Biochemical Lesions in Copper-Deficient Rats Caused by Secondary Iron Deficiency

DERANGEMENT OF PROTEIN SYNTHESIS AND IMPAIRMENT OF ENERGY METABOLISM

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Severe copper deficiency was induced in rats by rearing nursing dams and their offsprings on a semisynthetic diet comprising all the requisite nutrients and trace metals except copper. The copper-deprived rats exhibited growth retardation, severe anaemia, loss of caeruloplasmin, decrease of cytochrome oxidase, accumulation of salt-soluble collagen and a drastic decrease in iron in plasma and liver. Apart from these characteristic signs of deficiency, a marked inhibition of protein synthesis was found to occur both *in vivo* and in cell-free liver preparations. The curtailed ability to carry out endogenously coded amino acid incorporation into protein contrasted with the unimpaired poly(U)-acid-directed phenylalanine polymerization. This inhibition pattern, as well as the attendant disaggregation of the liver polyribosomes, suggested that the primary biosynthetic lesion was located at the stage of peptide-chain initiation. Concurrently with this alteration there was a pronounced depletion of the hepatic ATP content, associated with a parallel depression of mitochondrial respiration and an enhancement of ATPase activity. Supplementation of the copper-deficient diet with a 2–4-fold excess of iron (relative to the standard diet) prevented growth retardation and anaemia and restored normal energy metabolism, as well as unimpaired protein-synthesizing capacity. The conclusion that these disturbances were primarily determined by the secondary iron deficiency was also borne out by the finding that similar alterations occurred in rats maintained on a copper-sufficient but iron-deficient diet. On the other hand, the iron-fortified diet failed to reverse the other signs of copper deficiency, namely the loss of caeruloplasmin, the diminished rate of cytochrome oxidase and the increase of soluble collagen. The interrelations between the various biochemical lesions induced by deprivation of copper or iron are discussed and the possible role of ATP depletion in determining the derangement of protein synthesis is considered.

The multiple physiological functions of copper and its role as an indispensable micronutrient have been brought to light by the numerous descriptions in the literature of the various functional and structural alterations occurring in copper-deficient animals. These disturbances comprise growth retardation, anaemia, impaired utilization of iron and depletion of its body stores, depigmentation of hair and teeth, demyelination of the central nervous

system, defective formation of cross-linkages in collagen and elastin with resultant accumulation of soluble collagen, and appearance of bone deformities and aneurysms (Peisach *et al.*, 1966; Mills, 1970; Evans, 1973; O'Dell, 1976; Underwood, 1977).

The understanding of the biochemical lesions underlying the gross symptoms of copper deficiency, though still rather incomplete, has been greatly advanced by the extensive information accrued from enzymological studies on the role of copper as an integral constituent of a large variety of enzymes, such as caeruloplasmin (EC 1.16.3.1), cytochrome

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oxidase (EC 1.9.3.1), dopamine β -hydroxylase (EC 1.14.17.1), superoxide dismutase (EC 1.15.1.1), lysyl oxidase and others (O'Dell, 1976; Underwood, 1977).

The present paper describes some novel observations on the derangement of hepatic energy metabolism and inhibition of protein synthesis in copper-deficient rats and provides evidence that these alterations, as well as growth arrest and anaemia, are primarily determined by the severe iron deficiency induced by copper deprivation.

Experimental

Materials

The various nucleotides and enzymes were acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Radioactive compounds were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Ferritin was purchased from Nutritional Biochemicals Corp. (Cleveland, OH, U.S.A.). Complete Freund's adjuvant was obtained from Difco Laboratories (Detroit, MI, U.S.A.).

Anti-ferritin serum was prepared in rabbits by three weekly subcutaneous injections of 5 mg of ferritin suspended in 0.2 ml of Freund's adjuvant. Blood was collected 1 week after the last injection, the serum was separated and 0.24% (w/v) phenol was added as a preservative. All other chemicals were commercial products of analytical or reagent grade.

Copper-deficient and iron-deficient animals

Charles River Strong albino rats of local breed were used. In each cage one nursing dam with eight suckling offspring were housed together and supplied *ad libitum* with glass-distilled water and a semi-synthetic copper-deficient diet containing less than 0.8 p.p.m. of Cu^{2+} and compounded as described by Dutt & Mills (1960), except that casein was replaced by skim milk. Control rats received 20 p.p.m. of Cu^{2+} (in the form of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in the solid food and 20 p.p.m. in the drinking water. After weaning at the age of 21 days, nickel grids were introduced into the cages to prevent coprophagy. This precaution was deemed necessary in view of the fact that a large proportion of the ingested copper is known to be excreted in the faeces (Owen, 1964). Cages and grids were coated with plastic paint to obviate contact of the rats with metallic parts.

Iron-supplemented copper-deficient rats received 40–80 p.p.m. of Fe^{2+} (in the form of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) in the solid food and 20 p.p.m. in the drinking water.

Iron-deficient rats were reared on the same semisynthetic diet as above, except that the iron salt in the diet (Dutt & Mills, 1960) was replaced by 20 p.p.m. of Cu^{2+} and another 20 p.p.m. of Cu^{2+}

were added to the drinking water. The diet contained less than 6 p.p.m. of iron.

Methods

Assays of protein and RNA synthesis. Protein synthesis *in vivo* and *in vitro* was assayed as previously described (Glaser & Mager, 1972). RNA synthesis *in vivo* was measured by injecting intraperitoneally $1 \mu\text{mol}$ of $[6\text{-}^{14}\text{C}]\text{orotate}$ (25 Ci/mol)/kg. The animals were killed 80 min later and a 30% (w/v) liver homogenate in medium A (see legend to Table 2) was prepared. RNA was precipitated with cold trichloroacetic acid (final concn. = 5%, w/v) and hydrolysed by overnight incubation at room temperature in 0.25 M-NaOH. The radioactivity of the resultant nucleotides was measured in Bray's (1960) solution, with a Packard liquid-scintillation spectrometer, and corrected for quenching by use of an internal standard.

Measurement of oxidative phosphorylation. A 10% liver homogenate (wet wt.) in 0.25 M-sucrose containing 0.5 mM-EDTA was centrifuged in the cold for 10 min at 650g to sediment debris and nuclei. Mitochondria were pelleted by centrifugation for 10 min at 10000g, then washed with 10 vol. of 0.25 M-sucrose containing 5 mM-Tris/HCl, pH 7.4, and an amount equivalent to 1 g of liver was suspended in 1.5 ml of the same 0.25 M-sucrose/Tris solution. The reaction mixture contained, in a final volume of 2 ml, the following components: 25 mM-Tris/HCl, pH 7.4, 20 mM-potassium phosphate, pH 7.4, 12.5 mM-KCl, 10 mM-MgSO₄, 7.5 mM-NaF, 1.5 mM-ATP, 20 mM- β -hydroxybutyrate (sodium salt), 20 mM-glucose, 1 mg (150 units) of crystalline hexokinase (EC 2.7.1.1) and approx. 2.5 mg of mitochondrial protein.

The mixture was incubated with continuous shaking for 10 min at 30°C and the reaction was stopped by adding 8 ml of 5% (w/v) trichloroacetic acid. Oxidative phosphorylation (P/O ratios) was measured in the supernatant by determining the amounts of P_i that disappeared and acetoacetic acid formed.

Miscellaneous analytical procedures

Sucrose-gradient-centrifugation analyses were performed as previously described (Glaser & Mager, 1972). Ferritin was determined by precipitation with antiserum, and the iron-induced synthesis of ferritin was assayed by measuring the increase of the liver ferritin content 20 h after the intraperitoneal injection of 50 mg of iron-dextran/kg (Yoshimo *et al.*, 1968). Mitochondrial and microsomal ATPase activities were measured as described by Ernster *et al.* (1962). Mitochondrial cytochrome oxidase activity was assayed as described by Wharton & Tzagoloff (1967), by following spectrophotometrically the rate

of oxidation of ferrocytochrome *c*. Caeruloplasmin was determined as described by Houchin (1958), by measuring the oxidation of *p*-phenylenediamine in a 10-fold-diluted 0.1 ml sample of plasma during 30 min incubation at 37°C. The activity is expressed in terms of ΔA_{520} . Haemoglobin was measured by the method of Drabkin & Austin (1932). Skin soluble collagen was extracted as described by Tanzer & Gross (1964), hydrolysed with 6 M-HCl in sealed ampoules for 4 h at 130°C, and its hydroxyproline content was determined by the procedure of Woessner (1961). Liver ATP was extracted and determined by the luciferin-luciferase method as previously described (Freudenberg & Mager, 1971; Glaser & Mager, 1972). P_i was measured by the procedure of Fiske & Subbarow (1925). Acetoacetate was measured as described by Walker (1954). Copper and iron were determined in tissue digests [prepared in hot conc. H_2SO_4 containing 70% (w/v) $HClO_4$] and in plasma deproteinized with 10% (w/v) trichloroacetic acid with the aid of a Zeiss M-20 atomic absorption spectrometer equipped with suitable hollow-cathode lamps. Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Copper deficiency

Rats reared under the conditions of copper deprivation, as described in the Experimental section, exhibited at the age of 6–8 weeks severe symptoms of copper deficiency. As shown in Table 1, the copper contents in the plasma and liver of

these rats were about 6–7 times lower than the corresponding values in normal controls (Table 1). The copper deficiency was manifested also by growth retardation, severe anaemia (as reflected in the decrease in blood haemoglobin), obliteration of plasma caeruloplasmin, decrease of mitochondrial cytochrome oxidase and increase of salt-soluble collagen in the skin.

Protein synthesis, as measured *in vivo* by following the incorporation of labelled amino acids into the proteins of various organs, such as liver, kidney, heart and brain, showed a progressive decline in the course of the evolving copper depletion (Table 2). The degree of inhibition was essentially the same, when different ^{14}C -labelled amino acids were used as tracers, or when their concentrations were varied over a 50-fold range.

A 50–60% inhibition of protein synthesis was also demonstrable in the postmitochondrial fractions from copper-deficient rat livers. Here again, the degree of inhibition did not change appreciably when different tracer amino acids were used or when their concentrations were varied (Table 3). It seemed therefore justifiable to conclude that the decline in the amino acid incorporation into protein represented an intrinsic defect of the system, rather than isotope dilution owing to a possible increase of the internal amino acid pools.

In contrast with the decreased phenylalanine incorporation into protein coded by the endogenous mRNA, the poly(U)-directed phenylalanine polymerization was practically the same in postmitochondrial preparations from both normal and copper-deficient rat livers (Table 4). This charac-

Table 1. *Biochemical alterations induced in rats by copper-deficient and iron-deficient diets: effects of copper repletion and iron supplementation*

Each experimental group comprised five or six rats 7–8 weeks old. The rats were starved overnight before the experiment and killed by cervical dislocation. Where indicated, copper repletion was started at the age of 6 weeks and continued for 2 weeks. The iron-supplemented copper-sufficient diet contained a 4-fold higher amount of iron than the normal standard diet. The values found in the iron-supplemented control group did not differ essentially from those of the unsupplemented control. The data represent means of 8–12 experiments in each group. $\pm$ S.D. All the assays were run in duplicate.

Diet	Body weight (g)	Plasma copper (μ g/100 ml)	Liver copper (μ g/g dry wt.)	Plasma iron (μ g/100 ml)	Liver iron (μ g/g dry wt.)	Haemoglobin (g/100 ml of blood)	Caeruloplasmin (ΔA_{520} /0.1 ml of plasma)	Skin salt-soluble collagen (μ g of hydroxyproline/g fresh wt.)
Control	184 $\pm$ 21	134 $\pm$ 19	24 $\pm$ 3.7	263 $\pm$ 31	295 $\pm$ 23	13.8 $\pm$ 1.2	0.36 $\pm$ 0.02	160 $\pm$ 40
Copper-deficient	95 $\pm$ 25	29 $\pm$ 6.4	3.5 $\pm$ 0.9	57 $\pm$ 7	118 $\pm$ 15	3 $\pm$ 0.8	0	340 $\pm$ 25
Copper-deficient followed by copper-sufficient	178 $\pm$ 10	142 $\pm$ 11	38 $\pm$ 5.4	240 $\pm$ 18	240 $\pm$ 20	11.5 $\pm$ 0.7	0.32 $\pm$ 0.01	190 $\pm$ 18
Copper-deficient plus 4-fold excess of iron	192 $\pm$ 20	32 $\pm$ 5.8	3.6 $\pm$ 0.8	190 $\pm$ 25	294 $\pm$ 30	12.5 $\pm$ 0.6	0	325 $\pm$ 35
Iron-deficient	105 $\pm$ 15	145 $\pm$ 15	36 $\pm$ 4.2	60 $\pm$ 13	121 $\pm$ 35	2.8 $\pm$ 0.4	0.38 $\pm$ 0.02	116 $\pm$ 21

Table 2. *Progressive decline of protein synthesis in vivo in copper-deficient rats*

Protein synthesis was measured by the extent of incorporation of [^{14}C]leucine into the proteins of various organs. The radioactive amino acid was dissolved in 0.9% NaCl at 1 mM concn. (6 Ci/mol) and injected intraperitoneally at a dose of 1 ml/100 g body wt. The animals were killed 30 min after the injection, and the organs were excised and homogenized with 2 vol. of medium A (250 mM-sucrose, 25 mM-KCl, 1 mM-MgSO₄ and 50 mM-Tris/HCl buffer, pH 7.5). All the subsequent operations were performed as previously described (Glaser & Mager, 1972).

Organs tested	Time on copper-deficient diet . . .	Amino acid incorporation into protein (% of control)		
		3 weeks	5 weeks	8 weeks
Liver		80 $\pm$ 10	60 $\pm$ 14	44 $\pm$ 8
Kidney		95 $\pm$ 16	72 $\pm$ 8	52 $\pm$ 6
Heart		85 $\pm$ 12	65 $\pm$ 10	40 $\pm$ 10
Brain		90 $\pm$ 8	74 $\pm$ 6	48 $\pm$ 7

Table 3. *Inhibition of amino acid incorporation into protein in a postmitochondrial liver fraction from copper-deficient rats*

The reaction mixture contained, in a final volume of 0.1 ml, the following ingredients: 50 mM-Tris/HCl buffer, pH 7.4, 100 mM-KCl, 1 mM-ATP, 0.2 mM-GTP, 2.5 mM-MgSO₄, 20 mM-phosphocreatine, 40 μg of creatine kinase (EC 2.7.3.2), 0.02 ml of the liver postmitochondrial fraction (15 000 g supernatant) containing about 0.2 mg of protein, and the U- ^{14}C -labelled L-amino acids in the amounts indicated. The complete system was incubated at 30°C for 30 min. The radioactive proteins were isolated as described by Mans & Novelli (1961) and counted for radioactivity in a Packard liquid-scintillation spectrometer, corrected for quenching by use of an internal radioactive standard.

U- ^{14}C -labelled amino acids (mM)	Amino acid incorporation into protein		
	Control rats (c.p.m.) (A)	Copper-deficient rats (c.p.m.) (B)	B as % of A
Phenylalanine (0.4)	2515	1182	47
Phenylalanine (1.2)	3685	1547	42
Leucine (0.4)	1224	526	43
Leucine (1.2)	1630	749	46
Valine (0.2)	1024	460	45
Valine (1.8)	1680	772	46

Table 4. *Inhibition of endogenously coded incorporation of phenylalanine into protein and unimpaired poly(U)-directed phenylalanine polymerization in copper-deficient and iron-deficient rats; corrective effects of copper repletion and iron supplementation*

The reaction mixture was the same as described in Table 2. For measuring poly(U)-dependent phenylalanine incorporation the MgSO₄ concentration was increased to 10 mM and 10 μg of poly(U) was added. Copper repletion was started at the age of 6 weeks and continued for 2 weeks. The iron-supplemented copper-deficient diet contained a 2-fold excess of iron relative to the standard diet. Phenylhydrazine-treatment was initiated 1 week after weaning, with six daily subcutaneous injections of 50 mg/kg and was continued for another 2 weeks with daily injections of 30 mg/kg. Pair-feeding consisted in limiting the food intake of normal rats to that of their copper-deficient counterparts. The rats were killed at the age of 6–7 weeks.

Treatment	Body weight (g)	Haemoglobin (g/100 ml of blood)	[^{14}C]Phenylalanine incorporation (c.p.m./mg of protein)	
			Endogenously coded	Poly(U)-coded
Control	163 $\pm$ 34	12.8 $\pm$ 1.8	2076 $\pm$ 150	2240 $\pm$ 175
Copper-deficient diet	78 $\pm$ 14	3.0 $\pm$ 0.5	964 $\pm$ 45	2392 $\pm$ 124
Copper-deficient followed by copper-sufficient diet	145 $\pm$ 28	11.5 $\pm$ 1.4	2010 $\pm$ 85	2348 $\pm$ 186
Iron-supplemented copper-deficient diet	138 $\pm$ 22	10.2 $\pm$ 1.1	1951 $\pm$ 128	2385 $\pm$ 105
Iron-deficient diet	67 $\pm$ 18	2.5 $\pm$ 0.3	996 $\pm$ 67	2174 $\pm$ 88
Pair-feeding	82 $\pm$ 16	13.4 $\pm$ 2.1	2224 $\pm$ 165	2326 $\pm$ 125
Phenylhydrazine	110 $\pm$ 24	4.5 $\pm$ 1.2	1968 $\pm$ 134	2040 $\pm$ 148

Fig. 1. *Effect of various diets on the liver ribosome patterns*

The 30000g supernatant of the liver homogenates from the different experimental groups were diluted 10-fold with medium A and supplemented with sodium deoxycholate to a final concn. of 0.5%. Suitable samples were analysed in a 5 ml linear 15–40% (w/v) sucrose gradient in medium A by centrifuging for 60 min at about 200000g in a Spinco model SW 50.1 rotor. The A_{254} was recorded by pumping the gradient through a flow cell in the Isco model UA.2 analyser. Other experimental details were as previously described (Freudenberg & Mager, 1971; Glaser & Mager, 1972). Conditions otherwise were as in Tables 1 and 2. (a) Control; (b) copper-deficient; (c) copper-deficient, fortified with a 2-fold excess of iron (relative to the iron content of the standard diet); (d) iron-deficient. The liver ribosome profile from rats maintained on iron-supplemented control diet was indistinguishable from that of the control group. Note the virtual absence of polyribosomes in (b) and (d).

teristic pattern of inhibition correlated with an increase in the proportion of monoribosomes and a reciprocal decrease of polyribosomes in the liver ribosome populations, as measured by sucrose-gradient-centrifugation analysis (Fig. 1). The decline of the protein-synthetic activity was not accompanied by any significant alteration in the ability of the copper-deficient rats to carry out incorporation of [^{14}C]orotic acid into liver RNA.

It appeared possible that the inhibition of protein

synthesis in the copper-deficient rats was a secondary outcome of the diminished food intake or anaemia. This possibility, however, was not borne out by the results of the experiments, which showed that neither restricted food supply instituted by pair-feeding of normal rats together with the copper-deficient animals nor anaemia induced by phenylhydrazine treatment gave rise to any perceptible impairment of protein synthesis (Table 4).

Re-feeding of copper to the copper-deficient rats

resulted at the end of 2 weeks in complete restoration of the normal protein-synthetic activity (Table 4), along with the reversal of the other alterations induced by copper deprivation (Table 1).

Iron deficiency

In agreement with the earlier literature (Elvehjem, 1935; Chase *et al.*, 1952; Marston *et al.*, 1971), it was noticed in the course of these experiments that copper deficiency was attended by a marked decrease in the iron contents of the plasma and liver (Table 1) and a depletion of the hepatic ferritin content to about 30% of the control value (24 ± 10 and $77 \pm 19 \mu\text{g}$ of protein/g of liver respectively). It was also realized that intraperitoneal administration of iron, which is known to induce ferritin formation in the livers of normal rats (Drysdale & Munro, 1966), enhanced the synthesis of ferritin in the copper-deficient rats, so as to raise its content within 20h up to that of the similarly treated controls (about $160 \mu\text{g}$ of protein/g of liver). In extending this observation it was found that addition of a 2–4-fold excess of iron (over that present in the standard diet) to the diet of the copper-deficient rats resulted in resumption of normal food intake and growth and restoration of the normal blood haemoglobin content. The excess iron supply was also effective in reversing the derangement of the protein-synthetic machinery, as reflected in the restitution of unimpaired amino acid incorporation into protein (Table 4) and of the normal pattern of ribosome aggregation (Fig. 1). On the other hand, iron supplementation failed to affect the other characteristic signs of copper deficiency, namely the augmented amount of salt-soluble collagen, the

virtual loss of caeruloplasmin from the plasma and the strikingly diminished mitochondrial cytochrome oxidase activity (Tables 1 and 5).

These results suggested that the iron deficiency secondary to copper deprivation was primarily responsible for the inhibition of protein synthesis. The latter conclusion was strengthened by the finding that the liver postmitochondrial fraction from rats reared on a copper-sufficient but iron-deficient diet exhibited the same pattern of protein synthesis inhibition as that observed in the copper-deficient animals (Table 4, Fig. 1).

Hepatic ATP depletion

In a separate series of experiments it was found that livers of copper-deficient rats exhibited a progressive decrease of their ATP content. The decline was noticeable already in the weanling rats maintained on a copper-deficient diet and progressed until at the age of 4–6 weeks the liver ATP content was stabilized at about 40–50% of the normal value (Table 5). Here again, the causal role of the secondary iron deficiency was indicated by the finding that ATP depletion was largely reversed by adding a 2-fold excess of iron to the copper-deficient diet. On the other hand, nutritional iron deprivation induced a decrease in the liver ATP similar to that found in the copper-deficient rats (Table 5). In contrast, no significant change in the ATP content was seen in rats rendered anaemic by phenylhydrazine treatment or in pair-fed rats in which the food intake was restricted so as to match the quantity of food consumed by their copper-deficient counterparts (results not shown).

The ATP depletion appeared to correlate with a

Table 5. *Effect of dietary deprivation of copper or iron on hepatic ATP content, mitochondrial respiration and cytochrome oxidase activity*

Rats aged 7–8 weeks were used and the litters were randomized after weaning. Oxidative phosphorylation and cytochrome oxidase were determined as described in the Experimental section. Where indicated, copper re-feeding started at the age of 6 weeks and was continued for 2 weeks. The iron-supplemented diet contained a 2-fold excess of iron relative to the control diet. The data are means of six to eight experiments $\pm$ s.d. Conditions otherwise were as in Table 1.

Diet	Hepatic ATP content ($\mu\text{mol/g}$ wet wt.)	Uptake of P_i ($\mu\text{mol/h}$ per mg of mitochondrial protein)	Acetoacetate formed ($\mu\text{mol/h}$ per mg of mitochondrial protein)	P/O ratio	Cytochrome oxidase (μmol of ferrocytochrome c oxidized/min per mg of mitochondrial protein)
Control	1.6 ± 0.2	15 ± 2.3	5.4 ± 0.6	2.8	0.45 ± 0.08
Copper-deficient	0.7 ± 0.1	7.2 ± 2.2	2.4 ± 0.5	3.0	0.10 ± 0.03
Copper-deficient followed by copper-sufficient	1.2 ± 0.2	11.4 ± 2.0	4.2 ± 0.7	2.7	0.43 ± 0.06
Iron-supplemented copper-deficient	1.4 ± 0.2	13.8 ± 2.4	4.8 ± 0.4	2.9	0.12 ± 0.04
Iron-deficient	0.6 ± 0.2	6.6 ± 1.8	2.4 ± 0.3	2.8	0.47 ± 0.05

marked decrease in the respiratory activity of the rat liver mitochondria, which occurred both in copper-deficient (cf. Gallagher *et al.*, 1956) and in iron-deficient rats. The decay of the oxidase capacity of the mitochondria was attended by a parallel decrease in the rate of coupled phosphorylation, with no significant change in the P/O ratio. Furthermore, the impairment of the oxidative phosphorylation was prevented by iron supplementation of the copper-deficient diet (Table 5).

Of possible relevance to the underlying mechanism of ATP depletion was the additional observation that ATPase activity, as measured both in the liver mitochondria and in the postmitochondrial (microsomal) fractions of either copper-deficient or iron-deficient rats, exhibited a nearly 2-fold increase relative to the normal controls. In the copper-deficient animals this alteration could be corrected either by re-feeding with copper or by iron supplementation (Table 6).

The activity of the copper-containing cytochrome oxidase (Wainio *et al.*, 1959; Kuboyama *et al.*, 1972) was decreased in the liver mitochondria of copper-deficient rats to about 20–25% of the control value (cf. Gallagher *et al.*, 1956). The diminished rate of cytochrome oxidase, however, did not seem to play a role in limiting the rate of mitochondrial respiration and mediating ATP depletion, since, as mentioned above, the latter alterations occurred also in iron-deficient rats which showed no impairment of

cytochrome oxidase activity (Table 5). Furthermore, iron supplementation corrected both mitochondrial respiration and ATP depletion, with no concomitant restoration of normal cytochrome oxidase activity (Table 5).

Farber and his co-workers (Villa-Trevino *et al.*, 1963; Farber, 1967) have described the occurrence in ethionine-poisoned rats of a pronounced inhibition of hepatic protein synthesis coinciding with a drastic fall in the ATP content, owing to the trapping of its adenosine moiety in the form of S-adenosylethionine. They also showed that both lesions could be reversed by administration of adenine or its nucleotide derivatives.

We found that injection of AMP to copper-deficient rats (200 μmol of AMP/100g body wt.) had no significant effect on their liver ATP content, in contrast with the substantial ATP increase elicited in similarly treated controls (for details see Glaser & Mager, 1972). We inferred, therefore, that ATP depletion in the copper-deprived rats reflected an impaired capacity for energy generation, rather than a diminished availability of ATP precursors.

Discussion

The results of the present study clearly indicate that some of the major symptoms of nutritional copper deficiency are attributable to a secondary defect of iron metabolism, reflecting an impaired capacity for utilization of ingested iron (Chase *et al.*, 1952; Lahey *et al.*, 1953; Lee *et al.*, 1968). We found that growth retardation, anaemia, hepatic ATP depletion and inhibition of protein synthesis were prevented by supplementing the copper-deficient diet with an excess of iron and could be induced in copper-repleted rats by dietary iron deprivation. By contrast, the other characteristic signs of copper deficiency, namely accumulation of salt-soluble collagen and the decreases in cytochrome oxidase and caeruloplasmin were not affected by excess iron supply.

Frieden and his collaborators (Osaki *et al.*, 1966) postulated that the impaired ability of copper-deficient rats to utilize iron is due to the loss of caeruloplasmin, which normally functions by oxidizing ferrous iron to its ferric form and thereby facilitates the mobilization of the iron stores from the reticuloendothelial system into the plasma and the transferrin-mediated transport of the plasma iron to the haematopoietic tissues. As pointed out by Dowdy (1969), this concept does not account for the fact that the characteristic absence of caeruloplasmin in Wilson's disease (hepatolenticular degeneration) is usually not accompanied by anaemia or disturbed iron metabolism. More recently, Topham & Frieden (1970) discovered another copper-

Table 6. Enhancement of the ATPase activity of rat liver mitochondria and postmitochondrial fraction induced by deprivation of copper or iron

The reaction mixture contained, in a final volume of 1 ml, the following components: 50 mM-Tris/HCl buffer, pH 7.4, 5 mM-MgSO₄, 5 mM-ATP (sodium salt) and 0.1 ml of the mitochondrial or postmitochondrial preparations, containing about 1 mg of protein each. After incubation for 10 min at 37°C the reaction was terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid. The mixtures were as in Table 1.

Diet	ATP hydrolysis (μmol of P _i liberated/10 min per mg of protein)	
	Mitochondria	Postmitochondrial fraction
Control	0.42 ± 0.04	0.38 ± 0.03
Copper-deficient	0.68 ± 0.05	0.75 ± 0.06
Copper-deficient followed by copper-sufficient	0.44 ± 0.07	0.45 ± 0.03
Iron-supplemented copper deficient	0.40 ± 0.03	0.41 ± 0.05
Iron-deficient	0.76 ± 0.06	0.80 ± 0.07

containing enzyme (ferro-oxidase-II), which in their view may play a role in maintaining normal iron metabolism in the absence of caeruloplasmin. However, Frieden's hypothesis (Osaki *et al.*, 1966; Topham & Frieden, 1970), even in its revised version, cannot be readily reconciled with the present observation that the copper-deficiency anaemia could be prevented by supplying an excess of iron to the deficient rats, unless it is assumed that the supplementary iron acts by bypassing the need for the normal process of iron transport from the plasma to the tissues.

The hepatic ATP depletion observed in the copper-deficient or iron-deficient rats seems to be governed primarily by the impairment of mitochondrial respiration, whereas the concomitant increase in ATPase activity may play a contributory role. On the other hand, it is evident that the deterioration of the respiratory capacity, as well as the decrease in ATP, bear no relationship to the decrease of the mitochondrial cytochrome oxidase, since both disturbances could be corrected by iron supplementation, which had no effect on the activity of cytochrome oxidase. It therefore appears reasonable to surmise that impaired formation of some iron-containing component(s) of the mitochondrial electron-transport system, other than cytochrome oxidase, may be responsible for the derangement of the energy-yielding respiration.

The inhibition of protein synthesis in the copper-deficient (or iron-deficient) rats was found to occur not only in the liver but also in other organs tested, such as brain, heart and kidney, and seems therefore to be a widespread, systemic lesion. It appears likely that defective protein synthesis is also involved in the pathogenesis of the characteristic anaemia.

The postmitochondrial liver fraction from copper-deficient (or iron-deficient) rats exhibits a nearly complete disaggregation of the polyribosomes into monoribosomes. This alteration is indicative of a block at the stage of peptide-chain initiation, as the underlying cause of the inhibition of the amino acid incorporation into protein coded by endogenous mRNA. The latter interpretation accounts also for the unimpaired poly(U)-directed phenylalanine polymerization, which takes place at a relatively high Mg^{2+} concentration and does not involve the physiological mechanism of chain initiation (see Giloh & Mager, 1975).

A possible causal relationship of ATP depletion to the inhibition of protein synthesis is suggested by the work of Farber (1967) on the mechanism of ethionine toxicity, as mentioned in the Results section, as well as by the observations reported from our laboratory on the severe impairment of peptide-chain initiation occurring in ATP-starved rabbit reticulocytes (Giloh & Mager, 1975).

However, the precise molecular mechanism

mediating the deleterious effect of energy deprivation on the protein-synthetic system remains as yet to be elucidated.

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