DEPHOSPHORYLATION OF NUCLEOTIDES; THEIR ANALYSIS BY COUNTER-CURRENT DISTRIBUTION

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The analysis for the pyrimidine constituents of ribonucleic acids has long been a difficult problem, because of the stability of the ribosidic and phosphoryl bonds of the pyrimidine nucleotides. The nucleotides are very soluble in water and are difficult to separate. There have been several attempts in recent years to overcome the difficulties. Loring, Ordway, and Pierce (1) used ammonia at 140° for 4 to 8 hours to obtain pyrimidine nucleosides from ribonucleic acid; the nucleosides were assayed by the use of a strain of Neurospora. The authors indicated certain difficulties in the procedure. Vischer and Chargaff (2) described another method for hydrolysis under strenuous conditions. Chargaff et al. subsequently described a procedure (3) based upon a paper chromatographic separation of the nucleotides. Cohn (4) has described a procedure in which the nucleotides are fractionated by anion exchange. Tinker and Brown (5), in a study of the characterization of purines and pyrimidines by the method of counter-current distribution, made the statement, "and application of the technique for further characterization of nucleic acids must await studies of the hydrolysis of nucleic acids and their components."

Bamann and Nowotny (6) noted that the lanthanum salt of β-glycerophosphate is more unstable in alkaline solution than are its salts with other metallic ions. The ester β-glycerophosphate has a stability to hydrolysis similar to that of the pyrimidine nucleotides. On the basis of such observations, then, a new method has been developed for the dephosphorylation of the pyrimidine nucleotides. The procedure follows.

2 mg. of the sodium salt of a pyrimidine nucleotide in 1 ml. of water are placed in a centrifuge tube. 0.50 ml. of 0.05 N lanthanum nitrate and 0.65 ml. of 0.1 N sodium hydroxide are added.

The tube is placed in a boiling water bath. At intervals of 20 minutes, 0.06 ml. of N hydrochloric acid is added, the suspension is stirred a few moments to dissolve the precipitate, and then 0.06 ml. of N sodium hydroxide is added to reprecipitate the lanthanum. The heating is continued for a total of 100 minutes.

The tube is centrifuged for 10 minutes at 1200 X g. The supernatant is removed, and 2 ml. of wash water are added. Distilled water, which
has been adjusted to pH 10 by the addition of dilute sodium hydroxide, is used for the wash. The precipitate is washed twice, dissolved and reprecipitated once, and then washed once more.

The pH of the pooled supernatants is adjusted to near neutrality, and the solution is evaporated to less than 2 ml. on a steam bath. The solution is transferred to the first tube of a counter-current machine and the vessel is rinsed with aqueous buffer, which is used to fill the first tube. The material is distributed with forty to 50 transfers. The aqueous solvent which has been used in these studies is 1.0 M sodium chloride and 0.1 M phosphate, pH 7.1. The organic solvent is n-butanol. Reagent grade butanol is too highly absorbing in the ultraviolet; it is purified by contact with aluminum amalgam for several days, followed by distillation in an all-glass apparatus in an atmosphere of dry nitrogen.

The observed and theoretical distributions for the products obtained from the separate hydrolyses of highly purified samples of cytidylic and uridylic acids are presented in Figs. 1 and 2. Absorption measurements were made at 250, 260, 270, 280, and 290 m\(\mu\) in a Beckman model DU spectrophotometer. The measurements at different wave-lengths are dependent with respect to the operation of the machine, but independent
with respect to measurements of absorption. Consequently, the distributions at different wave-lengths afford partially independent measurements of $K$, the distribution coefficient.

The values of the distribution coefficients were computed by a hitherto unpublished method. A counter-current distribution has the property of a discrete frequency distribution. Such distributions may be characterized by their moments (7). For a counter-current distribution the first moment, or mean position, is given by the expression $\frac{\sum iD_i}{\sum D_i}$. The quantity $D_i$ is any measure of the amount of solute in the $i$th tube. The first tube, into which the solute is introduced, should be given the number zero ($i = 0, 1,$ etc.). The first moment of the binomial distribution is equal to $\frac{nK}{1 + K}$. Since observed distributions generally fit the expected binomial distributions quite well, the expressions for the observed and theoretical first moments may be equated.

$$\frac{\sum iD_i}{\sum D_i} = \frac{nK}{1 + K}$$

where $s$ is the number of the last tube which contains a measurable quantity of solute. The two sums can be calculated simultaneously in 2 or 3
minutes with any computing machine which has provision for cumulative multiplication. The method applies for any value of \( n \), just as long as the advancing limb of the distribution does not overtake the trailing limb during a multiple cycling of the machine. Equation 1 gives a maximum likelihood estimate of \( K \). The binomial expansion in \( K \) can be computed for comparison with the observed values. If \( T_i \) is the \( i \)th term of the binomial expansion, with the first term given the number zero, then the expected value of \( D_i \) is \( T_i \sum_{k=0}^{i} D_i \). Generalized statistical procedures are considered in detail elsewhere.\(^1\)

The values of \( K \) for cytidine, from the measurements at 260, 270, and 280 m\( \mu \), are 0.106, 0.107, and 0.108, as calculated from Equation 1. Tinker and Brown (5) reported the value 0.08, but a different aqueous buffer was used. The yield of cytidine was 95 per cent. The yield was calculated from the recovered absorption, summed over the tubes of the machine, in comparison to the absorption of an aliquot of the original solution of cytidylic acid after dilution with the same solvent which was used in the other absorption measurements. The slight difference between the absorptions of cytidylic acid and of cytidine was corrected by the data of Plocser and Loring (8).

The values of \( K \) for uridine, from the measurements at 250, 260, and 270 m\( \mu \), are 0.153, 0.154, and 0.154. The distribution for \( K \) equal to 0.157 fits the points a little better than that for \( K \) equal to 0.154, except for the first three points. It is assumed that 1 or 2 per cent of the uridylic acid remained unhydrolyzed. Tinker and Brown (5) reported the value 0.12. The yield of uridine was 93 per cent.

The nature of the systematic deviations between the theoretical and observed points of Figs. 1 and 2 will be discussed elsewhere.\(^1\)

DISCUSSION

The hydrolytic reaction is peculiar in some respects. Considerable data have been accumulated during the study of the reaction, all of which can be explained by one hypothesis; namely, that the reaction occurs in the solution and requires both lanthanum and hydroxyl ions. Lanthanum ions are removed by precipitation with the liberated phosphate, since all of the phosphate is recovered in the washed precipitate. Furthermore, lanthanum hydroxide is very insoluble. However, a freshly precipitated suspension of lanthanum hydroxide is supersaturated. The optimum pH for the reaction is approximately 10. Thus for a short time following the precipitation with sodium hydroxide, appreciable concentrations of the necessary ions remain in the supernatant. The nucleotide is only partly

\(^1\) Bacher, J. E., to be published.
coprecipitated. It is necessary to effect the hydrolysis as quickly as possible, since there is loss, to the extent of 2 or 3 per cent per hour, of the ultraviolet absorption either of the nucleotides or of the nucleosides under the conditions of the hydrolysis. The determination of phosphate according to the method of Fiske and Subbarow is not applicable to the hydrolysate since the hot alkali dissolves sufficient silicate from the glass vessel to augment seriously the development of blue color.

The absorption spectra found in the tubes of each distribution were identical on a relative scale and were identical to the absorption spectrum of the authentic nucleoside (8). There can be little doubt in regard to the identity and purity of the reaction products in the two cases. There were no indications of any free pyrimidine in either case.

SUMMARY

A method is given for the dephosphorylation of pyrimidine nucleotides in yields of 94 per cent. It is believed the method will work equally well for any stable phosphate ester.

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