

Carbohydrate metabolism in erythrocytes of copper deficient rats¹

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Abstract

Dietary copper deficiency is known to adversely affect the circulatory system of fructose-fed rats. Part of the problem may lie in the effect of copper deficiency on intermediary metabolism. To test this, weanling male Long-Evans rats were fed for 4 or 8 weeks on sucrose-based diets containing low or adequate copper content. Copper deficient rats had significantly lower plasma and tissue copper as well as lower plasma copper, zinc-superoxide dismutase activity. Copper deficient rats also had a significantly higher heart:body weight ratio when compared to pair-fed controls. Direct measurement of glycolysis and pentose phosphate pathway flux in erythrocytes using ¹³C NMR showed no differences in carbon flux from glucose or fructose to pyruvate but a significantly higher flux through the lactate dehydrogenase locus in copper deficient rats (approximately 1.3 times, average of glucose and glucose + fructose measurements). Copper-deficient animals had significantly higher erythrocyte concentrations of glucose, fructose, glyceraldehyde 3-phosphate and NAD⁺. Liver metabolite levels were also affected by copper deficiency being elevated in glycogen and fructose 1-phosphate content. The results show small changes in carbohydrate metabolism of copper deficient rats. © 2003 Elsevier Inc. All rights reserved.

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1. Introduction

Mild copper deficiency is reported to be a common condition in humans [1]. The U.S. National Academy of Sciences has recently established a new Recommended Daily Allowance for copper of 0.9 mg/day for adults [2]. The annual average aggregate daily intake of copper from food, beverages and drinking water was less than 0.9 mg/day for 60% of the participants in the National Human Exposure Assessment Survey in Maryland [3]. Copper deficiency results in anemia in a number of species including rats and humans [4, 5] and has been associated with increased tissue lipid peroxidation [6, 7]. In addition, copper deficiency alters the activities of a number of key enzymes in the pathways of energy metabolism. These include copperoenzymes such as cytochrome c oxidase [8] as well as enzymes not known to contain or directly require copper for their activity, such as glucose-6-phosphate dehydrogenase,

the main entry point to the pentose phosphate pathway (reviewed in [9]).

Interactions between copper deficiency and intermediary metabolism are well known. For example, copper deficiency alters energy substrate utilization in rats from carbohydrate to fat, as indicated by a reduction in respiratory quotient values [10]. In male rats, signs of copper deficiency are more severe when the basal diet contains sucrose or fructose as the principle source of carbohydrate energy, in place of starch or glucose [11]. In addition to physiological manifestations of copper deficiency, fructose-fed copper-deficient male rats accumulate sorbitol in selected tissues [12–14] suggesting that copper deficiency somehow affects the initial reactions of glycolysis. Fructose and glucose have tissue-dependent points of entry into glycolysis but can be inter-converted via the intermediate sorbitol. Since sorbitol production from either glucose or fructose uses reducing equivalents, cellular sorbitol accumulation has been suggested as one of the causes of the increased oxidative stress associated with copper deficiency. Dietary fructose may heighten the oxidative stress associated with copper deficiency because fructose metabolism can completely bypass

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the pentose phosphate pathway in liver and some other tissues [15] and this phenomenon may extend to erythrocyte metabolism.

The present study investigated the relationship between carbohydrate metabolism, cellular reduction potential and copper deficiency-related anemia by following carbohydrate metabolism in erythrocytes. We hypothesized that, similar to other tissues [12–14] erythrocytes of copper deficient rats fed high fructose diets may accumulate sorbitol and this may alter the cells' capacity to generate NAD(P)H by the mechanisms discussed above. We focused on erythrocytes as our model system because copper-deficient rats are anemic. Furthermore, the erythrocyte provides a relatively simple model system for investigation of effects on energy metabolism, having glycolytic and pentose phosphate pathways but no tricarboxylic acid cycle. Liver metabolism was also monitored as well as carbohydrate storage in skeletal muscle since overall carbohydrate metabolism is known to be affected by copper deficiency.

2. Methods and materials

2.1. Animals and diets

The study was approved by the Health Canada Animal Care Committee and the animals were housed and cared for in accordance with the guidelines of the Canadian Council on Animal Care. Weanling male Long-Evans rats (Charles River Canada, St. Constant, QC) were randomly assigned to two groups and fed sucrose-based modified AIN-93G diets (20% casein, 13.2% corn starch, 49.8% sucrose, 5% Solka floc, 7% soybean oil by weight plus AIN93GMX and AIN93VX) containing normal (Experiment 1: 5.41 ± 0.54 ; Experiment 2: 6.19 ± 1.24 mg Cu/kg dry weight; mean \pm SD, n 6) or low copper levels (Experiment 1: 0.3 ± 0.02 ; Experiment 2: 0.43 ± 0.03 mg Cu/kg dry weight; mean \pm SD, n 6). Food consumption by copper-deficient rats fed *ad libitum* was measured daily and control rats were pair-fed at the group mean feeding level of the copper-deficient rats. Animals were fed for 8 weeks (Experiment 1) or 4 weeks (Experiment 2), anesthetized using Isoflurane, killed by exsanguination and immediately necropsied. Body weights were measured weekly and at the time of killing.

2.2. Assessment of copper status

For all experiments, free-flowing blood was collected into heparinized capillary tubes for determination of packed cell volume, superoxide dismutase and glutathione peroxidase activities. The copper status of the animals was ascertained by measurement of: (a) diet and tissue copper levels (Experiment 1: liver, heart, aorta; Experiment 2: liver only); (b) heart weight:body weight ratios; (c) the activity of copper, zinc superoxide dismutase (Cu, Zn-SOD) in plasma (Experiment 1) or plasma and RBCs (Experiment 2) [16];

(d) serum ceruloplasmin (Experiment 1) [17]; and (e) RBC selenium-dependent glutathione peroxidase (SeGSH-Px, Experiment 2) [18]. In the first experiment, 2 of 12 rats fed the low copper diet died of cardiac complications before the end of the 8 week feeding period. This was a significant factor in the decision to use a 4 week feeding period in Experiment 2.

2.3. Measurement of erythrocyte, liver and skeletal muscle metabolites (Experiment 1)

Rats were killed at 8:00 h after overnight food deprivation. Red blood cells were isolated from abdominal aortic blood by rapid microcentrifugation (15 s at 13,000g) and frozen in liquid nitrogen. Liver and gastrocnemius muscle were rapidly dissected out and frozen with the aid of liquid nitrogen-cooled tongs. Glycogen concentrations were measured according to Brooks and Lampi [19]. Trichloroacetic acid extracts were prepared for the measurement of all other metabolites [20]. Glucose, glucose 1-phosphate, fructose 6-phosphate, 6-phosphogluconate and glucose 6-phosphate (G6P), were measured according to Passonneau and Lowry [20]. Fructose [21], fructose 1,6-bisphosphate [22], glyceraldehyde 3-phosphate [22], dihydroxyacetone phosphate [22], fructose 1-phosphate [23], sorbitol [24], and sorbitol phosphate [25] were measured according to published procedures. Nicotinamide dinucleotides were measured by enzyme cycling as described by Passonneau and Lowry [20] using a Fluorolite 1000 fluorescence microplate reader (Dynex Instruments, Chantilly, VA). Values are reported as $\mu\text{mol/g}$ wet weight (liver and skeletal muscle) or as mmol/L RBC. Metabolite concentrations expressed per g hemoglobin from Experiment 1 were converted to units of mmol/L using the mean cell hemoglobin content values determined for Experiment 2 (Table 1).

2.4. Preparation of washed erythrocytes (Experiment 2)

Rats were killed at 8:00 h or 13:00 h and blood was withdrawn from the abdominal aorta and transferred into tubes containing EDTA (Vacutainer™, Becton Dickinson and Company, Franklin Lakes, NJ), on ice. Pooled erythrocytes from two rats were washed 3 times in Buffer A (25 mmol/L Hepes, 120 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.0 mmol/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, pH 7.4 at 25°C) to remove the buffy layer and any broken cells. The final erythrocyte pellet was re-suspended in about 4.5 mL of Buffer A to give between 80% and 85% hematocrit. A 0.4 mL aliquot of this final solution was mixed with 10 mL of CHCl_3 and stored at -20°C for analysis of membrane fatty acids (see below). Viability (membrane integrity) of the pooled, washed erythrocyte preparations was assessed by a dye exclusion assay using Trypan Blue [26]. Viability was similarly assessed on several samples at the end of the NMR data collection period. In all cases (before or after NMR assays) cell viability was greater than 98%.

Table 1
Physiological and haematological parameters in control and copper deficient rats¹

	Indicator	Control	Copper deficient	P value
Experiment 1 (8 wk)	final bodyweight, g	420 ± 7	418 ± 10	N.S.
	heart/body weight, g/100 g bw	0.30 ± 0.01	0.49 ± 0.02	P < 0.001
	heart copper, nmol/g dry weight	338 ± 6	51 ± 2	P < 0.001
	aorta copper, nmol/g dry weight	53 ± 6	30 ± 2	P < 0.005
	liver copper ² , nmol/g dry weight	205 ± 7	42 ± 3	P < 0.001
	plasma Cu,Zn-SOD (U/mg protein)	45 ± 2	19 ± 1	P < 0.001
	serum ceruloplasmin (U/L)	117 ± 3	3.2 ± 0.05	P < 0.001
	haematocrit	0.40 ± 0.01	0.37 ± 0.01	N.S.
Experiment 2 (4 wk)	final body weight, g	255 ± 4	270 ± 7	N.S.
	heart/body weight, g/100 g bw	0.37 ± 0.01	0.48 ± 0.02	P < 0.001
	liver copper ² , nmol/g dry weight	193 ± 6	35 ± 3	P < 0.001
	plasma Cu,Zn-SOD (U/mg protein)	37 ± 2	24 ± 3	P < 0.05
	RBC Cu,Zn-SOD (U/mg hb)	35 ± 1	13 ± 1	P < 0.025
	RBC Se-GSH-Px (mU/mg Hb)	219 ± 4	293 ± 11	P < 0.05
	haematocrit	0.37 ± 0.01	0.33 ± 0.01	N.S.
	Hb (g/L)	131 ± 1	116 ± 3	P < 0.05
	mean cell volume (fL)	63 ± 2	58 ± 3	P < 0.05
	mean cell hemoglobin content (pg)	22.5 ± 0.7	20.4 ± 1.1	P < 0.05
	mean cell hemoglobin concentration (g/L)	357.6 ± 9.5	349.6 ± 11.6	N.S.
red blood cell distribution width (%)	13 ± 1	17 ± 3	P < 0.05	

¹ Values, represent mean ± SEM, n = 10 (copper deficient, Experiment 1), n = 6 (control, Experiment 1) or n = 18 (Experiment 2).

² Derived by calculation from absolute masses of copper and dry matter respectively, summed from analyses of individual liver portions.

Abbreviations: Cu,Zn-SOD, copper, zinc superoxide dismutase; Se-GSH-Px, selenium dependent glutathione peroxidase; Hb, haemoglobin, bw, body weight.

2.5. ¹³C-NMR spectroscopy (Experiment 2)

¹³C-NMR spectroscopy was performed using a Brüker AM-400 spectrometer (Brüker Spectrospin Canada, Milton, ON) equipped with an ASPECT 3000 computer with a 16 bit analog-digital converter. Samples were held in a 10 mm probe operating at 100.6 MHz. Free induction decays were collected using continuous composite-pulse broadband decoupling. The pulse width was 0.9 μs (9.2° rotation angle). Scans were collected in 1 h blocks of 8700 scans with no relaxation delay (0.41 s/scan). The temperature was held at 27°C. ¹³C-glucose utilization was measured by placing a 2.3 mL aliquot of re-suspended erythrocytes (in Buffer A) in a 10 mm NMR tube. A 2.5 mm (O.D.) capillary tube containing *p*-phenylenediamine (Aldrich Chemical Co., Milwaukee, WI) dissolved in D₂O was placed into the 10 mm tube as an internal standard and the instrument was shimmed and tuned. Either: (A) 100 μL of 200 mmol/L [2-¹³C]D-glucose (Omicron Biochemicals Inc., South Bend, IN) plus 100 μL of water, (B) 100 μL of 200 mmol/L [2-¹³C]D-glucose plus 100 μL of 50 mmol/L D-fructose, or (C) 100 μL of 200 mmol/L D-glucose plus 100 μL of 50 mmol/L [2-¹³C]D-fructose (Omicron Biochemicals Inc., South Bend, IN) was added to the 10 mm tube. This gave erythrocyte solutions containing either: (A) 8 mmol/L labeled glucose, (B) 8 mmol/L labeled glucose plus 2 mmol/L fructose, or (C) 8 mmol/L glucose plus 2 mmol/L labeled fructose. The initial glucose and fructose concentrations were based on previously measured fasting plasma concen-

trations of these compounds in copper-deficient or copper-adequate rats [27]. All labeled peaks were measured relative to the internal standard contained in the capillary tube. The glucose, fructose, C2-lactate and the 2,3-diphosphoglycerate peaks were integrated using a peak deconvolution algorithm from Brüker PC Software (Win-NMR, version 950901.1). The C3-lactate peak was integrated by hand. Concentrations were calculated by comparison with appropriate standards measured under the same conditions. Peaks were identified as previously described [28].

2.6. Fatty acid analysis (Experiment 2)

Lipids were extracted using 25 volumes of chloroform-methanol (2:1) and converted to fatty acid methyl ester derivatives. The methyl ester derivatives were prepared using 14% (w/v) boron trifluoride-methanol (Sigma-Aldrich Canada, Oakville, ON) and analyzed using a Hewlett-Packard 5890 Series 2 gas-liquid chromatograph (Palo Alto, CA) equipped with a flame ionization detector and an SP-2560 flexible fused silica capillary column (100 × 0.25 mm internal diameter, 20 μm film thickness; Supelco Inc., Bellefonte, PA). GLC peaks were identified using fatty-acid methyl ester standards (Nu Check Prep., Elysian, MN).

2.7. Elemental analyses

Diet and tissue samples were dry-ashed in a programmable furnace (Fisher Scientific, Nepean, ON, Canada, Model

497) at 450°C using concentrated nitric acid as an oxidizing agent. The ash was dissolved in 2.9 mol/L hydrochloric acid and analyzed for copper by flame atomic absorption spectrophotometry [29] using a Perkin Elmer 5100PC (Perkin-Elmer Corporation, Norwalk, CT). Analytical standards were prepared from certified single-element stock solutions (SPEX Chemical, Metuchen, NJ).

2.8. Statistics

Heart:body weight ratios, tissue copper concentrations, Cu,Zn-SOD activities, ceruloplasmin activities, hematocrit results and metabolite concentrations were analyzed by Student's *t*-test for independent samples with a Bonferoni correction applied to correct for the number of tests performed. The hematology results from Experiment 2 were analyzed using the non-parametric Mann-Whitney U test because of unequal variances. Metabolic rates were analyzed by ANOVA followed by LSD. Comparisons of fatty acid profiles between groups were performed by MANOVA. All statistical analyses were performed using STATISTICA for Windows (StatSoft, Inc., 1997, Tulsa, OK). When values from different groups were not statistically different, the values were pooled to give a single mean for all groups.

3. Results

3.1. Physiological parameters

Dietary copper levels did not affect body weight (Table 1) or food intake (data not shown). Copper deficient rats had a significantly higher heart:body weight ratio that was the result of significantly greater heart weights. Significantly lower heart and aorta (Experiment 1) and liver (Experiments 1 and 2) copper concentrations were also observed. Lower copper values corresponded with lower plasma or erythrocyte Cu,Zn-SOD activities and a significantly higher Se-GSX-Px activity in erythrocytes (Table 1).

Copper deficient rats also had altered hematology when compared to controls. The more detailed hematological measurements performed for Experiment 2 showed that copper deficient rats had lower total hemoglobin (Table 1), a reduced mean cell volume, mean cell hemoglobin, and an increased red blood cell distribution width. Red blood cell counts were not significantly different between these treatments (pooled value: $5.7 \pm 0.4 \times 10^{12}$ cells/L).

3.2. Erythrocyte utilization of glucose and fructose

Isolated erythrocytes were incubated in an NMR tube in the presence of externally added glucose (Fig. 1) or fructose (Fig. 2) to obtain rates of glucose and fructose disappearance as well as rates of lactate and 2,3-diPGA appearance. These rates were used to calculate the flux through the reactions of Fig. 3. The rates of glucose or fructose utiliza-

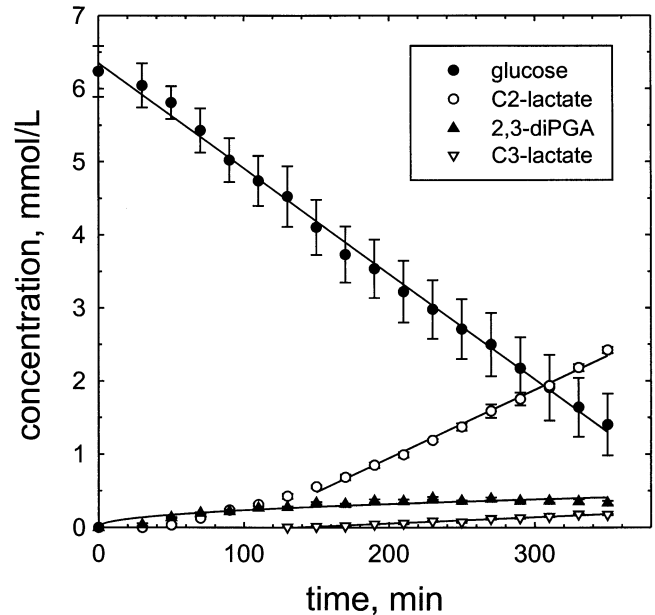


Fig. 1. Reaction progress curve of glucose utilization by erythrocytes. The erythrocytes were incubated with 8 mmol/L labelled glucose at 27°C in an NMR tube. The graph shows the mean \pm SEM concentrations of glucose (solid circles), 2, 3-diPGA (solid triangles), C2-lactate (open circles) and C3-lactate (open triangles) in mmol/L as a function of time for $N=3$ erythrocyte preparations from control rats calculated from NMR spectra. Solid lines represent linear regression analysis of progress curves (glucose, lactate) or a theoretical fit to Equation 1 (2, 3-diPGA).

tion as well as the rates of lactate appearance ($v_{C2\text{-lactate}}$ and $v_{C3\text{-lactate}}$) were obtained by linear regression of the reaction progress curves. The rate of 2,3-diPGA appearance ($v_{2,3\text{-diPGA}}$) were obtained by fitting the NMR data to Equation 1 [28]:

$$[2,3 - \text{diPGA}] = \frac{k_1 A_0}{k_2} (1 - e^{-k_2 t})$$

and taking the initial slope of the curve ($v_{2,3\text{-diPGA}} = k_1 A_0$). The individual metabolic fluxes were calculated as described in the legend to Table 2 where the calculated rates for each different experiment are presented: labeled glucose (A), labeled glucose plus fructose (B) or glucose plus labeled fructose (C).

The data of Table 2 show that erythrocytes preferentially utilize glucose as metabolic substrate. Fructose had no apparent effect on labeled glucose utilization; labeled fructose utilization in the presence of unlabelled glucose proceeded only after a delay (Fig. 2) and was lower than the rate of glucose utilization ($P = 0.02$, ANOVA treatment A or B vs. C). This difference in rates necessitated the inclusion of substrate utilization as a covariate when comparing the relative rates of flux for the various steps of Fig. 3. Even with substrate as covariate, a significant effect of substrate ($P = 0.0002$, ANOVA treatment A or B vs. C) and copper deficiency ($P = 0.015$, ANOVA normal vs. deficient) on the flux through LDH (J_{10}) was noted. Flux through the LDH

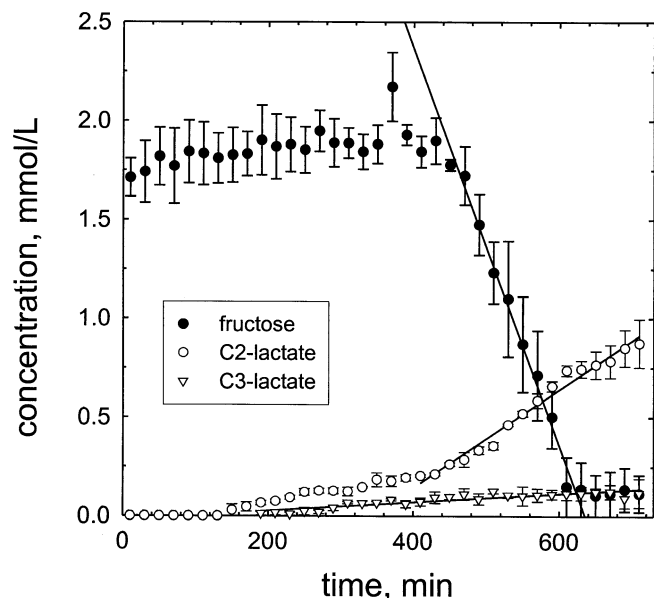


Fig. 2. Reaction progress curve of fructose utilization by erythrocytes. The erythrocytes were incubated with 8 mmol/L glucose and 2 mM labelled fructose at 27°C in an NMR tube. The graph shows the mean \pm SEM concentrations of fructose (solid circles), C2-lactate (open circles) and C3-lactate (open triangles) in mmol/L as a function of time for $N=3$ erythrocyte preparations from control rats calculated from NMR spectra. Solid lines represent linear regression analysis of progress curves (glucose, lactate).

locus was higher in copper deficient animals when compared to controls. In addition, when labeled fructose was added (treatment C), the flux through the LDH locus was

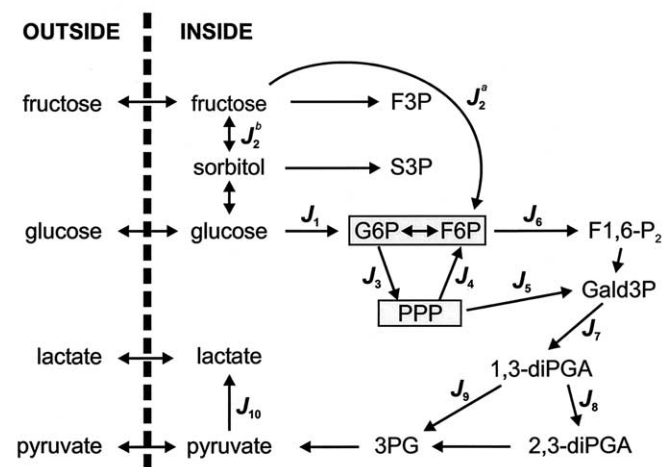


Fig. 3. Erythrocyte metabolic pathways of glucose and fructose utilization. Schematic shows reactions inside and outside the erythrocyte. Letters (J_1 - J_{10}) identify individual steps for which fluxes could be calculated. Fructose utilization represents the sum of the fluxes $J_2^a + J_2^b$. Abbreviations as follows: fructose 3-phosphate (F3P), sorbitol 3-phosphate (S3P), glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), pentose phosphate pathway (PPP), fructose 1, 6-bisphosphate (F1, 6-P₂), glyceraldehyde 3-phosphate (Gald3P), 1, 3-diphosphoglyceric acid (1, 3-diPGA), 2, 3-diphosphoglyceric acid (2, 3-diPGA), and 3-phosphoglyceric acid (3PG).

lower than when labeled glucose (treatments A and B) was added as substrate.

3.3. Metabolites

Copper deficient rats had significantly higher erythrocyte total NAD levels when compared to control rats and this correlated with higher NAD⁺ levels in copper deficient rats (Table 3). There were no significant differences in the levels of NADH in erythrocytes ($11 \pm 14 \mu\text{mol/L RBC}$, pooled value). No differences in liver NADH or NAD⁺ values were observed and no differences in NADPH, NADP⁺ or the NADPH/NADP⁺ ratio were observed in either erythrocytes or liver (data not shown).

The concentrations of glycolytic intermediates were also measured in erythrocytes and liver (Table 3). Glucose, fructose, and glyceraldehyde 3-phosphate contents were higher in erythrocytes of copper deficient rats. In liver, glycogen and fructose 1-phosphate contents were higher in copper deficient rats. Erythrocyte levels of sorbitol, 6-phosphogluconate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, and dihydroxyacetone phosphate were not affected by dietary copper. Glucose 1-phosphate and sorbitol-phosphate were not detected in erythrocytes. In liver, sorbitol, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, and dihydroxyacetone phosphate were unaffected by dietary copper levels. 6-phosphogluconate and sorbitol phosphate were not detected in liver samples. Skeletal muscle glycogen concentrations were not different when values for copper deficient rats ($1.22 \pm 0.44 \text{ mg/g wet weight}$; $N = 10$) were compared to pair fed animals ($0.85 \pm 0.34 \text{ mg/g wet weight}$; $N = 6$).

3.4. Fatty acid analysis

Erythrocyte stress was also indirectly assessed by following changes in membrane fatty acid content because lipid peroxidation is known to be associated with copper deficiency [6, 7]. MANOVA analysis of the data (data not shown) showed no statistical differences between copper deficient and control groups.

4. Discussion

In light of the present results, it is difficult to assign the hematological manifestations of the fructose \times copper deficiency synergistic effect [11] to fructose utilization by erythrocytes. Fructose itself was poorly utilized by rat erythrocytes when adequate external glucose concentrations were present. This was demonstrated by the absence of sorbitol and sorbitol 6-phosphate in erythrocytes (as shown by metabolite analysis) as well as by absence of sorbitol 3-phosphate and fructose 3-phosphate (as shown by NMR analysis) [30, 31] (data not shown). In addition, direct measurement of fructose metabolism by NMR (Fig. 1 & 2)

Table 2
Fluxes through erythrocyte metabolic pathways measured at 27°C expressed in $\mu\text{mol}/(\text{hr} \times \text{L})$ erythrocytes

Substrate	Cu	glc (J_1) or fru (J_2) utilization	Flux to PPP (J_3)	PPP \rightarrow F6P (J_4)	PPP \rightarrow Gald3P (J_5)	flux through PFK (J_6)	triose flux (J_7)	flux through 2,3-diPGA (J_8)	flux through PGK (J_9)	total flux to lactate (J_{10})	% of triose flux through LDH
(A) 8 mmol/L glc*	+	1020 \pm 50	153 \pm 6	102 \pm 4	51 \pm 2	970 \pm 50	1990 \pm 100	660 \pm 100	1.33 \pm 0.04	1510 \pm 40	75.8 \pm 2.2
	-	970 \pm 110	133 \pm 17	89 \pm 12	44 \pm 6	920 \pm 100	1890 \pm 210	490 \pm 130	1.41 \pm 0.22	1750 \pm 340	92.9 \pm 16.8
(B) 8 mmol/L glc* + 2 mmol/L fru	+	1010 \pm 210	149 \pm 34	99 \pm 22	50 \pm 11	960 \pm 200	1970 \pm 400	700 \pm 310	1.28 \pm 0.16	1460 \pm 260	75.8 \pm 15.9
	-	1180 \pm 170	152 \pm 23	101 \pm 15	51 \pm 8	1130 \pm 160	2310 \pm 330	780 \pm 250	1.53 \pm 0.09	1920 \pm 210	83.9 \pm 11.4
(C) 8 mmol/L glc + 2 mmol/L fru*	+	720 \pm 130	137 \pm 41	92 \pm 27	46 \pm 14	680 \pm 140	1400 \pm 270	ND	-	450 \pm 80	33.5 \pm 12.2
	-	700 \pm 380	153 \pm 49	102 \pm 33	51 \pm 16	650 \pm 400	1340 \pm 780	ND	-	580 \pm 60	52.4 \pm 24.4

Asterisks indicate ^{13}C labeled substrates. Metabolic steps as indicated in Fig. 3. ND, not detectable. Values are means \pm SD for N = 3 separate determinations. The proportion of carbon through the pentose phosphate pathway (PC) = $0.5 \times \alpha/(1-\alpha)$ where α = ^{13}C label in C3-lactate/ ^{13}C label in C2-lactate [28]. The flux through the pentose phosphate pathway (J_5) = PC \times (J_1 or J_2). The rates of glucose (J_1) or fructose (J_2) utilization were measured directly from reaction progress curves (see Figs. 1 and 2). The other rates were calculated as previously described [29] using the following equation: $J_3 = 3 \times J_5$, $J_4 = 2 \times J_5$, J_5 (see above), $J_6 = (J_1 \text{ or } J_2) - J_5$, $J_7 = 2 \times (J_1 \text{ or } J_2) - J_5$, $J_8 = J_7 \times v_{2,3\text{-dPGA}}/v_{\text{C2-lactate}}$, $J_9 = J_7 - J_8$, and $J_{10} = v_{\text{C2-lactate}} + v_{\text{C3-lactate}}$. Abbreviations as follows: glucose (glc), fructose (fru), pentose phosphate pathway (PPP), fructose 6-phosphate (F6P), glyceraldehyde 3-phosphate (Gald3P), phosphofructokinase (PFK), 2,3-diphosphoglyceric acid (2,3-diPGA), phosphoglycerate kinase (PGK), lactate dehydrogenase (LDH).

revealed that fructose utilization did not begin until external glucose concentrations were exhausted. The plasma glucose concentration has previously been measured in fasted rats fed for 2 weeks on either starch or fructose-based diets [27] to be approximately 8 mmol/L and the fructose concentration was measured at 1 to 2 mmol/L. The large excess of plasma glucose over fructose indicates that the copper deficiency related effects of dietary fructose cannot be the result of fructose metabolism in erythrocytes but must have another origin. The reasons for preferential glucose utilization are not related to fructose translocation into the erythrocyte since metabolite analysis showed an increased fructose concentration in the erythrocytes of copper-deficient rats. Previous studies have shown that fructose utilization

by erythrocytes has an overall estimated K_m of 30 mmol/L with respect to extracellular fructose [32]. This agrees with the known low affinity of erythrocyte hexokinase for fructose [33]. Thus, fructose utilization is inhibited by the presence of glucose, and erythrocytes, unlike other tissues, do not phosphorylate fructose to any significant extent under normal conditions. These observations agree with data from human erythrocytes that showed sorbitol accumulation in response to external glucose, but not fructose [34, 35] and show that an accumulation of sorbitol and sorbitol-phosphate could not account for the impairment of hematological parameters observed during copper deficiency.

A surprising result from this study is the increase in lactate dehydrogenase (LDH) flux in copper deficient rats and the decrease in LDH flux when fructose was used as the metabolic substrate. The non-cuproenzyme, lactate dehydrogenase, has not previously been recognized as a locus sensitive to copper deficiency [8] and the enzyme itself has no known requirement for copper. This suggests that other cellular parameters are responsible for the observation. Several different possibilities can be envisioned to explain this observation. Increased flux through LDH could result from an alteration in the rate of lactate and pyruvate export out of the erythrocyte to alter the ratio of the substrate/product pair at LDH. The increased flux through the LDH locus could also be linked to the decreased NADH/NAD⁺ ratio observed in erythrocytes from copper deficient rats. The LDH reaction re-generates NAD⁺ from NADH in anaerobic glycolysis, re-generating the oxidizing equivalents necessary to glyceraldehyde 3-phosphate dehydrogenase. In rat erythrocytes, which lack mitochondria, a correspondence between the activity of LDH and glyceraldehyde 3-phosphate dehydrogenase is expected, although other sources of oxidizing equivalents are available (the pentose phosphate pathway, for example). Changes in NAD⁺ concentrations may be expected to alter cellular reduction potentials but this should

Table 3
Cellular metabolite concentrations in copper deficient and copper sufficient rats (Experiment 1)

Metabolite	Erythrocytes (mmol/L RBC)		Liver ($\mu\text{mol}/\text{g}$ wet weight)	
	Control	Copper deficient	Control	Copper deficient
Total NAD	0.17 \pm 0.02	0.28 \pm 0.06	-	-
Oxidized NAD (NAD ⁺)	0.15 \pm 0.03	0.27 \pm 0.09	-	-
glycogen ¹	-	-	23.5 \pm 13.3	54.0 \pm 9.0*
glucose	4.2 \pm 1.0	5.7 \pm 1.5*	18.6 \pm 1.8	17.6 \pm 3.1
fructose	0.20 \pm 0.05	0.36 \pm 0.16*	0.11 \pm 0.07	0.12 \pm 0.04
fructose 1-phosphate	ND	ND	0.08 \pm 0.06	0.13 \pm 0.08*
glyceraldehyde 3-phosphate	0.02 \pm 0.01	0.03 \pm 0.01*	0.04 \pm 0.01	0.04 \pm 0.01

ND: not detectable.

¹ Reported as mg/g wet weight

Values are means \pm SEM for N = 6 (control) or N = 10 (copper deficient).

* Significantly different from control as determined by Student's t-test analysis at the P < 0.05 level.

also affect the other NAD-linked enzyme activities as well as the cellular NADPH/NADP⁺ ratio. NAD and NADP are linked through various substrate-level cycles and changes in one should be reflected by changes in the other. No change in this ratio was observed; the reasons for the changes in LDH activity remain to be investigated.

In addition to the changes in erythrocyte metabolism, significant elevations of liver glycogen and fructose 1-phosphate concentrations were also observed (Table 3). Increased fructose 1-phosphate concentrations have previously been noted in copper-deficient Sprague-Dawley male weanlings fed high fructose diets, although the absolute levels of fructose 1-phosphate differ between our study and that of Millo and Werman [14] possibly because of the difference in the fructose content of the two diets. Increased fructose 1-phosphate levels have been postulated to arise from a copper deficiency-associated decrease in aldolase B activity [14], the enzyme responsible for hydrolyzing fructose 1-phosphate to glyceraldehyde and dihydroxyacetone phosphate. It is possible that the changes in fructose 1-phosphate may also be linked to the higher liver glycogen content observed in the copper-deficient rats of the present study. This could come about because fructose 1-phosphate has been shown to inhibit glucose 6-phosphatase (G6Pase) and activate glucokinase [36]. This would divert carbon away from glucose formation towards glycogen synthesis. In addition to these effects, it is possible that total G6Pase activity decreases during copper deficiency [9]. Thus, two different effects may operate to decrease G6Pase activity in fructose-fed copper deficient rats: an inhibition by increased fructose 1-phosphate levels and a reduction in the total enzyme activity and these may lead to a higher glycogen accumulation in liver.

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