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Studies on the effects of copper deficiency on rat liver mitochondria. II. Effects on oxidative phosphorylation

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Effects of dietary copper deficiency in rats on respiratory enzymes of isolated rat liver mitochondria have been studied. After 2 weeks of Cu-depletion, cytochrome *c* oxidase (EC 1.9.3.1) activity had declined by 42% and between 4 and 8 weeks exhibited between 20 and 25% of the activity of control mitochondria. Activities of NADH cytochrome *c* reductase (EC 1.6.99.3) and succinate cytochrome *c* reductase (EC 1.3.99.1), were unaffected initially but declined by 32 and 46%, respectively, after 8 weeks of Cu-depletion. After 4 weeks there was a significant (34%) decline in succinate supported state 3 respiration with only a modest (18%) decline in state 4 respiration. The ADP:O ratio was unaffected by Cu-depletion after 6 and 8 weeks of dietary Cu-restriction. State 3 respiration was significantly reduced after 6 weeks when glutamate/malate or β-hydroxybutyrate were used as substrates, whereas state 4 respiration and ADP:O ratios were unaffected. The fall in state 3 respiration was of sufficient magnitude at 8 weeks to cause a significant decline in the respiratory control ratio with all substrates. Comparisons between the relative activities of cytochrome *c* oxidase and reductase activities in Cu-deficient preparations, the relatively specific effect of the deficiency on state 3 respiration with all substrates tested and the ability to increase significantly oxygen consumption in excess of maximal state 3 respiration by the uncoupler 2,4-dinitrophenol suggest that the defect in Cu-deficient mitochondria cannot be attributed solely to the decreased activity of cytochrome *c* oxidase.

Introduction

A decline in the activity of the cupro-enzyme cytochrome *c* oxidase (EC 1.9.3.1), in tissues of Cu-deficient animals of many species is well documented (for reviews see Refs. 1 and 2). However, even though many biochemical and pathological lesions associated with Cu-deficiency have been ascribed to reduced cytochrome *c* oxidase, including impaired hepatic phospholipid synthesis in rats [3], haem synthesis in pigs [4] and defective brain stem spinal myelination in lambs suffering enzootic neonatal ataxia (swayback) [5], few studies have attempted to determine whether reduced cytochrome oxidase activity impairs respiratory activity of intact Cu-deficient mitochondria.

In this study we report the effects of increasingly severe Cu-deficiency in rats on the activities in vitro of some key hepatic mitochondrial respiratory enzymes and their relationship to changes in mitochondrial functional integrity as assessed from respiratory activity and capacity to carry out oxidative phosphorylation.

Materials and Methods

Chemicals and reagents. All reagents were of analytical grade and purchased from either B.D.H. Ltd. (Poole, Dorset, U.K.) or Sigma Ltd. (Poole, Dorset, U.K.).

Animals and diets. Details concerning diets, animal management and slaughter procedures are

presented in the accompanying paper [6].

Preparation of mitochondria. Hepatic mitochondria-rich fractions were prepared by differential centrifugation of liver homogenates in 0.25 M sucrose/3.4 mM Tris/1.0 mM EGTA (pH 7.4) [7] as described previously [6].

Oxygen uptake by mitochondria. State 3 respiration (oxygen consumption in the presence of ADP) state 4 respiration, (oxygen consumption after depletion of exogenous ADP) respiratory control ratio; ratio of state 3 respiration: state 4 respiration) ADP:O ratios (μmol ADP added/μatoms O_2 utilized) were determined at 30°C in a Gilson Oxygraph (Gilson Medical Electronic Inc., WI, U.S.A.) fitted with a Clark electrode, by the methods of Estabrook [8].

Determination of mitochondrial cytochrome c oxidase, succinate cytochrome c reductase and NADH cytochrome c reductase activities. Suspensions of mitochondria were diluted to 400 μg mitochondrial protein/ml in a hypotonic ionic medium (0.05 M potassium phosphate buffer (pH 7.4)), to increase membrane permeability [7] to exogenous NADH and cytochrome *c*. Aliquots (40 μg protein of control mitochondria and 80 μg protein of Cu-deficient preparations were assayed for their cytochrome *c* oxidase activity by the method of Mills and Dalgarno [9] using either a Hilger Gilford reaction kinetics spectrophotometer (Rank Precision Industries Ltd., London, U.K.) or a Pye Unicam SP 8800 spectrophometer (Pye Unicam, Cambridge, U.K.).

To relate changes in cytochrome *c* oxidase activity quantitatively to activities of linked respiratory enzymes, a sequential assay was developed in which succinate cytochrome *c* reductase and NADH cytochrome *c* reductase could be assayed in the same preparation. In brief, the cytochrome *c* oxidase assay was allowed to proceed until approx. 0.1 μmol cytochrome *c* had been oxidized giving a ΔA_{550} of at least 0.6 units. Cytochrome *c* oxidase activity was then inhibited by addition of CN^- (final concentration 166 μM). Succinate (final concentration 12.5 mM) was subsequently added and succinate cytochrome *c* reductase activity monitored by following the ΔA_{550} due to cytochrome *c* reduction. This assay was terminated by adding malonate (final concentration, 16.6 mM) and NADH cytochrome *c* reductase was then determined by adding NADH (final concentration, 2.8 mM) and monitoring the further increase in cytochrome *c* reduction at 550 nm. Preliminary studies with mitochondrial preparations from both Cu-deficient and Cu-adequate rats demonstrated that results from sequential determinations of succinate and NADH cytochrome *c* reductase activities by this method were identical to those obtained from separate, individual assays.

Statistical analysis. Results were statistically analysed using unpaired Student's *t*-tests.

Results

Assessment of Cu status

A detailed description of changes in plasma and liver Cu and Fe concentrations and haematological status of the rats used in this study is presented in the accompanying paper [6]. To summarize, after only 2 weeks of dietary treatment rats receiving the Cu-deficient diet had significantly lower liver Cu and plasma Fe contents and elevated hepatic Fe stores. 4 weeks of dietary Cu-deficiency resulted in impaired weight gain, decreased plasma and liver Cu concentrations and the development of a frank anaemia as assessed from reduced blood haemoglobin concentrations and reduced haematocrits.

Effects of Cu-deficiency on cytochrome c oxidase succinate cytochrome c reductase and NADH cytochrome c reductase

The effects of increasing severity of Cu-deficiency on cytochrome *c* oxidase, succinate and NADH cytochrome *c* reductase activities are shown in Fig. 1. After 2 weeks of dietary treatment liver mitochondrial cytochrome *c* oxidase activity of rats receiving the Cu-deficient diet had fallen by 40% ($P < 0.001$) compared with Cu-adequate controls. From 4–8 weeks of treatment the mitochondrial cytochrome *c* oxidase of the Cu-deficient group plateaued to a value approx. 20–25% of the controls. Cu-deficiency was without significant effect on the activities of succinate cytochrome *c* reductase or NADH cytochrome *c* reductase during the first 6 weeks of the experiment. However, after 8 weeks, Cu-deficient liver mitochondria exhibited significantly reduced activities ($P < 0.01$) of both succinate cytochrome *c*

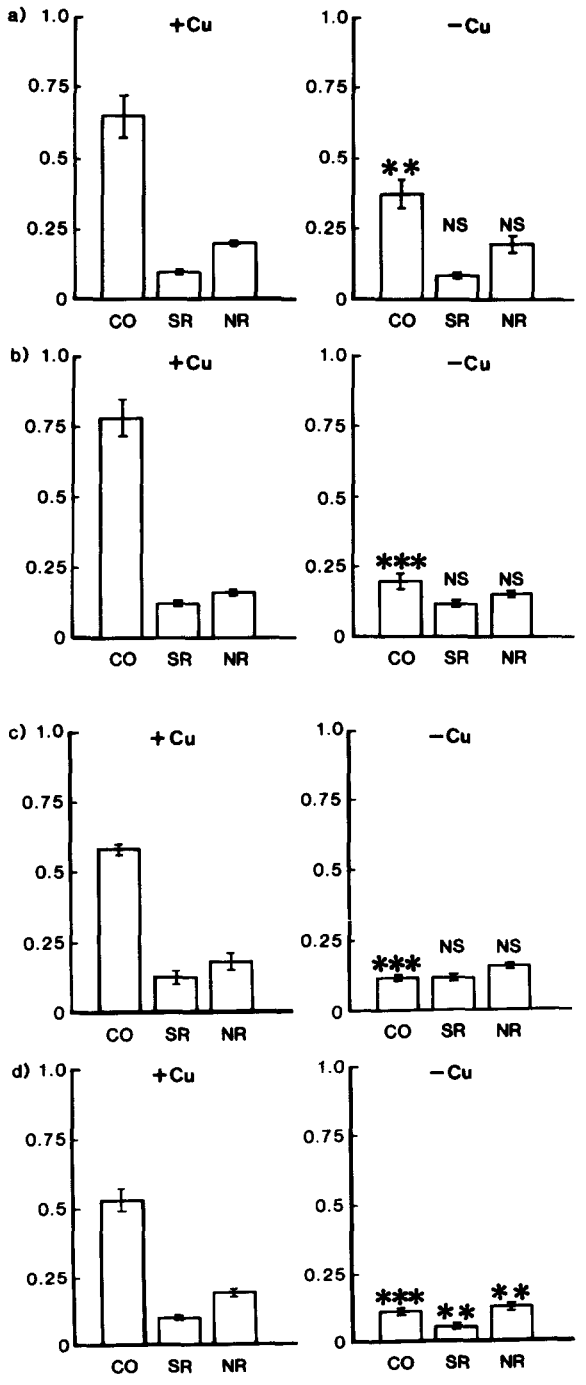

Fig. 1. The effect of Cu-deficiency on the activities of cytochrome *c* oxidase (CO), succinate cytochrome *c* reductase (SR) and NADH cytochrome *c* reductase (NR) in isolated rat liver mitochondria after (a) 2, (b) 4, (c) 6 and (d) 8 weeks of treatment. The activities were measured in swollen mitochondria by following either the oxidation or reduction of cytochrome *c* by changes in *A* at 550 nm, in sequential assays on the same preparation. Results; μmol cytochrome *c* reduced or oxidized per min per mg protein. ***, $P < 0.001$; **, $P < 0.01$.

reductase and NADH cytochrome *c* reductase as well as cytochrome *c* oxidase.

The effects of Cu-deficiency on mitochondrial respiratory function

The effects of Cu-deficiency on state 3 and state 4 respiration, respiratory control and ADP:O ratios by isolated mitochondria utilising succinate, glutamate/malate and β-hydroxybutyrate as substrates are shown in Tables I, II and III, respectively. After 4 weeks of dietary treatment succinate-supported state 3 respiration of liver mitochondria from Cu-deficient rats was 35% lower than that of control mitochondria ($P < 0.001$), whereas state 4 respiration was reduced by only 18% (Table I).

This marked inhibition of state 3 respiration increased to more than 50% in mitochondria from rats maintained on the Cu-deficient diet for 8 weeks. Although Cu-deficiency slightly reduced state 4 respiration from 4–8 weeks of dietary treatment, the substantially greater decrease in state 3 respiration resulted in significant declines in the respiratory control ratio.

Essentially similar findings were found when the two NADH-linked substrates, glutamate/malate and β-hydroxybutyrate, were tested (Tables II and III). After 4 weeks of dietary Cu-deficiency there was a trend ($P < 0.1$) towards a decline in state 3 respiration supported by glutamate/malate. After 6 and 8 weeks of Cu-depletion, state 3 respiration was reduced by 30% ($P < 0.01$) and 40% ($P < 0.001$), respectively, although with this substrate, no significant effects due to dietary treatment were observed on state 4 respiration. The respiratory control ratio was significantly reduced ($P < 0.05$) after 8 weeks of Cu-depletion.

Cu-deficiency provoked comparable defects in mitochondrial respiratory function when β-hydroxybutyrate was used as the test substrate (Table III). After 4 weeks state 3 respiration by mitochondria of the Cu-deficient group was significantly reduced ($P < 0.01$), whereas state 4 respiration was unaffected. Preparations from 8-week-Cu-deficient rats exhibited a 35% decline in β-hydroxybutyrate supported state 3 respiration ($P < 0.001$) whilst, as with glutamate/malate, no effect was observed on state 4 respiration, resulting in a significant decline in the respiratory control ratio ($P < 0.05$).

TABLE I

THE EFFECT OF Cu-DEFICIENCY ON RESPIRATORY ACTIVITY AND OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA WITH SUCCINATE AS SUBSTRATE

Mitochondrial respiration rates were determined polarographically at 30°C and are expressed in ngatoms O_2 consumed/min per mg protein. Values are means ± S.E., $n = 6$. RCR, respiratory control ratio.

	Weeks on diet:	2	4	6	8
ADP:O	+Cu	1.37 ± 0.06	1.50 ± 0.02	1.60 ± 0.02	1.54 ± 0.05
	−Cu	1.43 ± 0.04	1.40 ± 0.04 [a]	1.56 ± 0.03	1.42 ± 0.05
State 3 respiration	+Cu	65.61 ± 5.45	87.33 ± 2.81	83.99 ± 2.89	108.32 ± 2.85
	−Cu	55.93 ± 3.90	57.79 ± 4.51 ***	44.68 ± 2.28 ***	45.52 ± 4.61 ***
State 4 respiration	+Cu	24.59 ± 2.81	18.14 ± 1.07	22.86 ± 1.57	20.00 ± 1.20
	−Cu	20.64 ± 0.97	14.94 ± 0.54 *	15.00 ± 1.18 **	14.85 ± 0.36 **
RCR	+Cu	2.75 ± 0.24	4.88 ± 0.32	3.54 ± 0.15	5.51 ± 0.41
	−Cu	2.76 ± 0.27	3.85 ± 0.23 *	3.04 ± 0.23	3.06 ± 0.29 **

* $P < 0.05$ compared with values for +Cu controls.
** $P < 0.01$ compared with values for +Cu controls.
*** $P < 0.001$ compared with values for +Cu controls.
[a] $P < 0.1$ compared with values for +Cu controls.

Cu-deficiency had no effect on liver mitochondrial ADP:O ratios when succinate, glutamate/malate or β-hydroxybutyrate was used as substrate.

Discussion

This study confirms and extends the findings of Gallagher et al. [3,10], who demonstrated that

TABLE II

THE EFFECT OF Cu-DEFICIENCY ON RESPIRATORY ACTIVITY AND OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA WITH GLUTAMATE/MALATE AS SUBSTRATE

Mitochondrial respiration rates were determined polarographically at 30°C and are expressed in ngatoms O_2 consumed/min per mg protein. Values are means ± S.E., $n = 6$. RCR, Respiratory control ratio.

	Week of treatment:	2	4	6	8
ADP:O	+Cu	2.12 ± 0.12	2.15 ± 0.09	2.39 ± 0.04	2.24 ± 0.09
	−Cu	2.13 ± 0.06	2.11 ± 0.04	2.25 ± 0.07	2.10 ± 0.09
State 3 respiration	+Cu	52.66 ± 2.61	41.84 ± 3.29	48.86 ± 2.78	59.64 ± 2.39
	−Cu	44.26 ± 2.86 [a]	33.14 ± 2.94 [a]	33.10 ± 1.67 **	35.63 ± 2.44 ***
State 4 respiration	+Cu	15.71 ± 1.47	8.90 ± 1.65	10.88 ± 0.70	9.12 ± 1.08
	−Cu	12.33 ± 1.24 [a]	7.11 ± 0.59	9.34 ± 0.54	10.31 ± 1.28
RCR	+Cu	3.32 ± 0.18	4.87 ± 0.82	4.55 ± 0.35	6.97 ± 0.99
	−Cu	3.63 ± 0.14	4.72 ± 0.28	3.57 ± 0.18 *	3.74 ± 0.72 *

* $P < 0.05$ compared with values for +Cu controls.
** $P < 0.01$ compared with values for +Cu controls.
*** $P < 0.001$ compared with values for +Cu controls.
[a] $P < 0.1$ compared with values for +Cu controls.

TABLE III

THE EFFECT OF Cu-DEFICIENCY ON RESPIRATORY ACTIVITY AND OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA WITH β-HYDROXYBUTYRATE AS SUBSTRATE

Mitochondrial respiration rates were determined polarographically at 30°C and are expressed in ng atoms O_2 consumed/min per mg protein. Values are means ± S.E., $n = 6$. RCR, Respiratory control ratio.

	Weeks on diet:	2	4	6	8
ADP:O	+Cu	2.18 ± 0.09	2.04 ± 0.07	2.20 ± 0.07	2.26 ± 0.07
	−Cu	2.12 ± 0.11	2.04 ± 0.04	2.21 ± 0.05	2.15 ± 0.07
State 3 respiration	+Cu	38.19 ± 1.44	37.14 ± 0.92	36.67 ± 2.94	43.25 ± 1.05
	−Cu	34.67 ± 3.71	31.66 ± 0.97 **	31.96 ± 1.45	28.25 ± 1.57 ***
State 4 respiration	+Cu	14.24 ± 1.58	7.83 ± 0.34	9.69 ± 0.40	9.01 ± 0.76
	−Cu	11.69 ± 1.58	7.87 ± 0.65	9.05 ± 0.29	9.23 ± 0.91
RCR	+Cu	2.76 ± 0.25	4.79 ± 0.30	3.80 ± 0.31	4.91 ± 0.36
	−Cu	2.99 ± 0.09	4.11 ± 0.27	3.55 ± 0.19	3.19 ± 0.42 *

* $P < 0.05$ compared with values for +Cu controls.
** $P < 0.01$ compared with values for +Cu controls.
*** $P < 0.001$ compared with values for +Cu controls.

'moderate' Cu-deficiency was without effect on respiration measured manometrically by liver slices and homogenates, despite significant declines in cytochrome *c* oxidase and 'succinoxidase activities'. They ascribed the decreased 'succinoxidase' activity specifically to the reduced activity of cytochrome *c* oxidase since succinate dehydrogenase activity was normal. In contrast, in severe Cu-deficiency O_2 consumption by liver homogenates was significantly reduced when octanoate, malate, pyruvate, citrate, glutamate or α-keto-glutarate was used as substrate. This general decline in respiratory activity they attributed to the virtual total loss of cytochrome *c* oxidase activity. In addition, in severe Cu-deficiency these workers noted a decline in the activity of NADH cytochrome *c* reductase activity in liver homogenates.

It is difficult to compare, quantitatively, the results of this study with those of Gallagher et al. [3,10]. The latter workers employed diets of hydrogen sulphide-treated whole milk which, as pointed out in the previous paper [6], may have been nutritionally inadequate in respects other than simply Cu-deficient. Secondly, most of their studies of the effects of Cu-deficiency on respiratory enzymes were made on unfractionated liver homogenates rather than the relatively pure mitochondrial fractions used in this study.

Notwithstanding these differences, both studies have demonstrated at an early stage of Cu-deficiency a decline in liver cytochrome *c* oxidase activity without concomitant changes in succinate dehydrogenase or NADH cytochrome *c* reductase activities. Similarly, at later stages of deficiency, NADH cytochrome *c* reductase activity was significantly depressed in both studies. Our evidence, that following 8 weeks of Cu-depletion, a loss of 80% of cytochrome oxidase activity is also accompanied by reduced activities of NADH and succinate cytochrome *c* reductases, may well indicate that a generalized loss of mitochondrial integrity is induced by severe deficiency (Fig. 1). However, as demonstrated in our previous study [6], this cannot be associated with either any gross change in fatty acid composition of mitochondrial membranes or in a reduction in total Fe, flavin or cytochrome *b*, *c* or c_1 contents of Cu-deficient mitochondria.

The most important finding of the present study was that with all substrates tested a significant decline in state 3 respiration of mitochondria occurred between 4 and 8 weeks of Cu-depletion (Tables I, II and III). This was accompanied by either a slightly decreased rate of state 4 respiration with succinate as substrate, or no effect when β-hydroxybutyrate or glutamate/malate were oxidized. After 6–8 weeks of deficiency, these

changes were of sufficient magnitude to reduce significantly the respiratory control ratio regardless of the substrate. Although a reduction in RCR can be indicative either of artefactal or of damage in vivo to mitochondria, such loss of structural integrity appears unlikely from the absence of changes in ADP : O ratios [7]. Gallagher and Reeve [11] also failed to find effects of Cu-deficiency on the P : O ratio of isolated rat liver mitochondria respiring with glutamate as substrate. These observations indicate that Cu-deficiency results in some specific defect in the process(es) controlling the rate of mitochondrial respiration when linked to ADP phosphorylation.

Our data agree in some but not all respects with those of Kassabova and Russanov [12] who demonstrated that liver mitochondria prepared from Cu-deficient rats exhibited slightly impaired state 3 respiration when succinate or glutamate/malate were substrates, but not with β-hydroxybutyrate. Again, in contrast to our findings, they demonstrated that when glutamate/malate was substrate, Cu-deficient mitochondria exhibited a low ADP : O ratio. Such mitochondria exhibited low cytochrome *c* oxidase and succinate cytochrome *c* reductase activities but an increased NADH cytochrome *c* reductase activity.

It is important to emphasise that the studies of Kassabova and Russanov [12] and Gallagher et al. [3 and 10] both employed techniques for the induction of Cu-deficiency which must now be regarded as questionable. Although both used diets based on whole milk fortified with iron, neither attempted to rectify other nutritional limitations of such a diet. Moreover, it appears likely from related studies [13,14] that the Cu-adequate control animals of Kassabova and Russanov [12] received a totally different solid diet. The extent to which such nutritional limitations are related to discordant conclusions arising from these series of studies on mitochondrial respiratory activity cannot be resolved.

While it is tempting to speculate that the decline in state 3 respiration due to Cu-depletion observed in the present study may be due to the marked decrease in cytochrome *c* oxidase activity, some of our findings suggest that other processes associated with oxidative phosphorylation may also be involved. Thus, comparison of the changes in succinate cytochrome *c* reductase and cytochrome oxidase activities induced by Cu-depletion for 4–8 weeks strongly suggests that oxidase activity was unlikely to be the rate-limiting step in the oxidation of succinate by Cu-deficient mitochondria (Fig. 1). However, at these times there was clear evidence of impaired state 3 respiration with succinate. Furthermore, the reduction in cytochrome *c* oxidase activity of Cu-deficient mitochondria at 4, 6 and 8 weeks remained relatively constant (75, 80 and 80%, respectively), although the inhibition of state 3 respiration progressively increased, regardless of its substrate. That a defect other than the loss of cytochrome oxidase activity is influencing 'Cu-deficient' mitochondrial respiratory activity is again suggested by our evidence that a depression of state 3 respiration and of the respiratory control ratio occurred whether the substrate used was being oxidised rapidly (e.g., succinate, Table I) or slowly (β-OH butyrate, Table III). Even at late stages of Cu-depletion it is evident that sufficient cytochrome *c* oxidase activity remained to support normal rates of β-hydroxybutyrate oxidation. However, after 8 weeks Cu-depletion liver mitochondrial preparations exhibited a 35% decline in state 3 respiration with this substrate. Our additional finding that addition of the uncoupling agent 2,4-dinitrophenol to mitochondria from 4–8-week-Cu-deficient rats respiring with succinate as substrate increased the rate of O_2 consumption above the rate of state 3 respiration ($13 \pm 2.4\%$, $n = 11$, $P < 0.001$) again suggests that their lower cytochrome *c* oxidase activity could not be the sole reason for impaired respiratory function.

The concept that control of metabolite flux through a pathway may not be limited to one enzyme but shared by some or all enzymes on that pathway has been proposed by Kacser and Burns [15] and Heinrich and Rapoport [16]. Furthermore, by use of appropriate inhibitors, it is possible in some circumstances to determine the degree of control (control strength) exerted by individual enzymes on the metabolic flux. Recently, Groen et al. [17] have applied these principles to the flux of reducing equivalents to molecular oxygen along the electron transport chain of liver mitochondria from normal rats. They have shown that when mitochondria are respiring maximally in state 3

with succinate as substrate, cytochrome *c* oxidase exerts only minor regulatory control. Far greater control is exerted by the adenine nucleotide translocase. In view of this and the well-established observation that the mitochondrial nucleotide translocase inhibitor, atractyloside, specifically inhibits respiration linked to phosphorylation (state 3) [18], a study of the effects of Cu deficiency on mitochondrial adenine nucleotide translocation seems justified. Further support for such an investigation can be derived from a preliminary study by Gallagher and Reeve [19], which showed that ADP-binding to inner membrane preparations of liver mitochondria was depressed in Cu-deficiency.

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