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Some Observations Relating to Acyl Mobility in Aminoacyl Soluble Ribonucleic Acids*

B. E. Griffin, M. Jarman,† C. B. Reese, J. E. Sulston,† and D. R. Trentham

ABSTRACT: A technique, based on nuclear magnetic resonance spectroscopy, has been developed for determining the rate of acyl migration in ribonucleoside derivatives. In buffered dimethyl sulfoxide solution at 20°, the rate of conversion of 3′-*O*-formyladenosine into an equilibrium mixture of 2′ and 3′ isomers has been found to be *ca.* 7000 times faster than the equilibration rates of 3′-*O*-acetyladenosine and -uridine. The rate of hydrolysis of 2′(3′)-*O*-formyladenosine in 0.1 M phosphate buffer (pH 7) at 20° is close to that reported for an average aminoacyl derivative of adenosine or soluble ribonucleic acid (s-RNA), but *ca.* 230 times faster than that of 2′(3′)-*O*-acetyladenosine. The equilibration and hydrolysis rates of 2′(3′)-*O*-acyl derivatives of adenosine are slightly (10–15%) faster than those of the corresponding uridine derivatives. The respective half-times of hydrolysis and equilibration of 2′(3′)-*O*-acetyluridine in 0.1 M phosphate buffer at 20° are *ca.* 30 days and 7.5 sec; their ratio is 350,000.

From these data and a suitable temperature correction, the equilibration half-time of an average aminoacyl-s-RNA derivative has been estimated to be *ca.* 2×10^{-4} sec. Thus such derivatives are likely to exist in living organisms as mixtures of 2′ and 3′ isomers.

In the process of *in vivo* protein synthesis, it has been established (Zachau *et al.*, 1958; Preiss *et al.*, 1959; Hecht *et al.*, 1959) that each amino acid is esterified to one of the hydroxyl groups of the *cis*-2′,3′-diol system of the terminal adenosine residue of a specific soluble ribonucleic acid (s-RNA) molecule before it is transferred in the reaction or reactions leading to the formation of a peptide bond (step b). However, it is not known whether the aminoacyl group is attached to the 2′- or the 3′-hydroxyl function of the adenosine residue when this acylation reaction occurs. Indeed, it is not even clear that this is a meaningful question to consider.

$$\text{s-RNA} + \text{aminoacyl-AMP}^{1} \rightleftharpoons \text{aminoacyl-s-RNA} + \text{AMP} \quad \text{(a)}$$

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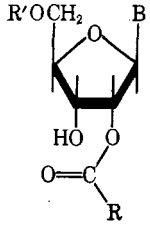
[1] Adenosine 5′-monophosphate is abbreviated as AMP. Aminoacyl-AMP represents the mixed anhydride of an amino acid and AMP; aminoacyl-s-RNA represents an aminoacyl derivative of s-RNA in which the amino acid is esterified to the 2′- (or 3′-) hydroxyl group of the terminal adenosine residue.

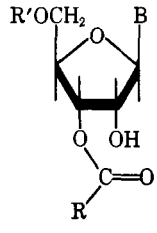
aminoacyl-s-RNA + peptide ⟶
aminoacyl peptide + s-RNA (b)

It may be argued, largely on the basis of the observed effect of puromycin on protein synthesis (Nathans and Neidle, 1963), that 3′-*O*-aminoacyl-s-RNA is more likely to be the active acylating species. Even if this were so, it does not follow that aminoacylation of s-RNA (step a) leads directly to the 3′ isomer (Zamecnik, 1962). There is evidence in the chemical literature (Brown *et al.*, 1956; Neilson, 1957) that the 2′-hydroxyl groups of both adenosine and uridine are more open to electrophilic attack than the 3′-hydroxyl groups. Thus the possibility of acyl migration between steps a and b must be considered.

Two distinct approaches have been made to this problem of aminoacyl-s-RNA orientation. The first approach, which has been investigated in several laboratories (Sonnenbichler *et al.*, 1963; Feldmann and Zachau, 1964; Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965a), involves a direct attempt at orientation by chemical and physical methods. For this to be valid, all reactions and measurements must be carried out under conditions which do not promote acyl migration. The second approach, followed by two of the above laboratories (Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965b), involves the estimation of the actual rate of acyl migration in model compounds, under carefully specified conditions. As it is well known that monoacyl derivatives of *cis*-1,2-diol systems readily isomerize (Ness and Fletcher, 1956; Kupchan *et al.*, 1962; Reese and Trentham, 1965b), it seemed to us that, before the problem of the actual orientation of aminoacyl-s-RNA could be undertaken with confidence, reliable estimates of the rates of aminoacyl migration under physiological conditions were required. In the present report, we wish to describe experiments which we believe enable satisfactory estimates of such rates to be made.



I

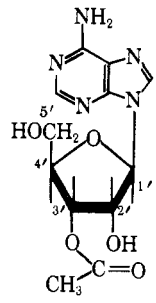


II

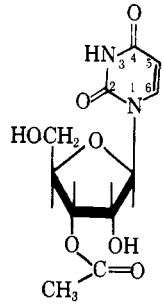
Recently a general method for the synthesis (Reese and Sulston, 1964) of 2′- and 3′-*O*-acyl ribonucleoside derivatives (I and II, respectively) has been developed. The orientation of such compounds has been established both by a simple chemical method (for uridine derivatives) (Reese and Trentham, 1965a) and, more generally, by proton magnetic resonance spectroscopy (Fromageot *et al.*, 1966). Very recently, Žemlička and Chládek (1965) have prepared 2′- and 3′-*O*-glycyl derivatives of uridine and adenosine by the above ortho ester exchange procedure, but these workers have not reported the isolation of a pure isomer.

In some previous acyl migration studies (Reese and Trentham, 1965b) which were carried out in anhydrous pyridine solution, it was observed that different acyl groups migrated at widely differing rates, but that the equilibrium ratios of 3′ to 2′ isomers varied only between *ca.* 1.4 and 2.0. Although these results were of interest, they did not allow an estimate of the rate of acyl migration in aminoacyl-s-RNA to be made. In order to achieve this end, it was necessary to carry out measurements in aqueous solution, and thus simulate physiological conditions more closely. Therefore, a water-soluble model compound, and a technique which would enable us to determine the compositions of mixtures of isomeric esters in aqueous solution at *ca.* pH 7, were required.

Results and Discussion



III



IV

As adenosine esters undergo solvolysis, and thus probably also equilibration, at much the same rates as the corresponding esters of adenosine 5′-monophosphate and s-RNA (Zachau *et al.*, 1958; Zachau, 1960), it seemed reasonable to use a 2′ or 3′ ester of adenosine as a model for aminoacyl-s-RNA. Furthermore, as acyl migration appears to be a general reaction of ribonucleoside 2′(and 3′)-carboxylate esters (Reese and Trentham, 1965b), it seemed justifiable to work with a less mobile model system than a 2′- or a 3′-*O*-aminoacyladenosine derivative, and then to make a suitable rate adjustment. In any case, it was possible that the rate of aminoacyl migration in aqueous solution at pH 7 would be too great to be measurable by any available technique.

The use of nuclear magnetic resonance spectroscopy for distinguishing between isomeric 2′- and 3′-ribonucleoside derivatives (Fromageot *et al.*, 1966) has now been developed into a technique for estimating the proportions of such isomers in a mixture, and also for measuring the rate of acyl migration in ribonucleoside systems. Previous work led to the formulation of two empirical rules for distinguishing between pairs of 2′ and 3′ isomers. The more general rule (the chemical shift rule) states that the glycosidic proton of the 2′ isomer is more deshielded (*i.e.*, the H(1′) resonance occurs at lower field) than that of the 3′ isomer. Figure 1a illustrates the nuclear magnetic resonance spectrum of an equilibrium mixture of 2′- and 3′-*O*-acetyl-

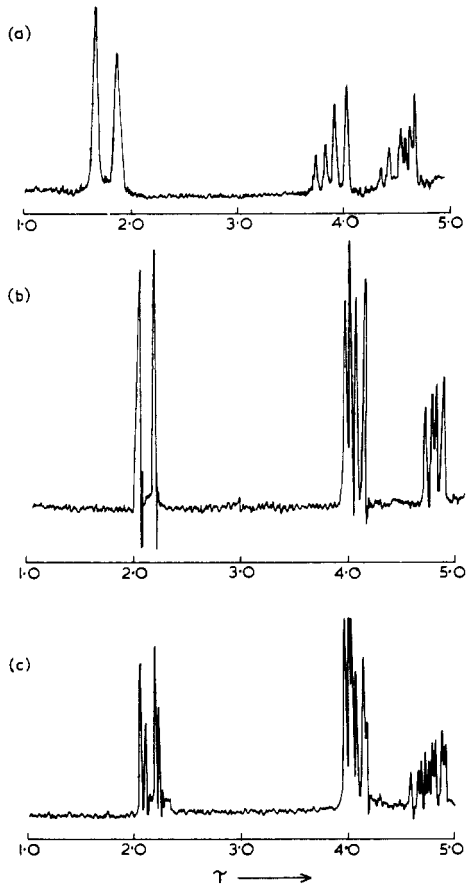
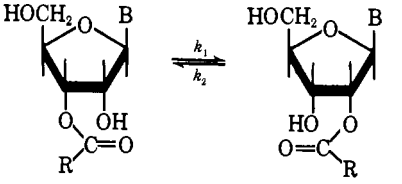


FIGURE 1: Nuclear magnetic resonance spectra (60 Mcycles/sec) of (a) equilibrium mixture of 2′(3′)-*O*-acetyladenosines in D_2O, (b) pure 3′-*O*-acetyluridine in D_2O (0.1 M with respect to acetic acid), and (c) equilibrium mixture of 2′(3′)-*O*-acetyluridines in D_2O.

adenosines in deuterium oxide solution; the less intense doublet corresponding to the H(1′) resonance of the 2′ isomer (at τ 3.80) is at lower field than that corresponding to the H(1′) resonance of the more abundant 3′ isomer (at τ 3.98).

In view of the above considerations, the readily available (H. P. M. Fromageot, B. E. Griffin, C. B. Reese, and J. E. Sulston, unpublished data) crystalline 3′-*O*-acetyladenosine (III) was considered as a suitable model compound for migration studies, but unfortunately (unlike the equilibrium mixture of 2′ and 3′ isomers; see Figure 1a) it was insufficiently soluble in aqueous buffer to permit satisfactory nuclear magnetic resonance spectra to be obtained. However, 3′-*O*-acetyluridine (IV), which was also readily available as a pure crystalline solid (Reese and Sulston, 1964), had the necessary solubility properties. The latter (IV) may be considered to be as suitable a model compound as 3′-*O*-acetyladenosine (III), since both compounds display similar isomerization rates in buffered dimethyl sulfoxide (see Table I and Figure 2) and similar hydrolysis rates in 0.1 M sodium phosphate buffer (pH 7) (see Table II and Figure 3).

The nuclear magnetic resonance spectra of deuterium oxide solutions of 3′-*O*-acetyluridine and the equilibrium mixture of 2′ and 3′ isomers are illustrated in Figure 1b and c, respectively. An examination of the τ 4 region of Figure 1b indicates that the H(1′) and H(5) resonance signals (see Fromageot *et al.*, 1966) of 3′-*O*-acetyluridine (IV) overlap, and an examination of the corresponding region of Figure 1c reveals an absorption pattern which is far too complex to allow an estimate of the proportions of 2′- and 3′-*O*-acetyluridines, in the mixture, to be made. However, the H(6) doublets for the two isomers (at τ 2.14 and 2.17) are separated by *ca.* 0.03 ppm, the doublet for the 2′ isomer being at higher field. It has been established (see Experimental Section) that the ratio of the heights of the low-field (or high-field) signals of the H(6) doublets can lead to a reliable estimate of the relative proportions of the 3′ and 2′ isomers. Figure 4 illustrates the H(6) resonance region of the nuclear magnetic resonance spectra of (a) pure 3′-*O*-acetyluridine, (e) the equilibrium mixture, and (b–d) other mixtures of isomers.



(1)

Acyl migration is an equilibration reaction, and therefore kinetic measurements lead to a value for the sum of the forward and backward rate constants ($k_1 + k_2$). If the equilibrium constant (k_1/k_2) is known, then the individual rate constants may be calculated. For such a reaction, the rate of equilibration has been shown to be proportional to the concentration of the substrate and to the concentration of hydroxide ions (see Wolfenden *et al.*, 1964); at constant pH, it is expected to follow a first-order law according to the equation (Frost and Pearson, 1953) $\ln [(A_0 - A_e)/(A - A_e)] = (k_1 + k_2)t$, where A_0, A_e, and A represent the respective concentrations of a particular isomer initially, at equilibrium, and after a time, t. All our experiments were carried out at pH 7 in order to approximate physiological conditions. As a preliminary experiment indicated that 3′-*O*-acetyluridine (IV) underwent equilibration extremely rapidly in pH 7 phosphate buffer, rate measurements were conducted at 20° rather than at 37°. The variation of ln Q, where $Q = [(A_0 - A_e)/(A - A_e)]$, with time for this reaction is illustrated in Figure 5a: the gradient of the straight-line plot obtained, 5.4 min^{-1}, is a measure of $k_1 + k_2$. From this result, it can be calculated that the half-time ($t_{1/2}$) of equilibration of 3′-*O*-acetyluridine in 0.1 M sodium phosphate buffer (pH 7) at 20° is *ca.* 7.5 sec (see Table I).

If an estimate of the equilibration rate of 3′-*O*-acetyluridine at 37° was required, it was necessary to

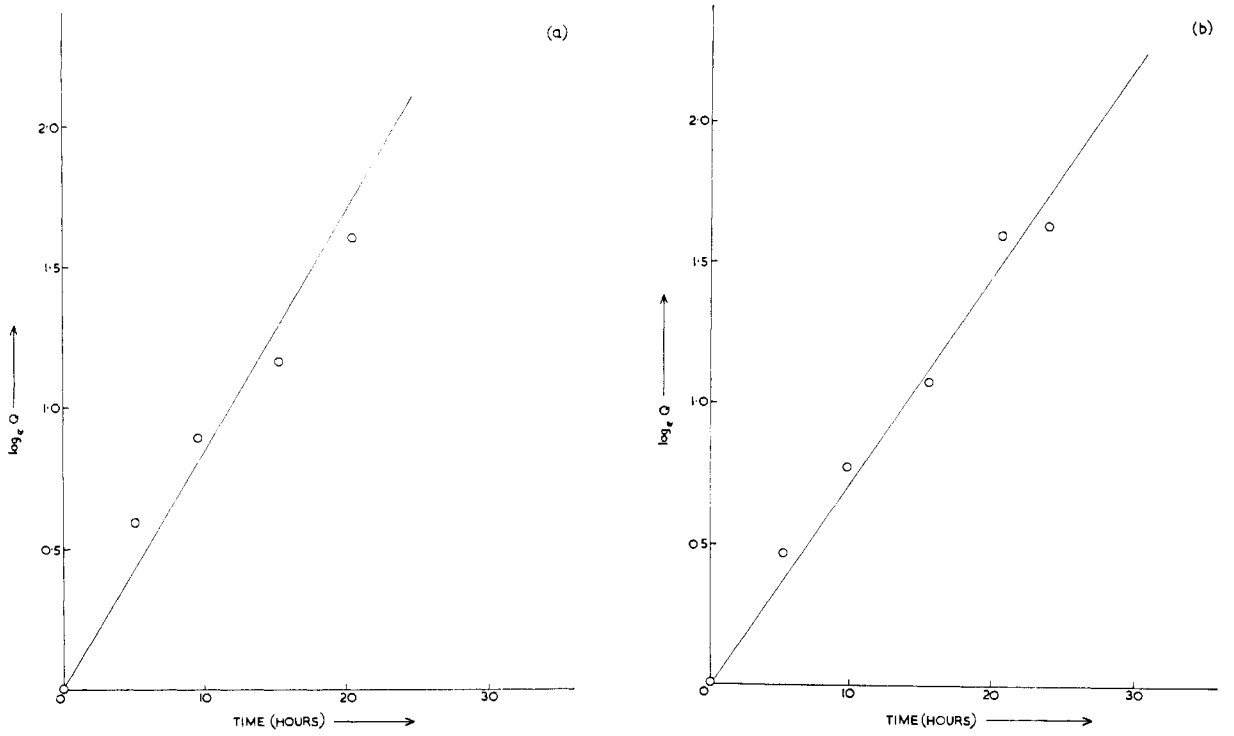


FIGURE 2: Rate plots of equilibration in dimethyl sulfoxide–0.2 M sodium phosphate buffer (pH 7) (8:1, v/v) at 20° of (a) 3′-*O*-acetyladenosine and (b) 3′-*O*-acetyluridine.

TABLE I: Rates of Equilibration of Acyl Ribonucleoside Derivatives.

Nucleoside Derivative	Solvent[a]	Temp (°C)	$k_1 + k_2$ (min^{-1})	$t_{1/2}$ (min)	k_2/k_1[b]
3′-*O*-Acetyluridine	0.1 M phosphate buffer (pH 7)	5	0.95	0.73	1.7
3′-*O*-Acetyluridine	0.1 M phosphate buffer (pH 7)	20	5.4	0.128	1.7
3′-*O*-Acetyluridine	Me_2SO–buffer	20	1.21×10^{-3}	5.7×10^2	1.7
3′-*O*-Acetyladenosine	Me_2SO–buffer	20	1.41×10^{-3}	4.9×10^2	2.7
3′-*O*-Acetyladenosine	Me_2NCHO–buffer	20	1.56×10^{-3}	4.4×10^2	3.0
3′-*O*-Formyladenosine	Me_2SO–buffer	20	8.82	0.079	4.3
3′-*O*-Formyladenosine	Me_2NCHO–buffer	20	11.3	0.061	4.6

[a] The organic solvent–buffer mixtures contain eight parts (v/v) of dimethyl sulfoxide or dimethylformamide and one part of 0.2 M phosphate buffer (pH 7). Buffer is added to avoid anomalous effects due to traces of acids or bases, and also to accelerate the reactions. [b] This is the reciprocal of the equilibrium constant of the reaction illustrated in reaction 1.

study its isomerization at an additional temperature; at 5°, its equilibration rate constant in 0.1 M sodium phosphate buffer (pH 7) was found to be 0.95 min^{-1} (see Figure 5b and Table I). If the Arrhenius equation is applied to the rates observed at 5 and 20°, the half-time of equilibration of 3′-*O*-acetyluridine (IV) in 0.1 M sodium phosphate buffer (pH 7) at 37° can be calculated to be *ca.* 1.4 sec.

In its present form, this nuclear magnetic resonance technique could not be used to determine the rates of equilibration reactions with half-times below *ca.* 5 sec, and so the limit of its applicability was almost reached in the study of the isomerization of 3′-*O*-acetyluridine (IV) in pH 7 phosphate buffer at 20°. It was therefore clear that a different solvent system would be required if the migration of more mobile

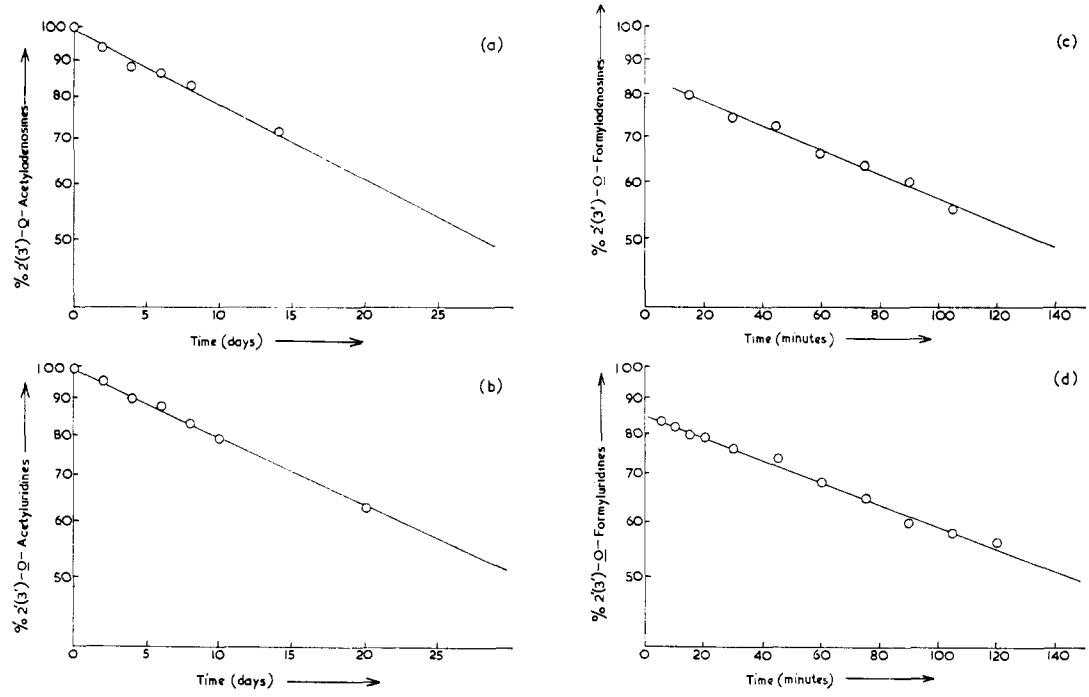


FIGURE 3: Rate plots of hydrolysis in 0.1 M sodium phosphate buffer (pH 7) at 20° of (a) 2′(3′)-*O*-acetyladenosines, (b) 2′(3′)-*O*-acetyluridines, (c) 2′(3′)-*O*-formyladenosines, and (d) 2′(3′)-*O*-formyluridines. The ordinate scales are logarithmic.

TABLE II: Rates of Hydrolysis of Acyl Ribonucleoside Derivatives at 20° and pH 7 (0.1 M Phosphate Buffer).

Nucleoside Derivative	Rate Constant (min^{-1})	$t_{1/2}$ (min)
2′(3′)-*O*-Acetyluridine[a]	1.54×10^{-5}	4.50×10^{4}
2′(3′)-*O*-Acetyladenosine[a]	1.71×10^{-5}	4.05×10^{4}
2′(3′)-*O*-Formyladenosine[a]	3.95×10^{-3}	1.75×10^{2}
2′(3′)-*O*-Formyluridine[b]	3.60×10^{-3}	1.92×10^{2}

[a] The pure 3′-*O*-acyl nucleosides were rapidly converted into equilibrium mixtures of 2′ and 3′ isomers. This is unimportant as hydrolysis is much slower than equilibration and is thus the rate-determining process. [b] This mixture of isomers was generated *in situ* by treating 2′,3′-*O*-methoxymethylideneuridine with 0.01 N hydrochloric acid.

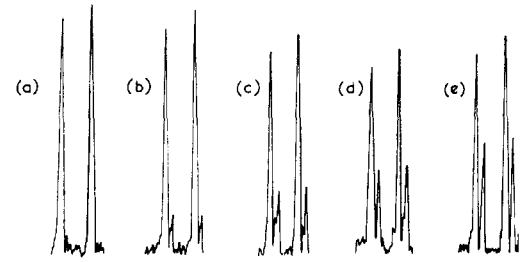


FIGURE 4: H(6) resonance signals, in acidified aqueous solutions of (a) pure 3′-*O*-acetyluridine, (b) mixture of isomers containing 10.5% 2′-*O*-acetyluridine, (c) mixture of isomers containing 22% 2′-*O*-acetyluridine, (d) mixture of isomers containing 27% 2′-*O*-acetyluridine, and (e) equilibrium mixture of isomers (containing 37% 2′-*O*-acetyluridine).

acyl groups were to be studied. Dimethyl sulfoxide, which is a good solvent for nucleoside derivatives, was selected, but as the rate of equilibration is pH dependent, it was necessary to add buffer to it. The first-order rate plots obtained for 3′-*O*-acetyladenosine (III) and 3′-*O*-acetyluridine (IV) in buffered dimethyl sulfoxide solution at 20° are illustrated in Figure 2. From Table I, it can be seen that, although the equilibration rates of the two compounds differ only by *ca.* 15%, they are both very much slower than that of 3′-*O*-acetyluridine in pH 7 aqueous buffer. This decrease in rate of *ca.* 4×10^{3} offered an opportuntiy of comparing much more mobile acyl groups with acetyl directly.

Although several mixed 2′ (and 3′)-*O*-aminoacyl derivatives of adenosine have been described (Zachau, 1960; Rammler and Khorana, 1963; Shabarova *et al.*,

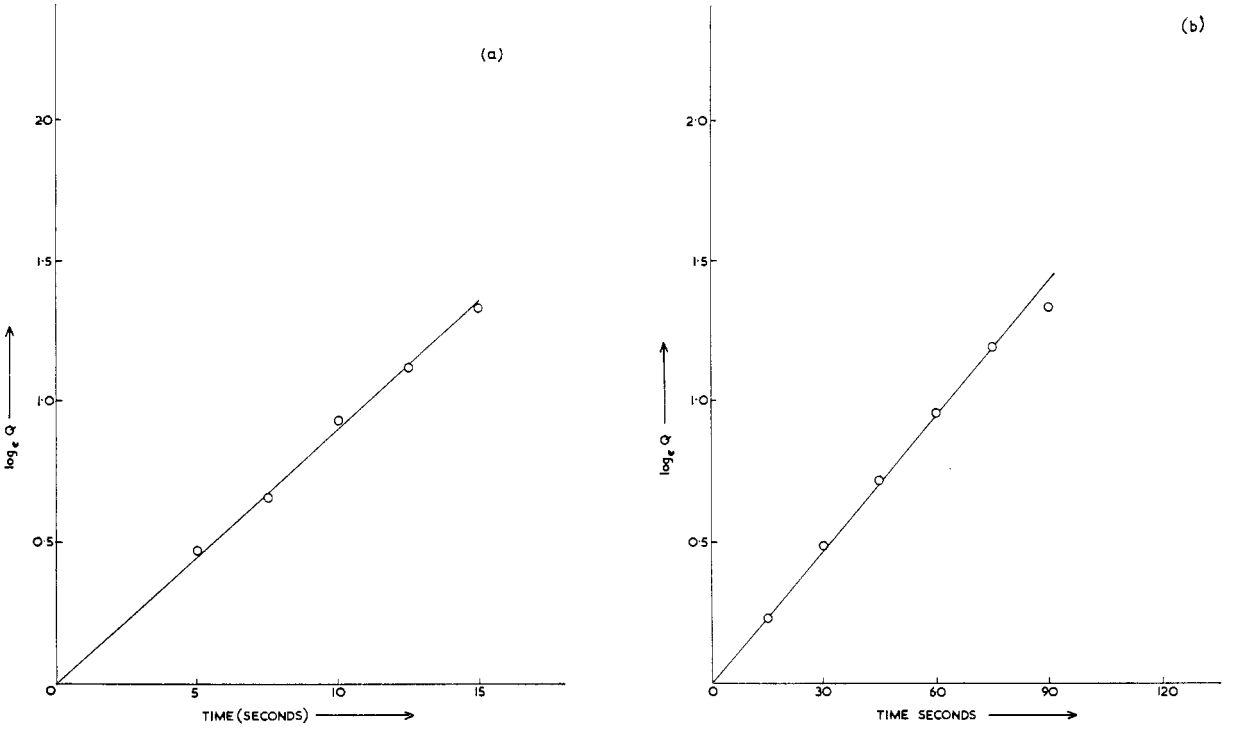
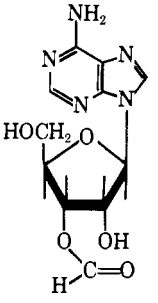


FIGURE 5: Rate plots of equilibration of 3′-*O*-acetyluridine in 0.1 M sodium phosphate buffer (pH 7) at (a) 20° and (b) 5°.



V

1964; Žemlička and Chládek, 1965), no pure isomers were available for acyl migration studies. However, it was possible to isolate pure crystalline 3′-*O*-formyladenosine (V) in 90% yield from the products of hydrolysis of 2′,3′-*O*-methoxymethylideneadenosine (Jarman and Reese, 1964) with 96% formic acid. This compound (V), the orientation and purity of which were established by nuclear magnetic resonance spectroscopy, underwent hydrolysis in 0.1 M phosphate buffer (pH 7) at a rate (see Table II and Figure 3c) within the range reported for aminoacyl-s-RNA and aminoacyladenosine derivatives (Coles *et al.*, 1962; Wolfenden, 1963; Rammler and Khorana, 1963), and thus it seemed possible that it would undergo acyl equilibration at a rate comparable with the latter systems (see discussion below). The first-order rate plot for the equilibration of 3′-*O*-formyladenosine (V) in buffered dimethyl sulfoxide at 20° is illustrated in Figure 6. It can be seen from Table I that the rate of this reaction is *ca.* 7000 times as fast as the rate of equilibration of 3′-*O*-acetyluridine (IV) under the same conditions. Additional evidence for the relatively high mobility of the formyl group comes from the observation (see Table I) that the ratios of the equilibration rates of 3′-*O*-formyl- and 3′-*O*-acetyladenosines (V and III, respectively) in buffered dimethylformamide and buffered dimethyl sulfoxide are of the same order.

If it is assumed that this formyl/acetyl rate ratio of *ca.* 7000 remains unchanged in 0.1 M phosphate buffer (pH 7) at 20°, then it follows that the half-time of equilibration of 3′-*O*-formyladenosine (V), in the latter system, would be *ca.* 0.001 sec. If it is further assumed that the ratio of the equilibration rates of 3′-*O*-formyladenosine (V) at 37 and 20° is the same as for 3′-*O*-acetyluridine (IV) (see above), then the estimated half-time of equilibration of the former (V) at 37°, in 0.1 M phosphate buffer (pH 7), would be 1.8×10^{-4} sec. Thus, if all the above approximations are made, the present studies lead to an estimated value of *ca.* 2×10^{-4} sec for the half-time of equilibration of an average aminoacyl-s-RNA system in a neutral buffered medium at 37°.

Studies with various aminoacyl-s-RNA derivatives and related systems (Coles *et al.*, 1962; Wolfenden, 1963; Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965b) have shown that their hydrolysis rates depend both on substrate and on hydroxide ion concentration and that hydrolysis, like acyl migration, displays first-order kinetics at constant pH. It has been assumed that the ratio of the rates of acyl equilibration and

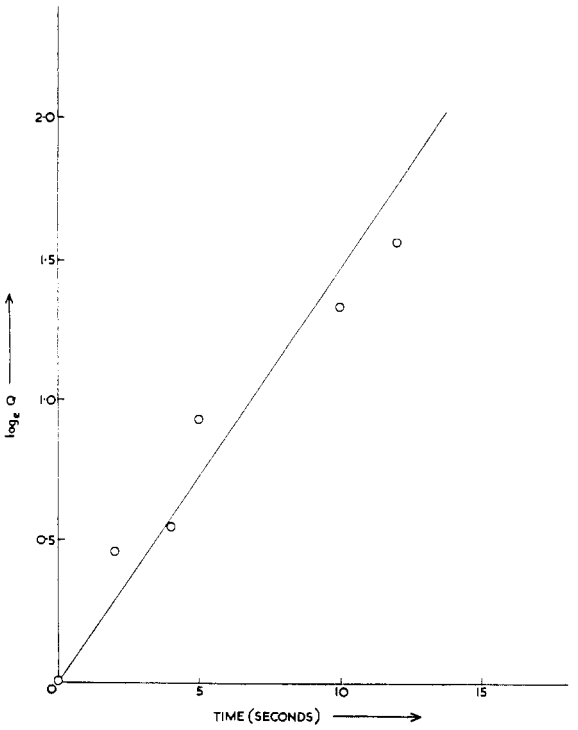
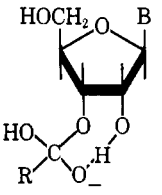


FIGURE 6: Rate plot of equilibration of 3′-*O*-formyladenosine in dimethyl sulfoxide–0.2 M sodium phosphate buffer (pH 7) (8:1, v/v) at 20°.

hydrolysis for a monoacyl derivative of a particular diol system might be independent of the nature of the acyl group (Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965b). If this were so, in order to determine the rate of acyl equilibration in any 2′- (or 3′-) *O*-acyl ribonucleoside derivative, it would only be necessary to determine the ratio of its rate of hydrolysis in 0.1 M phosphate buffer (pH 7) and that of 3′-*O*-acetyluridine (IV); the product of this ratio and the known rate of equilibration of IV would lead to the desired result. In order to examine the validity of this assumption, the rates of hydrolysis, in 0.1 M phosphate buffer at 20°, of 2′(3′)-*O*-acetyladenosines and -uridines, and the corresponding formyl derivatives, were determined. The results are listed in Table II, and the first-order rate plots obtained are illustrated in Figure 3.

It can be seen from Table II that the rate of hydrolysis of 2′(3′)-*O*-formyladenosines is *ca.* 260 times greater than that of 2′(3′)-*O*-acetyluridines. Thus, if the ratio of the rates of acyl equilibration and hydrolysis is assumed to be independent of the nature of the acyl group, then it follows that the half-time of equilibration of 3′-*O*-formyladenosine, at 20° in pH 7 phosphate buffer, would be *ca.* 0.03 sec, *i.e.*, 30 times greater than our original estimate. The choice of 3′-*O*-formyladenosine (V) as a model for an aminoacyl-s-RNA system is based on its half-time of hydrolysis (175 min in pH 7 phosphate buffer at 20°), which is longer than that reported for the corresponding phenylalanine ester (48 min in the same buffer at 25°) (Rammler and Khorana, 1963), but, if a correction is made for temperature and pH, most likely shorter than that reported for leucyl-s-RNA (27 min in pH 7.4 buffer at 37°) (Wolfenden, 1963).



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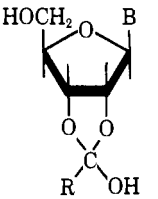

VII

The mechanisms of hydrolysis and acyl migration are likely to be different. It is well known that ester hydrolysis can be facilitated by the participation of a neighboring hydroxyl group (Bruice and Fife, 1962) which, it has been suggested (Kupchan *et al.*, 1962; Bruice and Fife, 1962), stabilizes the transition state following the attack on the substrate by an external nucleophile (*e.g.*, as in VI). However, it seems probable that acyl migration involves direct interaction between the neighboring hydroxyl group and the acyl carbonyl function, and that it proceeds *via* an ortho ester intermediate such as VII (Kupchan *et al.*, 1962). Although the rates of both hydrolysis and equilibration would be subject to the inductive effect of R, it is possible that the rate of one process would be more dependent on the size of R. If such were the case then the assumption that the ratio of the rates of acyl equilibration and hydrolysis is independent of the nature of the acyl group would not be valid. We therefore prefer our original estimate of *ca.* 2×10^{-4} sec for the half-time of equilibration of 3′-*O*-formyladenosine (V) in pH 7 aqueous buffer at 37°. However, if steric considerations are especially important in the transition states of acyl migration reactions, the comparatively unhindered 3′-*O*-formyladenosine (V) would not be a very satisfactory model for a corresponding aminoacyl system, despite its hydrolysis properties.

Before discussing the implications of the above results, it is of interest to compare them with those of other workers in this field. Wolfenden *et al.* (1964) studied the hydrolysis and isomerization of a model system, glycerol β-monoacetate, over a range of pH values, and observed pseudo-first-order kinetics for both these reactions. The rate constants obtained at pH 7 and 37° for base-catalyzed hydrolysis and isomerization were 0.86×10^{-5} and 0.056 min^{-1}, respectively. Thus the rate of hydrolysis of glycerol monoacetate at 37° is only about one-half that of 2′(3′)-*O*-acetyladenosine at 20°. This difference is possibly due to the more effective participation of the neighboring hydroxyl group in the hydrolysis of the latter more rigid system. As anticipated by Wolfenden *et al.* (1964), the difference is much more marked in the acyl migration reaction: the ratio of the rates of base-catalyzed migration and hydrolysis of glycerol β-monoacetate is 6500 at pH 7 and 37°, whereas the corresponding ratio for the rates of equilibration and hydrolysis of

2′(or 3′)-*O*-acetyluridine is *ca.* 350,000 at pH 7 and 20°. Thus studies with glycerol β-monoacetate have led to rather conservative estimates of the rates of acyl migration in aminoacyl-s-RNA derivatives. These estimates were based not only on a more flexible model diol system, but also on the questionable assumption (see above) that the ratio of the rates of migration and hydrolysis for an aminoacyl group is equal to that observed for the acetyl group.

The work most directly comparable with that presently under discussion involves studies on the isomerization and hydrolysis of a model system which is more closely related to aminoacyl-s-RNA. McLaughlin and Ingram (1965a,b) have stated that 2′(3′)-*O*-valyladenosines may be acetylated specifically on the valyl residue, and that the resulting 2′(and 3′)-*O*-(*N*-acetylvalyl)adenosines can be separated by thin layer chromatography. The orientation of these isomers is then based on their relative abundance in the equilibrium mixture. Although a certain amount of isomerization occurred during the isolation of the assumed 2′ isomer, these workers were able to study acyl migration in the pH range 5.0–7.0, and establish that the equilibration reaction displayed first-order kinetics at constant pH. The reaction rate at pH 7 (0.05 M phosphate buffer) and 15° was such that the half-time for equilibration was *ca.* 1.9 min. This rate is appreciably slower than that observed, under similar conditions,[2] for 3′-*O*-acetyluridine (IV), and thus that expected for 3′-*O*-acetyladenosine (III). This is a surprising result as *N*-acetylvaline is likely to be a much stronger acid than acetic acid and thus, on electronic grounds, the *N*-acetylvalyl would be expected to be more mobile than the acetyl group. Likewise, the reported rate of hydrolysis of 2′(3′)-*O*-(*N*-acetylvalyl)-adenosines was less than might be expected. This reaction was carried out in 0.05 M carbonate buffers (pH range 9.0–11.0) at 15°, and was found to follow a second-order law. From the appropriate pseudo-first-order rate constant, the half-time of hydrolysis may be calculated: at pH 7, it would be *ca.* 54 days, which is perhaps marginally greater than that of 2′(3′)-*O*-acetyladenosines (28 days, at 20°). It is further unexpected that the rate of hydrolysis of 2′(3′)-*O*-(*N*-acetylvalyl)adenosines should be more than 100 times slower than that of 2′(3′)-*O*-valyladenosines (McLaughlin and Ingram, 1965b), especially in the light of the report that the rate of hydrolysis of polyphenylalanyl-s-RNA is only 7 times slower than that of phenylalanyl-s-RNA (Gilbert, 1963). However, by making the assumption that the ratio of the equilibration and hydrolysis rates of a 2′- (or 3′-) *O*-acyladenosine derivative is constant (calculated to be 40,000), McLaughlin and Ingram (1965b) have estimated that at pH 7.25 and 37°, the equilibration half-times for glycyl- and valyl-s-RNA are 7×10^{-3} and 7×10^{-2} sec, respectively. Other aminoacyl groups are expected to have mobilities intermediate between those of glycyl and valyl.

Our approach to the study of acyl migration has the advantage of depending on a reasonably accurate and convenient physical method (*i.e.*, nuclear magnetic resonance spectroscopy) for the analysis of mixtures of acyl isomers. The only measurements which were likely to be subject to appreciable experimental error were the very fast equilibration rates of 3′-*O*-acetyluridine (IV) in phosphate buffer and of 3′-*O*-formyladenosine (V) in buffered organic media. However, we are confident that these rates are accurate to within *ca.* 20%. The difficulty of effecting rapid quenching may have led to some error, but this technique appeared to be satisfactory (see Experimental Section). We carried out all our migration studies with comparatively large quantities of pure crystalline 3′-*O*-acyl ribonucleoside derivatives, of definite composition and orientation. However, the methods used in the preparation and isolation of 2′(and 3′)-*O*-(*N*-acetylvalyl)adenosines (McLaughlin and Ingram, 1965a,b) discussed above were such that the characterization and orientation of these compounds were less rigorously established. Nevertheless, the results of McLaughlin and Ingram (1965b) are qualitatively similar to ours.

If a reliable technique is available for the study of acyl migration, the ultimate accuracy of the estimated rate of equilibration of an aminoacyl-s-RNA system depends on a suitable choice of model compounds and on the approximations made. The use of 3′-*O*-acetyluridine (IV) instead of the corresponding adenosine derivative III in the pH 7 phosphate buffer studies should introduce little error as both compounds undergo equilibration and hydrolysis at similar rates.[3] However, as indicated above, although 3′-*O*-formyladenosine (V) undergoes hydrolysis at a rate within the range reported for aminoacyl derivatives of adenosine, it is not clear whether it is an equally good model for equilibration studies, and thus an even more reliable estimate could be made if a pure 2′(or 3′)-*O*-aminoacyladenosine were available. The final assumption, that the ratio of the migration rates of two acyl groups would be the same in aqueous buffer as in buffered dimethyl sulfoxide, could only be avoided if a technique were available for the study of very fast acyl migration reactions with half-times of equilibration of the order of 0.001 sec.

Such are the approximations which have led to an estimate of *ca.* 2×10^{-4} sec for the half-time of equilibration of an average of aminoacyl-s-RNA derivative in pH 7 buffer at 37°. Unlike other workers we have measured all hydrolysis and equilibration rates at pH 7 (in aqueous buffer or buffered organic media). Indeed,

[2] For 3′-*O*-acetyluridine in 0.1 M phosphate buffer (pH 7) a 20°, $t_{1/2}$ = 0.13 min (see Table I). However, although the higher ionic strength would be expected to have little effect on the rate, a correction should be made for temperature; our Arrhenius plot indicates that $t_{1/2}$ = 0.23 min for 3′-*O*-acetyluridine at 15°.

[3] From Tables I and II it can be seen that the adenosine derivatives have equilibration and hydrolysis rates which are, respectively, *ca.* 15 and 10% higher than those of the corresponding uridine derivatives.

in the case of 3′-*O*-acetyluridine (IV), we were able to study equilibration and hydrolysis under conditions which differed only in the substrate concentrations, although the ratio of the rates of these processes was *ca.* 350,000.

It would appear from the present studies that it would be a futile exercise to undertake the orientation of an aminoacyl-s-RNA derivative by chemical or by any other methods. The equilibration rate is such that it would be impossible even to charge s-RNA *in vitro* under conditions which would not lead to an equilibrium mixture of 2′ and 3′ isomers. In this connection, the results of Zachau and co-workers (Sonnenbichler *et al.*, 1963, 1965; Feldmann and Zachau, 1964) call for some comment. These workers showed by nuclear magnetic resonance spectroscopy that equilibrium mixtures of synthetic 2′(3′)-*O*-valyl-, -alanyl-, -and methionyladenosines contained, as expected, *ca.* 30% of 2′ and 70% of 3′ isomer. However, the mixture of 2′(3′)-*O*-aminoacyladenosines, obtained by the enzymatic degradation of aminoacyl-s-RNA, appeared to contain over 90% of the 3′ isomer. It now seems likely that the s-RNA was charged under equilibrating conditions (Sonnenbichler *et al.*, 1963). As three of the six amino acids incorporated were valine, alanine, and methionine, these results do not appear to be self-consistent. These data could be rationalized if the aminoacyladenosines, isolated from aminoacyl-s-RNA, had undergone hydrolysis to adenosine before the nuclear magnetic resonance spectrum could be recorded. The H(1′) resonance regions of the spectra of adenosine and 3′-*O*-acyladenosines can be confused: acylation of adenosine on O(2′) causes the H(1′) resonance signal to shift *ca.* 0.2 ppm to lower field (Sonnenbichler *et al.*, 1965; Fromageot *et al.*, 1966), but acylation on O(3′) has a small or even negligible deshielding effect on H(1′).

In conclusion, if our estimate of the equilibration rate of an average aminoacyl-s-RNA system is correct, then equilibration would most likely be much faster than peptide bond formation (see Wolfenden *et al.* 1964; McLaughlin and Ingram, 1965b). If this were so, then the orientation (or equilibrium composition) of a particular species of aminoacyl-s-RNA would have little effect on its biological activity. Nevertheless, in an enzyme-controlled process such as protein synthesis, aminoacyl groups are likely to be transferred specifically from either the 2′ or the 3′ position. Similarly, aminoacyl groups are likely to be attached initially to a specific position which, due to the facility of acyl migration, may possibly be different.

Experimental Section

Nuclear magnetic resonance spectra were measured on a Perkin-Elmer spectrometer, operating at 60 Mcycles/sec, and on a Varian HA 100 spectrometer. Tetramethylsilane (for solutions in organic solvents) and *t*-butyl alcohol (for aqueous or D_2O solutions) were used as internal standards. Chemical shifts are expressed in parts per million on a τ scale; coupling constants (J) are expressed in cycles per second. The spectrophotometric estimations, in connection with the hydrolysis experiments, were made with a Zeiss Model PMQII ultraviolet spectrometer. Paper chromatography was conducted in the solvent system butan-1-ol–acetic acid–water (5:2:3). Ascending chromatography on Whatman No. 42 paper was used in the studies involving 2′(3′)-*O*-acetyl nucleosides; descending chromatography on Whatman No. 541 paper was used in the studies involving 2′(3′)-*O*-formyl nucleosides. Merck Kieselgel GF_{254} was used as the support for thin layer chromatography. Dimethyl sulfoxide and dimethylformamide, dried over No. 4A molecular sieves, were redistilled under reduced pressure (15 mm). The pH 7 buffer solutions were prepared by mixing 0.2 M (or 0.1 M) aqueous Na_2HPO_4 (6.1 ml) and 0.2 M (or 0.1 M) aqueous NaH_2PO_4 (3.9 ml); pH values were checked with a calibrated EIL Model 23A pH meter.

3′-O-Formyladenosine (*V*). 2′,3′-*O*-Methoxymethylideneadenosine (Jarman and Reese, 1964) (0.5 g) was dissolved in 96% formic acid (5 ml). After the resulting solution had been allowed to stand at 20° for 3 min, it was lyophilized to give a colorless glass, which was then dissolved in absolute ethanol (15 ml). The solution, which was filtered immediately, deposited 3′-*O*-formyladenosine (0.4 g, 90%) as colorless prisms, mp 166–167°. *Anal.* Calcd for $C_{11}H_{13}N_5O_5$: C, 44.7; H, 4.4; N, 23.7. Found (in material dried at 70° over KOH): C, 44.8; H, 4.7; N, 23.4. The ultraviolet absorption in dioxane showed λ_{max} 259 mμ (log ϵ 4.22); nuclear magnetic resonance spectrum of IIIb (0.06 g) in dimethyl sulfoxide–D_2O (0.4:0.05 ml) (1 N with respect to hydrogen chloride): τ 1.61 (a singlet, weight 1, assigned to H(2), 1.65 (a singlet, weight 1, assigned to H(8)), 1.79 (a singlet, weight 1, assigned to formyl proton), 4.04 (a doublet (J = 7.5 cycles/sec), weight 1, assigned to H(1′)), and 4.57 (a multiplet, weight 1, assigned to H(3′)).

Determination of Rates of Hydrolysis of 2′(3′)-O-Acyl Ribonucleoside Derivatives. A. 2′(3′)-*O*-Acetyladenosines and uridines. Solutions of the substrates (0.005 g) in 0.1 M sodium phosphate buffer (pH 7, 1 ml) were sealed in ampoules, which were kept at 20° in a thermostat. After suitable intervals of time, ampoules were opened and 3 N hydrochloric acid (0.03 ml) was added to their contents. The extent of hydrolysis, in each case, was determined in the following way: 0.025 ml of the acidified hydrolysate was applied to a paper chromatogram, which was then developed as described above. The spots corresponding to the starting material and the product (*i.e.*, adenosine or uridine) were cut out separately from the dried, developed chromatogram. An equivalent area of paper, which was required for a blank, was also cut out. Each of the three pieces of paper was cut into strips, and was allowed to stand with occasional shaking in a stoppered tube with 0.1 N hydrochloric acid (6 ml) for 24 hr at 20°. The optical densities of the two tubes containing nucleosidic material were then measured against the blank at λ_{max} (257 mμ for adenosine derivatives and 262 mμ for uridine derivatives). The rates of hydrolysis were

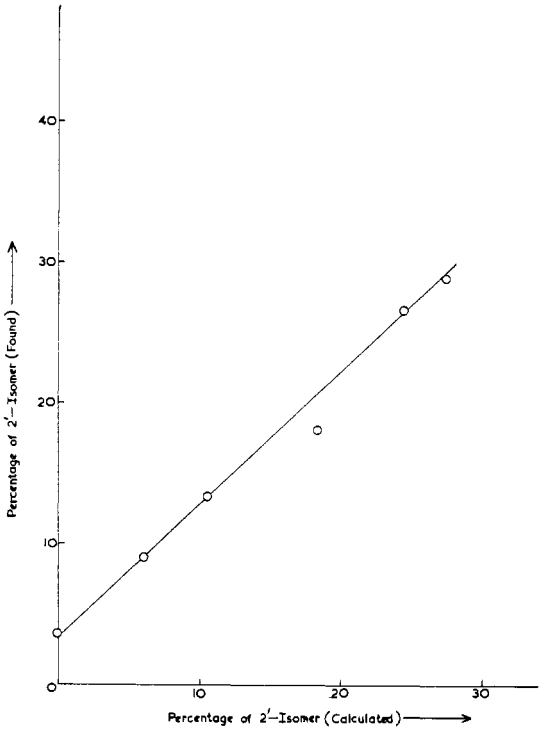

FIGURE 7: Plot of estimated (by nuclear magnetic resonance spectroscopy) against calculated percentages of 2′-*O*-acetyluridine in mixtures of 2′ and 3′ isomers.

then obtained from these data (see Table II and Figure 3). The pH of the contents of each ampoule was checked as soon as it was opened; it fell to 6.9 after *ca.* 50% hydrolysis had occurred.

B. 2′(3′)-*O*-FORMYLADENOSINES. Crystalline 3′-*O*-formyladenosine (0.04 g) was dissolved in 0.1 M sodium phosphate buffer (pH 7, 8 ml) and the solution was maintained at 20° in a thermostated bath. After suitable time intervals, aliquots (0.5 ml) were withdrawn and quenched with acetic acid (0.025 ml). The hydrolysis products were analyzed and the rate was determined as above (see Table II and Figure 3). After 120 min, the pH of the reaction solution had fallen to 6.9.

C. 2′(3′)-*O*-FORMYLURIDINES. 2′,3′-*O*-Methoxymethylideneuridine (Jarman and Reese, 1964) (0.04 g) was dissolved in 0.01 N hydrochloric acid (4 ml) and the solution was allowed to stand at 20° for 1 hr, after which 2′(3′)-*O*-formyluridines were the sole products (as indicated by thin layer chromatography). The pH was raised to 7 by the addition of 0.2 M sodium phosphate buffer (4 ml), and the reaction temperature was maintained at 20°. The course of the reaction was followed in the manner described above for 2′(3′)-*O*-formyladenosines. The results are indicated in Table II and Figure 3.

Determination of Rate of Equilibration of 3′-O-Acetyluridine in Aqeuous Solution at pH 7. To a magnetically stirred solution of 3′-*O*-acetyluridine (0.04 g) in 0.001 N hydrochloric acid (0.2 ml) at 20°, 0.2 M sodium phosphate buffer (pH 7, 0.2 ml), also at 20°, was added rapidly. After a suitable time interval (5–15 sec, see Figure 5a), the reaction was quenched by the rapid addition of 0.5 N hydrochloric acid (0.1 ml). The proportions of 2′ and 3′ isomers were then estimated in the manner described below. A separate experiment was conducted for each reaction time.

The procedure was repeated at 5°. The same technique and assay procedure (see below) were used, but the experiments were conducted in a cold room, maintained at 5°.

Quenching. Quenching by the addition of 0.5 N hydrochloric acid was found to be effective; no further migration (as indicated by nuclear magnetic resonance spectroscopy) and no hydrolysis (as indicated by thin layer chromatography) was detected after 2 hr at 25°. The products were assayed well within this period. Similarly, the original solution of 3′-*O*-acetyluridine in 0.001 N hydrochloric acid was found to be unchanged after it had stood for 30 min at 37°.

Assay Procedure. The method adopted involves the measurement of the relative peak heights of the resonance signals of the H(6) protons of the 2′ and 3′ isomers. The H(6) signal of the 2′ isomer consists of a doublet, with sharp bands at τ 2.10 and 2.24; the H(6) signal of the 3′ isomer consists of a similar doublet at τ 2.07 and 2.21 (see Figure 4). The peak height for each isomer is estimated by averaging the heights of both bands of an H(6) doublet over six separate runs. As a first approximation, it is assumed that the ratio of the average peak height of the doublet corresponding to the 2′ isomer (*i.e.*, the average of the bands at τ 2.10 and 2.24) to that of the 3′ isomer (*i.e.* the average of the bands at τ 2.07 and 2.21) is equal to the ratio of the respective proportions of the isomers in a particular mixture. A correction (see below) should then be applied to this result, especially for low proportions of 2′ isomer.

By assuming that the result obtained by the peak height method for the equilibrium mixture (*i.e.*, 1.7 parts of 3′ isomer to 1 part of 2′ isomer) is valid, a correction curve (Figure 7) has been obtained for mixtures poorer in 2′ isomer.[4] Two solutions were prepared as follows. Solution a: 3′-*O*-acetyluridine (0.04 g) in 0.001 N hydrochloric acid (0.2 ml) and 0.5 N hydrochloric acid (0.1 ml) was treated with 0.2 M sodium phosphate buffer (pH 7, 0.2 ml). Solution b: to 3′-*O*-acetyluridine (0.12 g) in 0.001 N hydrochloric acid (0.6 ml) was added 0.2 M sodium phosphate buffer (pH 7, 0.6 ml), and the solution was allowed to stand at 20° for 10 min before the addition of 0.5 N hydrochloric acid (0.3 ml). Both a and b were stable

[4] It can be seen from Figure 4a that the nuclear magnetic resonance spectrum of pure 3′-*O*-acetyluridine shows slight absorption in the region of the H(6) resonance of the 2′ isomer. It seems likely that the peaks at τ 2.07 and 2.10 (and at τ 2.21 and 2.24) would be of equal height for a 1:1 mixture of isomers (which cannot be obtained experimentally from pure 3′ isomer), and that strict proportionality of peak heights to composition would only then apply. However, the correction for the equilibrium mixture is likely to be small.

acidic solutions; a was an 8% solution of pure 3′-*O*-acetyluridine, and b was an 8% solution of the 2′(3′) equilibrium mixture.

If the composition of the equilibrium mixture is assumed to be as above, it is possible to calculate the percentages of 2′ isomer in solutions obtained by adding increasing volumes of solution b to 0.5 ml of solution a. In Figure 7, the percentage of 2′-*O*-acetyluridine estimated by the peak height method is plotted against the calculated percentage. A straight line is obtained, and it can be seen that larger corrections are necessary at low proportions of 2′ isomer. The plots for the isomerization of 3′-*O*-acetyluridine at 5 and 20°, illustrated in Figures 5a and b, have been corrected in this way.

Determination of Rate of Equilibration of 3′-O-Acetyluridine in Aqueous Dimethyl Sulfoxide. A solution of 3′-*O*-acetyluridine (0.06 g) in dimethyl sulfoxide (0.4 ml) and 0.2 M sodium phosphate buffer (pH 7, 0.05 ml), contained in a nuclear magnetic resonance tube, was maintained at 20°. The nuclear magnetic resonance spectrum (at 100 Mcycles/sec) of the reaction solution was measured after suitable intervals of time. The H(1′) resonance signal of 2′-*O*-acetyluridine (a doublet (J = 5.5 cycles/sec) at τ 4.11) was well separated from that of the 3′ isomer at τ 4.25 and from the H(5) resonance signals of both isomers (at τ 4.35 and 4.33, respectively).

The proportion of 2′ isomer was estimated by cutting out ahd weighing the area of paper under the τ 4.11 doublet, and by comparing its weight with: (a) that of the area under the signals assigned to the H(1′) resonance of the 3′ isomer and the H(5) resonances of both isomers, and (b) that of the area under the signals, centered at τ 4.93, assigned to the H(2′) resonance of the 2′ isomer and the H(3′) resonance of the 3′ isomer (this total area corresponds to one proton). These results are plotted in Figure 2b.

Determination of Rates of Equilibration of 3′-O-Acetyladenosine in Aqeuous Dimethyl Sulfoxide and Aqueous Dimethylformamide. A solution of 3′-*O*-acetyladenosine (0.06 g) in dimethyl sulfoxide or dimethylformamide (0.4 ml) and 0.2 M sodium phosphate buffer (pH 7, 0.05 ml), contained in a nuclear magnetic resonance tube, was maintained at 20°. The nuclear magnetic resonance spectrum (at 100 Mcycles/sec) of the reaction solution was measured after suitable intervals of time. The H(1′) resonance signal of 2′-*O*-acetyladenosine (a doublet at τ 3.81 (J = 6.0 cycles/sec) in dimethyl sulfoxide and at 3.66 (J = 6.0 cycles/sec) in dimethylformamide) was well separated from that of the 3′ isomer (a doublet at τ 4.05 (J = 7.2 cycles/sec) in dimethyl sulfoxide and at 3.90 (J = 7.2 cycles/sec) in dimethylformamide).

In both solvent systems, the proportion of 2′ isomer was estimated by cutting out and weighing the area of paper under the H(1′) doublet and comparing its weight with: (a) that of the area under the H(1′) doublet of the 3′ isomer, and (b) that of the area under the H(3′) quartet of the 3′ isomer (at τ 4.68 in dimethyl sulfoxide and at 4.53 in dimethylformamide). The results of the equilibration experiment in dimethyl sulfoxide solution are plotted in Figure 2a.

Determination of Rates of Equilibration of 3′-O-Formyladenosine in Aqeuous Dimethyl Sulfoxide and Aqueous Dimethylformamide. 3′-*O*-Formyladenosine (0.06 g) was allowed to dissolve in the anhydrous organic solvent (0.4 ml) at 20°. When complete solution had occurred (*i.e.*, after at most 2 min, and nuclear magnetic resonance indicated no isomerization after 5 min), 0.2 M sodium phosphate buffer (pH 7, 0.05 ml), also at 20°, was added rapidly and with stirring. After a suitable time interval (2–12 sec, see Figure 6), the reaction was quenched by the addition of 3 N hydrochloric acid (0.08 ml). A separate experiment was conducted for each reaction time. All nuclear magnetic resonance spectra were run within 10 min of the quenching process, which was found not to be absolutely effective inasmuch as a control containing pure 3′-*O*-formyladenosine underwent *ca.* 5% isomerization in 40 min.

The proportions of 2′- and 3′-*O*-formyladenosines in the reaction mixtures were estimated by the method described above for the corresponding acetyl derivatives. The respective chemical shifts of the H(1′) doublets of 2′- and 3′-*O*-formyladenosine were τ 3.76 (J = 5.4 cycles/sec) and 4.04 (J = 7.4 cycles/sec) in the dimethyl sulfoxide system, and 3.63 (J = 5.4 cycles/sec) and 3.91 (J = 7.4 cycles/sec) in the dimethylformamide system. The chemical shift of the H(3′) resonance of 3′-*O*-formyladenosine was τ 4.59 in the dimethyl sulfoxide and 4.43 in the dimethylformamide system.

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Reduction of Carotenoid Epoxides with Lithium Aluminum Hydride*

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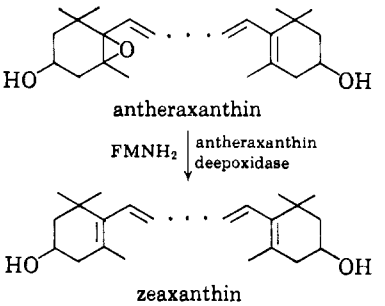
ABSTRACT: We have undertaken a study of the chemical reduction of antheraxanthin and neoxanthin, the epoxide carotenoids of *Euglena gracilis*, in an attempt to elucidate the mechanism of the enzymatic reductive deepoxidation of carotenoids (Bamji, M. S., and Krinsky, N. I. (1965), *J. Biol. Chem. 240*, 467). These epoxide carotenoids were treated with a large excess of $LiAlH_4$, and the resultant reaction mixtures were separated into several fractions by gradient elution from silica gel G–Celite (1:1). The individual fractions were characterized by absorption spectra, relative polarity values, and dehydration reactions with acidichloroform. The 5,6-epoxide groups of antheraxanthin and neoxanthin, upon reduction with $LiAlH_4$, yield, along with the expected 5-hydroxyl derivatives, equal amounts of the unexpected 5,6-olefins *via* a mechanism which does not involve dehydration of an hydroxylated intermediate. The mechanism of enzymatic deepoxidation may be like the $LiAlH_4$ reaction reported here. Based on our results, we suggest that the 3-hydroxyl and 5,6-epoxide groups of antheraxanthin and neoxanthin are in a *cis* configuration on the ionone ring.

The deepoxidation of epoxide carotenoids in photosynthetic tissue has been described as an anaerobic light-induced reaction (Sapozhnikov *et al.*, 1957; Yamamoto *et al.*, 1962b; Krinsky, 1964). Recently, Bamji and Krinsky (1965) have demonstrated that in *Euglena gracilis* the anaerobic deepoxidation of antheraxanthin to zeaxanthin can occur in the dark upon the addition of $FMNH_2$.[1]

These authors concluded that light functions in this type of reaction by generating reducing potential.



They have suggested that the deepoxidation of antheraxanthin might proceed *via* reduction of the 5,6-epoxide to an hydroxyl intermediate followed by dehydration to the 5,6-olefin. The proposed hydroxyl intermediate has not as yet been demonstrated as a participant in this reaction, nor has it been demonstrated as a naturally occurring component of *E.*

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[1] Abbreviation used: $FMNH_2$, reduced flavin mononucleotide.