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).

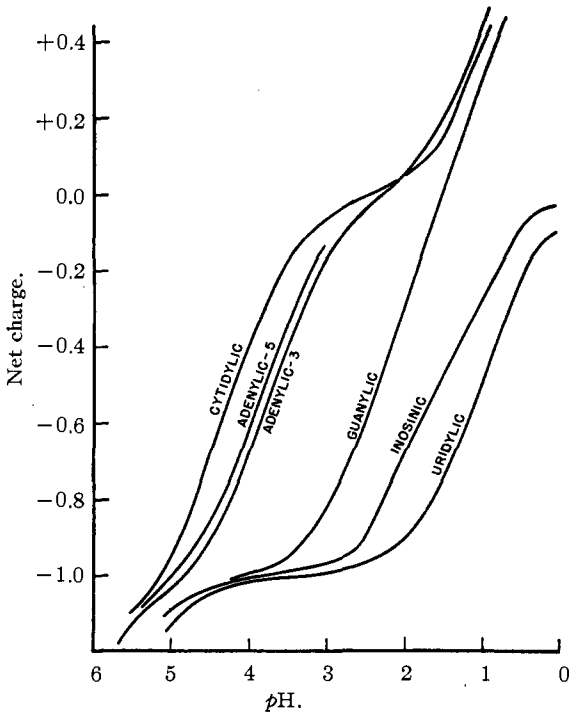


Fig. 1.—Net charge per molecule of ribonucleotide as a function of *p*H (calculated from data of Levene).

arations reported previously[6]; (3) at *p*H values above 5, all are strongly negative. From these observations, it would seem simplest to adsorb the mixture at *p*H values of 6 or greater, and to remove the nucleotides in the order cytidylic, adenylic, guanylic and uridylic acid by lowering the *p*H in a stepwise fashion, increasing the anion concentration with salt near the end if necessary to avoid strongly acid conditions which might hydrolyze the compounds. This was the plan of the experiments reported below.

Experimental

Apparatus.—The type of column is described by Tompkins.[3,8] An automatic sample changer was used to change receivers at fixed time intervals, thus permitting extended runs to be made conveniently. A Beckmann ultraviolet spectrophotometer was employed to assay and identify the samples, taking advantage of the characteristic ultraviolet spectra of the several substances being investigated. The dependency of the shape of these spectra upon *p*H offers a further method of identification. Paper chromatography also served as a means of identification.[10,11]

Ion-exchangers:—The experiments utilized principally 200–400 mesh Dowex-1,[12] a strong-base anion exchanger; Dowex 2[12] and Amberlite IRA-400[13] were also employed with similar results. The exchangers were washed free of fines by decantation, slurried into the columns and washed with acid and base before being converted to the salt form desired for a particular experiment. Columns were used repeatedly without change of resin, often without backwash or resuspension, until accident or change in type of experiment warranted discarding them. One has been used in thirty separations without noticeable loss in combining capacity or efficiency.

Test Substances.—As a source of mixed monoribonucleotides, barium hydroxide hydrolysates of yeast nucleic acid were used.[10,14] Commercial adenylic, guanylic and cytidylic acids were employed without purification as the impurities lent themselves to the investigations in hand. Solutions of uridylic acid were prepared by cation-exchange methods,[6] identified by spectral properties[15] and standardized by phosphorus analysis.[11]

Procedure.—The columns were prepared for use by washing with solutions of stronger eluting power than any which were to be used in the experiment (*e. g.*, 1 *N* hydrochloric acid if replacement by Cl^- was contemplated) until influent and effluent were identical in *p*H and optical density at 240–280 mμ. Following a water wash, the test substance, in amount usually less than 5% of the exchanger capacity, was adsorbed from a solution of low eluting power (*e. g.*, *p*H 8, anion concentration $\leq 0.01M$). The column was again washed with water and elution with the desired reagent was commenced. Zero volume was taken as the point when the effluent attained the same *p*H and ionic strength as the influent solution.

Results

Although many conditions, varying from *p*H 1.8 to 5.6 with various anions and varying anion concentrations, were studied, it is necessary to present only a few typical experiments at the various extremes before summarizing the data obtained. A rapid partial separation, utilizing a stepwise *p*H procedure as outlined above, is shown in Fig. 2. The spectrophotometric ratios (*i. e.*, ratios of optical densities at two wave lengths) of the fractions collected indicate a tendency for guanylic acid to follow uridylic acid, the reverse of the predicted order. The degree of sep-

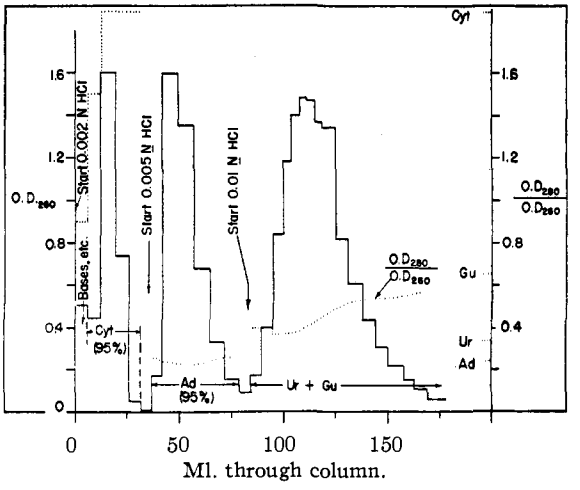


Fig. 2.—Rapid separation of nucleotides: exchanger, Dowex-1, 0.9 sq. cm. × 2 cm.; solution, hydrochloric acid at 0.8 ml./min.; test material, ~4 mg. of mixed nucleotides.

(10) We are indebted to Dr. C. E. Carter for chromatographic and other analyses[11] and also for the nucleic acid hydrolysates and other preparations used in these experiments.

(11) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(12) Obtained through the courtesy of Dr. L. A. Matheson, Dow Chemical Co., Midland, Michigan.

(13) Resinous Products and Chemicals Co., Washington Square, Philadelphia, Pa.

(14) H. S. Loring, P. M. Roll and J. G. Pierce, *J. Biol. Chem.*, **174**, 729 (1948).

(15) J. M. Ploeser and H. S. Loring, *ibid.*, **178**, 431 (1949).

aration of cytidylic and adenylic acids is apparent. Free bases (adenine, guanine, uracil, cytosine) or ribosides exhibit practically no affinity for anion-exchangers at *p*H values below 8 and hence are removed, if adsorbed at all, ahead of cytidylic acid.[6]

As predicted, separations at *p*H values above 4 are less satisfactory and it is necessary to use longer columns in order to achieve even a separation into pairs of nucleotides, as shown in Figs. 3 and 4. In addition, the affinity of the nucleotides for the exchanger here is so much greater than at *p*H 2-3 that the anion concentration must be increased considerably to achieve comparable elution rates, thus hindering readsorption for purposes of concentration (see below). Nevertheless, such a procedure is satisfactory for simple analysis as the spectrophotometric ratios are sufficiently different to permit calculation of the composition of each of the two fractions (pyrimidine nucleotides and purine nucleotides). In addition, satisfactory resolution of the isomers of adenylic and of inosinic acids can be obtained under these same conditions; this, as well as the proof that two kinds of adenylic acid exist in hydrolysates of yeast nucleic acid, is discussed elsewhere.[11,16]

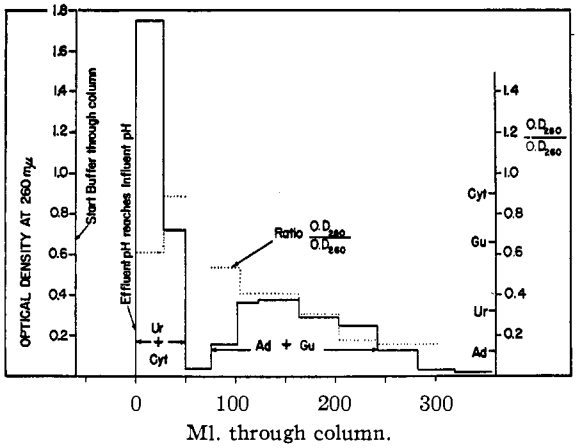


Fig. 3.—Rapid separation of mononucleotides: exchanger, Dowex-1 (Cl^-), 0.74 sq. cm. × 12 cm.; solution, 0.1 *M* sodium chloride in 0.01 *M* acetate buffer, *p*H 5.6, 0.7 ml./min.; test material, ~4 mg. of purified nucleotides.

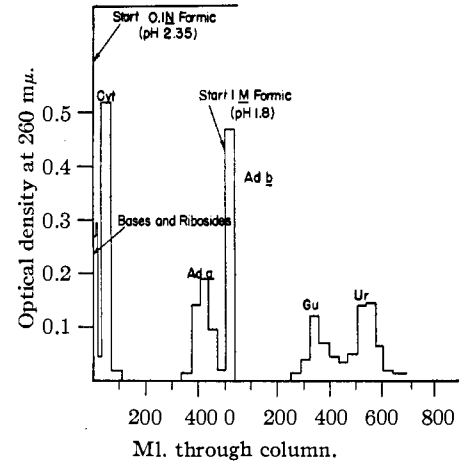


Fig. 5.—Separation of nucleotides: exchanger, Dowex-1 (formate), 0.74 sq. cm. × 13 cm.; solution, formic acid, 0.7 ml./min.; test material, 3 mg. of mixed nucleotides from barium hydroxide hydrolyzate of yeast nucleic acid.

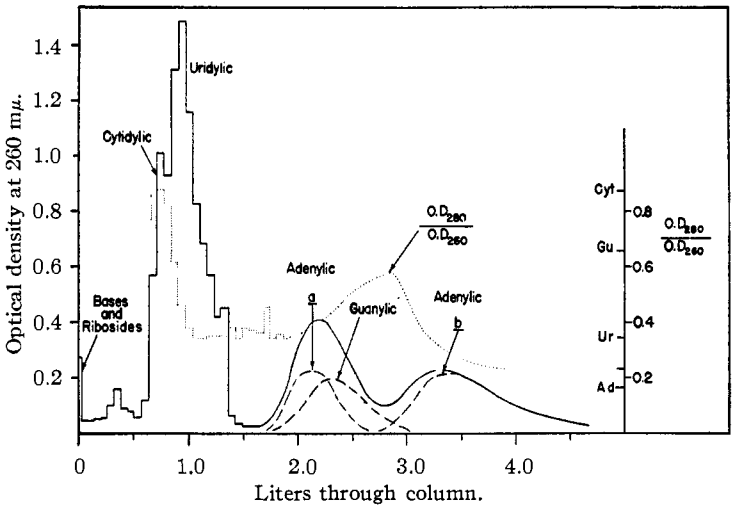


Fig. 4.—Separation of mixed monoribonucleotides: exchanger, Dowex-1 (Cl^-), 10 cm. × 0.94 sq. cm.; solution, 0.02 *M* sodium chloride in 0.01 *M* acetate buffer, *p*H 5.6, 1.4 ml./min.; test material, ~40 mg. of mixed nucleotides from barium hydroxide hydrolyzate of yeast nucleic acid.

Longer columns and higher distribution coefficients give better separations, as demonstrated in Fig. 5. Utilizing formic acid in place of hydrochloric (giving better *p*H control, more available anion and a lower specific replacement power than Cl^- on Dowex-1),[17] adenylic acid is completely separated from the other nucleotides and divided into two parts. (This experiment is the first in which practically complete resolution by ion-exchange of the two adenylic acid isomers derived from yeast nucleic acid was obtained; this separation was verified by paper chromatography and by many subsequent separations and tests.[11,16] It should also be noted that uridylic now follows guanylic, as originally predicted, contrary to the calculations of Fig. 2 (and of Fig. 6, below)).

The best separations of the nucleotides of ribonucleic acid with hydrochloric acid have been obtained at *p*H values

(16) C. E. Carter and W. E. Cohn, to be published; *Federation Proc.*, **8**, 190 (1949).

(17) L. A. Matheson, personal communication.

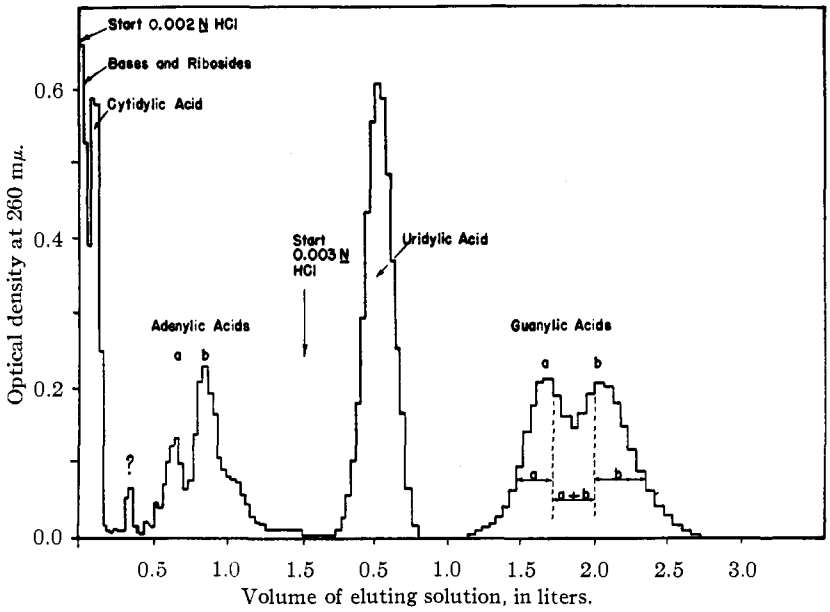


Fig. 6.—Separation of mononucleotides of rat liver ribonucleic acid by anion-exchange: exchanger, Dowex-1, 12.5 cm. × 0.74 sq. cm.; eluent, dilute hydrochloric acid, 0.5 ml./min.; test material, ~14 mg. of mixed nucleotides from barium hydroxide hydrolysis of rat liver nucleic acid in 10 ml. of water.

in the neighborhood of 2.7. A separation of a mixture of nucleotides obtained from a barium hydroxide hydrolysate of ribonucleic acid from rat liver at *p*H 2.7 is shown in Fig. 6. The two adenylic acid peaks are clearly shown while uridylic now precedes guanylic. Of particular interest is the observation of two peaks in the latter. The eluate was therefore divided into the three fractions (a, a + b, b) shown in Fig. 6. When these three fractions were chromatographed,[10,11] the samples a and b migrated to two slightly different positions, while sample a + b occupied both positions. All three fractions were degraded to guanine by acid,[10] which is taken as evidence that both peaks are due to guanylic acid. More evidence is necessary in order to establish the possible existence of two guanylic acids, analogous to the two adenylic acids.[11,16] The nature of the small peak between cytidylic and adenylic acid is not known; in amount, it was too small to be analyzed.

The removal of free base and ribosides ahead of (*i. e.*, at higher *p*H values than) cytidylic acid has been mentioned above in connection with Figs. 2, 4, 5 and 6. In Fig. 7, a separation of a mixture of several free bases and nucleosides from adenylic acid, representing the nucleotides, is shown to prove this point. In this experiment, the concentration of anion (formate) was kept constant throughout, the *p*H being shifted by changing the proportions of ammonium hydroxide and formic acid in the solution. Satisfactory resolution of the pairs shown can be attained by more careful adjustment of conditions; this has already been demonstrated for the five bases[6] and for cytidine and uridine.[18,19] (The probable positions of the latter two substances, obtained from experiments, conducted by Mr. J. X. Khym in this Laboratory, are shown in brackets in Fig. 7,

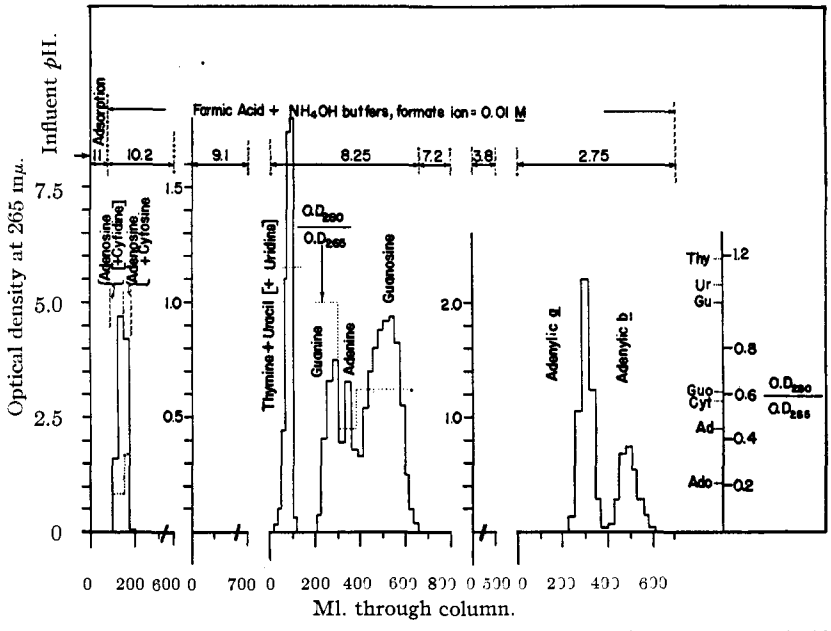


Fig. 7.—Separation of bases, ribosides and adenylic acid: exchanger, Dowex-1, 13 cm. × 0.74 sq. cm.; rate, 0.5 ml./min.

although they were not included in this particular run.) Thus, it is possible to separate most of the free bases, nucleosides and nucleotides of ribonucleic acid from one another in one column run; this procedure can, therefore, be used for the quantitative analysis of acid hydrolysates of nu-

(18) D. T. Elmore, *Nature*, **161**, 931 (1948).
(19) R. J. C. Harris and J. F. Thomas, *ibid.*, **161**, 931 (1948).

cleic acid, which yield adenine, guanine, cytidylic and uridylic acid,[20] in place of the laborious and less accurate precipitation methods, as well as for other types of hydrolysates.

Attempts to obtain complete separations in *p*H regions above 3 have not been successful as certain peaks are too close together. A complete summary of the data, covering the entire range from *p*H 1.8 to 5.6, is given by the points in Fig. 8. The points and the broken lines connecting them denote the positions of the several peaks, these positions being proportional to the distribution coefficients,[21] with respect to that of adenylic acid *a*. When used in conjunction with the absolute data on adenylic acid *a* given in Table I, the curves in Fig. 8 make it possible to estimate the

TABLE I

ML. TO PEAK OF ADENOSINE; PHOSPHATE (ADENYLIC ACID *a*) FOR DOWEX-1 COLUMN, APPROXIMATELY 0.74 SQ. CM. × 13.5 CM. (10 ML.)

Solution	Concentration (*M*)	*p*H	Ml. to peak
Formic acid	1	1.8	25
	0.25	2.2	100
	.1	2.4	340
HCl	.005	2.3	70
	.004	2.4	110
	.002	2.7	600
	.0016	2.8	900
0.01 *M* acetate buffer	.01	3.6	800
plus NaCl	.01	4.6	1800
	.02	4.6	800
	.02	5.6	2100
	.10	5.6	150

positions of the peaks of all ribonucleotides for a given column. The solid curves in Fig. 8 express the calculated net charge of the same nucleotides, again relative to adenylic acid, using the data summarized in Fig. 1; the curves for cytidylic and uridylic acid have, however, been normalized by the arbitrary factor 0.37, the significance of which remains to be determined. While use of this factor gives a good coincidence of experimental and calculated values for uridylic acid, the fit for cytidylic is less satisfactory. This may be due to the fact that cytidylic acid is always eluted first, at low volumes, with a corresponding large uncertainty in zero point (*p*H equilibrium) and hence position of peak.

The data in Fig. 8 indicate clearly the reversal of the relative positions of uridylic and guanylic acid which takes place at *p*H 2.0. Indeed, it is apparent that uridylic can be eluted simultaneously with guanylic acid at *p*H 2, with adenylic acids at *p*H 3.3–3.5, or with cytidylic acid at *p*H values above 4. Also apparent is the fact that the optimum separation of all nucleotides at a single *p*H occurs at about 2.9 (dotted line).

(20) G. Schmidt, R. Cubiles and S. J. Thannhauser, *Cold. Spr. Hbr. Sym. XII*, 161 (1947).

(21) E. R. Tompkins and S. W. Mayer, THIS JOURNAL, **69**, 2859 (1947).

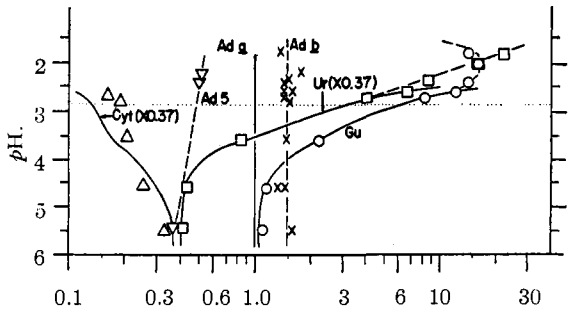


Fig. 8.—Observed relative distribution coefficients (points, broken lines) and calculated relative negative charges (solid lines) of nucleotides as functions of *p*H.

Although the data presented on separations in the region of *p*H 2.9 (*e. g.*, Figs. 6 and 7) indicate that the process may be a slow one and, therefore, somewhat impractical, it should be pointed out that the volume (and hence time) required to remove a given substance from the column at a fixed *p*H is dependent upon the anion concentration of the solution. While this point has not been thoroughly investigated, it appears, as might be expected, that the absolute peak distances (distribution coefficients) are approximately proportional to the (monovalent) anion concentration in the *p*H range of 2 to 5 where the nucleotides are monovalent anions. Therefore, the time of separation of a mixture into its component nucleotides may be reduced several-fold by increasing the anion concentration after the elution of each substance. Thus, each can be obtained in a small volume, comparable to those of cytidylic and uridylic acids in Fig. 6. Alternatively, as has been shown previously[7] and also in Figs. 2, 5 and 6, the *p*H can be changed at appropriate intervals.

The use of formic (or acetic) acids for elution in the *p*H 2–3 region has three advantages over dilute hydrochloric acid: (1) *p*H regulation is more exact; (2) the neutralization of the nucleotides, usually absorbed as divalent anions, is more rapidly achieved, eliminating large dead volumes; (3) simple evaporation may be used to recover the products. However, dilute hydrochloric acid has one very marked advantage in that subsequent concentration by ion-exchange, which requires the conversion of the effluent to a solution of *p*H 8–10 with low (< 0.01 *M*) anion concentration, is possible by the simple addition of a small amount of ammonium hydroxide. Such a solution can readily be stripped of its nucleotide content on 10-fold smaller columns, from which the product can be recovered in concentrated (1–10 mg./ml.) solution by elution with 0.02–0.1 *M* hydrochloric acid. In practice, it has been possible practically to saturate a 2 ml. column with adenylic acid (about 150 mg.) from 2 liters of 0.01 *M* Cl^- solution at *p*H 9; subsequent elution, with 0.1 *M* hydrochloric acid, yields a solution of 5–10 mg./ml., from which the adenylic acid crystallizes on standing. This

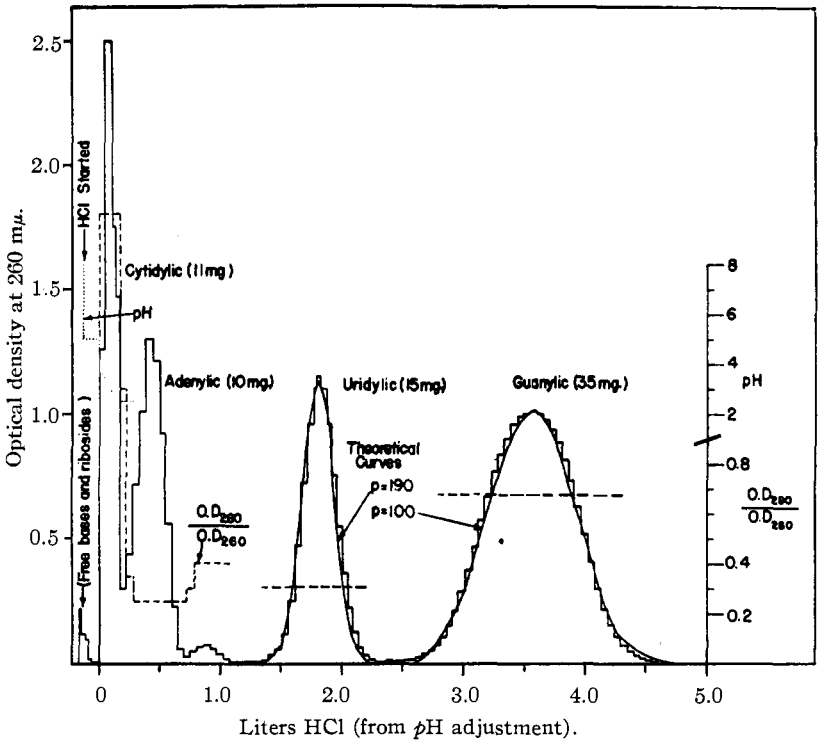


Fig. 9.—Separation of mixed ribonucleotides: exchanger, Dowex-2 (Cl^-), 0.94 sq. cm. × 12.5 cm.; solution, 0.003 *M* hydrochloric acid, 0.8 ml./min.

recycling procedure has been used to prepare gram amounts of the two adenylic acid isomers, in crystalline form, no other operations being necessary. Coupled with a recording flow spectrophotometer and a recording *p*H meter, this procedure, utilizing a 33 sq. cm. column, has afforded a simple method for isolating pure nucleotides on a gram scale; this will be described in detail elsewhere.

No attempt has been made to determine optimum flow rates. Rates up to 1 ml./sq. cm. minute yield elution curves that are apparently symmetrical and practically identical with the normal curve of error shape predicted by the equilibrium theory of Mayer and Tompkins[22] while rates of 2 and 4 do not (*cf.* Fig. 6 *versus* the curve given in[7]). It would seem, then, that flow rates of about 1 ml./sq. cm. min. (at room temperature) permit the attainment of equilibrium between the resin beds employed and the nucleotides.

Discussion

Although these experiments were undertaken for the purposes stated in the introduction, the results reported here and previously[6] have perhaps much greater significance for such basic problems of nucleic acid chemistry as the quantitative assay of known constituents, the detection and isolation of unknown constituents and the manufacture of both. Of considerable importance in any of these applications is the fact that the shape of the elution curve, within the range of conditions investigated, is that of the normal curve of error, as predicted by the equilibrium theory of Mayer and Tompkins.[22] It is, therefore, possible to utilize the mathematical relationships of the normal curve of error to predict quantitatively the conditions necessary for a given degree of separation of two substances (length of column, the ratio of the distribution coefficients), the recoveries at given volumes of effluent solution, the concentration at the maximum relative to the distribution coefficient (position of the peak), etc., as has already been demonstrated in the rare earth separations.[22] (A full exposition of these applications is given by Tompkins.[8])

The approach of elution curves for the nucleotides to the theoretical is demonstrated in Fig. 9, in which theoretical curves are superimposed upon the elution curves of uridylic and guanylic acids obtained in a single run at constant *p*H. The theoretical curves were calculated from the formula[22]

$$t = \sqrt{p}\,(F/C - 1) \qquad (1)$$

where F and C are the effluent volumes to a given point and to the maximum of the elution curve, respectively, while the constant p (number of plates) is obtained from a simple modification of the Mayer–Tompkins formula[22]

$p = 2\pi\,(C_p L_{max.})^2$, namely

$$p = 2\pi \left(\frac{\text{concentration at peak}}{\text{total solute}} \times \text{ml. to peak}\right)^2 \qquad (2)$$

Since the area under the normal curve of error (amount eluted) corresponding to a given value of t (ordinate of the curve of error) is obtainable from probability tables, only three values are necessary in order to solve, say, for total solute present: concentration at the peak, volume to peak, and p. Although p may vary, on a given column, from compound to compound (*cf.* Fig. 9) and from one eluting condition to another, both it and the volume to the peak are constant for a given set of conditions and a given compound and can be determined from a standardization experiment. Therefore, to solve for the total solute present in an unknown sample, as in a quantitative analysis, it is necessary to determine only the

(22) S. W. Mayer and E. R. Tompkins, THIS JOURNAL, **69**, 2866 (1947).

concentration at the peak or to calculate this value, using (1) and the probability tables, from the concentration after a known volume (F) of effluent has passed through the column. It is also possible, once p is known, to predict the fraction eluted when the effluent has arrived at a given fraction of the peak concentration. Thus, when the elution curve has dropped to 50% of its maximum, 88% of the solute has been removed; for a 95% recovery, it is only necessary to procee l to 25% of the peak concentration; etc. Since these relationships hold on the ascending portions of the curves as well, their application to determine the degree of overlap of two substances in a separation procedure is obvious.[8,22]

These useful applications, as well as the use of symmetry as a criterion of a single component, are possible only with elution procedures which result in theoretical curves. Since these cannot be obtained when conditions vary during the course of a separation, it is advisable, in quantitative work or when searching for new substances, to keep flow rate, pH, etc., constant throughout to avoid the asymmetry which results from variations in such conditions.

Of considerable interest, from a theoretical standpoint, is the constant divergence of the observed relative distribution coefficients of the pyrimidine nucleotides from the values predicted on the basis of the ionization constants of the phosphate and amino groups. The data (presented in Fig. 8) indicate that the pyrimidine nucleotides are three times less strongly adsorbed, per unit charge on the molecule, than the purine nucleotides or, conversely, that the latter are three times more strongly adsorbed than would be judged from net charge considerations alone. It would seem, in view of the similarities in molecular weight, constitution, valencies and concentrations of these ions, that degree of ionization would be the sole determining factor.

In searching for an explanation of the observed effect, two family differences between pyrimidine and purine nucleotides come to mind: the much greater solubility of the former[1a] and the presence of the extra imidazole ring in the latter. Evidence favoring the former over the latter as the cause comes from a few (as yet unpublished) observations on inosinic acid, which possesses the high solubility in water characteristic of the pyrimidine nucleotides but also the imidazole ring of the purine nucleotides. The inosinic acids[10] corresponding to the 5, a and b adenylic acids are eluted in that order[16] ahead of the corresponding adenylic acids at pH 5.6 but follow them, again in order, at pH 1.8; at the latter pH, they are scarcely separable from guanylic and uridylic acids. This behavior relates them to uridylic acid (which, like them, possesses no amino group). The factor necessary to normalize the theoretical curve for inosinic acid (based upon dissociation constants only as shown in Fig. 1) to fit the few points determined by elution seems to be about 0.5, which is not too far—considering the number and accuracy of the determinations—from the 0.37 factor noted for the pyrimidine nucleotides.

It should be recalled that the nucleotides were adsorbed from solutions of alkaline pH, where all are very soluble, in the topmost layers of the exchanger bed (as proved by the concentration data mentioned above). The resins employed have apparent combining capacities of about 2–3 meq. per gram of exchanger; thus, the concentration of nucleotide in the exchanger may be as high as one millimole per gram. The elutions were then carried out at lower pH values, between 5.6 and 1.8, and, therefore, amount to a process of dissociating a precipitated acid in a solution in which it is more (pyrimidine nucleotides) or less (purine nucleotides) soluble.

Indeed, it has been found possible, at very low pH and high chloride concentration (*e. g.*, 0.1 N hydrochloric acid), to elute adenylic acid from the exchanger at such a rate that it forms a supersaturated solution, from which it crystallizes in the receiver or, if the flow rate be slow enough, in the exchanger bed itself. In the latter case the crystals may easily be seen dispersed throughout the bed. This indicates that the concentration of nucleotide in the resin bed can exceed greatly its solubility in the aqueous medium, a necessary condition in the hypothesis that solubility is responsible for the differences in distribution coefficient noted.

These methods are being applied in the manner indicated to the analysis of the products of the enzymatic digestion of nucleic acid, to the production of uridylic acid and of the various nucleotide isomers discussed, and to the quantitative assay of the nucleotide content of nucleic acids from various sources. It is intended to apply them in similar fashion to the isolation of desoxyribonucleotides and other nucleotide material of interest in our investigations, including polynucleotides.

Acknowledgment.—It is a pleasure to acknowledge the continued advice and assistance of Dr. C. E. Carter in the course of these experiments, in particular for the chromatographic analyses and nucleic acid hydrolysates.

Summary

The nucleotides of ribonucleic acid have been separated from each other and from the corresponding free bases and ribosides by successive elution from strong base anion exchangers with weak acids and buffers of controlled pH and anion concentration. The order of elution at pH 2 is cytidylic, adenylic, guanylic and uridylic acid; at pH 2.5–3, uridylic precedes guanylic acid; at pH 5.5, uridylic and cytidylic are removed together as are adenylic and guanylic acid. In all cases, the bases and ribosides precede cytidylic acid

and may be separated from each other as well as from the nucleotides during the procedure. The three isomers of adenylic and of inosinic acids may be separated.

The applications of this technique to analysis, isolation, investigation and manufacture are indicated.

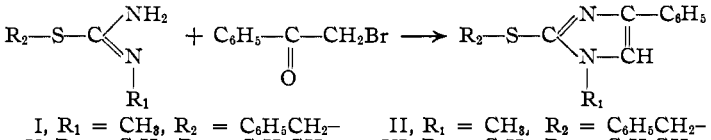
RECEIVED JULY 5, 1949

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA]

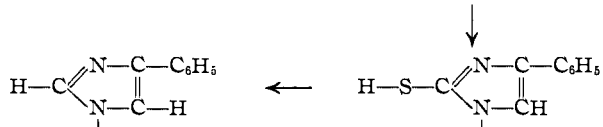
The Preparation of 2-Alkylthioimidazoles

BY R. M. DODSON AND FRANK ROSS[1]

It has recently been shown that 2-benzylthio-4(5)-phenylimidazole could be prepared by the reaction of S-benzylisothiourea with phenacyl bromide.[2] This reaction has now been extended to the preparation of 1-substituted-2-alkylthio-4-phenylimidazoles by the condensation of N-substituted-S-alkylisothioureas with phenacyl bromide. Thus, 1-methyl-2-benzylthio-4-phenylimidazole (II) was prepared in low yield from N-methyl-S-benzylisothiourea (I). Although two isomeric compounds, 1-methyl-2-benzylthio-4-phenylimidazole (II) and 1-methyl-2-benzylthio-5-phenylimidazole, could be expected from the reaction, only compound II was found. The structure of compound II was established by its cleavage to 1-methyl-2-thiol-4-phenylimidazole (III) with phosphorus and iodine in glacial acetic acid, followed by oxidation of III to the known 1-methyl-4-phenylimidazole (IV).[3]



I, $R_1 = CH_3$, $R_2 = C_6H_5CH_2-$ II, $R_1 = CH_3$, $R_2 = C_6H_5CH_2-$
V, $R_1 = C_6H_5$, $R_2 = C_6H_5CH_2-$ VI, $R_1 = C_6H_5$, $R_2 = C_6H_5CH_2-$
VII, $R_1 = C_6H_5$, $R_2 = CH_3-$ VIII, $R_1 = C_6H_5$, $R_2 = CH_3-$



IV, $R_1 = CH_3-$ III, $R_1 = CH_3-$
X, $R_1 = C_6H_5-$ IX, $R_1 = C_6H_5-$

1-Methyl-2-benzylthio-4-phenylimidazole (II) was independently synthesized by the direct methylation of 2-benzylthio-4(5)-phenylimidazole[2] with methyl sulfate and alkali. The odor of mercaptan indicated that some cleavage of the sulfide was taking place. Orientation in the methylation of 2-benzylthio-4(5)-phenylimidazole was similar to that in the methylation of 4(5)-phenylimidazole. Pyman and co-workers[3] found that 4(5)-phenylimidazole, on treatment with methyl sulfate yields 1-methyl-4-phenylimidazole and 1-methyl-5-phenylimidazole in the ratio of 4.8 to 1. We did not succeed in isolating any 1-methyl-2-benzylthio-5-phenylimidazole from our methylation.

By analogous reactions, N-phenyl-S-benzylisothiourea (V) and N-phenyl-S-methylisothiourea (VII) were condensed with phenacyl bromide to give 1,4-diphenyl-2-benzylthioimidazole (VI) and 1,4-diphenyl-2-methylthioimidazole (VIII), respectively, in yields of 51 and 40%. None of the 1,5-diphenyl-2-alkylthioimidazole was found in either of these preparations. Compounds VI and VIII were cleaved to the same 1,4-diphenyl-2-thiolimidazole (IX) with phosphorus and iodine in glacial acetic acid,[4] and the 2-thiolimidazole (IX) was then oxidized to 1,4-diphenylimidazole (X).

In order to establish the structures of compounds VI and VIII, 1,4-diphenyl-2-thiolimidazole (IX) was independently synthesized from N-phenacylaniline and potassium thiocyanate. This 1,4-diphenyl-2-thiolimidazole was then alkylated with benzyl chloride and with methyl iodide to produce 1,4-diphenyl-2-benzylthioimidazole (VI) and 1,4-diphenyl-2-methylthioimidazole (VIII), respectively.

A comparison of the above reactions of S-alkylisothioureas with the alkylation of N-substituted benzamidines indicates that the reactions are similar. Pyman[5] has shown that N-phenylbenzamidine monomethylated in good yield on the nitrogen holding the phenyl group. The isomeric product, N-phenyl-N[1]-methylbenzamidine was formed in only 0.6% yield. Analogously, N-phenyl-S-benzylisothiourea reacted with phenacyl bromide to form the imidazole VI in good yield; the isomer obtained corresponds to primary alkylation of the nitrogen holding the phenyl group with phenacyl bromide. On the other hand, N-methylbenzamidine monomethylated in poor yield and gave a mixture of the two expected isomers.[5] Similarly, S-benzyl-N-methylisothiourea reacted poorly with phenacyl bromide

(1) Abstracted from a thesis by Frank Ross presented to the Graduate Faculty of the University of Minnesota, in partial fulfillment of the requirements for the M.S. degree, June, 1949.

(2) R. M. Dodson, THIS JOURNAL, **70**, 2753 (1948).

(3) Hazeldine, Pyman and Winchester, *J. Chem. Soc.*, **125**, 1431 (1924).

(4) The cleavage of sulfides with phosphorus and iodine in glacial acetic acid may prove to be a general reaction. This possibility is being investigated.

(5) F. L. Pyman, *J. Chem. Soc.*, **123**, 3359 (1923).