Iron metabolism in copper-deficient rats

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1. The effects of ingestion of diets deficient in both copper and iron on storage of these elements and on the red cell indices have been studied in rats.

2. Injection of Cu into rats whose stores of Cu had been virtually exhausted resulted in a temporary increase in the concentration of plasma Fe and depletion of the Fe stored in the liver. Storage of Fe in the spleen seemed to be affected somewhat differently from that in the liver.

3. Fe injected into Cu-deficient rats was transported to storage sites but, although the plasma Fe concentration was presumably transiently increased thereby, there was no lasting effect.

4. The hypotheses that Cu mediates in the release of Fe from ferritin and that of Osaki, Johnson & Frieden (1966) that caeruloplasmin promotes the rate of Fe-saturation of apotransferrin are discussed.

The particular functions which copper assumes in the metabolism of iron have not been greatly clarified during the 40 years since Elvehjem and his co-workers noted that rats given a diet composed solely of milk responded to the administration of Fe only if Cu was provided in addition (Hart, Steenbock, Waddell & Elvehjem, 1928).

The following series of experiments was carried out in an attempt to throw some light on the problem. Rats were used as the experimental animals as deficiencies of both Cu and Fe may be relatively easily produced in them. A brief report of our findings has appeared (Marston & Allen, 1967).

EXPERIMENTAL AND RESULTS

Materials and methods

Animals. Male rats (Wistar strain) were weaned at 4 weeks of age, placed in individual cages and given *ad lib.* the milk diet detailed below. The cages for Expt 1 were of conventional design and made of tinned wire; those for Expt 2 consisted of an anodized aluminium cylinder with a stainless-steel band at the top, to prevent chewing of the edge, and brackets to hold the glass cover-plate. A chromed wire screen formed the bottom of the cage.

Supplements were provided as required for the individual experiments. Oral supplements, to which three drops of a saturated solution of sucrose were added, were offered in syracuse watch glasses. Water for drinking was glass-distilled. The animals were weighed weekly.

Diet. The ingredients were: full-cream powdered milk, 1320 g; sucrose, 595 g;

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sodium chloride, 43·5 g; cellulose, 40 g (filter paper, washed with 0·2 N-HCl and water, shredded and made into a pulp with the aid of water and a mechanical stirrer with a stainless-steel spindle and polyethylene blades); cod-liver oil, 7·5 g; mineral mixture, 20 ml ($ZnSO_4.7H_2O$, 8·82 g; $MnSO_4.4H_2O$, 4·05 g; $CoSO_4.7H_2O$, 2·4 g; each dissolved separately, mixed and 10 ml 6 N-HCl added before diluting with water to 1 l). Water was added to the diet to give a consistency which would drop fairly readily off a spoon.

Sulphided diet was treated with 3 ml of a 15% (w/v) solution of sodium sulphide/450 g diet to convert any Cu in the diet into a form which is unavailable to the rat (Summerson, 1938). The diet was left in a refrigerator, at least overnight, before using.

The diet contained *c.* 3 μg Cu and *c.* 4 μg Fe/g dry weight.

Analytical methods. All glassware was washed in hot chromic acid, soaked in 10% HCl, and rinsed first with distilled water and then with glass-distilled water.

Cu in tissues, blood and diet was estimated by complexing with diethyldithiocarbamate after wet digestion. Cu in plasma was estimated as described by Rice (1963).

Fe in tissues was estimated, after wet digestion, by complexing with α,α′-dipyridyl, using thioglycollic acid as reducing agent. Fe in diet, after wet digestion, and in plasma was estimated by complexing with bathophenanthroline (Giovanniello & Peters, 1963). The method of Giovanniello & Peters (1963) was also used for estimation of total Fe-binding capacity of plasma.

Haemoglobin was estimated by the cyanmethaemoglobin method (Dacie & Lewis, 1963). Haemoglobin in plasma was estimated by the benzidine reaction (Crosby & Furth, 1956). Samples in which a degree of haemolysis, detectable by eye, had occurred were discarded. Concentrations of plasma Fe were corrected for Fe arising from haemolysis in Expt 2 but not in Expt 1.

Packed cell volume was determined after centrifugation for 5 min in an Adam's micro-haematocrit centrifuge.

Red cell count: blood was diluted with Hayem's solution and the cells were counted in a Neubauer counting chamber.

Extraction and estimation of ferritin. Bone-marrow from four femurs and four tibias was scraped into a micro-beaker containing *c.* 0·5 ml of water and the beaker was sealed and stored in a deep-freeze unit. The marrow was subsequently triturated with a glass rod, homogenized in a total volume of 1 ml (made up with 0·03 M-NaCl) at pH 7·0 and the homogenate centrifuged. The supernatant fraction was heated with stirring for 5 min in a water bath at 70°, cooled, centrifuged and the supernatant fraction reduced in volume by evaporation under reduced pressure over P_2O_5. The residue was made to a convenient volume with water and samples were taken for the separation of ferritin by electrophoresis on cellulose acetate in 0·067 M-phosphate buffer at pH 6·4 (Darcel, 1961). Ferritin was stained by heating for 15 min at 56° in a freshly prepared mixture of equal quantities of 0·2 N-HCl and 2% (w/v) potassium ferrocyanide solution. The amount of ferritin present in the bone-marrow of a normal rat was estimated quantitatively by spectrophotometric scanning of the appropriately stained cellulose acetate strip and reference to the protein content of the original

supernatant fraction. The amounts of ferritin present in the marrows of the deficient rats were so small, and consequently the volume that it was necessary to apply to the cellulose acetate was so relatively large, compared with that used for normal rats, that clean separations from the brown contaminating material (haem?) were not possible; assessment was therefore made by visual inspection.

Collection of blood and perfusion: Expt 1. Rats were anaesthetized by administration of Nembutal by intraperitoneal injection; a facial mask saturated with diethyl ether was used if an animal showed any sign of returning consciousness. A carotid artery was exposed and freed from connective tissue to allow placing of a micro-clip, to prevent loss of blood during insertion of the cannula, and two lengths of thread. A very small nick was made in the artery with iridectomy scissors and a cannula containing heparin (1000 i.u./ml) was inserted and tied into position. A syringe was attached to the cannula, the clip released and the heparin gently mixed into the circulation. The artery above the cannula was then tied off, the syringe removed and blood collected direct from the cannula into a heparinized tube—about 10 ml of blood may be obtained from a fully grown rat. To perfuse the animal, the inferior vena cava was cut and 0·9% saline was passed through the cannula under pressure.

Collection of blood and perfusion: Expt 2. The tail was thoroughly cleaned and the rat was lightly anaesthetized with diethyl ether. As soon as the animal had lost consciousness its tail was placed in a beaker of distilled water at 40° for 5 min. The tail was dried with paper tissues, a thin coating of petroleum jelly applied to the section to be cut, the tip placed on a glass slide and about 1 cm cleanly removed by cutting with a surgical scalpel blade; a new blade was used for each cut. The tail was held in a vertical position and the blood (*c.* 1–1·5 ml) which dripped from it was collected in a 5 ml conical centrifuge tube which had been coated with paraffin wax and rinsed with water and which contained heparin—three drops of a 1000 i.u./ml solution dried under reduced pressure. Following initial anaesthesia, the rat was kept in a state of semi-consciousness by periodic use of a facial mask saturated with diethyl ether.

A sample of blood (0·02 ml) was removed for estimation of haemoglobin and the remainder spun at a temperature of 60° to separate the plasma. The most effective way of stopping the bleeding was to slip a rubber O-ring of suitable size on to the tail, so as to restrict the circulation, and leave it in position for 1–2 h.

Before perfusion, the rat was anaesthetized with Nembutal as described above. The thoracic aorta was exposed and isolated from connective tissue so that a cotton thread could be inserted for tying the cannula into position; heparin (0·05 ml of 1000 i.u./ml) was injected just below the kidneys and time allowed for it to circulate. A cannula was then inserted into the hole made by the needle used for injecting the heparin and tied into position. The greater part of the blood escaped via the cannula which was then connected to a separating funnel containing 0·9% saline, the artery below the cannula was clipped off with pressure forceps, the inferior vena cava cut and the animal perfused under pressure of *c.* 55 cm until the effluent fluid was free of blood. The livers were retained for analysis.

Expt 1. To determine the effects of (a) Cu, (b) Fe and (c) Cu and Fe deficiencies on rats

Eight groups of rats were chosen from forty-eight animals, divided, on the basis of body-weight (range 68–77 g), into six batches of eight evenly matched rats. Non-sulphided diet (i.e. diet to which sodium sulphide had not been added) was given *ad lib.* to groups 1A to 4A inclusive; sulphided diet (i.e. diet to which sodium sulphide had been added) was given *ad lib.* to groups 1B to 4B inclusive.

Treatments were as follows: group 1 (A and B), no supplement; group 2 (A and B), Fe, 500 μg/d, administered orally as $FeSO_4$ in 0·5 ml of solution thrice a week; group 3 (A and B), Cu, 25 μg/d, administered by intraperitoneal injection as $CuSO_4$ in 0·25 ml of solution thrice a week; group 4 (A and B), Fe, 500 μg/d orally, plus Cu, 25 μg/d parenterally, as described for groups 2 and 3.

After 8 weeks on this regimen blood was collected from each rat and the animals were perfused as described on p. 17. Results of the analyses on blood and tissues are reported in Tables 1 and 2.

Expt 2. To determine the effects of parenterally administered Cu and Fe on rats given a diet deficient in both Cu and Fe

This experiment was made up of a number of parts in each of which the rats were divided initially into two groups: one (deficient), members of which received no supplement, and one (positive control), whose members were given daily 0·5 ml of a supplement that provided 100 μg Cu + 500 μg Fe. The stock solution was prepared as follows: $CuSO_4.5H_2O$, 156 mg; $FeSO_4.7H_2O$, 1 g; ammonium citrate, 1 g; HCl, 1·55 ml of 6-N; the pH was adjusted to *c.* 4 with NH_4OH and the mixture diluted to 200 ml with water. Non-sulphided diet was given *ad lib.* Graphs of mean body-weights, typical for the experiment, are shown in Fig. 1.

Meticulous care during the preparation of diet and in the washing of apparatus decreased the period required to produce terminal symptoms of the deficiency from 8 (cf. Expt 1) to 4·5–6 weeks.

Effect of Cu. When the animals that had not received the supplement were in an advanced stage of the deficiency syndrome they were divided, as evenly as possible, according to extent of depigmentation and reduction in rate of growth, into the required number of groups. Blood was obtained from the tail of each animal, after which each was treated by intraperitoneal injection with either Cu, 200 μg in 0·25 ml of a solution of $CuSO_4$ in 0·9% NaCl, or the same volume of 0·9% NaCl. After an appropriate interval a second sample of blood was obtained from the tail. The animals were then perfused and the livers removed and retained for analysis.

Following parenteral administration of Cu to deficient rats, significant increases occurred in the concentrations of plasma Fe compared with those observed after injection of physiological saline (Table 3). Two hours after the injection the difference between the mean differences, after and before injection, was significant at $P < 0·05$ by the *t* test; at 18 h and 24 h it was significant at $P < 0·01$ and $P < 0·05$, respectively, both by the *d* test.

Table 1. *Body-weights, red cell indices and copper and iron concentrations of blood and plasma, respectively, of the rats of Expt 1. Supplements of Fe and Cu, when given, were administered thrice weekly*

Diet	Group (six rats)	Body-weight (g)	Hb (g/100 ml)	PCV (%)	RCC ($\times 10^{-6}$/mm^3)	MCV (μm^3)	MCH (pg)	MCHC (%)	Blood Cu (μg/ml)	Plasma Fe (μg/ml)
Non-sulphided, no supplement	1A	$\bar{x}$ 191	3·67	14·5	4·8	30	7·7	25	0·08	0·46
		s 13	0·27	1·2	0·5	2	0·4	1	0·05	0·22
+ 0·5 mg Fe/d *per os*	2A	$\bar{x}$ 229	12·3	37·7	9·0	42	14	33	0·09	0·87
		s 17	1·7	2·9	0·6	3	2	2	0·04	0·43
+25 μg Cu/d intra-peritoneally	3A	$\bar{x}$ 208	7·09	26·7	9·5	28	7·5	27	1·10	0·81
		s 10	0·93	3·0	1·5	3	0·8	1	0·18	0·52
+0·5 mg Fe/d *per os*	4A	$\bar{x}$ 270	15·7	46·0	9·5	49	17	34	1·02	1·76*
+25 μg Cu/d intra-peritoneally		*s* 10	1·4	1·6	0·8	4	2	1	0·25	0·44
Sulphided, no supplement	1B	$\bar{x}$ 170	3·14	11·5	3·2	36	10	28	ND	0·93
		s 13	0·27	1·2	0·5	3	1	1	—	0·22
+0·5 mg Fe/d *per os*	2B	$\bar{x}$ 189	5·60*	19·7*	5·6*	35*	9·9*	29*	0·03*	1·4†
		s 17	0·59	2·9	0·6	2	0·5	1	0·04	0·43
+25 μg Cu/d intra-peritoneally	3B	$\bar{x}$ 212	7·18	26·4	9·9	27	7·3	27	1·08	1·0
		s 10	0·93	3·0	1·5	3	0·8	1	0·18	0·52
+0·5 mg Fe/d *per os*	4B	$\bar{x}$ 255	16·5	47·5	9·6	50	17	35	0·78	3·19*
+25 μg Cu/d intra-peritoneally		*s* 10	0·5	1·6	0·8	4	2	1	0·25	1·37

Hb, haemoglobin; PCV, packed cell volume; RCC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; $\bar{x}$, group mean; *s*, standard deviation of a single observation; ND, not detectable; NS, not significant.

* Group of five rats. † Group of three rats. ‡ Test of significance by *d* test; otherwise by *t* test.

Body-weight, group 1B < group 1A, $P < 0·05$; group 2B < group 2A, $P < 0·01$; group 4B < group 4A, $P < 0·05$; difference between groups 3A and 3B NS; Hb, group 1B < group 1A, $P < 0·01$; group 2B < group 2A, $P < 0·01$‡; differences between groups 3A and 3B; 4A and 4B‡ NS; PCV, group 1B < group 1A, $P < 0·01$; group 2B < group 2A, $P < 0·001$; differences between groups 3A and 3B; 4A and 4B NS; RCC, group 1B < group 1A, $P < 0·001$; group 2B < group 2A, $P < 0·001$; differences between groups 3A and 3B; 4A and 4B NS; MCV, group 1B > group 1A, $P < 0·01$; group 2B < group 2A, $P < 0·01$; differences between groups 3A and 3B; 4A and 4B NS; MCH, group 1B > group 1A, $P < 0·01$‡; group 2B < group 2A, $P < 0·01$‡; differences between groups 3A and 3B; 4A and 4B NS; MCHC, group 1B > group 1A, $P < 0·01$; group 2B < group 2A, $P < 0·01$‡; group 3B > group 3A, $P < 0·05$; differences between groups 4A and 4B NS; Blood Cu, group 2B< group 2A, $P < 0·05$; differences between groups 3A and 3B; 4A and 4B NS; Plasma Fe, group 1B > group 1A, $P < 0·01$; differences between groups 2A and 2B; 3A and 3B; 4A and 4B‡ NS.

Table 2. *Copper and iron contents of organs of the rats of Expt 1, expressed on a dry-weight basis. Supplements of Fe and Cu, when given, were administered thrice weekly*

Diet	Group (six rats)		Liver Cu µg/g	Liver Cu Total (µg)	Liver Fe µg/g	Liver Fe Total (µg)	Kidney Cu µg/g	Kidney Cu Total (µg)	Kidney Fe µg/g	Kidney Fe Total (µg)	Spleen Cu µg/g	Spleen Cu Total (µg)	Spleen Fe µg/g	Spleen Fe Total (µg)	Heart Cu µg/g	Heart Cu Total (µg)	Heart Fe µg/g	Heart Fe Total (µg)
Non-sulphided,																		
no supplement	1A	$\bar{x}$	3·5	6·5	262	483	9·3	4·1	91	41	3·7*	0·73*	441	87	6·3	1·7	157	43
		s	0·6	0·9	76	163	0·9	0·5	5	4	—	—	35	16	1·2	0·3	11	4
+0·5 mg Fe/d *per os*	2A	$\bar{x}$	5·6	12·5	802	1788	11·7	4·8	122	51	3·3*	0·32*	669	63	8·7	1·7	220	43
		s	0·6	2·0	114	447	1·1	0·6	16	8	—	—	170	25	1·3	0·2	24	9
+25 µg Cu/d intraperitoneally	3A	$\bar{x}$	13·6†	23·3†	84	144	15·3	5·5	82	30	12·3*	2·0*	555	90	24·9	4·2	173	28
		s	1·5	2·6	5	10	2·0	1·0	4	2	—	—	126	36	2·8	0·6	54	6
+0·5 mg Fe/d *per os*	4A	$\bar{x}$	11·8	29·3	248	616	16·8	7·3	134	58	10·1*	0·90*	905	80	18·8	3·3	215	38
+25 µg Cu/d intraperitoneally		*s*	2·8	5·0	139	327	3·1	1·8	36	20	—	—	502	39	1·9	0·4	13	3
Sulphided,																		
no supplement	1B	$\bar{x}$	2·0	3·5	349	631	7·0	3·2	109	50	3·3*	0·37*	533	59	7·0	1·8	179	46
		s	0·6	0·9	76	163	0·9	0·5	5	4	—	—	117	16	1·2	0·3	11	4
+0·5 mg Fe/d *per os*	2B	$\bar{x}$	1·9	4·1	695	1545	8·2	3·6	134	60	2·5*	0·26*	626	69	7·2	1·4	195†	38†
		s	0·6	0·7	114	447	1·1	0·6	16	8	—	—	170	25	1·3	0·2	24	9
+25 µg Cu/d intraperitoneally	3B	$\bar{x}$	14·0	26·0	79	148	17·3	6·5	86	33	7·4*	1·2*	564	92	24·4	4·0	177	29
		s	4·9	8·5	5	10	2·0	1·0	11	5	—	—	126	36	2·8	0·6	13	6
+0·5 mg Fe/d *per os*	4B	$\bar{x}$	11·0†	27·0†	434	1073	17·9	8·0	171	76	12·1*	1·1*	1612	132	16·4	2·9	247	43
+25 µg Cu/d intraperitoneally		*s*	0·7	5·0	139	327	3·1	1·8	36	20	—	—	502	39	1·9	0·4	13	3

$\bar{x}$, group mean; *s*, standard deviation of a single observation; NS, not significant; † group of five rats; * spleens from either six or three rats were pooled; ‡ test of significance by *d* test; otherwise by *t* test.

Liver Cu, µg/g, group 1B < group 1A, $P < 0.001$; group 2B < group 2A, $P < 0.001$; group 1A < group 2A, $P < 0.001$; group 1A < group 4A, $P < 0.01$‡; group 1B < group 4B, $P < 0.001$; differences between groups 3A and 3B‡; 4A and 4B‡; 3A and 4A; 1B and 2B; 3B and 4B‡ NS; Liver Cu, µg total, group 1B < group 1A, $P < 0.001$; group 2B < group 2A, $P < 0.01$‡; differences between groups 3A and 3B‡; 4A and 4B NS; Liver Fe, µg/g, group 4B > group 4A, $P < 0.05$; group 1A > group 3A, $P < 0.01$‡; group 2A > group 4A, $P < 0.001$; group 1B > group 3B, $P < 0.01$‡; group 2B > group 4B, $P < 0.05$; differences between groups 1A and 1B; 2A and 2B; 3A and 3B; 1A and 4A‡; 1B and 4B NS; Liver Fe, µg total, group 4B > group 4A, $P < 0.05$; differences between groups 1A and 1B; 2A and 2B; 3A and 3B NS; Kidney Cu, µg/g, group 1B < group 1A, $P < 0.01$; group 2B < group 2A, $P < 0.001$; group 1A < group 2A, $P < 0.01$; group 1A < group 4A, $P < 0.01$‡; group 1B < group 4B, $P < 0.01$‡; differences between groups 3A and 3B; 4A and 4B; 3A and 4A; 1B and 2B; 3B and 4B NS; Kidney Cu, µg total, group 1B < group 1A, $P < 0.01$; group 2B < group 2A, $P < 0.01$; differences between groups 3A and 3B; 4A and 4B NS; Kidney Fe, µg/g, group 1B > group 1A, $P < 0.001$; group 1A > group 3A, $P < 0.01$; group 1A < group 4A, $P < 0.05$‡; group 1B > group 3B, $P < 0.01$‡; group 1B < group 4B, $P < 0.01$‡; differences between groups 2A and 2B; 3A and 3B‡; 4A and 4B; 2A and 4A‡; 2B and 4B NS; Kidney Fe, µg total, group 1B > group 1A, $P < 0.01$; differences between groups 2A and 2B; 3A and 3B‡; 4A and 4B NS; Spleen Fe, µg/g, group 4B > group 4A, $P < 0.05$; group 1A < group 3A, $P < 0.05$‡; group 1A < group 2A, $P < 0.01$‡; group 1A < group 4A, $P < 0.01$‡; group 3A < group 4A, $P < 0.05$‡; group 1B < group 4B, $P < 0.01$‡; group 2B < group 4B, $P < 0.05$‡; group 3B < group 4B, $P < 0.01$‡; differences between groups 1A and 1B‡; 2A and 2B; 3A and 3B; 2A and 3A; 2A and 4A; 1B and 3B; 1B and 2B; 2B and 3B NS; Spleen Fe, µg total, group 1B < group 1A, $P < 0.05$; group 4B > group 4A, $P < 0.05$; differences between groups 2A and 2B; 3A and 3B NS; Heart Cu, µg/g, group 1A < group 2A, $P < 0.01$; group 3A > group 4A, $P < 0.01$; group 1A < group 4A, $P < 0.001$; group 3B > group 4B, $P < 0.001$; group 1B < group 4B, $P < 0.001$; differences between groups 1A and 1B; 2A and 2B; 3A and 3B; 4A and 4B; 1B and 2B NS; Heart Cu, µg total, group 2B < group 2A, $P < 0.05$; differences between groups 1A and 1B; 3A and 3B; and 4A and 4B NS; Heart Fe, µg/g, group 1B > group 1A, $P < 0.01$; group 4B > group 4A, $P < 0.01$; group 1A < group 2A, $P < 0.001$; group 1A < group 4A, $P < 0.01$; group 1B < group 4B, $P < 0.001$; group 2B < group 4B, $P < 0.001$; group 3B < group 4B, $P < 0.001$; differences between groups 2A and 2B; 3A and 3B‡; 1A and 3A‡; 2A and 3A; 2A and 4A; 3A and 4A‡; 1B and 3B; 1B and 2B‡; 2B and 3B NS; Heart Fe, µg total, group 4B < group 4A, $P < 0.05$; differences between groups 1A and 1B; 2A and 2B; 3A and 3B NS.

The increases in the concentrations of plasma Fe that occurred 6, 12 and 18 h following injection of Cu were similar and much greater than that observed 2 h after treatment. Twenty-four hours after injection the increase was significant but results between experiments were more variable, and 48–72 h after treatment concentrations of plasma Fe were not significantly different from those before treatment (Table 4). Concentrations of haemoglobin were, in general, lower after than before injection (Table 4).

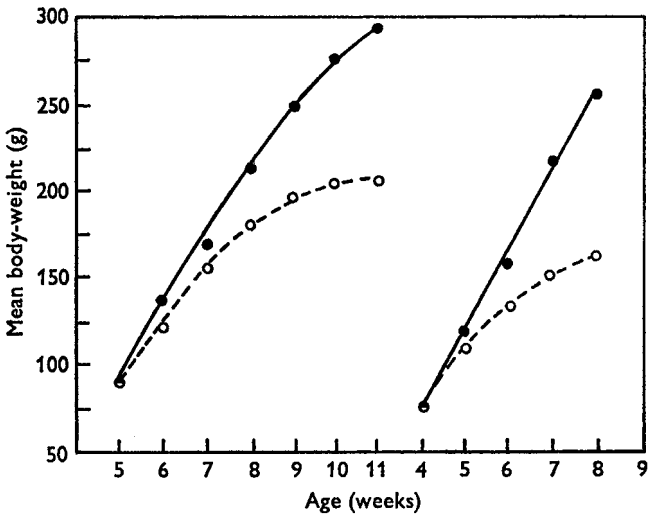

Fig. 1. Mean body-weights of rats given a diet deficient in Cu and Fe (○) and of the positive control animals (●) during Expt 2.

Table 3. *Effect on the concentrations of plasma iron of parenteral administration of copper to rats given a diet deficient in Cu and Fe*

(Mean values and standard deviations of a single difference, with numbers of observations in parentheses)

Treatment	Time interval after injection (h)	Plasma Fe (μg/ml) Pre-injection	Plasma Fe (μg/ml) Post-injection	SD
Cu*	2	0·25	0·29	0·06 (4)
NaCl†	2	0·41	0·33	0·06 (4)
Cu*	18	0·19	0·56	0·17 (12)
NaCl†	18	0·26	0·28	0·10 (15)
Cu*	24	0·21	0·52	0·24 (9)
NaCl†	24	0·26	0·32	0·09 (8)

* 200 μg Cu by intraperitoneal injection.
† 2·25 mg NaCl by intraperitoneal injection.

Concentrations of both plasma Fe and haemoglobin of rats in the positive control groups 18 and 24 h after injection of Cu were either not significantly different from or lower than those before injection.

The pre-injection mean concentrations and standard deviations of a single observa-

Table 4. *Concentrations of plasma iron and of haemoglobin before, and at various intervals after, parenteral administration of 200 μg Cu to rats given a diet deficient in Cu and Fe*

Time interval after injection (h)	Plasma Fe (ug/ml)			Haemoglobin (g/100 ml)			
	Pre-injection	Post-injection	Significance	Pre-injection	Post-injection	Significance
2	0·25 0·07 (4)	0·29 0·07 (4)	NS*	4·36 1·13 (4)	3·28 0·27 (3)	NS
6	0·27 0·06 (4)	0·90 0·30 (4)	$P < 0·01$	5·10 0·26 (3)	4·07 0·26 (4)	$P < 0·01$*
12	0·25 0·16 (4)	0·86 0·16 (3)	$P < 0·01$*	4·54 1·0 (4)	4·14 1·0 (4)	NS*
18	0·24 0·05 (4)	0·90 0·34 (4)	$P < 0·01$	5·31 0·31 (4)	4·50 0·31 (4)	$P < 0·05$*
18	0·19 0·03 (12)	0·56 0·15 (12)	$P < 0·01$	4·51 0·72 (12)	4·21 0·72 (12)	NS*
24	0·30 0·01 (3)	1·15 0·21 (3)	$P < 0·01$	3·83 0·18 (2)	3·56 0·18 (3)	NS*
24	0·21 0·06 (9)	0·52 0·26 (9)	$P < 0·01$	4·37 0·50 (10)	3·54 0·50 (10)	$P < 0·01$*
48	0·33 0·06 (3)	0·27 0·06 (3)	NS*	3·75 0·34 (3)	3·87 0·34 (3)	NS*
48	0·24 0·06 (8)	0·30 0·12 (8)	NS	3·57 0·43 (8)	3·55 0·43 (8)	NS*
72	0·28 0·05 (2)	0·29 0·05 (2)	NS*	4·01 0·31 (2)	3·97 0·31 (2)	NS*
72	0·24 0·03 (8)	0·30 0·16 (8)	NS	3·89 0·33 (8)	3·58 0·33 (8)	NS*

The values quoted are means and standard deviations of a single observation, with numbers of observations in parentheses.
* Test of significance by *t* test, otherwise by *d* test. NS, not significant.

tion, with numbers of rats in parentheses, were: plasma Fe, in μg/ml: deficient, 0·26, 0·09 (113); positive controls, 2·40, 0·84 (17); and haemoglobin, in g/100 ml: deficient, 4·25, 0·74 (109); positive controls, 14·6, 1·0 (20).

There was no indication of impairment of Fe-binding capacity in the plasmas of deficient rats.

Concentrations of Cu in the plasmas of deficient rats ranged from undetectable to < 0·05 μg/ml; 18, 48 and 72 h after injection of Cu the averages and standard deviations of a single observation, with numbers of animals in parentheses, were, respectively, 0·67, 0·12 (13); 1·06, 0·16 (8) and 1·42, 0·50 (8).

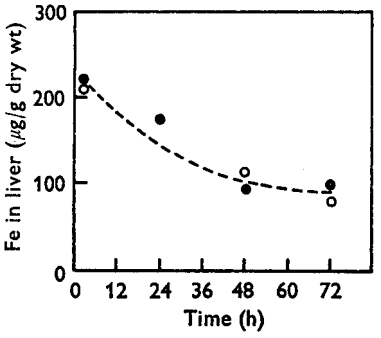

Fig. 2

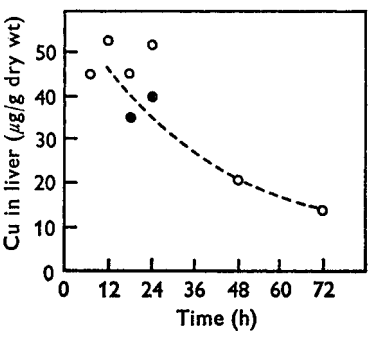

Fig. 3

Fig. 2. Reduction in the concentration of iron in the livers of rats given a diet deficient in Cu and Fe following intraperitoneal injection of 200 μg Cu at 0 h. The points plotted represent two experiments, and each point is the mean value of the concentrations of at least two livers.

Fig. 3. Reduction in the concentration of copper in the livers of rats following intraperitoneal injection of 200 μg Cu at 0 h. The points plotted are the mean values of the concentrations of at least three livers. ○, rats given a diet deficient in Cu and Fe; ●, positive control rats.

Fe stores in the livers of deficient rats gradually became depleted after administration of Cu (Fig. 2). Eighteen and 24 h after injection of Cu, mean concentrations of Fe in the livers of these animals were significantly less ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively, both by the t test) than those of similar rats injected with physiological saline.

Concentrations of Cu in the livers of both deficient and normal rats rose to abnormally high levels following the injection of Cu; 72 h later, however, the concentrations in deficient animals approximated to those found in the uninjected, positive controls (Fig. 3). The means and standard deviations of a single observation (with numbers of rats in parentheses) of Cu concentration in the liver (μg/g dry weight) were, for deficient rats, 3·11, 1·10 (39) and for positive controls 13·8, 3·1 (6).

Release of Fe in vitro. The animals used for this experiment were perfused with ice-cold Ringer-phosphate solution, the livers removed, blotted with acid-washed filter paper, placed in containers and transferred to a cold room where slices (*c.* 1 g/incubation) were cut as described by Deutsch (1936) and placed in 5 ml Krebs bicarbonate mixture (without phosphate). Anaerobic (95 % N_2, 5 % CO_2) and aerobic (95 % O_2, 5 % CO_2) incubations (1 h at 38°) were carried out, with shaking, with slices from each liver. At the end of 1 h the incubation medium was freed from solid particles by

centrifugation and Fe was estimated in the supernatant fraction after precipitation of proteins with trichloroacetic acid; a small sample of the supernatant fraction was retained for estimation of haemoglobin by the benzidine reaction.

The amount of Fe released into the medium during anaerobic incubation of slices of liver, less that released during aerobic incubation, seemed to bear little relationship to their Cu content; there was, however, a significant linear regression ($P < 0.001$) between the concentration of Fe and that released during incubation.

Table 5. *Comparison of the concentrations of iron in livers of rats with the amounts released during anaerobic incubation*

(Mean values and standard deviations of a single observation, with numbers of rats in parentheses)

Treatment	Time interval after injection* (h)	Iron: Concentration (μg/g dry wt)	Iron: Released (μg/g tissue)
Deficient+Cu	2	219 58 (3)	2·40 0·41 (4)
+NaCl	2	208 58 (4)	2·36 0·41 (4)
+Cu	24	182 24 (3)	2·73 0·31 (3)
+Cu	48	94 37 (3)	1·60 0·06 (3)
+Cu	72	97 < 1 (2)	1·57 0·42 (2)
Positive control, not injected		425 115 (5)	5·84 1·68 (5)

* At which rat killed.

Treatment with copper and NaCl was by administration of 200 μg and 2·25 mg, respectively, each by intraperitoneal injection. For details of incubation see p. 23.

Table 6. *Concentrations of copper and iron in livers of Cu-deficient and control rats and in Cu-deficient rats injected with Fe*

(Mean values and standard deviations of a single observation, with numbers of rats in parentheses)

Group	Cu (μg/g dry wt)	Fe (μg/g dry wt)
A, deficient	3·45 0·75 (6)	161 27 (6)
B, as A+Fe	2·42 0·35 (8)	397 72 (10)
C, as A, 6 d later	2·49 0·71 (11)	207 58 (11)
Positive control	13·8 3·1 (6)	339 54 (6)

1750 μg of Fe were administered parenterally to the rats of group B over a period of 5 d.

The amount of Fe released in vitro 2 h after injection of Cu into the live animal was not significantly different (by the *t* test) from that released during incubation of slices of liver from rats injected with saline. There was no significant difference (by the *t* test) between the mean concentrations of Fe in the livers of the two groups (Table 5).

Effect of Fe. At the beginning of the 5th week of this experiment blood samples and livers were taken from six deficient rats (group A) and ten others were injected with Fe (group B). A total of 1750 μg Fe was injected into the peritoneal cavity of each rat in three equally divided doses in 0·25 ml of solution on 3 alternate d; the Fe was in

the form of ferrous sulphate and the solution contained 0·1% (w/v) ammonium citrate.

Twenty-four hours after the third injection, blood samples and livers were obtained from these rats and from eleven deficient rats (group C) from the same experiment.

No significant differences in the mean concentrations of plasma Fe or haemoglobin occurred between groups A, B or C; the concentrations of Fe in the livers of the rats of group B were, however, increased to normal by parenteral administration of Fe.

The mean concentration of Cu in the livers of the rats of group A was significantly higher than those of the groups (B and C) killed 6 d later ($P < 0{\cdot}05$ by the *d* test and *t* test respectively) (Table 6).

Ferritin in bone-marrow. So little ferritin was present in the bone-marrows of deficient rats that it was not possible to detect whether changes in concentration resulted from injection of the animals with Cu. The amount of ferritin in the bone-marrows of rats injected with Fe (group B above) was certainly greater than that in those not so injected, although still very much less than that in normal animals.

The greater part of the material which stained with ferrocyanide after electrophoresis of extracts of bone-marrow from deficient rats either remained on the starting line or migrated only a short distance—very little was found in the position assumed by ferritin extracted from the spleen. Whether the material was in fact ferritin whose rate of migration had been altered by the presence of abnormally high concentrations of other materials or whether it was indicative of 'ageing' (a similar phenomenon developed in extracts of ferritin which had been stored for some months in a refrigerator) or whether it was another Fe-containing compound was not determined. Two molecular forms of ferritin have been reported to occur in bone-marrow (Alfrey, Lynch & Whitley, 1967; Gabuzda & Pearson, 1968), and an Fe-containing precursor, other than ferritin, has been described by a number of workers (cf. Salera, Magnanelli, d'Avino, Zecca & Matcovich, 1961; Greenough, Peters & Thomas, 1962; Zail, Charlton, Torrance & Bothwell, 1964); according to the first-mentioned group, however, the compound does not react with ferrocyanide to form a blue compound. Migration of ferritin extracted from the bone-marrows of rats in the positive control group was similar to that extracted from the spleen, although the material had been stored for the same length of time as that from the deficient animals.

The spleen, under conditions of anaemic stress, may become a site of extramedullary haemopoiesis and the spleens of all of the deficient animals were slightly larger than normal. In the last of this series of experiments the spleens of 25% of the deficient rats were grossly enlarged. No significant difference between the mean growth rate of these animals and that of the rest of the group was observed; however, the appearance of a histological section was suggestive of a non-specific inflammation.

DISCUSSION

The symptoms suffered by Cu-deficient animals have been described (Marston, 1952; Underwood, 1962). The first signs of the deficiency to appear in rats are depigmentation of both hair and teeth, harshness of the hair, decrease in the rate of

growth and anaemia. Depigmentation of the enamel of the teeth, which is known to contain Fe (Reith, 1961), was very marked in animals deficient in both Cu and Fe. In the terminal stages, oedema is a particularly noticeable feature, especially in older rats, although we have observed it in animals at 8 weeks of age. Oedema was apparent in only a few of the deficient rats in this series.

The mechanisms by which Fe is absorbed, becomes attached to apotransferrin and is subsequently transferred to apoferritin at storage sites, and is again mobilized as need arises, have interested research workers over many years (e.g. Beutler, Fairbanks & Fahey, 1963; Brown, 1963; Harris, 1963; Coons, 1964; Gross, 1964; Smith, Drysdale, Goldberg & Munro, 1968; Wynter & Williams, 1968).

Saltman and his collaborators have made a critical approach to the problem and, in recently published work (Bates, Billups & Saltman, 1967*a*, *b*; Billups, Pape & Saltman, 1967; Pape, Multani, Stitt & Saltman, 1968*a*, *b*), propose mechanisms which offer more logical and satisfactory explanations of these phenomena than any put forward formerly. They suggest that Fe enters the cell in the form of chelates produced by the binding of ferrous or ferric Fe with low molecular weight chelating agents of exogenous or endogenous origin. Once in the cell the Fe is either exchanged with other low molecular weight ligands or is bound in a storage form to macromoles such as apoferritin. Attachment of Fe to apotransferrin and release of Fe from transferrin are thought to be mediated by formation of a ternary complex of a low molecular weight chelator, Fe and apotransferrin, which breaks down to form chelator and transferrin, both reactions being reversible. Confirmation of some of these findings may be found in the work of Aisen, Aasa, Malmström & Vänngård (1967).

Cu may influence the metabolism of Fe in one or more of a number of ways of which the following are relevant: (1) binding to apotransferrin; (2) release from transferrin; (3) acceptance by apoferritin; (4) release from ferritin; (5) utilization, either direct or indirect, in any one of the innumerable functions of Fe in the body. Acceptance by apoferritin or apotransferrin, and release from transferrin are unlikely to be involved, for both in Cu-deficient rats (cf. Table 2) and in sheep (Marston, 1952) storage of Fe, provided Fe is available in the diet, is increased, and in rats (this paper) and sheep (Allen, 1956) the concentration of plasma Fe is decreased, while the total Fe-binding capacity remains unimpaired.

Fe injected into the peritoneal cavity is thought to be transported via the lymphatics and delivered to the venous circulation through the thoracic duct (Hedenstedt, 1947; Thirayothin & Crosby, 1962). A considerable proportion of the Fe injected into rats subsisting on a diet deficient in Cu and Fe reached the liver, for the concentration was thereby approximately doubled compared with that in rats not so injected (Table 6), and some was transported to the bone-marrow. The concentrations of Cu in the plasmas of these animals ranged from undetectable to 0·05 μg/ml, a range some ten times lower than that reported by Dreosti & Quicke (1968) for rats allegedly Cu-deficient. Osaki, Johnson & Frieden (1966) postulate a catalytic role for caeruloplasmin in converting ferrous into ferric Fe, thereby promoting its rate of incorporation into apotransferrin. If the intraperitoneally injected Fe was in fact transported by transferrin then either caeruloplasmin is not essential for incorporation of Fe into

transferrin or the amount required is extraordinarily small. Transferrin apparently functioned normally in the Cu-deficient rats under study, for the amount of Fe associated at any one time with transferrin, at least in the healthy animal, is small compared with the amount transported each day (Harris, 1963; Cheney, Lothe, Morgan, Sood & Finch, 1967). The release of Fe from ferritin and its subsequent utilization should therefore be considered in seeking the functions of Cu in the metabolism of Fe.

The values quoted in Table 1 for concentrations of plasma Fe were not corrected for Fe arising from haemolysis; not so much reliance can be placed on them, therefore, as on those quoted in subsequent tables; however, it is clear that, in the groups to which no supplements were administered, concentrations of plasma Fe were lower and of haemoglobin higher in animals given the non-sulphided diet (group 1A) than in those (group 1B) on the sulphided diet—the diet in which the small amount of Cu present had been rendered unavailable. Comparison of the groups supplemented with Fe (groups 2A and 2B) shows again that haemoglobin concentrations were higher in the animals that consumed the non-sulphided diet, indicating greater availability of Fe in those animals in which the concentration of Cu was less limited.

Packed cell volume was influenced by the relative deficiencies of Cu and Fe in a manner very similar to that of haemoglobin. The effects of the deficiencies on the other red cell indices are less clear; they confirm, however, findings from many laboratories that the anaemias produced are microcytic in nature.

Concentrations of Fe in the storage organs, liver and spleen, were affected differently by the relative deficiencies of Fe and Cu (Table 2). The concentrations of Fe in the spleens of the animals supplemented with Cu (group 3) were either not significantly different from, or greater than those of the unsupplemented animals (group 1). In the livers, on the other hand, concentrations of Fe were highly significantly lower in the animals given the Cu supplement compared with those on the unsupplemented basal diet. There is apparently a concentration of Fe in the liver of rats, *c.* 80 μg Fe/g dry weight, below which it is impossible for the animal to exist. This was the concentration to which the livers of the animals of group 3 (Table 2) were reduced and the concentration approached in deficient rats of Expt 2, 72 h after they had been injected with Cu (Fig. 2).

Concentrations of Fe in the spleens of rats given a supplement of Fe (group 2) were significantly higher than those in the animals not given the supplement only in those on the non-sulphided diet, whereas storage in the liver was increased in both groups 2A and 2B compared with that in the groups not given the supplement. The relatively small amount of Fe found in the spleen, even in the control animals, indicates that it is derived from the haemoglobin of aged red cells and that this organ contains little of the Fe absorbed direct from the intestinal tract. It is possible that Fe is stored in the spleen in a different form from that in the liver, or that its release is effected by a different mechanism, or that the concentration in the spleens of the deficient rats represented a minimum below which the animals could not survive; whatever the reason, the availability of Cu did not have the same dramatic effect on storage of Fe in the spleen that it did on that in the liver. Reduction, induced by

haemorrhage, of Fe stored in the livers and spleens of rats has, on the other hand, been reported (Morgan, 1961) to occur in the same relative proportions from these organs.

In the kidney, as in the liver, concentrations of Fe were lower in the groups supplemented with Cu. In the heart, in which Fe is probably associated primarily with enzyme systems and myoglobin, comparison of concentrations between the various groups, without dissection into the constituents, is less meaningful than where storage Fe is considered.

Concentrations of Cu were significantly decreased in liver, kidney and heart of basal, compared with positive control groups. It is not immediately apparent why the concentrations of Cu in all three organs of the rats on the non-sulphided diet should be less in the basal than in the group given the Fe supplement, or why, on both diets, the concentrations of Cu in the hearts of the animals given a supplement only of Cu should be higher than those of the groups given supplements of both Cu and Fe.

Further support for the hypothesis that Cu is needed for the release of Fe was obtained during the course of Expt 2. Injection of Cu into deficient rats was followed by a significant increase in the concentration of plasma Fe compared with that in rats injected with physiological saline solution (Table 3).

The increase in the concentration of plasma Fe that occurred in deficient animals 2–24 h after treatment with Cu and removal of a small quantity of blood may indicate that the Fe so released cannot immediately be utilized efficiently for production of haemoglobin. Similar treatment of control animals was followed either by a decrease, or a non-significant increase, of plasma Fe, indicating rapid utilization to replace the haemoglobin lost by bleeding. At least 20% of labelled Fe injected into normal rats may be detected in the red cells within 24 h (cf. Reichlin & Harrington, 1960).

Although, 48 and 72 h after a single injection of 200 μg of Cu into deficient rats, the concentrations of plasma Fe were not significantly different from those before injection (Table 4), Fe continued to be released from the liver throughout the period of observation (Fig. 2), from which it may be tentatively concluded that following treatment with Cu a period of more than 24 h was needed for efficient utilization of the Fe released into the circulation.

Injected Cu was transported fairly rapidly to the livers of deficient rats (Fig. 3) and, unlike Fe, the concentration in the plasma gradually increased over the following 72 h.

Fe (1750 μg) injected into the peritoneal cavities of Cu-deficient rats over a period of 5 d had no apparent influence, 24 h after the last injection, on the concentrations of either plasma Fe or haemoglobin; the concentration of Fe in the liver was, however, increased to normal by this procedure, and some increase was noted in the amount of ferritin in bone-marrow. Storage and transport of Fe are therefore able to proceed at tissue Cu concentrations which are too low to allow for release of Fe from ferritin in the liver.

Slices of liver incubated anaerobically have been shown to liberate more Fe into the medium than similar slices incubated aerobically (Mazur, Baez & Shorr, 1955). The amount of Fe released into the medium during anaerobic incubation of liver slices from both deficient and normal rats, less than released during aerobic incubation, was related more closely to the total Fe content of the liver than to the Cu

content (Table 5), in contradistinction to conditions in vivo where Fe is apparently not released when the Cu concentration is limiting. Results of experiments carried out in vitro are thus not always directly applicable to reactions occurring in the live animal.

The results presented suggest that a primary function of Cu in the metabolism of Fe is participation, either as a Cu-containing enzyme or as a co-factor in an enzyme system, in the reaction by which Fe is released from ferritin. This hypothesis is based on the assumption that Fe absorbed from the intestinal tract, and that injected intraperitoneally, is transported to the liver by transferrin.

The very large stores of Fe found in the livers of Cu-deficient sheep (Marston, 1952) and the apparent ease with which intraperitoneally injected Fe found its way to the livers (Table 6), and some to the bone-marrows, of rats given a diet deficient in both Cu and Fe, together with the low haemoglobin and plasma Fe concentrations of these animals, makes the postulate of Osaki *et al.* (1966), that caeruloplasmin mediates in the mechanism by which Fe is attached to apotransferrin, difficult to accept if absorbed Fe is in fact transported by transferrin. If, on the other hand, transport of absorbed Fe is effected by a complex other than transferrin, which seems unlikely, though it is doubtful if there is any proof that it is not so, and transferrin is active only in the transport of Fe from storage sites such as liver and spleen to tissues where it is required, then a virtual absence of caeruloplasmin could explain the non-availability of Fe, i.e. the reduction in plasma Fe (transferrin) concentrations in Cu-deficient animals.

Whether Cu also assumes a role in the utilization of Fe for production of haemoglobin has not been determined. 6-Aminolaevulic acid dehydrase, which catalyses the formation of porphobilinogen, was originally thought to be a Cu-containing enzyme, but recent findings do not support this claim (cf. Rimington, 1958; Harris, 1963), and abnormally high concentrations of free protoporphyrin have been found in the red cells of Cu-deficient sheep (Allen, 1956). The enzyme, ferrochelatase, which catalyses the production of haem from protoporphyrin and Fe, is said to be inhibited by Cu (Labbe & Hubbard, 1961).

The studies reported leave many questions unanswered; they do, however, indicate fields where further study may be profitably undertaken.

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REFERENCES

Aisen, P., Aasa, R., Malmström, B. G. & Vänngård, T. (1967). *J. biol. Chem.* **242**, 2484.
Alfrey, C. P. Jr, Lynch, E. C. & Whitley, C. E. (1967). *J. Lab. clin. Med.* **70**, 419.
Allen, S. H. (1956). *Biochem. J.* **63**, 461.
Bates, G. W., Billups, C. & Saltman, P. (1967*a*). *J. biol. Chem.* **242**, 2810.
Bates, G. W., Billups, C. & Saltman, P. (1967*b*). *J. biol. Chem.* **242**, 2816.
Beutler, E., Fairbanks, V. F. & Fahey, J. L. (1963). *Clinical Disorders of Iron Metabolism.* New York and London: Grune & Stratton.
Billups, C., Pape, L. & Saltman, P. (1967). *J. biol. Chem.* **242**, 4284.
Brown, E. B. (1963). *Am. J. clin. Nutr.* **12**, 205.
Cheney, B. A., Lothe, K., Morgan, E. H., Sood, S. K. & Finch, C. A. (1967). *Am. J. Physiol.* **212**, 376.
Coons, C. M. (1964). *A. Rev. Biochem.* **33**, 459.
Crosby, W. H. & Furth, F. W. (1956). *Blood* **11**, 380.
Dacie, J. V. & Lewis, S. M. (1963). *Practical Haematology* 3rd ed. London: J. & A. Churchill Ltd.
Darcel, C. le Q. (1961). *Can. J. comp. Med.* **25**, 129.
Deutsch, W. (1936). *J. Physiol., Lond.* **87**, 56*P*.
Dreosti, I. E. & Quicke, G. V. (1968). *Br. J. Nutr.* **22**, 1.
Gabuzda, T. G. & Pearson, J. (1968). *Nature, Lond.* **220**, 1234.
Giovanniello, T. J. & Peters, T. Jr (1963). *Stand. Meth. clin. Chem.* **4**, 139.
Greenough, W. B. III, Peters, T. Jr & Thomas, E. D. (1962). *J. clin. Invest.* **41**, 1116.
Gross, F. (editor) (1964). *Iron Metabolism.* International Symposium, Aix-en-Provence, France, 1963: sponsors Ciba.
Harris, J. W. (1963). *The Red Cell.* Cambridge, Massachusetts: Harvard University Press.
Hart, E. B., Steenbock, H., Waddell, J. & Elvehjem, C. A. (1928). *J. biol. Chem.* **77**, 797.
Hedenstedt, S. (1947). *Acta chir. scand.* **95**, suppl. 128.
Labbe, R. F. & Hubbard, N. (1961). *Biochim. biophys. Acta* **52**, 130.
Marston, H. R. (1952). *Physiol. Rev.* **32**, 66.
Marston, H. R. & Allen, S. H. (1967). *Nature, Lond.* **215**, 645.
Mazur, A., Baez, S. & Shorr, E. (1955). *J. biol. Chem.* **213**, 147.
Morgan, E. H. (1961). *Aust. J. exp. Biol. med. Sci.* **39**, 371.
Osaki, S., Johnson, D. A. & Frieden, E. (1966) *J. biol. Chem.* **241**, 2746.
Pape, L., Multani, J. S., Stitt, C. & Saltman, P. (1968*a*). *Biochemistry, Easton* **7**, 606.
Pape, L., Multani, J. S., Stitt, C. & Saltman, P. (1968*b*). *Biochemistry, Easton* **7**, 613.
Reichlin, M. & Harrington, W. J. (1960). *Blood* **16**, 1298.
Reith, E. J. (1961). *J. Cell Biol.* **9**, 825.
Rice, E. W. (1963). *Stand. Meth. clin. Chem.* **4**, 57.
Rimington, C. (1958). *Rev. pure appl. Chem.* **8**, 129.
Salera, U., Magnanelli, P., d'Avino, R., Zecca, I. & Matcovich, A. L. (1961). *Proc. 8th Congr. Eur. Soc. Haematol., Vienna* p. 240*a*.
Smith, J. A., Drysdale, J. W., Goldberg, A. & Munro, H. N. (1968). *Br. J. Haemat.* **14**, 79.
Summerson, W. H. (1938). *J. biol. Chem.* **123**, cxix.
Thirayothin, P. & Crosby, W. H. (1962). *J. clin. Invest.* **41**, 1206.
Underwood, E. J. (1962). *Trace Elements in Human and Animal Nutrition* 2nd ed. New York and London: Academic Press Inc.
Wynter, C. V. A. & Williams, R. (1968). *Lancet* ii, 534.
Zail, S. S., Charlton, R. W., Torrance, J. D. & Bothwell, T. H. (1964). *J. clin. Invest.* **43**, 670.

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