DISCUSSION

The analytical method reported above is suited to the detection of all ketones and aldehydes that react with the Girard T reagent. Their measurement is, however, the less accurate the lower a compound's molecular weight and the greater its number of reactive carbonyl groups and other polar oxygen functions (hydroxyl groups and unreactive oxo groups). Under the conditions employed, compounds of the androstane and pregnane series with one or two reactive carbonyl groups and with altogether not more than three polar oxygen functions are determined with acceptable accuracy (see Table 1). In contradistinction to other methods commonly used for the determination of oxo steroids, the present assay does not distinguish between different types of oxo groups (e.g. $\alpha\beta$ -unsaturated ketones and saturated ketones) provided that they react with the Girard T reagent. Hence the assay may be used for the determination of the number of reactive carbonyl groups in a molecule. An attractive feature of the method is that oxo compounds, after having been determined, can be separated from other constituents of the analytical sample and recovered in good yield. The scope of the method is not limited to the determination of ketones and aldehydes: indirect determination of secondary alcohols by their initial oxidation to ketones (cf. Menini & Norzymski, 1962) and assessment of the efficiency of Girard separations are two obvious applications. Though we had no opportunity of applying the assay to biological material, Dr R. A. Harkness informs us that he had used it with success for the determination of 5β -pregnane-3,20-dione derived from the oxidation of urinary pregnanediol and pregnanolone.

SUMMARY

1. Salts formed between the Girard derivatives of carbonyl compounds and bromothymol blue were preferentially extracted from aqueous solution with ethylene dichloride. Colorimetric measurement of the extracted salts gave an accurate estimate of compounds of the androstane and pregnane series with one or two reactive carbonyl groups and altogether not more than three polar oxygen functions.

2. The method outlined was adapted to the detection of oxo steroids on paper chromatograms.

We are indebted to Professor W. Klyne for gifts of compounds from the M.R.C. Steroid Reference Collection.

REFERENCES

- Auerbach, M. E. (1943). *Industr. Engng Chem. (Anal.)*, **15**, 492.
Barr, T., Oliver, J. & Stubbings, W. (1948). *J. Soc. chem. Ind., Lond.*, **67**, 45.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Eberlein, W. R. & Bongiovanni, A. M. (1955). *Arch. Biochem. Biophys.* **59**, 90.
Girard, A. & Sandulesco, G. (1936). *Helv. chim. acta*, **19**, 1095.
Jones, J. H. (1945). *J. Ass. off. agric. Chem., Wash.*, **28**, 398.
Lehman, R. A. & Aitken, T. (1943). *J. Lab. clin. Med.* **28**, 787.
Menini, E. & Norzymski, J. K. (1962). *Biochem. J.* **84**, 195.
Prudhomme, R. O. (1938). *Bull. Soc. Path. exot.* **31**, 929.
Roy, A. B. (1956). *Biochem. J.* **62**, 41.
Wheeler, O. H. (1962). *Chem. Rev.* **62**, 205.
White, E. C. & Acree, S. F. (1917). *J. Amer. chem. Soc.* **39**, 648.

Biochem. J. (1964), **93**, 14

The Isolation and Composition of Ribonucleic Acids from Leaves

By A. R. TRIM, JANET E. BAKER AND A. B. LEAH

Plant Breeding Institute, Maris Lane, Trumpington, Cambridge, and The Agricultural Research Council's Virus Research Unit, Huntingdon Road, Cambridge

(Received 10 December 1963)

The most extensive investigation of the nucleotide composition of plant ribonucleic acids is that of Vanyushin & Belozersky (1959), who analysed the whole RNA of seed, pollen and thallus material from a wide taxonomic range of plants by using a modified Schmidt & Thannhauser (1945) method. The work was handicapped by the interference of

ultraviolet-absorbing substances other than nucleotides which are present in most plant tissues. Partly because of this difficulty a preliminary separation of the nucleic acids from the plant organs is desirable. In the present paper, following earlier attempts to obtain a quantitative recovery of minimally degraded nucleic acid from leaves

(Trim, 1959), further developments of the method are described together with additional checks on its efficiency and chemical analyses of the products.

METHODS

Determination of dry weights. Nucleic acid solutions were freeze-dried, heated in an oven at 105° overnight and weighed. Other material was dried to constant weight in an oven at 105°.

Determination of total phosphorus. The method of Allen (1940) was used. Smooth digestion of the samples was ensured by using a mixture of 60% (w/w) HClO_4 , 18N- H_2SO_4 and water (2:3:1, by vol.) instead of 60% (w/w) HClO_4 (unpublished modification by C. S. Hanes). Samples with a very low phosphorus content were estimated by the method of Berenblum & Chain (1938).

Determination of nitrogen. This was estimated by the micro-Kjeldahl method.

Determination of protein. An approximate value for protein in fractions prepared from leaves was obtained by Osborne's method (Coghill, 1931) with the quantities recommended by Holden & Pirie (1955). A sample of serum albumin (fraction 5; Armour Laboratories, Eastbourne, Sussex) was used as a standard.

Determination of deoxyribonucleic acid. DNA was detected and estimated by Burton's (1956) modification of the Dische diphenylamine method, which, when calibrated over the range 10–100 μg . of DNA, gave results within $\pm 2\%$. Commercial herring-sperm DNA, which has a nucleotide composition close to that of higher plants, was used as a standard. Assuming the nucleotide composition found by Wyatt (1951), pure herring-sperm DNA should contain 26% of purine-bound deoxyribose. When checked against a pure sample of the monohydrate of the ammonium salt of deoxyadenosine 5'-monophosphate the DNA standard was found to contain 28% of purine-bound deoxyribose. After drying to constant weight at 100° *in vacuo* over P_2O_5 , the deoxyadenylate contained 8.8% of phosphorus (calculated value 8.5%). When heated for 30 min. in N-HCl at 100° it yielded 95% of the theoretical yield of adenine, identified by chromatography in solvent A (see below) and its spectra at pH 2, 7 and 12. It was found to be homogeneous with respect to the ultraviolet-absorbing component when subjected to electrophoresis at pH 3.5, and when chromatographed in solvent C (see below). Its spectral characteristics were very close to those given by Hayes, Michelson &

Todd (1955) and it was assumed to have a purity of about 95%.

Determination of nucleotides and purine bases. The purine bases and pyrimidine nucleotides in RNA were determined by the method of Smith & Markham (1950), i.e. after hydrolysis at 100° for 1 hr. in N-HCl and chromatography, either in solvent A, made by taking 700 ml. of 2-methylpropan-2-ol at 26°, adding 132 ml. of constant-boiling HCl and making to 1 l. at 20°, or in solvent B, made by taking 170 ml. of propan-2-ol, adding 41 ml. of conc. HCl (sp.gr. 1.19) and making to 250 ml. with water (Wyatt, 1951). The molar extinction coefficients given by Markham & Smith (1951) were used for the calculations.

Reference nucleotides. Pure commercial nucleotides (California Foundation) were dried to constant weight *in vacuo* at 100° over P_2O_5 , assumed to be anhydrous and used as standards. Their phosphorus content and spectral characteristics in acid, neutral solution and alkali were measured. They were chromatographed in solvent C [propan-2-ol-water (7:3, v/v); Markham & Smith (1952)] with which NH_3 is present in the vapour phase and in solvent D [satd. $(\text{NH}_4)_2\text{SO}_4$ -M-sodium acetate-propan-2-ol (79:19:2, by vol.); Markham & Smith (1951)], and subjected to electrophoresis in 0.1 M-ammonium formate buffer, pH 3.5 (Markham & Smith, 1952). They were hydrolysed for 1 hr. with N-HCl and chromatographed in solvents A and B. Spectra of the products in acid, neutral solution and alkali were measured after each separation.

The measured spectral characteristics of the untreated nucleotides* do not differ appreciably in this respect from values from the literature [values compiled by the California Foundation for Biochemical Research were used (Catalogue April 1962, p. 208)]. The phosphorus contents were close to the calculated values. Less than 5% of the ultraviolet absorption of the GMP was due to contaminants and these were mainly traces of guanine and related bases. Less than 5% of the ultraviolet absorption of the AMP was due to a contaminant, which was probably adenine. The CMP contained approx. 5% of UMP. The UMP appeared to be free from ultraviolet-absorbing contaminants. Chromatography in solvent D showed that all the samples contained a preponderance of one or other of the 2'- and 3'-isomers. The hydrolysis of each nucleotide by N-HCl at 100° was followed for 1 hr. (Table 1). Recoveries from an

* Abbreviations: AMP, GMP, CMP and UMP represent mixtures of the 2'- and 3'-isomers of adenylic acid, guanylic acid, cytidylic acid and uridylic acid respectively.

Table 1. Recoveries of reference nucleotides after acid hydrolysis and paper chromatography

The method of Smith & Markham (1950) was used. Solutions of each nucleotide were made in N-HCl, and samples were heated in sealed tubes, run in solvent A on Whatman no. 1 paper and estimated spectrophotometrically. In control runs 93.1 and 96% of AMP and GMP respectively were recovered when they were run in solvent A. The values for CMP and UMP were not corrected for the losses known to occur by hydrolysis to the corresponding nucleoside (Markham & Smith, 1951). Each value is the mean of four determinations.

Time of hydrolysis (min.)	Percentage of sample recovered			
	GMP (as guanine)	AMP (as adenine)	CMP	UMP
0	—	—	98.1 $\pm$ 0.2%	98.8 $\pm$ 0.3%
15	95.1 $\pm$ 0.3%	92.8 $\pm$ 0.2%	93.5 $\pm$ 0.2%	94.4 $\pm$ 0.3%
60	95.1 $\pm$ 0.2%	92.0 $\pm$ 0.4%	90.1 $\pm$ 0.3%	92.7 $\pm$ 0.2%

approximately equimolar mixture of the four reference nucleotides after the same treatment, and also after such treatment preceded by heating for 48 hr. in *N*-diethylamine at 55° (Crestfield & Allen, 1957) and removal of the diethylamine and water by evaporation *in vacuo*, are given in Table 2.

Identification of ribonucleotides from ribonucleic acid. To obtain suitable solutions for the spectrophotometric comparisons an approximately equimolar mixture of the four reference nucleotides was run in bands containing about 10 mg. of total nucleotides on strips of Whatman no. 3 MM paper 23 cm. wide with solvent E [propan-2-ol-water (3:2, v/v); Markham & Smith (1951)]. The nucleotide bands were eluted with water and combined, and samples were taken for electrophoresis at pH 3.5 and hydrolysis in *N*-HCl at 100° for 1 hr. followed by chromatography in solvent A. The products of both separations were eluted with water (0.1 *N*-HCl for guanine) and absorption spectra were measured in acid, neutral solution and alkali against appropriate paper blanks. The solutions were adjusted to have an extinction at the maximum of about 1.0. Total phosphorus was determined after the separation by electrophoresis.

Samples of the components separated by chromatography in solvent A were run separately in solvent B and spectral measurements were repeated on the eluted components.

All spectral characteristics were the same as those of the reference specimens of nucleotides and purines. ϵ_{max} and ϵ_{min} were not measured. Characteristic absorption spectra were plotted on the Cary model 14 recording spectrophotometer by using the log(density) slide wire (part no. 1480550). The treated reference nucleotides gave spectra that coincided with those of the untreated compounds at least over the range 240–280 $m\mu$ and usually well beyond in both directions, and the form of the spectra remained approximately the same from 220 to 300 $m\mu$. The characteristic absorption spectra of the guanine and adenine obtained by acid hydrolysis of the nucleotides coincided with those of the authentic bases at the three chosen pH values.

The nucleotides were obtained from RNA preparations by heating 20 mg. of material with *N*-diethylamine at 55° for 48 hr. (Crestfield & Allen, 1957), the reagent and surplus water being removed by evaporation *in vacuo* before examination. A sample of the mixture obtained by acid hydrolysis was then used to determine the nucleotide composition.

Pentose in nucleotides. Samples (5–10 mg.) of leaf RNA were hydrolysed with diethylamine for the determination of pentose. The nucleotides eluted after running in solvent E were concentrated to 0.60 ml. and centrifuged at high speed. Samples (10 μ l.) were taken for the estimation of purine-bound pentose by Miltzer's (1946) modification of Bial's orcinol method, by heating the sample for 8 min. with the reagent (Markham, 1955) and using xylose as a standard. Pyrimidine-bound pentose was labilized by diluting a 100 μ l. sample to 5 ml. with water and adding freshly made bromine water drop by drop until the solution just remained coloured (Cohn, 1956). Excess of bromine was removed by aeration for 5 min., 0.5 ml. of *N*-diethylamine added, and the solution kept at room temperature for 2 hr. and then evaporated *in vacuo*. Samples were taken for pentose determination, the difference between the pentose values for equivalent treated and untreated

Table 2. *Recovery of the four ribonucleotides from an equimolar mixture after acid hydrolysis and after alkaline hydrolysis followed by acid hydrolysis*

A solution of the four reference nucleotides, each about 2.5 mm, was made in distilled water from the anhydrous compounds. To obtain the values in row 1 weighed samples of such a solution were dried, hydrolysed with <i>N</i> -HCl for 1 hr. at 100° and run in solvent A for analysis by the method of Smith & Markham (1950). To obtain the values in row 2 weighed samples of such a solution were hydrolysed with <i>N</i> -diethylamine at 55° for 48 hr., dried, hydrolysed with <i>N</i> -HCl for 1 hr. at 100° and run in solvent A for analysis. Two samples of the mixture were hydrolysed in this way and two samples from each hydrolysate were analysed. The values in parentheses were calculated from the composition of the prepared mixture. The values for CMP and UMP were corrected for losses due to partial hydrolysis to the corresponding nucleosides by the <i>N</i> -HCl (Markham & Smith, 1951).									
Percentage of each nucleotide recovered				Percentage of total nucleotides recovered				Nucleotide composition (moles/100 moles)	
GMP (as guanine)	AMP (as adenine)	CMP	UMP	GMP	AMP	CMP	UMP	Purine pyrimidine nucleotide ratio	AMP + UMP GMP + CMP ratio
1. 93.2 ± 0.3%	93.6 ± 0.4%	93.0 ± 0.4%	100.3 ± 0.6%	24.5 ± 0.3% (25.0)	24.7 ± 0.4% (25.0)	24.4 ± 0.4% (25.0)	26.4 ± 0.4% (25.0)	0.97 (1.00)	1.04 (1.00)
2. 93.9 ± 0.4%	95.9 ± 0.2%	92.1 ± 0.4%	105.8 ± 0.5%	24.2 ± 0.2% (25.0)	24.7 ± 0.5% (24.9)	23.6 ± 0.2% (24.8)	27.6 ± 0.3% (25.3)	0.96 (1.00)	1.09 (1.01)
								0.96 (1.00)	0.96 (1.00)
								0.96 (1.00)	0.93 (0.99)

samples giving a value for the pyrimidine-bound pentose. Each pyrimidine nucleotide gave a high but variable and rarely quantitative recovery after the bromine treatment, whereas the recovery from AMP was quantitative and that from GMP about 50%.

For the preliminary identification of pentose 100 μ l. samples of the untreated nucleotide mixture and an equivalent quantity of the bromine-treated material were hydrolysed for 90 min. with $N-H_2SO_4$ at 100° in sealed tubes. The SO_4^{2-} ions were removed by adding 0.3 N -Ba(OH)₂ to give pH 7. The solution was centrifuged and concentrated at 55°, and samples were chromatographed in solvent F, made by shaking an ethyl acetate–water–acetic acid mixture (3:3:1, by vol.), allowing it to separate and using the top layer (Jermyn & Isherwood, 1949). Samples were also subjected to electrophoresis in sodium borate buffer, pH 9.2, on Whatman no. 3 MM paper, which separates the borate complexes of the four pentoses. By taking the mobility of lyxose as 1.0 the relative mobilities of the others were: ribose, 1.1; arabinose, 1.3; xylose, 1.4. The aniline hydrogen phthalate spray of Partridge (1949) was used to detect the sugar spots on the papers and an equimolar mixture of the four pentoses was used as a marker.

At 260 μ a solution of the mononucleotide mixture obtained by alkaline hydrolysis of leaf RNA, containing 30 μ g. of nucleotides/ml., has an extinction very near to 1.0 at pH 7.0. This value was used to calculate the amounts of purine-bound and total pentose in samples used for pentose analysis from their nucleotide composition and extinction at 260 μ .

Buffers. Buffers used were as follows: 0.1 M -glycine (in 0.1 M -NaCl)–HCl, pH 3; 0.1 M -ammonium formate–formic acid, pH 3.5; 0.05 M -tris–HCl, pH 7.2 and pH 8.0; 0.05 M -boric acid (in 0.05 M -KCl)–NaOH, pH 9.2; 0.1 M -glycine (in 0.1 M -NaCl)–NaOH, pH 10.

Calculations. The variation of replicated results has been given in the form of the percentage error, i.e. the S.E.M. expressed as a percentage of the mean.

Nucleic acid preparations. Nucleic acid preparations were made from leaf powders obtained from freshly harvested leaves heated in aq. 80% (v/v) ethanol (Trim, 1959). In later experiments changes in ethanol concentration caused by evaporation during the boiling were prevented by heating under reflux, the time of heating was increased to 5 min. and the volume of ethanol was increased to 600 ml./30 g. fresh wt. of leaves. The nucleic acids were extracted under various comparatively mild conditions that are not believed to hydrolyse internucleotide bonds.

Methods employing phenol (Kirby, 1956, 1957; Wecker, 1959; Georgiev, 1959) were much less effective than that using hot 1.7 M -NaCl (Trim, 1959), essentially the method of Clarke & Schryver (1917). In this method, as used in the present work, the powder from 30 g. fresh wt. of leaves was extracted four times with 50 ml. of boiling 1.7 M -NaCl in a conical beaker standing in a boiling-water bath. The boiling NaCl was stirred into the warmed contents of the beaker and the heating was continued for 2 min. with stirring. After each extraction the liquid was removed by centrifuging for 5–10 min. at 2000 rev./min. in an improvised basket centrifuge. The basket consisted of a small seamless bag of paper nylon suspended in a wide-mouthed 250 ml. centrifuge bottle with a rubber bung jamming the tapered mouth of the bag into the mouth of the bottle. If it was not intended to measure the phosphorus content of the

residue about 1 g. of kieselguhr was added as a filter aid. This kind of procedure was used in methods 1 and 2 (Trim, 1959). Two further procedures were introduced. One was method 3 in which 1.7 M -diethylamine hydrochloride, pH 7, was used in the place of 1.7 M -NaCl, in other respects the procedure remaining as in method 2. The solvent was prepared by neutralizing diethylamine dissolved in water at 0° by slowly stirring in conc. HCl to give pH 7. It was adjusted to $2 \times 1.7 M$ and diluted for use. The samples of leaf powder were extracted four times for 5 min. In method 4 the pH of the diethylamine was raised to pH 8 by adding an equal volume of tris buffer, pH 8, to the $2 \times 1.7 M$ -diethylamine hydrochloride and the extraction was done in an autoclave at 121° for 1 hr. Method 4 was only used to complete the extraction of nucleic acid after the use of methods 2 and 3. Equal leaf samples for comparing the efficiency of the methods were obtained by dividing the batches of leaves into two at the mid-rib. With some samples of young leaves method 3 was more effective than method 2, but with older leaves the two methods were equally effective. Only the young leaves yielded additional RNA when treated by method 4 after method 3, but treatment by method 4 was always necessary for the complete extraction of DNA.

Extraction of residual phosphorus compounds. The 30 g. fresh wt. samples of leaves usually yielded 10–50 mg. of polynucleotide by the above methods. Even after the most exhaustive extraction the residues contained 0.1–1.0 mg. of phosphorus, equivalent to about 1–15 mg. of polynucleotide. The ease with which this fraction could be removed varied widely with different samples. It was usually completely removed by heating with 0.5 N -HClO₄ at 70°, by heating with 0.17 M -EDTA at pH 7 for 30 min. to 1 hr. in an autoclave at 121° or under the same conditions with 0.1 M -sodium citrate, pH 8.9, which was the method usually used. This treatment decreased the phosphorus content of the residue from 30 g. fresh wt. of leaves to much less than 0.1 mg. Most of the work on this phosphorus fraction was done with sugar-beet leaves but similar results were obtained with the leaves of other plants.

Precipitation and recovery of nucleic acids from plant extracts. Extracts obtained by methods 3 and 4 were made 1.7 M with respect to NaCl by adding the calculated quantity of the solid. The extract obtained by method 4 was then adjusted to pH 3 with acetic acid. To these adjusted extracts and those obtained by method 2 were added 1 vol. of glycine buffer, pH 3, and 0.1 vol. of 0.1 M -Al₂SO₄·K₂SO₄·24H₂O. The mixture was kept overnight at 4° and the nucleic acid precipitate separated by centrifuging. In method 1 (Trim, 1959) the clarified extract in 1.7 M -NaCl was allowed to stand at 0° for 24 hr. when 70–80% of the nucleic acid was precipitated.

The precipitates were dissolved in glycine buffer, pH 10, until the solution was neutral. A final adjustment to pH 7 was made by adding an equal volume of tris–HCl buffer, pH 7. Then 3 vol. of ethanol and 1 drop of saturated NaCl were added and the mixture was kept at 0° overnight. The precipitate of nucleic acid was redissolved in water and reprecipitated twice. The final precipitate was dissolved in water, ethanol removed by evaporation *in vacuo*, and the polynucleotide made to a concentration of 2–10 mg./ml. in water and stored at –20° in 5 ml. serum bottles. Preparations obtained by method 4 contained a large proportion of non-nucleotide material, much of which was removed by

one treatment with phenol (Kirby, 1956). Phenol was removed from the solution by repeated evaporation *in vacuo*.

RESULTS

Efficiency of recovery of nucleic acid

Possible losses that might occur at five stages of the preparation procedure were investigated.

Loss of an ethanol-soluble fraction during the extraction of the leaves with 80 % ethanol. This needs further investigation but with sugar-beet leaves the extract contains no water-soluble components precipitable with Al^{3+} ions at pH 3.

Losses in the tissue residue. This possibility required the examination of the residual phosphorus extracted by citrate. Besides phosphorus these extracts contained nitrogen, pentose and substances that react with the biuret reagent, which were removed by one extraction with phenol (Kirby, 1956), without decreasing the amount of phosphorus in the aqueous layer. After the removal of phenol by distillation *in vacuo* the solutions had no specific absorption at wavelengths between 220 and 300 m μ , and so did not contain nucleotides.

Loss by incomplete precipitation with Al^{3+} ions at pH 3. It was often found that the supernatant solutions after the precipitation of the plant extracts with Al^{3+} ions contained substances with an ultraviolet-absorption spectrum similar to that of polynucleotides. These compounds were not precipitated by ethanol or cetyltrimethylammonium bromide (Aubel-Sadron, Beck, Ebel & Sadron, 1960), and did not sediment in the ultracentrifuge, and so appeared to have a low molecular weight. Compared with simple nucleotides they moved relatively very rapidly or very slowly in the chromatographic solvent C and could not be eluted with ethanolic ammonia (Bergkvist, 1956) after adsorption on to charcoal, confirming that they were not simple nucleotides such as would be produced by the action of plant ribonuclease on RNA.

Loss during reprecipitation with ethanol. Provided that a little sodium chloride was added precipitation of the nucleic acid was always complete.

Loss in the phenol layer during phenol treatment. When fractions obtained by method 4 were treated with phenol (Georgiev, 1959) the phenol layer contained substances giving a positive diphenylamine reaction which may have been DNA. The possibility that this fraction also contained RNA was not investigated, although phenol-soluble fractions of RNA have been reported (Yamana & Sibatani, 1960). Most of the samples of leaves investigated did not require treatment by method 4, but those that did release further RNA on treatment by method 4 after method 3 yielded only a small proportion of the total water-soluble RNA in that fraction.

Composition of ribonucleic acid preparations obtained from plants

The nucleotide composition of preparations made by the above-mentioned methods were usually determined after acid hydrolysis, which introduced errors arising from the presence of DNA in the preparations. These were allowed for as follows.

Herring-sperm DNA, which has a nucleotide composition (Wyatt, 1951) close to that reported for plant DNA (Brawermann & Chargaff, 1951; Thomas & Sherratt, 1956; Thomas, 1959), was used instead of the latter for this purpose. When hydrolysed for 1 hr. at 100° in *N*-hydrochloric acid and chromatographed in solvent A, it was separated into guanine, adenine overlapping the fluorescent derivative W.S. (Levy & Snellbaker, 1955) formed by the reaction between guanine and deoxyribose (Dunn, 1955), a component with the same R_f as UMP, and other less-well-defined ultraviolet-absorbing regions. When eluates of the regions of the chromatogram corresponding in position to the four main components given on acid hydrolysis of RNA were analysed as if they contained these compounds, the apparent values for the molar base proportions of herring-sperm DNA were G:A:C:U as 20.9:26.5:7.6:8.5 respectively, 63.5 % of the weight of the polynucleotide being accounted for in this way. A correction based on this 'nucleotide composition' and the measured value of the DNA present was applied for each ribonucleotide in the RNA sample.

The ribonucleotide composition of preparations made from sugar beet by method 1 (Trim, 1959) was determined by the method of Smith & Markham (1950) with solvent A for the chromatography. The values thus obtained are given in Table 3, and the calculated recoveries of nitrogen, phosphorus and dry matter are given in Table 4. These sodium chloride-precipitated samples, representing only 70–80 % of the polynucleotide extracted from the leaves, contained about 60 % of polynucleotide with a sedimentation constant, $S_{20,w}$, of 5–6S, and were the least degraded of all the preparations that have been analysed.

Most of the preparations were analysed by method 2. Nine such samples from sugar-beet leaves had very similar nucleotide compositions and contained about 8.5 % of phosphorus and 14 % of nitrogen. They included preparations from young and mature leaves of both healthy plants and plants heavily infected with a necrotic strain of sugar-beet-yellows virus. Some of the plants were grown in the soil under glass and others in small pots in the glasshouse. The preparations had low sedimentation constants and appeared to contain a considerable proportion of polynucleotide molecules with about 100 nucleotides per chain.

Table 3. Nucleotide composition and nucleotide ratios of ribonucleic acid from four varieties of sugar beet prepared by method 1

The nucleic acids were hydrolysed with N-HCl and analysed by the method of Smith & Markham (1950). Values (i) are corrected for the effect of the hydrolysis products of the DNA component. Values (ii) were obtained after hydrolysing with N-KOH for 24 hr. at 22°. The solution was then brought to pH 1 and the precipitate of DNA removed by centrifuging. The pH was brought to 0° and the temperature to 0° and the precipitate of KClO₄ removed by centrifuging. The supernatant solution was evaporated and hydrolysed with N-HCl for analysis as above. The values for DNA are the means of two determinations.

Beet variety	No. of hydrolyses	No. of determinations per hydrolysate	DNA (% of total nucleotides)	Nucleotide composition (moles/100 moles)					Purine pyrimidine nucleotide ratio	AMP + UMP		AMP + CMP GMP + UMP ratio
				GMP (as guanine)	AMP (as adenine)	CMP	UMP	AMP + UMP GMP + CMP ratio		AMP + UMP GMP + CMP ratio		
KDNB (<i>Beta vulgaris</i>)	2	2	4.8	30.4	25.0	23.7	20.9	1.24	0.85	0.95		
				±0.8%	±0.4%	±0.5%	±0.5%					
				(i) 30.3	24.5	24.0	21.2					
				(ii) 30.6	24.6	23.3	21.6					
Battle's (<i>Beta vulgaris</i>)	2	2	5.2	±0.4%	±0.4%	±0.6%	±0.9%	1.24	0.85	0.94		
				30.6	24.8	23.6	21.1					
				±0.3%	±0.2%	±0.4%	±0.4%					
				(i) 30.5	24.3	23.9	21.4					
Polyploid (<i>Beta vulgaris</i>)	2	2	5.4	(ii) 30.8	24.5	23.2	21.5	1.24	0.85	0.91		
				±0.7%	±0.1%	±0.0%	±0.9%					
				30.5	25.0	23.8	20.9					
				±0.2%	±0.2%	±0.3%	±0.2%					
<i>Beta maritima</i>	2	2	4.7	(i) 30.4	24.5	24.1	21.2	1.21	0.84	0.94		
				(ii) 30.5	24.4	23.2	22.0					
				±0.5%	±0.7%	±1.1%	±2.3%					
				30.9	24.4	23.3	21.4					
				±0.3%	±0.1%	±0.4%	±0.1%	1.21	0.84	0.90		
				(i) 30.8	23.9	23.6	21.7					
				(ii) 30.2	24.3	23.4	22.1					
				±0.3%	±0.9%	±1.0%	±0.6%					

Samples dialysed at 0° against distilled water for 4 days and then against saturated sodium chloride for the same period lost 1–3 % of material with an absorption spectrum like that of nucleotides. For analysis the samples were treated by Kirby's (1956) Cellosolve method to remove polysaccharide. No polynucleotide was lost. Analyses of three samples from healthy leaves are given in Tables 5 and 6.

The nucleotide composition of the RNA from three different tissues of the sugar beet (*Chenopodiaceae*) are set out in Table 7. Analyses of RNA preparations from the leaves of three other families of plants, namely wheat (*Gramineae*), kale (*Cruciferae*) and potato (*Solanaceae*), are given in Table 8.

Samples of leaf RNA were prepared from one representative of each of four families of plants: *Solanaceae*, potato (*Solanum tuberosum*); *Chenopodiaceae*, sugar beet (*Beta vulgaris*); *Gramineae*, wheat (*Triticum aestivum*); *Cruciferae*, kale (*Brassica oleracea*). All preparations were by method 3, followed by method 4 to ensure complete extraction

of nucleic acids. The preparations were severely degraded, having $S_{20,w}$ 2s and an increase of ϵ_{257} (P) (i.e. the molar extinction coefficient for nucleic acid based on 1 g. atom of phosphorus; Chargaff & Zamenhof, 1948) on hydrolysis of about 25 %. However, prolonged dialysis against water and saturated sodium chloride at 0° caused a very small loss of ultraviolet-absorbing substances. The preparations contained 10–20 % of peptide material which was decreased to about one-half after one treatment with phenol–sodium *p*-aminosalicylate at pH 7.4 (Georgiev, 1959). All leaf residues were extracted with sodium citrate to remove considerable quantities of residual phosphorus compounds, which were shown to be free from nucleotide material.

Since deproteinization with phenol might involve a loss of nucleic acid and in any case was not complete, the samples used for nucleotide analysis and identification were not treated in this way. To avoid interference by DNA and to obtain all the hydrolysis products in the nucleotide form, the samples were hydrolysed with base (*N*-diethylamine).

Table 4. Recovery of nitrogen, phosphorus and dry matter calculated from nucleotide analyses of ribonucleic acid from sugar-beet leaves

The corresponding nucleotide analyses are given in Table 3. The values obtained after acid hydrolysis were used, uncorrected for DNA.

Beet variety	N (%)	P (%)	N, P and dry matter in the sample calculated from the nucleotide determinations (% of the means of the same values obtained by direct measurements)		
			N	P	Dry matter
KDNB (<i>Beta vulgaris</i>)	14.7 ± 0.3 % (4)	8.6 ± 0.6 % (4)	85.5 ± 0.2 %	92.3 ± 0.1 %	82.9 ± 0.1 %
Battle's (<i>Beta vulgaris</i>)	15.0 ± 0.3 % (4)	8.4 ± 0.0 % (4)	83.4 ± 0.1 %	93.7 ± 0.1 %	82.7 ± 0.1 %
Polyploid (<i>Beta vulgaris</i>)	15.4 ± 0.2 % (4)	7.7 ± 0.4 % (4)	77.1 ± 0.3 %	98.0 ± 0.3 %	78.3 ± 0.3 %
<i>Beta maritima</i>	15.3 ± 0.0 % (4)	8.6 ± 0.6 % (4)	93.0 ± 0.5 %	93.7 ± 0.5 %	84.5 ± 0.5 %

Table 5. Nitrogen and phosphorus composition and other properties of sugar-beet-leaf ribonucleic acid obtained by method 2

Values given for measurements other than those of N and P are the means of duplicate determinations.

Plant material	Protein (%)	DNA (%)	N (%)	P (%)	N/P ratio	ϵ_{257} (P) at pH 7.0	Percentage increase of ϵ_{257} (P), measured at pH 7.0, on alkaline hydrolysis
1. Young leaves from healthy plants grown in the soil under glass	Approx. 1.8	1.3	14.6 ± 0.4 % (3)	8.4 ± 0.6 % (3)	3.8	8060	32
2. Mature leaves from healthy plants grown in the soil under glass	—	1.9	14.5 ± 0.0 % (3)	8.4 ± 0.1 % (3)	3.8	8040	27
3. Mature leaves from healthy plants grown under glass in small pots	Approx. 0.9	2.5	13.9 ± 0.4 % (3)	8.7 ± 0.4 % (3)	3.5	8200	25

Table 8. *Nucleotide composition of ribonucleic acid prepared from the leaves of three plant species*

Leaves approaching maturity were taken from healthy plants grown in pots in the glasshouse. A large sample of leaves from each plant was divided into three equal parts and the nucleic acid prepared from each of them by method 2. For the analyses two samples of each preparation were hydrolysed with acid and two determinations of the nucleotide composition were made on each hydrolysate by the method of Smith & Markham (1950). The values in parentheses are corrected for DNA.

Plant	Prep. no.	DNA (% of total nucleotide)	Nucleotide composition (moles/100 moles)				Purine pyrimidine nucleotide ratio	AMP + UMP GMP + CMP ratio	AMP + CMP GMP + UMP ratio
			GMP (as guanine)	AMP (as adenine)	CMP	UMP			
Wheat (<i>Triticum aestivum</i>)	1	4.3	30.3 ±0.8% (30.2)	25.2 ±0.1% (24.8)	23.9 ±0.2% (24.3)	20.6 ±0.2% (20.8)	1.25 (1.22)	0.85 (0.84)	0.97 (0.96)
	2	3.7	30.5 ±0.1% (30.4)	25.1 ±0.1% (24.7)	23.7 ±0.2% (24.0)	20.7 ±0.2% (20.9)	1.26 (1.23)	0.85 (0.84)	0.95 (0.95)
	3	3.4	30.3 ±0.2% (30.3)	25.0 ±0.2% (24.6)	23.9 ±0.1% (24.1)	20.8 ±0.4% (21.0)	1.24 (1.22)	0.85 (0.84)	0.96 (0.95)
Kale (<i>Brassica oleracea</i>)	1	5.8	29.7 ±0.2% (29.6)	25.6 ±0.2% (25.0)	23.6 ±0.2% (24.1)	21.1 ±0.4% (21.4)	1.24 (1.20)	0.88 (0.86)	0.97 (0.96)
	2	6.3	29.7 ±0.5% (29.6)	25.6 ±0.9% (24.9)	23.6 ±0.6% (24.1)	21.1 ±0.4% (21.5)	1.24 (1.20)	0.88 (0.86)	0.97 (0.96)
	3	5.2	29.2 ±0.5% (29.0)	25.5 ±0.0% (24.9)	24.0 ±0.3% (24.4)	21.4 ±0.4% (21.7)	1.21 (1.17)	0.88 (0.87)	0.98 (0.97)
Potato (<i>Solanum tuberosum</i>)	1	10.7	30.2 ±0.7% (30.0)	25.9 ±0.3% (24.8)	23.2 ±0.6% (24.0)	20.7 ±0.5% (21.2)	1.28 (1.21)	0.87 (0.85)	0.97 (0.95)
	2	14.9	30.2 ±0.9% (29.9)	26.2 ±0.6% (24.6)	23.1 ±0.5% (24.3)	20.4 ±0.7% (21.1)	1.30 (1.20)	0.87 (0.84)	0.97 (0.96)
	3	15.7	29.5 ±0.4% (29.1)	26.2 ±0.3% (24.4)	23.1 ±0.5% (24.3)	21.2 ±0.4% (22.1)	1.26 (1.15)	0.90 (0.87)	0.97 (0.97)

Table 9. Nucleotide composition of ribonucleic acid from the leaves of four species of plants

Approx. 20 mg. samples of nucleic acid prepared by methods 3 and 4 were hydrolysed to nucleotides by heating with *N*-diethylamine for 48 hr. at 55°. The diethylamine was removed by evaporation *in vacuo* and the nucleotide mixture run as bands on two 23 cm. strips of Whatman no. 3MM paper in solvent D. The nucleotide bands were eluted by irrigation with water, combined and concentrated. Samples containing 2-4 mg. of nucleotide were hydrolysed with *N*-HCl at 100° for 1 hr. for the determination of the nucleotide composition by the method of Smith & Markham (1950).

Nucleic acid sample from	No. of determinations	Nucleotide composition (moles/100 moles)					Purine pyrimidine nucleotide ratio	AMP + UMP GMP + CMP ratio	AMP + CMP GMP + UMP ratio
		GMP (as guanine) $\pm$ 1.3%	AMP (as adenine) $\pm$ 0.9%	CMP $\pm$ 0.9%	UMP $\pm$ 0.8%				
Potato by method 3	4	29.0	24.1	24.7	22.3		1.13	0.87	0.95
Potato by method 4 after method 3; no nucleic acid was extracted	—	—	—	—	—		—	—	—
Sugar beet by method 3	4	28.9 $\pm$ 0.1%	24.5 $\pm$ 0.3%	25.5 $\pm$ 0.8%	21.2 $\pm$ 0.8%		1.14	0.84	1.00
Sugar beet by method 4 after method 3	4	29.7 $\pm$ 0.2%	24.9 $\pm$ 0.1%	23.8 $\pm$ 0.4%	21.5 $\pm$ 0.4%		1.21	0.87	0.95
Wheat by method 3	4	28.7 $\pm$ 0.3%	25.2 $\pm$ 0.2%	24.8 $\pm$ 0.3%	21.4 $\pm$ 0.3%		1.17	0.87	1.00
Wheat by method 4 after method 3	4	30.2 $\pm$ 0.3%	24.6 $\pm$ 0.6%	24.6 $\pm$ 0.6%	20.6 $\pm$ 1.4%		1.21	0.82	0.96
Kale by method 3	4	29.2 $\pm$ 0.2%	24.7 $\pm$ 0.3%	23.9 $\pm$ 0.2%	22.2 $\pm$ 0.3%		1.17	0.88	0.94
Kale by method 4 after method 3; no nucleic acid was extracted	—	—	—	—	—		—	—	—

About 98 % of the ultraviolet-absorbing material that moved away from the origin in solvent E was located in the mononucleotide bands and separated into compounds with the chromatographic and electrophoretic properties of GMP, AMP, CMP and UMP only, guanine and adenine appearing instead of GMP and AMP after acid hydrolysis. The characteristic absorption spectra of the products corresponded exactly to those of the treated reference compounds at least over the wavelength range 240–280 $m\mu$ and usually well beyond in both directions. With CMP the range of coincidence was from 240 to 290 $m\mu$, except for the spectra measured at pH 2 for which the coincidence was exact from 250 to 300 $m\mu$. Beyond these ranges the correspondence was close. After electrophoresis the nucleotides contained 95–100 % of the phosphorus calculated from the extinction at λ_{max} . The treated reference nucleotides contained 95–97 % of the calculated proportion of phosphorus. The nucleotide compositions are given in Table 9.

The pentose component of both the purine and pyrimidine nucleotides obtained from all preparations corresponded chromatographically and electrophoretically with ribose to the extent of 98 %. Heavily loaded chromatograms showed traces of other components reacting like pentose and moving in the positions of xylose and arabinose. There were also traces trailing back to the origin. There was no evidence of the presence of aldohexoses and 6-deoxyaldohexoses. Pentose values determined by the orcinol reaction are given in Table 10.

DISCUSSION

It is now becoming possible and necessary to separate tissues into their subcellular fractions before isolating nucleic acids for study, and grosser procedures, used above, are declining in importance. But for some purposes, and particularly where plants are concerned, the latter continue to have certain advantages: for example, they are more quantitative than the procedures employing a preliminary fractionation of cell components and they yield products that are degraded to a limited, perhaps definite, extent.

A very high proportion of the whole RNA of leaves has been shown above to be composed of the four major ribonucleotides. This was established by the fullest use of methods employing chromatography and electrophoresis on paper. However, such evidence is not sufficient to preclude the presence of even quite large proportions of other constituents such as isomers and closely related derivatives of the known ribonucleotides. It is relevant that both of these types of compounds have already been found in alkaline hydrolysates of RNA: pseudouridylic acid, 5-ribosyluridine mono-

Table 10. *Pentose in nucleotides obtained by alkali hydrolysis of ribonucleic acid extracted from the leaves of four species of plants by methods 3 and 4*

Samples of the material used for the measurements recorded in Table 9 were analysed by the method for purine-bound and pyrimidine-bound pentose described in the text. Values for the means of duplicate determinations are given.

Nucleic acid sample from	Purine-bound pentose ($\mu\text{g./0.1 ml. sample}$)		Total pentose ($\mu\text{g./0.1 ml. sample}$)	
	Calc.	Measured	Calc.	Measured
Potato by method 3	265	280	499	446
Sugar beet by method 3	337	360	632	531
Sugar beet by method 4 after method 3	237	228	434	344
Wheat by method 3	300	323	556	500
Wheat by method 4 after method 3	249	270	454	419
Kale by method 3	337	343	626	532

phosphate (Davis & Allen, 1957; Cohn, 1959) and 2-*O*-methyl ribonucleotides in the alkali-stable dinucleotides separated by Smith & Dunn (1959). There is now preliminary evidence for the presence of all the known minor constituents of RNA in preparations from plant sources (Dunn, Hitchborn & Trim, 1963). This emphasizes the need for a more rigorous approach to the analysis of nucleic acids.

The preliminary evidence given above for the nature of the pentose is more than is usually accepted for identification of the sugar in RNA. It seems fairly certain that ribose may be obtained in approximately the correct stoichiometric proportions from both the purine and the pyrimidine nucleotides of leaf RNA. Counting the initial step whereby the RNA is separated from the nucleoprotein complex, in which most of it is combined in the native state, this separation is achieved by submitting the material to a number of comparatively drastic treatments that include hydrolysis with *N*-alkali at 55° for 48 hr. and hydrolysis with *N*-sulphuric acid at 100° for 90 min. Heating with the acid destroys some of the sugar so that it can no longer react with the pentose reagent. The possibility that this and other treatments could degrade ribose derivatives to ribose itself has yet to be taken into consideration in interpreting analyses of RNA. The fact that the 2-(or 3-)hydroxyl groups of certain of the ribose residues of RNA are not free, as in the amino acyl forms of transfer RNA (Hoagland, 1960) and the 2-(or 3-)*O*-methyl ribose residues shown by Smith & Dunn (1959) to occur in small amounts in a number of preparations of RNA, suggests the possibility that other (more or less stable) derivatives of RNA involving this group may exist.

The inadequacy of existing methods for the analysis of nucleic acids is further emphasized by the fact that all preparations that have been described so far contain non-nucleotide material. With little justification this is usually treated as a

contaminant. It should be made clear that there are no grounds for ignoring the repeated claims that peptides, firmly bound to polynucleotides, which are found in RNA preparations, may not be artifacts (Potter & Dounce, 1956; Medvedev & Zabolotski, 1958; Habermann, 1959). The analyses of nucleic acids discussed in the present paper would allow for the presence of at least a small proportion of peptides or other nitrogen compounds or both, a proportion of non-nucleotide phosphorus compounds and often a rather higher proportion of material containing neither nitrogen nor phosphorus. As far as nitrogen compounds are concerned, Johnson & Markham's (1962) discovery of bis-(3-aminopropyl)amine associated with RNA in certain plant viruses suggests that organic bases may be specifically bound to native RNA, possibly by covalent linkages.

In the present paper representative examples have been quoted from among a much larger number of analyses of different samples of plant RNA obtained from the leaves, and other organs, of higher plants placed in five different families that are widely separated in taxonomy. Both healthy and virus-infected leaves of different ages have been studied. The results are summarized in Table 11 and no biological significance is attached to the small differences in composition that were found.

Of comparable analyses of plant RNA already published only those of Vanyushin & Belozersky (1959) agree at all closely with those given in the present paper. Thomas & Sherratt (1956) analysed RNA from a number of plants but it is likely that their preparations were fractions of the whole RNA. In their work and that of Reddi (1957) insufficient precautions against degradation were taken.

A summary of some of the values obtained by Vanyushin & Belozersky, included in Table 11 for comparison, shows that they are close to those

Table 11. Mean values of measurements of the nucleotide composition of ribonucleic acid in higher plants

Nucleotide composition (moles/100 moles)				Purine pyrimidine nucleotide ratio	AMP + UMP GMP + CMP ratio	AMP + CMP GMP + UMP ratio
GMP	AMP	CMP	UMP			
30.1 ±0.4%	24.5 ±0.3%	24.2 ±0.2%	21.2 ±0.5%	1.20	0.84	0.95
30.0 ±0.6%	25.6 ±0.5%	23.0 ±1.4%	21.4 ±0.5%	1.25	0.89	0.95
29.0 ±0.4%	24.6 ±0.9%	24.7 ±1.3%	21.8 ±1.1%	1.15	0.86	0.97
29.4 ±0.6%	25.0 ±0.5%	24.9 ±0.5%	20.8 ±0.8%	1.19	0.84	1.00

Mean values for 27 samples from leaves representing five families of higher plants. Samples were prepared by method 2, and the composition was determined by the method of Smith & Markham (1950). Values are corrected for DNA, which ranged from 1 to 27% of the total polynucleotide

Mean values for five samples representing two families of plants. Samples were prepared by method 3, and the composition was determined by the method of Smith & Markham (1950). Values are corrected for DNA content

Mean values for four samples, representing four families of plants, prepared by method 3, hydrolysed with *n*-diethylamine (to nucleotides) followed by the method of Smith & Markham (1950)

Mean values for 19 samples from seeds and pollen of 18 families of dicotyledonous plants. Samples were analysed as nucleotides obtained by hydrolysis with KOH (Vanyushin & Belozersky, 1959)

reported in the present paper. Seeds and pollens of 18 species of dicotyledonous plants occurring in 18 different families were analysed by estimating the nucleotides released from the whole tissues by the action of alkali. It seems permissible to compare these two sets of results since it has been shown in the present work that similar values are obtained with either acid or alkaline hydrolysis and since there is no evidence of organ specificity in the nucleotide composition of the whole RNA of tissues. However, the agreement between the results obtained by the two methods might be less if account were to be taken of that portion of RNA, discussed by Potter & Dounce (1956), which is not hydrolysed to simple nucleotides on hydrolysis with alkali. The composition, proportion and distribution of this component in alkali hydrolysates of RNA is still uncertain. It has not been possible to assess the effect of hydrolysis in the presence of other tissue components on the yield of each nucleotide, and in the comparisons that have been made any such effect has been assumed to be negligible.

A quantitative and generally applicable procedure for the separation of DNA from plant tissues would be invaluable and attempts were made to devise one during the present work. Two main obstacles were encountered. Part of the DNA was found to be firmly attached to the insoluble tissue residue. It could be extracted by the most drastic of the procedures employed, namely method 4, but when obtained in this way it was mixed with a very large amount of other material from which it could not be easily or quantitatively separated. It was also found that part of the DNA became insoluble in water when salts were removed. These observations led to the conclusion that some other approach is necessary to obtain a simple and quantitative separation of DNA from plant tissues.

SUMMARY

1. Methods for the extraction of nucleic acids from leaves have been devised and their efficiency studied. The most efficient methods depend on extraction with 1.7M-sodium chloride or 1.7M-diethylamine hydrochloride at 100°.
2. About 80 % of the nucleic acid extracted with neutral 1.7M-sodium chloride was precipitated on standing at 0°. This nucleic acid, like that obtained by extraction with 1.7M-diethylamine hydrochloride at 100°, was precipitated without loss by dilute Al³⁺ ions at pH 3.
3. All preparations were composed mainly of polynucleotide, the greater proportion of which was RNA. The DNA content varied widely, from 2 to 27 % of the total polynucleotide.
4. The nucleotide composition of a large number of preparations, including some from sugar beet

heavily infected with a necrotic strain of sugar-beet-yellowings virus was determined, and no biological significance has been attached to the small variations of composition that were found.

5. The major nucleotide components of the RNA from the leaves of four species of plants have been more fully identified. The samples were extracted by the most exhaustive procedures for separating nucleic acid and the phosphorus compounds remaining in the leaf residues were extracted and shown to be free from nucleotides.

6. Electrophoresis and chromatography of the products of hydrolysis of these samples, followed by the measurement of spectral characteristics, showed the presence in quantity of only AMP, GMP, CMP and UMP (2'- and 3'-isomers), and there was no evidence of the presence of other nucleotides, or nucleosides, in more than trace quantities.

7. Preliminary studies by indirect methods suggest that effectively the whole of the purine-bound and pyrimidine-bound sugar is ribose.

This work was supported, in part, by a grant from the U.S. Public Health Service. The authors are indebted to Dr G. D. H. Bell, Director of The Plant Breeding Institute, and Dr R. Markham, F.R.S., Director of the Agricultural Research Council's Unit for Virus Research, for their interest and advice.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
- Aubel-Sadron, G., Beck, G., Ebel, J. P. & Sadron, C. (1960). *Biochim. biophys. Acta*, **42**, 542.
- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
- Bergkvist, R. (1956). *Acta chem. scand.* **10**, 1303.
- Brawermann, G. & Chargaff, E. (1951). *J. Amer. chem. Soc.* **73**, 4052.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Chargaff, E. & Zamenhof, S. (1948). *J. biol. Chem.* **173**, 327.
- Clarke, G. B. & Schryver, S. B. (1917). *Biochem. J.* **11**, 319.
- Coghill, R. D. (1931). *J. biol. Chem.* **90**, 57.
- Cohn, W. E. (1956). *Biochem. J.* **64**, 28 p.
- Cohn, W. E. (1959). *Biochim. biophys. Acta*, **32**, 569.
- Crestfield, A. M. & Allen, F. W. (1957). *Fed. Proc.* **16**, 168.
- Davis, F. F. & Allen, F. W. (1957). *J. biol. Chem.* **227**, 907.
- Dunn, D. B. (1955). *Biochim. biophys. Acta*, **18**, 317.
- Dunn, D. B., Hitchborn, J. H. & Trim, A. R. (1963). *Biochem. J.* **88**, 34 p.
- Georgiev, G. P. (1959). *Biokhimiya*, **24**, 472.
- Habermann, V. (1959). *Biochim. biophys. Acta*, **32**, 297.
- Hayes, D. H., Michelson, A. M. & Todd, A. R. (1955). *J. chem. Soc.* p. 808.
- Hoagland, M. B. (1960). In *The Nucleic Acids*, vol. 3, p. 349. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Holden, M. & Pirie, N. W. (1955). *Biochem. J.* **60**, 46.
- Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
- Johnson, M. W. & Markham, R. (1962). *Virology*, **17**, 276.
- Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
- Kirby, K. S. (1957). *Biochem. J.* **66**, 495.
- Levy, H. B. & Snellbaker, L. F. (1955). *Arch. Biochem. Biophys.* **54**, 549.
- Markham, R. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 246. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
- Markham, R. & Smith, J. D. (1951). *Biochem. J.* **49**, 401.
- Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.
- Medvedev, Zh. A. & Zabolotski, N. N. (1958). *Proc. Timiryazev agric. Acad.* **39**, 142.
- Militzer, W. E. (1946). *Arch. Biochem.* **9**, 85.
- Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
- Potter, J. L. & Dounce, A. L. (1956). *J. Amer. chem. Soc.* **78**, 3078.
- Reddi, K. K. (1957). *Biochim. biophys. Acta*, **23**, 208.
- Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
- Smith, J. D. & Dunn, D. B. (1959). *Biochim. biophys. Acta*, **31**, 573.
- Smith, J. D. & Markham, R. (1950). *Biochem. J.* **46**, 509.
- Thomas, A. J. (1959). *Arch. Biochem. Biophys.* **79**, 162.
- Thomas, A. J. & Sherratt, H. S. (1956). *Biochem. J.* **62**, 1.
- Trim, A. R. (1959). *Biochem. J.* **73**, 298.
- Vanyushin, B. F. & Belozersky, A. N. (1959). *Dokl. Akad. Nauk S.S.S.R.* **127**, 455.
- Wecker, E. (1959). *Virology*, **7**, 247.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
- Yamana, K. & Sibatani, A. (1960). *Biochim. biophys. Acta*, **41**, 295.