XXVI. THE GLYCERIDE FATTY ACIDS OF FORAGE GRASSES.

I. COCKSFOOT AND PERENNIAL RYEGRASS.

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Although grasses constitute the chief food of most herbivorous animals we believe we are correct in stating that no knowledge exists of the amount of true fats and phosphatides which they contain. The agricultural chemist, it is true, generally determines the "ether extract" when analysing samples of grasses, but it has been realised since the pioneer work of Kellner that this value does not by any means represent true fat, and in nutrition experiments it is generally converted into the corresponding "starch equivalent."

During the past two years a large amount of data has been collected in this laboratory concerning the composition of the ether extract of various forage grasses, and in particular the amount of true fats, phosphatides, unsaponifiable material (including sterols), waxes and pigments has been determined. Before presenting the general results of this research, which are of agricultural as well as chemical interest, it is proposed to describe in some detail the more extensive investigations which have been made into the chemical constitution of the above-mentioned groups of substances present in the well-known forage grasses, cocksfoot (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). Details of the wax investigation have already been published [Pollard *et al.* 1931]; the present paper deals with the glyceride fatty acids.

Preparation of the glyceride fatty acids. The ether extract from the fresh grass was prepared by the method of Chibnall and Channon [1927, 1] as recently modified in this laboratory [Pollard *et al.* 1931]. The extract was concentrated until it contained about 25 % of fatty material, when 2 volumes of warm acetone were added. The mixture was allowed to stand in the ice-chest for some hours, and the precipitated waxes and phosphatides were removed by filtration. The acetone-ether filtrate, which contained the glycerides, unsaponifiable material, pigments, *etc.*, was then treated by the method of Chibnall and Channon [1927, 2] to obtain the unsaponifiable material and the glyceride fatty acids.

THE GLYCERIDE FATTY ACIDS OF COCKSFOOT.

Batch P. 33 kg. of fresh grass (4·9 kg. dry wt.) gave 283 g. of ether extract, from which were obtained 107·5 g. of a semi-solid mixture of fatty acids. The yield was therefore 38 % of the ether extract and 2·2 % of the dry weight of the grass. The acids had an iodine value (I.V.) of 170 and a mean molecular weight of 278. Before saponification the glyceride fraction contained only 2·3 % of free fatty acids.

First separation of the saturated and unsaturated acids.

Chibnall and Channon [1927, 2] attempted to prove the presence of oleic acid in the glyceride fatty acids from cabbage leaves by using the method of Levene and Rolf [1922], which depends on the difference in solubility in various solvents of the barium and lead soaps of the saturated acids, oleic acid and the more unsaturated acids. No evidence for the presence of oleic acid was, however, obtained.

In the present case 107·5 g. of fatty acids were converted into the barium soaps and then treated with benzene containing 5 % of 95 % alcohol. The insoluble portion, which should have contained the barium soaps of the saturated acids and of oleic acid, was freed from barium and the acids were converted to the lead soaps. These were then treated with ether, in which solvent the soaps of the saturated acids are fairly insoluble and those of oleic acid are soluble. The soluble fraction was freed from ether by evaporation, the dry soaps were dissolved in hot benzene, and lead was removed by hydrogen sulphide. The recovered acids were then again put through the barium and lead soap treatments successively. The final fraction, which should have contained any oleic acid, weighed only 1·5 g., and had an I.V. of 192. It was clear from this result that the amount of oleic acid in the original mixed fatty acids must have been very small if, indeed, the acid was present at all. The lead soaps insoluble in ether gave 7·2 g. of solid acid of M.P. 49–50° and I.V. 12. Assuming that the I.V. is due to a small proportion of the unsaturated acids mentioned below the corrected amount of saturated fatty acid was 6·7 g., or 6·2 % of the original mixture. The unsaturated acids recovered from the various fractions of lead and barium soaps, together with the so-called oleic acid fraction of 1·5 g. mentioned above, were collected, and gave 82·5 g. of liquid acids of I.V. 185. This corresponds to 76·7 % of the original acid mixture, showing that there had been a loss in the series of operations of approximately 17 %. It will be shown later that this falls almost entirely on the unsaturated acids.

Identification of the unsaturated acids by bromination.

Hexabromo-derivatives. 10·4 g. of the unsaturated fraction mentioned above were brominated in anhydrous ether at 0° in the usual way. 5·89 g. of insoluble bromo-derivatives were formed, which were completely soluble in

boiling benzene, showing the absence of octabromo-derivatives. On cooling the benzene solution 5·1 g. of hexabromostearic acid crystallised out. (Found: M.P. 180–180·5°: C, 28·8; H, 4·1; Br, 63·0 %. $C_{18}H_{30}O_2Br_6$ (M.P. 180–181°) requires C, 28·5; H, 4·0; Br, 63·3 %.) The amount of α-linolenic acid was therefore 21·2 % of the unsaturated fraction or 16·3 % of the original fatty acid mixture.

Tetrabromo-derivatives. The bromination products soluble in ether were freed from excess bromine by treatment with a solution of sodium thiosulphate, the ethereal solution dried with anhydrous sodium sulphate, the ether removed by evaporation and the residue treated with cold light petroleum (B.P. 40–60°). The insoluble portion weighed 8·9 g. It was soluble in hot solvents, but on cooling separated as an oil, and no crystalline tetrabromostearic acid, M.P. 114°, could be obtained. Analysis showed that it was probably a mixture of isomeric tetrabromostearic acids. (Found: C, 34·3; H, 4·9; Br, 52·6. $C_{18}H_{32}O_2Br_4$ requires C, 36·0; H, 5·4; Br, 53·3 %.) That the acid, M.P. 114°, derived from ordinary linoleic acid (9 : 12-octadecadienoic acid) was present, although it could not be obtained crystalline, was shown by conversion of the tetrabromo-acids into the corresponding tetrahydroxy-acids. 1 g. of the tetrabromo-fraction was reduced by Rollet's method [1909] and the resulting unsaturated acids oxidised by the method of Haworth [1929]. 0·2 g. of mixed α- and β-sativic acids was obtained, M.P. 155–163°. (Found: C, 62·1; H, 10·4. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %.) In Table I this fraction is referred to as α-linoleic acid.

Bromo-derivatives soluble in light petroleum. This fraction should have contained any dibromostearic acid, any saturated acid which still remained in the unsaturated fraction now under discussion, and any tetra- and hexa-bromo-acids derived from corresponding unsaturated acids. Following the nomenclature of Kaufmann and Keller [1931] we shall refer to these unsaturated acids as β-linoleic and β-linolenic acids respectively. Both are probably mixtures of unknown isomerides, which have the property in common of giving —under the usual well-defined conditions—bromo-derivatives which have solubilities different from those of the known α-linoleic and α-linolenic acid respectively.

The bromine content of this light petroleum-soluble fraction was 45·8 %. To remove any residual saturated acid, 8·0 g. of the products were converted to the lead soaps, which were then dissolved in a small volume of warm ether and left overnight in the ice-chest. The insoluble soaps yielded 0·6 g. of saturated acid. This amount represents 5·7 % of the unsaturated fraction. The soluble lead soaps yielded a bromo-product giving the following analysis: C, 38·1; H, 5·7; Br, 51·5 %. It is shown later that Bertram's method of oxidation gave a higher value (12 % as against 5·7 %) for the saturated acids than the lead soap precipitation methods, so that the above fraction probably still contained saturated acid. As tetrabromostearic acid has 53·3 % Br, it follows that this fraction must have contained some hexabromostearic acid and but very

little dibromostearic acid. To investigate the fraction further 1 g. was debrominated by the method of Rollet [1909] and the resulting unsaturated acids oxidised by alkaline permanganate [Haworth, 1929]. A small amount of mixed α- and β-sativic acids was isolated, showing the presence of a tetrabromostearic acid in the bromo-fraction, but no dihydroxystearic acid was obtained, again showing the probable absence of oleic acid from the original mixed fatty acids.

The bromination experiments therefore suggest that the unsaturated fraction of the fatty acids had the composition shown in Table I. It is assumed

Table I. *Composition of the glyceride fatty acids of cocksfoot as suggested by bromination experiments.*

	Unsaturated fraction %	Original mixed fatty acids %
Saturated acids (Bertram)	12	15
Oleic acid	—	—
α-Linoleic acid	38·5	29·5
β-Linoleic acid	21·0	16
α-Linolenic acid	21·0	16
β-Linolenic acid	7·5	6
Total	100·0	82·5*

* There was a loss of 17 % in the initial fractionation into saturated and unsaturated acids.

that oleic acid is absent, and the amounts of β-linoleic acid and of β-linolenic acid have been calculated from the original I.V. (185·3) of the unsaturated fraction.

Identification of the unsaturated acids by oxidation.

1. *Method of Lapworth and Mottram [1925].* This method is known to give 95 % yields of dihydroxystearic acid from oleic acid, and was applied in the present case with the object of demonstrating, if possible, the presence of oleic acid in the unsaturated fraction. 11 g. of this fraction were dissolved in 1 litre of a 1 % solution of sodium hydroxide, which was then poured into 8 litres of ice-cold water. 800 cc. of 1 % solution of potassium permanganate were added quickly with stirring, and after 5 minutes a stream of sulphur dioxide was passed in until the mixture was colourless. Concentrated hydrochloric acid (150 cc.) was then added, and the white flocculent precipitate filtered off, washed, and dried *in vacuo* at 100°. Treatment with light petroleum (B.P. 40–60°) removed 1·54 g. of solid acid, M.P. 35–40°. The residue (M.P. 145°), weighing 0·9 g., was extracted with ether, which removed 0·4 g. of a gum from which no dihydroxystearic acid could be obtained. It is improbable therefore that any oleic acid was present in this unsaturated fraction. The material insoluble in both light petroleum and ether (0·45 g.) melted indefinitely at 163–165° with previous softening at 145–150°. It was a tetrahydroxystearic acid. (Found: C, 61·6; H, 10·4. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %.) 0·17 g. was heated with ethyl acetate and filtered hot. On cooling α-sativic acid (0·06 g.) crystallised out, M.P. 159–160°. The insoluble residue (0·1 g.) was β-sativic acid, M.P.

171·5–172·5°. According to Meyer and Beer [1912] α- and β-sativic acids fractionated in this way melt at 163° and 173° respectively.

The aqueous filtrate, after removal of the above-mentioned white flocculent precipitate, amounted to approximately 12 litres. 8 litres of this were concentrated *in vacuo* to 1 litre and filtered hot. The residue on the filter-paper was boiled with ethyl alcohol and again filtered hot. On cooling the filtrate deposited 0·25 g. of white material, M.P. 202°. It was recrystallised from water; M.P. 203–4°. (Found: C, 56·9; H, 9·2. $C_{18}H_{36}O_8$ requires C, 56·8; H, 9·5 %.) Hazura records a melting-point of 203–5° and Krzizan of 204–5° for linusic acid [Lewkowitsch, 1921].

2. *Method of Haworth [1929]*. This method differs from the former only in certain details of procedure, and was applied to confirm the absence of oleic acid. 9·6 g. of the unsaturated fraction gave insoluble oxidation products which, after extraction with light petroleum as before, yielded on extraction with ether 0·35 g. of a dark gum. No crystalline dihydroxystearic acid could be obtained from this material. (Found: C, 66·6; H, 9·3. $C_{18}H_{36}O_4$ requires C, 68·35; H, 11·5 %.)

These two oxidation methods therefore confirm the presence of linoleic and linolenic acid in the unsaturated fraction, and the probable absence of oleic acid.

Determination of the total saturated fatty acids.

Method of Twitchell [1921]. The procedure followed that recommended by Hilditch and Priestman [1931]. Two experiments were carried out, one in which the amount of lead acetate added was only slightly in excess of that required to convert the saturated acids into their lead soaps, and the other in which it was sufficient to convert all the acids present into their lead soaps.

In the first experiment 9·84 g. of the unsaturated fraction were dissolved in 30 cc. of 95 % (by weight) alcohol and 1·2 g. of lead acetate in 70 cc. of boiling 95 % alcohol added. The hot mixture was allowed to cool very slowly to 15–20° and then left overnight in an oven so that the temperature was strictly maintained within these limits. The crystalline lead soaps were then filtered off, washed with 95 % alcohol till the washings no longer became turbid on diluting with a drop of water, and then recrystallised as before from 100 cc. of hot 95 % alcohol containing 0·5 g. acetic acid. 0·63 g. of dark brown solid acid was recovered from the lead soaps; M.P. 48°, I.V. 13. Correcting for the I.V. this amount of acid corresponds to 6·0 % of the unsaturated fraction. In the second experiment the value was slightly higher, 6·8 %. Both of these results are of the same order as that given by the lead soaps in the bromination experiments previously described (p. 220) which was 5·7 %, but are definitely lower than that given by the Bertram oxidation described below.

Method of Bertram [1927]. The procedure followed that recommended by Hilditch and Priestman [1931]. 9·63 g. of the unsaturated fraction were dissolved in 200 cc. of a 5 % solution of potassium hydroxide. Water (1300 cc.)

containing 60 g. of potassium permanganate was then added, the temperature being maintained between 35° and 50°. After standing overnight the solution was decolorised with sulphur dioxide and exhaustively extracted with light petroleum (B.P. 60–80°). There was obtained 1·89 g. of a soft brown solid from which 0·34 g. of non-acidic material was removed in the usual way. The acidic products were precipitated twice as the magnesium soaps and on decomposition yielded 1·14 g. of recrystallised acid.

This yield is equivalent to 11·8 % of the unsaturated fraction, which is about 5·6 % higher than the corresponding value given by Twitchell's method. This large difference might be due to (1) the solubility of lead palmitate and higher homologues in alcohol being increased by the presence of relatively larger amounts of lead linoleate and linolenate; (2) the presence of saturated acids lower in the series than palmitic, which are known to be only partially precipitated, if at all, in the Twitchell process; (3) the fact that Bertram's method has given saturated acids lower than palmitic acid by oxidation of unsaturated acids with the double bonds in positions near the end of the carbon chain. It is shown in a later experiment (p. 233) that the amount of shorter acids pre-existing in the original mixed fatty acids was exceedingly small, and analysis does not suggest the presence of shorter acids in the Bertram oxidation products. (Found: C, 75·3; H, 12·6: mol. wt. by titration, 266. $C_{16}H_{32}O_2$ requires C, 74·9; H, 12·6; mol. wt. 256. $C_{18}H_{36}O_2$ requires C, 76·0; H, 12·8 % and mol. wt. 284.) Furthermore the shorter fatty acids are not readily precipitated as the magnesium soaps. We attribute the difference between the Twitchell and Bertram methods therefore to the residual solubility of lead palmitate, and accept the higher value given by the latter method as correct. It is interesting to note that Kaufmann finds that Bertram's method frequently gives results which are 1 to 2 units % higher than those given by the lead soap methods.

Identification of the saturated acids.

8·0 g. of recrystallised acids, M.P. 50°, were esterified with ethyl alcoholic hydrogen chloride in the usual way. The 8·4 g. of crude esters thus obtained were distilled at 0·3 mm. giving four fractions and a residue. Each of these was then saponified, and the recovered acids were crystallised repeatedly from acetone or pyridine. The collected analytical data are given in Table II, and the following conclusions can be drawn.

(*a*) Fractions 1 and 2 consist of nearly pure palmitic acid. There is no evidence of a lower acid (ethyl myristate melts at 10·5–11·5°, and myristic acid has mol. wt. 228).

(*b*) Fraction 5 is a mixture of longer fatty acids of mean molecular weight corresponding to $C_{26}H_{52}O_2$. It is clearly the usual "cerotic acid" which occurs extensively in plant fats and waxes, and which was shown by Francis, Piper and Malkin [1930] to be a mixture. A similar product, of slightly higher mean molecular weight, was found in the wax fraction of the cocksfoot ether extract

Table II. *Fractionation of the saturated acids from cocksfoot.*

Esters				Acids					
Fraction	B.P. at 0·3 mm.	Weight (g.)	M.P. (approx.)	Weight (g.)	Purification by recrystallisation from acetone (A) or pyridine (P)	M.P.	C (%)	H (%)	mol. wt. (titration)
1	124°	1·32	22–22°	1·1	Once from A	60·5–61·5°	74·9	12·7	258
					Five times from A	62·0–62·7°	75·0	12·6	—
2	124°	2·35	21–22°	2·1	Once from A	60–61°	75·15	12·65	258
3	130°	1·31	23–32°	1·2	Once from A	55·5–57·5°	75·1	12·5	263
					Five times from A	54–55°	—	—	—
4	135–145°	1·15	29–32°	1·05	Once from A	52·5–53·5°	75·7	12·6	293
					Three times from A	62·0–63·5°	77·5	13·0	—
5	Not distilled	1·96	38–42°	1·8	Once from A	67–68°	77·4	13·0	—
					Four times from A	72·5–73·5°	78·3	13·3	384
					(followed by)				
					Once from P and twice from A	79·5–80·5°	78·4	13·2	—
Ethyl stearate			34–35°	—	Palmitic acid requires	63°	74·9	12·6	256
Ethyl palmitate			25°	—	Stearic acid „	70°	76·0	12·8	284
Ethyl myristate			10·5–11·5°	—	"Cerotic acid" „	80–82°	78·7	13·2	396

[Pollard *et al.* 1931], and the small amount encountered in the present case may well be due to the residual solubility of the waxes in the ether-acetone used to precipitate the latter in the preparation of the glyceride fraction.

(*c*) Fractions 3 and 4 call for more extended discussion. On repeated recrystallisation the M.P. of the former acid was depressed slightly to 54–55°. It was clearly a mixture and the M.P. suggested 70 % palmitic acid with 30 % stearic acid. Such a mixture however was shown to yield without difficulty a higher-melting fraction when recrystallised three times from acetone. To obtain further evidence six mixtures, all of which had a mean molecular weight and carbon and hydrogen content similar to that of Fraction 3, were prepared, and their melting-points determined. Each was then recrystallised three times from acetone or acetone-pyridine, and the M.P. of the least soluble fraction determined. The data obtained are given in Table III. It will be seen that all the

Table III. *Melting-points of certain mixtures of fatty acids prepared from purified acids.*

No.	% of palmitic acid M.P. 61–61·7°	% of stearic acid M.P. 68–68·5°	% of eicosanic acid M.P. 75–75·2°	% of cerotic acid M.P. 77–78°	M.P. of mixture	M.P. of least soluble fraction after 3 recrystallisations from acetone
1	91	—	—	9	59·5–60·5°	84°
2	67	27	—	6	52·5–54·0°	66·5–68°
3	69	29	—	2	54–55°	60–61°
4	70	30	—	—	55–55·5°	61·5–63·5°
5	78	—	22	—	54·5–55·5°	74·5–75·0°
6	67	27	6	—	53·5–54°	54–54·5°

binary mixtures were readily separated into higher-melting fractions, especially those of palmitic acid with an acid higher in the series than stearic acid. The mixture composed of 67 % palmitic, 27 % stearic and 6 % eicosanic acids was the only one which was in any way analogous to Fraction 3, and there would seem to be no doubt that this represents fairly closely the composition of the latter. In the same way Fraction 4 is probably a similar mixture in which a small amount of "cerotic acid" replaces the eicosanic acid. The evidence from Tables II and III therefore shows that the saturated fraction consists of about 60 % palmitic acid, 20 % stearic acid and 20 % of a mixture of acids containing 20, 22, 24, *etc.*, carbon atoms, of which the higher ones constitute the so-called "cerotic acid."

Thiocyanometric analysis of the fatty acids.

Description of the method. The fact that oleic acid is always assumed to be present in glyceride fatty acid mixtures of the type under discussion made it imperative that more definite evidence for its presence or absence should be obtained. It was therefore decided to apply Kaufmann's thiocyanometric method of analysis, which is being increasingly used in researches into the composition of fatty acid mixtures. A list of references to Kaufmann's work [1925–31] on thiocyanometric analysis is given at the end of this paper, but

the one of chief interest in connection with mixtures containing linolenic acid is that of Kaufmann and Keller [1929]. The method of analysis is based on the observation that, whereas oleic, linoleic and linolenic acids absorb 2, 4 and 6 atoms of halogen, they absorb only 2, 2 and 4 CNS radicals respectively. The thiocyanogen number (CNS v.) is calculated for purposes of comparison as an iodine number (I.V.), and if S, O, L and Ln represent the percentages of saturated, oleic, linoleic and linolenic acids respectively, it can be readily shown that the composition of a mixture of fatty acids can be calculated from the following three equations.

$$S + O + L + Ln = 100,$$

$$O + 2L + 3Ln = \frac{100}{90{\cdot}6} \text{ I.V.},$$

$$O + L + 2Ln = \frac{100}{90{\cdot}6} \text{ CNS V.}$$

The validity of the method depends on Kaufmann's assumption that the CNS v. of linolenic acid is 183. The evidence for this is indirect, as the pure acid has not yet been prepared. This point will be discussed in greater detail after the results of our own analysis have been given.

Application of the method to the unsaturated fraction. The thiocyanogen solution was prepared by Kaufmann's method [1928] and a reaction period of 24 hours was used. The CNS v. was found to be 115·3. It has already been shown that the I.V. of this fraction was 185·3, that the amount of α-linolenic acid was 21·2 % (bromination experiments) and that the amount of saturated acid was 11·8 % (Bertram). Using these values in conjunction with the equations given above, the analysis given in Table IV was obtained. It will be

Table IV. *Thiocyanometric analysis of the glyceride fatty acids of cocksfoot and ryegrass.*

	Batch P. Cocksfoot		Batch N. Cocksfoot	Batch C. Ryegrass	
	Saturated acids by Twitchell's method %	Saturated acids by Bertram's method %	Saturated acids by Twitchell's method %	Saturated acids by Twitchell's method %	Saturated acids by Bertram's method %
Saturated acids	11·1	15·2	10·6	11·9	16·8
Oleic acid	12·5	8·9	16·5	22·5	17·6
Linoleic acid	33·4	29·8	30·9	26·1	21·2
Linolenic acid	25·8	29·5	42·0	39·5	44·4
Total	82·8*	83·4*	100·0	100·0	100·0
α-Linolenic acid by bromination	16·3	16·3	19·5	16·6	16·6

* There was a loss of 17 % in the initial fractionation into saturated and unsaturated acids.

seen that the method postulates the presence of 8·9 % of oleic acid in the original mixed fatty acids. After the evidence obtained from the bromination and oxidation experiments this result was unexpected, and we decided to repeat the analysis on a second sample of cocksfoot glyceride fatty acids.

Analysis of another sample of cocksfoot glyceride fatty acids.

Batch N (fresh weight, 1·84 kg.; dry weight, 276 g.) gave 18·0 g. of ether extract and 6·4 g. of glyceride fatty acids. The I.V. was 184·8 and the CNS v. was 119. 6·0 g. of the acids were separated into saturated and unsaturated acids by Twitchell's method. The weight of solid acids was 0·68 g.; M.P. 52–53°; I.V. 13. Correcting for the small amount of unsaturated acid present the yield of saturated acids was 10·6 %. Bromination of the unsaturated acids in anhydrous ether gave 3·1 g. of hexabromostearic acid, showing that the original mixed fatty acids contained 19·5 % of α-linolenic acid. Unfortunately sufficient original fatty acid was not available for a determination of the saturated acids by Bertram's method. The result of the thiocyanometric analysis is given in Table IV. The presence of a large amount of oleic or some other unsaturated acid containing one double bond is again suggested.

The low I.V. (13) of the saturated fraction obtained by Twitchell's method shows that this cannot have been due to petroselinic (6-octadecenoic) acid, which gives an alcohol-insoluble lead soap.

Discussion.

It will be convenient first of all to summarise in some detail the results of the oxidation and bromination experiments with Batch P.

(1) Bromination showed that 21 % of the unsaturated fraction was α-linolenic acid. From the amount of light petroleum-soluble hexabromo-derivatives it was concluded that the unsaturated fraction also contained about 7·5 % of the so-called β-linolenic acid. This may be a mixture of stereo-isomerides of the α-acid, or perhaps of acids with double bonds in different positions. On oxidation only a small amount of linusic acid was obtained, suggesting that the β-acid had been broken into quite short-chain water-soluble products.

(2) Oxidation gave small amounts of α- and β-sativic acids, showing the presence of α-linoleic acid in the unsaturated fraction. Bromination experiments suggested that β-linoleic acid was present, but on oxidation only short-chain water-soluble products were formed from it.

(3) Oxidation did not give any dihydroxystearic acid melting at either 95° or 132°. If oleic or eläidic acid were present in the unsaturated fraction the amount must have been well under 5 %. No evidence of the production of lauric acid was obtained, so that petroselinic (6-octadecenoic) acid was absent or present only in small amounts. The bromo-derivatives soluble in light petroleum had a bromine content of 51·5 %. If allowance be made for the residual amount of saturated acid known to be present, very little, if any, dibromostearic acid can have been present.

Reverting to the thiocyanometric method of analysis, Kaufmann has adopted two methods to show that linolenic acid takes up thiocyanogen at two

of its three double bonds. (1) He considers what would result if this acid acted towards CNS at one, two, or all three of its double bonds. If it acted at only one, then the CNS v. of a mixture containing oleic, linoleic and linolenic acids could never exceed 91. This was contrary to his experience with many unsaturated oils, hence the linolenic acid must act at more than one double bond. If the linolenic acid were assumed to act at all three double bonds, in the case of many linseed oils a large negative value for oleic acid was obtained. Hence Kaufmann concluded that it must act at two of its double bonds. (2) A sample of linseed oil was taken for analysis. The saturated acids were removed by dissolving the oil in pentane and leaving the solution for some time at − 18°, when the solid acids crystallised out. The oleic acid was then removed by the lithium soap method of Moore [1919], which is described later (p. 230). The remaining mixture consisted of linoleic and linolenic acids. Measurement of the I.V. enabled the amount of each constituent to be calculated. The CNS v. was then determined, and on the assumption that the linoleic acid absorbed at one and the linolenic acid at two double bonds, practically the same amount of each constituent was obtained by calculation as before.

We wish to make two criticisms of this experiment. (*a*) In the presence of large amounts of highly unsaturated acids it seems to us very doubtful if the saturated acids or the lithium oleate would be quantitatively precipitated under these conditions. If saturated acid or oleic acid still remained in the linoleic-linolenic mixture, then it is clear that the absorption coefficient used for one or both of these acids must be faulty.

(*b*) Assuming that these two acids do, as Kaufmann and Keller conclude, absorb at one and two double bonds respectively, this relationship may only hold for the particular isomerides of these acids with which they were dealing. This criticism, based on the large number of possible isomerides of both linoleic and linolenic acids, has already been put forward by Van der Veen [1931].

In the present research it has been shown quite clearly that the cocksfoot fatty acids contain large amounts of β-linoleic and β-linolenic acids. As has been stated above, these may both be mixtures of many isomerides. It is possible that isomerisation has taken place during bromination, as Van der Veen suggests, but the very low yield of sativic acid which we obtained by Haworth's method of oxidation, compared with that which the latter himself obtained from linoleic acid isolated from poppy seed and linseed oils, suggests that these isomerides were in large part already present in the original mixed fatty acids.

If we accept Kaufmann and Keller's supposition that these β-linoleic and β-linolenic acids absorb thiocyanogen at one and two double bonds respectively, then the cocksfoot fatty acids must have contained 8·9 % of oleic or some other one double bond acid. In the case of the ryegrass fatty acid we have applied Moore's lithium soap method to isolate any oleic acid (p. 230).

None was found, yet in this case the thiocyanometric method suggested 17·6 % of oleic acid.

We do not believe that our present meagre knowledge of the chemistry of the β-linoleic and β-linolenic acids is sufficient to warrant Kaufmann and Keller's assumption that the thiocyanometric method of analysis can be applied to all mixtures of fatty acids in which they are present. If we assume, for purposes of illustration, that no oleic or other one double bond acid is present in the unsaturated fraction from cocksfoot (Batch P), then the amounts of total linoleic and linolenic acids can be calculated from the I.V. alone, as has already been done in computing the composition given in Table I. The total linoleic acid is 59·5 % and the total linolenic acid is 28·5 %. Using Kaufmann and Keller's equations it is then possible to calculate the thiocyanogen absorption coefficient of either (*a*) the total linolenic acid, or (*b*) the total linoleic acid.

(*a*) The coefficient is 2·4. If the α-linolenic acid (21 %) has a coefficient of 2, then that of the β-linolenic acid (7·5 %) must be 3·5, an impossible value.

(*b*) The coefficient is 1·18. If the α-linoleic acid (38·5 %) has a coefficient of 1, then that of the β-linoleic acid must be 1·5, *i.e.* one-half of the total isomerides grouped together under this name must have a coefficient of 1 and the other half of 2.

We do not suggest that the present results provide definite evidence that certain isomeric octadecadienoic acids absorb thiocyanogen at both their double bonds because the amount of oleic or other one double bond unsaturated acid present in the mixed acids must remain uncertain.

We think, however, that our analytical methods prove that oleic acid cannot be present in cocksfoot and ryegrass glyceride fatty acids to the extent found by the thiocyanometric method. It is not to be inferred from this statement that we query the validity of this method when used by Kaufmann and Keller to determine the composition of mixed seed fatty acids. These acids, especially the less unsaturated acids, have been the subject of much research, and their constitution is known with a fair degree of certainty. But this is not so in the present case. Unlike the seed glycerides the leaf glycerides are not reserve material but are an integral part of the protoplasm of physiologically active cells; and, in the absence of confirming evidence, we have no more right to assume that the highly unsaturated acids derived from them have the same constitution as those derived from the seed glycerides than we have to assume, for instance, that the unsaturated acids of the liver are the same as those of adipose tissue. The discrepancy between the thiocyanometric and the other methods of analysis may be due to the presence of an unknown octadecenoic acid, but equally well to an unknown octadecadienoic or octadecatrienoic acid. Until further evidence for the constitution of the leaf glyceride fatty acids is available, therefore, we consider that the thiocyanometric method of analysis is unreliable.

To summarise, the results of the present research show that the cocksfoot glycerides contain palmitic, stearic, α-linoleic (9 : 12-octadecadienoic) and

α-linolenic acids. Very small amounts of higher saturated acids were found, but these were probably derived from the small amount of wax that was not removed from the ethereal solution of the glycerides by precipitation with acetone.

The glyceride fatty acids of ryegrass.

73 kg. of fresh grass (9·2 kg. dry weight) gave 415 g. of ether extract, from which were obtained 156 g. of mixed fatty acids. The yield was 37·5 % of the ether extract or 1·7 % of the dry weight of the grass. The acids had an I.V. of 175 and a CNS v. of 115·6.

Separation of saturated acids by the method of Bertram.

10·3 g. were taken for oxidation. The saturated acids were extracted as before by light petroleum (B.P. 60–80°) and precipitated twice as the magnesium soaps. The final weight was 1·73 g., equivalent to 16·8 % of the original mixed acids. (Found: C, 75·0; H, 12·6 %; mol. wt. 266. $C_{16}H_{32}O_2$ requires C, 74·9; H, 12·6 %; mol. wt. 256.) The I.V. was 1·6.

Separation of the saturated and unsaturated acids by the method of Twitchell.

10·3 g. of mixed acids treated with 1·7 g. of lead acetate gave 1·32 g. of solid acids of I.V. 13 and M.P. 51·3°. Correcting for the amount of unsaturated acid present the yield was 11·9 % of the original mixed acids. It will be seen that the result is again about 5 % lower than that given by Bertram's method.

α-Linolenic acid. The unsaturated fraction from Twitchell's method described above was brominated in the usual way. The amount of hexabromostearic acid obtained was 4·5 g., M.P. 180°, corresponding to 1·71 g. of α-linolenic acid. The yield was 16·6 % of the original mixed fatty acids.

Thiocyanometric analysis.

The data given above enabled the percentage of the various acids to be calculated, as shown in Table IV. It will be seen that the method suggests the presence of 17·6 % of oleic acid. This is nearly twice the amount which Kaufmann and Keller were able to remove quite readily from linseed oil by means of the lithium soap method of Moore [1919]. We therefore decided to apply this method in the present case.

Attempted separation of oleic acid as lithium oleate.

20·6 g. of the unsaturated acids obtained by Twitchell's method were dissolved in 51·5 cc. of absolute alcohol and treated with an equal volume of water containing slightly more than the theoretical amount of lithium hydroxide required to convert all the acids to the corresponding soaps. The mixture was allowed to stand overnight in the ice-chest. The yellow precipitate was

filtered off and washed with 50 % alcohol. It was recrystallised from the same solvent, the solution being left overnight in the ice-chest. The soaps thus separated were converted into the corresponding acids. 0·83 g. of semi-solid material was obtained, I.V. 92. On recrystallisation from acetone 0·5 g. yielded 0·18 g. of solid acid and 0·32 g. of liquid acid, I.V. 134. It is clear from this result that the amount of oleic acid isolated must have been less than 1 % of the unsaturated acids taken for analysis.

Oxidation of the unsaturated acids by the method of Hilditch.

As in the experiments with the cocksfoot fatty acids the method of alkaline permanganate oxidation gave no recognisable amount of a dihydroxystearic acid, we decided in the present case to apply the acid oxidation method of Hilditch [1926]. 26·5 g. of unsaturated acids separated by Twitchell's method were dissolved in 100 cc. of glacial acetic acid and treated with 4·4 g. of hydrogen peroxide (100 vol.), dissolved in a further 60 cc. of glacial acetic acid. The mixture was allowed to stand at room temperature for 14 days, when a small crystalline precipitate had settled and the containing liquid was otherwise homogeneous. The acetic acid was then removed by distillation in steam, and the residue saponified for 6 hours with 1 litre of *N* sodium hydroxide. 20 g. of dark solid products were thus obtained. Extraction with light petroleum removed 6·5 g. of yellow semi-solid material. The residue was a dark coloured liquid. This was dissolved in a small volume of ethyl acetate, which was then allowed to stand for some days in the ice-chest. The small crystalline precipitate was filtered off, and well washed with ether. The ethereal washings should have contained the dihydroxystearic acid, M.P. 95°, derived from any oleic acid present in the original unsaturated acids, but none was found. The material insoluble in the ether was recrystallised twice from ethyl acetate. The M.P. was 144° and it was not raised by repeated recrystallisation. It was a tetrahydroxystearic acid. (Found: C, 62·0; H, 10·5. $C_{18}H_{36}O_6$ requires C, 62·0; H, 10·4 %.) In the case of the ryegrass fatty acid therefore we are again unable to show the presence of any oleic acid.

Identification of the saturated acids.

Attention has been called above (p. 223) to the fact that the yield of saturated acids given by Twitchell's method is 5–6 % lower than that given by Bertram's method. This may be due to the presence of saturated acids lower in the series than palmitic acid, as the solubility of the lead soaps in alcohol increases rapidly with decrease in molecular weight. To test this supposition 54 g. of the unsaturated acids given by Twitchell's method were converted into the methyl esters in the usual way and then distilled from a Willstätter flask at a pressure of 0·6 mm. Two small fractions only were collected. The first weighed 1·35 g. (B.P. 130°, mol. wt. 244, I.V. 138) and the second 1·83 g. (B.P. 130–150°, mol. wt. 255, I.V. 167). As methyl myristate boils below 100°

Table V. *Fractionation of the saturated fatty acids from ryegrass.*

	Esters			Acids						
Fraction	B.P. 0·03 mm.	Weight g.	M.P. (approx.)	Weight g.	Crystalline fractions from acetone	Recrystallised from acetone (A), or from pyridine (P)	M.P.	C %	H %	mol. wt. (titration)
1	116°	1·32	20°	1·3	1st crop 0·62 g.	Once from A	59·5–61°	74·9	12·8	259
						Four times from A	61–61·5°	75·3	12·9	—
					2nd crop 0·3 g.	Once from A	54–55·5°	75·2	12·8	—
2	118°	2·34	22°	2·2	1st crop 1·3 g.	Once from A	60·5–61·5°	—	—	—
						Five times from A	61·8–62·2°	75·2	12·8	259
					2nd crop 0·6 g.	Once from A	60·5–61°	75·2	13·0	263
3	122°	2·63	22°	2·5	1st crop 1·5 g.	Once from A	60·5–61·3°	75·2	12·8	260
					2nd crop 0·6 g.	Once from A	58·5–59°	75·2	12·7	267
4	125–130°	2·89	23–24°	2·7	1st crop 1·7 g.	Once from A	54–56°	74·9	13·0	267
						Four times from A	52–54°	—	—	—
					2nd crop 0·7 g.	Once from A	55–57·5°	75·3	12·7	273
Residue	Not distilled	1·97	31–36°	1·6	1st crop 0·7 g.	Once from A	59·5–60·5°	—	—	—
						Once from P, twice from A	71·5–72·5°	78·2	13·3	353
					2nd crop 0·6 g.	Once from A	50–51·5°	75·8	12·8	282
Ethyl myristate			10·5–11·5°	—	—	Palmitic acid requires	63°	74·9	12·6	256
Ethyl palmitate			25°	—	—	Stearic acid ,,	70°	76·0	12·8	284
Ethyl stearate			34–35°	—	—	"Cerotic acid" ,,	80–82°	78·7	13·2	396

at this pressure it is clear from the boiling-points and I.V. recorded that no appreciable amount of shorter-chain saturated fatty acids was present in this unsaturated fraction. 10·8 g. of saturated acids separated by the Twitchell method were then converted into the ethyl esters in the usual way and distilled at 0·03 mm. The fractions were saponified and the resulting acids crystallised from acetone. Two crops were collected in each case, both of which were then further treated, as in the case of the corresponding acid fractions from cocksfoot. The results are recorded in Table V and can again be interpreted with the aid of the data given in Table III. The first three fractions are nearly pure palmitic acid; Fraction 4 is a mixture of palmitic and stearic acids with a small amount of a higher acid such as eicosanic acid, while the residue is a mixture of longer-chain fatty acids corresponding to "cerotic acid." The approximate analysis—palmitic acid, 70 %; stearic acid, 20 %; "cerotic acid," 10 %—is similar to that of the saturated acid fraction from cocksfoot.

Summary.

The glyceride fatty acids of two forage grasses, cocksfoot and perennial ryegrass, have been investigated in some detail.

The mixed acids are highly unsaturated, and contain a relatively low proportion of saturated acids.

The saturated acids are palmitic and stearic acids, together with a small amount of mixed higher acids similar to "cerotic acid," which may have been derived from wax esters.

The presence of α-linolenic acid was proved by bromination and oxidation, and of α-linoleic acid by oxidation.

Thiocyanometric analysis suggests the presence of oleic or an isomeric octadecenoic acid, but no confirmatory evidence could be obtained by oxidation experiments.

The thiocyanometric method of analysis is discussed in some detail, and the conclusion drawn that it may give misleading results in the case of mixed fatty acids containing large amounts of the so-called β-linoleic and β-linolenic acids. The mixed fatty acids from grasses are of this type.

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