at 11 mm.; yield 10.5 g., 62% of theoretical based on acid chloride.

γ-Phenylbutyraldehyde.—The Grignard was formed in ether with 50 g. of γ-phenylpropyl bromide; 37 g. of freshly distilled ethyl orthoformate was added and the mixture refluxed six hours. The ether was removed by distillation and 200 cc. of chilled 6% hydrochloric acid was added with stirring. The oily layer of acetal was separated and decomposed by distillation with 10% sulfuric acid; 25 g. of sodium bicarbonate in 50 ml. of water was added to the steam distillate and the mixture cooled to 5°. The aldehyde was filtered off and washed; yield 8 g. of impure aldehyde; m. p. 46–48° cor.; 2,4-dinitrophenylhydrazone, m. p. sinters 104°, melts 106–107° cor.

Phenylacetic Acid.—Eastman Kodak Co. (practical) crystallized from isopropyl alcohol; m. p. 76–77°.

Diphenylacetic Acid.—Prepared by the method of "Organic Syntheses," Coll. Vol. I, p. 224, using 120 g. of benzilic acid. The wet product was recrystallized from 600 cc. of 60% ethanol, yield 88.5 g., 72%; m. p. 143–144°. The product gave no red color with concd. sulfuric acid, indicating freedom from benzilic acid.

Dibenzyl.—Eastman Kodak Co. (practical) recrystallized from ethanol, m. p. 52° cor.

Phenylacetone.—Eastman Kodak Co. (practical) phenylacetone was converted to the semicarbazone and recrystallized three times from ethanol, m. p. 186–187°. The semicarbazone was hydrolyzed with 10% phosphoric acid and redistilled, b. p. 69–71° (3 mm.).

3-Phenylbutanone-2 (Phenyl Methyl Acetone).—Prepared by the method of Bruzau[7]: 39 g. of phenylmethylacetonitrile was allowed to react with 100 g. of methylmagnesium iodide in 250 ml. of ether. The ether was distilled off and 200 ml. of dry toluene added. The mixture was refluxed for two hours. Recovery of the ketone was as in the reference cited; yield 12.3 g., 28%; b. p. 78.0–78.5 at (1.5 mm.), $n^{25}D$ 1.5088. The semicarbazone was prepared and recrystallized four times; m. p. 156–158° cor. The semicarbazone was decomposed with 5% phosphoric acid, redistilled 106–108° (22 mm.), $n^{25}D$ 1.5085.

3-Methyl-3-phenyl-butanone-2 (Phenyldimethylacetone).—Prepared by the method of Bruzau,[7] 40 g. of phenyldimethylacetonitrile was allowed to react with 181 g. of methylmagnesium iodide (4-fold molal excess) following the same procedure as for 3-phenylbutanone-2; yield 27 g., 61% of theoretical; ketone distilled 76–77° (15 mm.), $n^{25}D$ 1.5078. The semicarbazone (m. p. 184.5–186.5° cor.) was decomposed with 5% phosphoric acid and redistilled, b. p. 72–74° (2 mm.), $n^{25}D$ 1.5074.

2,3-Diphenylbutanone-2.—Prepared by method of Sisido and Nozaki, THIS JOURNAL, **70**, 776 (1948): The semicarbazone was prepared and recrystallized 3 times, final m. p. 177–178° cor., after which it was decomposed by refluxing with 10% phosphoric acid; purified ketone, b. p. 132° (1 mm.), m. p. 39–40°.

Summary

The absorption spectra of some α-phenyl carbonyl compounds have been measured, including phenylacetaldehyde, phenylacetone, phenylmethylacetone (3-phenylbutanone-2), phenyldimethylacetone (3-phenyl-3-methylbutanone-2) *d,l*-methadon hydrochloride, and 3,3-diphenylbutanone-2. β-Phenylpropionaldehyde and γ-phenylbutraldehyde, phenylacetic acid and diphenylacetic acid have also been measured.

All of the α-phenylcarbonyl compounds have a band of unexpectedly high extinction in the 290–310 mμ region. Evidence is presented that neither ring formation nor enolization is responsible but that an interaction between the carbonyl and the phenyl group takes place through the saturated α-carbon atom. An explanation in terms of no-bond resonance has been given for the phenomenon. This behavior indicates that two chromphore groups do not always act independently when separated by a saturated carbon atom and that some resonance interaction takes place through such an atom.

SAN FRANCISCO, CALIFORNIA RECEIVED JANUARY 17, 1949

[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY][1]

Paper Chromatography of Purine and Pyrimidine Derivatives of Yeast Ribonucleic Acid[2]

BY C. E. CARTER

The application of paper chromatography to problems of nucleic acid chemistry was first reported by Vischer and Chargaff[3] who extended this technique to the quantitative analysis of purine and pyrimidine bases of nucleic acids.[4] 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[5] 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.

(1) Operated by Carbide and Carbon Chemicals Corporation under Contract No. W-7405-eng-26 for the Atomic Energy Commission, Oak Ridge, Tennessee.

(2) Since submitting this manuscript for publication two notes have appeared dealing with the detection of purines and pyrimidines by fluorescence on paper chromatograms (R. Markham and J. D. Smith, *Nature*, **163**, 250 (1949); and E. R. Holiday and E. A. Johnson, *ibid.*, **163**, 216 (1949)), and a note on paper chromatography of nucleotides employing an isobutyric acid solvent system (E. Chargaff, B. Magasanik, R. Doniger and E. Vischer, THIS JOURNAL, **71**, 1513 (1949)).

(3) E. Vischer and E. Chargaff, *J. Biol. Chem.*, **168**, 781 (1947).

(4) E. Vischer and E. Chargaff, *ibid.*, **176**, 703 (1948).

(5) R. D. Hotchkiss, *ibid.*, **175**, 315 (1948).

Materials and Methods

The detection of purine and pyrimidine compounds by fluorescence is readily accomplished by use of the Mineralight lamp (Model number V-41) manufactured by The Ultraviolet Products Corporation, Los Angeles, California. The successful application of this ultraviolet source, in contrast to several others which were tried, is probably due to the high intensity of its spectrum in the 240–260 mμ region.

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 *p*H 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 non-aqueous phase. The latter system which is a departure from the usual techniques of paper chromatography 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. non-aqueous 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 ribonucleotides. The butanol system described by Hotchkiss[6] 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 of the spot and resolution is poor. By placing a thin layer of non-aqueous 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 R_f 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 No. 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 micropipet 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[6] 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 was prepared by the ion exchange procedure of Cohn[7] and muscle adenylic acid 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 (less than 2%) of adenylic acid and adenine; in adenylic acid a detectable amount (less than 2%) of adenosine; in adenosine about 3.0% guanosine; in hypoxanthine about 20% adenine; and in uridine a trace of uracil. In all cases it was found that a single recrystallization did not significantly alter the purity of these compounds. Because of the ability to identify the contaminants in the standards employed by their position on the chromatograms and by ultraviolet absorption spectra on elution from the chromatogram, the impurities encountered do not invalidate the procedures described. However, any statement regarding the quantitative recovery of components from the chromatogram is limited by the absence of critically pure standards.

The quantitative aspects of paper chromatographic analysis have been discussed by Chargaff and Vischer[4] 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 the concentration of each constituent calculated from its optical density in the Beckman ultraviolet spectrophotometer, in comparison with a standard solution of the individual pure substances.

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 anion exchange procedure described by Cohn.[7] The purine nucleotides are eluted from the paper chromatogram and determined directly by spectrophotometry.

In Table I the R_f values for the mononucleotides of yeast ribonucleic acid in the dibasic sodium phosphate–isoamyl alcohol solvent system and the per cent. recovery of these components from the chromatogram are shown. These data demonstrate that yeast adenylic acid is resolved into two components. Several samples of commercial yeast adenylic acid have been chromatographed and all were resolved into two components having ultraviolet absorption spectra identical with adenylic acid when the *p*H of the buffered aqueous phase was between 5.2 and 10.0.

(6) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

(7) W. E. Cohn, THIS JOURNAL, **72**, 1471 (1950).

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 $D = 0.862$ in 5 cc. of water at 2600 Å. in the ultraviolet spectrophotometer was quantitatively put on the paper. Recovery of nucleotides based on total 2600 Å. adsorption was 106%: solvent system, 5% dibasic sodium phosphate–isoamyl alcohol; solvent front, 26.5 cm.

Standard[a]	R_f values standards	Recovery of standards, %		R_f values of components of nucleotide mixture obtained by barium hydroxide hydrolysis of yeast RNA	Amt. nucleotides in 0.025 cc. of nucleotide mixture derived from barium hydroxide hydrolysis of yeast RNA μg.
Cytidylic[b]	0.86	101		0.88	24.0
Uridylic	.86	98		.88	63.0
Guanylic[b]	.79	92		.81	22.0
Adenylic-a	.74	34	102	.75	16.6
Adenylic-b	.64	68		.65	24.3

[a] Standard solution contained 40 μg. of each nucleotide in a volume of 0.025 cc. of 1 *N* ammonium hydroxide. [b] Guanylic acid was eluted in 5 cc. of 1 *N* ammonium hydroxide; adenylic acids in 5 cc. of 0.01 *N* hydrochloric acid. Pyrimidine nucleotides eluted in 0.01 *N* hydrochloric acid and individually determined by anion exchange chromatography.

The chromatographic procedures exclude adenosine and adenine as the second component (see Table II), and when the two yeast adenylic acid components were eluted from the chromatogram and hydrolyzed ribose and phosphate in equimolar proportions could be demonstrated in each component. A mixture of the barium-insoluble salts obtained from yeast ribonucleic acid by the barium hydroxide hydrolysis procedure described by Loring, *et al.*,[8] when regenerated as free acids and chromatographed, also exhibited two components with R_f values and absorption spectra identical with the two components of yeast adenylic acid and in approximately the same ratio. The other components of the mononucleotide mixture exhibited R_f values and absorption spectra identical with the corresponding guanylic, uridylic and cytidylic acid standards. These components were eluted from the chromatogram and determined in the ultraviolet spectrophotometer and the composition of the mixture is recorded in Table I. It should be pointed out that these data refer to the barium-insoluble fraction only and do not represent a complete analysis of yeast ribonucleic acid.

TABLE II

SOLVENTS FOR PAPER CHROMATOGRAPHY OF YEAST NUCLEIC ACID DERIVATIVES R_f VALUES

	5% Am. citrate *p*H 3.6 –iso-amyl alcohol	5% Am. citrate *p*H 9.6 –iso-amyl alcohol	5% KH_2PO_4 –iso-amyl alcohol	5% Na_2HPO_4 –iso-amyl alcohol	5% KH_2PO_4 *p*H 7.0 –iso-amyl alcohol	Butanol–urea
Adenine	0.69	0.37	0.53	0.44	0.42	0.41
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.

As shown in Fig. 1, when yeast adenylic acid is resolved on the chromatogram the faster moving component has an R_f value which is the same as that of muscle adenylic acid. It was possible to exclude muscle adenylic acid (adenosine-5-phosphoric acid) as a component of yeast nucleic acid by the enzymatic procedure described by Kalckar[9] based on Schmidt's observation that muscle adenylic acid deaminase would not hydrolyze the amino group of yeast adenylic acid. Thus, as shown in Fig. 1, when muscle adenylic acid was incubated with the enzyme, inosinic acid was the product, whereas incubation under the same conditions with yeast adenylic acid did not alter either component. It was also found that the phosphate group of both yeast adenylic acid components was acid labile in contrast to the phosphate group of muscle adenylic acid in agreement with the data of Levene and Stiller.[10] Therefore, neither component of yeast adenylic acid is adenosine-5-phosphoric acid. If the two components of yeast adenylic acid were isomeric nucleotides differing in the position of the phosphate ester linkage on the ribose carbon chain, then both should be converted to adenosine and adenine on suitable treatment. Figure 1 illustrates the quantitative conversion of both adenylic acid components to adenine by heating with 1 *N* hydrochloric acid, and to adenosine by incubating with a kidney extract nucleotidase. The conversion to adenosine may also be effected by 1 *N* ammonium hydroxide at 100° but under these conditions about 10% adenine is also formed. These data indicate that the two components of yeast adenylic acid differ with respect to the phosphate ester linkage although isomerism involving the glucosidic linkage is not excluded.

Isolation of each of the two yeast adenylic acid components has been effected by fractional crystallization at −10° in 59% alcohol (the faster moving component being obtained in pure form by a single crystallization), and by an anion exchange

(8) H. E. Loring, P. M. Roll and J. C. Pierce, *J. Biol. Chem.*, **174**, 729 (1948).

(9) H. M. Kalckar, *ibid.*, **167**, 461 (1947).

(10) P. A. Levene and E. T. Stiller, *ibid.*, **104**, 299 (1934).

procedure.[7] (A detailed description of the physical chemical properties of the isomeric yeast adenylic acids and the methods of preparation will be presented in another publication.) It has been possible to take one of the components isolated by anion exchange procedures or fractional crystallization, run it on the chromatogram and demonstrate only one component, thus eliminating artefacts which might arise through chromatographic procedures as cause for the production of two components in the system.

Nucleosides

In Table II the R_f values for the purine and pyrimidine derivatives of yeast nucleic acid are recorded for several different solvent systems. As shown in this table, resolution of nucleosides can be 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 a one-dimensional chromatogram. For analysis of mixtures of the various 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 spots of adenosine and guanosine and to 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 citrate *p*H 3.6).

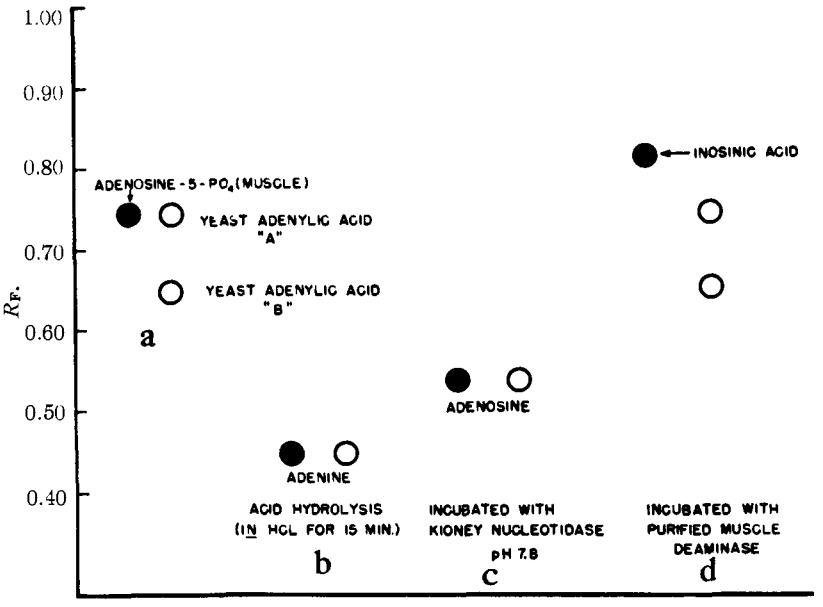

Fig. 1.—The enzymatic and chemical degradation of yeast adenylic acids. Solvent system: 5% dibasic sodium phosphate–isoamyl alcohol. Adenylic acids were employed in 0.5% solution. Aliquots for chromatography were 0.025 ml. The experiments illustrated, reading from left to right are: (a) standard samples of muscle adenylic (●) and yeast adenylic acids (○), (b) acid hydrolysis (1 *N* hydrochloric acid, twenty minutes at 100°) of muscle and yeast adenylic acid which effected a conversion (93–97% recovery on elution) of the adenylic acids to adenine in each case and liberated 93% of yeast adenylic acid phosphate and 12% of muscle adenylic acid phosphate as inorganic phosphate, (c) the enzymatic degradation of muscle and yeast adenylic acids to adenosine (90–92% recovery) by a purified kidney phosphatase[a] in digests which consisted 0.2 cc of a 0.5% solution of adenylic acid adjusted to *p*H 7.8 in 0.1 *M* ammonium citrate and 0.2 cc. of enzyme suspension incubated for 12 hours; (d) the selective enzymatic deamination of muscle adenylic acid to inosinic acid (98% recovery) by a purified muscle deaminase[7] in digests which consisted of 1 cc. of 0.5% muscle and yeast adenylic acids in solutions adjusted to *p*H 5.9 with dilute alkali and 0.2 cc. of enzyme solution incubated for five hours. Results illustrated were derived from three experiments in each group.

[a] A. Kornberg and O. Lindberg, *J. Biol. Chem.*, 176, 665 (1948).

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, μg.	Per cent. recovery[a] on elution with 1 *N* NH_4OH
Uridine	50	95.5
Cytidine	50	104.5
Adenosine	50	95.7
Guanosine	50	89.5

[a] 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 $D \frac{2800 \text{ Å.}}{2600 \text{ Å.}}$ in 1 *N* NH_4OH: Uridine = 0.40; cytidine = 0.89. Ratio $D \frac{2800 \text{ Å.}}{2600 \text{ Å.}}$ in unknown = 0.652. (0.89 − 0.652)/(0.89 − 0.40) = 0.49. Total 2600 Å. absorption of unknown, D = 0.680 × 0.49 = 0.335. Standard solution, 50 μg. uridine in 5 cc. 1 *N* NH_4OH, D = 0.350. Recovery = 95.5% uridine, by difference; 104.5% cytidine. [b] Purine nucleosides calculated directly from 2600 Å. absorption compared with standard solutions.

Purines and Pyrimidines.—These compounds have been thoroughly studied by Chargaff and Vischer[4] and Hotchkiss[5] 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 was 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

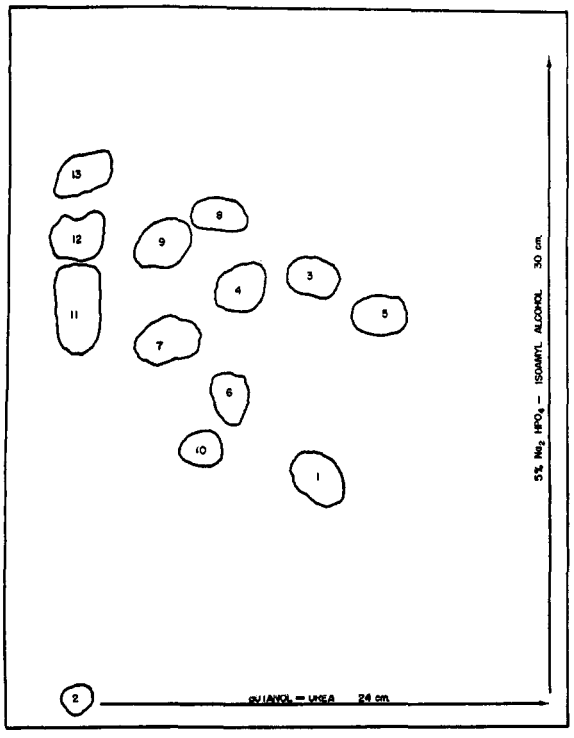

Fig. 2.—Two-dimensional chromatogram of yeast nucleic acid derivatives, R_F values in parentheses; twenty micrograms of each component were put on the starting spot: 1, adenine (butanol-urea −0.50, phosphate −0.38); 2, guanine (0, 0); 3, uracil (0.50, 0.71); 4, cytosine (0.35, 0.69); 5, thymine (not a component of yeast nucleic acid) (0.64, 0.64); 6, adenosine (0.31, 0.52); 7, guanosine (0.20, 0.58); 8, uridine (0.31, 0.83); 9, cytidine (0.20, 0.75); 10, xanthine (found as impurity) (0.27, 0.43); 11, adenylic acid *a* + *b* (0, 0.58, 0.70); 12, guanylic (0, 0.75); 13, cytidylic + uridylic (0, 0.87). The solvent front in the second dimension is irregular and advanced about 5 cm. in the region overlying the nucleotides.

adenylic acid "a," adenylic acid "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.

Recovery of components from the two dimensional chromatogram by elution in suitable solvent was between 82 and 115%.

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. 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 moiety 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, 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 diaminase (Fig. 1). It is therefore suggested that the two components are adenosine-2-phosphoric acid and adenosine-3-phosphoric acid, although an α,β-isomerism of the glucosidic linkage cannot be excluded.

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.[7] Preliminary experiments in the analysis of tissues indicate 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 differ with respect to the location of the phosphoric acid moiety on the ribose chain and are adenosine-2-phosphoric acid and adenosine-3-phosphoric acid, respectively.

RECEIVED MARCH 3, 1949

[CONTRIBUTION FROM THE BIOLOGY DIVISION OF OAK RIDGE NATIONAL LABORATORY[1]]

The Anion-Exchange Separation of Ribonucleotides

BY WALDO E. COHN

Investigations of nucleic acid chemistry, constitution and metabolism, particularly where tracers are employed, depend to a great degree upon the development of methods for the quantitative separation of the various chemical and enzymatic degradation products of the larger molecules. For greatest usefulness, these methods should be operable at the lowest detectable levels of concentration and amount of material and they should give adequate resolution—complete, if possible—of closely related substances. The classical methods[1a] are based principally upon precipitation which, because of solubility limitations and coprecipitation, do not, in general, meet such quantitative demands as well as do those methods based upon solvent extraction or ion-exchange, which have found such utility in the isolation of carrier-free radioisotopes.[2]

Previous work in the development of ion-exchange procedures for the quantitative separation of fission product ions, including the rare earth elements, from their mixtures at trace concentrations[3,4,5] led to the choice of ion-exchange as a likely mechanism to exploit in order to develop better separation methods for the nucleotides, nucleosides and free bases. The fact that nucleotides and their degradation products contain both acid and basic groups offered the possibility of using both anion and cation exchangers while the differences in pK from compound to compound[1a] indicated that these might prove as conclusive in effecting organic separations as are the complex dissociation constants and base strengths in the rare earth separations.

The experiments and results described below (and described in preliminary form earlier)[6,7] indicate that ion-exchange is a separation tool of the required accuracy and sensitivity and, as with any quantitative and sensitive separation method, it can serve also as a means of analysis, investigation and production.

Preliminary Considerations.—To effect an ion-exchange separation of the members of a family of ions, it is necessary to find a set of conditions under which each exhibits a different degree of affinity (distribution coefficient) with respect to the exchanger. While this affinity is governed by a number of variables,[8,9] it is reasonable to assume that, in a group as closely related as the monoribonucleotides, the net charge per ion will be the most important one. Since these substances possess both acid (phosphate) and basic (amino) groups, the pH of the medium determines the net charge by determining the degree of dissociation of these groups. Hence, a preliminary calculation was made of the degrees of dissociation of these groups as a function of pH, using the pK values quoted by Levene[1a]; these calculations are presented graphically in Fig. 1.

It is immediately apparent from Fig. 1 that (1) the order of increasing net negative charge per molecule is cytidylic, adenylic, guanylic and uridylic acid; (2) cytidylic and adenylic exhibit a net positive charge, and hence are cations, at pH values below about 2.5 and guanylic below 1.5, while uridylic remains anionic down to pH 0, which facts are consistent with the cation-exchange sep-

(1) Operated by Carbide and Carbon Chemicals Corporation under Contract No. W-7405-Eng-26 for the Atomic Energy Commission, Oak Ridge, Tennessee.

(1a) P. A. Levene and L. W. Bass, "Nucleic Acids," Chemical Catalog Co., Inc., New York, N. Y., 1931.

(2) W. E. Cohn, in "Advances in Biological and Medical Physics," Vol. I, Academic Press, New York, N. Y., 1949; also, *Naval Med. Bull. Suppl.*, Mar.-April, 1948, p. 42.

(3) E. R. Tompkins, J. X. Khym and W. E. Cohn, THIS JOURNAL, **69**, 2769 (1947).

(4) W. E. Cohn, G. W. Parker and E. R. Tompkins, *Nucleonics*, **3**, No. 5, 22 (1948).

(5) W. E. Cohn and H. W. Kohn, THIS JOURNAL, **70**, 1986 (1948).

(6) W. E. Cohn, *Science*, **109**, 377 (1949).

(7) W. E. Cohn, THIS JOURNAL, **71**, 2275 (1949).

(8) E. R. Tompkins, *J. Chem. Educ.*, **26**, 32, 92 (1949).

(9) R. Kunin, *Anal. Chem.*, **21**, 87 (1949).