was impractical for the mink ranchers to divide their mink into separate groups for control measures, whole ranches were placed on the ammonium chloride feeding program. The experimental period was from April 10 to June 1, 1953. Mink ranches feeding ammonium chloride at the 1 g level had small losses of mink with urinary calculi (less than .2%). Over half of those ranches which fed the ½ g level of the chemical had some significant losses of mink with urinary calculi (.5-3.0%).

Discussion. The addition of ammonium chloride to the diet of the mink at a level of 1 g per mink per day is a practical and effective means of preventing the formation of urinary calculi in mink. The feeding of ammonium chloride at a level of ½ g per mink per day has been found to be ineffective in preventing urinary calculi formation on many mink ranches. It should be emphasized that the ammonium chloride regimen for the control of urinary calculi disease in mink is only a prophylactic measure. The urinary pH produced by the recommended level of ammonium chloride feeding is not low enough for rapid dissolution of the mink calculi. Although small urinary calculi may be dissolved, the animal may die before the larger calculi have been dissolved by the slightly acid urine. Partially dissolved calculi have been observed in mink from mink ranches starting the ammonium chloride feeding program late in May, *i.e.*, towards the end of the critical period of urinary calculi formation in mink.

The effectiveness of the ammonium chloride feeding program in preventing calculi formation in mink emphasizes the importance of urinary pH in urinary calculi formation. Urinary pH may be altered by dietary as well as by chemical means. It may be possible to control urinary calculi disease in mink by dietary management. The low incidence of urinary calculi on ranches feeding high levels of fish would indicate that control of urinary calculi in mink by dietary management may be feasible.

Summary. The formation of magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$) urinary calculi in mink can be prevented by the addition to the mink's diet of 1 g of ammonium chloride (NH_4Cl) per mink per day. A level of ½ g of NH_4Cl per mink per day has been found to be ineffective in preventing urinary calculi formation in mink.

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Localization of Cu^{64} in Serum Fractions Following Oral Administration: An Alteration in Wilson's Disease. (20780)

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Previous work(1,2) has shown that the circulating serum iron is bound specifically to a β_1 globulin and that under normal conditions no binding occurs with any of the other plasma components. Experiments *in vitro,* however, have shown that this metal combining globulin will also form complexes with copper and zinc(2,3), and it was initially suggested that copper may be transported in the circulation attached to the β_1 metal combining globulin. More recently Holmberg and Laurell(4) have shown that copper in human serum is largely bound to a protein with a mobility of an α_2 globulin. This protein has been named ceruloplasmin and has been shown to exhibit oxidase activity towards a variety of substrates, the

best of which is paraphenylenediamine(5,6). A small percentage of the serum copper, however, does not exhibit oxidase activity, and it has been suggested that this copper represents transport copper(7). The precise serum fraction to which the non ceruloplasmin copper is bound and the form in which it is transported remains unknown.

In this report electrophoretic separation of the serum of man and the rat following the oral administration of radioactive Cu^{64} has enabled a more precise analysis of the roles of ceruloplasmin and non ceruloplasmin copper to be undertaken. Patients with Wilson's disease have also been studied since this disease is characterized by a deficiency of ceruloplasmin(8,9) associated with an increased urinary excretion of copper(10). Patients with cirrhosis of the liver exhibiting an increased ceruloplasmin level(11) were also studied.

Methods and materials. Radioactive copper was obtained from the Oak Ridge National Laboratory in the form of copper wire. This was converted to copper nitrate, and was administered orally(12). The rats received 2 mc containing 25.6 mg copper and in man a dose of 1 mc containing 12.8 mg copper was used. Samples of blood were removed at timed intervals and radioactivity measurements carried out using a Geiger Müller counter. Electrophoretic separation of 1-4 ml samples of serum was carried out in a starch supporting medium by a slight modification of the method described by Kunkel and Slater(13). To eliminate air spaces between the starch block and the material covering the block, wax paper was applied to the starch while still wet following pouring of the starch buffer mixture into a mold. The starch block was then dried by means of blotting paper at the ends and sides. The close contact of the wax paper to the starch block prevented condensation of liquid in the form of small bubbles beneath the wax paper during the electrophoresis experiment. The sample was applied through a flap in the wax paper. After the separation, the starch block was cut in segments and the liquid removed from each segment by displacement filtration(13). Aliquots were taken for measurements of radioactivity and also for the construction of a protein curve using

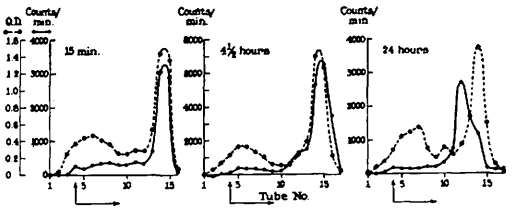

FIG. 1. Electrophoretic separation of rat serum following the administration of Cu^{64} by mouth. In this and succeeding 2 figures the solid line represents radioactivity and the dashed line represents the protein curve.

the modified Folin Tyrosine method(14).

Results. Observations on rats. Cu^{64} was added *in vitro* to rat serum which was then subjected to zone electrophoresis in a starch supporting medium. When 2.0 μg copper was added to the serum, it was found that the radioactivity was confined to the albumin component. The radioactive copper was also given orally to rats who were bled at timed intervals, and the serum obtained subjected to zone electrophoresis. Fifteen minutes after its administration, the radioactive copper was located primarily in the albumin fraction. At 4½ hours the majority of the radioactivity was still present in the albumin but a hump had occurred in the α_1 globulin region accounting for about 15% of the total activity in the serum. At 24 hours the albumin peak had decreased strikingly and over 70% of the total activity was now found in the α_1 globulin region. These results are graphically illustrated in Fig. 1. The close proximity of the globulin peak to the albumin peak made complete separation from the albumin peak difficult. However, the peak of maximum radioactivity at 24 hours was well separated from the albumin peak. The slight hump on the descending curve of the α_1 globulin copper peak represents, at least in part, copper remaining in the albumin fraction. Direct measurements of oxidase activity in rat serum employing paraphenylenediamine as the substrate localized all the ceruloplasmin activity to the α_1 globulin region. No oxidase activity was demonstrated in the albumin peak.

Observations in man. Experiments were carried out in control subjects, patients with cirrhosis of the liver and patients with Wilson's disease. Cu^{64} was added *in vitro* to normal serum, serum from patients with cir-

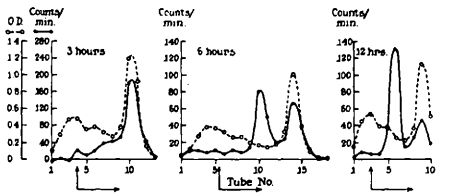

FIG. 2. Distribution of radioactive copper in the electrophoretically separated fractions of the serum of control subjects following the administration of Cu^{64} by mouth.

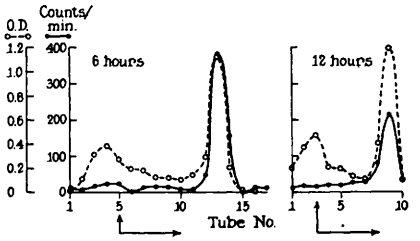

FIG. 3. Distribution of radioactive copper in the electrophoretically separated fractions of the serum from a patient with Wilson's disease following oral administration of Cu^{64}.

rhosis of the liver and serum from 2 patients suffering from Wilson's disease. Starch zone electrophoresis revealed that in all the groups studied when 0.25 μg copper was added to the serum the added copper became bound to the albumin fraction since all the radioactivity was confined to this component. Small amounts of radioactive copper appeared in other fractions when 2.5 μg copper was added to 1 cc of serum. In the *in vivo* experiments the low radioactivity found in the serum made it necessary to separate at least 2 ml serum.

Blood samples drawn 15 minutes after the oral administration of 1 mc of Cu^{64} showed that the radioactivity was again confined to the albumin peak. At 6 hours, in control subjects, a significant proportion of the radioactivity had been transferred to the α_2 globulin region (Fig. 2). In patients with Wilson's disease, however, the radioactivity was still confined to the albumin peak, and no radioactivity was present in the α_2 globulin fractions (Fig. 3). In control subjects blood drawn at 12 hours revealed that over 70% of the radioactivity in the serum was now located in the α_2 globulin fraction. In patients with Wilson's disease, however, the majority of the radioactivity was still confined to the albumin and the α_2 globulin fraction of the serum contained little or no radioactive material. The short half life of copper prevented long term studies being carried out, but serum drawn at 24 hours, in patients with Wilson's disease, still showed a considerable amount of radioactive copper associated with the albumin fraction with little or none in the ceruloplasmin. In normal subjects at 24 hours the major peak was now in the ceruloplasmin and only a very small amount of radioactive copper was still associated with the albumin component.

Serum from normal subjects who had not received radioactive copper, was also subjected to zone electrophoresis and confirmed that the majority of the serum copper was located in the α_2 globulin fraction (Fig. 4). A small peak present in the albumin fraction was due in part to contamination with trace amounts of copper since the height of this peak was dependent in large part on the care taken to eliminate contaminating copper from the starch and buffer materials(15). Moreover, over 90% of the total copper in the serum could be accounted for by the copper in the α_2 globulin peak. All the oxidase activity of the serum was confined to the α_2 globulin peak which therefore represents ceruloplasmin(15). The ceruloplasmin copper peak in rat serum migrates closer to the albumin than in the human and the oxidase activity is also located in this fraction.

An additional alteration noted in the patients with Wilson's disease following the oral administration of Cu^{64} was an increased urin-

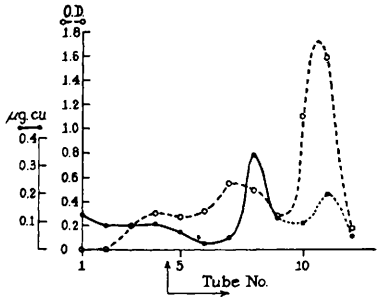

FIG. 4. Curves showing the distribution of chemically determined copper in normal serum. Ceruloplasmin, measured enzymatically, is localized in Tube 8.

ary excretion of Cu^{64} compared with normal subjects. This was also observed after intravenous administration. Fecal excretion of Cu^{64} was however diminished compared with normal subjects when the copper was given by the latter route.

Discussion. It has been shown by Klotz (16) that serum albumin is capable of binding copper *in vitro*. The results of this study show that under normal conditions copper added to serum is not bound to the metal combining globulin but is bound to the albumin fraction of serum. The possibility that some other protein migrating with the albumin fraction binds the copper appears remote. Following the oral administration of copper to rats, normal subjects and patients with Wilson's disease the copper initially is also bound to the serum albumin fraction. In normal subjects and patients with cirrhosis of the liver the copper appears relatively quickly in the ceruloplasmin and disappears from the albumin. In the rat the shift of the radioactivity to the α_1 globulin region was slower than that found in control subjects and is probably related to the difference in the amount of copper administered to the two groups. In patients with Wilson's disease, however, there is a specific deficiency of ceruloplasmin and the copper remains confined to the albumin component and no shift of the radioactive copper to other plasma components occurs. Earl and Selverstone (17) have used 50% precipitation with ammonium sulfate to fractionate the serum proteins following the oral administration of Cu^{64}. In normal subjects and patients with Wilson's disease the radioactivity first appeared largely in the supernatant. Later the radioactivity in normal subjects was found in the precipitate, whereas in patients with Wilson's disease the radioactivity still largely remained confined to the supernatant. The present work suggests that the serum component which is not precipitated by 50% ammonium sulfate and to which radioactive copper is bound in Wilson's disease is serum albumin, and no significant binding with any other plasma component occurs.

Since copper is first associated with the albumin fraction following absorption from the intestine it is probable that this fraction is concerned with the transport of copper to ceruloplasmin and also to various tissue copper proteins. Although the rate at which copper disappeared from the albumin component and appeared in the ceruloplasmin in both rat and man was partially dependent upon the dose administered, rapid uptake of copper in ceruloplasmin was apparent.

Summary. 1. The administration of Cu^{64} by mouth to the rat resulted in an immediate uptake of the copper by the albumin fraction of serum as indicated by experiments employing zone electrophoresis for the separation of the serum. Later the copper shifted to the major copper protein in rat serum, which has the approximate mobility of an α_1 globulin. 2. The administration of Cu^{64} by mouth to normal subjects and patients with cirrhosis of the liver also resulted in an immediate uptake of copper by the serum albumin fraction. The radioactivity shifted within a few hours to the α_2 globulin, ceruloplasmin, the major copper protein in human serum. 3. When radioactive copper was administered to patients with Wilson's disease, the radioactivity was confined to the albumin peak and little or no localization of the copper to the α_2 globulin fraction occurred.

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Autoradiographic Studies on Iron-59 Turnover by Erythroid Cells in Rat Bone Marrow. (20781)

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From the data on hemoglobin formation in the human bone marrow obtained by Thorell (1) with the aid of absorption microspectroscopy, it is known that "the endocellular synthesis of hemoglobin does not start before the ribose polynucleotide metabolism is finished" and that "the formation of the Fe-porphyrin component and probably also the simultaneous coupling to hemoglobin takes place chiefly during a later maturation phase." Since, from the data of Thorell(1), the ribose polynucleotide metabolism appears to be concluded within the basophilic phase of erythropoiesis, the polychromatic and orthochromatic erythroblasts appear to be the cells in which the Fe-porphyrin and hemoglobin appear first. He has evaluated the hemoglobin content in polychromatic erythroblasts to be 25%, in the orthochromatic 20-25%, and in the erythrocyte 33%, while the basophilic erythroblast either does not contain hemoglobin or the content is not measurable (less than 0.5%). These data, which agree with what is known about hemoglobin formation through the morphology and the staining of immature red cells, do not seem to be substantiated by the isotopic technic, since the rapidity with which the radioiron is taken up by the erythroid cell is so great that it is probable that the earliest stages of the erythroblasts (pro-erythroblasts and basophilic erythroblasts) are also actively involved in the formation of hemoglobin in the red cells. Clinical studies on patients with refractory anemias, showing uptake of iron in the bone marrow, but with little appearing in the peripheral red cells, also suggest that Fe must enter into the immature red cell precursors. This hypothesis can be verified only by using the qualitative procedure of autoradiography of bone marrow smears after radioiron administration. Other technics now available cannot discriminate between the different stages of erythropoiesis. There is no record of such an investigation with iron, although London and coworkers(2), Altman *et al.*(3), and Boyd and associates(4) have employed this technic to demonstrate that glycine labeled with C^{14} or N^{15} is taken up *in vivo* and *in vitro* by the immature red cells of the bone marrow and is incorporated into hemoglobin.

In the present study the stripping film autoradiographic technic, which gives the proper resolution for this purpose, was adopted to detect the first appearance of labeled iron in the earlier stages of erythropoiesis in the rat.

Method. A single dose of 80 μc of Fe-59-labeled ferric chloride in citrated solution was given intraperitoneally to 5 young rats of the Long-Evans strain, weighing about 160-180 g. The animals were then placed under ether narcosis, and sufficient bone marrow to make very thin smears was obtained from the femur or the tibia at specified intervals (½, 1, 3, and 6 hours). In order to obtain hyperplastic and hyperactive bone marrow, two additional animals were bled of half their

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