2. The spectrum shift and increase in the extinction maximum in the presence of high concentrations of formaldehyde was also shown by *N*⁵-formyl-4-hydroxy-6-methyltetrahydropteridine, 4-hydroxy-6-methylpteridine, pteroylglutamic acid and 2-deaminopteroylglutamic acid. The dissociation constants calculated from these spectrum changes ranged from 0.2 to 0.4 M.

3. These data are interpreted as indicating that 4-hydroxypteridines combine with formaldehyde when it is present at a high concentration to form *N*³-hydroxymethyl derivatives. This is confirmed by the fact that 2-hydroxypyrimidine behaves similarly but the spectrum of 2-methoxypyrimidine is unaffected by formaldehyde.

4. At neutral pH solutions of tetrahydropteroylglutamic acid rapidly decomposed as shown by spectrum changes. Chelating agents, thiols (especially 2:3-dimercaptopropan-1-ol) and formaldehyde retarded this oxidative decomposition.

5. In the presence of 2:3-dimercaptopropan-1-ol the reaction of tetrahydropteroylglutamic acid with formaldehyde resulted in an increase in the extinction maximum from 28 400 to 32 000 and shift in the wavelength of the absorption maximum from 298 to 294 mμ. The change was reversed by addition of bisulphite.

6. The dissociation constant for the product of this reaction was calculated to be $3.1 \pm 0.19 \times 10^{-5}$ M.

7. The product of the reaction, believed to be *N*⁵*N*¹⁰-methylenetetrahydropteroylglutamic acid, is also subject to oxidative decomposition at all pH values. Chelating agents retard this decomposition.

8. The changes produced by formaldehyde in the spectra of a number of other hydropteridines are also discussed.

I am greatly indebted to Dr D. J. Brown, who made much of this work possible by his generous gifts of pteridines and pyrimidines. I should also like to express my indebtedness to Dr H. P. Broquist for a gift of leucovorin; to Professor A. H. Ennor and Dr J. F. Morrison for assistance in the preparation of the manuscript; to Professor A. Albert for helpful suggestions concerning the ionisation of hydropteridines and to Mr J. McKeough for skilled technical assistance.

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Nucleoproteins of White Clover

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(Received 27 April 1959)

'Microsomal' ribonucleoproteins have been isolated from several sources, such as liver cells (Petermann & Hamilton, 1957), yeast cells (Chao & Schachman, 1956), pea shoots (Ts'o, Bonner & Vinograd, 1956) and *Escherichia coli* (Tissières & Watson, 1958). Preparations from leaf cells of higher plants have not been reported, although some earlier work has indicated that high-molecular-weight ribonucleoproteins are in fact present. Thus Pirie (1950, 1957) described the preparation from tobacco leaves of a nucleoprotein fraction which was sedimentable by centrifuging at 80 000g for 30 min. With clover the bulk of the ribonucleic acid of leaf extract could be sedimented at 100 000g in 40 min., and, when redissolved, this sediment was shown in the analytical ultracentrifuge to contain a component of sedimentation coefficient 65 s (Lyttleton, 1956).

The studies which have been made on the 'microsomal' ribonucleoprotein particles from the various sources mentioned above have indicated that these

particles are stable in solution only within a limited range of conditions, and it is probable that the lack of success in earlier attempts to isolate high-molecular-weight ribonucleoprotein from clover (Lyttleton, 1956) was due to a failure to recognize this difficulty.

With the knowledge now available of the conditions necessary to prevent degradation of the particles, their isolation has been re-investigated. This paper describes the successful isolation of 'microsomal' ribonucleoproteins from clover leaf. It gives details of their physical and chemical properties, which are shown in many respects to be similar to those of ribonucleoproteins isolated from other sources.

MATERIALS AND METHODS

Plant material. White clover (*Trifolium repens* L.) was grown in boxes in glasshouses. Three or four crops of mature leaves were taken before the plants were discarded for new

sowings. Field-grown clover was unsatisfactory because of the prevalence of infection with pea-wilt virus (Fry, 1959), which contaminated any preparation of ribonucleoprotein.

Extraction of leaves. The leaves were usually ground in an End Runner Mill as previously described (Lyttleton, 1956). Alternatively, the leaves, moistened with a suitable buffer solution, were pressed through an annular aperture (0.001 in.) in a chilled stainless-steel press (Pirie, 1956). The paste of disintegrated tissue thus produced was filtered through Whatman no. 3 MM paper in a basket centrifuge and the residue washed with buffer solution. This method was particularly useful when small amounts of concentrated extract were required, but the chloroplast material was so highly dispersed that its removal by fractional centrifuging was never as complete as from solutions produced by grinding.

Fractional centrifuging. This was carried out in a Spinco model L preparative ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, Calif., U.S.A.). Figures quoted for the centrifugal force are those tabulated for the average radius of rotation.

All manipulations were carried out at 4°, and a pre-cooled rotor was used in the centrifuge, to which refrigeration was applied during sedimentation. Resuspension of the pellets was effected with the aid of a magnetic stirrer, and could be made more rapid by filling and emptying a 10 ml. hypodermic syringe with the needle inserted below the level of the resuspending medium. The rapid flow of liquid through the rather narrow orifice was most effective in dispersing the gel-like sediment produced by high-speed centrifuging. A hypodermic syringe was also used to withdraw the supernatants from the clarifying stages, as the sediments produced here were not well packed.

Ultracentrifugal analyses. These were carried out in an air-driven ultracentrifuge constructed for this Division by the Dominion Physical Laboratory. It was fitted with an inclined-bar Philpot schlieren optical system, and operated under automatic-speed control at 42 000 or 60 000 rev./min. with a constancy of 1 part in 10 000. Sedimentation coefficients are expressed in Svedberg units (s).

Electrophoretic analyses. These were carried out at 0.5° in a Tiselius apparatus manufactured by LKB-Produkter Ltd. Mobilities are expressed in units of 10^{-5} cm.²/v sec.

Absorption spectra. These were measured on a Beckman DU quartz spectrophotometer. In describing the ultraviolet spectra of the nucleoprotein preparations, the ratio $E_{\max} : E_{\min}$ expresses the ratio of the extinctions at the maximum (near 260 m μ) and at the minimum (usually 230–240 m μ).

Ribonucleic acid. The ribonucleic acid (RNA) content of samples was estimated by precipitation with 5% (w/v) trichloroacetic acid, washing with 70% (v/v) ethanol and hydrolysing with 2N-KOH at 20° for 18 hr. The resulting nucleotides were separated by paper chromatography in propan-2-ol-aq. NH₃ soln. (Markham & Smith, 1952), and their concentration was determined after elution by means of their ultraviolet absorption.

Base analysis of ribonucleic acid. This was carried out by hydrolysis in 1N-HCl for 1 hr. at 100° followed by paper chromatography in propan-2-ol-HCl (Smith & Markham, 1950).

Phosphorus. This was estimated by the method of Allen (1940).

Buffer solutions. Phosphate buffers contained analytical-

grade Na₂HPO₄ and KH₂PO₄, and their concentration is expressed in terms of ionic strength (*I*). Reagent-grade 2-amino-2-hydroxymethylpropane-1:3-diol (tris) was used for making tris-HCl buffers. Other salts used were of analytical grade.

Direct examination of extracts

An extract from clover was prepared from 30 g. of leaf moistened with 5 ml. of water in the stainless-steel press. Examination of this extract, diluted with an equal volume of water, in the analytical ultracentrifuge revealed two heavy components (Fig. 1a) with apparent sedimentation coefficients 65s and 55s, as well as large concentrations of the two lower-molecular-weight components, fractions I and II (Singer, Eggman, Campbell & Wildman, 1952). Experiments which will be described later indicated that at least one, and probably both, of these two heavy components were ribonucleoproteins. Accordingly they will be referred to in the remainder of this paper as NP1 and NP2 respectively.

When the extract was diluted with phosphate buffer, pH 7.5, *I* 0.2, instead of with water, NP2 was no longer detectable and NP1 was somewhat reduced in concentration. After dialysis of the extract against phosphate buffer, pH 7.5, *I* 0.1, neither heavy component was detectable in the ultracentrifuge pattern.

Isolation of NP1 and NP2

Precipitation. Previous work (Lyttleton, 1956) had shown that leaf nucleoprotein could not be recovered after precipitation by addition of Na₂SO₄ or by lowering of the pH of the solution. Precipitation by addition of ethanol at –5° to a concentration of 20% (v/v) permitted recovery of the nucleoprotein. Attempts were therefore made to concentrate selectively NP1 and NP2 by ethanol precipitation at concentrations between 15 and 25%, but although good recovery of NP1 was obtained, the ratio of NP1 to fraction I was not increased. The second component, NP2, could not be recovered after ethanol precipitation.

Fractional centrifuging. Because of their high sedimentation rates, NP1 and NP2 may be selectively concentrated by centrifuging at high speeds. Ultracentrifuge examination showed that sedimentation at 105 000*g* for 60 min. removed all but a trace of these components from the supernatant solution. Accordingly the scheme outlined in Fig. 2 was used for isolating NP1 and NP2 from clover extracts. The terminology of Ts'o *et al.* (1956) is used, where DC1, DC2 and DC3 represent preparations obtained by differential centrifuging to one, two and three stages.

The choice of medium for extraction and resuspension was most important, an ideal medium being one which would produce good yields of intact NP1 and NP2, and prevent the production of artifacts. It was found that values between pH 6.0 and 7.5 were most satisfactory, as below pH 6.0 the components tended to aggregate, and above pH 7.5 they tended to break down to lower-molecular-weight material.

Even within this favourable range of pH values, NP1 and NP2 were unstable in the presence of buffers of ionic strength greater than 0.02. This instability was greatly reduced by the addition of low concentration of Mg²⁺ ions to the solution, and most preparations were made in the presence of mM-MgSO₄.

The media which were used during these investigations may be divided into three classes.

Low-ionic-strength medium. An extract prepared by grinding clover into water had a pH close to 6.0 when freshly prepared, though on standing it dropped rapidly. It was thus possible to fractionate the components of a clover extract with only water as extraction and resuspension medium. However, in DC2 and DC3 preparations made in this way, in addition to NP1 and NP2 the solution con-

tained a new component of sedimentation coefficient 40–42s (Fig. 1b). This component, not detectable in the original extract, was considered to be an artifact. The use of mM-MgSO₄ in place of water gave similar results, with a slightly reduced production of the 40s component.

High-ionic-strength medium. Provided that magnesium was present, it was found possible to use buffered 0.1M-NaCl to prepare NP1, though NP2 did not survive sedimentation in this medium. No new components were

Fig. 1. Ultracentrifuge diagrams. (a) Clover-leaf extract, diluted with an equal volume of water. (b) DC2 fraction prepared in water. (c) DC3 fraction prepared in 0.1M-NaCl-mM-MgSO₄-phosphate buffer (pH 7.5, *I* 0.02). (d) DC3 fraction prepared in mM-MgSO₄-phosphate buffer (pH 7.5, *I* 0.02). All diagrams were photographed 9 min. after running speed (42 000 rev./min.) was reached. Sedimentation is from left to right.

Fig. 2. Fractionation of clover extract by differential centrifuging. In some preparations, where a larger volume was to be handled, the clarification of the original extract and the first sedimentation were carried out in the no. 30 rotor for 20 min. at 20 000 rev./min. (35 000 g) and 120 min. at 30 000 rev./min. (78 000 g) respectively.

produced, and the best preparations of NP1 have been made in 0.1M-NaCl-mm-MgSO₄ buffered at pH 7.5 with phosphate (I 0.01). A DC3 preparation made with this medium was seen to contain only NP1 when examined in the analytical ultracentrifuge (Fig. 1c).

Medium-ionic-strength medium. In order to preserve NP2 and yet avoid the production of artifacts some compromise between low- and high-ionic-strength media seemed desirable. A range of these was tried, mainly with total ionic strength 0.02, made up of phosphate buffer, pH 6.5, 7.0 or 7.5, or tris buffer at pH 7.2, either with or without 0.01M-NaCl but always containing mm-MgSO₄. With these media the production of artifacts was avoided, but with none of them was a satisfactory recovery of NP2 found (Fig. 1d). Each stage of centrifuging led to a decrease in the ratio of concentrations NP2 : NP1, so that whereas in fresh extract this ratio was approx. 1 : 2, in DC3 preparations it dropped to approx. 1 : 10.

In all media of low or medium ionic strength which contained mm-MgSO₄, DC2 and DC3 preparations contained small amounts of a component of sedimentation coefficient 100–105s. A similar component has also been produced by fractional centrifuging of pea-shoot extracts (Ts'o *et al.* 1956) and of bacterial extracts (Tissières & Watson, 1958), and is apparently a dimer of NP1.

The method of preparing NP1 and NP2 depended upon the object of the experiment. For studies on the properties of NP1, sedimentation was carried out in buffered 0.1M-NaCl-mm-MgSO₄. Three stages of sedimentation were needed to remove fraction I completely from the final preparation. However, the two-stage preparation (DC2), containing about 10% of fraction I, was suitable for preliminary work on the dissociation of NP1. On the other hand, solutions of medium ionic strength, as mentioned above, were used in making preparations to study the properties of NP2, which was always accompanied by larger amounts of NP1.

It was difficult to remove the last traces of highly dispersed chloroplastic material even from DC3 preparations. If a high yield was sought by prolonged grinding of leaf tissue, or by passing larger amounts of leaf through the stainless-steel press, this was invariably accompanied by the production of chlorophyll-containing fragments with sedimentation rate only slightly greater than those of the desired components. Under these circumstances, even the four stages of clarification used in producing the DC3 preparation did not completely remove contaminating material. It should be noted, however, that this contamination did not hinder observation either in the analytical ultracentrifuge or in the electrophoresis apparatus.

Distribution of ribonucleic acid during fractionation

With the chromatographic isolation technique described above, estimations were regularly made of the RNA content of the clarified extract and of the final product, DC2 or DC3. In most cases between 25 and 35% of the RNA contained in the clarified extract was recovered in a DC3 preparation, and slightly more (30–40%) in a DC2 preparation. Usually the higher recoveries were associated with the use of medium ionic-strength extraction media; but the results were somewhat irregular, and differences were probably due to minor variations in manipulative techniques.

On several occasions a more complete balance sheet was made of the distribution of RNA during a fractionation, and a typical result is given in Fig. 3.

Ultraviolet spectrum

Whereas the ultraviolet spectrum of clarified clover extract showed a continually increasing absorption with decreasing wavelength, with a shoulder in the region 270–280 mμ, that of a DC2 or DC3 preparation showed a pronounced maximum at 260 mμ, indicating that one or both of the heavy components which were concentrated in these fractions contained nucleic acid, and were presumably nucleoprotein in nature.

The actual shape of the ultraviolet-absorption spectrum depended on the degree of contamination with non-nucleoprotein material, such as fraction I (in DC2 preparation) and residual chloroplast fragments. The best DC3 preparations gave a ratio $E_{\max} : E_{\min}$ of 1.5–1.55 : 1, with a minimum absorption at 233 mμ.

RESULTS

Properties of NP1 and NP2

Stability. All preparations have proved to be somewhat unstable even at 4°. This instability is revealed by ultracentrifugal examination, which shows a progressive loss of NP1 and NP2 with the production of degraded material of ill-defined sedimentation behaviour, some aggregated and sedimenting at low rotor speeds and some of low molecular weight not separating from the meniscus of the ultracentrifuge cell at 60 000 rev./min. The NP2 component is particularly labile, and rarely persists as long as 18 hr. after completion of a fractionation. The NP1 component, however,

Fig. 3. Distribution of RNA as determined by nucleotide chromatography during differential centrifuging of clover extract. This fractionation was carried out with phosphate buffer (pH 7.0, I 0.01)–0.01M-NaCl-mm-MgSO₄ throughout. The 'residues' referred to are the discarded sediments produced during the clarifying steps in the fractionation.

usually remains substantially unaltered for 24 hr. and may persist for 2 or 3 days at 4°.

Pirie (1950) found that nucleoprotein derived from tobacco leaf liberated acid-soluble phosphate on standing. Similarly the degradation of NP1 and NP2 is accompanied by a decrease in the amount of RNA and of phosphate which can be precipitated from the solution by cold 5% (w/v) trichloroacetic acid. In a typical case, from a freshly prepared DC3 preparation in tris-HCl buffer, 95% of the RNA and 97% of phosphate could be precipitated; these amounts decreased progressively until after 3 days at 4°, 71% of the RNA and 69% of phosphate were precipitable.

Apart from the requirement for magnesium to prevent dissociation in the presence of buffer salts, no definite relationship has been established between suspension medium and stability of NP1 and NP2, and no satisfactory way of decreasing breakdown of the components has been found. As a result of this, all investigations of the intact components have been carried out within 24 hr., and when possible within 2–3 hr. of their isolation.

Sedimentation. The sedimentation coefficient of NP1, determined in 0.1M-NaCl-mm-MgSO₄ buffered with phosphate at pH 7.5, *I* 0.01, showed a moderate dependence on concentration, rising from 76s at a concentration of 8 mg./ml. to an extrapolated value of 83s at infinite dilution. The lower value of 65s observed for NP1 in clover extracts is due to the fact that no correction was made for the high concentration of protein in which sedimentation was proceeding.

Measurement of the sedimentation coefficient of NP2 in DC3 preparations gave values of 62–66s. If the effect of concentration upon sedimentation is the same as with NP1, the value at infinite dilution might be expected to be between 68 and 70s.

Electrophoresis. Electrophoretic analyses of NP1 have normally been carried out in the buffer solution which was used for differential centrifuging,

thus eliminating the need for dialysis. In phosphate buffers, pH 6.0–7.5, *I* 0.01, containing 0.01M-NaCl and mm-MgSO₄, NP1 migrated as a single symmetrical boundary with a mobility of –9.8 units at pH 7.5 and –8.1 units at pH 6.0. Behind the main NP1 component moved a small boundary, accounting for 10–15% of the total area, apparently produced by contaminating dispersed chloroplastic material. The chlorophyll remaining in the solution migrated with this boundary (Fig. 4).

It is difficult to study the electrophoretic behaviour of NP2 because of its lability. Various preparations made with the object of discovering whether or not it migrated independently of NP1 led to inconclusive results, because ultracentrifugal examination of the residues after electrophoretic analysis showed that at most a trace of NP2 remained.

Such evidence as is available indicates that in fact NP1 and NP2 migrate together on electrophoresis. On no occasion were the boundaries produced from DC2 or DC3 preparations containing small residual amounts of NP2 observed to split, indicating the presence of more than one component. In one analysis of a DC3 preparation containing an appreciable amount of NP2, material migrating with the NP1 electrophoretic boundary was isolated from the ascending limb of the U-tube, and in the ultracentrifuge it was shown to contain a small amount of NP2 as well as a larger amount of NP1.

In an attempt to verify this finding, the product of the first step in the centrifugal fractionation (DC1), which contained a higher proportion of NP2 than later steps, was electrophoretically analysed immediately after its preparation. The electrophoretic pattern showed a boundary with a mobility approximately that of NP1, which split into two as analysis proceeded. The components forming this double boundary were isolated from the ascending limb of the U-tube, and examined in

Fig. 4. Electrophoretic analysis of NP1, in 0.01M-NaCl-mm-MgSO₄-phosphate buffer (pH 7.0, *I* 0.01). (a) Anode limb; (b) cathode limb.

the ultracentrifuge. Along with the expected NP1, this showed a new 40s component, possibly similar to the artifact produced by using water as medium during fractionation, but no NP2 remained. The ultraviolet spectrum showed a ratio $E_{\max} : E_{\min}$ of 1.28 : 1, much lower than that of good NP1 preparations, so the 40s artifact presumably contained some of the fraction I protein with which DC1 preparations are heavily contaminated.

Content of ribonucleic acid. On three occasions, preparations of NP1 were run in the electrophoresis apparatus, short intermediate cells being used to form the U-tube so that, at the conclusion of the run, electrophoretically pure samples of NP1 could be isolated in the upper section of the ascending limb.

Because the amount available was so small, these colourless pure samples were analysed for RNA by a non-destructive method, similar to that of Englander & Epstein (1957), by determining the extinction at 260 $m\mu$, and measuring the concentration of NP1 by freeze-drying and weighing. The results obtained for the three preparations were 53, 56 and 52 mg. of RNA/100 mg. of NP1. By contrast, DC3 preparations of NP1, which were shown by electrophoresis to contain 10–15% of contaminant, contained 40–45% of RNA.

The ultraviolet-absorption curve of the electrophoretically purified samples of RNA showed the expected maximum at 260 $m\mu$, and a ratio $E_{\max} : E_{\min}$ of 1.65–1.70 : 1 (cf. unpurified preparation 1.5–1.55 : 1).

Composition of ribonucleic acid base. The freeze-dried samples of electrophoretically purified NP1 were pooled and hydrolysed with *N*-HCl at 100° for 1 hr. Chromatography in propan-2-ol–HCl enabled the ratio of the base components of the RNA to be determined, with results given in Table 1. For comparison, the ratios determined on a hydrolysed sample of a DC3 preparation of NP1, before electrophoretic purification, are also given. It has not yet been possible to isolate NP2, so its composition is unknown.

Dissociation products of NP1

As with the ribonucleoprotein particles prepared from yeast (Chao, 1957), from pea shoot (Ts'o, Bonner & Vinograd, 1958) or from *E. coli* (Tissières & Watson, 1958), the leaf ribonucleoprotein NP1 will dissociate in certain solvents into smaller components. The various types of dissociation which have been studied in the analytical ultracentrifuge are considered here according to the solvent which produced them.

Phosphate buffers in the presence of magnesium. Provided that mm-MgSO_4 was present, NP1 was not affected by phosphate, pH 7.5, at *I* below 0.03. When *I* was 0.05, a small amount of material of low

molecular weight was formed, and at *I* 0.10 two new components were formed, with apparent sedimentation coefficients of 34s and 52s, as well as more low-molecular-weight material. At ionic strengths above 0.5, NP1 was completely dissociated to give a little 34s component, a larger amount of low-molecular-weight material and aggregated material which sedimented at low rates of centrifuging.

The distribution of the RNA in the completely dissociated NP1 was investigated by measuring the ultraviolet-absorption spectrum of the supernatants and redissolved sediments from the analytical ultracentrifuge cell at the conclusion of runs of sufficient length to sediment the 34s component fully. The results, given in Table 2, indicate that the bulk of the RNA after dissociation was found in the unsedimented low-molecular-weight material. The ratio $E_{\max} : E_{\min}$ for this latter was 1.82 : 1, indicating that less protein was present per unit of RNA than in the undissociated NP1.

Phosphate buffers in the absence of magnesium. If a solution of NP1 was prepared in the absence of magnesium by carrying out the final steps of the differential centrifuging in water, the addition of phosphate buffer, pH 7.5, *I* 0.025, produced a partial dissociation of NP1 into two well-defined smaller components (Fig. 5a). Their sedimentation rate indicated that they were the same as the 34s and 52s components described above; but in the absence of added magnesium they were produced at lower concentrations of phosphate, appeared less diffuse in the ultracentrifuge and were not accompanied by the formation of material of low molecular weight. The formation of these components was apparently very rapid, for whereas the presence of mm-MgSO_4 in solution prevented the dissociation of NP1 by phosphate, pH 7.5, *I* 0.025, the addition

Table 1. *Base composition of ribonucleic acid of NP1*

	Ratio of base components			
	Guanine	Adenine	Cytosine	Uracil
Electrophoretically pure NP1	1.32	0.95	0.84	0.88
DC3 preparation of NP1	1.26	0.94	0.88	0.92

Table 2. *Distribution of ribonucleic acid after centrifuging of NP1 in analytical cell before and after dissociation by phosphate*

Ionic strength of phosphate (pH 7.5)	E_{260} (supernatant)	E_{260} (sediment)
0.01	0.6	7.5
0.5	5.5	2.2

of MgSO_4 to this concentration immediately after the addition of phosphate did not appreciably reverse the dissociation.

Increase of phosphate concentration to $I\ 0.05$ did not give greater concentration of the 34s and 52s components; but they became less well defined in the ultracentrifuge, and a little material of low molecular weight was formed. This effect was also brought about by adding 0.1M-NaCl to the phosphate at $I\ 0.025$.

Higher concentrations of phosphate lead to progressive loss of NP1 and the production of low-molecular-weight material in a manner very similar to that observed in the presence of magnesium. The bulk of the RNA was again associated with the low-molecular-weight material.

Dialysis of a solution of NP1 against phosphate buffer, pH 7.5, $I\ 0.025$, for 18 hr. led to dissociation with the production of a higher proportion of the 34s component than was formed by simple addition of phosphate. Electrophoretic analysis of this dialysed solution revealed two well-defined boundaries, migrating with mobilities -12.5 and -9.8 units. The lower mobility agreed with that of undissociated NP1 in a buffer of the same pH and ionic strength which contained mm-MgSO_4 , so it appeared that the dissociation products moved in the more rapid boundary. It will be noted that no boundary was observed to move with a mobility typical of free RNA in this medium (-14 to -15 units) so the dissociation products were presumably still nucleoproteins. This observation agrees with the findings of Ts'o *et al.* (1958), whose ultracentrifuge studies with an ultraviolet-absorption optical system indicated that similar dissociation products of pea-shoot ribonucleoproteins were themselves nucleoprotein in nature.

Dissociation in tris-hydrochloric acid buffers. A solution of NP1 was prepared in tris-HCl buffer, pH 7.2, $I\ 0.02$, containing mm-MgSO_4 , then dialysed for 18 hr. against the same buffer without MgSO_4 . The NP1 component was completely dissociated into a component of sedimentation coefficient

approximately 50s, together with low-molecular-weight material (Fig. 5b). Electrophoretic analysis of this dialysed preparation in tris-HCl (pH 7.2, $I\ 0.03$)– 0.02M-NaCl revealed components with mobilities -9.0 and -14.0 units. The more rapidly moving component was isolated at the end of the run, and was shown to contain the low-molecular-weight material observed in the centrifuge. Its absorption spectrum showed a ratio $E_{\text{max.}} : E_{\text{min.}}$ of $2.35:1$. This was considerably higher than the ratio obtained for the low-molecular-weight material produced by dissociation with phosphate, indicating a higher proportion of RNA to protein.

With bacterial ribonucleoprotein particles it has been found that whereas dissociation occurs in the absence of magnesium, in tris-HCl buffers containing 5 mm-MgSO_4 the particles aggregated to produce a heavier component, presumably a dimer, of sedimentation coefficient approximately 110s (Tissières & Watson, 1958). A similar aggregation was not produced when preparations of NP1 were dialysed against tris-HCl buffer (pH 7.2, $I\ 0.03$)– 5 mm-MgSO_4 . The small amount of 105s component normally present in DC3 preparations was not increased by the higher concentration of magnesium.

Action of chelating compounds. Removal of magnesium from a solution of NP1 in phosphate buffer (pH 7.5, $I\ 0.01$)– mm-MgSO_4 by addition of a slight excess of sodium ethylenediaminetetraacetate brought about considerable loss of NP1, apparently by aggregation, but produced no recognisable products of dissociation. A similar effect was obtained with sodium citrate.

DISCUSSION

The experiments described in this paper indicate that clover-leaf cells contain a class of high-molecular-weight ribonucleoprotein particles (NP1) which have many properties in common with similar particles found in other cell extracts. The

Fig. 5. Ultracentrifuge diagrams. (a) NP1 in phosphate buffer, pH 7.5, $I\ 0.025$; (b) NP1 dialysed against tris-HCl buffer, pH 7.2, $I\ 0.02$. Both diagrams were photographed 9 min. after running speed (42 000 rev./min.) was reached. Sedimentation is from left to right.

RNA content of NP1 (52–56%) is intermediate between the values found for particles from *E. coli* (60–65%; Tissières & Watson, 1958) and those from yeast (40–45%; Chao & Schachman, 1956) and from pea shoots (35–40%; Ts'o *et al.* 1956). The extrapolated sedimentation coefficient of NP1 (83s) also agrees with those found for ribonucleoproteins from the sources mentioned. A further similarity lies in the stabilizing effect of Mg^{2+} ions on NP1, and in its dissociation into large subunits in the presence of phosphate buffers at pH 7.5.

The NP1 particles from clover leaf appear to be considerably less stable than those described from other sources. As it is known that ribonucleoproteins of this type are susceptible to the action of ribonuclease (Pirie, 1950; Ts'o *et al.* 1956), it is possible that the instability observed here is due to contamination with ribonuclease, which is known to occur in leaves of higher plants (Holden & Pirie, 1955). This would explain the production of acid-soluble RNA and phosphorus from the NP1 preparations on standing.

Earlier work on leaf proteins (Eggman, Singer & Wildman, 1953) had indicated that when fraction I was isolated by fractional centrifuging, the bulk of the soluble RNA was found to be associated with this component rather than with heavier molecules of the type found here. This may be accounted for by the fact that the preparations were sedimented and resuspended in buffer at pH 7.0 in the absence of magnesium, and were dialysed against similar buffers before ultracentrifugal analyses. These treatments might be expected to degrade the heavy ribonucleoproteins and to lead to contamination of fraction I. As shown previously (Lyttleton, 1956; Lyttleton & Ts'o, 1958), it is possible by mild methods to prepare fraction I free from RNA.

It is doubtful whether all of the soluble RNA of leaf cells is located in NP1 and NP2. The distribution observed during fractionation indicated that some 20% of the RNA in the clarified extract was not removed from the supernatant under conditions for which sedimentation of NP1 and NP2 was substantially complete. The rapidity with which the fractionation was carried out makes it unlikely that the unsedimented RNA was merely a product of attack by ribonuclease on the NP1 and NP2.

For the study of ribonucleoprotein particles of plant origin, leaf tissue has several disadvantages as a starting material. In addition to the instability discussed above, the purification of the ribonucleoproteins by differential centrifuging is made more difficult by the presence of large amounts of relatively high-molecular-weight protein (fraction I, sedimentation coefficient 17–18s) and of finely dispersed chloroplast fragments. Three stages of sedimentation are needed to remove fraction I, and

even then contamination with chloroplast fragments is often troublesome.

Extracts of leaf cells contain two soluble high-molecular-weight components, in place of the more usual single component observed in etiolated shoots and in roots. The presence of this second component is not confined to clover leaves; examination of extracts from leaves of green peas, Chinese cabbage, spinach, vegetable marrow and Italian ryegrass (Lyttleton, unpublished work) has revealed the presence of a pair of high-molecular-weight components similar to the NP1 and NP2 of clover leaf.

The nature of the second component, NP2, has not been established. During this work it has been considered probable that it is a ribonucleoprotein, but confirmation of this must await its isolation and analysis. So far the only clues as to its nature have been (a) its high sedimentation coefficient, which is similar to those for naturally occurring ribonucleoproteins, (b) some indication that its electrophoretic mobility is the same as that of NP1, and higher than that of other leaf proteins, and (c) its great instability in the presence of buffer salts. This instability explains the fact that it was not observed during previous work with clover-leaf extract, where extraction into buffers was the standard procedure (Lyttleton, 1956).

It is not likely that NP2 is a product of dissociation of NP1. It is observed immediately after extraction in leaf extracts, in which its concentration decreases rather than increases on standing. Its sedimentation coefficient and electrophoretic mobility do not agree with those of the larger fragments of NP1 obtained by dissociation in the presence of phosphate buffers. It appears to be a genuine constituent of leaf cells, though a very unstable one, and its isolation will present a difficult problem.

As has been pointed out by Pirie (1950), the presence in leaf cells of high-molecular-weight ribonucleoproteins of this type should be borne in mind when plant viruses are to be prepared from leaf extracts. Unless some steps are taken to remove these normal leaf constituents they are liable to contaminate any virus prepared by fractional centrifuging alone. The experiments described in this paper, along with other similar unpublished results obtained with other plants, indicate that solution in phosphate buffers of ionic strength greater than 0.2 at pH 7.5 should lead to complete degradation of the ribonucleoproteins from the leaves of most plants, whereas many viruses remain unaffected by this treatment. Alternatively, it should be possible to make use of chelating agents such as ethylenediaminetetra-acetic acid to remove Mg^{2+} ions from solution, hence rendering the ribonucleoproteins unstable.

SUMMARY

1. Ultracentrifugal examination of clover-leaf extracts has shown two components of high sedimentation coefficient to be present.

2. The heavier of these has been isolated and shown to be a ribonucleoprotein containing 50–55 % of ribonucleic acid. Its properties have been studied by ultracentrifuging and electrophoresis.

3. In common with similar ribonucleoproteins from pea shoots, yeast or liver cells, this component dissociates into subunits in the presence of certain buffer solutions.

4. The second component is considerably less stable, and has not yet been isolated. It is suggested that it may also be a ribonucleoprotein.

5. The possibility of contamination of plant-virus preparations with these normal constituents of leaf is discussed.

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The Estimation of Aminoacetone and δ -Aminolaevulinic Acid

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(Received 8 May 1959)

Since the discovery of δ -aminolaevulinic acid as a precursor of porphyrins (Shemin & Russel, 1953; Neuberger & Scott, 1953) two methods for the determination of this compound have been published. One of these involves the conversion of the amino ketone into a pyrrole by condensation with a second ketone such as acetylacetone or ethyl acetoacetate; the pyrrole so formed is then estimated by means of the Ehrlich reaction. Mauzerall & Granick (1956) used a modified Ehrlich reagent, which gave more stable colours and greater sensitivity than that described by Shuster (1956), and their procedure is generally the method of choice, particularly when small amounts of amino ketone are to be measured. It suffers, however, from the disadvantage that any excess of Ehrlich-reacting compounds such as porphobilinogen or indole derivatives must first be removed.

The other method for the determination of δ -aminolaevulinic acid is based on the observation (Shuster, 1956) that when the amino ketone is mixed with a solution of picric acid and excess of sodium hydroxide is added, a complex is formed

which gives a red-brown colour on subsequent acidification with hydrochloric acid. The mechanism of the reaction and the nature of the complex are not known.

During work on the production of aminoacetone by *Staphylococcus aureus* (Elliott, 1958, 1959), it was necessary to have a suitable assay method. Aminoacetone could be estimated by both procedures, but the picrate method was chosen because of its greater freedom from interference from other substances and because of its simplicity. However, the original procedure (Shuster, 1956) gave erratic results in the determination of both δ -aminolaevulinic acid and aminoacetone and the conditions of the estimation were therefore re-investigated.

The present paper describes a simple procedure for the determination of both of these metabolites; although it is less sensitive than the procedure of Mauzerall & Granick (1956), the method is valuable since it can be used in conditions where the latter is inapplicable. It has the additional advantage that, when combined with a simple resin treatment, only amino ketones, among biologically occurring