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Studies on the effect of copper deficiency on rat liver mitochondria. III. Effects on adenine nucleotide translocase

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Liver mitochondria from Cu-deficient rats exhibit impaired State 3 respiration (oxygen consumption in the presence of exogenous ADP) compared with Cu-adequate controls, whereas State 4 respiration (oxygen consumption after depletion of exogenous ADP) and ADP/O are unaffected. In view of previous observations (Davies, N.T., Lawrence, C.B., Mills, C.F. and Nicol, F. (1985) Biochim. Biophys. Acta 809, 351–361) it seemed that a decline in cytochrome *c* oxidase activity (EC 1.9.3.1) could not fully account for these findings. Cu deficiency resulted in a significant decline (40%, $P < 0.01$) in [^{14}C]ADP uptake by liver mitochondria which suggests there is a reduced activity of the adenine nucleotide translocase. The reduced translocase activity was not associated with any marked change in fatty-acid composition of either intact mitochondria or inner mitochondrial membranes. Inhibitor titrations with the irreversible inhibitor carboxyatractyloside showed that 'Cu-deficient' mitochondria required the same concentration of inhibitor to produce 100% inhibition of State 3 respiration as control mitochondria, suggesting that the amount of functional translocase enzyme present is unaffected. When the translocase assay was allowed to proceed until equilibrium was established between external and internal nucleotides, it was apparent that the exchangeable adenine nucleotide pool of Cu-deficient mitochondria was 36% lower than in controls. Analysis of mitochondria for their ATP, ADP and AMP contents showed that, whereas the AMP content was unaffected, ATP and ADP contents were 39 and 40% lower, respectively, which resulted in a significantly reduced pool of total adenine nucleotides (ATP + ADP + AMP) and a reduced 'energy charge' [(ATP + 0.5 ADP)/(ATP + ADP + AMP)]. These results are discussed in relation to current concepts of the regulation and control of mitochondrial respiration.

Introduction

Although many biochemical and pathological lesions in Cu-deficient animals have been ascribed to reduced cytochrome *c* oxidase (EC 1.9.3.1) (see Refs. 1 and 2), we have recently presented evidence that impaired respiratory activity of Cu-deficient rat liver mitochondria may result from disturbances in other processes involved in oxidative phosphorylation [3]. Thus, comparisons between the relative activities of cytochrome *c* oxidase and reductase activities in Cu-deficient mitochondrial preparations, the relatively specific effect of the deficiency on State 3 respiration regardless of whether substrates were rapidly or more slowly utilized and the ability to increase significantly

Abbreviations: Cyt, cytochrome; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

oxygen consumption in excess of State 3 respiration by the uncoupler 2:4 dinitrophenol all indicate that the defect in Cu-deficient mitochondria cannot be attributed solely to the decreased activity of cytochrome *c* oxidase.

In this study, we demonstrate that the decline in State 3 respiration by Cu-deficient mitochondria is accompanied by decreased activity of the adenine nucleotide translocase. Results of investigations are presented directed towards elucidation of the mechanism(s) responsible for reduced 'translocase' activity.

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

Weanling male rats of the Rowett Hooded Lister strain were randomly assigned to 'Cu-adequate control' or 'copper deficient' groups. Copper deficient rats were offered ad libitum a semisynthetic diet (less than 0.5 mg Cu/kg) similar in composition to that described earlier [4], except it contained casein (250 g/kg) instead of albumin (200 g/kg). Control rats were offered the same diet supplemented with $CuSO_4 \cdot 5H_2O$ to provide 5 mg Cu/kg. Rats were individually housed in glass–perspex cages and the controls were pair-fed to the Cu-deficient rats. Distilled water was supplied ad libitum.

Animals were killed after 7–9 weeks of dietary treatment by stunning and cervical dislocation; rats used for plasma analysis were anaesthetised with diethyl ether.

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, or 0.2 M sucrose/5.0 mM Hepes/0.5 mM EGTA, pH 7.4 (Ref. 5) as described previously [4].

Oxygen uptake by mitochondria

State 3 respiration (oxygen consumption in the presence of ADP), State 4 respiration (oxygen consumption after depletion of exogenous ADP), and respiratory control ratio were determined by the methods of Estabrook [6], as previously described [4].

Assessment of copper status

Plasma Cu and Fe concentrations and liver Cu content and mitochondrial cytochrome oxidase activities were performed by methods described earlier [4].

Assay of adenine nucleotide translocase activity

Adenine nucleotide translocase activity was assayed by the 'inhibitor stop' method of Duée and Vignais [3]. Mitochondria (5 mg protein) were incubated at 0°C in 110 mM KCl/20 mM Tris/1 mM EDTA (pH 7.4) in a final volume of 2 ml. The reaction was started by the addition of ADP (final concentration, 60 μM) containing 0.05 μCi [^{14}C]ADP (Amersham International, Bucks., U.K.). Either just before addition or 30, 60 or 120 s after addition of ADP, carboxyatractyloside was added (final concentration, 60 μM) and the incubation tube rapidly centrifuged (10000 $\times$ *g* for 10 min). The pellets were washed with the same incubation medium (without added ADP), recentrifuged and the pellet dissolved in 400 μl sodium dodecylsulphate (2.5% in distilled water). Solubilized mitochondria were transferred to counting vials, 10 ml scintillation fluid (lumax/xylene, Nuclear Enterprises Ltd., Edinburgh, U.K.) added and the [^{14}C]ADP retained by the mitochondria counted in a scintillation counter (Beckman LSR-345, Beckman, U.K.).

When the size of the mitochondrial exchangeable nucleotide pool was measured [8] incubation conditions were the same as described above, except the final ADP concentration was 100 μM and carboxyatractyloside (100 μM) was added either before or 2, 20 or 30 mins after the start of the incubation.

Preparation and fatty-acid analysis of inner mitochondrial membranes

Mitochondrial inner membrane fractions were isolated using the digitonin/lubrol technique of Greenawalt [9]. Methyl esters of inner membrane fatty acids were prepared from samples containing 7 mg membrane protein, saponified with KOH/

ethanol (42.5 g/l ethanol), extracted with 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) 152 × 6 mm column in a Perkin-Elmer F17 GLC (Perkin-Elmer Ltd., Beaconsfield, U.K.). Inlet pressures were 104 kPa for N_2 and 124 kPa for H_2. Flame ionization detection was used.

Carboxyatractyloside titration of State 3 respiration

The methods described by Gellerich et al. [10] to measure the 'control strength' of the adenine nucleotide translocator were adopted to assess the amount of carboxyatractyloside (per mg mitochondrial protein) needed for complete inhibition of State 3 respiration. Mitochondria (1.2 mg protein) were incubated in a Gilson oxygraph cell fitted with a Clark-type oxygen electrode (Gilson Medical Electronics Inc., Middleton, Wisconsin, U.S.A.) in a medium containing 110 mM sucrose/60 mM Tris/60 mM KCl/15 mM glucose/10 mM K_2HPO_4/5 mM $MgCl_2$/0.5 mM EDTA (pH 7.4). The medium was completed immediately before measurement of O_2 consumption by addition of 1 μM rotenone/10 mM succinate/1 mM ATP/0.9 Units yeast hexokinase (EC 2.7.1.1). After an initial maximal State 3 respiration rate was established, repeated additions of carboxyatractyloside were made until State 3 respiration was inhibited by 100%.

Determination of mitochondrial adenine nucleotide contents

Freshly prepared mitochondrial suspensions (50 mg protein) were deproteinized with ice-cold perchloric acid (0.6 M) and after centrifugation, the supernatant was assayed for ATP by the enzymic procedure of Bucher [11] using a Test-combination kit (Boehringer, Boehringer Corporation Ltd., London, U.K.). Aliquots of the perchloric acid supernatant were analysed for ADP and AMP by the enzymic methods of Jaworek et al. [12], using an 'assay kit' (Boehringer Corporation Ltd., London, U.K.).

Statistical analysis

Results were statistically analysed using unpaired Student's *t* tests

Results

Assessment of copper status

The rats receiving the low Cu diets for 8 weeks were judged to be Cu-deficient by the results shown in Table I. Plasma and liver Cu concentrations were reduced by 94 and 85%, respectively ($P < 0.001$) and liver cytochrome oxidase activity reduced by 63% ($P < 0.001$) compared with controls. Defects in Fe metabolism were evident as demonstrated by a significant (68%, $P < 0.001$) decline in plasma Fe concentration.

Effect of copper-deficiency on liver mitochondrial State 3 respiration and adenine nucleotide translocase activity

As we have previously demonstrated [3] 'Cu-deficient' mitochondria utilizing succinate as substrate exhibited a significant decline in State 3 respiration, whereas State 4 respiration and the ADP/O ratio were unaffected. The net result of these changes in respiration was a reduction in respiratory control ratio (Table II).

At first sight these results are compatible with the suggestion that the impaired State 3 respira-

TABLE I

THE EFFECT OF COPPER DEFICIENCY ON PLASMA COPPER AND IRON CONCENTRATION AND LIVER COPPER CONTENT AND CYTOCHROME *c* OXIDASE ACTIVITY

Results expressed as means ± S.E. For Copper deficiency, $P < 0.001$ (significantly different from control).

	Plasma Cu concn. (mg/l) (4 animals)	Plasma Fe concn. (mg/l) (4 animals)	Liver Cu concn. (mg/kg) (7 animals)	Liver cytochrome oxidase activity (μmol Cyt *c* reduced per min per mg protein) (4 animals)
Control	1.00 ± 0.027	2.07 ± 0.20	4.53 ± 0.106	1.47 ± 0.14
Cu-deficient	0.06 ± 0.013	0.67 ± 0.033	0.68 ± 0.046	0.54 ± 0.045

TABLE II

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

Mitochondrial respiration rates were determined polarographically at 30°C and are expressed as ngatoms O_2 consumed/min per mg protein. Values are mean ± S.E., $n = 4$.

	State 3 respiration	State 4 respiration	Respiratory control ratio	ADP/O
Controls	85.5 ± 8.4	19.8 ± 1.3	4.3	1.86 ± 0.08
Cu-deficient	56.1 ± 5.6 [a]	15.4 ± 1.7	3.7	1.86 ± 0.05

[a] $P < 0.05$ Cu-deficient compared with controls.

tion supported by succinate (Table I) and by glutamate/malate and β hydroxybutyrate [3] occurs as a direct consequence of reduced cytochrome oxidase. However, as previously discussed, some of our earlier findings [3] led us to suggest that impairment of some other process involved in oxidative phosphorylation, e.g., adenine nucleotide translocation, may also occur in Cu deficiency.

Adenine nucleotide translocase ('translocase') activity of liver mitochondria from Cu-deficient and control rats, assayed by monitoring uptake of [^{14}C]ADP, is shown in Fig. 1. At 0 s, when the translocase inhibitor was added to the incubations before starting the assay with [^{14}C]ADP, the amount of ADP bound was the same in the two preparations. This indicated that Cu-deficiency was without effect on non-specific adenine nucleotide binding. In contrast, at each of the subsequent times (30, 60 and 120 s), [^{14}C]ADP uptake by 'Cu-deficient' mitochondria was significantly lower than the controls. These results clearly demonstrate that at this stage of Cu-deficiency (8–10 weeks) there is reduced 'translocase' activity in rat liver mitochondria.

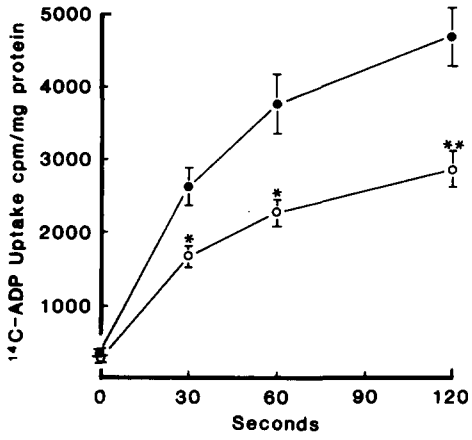

Fig. 1. The effects of Cu deficiency on adenine nucleotide translocase activity of rat liver mitochondria. Mitochondria (5 mg protein) were incubated at 0°C in the presence of 60 μM ADP labelled with 0.05 μCi [^{14}C]ADP. Either just (0 s) before or at 30, 60 or 120 s after addition of ADP, carboxyatractyloside was added (60 μM) and the mitochondria harvested by centrifugation, washed in the same medium without added ADP, recentrifuged and the pellet dissolved in a solution of sodium dodecylsulphate prior to scintillation counting from ^{14}C. Results: O——O, 'Cu-deficient' mitochondria; ●——●, 'Control' mitochondria. Mean values, vertical bars are 2 S.E., $n = 8$. *, $P < 0.05$; **, $P < 0.01$ Cu deficient compared with controls.

Investigations on the mechanism(s) responsible for reduced adenine nucleotide translocase activity. Effects on mitochondrial fatty acid composition

It has been suggested that the changes in translocase activity of rat liver mitochondria from cold stressed animals [13] and animals differing in thyroid status [8] may result from alterations in the degree of unsaturation of the fatty acids of the mitochondrial membranes.

In a previous study, we showed that except for changes in the content of docosapentaenoic acid (22 : 5), a minor component, the fatty acid composition of whole mitochondria was unaffected by Cu deficiency [14]. However, since the translocase enzyme is located specifically in the inner mitochondrial membrane we have examined the effects of Cu deficiency on the fatty acid composition of this mitochondrial component. Results are shown in Table III together with those of our previous study on whole mitochondria [4]. It is

TABLE III

THE EFFECT OF COPPER DEFICIENCY ON FATTY-ACID COMPOSITION OF RAT LIVER MITOCHONDRIAL INNER MEMBRANES

Results are mean fatty-acid contents in percent mole fraction ± S.E., $n = 4$ or 6. Analyses were carried out after 8 to 9 weeks of experimental treatment.

Dietary treatment	Inner membranes		Whole mitochondria [a]	
	with Cu	without Cu	with Cu	without Cu
Fatty acid				
16:0	11.34 ± 0.17	11.26 ± 0.35	15.17 ± 0.84	15.48 ± 0.50
16:1	3.23 ± 0.14	2.62 ± 0.34	2.59 ± 0.17	3.49 ± 0.49
18:0	13.68 ± 0.41	13.35 ± 0.33	14.65 ± 0.55	12.77 ± 0.89
18:1	13.57 ± 0.76	12.83 ± 0.12	10.03 ± 0.53	9.93 ± 0.48
18:2	17.69 ± 0.38	16.39 ± 0.81	14.20 ± 0.63	13.29 ± 0.51
20:4	24.83 ± 0.30	23.88 ± 0.57	25.48 ± 0.63	23.57 ± 0.45 [b]
Unknown	3.07 ± 0.71	3.48 ± 0.46	3.29 ± 0.44	4.09 ± 0.45
22:4	6.04 ± 1.15	6.93 ± 0.74	8.72 ± 1.02	11.33 ± 1.23
22:5	4.12 ± 0.18	6.67 ± 0.42 [c]	2.37 ± 0.26	3.27 ± 0.09 [c]
22:6	2.63 ± 0.24	2.40 ± 0.03	3.50 ± 0.14	3.10 ± 0.14
Unsaturation index [a]	212	219	211	213

[a] Fatty-acid content of whole mitochondria taken from Ref. 4.
[b] $P < 0.05$ Cu-deficient compared with controls.
[c] $P < 0.01$ Cu-deficient compared with controls.
[d] Unsaturation index is Σ (mole fraction × number of unsaturated bonds).

clear that the fatty acid composition of both the 'Cu-deficient' and 'control' inner membranes differ little from that of intact mitochondria. As with the latter, Cu deficiency significantly ($P < 0.01$) increased the inner membrane content of docosapentaenoic acid (22:5). There was only a small (3.5%) increase in the unsaturation index of the Cu-deficient preparation which could be fully accounted for by the increase in docosapentaenoic acid.

Carboxyatractyloside titration of State 3 respiration

The changes in fatty-acid composition of mitochondrial membranes described above were only minor and seemed unlikely to account for the decreased 'translocase' activity of the Cu-deficient mitochondria. To assess whether in Cu-deficiency the amount of functional 'translocase' enzyme in liver mitochondria was reduced, inhibitor titrations of State 3 respiration with carboxyatractyloside were carried out. Carboxyatractyloside is a quasi-irreversible inhibitor of adenine nucleotide translocase, thus the minimum amount of the inhibitor added which produces complete inhibition of State 3 respiration should give an indirect measure of the amount of enzyme present in mitochondrial preparations. The results of an experiment with a Cu-deficient and a control mitochondrial preparation are shown in Fig. 2. Throughout these experiments a high concentration of hexokinase plus glucose was added to the incubations as an 'ATP trap' in order to maintain a high and stable concentration of extramitochondrial ADP throughout the titration period, viz. conditions favourable for State 3 respiration. In the absence of added carboxyatractyloside, State 3 respiration (succinate as substrate) of the Cu-deficient preparation was, as demonstrated previously (Table II), lower than that of the control. Increments of carboxyatractyloside resulted in progressive inhibition of oxygen consumption described by sigmoidally shaped curves (Fig. 2). Maximum inhibition of ADP-stimulated respiration was achieved by approximately the same amount of carboxyatractyloside in both the Cu-deficient and control preparations. The rates of oxygen consumption when carboxyatractyloside inhibition was maximal were the same, indicating

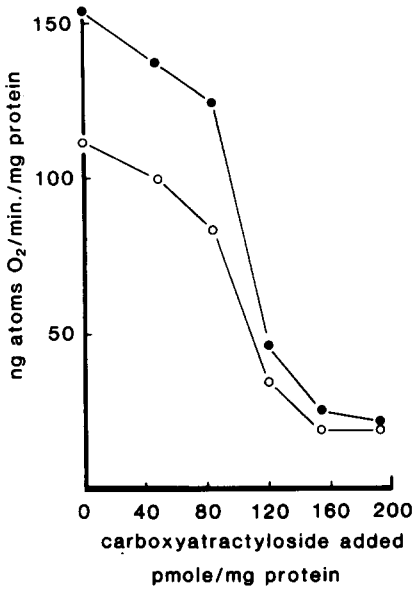

Fig. 2. Carboxyatractyloside inhibition of State 3 respiration. A plot of mitochondrial respiration rate (ng atom O_2/min per mg protein) against amount of added carboxyatractyloside (pmol/mg protein). Mitochondria (1.2 mg protein) were incubated in the presence of 1 mM ATP/0.9 Units yeast hexokinase/15 mM glucose/10 mM succinate. Increasing amounts of carboxyatractyloside were added (X axis) until state 3 respiration was maximally inhibited. Results, O——O, 'Cu-deficient' preparation; ●——●, 'Control' preparation.

that State 4 respiration was unaffected by Cu-deficiency.

In a series of similar titrations there was no significant difference between the two groups in the amount of carboxyatractyloside required for 100% inhibition of State 3 respiration the results being (pmol/mg protein, means ± S.E.) 198 ± 18(4) in Cu-deficient preparations compared with 187 ± 21(4) in controls ($P > 0.05$), suggesting that Cu-deficiency is without effect on the amount of functional 'translocase' present in rat liver mitochondria.

Effects of Cu-deficiency on the 'exchangeable' mitochondrial adenine nucleotide pool

The rate of mitochondrial adenine nucleotide translocation is affected by changes in the intramitochondrial pool size of the sum of the two exchangeable nucleotides, ATP and ADP [14]. An estimate of relative changes in exchangeable pool size can be obtained by allowing the 'translocase' assay of mitochondrial [^{14}C]ADP uptake to proceed to equilibrium. At equilibrium the intramitochondrial substrates for translocation (ATP + ADP) are labelled to the same specific radioactivity as the extramitochondrial ADP.

The results of an experiment to determine whether Cu deficiency affects the exchangeable nucleotide pool size of rat liver mitochondria are shown in Fig. 3. The time-courses of [^{14}C]ADP uptake by 'Cu-deficient' and 'control' mitochondria over the first 2 min of incubation were similar to those shown in Fig. 1. Thus there was no difference in the non-specific binding of ADP between the Cu-deficient and control preparations, but 'translocase' activity as assessed from [^{14}C]ADP uptake at 2 min was markedly lower in the Cu-deficient mitochondria. When the incubations were allowed to proceed to 20 or 30 min, by which time equilibrium had been established, the amount of ^{14}C-label in the Cu-deficient mitochondria was 36% lower than in the controls. These results suggest that the reduced activity of adenine nucleotide translocase may be a consequence of a diminished pool size of exchangeable nucleotides.

An apparently reduced exchangeable nucleotide pool size may result either from decreased absolute

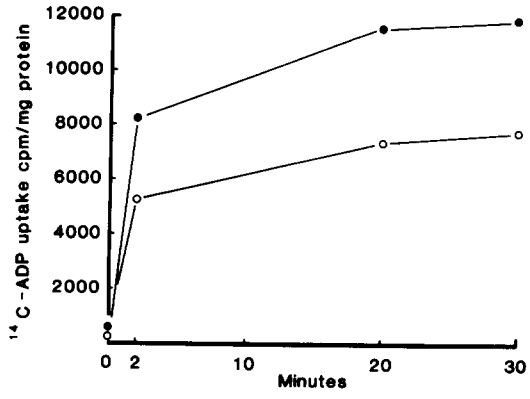

Fig. 3. The effect of Cu deficiency on the size of the exchangeable adenine nucleotide pool of rat liver mitochondria. A plot of the amount of [^{14}C]ADP taken up by mitochondria (cpm ^{14}C/mg protein) against time of incubation (min). Conditions as in Fig. 1, except 100 μM ADP was used and the incubations stopped at the time indicated by addition of carboxyatractyloside (100 μM). Results, O——O, 'Cu-deficient' mitochondria; ●——● 'Control' mitochondria. Mean values, $n = 3$.

TABLE IV

ENDOGENOUS ADENINE NUCLEOTIDE CONTENTS IN LIVER MITOCHONDRIA FROM Cu-DEFICIENT AND CONTROL RATS

Mitochondria were prepared from livers of control or 8–10 weeks Cu-deficient rats as described in Materials and Methods. Samples (50 mg/protein) were extracted with perchloric acid: the extracts were enzymically analysed for adenine nucleotide contents. Values are in nmol per mg protein, means ± S.E., $n = 7$.

	ATP	ADP	AMP	ATP + ADP ('exchangeable' pool) [a]	ATP + ADP + AMP ('total' pool)	$\frac{ATP + 0.5ADP}{ATP + ADP + AMP}$ (energy charge)
Control	4.80 ± 0.24	4.86 ± 0.66	2.63 ± 0.51	9.66 ± 0.71	12.30 ± 0.87	0.60 ± 0.024
Cu-deficient	2.92 ± 0.35 [b]	2.89 ± 0.31 [c]	2.94 ± 0.24	5.81 ± 0.36 [b]	8.75 ± 0.16 [d]	0.50 ± 0.024 [c]

[a] Pool of adenine nucleotides potentially available for translocation.
[b] $P < 0.001$ Cu-deficient compared with control.
[c] $P < 0.02$ Cu-deficient compared with control.
[d] $P < 0.005$ Cu-deficient compared with control.

amounts of ATP and ADP or from partitioning which reduces the proportion of these nucleotides available for translocation [8]. To investigate these possibilities, freshly prepared 'Cu-deficient' and 'control' mitochondria were extracted with perchloric acid and the extracts assayed for ATP, ADP and AMP. Results are shown in Table IV.

The ATP and ADP contents of 'Cu-deficient' mitochondria were respectively, 39% ($P < 0.001$) and 40% ($P < 0.05$) lower than controls. In contrast, there was no significant change in the AMP content of Cu-deficient mitochondria. However, the reduction in the size of the exchangeable nucleotide pool (ATP + ADP) was of sufficient magnitude ($P < 0.001$) to decrease significantly ($P < 0.01$) the 'total adenine nucleotide pool' (ATP + ADP + AMP). In addition, these changes reduced ($P < 0.05$) the 'energy charge' (viz. ATP + 0.5 ADP/ATP + ADP + AMP) of the Cu-deficient mitochondria compared with the controls.

Discussion

The experimental protocol in this investigation differed in two respects from that of our previous studies [3,4]. Firstly, casein (250 g/kg diet) was used in place of albumin (200 g/kg diet) as the protein source, since the Cu content of commercially available spray dried egg albumin was found to be variable and on occasions sufficiently high to result in diets containing more than 0.7 μg Cu/g. Supporting studies have shown that investigations of the type described here and elsewhere [3] only give consistent results if the basal diet contains less than 0.7 μg Cu/g. Secondly, since in this investigation studies were made on some regulatory aspects of mitochondrial adenine nucleotide metabolism, which are known to be affected by food restriction [15] the food intakes of the Cu-adequate control rats were matched to those of the Cu-deficients by pair-feeding.

Notwithstanding these differences in methodology, the changes in hepatic Cu content and cytochrome-*c*-oxidase activity and plasma Cu and Fe concentrations after 8 weeks of treatment were essentially the same as those observed previously. Similarly, the change in dietary protein source and in the pattern of food intake of the controls did not affect our previous conclusions on the effects of Cu-deficiency on the respiratory function of liver mitochondria [3]. Thus, Cu-deficiency did not affect the ADP/O ratio and rate of State 4 respiration of mitochondria utilizing succinate, but significantly reduced the rate of State 3 respiration (Table II).

On the basis of previous observations (see Ref. 3 and Introduction) it seemed unlikely that these changes in respiratory activity of Cu-deficient mitochondria could be fully accounted for by a decreased activity of cytochrome oxidase. Since the classical mitochondrial inhibitors oligomycin and atractyloside produce analogous changes in mitochondrial respiration to those we have observed in Cu-deficient liver preparations (the

former by inhibition of the F_1-ATPase and the latter by inhibition of the adenine nucleotide translocase) studies of the effect of Cu-deficiency on the function of these two mitochondrial enzymes seemed justified.

Since atractyloside insensitive [^{14}C]ADP binding to mitochondria was unaffected by Cu-deficiency, but atractyloside-sensitive [^{14}C]ADP uptake was significantly ($P < 0.01$) reduced this suggests that adenine nucleotide translocase activity of liver is sensitive to Cu-depletion. This conclusion is consistent with the proposal of Gallagher and Reeve [16] that Cu deficiency considerably decreases the affinity of mitochondrial adenine nucleotide binding sites on the inner membrane. They subsequently concluded [17] that "this defect, as with the inhibition by atractyloside, impairs the translocation of ATP to the outer membrane".

We question the validity of this conclusion on grounds of the methods used in this earlier study [17]. Thus, [^{14}C]ADP 'binding' was investigated with inner mitochondrial membranes prepared by Lubrol detergent treatment, membrane fractions were incubated for 20 min, harvested by centrifugation and the radioactivity of the unwashed pellet was subsequently counted. As these authors emphasise, no account was taken of [^{14}C]ADP trapped in the extra-membrane space of the pellet. Furthermore, the 'binding' of ADP to sites associated with adenine nucleotide translocation are by definition those that are sensitive to inhibition by atractyloside. In their study, Gallagher and Reeve [16] did not distinguish between specific ADP 'binding' to actractyloside-sensitive sites and atractyloside-insensitive 'binding'. In a later investigation [17] they showed that atractyloside-insensitive ADP binding accounted for 42–45% of total ADP-binding by inner-membrane mitochondrial fractions prepared from untreated rats. Clearly these workers did not, using their procedures, distinguish between a specific effect of Cu deficiency on the adenine nucleotide translocase per se and effects on non-specific ADP 'binding'.

Various studies on the effects of alterations of nutritional and hormonal status and environmental conditions have shown that changes observed in the mitochondrial respiratory function are associated with alterations in the degree of saturation of the fatty acids of the mitochondrial membranes. In general, increases in State 3 respiration, respiratory control and in some instances translocase activity appears to be related to increases in the unsaturation index. Examples include changes in thyroid status [18,19,8], pituitary status [20], cold stress [13] and dietary deficiency of unsaturated fat [21,22].

The minor changes we have found in fatty acid unsaturation index of intact mitochondria [3] and inner membranes appear unlikely to account for the decreased rate of State 3 respiration, respiratory control ratio and 'translocase' activity observed in Cu deficiency in this and our previous studies [3,4]. Indeed, the only consistent effect of Cu deficiency on fatty-acid composition that we observed in intact mitochondria as well as their inner membranes was a significant increase in the content of docosapentaenoic acid. Neither the biochemical mechanism(s) responsible for this effect not its relationships to our other findings are known.

Gallagher and Reeve [16] suggested that the decreased adenine nucleotide 'binding' to inner membranes of 'Cu-deficient' liver mitochondria may result from the observed increase in unsaturated fatty acids and the inhibitory action of free acids on translocase activity [23]. This explanation is unlikely, since Wojtzak and Zaluska [23] also showed that inhibitory concentrations of added oleate also uncoupled oxidative phosphorylation, yet neither we [4] nor Gallagher and Reeve [16] could show an effect of Cu deficiency on P/O ratios. It also seems unlikely that in vitro evidence of inhibition by fatty acids of translocase activity has physiological significance, since, during starvation and long-term exercise, when fatty acids are important energy sources, the demands upon translocase activity to deliver ADP to the respiratory chain and ATP to the energy-requiring sites in the cytosol are particularly high.

The minimum concentration of carboxyatractyloside needed to inhibit State 3 respiration of mitochondria incubated in the presence of high and stable concentrations of ADP is an indirect measure of the mitochondrial content of functional 'translocase' (reviewed by Klingenberg [14] and Stubbs [24]). Inhibitor studies (Fig. 2) provided no evidence of a decreased mitochondrial

content of functional enzyme. This conclusion is again at variance with that of Gallagher and Reeve [16,17] who suggested a specific effect of Cu deficiency on the ADP-binding sites of the 'translocase'. However, these differing conclusions may result from differences in methodology employed in addition to alternative interpretations of their data.

Adenine nucleotide translocase preferentially exchanges intra-mitochondrial $ATP_{(i)}$ for extra-mitochondrial $ADP_{(o)}$, although under appropriate conditions $ATP_{(i)}$–$ATP_{(o)}$, $ADP_{(i)}$–$ADP_{(o)}$ and $ADP_{(i)}$–$ATP_{(o)}$ exchanges can be demonstrated. In contrast, neither internal nor external AMP is exchangeable. Thus, changes in the relative affinities of internal and external ATP and ADP for the enzyme, their absolute and relative concentrations internally and externally and the state of energisation of the mitochondria may have marked effects on the measured activity of the enzyme in isolated mitochondria. A detailed review of the kinetic and thermodynamic control of translocase activity has recently been published [14].

With these considerations in mind, we investigated the possibility that the internal exchangeable mitochondrial adenine nucleotide pool, (i.e., ATP + ADP) may be decreased in Cu-deficient liver mitochondria. Direct assays of ATP, ADP and AMP (Table IV) showed that the ATP and ADP contents of 'Cu-deficient' mitochondria were significantly reduced by 39 and 40%, respectively, but AMP was unaffected. This proportional reduction of ATP + ADP was similar to the decrease (36%) in exchangeable nucleotide pool estimated from [^{14}C]ADP uptake (Fig. 3). The inference that essentially all of the mitochondrial ATP and ADP is exchangeable in both Cu-deficient and control mitochondria is consistent with other evidence of the complete exchangeability of ATP and ADP in rat liver mitochondria [25].

Our findings resemble in some respects the effects of hypothyroidism on mitochondrial function. The similarities include a decreased pool of exchangeable nucleotides [8] in addition to the changes in respiratory control ratio, translocase activity and State 3 rates of respiration [8,10]. However, it is unlikely that the effects we have observed in Cu deficiency occur secondarily to altered thyroid function, since in hypothyroidism the decrease in exchangeable nucleotide pool size is evident despite a 60% increase in total ATP + ADP content [8].

No explanation can be offered for the apparent specific decrease in ATP and ADP and maintenance of normal AMP contents in 'Cu-deficient' mitochondria. Changes in permeability of the inner mitochondrial membrane seem unlikely in view of the similar properties of 'aged' Cu-deficient and control mitochondria, the absence of effects on soluble cytochrome *c* and the failure of exogenously added NADH to stimulate respiration [3]. Our finding that the degree of coupling remained unchanged also supports the conclusion that increases in 'leakiness' or permeability of Cu-deficient mitochondria cannot account for their decreased ATP and ADP contents. Clearly, if the inner mitochondrial membrane were more permeable to such large and highly charged species as ATP^{4-} and ADP^{3-}, it is scarcely conceivable they could maintain a normal proton gradient and hence normal ADP/O ratios.

The reduced 'energy charge' [(ATP + 0.5 ADP)/(ATP + ADP + AMP)] observed in Cu-deficient mitochondria could obviously have important effects on cytosolic events due to reduced 'translocase' activity, and hence provision of ATP for energy-consuming reactions such as protein synthesis, gluconeogenesis, phospholipid synthesis and maintenance of ion gradients. In addition, it may also have signficiant effects on intra-mitochondrial processes which depend upon energy state, such as the synthesis of citrulline from bicarbonate, ammonia and ornithine [26], and Fe metabolism [2,27].

Recent controversy over the roles of various intra- and extra-mitochondrial factors, controlling respiration in vivo and in intact isolated cells and mitochondria has been reviewed by Stubbs (1981) [24]. Davis and Davis-Van Thienen [28] claim that respiration in isolated mitochondria is dependent on the extra-mitochondrial ATP/ADP ratio with adenine nucleotide translocase linking extra- and intramitochondrial equilibria processes and hence exerting a rate-limiting role. Others concluded that respiration is thermodynamically controlled by the extra-mitochondrial phosphate potential (ATP/ADP + P_i) [29], while Wilson et al. [30] have proposed that all control is exerted by the terminal

step viz. cytochrome *c* oxidase.

Recently, the concept that control of the rate of metabolite flux through a pathway may not be limited to one enzyme, but shared by some or all of the enzymes on that pathway [31,32] has been applied to the investigation of control of respiration by intact mitochondria [10,33]. These workers have attempted to quantify the amount of control (control strength) that the adenine nucleotide translocator and other intra- and extramitochondrial steps exert on the rate of respiration. The control strength (C) of a step in a metabolic pathway is given by the fractional change in steady-state flux (dJ/J) induced by a fractional change in the concentration of the enzyme under consideration (de/e) (see Refs. 31 and 32), i.e.,

$$C = \frac{dJ/J}{de/e}$$

Briefly, such studies [33,10] suggest that mitochondrial respiratory control is distributed among a variety of steps which include both nucleotide translocase and cytochrome oxidase. However, the relative control strengths of individual steps are influenced by rates of respiration and, particularly, by extra-mitochondrial processes, such as the nature of ATP-generating and ADP-regenerating reactions [10,33].

Provisional estimates of the control strength of cytochrome oxidase derived from data on changes in enzyme activity and the concomitant reduction in State 3 respiration in mitochondria of rats depleted of Cu for 2 and 4 weeks are presented in Table V. This analysis suggests that the control strength of cytochrome oxidase with all substrates is less than 1, indicating that the rate of respiration is not controlled solely by changes in activity of this enzyme. Even after 4 weeks of deficiency it appears to contribute at most 45% of the total control of succinate supported State 3 respiration. These estimates contrast strongly with the proposals of Wilson et al. [30] that all control is exerted by this enzyme. Such conclusions are tentative, since, even at these early stages of deficiency, changes other than in cytochrome-oxidase activity may influence respiratory function. In addition, the theory requires ideally extrapolation of the metabolic flux rate after removal of a minimal quantity of enzyme to that observed when the full complement of enzyme is present. In nutritional studies of this nature, this latter condition is impossible to satisfy fully. However, errors introduced in the analysis due to either of the reservations mentioned above would almost certainly overestimate control strength.

It would be unwise, in our view, to extrapolate too freely the conclusions drawn from these studies on rat liver to other tissues, for example, heart [34] or pancreas [35], which exhibit marked pathological changes in Cu deficiency. Doussière et al. [36] have shown, for example, that the distribution of control of respiration in heart mitochondria differs markedly from that of liver with the translocase enzyme having virtually no influence.

However, in view of our findings that adenine

TABLE V

THE PERCENTAGE DECLINE IN STATE 3 RESPIRATION AND CYTOCHROME *c*-OXIDASE ACTIVITY OF COPPER-DEFICIENT RAT LIVER MITOCHONDRIA AND ESTIMATED CONTROL STRENGTH OF CYTOCHROME OXIDASE ON RESPIRATION

Rat-liver mitochondria data are from Lawrence et al. [4] and Davies et al. [3]. Estimated control strength data are as defined by Kacser and Burns [31] and Heinrich and Rapoport [32]. The control strength of a step in a metabolic pathway is given by the fractional change in steady-state flux (dJ/J) induced by a fractional change in the concentration of the enzyme under consideration (de/e), i.e. $C = (dJ/J)/(de/e)$. In this case: dJ/J = % change State 3 respiration. de/e = % change cytochrome oxidase activity.

Number of weeks of deficiency	Percentage decline relative to controls			
	cytochrome oxidase	State 3 respiration		
		succinate	glutamate/ malate	β-OH butyrate
2	42	15	16	9
4	75	34	21	15
6	80	47	31	13
8	80	58	40	35

Estimated control strength [C] of cytochrome oxidase on State 3 respiration with succinate, glutamate/malate or β-hydroxybutyrate as substrates after 2 and 4 weeks Cu depletion.

	Succinate	Glutamate/ malate	β-Hydroxybutyrate
2 weeks	0.36	0.38	0.22
4 weeks	0.45	0.28	0.22

nucleotide metabolism, 'translocase' activity and cytochrome oxidase activity in rat-liver mitochondria are all sensitive to Cu depletion, detailed studies in more intact systems such as isolated cells and perfused organs may further our understanding of fundamental mechanisms involved in regulation of cellular energy metabolism as well as of clarifying biochemical aspects of the pathology of Cu deficiency.

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Note added in proof (January 30th, 1986)

Due to a misunderstanding in the Editorial Office, the fatty acid, 22 : 5, appeared in Biochim. Biophys. Acta 809 (1985) 351–361 (Ref. 4 in the present article) as docosapentenoic acid instead of docosapentaenoic acid, its proper designation.

References

1 Underwood, E.J. (1977) Trace Elements in Human and Animal Nutrition, 4th Edn, Academic Press, New York
2 Mills, C.F. (1980) in Biological Roles of Copper, Ciba Foundation Symposium 79, pp. 49–65, Excerpta Medica, Amsterdam
3 Davies, N.T., Lawrence, C.B. and Mills, C.F. (1985) Biochim. Biophys. Acta 809, 362–368
4 Lawrence, C.B., Davies, N.T., Mills, C.F. and Nicol, F. (1985) Biochim. Biophys. Acta 809, 351–361
5 Chappell, J.B. and Hansford, R. (1969) in Subcellular Components (Birnie, G.D. and Fox, S.M., eds.), pp. 43–56, Butterworths, London
6 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–51
7 Duée, E.D. and Vignais, P.V. (1969) J. Biol. Chem. 244, 3923–3931
8 Hoch, F.L. (1977) Arch. Biochem. Biophys. 178, 535–545
9 Greenawalt, J.W. (1974) Methods Enzymol. 31, 310–323
10 Gellerich, F.N., Bohnensack, R. and Kunz, W. (1983) Biochim. Biophys. Acta 722, 381–391
11 Bucher, T. (1947) Biochim. Biophys. Acta 1, 292–313
12 Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 2nd Edn., p. 2127, Academic Press, New York
13 Mak, I.T., Shrago, E. and Elson, C.E. (1983) Biochim. Biophys. Acta 722, 302–309
14 Klingenberg, M. (1979) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 3, pp. 383–438, John Wiley & Sons, London
15 Veech, R.L., Rayman, L. and Krebs, H.A. (1970) Biochem. J. 117, 499–503
16 Gallagher, C.H. and Reeve, V.E. (1971) Aust. J. Exp. Biol. Med. Sci. 49, 445–451
17 Gallagher, C.H. and Reeve, V.E. (1976) Aust. J. Exp. Biol. Med. Sci. 54, 593–600
18 Chen, Y.D. and Hoch, F.L. 91976) Arch. Biochem. Biophys. 172, 741–744
19 Babior, N.B.M., Creagan, S., Ingbar, S.H. and Kipnes, R. (1973) Proc. Nat. Acad. Sci. 70, 98–102
20 Clejan, S., Collip, P.J. and Maddaiah, V.T. (1980) Arch. Biochem. Biophys. 203, 744–752
21 Ito, T. and Johnson, R.M. (1964) J. Biol. Chem. 239, 3201–3208
22 Divakaran, P. and Venkataraman, A. (1977) J. Nutr. 107, 1621–1631
23 Wojtczak, L. and Zaluska, H. (1967) Biochem. Biophys. Res. Commun. 26, 76–81
24 Stubbs, M. (1981) in International Encyclopaedia of Pharmacology and Therapeutics. Section 107. Inhibitors of Mitochondrial Functions. (Erecinska, M. and Wilson, D.F., eds.), p. 283–301, Pergamon Press, Oxford
25 Pfaff, E., Heldt, H.W. and Klingenberg, M. (1969) Eur. J. Biochem. 10, 484–493
26 Wanders, R.J.A., Groen, A.K., Meijer, A.J. and Tager, J.M. (1981) FEBS Lett. 132, 201–206
27 Romslo, I. (1980) in Iron and Biochemistry and Medicine, II (Jacobs, A. and Worwood, M., eds.), pp. 325–362. Academic Press, London
28 Davis, E.J. and Davis-van-Thienen, W.I.A. (1978) Biochem. Biophys. Res. Commun. 83, 1260–1266
29 Holian, A., Owen, C.S. and Wilson, D.F. (1977) Arch Biochem. Biophys. 181, 164–171
30 Wilson, D.F., Owen, C.S. and Holian, A. (1977) Arch. Biochem. Biophys. 182, 749–762
31 Kacser, H. and Burns, J.A. (1973) in Rate Control of Biological processes (Davies, D.D., ed.), pp. 65–104, Cambridge University Press, London
32 Heinrich, R. and Rapoport, T.A. (1974) Eur. J. Biochem. 42, 97–105
33 Groen, A.K., Wanders, R.J.A., Westerhoff, H.V., Van der Meer, R. and Tager, J.M. (1982) J. Biol. Chem. 257, 2754–2757
34 Dallman, P.R. and Goodman, J.R. (1970) Blood 35, 496–505
35 Fell, B.F. (1981) Phil. Trans. R. Soc. Lond. B 294, 153–169
36 Dousière, J., Ligeti, E., Brandolin, G. and Vignais, P.V. (1984) Biochim. Biophys. Acta 766, 492–500