3. The organic complexes of copper in these extracts appeared to be stable in the presence of a cation-exchange resin after *in vitro* incubation at pH values above 2.5. Below pH 2.0 decomposition of complexes was appreciable.

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The Dietary Availability of Copper in the Form of Naturally Occurring Organic Complexes

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In the preceding paper (Mills, 1956) it was shown that the greater part of the copper in an aqueous extract of herbage was in the form of complexes which appear to be stable above pH 2.5. Earlier it had been found that such extracts promoted a more rapid recovery of the copper-deficient rat than a corresponding quantity of copper administered as an inorganic supplement (Mills, 1955).

The object of the present work was to investigate the availability of copper in herbage extracts treated with a cation-exchange resin to remove traces of ionic copper and copper from complexes of low stability and to determine whether the rapid response to such preparations in earlier biological trials was due to either: (1) the efficient utilization of any ionic copper present in the original extract or liberated from complexes during gastric digestion, or (2) the utilization of stable organic complexes of copper.

The use of the copper-deficient rat to determine the biological availability of dietary copper has been described elsewhere (Mills, 1955). The technique consists in depleting the weanling black-hooded rat of liver-copper reserves on a copper-deficient basal diet supplemented with vitamins and with a purified-iron salt. As the animal becomes copper-deficient a marked anaemia develops, with severe depigmentation of hair and failure of growth. The effect of a dietary supplement containing copper on haematopoiesis, melanin-pigment regeneration, growth and liver-copper storage is taken as an index of the biological availability of copper in the test material.

EXPERIMENTAL

Preparation of 'de-ionized' herbage extract. [The term 'de-ionized' herbage extract is in this paper used to indicate a herbage extract treated with Zeo-Karb 215 (H) to remove

ionic copper.] Milled, freeze-dried herbage (200 g.) was stirred vigorously for 18 hr. with 2 l. of glass-distilled water at 37°. Coarse solid material was filtered off with muslin and the filtrate centrifuged at 1500 g for 1 hr. at -0°. A 300 mm. x 18 mm. column of Zeo-Karb 215 was activated with 4 vol. of 2N-HCl (A.R.) and washed with glass-distilled water until the eluate was chloride-free. The centrifuged herbage extract was percolated through the cation-exchange resin at the rate of 5 ml./minute. The first samples of eluate were strongly acid (pH < 1), but after about 45 ml. of eluate had been run off the pH rose rapidly to 3.4 and the collection of a bulk sample was commenced. This extract was freeze-dried and then reconstituted with glass-distilled water so that 0.5 ml. of concentrate contained 3.0 µg. of Cu. This concentrate was stored under N₂ at +1°.

Production of copper-deficient rats. Rats of the Rowett Institute strain of black-hooded Lister were used for this work. (i) Pre-weaning treatment: fresh whole milk was gathered into acid-washed glass vessels and was supplemented with the following vitamins (mg./l.) before feeding *ad lib.* to dam and litter: thiamine 0.3, riboflavin 0.4, Ca pantothenate 2.0, folic acid 0.2, nicotinic acid 0.2, *p*-aminobenzoic acid 0.2 (added in aqueous solution); vitamin A (alcohol) 0.8, α -tocopherol acetate 5.0 (added in arachis oil). This supplemented milk was shaken vigorously before use to ensure uniform dispersion of arachis oil containing fat-soluble vitamins. The dam was removed to a separate cage for three 1 hr. periods daily for the feeding of a solid diet. (ii) Post-weaning depletion diet: a solid basal diet of the following composition was fed at the rate of 6 g./rat/day for the first two weeks after weaning and 10 g./rat/day thereafter: roller-dried full-cream milk (low Cu batch) 95%, sucrose (A.R.) 5%. To this the following water-soluble vitamins (mg./kg.) were added: thiamine 5.0, riboflavin 10.0, pyridoxine 5.0, nicotinic acid 20.0, Ca pantothenate 50.0, *p*-aminobenzoic acid 10.0, folic acid 11.0, inositol 400.0. The copper content of this diet was 0.18 µg./g. of D.M. A purified FeCl₃ supplement was administered orally to give 0.5 mg. of Fe²⁺/rat/day. The following fat-soluble vitamins were administered orally in 0.2 ml. of arachis oil: vitamin A (alcohol), 37 µg./rat/day;

α -tocopherol acetate, 2.4 mg./rat/day. A marked copper-deficiency anaemia developed after feeding this diet for 6 weeks, whereupon animals were randomized into experimental groups (see Table 2).

pH determinations on stomach contents. Animals receiving the diet described above were killed by decapitation. The stomach was removed under liquid paraffin and a glass-electrode system inserted through an incision in the stomach wall. All readings were obtained within 1 min. of death. Results are presented in Table 1.

Haemoglobin determinations. Blood samples were obtained by puncture of the coccygeal artery and vein. Haemoglobin was determined by a modification of the method of Nicholas (1951), the optical density of oxy-haemoglobin being measured at 541 m μ . in a Beckman spectrophotometer.

Hair-pigment assessment. A series of pelts were prepared from black-hooded rats receiving different levels of dietary copper. The pelts ranged in colour from black to pale silver-grey. Experimental animals were given a 'pigmentation index' by comparison with these pelts, an index of 10 indicating a fully pigmented head and an index of 1 a very severely depigmented animal.

Uptake of ^{64}Cu by the rat liver. A sample (0.04 g.) of irradiated Specpure Cu foil was dissolved in 1 ml. of 70% (w/w) HNO_3 (A.R.) and the excess of acid neutralized with 2N-NaOH while being stirred vigorously to prevent precipitation of $\text{Cu}(\text{OH})_2$. The uptake of ^{64}Cu by the rat liver was compared after feeding 10 μg . of the irradiated Cu [10 μg . of (^{64}Cu + inert ^{63}Cu) as received from A.E.R.E. Harwell] (i) as the free Cu^{2+} ion, and (ii) after combining the irradiated Cu with a reconstituted freeze-dried aqueous extract of 20 g. of herbage. (The product was freed from traces of the free $^{64}\text{Cu}^{2+}$ ion by passage through a H^+ -activated Zeo-Karb 215 column at pH 3.4 as described above.) Animals were depleted of copper reserves before this experiment, using the basal diet, vitamin and iron supplements described above. Food was withdrawn 3 hr. before feeding radioactive supplements. Animals were killed by coal gas. Livers were removed, washed in distilled water and prepared for liquid counting by the technique of Comar, Davis & Singer (1948). Samples were counted in a 10 ml. capacity Mullard ethanol-quenched liquid counter.

RESULTS

Biological availability trial. The physiological response obtained from the feeding of 3 μg . of copper/day as the stable copper complexes of a 'de-ionized' herbage extract was compared with that obtained from 3, 6, 10 and 15 μg . of the Cu^{2+} ion administered as cupric sulphate. The results are presented in Table 2. The group-mean haemoglobin levels at intervals throughout the trial and the standard deviations of these values are presented graphically in Fig. 1. There were significant differences in final haemoglobin levels between treatments ($P < 0.001$). The results fell into two groups with treatments 1 and 2 (3 μg . of Cu^{2+} and

Fig. 1. Haemoglobin recovery curves of copper-deficient rats fed on copper supplements (mean of results for group): $\times$ — $\times$, 3 μg . Cu^{2+} /day (6 animals); Λ — Λ , 6 μg . Cu^{2+} /day (6 animals); $\bullet$ — $\bullet$, 10 μg . Cu^{2+} /day (5 animals); $\odot$ — $\odot$, 15 μg . Cu^{2+} /day (8 animals); $\circ$ — $\circ$, 3 μg . copper/day in 'de-ionized' herbage extract (6 animals).

Table 1. pH of stomach contents of rats fed on copper-deficient basal diet

Time after feeding (hr.)	...	...	...	2	6	10	17
Range of pH values of stomach contents				4.3-4.0	3.8-3.5	3.8-2.8	3.6-3.4
Mean				4.1	3.7	3.3	3.5
(No. of animals)				(4)	(4)	(4)	(3)

Table 2. Group mean values and standard errors for haemoglobin level, weight gain, liver copper and change in pigmentation index of rats fed on ionic copper or a de-ionized extract of herbage for 30 days

Treatment	Final haemoglobin level (g./100 ml.)	Wt. gain during period (g.)	Total liver Cu (μg .)	Change in pigmentation index during period	No. of animals receiving treatment
(1) 3 μg . Cu^{2+} /day as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (A.R.)	9.4 $\pm$ 0.6	19 $\pm$ 4	9.7 $\pm$ 0.8	+1	6
(2) 6 μg . Cu^{2+} /day as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (A.R.)	9.2 $\pm$ 0.6	25 $\pm$ 4	10.9 $\pm$ 0.8	+1	6
(3) 10 μg . Cu^{2+} /day as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (A.R.)	11.8 $\pm$ 0.6	30 $\pm$ 4	12.8 $\pm$ 0.9	+2	5
(4) 15 μg . Cu^{2+} /day as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (A.R.)	11.8 $\pm$ 0.5	35 $\pm$ 4	13.0 $\pm$ 0.7	+2	8
(5) 3 μg . Cu/day as 'de-ionized' herbage extract	11.1 $\pm$ 0.6	30 $\pm$ 4	13.2 $\pm$ 0.9	+2	6

Table 3. *Uptake of ^{64}Cu by the rat liver when fed in combination with various supplements*

Group treatment	Cu recovered in liver (% of admin- istered dose)	No. of animals	Mean log* of liver copper
Expt. 1 (period 24 hr.)			
(i) 10 $\mu\text{g.}$ ^{64}Cu + carrier Cu fed as free ion + 2 g. sucrose	1.4	5	1.13
(ii) 10 $\mu\text{g.}$ ^{64}Cu + carrier Cu combined with aqueous extra of herbage fed + 2 g. sucrose	4.0	5	1.58
Standard error of difference of mean logs* = ± 0.09 (8 degrees of freedom).			
Expt. 2 (period 36 hr.)			
(i) 10 $\mu\text{g.}$ ^{64}Cu + carrier Cu fed as free ion + 2 g. sucrose	3.0	5	1.47
(ii) 10 $\mu\text{g.}$ ^{64}Cu + carrier Cu mixed with 5 g. dried milk basal diet + 2 g. sucrose before feeding	7.0	5	1.84
(iii) 10 $\mu\text{g.}$ ^{64}Cu + carrier Cu combined with aqueous extract of herbage fed + 2 g. sucrose	9.4	5	1.96
Standard error of difference of mean logs* = ± 0.06 (12 degrees of freedom).			

* To stabilize the variance, the log of liver-copper values were analysed rather than the values themselves.

6 $\mu\text{g.}$ of Cu^{2+} /rat/day) giving lower final haemoglobin levels than treatments 3, 4 and 5 (10 $\mu\text{g.}$ of Cu^{2+} , 15 $\mu\text{g.}$ of Cu^{2+} and 3 $\mu\text{g.}$ of Cu in 'de-ionized' herbage extract/rat/day). Neither the differences in weight gain or in liver-copper storage at the end of the 30 day experimental period were quite large enough to reach significance, but in each case the mean values obtained suggested a pattern similar to that of the haemoglobin results with the 3 $\mu\text{g.}$ of Cu^{2+} and 6 $\mu\text{g.}$ of Cu^{2+} /day treatments giving lower values than the other three treatments. This pattern of differences was repeated in the results for hair-pigment regeneration.

These results demonstrate that the 'de-ionized' herbage extract contains a source of copper which is readily available to the copper-deficient rat. The rate of recovery of the animals receiving 3 $\mu\text{g.}$ of copper daily in this product was superior in all measures to that obtained by feeding 3 $\mu\text{g.}$ of copper as cupric sulphate. The response to the feeding of herbage extract was approximately equivalent to that obtained with 10 $\mu\text{g.}$ /day of the free Cu^{2+} ion.

Uptake of ^{64}Cu by the rat liver. As shown in Table 3 (Expt. 1) approximately 2.8 times as much radioactive copper was absorbed and stored in the rat liver when fed in combination with the metal complex-forming ligands of an aqueous extract of herbage as when fed as the free Cu^{2+} ion. This difference was highly significant with $P < 0.005$. In this, as in all previous copper-availability trials with the rat, an attempt was made to minimize interactions between copper supplements and the constituents of the basal diet by feeding these materials at different times. In a separate experiment, the results of which are presented in Table 3

(Expt. 2), the effect of mixing the radioactive Cu^{2+} ion with the dried-milk basal diet was investigated. As in the previous experiment more copper was taken up by the liver when combined with a herbage extract than when fed as the free ion ($P < 0.001$). Although an increased uptake was obtained by allowing the free ion to combine with the milk basal diet before feeding, the results suggest that copper fed in this form is still not as efficiently utilized as that fed in combination with the herbage extract ($0.05 < P < 0.1$).

DISCUSSION

The results of pH determinations on the stomach contents of rats receiving the diet used in these experiments indicate that pH fluctuations in the range 4.3–2.8 might be expected. It has been shown (Mills, 1956) that only below pH 2.5–2.0 are appreciable quantities of ionic copper liberated from the copper complexes of an aqueous extract of herbage during *in vitro* incubation. To account for the observation that the copper of such extracts is readily utilized by the rat it is necessary to consider the possible fate of orally ingested copper in the digestive tract.

Albert (1951) has shown that the Cu^{2+} ion may combine readily with many biological materials to form complexes, and Albert & Gledhill (1947) and McNaught, Owen & Smith (1950) have shown that such complexes may form under physiological conditions. The solubility and stability of such complexes may vary greatly. Thus complex-formation with most amino and hydroxy acids results in soluble products, whereas the copper complexes formed with some proteins, with

porphyrins and with lignin are insoluble at normal physiological pH.

Evidence is however available to suggest that certain soluble complexes of copper may be more readily transported through biological membranes than the free ion. Thus Uzman (1953) has demonstrated that the urinary excretion of copper in hepatolenticular degeneration is associated with the excretion of specific copper oligopeptide complexes. The mobilization and renal excretion of body copper as the Cu-BAL complex has been demonstrated by Denny-Brown & Porter (1951). Saha & Guha (1941) found that an iron-copper nucleoprotein complex isolated from fish muscle promoted a more rapid regeneration of haemoglobin than equivalent quantities of ionic iron and copper fed to the copper- and iron-deficient rat. This complex was readily absorbed through the small intestine mucosa of the rabbit (Saha & Bannerjee, 1943). Seelemann & Baudissin (1954) obtained a greater stimulation of haematopoiesis and growth in the rabbit from the sodium salt of a copper allylthiourea-benzoic acid complex than from inorganic copper.

From the foregoing it appears possible that, in the present work, the rapid recovery of the copper-deficient rat fed on an aqueous extract of herbage treated with a cation-exchange resin may be due, in part, to the presence of soluble complexes which are stable at the pH encountered in the stomach and which are readily absorbed from the jejunum (the site of copper absorption according to Sachs, Levine, Hill & Hughes, 1943).

It is suggested that the transport of ionic copper through the intestinal mucosa may not be the only mechanism whereby this element is absorbed. If, as the results described above appear to indicate, copper may be absorbed as stable, soluble complexes, it is possible that such complexes could arrive at the site of absorption from two sources: (1) from specific complexes preformed in a biological food material (e.g. herbage); or (2) from the interaction of ingested Cu^{2+} ions with specific complex-forming ligands in the digestive-tract contents. It would be expected that since, in this last case, the free ion may alternatively combine with other constituents to form insoluble products or complexes not suitable for absorption, the utilization of copper in this form would be poor. This suggestion is supported by results in the present study and by Coulson, Remington & Lynch (1934), Comar *et al.* (1948) and Schubert, Maurer & Riezler (1948) who have all demonstrated the poor utilization of the orally administered Cu^{2+} ion.

SUMMARY

1. Copper-deficient black-hooded rats were used to study the availability of copper in 'de-ionized' aqueous extracts of herbage. Copper uptake by the rat was assessed by haematopoietic response, melanin-pigment regeneration, growth and measurement of liver-copper storage.

2. Aqueous extracts of herbage passed through a cation-exchange resin to remove the Cu^{2+} ion still contained copper in an available form. The stable, soluble complexes of copper remaining in these extracts promoted a more rapid physiological response and a greater storage of copper in the liver than the feeding of equivalent amounts of the Cu^{2+} ion.

3. These results were confirmed by comparing the uptake of ^{64}Cu by the rat liver after feeding the isotope as the $^{64}\text{Cu}^{2+}$ ion and after combining it with the complex-forming ligands of an aqueous herbage extract.

4. To account for the rapid utilization of the copper of the stable, soluble complexes of herbage it is suggested that copper may be transported through the intestinal mucosa in the form of such complexes.

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