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

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ABSTRACT: To investigate the 2′ (3′) isomerism of the amino acid in aminoacyl s-RNA, a series of chromatographic systems capable of separating several 2′-*O*-valyladenosine derivatives from the 3′-*O*-valyladenosine derivatives were applied to [^{14}C]valyladenosine isolated from [^{14}C]valyl s-RNA. Acyl migration occurred before and/or during the isolation procedure. The chromatographic studies indicate that in the case of 2′- (and 3′) *O*-(*N*-acetylvalyl)adenosine, 2′- (and 3′-) *O*-(*N*-acetylvalyl)adenosine-5′-phosphate, and 2′- (and 3′-) *O*-valyladenosine the equilibrium ratio is about 3:1 in favor of the 3′-ester.

Kinetic studies on the rate of migration in 2′-*O*-(*N* acetylvalyl)adenosine indicate that migration is a rapid base-catalyzed reaction. The $k_{2(obsd)}$ migration is 3,600,000 M^{-1} min^{-1} at 15° while the $k_{2(obsd)}$ hydrolysis is 89 M^{-1} min^{-1} at 15°. The ratio of $k_{2(obsd)}$ migration/ $k_{2(obsd)}$ hydrolysis is 40,000. Estimates of the rate of migration indicate that the rate of acyl migration of aminoacyl s-RNA is faster than the rate of aminoacyl s-RNA turnover even in rapidly growing bacteria. Therefore, aminoacyl s-RNA may exist *in vivo* as the equilibrium mixture of 2′- and 3′-esters.

The chemical approach to the determination of the position of the aminoacyl group, which was developed in the preceding paper (McLaughlin and Ingram, (1965), indicated that the aminoacyladenosine isolated from enzymatically prepared aminoacyl s-RNA was an equilibrium mixture of the 2′ and 3′ isomers. This result was unsatisfactory from a biological standpoint because in the absence of known 2′- or known 3′-aminoacyladenosine it was not possible to evaluate how much acyl migration occurred during the necessary manipulations in this system or in any of the other chemical systems (Feldmann and Zachau, 1964; Wolfenden *et al.*, 1964) developed to determine the position of the amino acid residue. Thus it was impossible to determine whether significant acyl migration had occurred during the incubation to form aminoacyl s-RNA, or during the isolation of the aminoacyladenosine, or during the blocking reaction. To overcome these difficulties inherent in the chemical approach, chromatographic systems capable of separating various 2′- and 3′-acyladenosine isomers have been developed. Particular attention was focused on the valyladenosine isomers since they would be the most sterically hindered amino acid esters and thus should have the slowest rate of acyl migration among the various aminoacyladenosines. Some of the results obtained have already been briefly reported (McLaughlin and Ingram, 1964).

Materials and Methods

The various preparative procedures were described in the preceding paper (McLaughlin and Ingram, 1965).

Thin-Layer Chromatography. The thin-layer plates were prepared with the thickness of 0.25 mm from a slurry of water and MN 300 G cellulose powder (Macherey Nagel and Co., Germany). This cellulose powder contained 10% $CaSO_4 \cdot 0.5$ H_2O as binder. Chromatography was carried out in a solvent-saturated atmosphere at 2°.

Chromatographic Separation of the 2′ and 3′ Isomers of Valyladenosine and Related Compounds. A series of chromatographic systems have been developed which are capable of separating the 2′ and 3′ isomers of 2′- (and 3′-) *O*-valyladenosine, 2′- (and 3′-) *O*-(*N*-acetylvalyl)adenosine, and the corresponding adenosine-5′-phosphate compounds. Thin-layer cellulose was used as the supporting medium, while the solvent systems are based on buffered ammonium sulfate solutions (Markham and Smith, 1951). Figure 1 shows some separation attainable with these systems. The *N*-acetylvalyladenosine isomers separate well over the broad *p*H range 0.0–5.0, while above *p*H 6.0 the two isomers do not resolve. A convenient solvent was saturated ammonium sulfate–1 M ammonium formate, *p*H 3.2 (80:20). The isomers of *N*-acetylvalyladenosine-5′-phosphate separate over a wide range of *p*H (this separation has not been tested at *p*H values higher than 4.0). Good separation of the 2′ and 3′ isomers

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has been observed in saturated ammonium sulfate adjusted to *p*H 2.0 or 3.0 with formic or sulfuric acid, but the separation is not greatly affected by the solvent composition. At *p*H 2.0 or *p*H 3.0, *N*-acetylvaline and AMP, the probable degradation products, have much higher mobilities and do not contaminate the *N*-acetylvalyladenosine-5′-phosphate spots.

The separation of the valyladenosine-5′-phosphate isomers is more difficult to achieve. At lower *p*H values the molecule, with three charged groups, has such a high mobility that the separations are very poor and the compounds move together with AMP. Free valine fortunately runs with the solvent front at all *p*H values observed. The two isomers cannot be separated above *p*H 4.0, where they have a sufficiently low mobility, because they begin to run as one band; presumably base-catalyzed acyl migration becomes too rapid. The best separation of the two isomers occurred in saturated ammonium sulfate–water (10:1) adjusted to *p*H 3.0 with formic acid (see Figure 1a). As in the case of the valyladenosine-5′-phosphate isomers, the valyladenosines run as a broad band at *p*H 4.0, and lower *p*H values have to be used. In contrast to the other compounds, however, the valyladenosines streak at *all* *p*H values tested. Addition of 2-propanol to the solvents caused even more streaking. The 2-propanol in the original solvent (Markham and Smith, 1951) had been left out, because the *N*-acetylvalyladenosine separations were better without it. The best solvent found for valyladenosine was saturated ammonium sulfate, *p*H 1.0 (see Figure 1a). It is very evident even in this solvent that the faster component streaks into the slower one.

Determination of the Equilibrium Distribution of the Acyl Group between the 2′ and 3′ Positions. The isolation of the compounds used has been described in a previous paper (McLaughlin and Ingram, 1965). Samples were stored frozen in dilute acetic or formic acid at *p*H 4 to 5. The equilibrium distribution was determined by measuring the proportions of band 1 (the slower component) and band 2 (the faster component) after thin-layer chromatography as described. The plates were partially dried in a current of air and the bands were located by ultraviolet light. The relevant bands and a suitable blank region were scraped off and eluted with 0.1 N acid. The spectra were determined on a Cary Model 14 spectrophotometer.

The radioactive spots were estimated by placing them in scintillation vials, adding 1 ml of water, and eluting the powder for 1 hour. After this treatment 10 ml of a dioxane-based scintillation fluid (Bray, 1960) was added and the samples were counted in a Nuclear Chicago liquid scintillation spectrometer. A series of ^{14}C standards, mixed with various areas of cellulose from the thin-layer plates, failed to show any quenching of ^{14}C by the cellulose. In any case, since the areas taken for bands 1 and 2 were about the same, quenching should not affect the relative amounts of radioactivity.

For a quantitative ninhydrin determination, the dry

[1] Abbreviations used in this work: ATP, adenosine triphosphate; CTP, cytidine triphosphate.

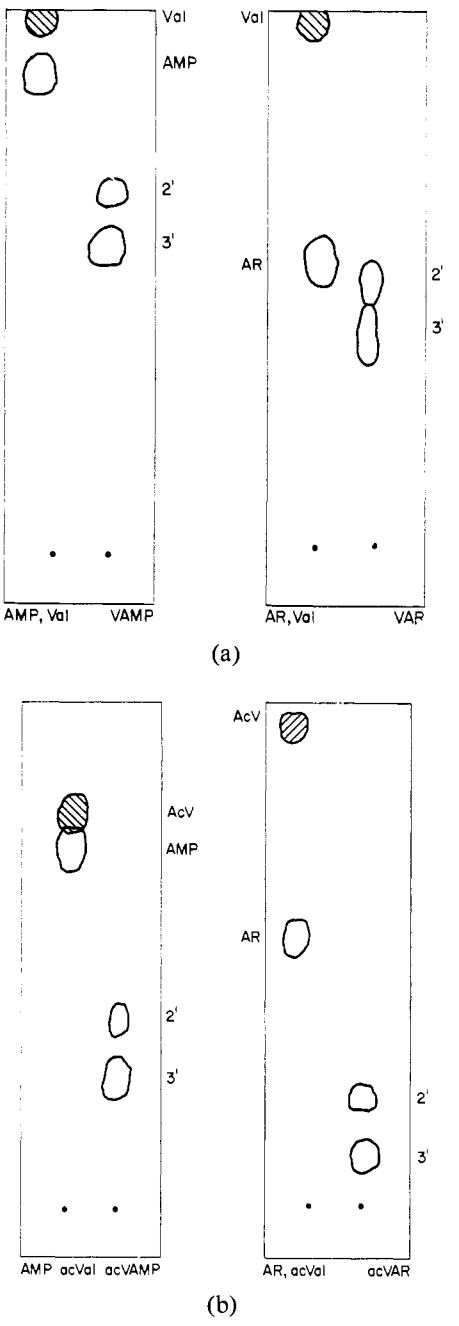

FIGURE 1: Thin-layer cellulose chromatograms. (a) 2′- (or 3′) *O*-Valyladenosine (VAR) and 2′- (or 3′-) O-valyladenosine-5′-phosphate (VAMP). Chromatographic solvent (VAMP): saturated ammonium sulfate–water (90:10) adjusted to *p*H 3.0 with concentrated formic acid. Chromatographic solvent (VAR):saturated ammonium sulfate adjusted to *p*H 1.0 with concentrated sulfuric acid. (b) 2′- (or 3′-) *O*-(*N*-Acetylvalyl)adenosine-5′-phosphate (acVAMP) and 2′- (or 3′-) *O*-(*N*-acetylvalyl)adenosine (acVAR).Chromatographic solvent (acVAMP): saturated ammonium sulfate adjusted to *p*H 2.0 with concentrated sulfuric acid. Chromatographic solvent (acVAR):saturated ammonium sulfate–1 M ammonium formate, *p*H 3.2 (80:20).

thin-layer plate was stained with ninhydrin (Heilman *et al.*, 1957). The plate was stored in a desiccator over P_2O_5 in a dark cupboard for 24 hours. The bands and a blank from a nearby region were scraped into fritted glass funnels and eluted with reagent grade methanol. Quantitative determinations were made by measuring the absorbancy at 500 mμ (see Table I).

TABLE I: Aminoacyl Equilibrium Distribution.

Substance	Band 1(3′) (%)	Band 2(2′) (%)	Measurement
N-Acetylvalyl-adenosine	76	24	[^{14}C]Acetyl
N-Acetylvalyl-adenosine	75	25	[^{14}C]Valyl[b]
N-Acetylvalyl-adenosine	78	22	Optical density (257 mμ)
N-Acetylvalyl-adenosine-5′-phosphate	73	27	[^{14}C]Acetyl
N-Acetylvalyl-adenosine-5′-phosphate	74	26	Optical density (257 mμ)
Valyladeno-sine-5′-phosphate	78	22	Quantitative ninhydrin
Valyladeno-sine-5′-phosphate	79	21	Optical density (257 mμ)
Valyladeno-sine[a]	(87)	(13)	[^{14}C]Valyl[b]

[a] All chromatograms streak. [b] [^{14}C]Valyl compounds were prepared from valyl-s-RNA.

The low value for 2′-valyladenosine is due to the streaking of this band into the 3′ isomer. Therefore the chromatogram only shows that a 2′ component is present and that its concentration is greater than 13%. The other values given in Table I are quite reproducible from preparation to preparation and from day to day determinations do not vary more than 2 or 3%. The differences among the compounds are small, but they may be real in the case of *N*-acetylvalyladenosine-5′-phosphate and valyladenosine-5′-phosphate. The assignment of band 1 to the 3′ isomer and band 2 to the 2′ isomer is based on a comparison of these equilibrium quantities with those obtained for *N*-acetylvalyladenosine and valyladenosine by phosphorylation (McLaughlin and Ingram, 1965).

Acetylation of 2′- (or 3′-) O-Valyladenosine. The reduced electron-withdrawing power of the *N*-acetyl derivatives vis-à-vis the protonated amino acid should lower both their rate of hydrolysis and their rate of acyl migration in the *p*H range where a substantial proportion of the free amino groups would be protonated. Since all the ribose hydroxyl groups and the N-1 and N-6 nitrogens of adenosine can be acetylated under various conditions (Gilham and Khorana, 1958; Ralph and Khorana, 1961; Stuart and Khorana, 1963) a series of experiments were undertaken to make sure that *N*-acetylvalyladenosine and *N*-acetylvalyladenosine-5′-phosphate prepared by the very gentle method described in the previous paper (McLaughlin and Ingram, 1965) were only acetylated on the α-amino group of the valyl residue.

N-Acetylvalyladenosine prepared from valyladenosine-5′-phosphate is indistinguishable from that prepared from valyladenosine on electrophoresis at *p*H 3.0 and chromatography in saturated ammonium sulfate–1 M ammonium formate, *p*H 3.5 (80:20). The spectrum of *N*-acetylvalyladenosine has the same maximum and general shape as adenosine in 0.1 N HCl. Electrophoresis in 0.05 M sodium borate, *p*H 9.2, of adenosine treated with acetic anhydride under the identical mild conditions indicated that neither the 2′- nor the 3′-hydroxyl group was acetylated. Acetylation of valyladenosine with [^{14}C]acetic anhydride demonstrated that 0.96 mole of acetate is added to each mole of valyladenosine, measuring the radioactivity and the absorbancy of the product. On partial alkaline hydrolysis of this *N*-[^{14}C]acetylvalyladenosine, only *N*-[^{14}C]acetylvaline and the starting material were detectable. Thus, it is clear that under our conditions only the α-amino group of the valine residue is acetylated and that it is acetylated quantitatively.

Acetylation of Valyl s-RNA. The amount of acetic anhydride necessary to acetylate completely the valyl amino group of valyl s-RNA was determined empirically. *N*-[^{14}C]Acetylvalyladenosine was prepared from [^{14}C]valyl s-RNA as described (McLaughlin and Ingram, 1965). The final purification was accomplished by electrophoresis at *p*H 3.0. When the product was examined by chromatography in saturated ammonium sulfate–1 M ammonium formate, *p*H 3.5, it was found that the equilibrium distribution of the aminoacyl group had already been obtained. Consideration of the isolation system used indicated that it surpassed the published isolation systems (Feldmann and Zachau, 1964; Wolfenden *et al.*, 1964) in minimizing acyl migration. However, to reduce acyl migration even further the following method was used. The incubation mixture contained: 200 μl (4 mg) s-RNA, 300 μl 1 M $MgCl_2$, 200 μl ATP-CTP[1] (containing 6 mg and 0.6 mg, respectively), 5 μmoles of each of the neutral amino acids except valine, 800 μl yeast amino acid–activating enzyme (16 mg protein; prepared as McLaughlin and Ingram, 1965), and 200 μl water. The mixture was adjusted to *p*H 6.0 with 1 N acetic acid; [^{14}C]valine (1 μc, 205 μc/mole) was added and the whole was incubated at 23° for 5 minutes in a *p*H-Stat. Acetic anhydride (2 μl) was added and the *p*H was allowed to drift to 5.6. After 2 minutes pancreatic RNAase (0.4 mg) was added. After 5 minutes the *p*H was lowered to 3.5 with formic acid. The solution was cooled to 0°

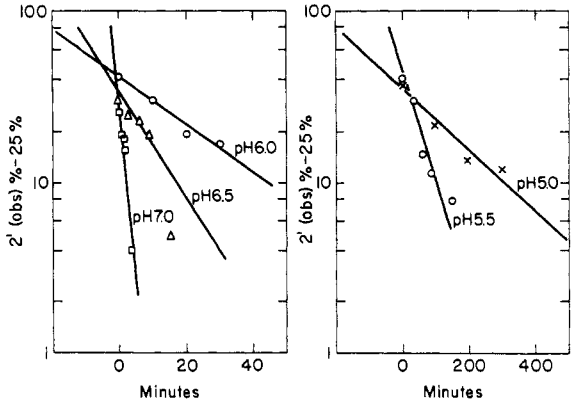

FIGURE 2: Rate of isomerization of 2′-*O*-(*N*-acetylvalyl)adenosine at various *p*H values. Temperature 15°; other experimental conditions are presented in the text.

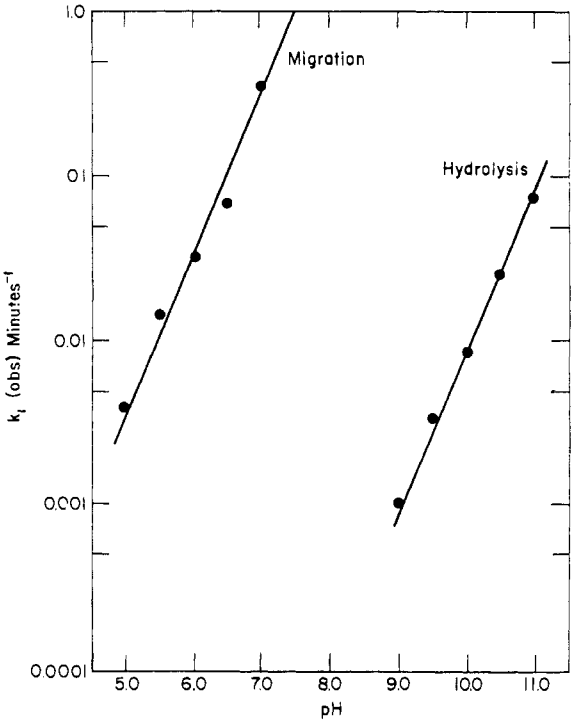

FIGURE 3: Rate of migration and hydrolysis of 2′-*O*-(*N*-acetylvalyl)adenosine as a function of *p*H. Temperature 15°; other experimental details are presented in the text. The solid lines are calculated from the second-order rate constants given in the text.

and the precipitate centrifuged off. A drop of amyl alcohol was added to the supernatant solution along with an equal volume of chloroform. The denatured protein interface was removed four times (time consumed, 0.5 hour); about one-fourth the aqueous solution was taken for chromatography. After scraping the spots off and counting them, 16% of the ^{14}C was in the 2′ spot and 84% was in the 3′ spot. This again indicates that the equilibrium mixture had been obtained. The drop in the 2′ component is probably entirely due to the fact that this chromatogram had to be overloaded to get a reasonable amount of the *N*-acetylvalyladenosine.

Results

Kinetic Studies on the Rate of Acyl Migration in 2′- (or 3′-) O-(N-Acetylvalyl)adenosine. This compound was selected for most of the experiments because it is easily obtained with ^{14}C in either the acetyl or the valyl group and because it has the lowest chromatographic mobility in the ammonium sulfate systems and is thus the least sensitive to salt effects in this solvent.

Saturated ammonium sulfate–1 M ammonium formate, *p*H 3.5 (80:20), was used to separate a mixture (containing [^{14}C]acetyl; 30,000 cpm) of the 2′ and 3′ isomers. The 2′ band was scraped off and eluted. The eluate was divided into five tubes and lyophilized to dryness. The following buffers were used to dissolve the samples: 100 μl of 0.1 M ammonium acetate, *p*H 5.0, 5.5, and 6.0; and 100 μl of 0.05 M potassium phosphate, *p*H 6.5 and 7.0. Each sample was checked with *p*H paper and was found to be at the *p*H of the original buffer. The zero time control was removed after dissolving the sample. The remainder of the material was then immediately transferred to a 15° bath for the rest of the experiment. Samples were removed at timed intervals and placed in tubes containing 5 μl of 1 M acetic acid and 0.3 ODU (260) of nonradioactive *N*-acetylvalyladenosine. The tubes were then frozen and reduced in volume prior to spotting. After chromatography, the isomers were located and counted. For each *p*H, a curve of log (% 2′obsd-25) versus time (Figure 2) was plotted because the % 2′ isomer at equilibrium = 25%. The first-order constants were calculated from the observed $t_{1/2}$ of migration which was determined graphically from the extrapolated lines as the time between log (100 − 25) and log $^1/_2$(100 − 25). The reaction follows pseudo-first-order kinetics between *p*H 5.0 and 7.0. Above *p*H 7.0 the rate becomes too fast to measure conveniently; it has also not been determined below *p*H 5.0. In Figure 3, the observed k_1 values are seen to be proportional to the *p*H. The second-order rate constant can be calculated from such a plot according to the equation $k_{2(obsd)} = k_{1(obsd)}/[OH^-]$. Such a calculation gives $k_{2(obsd)}$ = 3,600,000 M^{-1} min^{-1}. The second-order rate equation is:

observed rate of migration =
3,600,000 [*N*-acetylvalyladenosine] [OH], M^{-1} min^{-1}

The fact that the $2'_0$ and $3'_0$ values have never been observed to be 100% in the respective 2′, 3′ zero time controls is attributed to several necessary operations which have caused some migration. First, to locate the bands under ultraviolet light requires several-minutes exposure of the partially dried plate at room temperature. After elution the sample is lyophilized to dryness. This step is unfortunately necessary, because the buffer

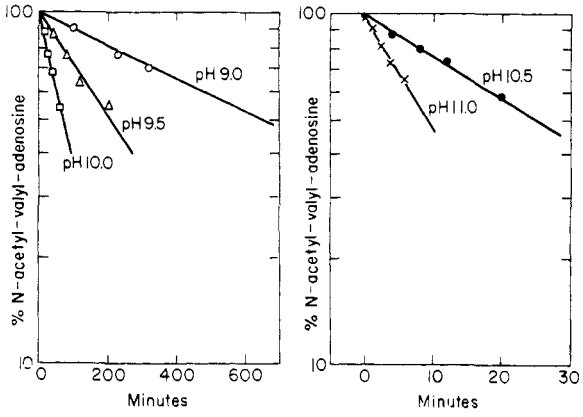

FIGURE 4: Rate of hydrolysis of 2′- (or 3′-) *O*-(*N*-acetylvalyl)adenosine at various *p*H values. Temperature 15°; other experimental details are presented in the text.

(ammonium formate) must be removed to allow one to fix the *p*H in the controlled migration step without resorting to highly concentrated buffers, which would interfere with the chromatographic analysis step. Although these chromatographic solvents are, comparatively speaking, not very salt sensitive, there is a limit to what they will tolerate. Finally, the samples are reduced almost to dryness and applied to the second chromatogram. This last application is done at room temperature taking the precaution of keeping the samples acid with formic and acetic acid. All these steps may involve some migration. The same k_1(obsd) is obtained regardless of the value of A_0 used. Although for accuracy it is best to have the initial value as close to 100% as possible, it is possible from a theoretical standpoint to have the zero time value at any value except equilibrium.

The chromatographic system itself has been eliminated as a source of migration. A sample of *N*-acetylvalyladenosine was applied as a spot in a corner of a 20- × 20-cm thin-layer plate. After development in one dimension for 6 hours, blotting paper strips were added without removing the plate from the cold room and the plate was developed for 12 hours in the other dimension. In spite of heavy loading, only a single 2′ spot and a single 3′ spot were seen without a trace of additional spots due to migration. Formation of a 3′ spot from the 2′ spot would be the most sensitive effect of migration, but it was not detected.

Base-catalyzed Migration in 2′-O-(N-Acetylvalyl)-adenosine-5′-phosphate, 2′-O-Valyladenosine-5′-phosphate, and 2′-O-Valyladenosine. The experimental procedure in each case was to isolate the 3′ and 2′ isomers by the appropriate chromatographic system. The spots were then eluted with 0.05 M formic acid. The solutions from each 2′ spot were divided in half after they had been concentrated to a few hundred μl. One-half of the 2′ solution along with all of the 3′ solution was lyophilized to dryness. These samples served as the zero time controls. Then the other half of the 2′ solution was carefully adjusted with dilute ammonia to *p*H 7.5 as judged by *p*H paper. After standing 1 minute at room temperature acetic acid was added to lower the *p*H and the solution was quickly frozen and lyophilized to dryness. Formic acid (0.1 M) was used to spot the samples for the second chromatography step. The valyladenosine and *N*-acetylvalyladenosine-5′-phosphate samples contained [^{14}C]valyl and [^{14}C]acetyl, respectively, and nonradioactive carrier was added to them before chromatography. No attempt was made to observe base-catalyzed migration in the 3′ compound, because the high equilibrium concentration of this isomer makes such experiments insensitive.

For *N*-acetylvalyladenosine-5′-phosphate the 3′ spot yielded 89% 3′ and 11% 2′; the untreated 2′ sample yielded 47% 3′ and 53% 2′; base-catalyzed migration due to the brief incubation at *p*H 7.5 shifted this figure to 68% 3′ and 32% 2′. The normal equilibrium for this system is 73% 3′ and 27% 2′ (Table I). For valyladenosine, the 3′ spot yielded 95% 3′ and 5% 2′; the untreated 2′ sample yielded 55% 3′ and 45% 2′; base-catalyzed migration shifted this figure to 89% 3′ and 11% 2′ (see Table I).

Quantitative data on valyladenosine-5′-phosphate was not obtained, since the compound was not radioactive. Qualitatively it resembled the two preceding cases in that the brief incubation at *p*H 7.5 caused extensive acyl migration. Thus it is clear that these compounds also undergo base-catalyzed acyl migration under very mild conditions.

Base-catalyzed Hydrolysis of 2′- (or 3′-) O-(N-Acetylvalyl)adenosine. The rate of hydrolysis of *N*-acetylvalyladenosine was studied to complement the migration rate experiments. *N*-Acetyl[^{14}C]valyladenosine was prepared from s-RNA as described previously. The final purification step was electrophoresis in 20% acetic acid, *p*H 3.0. About 50,000 counts (specific activity = 205 mc/mmoles) were divided equally between 5 tubes and lyophilized to dryness. The following 0.05 M $KHCO_3$–KOH buffers were prepared: *p*H 9.0, 9.5, 10.0, 10.5, and 11.0. Buffer (250 μl) was added to each tube and the zero time sample removed. The tube was immediately placed in the 15° bath and timed aliquots were removed. The *p*H of the reaction tube was checked with *p*H paper. The reaction was stopped by adding each aliquot to a tube containing 50 μl of 0.5 M acetic acid, 0.5 ODU (260) of cold *N*-acetylvalyladenosine, and 0.5 ODU (260) of each of the four nucleotides. The contents was frozen and lyophilized. Each sample was dissolved in a few μl of the electrophoresis buffer, 10% acetic acid–KOH, *p*H 3.0, applied as a spot, and submitted to electrophoresis for 3 hours at 1.25 kv. The *N*-acetyl[^{14}C]valyladenosine and *N*-acetyl[^{14}C]valine spots were located with the Vanguard chromatograph scanner. The *N*-acetylvalyladenosine compound was also located by the position of the synthetic carrier *N*-acetyl valyladenosine. The *N*-acetylvaline was located in relation to the nucleotide markers after a few guide strips were scanned to determine its mobility relative to these markers. Free valine was not detected by the Vanguard

scanner. The proper areas of the paper were cut out and counted in the scintillation counter as described in the method section. The results are plotted in Figure 4 as log per cent of *N*-acetylvalyladenosine as a function of time at each *p*H value. The first-order constant $k_{1(obsd)}$ was obtained from the equation: $k_1 = 0.693/t_{1/2}$. The $t_{1/2}$ values were obtained graphically as the time interval between the point log (*N*-acetylvalyladenosine = 100%) and the point log (*N*-acetylvalyladenosine = 50%). The $k_{1(obsd)}$ values obtained are plotted against *p*H in Figure 3, which shows the rate of hydrolysis to be proportional to the [OH^-]. The second-order constant, k_2, is calculated from the equation

$$k_2 = k_1/[OH^-]$$

The value obtained is $k_2 = 89\ M^{-1}/min^{-1}$. Thus, the rate expression is:

observed rate of hydrolysis
= 89 [*N*-acetylvalyladenosine][OH^-], $M^{-1}min^{-1}$.

It can be concluded that hydrolysis is a base-catalyzed reaction in the range *p*H 9.0–11.0.

The ratio of $k_{2(obsd)}$ migration/$k_{2(obsd)}$ hydrolysis = 40,000.

Discussion

Chromatographic Separation of the 2′ and 3′ Acyl Isomers. The identification of the upper and lower chromatographic spots as the 2′ and 3′ isomers, respectively, of *N-acetylvalyladenosine* follows from the following observations: The *N*-acetylvalyladenosine sample migrated as a single spot with the expected mobility on electrophoresis, and it was chromatographically homogeneous as jugded by five other solvent systems (McLaughlin, 1964). No acetylation of adenosine was observed under the acetylating conditions used. The ratio of the two chromatographic bands obtained in the modified Markham and Smith solvent is the same whether the ratio is determined on the basis of [^{14}C]valine, [^{14}C]acetyl groups, or optical density. The spectra of the two bands are practically identical, closely resembling the spectrum of the original mixed isomers (McLaughlin, 1964). Only one [^{14}C]acetyl group is found per molecule and *N*-acetylvaline is recovered after alkaline hydrolysis. The same chromatographic separation of the 2′ and 3′ isomers is observed when *N*-acetylvalyladenosine is prepared from valyladenosine or from valyladenosine-5′-phosphate. Finally, under certain conditions, the two isomers may be obtained again in the original 3:1 ratio from either band 1 or band 2.

The assignment of the 3′ isomer to band 1 and of the 2′ isomer to band 2 is based on the similarity of the observed ratio to the ratio determined by phosphorylation. In the latter method, *N*-acetylvalyladenosine gave 73% of 3′ isomer and 27% of 2′ isomer (see paper IV in this series, McLaughlin and Ingram, 1965); the chromatographically separated band 1 represents 75% and band 2 represents 25% of the original *N*-acetylvalyladenosine. Similar observations for valyladenosine, valyladenosine-5′-phosphate, and *N*-acetylvalyladenosine-5′-phosphate led to the conclusion that in all these cases the 2′ isomer has a higher R_F value than the 3′ isomer.

Kinetic Studies. The recognition that base-catalyzed acyl migration in compounds of this type might be a measurable effect under physiological conditions (i.e., *p*H 6–8 and 37°) is recent. The rate of acyl migration in several β-monoglycerides has been determined (Mattson and Volpenhein, 1962; Wolfenden *et al.*, 1964) under such conditions. The ratio $k_{2(obsd)}$ migration/$k_{2(obsd)}$ hydrolysis for glycerol β-monoacetate was reported as 6500 (Wolfenden *et al.*, 1964).

In the present experiments, the ratio of migration to hydrolysis in *N*-acetylvalyladenosine was found to be 40,000. This value differs from Wolfenden's, because in our case the 2′ and 3′ ribose hydroxyl groups are constrained in a more favorable position for this kind of migration (Anderson and Lardy, 1950; Tamelen, 1951). The actual rate of migration of *N*-acetylvalyladenosine (at 15° $k_{2(obsd)}$ = 3,600,000) is also much greater than the rate observed with glycerol β-monoacetate.

Relationship of Migration to Hydrolysis. Bender and Glasson (1959) have shown in a study of the simultaneous base-catalyzed hydrolysis and alcoholysis of esters that the rates vary in a parallel manner when the parameters are altered; that is, factors which increase the rate of hydrolysis similarly increase the rate of alcoholysis as judged by the relative proportion of ester interchange and hydrolysis. The alcohols used were ethanol and methanol. This finding is not unexpected since the mechanism of base-catalyzed alcoholysis and hydrolysis are the same (Taft *et al.*, 1950).

The work of van Lohuigen and Varkade (1960) shows that steric effects influence migration. It was found that the rate of migration paralleled the rate of hydrolysis in the acyl compounds studied. Migration is an intramolecular alcoholysis and the parallelism was expected. Polar effects in acid hydrolysis are small, while steric effects account for most of the rate differences (Gould, 1959). Thus, the parallelism of acid-catalyzed migration and hydrolysis means that the steric effects operate in a normal manner during migration. The transition states for acid- and base-catalyzed hydrolysis of esters differ by only 2 protons which have a negligible steric effect (Gould, 1959). Thus, steric factors should be equally important in acid-catalyzed and base-catalyzed migration. This fact, together with the observations of Bender and Glasson (1959) just mentioned, allows the use of the hydrolysis to migration ratio observed for *N*-acetylvalyladenosine in the calculation of migration rates from known hydrolysis rates of various aminoacyladenosines. This calculation should lead to a good estimate of the migration rate for each related molecular species for which the rate of hydrolysis is known.

The *pK* of the OH group in adenosine is 12.5. This low *pK* coupled with the very favorable steric position of the adjacent hydroxyl for attack on the carbonyl carbon presumably accounts for the high ratio of migration to

hydrolysis in these compounds. Intramolecular catalysts often show a high ratio of internal catalysis/external catalysis. A study of the ΔH^* and ΔS^* factors of such reactions shows that the ΔH^* factors are unchanged or nearly unchanged and that almost all of the kinetic effects are due to a change in the ΔS^* factor (Bender, 1960). Presumably migration is affected in the same way; ΔH^* is largely unchanged from the ΔH^* value of a similar alcoholysis, which itself is nearly the same as the ΔH^* of hydrolysis (Taft *et al.*, 1950). Thus, to a first approximation, it is likely that the ΔH^* for hydrolysis and ΔH^* for migration are approximately the same. No great error should occur when the 40,000 figure is used in the region 0–40°.

The chromatographic studies provide confirmation of the fact that inductive influences operate about equally on migration and hydrolysis. At *p*H 4.0 and at higher *p*H values the 2′ and 3′ isomers of valyladenosine and valyladenosine-5′-phosphate fail to separate. However, the 2′ and 3′ isomers of *N*-acetylvalyladenosine will separate up to *p*H 6.0. At this *p*H, the rate of migration is so high in comparison with the rate of travel on the chromatographic plate that no separation is possible. The estimated difference between the migration rate of valyladenosine and *N*-acetylvalyladenosine is approximately 100 by this method. The comparison of the rates of hydrolysis of *N*-acetylvalyladenosine and valyladenosine at *p*H 7.25 (McLaughlin and Ingram, 1965), where valyladenosine is largely protonated on the α-amino group, indicates that the rates differ by a factor of 110 (a Q_{10} of 2.4 must be assumed for this calculation [Rammler and Khorana, 1963]), in good agreement with the migration rate difference estimated from chromatography.

Aminoacyladenosine as Model Compounds for Aminoacyl s-RNA. No way of working directly with aminoacyl s-RNA has been devised, and it is necessary to inquire whether conclusions drawn from the model compounds are valid for aminoacyl s-RNA. The rates of alkaline hydrolysis of valyl s-RNA and valyladenosine are similar (McLaughlin and Ingram, 1965; Zachau, 1960).

In spite of the fact that space-filling molecular models indicate that there are differences among the model compounds studied in terms of possible hydrogen bonds and salt linkages that could be formed, there is very little difference in the equilibrium distribution of acyl groups (Table I). Thus, all of the valyladenosine compounds have an equilibrium distribution of about 3:1 in favor of the 3′ ester, which indicates that the equilibrium in valyl s-RNA is also about 3:1 in favor of the 3′ ester at *p*H values where the adenosine amino group is uncharged.

The evidence suggests that the majority of the amino acids have a similar distribution of isomers. Our work (McLaughlin and Ingram, 1965) and the work of Wolfenden, *et al.* (1964) show that the distribution of isomers as determined chemically for mixed neutral aminoacyladenosines, valyladenosine and phenylalanyladenosine, is the same in all cases (1:2). Almost the same ratio (2:1–3:1) has been obtained for valyladenosine in pentadeuteriopyridine by nuclear magnetic resonance spectroscopy (Feldmann and Zachau, 1964). Substantial modification in the valine moiety (see Table I) had little effect on the equilibrium. The results of the study of migration in the glycerol esters (Brandner and Birkmeir, 1960; Crossly *et al.*, 1959; Hatch and Adkins, 1937; Lohuigen and Verkade, 1960) indicate that the acyl substituent does not influence the equilibrium distribution. One might conclude that, unless specific steric or charge-charge interactions are important, the distribution of isomers at equilibrium will be close to 3:1, in favor of the 3′ isomer, for all of the aminoacyl s-RNA molecules.

Physiological Significance of Aminoacyl Migration in Aminoacyl s-RNA. From the extensive data on the hydrolysis of aminoacyl s-RNA one can derive an estimate of the rate of migration of various aminoacyl s-RNA species. Meister and co-workers (Coles *et al.*, 1962) have determined the rate of hydrolysis of five aminoacyl s-RNA molecules, including glycyl s-RNA and valyl s-RNA at *p*H 7.25 and 37°. From their data and from the ratio of migration to hydrolysis in *N*-acetylvalyladenosine determined in the present experiments, one can estimate that the average migration lifetime (the average lifetime is 1.44 times the half time) for glycyl s-RNA is 0.01 second and that for valyl s-RNA is 0.1 second under the above conditions. Since these two amino acids are respectively the least and the most sterically hindered, the migration lifetimes of other aminoacyl s-RNA molecules should be between these two values. Insofar as the *p*H at the site of protein synthesis is higher than *p*H 7.25, the migration lifetimes will be lower than the present estimate. One assumes that the *p*H dependence of the rate of migration will closely resemble that of the rate of hydrolysis (Wolfenden, 1963).

On the other hand, an estimate of the *in vivo* lifetimes of aminoacyl s-RNA molecules can be made, subject to some uncertainty. An upper limit for the lifetime of an aminoacyl s-RNA molecule can be obtained from the experimentally determined over-all rate of protein synthesis and the number of s-RNA molecules participating in the synthesis. This value would represent the actual lifetime of an aminoacyl s-RNA molecule if all of the turnover in aminoacyl s-RNA was due to protein synthesis, if all of the s-RNA molecules existed *in vivo* as aminoacyl s-RNA molecules, and if all of this estimated time was available for migration. In rapidly growing *E. coli* cells the first assumption appears to be largely correct, since Lacks and Gros (1959) found that the rate of incorporation of amino acids into aminoacyl s-RNA was largely, though not completely, dependent on the rate of protein synthesis. The second assumption, too, may be justified, because most of the s-RNA in pancreas (Acs *et al.*, 1959), liver (Zachau *et al.*, 1958), and *E. coli* (Lacks and Gros, 1959) can be isolated as aminoacyl s-RNA. Considerations of the regulation of RNA synthesis have led to the concept that ribosomal RNA synthesis only occurs when substantial amounts of aminoacyl s-RNA are present (Gros *et al.*, 1963; Kurland and Maaloe, 1962; Stent and Brenner, 1961)

The evidence indicates that, of the s-RNA in a cell, very little is closely associated with either a protein component (Tissières, 1959) or the ribosome fraction (Warner and Rich, 1964). This suggests that most of the aminoacyl s-RNA molecules are free as far as acyl migration is concerned. Table II lists the average lifetime of aminoacyl s-RNA molecules calculated for several organisms. These estimates represent the upper limit for the lifetime of an aminoacyl s-RNA molecule. It is difficult to arrive at an estimate of a lower limit for the lifetime of an aminoacyl s-RNA molecule, because such a limit depends on the extent to which the above three assumptions are not justified. However, it seems unlikely that the above estimate would be low by a factor of over a hundred. Thus it is possible that *in vivo* aminoacyl s-RNA exists in the equilibrium distribution of 2′ and 3′ isomers and that this distribution favors the 3′ isomer by a factor of about 3:1.

TABLE II: Lifetime of Aminoacyl s-RNA Molecules.

Systems	Lifetime of Amino-acyl s-RNA[a]	Rate of Peptide Bond Forma-tion[a]
Rabbit reticulocyte (Lingrel and Borsook, 1963)	20	0.4
Rabbit reticulocyte (cell free) (Allen and Schweet, 1962)	100	10.0
E. coli (Bolton, 1959)	1	0.03
Hela cells (Darnell *et al.*, 1963)[b]	10	0.4

[a] Time in seconds. [b] And personal communication.

Zachau and his co-workers (Sonnenbichler *et al.*, 1963; Feldmann and Zachau, 1964) have reported that isolation of mixed aminoacyladenosines on an IRC-50 column gave 90–95% of the 3′ isomer, as determined by chemical and nuclear magnetic resonance methods. The isolation technique involves exposure to 1 M acetic acid for almost a day. One can calculate from the observed migration that the half-life of migration of valyladenosine under these conditions would be 2 to 3 days. Most of the other aminoacyladenosines besides valine and even leucine and phenylalanine should have migrated substantially to a new equilibrium distribution under the isolation conditions. The proportions of isomers obtained after Zachau's isolation procedure may therefore not reflect the proportions originally present in the incubation. Neither of the explanations put forward by these authors is convincing, and it seems unlikely that the equilibrium at physiological *p*H can be 95% 3′, 5% 2′ isomers. Calculations indicate that for base-catalyzed migration our isolation system should cause over 100-fold less acyl migration than does the isolation system which Zachau and co-workers had developed to prevent acyl migration. For acid- or water-catalyzed migration the situation is much the same. However, the calculations also indicate that most of the base-catalyzed acyl migration occurs during the initial incubation rather than during isolation of the aminoacyladenosine. If it is assumed that aminoacyl migration will show the same *p*H dependence as does the hydrolysis of aminoacyl s-RNA (Wolfenden, 1963), then we feel that, so far as base-catalyzed migration is concerned, the initial incubation conditions for the present system and for that developed by Zachau and co-workers are similar. The initial incubation involved 45 minutes at *p*H 7.5 (35°), and under these conditions the estimated half-life of migration of valine on s-RNA is less than 0.1 second.

There is at the present time no experimental evidence on the initial site of acylation, but this is in any case not relevant, because rapid acyl migration would give the equilibrium distribution regardless of which acyl isomer was formed initially. Biologically, one would expect only one isomer to be effective at the *ribosomal* level. The unused isomer might not bind to the ribosome or a specific enzyme might be available to catalyze the migration at the ribosome. The rates of peptide bond formation listed in Table II suggest that the normal base-catalyzed rate of acyl migration might be rapid enough to supply the proper isomer without the necessity for an enzyme-catalyzed reaction. The time required to form a peptide bond is in all cases greater than or equal to the average lifetime for migration to equilibrium.

Isomerization (2′ ↔ 3′) of the growing peptide chain would occur at a slower rate than that of the aminoacyl s-RNA. The rate of hydrolysis of polyphenylalanyl s-RNA has been determined. Comparison of this rate of hydrolysis with that determined here for *N*-acetylvalyladenosine demonstrates that *N*-acetylvalyladenosine is a fairly good model compound for the growing peptide chain s-RNA compound, as expected on chemical grounds. At *p*H 7.25, the difference in migration rates between the aminoacyl s-RNA and the growing peptide chain s-RNA is estimated to be about 100-fold based on the comparison of the rate of hydrolysis of valyladenosine and *N*-acetylvalyladenosine at *p*H 7.25. At this *p*H, the migration lifetimes of the growing peptide chain s-RNA should lie somewhere between 2 and 20 seconds. Table II shows that the synthetic times of a peptide chain vary between 4 and 60 seconds *in vivo*. One would, therefore, expect an occasional migration to occur during chain synthesis. Here, the suggestion that an enzyme prevents isomerization, or rather shifts the equilibrium in favor of one isomer, becomes more likely.

Cell-free protein synthesis takes place at a much slower rate in the reticulocyte system (Table II). The rate of peptide bond formation is so slow (10 seconds/bond) that it is comparable to the predicted migration

lifetime of the growing peptide chain (2 to 20 seconds). The reason for the lower rate of peptide bond formation in such a system is not clear, but migration of the peptide chain s-RNA in the cell-free system is a possibility.

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