Effect of dietary copper deficiency in the rat on fatty acid composition of adipose tissue and desaturase activity of liver microsomes

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(Received 1 October 1974 – Accepted 4 November 1974)

1. Male rats were maintained from weaning to between 4 and 16 weeks of age on a semi-synthetic diet which was deficient in copper.

2. Methyl esters of fatty acids from adipose tissue of the rats were analysed by gas-liquid chromatography and the desaturase activity of liver microsomes, with [1-14C]stearic acid as the substrate, was determined. Liver and plasma Cu concentration, cytochrome c oxidase (EC 1.9.3.1) activity and caeruloplasmin activity were determined as indices of Cu status.

3. Cu deficiency was associated with decreased mono-unsaturated:saturated ratios for C16 and C18 fatty acids from subcutaneous adipose tissue and decreased desaturase activity for liver microsomes. When Cu-deficient rats were given free access to the Cu-adequate diet or were injected intraperitoneally with an aqueous solution of CuSO4, that is, when the animals were repleted with Cu, the indices of Cu status, and desaturase activity for liver microsomes returned to values found in control animals.

4. When Cu or a Cu-chelator (Neocuproine) was added to microsomes, there was no effect on the activity of the desaturase enzyme system; the stability of the desaturase was not affected by Cu.

5. These results are indicative of an involvement of Cu in the desaturase reaction. It is suggested that the site of this involvement could be the terminal component of the microsomal electron transport chain.

Pigs maintained on diets supplemented with 250 mg copper/kg diet show increased growth rates (see Braude, 1967). The increased dietary Cu is associated with softening of the adipose tissue (Taylor & Thomke, 1964) due partly to the increase in the proportions of 16:1 and 18:1 fatty acids relative to the corresponding saturated acids (Elliot & Bowland, 1968; Moore, Christie, Braude & Mitchell, 1969) and partly to changes in the stereospecific distribution of fatty acids within the triacylglycerol molecules (Christie & Moore, 1969; Moore et al. 1969).

Recent reports have shown that the fatty acid desaturase activity in liver (Ho & Elliot, 1973, 1974; Thompson, Allen & Meade, 1973) and adipose tissue (Ho & Elliot, 1973, 1974) is increased in pigs given Cu-supplemented diets and is probably responsible for the reported differences in fatty acid composition of the triacylglycerols. The addition of Cu ions in vitro is accompanied by increased activity and stability of desaturase in microsomes from pig liver (Thompson et al. 1973).

The purpose of the present work was to study the effect of dietary Cu deficiency in rats on (a) the fatty acid composition of triacylglycerols and (b) the desaturase activity of liver microsomes, in an attempt to find a possible Cu requirement for this reaction. A preliminary account of this work has been given (Wahle & Davies, 1974).
MATERIALS AND METHODS

Animals and diets

Male rats of the Hooded Lister strain were maintained from weaning to between 4 and 16 weeks of age on a semi-synthetic diet (Williams & Mills, 1970) containing (kg): 200 g albumin, 600 g sucrose, 100 g arachis oil, and 0.6 (deficient), 3, 12 or 25 (adequate) mg Cu as CuSO₄·5H₂O, and sufficient vitamins and minerals. Animals were housed in polypropylene cages and only in pair-feeding experiments were the rats caged individually.

Tissue preparations and assays

Animals were anaesthetized by intraperitoneal injection of Nembutal (Abbot Laboratories Ltd, Agro-Vet Division, Queenborough, Kent ME11 5EL) (45 mg/kg body-weight); blood was taken by cardiac puncture and the livers and samples of perinephric and subcutaneous adipose tissue were removed.

Desaturase estimation. The preparation of the microsomes and the estimation of fatty acid desaturase activity were done as described previously (Wahle, 1974).

Fatty acid determination. Extraction of adipose tissue triacylglycerols and the preparation and determination of their main constituent fatty acids as methyl esters by gas–liquid chromatography were done using the method of Duncan & Garton (1967).

Plasma Cu concentrations. The heparinized blood samples were centrifuged and 1 ml portions of plasma were mixed with 1 ml trichloroacetic acid (TCA; 100 g/l) and centrifuged again to remove precipitated protein. The Cu concentrations were determined by atomic absorption spectrophotometry. Standard Cu solutions were prepared in TCA (50 g/l).

Tissue Cu concentrations. Liver (0.5 g wet weight) was freeze-dried, ground to a powder and approximately 50 mg was digested in conc. HNO₃-conc.HClO₄-conc. H₂SO₄ (5:1:0.5, by vol.). The conc. HNO₃-conc. HClO₄ was removed by boiling and the residual H₂SO₄ diluted to 10 ml. The Cu content was determined by atomic absorption spectroscopy using Cu standards prepared in a solution of 50 ml conc. H₂SO₄/l.

Cytochrome c oxidase activity. The liver samples were homogenized (1:100, w/v) in 5 mM-EDTA–Tris buffer, pH 7.4, using a glass homogenizer and Teflon pestle. The resulting homogenates were centrifuged at 1000 g and the supernatant fractions used for the estimation of cytochrome c oxidase activity using the method of Mills & Dalgarno (1970).

Caeuruloplasmin activity. The method of Houchin (1958) as modified by Rice (1960) was used and the results were expressed in IU as defined by Rice (1962).

Protein concentration. This was determined using the method of Miller (1959).

Chemicals. Neocuproine (2,9-dimethyl-1,10-phenanthroline hemihydrate), ATP, NADH, coenzyme A, 1-glycerol-3-phosphate and N-acetylcysteine were obtained from Sigma (London) Chemical Co. Ltd, Kingston-upon-Thames, Surrey. [1-¹⁴C]-
stearic acid was supplied by The Radiochemical Centre, Amersham, Bucks. All other chemicals were of AR grade, purchased from BDH Chemicals Ltd, Poole, Dorset.

**EXPERIMENTAL AND RESULTS**

The mean mono-unsaturated:saturated ratios for C\textsubscript{16} and C\textsubscript{18} fatty acids were significantly lower in the triacylglycerols of subcutaneous adipose tissue from rats maintained on the Cu-deficient diet compared with those maintained on the Cu-
Table 2. Desaturation of [1-14C]stearic acid by liver microsomes from rats maintained on semi-synthetic diets* differing in copper content, incubated with and without the Cu-chelator Neocuproine (10 μmol/ml incubation medium) (Mean values for two determinations)

<table>
<thead>
<tr>
<th>Dietary Cu content (mg/kg)</th>
<th>0.6 (deficient)</th>
<th>3.0</th>
<th>12.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desaturase activity†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without Neocuproine</td>
<td>51.6</td>
<td>109.5</td>
<td>106.5</td>
</tr>
<tr>
<td>With Neocuproine</td>
<td>nd</td>
<td>112.8</td>
<td>115.5</td>
</tr>
</tbody>
</table>

nd, Not determined.

* For details of diets, see p. 106.
† nmol 18:0 fatty acid converted to 18:1/30 min per mg protein; for details of procedures, see Wahle (1974).

Table 3. Effect of repletion of copper-deficient rats by feeding with a Cu-adequate diet (25 mg Cu/kg) or by intraperitoneal injection of doses of 300 μg Cu as CuSO4 in 1 ml saline (9 g NaCl/l), 2 and 7 d before killing, on fatty acid desaturase activity of liver microsomes and indices of Cu status (Mean values with their standard errors for three animals/group)

<table>
<thead>
<tr>
<th>Cu-adequate (control)</th>
<th>Cu-adequate + injected Cu</th>
<th>Cu-deficient</th>
<th>Cu-deficient + injected Cu</th>
<th>Cu-deficient fed Cu-adequate diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desaturase activity</td>
<td>Mean ± se</td>
<td>Mean ± se</td>
<td>Mean ± se</td>
<td>Mean ± se</td>
</tr>
<tr>
<td>(nmol 18:0 fatty acid</td>
<td>48.3 ± 7.2</td>
<td>44.1 ± 20.4</td>
<td>16.5 ± 4.8</td>
<td>42.3 ± 6.0</td>
</tr>
<tr>
<td>converted to 18:1/30 min per mg protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver Cu concentration</td>
<td>14.7 ± 0.7</td>
<td>32.0 ± 8.1</td>
<td>5.8 ± 1.4</td>
<td>24.3 ± 4.5</td>
</tr>
<tr>
<td>(mg/kg dry wt)</td>
<td></td>
<td></td>
<td></td>
<td>15.9 ± 0.5</td>
</tr>
<tr>
<td>Liver cytochrome c oxidase activity (μmol</td>
<td>0.54 ± 0.07</td>
<td>0.51 ± 0.02</td>
<td>0.24 ± 0.06</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>oxidized/min per mg protein)</td>
<td></td>
<td></td>
<td></td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Plasma Cu concentration</td>
<td>1.41† (1.14, 1.67)</td>
<td>1.58 ± 0.10</td>
<td>0.4 ± 0.14</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>(μg/ml)</td>
<td></td>
<td></td>
<td></td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Califusmin activity</td>
<td>43.0† (41.9, 44.0)</td>
<td>63.5 ± 3.5</td>
<td>1.5 ± 1.0</td>
<td>68.5 ± 9.8</td>
</tr>
<tr>
<td>(IU/l)</td>
<td></td>
<td></td>
<td></td>
<td>51.3 ± 4.4</td>
</tr>
</tbody>
</table>

For details of experimental procedures, see pp. 109 and 110.
* For definition of unit, see Rice (1962).
† Mean values for two animals.

adequate diet (Fig. 1). In perinephric adipose tissue there were no differences between the Cu-deficient and Cu-adequate rats with respect to these ratios for C16 and C18 fatty acids.

The desaturation of [1-14C]stearic acid to oleic acid was substantially decreased in microsomes from the liver of Cu-deficient rats compared with control (Cu-adequate) animals (Table 1). The lower food intakes and consequently decreased carcass weights
Cu deficiency and fatty acid metabolism

Fig. 2. The in vitro activity and stability of fatty acid desaturase of liver microsomes from rats maintained on Cu-adequate (-----) or Cu-deficient (----) semi-synthetic diets (0.6 and 25 mg Cu/kg respectively), incubated with (○) or without (●) added cupric ions (30 nmol CuSO₄/mg microsomal protein); for details of procedures, see Wahle (1974). Each point represents the mean value for two determinations.

of the Cu-deficient animals compared with the control animals may have effected the decreased desaturase activity in these animals. Control rats were therefore pair-fed with Cu-deficient animals with the result that carcass weights were not significantly different, but the decreased desaturase activity was still evident.

Microsomes from livers of 8.5-week-old rats, maintained from weaning on diets containing 0.6 mg Cu/kg (Cu-deficient), had desaturase activities approximately 50% lower than those from rats given diets containing 3.0 or 12.0 mg Cu/kg (Table 2). The carcass weights of these animals were similar. These results suggest that the decreased desaturase activity was due to the Cu deficiency rather than decreased food intake. The addition of Neocuproine (10 µmol/ml incubation medium), a specific Cu-chelating agent, to the liver microsomes in vitro did not affect their desaturase activity.

Cu-deficient rats were repleted with Cu either by allowing them access to the Cu-adequate diet (25 mg Cu/kg) for 12 d before killing or by injecting intraperitoneally doses of 300 µg Cu as CuSO₄ in 1.0 ml sterile saline solution (9 g NaCl/l) 7 and 2 d before killing; a group of control (Cu-adequate) rats was similarly injected with Cu. The desaturase activity of liver microsomes, liver Cu concentrations and cytochrome c oxidase activities are shown in Table 3. A reduced desaturase activity was again found for microsomes from the Cu-deficient rats, but this was restored to 'control' values
by both methods of Cu-repletion. The injection of Cu into control rats did not increase
the desaturase activity of liver microsomes, although the liver Cu concentrations were
approximately twice those found for untreated control animals. The Cu injections
also increased liver Cu concentrations for the Cu-deficient rats to values greater than
those found for control rats. Liver cytochrome c oxidase (a known Cu-containing
enzyme) showed the same decrease and restoration of activity with Cu depletion and
repletion as desaturase activity; plasma Cu concentrations and caeruloplasmin
activities followed the same pattern.

The addition of CuSO₄ (30 nmol/mg microsomal protein) to the incubation medium
had no effect on the in vitro activity or stability of the desaturase activity of liver
microsomes from Cu-deficient or control rats (Fig. 2). CuSO₄ concentrations of
200 nmol/mg microsomal protein, or above, inhibited desaturase activity, the inhibi-
tion being greater in microsomes from the livers of control rats. The addition of FeCl₃
at various concentrations to the incubation medium had no effect on the desaturase
activity of microsomes in vitro.

**DISCUSSION**

Dietary Cu deficiency resulted in decreased mono-unsaturated:saturated ratios for
C₁₆ and C₁₈ fatty acids of the triacylglycerols from subcutaneous tissue when com-
pared with those for control rats. The apparent decrease in the capacity of these
animals to effect the mono-desaturation of long-chain saturated fatty acids was reflected
in the decreased extent of desaturation of stearic acid to oleic acid by liver microsomes.
Palmitic acid and stearic acid are desaturated in animals by the same enzyme system
except that the enzyme has a lower affinity for the former (Brett, Howling, Morris &
James, 1971). The higher mono-unsaturated:saturated ratios for C₁₆ and C₁₈ fatty
acids of the triacylglycerols of subcutaneous adipose tissue from pigs given Cu-supple-
mented diets (Ho & Elliot, 1973; Thompson et al. 1973) and the increased desaturase
activity for microsomes from liver (Thompson et al. 1973) and from adipose tissue
(Ho & Elliot, 1973) when compared with those for control pigs would appear to com-
plement the findings for Cu-deficient rats. However, the extent to which increased
food consumption of the Cu-supplemented pigs influenced their fatty acid desaturase
activity and consequently the fatty acid composition of the triacylglycerols of adipose
tissue was not investigated. The pair-feeding of control (Cu-adequate) rats to Cu-
deficient animals precluded influences on desaturase activity ascribable to differences
in food intake (see Gellhorn & Benjamin, 1964) as found for non-pair-fed animals.
Decreased desaturase activity for the Cu-deficient rats compared with their pair-fed
controls can therefore be regarded as being due to Cu deficiency per se and not to
decreased food intake. Differences in desaturase activity between control rats pair-fed
to Cu-deficient animals and control rats fed to appetite on the Cu-adequate diet
probably reflected the influence of differences in dietary intake on the desaturase
enzyme system. A concentration of 3 mg Cu/kg diet seemed adequate for maintenance
of the desaturase activity in rat liver microsomes, since higher concentrations had no
further effect.

The reason for the lack of effect of Cu deficiency in the rat on the fatty acid com-
position of perinephric adipose tissue similar to that found for subcutaneous adipose tissue is not clear. It is noteworthy that the feeding of Cu-supplemented diets to pigs similarly had little effect on the fatty acid composition of the triacylglycerols of the perinephric adipose tissue (Thompson et al. 1973). Microsomes from adipose tissue of pigs had greater desaturase activity than did those from liver; however, increased dietary Cu enhanced desaturase activity in both tissues (Ho & Elliot, 1973). In rats, the desaturase activity for liver microsomes was greater than that for adipose tissue (Wahle, 1974); the former were therefore used for subsequent desaturase determinations. However, it was assumed, by analogy with the pig, that the desaturase activity of subcutaneous adipose tissue from the rat was also influenced by dietary Cu-deficiency in a similar manner to liver microsomes.

The restoration, either by dietary Cu or intraperitoneal injection of Cu, of the decreased activities for liver desaturase and cytochrome c oxidase (a known Cu enzyme), as well as the restoration of other indices of Cu status in the Cu-deficient rats, strongly indicated that Cu may be involved in the desaturase reaction. When Cu was injected intraperitoneally into Cu-deficient rats, liver Cu levels in excess of control values were found, but liver desaturase activity was not greater than that for control animals as it was for pigs given Cu-supplemented diets. Similarly, the addition of CuSO$_4$ in vitro to liver microsome preparations from rats did not enhance desaturase activity as it did for those from pigs (Thompson et al. 1973).

Although these findings are indicative of an involvement of Cu in the microsomal desaturase reaction neither its location within the sequence of reactions constituting the desaturase reaction nor its function therein is known.

The desaturase reaction requires a functional microsomal electron transport chain (ETC), the terminal component of which is inhibited by cyanide and is regarded as the desaturase enzyme (Oshino, Imai & Sato, 1966; Oshino & Sato, 1972) (Fig. 3). Electrons from reduced pyridine nucleotides pass via flavoproteins and cytochrome $b_5$ to the cyanide-sensitive factor (CSF) which, in its reduced state, activates molecular oxygen and interacts with long-chain acyl-CoA derivatives to produce the corresponding mono-unsaturated derivatives (Oshino, Imai & Sato, 1966, 1971). The CSF
is capable of reacting direct with phenols such as $p$-cresol (Oshino & Sato, 1971) which are the usual substrates for the enzyme phenolase in plants, or tyrosinase in mammalian tissues. This is an established Cu enzyme incorporating a cuprous–cupric valency shift in its reaction mechanism. Such Cu enzymes can usually only use molecular oxygen as the electron acceptor, making them ideally suited as terminal components of electron transport chains (see Mahler & Cordes, 1966). These properties, along with the indications that the CSF may not be a haem-protein (Shimakata, Mihara & Sato, 1972), led us to postulate that Cu could be involved in the terminal stage of the microsomal ETC in a manner analogous to that of cytochrome oxidase in the mitochondrial ETC (see Fig. 3). Preliminary determinations of the Cu concentration in the components of the microsomal ETC, isolated using the method of Shimakata et al. (1972), indicated a higher Cu content in the CSF than in the other components (Davies & Wahle, unpublished results). However, the preparations were not homogeneous and the preliminary nature of these findings must be emphasized. Further work is in progress to purify and analyse the CSF in detail.

We wish to thank Miss A. R. Burnett and Mr A. Flett for their able technical assistance and Mr R. B. Williams for supplying some of the rat tissues.

REFERENCES