Selective Inhibition of Aminoacyl Ribonucleic Acid Synthetases by Aminoalkyl Adenylates*

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ABSTRACT: Stable and unreactive structural analogs of several aminoacyl adenylates were prepared by condensing the corresponding amino alcohols with adenosine 5′-phosphate. The effect of these aminoalkyl adenylates on the activation of amino acids catalyzed by the appropriate aminoacyl ribonucleic acid synthetases was examined by means of the ATP–$P^{32}P_i$-exchange reaction and the reaction leading to aminoacyl-RNA formation. Each of the aminoalkyl adenylates examined was shown to be a remarkably potent inhibitor of the activation of its corresponding amino acid, competing with the amino acid and with adenosine triphosphate (ATP) for attachment to the substrate binding site of the enzyme. Furthermore, the comparative study of the inhibitory effect of an aminoalkyl adenylate and of the corresponding amino alcohol on the activation of the homologous amino acid indicates that, while both behave as competitive inhibitors, the value of the inhibitor constant K_i of the aminoalkyl adenylate is three orders of magnitude lower than that of the amino alcohol. These findings clearly demonstrate that the adenosine monophosphate (AMP) moiety of the aminoalkyl adenylate participates in the binding of the inhibitor to the active site of the enzyme, and support the view that the aminoalkyl adenylate occupies the same site on the enzyme as the corresponding aminoacyl adenylate.

In order to evaluate their specificity as inhibitors of aminoacyl-RNA synthetases, each of the ten available aminoalkyl adenylates was examined for its effect on the amino acid dependent ATP–$P^{32}P_i$-exchange reaction catalyzed by partially purified methionyl-, valyl-, and isoleucyl-RNA synthetases. The results demonstrate that each enzyme was very nearly quantitatively inhibited by its homologous aminoalkyl adenylate at a concentration at which none of the nine heterologous aminoalkyl adenylates were effective. However, when relatively higher concentrations of the heterologous derivatives were tested, a few cases of unspecific inhibition were encountered.

Thus, aminoacyl-RNA synthetases exhibit far greater affinity for their homologous aminoalkyl adenylate than for any of the heterologous aminoalkyl adenylates.

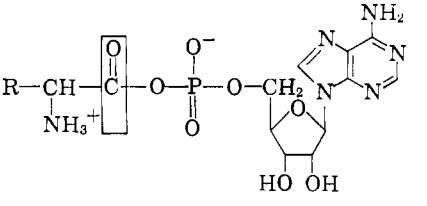
The first step in the biosynthesis of proteins starting from amino acids is the formation of aminoacyl adenylates (I) by a relatively specific reaction involving amino acids, adenosine triphosphate, and the appropriate aminoacyl-RNA synthetases. The amino acids,

$$\text{enzyme} + \text{amino acid} + \text{ATP}^1 \rightleftharpoons \text{enzyme}\cdot\text{aminoacyl adenylate} + \text{PP}_i$$

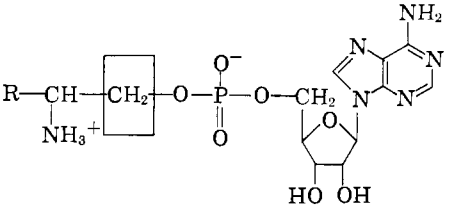
thus activated, are subsequently transferred to their corresponding tRNA through a highly specific reaction catalyzed by the same enzymes.

$$\text{enzyme}\cdot\text{aminoacyl adenylate} + \text{tRNA} \rightleftharpoons \text{aminoacyl-RNA} + \text{enzyme} + \text{AMP}$$

The present paper deals with the synthesis and properties of structural analogs of aminoacyl adenylates in which the highly labile, mixed anhydride linkage has been replaced by a stable and unreactive ester



aminoacyl adenylate (I)



aminoalkyl adenylate (II)

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[1] Abbreviations used: AMP and ATP, adenosine mono- and triphosphates; GSH, glutathione; TCA, trichloroacetic acid.

bond. The work was undertaken with the expectation that this structural modification would not impair the binding of these aminoalkyl adenylates (II) to the active site of their respective aminoacyl-RNA synthetases, thereby providing a class of compounds which might prove valuable as inhibitors of amino acid activation and as stable pseudo-substrates for studies concerned with enzyme–substrate interactions. The experiments have confirmed these expectations. In this report, we describe the inhibitory effect of several aminoalkyl adenylates on the amino acid dependent ATP–$P^{32}P_i$-exchange reaction and on aminoacyl-RNA formation catalyzed by aminoacyl-RNA synthetases.

Methods

Synthesis of Aminoalkyl Adenylates. The adenosine 5′-phosphate esters of amino alcohols were prepared by a general procedure involving condensation of the N-protected amino alcohol with $N^6,O^{2'},O^{3'}$-triacetyladenosine 5′-phosphate in the presence of *N,N′*-dicyclohexylcarbodiimide, followed by removal of the protecting groups. A detailed description of the synthesis and chemical characterization of L-isoleucinyl, L-leucinyl, L-valinyl, L-tyrosinyl, L-phenylalaninyl, L-prolinyl, DL-alaninyl, and glycinyl adenylates has been published (Sandrin and Boissonnas, 1966). The other two aminoalkyl adenylates used in this study (*i.e.*, L-methioninyl and L-lysinyl adenylates) were prepared as follows.

N^α,N^ϵ-Dibenzyloxycarbonyl-L-lysinol (3 equiv) (mp 50°, $[\alpha]_D^{22}$ −9.6° (*c* 2, methanol)) or *N-t*-butyloxycarbonyl-L-methioninol (mp 50°, $[\alpha]_D^{22}$ −18.7°, *c* 2, methanol) was condensed in dry pyridine with 1 equiv of $N^6,O^{2'},O^{3'}$-triacetyladenosine 5′-phosphate in the presence of 8 equiv of dicyclohexylcarbodiimide at 25° during 10 days. After filtration of the formed dicyclohexylurea and evaporation of the pyridine, the residue was washed successively with petroleum ether (bp 30–60°) and ethyl ether and dissolved in 9 N aqueous ammonia. After 1 day at 25°, the solution was evaporated to dryness and the residue was dissolved in a minimum amount of methanol. Addition of ethyl ether yielded the adenylates of the two different N-protected amino alcohols. The benzyloxycarbonyl groups were removed by hydrogenation (palladium catalyst) in methanol in the presence of 1.5 equiv of acetic acid. Cleavage of the *t*-butyloxycarbonyl group was achieved by a 5-min treatment with anhydrous trifluoroacetic acid. After evaporation of the solvents, both products were purified by chromatography on a silica gel column using the solvent system acetone–water (8:2). Dissolution of the column-purified material in a minimum amount of methanol and precipitation with ethyl ether yielded L-lysinyl adenylate (mp 180° dec, $[\alpha]_D^{22}$ −12.3° (*c* 2 water); log ϵ 4.15 at λ_{max} 258.5 nm) and L-methioninyl adenylate (mp 123°, $[\alpha]_D^{22}$ 9.7° (*c* 2, in water), log ϵ 4.08 at λ_{max} 258 nm), respectively. Both products gave correct elementary analyses and were homogeneous on paper electrophoresis (pH 1.9 and 5.8) and on thin layer chromatography on silica gel in the solvent systems acetone–water (8:2, 5:5, and 2:8) and methanol–water (8:2, 5:5, and 2:8), after visualization with ninhydrin, chlorine–potassium iodide–starch, and molybdate reagents (Sandrin and Boissonnas, 1966).

Stability of Aminoalkyl Adenylates. In order to assess their stability under the conditions used for the assay of amino acid activation, each aminoalkyl adenylate was examined by paper chromatography after incubation at 37° for 30 min in a mixture composed of 100 μmoles of Tris-HCl buffer at pH 8.0, 5 μmoles of $MgCl_2$, 10 μmoles of KF, 10 μmoles of 2-mercaptoethanol, and 4 μmoles of aminoalkyl adenylate in a volume of 1 ml. Aliquots were applied on Whatman No. 1 paper and submitted to descending chromatography using the solvent systems I and II (Table I)

TABLE I: R_F Values of Aminoalkyl Adenylates.[a]

Compound[b]	R_F Values	
	Solvent I	Solvent II
DL-Ala-ol-AMP	0.13	0.21
Gly-ol-AMP	0.09	0.14
L-Ile-ol-AMP	0.34	0.37
L-Leu-ol-AMP	0.39	0.39
L-Lys-ol-AMP	0.05	0.08
L-Met-ol-AMP	0.24 (and traces[c] at 0.07)	0.27 (and traces[c] at 0.16)
L-Phe-ol-AMP	0.41	0.33
L-Pro-ol-AMP	0.16	0.28
L-Tyr-ol-AMP	0.25	0.25
L-Val-ol-AMP	0.24	0.30
Adenosine 5′-phosphate	0.09	0.41

[a] Each derivative (40 mμmoles) was submitted to descending chromatography on Whatman No. 1 paper at 26°. Spots were located by ultraviolet absorption and reaction to ninhydrin. Solvent I: 1-butanol–acetic acid–water (4:1:5, v/v); 14-hr run. Solvent II: isopropyl alcohol–concentrated HCl–water (170:41:39, v/v); 16-hr run. [b] DL-Ala-ol-AMP stands for DL-alaninyl adenylate, etc. [c] Corresponding to the sulfoxide derivative.

capable of resolving each of the adenylates from any adenosine 5′-phosphate released through hydrolysis. The ten aminoalkyl adenylates used in this study were found to be completely stable after this incubation, as assessed by failure to detect any trace of ultraviolet absorption or ninhydrin-positive reaction other than that of the original, homogeneous material.

Assay Procedures. Amino acid activation was measured by the amino acid dependent [^{32}P]pyrophosphate–ATP-exchange reaction described by Berg (1956). The standard incubation mixture contained, in a

TABLE II: Specificity of Partially Purified Methionyl-, Isoleucyl-, and Valyl-RNA Synthetases for Aminoacyl Adenylate and Aminoacyl-RNA Formation.[a]

Amino Acid Tested	Methionyl-RNA Synthetase		Isoleucyl-RNA Synthetase		Valyl-RNA Synthetase	
	ATP–$P^{32}P_i$ Exchange	Aminoacyl-RNA Formation	ATP–$P^{32}P_i$ Exchange	Aminoacyl-RNA Formation	ATP–$P^{32}P_i$ Exchange	Aminoacyl-RNA Formation
L-Methionine	100	100	8.8	0	0.2	0
L-Isoleucine	1.8	0.2	100	100	2.0	0
L-Valine	0.7	0	54	0.9	100	100
L-Leucine	0	0	8.7	1.7	0.4	0

[a] Assays were carried out as described in Methods, with 2×10^{-3} M L-amino acid in the ATP–$P^{32}P_i$-exchange reaction and 2×10^{-5} M L-[^{14}C]amino acid in the assay for aminoacyl-RNA formation. The results obtained with the corresponding amino acid are expressed as 100, and all others relative to that figure. The value of 100 corresponds to the formation of 38 μmoles of AT^{32}P/mg of protein in 15 min at 37° and 208 mμmoles of methionyl-RNA/mg of protein in 10 min at 30° with methionyl-RNA synthetase; to 28 μmoles of AT^{32}P/mg of protein in 15 min at 37° and 57 mμmoles of isoleucyl-RNA/mg of protein in 10 min at 30° with isoleucyl-RNA synthetase; and to 109 μmoles of AT^{32}P/mg of protein in 10 min at 37° and 1400 mμmoles of valyl-RNA/mg of protein in 10 min at 30° with valyl-RNA synthetase.

volume of 1 ml, 100 μmoles of Tris-HCl buffer at pH 8.0, 5 μmoles of $MgCl_2$, 10 μmoles of KF, 10 μmoles of 2-mercaptoethanol, 2 μmoles of ATP (Na), 2 μmoles of L-amino acid, 2 μmoles of $P^{32}P_i$ (approximately 2×10^5 cpm), and limiting amounts of the appropriate enzyme. Aminoalkyl adenylates, which are readily soluble in water, were supplied as a neutral, aqueous solution at the concentrations specified in the text. After incubation at 37° for 15 min (with the exception of assays involving valyl-RNA synthetase, where incubation lasted for 10 min), the reaction was stopped by the successive addition of 0.5 ml of 7% perchloric acid, 0.4 ml of an aqueous suspension of Norit (100 mg/ml), and 1.8 ml of water, and the suspension was thoroughly mixed and centrifuged. The Norit was then washed with three 6-ml portions of water, and the residue from the last centrifugation was resuspended in 2.0 ml of 0.3 M ammonium hydroxide in 50% ethanol. After recentrifugation, an aliquot of the eluate was dried and counted in a thin-window, gas-flow counter.

[^{14}C]Amino acid attachment to tRNA was measured by a procedure similar to that used by Fangman and Neidhardt (1964). The reaction mixture contained, in a volume of 0.5 ml, 50 μmoles of Tris-HCl buffer at pH 7.4, 5 μmoles of $MgCl_2$, 5 μmoles of KCl, 1 μmole of GSH, 1 μmole of ATP (Na), 0.01 μmole of L-[^{14}C]-amino acid, 0.5 mg of tRNA, and limiting amounts of the appropriate enzyme. After incubating for 10 min at 30°, the reaction was stopped by the addition of 3.5 ml of a cold solution of 6% TCA. The resulting suspension was filtered through a Millipore filter in the cold (4°) and washed with 5% TCA containing 0.5% of the corresponding DL-[^{12}C]amino acid. The filter was then placed on a planchet, dried, and counted in a thin-window, gas-flow counter.

Enzyme Preparations. The enzymes were isolated from *Escherichia coli* B cells grown exponentially in synthetic medium "63" (Cohen and Rickenberg, 1956). The enzyme preparation used for the activation of tyrosine, phenylalanine, and alanine was an unfractionated mixture of aminoacyl-RNA synthetases obtained by the procedure of Martin *et al.* (1963).

The methionyl-, isoleucyl-, and valyl-RNA synthetases used throughout this work were partially purified according to Bergmann *et al.* (1961). This procedure was followed in all details with the exception that in the purification of methionyl-RNA synthetase, the step involving adsorption on alumina C_γ gel was omitted. The three enzymes were purified 27-, 42-, and 30-fold, respectively. To evaluate their specificity, each was tested for its ability to catalyze the ATP–$P^{32}P_i$-exchange reaction and the formation of aminoacyl-RNA in the presence of several L-amino acids (Table II). The use of these partially purified enzymes made it possible to distinguish between valine activation catalyzed by isoleucyl-RNA synthetase and valyl-RNA synthetase, respectively, and also minimized the hazards of possible contamination by other amino acids of the methionine and isoleucine used in the ATP–$P^{32}P_i$-exchange assays.

Materials

The chromatographically homogeneous L-amino acids used in the biochemical assays were purchased from the following sources: methionine, valine, and isoleucine from Mann Research Laboratories, alanine from California Corp. for Biochemical Research, and phenylalanine and tyrosine from Fluka AG., Switzerland. [^{32}P]Sodium pyrophosphate and L-[^{14}C]-amino acids were obtained from the Commissariat à

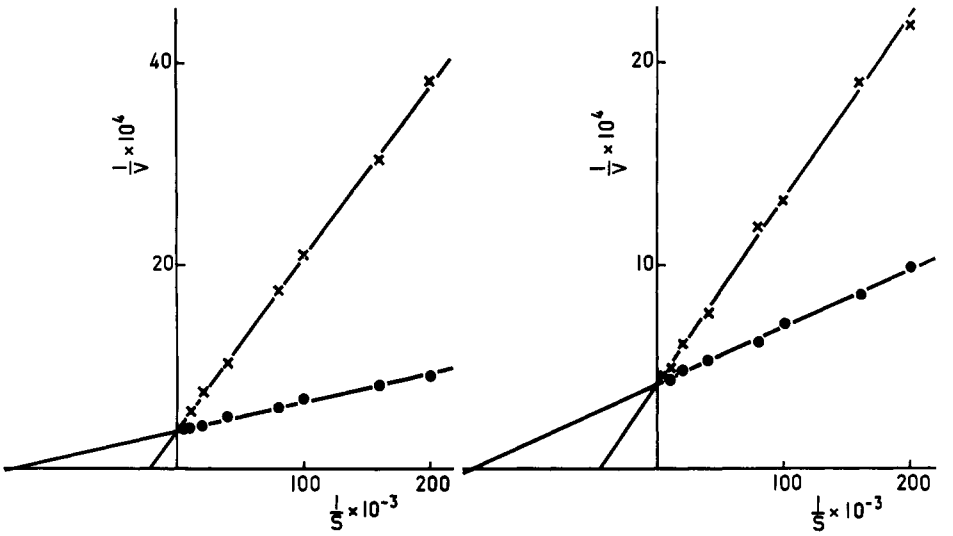

FIGURE 1: Lineweaver–Burk plots of the inhibition of the isoleucine-dependent ATP–$P^{32}P_i$-exchange reaction by 3.3×10^{-8} M L-isoleucinyl adenylate (a) (left) and 5×10^{-5} M L-isoleucinol (b) (right), measured by varying the amino acid concentration in the presence of saturating amounts of ATP. Incubation conditions are those described in Methods.

TABLE III: Comparison of the K_m and K_i Values Derived from the Lineweaver–Burk Plots of the Data Obtained in the ATP–$P^{32}P_i$-Exchange Assay.

Substrate	Inhibitor	K_m (M)	K_i (M)
L-Methionine	DL-Methioninol	4.0×10^{-5}	1.7×10^{-5}
L-Methionine	L-Met-ol-AMP	4.0×10^{-5}	8.6×10^{-9}
L-Isoleucine	L-Isoleucinol	7.1×10^{-6}	2.3×10^{-5}
L-Isoleucine	L-Ile-ol-AMP	7.1×10^{-6}	7.4×10^{-9}
L-Valine	L-Val-ol-AMP	1.6×10^{-4}	2.9×10^{-8}
L-Tyrosine	L-Tyr-ol-AMP	8.4×10^{-5}	2.9×10^{-8}
L-Phenylalanine	L-Phe-ol-AMP	1.9×10^{-4}	2.5×10^{-6}
L-Alanine	DL-Ala-ol-AMP	7.2×10^{-4}	9.1×10^{-6}

l'Energie Atomique, Saclay, France. The [^{14}C]amino acids were further purified by chromatography in the solvent system 2-butanol–3% ammonia (3:1, v/v), on Whatman 3MM paper prewashed with 33% acetic acid. *E. coli* B tRNA was from General Biochemicals.

Results

Inhibition of the Amino Acid Dependent ATP–$P^{32}P_i$-Exchange Reaction by Aminoalkyl Adenylates. None of the ten aminoalkyl adenylates examined promoted ATP–$P^{32}P_i$ exchange when assayed at 2×10^{-3} M concentration in the absence of amino acids. Under standard assay conditions in the presence of the appropriate enzyme and 2×10^{-3} M concentrations of amino acid and ATP, the ATP–$P^{32}P_i$-exchange reaction was 50% inhibited by the following concentration of the *homologous* (*i.e.*, the aminoalkyl adenylate in which the amino alcohol moiety corresponds to the amino acid tested) aminoalkyl adenylate: 5×10^{-7} M L-methioninyl, L-valinyl, and L-tyrosinyl adenylates, 10^{-6} M L-isoleucinyl adenylate, and 10^{-5} M L-phenylalaninyl and DL-alaninyl adenylates. The nature of this inhibition was examined in detail by varying the amino acid concentration at a fixed aminoalkyl adenylate concentration, in the presence of saturating amounts of ATP. The inhibition was found to be fully competitive in all six cases, as assessed by the Lineweaver–Burk (1934) and Eadie (1952) methods of plotting. The graphical representation of the inhibition of isoleucyl-RNA synthetase by L-isoleucinyl adenylate is shown in Figure 1a. The values for K_m and K_i derived from the Lineweaver–Burk plots are summarized in Table III.

The nature of the inhibition with respect to ATP utilization was examined by varying the ATP concentration at a fixed aminoalkyl adenylate concentration, in the presence of saturating amounts of the corre-

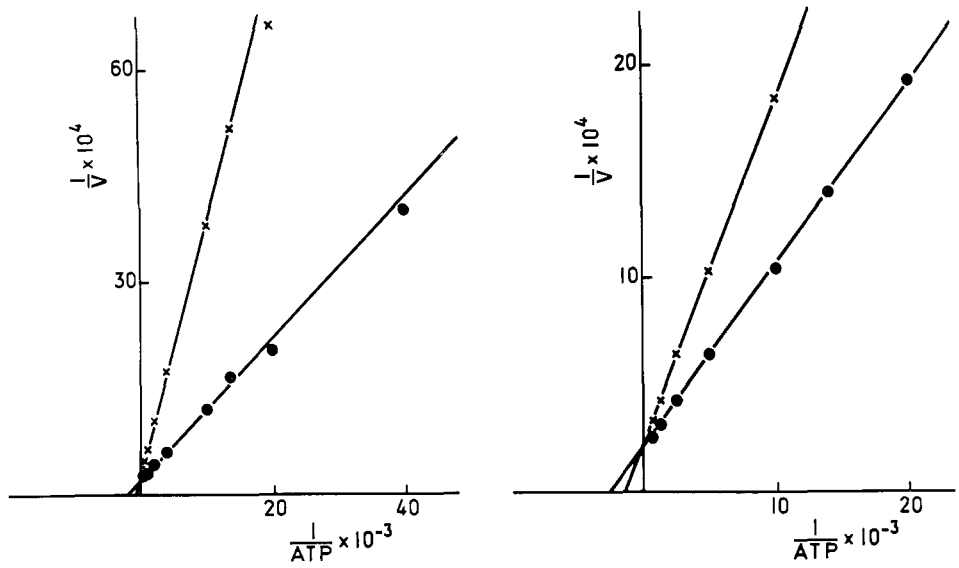

FIGURE 2: Lineweaver–Burk plots of the inhibition of the amino acid dependent ATP–$P^{32}P_i$-exchange reaction by aminoalkyl adenylates, measured by varying the ATP concentration in the presence of saturating amounts of the amino acid. (a) (left) Inhibition of methionine-dependent ATP–$P^{32}P_i$ exchange by L-Met-ol-AMP at a concentration of 10^{-7} M. Incubation conditions are those described in Methods, except that 10^{-3} M methionine and varying amounts of ATP were used. The Michaelis constant for ATP and the inhibitor constant are 4.7×10^{-4} and 4×10^{-8} M, respectively. (b) (right) Inhibition of isoleucine-dependent ATP–$P^{32}P_i$ exchange by L-Ile-ol-AMP at a concentration of 3.3×10^{-8} M. A saturating concentration of isoleucine (2×10^{-4} M) and varying amounts of ATP were used. The Michaelis constant for ATP and the inhibitor constant are 4×10^{-4} and 5×10^{-8} M, respectively.

sponding amino acid. The results of experiments with methionyl- and isoleucyl-RNA synthetases (shown in Figure 2) indicate that in both cases the utilization of ATP was competitively inhibited by the *homologous* aminoalkyl adenylate.

It was of interest to compare the relative efficiency of aminoalkyl adenylates and the corresponding amino alcohols in causing inhibition of amino acid activation. For this purpose, the effect of DL-methioninol and L-isoleucinol on the ATP–$P^{32}P_i$ exchange promoted by methionine and isoleucine, respectively, in the presence of the appropriate enzyme was examined. Under standard assay conditions, the methionine-dependent exchange catalyzed by methionyl-RNA synthetase was 50% inhibited at a concentration of 10^{-3} M DL-methioninol. Equivalent inhibition of isoleucine-dependent exchange by isoleucyl-RNA synthetase required 8×10^{-3} M L-isoleucinol. Both amino alcohols behaved as fully competitive inhibitors of their corresponding aminoacyl-RNA synthetase. The Lineweaver–Burk plot of the inhibition of isoleucyl-RNA synthetase by L-isoleucinol is presented in Figure 1b. The inhibitor constants derived from these plots (Table III) are of the same order of magnitude as the Michaelis constants for the corresponding amino acids, and are approximately three orders of magnitude higher than the K_i values for the corresponding aminoalkyl adenylates.

In order to evaluate the *specificity* of aminoalkyl adenylates, the effect of each of the ten available compounds on the activation of methionine, isoleucine, and valine by partially purified methionyl-, isoleucyl-, and valyl-RNA synthetases, respectively, was examined by the ATP–$P^{32}P_i$-exchange assay in the presence of 2×10^{-3} M concentrations of amino acid and ATP. The results in Table IV indicate that, with very few exceptions, the *heterologous* aminoalkyl adenylates were ineffective on amino acid activation at concentrations at which the homologous aminoalkyl adenylate quantitatively inhibited the reaction. Thus, methionine-dependent ATP–$P^{32}P_i$ exchange catalyzed by methionyl-RNA synthetase was nearly quantitatively inhibited by 10^{-5} M L-methioninyl adenylate, while none of the nine heterologous adenylates, at concentrations up to 10^{-4} M, had a significant effect on this reaction. Similarly, valine-dependent ATP–$P^{32}P_i$ exchange catalyzed by valyl-RNA synthetase was inhibited nearly quantitatively and completely specifically by 10^{-5} M L-valinyl adenylate. At a concentration of 10^{-4} M, only L-isoleucinyl and DL-alaninyl adenylates caused minor, yet significant, unspecific inhibition. Isoleucine-dependent ATP–$P^{32}P_i$ exchange by isoleucyl-RNA synthetase was quantitatively inhibited by 10^{-4} M L-isoleucinyl adenylate. At this concentration, none of the heterologous aminoalkyl adenylates other than L-valinyl adenylate inhibited the reaction.

Inhibition of the activation of isoleucine by L-valinyl adenylate was expected, in view of the ability of isoleucyl-RNA synthetase to activate valine in addition to isoleucine (Bergmann *et al.*, 1961). This

TABLE IV: Specificity of Inhibition of the Amino Acid Dependent ATP-$P^{32}P_i$-Exchange Reaction by Aminoalkyl Adenylates and Amino Alcohols.[a]

Additions (M)	Methionyl-RNA Synthetase		Isoleucyl-RNA Synthetase		Valyl-RNA Synthetase	
	Cpm	% Control	Cpm	% Control	Cpm	% Control
None (control)	916	–	911	–	1145	–
L-Met-ol-AMP						
10^{-8}	916	100	956	105	1170	102
10^{-7}	824	90	916	100	1170	102
10^{-6}	300	33	911	100	1126	99
10^{-5}	42	5	914	100	1222	107
10^{-4}	0	0	876	96	1202	105
L-Ile-ol-AMP						
10^{-8}	930	101	861	94	1180	103
10^{-7}	880	96	851	93	1224	107
10^{-6}	912	100	531	58	1210	106
10^{-5}	900	98	154	17	1190	104
10^{-4}	914	100	9	1	934	81
L-Val-ol-AMP						
10^{-8}	870	95	916	100	1100	96
10^{-7}	870	95	958	106	1020	89
10^{-6}	956	104	868	95	420	37
10^{-5}	946	103	928	101	54	5
10^{-4}	910	99	547	61	4	0
DL-Methioninol						
4×10^{-6}	940	102	903	99	1146	100
4×10^{-5}	940	102	895	98	1116	98
4×10^{-4}	676	74	966	106	1094	96
4×10^{-3}	192	21	871	96	1140	100
L-Isoleucinol						
1×10^{-5}	878	96	883	97	1120	98
1×10^{-4}	900	98	934	102	1048	92
1×10^{-3}	860	94	771	85	1144	100
3×10^{-3}	890	97	631	69	1050	92

[a] The effect of each aminoalkyl adenylate and amino alcohol (at the concentrations indicated) on the activation of 2×10^{-3} M L-methionine, L-isoleucine, and L-valine by their corresponding partially purified aminoacyl-RNA synthetase was examined under the conditions described in Methods. Glycinyl, L-leucinyl, L-lysinyl, L-phenylalaninyl, L-tyrosinyl, L-prolinyl, and DL-alaninyl adenylates, tested at concentrations up to 10^{-4} M, had no discernible inhibitory effect on the activation of methionine, isoleucine, and valine (with the only exceptionth at DL-alaninyl adenylate caused 16% inhibition of the activation of valine catalyzed by valyl-RNA synthetase). The amount of $AT^{32}P$ formed in the absence of inhibitor corresponds to 37 μmoles/mg of protein in 15 min at 37° by methionyl-RNA synthetase, 65 μmoles/mg of protein in 15 min at 37° by isoleucyl-RNA synthetase, and 106 μmoles/mg of protein in 10 min at 37° for valyl-RNA synthetase. A ±5% deviation from these control values is not considered significant.

case was examined in greater detail by comparing the relative efficiency of L-isoleucinol, L-isoleucinyl adenylate, and L-valinyl adenylate in causing inhibition of the activation of isoleucine and valine catalyzed by isoleucyl-RNA synthetase. The results, expressed in per cent inhibition of original activity, are shown in Figure 3. Under the standard assay conditions used, approximately 10^{-6} M L-isoleucinyl adenylate was required to inhibit the activation of isoleucine by 50%. The concentration of L-valinyl adenylate and isoleucinol required to produce equivalent inhibition were approximately 50- and 4000-fold higher, respectively. When the activation of valine by isoleucyl-RNA synthetase was examined, the *relative* efficiency of the

TABLE V: Specificity of the Inhibition of Aminoacyl-RNA Formation by Aminoalkyl Adenylates.[a]

Additions (M)	Methionyl-RNA Synthetase		Isoleucyl-RNA Synthetase		Valyl-RNA Synthetase	
	Cpm	% Control	Cpm	% Control	Cpm	% Control
None (control)	1700	—	1409	—	1520	—
L-Met-ol-AMP 10^{-9}	1595	94	1414	100	1518	100
L-Met-ol-AMP 10^{-8}	1265	74	1415	100	1538	101
L-Met-ol-AMP 10^{-7}	372	22	1408	100	1558	102
L-Met-ol-AMP 10^{-6}	55	3	1454	103	1560	102
L-Met-ol-AMP 10^{-5}	0	0	1444	102	1491	98
L-Met-ol-AMP 10^{-4}	0	0	1217	86	1246	82
L-Ile-ol-AMP 10^{-9}	1662	98	1389	99	1527	100
L-Ile-ol-AMP 10^{-8}	1695	100	1339	95	1568	103
L-Ile-ol-AMP 10^{-7}	1675	99	851	60	1528	100
L-Ile-ol-AMP 10^{-6}	1615	95	174	12	1509	99
L-Ile-ol-AMP 10^{-5}	1547	91	17	1	1188	78
L-Ile-ol-AMP 10^{-4}	1570	93	0	0	387	25
L-Val-ol-AMP 10^{-9}	—	—	1442	102	1548	102
L-Val-ol-AMP 10^{-8}	1600	94	1414	100	1298	85
L-Val-ol-AMP 10^{-7}	1625	95	1457	103	594	39
L-Val-ol-AMP 10^{-6}	1647	97	1334	95	79	5
L-Val-ol-AMP 10^{-5}	1604	94	878	62	31	2
L-Val-ol-AMP 10^{-4}	1600	94	189	13	0	0

[a] The effect of each aminoalkyl adenylate (at the concentrations indicated) on aminoacyl-RNA formation by partially purified methionyl-, isoleucyl-, and valyl-RNA synthetases in the presence of 2×10^{-5} M L-[^{14}C]methionine, L-[^{14}C]isoleucine, and L-[^{14}C]valine, respectively, was examined as described in Methods. The amount of aminoacyl-RNA formed in 10 min at 30° in the absence of inhibitor corresponds to 207 mμmoles/mg of protein for methionyl-RNA synthetase, 52 mμmoles/mg of protein for isoleucyl-RNA synthetase, and 1400 mμmoles/mg of protein for valyl-RNA synthetase.

three inhibitors remained virtually unchanged, while the absolute concentration of each inhibitor required to inhibit valine activation to the same extent as isoleucine activation decreased by a factor of approximately 70. This shift reflects the lower affinity of the active site of the enzyme for valine as compared to isoleucine (K_m 1.4×10^{-4} and 3×10^{-6} M, respectively; Loftfield and Eigner, 1965). Further examination of the results reported in Figure 3 and Table IV shows that under the assay conditions used, 10^{-5} M L-isoleucinyl adenylate totally suppressed valine-dependent ATP-$P^{32}P_i$ exchange by isoleucyl-RNA synthetase, without affecting valine-dependent exchange catalyzed by valyl-RNA synthetase. This should provide a practical means for distinguishing between these two activities when unfractionated enzyme preparations are assayed.

Inhibition of Aminoacyl-RNA Formation by Aminoalkyl Adenylates. The effect of aminoalkyl adenylates on aminoacyl-RNA formation was examined in the presence of saturating concentrations of L-[^{14}C]amino acid (2×10^{-5} M) and ATP (2×10^{-3} M). Under these conditions, the formation of methionyl-, isoleucyl-, and valyl-RNA by the appropriate aminoacyl-RNA synthetases was 50% inhibited by the following concentration of the *homologous* aminoalkyl adenylate: 3×10^{-8} M L-methioninyl adenylate, 6×10^{-8} M L-valinyl adenylate, and 1.5×10^{-7} M L-isoleucinyl adenylate. The competitive nature of this inhibition with respect to amino acid utilization is demonstrated by the Lineweaver–Burk plot of the data on methionyl-RNA formation catalyzed by methionyl-RNA synthetase in the presence of methioninyl adenylate (shown in Figure 4).

In order to evaluate the *specificity* of aminoalkyl adenylates in this reaction, the effect of methioninyl, isoleucinyl, and valinyl adenylates on aminoacyl-RNA formation catalyzed by each of the three partially purified enzymes was examined. These results are summarized in Table V. The formation of methionyl-RNA was quantitatively inhibited by 10^{-6} M methioninyl adenylate, whereas neither of the heterologous adenylates were effective at concentrations up to 10^{-4} M. Isoleucyl-RNA formation was specifically inhibited approximately 90% in the presence of 10^{-6} M isoleucinyl adenylate. At higher concentrations, valinyl adenylate

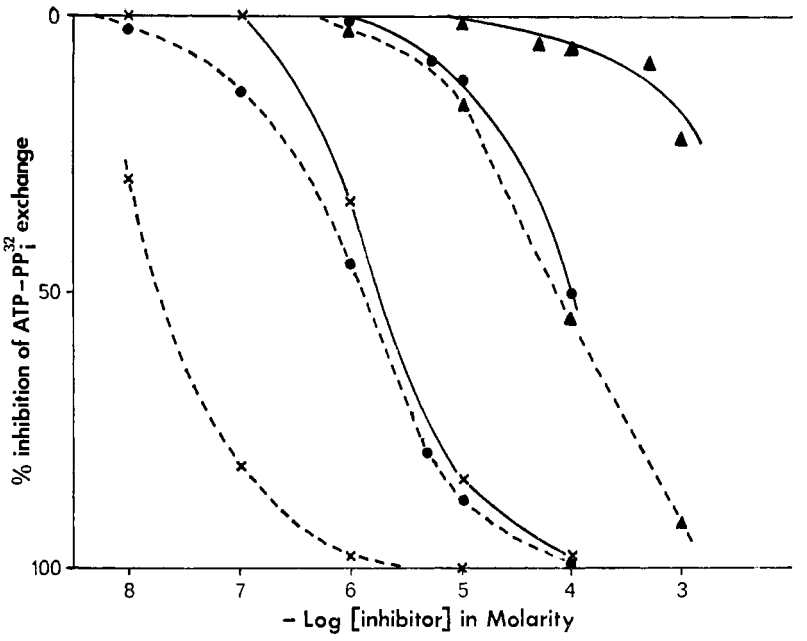

FIGURE 3: Inhibitory effect of L-isoleucinol (▲), L-Ile-ol-AMP (×), and L-Val-ol-AMP (●) on the activation of isoleucine (———) and valine (– – –) by isoleucyl-RNA synthetase. Activation was assayed by the ATP–$P^{32}P_i$-exchange reaction in the presence of 2×10^{-3} M concentration of amino acid and ATP. In the absence of inhibitor, isoleucine- and valine-dependent ATP–$P^{32}P_i$ exchange yielded 50.1 and 23.2 μmoles of AT$^{32}P_i$/mg of protein per 15 min at 37°, respectively.

also inhibited this reaction, as expected. Valinyl adenylate inhibited valyl-RNA formation quantitatively and specifically at a concentration of 10^{-6} M. Above this concentration, isoleucinyl adenylate affected the reaction, causing 75% inhibition at a concentration of 10^{-4} M. This unspecific inhibition of valyl-RNA synthetase was also observed in the ATP–$P^{32}P_i$-exchange assay (Table IV), where 20% inhibition occurred in the presence of 10^{-4} M isoleucinyl adenylate. The more pronounced inhibitory effect on aminoacyl-RNA formation results from the 100-fold lower concentration of amino acid present in this assay.

Discussion

The results reported in this paper indicate that structural analogs of aminoacyl adenylates in which the mixed anhydride linkage has been replaced by an ester bond behave as potent inhibitors of the activation of their corresponding amino acid by competing with the amino acid and ATP for attachment to the substrate binding site of the enzyme. In addition to a remarkably high affinity for their respective aminoacyl-RNA synthetases, these inhibitors possess the valuable features of stability as well as inability to participate in the reactions catalyzed by the enzymes.

Information on the mode of binding of aminoalkyl adenylates to their respective enzymes was obtained by comparing the inhibitory effect of an amino alcohol and its adenosine 5′-phosphate ester derivative on the activation of the corresponding amino acid. While both compounds behaved as competitive inhibitors, the inhibitor constant of the aminoalkyl adenylate was three orders of magnitude lower than that of the corresponding amino alcohol, in two cases examined. These results clearly indicate that the AMP moiety of the aminoalkyl adenylate participates in the binding of the inhibitor to the active site of the enzyme, and supports the view that the aminoalkyl adenylate occupies the same site on the enzyme as the corresponding aminoacyl adenylate. Furthermore, the results demonstrate that the mixed anhydride linkage of the aminoacyl adenylate and the carboxyl group of the amino acid are not essential for the binding of these molecules to the active site.

In order to evaluate the *specificity* of aminoalkyl adenylates as inhibitors of aminoacyl-RNA synthetases, the effect of each of the ten available compounds on the ATP–$P^{32}P_i$-exchange reaction catalyzed by methionyl-, isoleucyl-, and valyl-RNA synthetases was examined (Table IV). The results of these assays, carried out in the presence of a 2×10^{-3} M concentration of the appropriate amino acid, demonstrate that each enzyme was very nearly quantitatively inhibited by its homologous aminoalkyl adenylate at a concentration at which none of the nine heterologous derivatives were effective. At the highest concentration of aminoalkyl adenylate tested (*i.e.*, 10^{-4} M), the only significant unspecific inhibitions encountered were those of isoleucyl-RNA synthetase by valinyl adenylate

(*ca.* 40% inhibition) and of valyl-RNA synthetase by isoleucinyl and alaninyl adenylates (*ca.* 20% inhibition in each case). For comparative purposes, the effect of methioninyl, isoleucinyl, and valinyl adenylates on each of the three enzymes was also examined by the reaction leading to aminoacyl-RNA formation. In this reaction, performed in the presence of 2×10^{-5} M amino acid, specific and nearly quantitative inhibition by the homologous aminoalkyl adenylate was achieved at a concentration approximately one order of magnitude lower than that required in the ATP–$P^{32}P_i$-exchange assay (Table V). A parallel increase in the unspecific inhibition caused by heterologous adenylates was also observed. Thus, at the highest concentration of aminoalkyl adenylate tested (*i.e.*, 10^{-4} M), the unspecific inhibition of isoleucyl-RNA synthetase by valinyl adenylate, and of valyl-RNA synthetase by isoleucinyl adenylate increased to 87 and 75%, respectively, while methioninyl adenylate, which had no discernible effect on the ATP–$P^{32}P_i$-exchange reaction, inhibited both enzymes to a minor, yet significant, extent. From these observations, it is clearly apparent that while aminoacyl-RNA synthetases exhibit far greater affinity for their homologous aminoalkyl adenylate, they are also able to bind heterologous adenylates, with varying affinities. However, while each of the possible enzyme–heterologous aminoalkyl adenylate interactions will potentially lead to inhibition of amino acid activation through occupation of the active site of the enzyme, the extent to which inhibition occurs at any concentration of the adenylate is necessarily dependent on the amino acid concentration present during the reaction. Raising the amino acid concentration in the test will result in the progressive displacement of the heterologous aminoalkyl adenylates from the active site, in the order of increasing affinity, hence accounting for the fact that only very few cases of unspecific inhibition were observed in the ATP–$P^{32}P_i$-exchange assay in the presence of 2×10^{-3} M amino acid (Table IV).

The ability of aminoacyl-RNA synthetases to combine with heterologous aminoalkyl adenylates is not unexpected, in view of the demonstration (Novelli, 1958; Karasek *et al.*, 1958; Berg, 1958; Krishnaswamy and Meister, 1960) that these enzymes can catalyze ATP synthesis from a variety of externally supplied aminoacyl adenylates in addition to the homologous aminoacyl adenylate, in the presence of pyrophosphate. However, the particularly broad specificity observed in this reaction may be attributed to the fact that the enzyme–heterologous aminoacyl adenylate interactions leading to ATP synthesis take place in the absence of the competing homologous amino acid.

It should be pointed out that in addition to their ability to inhibit selectively the activation of their corresponding amino acid, aminoalkyl adenylates constitute, by virtue of their high affinity, their stability, and their inability to participate in the reactions catalyzed by the enzyme, a valuable tool in connection with studies concerned with enzyme–substrate interactions. Applications of aminoalkyl adenylates in such studies will be reported elsewhere.[2]

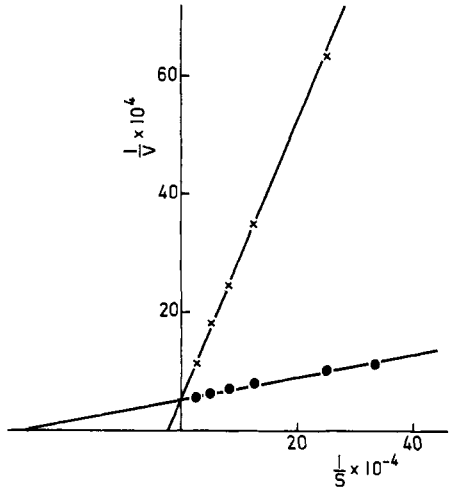

FIGURE 4: Lineweaver–Burk plot of the inhibitory effect of L-Met-ol-AMP on methionyl-RNA formation catalyzed by methionyl-RNA synthetase. The concentration of amino acid was varied in the presence of a fixed concentration of inhibitor (5×10^{-8} M) and saturating amount of ATP and sRNA. Incubation conditions are those described in Methods. The values for K_m and K_i derived from this plot are 3.4×10^{-6} and 4.7×10^{-9} M, respectively.

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The Specificity of Lincomycin Binding to Ribosomes*

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ABSTRACT: The binding of [^{14}C]lincomycin to ribosomes of *Bacillus stearothermophilus* was studied with respect to ribosomal subunit specificity and the stability of the lincomycin–ribosome complex. Ammonium or potassium, but not calcium or magnesium ions, are required for optimal binding. Lincomycin binds to the 50S subunit of *B. stearothermophilus*, but not to the 30S subunit, nor to the 70S ribosome of *Escherichia coli*. Between 0 and 37° both the rate and extent of lincomycin binding by ribosomes increase, whereas only the rate (but not the final extent) of [^{14}C]phenylalanyl soluble ribonucleic acid binding increases. Thus at 25° lincomycin is a more effective inhibitor of phenylalanyl soluble ribonucleic acid binding than at 15°, although the capacity of the ribosomes to bind phenylalanyl soluble ribonucleic acid is the same at these two temperatures. The [^{14}C]lincomycin–ribosome complex dissociates partially upon dilution indicating that, at least, one phase of the binding reaction is reversible. [^{14}C]-Lincomycin can be displaced from the ribosomes by [^{12}C]lincomycin or erythromycin, but not by chlortetracycline. These results suggest that the antagonism between erythromycin and lincomycin observed in intact cells may be owing to a breakdown of the lincomycin–ribosome complex in the presence of erythromycin.

Lincomycin, at low concentrations, inhibits protein synthesis in Gram-positive, but not Gram-negative, organisms (Josten and Allen, 1964). It has been shown to act on the 50S subunit of the ribosomes of a Gram-positive organism but not on the corresponding subunit of a Gram-negative organism (Chang *et al.*, 1966). In order to characterize further the model of action of this antibiotic, kinetics, ionic requirements, and subunit specificity of the binding of [^{14}C]lincomycin to ribosomes were studied.

Lincomycin-sensitive, erythromycin-resistant strains of *Staphylococcus aureus* are (phenotypically) lincomycin resistant when grown in the presence of erythromycin (Barber and Waterworth, 1964; Griffith *et al.*, 1965). In the present studies, the interaction of lincomycin and erythromycin with *Bacillus stearothermophilus* ribosomes was examined in cell-free preparations. The reversibility of the [^{14}C]lincomycin–ribosome complex in the presence of erythromycin suggests a mechanism for the antagonism between lincomycin and erythromycin which has been reported for intact cells *in vitro*.

Materials and Methods

Bacterial cells, ribosomes, ribosomal subunits, and aminoacyl-sRNA were prepared as described previously (Chang *et al.*, 1966). For the strain of *B. stearothermophilus* used, the minimum inhibitory concentrations (by tube dilution assay) were 10^{-8} to 10^{-7} M for erythromycin and 10^{-7} to 10^{-6} M for lincomycin. The nitrocellulose membrane assay (Nirenberg and Leder, 1964) was used as described below. Under these conditions, at least four A_{260} units of ribosomes of *B. stearothermophilus* or *Escherichia coli* could be adsorbed from a solution of 3 ml with an efficiency greater than 85%. This was verified using [^{14}C]uracil-labeled *E. coli* ribosomes as well as by determining the A_{260} recoverable from the nitrocellulose membrane filter by extraction with 5% TCA[1] for 30 min at 90°. Incubation of *E. coli* or *B. stearothermophilus* ribosomes with lincomycin had no effect on the amount of A_{260} subsequently recovered from the membrane filter by hot TCA extraction. In calculating the stoichiometry of binding, one A_{260} unit of 70S ribosomes corresponds to 24 $\mu\mu$moles and 100 cpm of lincomycin corresponds to 22 $\mu\mu$moles.

[^{14}C]Lincomycin (specific activity, 5 μc/μmole), prepared by reductive alkylation of *N*-demethyllincomycin treated with [^{14}C]formaldehyde, was generously provided by Drs. B. Margerlein and D. J. Mason of the Upjohn Co. *N*-Demethyllincomycin, a presumed precursor of lincomycin which lacks the methyl substituent on the ring nitrogen, is obtained as a by-

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[1] Abbreviations: PEP, phosphoenolpyruvate; TCA, trichloroacetic acid; ATP and GTP, adenosine and guanosine triphosphates.