneficial in terms of reducing hypercholesterolaemia, aortic atherosclerosis and deposition of tissue cholesterol in man

(Malinow *et al.* 1980). However, there is some indication in the literature that certain saponins may adversely influence mineral absorption. West *et al.* (1978) clearly demonstrated that Fe and Zn are complexed and precipitated by lucerne (*Medicago sativa*) root saponin *in vitro*, whilst animal studies showed that feeding lucerne plant saponins increased faecal Fe and magnesium loss in rats (Cheeke, 1980), and decreased plasma calcium and Zn in pigs (Pond & Yen, 1984). The present study was undertaken, therefore, to determine whether there was any indication of impaired mineral status in rats following prolonged consumption of diets containing a level of saponin generally considered to have little or no toxic effect (Gaunt *et al.* 1974; Reshef *et al.* 1976). Since Fe and Zn appeared to be strongly complexed by certain saponins (West *et al.* 1978), the investigation concentrated on these two essential minerals.

Chemically pure saponins are difficult to obtain in amounts necessary to conduct long-term feeding experiments, consequently the material used in the present study was a commercially available extract of *Gypsophila* root which consists of a mixture of acidic triterpenoid saponins and is of variable purity. The material used in the present study was determined to contain 604 g saponins/kg crude extract.

The body-weight gain of animals in the study was generally similar, when food intake was equalized between the 'saponin' and 'non-saponin' groups. There was, however, a small but significant growth depression in rats given saponin incorporated into a low-Zn diet. A reduction in the availability of Zn to the body, coupled with the lower Zn concentration of the diet given to the low-Zn groups of animals, might be expected to result in some growth retardation in young rats. However, femur Zn concentration indicated that the Zn status of each of the three 'saponin' groups of rats was as good as their respective control group, and possibly even better in the case of the low-Fe + saponin rats. At present it is difficult to interpret the difference in growth between the low-Zn and low-Zn + saponin animals, but it seems unlikely to be associated with a reduction in Zn absorption.

Rats consuming saponin added to a low-Fe and a mineral-replete diet showed evidence of a significant reduction in Fe status compared with their control group and, although values for the low-Zn and low-Zn + saponin groups were not significantly different, both the mean liver Fe concentration and total liver Fe were lower in the animals consuming saponin. The haemolytic properties of saponins are well known, but it is generally agreed that they are not readily absorbed, and that the normal environment of the erythrocytes counteracts their haemolytic activity (Bondi *et al.* 1973; Cheeke, 1980). It is more likely, therefore, that the lower Fe status observed in rats consuming saponins resulted from reduced Fe absorption, rather than a metabolic disturbance caused by saponins entering the bloodstream. One possible explanation for differences in absorption as suggested by the work of West *et al.* (1978) is that acidic triterpenoid saponins form complexes with dietary Fe thus rendering it unavailable for absorption. Alternatively, it is possible that the long-term consumption of these saponins produces changes in mucosal function such that Fe transport is impaired. It is well-known that saponins have a high affinity for cholesterol and so can become incorporated into the structure of the cell membrane to form stable pore-like structures (Seeman, 1974). *Gypsophila* saponins readily render permeable intestinal absorptive cells *in vitro*, and destroy their capacity for the accumulation of sugars (Johnson *et al.* 1986). It is possible therefore that dietary saponins may exert a similar effect in the intact animal, and hence reduce the efficiency of nutrient absorption, in which case it would be expected that Zn as well as Fe absorption would be affected. Since there was no indication of reduced Zn status in the 'saponin' groups of rats it appears that the first of these two possibilities is the more likely.

The present study also demonstrates a hypocholesterolaemic effect of *Gypsophila*

saponins, which was apparently influenced by the Fe content of the diet, or possibly the Fe status of the animal itself. The hypocholesterolaemic property of certain saponins is well documented (Cheeke, 1971) but the significant reduction in plasma cholesterol observed in rats given the 'saponin-free' low-Fe diet compared with the other control groups, and the increased potency of the saponin when incorporated into this diet, is puzzling and suggests an interesting saponin-mineral interaction which has not previously been documented.

In the context of the present study the effect of saponin on mineral status was limited to Fe. In view of suggestions that the consumption of saponins by man should be positively encouraged, and the fact that frank and occult Fe deficiency is a relatively common problem in the human population (Monsen *et al.* 1978), it is important that the nature of this effect is investigated further, particularly in relation to the levels and sources of saponin in the human diet. Obtaining pure samples of saponin from any source is difficult (Price *et al.* 1987*a*), consequently crude extracts, or foods rich in saponin, are routinely used to prepare experimental diets. The possibility that the 'non-saponin' fraction of these materials is in some way responsible for the physiological effects reported in the present and other studies has been considered, but thought unlikely. Nevertheless, future work will take account of this possibility.

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