gland was somewhat more active than the preparation of specific activity 3800 units/mg. N obtained by Caputto & Marsal (1941). However, further purification of the enzymes from kidney, liver and mammary gland was not pursued. An essentially homogeneous preparation of this class of alkaline phosphatases, which are distinct from intestinal alkaline phosphatases (Morton, 1952), had already been obtained from milk (Morton, 1953c). It has a specific activity of 15 300 units/mg. N.

SUMMARY

1. The alkaline phosphatases of calf intestinal mucosa and of cow mammary gland are associated with microsomal particles.

2. By treatment with *n*-butanol at 35° the enzyme is readily obtained in true solution. On the basis of this effect a convenient method for assay of alkaline phosphatase of animal tissues is proposed.

3. The purification of alkaline phosphatase from calf intestinal mucosa is described. It involves butanol treatment of washed microsomes, followed by acetone precipitation, heating and charcoal adsorption.

4. The most active preparation had an activity (with β -glycerophosphate) of 83 500 units/mg. N (Q_p 579 000). This represents a purification of over 1000 times and thus is the most active phosphatase preparation as yet obtained.

5. The alkaline phosphatases of kidney, liver and mammary gland have been partially purified by similar methods.

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Copper Complexes in Grassland Herbage

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Copper deficiency disorders of cattle and sheep in Britain frequently exist in areas where the dietary copper intake of the grazing animal appears to be adequate. In areas where neonatal ataxia of lambs (Swayback) is found (a condition which can be prevented by copper therapy) herbage copper values

are appreciably higher than those of areas in Australasia where the apparently identical syndrome, enzootic ataxia, is common. Thus Bennetts & Beck (1942) reported outbreaks of enzootic ataxia in Western Australia on pastures ranging in copper content from 1.1 to 6.1 p.p.m. (on dry matter) with a mean of 3.3 p.p.m. Cunningham (1944) described a copper deficiency in New Zealand where sheep

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have been grazing pastures with a copper content between 2 and 5 p.p.m. Beck (1941) has suggested that under Australian conditions pastures containing less than 5 p.p.m. copper are probably inadequate.

The following ranges have been quoted for the copper content of Swayback pastures in Britain, 14–27 p.p.m. (Innes & Shearer, 1940), 8–13 p.p.m. (Shearer, Innes & McDougall, 1940), 11–22 p.p.m. (Shearer & McDougall, 1944) and 6–13 p.p.m. (Mills, 1951). None of these workers have been able to show differences between the herbage copper values of affected and non-affected areas. Jamieson & Allcroft (1950), and Allcroft & Parker (1949) have described deficiency conditions in cattle which respond to copper therapy despite the fact that the copper levels in pasture ranged from 6.8 to 23 p.p.m.

From these results and from work which has been reviewed elsewhere (Allcroft, 1952; Marston, 1952) it is seen that many of the cases of copper deficiency in ruminants in Britain must be attributed to factors which either interfere with the copper metabolism of the animal or else affect the 'digestibility' of copper in the alimentary tract. The influence of molybdenum on the absorption and utilization of copper has been reviewed by Marston (1952). Dick & Bull (1945) have suggested that a high molybdenum intake may be a causative factor in conditioned copper deficiency disorders, but Stewart, Farmer & Mitchell (1946) have pointed out that pasture molybdenum values of affected areas of Britain are rarely above normal.

It was the object of the present work to investigate the possibility that the copper in herbage from affected areas may be present in a non-available form owing to the formation of stable organic complexes which the animal may be unable to absorb.

Many intermediates and products of plant and animal metabolism are structures with which copper can form complexes of considerable stability. Among such compounds are proteins (Mann & Keilin, 1938; Macheboeuf & Viscontini, 1943; Holmberg & Laurell, 1948; Klotz, 1952), amino acids (Albert, 1950*a*, 1952), porphyrins (Corwin, 1950), purines, pterins and flavins (Albert, 1950*b*, 1953; Mahler, 1953), catechol and its derivatives (Weinland & Walter, 1923) and tannins and lignin (Rademacher, 1936).

If copper is rendered unavailable to the ruminant by complex formation, the complexes produced must be sufficiently stable to resist breakdown by rumen micro-organisms and by the acid secretions of the abomasum. Furthermore, they must either be insoluble in water and fat or, if soluble, be of such large molecular size that passage through the wall of the digestive tract is prevented. That acid-stable

complexes of copper exist in biological material has been demonstrated by Neish (1939) and Macheboeuf & Viscontini (1943). Schultze, Elvehjem & Hart (1934) have shown that a number of organic copper complexes are not utilized as sources of copper by the rat.

In the work to be described comparison has been made in all cases between herbage from normal areas and from areas where outbreaks of Swayback are common. Comparisons have been based on:

(1) The solubility of herbage copper in a series of organic solvents of increasing dielectric constant.

(2) The solubility of copper in water and in dilute solutions of tartaric acid and glycine. The object of using the last two solvents has been to enable a qualitative estimate to be made of the stability of the naturally occurring complexes by providing an excess of organic ligands with which the bound copper may be induced to chelate to form a soluble complex. Soluble copper has been separated from the herbage residue by dialysis.

(3) In a limited number of cases, water-soluble fractions of herbage have been examined by a chromatographic separation of ionic copper from copper present in the form of soluble complexes.

EXPERIMENTAL

Sampling and preparation of herbage. Samples were taken by hand plucking from fields on a Derbyshire farm where Swayback occurs regularly if copper administration to pregnant ewes is not carried out. Samples from a non-affected area were taken from a farm in Kent where this disorder was unknown. Herbage was rinsed in glass-distilled water as soon as possible after gathering to minimize soil contamination and then dried for 3.5 days at 60° in a current of air. The dried material was ground in a Christy & Norris Laboratory Hammer mill (screen 64) that had been carefully cleaned before use by milling filter paper. Ground samples were vacuum-packed in glass jars for storage. Glassware was rinsed twice with 70% (w/w) HNO₃, washed with glass-distilled water and tested for freedom from heavy metals by rinsing with a solution of diphenylthiocarbazon in CCl₄, prepared by the method of Parkes (1943).

Copper analyses. Cu was estimated colorimetrically as copper diethyldithiocarbamate using an adaptation of the method of Eden & Green (1940). Approximately 1 g. of dried plant material was taken for each analysis and digested in Pyrex glassware with 2 ml. 70% (w/w) HNO₃ (redist. A.R.), 1 ml. 98% (w/w) H₂SO₄ (A.R.) and 1 ml. 72% (w/w) HClO₄ (low Cu batches A.R.). After digestion 10 ml. 4% Na₃P₄O₇, 10H₂O (four times recryst.) were added followed by 5 ml. NH₃ solution (sp.gr. 0.880) and distilled water to bring to 25 ml. vol. 2 ml. 0.5% sol. of sodium diethyldithiocarbamate were added and the yellow copper complex was extracted into 2 ml. amyl alcohol (redist. A.R.) the alcohol layer being subsequently centrifuged at 1085 g for 5 min. to remove suspended water droplets. Colour measurement was carried out using a 'Spekker' absorptiometer (model H 760) with Ilford Spectrum Blue-Green filters no. 603 (max. transmission at 490–500 mμ.). Readings

were compared with a calibration curve obtained with standard solutions in a 1 cm. microcell. All analyses were duplicated and reagent blanks were performed with each batch.

Extraction of herbage with organic solvents. Seamless extraction thimbles were freed from Cu contamination by continuous washing for 24 hr. in 0.5N-HCl (A.R.) and finally rinsed in glass-distilled water. Herbage (5 g.) was weighed into a dried thimble and placed in an all-glass Soxhlet apparatus. Serial extraction of each sample was carried out for 24 hr. periods with light petroleum (b.p. 40–60°), ether, CHCl_3 , ethyl acetate, butanol and water in that order. The Cu extracted by each solvent and in the herbage residue was estimated as described. 'Water-soluble Cu' had to be determined by difference owing to losses due to frothing during concentration. All organic solvents were purified by distillation, fractions distilling outside a range of $\pm 0.5^\circ$ of the expected boiling point being rejected. Light petroleum and ether were dried initially with P_2O_5 and stored over sodium. CHCl_3 was dried and stored over P_2O_5 in a dark vessel. Ethyl acetate and butanol were stored over anhydrous CaSO_4 .

Dialysis in aqueous solvents. Collodion dialysis thimbles were prepared by the method of Duckworth (1935). The membranes produced by this method were of satisfactory uniformity and proved to be equally permeable to cupric, cupriglycinate and cupritartrate ions. Membranes were stored under glass-distilled water containing a trace of toluene.

Duplicate samples of herbage were weighed into thimbles containing the following aqueous solvents. In the case of 'water-soluble Cu', 1 g. herbage was mixed with 20 ml. water in each thimble and dialysed for 36 hr. In the case of 'glycine-soluble Cu', 1 g. herbage was mixed with 20 ml. 0.02M glycine and the thimble and contents were immersed in 80 ml. of this solvent for 24 hr. In the case of 'tartaric acid-soluble Cu' the procedure was the same as for the 'glycine-soluble Cu' except that the solvent consisted of 0.02M tartaric acid. Dialysis was carried out with continuous stirring, against glass-distilled water in an all-glass apparatus through which a rate of flow of 5 ml./min. was maintained. In all cases the decrease in the content of Cu during dialysis was determined by analysis of the non-dialysable residue.

The chromatography of water-soluble components. The work of Dent (1949) and Crumpler & Dent (1949) in resolving α -amino acids as their copper complexes, and of Arden, Burstall, Davis, Lewis & Linstead (1948), Burstall, Davis & Wells (1949), Burstall, Davis & Linstead (1951) and Lederer (1948) on the filter-paper chromatography of inorganic ions suggested that a filter-paper strip technique might be developed for the chromatographic separation of free cupric ions and Cu organic complexes in aqueous herbage extracts. The suitability of a number of solvents for the separation of a range of Cu complexes and salts was examined. Butanol-5% acetic acid gave good resolution, but heavy 'tailing' of spots indicated that complex decomposition had been taking place during the run in the acid solvent. Benzene-water gave no evidence of decomposition but rather poor resolution. Phenol-water provided good resolution, and decomposition only occurred in runs with weak complexes. The results of separations achieved on a number of 18 hr. (descending) runs on Whatman no. 1 filter paper are given in Table 1.

Preparation of herbage extracts for chromatography. 2 g. samples of dried herbage were weighed directly into centrifuge tubes containing 50 ml. distilled water. Tubes were stoppered and shaken vigorously for 4 hr. on a 'Microid' flask shaker and then centrifuged for 18 hr. at 510 g. The clear supernatant was reduced to 1 ml. by drying in vacuum, first over CaCl_2 and finally over P_2O_5 . 0.2 ml. of this concentrate was applied to the filter paper by means of an 'Agla' micrometer syringe on to forty marked spots. The solvent was allowed to descend for 18 hr. and the paper was then dried at 60°. Results were assessed by two methods, (i) by spraying with a reagent consisting of 4% $\text{Na}_2\text{P}_2\text{O}_7$, 10H₂O, and 0.5% sodium diethyldithiocarbamate in NH_3 solution (sp.gr. 0.880); the position of the golden-yellow spots due to the Cu complex was marked and R_F values noted; (ii) by cutting the dried papers at right angles to the direction of solvent travel into five strips each of 0.2 unit of R_F ; each strip was ashed in a muffle furnace and the ash extracted with 0.5 ml. 35% (w/w) HCl. The Cu content of this extract was determined by adding 1 ml. NH_3 solution (sp.gr. 0.880), 0.2 ml. 4% $\text{Na}_2\text{P}_2\text{O}_7$, 10H₂O and 0.2 ml. sodium diethyl dithiocarbamate, the Cu complex being extracted into 0.75 ml. amyl alcohol. Although method (ii) gave only a roughly quantitative assessment owing to the very small amounts of copper present, the results were of value in supplementing those obtained by visual examination of the chromatograms.

Table 1. R_F values of copper complexes or salts in phenol/water as descending solvent on Whatman no. 1 paper (18 hr. at 18°)

Organic ligand	R_F of copper complex or salt
Histidine	0.98
Tryptophan	0.98
Creatine	0.97
β -Phenylalanine	0.96
Proline	0.96
Methionine	0.95
Leucine	0.94
Alanine	0.90
Tyrosine	0.84
Glycine	0.60
Asparagine	0.59
Glutamic acid	0.39
Pyridoxin	0.94
o-Coumaric acid	0.93
Ethylenediamine	0.86
Catechol	0.83
Malic acid	0.63*
Pyruvic acid	0.62*
Ethylenediamine	0.35
Malonic acid	0.20*
Lactic acid	0.08*
Tartaric acid	0.08
Acetic acid	0.05
Citric acid	0.05
Oxalic acid	0.05
Succinic acid	0.05
Inorganic anion	R_F of copper salt
Nitrate	0.06
Sulphate	0.05
Chloride	0.05

* Indicates 'tailing' observed on all runs probably due to slow decomposition of complex during run.

RESULTS

Organic solvent extraction. The solubility of a compound in a solvent is largely related to the electrovalent or covalent character of the solute molecule and the dielectric constant of the solvent. As the electrovalent characteristics of a series of copper compounds decrease, their solubility in solvents of high dielectric constant also tends to decrease providing strongly hydrophilic groups are not introduced into the molecule. Thus if cupric ions in a biological system enter into organic-complex formation, the solubility of that copper in water may fall, while that in solvents of low dielectric constant may be significantly increased. Failing this, the complex will be insoluble in both aqueous and organic solvents.

The solvent distribution of dissolved herbage copper from 'normal' and 'affected' herbage (i.e. samples from Swayback areas) is given in Table 2. In all samples examined, a substantial proportion of the total copper remained insoluble in organic solvents and water. The insoluble residue from samples of normal herbage contained, on the average, 64 % of the total copper, the corresponding figure for affected samples being 67 % (calculated from Table 2). The mean values for copper soluble in light petroleum showed no significant differences between normal and affected samples (3.8 % as

compared with 4.5 %). Ethyl acetate also showed no important differences (5.1 and 5.0 %). Extractions with ethyl ether dissolved about 0.6 % of the total copper in normal samples as compared with 2.3 % in affected samples. This difference is due to the slightly higher solubility of copper in samples gathered from affected pastures in early spring (samples A1 to A3). A similar seasonal variation was apparent in the figures for the chloroform-soluble copper of affected herbage at different stages of growth. Samples gathered in January showed a mean solubility of 3.5 % of total copper as compared with 0.5 % in June. No marked seasonal variations of the chloroform-soluble copper content of normal pasture was observed. The parallel variations in the figures for water and butanol extractions are not surprising since butanol with its high dielectric constant (17) would be expected to dissolve many water-soluble components of the herbage. Butanol and water together extracted more copper from normal samples (24 %) than from affected samples (17 %).

Considering all the samples together, approximately 13 % of the total copper in the herbage was dissolved by solvents of dielectric constant less than 10, a further 21 % of the total being extracted by butanol and water. This suggests that much of the readily soluble copper in herbage is combined with organic residues and is not in the form of inorganic salts.

Table 2. *The solubility of herbage copper in organic solvents*

Values for $\mu\text{g. soluble Cu/g. dry matter}$. Figures in parentheses express the percentage of the total Cu content.

'Normal' herbage

Sample (no.)	Month of sampling	Total Cu	Light petroleum	Ether	Chloroform	Ethyl acetate	Butanol	Water (by difference)	Insoluble residue
N1	February	6.50	0.25 (3.9)	0.08 (1.2)	0.04 (0.6)	0.46 (7.1)	0.85 (13.1)	0.99 (15.2)	3.83 (58.9)
N2	March	7.25	0.27 (3.7)	0.05 (0.7)	0.08 (1.1)	0.36 (4.1)	0.90 (12.4)	1.05 (14.5)	4.54 (62.6)
N3	March	8.75	0.30 (3.4)	0.04 (0.5)	0.09 (1.0)	0.22 (2.5)	1.04 (11.9)	1.00 (11.4)	6.06 (69.3)
N4	May	7.30	0.44 (6.0)	0.03 (0.4)	0.20 (2.7)	0.35 (4.8)	0.70 (9.6)	0.52 (7.6)	5.06 (68.9)
N5	June	5.75	0.11 (1.9)	0.03 (0.5)	0.16 (2.8)	0.39 (6.8)	0.98 (17.0)	0.58 (10.1)	3.50 (60.8)

'Affected' herbage

A1	January	8.10	0.26 (3.2)	0.14 (1.7)	0.20 (2.5)	0.37 (4.6)	1.24 (15.3)	0.28 (3.5)	5.61 (69.2)
A2	January	6.25	0.28 (4.5)	0.27 (4.3)	0.38 (6.1)	0.27 (4.3)	0.64 (10.2)	0.46 (7.4)	3.95 (63.2)
A3	January	5.50	0.33 (6.0)	0.24 (4.4)	0.36 (6.5)	0.21 (3.8)	0.55 (10.0)	0.37 (6.7)	3.44 (62.5)
A5	June	7.30	0.44 (6.0)	0.04 (0.5)	0.06 (0.8)	0.32 (4.4)	0.80 (11.0)	0.20 (2.7)	5.44 (74.5)
A6	June	7.10	0.21 (3.0)	0.04 (0.6)	0.05 (0.7)	0.56 (7.9)	0.76 (10.7)	0.56 (7.9)	4.92 (69.3)

Dialysis in aqueous solvents. Extraction of herbage samples with aqueous solutions was carried out by two methods:

(1) Extraction and dialysis with water alone. This procedure would be expected to remove any ionic copper which had escaped adsorption on insoluble cell materials and organic copper complexes that are readily soluble in water.

(2) Extraction with an excess of complex-forming agent followed by dialysis with water to provide qualitative information regarding the stability of copper complexes not extracted by procedure (1). This principle of competitive complex formation has been used by a number of workers to determine the order of affinity of a number of metal ions for an organic ligand (Pfeiffer, Thielert & Glaser, 1939; Ackermann, Prue & Schwarzenbach, 1949; Mellor & Maley, 1948) and of organic ligands for copper in an insoluble inorganic complex (Wain & Wilkinson, 1943).

If a complex-forming molecular species is introduced into a biological system and a water-soluble copper complex is formed which is then removed by dialysis, the extent to which copper is withdrawn from the system is governed largely by the relationship between the stability constants of the naturally

occurring copper complexes and that of the complex formed with the added ligand (equation i).

$$\left[\frac{K_s \text{ Cu } B}{K_s \text{ Cu } A} \right]$$

where A = naturally occurring ligands, and B = added ligand.

If 'bound' herbage copper remains insoluble in the presence of molecules with which it can form a stable complex it is an indication that the naturally occurring complexes are of great stability at that particular pH. Similarly, if the added ligand forms a weak complex and yet is capable of withdrawing copper from the system the preformed complexes must also be of low stability. In making comparisons of the performance of chelating agents in this way it is essential that the added solutions have the same effect on the pH of the system, otherwise the situation becomes complicated by changes in the degree of ionization of the organic ligands competing for the metal ion. Although this method does not enable an accurate assessment to be made of the stability of copper complexes in herbage it does

Table 3. *The solubility of herbage copper on extraction with water, glycine and tartaric acid*

Values for $\mu\text{g. soluble Cu/g. dry matter}$. Figures in parentheses express the percentage of the total Cu content.

			Diffusible copper in		
			Water extraction	Glycine extraction	Tartaric acid extraction
'Normal' herbage					
Sample (no.)	Month of sampling	Total copper			
N1	February	6.50	1.43 (22)	4.00 (62)	3.58 (55)
N2	March	7.25	1.38 (19)	2.05 (28)	1.74 (24)
N3	March	8.75	1.48 (17)	1.75 (20)	1.58 (18)
N4	May	7.30	1.10 (15)	4.24 (58)	2.20 (30)
N5	June	5.75	1.04 (18)	3.80 (66)	1.50 (26)
'Affected' herbage					
A1	January	8.10	0.49 (6)	2.50 (31)	2.11 (26)
A2	January	6.25	0.55 (9)	3.30 (53)	2.70 (43)
A3	January	5.50	0.49 (9)	2.66 (48)	2.36 (43)
A4	January	7.80	1.02 (13)	4.22 (54)	3.80 (49)
A5	June	7.30	0.60 (8)	1.97 (27)	1.38 (19)
A6	June	7.10	0.92 (13)	2.28 (32)	1.99 (28)

present a qualitative picture of the overall stability of these compounds.

The extractants used in these experiments were 0.02M glycine (log K_f of Cu complex 15.4, Albert, 1950a) and 0.02M tartaric acid (log K_f of Cu complex about 9-10, from results of Kahlenberg, 1895). Results are presented in Table 3. It will be seen that slight but consistent differences occur between the figures for water soluble copper in normal and affected samples. The average figure for normal samples is 1.29 $\mu\text{g./g.}$ (or 18.2 % of the total), that for affected samples being 0.68 $\mu\text{g./g.}$ (9.7 % of the total). No consistent variation in the water-soluble copper content was noted at different stages of growth. The average glycine-soluble copper content of normal samples in early spring (2.70 $\mu\text{g./g.}$) is approximately equal to that for affected samples at this stage of growth (3.22 $\mu\text{g./g.}$), but as the herbage matures differences become apparent as is shown in the comparison of results for samples N4 and N5 with A5 and A6, all of which were samples of late spring growth.

No appreciable differences appear to exist in the quantity of copper extracted by tartaric acid from normal and affected samples. Seasonal effects are slight, a small increase in the quantity of tartaric acid soluble copper being noted in the early spring samples A1, A2 and A3.

The data in Table 3 suggest that, in the normal pastures examined, there is a pronounced variation in the stability of herbage complexes at different stages of growth. Examining this effect in detail it is seen that at a very early stage of growth (N1) glycine and tartaric acid both extract an appreciable proportion of the total copper. As growth proceeds these extractants withdraw less copper, particularly in the case of sample N3 (sampled March 1951 during a phase of rapid growth). In more mature herbage (N4 and N5) the solubility of herbage copper in glycine has increased much more than in tartaric acid. If these variations had been due to appreciable alterations in the free cupric ion concentration of cell constituents it would be expected that parallel changes would have been noted in the figures for water-soluble copper. As this was not the case it must be concluded that the changes are due to the formation of copper complexes within the herbage and that the stability of these complexes varies with the stage of growth of the plant. That variations of similar magnitude were not noted in affected samples is probably due to the fact that there was no period in the growth of this moorland herbage which corresponded to the 'lush' spring growth of the ley herbage from which normal samples were drawn.

Chromatography of water-soluble components. The results of an examination of the water-soluble fractions of two normal and two affected samples

are presented in Fig. 1 and Table 4. The existence of a number of water-soluble copper complexes was demonstrated in each herbage examined. It appeared that at least eight compounds were present in the extracts of normal herbage as compared with five in the affected samples examined, the compounds giving spots of R_f 0.23, 0.37 and 0.77 being absent in the latter. From the data presented in Table 1 it is seen that inorganic copper did not give spots of R_f greater than 0.05, while of the twenty-eight water soluble complexes examined none had an R_f value of less than 0.08. Of the pasture samples examined N2 and N4 gave responses in the 0 to 0.05 range but such spots were almost entirely absent from samples A5 and A4. On the limited evidence available it would be premature to suggest that this indicated a low concentration of ionic copper in the extract of affected herbage although this may well be the case.

Fig. 1. Diagram of chromatograms obtained by running concentrate of aqueous extract of herbage with phenol-water (18 hr. at 18°). Sprayed with pyrophosphate-diethyldithiocarbamate-ammonia reagent. Intensity of shading indicates intensity of response.

Table 4. Copper content of segments dissected from chromatograms ($\mu\text{g. Cu} \times 10^3$)

+ Indicates that a positive response obtained but less than 0.03 $\mu\text{g. Cu}$ present, and below the lower limit of detection using the Spekker absorptiometer.
- Indicates no response for Cu obtained.

Sample	R_f of segment analysed				
	0.0-0.2	0.2-0.4	0.4-0.6	0.6-0.8	0.8-1.0
N2	3	+	6	5	8
N4	3	+	5	3	9
A4	+	-	4	+	10
A5	-	-	+	+	12

It is of interest to compare these results with those of Dawson & Nair (1950) who, using the copper-amalgam electrode have demonstrated the existence of two primary and four secondary complexes of copper in 'homogenates' of grass tissue.

DISCUSSION

The experimental data presented has shown that the greater part of the copper in herbage exists in a bound form and that much of this copper is not extractable by organic solvents or by dilute aqueous solutions of organic chelating agents. In no case have differences of great magnitude been demonstrated when comparing results for normal and 'swayback' herbage. The differences found have been small but reproducible and indicated that affected herbage contains less water-soluble copper probably both as cupric ions and as water-soluble complexes than normal herbage.

The extent to which the insoluble copper compounds of herbage would be affected by the hydrolytic and proteolytic activities of the digestive tract secretions and the action of rumen micro-organisms in the grazing animal is not known. If it is presumed that stable insoluble complexes are of low availability, the importance of small variations in the soluble copper content of the ingesta becomes more apparent. The problem therefore becomes reduced to these terms: is the lower content of soluble copper in affected herbage sufficient to induce a copper deficiency in the sheep? Before this question can be answered a number of points require investigation. Much more information is needed concerning the physiological availability of soluble copper complexes to the ruminant. Is cupric ion liberation from a complex a prerequisite to the absorption of copper from the digestive tract or can the intact molecule be utilized?

Comar, Davis & Singer (1948) and Mills (unpublished results), using ^{64}Cu , have demonstrated the very poor utilization of ionic copper administered orally to cattle and sheep. Havinga & Bykerk (1947) have shown that more copper is stored in the liver of calves after injection of stable soluble complexes of copper than after injection of equivalent quantities of copper as the sulphate.

In view of such observation it may well be that cupric ions are not as readily utilized as soluble complexes, for it is difficult to imagine that the free ion can be absorbed from the alkaline milieu of the small intestine rich in copper-binding groups and then pass through into the bloodstream without combining with immobile structural protein of the digestive tract wall. The work of Albert (1950*a*, *b*, 1951, 1952, 1953) has clearly demonstrated that in any biological system competition such as this must exist between metal ions and chelating groups of tissues and cell fluids.

Thus before full conclusions can be drawn from the results of this investigation the fundamental aspects of copper absorption and utilization by the animal require to be more fully understood. A study is being made of the dietary availability of copper complexes to the copper-deficient animal and the results of this work will be reported later.

SUMMARY

1. Herbage samples from both Swayback and normal pastures were subjected to organic solvent extraction with a series of solvents of gradually increasing dielectric constant. The quantities of copper extracted by different solvents are compared and the results discussed.

2. The solubility of herbage copper in water was investigated by the use of a dialysis procedure. The results indicate that rather less water-soluble copper is present in the Swayback pastures than in normal herbage.

3. Samples were extracted with dilute aqueous solutions of chelating agents. Seasonal variations in the solubility of herbage copper were demonstrated particularly in the case of the normal samples examined. The greater part of the copper in both Swayback and normal pastures appears to be bound in a form which is more stable than the copper-glycine complex.

4. The copper complexes present in the water soluble component of herbage were examined by the use of filter-paper partition chromatography.

5. The possible significance of the above findings is discussed in relation to the question of the availability of organic copper complexes to the ruminant.

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The Lipids of Fish

5. THE LIPIDS REMAINING IN THE FLESH OF THE HADDOCK AFTER EXTRACTION BY ACETONE AND ETHANOL-ETHER

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In the first paper of this series (Lovern, 1953) was described the preparation of a series of extracts from the flesh of the haddock, using solvents of presumed successively increasing effectiveness for lipids. The extracts obtained with acetone and ethanol-ether (3:1 by vol.), respectively, both at room temperature, have been discussed in earlier papers of this series (Lovern & Olley, 1953*a*, *b*; Olley & Lovern, 1953) and their extensive contamination with non-lipids was shown. The later extracts were obtained with ethanol-benzene (2:1 by vol.), chloroform-methanol (1:1 by vol.), both at the boiling point, and finally pyridine at 100°. Examination of these three extracts showed them to be overwhelmingly non-lipid in composition, and it was decided to present the results in a single paper. Moreover, in order to obtain sufficient lipid for detailed study, comparable extracts, available from an earlier series of experiments and since stored at -30° under nitrogen, were combined with the current series of ethanol-benzene and chloroform-methanol extracts.

ANALYTICAL METHODS

In general the analytical methods employed were as described in the previous papers. New features are given below.

Hydrolysis. The unsatisfactory hydrolysis of sphingomyelin with 0.5*N* ethanolic KOH (Lovern & Olley, 1953*b*) led to the use in the present work of 6*N* methanolic HCl for the hydrolysis (8 hr. refluxing) of fractions likely to contain over 5% of sphingosine. This reagent does not permit the separate isolation of unsaponifiable matter and plasmals, and the latter were assayed after such hydrolysis colorimetrically (Feulgen & Grünberg, 1938) on the unsaponifiable matter, separated from the methyl esters by a simplification of the procedure of Klenk & Friedrichs (1952). In all cases, however, only traces of plasmal were found, in contrast to the appreciable yields of supposed plasmal regularly isolated as a separate fraction after alkaline hydrolysis. Methanolic acid hydrolysis of ox-brain phospholipids readily gave the plasmal fraction in the form of dimethylacetals in the unsaponifiable fraction. This finding strengthens the doubts expressed in the last paper (Lovern & Olley, 1953*b*) whether the material isolated gravimetrically was genuinely plasmal. Its real nature remains obscure.