The Nucleic Acids: Separation by Chromatography on Filter Paper. [Papers]

Publication/Creation

9 January 1953 - 23 June 1955

Persistent URL

https://wellcomecollection.org/works/mc7qhtft

wellcome collection

Wellcome Collection
183 Euston Road
London NW1 2BE UK
T +44 (0)20 7611 8722
E library@wellcomecollection.org
https://wellcomecollection.org

TELE {GRAMS : GIRI, "SCIENCE," BANGALORE
PHONE : 2022, BANGALORE

INDIAN INSTITUTE OF SCIENCE

BANGALORE 3 (INDIA)

DR. K. V. GIRI, D.Sc., F.A.Sc., F.R.I.C.
PROFESSOR AND HEAD OF THE DEPARTMENT

Department of Biochemistry

Date 18th May 1954.

Ref. B.C. 151/54-3783.

Dr.G.R. Wyatt,
Laboratory of Insect Pathology,
Sault Ste., Marie, Ont., Canada.

Dear Dr. Wyatt,

I am indeed obliged to you for your kind letter dated the 10th of May, and for the interest evinced in our work on the Circular Paper Chromatographic analysis of Purine and and pyrimidine derivatives.

I enclose herewith a reprint of our paper published on the "Separation and identification of degradation products of purines and nucleic acids by Circular Paper Chromatography". Since the publication of this paper, we have confirmed the formation of 4(5) amino 5(4) imidazolecarboximidine by isolating it in crystaline form from the acid hydrolysates of adenine and determining its physical constants. Also, work is in progress to study the further degradation products of this compound and their biochemistry by employing the same technique.

Yours sincerely,

(K.V.Giri)

Encl: one)

Separatum
EXPERIENTIA
VERLAG BIRKHÄUSER, BASEL, SCHWEIZ

Vol. IX/8, 1953 – pag. 296

Separation and Identification of the Degradation Products of Purines and Nucleic Acids on Acid Hydrolysis by Circular Paper Chromatography

In the course of our investigations on the separation and identification of purine and pyrimidine bases by circular paper chromatography[1], we investigated the nature of the products formed after acid hydrolysis of purines and nucleic acids, using the reagents, sodium 1:2 Naphthoquinone 4-sulphonate (FOLIN's reagent) in acetone[2], ninhydrin in acetone[1] and the contact printing technique using photographic paper and filtered ultraviolet light[3] for the identification of the substances separated on the chromatogram. While this work was in progress, FRICK[4] reported the formation of amino acids in acid hydrolysates of adenine by paper chromatography. He also observed the formation of two ultraviolet spots in addition to the strong ninhydrin-positive spot in the position of glycine after acid hydrolysis of adenine. In view of the above findings of FRICK and our observations on the marked differences observed in the hydrolysis products of adenine, nucleic acids, adenosine, adenylic acid, adenosine-3-phosphoric acid on the one hand and those of the other purines and pyrimidines on the other prompted us to report our observations.

Adenine (25 mg) was hydrolysed with (3 cm^3) 6 Normal hydrochloric acid by autoclaving at 15 lbs. pressure for 6 h. After removing the acid, the solution was spotted in 3 aliquots of 5 μl on the circumference of

[1] K. V. GIRI and N. A. N. RAO, Nature *169*, 923 (1952); J. Ind. Inst. Sci. *34*, 95 (1952).

[2] K. V. GIRI and A. NAGABHUSHANAM, Naturwissenschaften *23*, 548 (1952).

[3] R. MARKHAM and J. D. SMITH, Biochemical J. *45*, 294 (1949).

[4] G. FRICK, Nature *169*, 759 (1952).

a circle drawn at the centre of the filter paper (Whatman No. 1, 24 cm diameter) and developed with n-butanol-acetic-acid-water according to the procedure described by GIRI and RAO[1]. After drying, the chromatogram was cut into three sectors, each containing the hydrolysis products of adenine separated. One of the sectors was sprayed with 0·2% ninhydrin in acetone[2] and the other with FOLIN's reagent[3]. The third sector was placed on a photographic paper and exposed to filtered ultraviolet light and developed according to the procedure described by MARKHAM and SMITH[4] (Fig. 1).

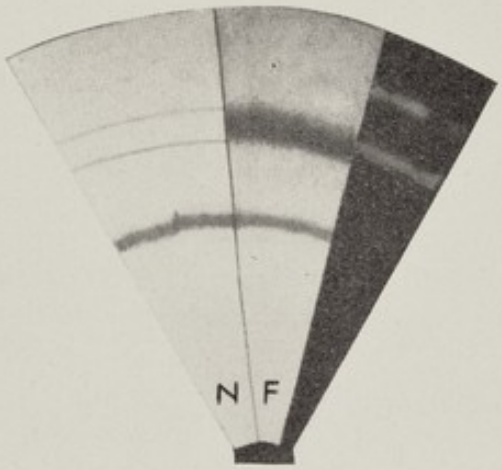


Fig. 1.—Different sectors of circular paper chromatogram of adenine hydrolysate treated as indicated below. *N* Sprayed with ninhydrin. *F* Sprayed with FOLIN's reagent. *U* Contact print of the sector taken in the ultraviolet.

It was observed that the adenine hydrolysate gives three clear bands on the chromatogram: (1) The band with the lowest R_f value (0·38) gives purple colour with ninhydrin and green colour with FOLIN's reagent and occupies the position of glycine on the chromatogram; (2) the second band with R_f value (0·69) higher than the first band gives faint yellow colour with ninhydrin (out-

[1] K. V. GIRI and N. A. N. RAO, Nature *169*, 923 (1952); J. Ind. Inst. Sci. *34*, 95 (1952).

[2] K. V. GIRI and N. A. N. RAO, Nature *169*, 923 (1952); J. Ind. Inst. Sci. *34*, 95 (1952).

[3] K. V. GIRI and A. NAGABHUSHANAM, Naturwissenschaften *23*, 548 (1952).

[4] R. MARKHAM and J. D. SMITH, loc. cit.

lined with pencil in Fig. 1) and intense purple with FOLIN's reagent. It is also characterised by ultraviolet absorption as it appears on the contact print; and (3) the third band which appears on the sector obtained by contact printing technique is due to unhydrolysed adenine. Figure 2 is the reproduction of the chromatogram of adenine and adenine hydrolysate obtained by contact printing technique. It shows the formation of another band characterised by the ultraviolet light absorption, in addition to the one relating to adenine.

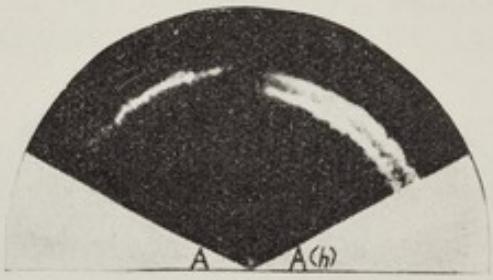


Fig. 2.—A contact print of a sector of a circular paper chromatogram of adenine and adenine hydrolysates taken in the ultraviolet using a chlorine filter. *A* Adenine, *A* (*h*) Adenine hydrolysate.

Other compounds of adenine and nucleic acids were similarly subjected to acid hydrolysis and chromatogramed. Figure 3 is the reproduction of the chromatogram showing the bands of the substances separated on the filter paper, present in the hydrolysates of adenine, ribonucleic acid (R.N.A.), Desoxyribonucleic acid (D.N.A.), Adenosine, Adenylic acid, and Adenosine-3-Phosphoric acid. It was observed that all the substances containing adenine give, in addition to the band occupying the position of glycine band, another band with higher R_f value than glycine band, giving an intense purple colour with FOLIN's reagent and faint yellow colour with ninhydrin reagent and characterised by ultraviolet absorption. This band was not present on the chromatograms of other purine and pyrimidine hydrolysates investigated, viz., Guanine, Xanthine, Cytosine, Uracil, Thymine, Hypoxanthine, Uric acid, etc., which gave a band only corresponding to glycine.

The substances formed in the acid hydrolysates of nucleic acids, adenine and adenine compounds, giving intense purple colour with FOLIN's reagent, was identi-

fied tentatively as 4(5) amino-5(4) imidazole carboxamidine as its absorption maximum (λ_{max} – 287 mμ) is in agreement with that given by CAVALLIERI *et al.*[1], who prepared it in 10% yield by hydrolysing adenine sulphate with 6 N · HCl for 2 h at 150°C and established its constitution unequivocally by its conversion to isoguanine. The formation of this compound from adenine and nucleic acids even under mild conditions of hydrolysis (with N · HCl for 1 h at 100°C) was an another interesting observation made during the course of our investigations.

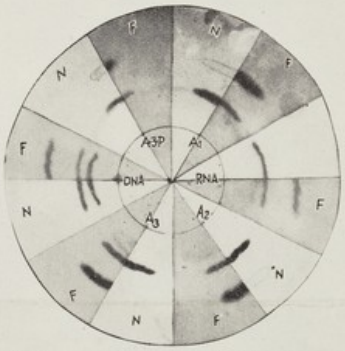


Fig. 3.—Circular paper chromatogram of acid hydrolysates of adenine (A_1), R.N.A., D.N.A., Adenosine (A_2), adenylic acid (A_3), and Adenosine-3-Phosphoric acid (A.3.P). *N* Sectors sprayed with ninhydrin. *F* Sectors sprayed with FOLIN's reagent.

The appearance of this band on the chromatogram is specific for adenine compounds and nucleic acids. From the foregoing, therefore, it will be seen that the observed reaction of adenine and its compounds and nucleic acids on acid hydrolysis, is of importance in the analysis of nucleic acids, particularly in the determination of the component purines in a nucleic acid. These observations seem to call for greater care in the interpretation of the results obtained by spectro-photometric determination of purines after acid hydrolysis of nucleic acids.

K. V. GIRI, P. R. KRISHNASWAMY,
G. D. KALYANKAR, and
P. L. NARASIMHA RAO.

Departement of Biochemistry, Indian Institute of Science, Bangalore 3, India, April 15, 1953.

Zusammenfassung

Die Degradationsprodukte, die sich bei der Hydrolyse der Purine, der Pyrimidine und der Nukleinsäuren bilden, werden mittels runder Filterpapierscheiben chromatographisch untersucht. Adenine, Nukleinsäuren und Adeninverbindungen zeigen bei einer Säurehydrolyse ausser einem Band, das den Platz des Glycin einnimmt, noch ein weiteres Band. Dieses Band ist versuchsweise als 4(5)-amino-5(4)-imidazol Carboxamidin charakterisiert worden. Die Bedeutung dieser Ergebnisse für die quantitative Analyse der Nukleinsäuren und Adeninverbindungen wird in der vorliegenden Arbeit erörtert.

[1] L. F. CAVALLIERI, J. F. TINKER, and G. B. BROWN, J. Amer. Chem. Soc. *71*, 3973 (1949).

THE NUCLEIC ACIDS
CHEMISTRY AND BIOLOGY

EDITORS:
ERWIN CHARGAFF
College of Physicians and Surgeons
Columbia University
630 West 168 Street
New York 32, New York
J. N. DAVIDSON
Biochemistry Department
The University
Glasgow W 2, Scotland

June 23, 1955

PUBLISHERS:
ACADEMIC PRESS INC.
125 East 23 Street
New York 10, New York

Dr. G. R. Wyatt
Department of Biochemistry
Yale University
New Haven, Conn.

Dear Doctor Wyatt:

Dr. Davidson and I would not want to let pass the occasion of the publication of the second volume of the Nucleic Acid book without a note of thanks to all contributors who have made possible the completion of this work. At the same time, we should like to ask you to note whatever misprints and errors of commission and omission you may encounter, both in your chapter and in the rest of the book, and to send us such lists whenever it is convenient. Though it is too early to say whether a supplement or a second printing with revisions may be forthcoming eventually, it would certainly be most helpful to us to receive whatever remarks you may wish to make.

With best wishes,

sincerely yours,

Erwin Chargaff

EC:eb

SFPPC-D

DUPLICATE SET

FOR Eisenhower I ORDER NO. 8510

PAGES 243 TO 266 9-29 1954

PAGE PROOF

FROM

WAVERLY PRESS, INC.

BALTIMORE

IMPORTANT

PLEASE OBSERVE:

Any instructions to Printer should be written on Proof, as first class postage will be required if instructions are written on separate sheets.

Please read proof carefully, giving particular attention to spelling of names, dates and figures. If cuts appear see that they are in their proper places.

An extra charge of $5.45 per hour is made for all changes from copy after galley proof is submitted.

If any errors are found that have been passed by our Proof Reader, mark them in red ink.

Mark any changes from copy in black ink. This enables us to keep separately the time for making each sort of correction. Each sort is handled by a different operator, and the time charged for alterations is accurate and not estimated.

Unless ordered, this type will not be kept standing. If ordered to be kept standing there is a charge for doing so.

This proof is a rough proof and not on the paper for which the order calls.

CHAPTER 7

Separation of Nucleic Acid Components by Chromatography on Filter Paper

G. R. Wyatt

Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada

I. Introduction

The feasibility of separating nucleic acid components by chromatography on filter paper was first demonstrated in 1947–1948 by Vischer and Chargaff[1] and by Hotchkiss.[2] Since then progress has been rapid and paper chromatography as a quantitative technique has now attained more success with this group of compounds than with any other. This is due largely to the intense absorption of ultraviolet light by purine and pyrimidine derivatives, which facilitates their detection on paper and makes possible their direct estimation, once separated, by spectrophotometry. The nitrogenous bases may be accurately estimated by ordinary methods of paper chromatography from less than 0.5 mg. of nucleic acid, or, with special refinements of technique, from as little as a few micrograms. Application of these methods has led to knowledge of the quantitative composition of nucleic acids from a variety of sources, to recognition of two pyrimidine bases not previously known to occur in nucleic acids, and, together with

[1] E. Vischer and E. Chargaff, *J. Biol. Chem.* **168**, 781 (1947).
[2] R. D. Hotchkiss, *J. Biol. Chem.* **175**, 315 (1948).

chromatography on ion-exchange columns and electrophoresis on filter paper, is playing an important part in investigations into the specificity of nucleases and the molecular structure of nucleic acids.

The present article aims to provide a practical guide to paper chromatographic methods for quantitative analysis of nucleic acids and for separation and estimation of their components and related substances.

II. General Technique of Paper Chromatography

The general procedures used in chromatography on filter paper are by now well known and are covered by several reviews and books.[3-6a] Both the original descending technique (in which the upper edge of the paper dips into a trough containing the solvent[7]), and the ascending technique (in which the paper, rolled into a cylinder, stands in a dish of the solvent[8]) have been widely used, and give similar results. The ascending method is convenient for small two-dimensional chromatograms but becomes impractical when the solvent is required to flow more than about 25–30 cm.; the descending method has the advantages that the solvent may be allowed to flow an indefinite distance and that the solvent in the trough may be renewed while retaining the vapor in the tank.

The troughs required for descending chromatography may be made of plastic or stainless steel, but for chemical reasons glass is to be preferred, and it is perhaps worth describing an especially simple method of making glass troughs.[9] Using a cutting diamond mounted at the end of a steel rod,[10] two longitudinal scratches are made inside a length of glass tubing about 2.5 cm. in diameter, separated from one another by about 90° around the circumference of the tube. The tube is then gently tapped on the outside with a piece of metal following the lines of the scratches, and will crack along them producing a trough whose ends can be sealed in a flame.

The grade of filter paper most frequently used has been Whatman No. 1, which is satisfactory for quantitative and qualitative work with nucleic acid derivatives. Whatman No. 4 is a faster running paper; in some solvent systems, however, this may result in poorer resolution. Schleicher and Schüll No. 597 paper has also been used with similar results, although different grades of paper may give different mobilities with the same solvent system. When larger quantities of material are to be sepa-

[3] A. J. P. Martin, *Ann. Rev. Biochem.* **19**, 517 (1950).
[4] R. J. Block, R. Le Strange, and G. Zweig, "Paper Chromatography." Academic Press, New York, 1952
[5] J. N. Balston and B. E. Talbot, "A Guide to Filter Paper and Cellulose Powder Chromatography." Reeve Angel and Co., London, and W. and R. Balston Ltd., Maidstone, 1952.
[6] F. Cramer, "Papierchromatographie," 2nd ed. Verlag Chemie, Weinheim, 1953.
[6a] E. and M. Lederer, "Chromatography." Elsevier Publishing Co., Amsterdam, 1953.
[7] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.* **38**, 224 (1944).
[8] R. J. Williams and H. Kirby, *Science* **107**, 481 (1948).
[9] This process was first demonstrated to the writer by Dr. R. Markham.
[10] Glass-cutting tool no. 6686, obtainable from A. Gallenkamp and Co., Ltd., London.

rated, the thick papers Whatman No. 3 (grained surface) and No. 3MM (smooth surface) are useful. No. 3 is approximately 2.2 times as thick as No. 1, and more than 10 mg. of a substance can be separated from a mixture applied as a band across a single sheet (18¼" x 22½"). 5-Hydroxymethylcytosine was isolated from bacteriophage DNA on this type of chromatogram in amounts adequate for crystallization and analysis.[11]

Filter papers contain a certain amount of ultraviolet-absorbing material which may be eluted by a chromatographic solvent, especially if the latter is acid, and collect in a band at or behind the solvent front. This can be removed by prior washing of the paper, which is desirable in preparative work. In quantitative work, it may generally be allowed for by taking appropriate blanks. For successful chromatography of phosphoric esters in many solvents, however, thorough washing of the paper is essential, as otherwise the presence of metallic ions may cause streaking or double spots.[11, 12]

With a given solvent system, precise R_F values[14] are influenced by (a) the composition of the vapor phase in the chromatography vessel, which, ideally, should be in equilibrium with the solvent mixture before a run is started, (b) the temperature, which affects partition coefficients, (c) the direction (ascending or descending) and length of run, since the composition of the solvent may change during its passage through the paper,[15] and (d) the paper, of which some variation is found even between batches of one grade. If these conditions are adequately controlled R_F values can be accurately reproduced.[16] However, as has frequently been observed, a characteristic pattern of the spots can be maintained despite considerable variation in absolute values of R_F, so that precise control is usually unnecessary. The sensitivity to environmental conditions depends on the particular solvent system.

III. Detection of Purine and Pyrimidine Derivatives on Filter Paper

1. Purine and Pyrimidine Bases

In the earlier experiments, a variety of means were tried for determining the positions of nucleic acid derivatives on filter paper. Hotchkiss[2] cut the paper into narrow bands, each of which was eluted for measurement of its ultraviolet extinction in the spectrophotometer. Vischer and Chargaff[1, 17] treated the paper with salts of mercury, and, after the excess had been washed out, the mercury fixed by the purine and pyrimidine bases was made visible by conversion to black mercuric sulfide. Another chemical

[11] G. R. Wyatt and S. S. Cohen, *Biochem. J.*, **55**, 774 (1953).
[12] C. S. Hanes and F. A. Isherwood, *Nature* **164**, 1107 (1949).
[13] K. C. Smith and F. W. Allen, *Federation Proc.* **12**, 269 (1953).
[14] Defined[7] as $R_F = \frac{\text{movement of band}}{\text{movement of advancing front of liquid}}$
[15] L. Horner, W. Emrich, and A. Kirshner, *Z. Elektrochem.* **56**, 987 (1952).
[16] E. C. Bate-Smith, *Biochem. Soc. Symposia (Cambridge, Engl.)* **No. 3**, 62 (1949).
[17] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176**, 703 (1948).

method of detecting purines[18] depends on their conversion to silver salts. Purines may also be made visible by staining their mercury complexes with eosin or bromphenol blue, or by their fluorescence after exposure to chlorine.[19] A microbiological method for detecting purines and pyrimidine nucleosides on paper chromatograms with the aid of deficient strains of *Ophiostoma* has been used by Fries *et al.*[20] In general, however, the most convenient techniques are those taking advantage of the absorption of ultraviolet light by the nucleic acid bases.

When a paper chromatogram is examined under an ultraviolet lamp having a high emission in the range of maximal nucleic acid absorption, and with visible light efficiently filtered out, spots of nucleic acid components appear as dark regions against the background fluorescence of the filter paper.[21-23] A low-pressure mercury resonance lamp is suitable, and it is reported that 0.2 μg. of adenine spread over a circle 1.5 cm. in diameter can be detected.[24]

Another procedure of approximately equal sensitivity, which is more laborious but provides a permanent record of each chromatogram, consists in making photographic contact prints in ultraviolet light.[25, 26] A medium- or high-pressure mercury lamp is used, with a filter system which isolates the 253.7-mμ and 265-mμ emission lines.[27] The dried paper chromatogram is pinned over a sheet of photographic paper (a contact document or photostat paper is suitable) on a board and exposed to the lamp for an appropriate time (usually less than a minute). In the developed print,

[18] R. M. Reguera and I. Asimov, *J. Am. Chem. Soc.* **72,** 5781 (1950).

[19] H. Michl, *Naturwissenschaften* **40,** 390 (1953).

[20] N. Fries and U. Bjorkman, *Physiol. Plantarum* **2,** 212 (1949); N. Fries and B. Forsman, *ibid.* **4,** 410 (1951).

[21] E. R. Holiday and E. A. Johnson, *Nature*, **163,** 216 (1949).

[21a] E. Chargaff, B. Magasanik, R. Doniger, and E. Vischer, *J. Am. Chem. Soc.* **71,** 1513 (1949).

[22] C. E. Carter, *J. Am. Chem. Soc.* **72,** 1466 (1950).

[23] T. Wieland and L. Bauer, *Angew. Chem.* **63,** 511 (1951).

[24] Marshak[84] reports this sensitivity using a General Electric Co. lamp No. GST5 equipped with Corning filter No. 9863. "Mineralight" lamps have been widely used, but are rather less sensitive. The effect has been photographed by J. P. Goeller and S. Sherry, *Proc. Soc. Exptl. Biol. Med.* **74,** 381 (1950).

[25] R. Markham and J. D. Smith, *Nature* **163,** 250 (1949).

[26] R. Markham and J. D. Smith, *Biochem. J.* **45,** 294 (1949).

[27] Markham and Smith[26] recommend the Mazda MB/V lamp with the glass bulb removed, and a filter system made with two 25-ml. fused silica round-bottomed flasks containing, respectively, a solution of cobalt and nickel sulfates ($CoSO_4 \cdot 7H_2O$ 10 g. and $NiSO_4$ 35 g. per 100 ml.) and dry chlorine gas. The writer has found satisfactory a General Electric AH-4 lamp equipped with this filter system. The chlorine gas filter may be replaced by a 1-cm. layer of chlorine dissolved in carbon tetrachloride.[25] The system transmits too much visible light to be satisfactory for making spots visible by fluorescence quenching, but by adding a Corning filter No. 9863 or by viewing the chromatograms through a blue filter, one may use it in this way too.

the positions of substances absorbing ultraviolet light of the wavelength used appear as white areas on a dark background. These spots may then be traced on the chromatogram itself. Slightly greater sensitivity is claimed for a technique identical in principle but utilizing the 257-mμ and 275-mμ emission lines from a cadmium arc.[28]

It has been pointed out by Smith and Markham[29] that guanine and compounds containing it fluoresce quite strongly in light of wavelengths 253.7 and 265 mμ, and are thus easily differentiated from other nucleic acid derivatives. Acid conditions are required, and may be created by exposing the chromatogram to fumes of hydrochloric acid. Xanthine behaves similarly.[29a] The effect may be recorded photographically by inserting between the chromatogram and the photographic paper a sheet of cellulose nitrate, which transmits only the fluorescent light. 8-Azaguanine and its compounds fluoresce under both acid and basic conditions,[29a] and may be detected on chromatograms by their fluorescence with greater sensitivity than by their absorption of ultraviolet light.[30]

With the device described by Paladini and Leloir,[31] in conjunction with the Beckman spectrophotometer, a continuous record may be obtained of the ultraviolet absorption of strip chromatograms.

2. Nucleosides and Nucleotides

The techniques using ultraviolet light described above are of course also applicable to nucleosides and nucleotides. Some color reactions of the sugar and phosphate portions of these compounds are also useful on occasion, as in identification of unknowns or where it is necessary to use a chromatographic solvent which itself absorbs in the ultraviolet range.

Buchanan, Dekker, and Long[32] have developed means of detecting both ribo- and deoxyribonucleosides on chromatograms by reactions of the sugars. The *cis*-glycol structure present in ribosides may be oxidized either with periodate, the resulting aldehydes being made visible with Schiff's reagent, or with lead tetraacetate, in which case white spots remain when the uncombined lead on the paper is converted to lead dioxide. Other substances having this configuration, including adenosine-5′-phosphate, react, but riboside-2′- and -3′-phosphates and deoxyribosides do not. The sensitivity of both methods is reported as about 20 μg. of nucleoside. Deoxyribosides on paper can be detected by adaptations of the Dische diphenylamine reaction or of the Feulgen reaction, or of the reaction with cysteine.[33] The last method is sensitive to 10 μg. of deoxyriboside. [Cf. Chapters 9 and 17.]

The positions of deoxyribosides on paper chromatograms have also been determined by virtue of their ability to promote growth of *Lactobacillus leichmannii*.[34]

[28] J.-E. Edström, *Nature* **168**, 876 (1951).
[29] J. D. Smith and R. Markham, *Biochem. J.* **46**, 509 (1950).
[29a] J. Kream and E. Chargaff, *J. Am. Chem. Soc.* **74**, 4274 (1952).
[30] R. E. F. Matthews, *Nature* **171**, 1065 (1953).
[31] A. C. Paladini and L. F. Leloir, *Anal. Chem.* **24**, 1024 (1952).
[32] J. G. Buchanan, C. A. Dekker, and A. G. Long, *J. Chem. Soc.* **1950**, 3162.
[33] J. G. Buchanan, *Nature* **168**, 1091 (1951).
[34] V. Kocher, R. Karrer, and H. R. Muller, *Intern. Z. Vitaminforsch.* **21**, 403 (1950).

Nucleotides and other phosphoric acid esters on paper chromatograms can be detected by spraying with an acid molybdate solution, partial hydrolysis of the ester, and reduction of the resulting phosphomolybdate complex to a blue-colored compound.[35] The necessary hydrolysis may be effected by heating the papers after spraying, by ultraviolet irradiation,[36] or by previous spraying with a solution of phosphatase.[37, 38] It is claimed that 0.1 μg. P can be detected. If the water in the reagent is partially replaced by acetone[39] the papers may be dipped in it instead of sprayed. Nucleotides can also be detected by fixation of uranium,[40] and phosphates, by fixation of ferric iron;[41] these reactions avoid the need for hydrolysis.

IV. Solvent Systems

1. General and Theoretical Considerations

The solvent systems with which successful separations were first obtained on paper chromatograms consisted of organic fluids saturated with water. Their effect was satisfactorily interpreted as resulting from the partition of solutes between a water-poor mobile phase and a water-rich phase held by the strongly hydrophilic cellulose fibers; and for a number of amino acids and carboxylic acids, partition coefficients calculated from R_F values on the basis of this theory agree well with the coefficients directly measured.[7, 42] A minor role may be played by adsorption and ion exchange, since the cellulose fibers are electronegative in water, and carry a small number of aldehyde and carboxyl groups.[5] It was subsequently found that the solvent need not be saturated with water, since the binding of water by the cellulose results in a partition effect with miscible solvents just as with water-saturated ones.[43] In a further innovation, it was discovered that separations may be obtained in the absence of any organic solvent, using salt solutions, or even with water alone. Separations with water as the solvent, apparently due to adsorption by the paper, may also be interpreted as the result of partition between water and a water–cellulose complex. [See below, Section IV.2.]

The influence of the composition of the solvent system on the movement

[35] Hanes and Isherwood[12] spray the chromatograms at a rate of 1 ml. per 100 cm.^{2} with a solution containing: 5 ml. 60% $HClO_4$, 10 ml. *N* HCl, 25 ml. 4% $(NH_4)_2MoO_4$, and water to 100 ml.; then heat them to 85° for 7 min., and subsequently expose them to H_2S. Benson *et al.*[42] describe a similar reagent.

[36] R. S. Bandurski and B. Axelrod, *J. Biol. Chem.* **193,** 405 (1951).

[37] N. G. Doman and Z. S. Kagan, *Biokhimiya* **17,** 719 (1952), seen only in abstract, *Chem. Abstracts* **47,** 4795 (1953).

[38] E. Fletcher and F. H. Malpress, *Nature* **171,** 838 (1953).

[39] S. Burrows, F. S. M. Grylls, and J. S. Harrison, *Nature* **170,** 800 (1952).

[40] B. Magasanik, E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem.* **186,** 37 (1950).

[41] H. E. Wade and D. M. Morgan, *Nature* **171,** 529 (1953)

[42] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, *J. Am. Chem. Soc.* **72,** 1710 (1950).

[43] H. R. Bentley and J. K. Whitehead, *Biochem. J.* **46,** 341 (1950).

of substances has been discussed in terms of partition theory by Martin.[3, 44] The following factors are among the most important which may be utilized in preparing solvents to effect desired separations and in using paper chromatography in the identification of unknown substances.

a. Water content

By using a miscible organic solvent, the water content may be varied over a wide range. As water is added to the moving phase, the rate of migration of solutes will increase in proportion to their polarity. For example, by altering the water content of propanol–water mixtures, the relative positions of adenine and adenylic acid[26] or 5-methylcytosine and 5-hydroxymethylcytosine[45] may be reversed.

b. pH

Since ionization will alter the partition of a solute in favor of the aqueous phase, one can regulate the relative rates of movement of ionizable substances by control of pH, having regard to their dissociation constants. [Cf. *Cohn*, Chapter 6, and *Jordan*, Chapter 13.] Thus, in neutral aqueous *n*-butanol uracil migrates more rapidly than cytosine, but if the solvent is made basic by the presence of sufficient ammonia the movement of uracil and thymine may be slowed until the former has an R_F less than that of cytosine. This may be explained by ionization of enolic hydroxyls (pK_1 = 9.5 and 9.9 for uracil and thymine, respectively[46]), the hydroxyl of cytosine having too high a pK (12.2) to be more than slightly affected by ammonia. The relative mobilities are of course sensitive to the precise concentration of ammonia (cf. Table I, solvents *a*, *b*, and *c*). Addition of strong acid or base to a chromatographic solvent may have further effects on partition coefficients; for example, hydrochloric acid added to aqueous alcohols decreases the R_F's of all the purine and pyrimidine bases, but the effect is differential and at about 1 N HCl the relative positions of adenine and cytosine are interchanged.[47]

c. Nature of the Organic Components

The importance of van der Waals' forces and of hydrogen bonding in determining the partition of a system has been stressed by Martin.[44] When solvent and solute are similar in structure, for example both aromatic or both aliphatic, closer fit between their molecules, resulting in greater van der Waals' forces and higher R_F values, may be expected. This principle is of limited utility in separating nucleic acid bases, where we are concerned

[44] A. J. P. Martin, *Biochem. Soc. Symposia (Cambridge, Engl.)*, **No. 3**, 4 (1949).
[45] G. R. Wyatt and S. S. Cohen, *Nature* **170**, 1072 (1952).
[46] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9**, 199 (1952).
[47] G. R. Wyatt, *Biochem. J.* **48**, 584 (1951).

exclusively with two similar ring systems, but does account for such facts as the better separation of 5-methylcytosine from cytosine with increasing chain length of the alcohol used.[48] The nucleic acid derivatives have numerous possibilities for hydrogen bonding, which undoubtedly contributes to the different order of movement of the nucleotides given by solvents active in forming hydrogen bonds, such as phenol and butyric acid, than by aliphatic alcohols.

d. Salt Content

Salt will generally decrease the mutual solubility of water and an organic solvent, and will thus alter the partition of a chromatographic solvent system, and for this reason the local presence of salt may cause distorted spots. Addition of salt to a system selectively slows the movement of solutes, and relatively strong salt solutions containing little or no organic solvent may be used to obtain chromatographic separations on filter paper. Hagdahl and Tiselius[49] have separated amino acids using 3 *M* phosphate buffer as the solvent; they term this "salting-out chromatography" and attribute the effect to reductions in solubility due to the presence of salt, with consequent increase in apparent adsorption. With similar "salting-out solvents" (5% ammonium citrate or sodium or potassium phosphate overlayered with isoamyl alcohol, which is slightly soluble in water and has the effect of producing more compact spots), Carter[22] has obtained separations of nucleic acid components. The principle has been further applied by Markham and Smith,[50] who used 0.8 saturated ammonium sulfate containing 2% isopropanol. It is notable that these systems separate substances in a different order from the more usual organic systems, and that they are capable of resolving nucleoside-2′- and -3′-phosphates, a separation not accomplished in any system of the more usual type.

Two practical considerations to be borne in mind when preparing solvent systems are: (a) it is preferable to select volatile substances (ammonium sulfate might with advantage be replaced by ammonium carbonate) and (b) when working with nucleic acid derivatives it is of course particularly desirable to avoid substances absorbing in the ultraviolet range.

2. Separation of Purine and Pyrimidine Bases and Nucleosides

At the time of writing, over eighty solvent systems have been described for separation of nucleic acid components on paper chromatograms. Fortunately for the reviewer and for those using this technique, relatively few of these show real advantages over others, and some of the simplest mix-

[48] G. R. Wyatt, *Biochem. J.* **48**, 581 (1951).
[49] L. Hagdahl and A. Tiselius, *Nature* **170**, 799 (1952).
[50] R. Markham and J. D. Smith, *Biochem. J.* **49**, 401 (1951).

tures remain the most valuable. In Table I are listed the reported R_F values of some purine and pyrimidine bases and nucleosides in a number of solvent systems selected to include (a) those which, in the writer's estimation, have proved the most broadly useful, and (b) representatives of different types of mixture which have been tried with some degree of success. It is obvious that for some purposes other solvents will be preferable to those listed. For completeness, the R_F values reported by the original authors have been supplemented with some determined by the writer.

Aqueous *n*-butanol, with and without added ammonia, was one of the first solvents tested for separation of nucleic acid derivatives[217] and remains among the most useful (Table I, solvents *a*, *b*, and *c*). Butanol saturated with water has usually been used; however, the solution is most conveniently made up at a fixed percentage composition slightly under-saturated, thus making its composition independent of temperature.[51] Since R_F values are rather low, it is advantageous to use the descending method and to cut the end of the paper to a number of teeth, from which the solvent is allowed to drip.[26] Ammonia may be added either to the solvent irrigating the paper or to that in the bottom of the tank; owing to the volatility of ammonia, the latter practice affords the more constant conditions. It has the effect of slowing the movement of substances with acidic substituents, and the results given by two different concentrations are illustrated in Table I (solvents *b* and *c*). With butanol–water–ammonia, all of the purine and pyrimidine bases known to occur in nucleic acids (except 5-hydroxymethylcytosine, which runs close to guanine) may be resolved from one another. In addition to the authors cited in the Table, Chargaff, *et al.*[52, 52a] Marshak and Vogel,[53] and others have used butanol–ammonia mixtures for quantitative separation of nucleic acid components.

Addition of formic acid to aqueous butanol (solvent *d*) results in more rapid movement of acidic substances such as uracil, thymine, xanthine, and hypoxanthine.

If butanol is saturated with a saturated solution of boric acid instead of with water, ribosides, by virtue of their *cis*-diol configuration, form borate complexes and do not move. Complete separation of free bases from ribosides may thus be obtained.[54]

Admixture of various other substances with butanol–water systems has been tried, generally without much advantage. Butanol may be saturated with a 10% solution of urea, instead of water, with similar results.[22] Mixing morpholine or diethylene

[51] *n*-Butanol satd. with water at 20° contains 84% by vol. of butanol, calcd. from data given by A. Seidell, "Solubilities of Organic Compounds," Vol. 2, p. 266. Van Nostrand Co., New York, 1941.

[52] E. Chargaff, R. Lipshitz, C. Green, and M. E. Hodes, *J. Biol. Chem.* **192**, 223 (1951).

[52a] C. Tamm, H. S. Shapiro, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **203**, 673 (1953).

[53] A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189**, 597 (1951).

[54] I. A. Rose and B. S. Schweigert, *J. Am. Chem. Soc.* **73**, 5903 (1951).

TABLE I

R_F VALUES OF PURINE AND PYRIMIDINE BASES AND NUCLEOSIDES

	Solvent									
	n-Butanol[a]	n-Butanol–NH_3[b]	n-Butanol–NH_3[c]	n-Butanol–formic acid[d]	Isopropanol–NH_3[e]	Isopropanol–HCl[f]	Collidine–quinoline[g]	Isobutyric acid–NH_3[h]	Na_2HPO_4–isoamyl alc.[i]	Water (pH 10)[j]
Adenine	0.38	0.28	0.40	0.33	0.37	0.32	0.34	0.83	0.44	0.37
Guanine	0.15	0.11	0.15	0.13	0.16	0.22	0.22	0.70	0.02	0.40
Hypoxanthine	0.26	0.12	0.19	0.30	0.16	0.29	0.44	0.69	0.57	0.63
Xanthine	0.18	0.05	0.01	0.24	0.11	0.21	0.62	0.60	0.49	0.62
Uracil	0.31	0.19	0.33	0.39	0.38	0.66	0.74	0.67	0.73	0.76
Thymine	0.52	0.35	0.50	0.56	0.52	0.76	0.84	0.78	0.73	0.74
Cytosine	0.22	0.24	0.28	0.26	0.32	0.44	0.21	0.80	0.73	0.70
5-Methylcytosine	0.29	0.27	0.36	—	0.37	0.52	—	—	—	0.73
5-Hydroxymethylcytosine	0.13	0.12	—	—	0.25	0.44	—	—	—	0.75
Adenosine	0.20	0.22	0.33	0.12	0.31	0.34	—	0.91	0.54	0.49
Guanosine	0.15	0.03	0.10	0.17	0.13	0.30	—	0.59	0.62	0.68
Inosine	—	0.03	0.08	—	0.14	0.30	—	—	—	0.81
Uridine	0.17	0.08	—	0.25	0.31	0.64	—	0.60	0.79	0.84
Cytidine	0.12	0.11	0.15	0.18	0.28	0.45	—	0.73	0.76	0.76
Adenine DR[k]	0.35	—	0.41	—	—	—	—	0.91	0.55	0.47
Guanine DR	0.21	—	0.18	—	—	—	—	0.67	0.62	—
Hypoxanthine DR	0.23	—	0.17	—	—	—	—	0.70	0.70	0.80
Uracil DR	0.38	—	0.34	—	—	—	—	0.67	0.79	0.83
Thymine DR	0.51	0.40	0.48	—	0.57	0.81	—	0.75	0.78	0.77
Cytosine DR	0.23	—	0.26	—	—	0.60	—	0.83	0.77	0.75
5-Methylcytosine DR	0.25	—	—	—	—	—	—	—	0.76	—

[a] 80% (vol./vol.) aq. *n*-butanol; Whatman No. 1 paper, descending; Markham and Smith, *Biochem. J.* **45**, 294 (1949). Values for deoxyribosides are from Buchanan,[23] using *n*-butanol satd. with water; values for methylcytosine and hydroxymethylcytosine detd. by the reviewer.

[b] 86% (vol./vol.) aq. *n*-butanol, with 5% by vol. of coned. NH_3 soln. (sp. gr. 0.880) added to solvent in bottom of tank; Whatman No. 1, descending; Markham and Smith, *op. cit.* Values for methylcytosine, hydroxymethylcytosine, inosine, and thymine deoxyriboside detd. by the reviewer.

[c] *n*-Butanol satd. with water at about 23° 100 ml., 15 *N* NH_4OH 1 ml.; Whatman No. 4, ascending; MacNutt, *Biochem. J.* **50**, 384 (1952). Values for xanthine, adenosine, and guanosine are from Hotchkiss, *J. Biol Chem.* **175**, 315 (1948), with a similar solvent system.

[d] *n*-Butanol 77%, water 13%, formic acid 10% by vol.; Whatman No. 1, descending; Markham and Smith, *op. cit.*

[e] Isopropanol 85 ml., water 15 ml., coned. (28%) NH_3 soln. 1.3 ml.; Whatman No. 1, descending; Hershey *et al.*, *J. Gen. Physiol.* **36**, 777 (1953). R_F values at 20–23° detd. by the reviewer.

[f] Isopropanol 170 ml., coned. HCl (sp. gr. 1.19) 41 ml., water to make 250 ml.; Whatman No. 1, descending; Wyatt, *Biochem. J.* **48**, 584 (1951). Values redetd. at 20–23° by the reviewer.

[g] Collidine 1 vol., quinoline 2 vol., mixt. satd. with 1.5 vol. water; Schleicher and Schüll No. 597 paper, descending, at about 22°; Vischer and Chargaff, *J. Biol. Chem.* **176**, 703 (1948).

[h] Isobutyric acid 400 ml., water 208 ml., 25% NH_3 soln. 0.4 ml.; Whatman No. 4, descending, at 22°; Lödgren, *Acta Chem. Scand.* **6**, 1030 (1952); excepting deoxyribosides, for which solvent and conditions are as in footnote *e*, Table II, and R_F values are scaled, from the relative mobilities given by Tamm *et al.*, *J. Biol. Chem.* **203**, 673 (1953), taking the R_F of thymidine as 0.75.

[i] 5% aq. Na_2HPO_4 satd. with isoamyl alc., both aq. and nonaq. phases being present in the trough; Whatman No. 1, descending; Carter, *J. Am. Chem. Soc.* **72**, 1466 (1950). Values for deoxyribosides are from Buchanan, *Nature* **168**, 1091 (1951).

[j] Water adjusted to pH 10 with *N* NH_4OH; Whatman No. 1, ascending, 22–23°; Levenbook, personal communication, 1953. Values for methylcytosine and hydroxymethylcytosine detd. by the reviewer.

[k] DR = deoxyriboside.

glycol with butanol permits addition of more water and gives higher R_F values, without markedly altering the order of separation of the bases.[37] Addition of ethanol gives higher R_F values with some loss of resolution.[38] Dioxane[38] and 2-methoxyethanol (methyl cellosolve)[54] have been added to butanol–water mixtures, and also lead to increased R_F values. Water-saturated *n*-butanol has been mixed with acetic acid, with ethyl acetate and morpholine, and with methyl glycol and morpholine with some success in separating deoxyribosides.[56]

Amyl alcohol saturated with water gives inconveniently low R_F values for most substances.[56] Isopropanol–water–ammonia (Table I, solvent *e*) has been found a useful mixture by Hershey, *et al.*:[57] it resolves 5-hydroxymethylcytosine from the other bases, but adenine and uracil run together, and the bases are less well separated from their ribosides than in butanol. Mixtures of tetrahydrofurfuryl alcohol with propanol and amyl alcohol buffered at different pH's have also been tried,[58] with some success in separation of nucleotides, but for separation of the bases and nucleosides they appear to be inferior to simple butanol solvents. Several other mixtures of alcohols with NH_3 and HCl have also been tested on a limited range of substances,[59] and the R_F values of orotic acid[60] and of uric acid and its riboside[61] in several solvents have been recorded.

Solvent systems based on collidine and quinoline instead of alcohols have been tested by Vischer and Chargaff.[17] In these (e.g., Table I, solvent *g*) the bases migrate in a different order, xanthine and hypoxanthine running more rapidly and cytosine much more slowly. However, the strong absorption of ultraviolet light by these solvents is a serious drawback.

Isobutyric acid mixed with water and ammonia, as tested by Löfgren,[62] distributes the bases and nucleosides in a different order from other solvents, as shown in Table I (solvent *h*), although R_F values are grouped undesirably close together. (Compare also Tamm *et al.*[32a]) The effect is partially retained, with better spread of R_F values, in a solvent containing *n*-butanol (75 ml.), isobutyric acid (37.5 ml.), water (25 ml.), and ammonia (2.5 ml. of 25% soln.). A mixture containing piperidine, tried by the same author, gave rather poor separations.

A limitation of all these neutral or weakly basic or acidic solvent systems for quantitative analysis of nucleic acids is their low capacity for guanine. Because of its insolubility, guanine in amounts of more than a few micrograms tends to form "tails" or double spots, or to remain partly at the origin. This difficulty may be avoided by use of solvents containing relatively high concentrations of hydrochloric acid. Such a system was first used by Smith and Markham[29] for separation of the purine bases and pyrimidine nucleotides obtained by mild acid hydrolysis of PNA (Table II, solvent *a*). A mixture containing isopropanol and hydrochloric acid (Table I, solvent *f*) was subsequently developed for separation of the bases from DNA.[47]

[54] S. G. Laland, W. G. Overend, and M. Webb, *J. Chem. Soc.* **1952**, 3224.
[56] W. S. MacNutt, *Biochem. J.* **50**, 384 (1952).
[57] A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36**, 777 (1953).
[58] D. C. Carpenter, *Anal. Chem.* **24**, 1203 (1952).
[59] B. Bheemeswar and M. Sreenivasaya, *Current Sci.* (*India*) **20**, 61 (1951).
[60] E. Leone and E. Scala, *Boll. soc. ital. biol. sper.* **26**, 1223 (1950).
[61] E. Leone and D. Guerritore, *Boll. soc. ital. biol. sper.* **26**, 609 (1950).
[62] N. Löfgren, *Acta Chem. Scand.* **6**, 1030 (1952).

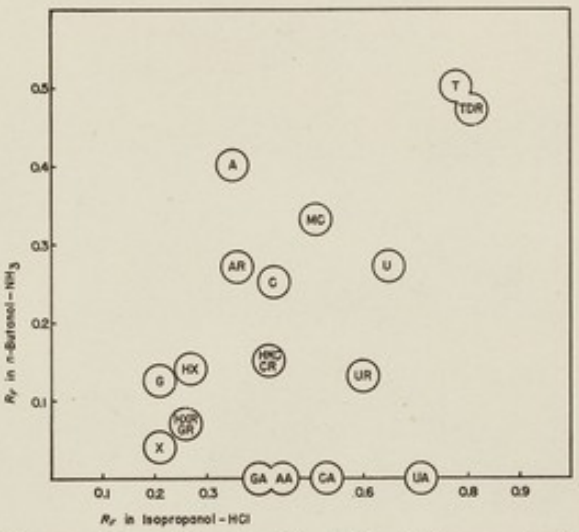


FIG. 1. Diagram of the positions of nucleic acid derivatives on a two-dimensional chromatogram run on Whatman No. 1 paper by the descending technique first in solvent *f* (Table I), then in solvent *e* (Table I; however, in the large chromatogram tank used here, the effective NH_3 concentration is reduced). A, adenine; AA, yeast adenylic acid; AR, adenosine; C, cytosine; CA, cytidylic acid; CR, cytidine; G, guanine; GA, guanylic acid; GR, guanosine; HMC, 5-hydroxymethylcytosine; HX, hypoxanthine; HXR, inosine; MC, 5-methylcytosine; T, thymine; TDR, thymidine; U, uracil; UA, uridylic acid; UR, uridine; X, xanthine.

This solvent resolves up to 75 μg. per spot of each of the DNA bases in 35 cm. movement of the front. Uracil is also resolved; 5-hydroxymethylcytosine, however, runs together with cytosine. Ribosides run at similar rates to their bases, and deoxyribosides rather faster; purine deoxyribosides are decomposed by the acid.

When using solvents containing a high proportion of hydrochloric acid, the acid must be thoroughly removed from the paper at the conclusion of the run by evaporation at not too high a temperature (to avoid charring). Residual acid may damage photographic paper used for printing the chromatograms and, according to Schramm and Kerekjarto,[63] on exposure to ultraviolet light may liberate chlorine which destroys cytosine by oxidation.

For mixtures too complex to be resolved in one dimension, a useful two-dimensional system is the isopropanol–HCl solvent in combination with *n*-butanol–NH_3 or with isopropanol–NH_3. Better movement of guanine is

[63] G. Schramm and B. von Kerekjarto, *Z. Naturforsch.* **7b**, 589 (1952).

obtained if the acid solvent is used first; the rate of flow of the second solvent is then more rapid than in untreated paper. The spacing of spots with such a system is shown in Figure 1.

Carter's dibasic sodium phosphate system is also included in Table I (solvent *i*). Pyrimidines are well separated from purines, but within each class there is little resolution. The use of water, or 0.01 *M* phosphate buffer, to bring about rapid separation of pyrimidines from purines was first described by Zamenhof *et al.*[64] Slightly greater dispersion of R_F values is given by water adjusted to pH 10, according to Levenbook,[65] whose data on this system are included in Table I (solvent *j*). The chief virtue of water as a solvent is its rapid flow, so that purine bases can be completely separated from pyrimidines in 3 hours.

3. Separation of Nucleotides

Because of the strong polarity of their phosphoryl groups, nucleotides do not move at appreciable rates in relatively nonpolar solvent systems such as water-saturated *n*-butanol. Their movement may be accelerated by increasing the content of water or other polar components in the system, or by suppressing phosphoryl dissociation by addition of acid. These conditions prevent full advantage being taken of the ionic differences in the constituent purine and pyrimidine bases, and separation of the nucleotides by paper chromatography has proven somewhat difficult. Effort has been expended on the problem in a number of laboratories, and some of the more satisfactory solvents which have been developed are shown in Table II. None, however, is entirely satisfactory for separation of the nucleotides of the four bases from PNA in a single run, although these may be resolved by two-dimensional chromatography or by electrophoresis on filter paper. [Cf. *Smith*, Chapter 8.]

The two solvent systems containing hydrochloric acid (Table II, solvents *a* and *b*) both separate the pyrimidine ribonucleotides and the purine bases excellently from one another. In solvent *a* cytosine and thymine deoxyriboside diphosphates are also resolved from the pyrimidine ribonucleotides.[66] The purine nucleotides are not satisfactorily resolved in either solvent. The isopropanol mixture is the faster running of the two. A mixture of *n*-butanol, ethanol, and 5 *N* HCl (3:2:2 by vol.) resolves purines and pyrimidine nucleosides and nucleotides, with the following R_F values;[67] guanine, 0.24; adenine, 0.35; cytidine, 0.43; cytidylic acid, 0.54; uridine, 0.63; uridylic acid, 0.78.

Solvent *c* is one of several mixtures of acetone with carboxylic acids tested by Burrows *et al.*[23] It affords good separation of guanylic, cytidylic, and uridylic acids;

[64] S. Zamenhof, G. Brawerman, and E. Chargaff, *Biochim. et Biophys. Acta* **9**, 402 (1952).
[65] L. Levenbook, personal communication, 1953.
[66] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **202**, 745 (1953).
[67] J.-E. Edström, *Biochim. et Biophys. Acta* **9**, 528 (1952).

the R_F value of yeast adenylic acid is not recorded. Adenosine-5′-phosphate, adenosine diphosphate, and adenosine triphosphate are also separable from one another in this system, or, with rather better spacing, in a mixture of 65 vol. of acetone with 35 vol. of 15% trichloroacetic acid.

From the published R_F values, the buffered isoamyl alcohol–tetrahydrofurfuryl

TABLE II

R_F VALUES OF NUCLEOTIDES

	Solvent							
	tert-Butanol–HCl[a]	Isopropanol–HCl[b]	Acetone–trichloroacetic acid[c]	Isoamyl alc.–tetrahydrofurfuryl alc.–buffer[d]	Isobutyric acid–NH_3[e]	Phenol–*tert*-butanol–formic acid[f]	Na_2HPO_4–isoamyl alc.[g]	$(NH_4)_2SO_4$–buffer–isopropanol[h]
Adenosine-2′-phosphate[i]	0.50	0.48	—	0.35	0.49	0.70	0.74	0.26
Adenosine-3′-phosphate[i]							0.67	0.16
Guanosine-2′-phosphate	0.46	0.43	0.20	0.67	0.24	0.46	0.79	0.50
Guanosine-3′-phosphate								0.40
Uridylic acid	0.80	0.77	0.51	0.43	0.24	0.35	0.85	0.73
Cytidylic acid	0.56	0.58	0.34	0.26	0.37	0.57	0.85	0.73
Adenosine-5′-phosphate	—	0.43	0.37	0.28	0.43	—	0.69	—
Adenosine diphosphate	—	—	0.10	0.07	—	—	0.77	—
Adenosine triphosphate	—	—	0.04	0.08	—	—	0.83	—
Deoxycytidylic acid	—	0.64	—	—	—	—	—	—
Thymidylic acid	—	0.81	—	—	—	—	—	—
Orthophosphate	0.90	0.84	0.61	—	—	0.22	—	—

[a] *tert*-Butanol at 26° 700 ml., const.-boiling HCl 132 ml., water to make 1 liter; Whatman No. 1, descending; Smith and Markham, *Biochem. J.* **46,** 509 (1950). R_F values (20–22°) are from Boulanger and Montreuil, *Bull. soc. chim. biol.* **33,** 784, 791 (1951).

[b] See footnote *f*, Table I. The value for orthophosphate is from Markham and Smith, *Biochem. J.* **49,** 401 (1951).

[c] Acetone 75 vol., 25% (wt./vol.) trichloroacetic acid 25 vol.; Whatman No. 1 paper washed in 2 *N* HCl and water, ascending, at 4°; Burrows *et al.*, *Nature* **170,** 800 (1952).

[d] Isoamyl alc. 1 vol., tetrahydrofurfuryl alc. 1 vol., 0.08 *M* potassium citrate buffer (pH 3.02) 1 vol.; Whatman No. 1, descending, 20–25°; Carpenter, *Anal. Chem.* **24,** 1203 (1952).

[e] Isobutyric acid 10 vol., 0.5 *N* NH_4OH 6 vol., pH 3.6–3.7; Schleicher and Schüll No. 597, descending, 21–25°; Magasanik *et al.*, *J. Biol. Chem.* **186,** 37 (1950). R_F values calcd. from the published relative mobilities and figures.

[f] 90% aq. phenol 84 vol., *tert*-butanol 6 vol., formic acid 10 vol., water 100 vol.; after sepn. at a temp. 2–3° below that of the chromatography room, the nonaq. phase is used and the aq. phase is placed in the bottom of the tank; Whatman No. 1 paper, descending, 20–22°; Boulanger and Montreuil, *op. cit.*

[g] See footnote *i*, Table I. Values for adenosine-5′-phosphate, diphosphate, and triphosphate are from W. E. Cohn and C. E. Carter, *J. Am. Chem. Soc.* **72,** 4273 (1950).

[h] Satd. $(NH_4)_2SO_4$ in water 79 vol., 0.1 *M* buffer soln. (pH 6) 19 vol., isopropanol 2 vol.; Whatman No. 1 paper, descending; Markham and Smith, *op. cit.* R_F values are measured from the published figure.

[i] The "a" and "b" nucleotides of Carter, *J. Am. Chem. Soc.* **72,** 1466 (1950), and subsequent authors are now identified as the 2′-phosphates and 3′-phosphates, respectively, of the ribosides.

alcohol system of Carpenter[58] (solvent *d*) should give adequate separation of the nucleotides from PNA. Use of this mixture in analytical work has not yet been reported.

The isobutyric acid–ammonium isobutyrate solvent of Magasanik *et al.*[46] (solvent *e*) has been used for quantitative analysis of PNA's.[68] [Cf. *Magasanik*, Chapter 11.] Since guanylic and uridylic acids occupy the same position on the chromatogram, however, the amount of each must be calculated from extinction measurements taken at two wavelengths. It is reported[52a, 68] that the five deoxynucleotides from thymus DNA are completely resolved from one another in this solvent, the respective distances travelled (relative to deoxyadenylic acid taken as 100) being: deoxyguanylic acid, 53; thymidylic acid, 66; deoxycytidylic acid, 80; deoxyadenylic acid, 100; deoxy-5-methylcytidylic acid, 137.

The phenolic solvent system of Boulanger and Montreuil[70] (solvent *f*) is capable of separating the ribonucleotides with excellent spacing, and was used for quantitative analysis of PNA's. Because of the ultraviolet absorption of the phenol, however, it is necessary to detect and estimate the nucleotides by reactions of the phosphoryl group, and any other nucleic acid derivatives would of course be missed.

For fractionation of the products of digestion of PNA by ribonuclease, Markham and Smith[71, 72] have used (1) 70% (by vol.) aqueous isopropanol and (2) 70% aqueous isopropanol with concentrated ammonia added to the solvent in the bottom of the tank at the rate of 0.35 ml. for each liter of gas space. The latter system proved especially useful for initial fractionation (prior to electrophoresis or chromatography in other solvents) of the mixture of mono-, di-, and trinucleotides; in it the cyclic nucleotides (riboside-2′3′-monohydrogen phosphates) move more rapidly than the riboside-2′- and -3′-phosphates. It also resolves nucleotides from their benzyl esters.[73]

With systems based on salt solutions instead of organic solvents (Table II, solvents *g* and *h*), the purine ribonucleotides, but not those of the pyrimidines, have been separated. Carter's[23] sodium and potassium phosphate systems resolve adenosine-2′-phosphate and -3′-phosphate. The resolution of adenosine phosphates in dibasic sodium phosphate is said to be improved by substituting for isoamyl alcohol 0.5% lauryl amine in *n*-amyl alcohol.[74] The ammonium sulfate mixture of Markham and Smith[59] gives better spacing of the isomeric nucleotides and separates also those of guanylic acid and 8-azaguanylic acid;[59] in this solvent the cyclic nucleotides move more slowly than the corresponding nucleoside-2′- and -3′-phosphates.[71]

With water as the solvent, the R_F values of all the nucleotides are close to 0.9.[68]

V. Quantitative Estimation of the Nitrogenous Components of Nucleic Acids

1. Hydrolysis of Deoxypentose Nucleic Acids

The purine and pyrimidine components of deoxypentose nucleic acids are satisfactorily estimated as free bases, since these may be obtained in

[68] E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger, and D. Elson, *J. Biol. Chem.* **186**, 51 (1950).
[69] E. Chargaff, *Federation Proc.* **10**, 654 (1951).
[70] P. Boulanger and J. Montreuil, *Bull. soc. chim. biol.* **33**, 784, 791 (1951).
[71] R. Markham and J. D. Smith, *Biochem. J.* **52**, 552 (1952).
[72] R. Markham and J. D. Smith, *Biochem. J.* **52**, 558, 565 (1952).
[73] D. M. Brown and A. R. Todd, *J. Chem. Soc.* **1953**, 2040.
[74] O. Snellmann and B. Gelotte, *Nature* **168**, 461 (1951).

virtually quantitative yield by appropriate acid hydrolysis. Good yields of nucleosides or nucleotides have not been obtained from DNA by any means of hydrolysis with acid or alkali. Nucleotides may be obtained enzymically,[75] but DNA containing 5-hydroxymethylcytosine resists this treatment[76] and chemical methods are preferable for routine work. [Cf. *Chargaff*, Chapter 10.]

a. Hydrolysis with hydrochloric acid

The purines are completely released from DNA by very mild acid treatment (pH 1.6 at 37° for 24 hr., or pH 2.8 at 100° for 1 hour[77]). More drastic hydrolysis with hydrochloric acid (10 mg. nucleic acid in 2 ml. 6 *N* HCl at 120° for 2 hours[78, 79]) gives a good yield of pyrimidines but causes some destruction of purines. Nearly quantitative yields of all the bases (including 5-hydroxymethylcytosine but with evidence for slight destruction of adenine) have been obtained by Hershey *et al.*[57] by subjecting the partially hydrolyzed nucleic acid extracted from bacteriophage T2 with hot trichloroacetic acid to further hydrolysis with 3 ml. redistilled 6 *N* HCl under CO_2 in sealed tubes at 100° for 3 hours.

b. Hydrolysis with perchloric acid

The use of concentrated perchloric acid for liberation of bases from nucleic acids was introduced by Marshak and Vogel.[52, 80] With DNA, either 7.5 *N* or 12 *N* (70%) $HClO_4$ may be used, and maximal yields are obtained in 1 hour at 100°. Loss of a small percentage of the thymine has been noted during hydrolysis with 70% $HClO_4$, which may be minimized by using not more than 15 μl. $HClO_4$ per mg. DNA, although if much protein is present the amount may be increased.[81] 5-Hydroxymethylcytosine is extensively destroyed during hydrolysis of bacteriophage DNA with perchloric acid, although the isolated pyrimidine is apparently not attacked by this acid.[11, 57] By hydrolyzing with $HClO_4$ it is possible to estimate the total purine and pyrimidine bases from a mixture containing both DNA and PNA, and to estimate the bases without isolation of nucleic acid from some biological materials. Non-nucleic acid components are degraded to products which interfere very little on the chromatograms. Perchloric acid hydrolysates may be diluted with water and applied directly to paper for chromatography. If phosphorus is to be estimated in samples of the hydrolysate, the insoluble residue from the nucleic acid carbohydrate, which tends to adsorb phosphate, should first be brought into suspension.[82]

[75] R. O. Hurst, J. A. Little, and G. C. Butler, *J. Biol. Chem.* **188**, 705 (1951).
[76] S. S. Cohen and G. R. Wyatt, unpublished results, 1953
[77] C. Tamm, M. E. Hodes, and E. Chargaff, *J. Biol. Chem.* **195**, 49 (1952).
[78] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **33**, 497 (1950).
[79] N. I. Gold and S. H. Sturgis, *J. Biol. Chem.* **196**, 143 (1952).
[80] A. Marshak and H. J. Vogel, *Federation Proc.* **9**, 85 (1950).
[81] G. R. Wyatt, *J. Gen. Physiol.* **36**, 201 (1952).
[82] Levenbook[65] has obtained evidence for differential adsorption of purine and pyrimidine bases also to the charcoal from nucleic acid samples contaminated with carbohydrates.

c. Hydrolysis with formic acid

The value of formic acid for liberating purine and pyrimidine bases from nucleic acids without destruction was first shown by Vischer and Chargaff,[83] and this acid has proved very satisfactory for the quantitative hydrolysis of DNA. The conditions used in Chargaff's laboratory have been 98–100 % formic acid at 175° for 2 hours. Shorter periods of heating have also been employed.[47, 55] Recently,[11] good results were obtained with DNA from bacteriophages and other sources using 88 % formic acid at 175° for 30 minutes, and it was found that whether 88 % or 98 % formic acid is used, recoveries of guanine and hydroxymethylcytosine are substantially increased by using a relatively large volume of it (500 μl. per mg. DNA) and by avoiding excessive oxidizing atmosphere while heating. The hydrolysis is carried out in sealed Pyrex glass bomb tubes, and decomposition of the formic acid produces considerable pressure, which it is advisable to release by melting the tips of the tubes in a flame before opening them. The hydrolysate is evaporated under reduced pressure to dryness, then redissolved in a small volume of *N* HCl, and samples are taken for chromatography and for estimation of phosphorus. Because of the high recoveries and the absence of insoluble residue in the hydrolysate, this is probably the method of choice for accurate microanalysis of purified preparations of DNA.

2. Hydrolysis of Pentose Nucleic Acids

The ribosides of both purines and pyrimidines are split with more difficulty than their deoxyribosides, and the resistance of the pyrimidine ribosides is such that it is very difficult to get quantitative yields of the free bases from PNA. However, the ease with which mononucleotides are obtained from PNA makes it possible to estimate the pyrimidines, or all of the bases, as nucleotides. [Cf. *Loring*, Chapter 5, and *Magasanik*, Chapter 11.]

a. Hydrolysis to purine and pyrimidine bases

Concentrated formic acid at 175° for 2 hours has been used for liberation of pyrimidines from PNA;[83] however, the yield of uracil is very low and the method has been found unsatisfactory for quantitative hydrolysis of uridylic acid.[68] Nearly quantitative yields of all the bases can be obtained with 70–72% perchloric acid at 100° for 1 hour,[42, 43] and this method has been used for analysis of PNA's.[84]

b. Hydrolysis to purine bases and pyrimidine nucleotides

The purine bases are liberated from PNA by relatively mild acid hydrolysis, e.g., *N* H_2SO_4[83] or *N* HCl[84] at 100° for 1 hour. The latter treatment was selected by Smith

[83] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176**, 715 (1948).
[84] A. Marshak, *J. Biol. Chem.* **189**, 597 (1951).
[85] Smith and Markham[59] include a critical discussion of previously used methods of hydrolysis.

and Markham for quantitative analysis of PNA's: pyrimidines are estimated as nucleotides, a correction of 5% being applied to allow for degradation to nucleosides.[56] Abrams,[84] using isotope dilution to test comparable hydrolytic conditions, has demonstrated about 7% destruction, probably deamination, of adenine and guanine.

c. Hydrolysis to nucleotides

As has long been known, PNA, unlike DNA, is readily hydrolyzed to mononucleotides. [Cf. Chapter 11, and also *Brown and Todd*, Chapter 12.] This may be achieved with *N* NaOH or *N* HCl at room temperature; the latter is less convenient because the nucleic acid dissolves only slowly and there is danger of splitting purine glycosidic linkages.[56] Chargaff *et al.*[68] used pH 13 to 14 at 30° overnight to degrade PNA to nucleotides prior to chromatographic separation. Boulanger and Montreuil[79] used 0.5 *N* NaOH at 20–22° for 18 hours, or concentrated ammonia solution ($D^{\circ} = 0.925$) at 45° for 8 days, with good results, although ammonia under other conditions is liable to produce nucleosides. At 37°, *N* alkali causes partial deamination of cytidylic acid, but 0.3 *N* alkali does not.[87] This was confirmed by Davidson and Smellie,[88] who used 0.3 *N* KOH at 37° for 18 hours to convert PNA to nucleotides, the potassium being removed as the perchlorate prior to separation of nucleotides by electrophoresis on filter paper. Crosbie *et al.*[89] analyzed a number of samples of PNA by this method as well as by hydrolysis in *N* HCl and in concentrated $HClO_4$ in conjunction with paper chromatography: alkaline hydrolysis gave consistently higher results for uracil, for cytosine, and for total recovery in terms of phosphorus than either of the other procedures. Alkaline hydrolysis followed by electrophoretic separation of nucleotides appears to be the most reliable method yet devised for microanalysis of PNA. [Cf. *Smith*, Chapter 8.]

3. Quantitative Technique

Small volumes of hydrolysates or other solutions can be measured on to filter paper for quantitative chromatography with a variety of micropipets and burets. Especially convenient are self-filling capillary pipets,[90] which are not difficult to make; those available commercially often have excessively thick tips and a bore so fine that they are liable to clogging and drainage error. The volume which may be placed on the paper in one application depends on the scale of working: in the range of 10 to 50 μg. of each purine or pyrimidine base, to be run in one dimension for about 18

[84] R. Abrams, *Arch. Biochem.* **30**, 44 (1951).
[87] D. H. Marrian, V. L. Spicer, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **189**, 533 (1951).
[88] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52**, 594 (1952).
[89] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54**, 287 (1953).
[90] P. L. Kirk, "Quantitative Ultramicroanalysis," p. 22. John Wiley and Sons, New York, 1950.

hours, a suitable volume is about 10 μl., and using less does not result in significantly smaller final spots.

A substance may be efficiently eluted in a small volume by cutting out a pointed band of paper including the spot, and allowing water to diffuse through it toward the point. The solute is carried with the water, which may be collected from the point of the paper in a small vessel,[91] in a pipet[92] or directly on another sheet of filter paper for re-chromatography.[93] This technique has been used to concentrate minor components such as 5-methylcytosine from nucleic acids for estimation.[48]

For quantitative estimation, spots are cut out and eluted by soaking in a volume of liquid appropriate for the cells in which the extinctions are to be read (4–5 ml. for the 1-cm. cells of the Beckman or similar spectrophotometer). 0.1 *N* HCl is an eluent[37] in which the nucleic acid bases are sufficiently soluble, and in which some of them have higher extinction coefficients than in neutral or alkaline solutions. Standing overnight at room temperature, with shaking before and after, effects quantitative elution. To allow for ultraviolet-absorbing substances in the paper, blanks are cut equal in area to the spots and at equal distances from the starting line, and are eluted and read at the same wavelengths as the corresponding spots. As a further precaution against error due to ultraviolet-absorbing contaminants, Vischer and Chargaff[17] read the extinctions of all eluates both at the absorption peak of the substance being estimated and at another reference wavelength where its absorption is low, basing their calculations on the difference. The same procedure was adopted by Crosbie *et al.*[59] in analyzing HCl and $HClO_4$ hydrolysates, and it is evident that it will generally reduce the error from contaminants, although their absorption at the two wavelengths will rarely be precisely equal. Spectral data for use in estimating nucleic acid derivatives by the absorption at their maxima and by the difference method are given in Tables III and IV, respectively. [Cf. *Beaven, Holiday and Johnson*, Chapter 14.] Hotchkiss[2] gives tables of the absorption of nucleic acid derivatives at 5-mμ wavelength intervals, which may be used to compute the composition of binary mixtures from density readings at two or more wavelengths.

The methods which have been described are suitable for estimation of purine and pyrimidine derivatives in the range of 5 to 100 μg. per spot. Quantitative analyses in duplicate have been conveniently carried out on samples of 0.3 to 1.0 mg. of nucleic acid by making up hydrolysates to a volume of 25 μl., from which two 8-μl. portions are taken for chromatography and two 2-μl. portions for phosphorus estimation.[11, 81] In an ultra-micro adaptation of paper chromatography recently described by Edström,[62] only one hundredth of this amount of material is required. The hydrolysate

[91] C. E. Dent, *Biochem. J.* **41**, 240 (1947).
[92] R. Consden, A. H. Gordon, and A. J. P. Martin, *Biochem. J.* **41**, 590 (1947).
[93] A. M. Moore and J. B. Boylen, *Science* **118**, 19 (1953).

TABLE III

ULTRAVIOLET ABSORPTION DATA ON PURINE AND PYRIMIDINE DERIVATIVES

Substance	Normality of HCl	Wavelength, mμ[a]	Millimolar extinction coefficient	$E_{1\text{ cm.}}^{0.001\%}$
Adenine[47]	0.1	260	13.0	0.96
Guanine[47]	0.1	250	11.0	0.73
Uracil[47]	0.1	260	7.9	0.705
Thymine[47]	0.1	265	7.95	0.63
Cytosine[47]	0.1	275	10.5	0.95
5-Methylcytosine[47]	0.1	283	9.8	0.785
5-Hydroxymethylcytosine[11]	0.1	279	9.7	0.685
Adenylic acid[94]	0.01	260	13.9	0.401
Guanylic acid [94]	0.01	260	11.8	0.325
Uridylic acid[95]	0.01	262	9.89	0.305
Cytidylic acid[95]	0.01	278	12.72	0.393

[a] Not, in all cases, the precise absorption maximum.

TABLE IV

ULTRAVIOLET ABSORPTION DATA ON PURINE AND PYRIMIDINE DERIVATIVES

Substance	Solvent	Absorption Maximum, mμ	Reference wavelength, mμ	Difference in $E_{1\text{ cm.}}^{0.001\%}$ at the two wavelengths
Adenine[89]	0.1 *N* HCl	262.5	290	0.935
Guanine[89]	1.6 *N* HCl	249	290	0.539
Cytosine[89]	0.1 *N* HCl	275	290	0.546
Uracil[89]	0.1 *N* HCl	259	280	0.609
Thymine[17]	Water	264.5	290	0.545
Cytidylic acid[89]	0.01 *N* HCl	278	300	0.331
Uridylic acid[89]	0.1 *N* HCl	260.5	280	0.814

of 1.5 to 5 μg. of nucleic acid is applied in a volume of about 0.5 μl. to a strip of filter paper 0.5 to 1 mm. broad, which is subjected to chromatography in the ordinary way. A contact print of the dried strip chromatogram is made on a process plate with light of wavelengths 257 and 275 mμ from rotating cadmium electrodes, then the darkening of the plate is measured with a recording microphotometer, the image of a rotating sector serving for calibration. This refined technique will undoubtedly be of value in certain biological problems; however, the equipment is not available in the average laboratory, and one may note that by using narrow strips of paper as described by Edström to prevent spreading of the spots, and eluting the bands for extinction measurement in microcells[96] on the spectrophotometer, comparable sensitivity could

[94] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).
[95] J. McT. Ploeser and H. S. Loring, *J. Biol. Chem.* **178,** 431 (1949).
[96] O. H. Lowry and O. A. Bessey, *J. Biol. Chem.* **163,** 633 (1946).

TABLE V

R_F VALUES OF PENTOSES

	Solvent			
	Phenol[a]	Methyl ethyl ketone[b]	Butanol–ethanol[c]	Butanol–pyridine[d]
Arabinose	0.54	0.075	0.12	—
Xylose	0.44	0.090	0.15	—
Lyxose	—	0.125	0.19	—
Ribose	0.59	0.165	0.21	0.49
Rhamnose	0.59	0.180	0.30	0.56
2-Deoxyribose	0.73	—	0.44	0.60

[a] Phenol satd. with water at 20°, with 1% NH_3 and a few crystals KCN in soln. in bottom of tank; Whatman No. 1 paper; Partridge, *Biochem. J.* **42,** 238 (1948).

[b] Methyl ethyl ketone satd. with water at 20°, with 1% NH_3 in soln. in bottom of tank; Whatman No. 1; Partridge, *op. cit.* The value for lyxose is calcd. from the data of Crosbie *et al.*, *Biochem. J.* **54,** 287 (1953).

[c] *n*-Butanol 50 vol., ethanol 10 vol., water 40 vol., the upper layer being used; Whatman No. 1. R_{TG} values (movement relative to that of 2,3,4,6-tetramethylglucose taken as 1.00) are shown. E. L. Hirst and J. K. N Jones, *Discussions Faraday Soc.* **7,** 271 (1949).

[d] To the upper layer resulting from the mixture of 1 vol. pyridine, 1.5 vol. water, and 3 vol. *n*-butanol. at about 28°, is added 1 vol. pyridine; Schleicher and Schüll No. 597 paper. Chargaff *et al.*, *J. Biol. Chem.* **177,** 405 (1949).

be achieved. The bases from 5 μg. of yeast PNA, each eluted in 0.1 ml. and examined in a cell of 1 cm. path length, would give optical densities in the range 0.25 to 0.5, so that it should be entirely practical to work on this scale without special equipment.

VI. Chromatography of Nucleic Acid Sugars

This account would be incomplete without reference to the identification of nucleic acid carbohydrates by paper chromatography. However, since the methods do not differ from those used for other carbohydrates, which have been adequately reviewed,[4-6, 97] and since the results with nucleic acid components have been limited in scope, this description will be kept very brief.

Numerous spray reagents have been described for detection of sugars on paper chromatograms. Ammoniacal silver nitrate, used in the original investigations in this field,[98] is sensitive but relatively nonspecific and subject to interference by impurities. *m*-Phenylenediamine[99] forms with a wide range of aldoses and ketoses derivatives which fluoresce in ultraviolet light, and has been used in Chargaff's laboratory for recognition of nucleic acid sugars. Aniline hydrogen phthalate[100] is a sensitive and convenient reagent

[97] S. M. Partridge, *Biochem. Soc. Symposia* (*Cambridge, Engl.*) **No. 3,** 52 (1949).

[98] S. M. Partridge, *Biochem. J.* **42,** 238 (1948).

[99] E. Chargaff, C. Levine, and C. Green, *J. Biol. Chem.* **175,** 67 (1948).

[100] Aniline, 0.93 g., and phthalic acid, 1.66 g., are dissolved in 100 ml. water-saturated butanol. The papers are sprayed and then heated to 105° for 5 min. S. M. Partridge, *Nature* **164,** 443 (1949).

for aldoses, including ribose and deoxyribose, but not for ketoses; increased range of sensitivity is reported for aniline hydrogen phosphate.[101] The reagents mentioned earlier (Section III.2) for ribosides and deoxyribosides react also with the free sugars.

The R_F values of the pentoses, along with rhamnose and 2-deoxyribose, in several chromatographic solvents are shown in Table V. Improved separations of a number of sugars are reported by Jermyn and Isherwood[102] using ethyl acetate–pyridine–water (2:1:2) and ethyl acetate–acetic acid–water (3:1:3) as solvents; however, R_F values of ribose and deoxyribose were not determined. 2-Deoxyribose, 3-deoxyribose, and 4-deoxyribose are separable with a butanol–ethanol mixture.[103] The D- and L-isomers of sugars have not been separated on paper chromatograms.

The purine-bound sugar from pentose nucleic acids may be liberated by hydrolysis in N H_2SO_4 at 100° for 1 hour,[83] and the hydrolysate can be applied to paper for chromatography without removal of the acid.[104] From DNA, because of the lability of the sugar and the stability of the internucleotide linkages, enzymic hydrolysis is required: Chargaff *et al.*[105] used deoxyribonuclease together with a phosphatase derived from *Aspergillus* to give nucleosides, after which the purine nucleosides were split by heating at pH 1.5 to 100° for 12 minutes.

The sugar components of all pentose nucleic acids yet examined by these methods have proved to be chromatographically identical with ribose, and those of deoxypentose nucleic acids, with 2-deoxyribose. [Cf. Chapters 2, 10, and 11.]

VII. Addendum

The following reports have appeared, or have come to the writer's attention, since preparation of this chapter.

A solvent composed of 7 vol. 95% ethanol plus 3 vol. M ammonium acetate buffer of pH 3.8, used in the study of uridine diphosphate glucose, gives fair resolution of the nucleotides of PNA.[106] Several systems suitable for resolution of deoxyribosides have been described by Tamm *et al.*[52e] Caldwell[107] has demonstrated resolution of adenosine-5′-phosphate, diphosphate, and triphosphate with Hanes and Isherwood's solvent containing 60 cc.

[101] N Aniline in butanol, 1 vol., mixed with 2 N H_3PO_4 in butanol, 2 vol.; J. L. Bryson and T. J. Mitchell, *Nature* **167**, 864 (1951).
[102] M. A. Jermyn and F. A. Isherwood, *Biochem. J.* **44**, 402 (1949).
[103] P. F. V. Ward and P. W. Kent, *Nature* **170**, 936 (1952).
[104] B. D. E. Gaillard, *Nature* **171**, 1160 (1953).
[105] E. Chargaff, E. Vischer, R. Doniger, C. Green, and F. Misani, *J. Biol. Chem.* **177**, 405 (1949).
[106] A. C. Paladini and L. F. Leloir, *Biochem. J.* **51**, 426 (1952).
[107] P. C. Caldwell, *Biochem. J.* **55**, 458 (1953).

n-propanol, 30 cc. concentrated ammonia solution, and 10 cc. water. He has also found useful for detection of ultraviolet absorbing spots a sheet of paper impregnated with proflavine as a fluorescent screen.

The circular method of paper chromatography of Rutter[108] and others, for which improved resolution is claimed, has been applied to nucleic acid derivatives.[109]

A comprehensive tabulation of ultraviolet absorption data, including molar extinction coefficients and the ratios of extinctions at different wavelengths, measured at three pH values, has been prepared by Volkin and Cohn.[110]

[108] L. Rutter, *Nature* **161,** 435 (1948).

[109] K. V. Giri, P. R. Krishnaswamy, G. D. Kalyankar, and P. L. N. Rao, *Experientia* **9,** 296 (1953).

[110] E. Volkin and W. E. Cohn, *in* "Methods of Biochemical Analysis," (D. Glick, ed.) Vol. 1, Interscience Publishers, New York (1954), and *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, Academic Press, New York (in press).

* Contribution No. 116, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

n-propanol, 30 cc. concentrated ammonia solution, and 10 cc. water. He has also found useful for detection of ultraviolet absorbing spots a sheet of paper impregnated with proflavine as a fluorescent screen.

The circular method of paper chromatography of Rutter[108] and others, for which improved resolution is claimed, has been applied to nucleic acid derivatives.[109]

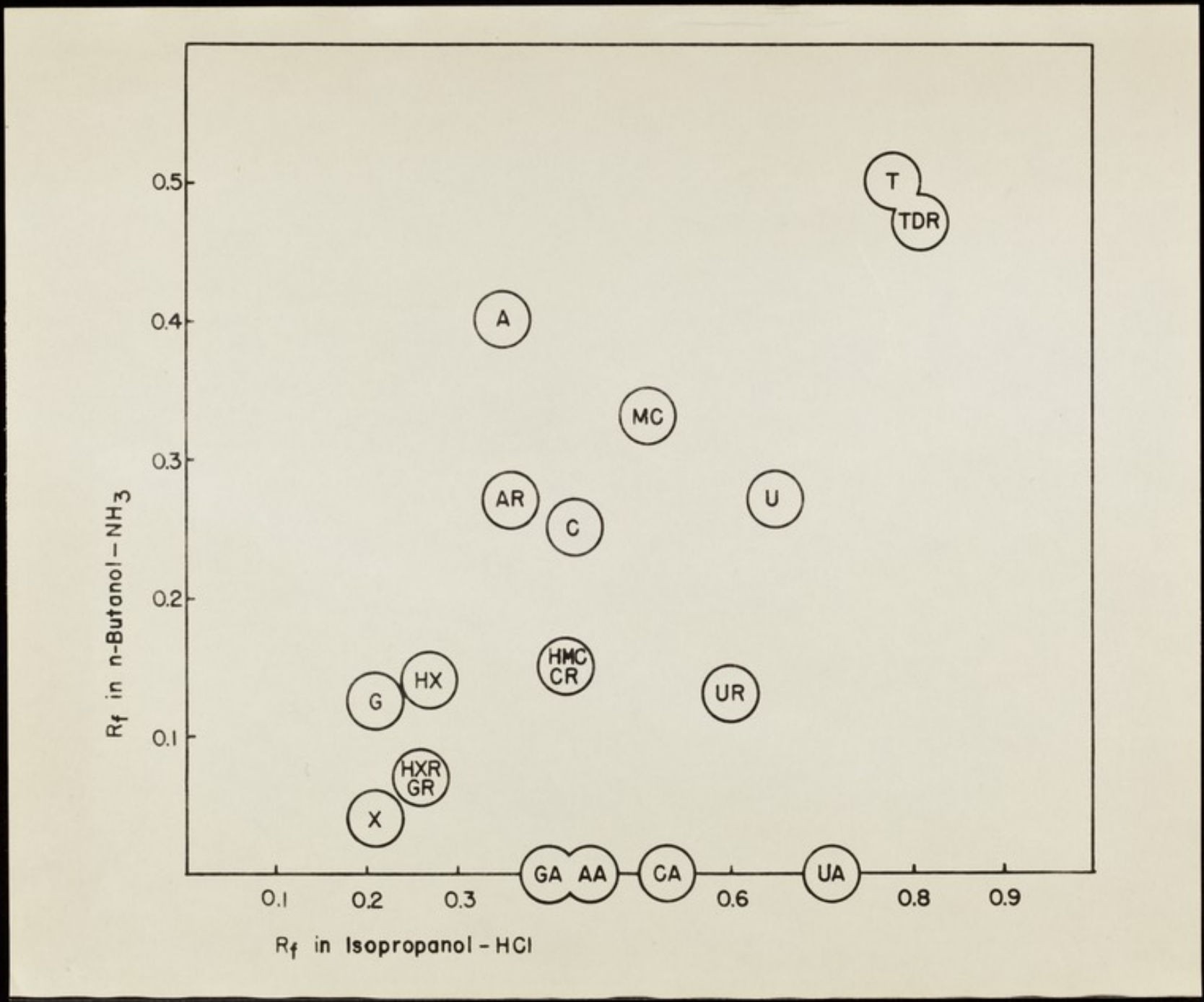
A comprehensive tabulation of ultraviolet absorption data, including molar extinction coefficients and the ratios of extinctions at different wavelengths, measured at three pH values, has been prepared by Volkin and Cohn.[110]

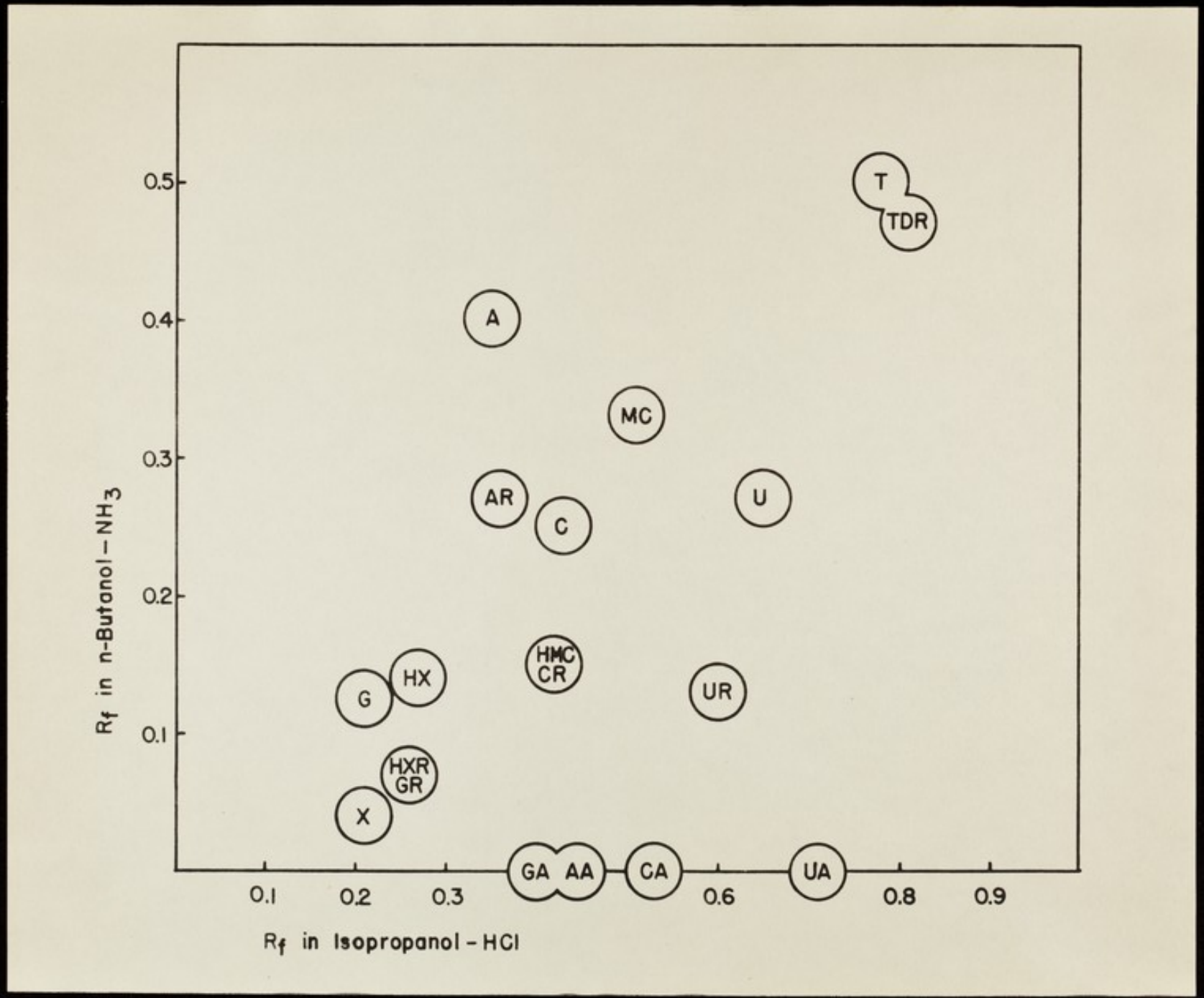
[108] L. Rutter, *Nature* **161**, 435 (1948).

[109] K. V. Giri, P. R. Krishnaswamy, G. D. Kalyankar, and P. L. N. Rao, *Experientia* **9**, 296 (1953).

[110] E. Volkin and W. E. Cohn, *in* "Methods of Biochemical Analysis," (D. Glick, ed.) Vol. 1, Interscience Publishers, New York (1954), and *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. 2, Academic Press, New York (in press).

* Contribution No. 116, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.


T
TDR
A
MC
AR
C
U
HMC
CR
HX
G
UR
HXR
GR
X
GA
AA
CA
UA
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
0.6
0.8
0.9
Rf in n-Butanol – NH3
Rf in Isopropanol – HCl


T
TDR
A
MC
AR
C
U
HMC
CR
HX
G
UR
HXR
GR
X
GA
AA
CA
UA
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
0.6
0.8
0.9
Rf in n-Butanol – NH3
Rf in Isopropanol – HCl

ACADEMIC PRESS INC.

PUBLISHERS

125 EAST 23RD STREET, NEW YORK 10, N. Y.

WALTER J. JOHNSON: PRESIDENT
KURT JACOBY: VICE-PRESIDENT

OREGON 7-6713

January 9, 1953

Dr. R. G. Wyatt
Department of Agriculture
Laboratory of Insect Pathology
Sault-Ste. Marie, Ontario, Canada

Dear Dr. Wyatt:

We are very happy to learn from Drs. Erwin Chargaff and J. N. Davidson that you have agreed to prepare a contribution for

THE NUCLEIC ACIDS: Chemistry and Biology.

We are very pleased with your decision in this matter, and we wish to thank you for your willing cooperation. Our arrangements with the contributing authors are rather informal. May we suggest the following terms:

1. The publishers will pay to the authors a royalty of ten per cent of the retail price of the book on all copies sold, and, for sales outside of the United States, two-thirds of the royalties in force on the United States sales. The royalty will be distributed among the authors in proportion to the number of pages contributed by each author. Payments will be made on or about March 1st for sales made through December of the previous year. When the number of copies sold per year falls below one hundred, the publishers will no longer be under obligation to continue payment of royalties.

2. One copy of the book will be presented to the author free of charge, as well as several tear sheets of his article.

3. The author will pay or permit to have charged against royalties the amount of expense incurred by the publishers because of the author's changes and/or additions in excess of ten per cent of the original cost of composition.

4. The author agrees to deliver two copies of his complete manuscript of about ..... printed pages before June 30, 1953 ..... The author will send a detailed table of contents of his article to the Editors by February 15.

Details of the preparation of the manuscript are given in the "Notice to Contributors" which is enclosed.

*** Please sign and return one copy of this letter.

Sincerely yours,

ACADEMIC PRESS INC.

Name .....

Date .....

Walter J. Johnson

Walter J. Johnson