favoured that at least the greater proportion of these linkages occur in separate homologous groups in the β -glucosan of barley.

SUMMARY

1. Cell-free culture filtrates of *Myrothecium verrucaria* contain a cellulase, producing cellobiose and glucose from complex and single-chain cellulose molecules, which is partially inactivated by heating at 60° for 10 min.

2. No evidence has appeared of the existence, in these filtrates, of a further enzyme breaking down complex cellulose into straight-chain fragments.

3. They also contain, though not always, cellobiase, hydrolysing cellobiose to glucose, which is completely inactivated in 10 min. at 60°.

4. When cellobiase is absent there is evidence of transglycosidase activity, producing glucose and cellobiose from cellobiose. This activity is also completely abolished by heating for 10 min. at 60°.

5. In addition to β -1:4-glucosidases, culture filtrates of *M. verrucaria* contain β -1:3-glucosidases, hydrolysing laminarin and laminaribiose.

6. When β -1:3- and β -1:4-ases act together on barley β -glucosan the products are cellobiose, laminaribiose and glucose.

7. The results obtained after the filtrate has been heated at 60° for 10 min. suggest that the β -1:3- and β -1:4-linkages occur separately in distinct groups in the barley β -glucosan.

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Protein of Pasture Plants

CYTOPLASMIC PROTEIN OF WHITE CLOVER AND ITALIAN RYEGRASS

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Studies of the proteins of the leaves of pasture plants have in general been confined to estimation of the amount of crude protein present, and analysis of this crude protein in terms of amino acid content. (For reviews see Chibnall, 1939; Lugg, 1949.) For this type of work, quantitative separation of the total protein of the leaf is desired, and the physical state of the extracted protein is relatively unimportant. The methods used frequently lead to denaturation, and very little has been learned of the properties of the native protein of the leaf cells.

The methods of extraction which are used when the protein is to be recovered without denaturation have recently been reviewed by Wildman & Jagendorf (1952), who discuss at length the work of

the Californian school on the physical chemistry of the leaf proteins of several higher plants. They draw attention to the compromise aimed at in devising methods severe enough to cause rupture of a reasonably large percentage of leaf cells, yet not so severe as to denature the soluble protein within these cells. The essence of the methods used is cell rupture by mechanical stress, the acid of the liberated vacuolar fluid being neutralized by extracting into buffer solution. The protein is thereby subjected to no chemical denaturant in extraction, and is protected from acid precipitation when liberated; but no safeguard can yet be offered against changes in protein structure brought about by the disorganization of cell contents, soluble and in-

soluble, which results from the severity of the method.

These limitations have been accepted in this work, in which a study is made of the soluble proteins extracted from pasture plants, mainly white clover (*Trifolium repens*) and to a less extent Italian ryegrass (*Lolium multiflorum*).

MATERIALS AND METHODS

Clover. White clover was grown in plots out of doors under normal pasture conditions. Plants more than 6 months old were not used. In cutting the clover, as little stalk as possible was included with the leaves.

Ryegrass. Italian ryegrass was grown in boxes in open frames, and kept watered with adequate nutrient solution. Grass was taken for several regrowths, but new boxes were regularly planted and old plants were not used.

Electrophoresis. Extracts and fractions were analysed by electrophoresis at 0.5° in the Tiselius-type apparatus manufactured by LKB-Produkter Ltd. Unless specified, all runs were carried out in phosphate buffer (Na_2HPO_4 , KH_2PO_4), pH 7.5, ionic strength (I) 0.1. (Mobilities are expressed in units of $10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$.) When particularly densely coloured solutions were run, it was found convenient to use a tungsten incandescent lamp in conjunction with an infrared filter (Ilford no. 207) and Kodak 35 mm. infrared film. So that the pattern could be observed by visible light, the infrared filter was placed immediately in front of the camera shutter, behind the reflex mirror. No refocusing of the optical system was necessary.

Dialysis of extracts before electrophoresis was carried out at 2° in Cellophane tubing on a rocking dialyser against repeated changes of buffer. In all cases the establishment of equilibrium was confirmed by conductivity measurement.

Paper electrophoresis. This was carried out in an apparatus somewhat similar to that of Vesselinovitch & Funnell (1954), in which the paper lies in a horizontal plane between two flat plates, supported by a series of insulated points.

Diffusion constants. These were measured in the LKB electrophoresis apparatus, the change in shape of the schlieren curve being followed for 3 days at 5°.

Ultracentrifugal analyses. Centrifuging for analytical and preparative purposes, at 50 000–200 000 g , was carried out in an air-driven ultracentrifuge constructed for this Division by Dominion Physical Laboratory.

Absorption spectra. Visible and ultraviolet absorption spectra were measured in a Beckman DU quartz spectrophotometer.

Determination of N and P. N was determined by micro-Kjeldahl estimation, with CuSO_4 as a catalyst in the digestion mixture. P was determined by the method of Allen (1940).

Phosphatase activity. This was estimated by measuring the liberation of inorganic phosphate in 30 min. from 1% (w/v) β -glycerophosphate at pH 6 in 0.15M-KOH-maleic acid buffer at 30°.

Ribonucleic acid (RNA). The RNA content of samples was estimated by precipitation with trichloroacetic acid, washing, and extraction for 18 hr. in the cold in 10% (w/v) HClO_4 , according to the method of Ogur & Rosen (1950). The absorption spectrum of the extract was measured in the range 300–240 $m\mu$, to confirm the presence of the typical

curve for RNA with maximum at 260 $m\mu$. The concentration of RNA was then expressed directly as the optical density at the maximum (D_{260}) of the extract derived from 1 ml. of sample, after correction for dilution during extraction and measurement.

Preparation of extracts

In the method finally adopted, the leaves were ground into buffer solution by mechanical pestle and mortar action in an end-runner mill (Pascall Engineering Co., Ltd.) operated in a cold room at 2–3°. This method, which has been used by Crook (1946), was relatively rapid, and did not lead to local heating or severe frothing, as can occur with blending or extraction with a colloid mill. The convenient capacity of the end-runner mill was 100–300 ml. of buffer, and it was found most efficient to grind fresh leaves of about half the weight of buffer in a single stage. After 5–10 min. the leaves were reduced to a slurry, which was filtered in the cold through a basket centrifuge lined with Whatman 3MM filter paper. This extract, free from cell-wall debris and gross solid matter, could be returned to the mill for a second load of leaves. By this means, even without the addition of abrasive materials such as sand, 80–90% of the total nitrogen of the leaf was regularly liberated into the extract; when two stages of extraction were used, a concentration of soluble protein suitable for electrophoretic and ultra-centrifugal examination was readily obtained.

In most cases phosphate buffer (Na_2HPO_4 – KH_2PO_4 , pH 7.5; I 0.2) was used for extraction, though 0.15M-KOH-maleic acid buffer (pH 7.4) and borate buffer (H_3BO_3 – NaOH – NaCl , pH 8.0; I 0.1) were occasionally used. Provided that the pH remained above 6.5 during extraction, no significant difference in yield or in any measured property of the extract was observed.

After the leaves had been ground and the slurry filtered, the resultant extract from both clover and ryegrass was an opaque green liquid, heavily contaminated with insoluble matter that was largely derived from the fragmentation of chloroplasts during extraction. The bulk of this could be removed by centrifuging at 25 000 g at 0° for 40 min. (International Model PR2, high-speed head), and this centrifuging was regularly carried out immediately after extraction. For the remainder of this paper, the terms 'clover extract' and 'ryegrass extract' refer to the supernatant produced by this centrifuging. The nitrogen thus removed as insoluble material was between 15 and 25% of the total of the crude extract. The range in amount of this loss was due to different degrees of prior removal of solids by the basket centrifuge, as the cake of cell-wall debris acted as a filter of variable efficiency. If the slurry of ground leaves was pressed through cheesecloth instead of passing through the basket centrifuge, the nitrogen removed at 25 000 g rose to 28–34%.

RESULTS

Clover extract. This was a green-brown transparent solution which exhibited strong scatter of light caused by highly dispersed insoluble matter. This could be removed by centrifuging at 50 000 g for 15 min. with loss of a further 3–4% nitrogen, yielding a clear brown liquid, but in most cases this step was not taken. Attempts were made to reduce contamination with insoluble green material by

grinding into buffer containing 0.5M sucrose to reduce osmotic damage to the chloroplasts (Neish, 1939). The chlorophyll content of the extract fell by 25–30 %, but the efficiency of extraction was also reduced, so that the ratio of chlorophyll to nitrogen fell by only 5–8 %. The mechanical stresses produced during grinding are probably sufficient to disrupt a large proportion of the chloroplasts, despite protection from osmotic damage. In view of the small improvement produced, and the difficulties introduced by the high concentration of sucrose, plain buffers were regularly used for extraction.

The brown colour of the extract appeared to be due to atmospheric oxidation of soluble constituents liberated from the cell, and could be reduced by working rapidly in the cold. On standing in air, even at 2°, the solutions darkened perceptibly at the surface over a period of hours. This oxidation could be almost completely prevented by extracting into buffer containing either sodium dithionite (0.05M) or ascorbic acid (0.05M). The extracts in these cases were of a pale-green colour, and browning at the air surface was markedly inhibited. In the experiments described in the remainder of this paper, however, these agents were not included in the extraction medium.

When reducing agents were not used, clover extract after removal of solids at 50 000 g produced an absorption spectrum as shown in Fig. 1. The slight maximum at 280 m μ ., and the rapid rise at 240 m μ ., are typical of proteins containing aromatic amino acids, while the general rise in the absorption with decreasing wavelength in the region 400–300 m μ . appeared to be associated with the brown pigment produced by oxidation. Unfortunately both sodium dithionite and ascorbic acid absorb strongly in the ultraviolet region, so that spectra of extracts in these media could not usefully be compared with those of normal extracts.

Fig. 1. Ultraviolet absorption spectrum of clover extract.

It was found that the height of the maximum at 280 m μ . was a useful measure of removal of pigmented material during fractionation of the extract. This height was expressed as the ratio of the optical densities at 280 and 310 m μ . (D_{280}/D_{310}). The value of this factor decreased from about 1.6 for fresh clover extract to 1.4 as oxidative darkening proceeded.

The protein contained in clover extract was apparently relatively stable, and at pH values between 6.5 and 7.5 could be stored at –20° for a few weeks or at +2° for a few days without becoming insoluble. Storage of the extract at –20° led to aggregation of the remaining insoluble matter, which could then readily be removed at 5000 g.

From 80 to 90 % of the nitrogen of clover extract was precipitable with 10 % (w/v) trichloroacetic acid. This percentage dropped to 70–75 % after 4 days at 2°, owing presumably to the breakdown of high-molecular-weight material. It was therefore necessary to work quickly when fractionating the extract, and fractionation was normally complete within 2–4 hr. of extraction.

Clover extract contained RNA in solution. Its concentration was estimated after precipitation in terms of the ratio D_{260}/N , this being the ratio of the optical density at 260 m μ . of the extracted RNA to the nitrogen content in mg./ml. of the solution from which this was taken. The value of the ratio for fresh clover extract lay between 4 and 6. If the extract was kept at 2°, the amount of RNA which could be estimated by extraction from a trichloroacetic acid precipitate progressively decreased. This was presumably due to hydrolytic breakdown into soluble units, as in 0.15M potassium maleate buffer (pH 7.5) the loss of RNA was accompanied by release of inorganic phosphate. With different samples of extract the apparent rate of hydrolysis was not regular, between 40 and 70 % of the RNA disappearing in 4 days. With uncentrifuged clover extract, the amount of RNA extractable from the trichloroacetic acid precipitate, which included insoluble matter as well as components from solution, fell more rapidly than with centrifuged extract, and the rate of fall was further increased if the integrity of cell components was partially preserved by extracting into buffer which contained sucrose.

The value of the ratio D_{260}/N gave an estimate of the percentage of nitrogen present as RNA. According to Ogur & Rosen (1950) the extinction at 260 m μ . per mole of P of RNA treated with perchloric acid is 10 800. This corresponds to a value for D_{260}/P of 348; if the N:P ratio of 15.3:8.0 for purified yeast RNA is used (Vischer & Chargaff, 1948), D_{260}/N for the pure acid is 182. The values found here show that in clover extract 2–3.5 % of the nitrogen is present in the form of RNA.

After dialysis, clover extract in the electrophoresis apparatus revealed a single component which was rather asymmetric, particularly in the descending boundary (Fig. 2*a, b*), with a mobility of 5.5–6.0 units in phosphate buffer at pH 7.5 (*I* 0.1). This mobility decreased with pH, falling to 4.2 units at pH 6.0, below which the protein tended to precipitate during the run. The bulk of the brown pigment migrated with the main boundary, though a small fraction regularly ran ahead of the ascending boundary with mobility of 12–13 units. The chloroplast fragments remaining in the extract also migrated with the main boundary, slightly behind its maximum ordinate.

The abnormally large boundary anomalies found for plant cytoplasmic proteins in phosphate buffers by Wildman, Cheo & Bonner (1949) were observed, and associated with these was an abnormally high ratio of ascending to descending mobility. These abnormalities decreased if the extract was dialysed for several days before electrophoresis, and were eliminated if the sample examined was reconstituted after freeze-drying.

In the ultracentrifuge, clover extract separated into three components (Fig. 2*c, d*): a small fraction (3–5 %) with sedimentation constant (S_{20}) 65–70 Svedberg units (referred to subsequently as *H*), a monodisperse fraction (35–40 %) with S_{20} approx. 17 units (fraction *I*), and a polydisperse residuum of material of lower molecular weight (52–58 %) with

mean S_{20} approx. 4 units (fraction *II*). The proportions of these components found by analysis of ten extracts from mature clover were within the ranges quoted. It was evident that the brown pigment was associated primarily with fraction *II*, no change in colour occurring at the boundaries due to fraction *I* or the *H* component.

The ultracentrifuge thus proved to be more useful than the electrophoresis apparatus in following the fractionation of extracts. It also revealed that the apparent stability of the extracts at 2° was deceptive. Although the *H* fraction was stable for several days, it was rapidly lost on dialysis. At pH values between 6.5 and 7.5 the concentration of fraction *I* slowly decreased (20 % loss in 4 days), presumably because of enzymic decomposition, as no aggregation was evident. At pH 6.0, however, aggregation occurred, leading to 50 % loss of fraction *I* in 3 days. Similarly storage at –20° led to considerable loss of fraction *I* and *H* component due to aggregation, particularly if the extract was frozen before centrifuging at 25 000 *g*.

Extraction of clover leaves into buffer containing sodium dithionite to inhibit oxidation resulted in complete loss of *H* and 16 % loss in fraction *I*. The effect of using buffer containing ascorbic acid was not determined.

Ryegrass extract. The properties of ryegrass extract after centrifuging at 25 000 *g* were broadly similar to those of clover extract. The brown pigmentation was much more prominent, however, obscuring the green of the contaminating chloroplast fragments. By extracting into buffer containing 1 % of sodium dithionite the browning could be almost completely prevented, but this agent was not used in experiments described subsequently.

Ryegrass extracts were less stable than those from clover. Obvious aggregation took place in neutral buffer after a few days at 2°, and after a week at –20° the bulk of the protein could be sedimented at 25 000 *g*. The extracts contained RNA in solution, with values of D_{260}/N between 4 and 7. This RNA broke down at much the same rate as in clover extract.

Electrophoresis revealed a pattern similar to that found with clover, i.e. an asymmetric single peak with a mobility of 6.5–7 units. The bulk of the pigment migrated with the main peak, a small amount migrated ahead of the ascending boundary with a mobility of 12–13 units. The ultracentrifuge pattern (Fig. 2*e*) revealed components corresponding to fraction *I* (35–45 %) with S_{20} approximately 16 Svedberg units and to fraction *II* (55–65 %) with mean S_{20} between 3 and 4 units. A component corresponding to *H* was observed only occasionally, and then only as a trace. The aggregation of protein of the extract on standing at 2°, or on storing at –20°, led to rapid decrease in fraction *I*.

Fig. 2. Electrophoresis and ultracentrifuge diagrams of extracts. (a) and (b), electrophoresis diagrams of clover extract, ascending and descending boundaries respectively. 5.5 v/cm. for 210 min. (c) and (d), ultracentrifuge diagrams of clover extract, at 45 000 rev./min. during acceleration, and after 10 min. at 60 000 rev./min. respectively. (e), ultracentrifuge diagram of ryegrass extract, after 10 min. at 60 000 rev./min.

Table 1. *Ultracentrifugal fractionation of clover extract*For definition of D_{200} and D_{200}/N see text.(a) *S1* produced by clarification of clover extract at 100 000 *g*; fractionated at 200 000 *g*

Fraction	Vol. (ml.)	N (mg./ml.)	D_{200}/N	Fraction II*	% Fraction I†	Component <i>H</i>
<i>S1</i>	49	2.01	3.8	2.57	41	+
<i>S2a</i>	28	1.01	0.7	1.72	7	—
<i>S2b</i>	20	2.57	1.7	2.90	39	—
<i>S2c</i>	5	2.30	2.3	0.84	82	—
<i>P2</i>	5	1.31	18.3	0.50	81	++

(b) *S1* produced by clarification of clover extract at 50 000 *g*; *S1* fractionated at 100 000 *g*, and *S2* at 200 000 *g*

Fraction	Vol. (ml.)	N (mg./ml.)	D_{200}/N	Fraction I*	Component <i>H</i> *
<i>S1</i>	73	2.64	5.3	—	—
<i>S2</i>	72	2.44	3.1	—	—
<i>P2</i>	2.8	3.58	46	0.96	2.01
<i>S2</i>	65	2.44	3.1	—	—
<i>S3a</i>	50	1.53	0.9	—	—
<i>S3b</i>	13.5	3.44	1.5	—	—
<i>S3c</i>	4	3.50	1.6	4.84	0
<i>P3</i>	3	4.34	19.5	6.15	1.05

* Expressed in arbitrary units derived from areas on ultracentrifuge diagrams.

† Expressed as percentage of total area.

Ultracentrifugal fractionation

Clover extract. An attempt was made to separate fractions I, II, and the *H* component by centrifuging at high speeds. An extract of clover in phosphate buffer (resultant pH 6.8, *I* 0.2) was spun at 100 000 *g* for 15 min. The resulting supernatant (*S1*) was spun at 200 000 *g* for 50 min., after which the centrifuge tubes contained a transparent sediment of gel-like material as well as a small well-defined layer of dense solution at the bottom. The tube contents were separated into four fractions: the upper three-fifths (*S2a*), the lower two-fifths (*S2b*), the dense layer (*S2c*) and the sediment (*P2*). The dense layer was diluted with buffer and the sediment dissolved by shaking for 30 min. into buffer, giving a solution from which a small precipitate could be removed at 10 000 *g*. The results of analyses of these fractions are set out in Table 1 (a). A similar fractionation carried out on clover extract in 0.5M potassium maleate buffer (pH 7.5) gave almost identical results.

The expected partial separation of fractions I and II was achieved, but recovery of *H* was very poor. Only in *P2* was it observed, and here in a partially degraded form. The recovery of RNA was only 67%. It was evident that some RNA, and probably some *H* component, were lost during clarification at 100 000 *g*. This loss was prevented by clarifying clover extract at 50 000 *g* for 15 min. and withdrawing the upper four-fifths of the supernatant (*S1*), thus avoiding contamination with larger particles. This *S1* was spun at 100 000 *g* for 40 min., giving a sediment of gel-like material (*P2*), after which the supernatant (*S2*) was spun at

Fig. 3. Ultracentrifuge diagrams of fractions prepared by centrifuging (see Table 1). (a), clover *P2*; 42 000 rev./min. 10 min. (b), clover *P3*; 42 000 rev./min. 10 min. (c), clover *S3c*; 60 000 rev./min. 10 min. (d), ryegrass *S2c*; 60 000 rev./min. 10 min.

200 000 *g* for 40 min., yielding fractions *S3a*, *b*, *c* and *P3* corresponding closely to those of the first method. Both sediments redissolved in buffer when the suspensions were shaken for 30 min. The results of the analysis of these fractions are given in Table 1 (b).

In this case 97% recovery of RNA was obtained in the fractionation of *S1*, and 87% in fractionation of *S2*. In the ultracentrifuge, *P2* showed a high concentration of *H*, as well as some material sedimenting between this and fraction I. *P3* showed a much smaller proportion of *H*. Fig. 3 shows the ultracentrifuge patterns produced by *S3*, *P2* and *P3*.

The ultraviolet absorption spectrum of *P2* and *P3* showed a broad maximum at 260 mμ., but the

spectrum of *S3c* was similar to that of unfractionated extract, with a somewhat enhanced maximum at 280 m μ . ($D_{280}/D_{310} = 2.2$).

Samples of *S3c*, *P2* and *P3* were analysed by paper electrophoresis, with Whatman 3MM filter paper, and sodium phosphate-potassium phosphate buffer (pH 7.5, *I* 0.05). A field of approximately 2v/cm. was applied for 16 hr., after which the paper was cut laterally into strips and eluted, and the absorption spectra of the eluates were analysed for RNA and for protein. Although RNA has a much higher specific absorption than protein, the ratio of the optical densities at 260 and 280 m μ . gave a qualitative estimate of the proportion of RNA to protein. Protein was clearly demonstrated in *S3c* and *P3*, but only a trace was found in *P2*; RNA was found only in *P2* and *P3*.

Some tailing of protein occurred, but the bulk had migrated 10 cm. under the conditions of the experiment. The RNA migrated slightly ahead of this, overlapping at about 10 cm.

Ryegrass extract. This was fractionated at 200 000 *g* by the first method used with clover. The results were similar, the expected concentration of fraction I occurring in the dense layer at the bottom of the tube (*S2c*), as illustrated in Fig. 3 (*d*). For the sediment (*P2*) the value of D_{280}/N was 18; for all other fractions it was below 2.

Salt fractionation

Clover and ryegrass extracts were fractionated at pH 6.8 by precipitation with sodium sulphate. As the concentration of added salt increased, the precipitated nitrogen increased without any abrupt discontinuity, but it was found that precipitation with 12, 18 and 25 % Na_2SO_4 (w/v) gave convenient separation into sufficiently well-marked fractions. The precipitates were named *P1*, *P2* and *P3* respectively, and the final supernatant *S3*. Each precipitate was shaken with enough phosphate buffer (pH 7.5, *I* 0.1) to give a volume one-tenth of that of the original extract, and centrifuged at 5000 *g* to remove insoluble material. The precipitates dissolved readily, with the exception of *P1*, from which a green sediment of aggregated particles

was obtained. The results of typical experiments with clover and ryegrass are given in Table 2.

Poor recovery (18 %) of RNA was obtained, and no trace of *H* was found in any fraction. It was evident from electrophoretic analysis of the precipitates that no separation of fractions I and II took place during migration, and the pattern gave no clear indication of their relative proportions. A high proportion of fraction II was evident only from an increased spreading of the peak. This spreading could be reversed by reversing the direction of the current in the cell, so it was due to inhomogeneity rather than to true diffusion.

After concentration, *S3* (clover) showed on electrophoresis a diffuse peak with a mobility of approximately 7 units, with a large amount of polydisperse material behind a small peak with a mobility of 12 units.

An indication of the change which takes place in clover extract on standing was given by the different distribution which resulted from salt precipitation carried out after 3 days at 2°. *P1* then contained 11 % of the nitrogen in the form of heterogeneous material which sedimented at 100 000 *g* in the ultracentrifuge, and a high proportion of fraction I occurred in *P3* rather than *P2*. Despite the obvious change of character of *P1*, it migrated as a single symmetrical peak on electrophoresis.

Acid fractionation

Extracts of clover and ryegrass were fractionated by addition of successive amounts of 0.2N-HCl at 2°. Each precipitate was shaken with a volume of phosphate buffer (pH 7.5, *I* 0.2) equal to one-tenth of that of the original extract, and the mixture was spun at 5000 *g* to remove insoluble material. The results are set out in Table 3.

As with salt precipitation, no well-marked discontinuity of precipitation was noted as the pH was lowered; the fractions selected in Table 3 are typical of the separation obtained by this method.

The recoveries of RNA were 72 % with ryegrass and 24 % with clover. Only traces of *H* were found in the clover fractions; none was observed in ryegrass fractions.

Table 2. Salt fractionation of clover and ryegrass extracts

For conditions see text.									
Fraction	Clover					Ryegrass			
	Concn. of Na ₂ SO ₄ (%, w/v)	N as % of total	<i>D</i> ₂₈₀ / <i>N</i>	<i>D</i> ₂₈₀ / <i>D</i> ₃₁₀	Fraction I (%)*	Concn. of Na ₂ SO ₄ (%, w/v)	N as % of total	<i>D</i> ₂₈₀ / <i>N</i>	Fraction I (%)*
Extract	0	100	4.8	1.5	38	0	100	4.4	45
<i>P</i> 1	12	4.5	0.6	1.3	27	12	24	0.7	42
<i>P</i> 2	18	34	0	2.2	71	18	12.5	0	57
<i>P</i> 3	25	8.5	0	1.4	21	25	24	0	67
<i>S</i> 3	—	24	2.4	1.2	—	—	35	1.9	—

* Expressed as a percentage of the total area on ultracentrifuge diagrams.

Table 3. *Acid fractionation of clover and ryegrass extracts*

For conditions see text.									
Clover					Ryegrass				
Fraction	pH	N as % of total	D_{260}/N	Fraction I (%)*	Fraction	pH	N as % of total	D_{260}/N	Fraction I (%)*
Extract	7.0	100	5.8	38	Extract	7.0	100	6.5	45
P1	5.25	37	2.3	58	P1	5.2	1.5	3.0	—
P2	4.6	14	3.6	24	P2	4.4	15	4.1	40
S2	—	31	0	—	P3	4.0	35	10.6	47
					S3	—	40	2.5	—

* Expressed as a percentage of the total area on ultracentrifuge diagrams.

Table 4. *Ethanol fractionation of clover and ryegrass extracts*

For conditions see text.					
Fraction	Ethanol (% by vol.)	N as % of total	P as % of total	D_{260}/N	Fraction I (%)*
Clover					
Extract	0	100	100	4.0	39
P1	11.3	12.5	11.0	3.6	33
P2	13.9	9.0	7.0	5.1	32
P3	16.5	14.0	10.0	6.2	41
P4	19.1	15.0	10.0	6.7	50
P5	21.6	11.5	7.0	6.3	63
P6	26	8.0	4.5	5.4	59
S6	—	22.5	45.0	0	—
Ryegrass					
Extract	0	100	100	5.5	35
P1	11.0	3.5	2.0	4.5	—
P2	16.0	13.5	5.5	5.4	47
P3	20.5	18.0	6.5	12.1	50
P4	24.5	10.0	6.0	19.4	52
P5	32	10.0	12.0	12.0	35
S5	—	39.0	61.5	1.5	—

* Expressed as a percentage of the total area on ultracentrifuge diagrams.

Ethanol fractionation

Fractionation of protein solutions by precipitation with organic solvents at low temperatures has been recognized in recent years as a powerful and flexible technique. So far, it does not seem to have been used with proteins extracted from plant leaves. Experiments were accordingly carried out, with ethanol as a precipitant, to find conditions under which satisfactory fractionation of clover and ryegrass extracts could be achieved.

As expected, it was necessary to keep the temperature below 0° if undenatured precipitate was to be obtained. At 2–3°, most of the precipitate formed would not redissolve, but if the temperature was lowered to –5° as the ethanol was added, the precipitate seemed undenatured, and redissolved readily in buffer. It was also necessary to work at a pH below 6.3 to give precipitation at reasonable concentrations of ethanol. At pH 6.8–7.0 no precipitate formed at ethanol concentration as high

as 70 % by volume. If the pH of a solution containing this concentration of ethanol was lowered, protein was precipitated in a denatured form even at –8°, and chlorophyll which was present in the extract in dispersed chloroplast fragments remained in solution.

Proteins from clover and ryegrass extract in 0.15M potassium maleate buffer were precipitated at pH 6.1 by addition of successive amounts of 95 % (v/v) ethanol, the temperature of the extract being lowered to –5° during the first addition, and being maintained there for subsequent additions. The precipitates were sedimented at 4000 *g* with the centrifuge rotor chamber at –10°, and redissolved in one-tenth of the original volume of 0.15M potassium maleate buffer, pH 7.5, at 2°. The results are set out in Table 4.

Complete recovery of RNA was obtained, with good recovery of N and P. Peaks corresponding to *H* in the ultracentrifuge diagrams of precipitates 2–5 (clover) were observed, but these were too small to measure accurately.

Purification of fraction I

Two samples of fraction I from clover extract, each of which contained less than 3% of fraction II, were prepared by a single stage of precipitation, followed by two stages of fractional centrifuging.

In the first method, an ethanol precipitate equivalent to $P_4 + P_5 + P_6$ of Table 4 was prepared, redissolved in phosphate NaCl buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$, I 0.1; NaCl, I 0.2; pH 7.5), and centrifuged at 200 000 g for 45 min., giving a sediment and a layer of concentrated solution at the base of the tube similar to that found in the ultracentrifugal fractionation of clover extract. This concentrated solution was withdrawn, diluted with phosphate–NaCl buffer, and centrifuged a second time at 200 000 g for 45 min., giving a trace of sediment and a layer of concentrated solution. This solution, diluted with buffer, gave the ultracentrifuge pattern shown in Fig. 4*a*.

The second method was similar, except that a salt precipitate equivalent to P_2 of Table 2 was used as the starting material. The resulting fraction I appeared identical with that of the first method. Neither protein contained measurable amounts of RNA. The salt-precipitated preparation showed the value of 3.65 for D_{280}/D_{310} from the absorption spectrum, and the electrophoretic mobility was 5.8 units. A slight amount of reversible spreading took place during migration, showing that the preparation was not homogeneous. The stability of this purified fraction I was considerably greater than in clover extract, and it could be stored at -20° for 10 days without change.

The sedimentation constants (S_{20}) in Svedberg units at 0.5% (w/v) protein concentration for the two preparations were 16.4 (ethanol precipitate) and 16.7 (salt precipitate). In calculating these

results, the value of 0.75 was assumed for the partial specific volume of the protein, and no extrapolation to infinite dilution was made. A single measurement of S_{20} at 2% (w/v) protein concentration ($S_{20} = 16.2$) differed only slightly from that at 0.5%, so that the error in omitting this extrapolation is small. The diffusion constant for each preparation was determined at 0.5% (w/v) concentration, with the following results for D ($\text{cm}^2 \text{sec}^{-1}$): 2.60×10^{-7} , for the ethanol precipitate; 2.75×10^{-7} , for the salt precipitate.

Ultracentrifugal analyses were made before and after diffusion to confirm absence of aggregation or breakdown into smaller units. From these results, the value 0.75 again being used for partial specific volume, the molecular weights were calculated to be 6.2×10^5 for the ethanol precipitate and 5.9×10^5 for the salt precipitate.

In view of the lack of precision in determining the diffusion constant by the schlieren-curve method without a specially designed cell, the difference between these values is probably not significant.

Concentration of the H component

Fractions containing a high proportion of component H were prepared by adding ethanol to a concentration of 21% (by vol.) to clover extract at pH 6.1, producing a precipitate equivalent to $P_1\text{--}P_5$ of Table 4. After the precipitate had been redissolved in buffer at pH 7.5, the solution was clarified at 50 000 g for 15 min., and H sedimented by centrifuging at 100 000 g for 50 min. The sediment contained 52% of H (Fig. 4*b*) and a high proportion of RNA ($D_{280}/N = 40$). Analysis of this preparation by paper electrophoresis under the conditions described above for P_2 of Table 1 (*b*) revealed a large amount of RNA migrating from 10 to 16 cm. from the origin, and a trace of protein at 10 cm.

In establishing a suitable ionic content of the solution to carry out electrophoresis of this preparation in the Tiselius apparatus, it was necessary to avoid dialysis, which causes rapid breakdown of H . Accordingly, the once-sedimented preparation was diluted with a relatively large volume of the buffer to be used ($\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$, pH 7.5, I 0.1), and recentrifuged at 100 000 g for 50 min. This twice-sedimented preparation contained, besides a high content of H and RNA, a new major component with sedimentation constant of 34 Svedberg units (Fig. 4*c*). Electrophoresis produced two distinct boundaries with mobilities of 5.5 and 8.3 units.

Qualitative analysis of this fraction for pentoses by the method of Ceriotti (1955) indicated the presence of ribose, but no detectable deoxyribose.

Fig. 4. Ultracentrifuge diagrams of fractions prepared from clover extract. (The approximate sedimentation constants are indicated in each diagram.) (a), Fraction I; 60 000 rev./min. 10 min. (b), component, once sedimented; 42 000 rev./min. 10 min. (c), H component, twice sedimented; 42 000 rev./min. 10 min.

Phosphatase activity

Fractions of clover and ryegrass extracts in 0.15M potassium maleate buffer, prepared by centrifuging as in Table 1 (a) and by ethanol precipitation as in Table 4, were examined for phosphatase activity. No great concentration of activity was found in any one fraction; high activity was associated with high content of fraction II, but the relationship was not linear. Complete recovery of activity was found in the centrifuged fractions; for ethanol precipitation the supernatant with a high content of ethanol was not assayed, but the precipitates contained 77 % (for clover) and 74 % (for ryegrass) of the activity.

DISCUSSION

One of the most striking points which emerges from this work is the similarity in many respects with the results obtained by Wildman and co-workers (Singer, Eggman, Campbell & Wildman, 1952; Eggman, Singer & Wildman, 1953) in their studies on a range of higher plants, chiefly tobacco and spinach. The physicochemical properties of proteins obtained from the leaf cells of these plants were remarkably similar, and the similarity extends to the pasture plants considered in this paper.

On the other hand, it has been suggested (Wildman & Jagendorf, 1952), on the basis of the properties of extracts from corn and oat leaves, that monocotyledons might differ from dicotyledons in that no fraction I protein was found in their leaves. The results given here for Italian ryegrass, as well as ultracentrifugal analysis of extracts from short-rotation ryegrass, perennial ryegrass, cocksfoot and wheat seedlings, show that in these monocotyledons a protein corresponding to fraction I is present to as great an extent as in dicotyledonous leaves. Thus although some genera of monocotyledons may not contain fraction I, there is no fundamental difference between the two classes in this respect.

The sedimentation constant of fraction I from clover falls within the range given for plants studied by Singer *et al.* (1952). Although these workers did not obtain this protein in a form sufficiently pure to enable the diffusion constant to be measured, their estimate of 600 000 for the molecular weight agrees well with that found here.

Components corresponding to *H* have not been described in extracts from other plants, though material with the considerably lower sedimentation constant of 25 units has been described (Singer *et al.* 1952). Components of high sedimentation constant have been produced as artifacts by polymerization of fraction I as a result of repeated sedimentation (Eggman *et al.* 1953), but the *H* component, present in uncentrifuged clover extract, does not arise in

this way. Artifacts of this type have been observed in this work (e.g. Figs. 3a; 4b, c), but the amount of *H* in these fractions may be accounted for by concentration of material already present.

When prepared by the methods used here, a fraction rich in *H* has always contained a high concentration of RNA, and it was thought that *H* might be a nucleoprotein of high molecular weight, possibly corresponding to the fraction NP of tobacco leaves described by Pirie (1950). Electrophoresis of concentrates showed two boundaries, as with Pirie's fraction, but attempts to isolate and characterize the components producing the two boundaries have not been successful. Thus the nature of *H* has not been established. It has not been prepared in a pure form, and has been concentrated only by methods involving fractional centrifuging, so that the association with RNA may be due only to similar sedimentation rates. Although both *H* and RNA are unstable, RNA persisted in solution under conditions which led to loss of *H*. This observation, in conjunction with the fact that ryegrass extracts have a high RNA content, yet often no *H* component, suggests that *H* and RNA may not be related.

The distribution of RNA found in clover and ryegrass extracts agrees with that found by Pirie (1950) for tobacco in that the bulk is sedimentable with centrifugal fields of the order of 100 000 *g*. With the possible exception of the *H* component, no homogeneous fraction identifiable with his nucleoprotein NP has been found by ultracentrifugal or electrophoretic analysis. The RNA appears to be extracted as polydisperse material of high molecular weight.

It has been suggested by Wildman & Jagendorf (1952) that Pirie's NP results from aggregation of fraction I in the acid conditions brought about by extraction without the use of buffers. Aggregation of this type has been successfully avoided in this work, yet the bulk of the RNA readily sediments in centrifugal fields which will sediment fraction I only very slowly.

Complete removal of RNA at 100 000 *g* was not expected; it is known that leaf extracts contain a ribonuclease (Parker, 1952) by which initially large molecules of RNA are progressively broken down to fragments, and during extraction of cell contents this will take place to some extent. Only by working rapidly in the cold has it been possible to sediment a high proportion of RNA; delay of even a few hours greatly reduces the yield.

The abnormally large boundary anomalies and high ratio of ascending to descending mobility observed in electrophoretic analysis of clover extracts in phosphate buffer are possibly due to the presence of RNA in these extracts. The bound phosphate groups of RNA, dissociated at pH 7.5,

would be expected to reduce the conductivity established by Donnan equilibrium on dialysis, thus producing the abnormalities observed. Support for this is found in the fact that the abnormalities disappear when RNA is absent. The difference between the conductivity of the extract and that of the dialysate at equilibrium was also found to depend on the presence of RNA, the difference decreasing as RNA disappeared. On the basis of this suggestion, the finding of Wildman *et al.* (1949) that electrophoresis of extracts in buffer such as cacodylate showed no abnormalities might be explained as due to accelerated enzymic removal of bound phosphate groups in the absence of inhibition by excess of phosphate ions in the buffer.

Eggman *et al.* (1953) described fraction I, prepared from spinach and tobacco extracts by fractional centrifuging, as a nucleoprotein, and believe that all the soluble RNA of the extracts is associated with this protein. The results obtained here indicate that fraction I from clover is not associated with significant amounts of RNA. The evidence for this may be outlined as follows.

In fractional centrifuging, the RNA content of the fractions bears no relationship to the content of fraction I. This is most clearly seen in fractions P2, P3 and S3c of Table 1 (b), where the quotients RNA/fraction I (expressed as D_{260} /fraction I area) are 172, 14 and 1.2 respectively. Residual RNA in other fractions is probably due to contamination with lower-molecular-weight products of ribonuclease digestion.

The sedimentation constants of fraction I preparations did not vary with the RNA content of the samples. Thus the mean value for the two most highly purified samples, containing no RNA, was 16.55; that of three samples prepared by single-stage centrifuging [equivalent to S2c of Table 1 (a)] was 16.5; and the value determined for the fraction I component of P2 (Fig. 3a) with a very high RNA content was 16.2. This constancy of value indicates lack of association between fraction I and RNA molecules.

In preparing samples of fraction I for analysis, Eggman *et al.* (1953) separated a fraction by centrifuging which was apparently equivalent to P2 of Table 1 (a). This sediment, containing both RNA and fraction I, produced aggregated polymers of fraction I on recentrifuging. Such polymers have not been produced in this work on recentrifuging of fraction I preparations, provided contamination with large amounts of RNA was avoided. Recentrifuging of a preparation rich in RNA produced aggregation of this type (Fig. 4c); in this case the polymer was considerably in excess of the unaggregated form. Indication of formation of smaller amounts of similar aggregates is seen in Fig. 3a, b. Paper electrophoresis of fractions rich in RNA

indicated that the protein component (fraction I) did not migrate in close association with RNA, although the leading edge of the protein band overlapped the rear of the RNA. From all these considerations it is concluded that fraction I of clover is not a nucleoprotein.

Of the various methods of fractionation used in this work, centrifuging is that least liable to modify the various components of cytoplasmic extracts. It does not, however, lend itself readily to fractionation of large quantities with high yields. Salt precipitation is a convenient method of producing quantities of partially purified fraction I, but RNA and the H component are not recovered. Multiple-stage salt precipitation leads to decreasing recovery, and even in a single stage Wildman & Bonner (1947) found considerable loss of phosphatase activity, so partial modification of the proteins seems likely.

Acid precipitation has been found by some workers to lead to denaturation (Chibnall, 1939; Wildman & Gordon, 1942), but under conditions used here reasonably good recoveries have been obtained. The best recoveries of total soluble nitrogen and phosphorus, as well as of the more labile components of the extract, resulted from ethanol fractionation at low temperature. Phosphatase activity was also preserved, so little modification of the proteins seems to have occurred. Under the conditions used here, the separation of the main fractions was not as great as with salt precipitation, but the flexibility of the method, with independent variability of ionic strength, pH and ethanol concentration may enable improvements to be made in this respect.

Despite their similarity of appearance in the ultracentrifuge, the components of clover and ryegrass extracts showed considerable difference in solubility. In general, the ryegrass proteins were less readily precipitated; this is seen from Tables 2, 3 and 4. This higher degree of solubility may be related to the higher charge on the ryegrass proteins, which is evident from the higher electrophoretic mobility.

SUMMARY

1. The soluble cytoplasmic proteins extracted from the leaf cells of white clover and Italian ryegrass have been studied by electrophoresis and ultracentrifuging.

2. These extracts have been fractionated by high-speed centrifuging, salt precipitation, acid precipitation and ethanol precipitation.

3. A protein fraction which was monodisperse on electrophoresis and in the ultracentrifuge has been isolated from clover leaf, and its molecular weight established as approximately 600 000.

4. Close similarity is found to the types of protein reported in extracts from other plants. However, the monodisperse fraction from clover differs from that described from spinach and tobacco in that it is not associated with ribonucleic acid.

5. Leaves of both monocotyledonous and dicotyledonous plants appear to contain the same types of soluble cytoplasmic protein.

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The Metabolism of δ -Aminolaevulic Acid

1. NORMAL PATHWAYS, STUDIED WITH THE AID OF ^{15}N

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On the basis of results obtained with *in vitro* systems, δ -aminolaevulic acid (ALA) has been found by several groups of workers to be a biological precursor of porphyrins and haem. The following reactions are believed to occur (Shemin & Russell, 1953; Neuberger & Scott, 1953):

The last compound, porphobilinogen (PBG), has been shown by Falk, Dresel & Rimington (1953) to be converted enzymically into porphyrins *in vitro*. Employing isotopically labelled glycine or ALA,

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Shemin & Russell (1953) and Shemin, Russell & Abramsky (1955) have demonstrated that ALA is used in preference to glycine and succinate for haem synthesis by avian red cells. Shemin, Abramsky & Russell (1954) showed that a soluble enzyme system, obtained by centrifuging lysed

avian red cells, would convert ALA into haemin. Gibson, Neuberger & Scott (1954, 1955) have isolated a widely distributed enzyme which catalyses the conversion of ALA into PBG.

Net synthesis of haem or porphyrin from glycine in a lysed avian red-cell system is very slight, but large quantities of porphyrins are formed when PBG is added in place of glycine (Falk *et al.* 1953). ALA also was found to give rise to free porphyrins under these conditions (Neuberger & Scott, 1953; Dresel & Falk, 1953), and was shown by the latter