Effects of dietary iron deficiency and tungsten supplementation on ^{59}Fe absorption and gastric retention from ^{59}Fe compounds in rats

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1. In vivo ^{59}Fe absorption from intrinsically labelled Fe-containing fractions of liver and blood were measured in rats by intragastric dosing. All rats were fed on a low-Fe diet for 3 d before dosing in order to standardize the Fe status of the intestinal mucosal cells.

2. An increase in digestion time from 2 to 12 h increased ^{59}Fe absorption ($P < 0.01$) from all fractions except ferritin.

3. Fe-deficient rats when compared with essentially Fe-replete rats showed decreased gastric retention for all fractions, but increased ^{59}Fe absorption over 2 h only from ferritin. Ferritin showed several unusual absorption characteristics.

4. Dietary tungsten supplementation of Fe-deficient rats reduced the ferroxidase activity of intestinal mucosal xanthine oxidase. In addition, gastric retention and ^{59}Fe absorption ($P < 0.05$) from all fractions were increased.

The high iron availability from meat is widely recognized (Martinez-Torres & Layrisse, 1971), but the exact mechanism and form of meat-Fe absorption and an understanding of the 'meat effect' on non-haem-Fe absorption are still unclear (Hazell *et al.* 1978). Whilst the high Fe availability from meat is often assumed to be due to the presence of haem-Fe from haem proteins, there exists also a substantial proportion of non-haem-Fe in meat, i.e. 26·7% in beef to 71·5% in chicken (Hazell, 1982). Earlier work had suggested that the Fe from the non-haem fractions from muscle (ferritin and haemosiderin) was poorly absorbed in rats (Bogunjoko *et al.* 1983; Latunde-Dada & Neale, 1986), but in these studies test-meal-protein concentrations were not constant and this could have affected Fe absorption dramatically (Bogunjoko *et al.* 1983). The present studies therefore, extend this earlier work with the various Fe fractions, and in particular investigate in rats the rate of Fe absorption from the different liver and blood fractions and the effect of mucosal Fe setting (Fairweather-Tait & Wright, 1984) on Fe absorption. In addition, earlier studies of Topham *et al.* (1982) have shown dietary tungsten supplementation to have a specific inhibitory effect on small intestinal mucosal ferroxidase activity, and their studies provide indirect evidence for a decrease in Fe absorption being linked to decreased ferroxidase activity. The present studies extend the work of Topham *et al.* (1982) to look more directly at the role of W on Fe absorption from various meat fractions in both Fe-replete and Fe-deficient rats, and in particular investigate the possibility that the effects on absorption so observed by Topham *et al.* (1982) were due to changes in gastric retention (stomach emptying) of oral Fe and not mucosal effects on absorption.

METHODS

Preparation of test meals

Male specific pathogen-free (SPF) Wistar rats (approximately 150 g) were injected intraperitoneally with approximately 25 μCi ^{59}FeCl$_3$ (Amersham International plc, Amersham, Bucks). The FeCl$_3$ solution was neutralized with sodium hydroxide and supplemented with approximately 5 mg ascorbic acid before injection to aid Fe solubility.

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After 7 d feeding on standard laboratory rat chow the rats were killed and the livers excised, pooled and homogenized in twice their weight of distilled water. Following centrifugation for 20 min at 17000 g, the residue was resuspended in distilled water and the homogenization and centrifugation steps repeated twice. The residue finally obtained was termed the 'haemosiderin-containing fraction' and is subsequently referred to as 'haemosiderin' for ease of reference, although other compounds were probably also present. The pooled supernatant fractions were dialysed at 4° for 4 d against distilled water using dialysis tubing of molecular weight cut-off 12000–14000 (Medicell International Ltd, London) to separate out the low-molecular-weight Fe compounds (volume of supernatant fraction: dialysing water, 1:35). The dialysate was concentrated by rotary vacuum evaporation at 30°. The solution remaining within the dialysis sacs was heated to 80° to precipitate out haem proteins leaving a ferritin-containing fraction, subsequently referred to as ferritin. Unheated haem proteins, prepared from blood, were used for the dosing. Cardiac blood cells were washed by suspending and centrifuging in saline (9 g sodium chloride/l) several times. The erythrocytes were lysed by suspending in distilled water and, following a final centrifugation to pellet stroma, a solution of haemoglobin was obtained. This haemoglobin solution was assumed to be representative in behaviour also of myoglobin which contributes significantly to total meat haem protein.

Dilutions of each fraction were performed with water so that the mean ^{59}Fe activity measured in 1 ml portions were the same between fractions.

The dry weights of the four fractions thus prepared were assumed to be equivalent to their protein content. Bovine serum albumin (BSA) was added as required to each diluted fraction to standarize dry weight and, therefore, protein concentrations at 20·4 g/l. The ^{59}Fe activity:protein ratios in all samples were therefore equal, although the total Fe:protein ratios were different. Fe:protein (mg Fe/g protein) in test meals fed to rats were as follows: haemoglobin 22·9, haemosiderin 13·1, ferritin 142·1, low-molecular-weight Fe compounds 98·0.

The composition of the low-Fe diet (7 mg Fe/kg) is shown in Table 1. The Fe-replete diet (approximately 300 mg Fe/kg) was prepared by the additional inclusion of 1·50 g finely ground $FeSO_4.7H_2O$/kg to the low-Fe diet which is approximately the same as the Fe content of the standard laboratory chow used in Nottingham. This compares with other commercial rat diets (e.g. Oxoid, Pilsbury) which contain approximately 200 mg Fe/kg.

The low-Fe, W-supplemented diet was prepared by the additional inclusion of 0·70 g sodium tungstate/kg low-Fe diet (0·39 g W/kg diet) as also used by Topham *et al.* (1982).

Pretreatment of experimental animals

Weanling SPF rats were used throughout and fed *ad lib.* on the relevant diet up until the time of dosing, according to the following regimens, to give three pretreatment dietary groups:

(1) Fe-deficient rats were fed on the low-Fe diet for 3 weeks and rendered Fe-deficient.

(2) Essentially-Fe-replete rats were fed on the Fe-replete diet for 18 d followed by the low-Fe diet for 3 d (3 d on a low-Fe diet does not deplete the liver Fe stores but ensures the mucosal Fe status is unchanged from the Fe-deficient group, the potential importance of which is outlined by Fairweather-Tait *et al.* (1985)).

(3) Fe-deficient, W-supplemented rats were fed on the low-Fe, W-supplemented diet for 3 weeks.

Intragastric dosing and measurement of ^{59}Fe absorption

Rats of 105–151 g body-weight from the different pretreatment dietary groups were dosed under light diethyl ether anaesthesia with 3 ml samples of ^{59}Fe-labelled haemoglobin,

Table 1. *Composition (g/kg) of the low-iron diet given to rats*

Casein	167
Glucose	333
Maize starch	432
DL-Methionine	1
Low-Fe mineral mix*	56
Vitamin mix†	11

* Mineral mix (g/kg): NaCl 303, KH_2PO_4 303, $CaCO_3$ 297, $MgSO_4.7H_2O$ 91·9, $MnSO_4.4H_2O$ 4·48, KI 0·584, $ZnSO_4.7H_2O$ 1·95, $CuSO_4.5H_2O$ 0·370, $CoCl_2.6H_2O$ 0·019.

† Vitamin mix (g/kg): thiamin 1, riboflavin 1, pyridoxine 1, calcium pantothenate 6, nicotinic acid 20, inositol 40, *p*-aminobenzoic acid 60, biotin 0·1, folic acid 0·5, vitamin B_{12} (in mannitol) 5, choline 120, menadione 0·1, Rovimix A_{500} 2·0, Rovimix D_3 500 1·5, Rovimix E_{50} 15, maize starch 727.

haemosiderin, ferritin or low-molecular-weight Fe compounds using flexible rubber catheter tubing (Chiltern Surgical Ltd, Bucks). After 2 or 12 h digestion periods the rats were killed by asphyxiation with carbon dioxide, and a 20 μl cardiac blood sample was removed for the determination of blood haemoglobin concentration. After ligaturing, the stomach, small intestine and large intestine were excised and placed on ice in separate vials. The ^{59}Fe (γ) activity of each sample was measured using a Scaler Timer ST7 (Nuclear Enterprises, Edinburgh), incorporating a demountable detector assembly with a 75 mm diameter and 75 mm well-type sodium iodide crystal.

Apparent ^{59}Fe absorption was calculated as:

$$\frac{(\text{initial } ^{59}\text{Fe dose} - \text{gastric } ^{59}\text{Fe} - \text{intestinal } ^{59}\text{Fe})}{\text{initial } ^{59}\text{Fe dose}} \times 100,$$

where intestinal ^{59}Fe is the sum of the ^{59}Fe activity in the small and large intestines. Some ^{59}Fe may remain in the stomach at the end of the digestion period and, since it is not available for absorption until it passes into the duodenum, a correction must be made to calculate the true percentage ^{59}Fe absorption from the available ^{59}Fe. Apparent ^{59}Fe absorption thus equals true absorption only if gastric emptying is complete.

$$\text{True \% } ^{59}\text{Fe absorption} = \frac{(\text{initial } ^{59}\text{Fe dose} - \text{gastric } ^{59}\text{Fe} - \text{intestinal } ^{59}\text{Fe})}{(\text{initial } ^{59}\text{Fe dose} - \text{gastric } ^{59}\text{Fe})} \times 100.$$

Percentage gastric retention was calculated as:

$$\frac{\text{gastric } ^{59}\text{Fe}}{\text{initial } ^{59}\text{Fe dose}} \times 100.$$

^{59}Fe absorption is true ^{59}Fe absorption unless apparent ^{59}Fe absorption is specified.

There was some variation between fractions and between treatments in the absolute amount of Fe given in the various test meals. This was not caused intentionally, but arose as a result of the primary need to keep protein contents as constant as possible for each test meal, as they varied considerably in their Fe:protein ratios. In addition the variation in Fe content with the same type of Fe fraction was caused by ^{59}Fe decay over time, which necessitated higher amounts of Fe to be given. The work of Bannerman (1965) demonstrated constant percentages of Fe absorbed over a wide range of Fe doses of both haem- and non-haem-Fe in Fe-deficient rats, and suggests that Fe absorption mechanisms are not saturated at these low dose levels. Variations described here in Fe dose given are insignificant in interpretation of the true effects on percentage Fe absorption, assuming all Fe fractions are absorbed in a linear manner as described by Bannerman (1965).

Determination of blood haemoglobin concentration

This was achieved by the cyanmethaemoglobin method (Kampen & Zijlstra, 1961) using a kit from Boehringer Corporation (London).

Determination of mucosal ferroxidase activity

The mucosal ferroxidase (*EC* 1.16.3.1) activity was determined for each rat. Following measurement of the ^{59}Fe activity in the small intestine, the contents of the lumen were gently flushed out using 30 ml distilled water and the entire length of the small intestine slit open. The mucosal cells were gently scraped off using a microscope slide and homogenized in an Ika-Werk turrax homogenizer in approximately 2 ml *N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer at pH 7·5 (0·05 M) for 45 s. The mucosal samples were then centrifuged at 115000 *g* for 1 h at 4°. The intestinal ferroxidase being almost exclusively contained in the supernatant fraction, the ferroxidase activity was determined at room temperature using a Unicam SP1800 ultraviolet spectrophotometer and Unicam AR25 linear recorder according to the method of Topham *et al.* (1981). All solutions were prepared using deionized, distilled water and all glassware was acid-treated to prevent trace Fe contamination. Protein was determined using a method based on that of Lowry *et al.* (1951).

Statistics

Statistical analysis of the results for true Fe absorption was carried out by analysis of variance, with approximate *t* values calculated from the standard error of the difference of the means using the residual mean square.

RESULTS

Table 2 compares the true and apparent ^{59}Fe absorption and ^{59}Fe gastric retention between various meat fractions and the different dietary pre-treatments. The Fe-deficient rats were moderately anaemic and showed some differences in ^{59}Fe absorption compared with the essentially Fe-replete animals. The ^{59}Fe absorption from ferritin was higher ($0·01 > P > 0·001$) in Fe-deficient rats, although the absorption of ^{59}Fe from haemoglobin, haemosiderin and low-molecular-weight Fe did not differ significantly between groups of rats. Gastric retention was much higher in Fe-replete rats, which makes comparison of the apparent ^{59}Fe absorptions difficult. In general the absorption of haemoglobin-Fe in rats was shown to be significantly lower ($P < 0·01$) compared with the other non-haem-Fe fractions, although unpublished results (G. E. Shears, R. J. Neale and D. A. Ledward) showed that addition of a 20-fold excess of protein in the form of BSA to the haemoglobin fraction, such that the test-meal composition resembled meat more closely, significantly increased ^{59}Fe absorption by greater than 100 %, indicating that protein aids the release and possible chelation of Fe from haemoglobin.

Comparison of ^{59}Fe absorption from the Fe compounds in the Fe-deficient rats over a 2 and 12 h period revealed that, although gastric retention was expected to be reduced after 12 h, it was unchanged with ferritin and showed an anomalous increase with the low-molecular-weight fraction.

As expected true ^{59}Fe absorption increased significantly ($P < 0·01$) in all cases between the 2 and 12 h time-period, except for the ferritin fraction. This agrees with studies using Fe-deficient rats by Bogunjoko *et al.* (1983), although their work measured apparent ^{59}Fe absorption. The percentage of the total 12 h ^{59}Fe absorption occurring within the first 2 h is shown in Fig. 1. Ferritin had the highest and haemoglobin the lowest absorption in the first 2 h.

Table 2. *Blood haemoglobin (Hb), ^{59}Fe absorption and gastric ^{59}Fe retention 2 or 12 h after intragastric dosing of various ^{59}Fe-labelled Fe-containing compounds in rats with different dietary pre-treatments**

(Values are means with standard errors for four rats except where otherwise stated)

Dietary pre-treatment	Iron compound†	Blood Hb (g/l)		Apparent ^{59}Fe absorption (%)		True ^{59}Fe absorption (%)		Gastric ^{59}Fe retention (%)		Dose Fe given (μg)
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Essentially Fe-replete, 2 h digestion	Hb	148	4	5·4	0·4	9·3	1·0	41·2	2·3	1·4
	Hs	143	4	13·4	0·5	35·9	8·5	56·3	9·8	0·9
	F	144	1	11·0	0·9	18·4	2·4	37·5	7·4	8·7
	LMW	—	—	19·3	1·7	52·0	6·3	61·0	5·8	6·0
Fe-deficient, 2 h digestion	Hb	94	7	4·8	0·6	6·9	0·9	27·1	10·7	1·4
	Hs	97	10	30·8	3·9	37·6	1·8	19·0	7·1	0·8
	F	97	5	29·1	2·3	32·4	2·4	10·2	2·8	21·3
	LMW	110	5	47·5	6·4	49·5	6·1	4·5	2·2	17·4
Fe-deficient, 12 h digestion	Hb‡	95	5	29·6	4·2	29·6	4·2	1·1	0·5	1·4
	Hs	104	4	53·2	5·9	56·9	5·3	6·8	3·9	0·8
	F	105	6	31·5	8·0	32·8	6·8	9·3	9·0	21·3
	LMW	98	8	58·4	5·2	67·2	1·3	13·5	6·4	17·4
Tungsten-supplemented, Fe-deficient, 2 h digestion	Hb‡	92	7	8·6	1·6	14·2	2·2	38·7	6·7	1·4
	Hs	86	9	22·6	2·9	45·8	2·3	50·7	5·6	0·9
	F	78	7	13·5	1·6	42·0	1·7	68·0	2·3	8·7
	LMW	83	7	34·6	4·4	69·5	5·8	49·4	7·8	6·0

Hs, haemosiderin; F, ferritin; LMW, low-molecular-weight Fe compounds.

* For details of pretreatment dietary groups and diets, see p. 574 and Table 1.
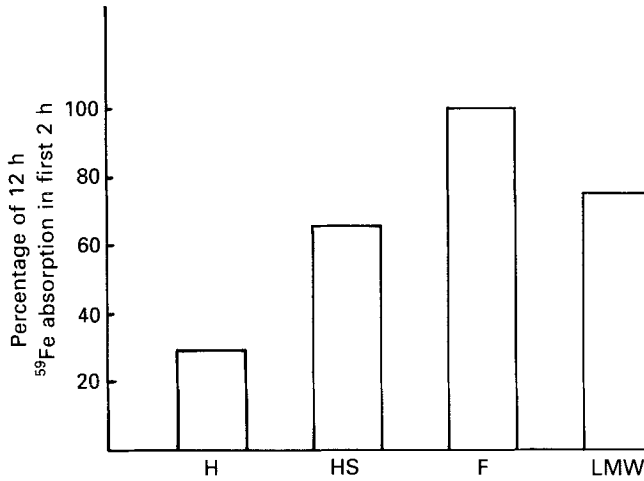
† For details, see p. 574.

‡ Three rats.

Fig. 1. Mean percentage of 12 h true ^{59}Fe absorption taking place in the first 2 h from various ^{59}Fe-labelled Fe compounds in Fe-deficient rats. For details of dietary regimen and diet, and procedures for determining ^{59}Fe absorption, see p. 574. Hb, haemoglobin; HS, haemosiderin; F, ferritin; LMW, low-molecular-weight Fe compounds.

Table 3. *Small intestinal mucosal ferroxidase (EC 1.16.3.1) activity in essentially iron-replete, Fe-deficient and tungsten-supplemented Fe-deficient rats†*

(Values are means with their standard errors; no. of rats in parentheses)

Fe status of rats	Small intestinal mucosal ferroxidase activity (μM Fe(III)-transferrin/ min per mg protein)	
	Mean	SE
Essentially Fe-replete	2·67	0·39 (4)
Fe-deficient	2·27	0·18 (3)
W-supplemented, Fe-deficient	1·50*	0·06 (4)

* Mean value was significantly different from the other values ($P < 0.05$).
† For details of dietary regimens and diets, see p. 574 and Table 1.

Analysis of variance showed that true ^{59}Fe absorption was significantly higher for all fractions ($P < 0.01$) in the Fe-deficient, W-fed rats compared with the non-W-fed group. Lack of correction for gastric retention showed that apparent ^{59}Fe absorption was lower in the W-fed rats. This shows how very important it is to correct for gastric retention, using this technique, to obtain the true effects on Fe absorption. Table 3 shows that W supplementation reduced ferroxidase activity by 34 and 44% compared with Fe-deficient and essentially Fe-replete rats respectively.

DISCUSSION

The interpretation of these results revolves around the understanding that the percentage ^{59}Fe absorbed is constant for any given form of Fe for all doses of Fe given up to 200 μg

total Fe. While we have not confirmed this in our studies, extensive work by Bannerman (1965) showed that in Fe-deficient rats the percentage absorption of dietary Fe remained constant for both haem- and non-haem Fe over the 0–200 μg Fe dose range. This implies that saturation of the Fe absorption mechanism(s) does not occur in Fe-deficient rats up to this level (200 μg), and varying amounts of total dietary Fe will be absorbed freely up to a point when eventually saturation kinetics will begin to be shown. Within this dose range a change in percentage Fe absorption affected by either Fe status, type of dietary Fe given or presence of W must reflect a change in affinity of the absorption mechanism(s) for Fe.

The relation between ^{59}Fe absorption over the 2 h compared with the 12 h digestion periods for various fractions of meat Fe (Table 2 and Fig. 1) is complex. The early phase of Fe absorption is extremely important for the non-haem forms of Fe with, for example, most of the ferritin-Fe disappearing within 2 h. This agrees with some of the earlier work of Wheby & Crosby (1963) who showed a rapid and delayed phase of Fe absorption, 60–80 % of total Fe absorption taking place in the first 2 h (Fig. 1). Unfortunately the total Fe contents of the test meals were not constant, and further work is required to measure the kinetics of uptake of Fe from these various chemical forms, as affected both by total Fe given in the test meal and the time characteristics of absorption. It is quite clear from this and previous work (Wheby & Crosby, 1963; Bogunjoko *et al.* 1983) that the absorption characteristics of Fe-replete rats (not standardized as regards mucosal setting) differ very markedly from both essentially Fe-replete and Fe-deficient rats, in particular in regard to the percentage Fe absorption over the various time-periods.

This important role of mucosal Fe setting on Fe availability was earlier described by Fairweather-Tait & Wright (1984) and Fairweather-Tait *et al.* (1985) using whole-body counters for rats to determine bioavailability of Fe. It is quite clear, however, that our technique, which essentially measures Fe absorption, also demonstrates the role of mucosal setting to low-Fe diets on subsequent Fe absorption. Thus compared with Fe-deficient rats the essentially Fe-replete rats with low gut Fe status but normal reserves showed similar Fe absorption for all meat fractions except ferritin, which was significantly greater ($P < 0.01$) in Fe-deficient rats. These studies demonstrate, therefore, that at least in the rat, mucosal Fe setting (status) appears to exercise far greater control over Fe absorption than body Fe stores. Alternate high-Fe and then low-Fe diets given to rats over periods of weeks or months could create a potentially hazardous situation for the Fe-replete animal, leading to possible Fe overload (or toxicity). It is not known if this occurs but unpublished results from our laboratory (G. E. Shears, R. J. Neale, D. A. Ledward and M. Davenport) show that the levels of mucosal transferrin (which are thought to be involved in the control of Fe absorption at the mucosa) peak 3 d after a low-Fe diet is given to Fe-replete rats, and then subsequently decline. Further work is needed to investigate the effect of low-Fe diets on the control of Fe absorption in the intestine in Fe-replete rats.

Gastric retention over the short (2 h) time-periods was in general much smaller for Fe-deficient rats compared with essentially Fe-replete rats (Table 2), i.e. stomach emptying was faster in Fe-deficient rats in these studies. This is in contrast to the earlier work of Bogunjoko *et al.* (1983) who showed delayed stomach emptying in Fe-deficient rats compared with Fe-replete rats, a phenomenon which was suggested as being advantageous for the Fe-deficient rat in maximizing Fe absorption. The present study and that of Bogunjoko *et al.* (1983) are not entirely comparable, however, as in the latter study the Fe-replete rats were fasted, the Fe content of the previous diet was not standardized and the protein contents of the test meals were dissimilar. The exact significance of the change in stomach emptying in Fe-deficiency anaemia remains to be elucidated.

Effect of W

Fe is absorbed as ferrous-Fe but transported in the blood bound to transferrin as ferric-Fe (Seelig, 1972). It is, therefore, possible that the mucosal ferroxidase activity (the ability to convert Fe^{2+} to Fe^{3+} is important in Fe absorption by aiding the binding of Fe to plasma transferrin and increasing the transport of Fe to the blood. W inactivates the mucosal ferroxidase activity by specifically inhibiting the incorporation of molybdenum into newly synthesized enzyme (Johnson et al. 1974). Topham et al. (1982) claimed that the effect of W in rats is extremely specific and no signs or symptoms of toxicity occur.

W supplementation produces an effective Mo deficiency, which interferes with the absorption of Fe resulting in a secondary anaemia. That this appears to have occurred in the W-supplemented animals is seen in Table 2. Mean blood haemoglobin concentrations in the rats given the low-Fe diet were consistently above 93 g/l blood, whereas the W-supplemented rats all showed haemoglobin concentrations lower than this value. As the decrease in blood haemoglobin concentrations seen previously in Fe-deficient rats compared with essentially Fe-replete rats did not (with the exception of ferritin) result in enhanced ^{59}Fe absorption, it can be seen that the decrease in blood haemoglobin concentration cannot alone be an explanation for the effect of W.

W supplementation reduced the ferroxidase activity to 66% of its control value in Fe-deficient rats (Table 3). An inhibition of apparent ^{59}Fe absorption occurred with W supplementation (in agreement with Topham et al. 1982), but after correcting for the increased gastric retention occurring with all fractions the true ^{59}Fe absorption values increased for all fractions ($P < 0.05$).

As this increase in true ^{59}Fe absorption accompanied an inhibition of ferroxidase activity, the exact role, and indeed importance, of ferroxidase in the regulation of Fe absorption, at least in the manner suggested by Topham et al. (1982), is questioned. The mechanism by which W brought about increases in gastric retention is unknown, but it is clear that the importance of considering the effects on apparent absorption values of varying degrees of gastric retention produced under different experimental conditions cannot be over-emphasized. The experiments of Topham et al. (1982) were possibly affected by changes in gastric retention, of which no account was taken.

The possibility that the distal region of the small intestine has enhanced Fe absorptive capacity in Fe-deficient rats (Wheby et al. 1970; Bogunjoko et al. 1983) suggests also that a digestion period substantially longer than the 10 min used by Topham et al. (1982) would be beneficial should any such change in regional absorptive capacity occur with W-supplemented animals. Temporary unexplained changes in gastric retention occurring 2 h after intragastric dosing have also been reported by Fields et al. (1986) with complex carbohydrate and simple sugars.

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