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PAPER CHROMATOGRAPHY OF PURINE AND PYRIMIDINE DERIVATIVES OF YEAST NUCLEIC ACID

C.E. CARTER

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PAPER CHROMATOGRAPHY OF PURINE AND PYRIMIDINE DERIVATIVES OF YEAST NUCLEIC ACID

C. E. Carter

The application of paper chromatography to problems of nucleic acid chemistry was first reported by Vischer and Chargaff (1) who extended this technique to the quantitative analysis of purine and pyrimidine bases of nucleic acids (2). The procedure employed consisted of locating the purines and pyrimidines on chromatograms developed with several organic solvents by forming their mercury salts on guide strips and converting this salt to the sulfide. The resultant black spot thus served as a guide for the elution of purines and pyrimidines from untreated areas of the chromatogram. The chromatographic procedure described by Hotchkiss (3) entails the elution of successive areas of the chromatogram developed with butanol and determination of ultraviolet absorption spectra in solutions from these areas for location and quantitation of purines, pyrimidines, and several nucleosides.

This paper describes chromatographic techniques for resolving mixtures of the purines, pyrimidines, nucleosides and nucleotides of yeast nucleic acid and a method for locating these compounds on chromatograms by ultraviolet fluorescence.

Materials and Methods

The detection of purine and pyrimidine compounds by fluorescence is readily achieved by using a high energy ultraviolet light source with a filter which transmits at 3650 A. and which excludes visible light. A satisfactory lamp for this purpose is the Mineralight manufactured by the Ultraviolet Products Corporation, Los Angeles, California. Several conventional light sources for the detection of fluorescence were examined and found inadequate.

The following solvent systems for the development of the paper chromatograms were employed: (a) Butanol saturated with a 10% aqueous solution of urea, (b) 5% monobasic potassium phosphate or 5% dibasic sodium phosphate, and (c) 5% citric acid adjusted to desired pH with concentrated ammonium hydroxide. The butanol-urea solution was used in an atmosphere saturated with water vapor and the aqueous salt solutions were used with isoamyl alcohol as an overlying nonaqueous phase. The latter system is a departure from the usual techniques of paper chromatography, and consists of saturating the salt solution with isoamyl alcohol, allowing the layers to separate, then, using a vessel of sufficient capacity so that both phases exist as thin layers (1.0 cm. aqueous phase, 0.5 cm. nonaqueous phase), the paper is introduced into the solution so as to pass through both phases.

A limitation of previously used solvent systems for chromatographic separation of nucleic acid derivatives has been the inability to move nucleotides. The butanol system described by Hotchkiss[3] and the butanol urea system described above is adequate for chromatography of nucleosides but not for nucleotides. Although an aqueous salt solution will distribute the nucleotides characteristically on a chromatogram, there is considerable diffusion on the spot and resolution is poor. By placing a thin layer of nonaqueous solvent, isoamyl alcohol, over the aqueous layer and developing the strip with this two-phase system, good resolution is achieved and diffusion of the spot is minimized. Absolute RF values vary with the relative thickness of the solvent layers although the relation of spots to one another is the same.

Chromatograms were developed in 18-inch cylindrical jars with desiccator lid tops. Strips of Whatman # 1 filter paper were suspended from

glass racks and allowed to dip into the solvent system which was contained in glass dishes in the bottom of the jar. The solution containing the compounds to be chromatographed was quantitatively put on the paper by means of a micropipette approximately 1.5 cm. above the solvent line and dried under an infrared lamp previous to immersion in the solvent. The volume of solution put on the paper is not critical as long as the area of the original spot is small. This may be achieved with dilute solutions, by successively drying repeated applications of small volumes. When two-dimensional chromatograms were run the technique described by Williams and Kirby (4) was employed. This consisted of making a cylinder of a rectangular sheet of filter paper which then supports itself in an upright position in the solvent, running the chromatogram in the first dimension, then turning the paper 90° and conducting the process in the second dimension. In both these procedures the chromatograms are developed by capillarity. This simplifies the apparatus required and gives the same degree of resolution as techniques employing suspension of the paper from a trough and running the solvent down the strip.

Following development of the chromatogram the paper was dried in air at 60° and the purine and pyrimidine compounds located by fluorescence using transmitted light. The lower limit of detection with the lamp used was 2 to 5 µg. of each component. The optimal amount of components for analysis is between 20 to 50 µg. (0.2% to 0.5% with respect to each component) although 100 to 200 µg. amounts may be resolved satisfactorily.

The components used in these studies were obtained from Schwarz Laboratories with the exception of cytidine, uridine and cytosine, which were generously supplied by Dr. M. Kuna, uridylic acid which was prepared by the ion exchange procedure of Cohn (5), and muscle adenylic acid which was obtained from the Sigma Chemical Company. In these compounds the following

impurities were encountered: Adenine and guanine mutually contaminated to an extent of about 10%; in thymine, about 5% uracil; in guanylic acid a detectable amount of adenylic acid and adenine; in adenylic acid a detectable amount of adenosine; in adenosine about 10% guanosine; in hypoxanthine about 20% adenine; and in uridine a trace of uracil. The absence of sufficiently pure standards seriously limits any statement regarding the quantitative nature of the chromatographic procedures employed in these studies.

The quantitative aspects of paper chromatographic analysis have been discussed by Chargaff and Vischer[2] and by Hotchkiss[3]. The principles elaborated in these papers apply to the systems described herein. For elution of the spots from the chromatograms 0.01 N hydrochloric acid was used in the case of uracil, thymine, and cytosine; 1.0 N hydrochloric acid for adenine and guanine; and 1 N ammonium hydroxide for adenosine, guanosine, uridine, cytidine, adenylic, guanylic, cytidylic, and uridylic acids. In the latter group 0.01 N hydrochloric acid may be used except for guanylic acid and guanosine where better recoveries are made with 1 N ammonium hydroxide elution. The spots were extracted for twenty-four hours in 5 cc. of solution and quantitated in the ultraviolet spectrophotometer.

Results

Nucleotides. - Buffered aqueous systems with an overlying thin layer of isoamyl alcohol adequately resolve mixtures of purine nucleotides. The pyrimidine nucleotides, cytidylic and uridylic acid, cannot be resolved with any buffer system so far studied, but can be separated from purine nucleotides. The pyrimidine nucleotide spot may then be eluted and the ultraviolet spectrum resolved into the two components of the mixture by determining the ratios of absorption at several wave lengths and calculating the composition of the mixture from known standards of uridylic and cytidylic acid. If

actual separation of uridylic and cytidylic acid is desired, the nucleotide pair may be eluted and quantitatively recovered by employing the cation exchanger Dowex-50 and acetic or citric acid as described by Cohn[5]. The purine nucleotides are eluted from the paper chromatogram and determined directly by spectrophotometry.

Application of these techniques to analysis of an hydrolysate of yeast nucleic acid obtained by procedures described by Loring (6) is illustrated in Table I. It should be emphasized that the barium hydroxide hydrolysate does not represent a quantitative conversion of yeast nucleic acid to its composite mononucleotides. The low solubility of barium guanylate makes recovery of this component difficult and the low concentrations of guanylic acid in such nucleotide mixtures is recognized[6]. In Table I the RF values of the nucleotides of yeast nucleic acid are given with the per cent recovery by elution from the paper and determination by spectrophotometry. This serves as a basis for comparison of the chromatogram of the nucleotides derived by barium hydroxide hydrolysis of yeast nucleic acid.

As indicated in Table I, yeast adenylic acid is resolved by the chromatographic procedures employed into two components, and the barium hydroxide hydrolysate of yeast nucleic acid contains the same two adenylic acid components. Several preparations of yeast adenylic acid, both from commercial sources and preparations made in this laboratory by ion exchange procedures, have been examined and all have exhibited two components both having the same ultraviolet spectrum and both being degraded to adenine by heating in 1 N hydrochloric acid for fifteen minutes, at 100° (7). (Fig.1) Conditions for the quantitative conversion of adenylic acid to adenosine by alkaline degradation have not been found. Considerable amounts of adenine are always formed simultaneously. However, an extract of rabbit kidney

Table I

Analysis of nucleotide mixture obtained from known standards and from the barium hydroxide hydrolysate of yeast nucleic acid.

Of the latter solution, 0.025 cc. containing nucleotides equivalent to D = 862 at 2600 A. in the ultraviolet spectrophotometer was quantitatively put on the paper. Recovery of nucleotides based on total 2600 A. absorption was 106%.

Solvent system: 5% dibasic sodium phosphate - isoamyl alcohol.

Solvent front: 26.5 cm.

Standard[1]	RF	Recovery	Mixed Nucleotides RF	Amt. Nucleotides in 0.025 cc. of mixture
				µg.
Cytidylic[2]	0.86	101%	0.88	24.0
Uridylic	0.86	98%	0.88	63.0
Guanylic[3]	0.79	92%	0.81	22.0
Adenylic-a	0.74	34% } 102%	0.75	16.6
Adenylic-b	0.64	68% }	0.65	24.3

1. Standard solution contained 40 µg. of each nucleotide in a volume of 0.025 cc. of 1 N ammonium hydroxide.

2. The pyrimidine nucleotide spot was analyzed by special ratios according to the following calculations:

Spot eluted in 5 cc. of .01 N hydrochloric acid, ratio optical density $\frac{2800\ A.}{2600\ A.} = 0.85$. Uridylic acid $\frac{2800\ A.}{2600\ A.} = 0.35$; cytidylic acid = 1.95. $\frac{1.95 - .80}{1.95 - .35} = 68\%$ uridylic acid. The eluate was then resolved and quantitated by ion exchange using the procedures described by Cohn[5], and these results, recorded in the table above, show 72% uridylic acid in the pyrimidine nucleotide mixture.

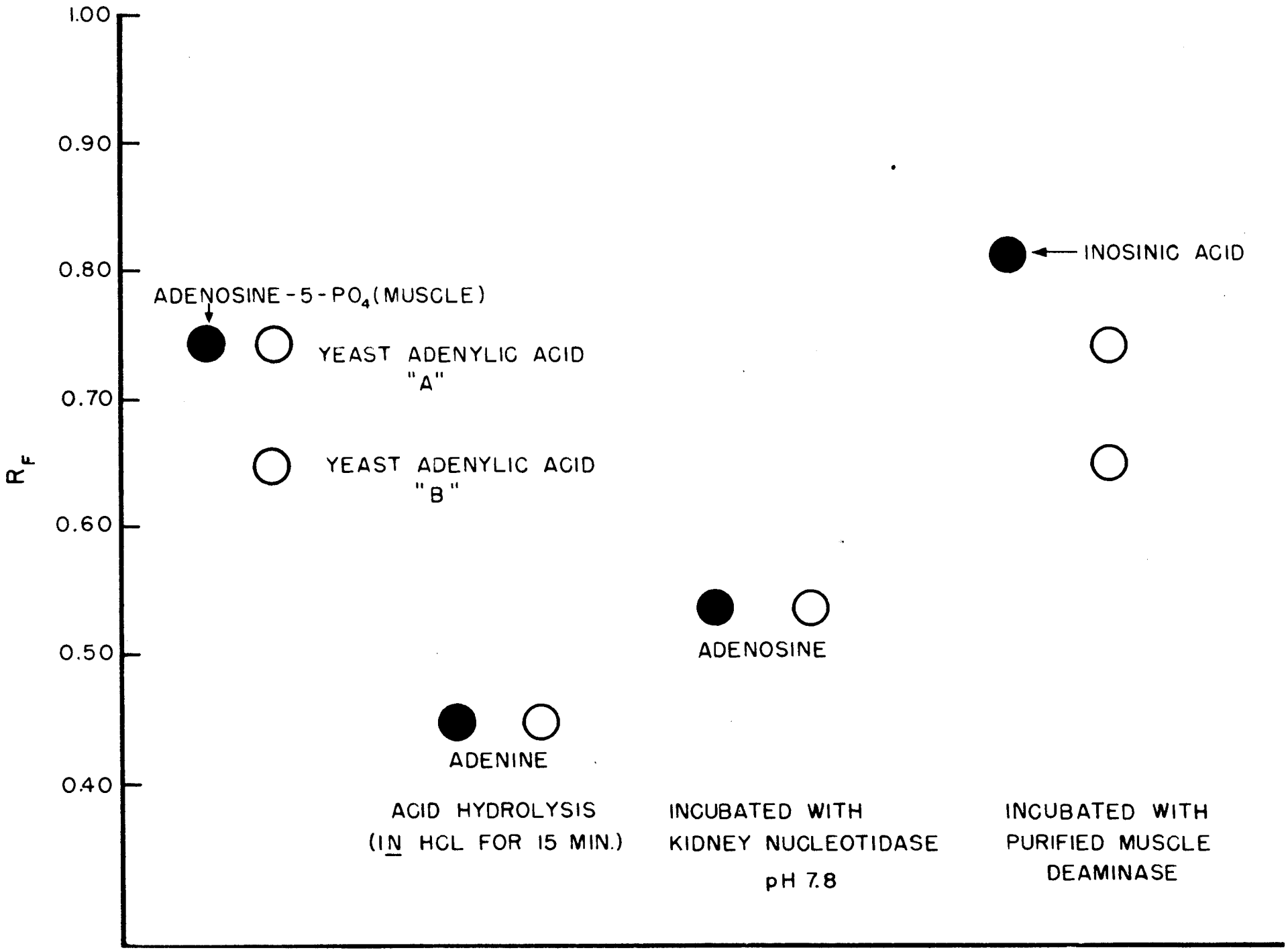
3. Guanylic acid was eluted in 5 cc. of 1 N ammonium hydroxide; adenylic acids in 5 cc. of .01 N hydrochloric acid.

Figure 1

The enzymatic and chemical degradation of muscle and yeast adenylic acids. Solvent system: 5% dibasic sodium phosphate-isoamyl alcohol. Muscle adenylic acid deaminase was prepared according to Kalckar's procedure[a]. Both yeast and muscle adenylic acid were employed in 0.5% concentration. For enzymatic deaminase degradation, 1 cc. of this solution was adjusted to pH 5.9, incubated five hours with 0.2 cc. purified enzyme, and 0.025 cc. of the digest chromatographed. Acid hydrolysis (1 N hydrochloric acid, twenty minutes at 100°) liberated 93% of yeast adenylic acid phosphate as inorganic phosphate and 12% of muscle adenylic acid phosphate as inorganic phosphate. The conversion of muscle and yeast adenylic acids to adenosine was achieved by employing the nucleotidase present in the supernatant of the kidney particle preparation described by Kornberg and Lindberg[b] in digests consisting of 0.2 cc. enzyme solution, and 0.2 cc. of 0.5% solution of muscle and yeast adenylic acids adjusted to pH 7.8 in 0.1 M ammonium citrate. The incubation period was twelve hours.

[a] H. M. Kalckar, J. Biol. Chem., 167, 461 (1947).

[b] A. Kohnberg and O. Lindberg, J. Biol. Chem., 176, 665 (1948).


1.00
0.90
0.80
0.70
0.60
0.50
0.40
R_F
ADENOSINE-5-PO_4(MUSCLE)
YEAST ADENYLIC ACID "A"
YEAST ADENYLIC ACID "B"
ADENINE
ADENOSINE
INOSINIC ACID
ACID HYDROLYSIS (IN HCL FOR 15 MIN.)
INCUBATED WITH KIDNEY NUCLEOTIDASE pH 7.8
INCUBATED WITH PURIFIED MUSCLE DEAMINASE

obtained by homogenizing the tissue in 0.1 M potassium phosphate buffer at pH 7.8 and centrifuging to remove nuclei and particulate matter, quantitatively converts both yeast adenylic acids and muscle adenylic acid to adenosine. This extract possesses no nucleosidase activity and deamination of adenosine is prevented by adequately buffering the solution at pH 7.8. Using an anion exchange procedure yeast adenylic acid has been resolved into two components and separated from muscle adenylic acid, by Cohn and Carter (8). This separation, in addition to the fact that muscle adenylic acid deaminase does not attack either of the yeast adenylic acids, (Fig. 1) eliminates the C'5 nucleotide as a possible component of yeast adenylic acid.

Nucleosides

In Table II the RF values for the purine and pyrimidine derivatives of yeast nucleic acid are recorded for different solvent systems. As shown in this table, resolution of nucleosides is achieved by developing the chromatogram with several solvents. It is apparent also that complete separation of nucleosides from one another and from interfering nucleotides, purines and pyrimidines, cannot be achieved in the one-dimensional chromatogram. For analysis of this class of compounds the two-dimensional chromatogram described below is necessary. However, as shown in Table III the four nucleosides may be resolved in the 5% dibasic sodium phosphate-isoamyl alcohol system to adenosine, guanosine and one spot containing the pyrimidine ribosides, uridine and cytidine. These spots may be eluted from the one-dimensional chromatogram, the purine ribosides determined separately, and the pyrimidine ribosides determined by resolving the spectrum of the mixture into that of each component by calculations involving the ratios of absorption at several wave lengths (see Table III), or by running a duplicate chromatogram in one of the solvent systems where the pyrimidine ribosides are resolved (ammonium

Table II

Solvents for Paper Chromatography of Yeast Nucleic Acid Derivatives RF Values

	5% Am. Citrate-pH 3.6 -Isoamyl Alcohol	5% Am. Citrate-pH 9.6 -Isoamyl Alcohol	5% KH_2PO_4 -Isoamyl Alcohol	5% Na_2HPO_4 -Isoamyl Alcohol	5% KH_2PO_4 pH 7.0 -Isoamyl Alcohol	Butanol-Urea
Adenine	.69	.37	.53	.44	.42	.41
Guanine	.50	.37	0	.02	.02	.05
Uracil	.72	.72	.78	.73	.74	.35
Cytosine	.83	.72	.79	.73	.74	.29
Thymine[a]	.72	.72	.77	.73	.74	.52
Adenosine	.68	.52	.58	.54	.53	.28
Guanosine	.66	.59	.68	.62	.64	.17
Uridine	.80	.80	.88	.79	.80	.23
Cytidine	.86	.77	.88	.76	.80	.17
Adenylic "a"	.74	.65	.72	.74	.74	0
Adenylic "b"	.74	.60	.81	.67	.63	0
Guanylic	.80	.73	.87	.79	.78	0
Cytidilic	.89	.82	.93	.85	.86	0
Uridylic	.89	.82	.93	.85	.86	0
Hypoxanthine	.63	.49	.52	.57	.59	.29
Xanthine	.52	.45	.56	.49	.42	.12

[a] Thymine is not a derivative of yeast nucleic acid.

Table III

Recovery of Nucleosides from Paper Chromatogram Developed with 5% Dibasic Sodium Phosphate-Isoamyl Alcohol System

Standard Solutions of Ribosides	Amount put on Chromatogram	Per Cent Recovery[1] on Elution with 1 N NH_4OH
Uridine	50 µg.	95.5%
Cytidine	50 µg.	104.5%
Adenosine	50 µg.	95.7%
Guanosine	50 µg.	89.5%

[1] Calculations based on absorption curves of standard nucleoside solution in 1 N ammonium hydroxide.

a. The pyrimidine nucleosides are not sufficiently resolved in the dibasic sodium phosphate-isoamyl alcohol solvent system to be separately determined; hence, the pyrimidine nucleosides are eluted as one spot and quantitated according to the following calculation:

Ratio D $\frac{2800\ A.}{2600\ A.}$ in 1 N NH_4OH: Uridine = .40; cytidine = .89.

Ratio D $\frac{2800\ A.}{2600\ A.}$ in unknown = .652

$$\frac{.89 - .652}{.89 - .40} = .49 \text{ or } 49\% \text{ uridine}$$

total 2600 A. absorption of unknown, D = .680 x .49 = .335

standard solution, 50 µg. uridine in 5 cc. 1 N NH_4OH, D = .350

Recovery = 95.5% uridine, by difference; 104.5% cytidine.

b. Purine nucleosides calculated directly from 2600 A. absorption compared with standard solutions.

citrate pH 3.6).

Purines and Pyrimidines. - These compounds have been thoroughly studied by Chargaff and Vischer[2] and Hotchkiss[3] and the solvent systems employed by these workers give excellent resolution of mixtures of purines and pyrimidines. The quantitative aspects of their analytical schemes have been confirmed in this laboratory and extended to the solvent systems described above.

Two-Dimensional Chromatography

The two-dimensional chromatogram illustrated in Fig. 2 was developed first in the butanol-urea system, then in 5% dibasic sodium phosphate isoamyl alcohol. Good resolution of purines, pyrimidines, and nucleosides is thus achieved. Resolution of nucleotides is adequate for analysis but diffusion is much greater than in the one-dimensional system yielding three poorly defined spots, pyrimidine nucleotides, guanylic acid, and adenine nucleotides. By reversing the order of solvent development the nucleotides are well resolved into adenylic acid "a", and adenylic "b", guanylic acid, and pyrimidine nucleotides. However, in the latter chromatogram resolution of ribosides and bases is not as satisfactory as when the butanol-urea dimension is run first.

In two-dimensional chromatography it is essential that the original starting spot be small. This is easily achieved by successive applications of volumes not larger than 0.01 cc. each. Nucleotides and nucleosides were put on the paper in 1 N ammonium hydroxide, the purines and pyrimidines in 1 N hydrochloric acid. To minimize diffusion of the spots high concentrations of salt and alkali in the solution should be avoided.

In Table IV, recovery of components from the chromatogram are reported. Uncertainty in regard to quantitative aspects of the method arise from the impurity of the standard solutions as well as the difficulty of running an

Figure 2

Two-dimensional chromatogram of yeast nucleic acid derivatives - RF values in parentheses.

1. Adenine (Butanol-urea, - .50, Phosphate - .38)
2. Guanine (0,0)
3. Uracil (.50, .71)
4. Cytosine (.35, .69)
5. Thymine (not a component of yeast nucleic acid)(.64, .64)
6. Adenosine (.31, .52)
7. Guanosine (.20, .58)
8. Uridine (.31, .83)
9. Cytidine (.20, .75)
10. Xanthine (found as impurity) (.27, .43)
11. Adenylic acid a + b (0, .58, .70)
12. Guanylic (0, .75)
13. Cytidylic + uridylic (0, .87)

The solvent front in the second dimension is irregular and advanced about 5 cm. in the region overlying the nucleotides.

Drawing # 6369

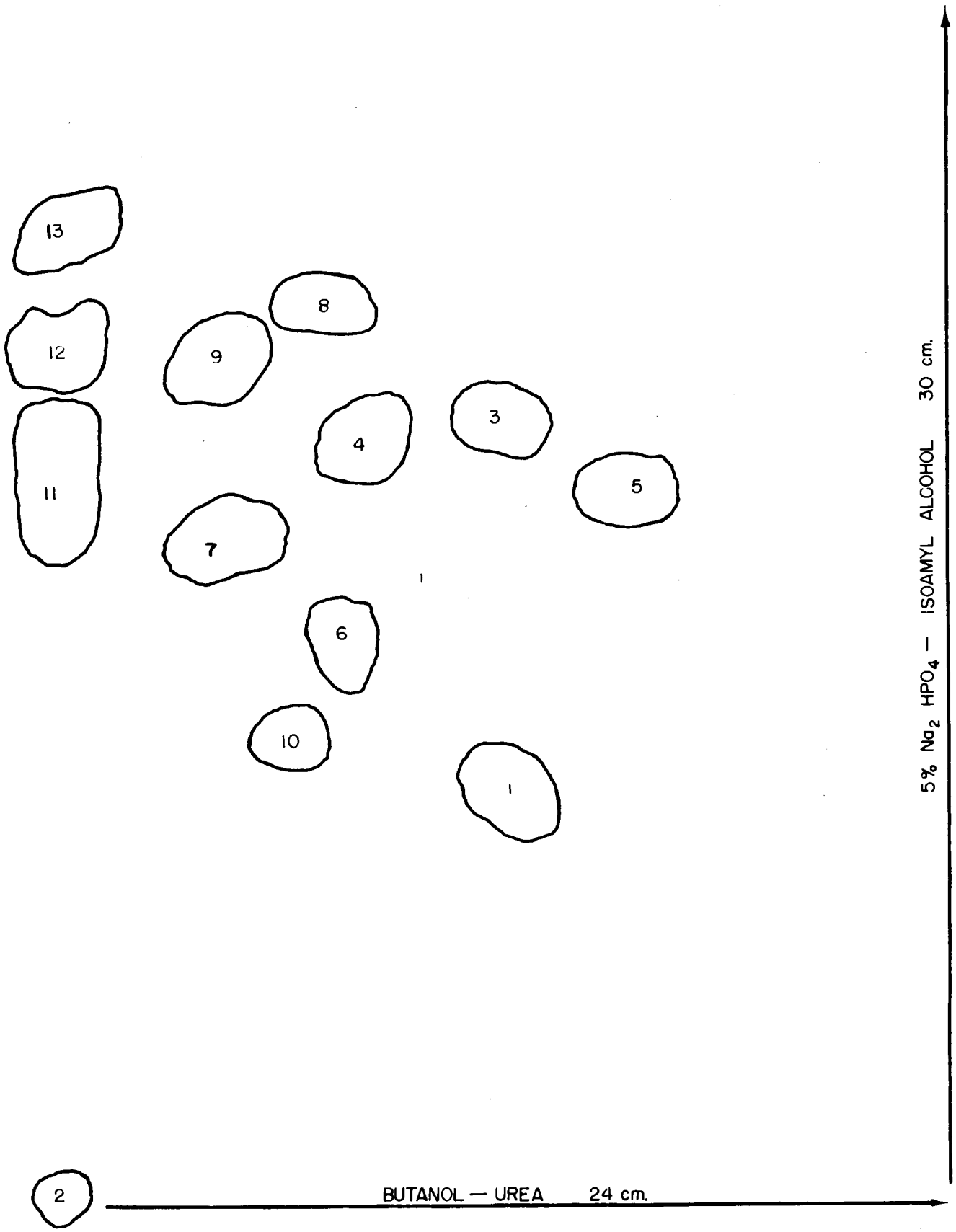


Table IV

Recovery of Components from Two-Dimensional Chromatograms (Fig. 2).

Components eluted from paper as described in text and quantitated on the basis of ultraviolet absorption. Twenty µg. of each component put on starting spot.

Components	Per Cent Recovery	Wave Length A.
Adenine	115	2600
Guanine	92	2600
Uracil	88	2600
Cytosine	109	2700
Thymine	82	2600
Adenosine	85	2600
Guanosine	91	2600
Uridine	89	2600
Cytidine	102	2700
Xanthine	trace	2600
Adenylic (a + b)	91	2600
Guanylic	85	2600
Cytidylic + uridylic	102	2700

adequate set of duplicates.

Discussion

The use of solvent systems suitable for the paper chromatography of nucleotides has resulted in the finding of two components in adenylic acid derived from yeast nucleic acid. The implications of this finding in regard to the structure of the two components are more fully discussed by Cohn and Carter[8]. Briefly, the following evidence indicates that the two adenylic acid components are isomers with respect to the position of the ester phosphate linkage on the ribose moity of the molecule: (a) both components are degraded to adenine by acid hydrolysis; (b) both components are enzymatically degraded to adenosine by kidney nucleotidase. Possibilities for isomerization of the phosphate ester linkage are limited by exclusion of the number one and four carbon atoms on the ribose chain by the nucleoside and furanose structures respectively (9), and the possibility of one of the components being adenosine-5-phosphoric acid is excluded by the acid labile nature of the phosphate of both components and the fact that neither is degraded to inosinic acid by Schmidt's muscle adenylic deaminase (Fig. 1). It is therefore suggested that the two components are adenosine-2-phosphoric acid and adenosine-3-phosphoric acid.

Although the chromatographic procedures described in this paper are suitable for analysis of complex mixtures of yeast nucleic acid derivatives, the methods have been principally used in this laboratory for following the enzymatic or chemical degradation of known purine and pyrimidine compounds and as an adjunct to ion exchange procedures[5]. Preliminary experiments in the analysis of tissues indicates that chromatographic procedures are most useful when combined with chemical separations of the various classes of purines and pyrimidines, this step effecting both concentration and partial separation

prior to chromatography.

The procedure is not presented as an inclusive quantitative analytical method for the components of nucleic acids by resolution in a two-dimensional paper chromatogram, although more rigorous examination may establish this application of the techniques described.

Presumably the nucleotides and nucleosides of thymus nucleic acid may be analyzed by these procedures; however, adequate standards are not yet available to test this application.

Summary

Solvent systems for paper chromatography of purine and pyrimidine derivatives of yeast nucleic acid are described and the use of the method for analysis of complex mixtures of these compounds by two-dimensional chromatography reported. Location of compounds on the paper chromatogram is achieved by ultraviolet fluorescence.

It has been found that the adenylic acid derived from yeast nucleic acid is resolved by chromatographic procedures into two components which may be chemically and enzymatically degraded to adenine and adenosine. It is therefore suggested that the two adenylic acids are isomers with respect to the location of the phosphoric acid moiety on the ribose chain: adenosine-2-phosphoric acid and adenosine-3-phosphoric acid.

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