XXXI. SULPHUR IN ORGANIC AND INORGANIC COMBINATION IN PASTURE GRASSES.

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THE sulphur content of pastures has recently become a subject of considerable importance both from the point of view of sulphur fertilisation of soils [Alway, 1927] and in relation to the sulphur requirements of sheep for wool-production [Robertson and Marston, 1928]. The question of sulphur deficiency in soils has led to very thorough surveys of the total sulphur content of pastures in different parts of the world, but the organic sulphur content of pastures has received little or no attention. Nevertheless the separation of the total sulphur content of grasses into organic and inorganic components is important in seeking a correlation between the sulphur content of pastures and their value for purposes of wool-production, as only organic sulphur can be made use of by animals in biological synthesis. In the present paper, a method is described whereby the organic sulphur of pastures can be readily determined. It is assumed that all the inorganic sulphur in green leaves is in the form of sulphate, whether free or as ethereal sulphate. This sulphate-sulphur is therefore estimated and deducted from the total sulphur, giving a value for the organic sulphur.

As regards the nature of the organic sulphur compounds in grass, they probably occur as constituents of protein. Woodman [1926] finds 27.92 % crude protein in autumn cuts of English grass, while Rigg and Askew [1929] find an average value of 33.37 % crude protein for eight samples of New Zealand pasture. "Crude protein" includes chlorophyll and other non-protein nitrogen compounds, but the figures are of the right order to account for the organic sulphur present in grass, assuming that grass-protein contains the average amount of sulphur. In this connection it is to be noted that a water-soluble protein prepared from grass, as subsequently described, by the method of Chibnall and Schryver [1921] did not contain sulphur. This substance was a proteose, however, not a native protein, and it represented only a small proportion of the total nitrogen of the grass.

Since protein-sulphur in grass would have to form part of an amino-acid, the question of the identity of this sulphur amino-acid immediately arises. Robertson and Marston [1928] apparently make a tacit assumption that the acid is cystine, this being the sulphur constituent of wool and also of widespread occurrence elsewhere in nature [Jones, Gersdorff and Moeller, 1924].

Such cystine could be directly assimilated by sheep and used in the growth of wool. However, it is significant that Mueller [1923] finds that methionine, a sulphur amino-acid isolated from caseinogen, occurs in wool along with cystine. In the experimental section of this paper, attempts to find cystine in grass by the ordinary precipitation and colorimetric methods are described. Though these were greatly hampered by the difficulty of decolorising the hydrolytic products from grass, the results obtained were consistently negative, from which it would appear that most of the organic sulphur of grass is not in the form of cystine.

EXPERIMENTAL.

Preliminary treatment of the grass.

The following grasses were analysed for sulphur: Cocksfoot (*Dactylis glomerata*), Perennial Rye (*Lolium perenne*), Prairie Grass (*Bromus unioloides*), Yorkshire Fog (*Holcus lanatus*), and Hair Grass (*Festuca bromoides*). Only the blades were collected as these give a more homogeneous sample than is obtained by the inclusion of stalks and heads, which are drier and less palatable to stock. The grass was freed from colourless and withered leaves and foreign matter, rinsed with water and spread on wire netting to dry. It was then reduced to a handy form for analysis by toasting on a hot plate and passing through a mincer. The arrangement consisted of a galvanised iron tray 3 ft. by 2 ft. standing on large tripods; 2 inches below this, a sheet of iron was supported on smaller tripods and heated by four Bunsen burners, the hot air from its upper surface maintaining the bottom of the tray at about 120°. The grass was turned continually till dry and then clipped into short lengths. When sufficiently brittle it was put through a mincer and readily reduced to a fine powder. The powder was placed in bottles which were heated to 110° in a toluene-oven and carefully stoppered.

In order to determine the moisture content of the grasses, quantities (about 8 g.) of the fresh, superficially dry specimen were placed in two beakers. One sample was dried in a toluene-oven at 110°, the other in an air-oven at 120°. The values obtained generally agreed within 0.3 %.

Estimation of total sulphur in powdered grass.

The estimation of sulphur in grass by means of sodium peroxide as used at the Rowett Institute, Aberdeen, apart from its rather difficult technique, has the disadvantage that occasionally sudden ignition occurs, rendering the estimation worthless, and also that the sodium peroxide rapidly destroys the nickel crucibles. A method was therefore devised by which the Benedict-Denis reagent could be used for the oxidation, and this proved both rapid and satisfactory. After several trials, the following procedure was adopted. 1-1.5 g. of grass powder is transferred from a weighing bottle to a 4 in. porcelain dish. 50 cc. of the Benedict-Denis reagent (125 g. crystallised copper nitrate, 125 g. sodium chloride, 50 g. ammonium nitrate, 500 cc. water, all free from sulphate)

is then added and the mixture evaporated to dryness on a curved asbestos gauze. It is then brought to a dull red heat at which the copper oxide formed completely oxidises the grass. The product is taken up with 50 cc. of 2N HCl, gently evaporated to dryness, and finally extracted by warming with successive portions of 2N HCl, 100 cc. being used for the purpose. Extracts and washings are made up to 250 cc. This is filtered by suction and portions of 100 cc. are taken in which sulphate is estimated by precipitation with barium chloride. In the precipitation of small amounts of barium sulphate, easy filtration is ensured by carrying out the precipitation at the boiling point in 3 % hydrochloric acid, using a large excess of barium chloride. The solution will clear in less than 3 hours and the precipitate is crystalline.

Three grasses in which sulphur was estimated, both by means of sodium peroxide and the Benedict-Denis reagent, gave the following results:

	Cocksfoot	Perennial Rye	Yorkshire Fog	} % sulphur
Sodium peroxide method	0.438	0.460	0.391	
Benedict-Denis method	0.442	0.462	0.390	

Estimation of sulphate-sulphur in powdered grass.

Attempts to extract the sulphates present in powdered grass by simply boiling with dilute hydrochloric acid gave rise to solutions containing colloids which were liable to be absorbed when the sulphates were precipitated with barium chloride. To overcome this, experiments were made in which the grass was first subjected to fusion in nickel crucibles with such agents as sodium carbonate, sodium acetate, borax, etc. and then extracted. This certainly obviated the formation of colloids, but apparently led to reduction of some of the sulphate, as low and variable results were obtained. The method of Rosenheim and Drummond, using benzidine hydrochloride as a precipitant, was then tried on a grass extract, the presence of colloids being disregarded as the estimation depends on a titration with alkali. Low values were obtained, however, as the alkali reacted very slowly with the precipitate, protected as it was with colloidal material. Finally it was discovered that decolorising charcoal could be conveniently used to remove colloids yielding a solution quite suitable for gravimetric estimation of sulphate. After some preliminary experiments, the following method was found to give satisfactorily constant results. 2-3 g. of grass powder is transferred from a weighing bottle to a 600 cc. beaker and boiled gently for half an hour with 100 cc. of 2N NaOH. 200 cc. of 2N HCl are then added and the mixture is boiled for a further half-hour. 2 g. of decolorising charcoal, previously freed from sulphate by repeated extraction with boiling hydrochloric acid, are added and the boiling continued for about 20 minutes. Finally, the product is filtered through a 4 in. Büchner filter, and the precipitate washed several times with dilute hydrochloric acid so as to leave no adhering sulphate. The extract is made up to 500 cc. and sulphate is estimated in 200 cc. portions by precipitation with barium chloride.

Results of sulphur estimations.

Examination of five common grasses yielded the results shown in Table I.

Table I.

Grass	Date of sampling	% water	% total sulphur	% sulphate-sulphur	% organic sulphur (by difference)
Cocksfoot	May 10	75.0	0.44	0.21	0.23
Perennial Rye	Aug. 15	77.1	0.46	0.23	0.23
Prairie Grass	Aug. 21	80.0	0.59	0.34	0.25
Yorkshire Fog	Aug. 21	77.0	0.39	0.13	0.26
Hair Grass	Aug. 21	73.3	0.35	0.10	0.25

It will be seen that there is no great variation in the organic sulphur content of these grasses. Indeed, Yorkshire Fog and Hair Grass, which are worthless as pasture, are slightly higher in organic sulphur than the valuable grasses Cocksfoot and Perennial Rye. It follows that the organic sulphur content of grasses does not necessarily bear any relation to their palatability and nutritive value.

On the other hand, a great variation is observed in the sulphate-sulphur content of the different species, possibly due to different amounts of sulphate in the soils on which the grasses were growing. It seems probable that the increase in sulphur content of pastures observed on making sulphur applications to soil is due to an increase in the inorganic sulphur of the grasses.

The water-soluble protein of grass.

In investigating the question of the occurrence of cystine in grass, an obvious point of attack is the separation and hydrolysis of the leaf protein. Hence it was decided to prepare and examine the leaf protein described by Chibnall and Schryver [1921]. This was carried out as follows. About 6 lb. of fresh Perennial Rye grass was minced first with a coarse, then with a fine cutter, and treated with an equal weight of water saturated with ether. After allowing 20 minutes for cytolysis to take place, the mixture was expressed through silk muslin, a green colloidal solution being obtained. This was filtered by means of a number of large funnels, as the ether present makes suction filtering disadvantageous. The resulting clear solution was light brown in colour. The water-soluble protein was precipitated by adding sufficient $2N\ H_2SO_4$ to make the whole solution $N/12$. The flocculent white precipitate was separated by siphoning off the supernatant liquid, filtering, and washing the precipitate with alcohol and ether. Some 10 g. of crude protein was thus obtained. This was dissolved in 0.3 % solution of KOH in 60 % alcohol, as used by Osborne and Wakeman [1920], and precipitated by careful addition of dilute HCl. The process was repeated twice, after which the isoelectric point was determined and found to be at $p_H\ 7.3$. The protein was found to be soluble in 0.4 % ammonia in 60 % alcohol, and this was then used instead of KOH to treat the crude protein. The protein gave both the xanthoproteic and the biuret reactions, though Chibnall and Schryver could get no protein colour

reactions with the similar material which they isolated from cut grass. The rose tint observed in the biuret test and the turbidity produced when the aqueous solution was treated with ammonium sulphate indicated that the substance was a simple proteose or hydrolysed protein. The purified material was carefully tested and found to contain no sulphur. It was concluded that the organic sulphur must have remained in the solid residue from the water-soluble products. A portion of this was analysed and found to contain 0.42 % of sulphur. Hence it was decided to hydrolyse the whole grass and test the product for cystine.

Testing the products of hydrolysis of grass for cystine.

Folin's method for the isolation of cystine was first applied to grass powder as follows. 500 g. of powdered Cocksfoot grass were hydrolysed with 2 litres of 20 % HCl on the water-bath for 144 hours. The liquid was filtered, partially neutralised with 150 cc. of 40 % NaOH and evaporated to low volume at 10 mm. pressure. Hot saturated sodium acetate was added till there was no mineral acid reaction to Congo red, and after standing for two days the precipitate was filtered by suction, dissolved in 100 cc. of 5 % HCl and boiled with successive quantities of decolorising charcoal till it was faintly yellow in colour. The solution was then brought to p_H 5 and allowed to stand. A white amorphous precipitate containing a good deal of calcium oxalate was formed and this was tested for cystine as described by Hunter and Eagles [1927] but without result. Finally the precipitate was tested with nitroprusside and found to contain no sulphur. In view of the quantity of grass taken, such a result throws considerable doubt on the assumption that the whole of the organic sulphur in grass can be represented as cystine.

It was now decided to test the hydrolysis products colorimetrically for cystine. The method of Sullivan [1925, 1926] was adopted as this is supposed to be the most specific estimation short of direct isolation. The test solution was prepared by boiling 500 g. of grass powder with conc. HCl for 6 hours, filtering, distilling off most of the acid *in vacuo*, taking up with water and decolorising by means of charcoal. Finally it was concentrated to about 200 cc., cooled, rendered slightly alkaline with 10 % ammonia [Okabe, 1928], filtered and reacidified. On testing 2 cc. with sodium 1:2-naphthaquinone-4-sulphonate, as described by Rimington [1929], no red colour stable to hydro-sulphite could be produced. Thus the colorimetric method also yielded a negative result. A point to be considered, however, is that great difficulty is experienced in decolorising the hydrolysis products from grass, which almost resemble ink in appearance, the colour being perhaps due to combination of tannin with the iron present in the grass. It has been shown by Folin and Marenzi [1929] that cystine is considerably adsorbed by decolorising charcoal, but it is hardly conceivable that the whole of the cystine could be thus adsorbed. Again, the toasting process might have produced chemical changes in the cystine, which is not as stable in protein as in the free state [Brand and

Sandberg, 1926]. Hence fresh grass was used instead of grass powder. Several pounds of fresh, minced Cocksfoot were hydrolysed with concentrated HCl in a large silica basin for 6 hours. The liquid was filtered, concentrated *in vacuo* nearly to dryness, taken up with water, treated with 30 % sodium acetate solution till neutral to Congo red, allowed to stand for 48 hours and filtered. Precipitate A was so obtained. Half of the filtrate was then treated by Gaskell's [1908] method for the isolation of cystine from urine: the solution was rendered slightly alkaline with 10 % ammonia, treated with an equal volume of acetone and just acidified with acetic acid, when a precipitate settled out and was filtered off (Precipitate B). Precipitates A and B were united, dissolved in dilute HCl, decolorised with charcoal and tested for cystine by Sullivan's method. The result was negative. The remaining half of the filtrate was then treated with 50 cc. of a 30 % solution of phosphotungstic acid in 5 % H_2SO_4 . A gummy brownish precipitate was obtained (Precipitate C). This was washed with dilute phosphotungstic acid solution and then dissolved in dilute NaOH. Phosphotungstic acid was removed by means of barium chloride and the barium by means of sulphuric acid. The resulting solution was decolorised with charcoal and tested by Sullivan's method. This time a slight colour was obtained, but comparison in a Kober colorimeter with a 0.01 % solution of cystine showed that it represented only a trace of cystine and indeed might have been due to tyrosine.

A final experiment was carried out, using Perennial Rye grass instead of Cocksfoot. The sulphate-sulphur was first removed by repeated extraction with dilute HCl, and the grass then hydrolysed with concentrated HCl in a silica basin. That hydrolysis proceeds slowly was shown by the fact that after 3 hours the insoluble residue still contained 0.20 % of sulphur. The product was examined by Gaskell's method and with phosphotungstic acid as described above, but only negative results were obtained. To sum up, no evidence was obtained from the experiments on the hydrolysis products of grass in support of the assumption that the organic sulphur of grass is present in the form of cystine.

SUMMARY.

1. The Benedict-Denis method of oxidation has been applied to the determination of the total sulphur content of grasses.
2. A method has been devised for the estimation of sulphate-sulphur in grass or green leaves.
3. The analytical results for five grasses show no relation between the nutritive value and the organic sulphur content of grasses.
4. The water-soluble protein obtained from grass did not contain sulphur.
5. The occurrence of cystine in quantity corresponding to the organic sulphur content of grass could not be demonstrated.

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