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Ceruloplasmin
Cohn fractionation
copper
cord blood
electron spin resonance spectroscopy
L-histidine

Abnormal Copper Metabolism in Menke's Steely-Hair Syndrome

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Summary

Copper (Cu) metabolism was selectively studied in seven infants with Menke's steely-hair syndrome (SHS). A daily oral regimen of $CuSO_4$ (584 μg Cu/kg) and L-histidine (100 mg/kg) in three infants produced an increase in serum Cu concentrations ranging from 33-95% of normal, but without the formation of ceruloplasmin. Cohn serum protein fractionation after oral Cu/L-histidine loading showed a disproportionate accumulation of Cu in the albumin fraction (V). The electron spin resonance spectrum of fraction V showed a heightened signal for the SHS patients, suggesting that an increased concentration of a radical Cu species is present after oral loading. The Sephadex G-150 chromatographic profile of serum fraction V in SHS did not differ significantly from controls. These results suggest that, in SHS, Cu absorbed in the presence of L-histidine is in an abnormal complex involving albumin, which does not allow for holoceruloplasmin biosynthesis. Cu and ceruloplasmin concentrations in the cord blood specimen of an infant who went on to develop SHS were normal, a finding which may account for the transient period of seemingly normal development after birth in SHS patients. An almost 6-fold difference in mean Cu concentration was observed in SHS fibroblasts compared to controls. Fibroblast Cu concentration was elevated in one of two possible maternal heterozygotes, a finding which may permit diagnosis of the carrier state for some SHS heterozygotes.

Speculation

The basic defect in SHS may be an abnormality in an intracellular Cu binding or transport protein, which is present in multiple tissues. This suggestion is supported by data from intestine and skin fibroblasts and may, in part, explain the common failure of simple Cu replenishment to alter the clinical course of SHS.

In 1972, Danks *et al.* (3) demonstrated a deficiency in the intestinal absorption of Cu in SHS and suggested that a general dysfunction in Cu containing enzymes might account for the keratin and collagen abnormalities, cerebrovascular disease, scorbutic bone changes, and hypothermia commonly seen in the disorder. Although Cu deficiency, as reflected by the low levels of serum Cu and ceruloplasmin, has been found to be a universal feature of the disease, emerging evidence suggests that defective intestinal absorption of the metal may be only part of a more widespread disorder of Cu transport and/or binding. Danks *et al.* (3) found that erythrocyte Cu concentrations were normal in four SHS infants, suggesting a preferential retention of Cu by red blood cells. Elevated Cu concentrations have also been demonstrated in liver (5, 16), skin fibroblasts (4, 14), amniotic fluid cells (19), and small intestine (2, 3) in the disorder. Excessive urinary (11) and fecal (6) excretion of Cu has been reported in SHS, along with unresponsiveness of Cu metalloenzymes (12). Replenishment of Cu deficiency through parenteral supplementation has not yielded impressive clinical results. At best, there is a mild subjective improvement (2, 3, 6) or a partial arrest of symptomatic psychomotor deterioration (17), but a recent report fails to demonstrate even these modest gains (13). We have presented morphologic evidence (28) through Golgi impregnation of brain that a prenatal developmental anomaly exists in Purkinje cell dendrites. These studies suggest that SHS is associated with a widespread and possibly prenatal abnormality in Cu metabolism, which is likely to involve aberrant binding and/or transport of the metal.

The participation of the small intestine in the proposed Cu binding transport defect in SHS and its accessibility for functional testing allows for the study of Cu metabolism under clinical conditions of oral Cu loading. In order to investigate the nature of the absorption defect, we have previously administered a high dose of oral Cu supplement to an infant with SHS in the presence of L-histidine (22), an amino acid known to be involved in Cu transport (20). A regimen containing a 5-fold increase in the normal recommended daily allowance of Cu resulted in absorption of the metal, as judged by elevated plasma levels of Cu, but failed to produce any detectable ceruloplasmin. Ceruloplasmin appeared in the blood after intravenous Cu infusion.

In the present investigation, we extend the observations on abnormal Cu metabolism in SHS through serum protein fractionation, electron spin resonance studies of Cu containing proteins and through analysis of Cu concentrations in cord blood and skin fibroblasts.

MATERIALS AND METHODS

PATIENTS

The diagnosis of SHS in seven patients was made on the basis of the findings cited by French (10) in his review of 37 cases. These criteria included growth failure, abnormal hair (pili torti, monilethrix, and/or trichorrhexis nodosa), seizures, developmental retardation, roentgenographic metaphyseal abnormalities in the form of cupping, spurring, or periosteal new bone changes, hypothermia, serum Cu less than 25% normal, and hypoceruloplasminemia.

CU AND CERULOPLASMIN

Cu was determined by atomic absorption spectrophotometry by using either a method previously employed (7), in some determinations, or by employing a Varian Techtron Model AA-5 instru-

ment using a carbon rod atomizer (Model 63). Steps were taken to exclude Cu contamination by soaking all glassware in nitric acid, followed by thorough rinsing in doubly distilled and deionized water. Ceruloplasmin was determined in aliquots of serum by p-phenylenediamine oxidase activity and confirmed by use of o-dianisidine substrates (21). Qualitative confirmation of the presence of ceruloplasmin was achieved by an Ouchterlony immunodiffusion assay.

SERUM PROTEIN FRACTIONATION AFTER CU LOADING

Three groups of serum were separated by the method of Cohn *et al.* (1) into five fractions. The first sample was a pooled collection of serum obtained from patient 1 while he was receiving oral Cu (584 μg Cu/kg) and L-histidine (100 mg/kg). The second sample consisted of serum from this patient while he was receiving iv Cu (20 μg/kg/day for 5 days). The third serum group comprised specimens from three controls who were receiving normal dietary Cu. A 2 ml sample of Cohn fraction V containing 40–50 mg/ml protein was applied to a Sephadex G-150 column (1.5 × 100 cm), which had been preequilibrated with 0.1 M tris buffer, pH 8.0, containing 1 M NaCl. The same buffer was employed to elute the column. Fractions of 2 ml were collected and monitored for protein by measuring absorption at 280 nm. Protein was determined by the method of Lowry *et al.* (23).

ELECTRON SPIN RESONANCE SPECTROSCOPY

Cohn fractions V were prepared as indicated above from serum of patients 1 and 2. The samples were lyophilized and then subjected to electron spin resonance determination. The spectra were recorded on a Varian E-9 instrument equipped with a standard variable temperature accessory, a 100 KHz, a low frequency modulation unit, and a Systron Donner frequency counter (Model 1017/1292). All spectra were recorded at X-Band frequencies. The *g*-value was measured relative to diphenylpicrylhydrazyl ($g = 2.0036$), which was attached to the sample by a small piece of tape. A normal control sample was prepared and analyzed for comparative purposes.

CORD BLOOD CU AND CERULOPLASMIN DETERMINATIONS

A filter paper (Schleicher-Schull No. 903) impregnated with whole umbilical cord blood from patient 1 was retrieved from the files of the Massachusetts State Screening Program for newborn infants. Control specimens were obtained from 10 normal neonates whose cord blood was similarly collected during the same week as the patient's birth, and blank filter paper identical in area to the cord blood sample served as a blank. Each blood sample was eluted individually by immersing the papers in 20 ml of 0.9% saline for 24 hr at 37°C. The papers were removed by centrifugation. All measurements were made on the whole eluate. Cu and ceruloplasmin concentrations were determined on the eluates according to the procedures described previously after removal of the papers by centrifugation.

FIBROBLAST CU CONTENT

Cu concentration was determined in skin fibroblasts form five patients with SHS, two possible heterozygotes (negative family history and a single effected son), and a fetus at risk. Skin fibroblast cultures were established from biopsies by methods that have been described previously (26). The Cu concentration of the fibroblast tissue culture medium was 23.5 ng/ml. Amniotic fluid cell cultures were initiated and grown also as noted previously (25). All were harvested when confluent, and the cell pellets washed twice in normal saline and frozen to await assay. All the fibroblast lines utilized had been previously passaged between 8–30 times, except for the amniotic fluid cell lines, which had been passaged less than eight times. The cells were suspended in deionized water and homogenized by ultrasonication. Aliquots were taken for protein determinations (23), which ranged between 1–2 mg/ml for the various cell lines. The results are expressed as nanograms of Cu/mg of protein in order to account for the slight differences in total protein reflecting some variability in cell confluency.

RESULTS

ORAL CU/L-HISTIDINE LOADING

In patient 1, less than 1% of orally administered ^{64}Cu was absorbed in 24 hr. Patients 1–3 were administered a daily regimen of Cu sulfate (584 μg Cu/kg body weight) and L-histidine (100 mg/kg) for varying time periods. The results are shown in Figure 1. An increase in serum Cu to low normal levels was seen in patient 2 by day 7 of the oral regimen, whereas serum Cu in patient 1 rose to approximately 75% of the normal values at this and subsequent time points of analysis. By contrast, the serum Cu values for patient 3 remained at less than 1/3 normal for the same period. A doubling of the daily Cu sulfate (1.2 μg/kg Cu) and L-histidine (200 mg/kg) dose in patient 3 for an additional 3-wk period failed to raise serum Cu concentration beyond 29 μg/100 ml. Despite the increase in serum Cu to substantive levels in patients 1 and 2, no significant rise in serum ceruloplasmin concentration was detectable biochemically. The appearance of a faint and diffuse immunoprecipitin band for ceruloplasmin was observed in patient 2 at day 7 of oral Cu/L-histidine loading when serum Cu concentration was 97.8 μg/100 ml in comparison to the sharp immunoprecipitin bands observed in two control samples (Fig. 2). When a subcutaneous infusion of Cu sulfate (2.5 mg/infusion daily for 5 days) was added to the regimen of oral Cu and L-histidine, serum ceruloplasmin rose to 20.5 mg/100 ml, and a sharp immunoprecipitin band for ceruloplasmin was clearly observed.

SERUM PROTEIN FRACTIONATION AFTER ORAL CU/L-HISTIDINE LOADING

Sera from multiple time-point collections for patient 1 after oral Cu loading were pooled, lyophylized, and subjected to serum protein fractionation (Table 1), as indicated in *Methods.* Fraction V of Cohn contained 55% of recoverable Cu for the SHS patient

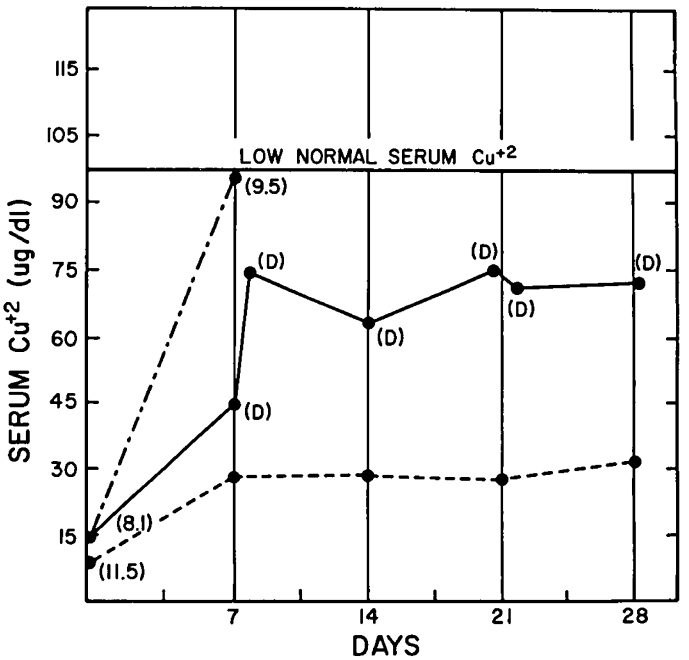

Fig. 1. Serum Cu concentrations on oral $CuSO_4$ (584 μg/kg Cu/day) and L-histidine (100 mg/kg) loading for patients 1 (●——●); 2 (————); and 3 (- - - - - - -). Numbers in parentheses denote serum ceruloplasmin concentration (mg/100 mg) and (D) refers to a diffuse precipitin band for ceruloplasmin on Ouchterlony diffusion assay.

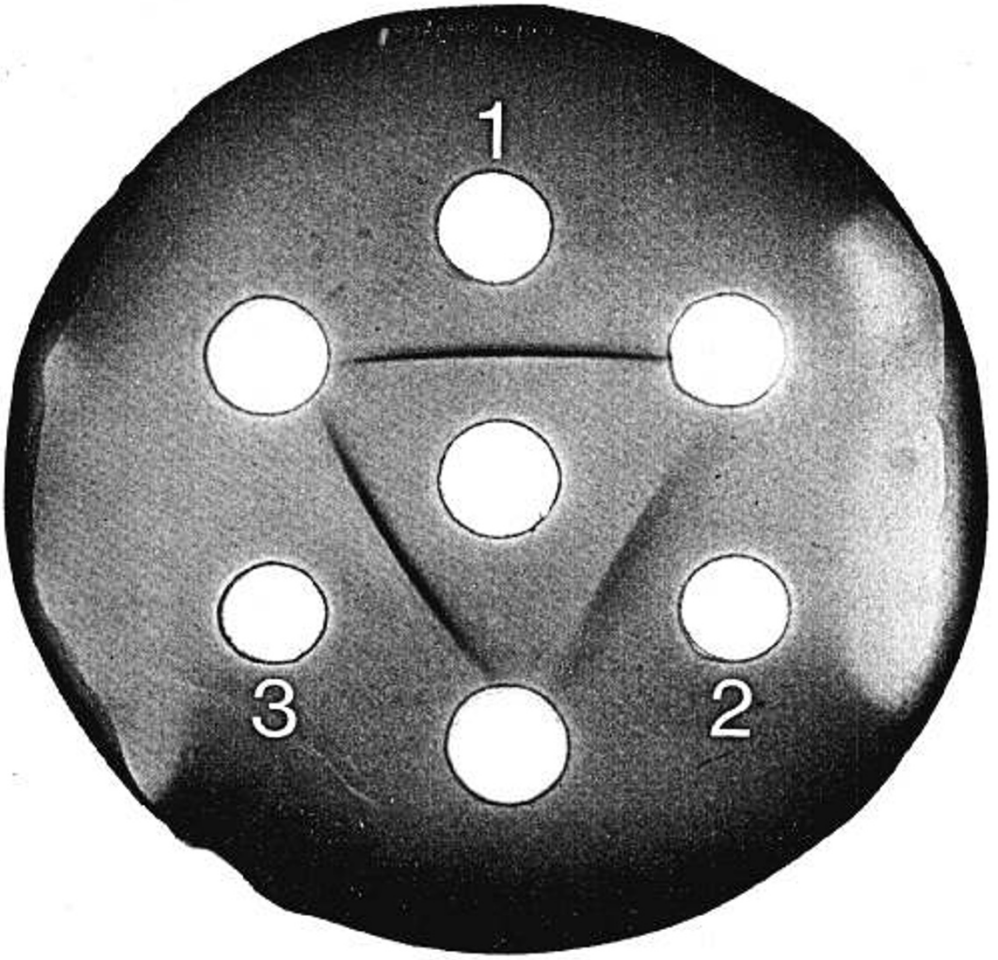

Fig. 2. Ouchterlony immunodiffusion assay for ceruloplasmin. The central well contains undiluted goat antibodies to native human ceruloplasmin. Well 1 contains serum from SHS patient 2 on the 5th day of subcutaneous $CuSO_4$ infusion (2.5 mg/infusion/day) when serum ceruloplasmin was 20.5 mg/100 ml. Well 2 contains serum from the same patient on the 7th day of oral $CuSO_4$ and L-histidine loading. Well 3 contains serum from an age-matched male control.

Table 1. *Cu distribution after Cohn fractionation of serum*

	Total recoverable Cu (μg)	% Cu in fraction				
		I	II + III	IV-1 cp	IV-4	V Alb.
Normal 1	5.17	8.7	28.6	27.9	10.1	24.8
Normal 2	4.51	14.2	24.2	29.5	10.0	22.2
Normal 3	2.41	10.6	24.3	39.3	6.5	19.0
Oral Cu	4.38	9.9	10.6	14.9	9.9	54.9
IV Cu	6.45	7.0	17.1	32.6	7.8	35.7

after oral administration, whereas controls receiving normal dietary Cu had 22 ± 2.4% Cu in this fraction. Fraction IV-1, consisting largely of α globulins including ceruloplasmin, was found to contain 15% of recoverable serum Cu in the patient sample. This was about half the amount found in comparable samples from the controls. After iv Cu infusion in SHS (20 μg/kg/day for 5 days), the amount of Cu present in the albumin fraction was decreased from 54.9 to 35.7, while the amount of Cu in the ceruloplasmin fraction (IV-1) was increased from 14.9 to 32.6%.

The Cohn fraction V obtained from patient 1 after oral administration was subjected to Sephadex G-150 column chromatography and compared to controls receiving normal dietary Cu. Aliquots of each fraction were analyzed for Cu and protein content. Cu was present in a single major and secondary smaller protein peak in the control. Fraction V from the patient showed a similar distribution in both Cu and protein.

ELECTRON SPIN RESONANCE DETERMINATION

Lyophylized fraction V was examined using electron spin resonance spectroscopy. At 110° K, a broad spectrum was revelaed, $g = 2.0041 \pm 0.0003$, without hyperfine structure. The signal could be saturated at a microwave power over 5 m watts. The magnitude of the signal from patients 1 and 2 was significantly higher than the signal observed from the control sample, suggesting that an increased concentration of a radical species was present in fraction V from both SHS patients (Fig. 3).

CU AND CERULOPLASMIN CONCENTRATIONS IN CORD BLOOD FROM AN SHS PATIENT

The concentration of Cu in cord blood extracted from filter paper specimens ranged from 26–169 μg/g protein for controls (mean value ± SD = 75.2 ± 44.9) compared with a value of 146 μg/g protein in the blood sample from patient 1 (Fig. 4). No Cu was dectable in the filter paper blank. Ceruloplasmin activity in both patient and control samples was demonstrable colorimetrically. Ceruloplasmin, as determined by its oxidase activity, was 0.403 in the patient's sample, which fell in the upper normal range.

FIBROBLAST CU CONTENT

Cu concentrations in fibroblasts derived from five patients with SHS, two mothers of affected children, one fetus at risk, and 14

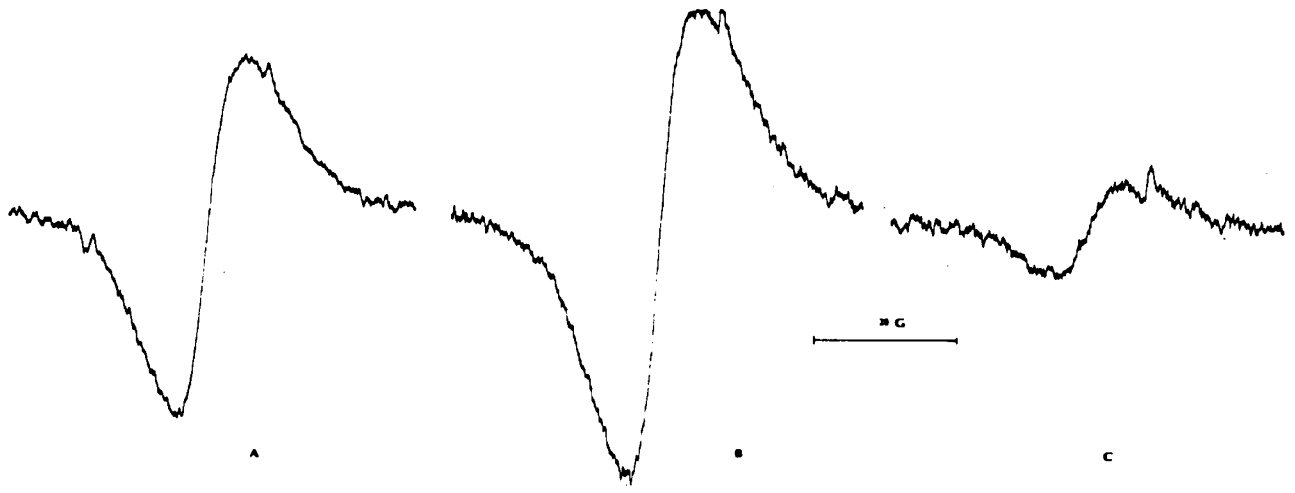

Fig. 3. Electron spin resonance spectra of Cohn fraction V at 110° K, V = 9.6 GHZ. A: spectrum from patient 1. B: spectrum from patient 2. C: spectrum from age-matched controls.

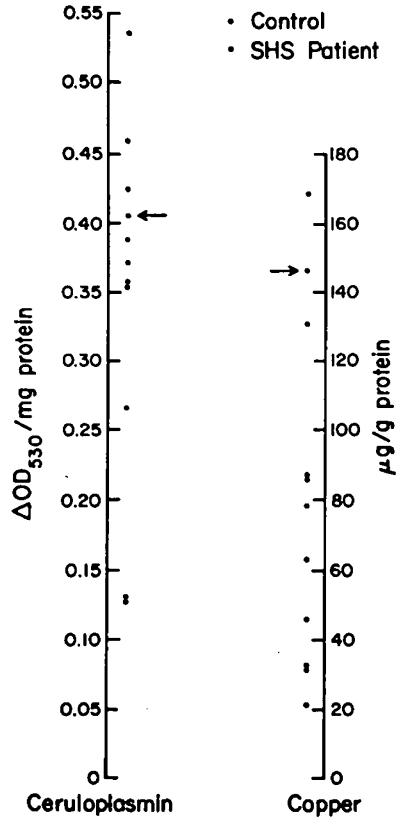

Fig. 4. Concentration of ceruloplasmin and Cu in cord blood of SHS patient (open circle) and 10 controls (closed circles).

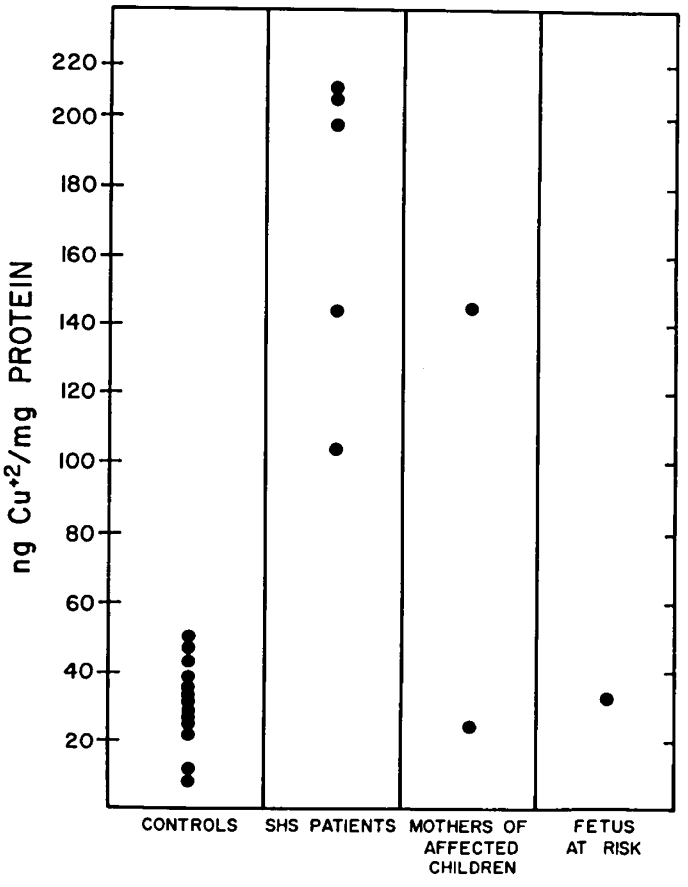

Fig. 5. Cu concentrations (ng Cu^{+2}/mg protein) in cultured skin fibroblasts from 14 controls, 5 SHS patients, 2 maternal heterozygotes, and a fetus at risk.

controls are shown in Figure 5. The results are expressed as ng Cu/mg protein in order to account for the slight differences in total protein reflecting some variability in cell confluency. Each value represented the mean of three estimations for each culture. An almost 6-fold difference in mean Cu concentration was observed in SHS fibroblasts compared to controls. An elevated level of intracellular Cu was observed in fibroblasts from one of the two possible heterozygotes. Cells from the fetus at risk, studied postabortion, had a normal intracellular Cu concentration. We have also studied a single permanent lymphoid cell line established from a child with SHS. This cell line had a normal intracellular Cu concentration when compared to a control lymphoid line, 25.5 and 20.8 ng/mg, respectively.

DISCUSSION

We have shown that, in three SHS patients, the intestinal block in Cu absorption may be partially overcome by oral loading of inorganic Cu salt in the presence of L-histidine. The resultant serum Cu level after this regimen varied from 33% of normal (patient 3) to 95% normal (patient 2), with patient 1 showing intermediate values. The dose of elemental Cu in the Cu-histidine regimen was approximately 10 times the normal daily requirement for infants (0.05–0.10 mg/kg Cu). The oral loading data in patients

2 and 3 confirm our findings reported in patient 1 (22) and indicate that the absorption defect in SHS may be incomplete in some patients. Dekaban *et al.* (5) have found approximately 25% residual absorption in three patients, and Danks *et al.* (3) cited two cases which showed increases in serum Cu after Cu sulfate loading, commensurate with our findings in case 3. Walker-Smith *et al.* (27) noted a rise in serum Cu concentration from 5 to 37 μg/100 ml after 6 months of oral Cu sulfate therapy. In the present study, L-histidine was employed as a chelating agent because of its tendency to form Cu amino acid complexes during intestinal absorption (8). In SHS, EDTA has not enhanced Cu absorption, but Cu-trisodium nitrilotriacetate has raised serum Cu to approximately 75% of normal (15, 16). Both this latter compound and L-histidine failed to result in an increase in ceruloplasmin, even though serum Cu was elevated to a level where ceruloplasmin is normally detectable. These findings suggest that the from in which Cu is absorbed from the intestinal muocsa in SHS does not permit the biosynthesis of ceruloplasmin by the liver.

Further study of the absorbed Cu after oral loading was carried out in patient 1 by Cohn fractionation of serum. Cu was disproportionately localized to fraction V and relatively decreased in fraction IV-1 (containing ceruloplasmin) compared to age-matched controls receiving normal dietary Cu. A nonspecific scattering of Cu throughout the remaining fractions in both patients and control may have represented contamination by ceruloplasmin, which was unrecovered during the fractionation procedure. There were no major differences in the G-150 gel filtration distribution patterns between control and diseased fraction V samples. Electron spin resonance spectroscopy is a highly sensitive method which has been widely used to investigate the nature of interactions of metal ions with various organic molecules. The lack of Cu hyperfine structure, ease of saturation of the signal by microwave power (less than 5 milliwatts) and $g = 2$, as shown in *Results*, indicates that the signal is due to an organic radical of unknown origin involving Cu. It is noteworthy that both SHS patients demonstrated an increased concentration of this radical species as measured by electron spin resonance spectroscopy. This finding suggests that the Cu present in fraction V is in an unusual oxidative state and that it may be involved in abnormal binding. It thus, seems likely that, in SHS, when Cu is forced across the intestine in the presence of L-histidine, an abnormal complex of the metal results, which may contain albumin along with another metalloprotein. It seems likely that the form in which Cu is absorbed after oral loading with L-histidine makes the metal unavailable to the liver for holoceruloplasmin biosynthesis. Apoceruloplasmin concentration in SHS has been shown to be normal in the presence of serum Cu deficiency (24).

Normal Cu absorption may be protein mediated. As reviewed by Evans (8), a Cu binding protein similar in properties to human and equine metallothionein has been identified in chick intestine and in rat and beef duodenum. Recent data, however, have indicated that the Cu binding protein in rat liver (named Cu-chelatin) is distinct from metallothionein (29). Such metalloproteins may play a role in Cu absorption by providing a temporary storage site for ingested Cu. In SHS, the mechanism of the basic defect in Cu absorption is not known. Duodenal mucosa from diseased infants has been shown to contain increased amounts of Cu, suggesting that an abnormal metal binding protein in mucosal cells may exist which has an increased avidity for Cu (4). Alternatively, the mechanism for Cu transport to the serosal surface, with subsequent release of the metal into the blood stream, may be altered in the disorder.

The level of ceruloplasmin has been shown to be lower in fetal blood than in maternal blood, while the level of nonceruloplasmin Cu is the same (9). This implies that ceruloplasmin does not cross the placenta from mother to child, and the presence of ceruloplasmin in cord blood is therefore a result of fetal synthesis. The cord blood sample obtained from patient 1 contained Cu and ceruloplasmin levels which were within the range of controls, suggesting that a possible defect in Cu transport observed in infants with SHS is not present in placenta. This finding probably explains why symptoms of Cu deficiency are not present at birth in SHS and could account for the transient period of seemingly normal development which has been reported in most cases. It thus, seem unlikly that cord blood Cu and ceruloplasmin determinations will suffice for early detection of SHS.

The observation of elevated intracellular Cu in cultured skin fibroblasts from patients with SHS provides a genetic marker in cell culture for this disorder. Our observations confirm those made by Goka *et al.* (14) and Horn (19). Of interest is the elevated fibroblast Cu concentration in one of two possible maternal heterozygotes. This is the first observation supporting the suggestion that the heterozygote might be detectable by this method, the result in the other possible heterozygote being consistent with either random x-inactivation or with a new mutation. Amniotic fluid cells were obtained at 16 wk from a mother at risk for having a second child with SHS and showed normal intracellular Cu concentration. These studies were performed only after elective abortion and suggest that the fetus was in fact free of SHS. Abnormal tissue distributions of Cu have been shown to occur in a male fetus suspected of SHS (18). At present, it would appear likely that the prenatal diagnosis of SHS can be made reliably. Studies are still required to demonstrate whether radiolabeled Cu incorporation and total intracellular Cu estimations in cultured amniotic fluid cells are equally reliable methods for diagnosis. Fibroblasts appear to behave similarly to the intestinal mucosal cell in SHS, which has been shown to have an abnormal affinity for Cu (4). It is not yet clear whether the basic defect in SHS is in membrane transport of Cu or related to an intracellular storage protein which has an increased affinity for the metal.

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