Biochimica et Biophysica Acta 809 (1985) 351–361
Elsevier

BBA 41847

Studies on the effects of copper deficiency on rat liver mitochondria. I. Changes in mitochondrial composition

C.B. Lawrence, N.T. Davies *, C.F. Mills and F. Nicol

Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB (U.K.)

(Received March 25th, 1985)

Key words: Cu deficiency; Cytochrome *c* oxidase; Mitochondrial membrane; Fatty acid composition; (Rat liver mitochondria)

As part of an investigation of the lesions of copper (Cu) deficiency a study was undertaken of the copper, iron, cytochrome and fatty acid composition of liver mitochondria from Cu deficient and Cu-adequate control rats. Cu concentrations were significantly decreased in whole liver, liver mitochondria and in blood plasma. Total iron was significantly increased in whole liver but remained at the normal level in mitochondria. Cytochrome *c* oxidase (EC 1.9.3.1) and its component cytochromes *a* and a_3 were significantly reduced in liver mitochondria from Cu-deficient rats, whereas there was no effect on the concentration of cytochromes *b*, c_1 and *c*. Evidence from comparisons between cytochrome *c* oxidase activity and the amount of enzyme present, as assessed from the mitochondrial cytochrome *a* and a_3 content, suggests that in addition to an absolute loss of enzyme, Cu-deficiency adversely affects the efficiency of the residual enzyme. Severe Cu deficiency had no effect on 'ageing' or 'swelling' properties of liver mitochondria, indicating no marked effects on fatty acid composition. Fatty acid analyses demonstrated a slight but significant increase in docosapentenoic acid (22:5) of Cu-deficient mitochondria, but since this represents a minor component there was no change observed in the 'unsaturation index'. It was concluded that, in contrast to previous reports, Cu deficiency of the severity reported did not have a deleterious effect on the integrity and permeability of the inner mitochondrial membrane as exemplified by any qualitative modification of fatty acid constitution per se.

Introduction

The occurrence of widespread pathological lesions in many tissues of severely copper-deficient animals have in many instances been ascribed to decreased activities of a number of cupro-enzymes. Examples include defective cross-linking of elastin and collagen in vascular tissue due to decreased activity of lysyl oxidase [1], impaired iron metabolism resulting in hepatic Fe accumulation and anaemia due to decreased plasma caeruloplasmin and its associated ferroxidase activity (EC 1.16.3.1) [2] and skin and hair depigmentation resulting from a decline in activity of cupro-enzyme(s) involved in melanogenesis. In some instances, the marked reduction in activity of the mitochondrial enzyme cytochrome *c* oxidase (EC, 1.9.3.1) in many tissues from Cu-deficient animals has led authors to suggest that a resultant decline in respiratory activity may be the primary cause of tissue pathology [3,4]. However, there has been no recent comprehensive and systematic study of the effects of copper deficiency on the relationship between a decline in cytochrome *c* oxidase activity and its possible effects on mitochondrial respiratory function and composition

* To whom correspondence should be addressed.

In this paper we report on the effects of increasingly severe Cu-deficiency on some aspects of the composition of whole rat liver and rat liver mitochondrial rich fractions and on the activity of hepatic cytochrome *c* oxidase.

Experimental

Animals and diets

80 Male rats of the Rowett Hooded Lister strain were randomly assigned in two separate experiments either to copper-adequate 'control' or 'copper-deficient' groups. After weaning, the animals were housed in polypropylene-stainless steel cages and fed their respective diets ad libitum. 'Control' rats were offered a semi-synthetic diet similar to that described by Williams and Mills [5] but containing approx. 5 mg Cu/kg dry matter and supplemented with additional trace elements [6]. Copper-deficient animals were offered the same diet from which the Cu supplement had been omitted; it supplied 0.5 mg Cu/kg dry matter. De-ionised drinking water was supplied ad libitum.

Experimental protocol and tissue sampling

Animals were killed after 2, 4, 6 and 8 weeks on diet. Tissue for trace element analyses and blood for haematological studies were obtained from animals under diethyl ether anaesthesia.

Liver, required for the preparation of mitochondria, was excised from rats killed by stunning and cervical dislocation. At each time-point, five 'control' and five 'deficient' rats for mitochondrial studies, plus at least three 'control' and three 'deficient' rats for other studies, were utilised.

Chemicals

All biochemicals and reagents were of analytical grade quality and purchased from either BDH (British Drug Houses, Poole, Dorset, U.K.) or Sigma (Sigma Ltd., Poole, Dorset, U.K.).

Sample analysis; mitochondrial preparation and analysis

Plasma Cu and iron (Fe) concentrations were measured by atomic absorption spectrometry , using a Varian 1000 atomic absorption spectrophotometer (Varian Pty., Melbourne, Australia), on the supernatant fraction after protein had been precipitated by mixing equal volumes of plasma and trichloroacetic acid (100 g/l). Liver samples were wet ashed using a concentrated HNO_3/H_2SO_4/$HClO_4$ (3 : 0.5 : 1, v/v) mixture, then analysed for Cu and Fe in H_2SO_4 (final concentration 50 ml/l) in double-distilled water using atomic absorption spectrometry. Blood haemoglobin was determined by the standard cyan-met technique.

Liver mitochondria were prepared by tissue homogenization and differential centrifugation in a 0.25 M sucrose/3.4 mM Tris-HCl/1.0 mM EGTA medium (pH 7.4) [7]. Homogeneity of mitochondrial fractions was monitored by assessing the presence of contaminating DNA (nuclear) [8], acid phosphatase (lysosomal) [9] (with the Boehringer-assay kit) and rotenone-insensitive NADPH cytochrome *c* reductase (microsomal) (Davies, unpublished results). The concentration of DNA determined 0.5 μg per mg mitochondrial protein was similar to that reported as occurring specifically in mitochondria [11]. Mitochondrial acid phosphatase activity was found in control or deficient animals to be less than 5% of that in liver homogenate, and rotenone-insensitive NADPH cytochrome *c* reductase was barely detectable. There was no evidence, therefore, of significant contamination.

Protein was estimated using the Biuret reaction [12]. Oxygen uptake by mitochondria was determined in a Gilson Oxygraph cell (Gilson Medical Electronics Inc., Middleton, WI, U.S.A.), fitted with a Clark oxygen electrode in the presence of various substrates. Rates of consumption, calculated in a manner similar to that of Estabrook [13] were also monitored with ADP added (state 3 respiration) and after depletion of exogenous ADP (state 4 respiration). Ageing studies were conducted on mitochondria kept at 30°C (for 0, 15 and 30 min) as described by Ito and Johnson [14]. Studies of mitochondrial swelling were made by challenging suspensions (5 mg protein) in phosphate buffer with either Cu ($3 \cdot 10^{-5}$ M) or silver ($3 \cdot 10^{-6}$ M) and measuring changes in absorbance at 520 nm over a period of 30 min [15]. Mitochondrial Cu and Fe contents were measured by graphite rod atomic absorption spectrometry

(Perkin Elmer PE 460 with an HGA 76 attachment; Perkin Elmer, Beaconsfield, Bucks., U.K.) after wet digestion in concentrated HNO_3/H_2SO_4/$HClO_4$ (4 : 0.5 : 1, v/v).

Oxidised and reduced spectra of cytochromes *a*, *b*, c_1 and *c* (5 mg mitochondrial protein, clarified using a 20 g/l deoxycholate solution) were obtained on a Cecil CE 505 double-beam spectrophotometer (Cecil Instruments, Cambridge, U.K.). Concentrations of cytochromes were determined by the method of Williams [16]. Cytochrome a_3 was quantified in the same sample [17,18]. Total flavin content of mitochondria was determined spectrophotometrically by the method of Chance [18]. Cytochrome oxidase activity in liver mitochondria was measured by the method of Mills and Dalgarno [19]. Methyl esters of mitochondrial fatty acids were prepared from a sample of 7.0 mg mitochondrial protein, saponified with KOH/EtOH (42.5 g/l EtOH), extracted with diethyl ether and treated with diazomethane. Aliquots were fractionated isothermally (at 187°C on a 3% EGS P-Z (on Gas-Chrom Q, 100–120 mesh) 6 ft × 1/4 inch column in a Perkin-Elmer F17 GLC (Perkin, Elmer, U.K.). Injector temperature was 200°C and nitrogen flow 30 units. Inlet pressures were 15 lb/inch^2 for nitrogen and 18 lb/inch^2 for hydrogen. Flame ionisation detection was used.

Statistical Analysis

All statistical analyses carried out using an unpaired Students *t*-test.

Results

Effects of Cu-deficiency on weight gain, plasma and liver Cu and Fe concentrations and haematological status

After 4 weeks, rats receiving the Cu-deficient diet exhibited slight, but significantly reduced rates of weight gain ($P < 0.01$) and also showed depigmentation compared with control rats. These effects, due to Cu-deficiency became more marked as the experiment progressed such that after 8 weeks the weight gain of the Cu-deficient rats was 54% of the controls ($P < 0.001$). The low Cu status of rats receiving the Cu-deficient diet was demonstrated by blood, plasma and liver analyses (Table I). After 2 weeks of dietary treatment, liver copper content had declined by 62% ($P < 0.001$) compared with controls and after 4 weeks plasma Cu concentration was significantly reduced ($P < 0.001$). After as little as 2 weeks of dietary treatment, disturbances of Fe metabolism were evident in the Cu-deficient rats as shown by significantly reduced plasma Fe concentration ($P < 0.05$) and hepatic Fe accumulation (Table I). Further evidence for defects in Fe metabolism was demon-

TABLE I

THE EFFECT OF Cu DEFICIENCY ON PLASMA AND LIVER COPPER AND IRON CONCENTRATIONS

Results: mean ± S.E. Numbers of animals in parentheses.

Weeks on diet	Plasma Cu (mg/l)		Plasma Fe (mg/l)		Liver Cu (mg/kg wet tissue)		Liver Fe (mg/kg wet tissue)	
	copper deficient	control	copper deficient	control	copper deficient	control	copper deficient	control
2	0.36 ± 0.12(3)	0.60 ± 0.12(3)	0.75 ± 0.11(3) [a]	1.67 ± 0.08(3)	1.87 ± 0.22(6) [b]	4.27 ± 0.18(6)	106 ± 7.3(6) [a]	72.7 ± 8.7(6)
4	0.41 ± 0.07(3) [b]	1.48 ± 0.02(3)	0.62 ± 0.04(3) [b]	2.40 ± 0.11(3)	0.62 ± 0.04(3) [b]	4.29 ± 0.10(3)	95.6 ± 1.0(3) [c]	73.6 ± 2.4(3)
6	0.03 ± 0.001(3) [b]	0.93 ± 0.01(3)	1.06 ± 0.05(3) [b]	2.29 ± 0.06(3)	1.28 ± 0.57(3) [c]	5.05 ± 0.45(3)	165 ± 10.6(3) [a]	118 ± 9.0(3)
8	0.01 ± 0.001(3) [b]	1.06 ± 0.03(4)	0.64 ± 0.07(3) [b]	2.02 ± 0.14(4)	0.62 ± 0.23(4) [b]	4.97 ± 0.41(4)	168 ± 19.0(4) [a]	107 ± 4.0(4)

Significance of the difference between deficient and control data:

[a] $P < 0.05$.

[b] $P < 0.001$.

[c] $P < 0.01$.

TABLE II

MITOCHONDRIAL COPPER AND IRON CONCENTRATIONS

Results are mean ± S.E. Each value is the mean of 4–6 mitochondrial preparations.

Weeks on diet	Cu (ng/mg protein)		Fe (ng/mg protein)	
	copper deficient	control	copper deficient	control
2	29.1 ± 3.12 [a]	49.4 ± 1.89	286 ± 15.5	297 ± 16.4
4	8.3 ± 1.03 [a]	18.3 ± 0.79	185 ± 15.4	166 ± 11.4
6	7.68 ± 1.50 [b]	16.8 ± 1.31	161 ± 15.0	148 ± 19.8
8	6.18 ± 0.65 [a]	16.2 ± 0.85	147 ± 12.7	160 ± 14.3

Significance of the difference between deficient and control data:
[a] $P < 0.001$.
[b] $P < 0.01$.

strated from haematological examination which showed that, after only 2 weeks of treatment, rats receiving the Cu-deficient diet had significantly lower blood haemoglobin concentrations than control animals ($P < 0.001$), which declined further as the experiment progressed. After 8 weeks the Cu-deficient rats were frankly anaemic, with blood haemoglobin concentrations and haematocrits 53% and 59%, respectively, of that of the controls.

Mitochondrial Cu, Fe, cytochrome and flavin contents and cytochrome c oxidase activity

Liver mitochondrial Cu and Fe contents of Cu-deficient and control rats are shown in Table II. Between 2 and 4 weeks of dietary treatment, mitochondrial Cu contents of both groups declined markedly, although, as early as 2 weeks, the Cu content of the Cu-deficient group was 41% lower ($P < 0.001$) than that of controls. After 4 weeks the mitochondrial Cu contents of both groups remained relatively constant, with the mitochondria from the deficient group having a content 40–45% of copper adequate controls. Liver mitochondrial Fe contents of both groups declined significantly between week 2 and 4 of the experiment and thereafter remained at relatively constant levels. However, at no time during the experiment was there evidence that the increased Fe stored in the livers of the Cu-deficient animals (Table I) had accumulated in the mitochondrial fraction.

There were no significant effects due to Cu-deficiency on mitochondrial contents of total flavins, or cytochromes *b*, *c* and c_1 (Table III). However, cytochrome *a* and a_3 contents of mitochondria from Cu-deficient rats were significantly lower than those of Cu-adequate animals after 2 weeks of dietary treatment, and thereafter they declined to relatively constant levels of approx. 30–40% of the controls. These changes induced by Cu-deficiency were reflected in a parallel decline in activity of mitochondrial cytochrome *c* oxidase activity (Table III).

Mitochondrial ageing, swelling and fatty acid composition

Gallagher and Reeve [20] reported that Cu-deficiency results in changes in fatty acid composition of rat liver mitochondria. Since changes in fatty acid composition may be reflected in ageing [21] and swelling [15] properties of liver mitochondria, these phenomena were investigated in this study. The effects of ageing of mitochondrial preparations on state 3 respiration using succinate and β-hydroxybutyrate as substrates are shown in Fig. 1. State 3 respiration of preparations from 8-week-Cu-deficient rats tended to be lower with both substrates. Although state 3 respiration of mitochondria, from Cu-deficient rats aged for 30 min, was significantly lower than controls when either succinate or β-hydroxy-butyrate was used, the relative rates of decline, with time of ageing, were unaffected by dietary treatment.

A similar lack of effect of Cu-deficiency was observed on the swelling properties of liver mitochondria in response to a challenge with either

TABLE III

CYTOCHROME AND TOTAL FLAVIN CONCENTRATIONS AND CYTOCHROME OXIDASE ACTIVITY IN LIVER MITOCHONDRIA

Results are mean ± S.E. 4–6 animals per group.

	Cytochromes (nM/mg protein)							
	Weeks on diet: 2		4		6		8	
Dietary treatment:	+Cu	−Cu	+Cu	−Cu	+Cu	−Cu	+Cu	−Cu
Cytochrome *a*	0.152 ± 0.013	0.081 ± 0.012	0.109 ± 0.01	0.022 ± 0.01	0.108 ± 0.011	0.017 ± 0.006	0.127 ± 0.008	0.040 ± 0.005
Cytochrome a_3	0.136 ± 0.04	0.082 ± 0.010	0.138 ± 0.009	0.055 ± 0.006	0.139 ± 0.006	0.038 ± 0.005	0.141 ± 0.10	0.067 ± 0.016
Total: $a + a_3$	0.288	0.164*	0.247	0.077***	0.247	0.055***	0.268	0.107***
b	0.164 ± 0.016	0.168 ± 0.008	0.143 ± 0.004	0.156 ± 0.005	0.153 ± 0.014	0.143 ± 0.008	0.176 ± 0.008	0.175 ± 0.017
c_1	0.107 ± 0.004	0.114 ± 0.007	0.093 ± 0.002	0.099 ± 0.010	0.103 ± 0.009	0.09 ± 0.009	0.125 ± 0.003	0.123 ± 0.016
c	0.136 ± 0.002	0.135 ± 0.005	0.119 ± 0.003	0.129 ± 0.004	0.125 ± 0.005	0.122 ± 0.010	0.145 ± 0.005	0.140 ± 0.017
flavins	0.671 ± 0.003	0.722 ± 0.046	0.702 ± 0.025	0.686 ± 0.039	0.805 ± 0.046	0.903 ± 0.089	0.931 ± 0.073	0.895 ± 0.046
	Cytochrome oxidase (μM/min per mg protein)							
Copper-deficient	0.378 ± 0.050**		0.199 ± 0.028***		0.114 ± 0.009***		0.109 ± 0.013***	
Control	0.651 ± 0.074		0.779 ± 0.060		0.579 ± 0.020		0.532 ± 0.039	

Significance of the difference between deficient and control data:

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$.

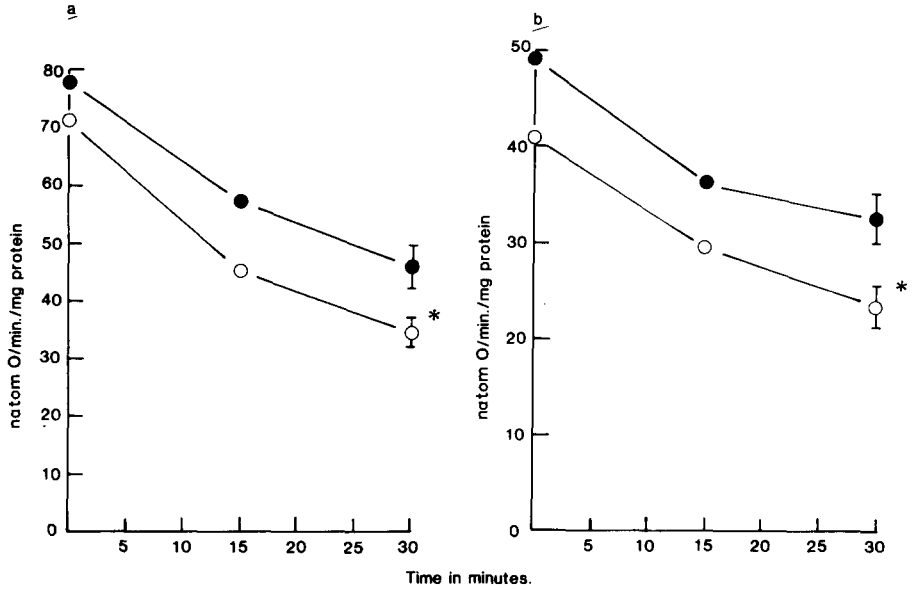

Fig. 1. The effects of mitochondrial ageing. (a) The effects of ageing liver mitochondria at 30°C for 15 and 30 min on state 3 respiration using succinate as substrate. O——O, Preparations from 8-week-Cu-deficient animals; ●——●, preparations from Cu-adequate controls. (b) As in (a) except that β-hydroxybutyrate was used as substrate.

Cu^{2+} or Ag^{+} (Fig. 2). Thus the changes, with time, in absorbance at 520 nm, of mitochondrial suspensions prepared from livers of 9-week-Cu-deficient rats were the same as their respective controls when swelling was provoked with either metal ion.

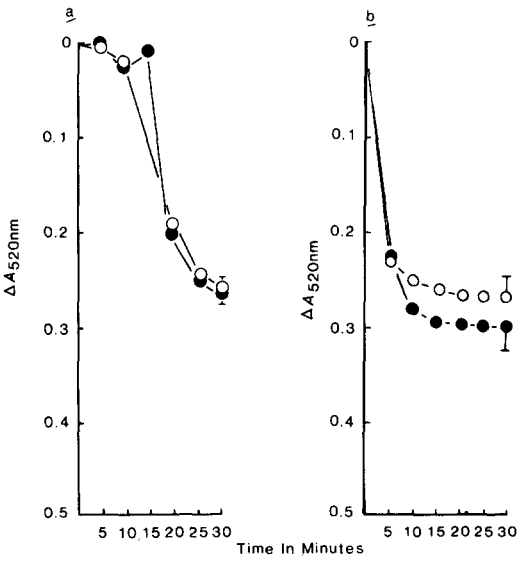

Fig. 2. The swelling properties of mitochondria. The change in absorption at 520 nm with time (swelling) of mitochondrial preparations (7 mg protein) after a challenge with (a) Cu^{2+} $3 \cdot 10^{-5}$ M, (b) Ag^{+} $1 \cdot 10^{-6}$ M. O——O, preparations from 9-week-Cu-deficient animals, ●——●, control preparations.

The fatty acid profile of liver mitochondria from rats depleted of Cu for 8 weeks showed only minor differences from that of control animals (Table IV). For comparison, the fatty acid composition of liver mitochondria from studies by other workers are also shown. There was a slight, but significant reduction in the proportion of arachidonic acid (20 : 4) and a significant increase in docosapentenoic acid (22 : 5). However, in two further experiments (results not shown), no effects due to Cu-deficiency were found on mitochondrial arachidonic acid content, although significant increases in docosapentaenoic were again observed.

Discussion

The diminished rate of liveweight gain and low plasma and hepatic Cu concentration in rats receiving the Cu-deficient diet demonstrate that these animals were of low Cu status. In addition, reduced plasma Fe concentration, marked hepatic Fe accumulation and the development of anaemia associated with Cu-deficiency confirm the findings of many other groups of works [22,23]. However, the recent findings of Weisenberg et al. [24] are at

variance with the present study and others in that they observed a decreased liver Fe content in Cu-deficient rats. No satisfactory explanation can be offered to account for these disparate findings.

The fall in Cu content of the liver mitochondria of Cu-adequate control animals over the first 4 weeks of experiment was probably an age-related rather than diet-associated phenomenon. During the latter stages of pregnancy, foetal livers accumulate Cu to high concentrations, much of which is deposited in the mitochondria bound to a protein 'mitochondrial hepatocuprein', which has subsequently been identified as a polymeric species of metallothionein [25]. Since milk has a low Cu content, it is likely that this protein may act as a Cu reserve upon which the new-born animals may draw to satisfy requirements for rapid growth during the lactation period. It was clear however, that even after 2 weeks the mitochondrial Cu content of rats receiving the Cu-deficient diet had declined more markedly than the controls, and by 4 weeks, at which time growth was impaired, it had plateaued at 40–45% of the control value. No evidence was obtained to suggest that the mitochondrial Cu pool was preferentially spared, since the percentage decline in Cu content of the mitochondria from Cu-deficient animals from 2 to 4 weeks of the experiment (71%) was little different from that of whole liver (67%) (Tables I and II).

TABLE IV

THE EFFECTS OF Cu-DEFICIENCY ON FATTY ACID COMPOSITION OF LIVER MITOCHONDRIA AFTER 8 WEEKS OF DIETARY TREATMENT

Results: mean ± S.E. 5–6 animals per group.

	Percentage of total fatty acid present							
	Mitochondria (week 8 on diet)		Mitochondrial 'control' FA data (other authors)		Mitochondria (milk diet)		Mitochondria (in P.U.F.A. deficiency)	
Dietary treatment: Fatty acid	+Cu	−Cu	$A^{1.a}$	$B^{1.b}$	$C^{1.c}$(+Cu)	C^2(−Cu)	A^2	B^2
16:0	15.17 ± 0.84	15.48 ± 0.50	23.9	14.8	17.6	17.6	29.2	15.5
16:1	2.59 ± 0.17	3.49 ± 0.49		3.4				8.2
18:0	14.65 ± 0.55	12.77 ± 0.89	13.6	19.1	19.2	18.8	12.6	19.6
18:1	10.03 ± 0.53	9.93 ± 0.48	12.6	10.6	17.8	14.6	26.0	24.0
18:2	14.20 ± 0.63	13.29 ± 0.51	20.5	20.3	8.3	11.7	11.5	1.6
20:4	25.48 ± 0.63	23.37 ± 0.45 *	18.5	30.0	11.0	12.6	13.0	8.9
unknown	3.29 ± 0.44	4.09 ± 0.45						
22:4	8.72 ± 1.02	11.33 ± 1.23						
22:5	2.37 ± 0.26	3.27 ± 0.09 **						
22:6	3.50 ± 0.14	3.10 ± 0.14	6.9		11.6	13.7	3.5	
Unsaturation index (total) [d]	211	213	169	175	148	171	122	
Unsaturation index (polyunsaturated)	198	200	156	161	130	156	96	

Significance of the difference between control and deficient data

* $P < 0.05$ (Significant in only one experiment).

** $P < 0.01$

[a] A^1 and A^2 [37].

[b] B^1 and B^2 [36].

[c] C^1 and C^2 [20].

[d] Mole fraction fatty acid × number of double bonds.

During the initial period of the experiment (2–4 weeks), liver mitochondrial Fe content of both Cu-deficient and control rats declined to a similar extent and thereafter remained at relatively constant values. It was clear that the significantly elevated Fe stores in whole liver of the Cu-deficient rats (Table I) were not present in the mitochondrial fraction. This finding is in contrast to the observations of Williams et al. [26,27] who demonstrated increased liver mitochondrial Fe content induced by Cu-deficiency. These authors, however, make no comment concerning the purity of their mitochondrial preparations and it is possible, therefore, that the apparent increase in Fe content may have been associated with contaminating lysosomes. In the present study lysosomal contamination of the mitochondrial fractions was minimal (less than 5% of liver total) as assessed by activity of the lysosomal marker enzyme, acid phosphatase.

Copper-deficiency was without effect on the mitochondrial contents of total flavin, or cytochromes *b*, c_1 and *c*. Although the latter observation conflicts with the results of a very limited study by Wohlrab and Jacobs [28], who found less KCl-water-extractable cytochrome *c* in liver mitochondria from two Cu-deficient rats than from two Cu-adequate controls, it is consistent with other evidence that tissue cytochrome *c* content is not affected by Cu-deficiency [3].

The most striking feature noted in this study was the rapid and marked decline in the mitochondrial contents of cytochromes *a* and a_3 in the Cu-deficient rats. Thus, after only 2 weeks of dietary treatment, Cu-deficiency resulted in 40–45% depletion of both cytochromes. From 4–8 weeks of treatment the liver mitochondrial contents of both cytochrome *a* and a_3 of the Cu-deficient animals remained at values 30–40% of their appropriate controls. These findings confirm and extend the earlier observations of Gallagher et al. [29] that Cu depletion in rats causes a significant fall in the amount of haem α (the prosthetic group of cytochrome *a* and a_3) extractable from liver mitochondria.

Cytochrome *c* oxidase, contains 1 mol each of cytochromes *a* and a_3 and 2 mol Cu per mol enzyme [30]. It is therefore not surprising that the decline in cytochrome *a* and a_3 content in liver mitochondria of the Cu-deficient rats was reflected in a comparable decline in mitochondrial cytochrome *c* oxidase activity. A decline in liver cytochrome *c* oxidase activity is a consistent feature of Cu-deficiency in all species studied [3]. However, there was an indication that the decline in activity was greater than the fall in the combined contents of cytochromes *a* and a_3. Thus, after 8 weeks of dietary Cu-deficiency liver mitochondrial cytochrome *c* oxidase activity had declined to 20% of control values, whereas the combined cytochrome *a* and a_3 content was reduced to only 40% of that of Cu-adequate controls. This apparent decrease in turnover number of liver mitochondrial cytochrome *c* oxidase (enzyme activity per mole of enzyme) induced by Cu-deficiency is demonstrated in Fig. 3, in which activities of preparations from Cu-deficient rats after 2–6 weeks of study are

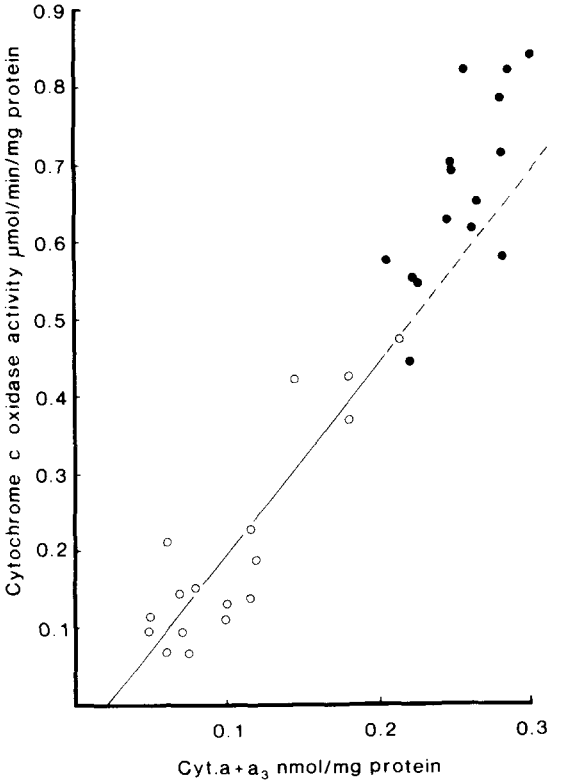

Fig. 3. Relationship between cytochrome *c* oxidase activity and cytochrome *a* and a_3 content of liver mitochondria from rats maintained on a Cu-deficient diet for 2–8 weeks (○) or a Cu-adequate diet (●). Solid line represents the linear regression for Cu-deficient rats; broken line represents the extrapolation of this regression.

plotted against their respective cytochrome *a* and a_3 contents. The cytochrome *c* oxidase activity of mitochondria from Cu-depleted rats (y) was related to mitochondrial cytochrome *a* and a_3 content (x) by the equation:

$$y = 2.452x - 0.053 \; (P < 0.001)$$

However, extrapolation of the regression line describing this relationship to preparations from Cu-adequate control rats revealed that only 2 out of 15 values were on or below this line, suggesting that the activity of cytochrome *c* oxidase per unit amount of enzyme was depressed in Cu-deficient animals compared with controls.

At least two explanations can be offered to account for the decline in activity of liver mitochondrial cytochrome *c* oxidase activity and cytochrome *a* and a_3 contents of Cu-deficient rats. Firstly, as suggested by Gallagher et al. [29], Cu-deficiency may adversely affect the synthesis of haem α, if so, it is implicit that the synthetic step, sensitive to Cu-deficiency, must be involved in side-chain modification of the porphyrin nucleus. Since no such role for Cu is yet known, a further possibility meriting consideration is that the $[haem]_2$-protein complex is destabilized by conformational changes arising either from the failure of Cu incorporation or from the ostensibly minor but consistent change in membrane fatty acid composition we have observed. Indications that anomalies develop in the relationship between cytochrome a/a_3 content and enzyme activity during Cu deficiency may well support the latter possibility. Further evidence may be inferred from a brief report by Wohlrab and Jacobs [31] (cited in Ref. 28) which indicated that a haem-free apo-cytochrome oxidase lipoprotein accumulates in yeast grown on a Cu-deficient medium.

The effect of Cu-deficiency on mitochondrial ageing, swelling and fatty acid composition

Gallagher and Reeve [32] reported liver mitochondria from Cu-deficient rats exhibited impaired phospholipid synthesis and contained significantly more polyunsaturated fatty acids than those of Cu-adequate controls [20]. They suggested that these phenomena may account for the permeability defects in 'aged' Cu deficient mitochondrial preparations observed in an earlier study [29].

The results of this present study contrast sharply with these earlier findings although the discrepancies may be at least partly explained by differences in methodology. In the 'ageing' studies of Gallagher et al. [29] mitochondrial preparations were aged at 0°C for periods up to 5 h, after which time Cu-deficient preparations exhibited impaired respiratory activity, with all substrates tested, compared with Cu-adequate controls. The authors ascribed these effects to increased mitochondrial permeability and the resultant leakage of essential co-factors needed to support maximal rates of respiration. In the present study, mitochondria were aged by incubation at 30°C for considerably shorter periods of time (15 and 30 min). Under these experimental conditions, no differences were observed due to Cu-deficiency in the rates of decline of state 3 respiration when either β-hydroxybutyrate or succinate were used as substrates, indicating that there was no significant loss of essential co-factors required for mitochondrial respiration. Further evidence that there was no marked increase in hepatic mitochondrial membrane permeability in response to Cu-deficiency may be inferred from the observation that the cytochrome *c* content of Cu-deficient mitochondria, a soluble mitochondrial component readily lost when membrane permeability is compromised [33], was unaffected by the Cu status of the animals (Table III). In addition, our failure to demonstrate increased respiratory activity in response to the addition of NADH to mitochondrial preparations from either control or Cu-deficient rats again suggests that Cu-deficiency results in no marked effects on mitochondrial permeability.

Studies by Century and Horwitt [15] indicate that varying the source of dietary fat results in altered fatty acid composition of liver mitochondria, which may be reflected in mitochondrial swelling properties. The failure in this present study to demonstrate any significant effects due to Cu-deficiency on either swelling characteristics or ageing properties of liver mitochondria indicated there were no substantial changes in mitochondrial fatty acid composition.

Direct fatty acid analyses of liver mitochondria support this suggestion. The only consistent change

in Cu-deficient preparations was an elevated content of docosapentenoic acid (22 : 5). However, this is only a minor component of the total fatty acid content and thus there were no significant changes in the total 'unsaturation index' (mol fraction fatty acid × number of double bonds) or polyunsaturated fatty acid unsaturation index. Whether or not a change in content of the 22 : 5 acid of a magnitude observed in this study, at a specific site in the mitochondrial membranes, could confer functional changes as, for example, decreased efficiency of cytochrome *c* oxidase, cannot be ascertained.

In this study, we were clearly unable to confirm the findings of Gallagher et al. [29] with respect to mitochondrial fatty acid composition and its modification by Cu deficiency. Possibly relevant to these discrepancies is the fact that Gallagher et al. offered rats a basal diet which, even when Cu supplemented, supports only slow growth [35]. This diet consisted primarily of whole liquid milk, notable for its low content of essential fatty acids. Thus it is of interest that the fatty acid composition of the mitochondria even of control rats of Gallagher et al. resembled that of rats given essential-fatty-acid-deficient diets [36,37] (Table IV). The full implications of these differences for the integrity of mitochondrial membrane stability in normal and low-Cu rats are as yet unclear. However, since increased mitochondrial permeability was not demonstrable in the Cu-adequate rats in the original study of Gallagher et al. [29], despite possible inadequacies in dietary supply of essential fatty acids, it may well be that under some circumstances Cu exerts a hitherto unrecognized role in polyunsaturated fatty acid metabolism.

In view of some apparently contradictory findings between this study and those of earlier studies [20,29], detailed investigations on the influence of Cu-deficiency on mitochondrial respiratory functions are clearly warranted.

Acknowledgements

The expert care of the experimental animals by the staff of the Small Animal House is gratefully acknowledged.

References

1 Siegel, R.C., Pinell, S.R. and Martin, G.R. (1970) Biochemistry 9, 4486–4492
2 Osaki, S., Johnson, D.A. and Frieden, E. (1966) J. Biol. Chem. 241, 2746–2751
3 Underwood, E.J. (1977) in Trace Elements in Human and Animal Nutrition 4th Ed. Academic Press, New York
4 Mills, C.F. (1980) in Biological Roles of Copper, p. 49–65, Ciba Foundation Symposium 79, Excerpta Medica, Amsterdam
5 Williams, R.B. and Mills, C.F. (1970) Br. J. Nutr. 24, 989–1003
6 Davies, N.T. and Reid, H. (1979) Br. J. Nutr. 41, 579–589
7 Chappell, J.B. and Hansford, R.G. (1969) in Subcellular Components, (Birnie, G.D. and Fox, S.M. Eds.), pp. 43–56, Butterworths, London
8 Burton, C. (1956) Biochem. J. 62, 315–323
9 Hillman, G. (1971) Z. Klin. Chem. Klin. Biochim. 9, p. 273
10 Reference deleted
11 Naas, S., Naas, M.K. and Hennix, U. (1969) Biochim. Biophys. Acta 95, 426–434
12 Miller, G.L. (1959) Anal. Chem. 31, 994–998
13 Estabrook, R.W. (1967) Methods Enzymol 10, 41–51
14 Ito, T. and Johnson, R.M. (1964) J. Biol. Chem. 239, 3201–3208
15 Century, B. and Horwitt, M.K. (1963) J. Nutr. 80, 145–149
16 Williams, J.N. (1964) Arch. Biochem. Biophys. 107, 537–543
17 Maddaiah, V.T., Weston, C.L., Chen, S.Y. and Collipp, P.J. (1976) Arch. Biochem. Biophys. 173, 225–230
18 Chance, B. (1957) Methods in Enzymology 4, 273–329
19 Mills, C.F. and Dalgarno, A.C. (1970) in Trace Element Metabolism in Animals, (Mills, C.F., ed.), pp. 456–459 Livingstone, London
20 Gallagher, C.H. and Reeve, V.E. (1971) Aust. J. Exp. Biol. Med. Sci. 49, 453–461
21 Johnson, R.M. (1963) J. Nutr. 81, 411–416
22 Evans, J.L. and Abrahams, P.A. (1973) J.Nutr. 103, 196–201
23 Williams, D.M., Kennedy, F.S. and Green, B.G. (1983) Br. J. Nutr. 50, 653–660
24 Weisenberg, E., Halbreich, A. and Mager, J. (1980) Biochem. J. 188, 633–641
25 Porter, H. (1974) Biochem. Biophys. Res. Commun. 56, 661–668
26 Williams, D.M., Barbuto, A.J., Atkin, C.L. and Lee, G.R. (1978) Prog. Clin. Biol. Res. 21, 539–549
27 Williams, D.M., Loukopoulos, D., Lee, G.R. and Cartwright, G.E. (1976) Blood 48, 77–85
28 Wohlrab, H. and Jacobs, E.E. (1967) Biochem. Biophys. Res. Commun. 28, 998–1002
29 Gallagher, C.H., Judah, J.D. and Rees, K.R. (1956) Proc. R. Soc. B. 145, 134–150
30 Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605
31 Wohlrab, H. and Jacobs, E.E. (1967), Proc. 7th Int. Congr. Biochem. The Science Council of Japan, Tokyo. (Abstr. cited by Wohlrab and Jacobs, [28])

32 Gallagher, C.H. and Reeve, V.E. (1971) Aust. J. Exp. Biol. Med. Sci. 49, 21–31
33 Jacobs, E.E. and Sanadi, D.R. (1960) J. Biol. Chem. 235, 531–543
34 Paul, A.A. and Southgate, D.A.T. (1978) in The composition of Foods (McCance and Widdowson, eds.), H.M.S.O. London
35 Mills, C.F. (1955) Br. J. Nutr. 9, 398–409
36 Hayashida, T. and Portman, O.W. (1960) Proc. Soc. Exp. Biol. Med. 103, 656–659
37 Divakaran, P. and Venkataraman, A. (1977) J. Nutr. 107, 1621–1631