Iron availability from meat

BY T. HAZELL, D. A. LEDWARD AND R. J. NEALE

Food Science Laboratories, Department of Applied Biochemistry & Nutrition, University of Nottingham, Sutton Bonington, Loughborough, Leics. LE12 5RD

(*Received* 20 *May* 1977 – *Accepted* 7 *October* 1977)

1. The distribution of radioactive iron in ^{59}Fe-labelled rat muscle extract was determined using ge filtration. This showed that most (approximately 70%) of the radioactivity was associated with the heamatin compounds; myoglobin and haemoglobin.

2. Raw beef and freeze-dried rat muscle were digested in vitro, under simulated physiological conditions, and after centrifugation the supernatants fractionated by gel filtration. The soluble products were haematin Fe complexes of molecular weight above 10000 and non-haematin Fe compounds of molecular weight below 6000, the major products being the non-haematin Fe complexes. The soluble compounds were also separated by dialysis and, in rat muscle, it was found that the low-molecular-weight non-haematin compounds accounted for more than 80% of the total soluble iron.

3. In vivo absorption studies with rats showed the Fe in a digested muscle dialysate to be more readily absorbed than that from an aqueous muscle extract which itself was more readily absorbed than the Fe from whole blood.

4. It may not, therefore, be the haemoproteins per se which are responsible for the high availability of Fe in meat, but rather the nature of their degradation products, formed by digestion within the meat environment.

It is well established that meat is an important dietary source of iron (Moore, 1961; Heinrich, Gabbe, Kugler & Pfau, 1971; Layrisse & Martinez-Torres, 1971). The absorption of Fe from meat has been widely tested by measuring haemoglobin absorption (Walsh, Kaldor, Brading & George, 1955; Callender, Mallett & Smith, 1957; Turnbull, Cleton & Finch, 1962; Conrad, Benjamin, Williams & Foy, 1967; Hallberg & Solvell, 1967; Heinrich, Gabbe & Kugler, 1971). This is presumably because haemoglobin is a uniform, well-defined compound that is easily labelled and isolated and, as a haemoprotein, can be said to typify much of the Fe in meat. These studies have led to the general belief that the superior availability of meat Fe compared with that from plant foods is due to a special mechanism involving the uptake into, and possible transfer of, intact haematin across the intestinal mucosa (Conrad, Cortell, Williams & Foy, 1966; Conrad, Weintraub, Sears & Crosby, 1966; Wheby, Suttle & Ford, 1970); the haematin complex being a digestion product of meat myoglobin and haemoglobin (Jacobs, 1976). Naish, Kimber & Deller (1973) have looked at the digestion of liver Fe, but little information is available concerning the physio-chemical changes caused by digestion of meat or 'muscle' Fe in whole tissue and the effect these changes might have upon the availability of Fe for absorption. The present study was designed to gain insight into the nature of the Fe compounds that could form during the in vivo digestion of meat and the possible relationship between these products and Fe availability.

MATERIALS AND METHODS

Preparation of labelled feeding material

The haemoproteins of rat blood and muscle were labelled by tail vein injection of about 1 ml containing 50 μCi ^{59}Fe citrate (2·7 μg Fe/ml) (Radiochemical Centre, Amersham, Bucks.) into 60 g male Wistar rats and allowing them to grow to 250–300 g before slaughter. ^{59}Fe-labelled meat was obtained by pooling post-mortem samples of limb, back and abdominal muscles. ^{59}Fe-labelled blood was obtained from the descending aorta. The meat

and blood from the rats gave respective counts of approximately 300 and 360 counts/min above background, per μg Fe. The meat and blood were freeze-dried to facilitate storage and subsequent solubilization.

Chemical analysis

^{59}Fe concentrations were determined by γ-counting (Nuclear Enterprises Model ST3 γ-counter) and total Fe contents by atomic absorption spectrophotometry (EEL 240). Spectral scans from 250 to 290 nm and 390 to 430 nm were used to estimate the protein (peak approximately 280 nm) and haemoprotein (peak approximately 410 nm) concentrations of solutions.

Distribution of radioiron in rat muscle

20 g of freeze-dried rat muscle was shaken for 2 h with 200 ml of distilled water and then centrifuged at 4000 ***g***. The supernatant was concentrated by freeze-drying and redissolved in 0·4 M-phosphate buffer (pH 7·0).

The soluble meat preparation was fractionated on Sephadex G100 (Pharmacia Fine Chemicals, Uppsala, Sweden) (height 270 mm, bed volume 100 ml and flow rate about 12 ml/h) equilibriated with 0·4 M-phosphate buffer (pH 7·0). Fractions were collected by counting 52 drops (approximately 1·4 ml) and analysed for ^{59}Fe, protein and haemoprotein. The column was calibrated with ferritin, haemoglobin and myoglobin.

In vitro digestion

A typical digestion procedure involved mixing either fresh or freeze-dried finely ground meat with approximately 0·01 M-HCl to yield a slurry containing about 15% protein at pH 2·0–2·5 to which pepsin was added in the ratio of about 1 part to 100 parts protein. Following gentle agitation at 37° for several hours (6–24 h), the pH of the slurry was adjusted to 7·0–7·5 with dry sodium bicarbonate. Trypsin (enzyme:protein approximately 1:500) and pancreatin (approximately 1:100) were added and the resulting mixture gently agitated at 37° for several hours (12–48 h). Finally the supernatant and undigested residue were separated by centrifugation at approximately 2000 ***g***. (All proteolytic enzymes used were porcine in origin and obtained from the Sigma Chemical Company, Kingston-upon-Thames, Surrey).

The supernatants from the beef digests were very viscous and some of the proteins were therefore precipitated by the addition of an equal volume of saturated ammonium sulphate (< 2 ppm iron) and the diluted supernatant dialysed free of salt and low molecular weight components (including the non-haematin Fe compounds), concentrated by freeze-drying and a concentrated but non-viscous solution made up for fractionation. This procedure was followed in preference to direct dilution as the haemoproteins survive this salting out procedure far better than the non-haematin containing proteins. No dilution or ammonium sulphate treatment of the extract from rat muscle was necessary.

The supernatants were fractionated on either Sephadex G-50 or Sephadex G-75. For Sephadex G-75 the column (height 400 mm, bed volume 150 ml, flow rate approximately 22 ml/h) was eluted with 0·05 M-Tris-HCl buffer (pH 7·0) and fractions were collected at 5 min intervals (volume 5·3 $\pm$ 0·2 ml). For Sephadex G-50 the column (height 400 mm, bed volume 220 ml, flow rate approximately 15 ml/h) was eluted with 0·4 M-potassium orthophosphate (pH 7·0) and 3·2 ml fractions were collected. The fractions were analysed for protein, haemoprotein and ^{59}Fe. Both columns were calibrated with standard proteins (e.g. myoglobin, chymotrypsinogen and glucagon).

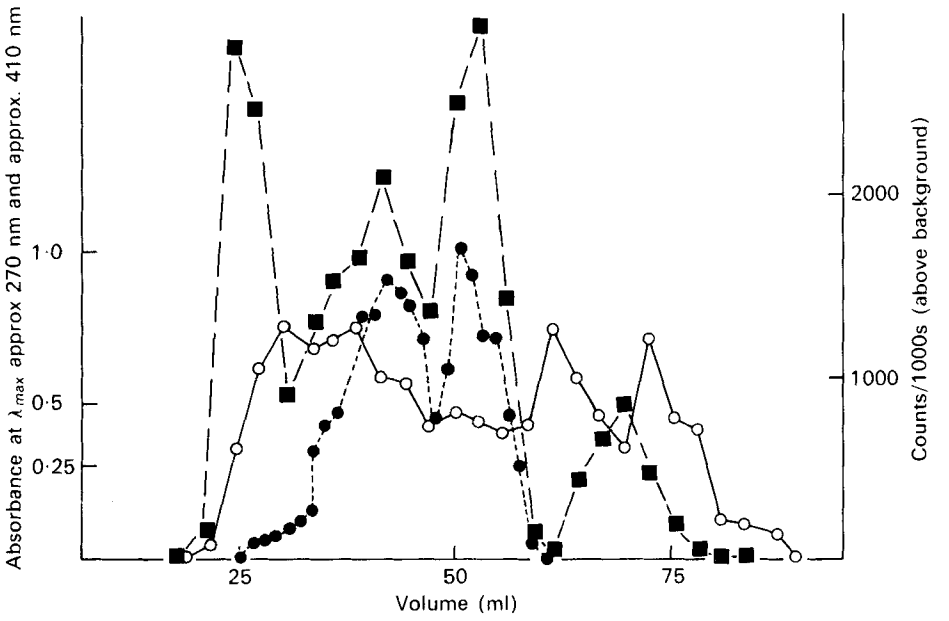

Fig. 1. Chromatogram of 2 ml of concentrated aqueous rat muscle extract obtained from 20 g of freeze-dried muscle, fractioned on Sephadex G100. The fractions were scanned from 390 to 430 nm for haemoproteins (●– –●), λ_{max} approximately 410 nm and from 250 to 290 nm for protein (○—○), λ_{max} varying from 265 to 280 nm and counted with a γ-counter for ^{59}Fe activity (■—■). The background count was 6550 for 1000 s.

In vivo absorption of ^{59}Fe

After digestion of rat muscle in vitro and dialysis of the supernatant against distilled water, the diffusate, which contained the low-molecular-weight ^{59}Fe complexes, was concentrated by freeze-drying. After solubilization in water and centrifugation at 100000 ***g*** for 30 min equal 1 ml quantities of radioactivity from this extract, a simple aqueous extract of rat muscle (prepared by shaking 1 g of ground freeze-dried muscle with 10 ml water and centrifuging) or a solution of freeze-dried whole blood were injected into ligated 100 mm loops of duodenum from unstarved adult rats (approximately 300 g) in vivo under Nembutal anaesthesia for periods of 0, 15, 30, 60 and 120 min. At the end of the time periods, loops were excised, the contents washed out with distilled water and both loop and contents counted for ^{59}Fe. The disappearance of ^{59}Fe from the ligated loops was used as the measure of ^{59}Fe absorption. All samples prepared for loop injection contained 3·5 μg Fe/ml and gave readings of about 660 counts/min (the background count being about 330 counts/min).

RESULTS

Characteristics of labelled rat muscle

Two assays were performed to determine the distribution of radioactive Fe between the Fe compounds of a rat muscle extract, in which at least 60% of total muscle iron was extracted. In both cases, the recovery of radioactivity was about 86%. Fig. 1 demonstrates that the radioactivity is distributed between four main components. About 21% of the activity is associated with the first peak corresponding to the exclusion volume of the column (molecular weight > 100000) and presumably consists mainly of ferritin and transferrin. The two intermediate radioactive peaks account for about 68% of the ^{59}Fe and correspond to

haemoglobin and myoglobin as shown by their coincidence with the two 410 nm absorbance peaks, the second peak corresponding to haemoglobin (molecular weight approximately 68000) and the third to myoglobin (molecular weight approximately 17000). The last radioactive peak accounts for 11% of the radioactivity and represents low-molecular-weight iron compounds.

Chemical analysis showed that 24% of Fe (approximately 7 μg) was present in the high-molecular-weight fractions, 62% (approximately 19 μg) was associated with the intermediate molecular weights (the haemoproteins) and 14% (approximately 4 μg) of the Fe was present in the low-molecular-weight fractions.

In vitro digestion

The percentage of Fe in the supernatants after digestion varied from experiment to experiment, the average recovery from rat muscle being around 60% and that from beef muscle about 25%. With the beef digest, approximately 15% of soluble Fe was lost during the ammonium sulphate precipitation step. The absence of the low-molecular-weight Fe complexes in the beef digest used for Sephadex chromatography (Fig. 2) is a consequence of the inclusion of a dialysis step in the preparation of the sample. The soluble Fe compounds, however, were similar in all experiments and could be readily separated by dialysis or Sephadex chromatography into two groups. The large compounds were haemoproteins ranging in molecular weight from about 10000 to 20000 (Fig. 2). They were identified as haemoproteins by their characteristic absorption peak in the Soret region (about 410 nm) and by reaction with benzidene (Lemberg & Legge, 1949). The second group of Fe-containing digestion compounds were smaller (molecular weight < 6000) (Fig. 2) and could be separated from the larger haemoproteins by dialysis against distilled water yielding a pale straw-coloured solution. These Fe compounds were not haematin complexes as their absorption spectra showed no characteristic Soret peak, there merely being an increase in absorption with decreasing wavelength. They also failed to give a positive reaction with benzidene. These low-molecular-weight compounds were the major Fe-containing soluble products from the meat digests accounting for more than 80% of the total soluble Fe. Although it may be argued that these low-molecular-weight complexes are derived from the ferritin–transferrin fraction, the total amount of Fe associated with these complexes (approximately 50% of the total) suggests that they could not have originated from this fraction alone as the haemoproteins account for about 70% of the Fe in meat. Therefore, it would appear that the degraded haemoproteins are the probable intermediates in their formation. It is interesting to note that these low-molecular-weight complexes elute in a similar volume to the low-molecular-weight Fe compounds in a meat extract, but it is impossible, as neither of these compounds has been characterized, to comment on the relationship between them.

When either a 1% solution of purified horse heart metmyoglobin or bovine haemoglobin was subjected to the in vitro digestion procedure there was no evidence for the formation of any non-haematin Fe complexes. The only products were haematin complexes (a small amount being dialysable) with characteristic Soret absorption maxima at about 390 nm (Lemberg & Legge, 1949).

Rat absorption studies

Table 1 shows the results of the ^{59}Fe absorption experiments. At all time intervals, ^{59}Fe serosal transfer was highest from the digested muscle dialysate, followed by the undigested aqueous muscle extract, with the lowest ^{59}Fe serosal transfer from whole blood. Instantaneous (zero time) serosal transfer was very high for the digested dialysate, $19{\cdot}3 \pm 0{\cdot}30\%$ compared with $6{\cdot}2 \pm 0{\cdot}25\%$ for the aqueous extract and $4{\cdot}2 \pm 0{\cdot}25\%$ for whole blood.

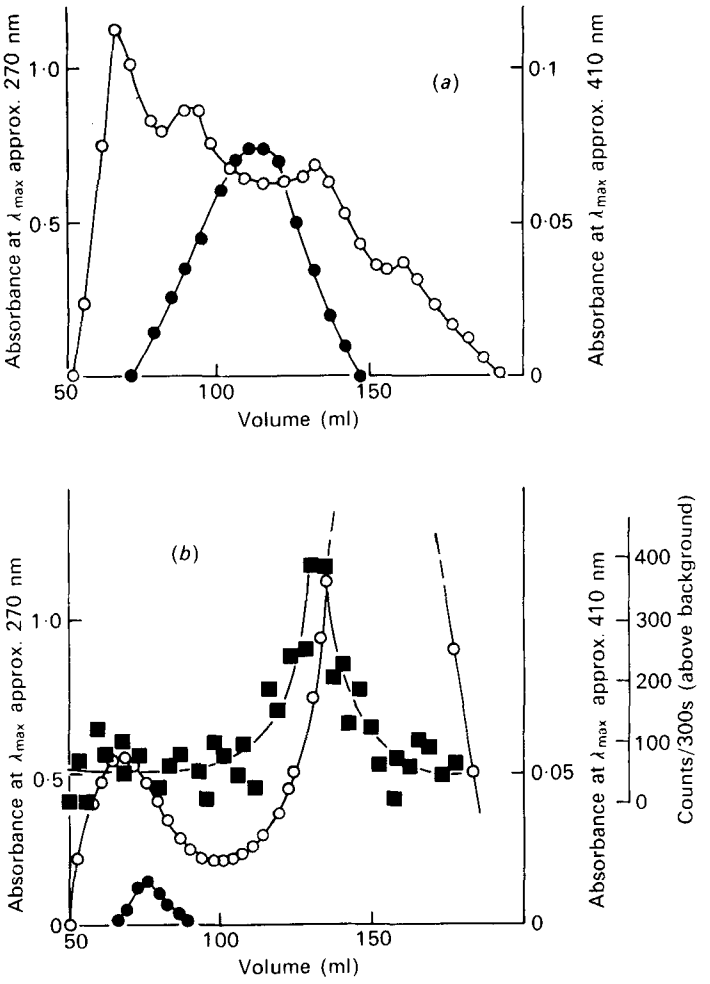

Fig. 2. (*a*) Chromatogram of 2 ml of the supernatant from raw beef after in vitro digestion at 37° and fractionation on Sephadex G-75. (*b*) Chromatogram of 3 ml of the supernatant from a ^{59}Fe-labelled rat-meat digest following in vitro digestion at 37° and fractionation on Sephadex G-50. The fractions were scanned from 390 to 430 nm for haemoproteins (●—●), λ_{max} approximately 410 nm and from 250 to 290 nm for protein fragments (○—○), λ_{max} varying from 265 to 275 nm and counted with a γ-counter for ^{59}Fe activity (■—■). The background count was 2081 for 300 s.

With all three samples, both mucosal uptake and serosal transfer can clearly be seen to increase regularly with increasing time. However, in all cases the mucosal retention of ^{59}Fe by the gut wall appears to level off after the initial increase.

DISCUSSION

The dose-response of Fe absorption in rat duodenum has been found to follow an exponentially linear (log–log) relationship (Terato, Hiramatsu & Yoshino, 1973), the amount of Fe absorbed being very sensitive to small changes in dosage at low dose levels. Although the level of Fe used in this study (3·5 μg/ml) is much lower than is usually administered in clinical studies, it does nevertheless more accurately reflect the amounts of iron that are tc be found in the gastrointestinal tract under true physiological conditions. For example

Table 1. *Rate of mucosal uptake, mucosal retention and serosal transfer of ^{59}Fe from a dialysate of digested ^{59}Fe-labelled rat muscle, an aqueous extract of undigested ^{59}Fe-labelled rat muscle and ^{59}Fe labelled rat blood*

(Values are single values or means ± SE of the no. of experiments in parentheses)

	Period in loop (min)	^{59}Fe Radioactivity absorbed: Mucosal uptake (% dose given)	Mucosal retention (% dose given)	Serosal transfer (% dose given)
Dialysate of digested muscle	0*	30·8 ± 1·80	11·5 ± 2·10	19·3 ± 0·30 (2)
	15	35·3 ± 3·20	18·1 ± 1·13	17·2 ± 2·05 (2)
	30	41·0	23·2	17·8
	60	57·4	23·2	34·2
Aqueous muscle extract	0*	22·4 ± 0·30	16·2 ± 0·10	6·2 ± 0·25 (2)
	15	36·9 ± 0·10	26·0 ± 3·05	10·9 ± 2·95 (2)
	30	39·5	26·4	13·1
	60	50·9 ± 2·95	22·6 ± 2·80	28·3 ± 5·75 (2)
Whole blood	0*	28·6 ± 1·05	24·4 ± 1·30	4·2 ± 0·25 (2)
	30	45·1 ± 0·34	35·4 ± 1·96	9·7 ± 2·29 (3)
	60	46·4 ± 2·10	32·9 ± 1·95	13·5 ± 0·10 (2)
	120	51·1 ± 2·54	34·3 ± 1·36	16·8 ± 2·43 (3)

* Zero time was approximately 30–60 s after administration.

Jacobs & Miles (1969) found the mean concentration of Fe in the gastric juice of human subjects to be 0·145 μg/ml. Thus the high levels of Fe used in most studies, although surmounting the problem of sensitive dose-response relationships found at low dosage levels, do not represent the physiological quantities normally present. However, in spite of the low dose levels used, because the quantities of Fe administered in the present studies are the same in all three treatments, results are directly comparable.

It is generally recognized that myoglobin and haemoglobin account for most of the chemical Fe found in meat and Fig. 1 shows that most of the radioactivity in the ^{59}Fe-labelled meat extract is associated with these two haemoproteins. Martinez-Torres & Layrisse (1971) and Martinez-Torres, Leets, Renzi & Layrisse (1974) obtained similar Fe distribution profiles for veal muscle and liver, and although large amounts of non-haematin Fe have been found in human muscle this is thought to be a peculiar characteristic of humans (Torrance, Charlton, Schmaman, Lynch & Bothwell, 1968).

Fig. 2 shows that with complete digestion, two groups of Fe compound are formed from meat; a group of haematin complexes with molecular weights between about 10000 and 20000 and a group of low molecular weight Fe-containing compounds possessing no haematin character. These low-molecular-weight Fe compounds appeared to be very well absorbed (Table 1). The undigested muscle extract was not as well absorbed although the difference between the predigested and untreated extracts decreased with time. This was not unexpected as the aqueous muscle extract should contain everything necessary for the formation of the readily absorbed low-molecular-weight non-haematin Fe compounds, and thus with digestion (within the duodenal loop) would form these. Absorption of Fe from undigested haemoglobin, in whole blood, was lower at all time intervals, and especially so when compared with the other treatments after 1 h. Various workers have shown that there is little release of 'free' Fe from haemoglobin following digestion (Callender *et al.* 1957; Kaldor, 1957; Sanford, 1960; Naish *et al.* 1973), small molecular weight haematin complexes being the major breakdown products (Conrad, Weinbtraub *et al.* 1966). Such an observation

was also made in this study, when either purified myoglobin or haemoglobin were subjected to the in vitro digestion procedure.

In human studies, it has been shown that meat Fe is better absorbed than Fe from vegetables or haemoglobin (Moore, 1961; Heinrich, Gabbe & Kugler, 1971; Layrisse & Martinez-Torres, 1971) and the present study suggests that the complete digestion of muscle causes the formation of a range of non-haematin Fe compounds in which the Fe is bound to some of the peptide and possibly pyrrole fragments resulting from the digestion. This binding of the Fe to the digestion products keeps it in solution and presumably protects it from inhibitors that may be present in the diet yet allows it to be efficiently absorbed into, and transferred across, the mucosal membrane. Other workers have also suggested that the absorption of both haematin Fe (Conrad, Cortell *et al.* 1966; Conrad, Weintraub *et al.* 1966; Martinez-Torres & Layrisse, 1971) and inorganic Fe (Kroe, Kinney, Kaufman & Klavins, 1962; Van Campen, 1972, 1973) is aided by the presence of protein digestion products and amino acids. These digestion products are also probably able to bind 'free' inorganic Fe from other foods and thus aid the absorption of Fe from plant material (Layrisse, Martinez-Torres & Roche, 1968). Thus the products of haemoprotein digestion in meat and isolated haemoglobin appear to be different, the Fe from meat being more efficiently absorbed than that from haemoglobin. Thus it is probable that the in vivo digestion of dietary haemoproteins which normally occurs within the intestine is more accurately represented by the in vitro digestion of meat than it is by either in vitro or in vivo digestion of purified haemoglobin or myoglobin.

Using both in vivo and in vitro techniques (with mouse and rat intestinal loops), Terato, Fujita & Yoshino (1973) and Terato, Hiramatsu & Yoshino (1973) have shown that saturation of mucosal Fe retention occurs after a short time with a range of Fe compounds of molecular weights of less than 100–9000. They suggest that transfer from lumen to mucosa is diffusion controlled and depends upon luminal concentration and it appears that the chemical nature and molecular weight of the Fe compound is not important. Transfer from the mucosa to the body, i.e. serosal transfer, is, however, probably influenced by the physical and chemical nature of the Fe compound, especially the molecular weight. In the present study, a mucosal saturation phenomenon was also observed, percentage mucosal retention remaining constant after 15–30 min (Table 1). Both mucosal uptake and serosal transfer, however, continued to increase at all time intervals. Thus the present results offer tentative support for Terato and co-workers' hypothesis as it appears that the mucosa will bind both the haematin Fe compounds from blood (i.e. haemoglobin) and the non-haematin Fe compounds from meat, although subsequent serosal transfer is greatly facilitated if low-molecular-weight (i.e. digested) fragments are present. This could explain how the presence of meat has such a marked enhancing effect on the absorption of Fe from haemoglobin as some of the ingredients in meat appear to be required to produce low-molecular-weight nonhaematin compounds from the haemoproteins. It may well be that the breakdown of haemoglobin and myoglobin within the meat environment is similar to the metabolic degradation of haemoglobin that occurs within the liver, and which does not itself lead to the formation of haematin complexes (Lemberg & Legge, 1949).

We are at present attempting to characterize the major products at all stages of the in vitro digestion procedure, especially the ultimate non-haematin compounds.

Mr T. Hazell thanks the S.R.C. for the award of a postgraduate studentship and the authors thank Professor D. E. Goldbloom of the University of California, Fresno, for his interest and assistance in this work.

REFERENCES

Callender, S. T., Mallett, B. J. & Smith, M. D. (1957). *Br. J. Haemat.* **3**, 186.
Conrad, M. E., Benjamin, B. I., Williams, H. L. & Foy, A. L. (1967). *Gastroenterology* **53**, 5.
Conrad, M. E., Cortell, S., Williams, H. L. & Foy, A. L. (1966). *J. Lab. clin. Med.* **68**, 659.
Conrad, M. E., Weintraub, L. R., Sears, D. A. & Crosby, W. H. (1966). *Am. J. Physiol.* **211**, 1123.
Hallberg, L. & Solvell, L. (1967). *Acta. Med. Scand.* **181**, 335.
Heinrich, H. C., Gabbe, E. E. & Kugler, G. (1971). *Eur. J. clin. Invest.* **1**, 321.
Heinrich, H. C., Gabbe, E. E. Kugler, G. & Pfau, A. A. (1971). *Klin. Wschr.* **49**, 819.
Jacobs, A. (1976). *Proc. Nutr. Soc.* **35**, 159.
Jacobs, A. & Miles, P. M. (1969). *Br. Med. J.* **4**, 778.
Kaldor, I. (1957). *Aust. Ann. Med.* **6**, 244.
Kroe, D., Kinney, T. D., Kaufman, N. & Klavins, J. V. (1962). *Blood* **21**, 546.
Layrisse, M. & Martinez-Torres, C. (1971). In *Progress in Haematology VII*, p. 137 [E. B. Brown and C. V. Moore, editors]. New York: Grune and Stratton.
Layrisse, M., Martinez-Torres, C. & Roche, M. (1968). *Am. J. clin. Nutr.* **21**, 1175.
Lemberg, R. & Legge, J. W. (1969). *Haematin Compounds and Bile Pigments*. New York: Interscience.
Martinez-Torres, C., Leets, I., Renzi, M. & Layrisse, M. (1971). *Am. J. clin. Nutr.* **24**, 531.
Martinez-Torres, C. & Layrisse, M. (1971). *Am. J. Clin. Nutr.* **24**, 531.
Martinez-Torres, C. & Layrisse, M. (1974). *J. Nutr.* **104**, 983.
Moore, C. V. (1961). *Harvey Lect.* **55**, 67.
Naish, R., Kimber, C. L. & Deller, D. J. (1973). *Br. J. Haemat.* **26**, 459.
Sanford, R. (1960). *Nature, Lond.* **185**, 533.
Terato, K., Fujita, T. & Yoshino, Y. (1973). *Dig. Dis.* **18**, 121.
Terato, K., Hiramatsu, Y. & Yoshino, Y. (1973). *Dig. Dis.* **18**, 129.
Torrance, J. D., Charlton, R. W., Schmaman, A., Lynch, S. R. & Bothwell, T. H. (1968). *J. clin. Path.* **21**, 495.
Turnbull, A., Cleton, F. & Finch, C. A. (1962). *J. clin. Invest.* **41**, 1897.
Van Campen, D. (1972). *J. Nutr.* **102**, 165.
Van Campen, D. (1973). *J. Nutr.* **103**, 139.
Walsh, R. J., Kaldor, I., Brading, I. & George, E. P. (1955). *Aust. Ann. Med.* **4**, 272.
Wheby, M. S., Suttle, G. E. & Foid, K. T. (1970). *Gastroenterology* **58**, 647.

Printed in Great Britain