On the Site of Esterification of Amino Acids to Soluble RNA*

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Aminoacyladenosine was isolated from aminoacyl-RNA by ribonuclease digestion and ion exclusion on DEAE-cellulose. To determine the position of esterification of the amino acid, 2,3-dihydropyran was used to block the unoccupied hydroxyl groups of the aminoacyl nucleoside, the amino acid was removed in mild alkali, and the hydroxyl group originally occupied by the amino acid was phosphorylated. Each step in the reaction sequence was carefully evaluated with model compounds. After removal of the protecting groups, 2′ and 3′ adenylic acids were isolated in a molar ratio of 1:2. A study of the comparative rates of acyl migration and hydrolysis in esters of glycerol, as models, shows that acyl equilibration between α and β positions is rapid, exceeding the rate of hydrolysis by a factor of more than 6000 in the neutral and alkaline range. Tentative extrapolation to aminoacyl-RNA suggests that aminoacyl 2′ and 3′ esters may be in base-catalyzed tautomeric equilibrium at *p*H values near neutrality.

The binding of amino acids to soluble RNA (s-RNA) (Hoagland *et al.*, 1957) has been shown to involve attachment to the 2′ or 3′ hydroxyl group of the adenosine residue which terminates the nucleic acid chain (Zachau *et al.*, 1958; Preiss *et al.*, 1959). Attachment by ester linkage was inferred from the ease of hydroxylaminolysis and alkaline hydrolysis of these compounds at relatively low *p*H and from the electrophoretic behavior of aminoacyladenosine derivatives prepared from amino acid derivatives of s-RNA by ribonuclease digestion. Such esters (Fig. 1) have been prepared synthetically by methods which may lead to esterification of either the 2′ or 3′ hydroxyl group (Shabarova *et al.*, 1959; Rammler and Khorana, 1960, 1963; Wieland *et al.*, 1960). The unusual reactivity of the ester linkage with nucleophilic reagents, as well as its high group potential, have been shown to arise mainly from the influence of the α-amino group which is protonated at physiological *p*H, greatly enhancing the rate of attack of nucleophilic reagents (Wolfenden, 1963), and reducing the thermodynamic stability of amino acid esters relative to the products of their hydrolysis (Jencks *et al.*, 1960).

The present paper describes an attempt to ascertain which of the two terminal hydroxyl groups in aminoacyl-RNA is involved in ester linkage with amino acids, an important question with regard to the stereochemical requirements for peptide bond formation. Attempts to provide an answer (Frank and Zachau, 1963; McLaughlin and Ingram, 1963) have been hindered by two main difficulties: the possibility of amino acid migration during structure determination and the absence of known amino acid esters possessing the two alternative structures. A similar question has arisen in the determination of the exact structure of the amino acid esters of the ribitol teichoic acids (Armstrong *et al.*, 1961; Baddiley *et al.*, 1961; Archibald, 1962).

In order to fix the position of the amino acid on adenosine, 2,3-dihydropyran was used to block the unoccupied positions in the aminoacyl nucleoside. This very rapid reaction produced base-stable acetals which allowed removal of the amino acid by treatment with ammonia. The hydroxyl group formerly occupied by the amino acid was then phosphorylated, yielding a protected adenylic acid derivative. After removal of the protecting groups the resulting 2′ and 3′ adenylic acids could be distinguished by known methods. Before application of these methods to the natural product, each step in the reaction sequence was carefully evaluated using chemically synthesized phenylalanyladenosine and other model compounds. The products of each of the steps in the reaction sequence described for aminoacyladenosine were isolated and characterized.

Despite the extreme precautions taken, ambiguous results were obtained, suggesting that equilibration of the amino acid had occurred to produce a mixture of 2′ and 3′ isomers. To explore the acyl migration more closely, we chose to study acetyl equilibration in the relatively easily prepared glycerol-β-monoacetate, where acetyl shift can be followed by the appearance of periodate-reactive neighboring hydroxyls. Because ester hydrolysis is generally more easily approached experimentally than intramolecular acyl shift, attention was directed towards establishing a background value of the ratio of acyl migration to hydrolysis by using glycerol-β-monoacetate as a convenient model system. Migration was expected to be faster than hydrolysis, and acetyl shift was indeed found to be more than 6000 times faster than ester hydrolysis at neutral *p*H. This seemed to suggest that under physiological conditions aminoacyl-RNA, when not protected—as is possible—by attachment to enzyme, may undergo a very rapid equilibration. This may also explain why an equilibrium mixture of 2′- and 3′-adenylic acids was invariably obtained from aminoacyladenosine in spite of reasonably adequate precautions to prevent equilibration during the procedure for blocking the free hydroxyl groups.

RESULTS AND DISCUSSION

Isolation of Aminoacyladenosine

s-RNA from *E. coli* was esterified with amino acids and the product was isolated by phenol extraction, ethanol precipitation, and gel filtration. Base-catalyzed hydrolysis of aminoacyl-RNA has been shown to proceed fairly rapidly even at neutrality (Wolfenden, 1963), and the possibility of acyl migration under acidic conditions has been indicated by the ease of migration of carbobenzoxyphenylalanyl esters of adenosine (Rammler and Khorana, 1963). In order to limit these reactions as much as possible, all procedures following enzymatic esterification at *p*H 7.4 were carried

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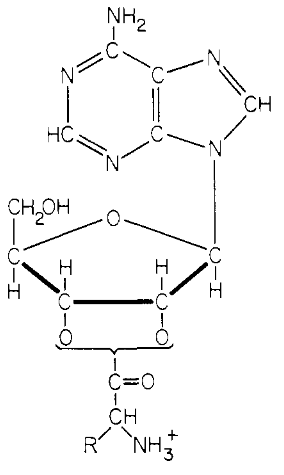

FIG. 1.—Aminoacyladenosine.

out at 5° at a pH near 5. In order to further eliminate the possibility of side reactions of 2,3-dihydropyran with multifunctional amino acids and yet maintain a large degree of esterification of the total RNA, the amino acids esterified with s-RNA were limited to those bearing a hydrocarbon side chain.

The isolation of amino acid esters of adenosine after ribonuclease digestion was achieved by column chromatography on DEAE-cellulose. In speed and recovery this method proved superior to electrophoresis or cation-exchange chromatography, and gave complete separation of the esters from the free adenosine released from nonesterified s-RNA (Fig. 2). The relative size of the two ultraviolet peaks provides one convenient measure of the degree of esterification of s-RNA without the need for radioactivity measurement. Free adenosine is weakly adsorbed, while the positively charged esters are rapidly eluted, presumably because of ion exclusion. Methyl esters of amino acids and synthetic phenylalanyladenosine were shown to behave similarly under the same conditions, emerging from the column ahead of adenosine.

The material constituting the aminoacyladenosine peak was immediately lyophilized to dryness from 0.02 M triethylammonium formate (pH 4.7), the buffer used to elute the esters from the column. An aqueous solution of the lyophilized product had a pH of 5.0, and control experiments showed that no serious shift of pH occurred during lyophilization.

Structure Determination.—Several reactions are available for blocking the free hydroxyl groups on aminoacyladenosine. In any method used, however, the possibility of migrations occurring during the blocking or during the subsequent removal of the blocking group must be avoided in order to render the proof unequivocal. The use of acetyl and benzoyl esters as blocking groups was excluded because of the chance of migration of these groups during the mild alkaline conditions necessary for the removal of the amino acid from the fully esterified adenosine derivative (Rammler and Khorana, 1962). The problem of migration of ester blocking-groups can be avoided by esterification with toluene-p-sulfonyl chloride or dibenzylphosphorochloridate in pyridine (Baddiley and Todd, 1947; Frank and Zachau, 1963); however, the likelihood of base-catalyzed isomerization of the amino acid during the time required for complete esterification cannot be excluded and made these routes unattractive. Direct phosphorylation with dicyclohexylcarbodiimide and β-cyanoethyl phosphate (Tener, 1961), with subsequent alkaline hydrolysis of the amino acid ester and the β-cyanoethyl groups, would readily provide the known diphosphates of adenosine (McLaughlin and Ingram, 1963), but, in addition to the problem of migration occurring during the long time required for complete phosphorylation, hydrolysis of the amino acid might well lead to the rapid cyclization and isomerization of the 2′ or 3′ β-cyanoethyl phosphate derivatives, yielding a mixture of diphosphate isomers (Brown and Todd, 1952).

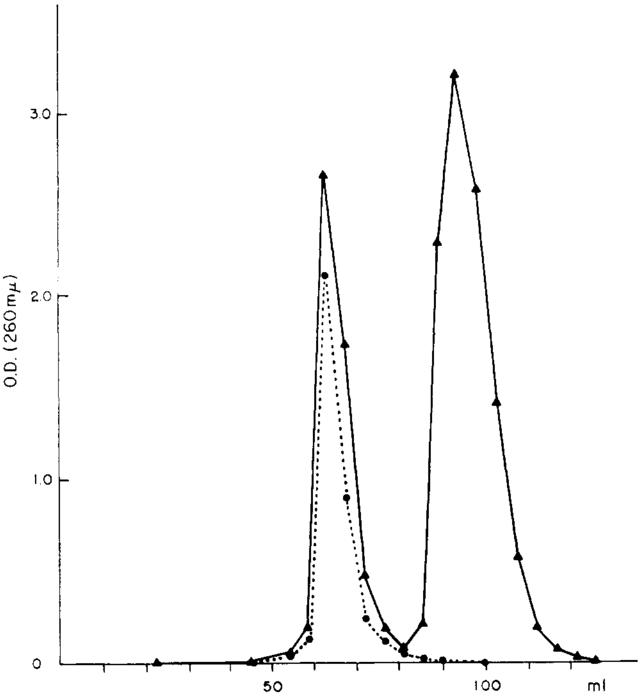

FIG. 2.—Column chromatography on DEAE-cellulose of a ribonuclease digest of 100 mg aminoacyl-RNA, esterified with ^{14}C-leucine and 5 nonradioactive amino acids (see text). Column-bed volume, 100 ml; elution buffer, triethylammonium formate (0.02 M, pH 4.6). The digest was applied in a volume of 2 ml. Solid line represents optical density; dotted line represents radioactivity.

To circumvent these problems and preserve the advantage of ending with easily identifiable adenylic acid derivatives, we had recourse to etherification with 2,3-dihydropyran. The resulting acetals were stable to alkali yet easily hydrolyzed under mildly acidic conditions (Smith *et al.*, 1962). The reaction sequence is illustrated in Figure 3 (the sequence is detailed only for the 2′ ester). In order to evaluate each of the steps indicated in Figure 3, all reaction conditions and procedures for product isolation were first tested on chemically synthesized phenylalanyladenosine. Using this substance the formation of 2′(3′),5′-di-*O*-tetrahydropyranyl-phenylalanyladenosine was shown to be rapid and quantitative, there being little or no hydrolysis of the amino acid when the reaction was carried out at room temperature using trifluoroacetic acid as catalyst. The product of this reaction could be isolated by paper electrophoresis or thin-layer chromatography on silicic acid. Adenosine so treated yielded exclusively the tri-*O*-tetrahydropyranyladenosine derivative. Ethyl esters of leucine and phenylalanine were exposed to the same reaction conditions and then hydrolyzed in dilute ammonia; complete recovery of the free amino acid showed that no pyranylation occurs at the amino group of the amino acid esters. On mild ammoniacal hydrolysis the protected aminoacyl nucleoside yielded phenylalanine and di-*O*-tetrahydropyranyladenosine as the sole products. The adenosine derivative was shown by paper chromatography on silicic acid to be identical with the product obtained by pyranylation of adenosine-2′(3′) phosphates, after removal of the phosphate group by bacterial alkaline phosphatase (also D. H. Rammler and H. G. Khorana, unpublished observations). Phosphorylation of this product, followed by removal of protecting groups, yielded the desired adenylic acid isomers.

Provided with these assurances, the reaction sequence described in Figure 3 was carried out with aminoacyladenosine obtained from s-RNA by ribonuclease digestion. Following pyranylation, the fully blocked aminoacyl nucleoside, (II) (only the 2′ isomer is illustrated in Figure 3), was isolated by paper electrophoresis or silicic acid chromatography. Mild hydrolysis with ammonia yielded di-*O*-tetrahydropyranyladenosine (III), which was isolated by paper chromatography or chromatography on silicic acid. Phosphorylation of compound III with β-cyanoethyl phosphate and dicyclohexylcarbodiimide in pyridine (Tener, 1961) yielded di-*O*-tetrahydropyranyladenosine β-cyanoethyl phosphate (IV). Removal of the cyanoethyl group by alkaline hydrolysis gave di-*O*-tetrahydropyranyladenosine phosphate (V), which upon hydrolysis in mild acid yielded adenylic acid (VI), in which the position of phosphate would correspond to the position originally occupied by the amino acid. The acidic conditions necessary for removal of the tetrahydropyranyl groups have been shown not to cause phosphoryl migration (Rammler and Khorana, 1962).

An important characteristic of this reaction sequence is that the speed of etherification minimizes the possibility of aminoacyl migration during the blocking of the free hydroxyl groups. Thus, pyranylation was complete and the same results were obtained regardless of the time allowed for reaction, even at the shortest times measured (4 minutes). No isomerization occurred in glycerol-β-monoacetate exposed to the same conditions of acidity as those required for pyranylation, but in the absence of dihydropyran. Adenosine-2′- and adenosine-3′-phosphate, pyranylated separately under the same conditions, dephosphorylated with bacterial alkaline phosphatase, rephosphorylated and isolated as described for di-*O*-tetrahydropyranyladenosine from aminoacyladenosine, gave the starting isomer in essentially quantitative yield in both cases, indicating that no phosphoryl migration had occurred during either the blocking procedure or the final removal of tetrahydropyranyl groups (also, Rammler and Khorana, unpublished observations). In addition, because the adjacent hydroxyl function is protected, no base-catalyzed isomerization via cyclic phosphate (Brown and Todd, 1952) can occur during β-elimination of the cyanoethyl group.

The final product in each of five experiments on aminoacyladenosine derived from s-RNA was an approximately 35:65 mixture of adenosine-2′-phosphate and adenosine-3′-phosphate. Synthetic phenylalanyladenosine gave the same products. Subjected to the same sequence of reactions, adenosine gave exclusively the starting material.

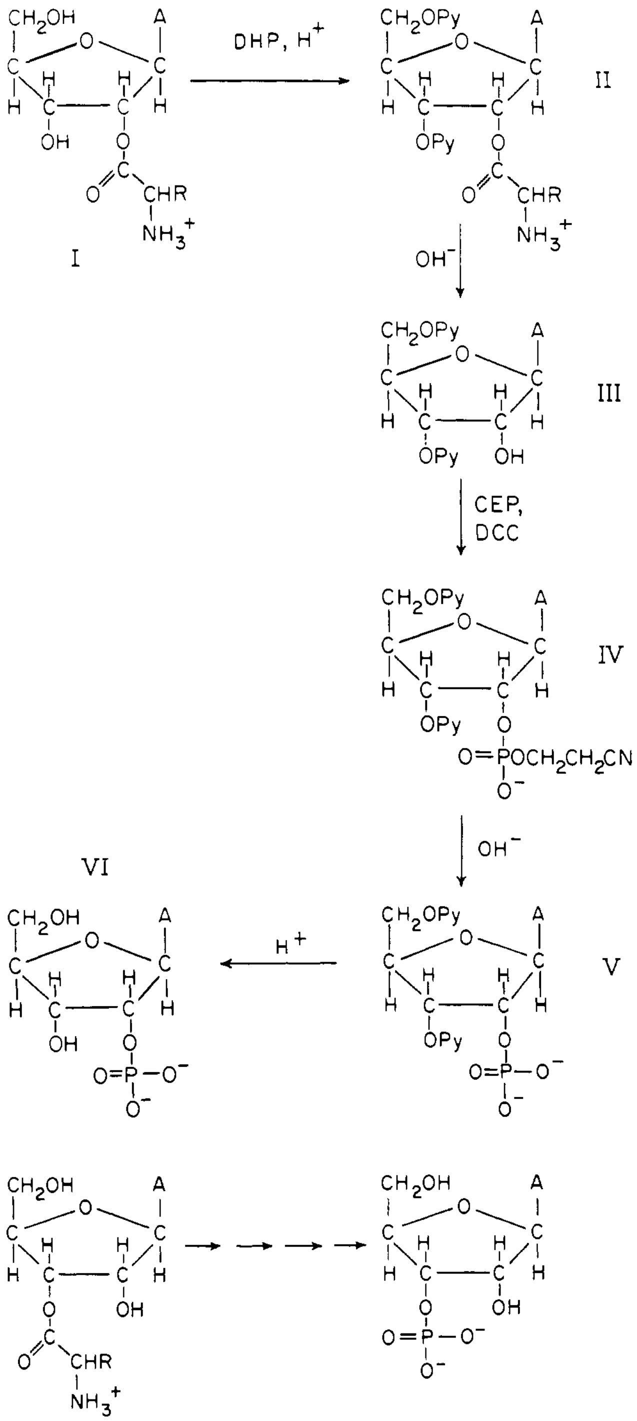

FIG. 3.—Sequence of reactions used for determination of the structure of aminoacyladenosine. The complete sequence (I to VI) is illustrated for the 2′ isomer. Only the starting material (Ia) and the final product (VIa) are illustrated for the 3′ isomer. The abbreviations used are the following: A = adenine, R = amino acid side chain, DHP = 2,3-dihydropyran, Py = tetrahydropyranyl group, CEP = cyanoethyl phosphate, DCC = dicyclohexylcarbodiimide.

Amino Acid Isomerization.—One is inclined to assume that there is a specific site of attachment of amino acid to s-RNA by the amino acid activating enzyme, and also that a specific site of attachment is required by the polymerizing enzyme that forms polypeptides from aminoacyl-RNA. It appears unlikely that the mixture of 2′ and 3′ adenylic acids derived from aminoacyl-RNA reflects the existence of a *mixture of stable isomers* in the original aminoacyl-RNA, because exactly the same mixture of adenylic acid isomers is obtained from chemically synthesized aminoacyladenosine (which is acylated on the 2′ and 3′ positions in a ratio that is determined by chemical rather then enzymatic factors; see also Frank and Zachau, 1963). A further argument against the existence of a mixture of stable isomers in aminoacyl-RNA may be that the hydrolysis of aminoacyl-RNA goes to completion with strictly first-order kinetics

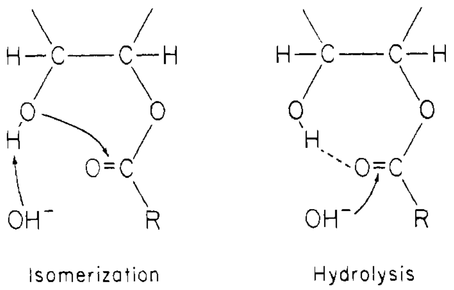

FIG. 4.—Mechanisms of base-catalyzed isomerization and hydrolysis of acyl esters of diols. The neighboring alkoxide ion, created by rapid proton shift, is presumably the attacking species in isomerization. The hydroxide ion is included to illustrate the origin of hydroxide-ion catalysis and not to imply "concerted" mechanism for isomerization.

(Wolfenden, 1963), whereas acyl groups on the 2′ and 3′ positions would be expected to show a measurable difference in kinetic behavior because of the proximity of the 2′ position to the purine base and to the potential carbonyl group (see Sugihara, 1953). It should be noted, however, that the thermodynamic difference between the two isomers is probably slight.[1]

Isomerization during the blocking procedure is a possibility that cannot be rejected. Nonetheless, glycerol monoacetate was shown not to isomerize under the conditions of acidity required for pyranylation, even in the absence of dihydropyran. Also, the rapidity of the pyranylation reaction and the fact that the adenylic acids, which undergo phosphate migration in acid, do not isomerize under the conditions employed here for pyranylation (see also Rammler and Khorana, 1962), appear to make this unlikely.

An orthoester structure for aminoacyl-RNA is highly improbable, since one would expect such an orthoester to have a much higher free energy of hydrolysis than that found experimentally for aminoacyl-RNA, whose free energy of hydrolysis is typical of normal amino acid esters (see the introductory paragraphs). Although acidic orthoesters would serve as intermediates in acyl isomerization (see Pacsu, 1945), it is not likely that such a structure could represent an appreciable proportion of acyl esters of this type (Bourne *et al.*, 1953; Ness and Fletcher, 1956). H. G. Zachau (personal communication) has recently obtained direct proof of this point by showing that synthetic aminoacyladenosine exhibits a normal ester carbonyl-absorption band in the infrared.

The results suggested the possibility that a rapid transesterification equilibrium might exist between the 2′ and 3′ positions of aminoacyladenosine and aminoacyl-RNA under neutral conditions. If this were the case, it would be nearly impossible to maintain an initial specific position of attachment of amino acid at physiological *p*H. This would explain the similarity of the mixture of 2′ and 3′ adenylic acids obtained as a result of formation of derivatives of aminoacyladenosine obtained from s-RNA to that obtained by subjecting chemically synthesized aminoacyladenosine to the same procedures. It would also explain the gross similarity of our results to those obtained by Frank and Zachau (1963), where base-catalyzed isomerization might well have occurred during their necessarily lengthy exposure of aminoacyladenosine to pyridine. This explanation would further account for the first-order kinetics observed for saponification of leucyl-RNA over a wide *p*H range (Wolfenden, 1963). A rapid pre-equilibration between the two positions would produce such kinetics even if the rate constants for hydrolysis of the two isomers were quite different. A rapid pre-equilibration would also explain the finding (Wolfenden, unpublished observations) that prolonged treatment of phenylalanyl-RNA with pyridine, with dilute acetic acid (homogeneous solution at *p*H 2.8), or with 5% trichloroacetic acid (in which the s-RNA is precipitated), followed by prolonged dialysis, results in no decrease in the transfer of RNA-bound phenylalanine to *E. coli* ribosomes in the presence of polyuridylic acid and the purified transfer factor of Nathans and Lipmann (1961). Approximately 65% transfer in a 5-minute incubation was observed with both an untreated control and aminoacyl-RNA treated with these solvents. These experiments were performed to detect any slow acid- or base-catalyzed migration of the amino acid to produce an isomer incapable of transferring amino acid to ribosomes.

Since base-catalyzed hydrolysis of aminoacyl-RNA occurs rapidly at physiological *p*H, one is led to inquire as to the relative rates of migration and hydrolysis in esters in which the alcoholic moiety bears an α-hydroxyl group. It has been established (Lohuizen and Verkade, 1960) that acid-catalyzed migration occurs a great deal more rapidly than acid-catalyzed solvolysis in monoglycerides. Indications that this might also be true for base-catalyzed migration were, first, that the same rate is observed for the saponification of both α and β glycerol monoacetates (Smith, 1922), and second, that migrations of oleic and stearic acids on glycerol have been shown to occur rapidly under mildly alkaline conditions which apparently produce no detectable solvolysis (Daubert and King, 1938; Mattson and Volpenhein, 1962). Numerous examples exist in carbohydrate chemistry of the use of base-catalyzed migrations in the preparation of the more stable of two such isomers (see Bourne *et al.*, 1953).

To obtain a more exact idea of the relative rates of base-catalyzed hydrolysis and base-catalyzed migration of acyl groups to a neighboring hydroxyl function (Fig. 4), the behavior of glycerol-β-monoacetate was examined. In this compound acetyl migration can be followed by the appearance of the α isomer which reacts with 1 mole of periodic acid. Hydrolysis may be followed by the disappearance of hydroxylamine-reacting material. Monoglycerides were chosen as model compounds because of their susceptibility to accurate kinetic measurement. By contrast with esters of nucleosides, the presence in glycerol monoesters of two hydroxyl groups to which migration can occur might assist migration relative to hydrolysis. However the presence of free rotation in the monoglyceride would more than compensate for this effect (see below), rendering it a conservative model for comparison with migration–hydrolysis rate ratios in esters of nucleosides. Nucleoside monoesters of defined structure were not available or amenable

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[1] The difference in free energy between isomers of diol esters is slight, as might be expected. In adenosine derivatives, the equilibrium ratio of adenosine-3′-phosphate to adenosine-2′-phosphate is 2.5 (Khym and Cohn, 1954). Similarly, the equilibrium ratio of the 3′ to the 2′ amino acid esters of adenosine and of s-RNA indicated by the present findings is 2.0. In glycerol monoacetates, at equilibrium, the ratio of acetate attached to one of the two α-hydroxyl groups to acetate attached to the β-hydroxyl was found here to be 2.8 (see also Verkade, 1953; Martin, 1953; also relevant is a comparative study of esterification equilibria in acetates of simple primary and secondary alcohols by Hatch and Adkins, 1937). Thus the free-energy differences in these cases are small, i.e. approximately 0.5 kcal.

to such a study, and the use of other cyclic carbohydrate esters would present special conformational difficulties.

1,3-Benzylidene-2-monoacetylglycerol was prepared by the method of Bergmann and Carter (1930) and the benzylidene group was removed by the borate replacement method of Martin (1953), yielding the water-soluble glycerol-β-monoacetate. Reaction of this product with periodic acid and hydroxylamine before and after alkaline hydrolysis showed that it was entirely monoglyceride, consisting of more than 97% of the β isomer. Exposure of the product to very dilute alkaline solution (*p*H 8.0) for 1 hour at room temperature resulted in the attainment of complete isomerization equilibrium with no detectable hydrolysis. At equilibrium the α and β isomers were found to be present in a ratio of 85:15, in reasonable agreement with equilibrium ratios of approximately 88:12 recorded for various monoglycerides of long-chain fatty acids (Verkade, 1953; Martin, 1953).

An attempt to prepare the analogous β-monoglyceride of phenylalanine yielded a monoglyceride which underwent hydrolysis, as expected, at a rate intermediate between those of the corresponding amino acid esters of s-RNA and of ethanol. However it was found that isomerization had occurred spontaneously during removal of the carbobenzoxy group from 1,3-benzylidene-2-carbobenzoxyphenylalanylglycerol by catalytic reduction. Hence it was not possible to obtain rate data for amino acid isomerization on glycerol, which must be rapid indeed.

Migration and hydrolysis of glycerol-β-monoacetate were measured as a function of *p*H at 37° and ionic strength 0.30. Both reactions exhibited good first-order kinetics at all *p*H values at which measurements were made. Hydrolysis was so much slower than migration that none was detectable during the time required for complete isomerization, even at *p*H 8.5, above which migration becomes too fast to measure by ordinary chemical methods. This simplifies kinetic analysis, since hydrolysis releases glycerol, which reacts with 2 moles of periodate and would complicate measurements of the rate of isomerization. Both migration and hydrolysis proved to be base-catalyzed reactions, exhibiting proportionality of rate to hydroxide-ion concentration throughout the range studied (Fig. 5). The second-order rate constant for hydroxide-ion-catalyzed hydrolysis under these conditions was 86 M^{-1} min^{-1}. The second-order rate constant for hydroxide-ion-catalyzed migration was 560,000 M^{-1} min^{-1}. Hence at any *p*H value base-catalyzed migration occurs at 6500 times the rate of base-catalyzed hydrolysis.

Leucyl-RNA, one of the more stable amino acid esters of s-RNA, exhibits a half-time of 27 minutes for base-catalyzed hydrolysis at *p*H 7.4 at 37° and ionic strength 0.30 (Wolfenden, 1963). Using the given ratio of the rate of base-catalyzed migration to the rate of base-catalyzed hydrolysis in a carboxylic acid ester bearing a neighboring hydroxyl group, a rough estimate of the half-time for isomerization of leucyl-RNA under these conditions would be 0.3 seconds. These are approximately the optimal conditions used *in vitro* for enzymatic esterification of amino acids with s-RNA, although they undoubtedly do not correspond exactly with those present within the cell. The true ratio of the rate of base-catalyzed migration to base-catalyzed hydrolysis may be much greater than 6500 in aminoacyl-RNA; there is only one group to which migration can occur, but this group is confined to the *cis* configuration, which markedly facilitates migrations of this type (Anderson and Lardy, 1950; Tamelen, 1951). Since the presence or absence of the polynucleotide chain has but slight influence on the chemical reactivity of aminoacyladenosine (Zachau, 1960), there is no indication that the polynucleotide chain could exert an appreciable retarding influence on amino acid migration.

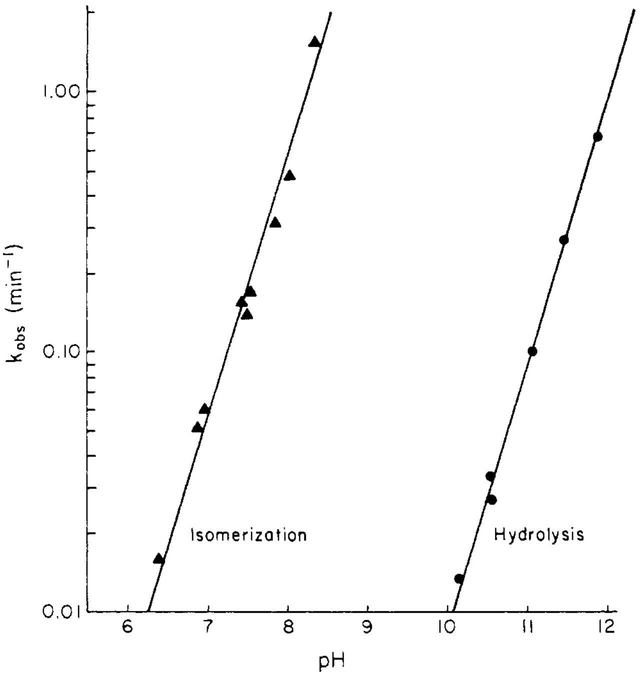

FIG. 5.—Rate of isomerization and hydrolysis of glycerol-β-monoacetate, extrapolated to zero buffer concentrations, as a function of *p*H, at 37° and ionic strength 0.30. The solid lines are calculated from rate equations presented in the text.

Glycerol monoacetate was used in these kinetic studies with the limited objective of providing an indication of the relative tendency toward base-catalyzed isomerization and hydrolysis of carboxylic esters bearing neighboring hydroxyl groups. Although it is an imperfect model (and the above calculation is subject to improvement if a better model becomes available), it serves to indicate that aminoacyl-RNA probably isomerizes virtually as soon as it is formed enzymatically, at least *in vitro*.[2] By the same considerations a base-catalyzed tautomeric equilibrium at physiological *p*H might also be expected to exist in the amino acid esters of the ribitol teichoic acids, whose general structure has been elucidated by Baddiley and his colleagues (see the introductory paragraphs). Here the rate of amino acid migration should be somewhat slower than in aminoacyl-RNA since the ribitol hydroxyl groups would not be held in *cis* configuration, i.e., there should be a closer resemblance to the glycerol model described at present.

CONCLUSIONS

It is biologically unlikely that more than one isomer of aminoacyl-RNA is synthesized by the activating enzyme or that more than one isomer is used in peptide-bond formation. These isomers may be, but need not be, the same. Energetically they are very similar.[1]

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[2] *Note added in proof.* A model compound bearing a more direct resemblance to aminoacyladenosine has recently been studied by C. S. McLaughlin and V. M. Ingram. The isomerization data obtained by them corroborate the present comparison. We are grateful to these workers for communicating their results to us as the present article went to press.

Puromycin, an inhibitor of enzymatic peptide bond formation with aminoacyl-s-RNA as the "active" amino acid, resembles aminoacyl esters of adenosine except that its amino acid is bound by amide linkage to a 3′-amino group on the ribose moiety. The 2′-puromycin analog tested by Nathans *et al.* (1963) was found to be virtually inactive. Since puromycin seems to compete with aminoacyl-RNA, as indicated by the findings of Allen and Zamecnik (1962), this constitutes reasonable evidence that the peptide-bond-forming enzyme requires 3′-esters of aminoacyl-RNA.

"Active" amino acids are used very rapidly in protein synthesis. It has been estimated that the time required for completion of a protein of mw 20,000 is approximately 5–10 seconds in rapidly growing bacteria (McQuillen *et al.*, 1959). Assuming an average molecular weight of 110 per amino acid residue (calculated from the composition of total *E. coli* protein, see Roberts *et al.*, 1957), the average time for incorporation of each amino acid into the growing peptide chain is of the order of 0.05 second. This is less than the presently estimated half time of 0.3 second for isomerization in leucyl-RNA at *p*H 7.4. The rate of base-catalyzed isomerization is, of course, highly sensitive to *p*H, and if more acidic conditions prevail in the environment of the activating enzymes and the ribosome, the half-time of isomerization would be appreciably larger, e.g., 1 second at *p*H 6.9 instead of 0.3 second at *p*H 7.4; the opposite would be true for more alkaline conditions.

If the aminoacyl-RNA isomer synthesized by the activating enzyme and the isomer used in peptide bond formation are different, it is possible that a specific isomerase exists for catalyzing this conversion in the living cell. If the isomer synthesized and the isomer used are the same, it may be that much of the aminoacyl-RNA remains enzyme-bound and is thus prevented from isomerizing within the cell. Although these possibilities must be recognized, equilibration between the 2′ and 3′ isomers of aminoacyl-RNA may be sufficiently rapid to supply whichever one is required by the enzymes involved in peptide-bond formation.

EXPERIMENTAL

Preparation of Amino Acid Esters of Soluble RNA.—A paste of frozen *E. coli*, strain B (500 g), grown on the medium of Littauer and Kornberg (1957) to the end of logarithmic phase, was homogenized with Tris-HCl buffer (0.01 M, *p*H 7.4, 1000 ml) for 1 minute. Redistilled phenol (88%, v/v) in water (1200 ml) was added with stirring and the mixture was stirred mechanically for 3 hours at room temperature. The mixture was centrifuged at maximum speed in a Lourdes VRA rotor for 2 hours at 5°. The aqueous upper phase was decanted and mixed with 20% potassium acetate (0.1 vol) and cold ethanol (2 vol). After the mixture had remained 2 hours in the cold, the precipitate was recovered by centrifugation and suspended and stirred for 3 hours in the cold with sodium chloride (1.0 M, 1500 ml). Material remaining insoluble was removed by centrifugation. To the supernatant was added cold ethanol (2 vol), which was allowed to stand for 2 hours in the cold. The precipitate was then recovered by centrifugation and suspended in Tris-HCl buffer (0.1 M, *p*H 7.4, containing 0.01 M magnesium chloride) to a volume of 300 ml. Deoxyribonuclease (0.6 mg, Worthington Biochemical Corp.) was added and the mixture was stirred for 1 hour in the cold. The *p*H was then adjusted to 9.5 with dilute ammonia and the mixture was incubated for 2 hours at 50° to saponify any remaining aminoacyl-RNA esters. Any material remaining insoluble after this treatment was removed by centrifugation and discarded. The clear supernatant was adjusted to *p*H 7.4 with dilute acetic acid and passed onto a column of DEAE-cellulose (300 g, Schleicher and Schuell Co., "Selectacel") pre-equilibrated with Tris-HCl (0.1 M, *p*H 7.4) according to the procedure of Holley *et al.* (1961). After the column had been washed with the same buffer (1500 ml), the s-RNA was eluted with 1 M sodium chloride in the same buffer (500 ml). The product was dialyzed against several changes of distilled water over a period of 24 hours in the cold. Dialysis tubing and vessels subsequently used for freeze-drying were boiled or heat-sterilized to prevent bacterial contamination.

The dialyzed product, which was stored either in solution or as the lyophilized powder, was completely stable in either form for at least 12 months. It consisted of approximately 2 g (42,000 OD units measured at 260 mμ in neutral solution) of RNA with less than 1% contamination with protein, DNA, or polysaccharide. It released 1 mole of adenosine per 62 moles of nucleotide upon alkaline hydrolysis (24 hours in 0.1 M KOH) and had an acceptor activity of 1 mole of leucine per 650 moles of nucleotide.

s-RNA so prepared (500 mg) was incubated for 5 minutes at 37° in a volume of 100 ml with ATP (1000 μmoles), DL-leucine-^{14}C (10 μmoles, New England Nuclear Corp.), glycine, L-alanine, L-isoleucine, L-valine, and L-phenylalanine (100 μmoles each), magnesium chloride (1000 μmoles), Tris-HCl buffer (*p*H 7.4, 10 mmoles), and 3 ml of enzyme solution. Enzyme solution was prepared by grinding *E. coli* in the cold with 3 times their weight of alumina and suspending in Tris-HCl buffer (0.01 M, *p*H 7.4, 2 vol) containing 0.01 M magnesium acetate and 0.01 M β-mercaptoethanol. This suspension was centrifuged at low speed and then for 3 hours at 105,000 × *g*, and was finally dialyzed against the same buffer overnight.

After incubation was complete, 88% redistilled phenol (100 ml) was added and the mixture was shaken in the cold. The aqueous upper phase was removed after centrifugation at low speed and combined with two subsequent water washes of the phenol phase. Potassium acetate (20%, 0.1 vol) adjusted to *p*H 5.1 with acetic acid was added, together with cold ethanol (2 vol). The flocculent precipitate was recovered by centrifugation, dissolved in triethylammonium formate buffer (0.02 M, *p*H 4.6, 7 ml) and passed onto a column of Sephadex G-25 (coarse grade, 100-ml bed volume; Pharmacia, Inc.) which had been equilibrated with the same buffer. Elution with the same buffer yielded aminoacyl-RNA, purified of low molecular weight contaminants including nonvolatile salt (see also Boman and Hjertén, 1962). RNA (450 mg) was recovered by this procedure, and a comparison of the leucine-^{14}C content of the final product with that of a sample precipitated with 5% trichloroacetic acid immediately after the enzyme incubation showed that negligible deacylation occurred during purification.

Preparation of Amino Acid Esters of Adenosine from s-RNA.—Amino acid esters of adenosine were prepared from aminoacyl-RNA by ribonuclease digestion followed by chromatography of the products on DEAE-cellulose with triethylammonium formate buffer (*p*H 4.6). Under these conditions the amino acid esters bear a positive charge and are rapidly eluted. The amino acid ester peak is closely followed by a second peak which consists of free adenosine released from nonesterified s-RNA. Nucleotides and oligonucleotides are retained. The elution pattern of a ribonuclease digest of s-RNA, esterified as described above, is represented in Figure 2.

Aminoacyl-RNA (100 mg), esterified as described above with six amino acids and redissolved in triethylammonium formate buffer (*p*H 4.6, 0.02 M, 2 ml) was incubated for 5 minutes at room temperature with crystalline ribonuclease (0.5 mg, Worthington Biochemical Corp.). The reaction mixture was then applied to a column of DEAE-cellulose (100-ml bed volume; Schleicher and Schuell, "Selectacel") pre-equilibrated with the same buffer. Upon elution with the same buffer, the aminoacyladenosine formate salts emerged at between 65 ml and 70 ml of eluate. The free nucleoside was eluted at between 80 ml and 120 ml of eluate. Both peaks were identical in ultraviolet absorption spectrum with adenosine in neutral solution. The first peak contained 25.5 OD units (260 mμ), while the second contained 45.5 OD units. All the amino acid (1.7 μmoles, or 1 mole per mole of adenosine) was contained in the first peak, which gave no reaction with periodic acid (Zachau *et al.*, 1958).

The different amino acid esters of adenosine do not emerge at exactly the same point. Synthetic phenylalanyladenosine, for example, was found to be slightly retarded on DEAE-cellulose compared with leucyladenosine derived from s-RNA; this retardation could be seen more clearly by the use of longer columns. The same was true for the methyl esters of tryptophan and phenylalanine. Although this retardation is insufficient to produce significant overlap with the adenosine peak, it is sufficient to produce a slight shift in the specific activity over the ester peak when a single amino acid ester is measured in the presence of others, as may be seen for leucine radioactivity in Figure 2 (dotted line). In control experiments performed with s-RNA esterified with a single amino acid, the specific radioactivity over the peak remained constant, with a ratio of 1 between amino acid and adenosine. Although some contamination of the ester peak with free amino acids occurs when ester hydrolysis has been allowed to occur before chromatography, this is negligible when the aminoacyl-RNA has been freshly dialyzed or submitted to gel filtration immediately before ribonuclease digestion. The non-esterified nucleoside obtained from *E. coli* s-RNA consisted entirely of adenosine, whereas a mixture of cytosine and adenosine was obtained from yeast s-RNA (McLaughlin and Ingram, 1963). It should be noted that the ratio of sample volume to column volume is critical in separations of this type, which may account for the inability of Frank and Zachau (1963) to separate the esters from free adenosine on DEAE-cellulose.

The chromatographic fractions constituting the aminoacyladenosine formate salt peak were pooled and lyophilized, yielding a dry salt-free powder. A solution of this powder in water (1 ml) had a *p*H of 5. When lyophilization was discontinued at various times before completion, it was found that the *p*H remained between 4.0 and 5.0.

2′,3′,5′-tri-O-Tetrahydropyranyladenosine.—Adenosine (15 mg) was dissolved in 1 ml of a cooled standard pyranylation solution prepared by mixing, in this order, freshly distilled dimethylformamide (1 ml), trifluoroacetic acid (0.1 ml), and 2,3-dihydropyran (Quaker Oats Co.) freshly distilled from KOH (1 ml). Care should be taken in making this mixture, which must be cooled before addition of dihydropyran and before addition of the compound to be pyranylated. After standing 5 minutes at room temperature, the reaction mixture was carefully evaporated to an oily residue and, after cooling, was made alkaline by the addition of concentrated ammonia (1 ml). Most of the solvent was removed by careful evaporation under reduced pressure. The gummy residue was applied to a standard silicic acid column (see below). The product was isolated as a single peak, emerging from the column with 3% methanol in chloroform. Material from this peak gave a single band, R_f 0.83, upon chromatography in solvent A (Table I). In ethanol this product showed an absorption-spectrum characteristic of adenosine, $\lambda_{\max}$ 258 mμ, $\lambda_{\min}$ 235 mμ. The yield was essentially quantitative. Hydrolysis in 80% acetic acid yielded, on paper chromatography in solvent A, only adenosine as the major ultraviolet absorbing band.

TABLE I
PAPER CHROMATOGRAPHY[a]

	Solvent A	Solvent B	Solvent C
Adenosine	0.45	0.24	0.15
2′,5′-di-*O*-Tetrahydropyranyladenosine 3′,5′-di-*O*-Tetrahydropyranyladenosine	0.72	0.82	0.62
2′,3′,5′-tri-*O*-Tetrahydropyranyladenosine	0.82	0.88	0.90
Adenosine-2′-phosphate Adenosine-3′-phosphate	0.08		
2′,5′-di-*O*-Tetrahydropyranyladenosine-3′-phosphate 3′,5′-di-*O*-Tetrahydropyranyladenosine-2′-phosphate	0.50		

[a] R_F values by descending chromatography on Whatman No. 40 paper: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2); solvent B, 1% concentrated ammonia in water, saturated with *n*-butanol; solvent C, *n*-butanol-water (86:14).

2′,5′-di-O-Tetrahydropyranyladenosine-3′-phosphate and 3′-5′-di-O-Tetrahydropyranyladenosine-2′-phosphate.—To a lyophilized powder of adenosine-2′-phosphate or adenosine-3′-phosphate (15 mg free acid) was added 1 ml of the standard pyranylation solution (see above). The reaction mixture was shaken until the nucleotide was completely dissolved (about 5 minutes) and allowed to stand an additional 20 minutes at room temperature. The reaction mixture was carefully evaporated to an oily residue under reduced pressure and, after cooling, was made alkaline by the addition of concentrated ammonia (1 ml). This solution was evaporated to a small volume, and the residue was dissolved in a small amount of 50% aqueous-ethanol and chromatographed on Whatman No. 3 MM paper in solvent A (Table I). The band with an R_F of 0.49 was eluted with water containing a drop of ammonia. The yield of dipyranyladenylic acid derivatives was quantitative, there being no starting materials detectable on the chromatograms. The product had a $\lambda_{\max}$ in each case at 258 mμ and was recovered in 90% yield.

2′,5′- and 3′,5′-di-O-Tetrahydropyranyladenosine.—Alkaline phosphatase from *E. coli* (0.25 mg), kindly provided by Dr. J. H. Schwartz, was added to a solution of either 2′,5′-di-*O*-tetrahydropyranyladenosine-3′-phosphate or 3′,5′-di-*O*-tetrahydropyranyladenosine-2′-phosphate (15 μmoles) in 1 ml ammonium carbonate buffer (0.1 M, *p*H 8.5). After 12 hours' incubation at 37°, the reaction solution was evaporated to a small volume and chromatographed on Whatman No. 40 paper in solvent A. A single band having an R_F of 0.72 was obtained (Table I). The product had a $\lambda_{\max}$ of 258 mμ in ethanol.

Phosphorylation of the pyranylated nucleoside, obtained by dephosphorylation of 2′,5′-di-*O*-tetrahydropyranyladenosine-3′-phosphate and of 3′,5′-di-*O*-tetrahydropyranyladenosine-2′-phosphate, gave, after removal of the pyranyl groups (see below), adenosine-3′-phosphate or adenosine-2′-phosphate as shown by chromatography in the system of Markham and Smith (1951) on Whatman No. 40 paper. In each case, the phosphate isomer obtained corresponded exclusively to the phosphate isomer originally pyranylated.

Substitution Reactions with Aminoacyladenosine.—Aminoacyladenosine from s-RNA or chemically synthesized (0.33 μmole) was dissolved in 0.5 ml of the standard pyranylation solution (see above). At the end of the reaction interval (4 minutes to 1 hour), the acid and excess dihydropyran were removed by evaporation under reduced pressure, leaving an oily residue.

In preliminary experiments, this residue was subjected to electrophoresis on Whatman No. 3 MM paper for 1 hour at 2000 v in triethylammonium bicarbonate buffer (0.01 M, *p*H 7.5). The major nucleoside band, which, in the case of ^{14}C-aminoacyladenosine from s-RNA, contained all the radioactivity, moved slightly toward the cathode. The total nucleoside material was eluted with water–ethyl alcohol (7:3, v/v) containing a drop of concentrated ammonia, evaporated to dryness, and treated with ammonia (0.5 ml) for 30 minutes at 60°. It was then evaporated to dryness once again and chromatographed in solvent B (Table I). Three ultraviolet-absorbing bands were detected. The major band corresponded in R_F (0.82) to 2′,5′- and 3′,5′-di-*O*-tetrahydropyranyladenosine markers. In addition, there were present a small amount of a faster-moving component (R_F 0.88) corresponding to 2′,3′,5′-tri-*O*-tetrahydropyranyladenosine, presumably originating from some deacylated material present in the original preparation, and a trace of slower-moving ultraviolet-absorbing material. The major component was eluted with solvent A, evaporated to dryness, and phosphorylated (see below).

Thin-layer silicic acid chromatography was used as an alternative method for isolating the protected aminoacyl nucleoside. When the pyranylation reaction mixture was subjected to thin-layer silicic acid chromatography (Research Specialties Co.) using 5% methyl alcohol in chloroform as the eluent, the main ultraviolet and radioactive band remained at the origin while tripyranyladenosine had an R_F of 0.69 and 3′,5′-di-*O*-tetrahydropyranyladenosine had an R_F of 0.55. The material at the origin was extracted with solvent A (3 × 5 ml) and kept at room temperature for 16 hours. Then the solvent was removed and part of the product was chromatographed again on thin-layer silicic acid plates using the same eluent. A single ultraviolet-absorbing band was detected having an R_F of 0.56. The remaining portion was chromatographed on Whatman No. 40 paper in solvent A. One ultraviolet band was detected having an R_F identical with dipyranyladenosine. In addition, ninhydrin-positive material was detected having an R_F similar to leucine, indicating that the material originally eluted from the silicic acid plates contained the amino acid ester. Similar results were obtained when synthetic phenylalanyladenosine was treated in the same manner.

To eliminate the possibility of acid-catalyzed loss of pyranyl groups which might occur during these procedures with the reaction mixture, in subsequent experiments ammonia (1 ml) was added directly at the end of the pyranylation reaction. This mixture was evaporated to a syrup and dissolved in a mixture of ammonia and ethanol (1:2, v/v). After 18 hours' standing at room temperature the solution was evaporated to a syrup, dissolved in methanol, and added to a small amount of a silicic acid slurry in chloroform. This mixture was evaporated to dryness at reduced pressure, additional chloroform (10 ml) was added, and the mixture was evaporated to dryness again. The residue was slurried in chloroform and placed on top of a column of silicic acid (13 × 1 cm; Mallinckrodt 100 mesh) packed in chloroform. Elution with 3% methanol in chloroform gave a small amount of tripyranyladenosine (presumably originating from some deacylated adenosine present in the original preparation); the dipyranyl derivative was eluted as the major product with 4% methanol in chloroform. No other nucleoside material was obtained. The purity of the products obtained by silicic acid chromatography was confirmed by paper chromatography (Table I). Their ultraviolet spectra in ethanol were identical with that of adenosine ($\lambda_{\max}$ 258 mμ).

The amorphous dipyranyladenosine isolated by either chromatographic procedure was dissolved in a small amount of dry pyridine (1 ml) containing β-cyanoethyl phosphate (50 μmoles; Tener, 1961). The mixture was evaporated to dryness. In order to remove last traces of moisture, dry pyridine (2 ml) was added, and the mixture was evaporated to dryness again. This procedure was repeated twice. To the residue were added dry pyridine (0.2 ml), and dicyclohexylcarbodiimide (50 mg).

After 2 days at room temperature, water (1 ml) was added and the mixture was kept at room temperature for 3 hours. Concentrated ammonia (1 ml) was then added and the mixture was heated for 1 hour at 60°. Excess dicyclohexylcarbodiimide was extracted with ether (2 × 1 ml), and the aqueous phase, containing dicyclohexylurea, was evaporated to dryness. The residue was re-evaporated to dryness several times with ethanol (2 ml) to insure complete removal of ammonia. The dry residue was dissolved in 80% acetic acid and kept at room temperature for 4 hours. After removal of the acetic acid by evaporation, the product was chromatographed in solvent A on Whatman No. 40 paper. The major ultraviolet-absorbing band corresponded in R_F (0.1) to adenylic acid; in addition, there was a very small amount of a faster-moving component (R_F 0.5) corresponding to adenosine. The nucleotide band was eluted and subjected to chromatography in the system of Markham and Smith (1951) (saturated ammonium sulfate; 0.1 M sodium acetate buffer, *p*H 6; isopropanol; 79:19:2). Two mononucleotide bands were detected, corresponding to adenosine-2′-phosphate (R_F 0.25) and adenosine-3′-phosphate (R_F 0.15). The products were present in a ratio of 1:2. Elution from the paper gave 0.25 OD unit of adenosine-2′-phosphate and 0.49 OD unit of adenosine-3′-phosphate.

The same result was obtained using either aminoacyladenosine derived from aminoacyl-RNA or phenylalanyladenosine prepared by the method of Rammler and Khorana (1963) and subjected to the procedures described for the natural product. In a series of five experiments carried out with aminoacyladenosine prepared from different batches of aminoacyl-RNA, no appreciable variation in the ratio of products was found. Variation of the time allowed for pyranylation from 4 minutes to 1 hour did not affect the result.

Glycerol-β-monoacetate.—2-Acetyl-1,3-benzylidene glycerol was prepared from 1,3-benzylidene glycerol (mp 82°; Verkade and Roon, 1942) by the method of Bergmann and Carter (1930) and gave mp 101° (uncorr.). To remove the benzylidene group, 2-acetyl-1,3-benzylidene glycerol (1 mmole) was dissolved in triethyl borate (1.3 ml), and granular boric acid (1.85 mmole) was added. The mixture was warmed for 5 minutes until the boric acid had dissolved completely, and was then evaporated under reduced pressure at 100° for 10 minutes (Martin, 1953). After cooling, the residue was dissolved in methanol (4 ml) and evaporated to dryness under reduced pressure at room temperature. To remove borate, the residue was once again dissolved in methanol and evaporated to dryness; this process was repeated twice more, after which a flame test showed that no borate remained (Zill *et al.*, 1953). The residue was dissolved in water (20 ml) and extracted with ether to remove benzaldehyde; ether was then removed from the aqueous phase by nitrogen bubbling. The resulting ester solution had a *p*H of 4.7. Analysis of this solution with hydroxylamine and periodic acid, as described below, showed that it contained 0.95 mmole of acetate ester and consumed less than 0.03 mmole of periodate. After treatment with 50% concentrated ammonia for 1 hour at 37°, no ester remained unhydrolyzed; 1.87 mmoles of periodate were now consumed. This showed that the original ester preparation was almost entirely β-monoglyceride, with less than 3% of the α-monoglyceride.

After treatment of the ester for 1 hour with 0.1 M glycylglycine buffer, *p*H 8.0, at room temperature, 0.95 mmole of ester still remained, indicating that no hydrolysis had occurred; however, the product now consumed 0.81 mmole of periodate. A rate study showed that this represents complete isomerization equilibrium, corresponding to an 85:15 mixture of α-monoglyceride and β-monoglyceride. By contrast, no detectable isomerization or hydrolysis occurred when glycerol-β-monoacetate was exposed to 5% trifluoroacetic acid in dimethylformamide at room temperature for 15 minutes, with conditions of acidity similar to those used in the pyranylation reactions described above.

Isomerization and Hydrolysis of Glycerol-β-monoacetate.—Buffer solutions for isomerization and hydrolysis contained potassium phosphate, glycylglycine, carbonate, and diethylamine buffers, 0.10 M (final concentration), together with sufficient potassium chloride to make the final ionic strength 0.30. All components of the reaction mixture were brought to 37°, and the reaction was initiated by addition of an aqueous solution of glycerol-β-monoacetate to produce a final ester concentration of 0.004 M. Aliquots of 1 ml were removed at timed intervals and tested for isomerization and hydrolysis. Determinations of *p*H were made at the beginning and end of each reaction using a glass electrode (Radiometer Type B), and a Radiometer Model 25 *p*H meter, and showed that no significant change in *p*H occurred during the course of the reactions.

Isomerization was followed by the appearance of periodate-reacting material. Aliquots of 1.0 ml of reaction mixture removed at various timed intervals were added to 2.0 ml of a mixture of sodium acetate buffer (1.25 ml, *p*H 4.3, 1.0 M) and sodium periodate (0.75 ml, 0.025 M) and allowed to stand for 20 minutes at room temperature, by which time reaction with periodate was complete. Periodate consumption was followed by disappearance of ultraviolet absorption at 300 mμ, measured with a Zeiss PMQ II spectrophotometer (Rammler and Rabinowitz, 1962).

Hydrolysis was followed by the disappearance of hydroxylamine-reacting material, measured by the method of Lipmann and Tuttle (1945) as modified by Jencks and Carriulo (1961). Aliquots of 1.0 ml of the reaction mixture were added to 1.0 ml of a mixture of hydroxylamine hydrochloride (0.4 ml, 4 M), sodium hydroxide (0.5 ml, 3.5 M), and water (0.1 ml), and allowed to stand for 6 minutes at room temperature, by which time reaction with hydroxylamine was complete. Ferric chloride solution was added (4.0 ml; 10% $FeCl_3 \cdot 6\ H_2O$ in 0.3 M HCl) and the color density was measured after 5 minutes with a Klett-Summerson photoelectric colorimeter using a No. 54 filter. After subtraction of the reading of a water blank, the result was compared with that given by an acethydroxamic acid standard.

All rate determinations were made in duplicate and reactions were followed for at least two half-times with at least four measurements. Good first-order kinetics were observed at all *p*H values for both isomerization and hydrolysis, and pseudo-first-order rate constants were calculated from the equation $k_{obs} = 0.693/t_{1/2}$. Kinetic analysis of isomerization was simplified by the fact that no detectable hydrolysis occurred even at *p*H values above which isomerization becomes too fast to measure. At each *p*H value a slight correction for buffer catalysis was made by extrapolation to zero buffer concentration using the values obtained in 0.10 and 0.05 M buffer, yielding corrected observed rate constants at zero buffer concentration and ionic strength 0.30. This correction never exceeded 10% of the observed rate in 0.10 M buffer and was usually much less.

The observed rate constants for isomerization and hydrolysis of glycerol-β-monoacetate at 37° and ionic strength 0.30, extrapolated to zero buffer concentration, were plotted as a function of *p*H (Fig. 5). The rates of both reactions were found to be strictly proportional to hydroxide-ion concentration, except at low *p*H values (well below the scale of the graph) where there appeared to be an additional contribution to the rate of isomerization from a very slow uncatalyzed or acid-catalyzed reaction. From these data, the base-catalyzed isomerization and hydrolysis of glycerol-β-monoacetate follow the rate laws:

$$k_{\text{isomerization}} = 560{,}000[\text{ester}][\text{OH}^-]\ \text{M min}^{-1}$$

$$k_{\text{hydrolysis}} = 86[\text{ester}][\text{OH}^-]\ \text{M min}^{-1}$$

The solid lines in Figure 5 are calculated from these expressions.

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Kinetic Studies of Glutamic Oxaloacetic Transaminase Isozymes*

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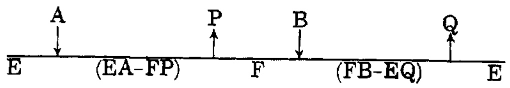
Initial velocity and product inhibition studies have been carried out on the anionic and cationic isozymes (separated by ion-exchange chromatography) of pig heart glutamic oxaloacetic transaminase at *p*H 7.4 and 37°, and Michaelis and inhibition constants for all reactants have been determined for both isozymes. The kinetic data are consistent only with a Ping Pong Bi Bi mechanism (an α-amino acid is bound to the enzyme and the corresponding α-keto acid is released, followed by binding of the second α-keto acid and subsequent release of the second α-amino acid). The measured kinetic constants for both isozymes are consistent with the predicted Haldane relationships for this mechanism. In addition, a dead-end α-ketoglutarate-enzyme complex is formed at high α-ketoglutarate concentrations. The dissociation constant for this dead-end complex is different for the two isozymes; the other kinetic constants are nearly the same. Substrates other than α-ketoglutarate do not show substrate inhibition.

The results of a number of workers (Schlenk and Fisher, 1947; O'Kane and Gunsalus, 1947; Nisonoff and Barnes, 1952; Meister *et al.*, 1954; Snell and Jenkins, 1959; Jenkins and Sizer, 1960; Turano *et al.*, 1960; Velick and Vavra, 1962) suggest that glutamic oxaloacetic transaminase has a Ping Pong Bi Bi mechanism (Mechanism 1):[1]



(1)

where E is the pyridoxal form of the enzyme, F is the pyridoxamine enzyme form, and A, B, P, and Q are L-aspartate, α-ketoglutarate, oxaloacetate, and L-glutamate, respectively. Recently, however, Evangelopoulos and Sizer (1963) proposed on the basis of spectral studies a sequential mechanism (both substrates must bind to the enzyme before release of either product).

Fleisher *et al.* (1960) observed in a crude pig heart preparation two electrophoretic fractions with trans-

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[1] Kinetic nomenclature used in this paper is that of Cleland (1963a,b).