Ceruloplasmin is an abundant serum glycoprotein containing greater than 95% of the copper found in the plasma of vertebrate species. Although this protein is known to function as an essential ferroxidase, the role of ceruloplasmin in copper transport and metabolism remains unclear. To elucidate the role of ceruloplasmin in copper metabolism, the kinetics of copper absorption, transport, distribution, and excretion were examined utilizing $^{64}$Cu in wild-type and aceruloplasminemic mice. No differences in gastrointestinal absorption, hepatic uptake, or biliary excretion were observed in these animals. Furthermore, steady state measurements of tissue copper content utilizing $^{64}$Cu and atomic absorption spectroscopy revealed no differences in the copper content of the brain, heart, spleen, and kidney. Consistent with these findings, the activity of copper-zinc superoxide dismutase in these tissues was equivalent in wild-type and ceruloplasmin-deficient mice. Hepatic iron was elevated 3.5-fold in aceruloplasminemic mice because of the loss of ferroxidase function. Hepatic copper content was markedly increased in aceruloplasminemic mice. As no differences were observed in copper absorption or biliary copper excretion, these data suggest that in these animals, hepatocyte copper intended for ceruloplasmin incorporation is trafficked into a compartment that is less available for biliary copper excretion. Taken together, these data reveal no essential role for ceruloplasmin in copper metabolism and suggest a previously unappreciated complexity to the subcellular distribution of this metal within the hepatocyte secretory pathway.

Ceruloplasmin is a 132-kDa serum $\alpha$-2 glycoprotein containing greater than 95% of the total copper found in the plasma of vertebrate species (1). This protein is synthesized and secreted by hepatocytes as a holoprotein with six atoms of copper tightly incorporated during its biosynthesis (2). In addition to the plasma form of ceruloplasmin, an alternative splice product yields a glycosyl phosphatidylinositol membrane-anchored form that is present in specific tissues (3–6). Although copper has no effect on the rate of apoceruloplasmin synthesis or secretion (7), the plasma half-life of apoceruloplasmin is shorter than holoceruloplasmin and makes the serum concentration of this protein and the loss of oxidase activity a sensitive marker for copper deficiency (8, 9). Given the abundance of ceruloplasmin in serum, the considerable copper content of this protein, and the detection of membrane receptors, many investigators have proposed a role for this protein in copper transport and metabolism (10–13).

Ceruloplasmin is a member of the multicopper oxidase family of enzymes and functions in humans as an essential ferroxidase (14). Aceruloplasminemia is an autosomal recessive disorder of iron metabolism manifesting in adulthood as insulin-dependent diabetes mellitus, retinal degeneration, and neurodegeneration attributable to mutations in the ceruloplasmin gene (15, 16). Affected individuals have a complete absence of circulating serum ceruloplasmin and present in the fourth to fifth decade of life with iron accumulation in the liver, spleen, pancreas, and basal ganglia resulting in tissue injury and necrosis (17, 18). Targeted disruption of the murine ceruloplasmin gene results in a murine model of this disorder, and ferrokinetic studies in this animal reveal a critical role for ceruloplasmin in determining the rate of iron efflux from the reticuloendothelial system and other cell types with mobilizable iron stores (19). This current study utilizes this unique murine model to directly examine the role of ceruloplasmin in copper transport and metabolism.

EXPERIMENTAL PROCEDURES

Materials—$^{64}$CuCl (specific activity = 30 mCi/mg) was obtained from the Mallinckrodt Institute of Radiology/Division of Radiologic Sciences, Washington University School of Medicine, St. Louis, MO. Unless otherwise indicated, all other chemicals were from Sigma-Aldrich.

$^{64}$Cu Plasma Kinetics and Tissue Distribution—To determine $^{64}$Cu plasma kinetics, aceruloplasminemic mice (Cp−/−) and wild-type littermates (Cp+/+) were intravenously injected via tail vein with 5 μCi of $^{64}$Cu (equivalent to 180 ng of copper). 20 μl of whole blood was obtained by retroorbital phlebotomy at 5, 10, 20, 30, 60, 120, 180, 240, 300, 360, and 400 min following $^{64}$Cu injection. $^{64}$Cu was determined in 10 μl of serum in a Packard Cobra II γ counter. To determine the $^{64}$Cu tissue distribution 24 h following the radiocopper injection, mice were exsanguinated and perfused with phosphate-buffered saline (PBS) via cardiac puncture to remove blood from organs, and radioactivity was measured in weighed tissue samples. Tissues were well perfused prior to γ counting to eliminate any possible blood contamination. 20 μl of serum obtained from each mouse at the time of exsanguination was measured for $^{64}$Cu.

Atomic Absorption Spectroscopy—Mice were exsanguinated and perfused with PBS via cardiac puncture to remove blood from organs. Organs (liver, brain, heart, kidney, and spleen) were wet-weighed, nitric acid-digested, and analyzed by flame atomic absorption spectroscopy for copper and iron as described elsewhere (20).
Copper Transport and Metabolism in Aceruloplasminemic Mice

64Cu Gavage Absorption Studies and Organ Distribution—To determine 64Cu plasma kinetics following oral radiocopper delivery, aceruloplasminemic mice (Cp−/−) and wild-type littermates (Cp+/+) were fasted for 12 h and then gavage-fed 1 μCi of 64Cu (equivalent to 32 ng of copper). 20 μl of whole blood was obtained by retroorbital phlebotomy at 30, 60, 150 min and 24 h following gavage. 64Cu was determined in 5 μl of serum in a Packard Cobra II γ counter. To determine the 64Cu tissue distribution, including gallbladder, following the radiocopper gavage injection, mice were fasted prior to exsanguination and perfused with phosphate-buffered saline via cardiac puncture to remove blood from organs, and radioactivity was measured in weighed tissue samples. Organs from the mice were harvested at 2½ and 24 h following radiocopper gavage based on previous experiments suggesting that these are critical time points in biliary excretion (21). Gallbladder radiocopper content was used as a measurement and accurate reflection of biliary 64Cu copper excretion. Gallbladders were identified, the common bile duct was ligated, and the organ was removed in total. All organ results represent 64Cu cpm/g dry tissue weight. Feces were collected from below the cage inserts, and 64Cu content was measured.

Cell Culturing and Bioinformatic Labeling—Primary hepatocytes were isolated from wild-type and aceruloplasminemic mice (22, 23) and incubated overnight at 37 °C in humidified air with 5% CO2 on disposable 35-mm plastic tissue culture dishes containing Williams E medium (Life Technologies, Inc.) supplemented with fetal bovine serum, dexamethasone, insulin, nicotinamide, and penicillin/streptomycin. Prior to radiocopper incubation, confluent cells were washed with sterile PBS, and the medium was replaced with serum-free Opti-mem medium (Life Technologies, Inc.) for 1 h. Cells were subsequently incubated with 100 μCi of 64Cu (equivalent to 3 μg of copper) for 3 h. Following labeling, the medium was removed and concentrated using Amicon Centricon filters (Millipore, Bedford, MA). The cell monolayer was washed three times with PBS, and the cells were lysed by freeze-thawing in a HEPES/Nonidet P-40 protein lysis buffer with protease inhibitors. 50 μg of protein from both lysate and medium, as determined by the Bradford-Lowry method (modified from Beauchamp and Fridovich (25)), Cu,Zn-SOD activity was assayed qualitatively utilizing the nitro blue tetrazolium (NBT) in-gel assay. Protein was isolated in a HEPES/Nonidet P-40 buffer containing aprotonin, phenylmethylsulfonyl fluoride (Roche Molecular Biochemicals), leupeptin, and pepstatin. 50 μg of protein isolated from Cp−/− and Cp+/+ mouse, as determined by the Bradford-Lowry method (24)(Bio-Rad), was run on a 10% native gel. The gel was incubated in a 2.45 mM NBT buffer for 30 min, rinsed in deionized water, and submerged in a TEMED/riboflavin/potassium phosphate buffer for 20 min. Oxidase active bands were visualized following placement of the gel on a light source for 15 min.

Animal Husbandry and Data Analysis—Mice were housed on a 12:12 light/dark cycle with ad libitum access to rodent chow (Purina Pico 5052, 0.02% w/w iron, 0.002% w/w copper). Cp−/− mice were maintained on an outbred genetic background (Black Swiss Webster) with wild-type littermates serving as controls. Newborn pups were genotyped at 3 weeks of age as described (19). For all animal experiments, a minimum of six animals in each group were analyzed. All mice were between 10 and 12 weeks of age for all experiments. For the radiocopper gavage and injection experiments, mice were housed on cage inserts to prevent coprophagia. Statistical analysis was by Student’s unpaired t test with significance defined as p < 0.01. All mouse protocols were in accordance with the National Institutes of Health guidelines and approved by the Animal Care and Use Committee of Washington University School of Medicine.

RESULTS

Copper kinetics were examined in serum from wild-type (Cp+/+) and aceruloplasminemic (Cp−/−) mice for 6.6 h following intravenous administration of 64Cu. As can be seen in Fig. 1, no differences were observed between mice during the first 4 h following 64Cu injection. However, by 300 min (5 h) following injection, 64Cu remained detectable in the serum of the wild-type mice and was entirely absent from the serum of aceruloplasminemic mice (p < 0.001). Total blood volume removed at this point was equivalent to ½ the total circulating blood volume of each mouse. Serum was again assayed for 64Cu 24 h following injection when the organs were harvested. Serum 64Cu was never detected in the aceruloplasminemic mice following the 4-h time point. The persistent 64Cu measured in the serum of wild-type mice is incorporated in ceruloplasmin, as confirmed by Western blot analysis (data not shown).

To examine the role of ceruloplasmin in tissue copper distribution, 64Cu cpm/g dry tissue weight in liver, spleen, kidney, pancreas, and brain 24 h following radiocopper intravenous injection was determined. As can be seen in Fig. 2, there was no difference detected between the aceruloplasminemic and wild-type mice. The total 64Cu cpm/g of organ weight was equivalent between all the organs tested. Further examination of the brain revealed no differences in the 64Cu cpm/g dry tissue weight between cortex, basal ganglia, cerebellum, and brainstem (data not shown). Only an aliquot of serum, counted 24 h following radiocopper injection, revealed a difference in detectable 64Cu counts between wild-type and aceruloplasminemic mice, consistent with its incorporation into ceruloplasmin (p < 0.001).

To determine the total organ copper content, tissue samples were analyzed by atomic absorption spectroscopy (Table I). Brain, heart, kidney, and spleen copper contents were not significantly different between wild-type and aceruloplasminemic mice. The total copper content in these organs was not significantly different between wild-type and aceruloplasminemic mice. The total copper content in these organs was not significantly different between wild-type and aceruloplasminemic mice.
Copper Transport and Metabolism in Aceruloplasminemic Mice

**TABLE I**

Organ copper and iron content in wild-type and aceruloplasminemic mice

<table>
<thead>
<tr>
<th>Copper</th>
<th>Liver</th>
<th>Brain</th>
<th>Heart</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.63 ± 0.2</td>
<td>4.7 ± 0.1</td>
<td>4.95 ± 0.1</td>
<td>3.75 ± 0.1</td>
<td>0.78 ± 0.1</td>
</tr>
<tr>
<td>Cp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>4.28 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>4.03 ± 0.1</td>
<td>0.84 ± 0.1</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Iron</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>85.3 ± 27.4</td>
<td>13.1 ± 0.7</td>
<td>56.5 ± 0.5</td>
<td>35.1 ± 6.8</td>
<td>477 ± 179</td>
</tr>
<tr>
<td>Cp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>304 ± 73.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5 ± 1.2</td>
<td>55.8 ± 6.3</td>
<td>32.9 ± 2.0</td>
<td>538 ± 65</td>
</tr>
</tbody>
</table>

<sup>a</sup> *p < 0.01.

<table>
<thead>
<tr>
<th>2&lt;sup&gt;1/2&lt;/sup&gt; h</th>
<th>gallbladder</th>
<th>liver</th>
<th>spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>42,392 ± 9,000</td>
<td>102,917 ± 11,000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16,266 ± 6,000</td>
</tr>
<tr>
<td>Cp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>34,608 ± 6,003</td>
<td>67,767 ± 19,300</td>
<td>9,506 ± 4,100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>24 h</th>
<th>cpm/g</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>26,203 ± 6,008</td>
<td>20,303 ± 4,000</td>
<td>6,021 ± 775</td>
</tr>
<tr>
<td>Cp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>28,717 ± 5,000</td>
<td>26,444 ± 2,600</td>
<td>7,020 ± 362</td>
</tr>
</tbody>
</table>

<sup>a</sup> *p < 0.01.

To confirm this, the activity of other copper-protein was unaffected by a lack of serum ceruloplasmin, the activity of Cu,Zn-SOD was examined employing a modified O<sub>2</sub> scavenging activity assay (21). Cu,Zn-SOD activity was measured utilizing the NBT in-gel assay. NBT yields a dark blue diimazo pigment in the presence of a reducing agent. Hence, the absence of Cu,Zn-SOD is active, a lack of pigment is identified. No qualitative differences were detected in Cu,Zn-SOD activity between protein isolated from liver, kidney, heart, spleen, and brain from wild-type (Cp<sup>+</sup>) and aceruloplasminemic (Cp<sup>-</sup>) mice (Fig. 4). Hence, in the absence of ceruloplasmin, cells were capable of incorporating copper, trafficking copper, and synthesizing an enzymatically active holoCu,Zn-SOD.

**DISCUSSION**

The results of this study indicate that copper absorption, transport, distribution, and excretion are unaffected by the absence of ceruloplasmin. Northern blot analysis of tissue from the ceruloplasminemic mouse reveals an absence ceruloplasmin mRNA, and thus this mouse is devoid of all forms of ceruloplasmin, regulating and glycosyl phosphatidylinositol-linked (19). Data from the current experiments indicate no essential role for ceruloplasmin in copper metabolism and are consistent with earlier nutritional studies that suggested that the exchange of copper measured from this protein was too slow to be consistent with a role for ceruloplasmin in copper transport (26). If ceruloplasmin has a role in copper transport, it

statistically different between aceruloplasminemic and wild-type mice. Liver copper content revealed a 2-fold increase in the Cp<sup>-</sup> mice as compared with Cp<sup>+</sup> littermates. To evaluate the loss of ferroxidase activity that defines the defect in aceruloplasminemic mice, total iron content was similarly measured by atomic absorption spectroscopy in all the tissues isolated. Liver iron results (μg/g) confirmed the recognized iron overload phenotype associated with the Cp<sup>-</sup> mice. Iron concentration in other organs was not altered by the loss of ceruloplasmin at this age.

To determine whether the increased liver copper concentration in the Cp<sup>-</sup> mice was the result of either increased copper absorption or decreased biliary excretion, 64<sup>Cu</sup> kinetics were studied in mice following oral radiocopper administration (Table II). Serum was sampled 30, 60, and 150 min and 24 h following gavage. The 30 and 60 min values (64<sup>Cu</sup> cpm/μl of serum) confirm that equivalent amounts of copper were absorbed across the gastrointestinal tract (data not shown). Total radiocopper content of liver, spleen, kidney, and gallbladder was examined 2<sup>1/2</sup> and 24 h following gavage. Gallbladder radiocopper accumulation was measured to reflect biliary excretion and was measured by excising the entire gallbladder and determining the 64<sup>Cu</sup> cpm/g of organ. At 2<sup>1/2</sup> h following 64<sup>Cu</sup> gavage, no difference in cpm/g of tissue was identified between Cp<sup>+</sup> and Cp<sup>-</sup> gallbladder, spleen, and kidney (data not shown). Cp<sup>-</sup> hepatic 64<sup>Cu</sup> cpm/g of tissue was 50% that of Cp<sup>+</sup><sup>a</sup> and could be accounted for by the presence of newly synthesized copper-ceruloplasmin (data not shown) (*p < 0.005). There were no differences in the radiocopper detected in organs at 24 h following gavage.

To address the concern that ceruloplasmin, despite not being essential for copper absorption, uptake, and distribution (Figs. 1 and 2), was essential for intrahepatic cellular copper trafficking, primary hepatocytes from wild-type and aceruloplasminemic mice were isolated, and copper metabolism was examined using biosynthetic 64<sup>Cu</sup> labeling. Copper protein biosynthesis was examined in these primary hepatocytes following a 1-h starvation in serum-free medium and incubation for 3 h in 100 μCi of 64<sup>Cu</sup>/ml medium. A lower radioactive band corresponding to Cu,Zn-SOD is identified in both Cp<sup>+</sup> and Cp<sup>-</sup> lysate and media lanes (Fig. 3) run on a 10% native gel. This band is qualitatively unchanged between wild-type and aceruloplasminemic primary hepatocyte samples, suggesting that 1) ceruloplasmin plays no role in providing copper for Cu,Zn-SOD production, 2) other copper-dependent proteins are unaffected by a lack of ceruloplasmin, and 3) copper destined for ceruloplasmin is not a cellular toxin in that nascent protein synthesis continues to take place. The upper band, corresponding to ceruloplasmin, is present in Cp<sup>+</sup> lysate and media and absent from Cp<sup>-</sup> samples.
Oxidase-active Cu,Zn-SOD bands are visualized in equivalent intensity. Cu,Zn-SOD (labeling and SOD activity gels revealed normal 64Cu incorporated in either ceruloplasmin (Cu,Zn-SOD) or Cu,Zn-SOD (Cu,SOD) is detected after a 12-h exposure. 64Cu-SOD bands are detected in all lanes. 64Cu-Cp (highlighted by *) was observed only in the Cp-/- lanes.

**FIG. 3. Biosynthetic 64Cu labeling of isolated primary hepatocytes.** Primary hepatocytes isolated from Cp +/- and Cp +/- mice were radiolabeled with 100 µCi/ml for 3 h, and lysate and media samples were run on a 10% native gel under nondenaturing conditions. Radiographic measurement of 64Cu incorporated in either ceruloplasmin (64Cu-Cp) or Cu,Zn-SOD (64Cu-SOD) is detected after a 12-h exposure. 64Cu-SOD bands are detected in all lanes. 64Cu-Cp (highlighted by *) was observed only in the Cp-/- lanes.

**FIG. 4. Cu,Zn-SOD activity assay.** Using an in-gel NBT assay, oxidase-active Cu,Zn-SOD bands are visualized in equivalent intensity in protein isolated from Cp +/- and Cp +/- organs and run on a 10% native gel. Control-purified human Cu,Zn-SOD (hSOD) can be seen in both panels. Oxidase activity correlates with the absence of reduced dark blue diformazan background.

must be biologically redundant. The biochemical nature of copper transport remains unknown.

Furthermore, if ceruloplasmin were truly essential for copper transport, altered copper prooprotein metallaition would be anticipated in the absence of ceruloplasmin. Cu,Zn-SOD is a ubiquitously expressed and highly conserved copper prooprotein responsible for the disproportionation of superoxide anions to hydrogen peroxide and oxygen (27). Primary hepatocyte 64Cu labeling and SOD activity gels revealed normal 64Cu incorporation into Cu,Zn-SOD in both wild-type and aceruloplasminemic mice (Figs. 3 and 4) and confirm that a lack of ceruloplasmin had no effect Cu,Zn-SOD synthesis and activity. This contrasts with the seminal work of Marceau and Aspin (28), who suggested the ceruloplasmin-derived copper preferentially goes to Cu,Zn-SOD.

A biphasic response to radiolabeled copper has been observed previously with an initial peak following injection that relates to redistribution of the radiocopper followed by hepatic uptake and a secondary rise in plasma 64Cu coincident with the synthesis and secretion of 64Cu/ceruloplasmin (29). This pattern was observed in our wild-type Cp +/- mice, whereas only the initial peak was observed in the aceruloplasminemic Cp-/- mice. Organ distribution was as predicted with liver and kidney expressing the greatest cpm/g of tissue weight (21). Spleen, brain, and pancreas were examined further for copper organ distribution because of their presumed involvement in iron overload in aceruloplasminemia. No differences in organ distribution were detected between wild-type and aceruloplasminemic mice as seen in Fig. 2.

Recognizing that trace radiocopper would represent only a single pool of molecules and might not accurately reflect total copper content, atomic absorption spectroscopy was performed to measure total organ copper content. Total organ iron content was also measured (Table I). Total iron content allowed for confirmation of the previously recognized iron overload phenotype in the aceruloplasminemic mice. Total liver iron was 3.5-fold higher in the aceruloplasminemic mice as compared with wild-type littermate controls. No difference in total copper content was measured in brain, heart, kidney, and spleen, all organs rich in copper proproteins.

Interestingly, liver copper content was markedly higher in the Cp-/- mice as compared with Cp +/-, and similar findings have been revealed in a human patient with aceruloplasminemia (18). This result could either represent increased copper uptake or decreased copper efflux. Recent studies reveal that CTR1, the plasma membrane copper transporter, is essential for mammalian copper uptake (30). Unlike yeast genes encoding for the high affinity copper transport proteins, CTR1 and CTR3, which are tightly regulated by copper content (31-34), recent characterization of the mouse Ctr1 gene reveals that mammalian Ctr1 mRNA expression is not regulated by copper concentration (35). Our hepatic uptake experiments (Fig. 1) and the 64Cu gavage experiments reveal no abnormalities in copper absorption in the aceruloplasminemic mouse (Table II). Thus the data suggest that a defect in hepatic copper excretion exists in the absence of ceruloplasmin. This would explain the increased hepatic copper content in the aceruloplasminemic mouse.

Biliary excretion represents the sole physiologic mechanism for copper excretion and is nonsaturable: the more copper injected, the greater the copper excreted into the bile (36). Interestingly, our data reveal, by measuring trace radiocopper in the whole gallbladder as a reflection of total biliary copper, that there is no difference in biliary copper excretion between wild-type and aceruloplasminemic mice (Table II). The increased hepatic copper content in the aceruloplasminemic mouse suggests that copper intended for ceruloplasmin incorporation enters a predetermined pool that, in the face of absent ceruloplasmin, is trafficked less efficiently than copper committed for biliary excretion.

We propose that copper intended for ceruloplasmin incorporation enters a unique, previously unidentified compartment and in the absence of ceruloplasmin results in the increased hepatic copper content seen in the aceruloplasminemic human and mouse. The hepatic copper content in the aceruloplasminemic human (17, 18) and mouse remains far less than observed in patients with Wilson disease and appears to result in no toxic hepatic copper accumulation or hepatic cellular necrosis (15, 17-19). In further contrast to Wilson disease, there is no extrahepatic copper accumulation in the brain of the aceruloplasminemic human (18) or mouse (Table I), consistent with...
the normal hepatocyte architecture observed histologically and presumed lack of hepatocyte copper leakage.

The aceruloplasminemic human and mouse have no stigmata of altered copper metabolism. In fact, even though mice lack ferroxidase activity and accumulate excess hepatic iron, anemia is not observed (19). Matings of Cp−/− mice reveal no abnormalities in fecundity, litter size, or pup development, indicating that placental copper transport is unaffected by a lack of ceruloplasmin (19). The data presented in this paper clearly reveal that ceruloplasmin is not essential for copper absorption or copper delivery under conditions of copper sufficiency. Ceruloplasmin appears to be a powerful marker of total body copper stores with the primary function of a ferroxidase critical for normal iron metabolism and not a copper transport protein.

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REFERENCES