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# Chemical Studies on Amino Acid Acceptor Ribonucleic Acids. IV. Position of the Amino Acid Residue in Aminoacyl s-RNA: Chemical Approach*

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ABSTRACT: A chemical method for determining the position of the aminoacyl group in the aminoacyladenosines isolated from s-RNA after pancreatic RNAase treatment was developed, based on the use of 2-cyanoethyl phosphate to phosphorylate the nonacylated hydroxyl groups. It was found that the 2-cyanoethyl group is removed by mild alkaline hydrolysis without formation of a cyclic phosphate intermediate even when a *cis*-hydroxyl group is present. The use of the reagent revealed that 65% of the aminoacyl groups in aminoacyladenosine isolated from aminoacyl s-RNA were 3′ while 35% were 2′.

The observation that the activation of amino acids for protein synthesis involved a ribonuclease-sensitive step (Holley, 1957) and that the amino acids were in fact bound to s-RNA in an intermediate step (Hoagland *et al.*, 1957, 1958; Ogata and Nohara, 1957; Ogata *et al.*, 1957) quickly led to the recognition that the amino acids were attached to either the 2′- or the 3′-hydroxyl of the terminal adenosine residue of the s-RNA molecule (Zachau *et al.*, 1958; Preiss *et al.*, 1959; Hecht *et al.*, 1959). The determination of the exact site of amino acid attachment, i.e., whether 2′ or 3′, is an important problem in the elucidation of the detailed mechanism of protein synthesis. The site of amino acid attachment is also of interest in the study of the action of inhibition of protein synthesis by puromycin which is thought to act as an analog of aminoacyl s-RNA (Yarmolinsky and De la Haba, 1959). The present paper and number V in this series describe experiments which were designed to define the site of amino acid attachment; some of the results obtained have already been briefly reported (McLaughlin and Ingram, 1963).

The *initial* site of attachment of an amino acid has been the subject of some speculation. It was predicted on the basis of the reactivity of the two hydroxyl groups of the terminal adenosine residue that the attachment would be to the 2′-hydroxyl group (Zamecnik, 1962), while on the basis of stereochemical considerations it was thought that the initial site would be 3′ (Urry and Eyring, 1962). An experimental approach to the problem has been the development of chemical methods to distinguish between the 2′- and 3′-aminoacyl esters of the adenosine end group of the s-RNA molecule (Frank and Zachau, 1963; McLaughlin and Ingram, 1963; Feldman and Zachau, 1964; Wolfenden *et al.*, 1964).

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[1] Abbreviations used in this work: ATP, adenosine triphosphate; CTP, cytidine triphosphate; pAp-3′, 3′,5′-adenosine diphosphate; and pAp-2′, 2′,5′-adenosine diphosphate.

It is first necessary to liberate and isolate the aminoacyladenosine fragment by digestion with pancreatic ribonuclease of aminoacyl s-RNA, because the RNA molecule has too many reactive sites for the subsequent chemical treatment. Next, the unoccupied hydroxyl groups (the 5′- and the 2′- or 3′-hydroxyls of the aminoacyladenosine) are blocked by a suitable reagent, a reaction which should take place under mild conditions to avoid acyl migration or degradation. The blocked derivative should itself be stable to migration and degradation. Next, the amino acid is removed; the product should allow ready characterization in terms of the original 2′ or 3′ position of the amino acid residue. In the present paper, the blocking reagent used was 2-cyanoethyl phosphate (Tener, 1961).

## Materials and Methods

*Preparation of the Amino Acid-activating Enzyme System.* The method of preparation is derived from that of Zamecnik *et al.* (1960). All operations were carried out at or below 4° unless otherwise noted. Bakers' yeast (50 g) (kindly supplied by Anheuser Busch, Inc.) was crumbled into liquid nitrogen. The frozen crumbs were pressed in a 50-ml Hughes press which had a starting temperature of −20°. The paste was suspended in 40 ml of a cold solution containing 0.14 M KCl, 0.001 M $MgCl_2$, and 0.01 M Tris, *p*H 7.2, in which ribosomes remain intact. A small crystal of pancreatic DNAase (Worthington Biochemical Corp., Freehold, N.J.) was added and the suspension stirred for 10 minutes without cooling, so that the temperature rose to about 4°. Centrifugation at 0° for 20 minutes in a Servall centrifuge at 14,000 rpm removed the gross cell debris. The supernatant fluid was adjusted to *p*H 7.5 with dilute ammonia and the solution was centrifuged at 40,000 rpm in a Spinco Model L centrifuge for 6 hours. Dialysis of the solution overnight against a solution of 0.05 M Tris, *p*H 7.5, 0.001 M $MgCl_2$, and 0.01 M mercaptoethanol (0.75 ml/liter) was followed by centrifugation for 6 additional hours in the Spinco centrifuge. The supernatant fluid was passed through a Sephadex G-25 column (5 × 40 cm) which had been equilibrated with a solution of 0.05 M Tris–0.001 M $MgCl_2$, *p*H 7.5. The main protein peak, now free of mercaptoethanol, contained typically 22 mg of protein/ml and 0.5 mg of nucleic acid/ml estimated spectrophotometrically (Warburg and Christian, 1942). The enzyme preparation is relatively stable when frozen.

*Preparation of Aminoacyladenosine from s-RNA.* Aminoacyl s-RNA was formed enzymatically and then incubated with pancreatic RNAase to liberate the aminoacyladenosine which was isolated by electrophoresis or chromatography on a Dowex-1-acetate column. The incubation mixture (25 ml) contained in 0.3 M Tris and 0.05 M $MgCl_2$ (*p*H 8.45): 40 mg (1.6 μmoles) of yeast s-RNA (General Biochemicals Inc., Chagrin Falls, Ohio); 80–120 mg of protein of the amino acid-activating enzyme system described above; 240 mg (386 μmoles) of ATP; 24 mg (40 μmoles) of CTP; 125 μmoles of each of the following amino acids: alanine, glycine, leucine, isoleucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan; also 60 μmoles of [$^{12}$C]valine and tracer quantities of [$^{14}$C]valine.

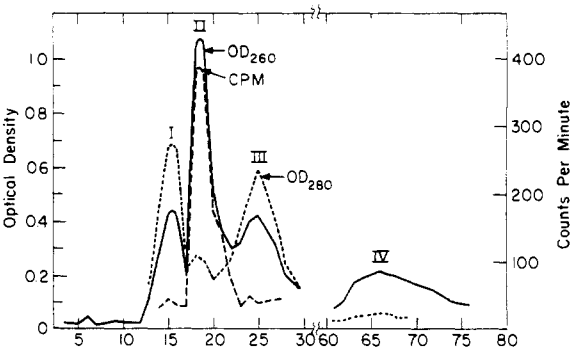


FIGURE 1: Fractionation of pancreatic RNAase–treated aminoacyl s-RNA on Dowex-1-acetate. Eluent: 0.0075 M acetic acid. s-RNA was esterified with [$^{14}$C]valine and other nonradioactive amino acids as described in the text. Peak I was unabsorbed protein and represents the void volume of the column. Peak II was aminoacyladenosine. Peak III was identified as cytidine by its spectrum and electrophoretic mobility at *p*H 3.0. The spectrum and electrophoretic mobility of peak IV were identical with those of adenosine.

The incubation period was 20 minutes at 37°. The reaction was stopped by chilling the vessel in ice. The aminoacyl s-RNA was separated from excess amino acids and ATP on a Sephadex G-50 column (2.5 × 40 cm) equilibrated with 0.01 M ammonium acetate, *p*H 5.5. The first 30 ml which corresponded to the void volume of the column was discarded and the next 40 ml taken. Pancreatic ribonuclease (4 mg) was added and the mixture incubated for 15 minutes at 37°. Preliminary experiments had shown this RNAase treatment to be sufficient to release most of the aminoacyladenosine. The *p*H was adjusted to 4.5 with acetic acid and 2 volumes of cold ethanol was added. The mixture stood in ice for 15 minutes and the fluffy white precipitate was removed by centrifugation. The ethanolic supernatant was reduced in volume to 2 or 3 ml and this solution was loaded onto a Dowex-AG-1-X-8 column (minus 400 mesh, California Corp. for Biochemical Research), 2.5 × 40 cm, which had been equilibrated at 4° with 0.0075 M acetic acid. The column was washed with a few ml of water and eluted with 0.0075 M acetic acid. Figure 1 shows a typical elution pattern. The aminoacyladenosine, peak II, was located by the ultraviolet absorption spectrum characteristic of adenosine and by its radioactivity. The peak tubes were pooled and the volume was reduced first in the rotary evaporator and then by lyophilization. The average yield of aminoacyladenosine based on the total amount of nucleoside recovered from the column was 22%. For the preparation of smaller amounts of a radioactive

aminoacyladenosine, electrophoresis in a *p*H 2.7, 20% acetic acid–ammonia buffer was more convenient than the Dowex-1 column.

*Preparation of 2'- (or 3'-) O-Valyladenosine-5'-phosphate and Related Compounds.* The synthesis of 2'- (or 3'-) *O*-valyladenosine-5'-phosphate was accomplished by the method of Wieland and co-workers (Wieland and Schäfer, 1952; Wieland *et al.*, 1956; Wieland *et al.*, 1960). The compound was purified by paper electrophoresis in 20% acetic acid adjusted to *p*H 1.9 with formic acid. To prepare 2'- (or 3'-) *O*-valyladenosine, the 5'-phosphate group was removed by incubating the valyladenosine-5'-phosphate (*ca.* 1 mg in 5 ml of 0.1 M ammonium acetate, *p*H 5.5) with *Escherichia coli* alkaline phosphatase at 2° for approximately 10 hours. An enzyme:substrate ratio of 1:10 was used.

Acetylation of the α-amino group of the aminoacyl moiety was carried out by dissolving 2 μmoles of the parent compound, either valyladenosine or valyladenosine-5'-phosphate, in 0.3 ml of 0.1 M sodium acetate solution previously chilled to 0°, followed by 6 μl of acetic anhydride (Eastman Organic Chemicals, redistilled). The mixture was kept at 0° with shaking for 2 minutes and was then immediately streaked onto 8–10 in. of an electrophoresis paper buffered at *p*H 2.7 with 20% acetic acid–ammonia. The desired reaction product was obtained pure after electrophoresis.

*2-Cyanoethyl phosphate* was synthesized as described by Tener (1961).

*5'-Trityl Nucleosides.* These compounds were synthesized as described by Gilham and Khorana (1958).

*Synthesis of 2-Cyanoethylthymidine-3'-phosphate.* Starting with thymidine (500 mg, 2 mmoles) 5'-*O*-tritylthymidine was synthesized (yield 220 mg after recrystallizing from benzene). The melting point was 115°; reported: 115–125° (Ralph and Khorana, 1961). The product (0.45 mmole) was phosphorylated for 1 week as below with 0.20 ml (0.20 mmole) of 2-cyanoethyl phosphate reagent. After the pyridine had been removed, the residue was dissolved in 10 ml of 80% acetic acid and set aside for a day to remove the trityl group. After removing most of the acetic acid with the rotary evaporator, the sample was lyophilized.

*Synthesis of 2-Cyanoethyluridine-2' (or 3')-phosphate.* 5'-*O*-Trityluridine was synthesized from uridine (750 mg, 3 mmoles). The product was not recrystallized since borate electrophoresis of the end product provided a sensitive assay for 5'-phosphate groups in case of incomplete tritylation. The yield was 390 mg (1.6 mmoles). The phosphorylation reaction, using 0.8 ml (0.8 mmole) of 2-cyanoethyl phosphate, and the removal of the trityl group were described above. Tener (1961) observed complete reaction of 2-cyanoethyl phosphate in 2 days, when the ratio of nucleoside to 2-cyanoethyl phosphate was 2:1. The same should apply under our conditions.

*Preparation of Nucleoside Cyclic Phosphate.* Sodium uridine-2',3'-cyclic phosphate was prepared from the barium salt (Schwarz BioResearch, Inc.). About 10 ml of a slurry of Dowex-50-X-8 resin, $Na^+$ form, was added to 50 mg of the barium salt in a few ml of water. After standing for 0.5 hour the mixture was poured through a Dowex-50, $Na^+$ form, column (0.9 × 10 cm). The column was washed with a few ml of water and the solution reduced to a convenient volume. Sodium cytidine-2',3'-cyclic phosphate was prepared exactly as described above from barium cytidine-2',3'-cyclic phosphate.

*Phosphorylation.* Although the proportions of the reactants varied, the same general procedure (Tener, 1961) was followed in all cases. Reagent grade pyridine (Mallinckrodt Chemicals) was stored over calcium hydride to maintain anhydrous conditions. A specified amount of 2-cyanoethyl phosphate stock solution was added to the nucleoside or nucleoside derivative together with 5 ml of pyridine (10 ml if more than 100 mg of nucleoside was used). As soon as the nucleoside dissolved, the pyridine solution was immediately reduced to a gum or an oil with a rotary evaporator at room temperature. Then 5 ml of pyridine was added, the solution again reduced to a gum, and the whole process repeated. Finally, the gum was dissolved in 5 ml of pyridine and dry *N,N'*-dicyclohexylcarbodiimide (Schwarz BioResearch, Inc.) was added (two-or threefold molar excess in terms of the 2-cyanoethyl phosphate added). The reaction was carried out in stoppered flasks at room temperature for varying times, from overnight to 1 week. It was stopped by adding 1 ml of water. Extraction with pentane removed most of the dicyclohexylurea and any unreacted dicyclohexylcarbodiimide. The pyridine-water layer was filtered and taken to dryness repeatedly with small volumes of water, using a rotary evaporator.

To remove the 2-cyanoethyl group, the product was dissolved in 5–10 ml of water in a *p*H-Stat (Radiometer Inc., Copenhagen) and the *p*H was raised in 0.5 *p*H unit steps to *p*H 10 over a period of 0.5 hour. It was then raised 0.5 *p*H unit every 0.5 hour until *p*H 11.5 was reached. This *p*H was maintained until the uptake of base decreased. When only small quantities of phosphorylating reagents had been used, the reaction mixture could be taken directly for chromatography or electrophoresis. However, in the case of the aminoacyladenosines where a 1000-fold excess of reagent had been used a desalting procedure was necessary. The nucleotides were adsorbed at *p*H 4.5 (acetic acid) onto acid-washed (Lane and Butler, 1959) charcoal (Darco G-50, Atlas Powder Co.), which was centrifuged and washed once with deionized water. The nucleotides were eluted from the charcoal with a mixture of ethanol, water, and concentrated $NH_4OH$ (50:50:2).

*Chromatography: Paper or Thin-Layer Cellulose.* Chromatography was carried out at room temperature using the solvent saturated ammonium sulfate–1 M ammonium acetate–2-propanol (80:18:2 by volume).

## Results

*Mechanism of Hydrolysis of 2-Cyanoethyl Nucleoside Phosphates.* To investigate whether the alkaline hydrolysis of the 2-cyanoethyl ribonucleoside phosphate

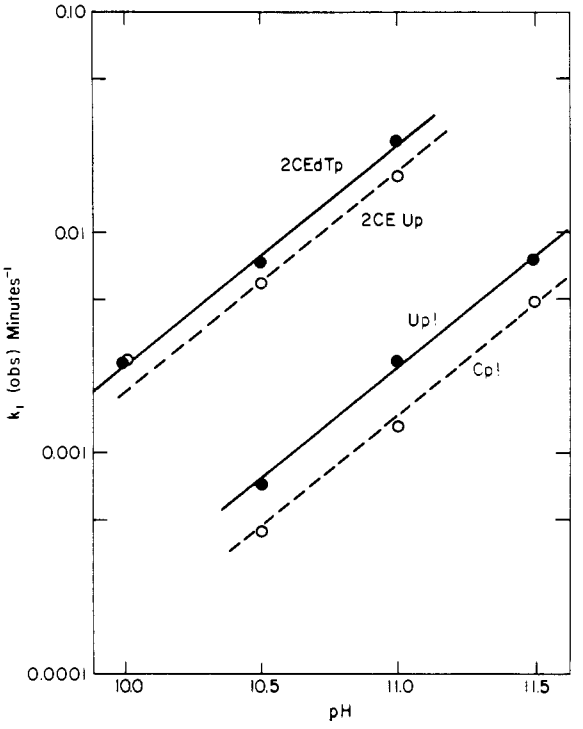


FIGURE 2: Rate of hydrolysis of 2-cyanoethylthymidine-3′-phosphate (2-CEdTp), 2-cyanoethyluridine-2′- (or 3′-) phosphate (2-CEUp), uridine-2′,3′ cyclic phosphate (Up!), and cytidine-2′,3′ cyclic phosphate (Cp!) as a function of *p*H. Temperature 37°. The lines are calculated from the rate constants presented in the text.

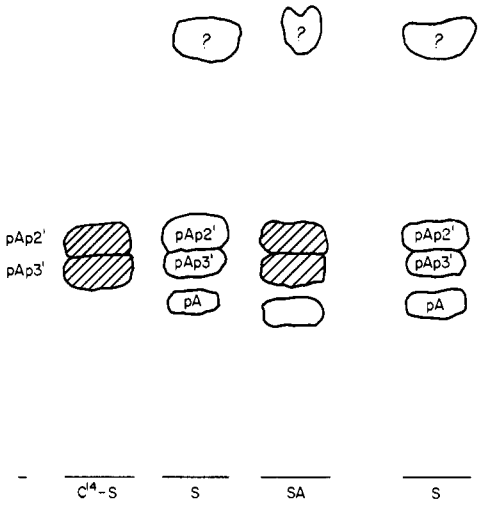


FIGURE 3: Chromatogram of the products of the phosphorylation of synthetic 2′- (or 3′-) *O*-valyladenosine (VAR). S is the standard phosphorylated adenosine mixture containing pA and pAp. $^{14}$C-S is the pAp-$^{14}$C standard. SA is the sample from VAR containing the internal pAp-$^{14}$C standard. The shaded areas represent the pAp-$^{14}$C areas located by radioautography. The area marked ? has not been identified. Ultraviolet spectra of this region show no distinct absorption maximum.

proceeds through a ribonucleoside-2′,3′-cyclic phosphate intermediate, the rates of alkaline hydrolysis of the following compounds were compared: 2-cyanoethylthymidine-3′-phosphate (the 2′-deoxy compound), 2-cyanoethyluridine-2′- (or 3′-) phosphate.

The hydrolysis of these model compounds was followed in the *p*H-Stat at 37° under nitrogen by measuring the rate of base uptake as a function of *p*H. The initial concentrations of nucleoside phosphate were determined spectrophotometrically. The concentration of cyanoethyl phosphate was corrected for prior breakdown to the phosphomonoester by estimating the secondary phosphate present. This estimation is based on a formula developed from the Henderson-Hasselbach equation by Schmidt (1955). The corrections amounted to about 10%.

Before the 2-cyanoethyl groups were removed, an aliquot of the 2-cyanoethyl nucleoside phosphate was chromatographed on thin-layer cellulose (see Methods). This chromatogram revealed no free nucleotide in the sample. The major spots were presumed to be unreacted nucleoside, which had been in excess in the reaction, and the protected nucleotide standards.

After complete removal of the 2-cyanoethyl group, aliquots of the hydrolysis mixture were electrophoresed in 0.05 M sodium borate, *p*H 9.2. The nucleoside and nucleotide regions were eluted and the quantities determined. The original quantity of 2-cyanoethyl derivative was calculated from the ratio of nucleotide to nucleoside plus nucleotide. The sample of 2-cyanoethyluridine-2′- (or 3′-) phosphate contained 54% uridine, and the 2-cyanoethylthymidine-3′-phosphate contained 34% thymidine. No uridine-5′-phosphate was detected in the electrophoreses.

The first-order rate constants were calculated from the equation $(\Delta A/\Delta T)/A = k_1$, where $A$ is the amount of the 2-cyanoethyl derivative present. Figure 2 shows a plot of the $k_1$ values against *p*H. The lines are calculated from the equation $k_1 = k_2(OH)$. The $k_2$ values (liters mole$^{-1}$ min$^{-1}$) used are as follows: cytidine-2′,3′-cyclic phosphate, 1.5; uridine-2′,3′-cyclic phosphate, 2.5; 2-cyanoethyluridine-2′- (or 3′-) phosphate, 19.0; and 2-cyanoethyluridine-3′-phosphate, 25.0.

Any intermediate in a hydrolysis reaction must be consumed with a rate which is no lower than the overall hydrolysis rate. Since the nucleoside cyclic phosphates hydrolyze at an almost tenfold slower rate than the 2-cyanoethyl nucleoside phosphates under the same conditions, it is clear that the cyclic phosphates are not intermediates in the hydrolysis of 2-cyanoethyl nucleoside phosphates. The fact that the 2-cyanoethyl phosphate derivative of the 2′-deoxy compound thymidine hydrolyzes at a rate substantially the same as the ribose compound also indicates that a cyclic form is not an intermediate.

*Determination of the Acyl Position in Adenosine Esters by Use of the Phosphorylation Reaction.* A solution of a few optical density units (at 260 mμ) of the

aminoacyladenosine compound was taken to dryness in a pear-shaped flask. Phosphorylation was carried out as described previously, shaking overnight at room temperature and using 1000-fold excess of 2-cyanoethyl phosphate.

The desalted products were examined in the standard 2′- (or 3′-) 5′-adenosine diphosphate (pAp) as internal standard. A standard phosphorylation mixture of adenosine was included in each chromatogram (Figure 3) to check the chromatography. The quantities of pAp were determined spectrophotometrically; in all cases the standards were within ±3% of the average figure of 50% pAp-3′ and 50% pAp-2′. If [$^3$H]adenosine was present, a pAp standard of 2.0 optical density units was added as an internal standard. This is the case in experiment 3 in Table I, which summarizes the results of the phosphorylation experiments. Electrophoresis of an aliquot of the phosphorylation reaction mixture before the protecting groups were removed showed that the aminoacyl group was stable under the conditions of the phosphorylation reaction.

TABLE I: Acyl Position by Phosphorylation.

| Substance | Acyl 3′ (%) | Acyl 2′ (%) |
|---|---|---|
| Aminoacyladenosine | 70 | 30 |
| Aminoacyladenosine | 71 | 29 |
| Aminoacyl[H$^3$]adenosine | 58 | 42 |
| Average | 66 | 34 |
| Valyladenosine (synthetic) | 67 | 33 |
| *N*-Acetylvalyladenosine (synthetic) | 73 | 27 |

## Discussion

The characterization of peak II from the Dowex-1 column (Figure 1) produced new experimental verification of the accepted 2′- or 3′-ester structure for aminoacyl s-RNA. Electrophoretic titration (McLaughlin, 1964) showed the two expected proton-accepting groups, namely the α-amino group of the aminoacyl group and the adenine amino group with $pK = 3.2$. Borate electrophoresis demonstrated that either the 2′- or the 3′-hydroxyl is unavailable for the formation of the borate-*cis*-hydroxyl complex (McLaughlin, 1964). The rate of hydrolysis of valyladenosine was similar to that of valyl s-RNA (McLaughlin, 1964) under conditions where the protonated amino acid ester was the main species contributing to the rate of hydrolysis.

The choice of a suitable blocking reagent is a critical step in the chemical determination of the aminoacyl position. Phosphorylation with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide was selected primarily because it permitted the ready identification of the products. The pAp-2′ and pAp-3′ isomers expected from the phosphorylation of aminoacyladenosine had been synthesized and identified (Baddiley *et al.*, 1958). The reaction conditions, overnight at room temperature in anhydrous pyridine, were thought to be sufficiently mild. Migration of the reagent should not occur once the 2-cyanoethyl group was removed, since monoesterified phosphates are only known to migrate under fairly acidic conditions (Cohn, 1950) which could easily be avoided. However, conceivably phosphate migration might occur during the removal of the 2-cyanoethyl group, since the amino acid ester with its known base lability could be expected to cleave before the 2-cyanoethyl group was removed. Normally, ribonucleoside phosphodiesters cleave through the formation of a cyclic phosphate intermediate (Lipkin *et al.*, 1954) which subsequently hydrolyzes to give about 60% 3′- and 40% 2′-phosphate (Carter, 1950).

By contrast, 2-cyanoethyl phosphate itself cleaves by β-elimination (Cherbuliez and Rabinowitz, 1956) and the 2-cyanoethyl group is also removed by β-elimination from simple alcohol phosphodiesters by a mild alkaline treatment with $Ba(OH)_2$ (Witzel *et al.*, 1960).

On the basis of these considerations, an experiment was designed to test whether the hydrolysis of 2-cyanoethyluridine-2′- (or 3′-) phosphate proceeds through the cyclic phosphate intermediate. The rates of hydrolysis of the various model compounds indicate that the hydrolysis of a 2-cyanoethyl nucleoside phosphate does *not* proceed through a cyclic intermediate, even when a *cis*-hydroxyl group is present. Thus there should be no phosphate migration during removal of the 2-cyanoethyl group, since phosphate migration at the diester level has not been observed under alkaline conditions (Brown *et al.*, 1956).

In fact, other data (Lane and Butler, 1959) indicate that normally during the hydrolysis of ribonucleoside phosphodiesters the rate-limiting step is the attack by the vicinal hydroxyl to form the cyclic phosphate rather than its subsequent cleavage. Therefore the rate difference for the β-elimination of the 2-cyanoethyl group and the more usual hydrolytic mechanism is even greater than the tenfold difference indicated by the rate of cyclic phosphate cleavage.

Application of the phosphorylation reaction to aminoacyladenosine showed that the aminoacyladenosine was an equilibrium mixture of the two acyl isomers (2′ and 3′). The ratio of isomers obtained was the same in the case of the mixed aminoacyladenosine from s-RNA and synthetic valyladenosine. The results indicate that two-thirds of the acyl groups was 3′ and one-third was 2′ under these conditions. The equilibrium distribution obtained by phosphorylation of *N*-acetylvalyladenosine closely resembles that obtained by direct chromatography of the *N*-acetylvalyladenosine (phosphorylation: 73% 3′, 27% 2′; chromatography: 75% 3′, 25% 2′) (McLaughlin and Ingram, 1965). It would appear that phosphorylation with 2-cyanoethyl phosphate is a valid and convenient way to determine

acyl position in nucleosides or other compounds in which the position of the phosphate residue can be easily evaluated.

All of the chemical methods developed to determine acyl position give the same equilibrium distribution for synthetically prepared aminoacyladenosines (Feldman and Zachau, 1964; McLaughlin and Ingram, 1963; Wolfenden *et al.*, 1964). Two of the methods (McLaughlin and Ingram, 1963; Wolfenden *et al.*, 1964) give the same equilibrium distribution for the mixed aminoacyladenosine from s-RNA as for the synthetic material. However, none of these chemical methods give definite information on the position of the aminoacyl group in s-RNA, because, in the absence of a pure 2′- or pure 3′-aminoacyladenosine as a standard, it has been impossible to evaluate quantitatively how much of the acyl migration to equilibrium took place during the blocking reaction and how much is due to aminoacyl migration before the blocking reaction.

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