Phosphorus Compounds in the Cell

2. THE SEPARATION BY IONOPHORESIS ON PAPER OF THE CONSTITUENT NUCLEOTIDES OF RIBONUCLEIC ACID

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In the first paper in this series (Davidson, Frazer & Hutchison, 1951) it was emphasized that in experiments on the incorporation of radioactive phosphorus (^{32}P) into the nucleic acids and their derivatives in the cell, it was essential, in order to obtain true specific activities, to separate the nucleotide fractions in a form free from all traces of highly active contaminants. The procedure of ionophoresis of nucleotides now to be described is eminently suitable for this purpose.

The separation of ribonucleotides by ion-exchange chromatography on columns has been described by Cohn (1949, 1950) and by Cohn & Carter (1950), and a chromatographic separation on filter paper has been described by Magasanik, Vischer, Doniger, Elson & Chargaff (1950), by Carter (1950), by Markham & Smith (1951*a*) and by Boulanger & Montreuil (1951*a, b*).

While these methods have much to commend them, they nevertheless suffer from certain disadvantages. In the method of Magasanik *et al.* (1950), for example, guanylic and uridylic acids share the same position on the chromatogram, while in Carter's (1950) and Markham & Smith's (1951*a*) procedure, the pyrimidine nucleotides share a spot. It has therefore occurred to us that a method utilizing the ionophoretic mobility of nucleotides might prove satisfactory, and the technique of ionophoresis as originally described by Consden, Gordon & Martin (1946) has accordingly been adapted for nucleotide separation. A preliminary account of this work has already been given (Smellie & Davidson, 1951).

EXPERIMENTAL

Initial experiments using silica gel as the medium for ionophoresis were not successful. Agar gel, as used by Gordon, Keil, Sebesta, Knessl & Sorm (1950) in the apparatus of Consden *et al.* (1946), was more satisfactory. The position of the nucleotides after separation was established by inspecting the surface of the gel in light from an ultraviolet lamp fitted with a filter, as described by Holiday & Johnson (1949). Elution was not attempted, but the areas of gel shown to contain nucleotides were cut out and the excised portions dissolved in water for the determination of the ultraviolet absorption spectra of the nucleotides. The agar

solution containing the nucleotides was then digested for the determination of P.

This method, while moderately satisfactory, is rather cumbersome and has been superseded by the use of the method of ionophoresis on paper described by Durrum (1950) for amino-acids. The procedure is as follows: an accurately measured volume of the material to be analysed, containing about 80 μg . P, is applied in a narrow band about 3.5 cm. long, about 5 cm. from one end of a strip of Whatman 3 MM paper, 72 $\times$ 7 cm. The application is made from an Agla micrometer syringe (Burroughes Wellcome Ltd.), and by holding the paper in a stream of warm air it is not difficult to spot up to 500 μl . Excessive heat must be avoided since it causes spontaneous formation of isomeric nucleotides. The strip is then moistened with 0.02M-citric acid/trisodium citrate buffer, pH 3.5 (obtained by a 50-fold dilution of a mixture of 3 vol. M-citric acid and 1 vol. M-trisodium citrate) and suspended over a glass rod so that the two ends are immersed to a depth of 1 cm. in two separate dishes containing about 0.5 l. of the same buffer. Carbon electrodes placed in each dish are connected to a source of direct current so that the anode is in the dish further away from the spot on the paper. The direct current supply consists of a full-wave valve rectifier capable of a smoothed output of 600 V. at 300 ma., which on a small load actually delivers about 800 V. The output is controlled by means of a Variac transformer in the input side which makes possible a complete range of 100–800 V.

Complete separation of adenylc, cytidylic, guanylic and uridylic acids is obtained in amounts of the order of 200–300 μg . of each by running at a potential gradient of 11 V./cm. for 18 hr. The apparatus is covered during the run with a glass case to minimize evaporation, and although it is possible to obtain the same separation in a shorter time by increasing the applied voltage, this procedure results in increased evaporation so that water condenses on the case and is liable to drip on to the paper. The apparatus is of such a size that several papers can be used simultaneously.

On completion of the run, the paper is dried by an infrared lamp, the bands located in ultraviolet light according to the procedure of Holiday & Johnson (1949) and marked lightly in pencil. If a permanent record is desired a photograph may be made by the method of Markham & Smith (1949). The bands are then cut out and the nucleotides eluted by the method of Consden, Gordon & Martin (1947) into graduated Pyrex tubes. The volume of the eluate is adjusted to 5 ml. with distilled water, sufficient 5N-HCl having been added to give a final concentration of 0.1N. The ultraviolet absorption spectrum of each eluate is determined on the Beckman Model DU spectrophotometer or a Unicam SP 500 quartz spectrophotometer. Identification is carried out first from

the positions of the bands, and secondly by relating the ultraviolet absorption spectra of the eluates to those of the pure nucleotides at the same pH.

As a further check on the identity of the nucleotides, occasional samples of the eluates have been hydrolysed with HClO_4 according to the method of Marshak & Vogel (1950, 1951) and the bases separated by paper chromatography by the procedure of Wyatt (1951) and characterized by their ultraviolet absorption. In every case only the appropriate base has been found. These separations were kindly carried out by Dr G. Crosbie.

P was estimated in the eluates by a micromodification of the method of Allen (1940).

The method of ionophoresis has been applied to the analysis of various specimens of ribonucleic acid (RNA). A commercial preparation of yeast RNA made by British Drug Houses Ltd. was purified by the method of Smith & Markham (1950) followed by precipitation from glacial acetic acid. Liver RNA was isolated by the method of Davidson *et al.* (1951). The hydrolysis of these purified materials was carried out by incubation with 0.3 N-KOH for 18 hr. at 37°. The volume of alkali used was 1 ml./5 mg. of RNA. The alkaline digest was brought to pH 1 in the cold by the addition of 60% (w/w) HClO_4 , insoluble KClO_4 was centrifuged out and washed, and the supernatant fluid and washings adjusted to pH 4 with N-KOH. P was estimated in one portion of this solution while a second portion was applied to the paper in amounts corresponding to approximately 80 μg . P.

Since the isolation procedure of Davidson *et al.* (1951) is tedious and time-consuming, the following modification has been adopted. A homogenate of liver tissue was extracted with trichloroacetic acid and lipid solvents as described by Davidson *et al.* (1951). The dry powder so obtained was extracted three times for 1 hr. each with 10% (w/v) NaCl solution at 100°. The combined extracts were treated with 2 vol. ethanol, and the precipitate of sodium nucleate was washed with ethanol and ether, and dried. The dry powder in the proportion of 20 mg./ml. was incubated with 0.3 N-KOH at 37° for 18 hr. and brought to pH 1 in the cold by addition of 60% HClO_4 as described above. The precipitate of deoxyribonucleic acid (DNA), protein and KClO_4 was spun down and removed and the acid extract applied to the paper.

This application of the Schmidt & Thannhauser (1945) principle was found to give material, which, while not as pure as an isolated nucleic acid, was nevertheless eminently suitable for ionophoresis. For reasons to be discussed in the next paper the use of the RNA fraction obtained by application of the Schmidt & Thannhauser (1945) method to the tissue itself is complicated by the presence of interfering factors for which special allowance must be made.

In preliminary tests samples of all four purified nucleotides manufactured by Nutritional Biochemicals Inc. were used. Guanylic and cytidylic acids supplied by British Drug Houses Ltd., and adenylic acid by Ernst Bischoff Co. Inc. were also employed.

RESULTS

The separation of pure nucleotides by ionophoresis is illustrated in Fig. 1, which is a photograph taken in ultraviolet light by the method of Markham & Smith (1949) of a sheet of paper to which all four nucleotides had been applied separately and as a

mixture, and subsequently subjected to ionophoresis. It is obvious that adequate separation can be achieved by this method and that the nucleotides separate in the order uridylic acid, guanylic acid

Fig. 1. Ionophoretic runs of single nucleotides and of a mixture of nucleotides. The paper has been photographed on Ilford reflex document paper by ultraviolet light according to the method of Markham & Smith (1949).

Table 1. *Recovery of pure nucleotides after ionophoresis*

(Each nucleotide was applied to the paper singly, run and eluted.)

Nucleotide	P applied (μg .)	P recovered (μg .)	Recovery (%)
Cytidylic acid	25.0	24.6	98.5
	19.2	19.0	99.0
	25.4	24.1	95.5
Adenylic acid	20.7	20.0	97.0
	20.6	20.1	97.5
	20.7	19.8	96.0
Guanylic acid*	19.6	19.0	97.0
	19.6	19.2	98.0
	10.0	9.4	94.0
Uridylic acid	24.7	23.7	96.5
	23.2	22.8	98.5
	23.3	23.7	101.0

* Previously purified by ionophoresis.

adenylic acid and cytidylic acid in decreasing order of mobility. From Table 1 it is seen that the recovery of pure, single nucleotides applied to filter paper, run and eluted is adequate; Table 2 shows that the recoveries from a mixture of the four pure nucleotides are also satisfactory.

Table 2. *Recovery of pure nucleotides from a mixture separated by ionophoresis on paper*

Nucleotide	P applied (μ g.)	P recovered (μ g.)	Recovery (%)
Cytidylic acid	19.2	18.3	95.5
	25.0	24.6	98.5
	25.4	25.0	98.5
Adenylic acid	20.6	19.4	94.5
	20.7	19.6	94.5
	20.7	19.7	95.0
Guanylic acid*	10.0	10.2	102.0
	10.0	9.9	99.0
	10.0	9.8	98.0
Uridylic acid	23.2	22.0	95.0
	24.7	25.4	102.5
	23.3	21.8	93.5

* Previously purified by ionophoresis.

Table 3. *Analysis of a mixture of pure nucleotides (a) by ionophoresis and (b) by chromatography of the bases derived from the nucleotides by perchloric acid hydrolysis and separated by the method of Wyatt (1951)*

(The results are expressed as molar ratios relative to adenylic acid as 10.)

Nucleotide	Ionophoresis	Chromatography
Cytidylic acid	10.8	10.8
Adenylic acid	10.0	10.0
Guanylic acid*	9.0	9.3
Uridylic acid	13.4	12.3

* A correction has been made for 4% free guanine present in the sample of guanylic acid used.

A check on the validity of the ionophoretic procedure is shown in Table 3 which gives the results of analysis of a mixture of pure nucleotides by two different methods. One portion of the solution was submitted to ionophoresis and phosphorus determined in the eluted nucleotides. A second portion of the solution was evaporated to dryness, digested with perchloric acid, the bases separated by paper chromatography (Wyatt, 1951) and determined by ultraviolet absorption measurements. There is, in general, good agreement except for uridylic acid for which it was invariably found that the figure obtained by ionophoresis was slightly higher than that found by chromatography.

Marrian, Spicer, Balis & Brown (1951) have shown that, when cytidylic acid is incubated in *N*-alkali at 37° for 18 hr., partial deamination occurs with the formation of uridylic acid. This observation has been confirmed by taking portions of cytidylic acid which had been incubated for 18 hr. in *N*-KOH at 37° and 25° and in 0.3*N*-KOH at 37° and submitting them to ionophoresis. Only after incubation in *N*-KOH at 37° was any detectable amount of uridylic acid produced. The quantity involved was about 10% of the original cytidylic acid.

A mixture containing all four nucleotides was then divided into four portions, one of which was maintained as a control while the others were incubated in *N*-KOH at 37° and at 25° and in 0.3*N*-KOH at 37°, respectively. The pH of each fraction was adjusted to 4 with 60% perchloric acid, the precipitate of potassium perchlorate was centrifuged out and portions of the supernatant fluid applied to paper in the usual way. After ionophoresis the nucleotides were eluted and phosphorus determined in each eluate. The results of this experiment are illustrated in Table 4 which shows that no deamination occurs after incubation in 0.3*N*-KOH at 37° although deamination is appreciable in *N*-KOH at 37°.

Table 4. *Analysis of a mixture of four nucleotides before and after incubation in 0.3N-KOH and N-KOH at 25° and 37°*

(The mixture after acidification was submitted to ionophoresis and P determination on the eluates. The results are expressed as molar ratios relative to adenylic acid as 10.)

Nucleotide	Un- incubated	0.3N-KOH at 37°	N-KOH at 25°	N-KOH at 37°
Cytidylic acid	11.0	10.8	10.3	9.9
Adenylic acid	10.0	10.0	10.0	10.0
Guanylic acid	9.1	9.0	9.0	9.4
Uridylic acid	13.1	13.4	13.0	15.0
P recovered (%)	98.0	98.5	100.0	92.0

The method of ionophoresis was then applied to the separation of the ribonucleotides obtained by digestion of specimens of purified RNA with 0.3*N*-KOH at 37°. The extent of liberation of acid-soluble phosphates from RNA by this procedure is illustrated in Table 5, from which it is clear that all

Table 5. *Liberation of acid-soluble phosphates from a specimen of yeast ribonucleic acid by alkaline hydrolysis followed by acidification*

(In each case 37.5 mg. RNA were incubated with 5 ml. 0.3*N*-KOH at 37° for 18 hr.)

Total P in alkaline digest (μ g.)	Total P in acid supernatant (μ g.)	Recovery (%)
3150	3070	97.5
3128	3025	96.7
3143	3035	96.6

the phosphorus of the RNA is liberated in the form of acid-soluble phosphates. The fact that only about 97% of the RNAP is recovered in the acid supernatant is almost certainly due to small losses incurred in the process of acidifying, removing the precipitated potassium perchlorate and washing the precipitate.

Specimens of isolated nucleic acid from yeast and from rat liver were then submitted to analysis by ionophoresis of the nucleotides obtained by alkaline incubation, with subsequent estimation of phosphorus in the eluates, and also by paper chromatography of the bases liberated by digestion of the

Table 6. *Analysis of purified yeast and liver ribonucleic acids (a) by ionophoresis of the nucleotides obtained by alkaline incubation with subsequent estimation of P in the eluates and (b) by paper chromatography of the bases after digestion with perchloric acid*

(The results are expressed as molar ratios with respect to adenylic acid or adenine as 10.)

Nucleotide	Yeast RNA		Rat-liver RNA	
	(a)	(b)	(a)	(b)
Cytidylic acid	8.8	8.1	14.5	13.9
Adenylic acid	10.0	10.0	10.0	10.0
Guanylic acid	12.4	11.5	17.6	17.5
Uridylic acid	10.4	7.9	10.1	8.1
P recovered (%)	93.5	—	93.8	—

RNA with perchloric acid. The results are shown in Table 6, while an ultraviolet photograph of the strip of paper used in the separation of the nucleotides in the sample of yeast RNA is shown in Fig. 2. The relative amounts of the four nucleotides are similar to those quoted by other workers (Chargaff, 1950; Chargaff *et al.* 1950; Smith & Markham, 1950). The liver nucleic acid differs from the yeast RNA in its higher contents of cytidylic and guanylic acids. In each case, however, the figure obtained for uridylic acid was appreciably higher by the ionophoresis of nucleotides than by chromatography of the bases.

The results of the analysis of specimens of RNA obtained by the modified isolation procedure in which the dry tissue powder is extracted with 10% sodium chloride solution and the ribonucleic acid so obtained submitted to alkaline hydrolysis are shown in Table 7.

The figures obtained by this method are very similar to those shown in Table 6 for isolated rat-liver RNA. Rat and rabbit livers give values of the same order, while the results obtained for rat-liver mitochondria and microsomes prepared by the method of Schneider (1948) do not differ appreciably from those for whole liver tissue.

Table 7. *Composition of ribonucleic acid from liver tissue as determined by ionophoresis of the ribonucleotide fraction obtained by alkaline incubation of the nucleic acid extracted with 10% NaCl*

(The results are expressed relative to adenylic acid as 10.)

Nucleotide	Rat liver 1	Rat liver 2	Rabbit liver	Rat-liver mitochondria	Rat-liver microsomes
Cytidylic acid	13.9	14.3	14.6	13.4	14.8
Adenylic acid	10.0	10.0	10.0	10.0	10.0
Guanylic acid	17.5	17.6	16.9	17.3	18.2
Uridylic acid	10.9	10.8	10.3	10.8	11.1

DISCUSSION

In any analytical procedure designed for the separation of the constituents of the nucleic acids, the chief difficulty is to establish a method of hydrolysis which will bring about adequate

Fig. 2. Ultraviolet print showing separation by ionophoresis on paper of the four constituent nucleotides obtained from yeast RNA by alkaline digestion.

cleavage without partial destruction of any component or interconversion of components. While the separation of the purine and pyrimidine bases by chromatography is straightforward with suitable solvents, the complete and quantitative hydrolysis of a nucleic acid to the bases is more difficult. Hydrolysis of the RNA to purine bases and pyrimidine nucleotides with *N*-hydrochloric acid also

appears to give rise to difficulties since Markham & Smith (1951a) have found it necessary to apply a correction for partial hydrolysis of pyrimidine nucleotides. Hydrolysis of RNA by alkali to the constituent nucleotides on the other hand is simple and quantitative and involves no destruction of base, provided that conditions are mild enough to avoid deamination of cytidylic acid. The quantitative analysis of a mixture of nucleotides, however, presents certain difficulties to which, it is now clear, the procedure of ionophoresis on paper offers a convenient solution. Ionophoresis is simple and rapid to carry out and has the advantage that the result can be quickly demonstrated by visual inspection in ultraviolet light. Chromatography with certain solvents, e.g. phenol as used by Boulanger & Montreuil (1951a, b) renders such inspection impossible, and in any case most solvent systems used in the chromatography of nucleotides on paper fail to give adequate separation of at least two of them. The electrophoretic separation of nucleotides under conditions rather different from ours has been used by Markham & Smith (1951b) for the separation of the di- and tri-nucleotides obtained by partial hydrolysis of RNA.

The separation of all the known ribonucleotides can be achieved by chromatography on columns of ion-exchange resins, but ionophoresis on paper is more convenient to carry out and is more suitable for small quantities.

In the analysis of samples of purified RNA the results for the base ratios obtained by ionophoresis are similar to those obtained by other workers using paper chromatography (Magasanik *et al.* 1950; Markham & Smith, 1951a) or column chromatography (Volkin & Carter, 1951). When the results of ionophoresis of nucleotides are compared for the same specimen of RNA with those obtained by paper chromatography (Table 6) after hydrolysis to the bases with perchloric acid by Marshak & Vogel's (1951) method, a higher figure for uridylic acid is invariably given by the former procedure. The same discrepancy is found with mixtures of pure nucleotides and is probably due to some destruction of uracil by the perchloric acid. Recovery of uridylic acid based on phosphorus estimations and ultraviolet determinations on eluates from runs has shown that the amount of

uridylic acid recovered is the same and accounts for all the uridylic acid applied to the paper. This problem will be discussed in greater detail in a separate communication.

Although the hydrolysis of RNA to nucleotides yields acid-soluble phosphates quantitatively, the nucleotides separated by ionophoresis account for not more than 94% of the total phosphorus in the hydrolysate (Table 6). While some of the remaining 6% can no doubt be accounted for by the slight experimental losses inherent in any such procedure as this, the presence of small amounts of non-nucleotide phosphorus cannot be excluded. Experiments with isolated RNA labelled with ^{32}P suggest that such, in fact, is the case.

Although the procedure of ionophoresis of nucleotides is eminently suitable for the analysis of purified specimens of RNA, the isolation of RNA by such procedures as that described by Davidson *et al.* (1951) is a lengthy process. The modified procedure described above in which the nucleic acids are extracted from the tissue powder with 10% sodium chloride and are subsequently submitted to a Schmidt-Thannhauser separation yields satisfactory results in a much shorter time. When the usual Schmidt & Thannhauser (1945) separation procedure is employed in which the dry lipid-free powder is incubated in alkali to hydrolyse RNA, the ribonucleotide fraction obtained by acidification of the alkaline digest is found to contain several other phosphate derivatives which greatly complicate the picture. They are discussed in the following paper.

SUMMARY

1. A method is described for the separation of ribonucleotides by ionophoresis on paper.

2. It can be applied to the analysis of a ribonucleic acid after alkaline digestion or to the ribonucleotide fraction obtained from a tissue by a modification of the method of Schmidt & Thannhauser.

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Phosphorus Compounds in the Cell

3. THE INCORPORATION OF RADIOACTIVE PHOSPHORUS INTO THE RIBONUCLEOTIDE FRACTION OF LIVER TISSUE

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In previous work on the distribution of radioactive phosphorus among the protein-bound phosphorus fractions of animal tissues (Davidson, Frazer & Hutchison, 1951) it has been pointed out that the removal of the last traces of contaminating acid-soluble phosphate from such fractions is an extremely difficult process, and the conclusion has been reached that it is unjustifiable to assume that the specific activities of the nucleic acid fractions obtained by the separation procedure of Schmidt & Thannhauser (1945) represent the true activities of the nucleic acids. In such experiments it is essential to eliminate the possibility of the presence of contaminating inorganic phosphate by a rigorous purification of each fraction. In the Schmidt-Thannhauser procedure the fraction most susceptible to interference is that containing the acid-soluble nucleotides formed from ribonucleic acid (RNA) by alkaline hydrolysis and subsequent acidification. A method for the separation and purification of these nucleotides in small quantities would therefore be of great value. The method of ionophoresis described by Smellie & Davidson (1951) and Davidson & Smellie (1952) has proved satisfactory for the purpose and its application will now be described.

METHODS

The technique of ionophoresis was employed as described in the previous paper (Davidson & Smellie, 1952).

Two separate methods were adopted for preparing material for ionophoresis.

Procedure 1. A modification of the Schmidt & Thannhauser (1945) method for the separation and estimation of nucleic acids was used. The tissue homogenate was extracted with trichloroacetic acid (TCA) and with lipid solvents to remove acid-soluble P and lipid P as described by Davidson *et al.* (1951). Water was then added in the proportion of 1 ml. to 100 mg. of the dry lipid-free residue and 5N-KOH to give a final concentration of 0.3N. The mixture was then incubated at 37° for 18 hr. The resulting digest was brought to pH 1 with 60% (w/w) HClO₄ and the precipitate of protein, deoxyribonucleic acid (DNA) and KClO₄ centrifuged down, the supernatant fluid plus washings being used for ionophoresis as the fraction A₂S of Davidson *et al.* (1951) after adjusting the pH to 4.

Procedure 2. Since the isolation of RNA from animal tissues by the method of Davidson *et al.* (1951) is tedious and time-consuming, the following modification was adopted. The tissue homogenate was extracted with TCA and lipid solvents as described in procedure 1. The residue was extracted three times for 1 hr. each at 100° with 10% (w/v) NaCl. The extracts were pooled and treated with 2 vol. ethanol. The precipitate so formed was washed with ethanol and ether and dried. It was hydrolysed to mononucleotides by digestion with 0.3N-KOH for 18 hr., using 1 ml. alkali/20 mg. powder. The alkaline digest was brought to pH 1 by adding 60% HClO₄. DNA, protein and KClO₄ were centrifuged out and washed, and the supernatant fluid and washings adjusted to pH 4 with N-KOH.

Radioactive phosphate supplied by the Atomic Energy Research Establishment, Harwell, as H₃PO₄ was submitted to 10 min. hydrolysis with N-HCl before use to destroy any traces of pyro- or poly-phosphates.