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ACTIVE INSOLUBILIZATION OF BIOLOGICAL COMPOUNDS

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ACTIVE INSOLUBILIZATION OF BIOLOGICAL COMPOUNDS

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ABSTRACT

Part I: The mechanism of the pyrophosphate exchange reaction catalyzed by L-isoleucine tRNA synthetase was investigated by studying the binding of substrate analogs. We found that the enzyme has a rigidly defined active conformation. Binding of any substrate suffices to induce this conformation totally. Conversion to the active conformation is linked to the opening of an ion pair in the active site. ATP was found to be bound in a strained, high energy conformation. This serves to promote the formation of the aminoacyl adenylate. Certain topographical features of the active site were determined.

We further determined that adsorbents for affinity chromatography might best be prepared through attachment of L-isoleucine analogs via tails on C-1 or by attachment of L-isoleucyl adenylate via tails on C-1 of the isoleucyl moiety or on the 6-amino group of the purine moiety. Adsorbents using L-isoleucine esters as the insolubilized inhibitor proved unsuccessful in effecting separation of the synthetase from contaminating proteins. Successful purification was achieved using L-isoleucinol adenylate attached to

agarose via an ethylaminosuccinylaminopropylaminopropyl tail on the 6-amino position.

Part II: An attempt to purify RNA-directed DNA polymerase by affinity chromatography using an adsorbent carrying N-amino-N-desmethylrifampicin as the insolubilized ligand gave inconclusive results.

Part III: The binding of L-alanine, poly-D,L-alanine, AMP, and polyadenylic acid to sodium, calcium, and aluminum montmorillonite was studied. Very low levels of binding were found in all cases. The montmorillonite mediated polymerization of L-alanyl adenylate was investigated.

INTRODUCTION

The title of this thesis, "Active Insolubilization of Biological Compounds," refers to the attachment of biologically active compounds to insoluble supports (either by adsorption or through the formation of covalent bonds) in such a way that some or all of the biological activity is retained. This is an enormous field and we have made no attempt to cover it here. Rather, this title was chosen because it is a dominant motif which links together the three projects described herein (also, because our aspirations at the time at which the title was selected were considerably greater than our achievements have been).

The first two parts involve affinity chromatography, a technique based on the ability of many biological molecules to recognize and bind to other molecules. The success of the technique is dependent upon the insolubilization of such a molecule in such a way that its ability to recognize or be recognized is not impaired.

It is also proposed that some of the synthetic techniques developed in Part I would be suitable for the insolubilization of NAD^+ or NADP in such a way that they retain their ability to function as coenzymes.

In Part III, L-alanyl adenylate is insolubilized by adsorption to montmorillonite clay. Such adsorptions have been postulated as mechanisms for concentration of organic molecules in

the prebiotic ocean. Upon insolubilization, the ability of the adenylate to polymerize to yield polypeptides is not only maintained, but enhanced.

Other areas of active insolubilization, such as the preparation of insolubilized enzymes, are not considered to a significant extent in this work.

PART I: THE PURIFICATION OF L-ISOLEUCINE tRNA SYNTHETASE FROM *E. COLI B* BY AFFINITY CHROMATOGRAPHY

INTRODUCTION

L-isoleucine tRNA synthetase (IRS)* is one of a family of enzymes known as aminoacyl tRNA synthetases. These enzymes are responsible for charging molecules of tRNA with the appropriate amino acid. There is a separate enzyme for each of the naturally occurring amino acids, except glutamine.[1]

It has been shown that the protein synthesizing system recognizes only the tRNA portion of the aminoacyl tRNA and that misacylation of a tRNA will result in the incorporation of a wrong amino acid into the growing protein.[2] The fidelity of protein synthesis is totally dependent on the correct attachment of amino acids to tRNA's by the synthetases.

It has been estimated that the frequency with which an incorrect amino acid is introduced into a protein *in vivo* is one in ten thousand.[1] Aminoacyl tRNA synthetases would therefore be expected to show quite extraordinary specificity in selecting the correct amino acids and tRNA's. Studies on the synthetases *in vitro* have generally born out this presumption. However, under certain conditions mischarging has been observed,[3-5] especially with IRS.[6-8]

Our interest in IRS was predicated on this latter finding. Frequent mischarging due to unusual conditions *in vivo*, whether by accident or design, might have significant implications for the biochemistry of aging, of neoplasia, or learning and memory, among other areas. In this lab there was particular interest in the

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*Abbreviations used in this work are listed in Appendix A.

possibility that the passage of nerve impulses could alter the nerve cell micro-environment in such a way as to induce specific misacylation, thus providing a possible mechanism for memory coding.

Although the latter hypothesis is admittedly quite tenuous, the information which would be obtained from studies of IRS would be of value in increasing our knowledge of a phase of protein synthesis which is only beginning to be understood.

In order to carry out such studies, it was necessary to obtain reasonable quantities of purified enzyme. The isolation of the enzyme by classical techniques was quite slow and tedious and gave very poor recoveries.

We decided to try to take advantage of the high specificity which the enzyme showed for its substrate, L-isoleucine, in order to effect a simple, one-step purification. That is, we would attach L-isoleucine by suitable means to an insoluble support. When cell sap containing IRS was passed through a column of such material, the IRS would bind to the L-isoleucine and be retarded or stopped in its passage through the column, while all of the impurities would pass directly through. If the IRS was sufficiently strongly bound so as to be retained on the column, it could be released by passage of an eluent containing free L-isoleucine. The IRS would partition between the soluble and insoluble substrate and be thereby eluted. This technique has since become known as affinity chromatography.

This was initially conceived as being a short term project. In practice, it turned out to be considerably more complicated than on

paper, and became the major portion of my research. A number of binding studies were also undertaken on the synthetase, but even these were primarily directed toward determining how to best approach the purification of the enzyme. They did provide considerable useful information on the geometry of the active site as well.

The binding studies and the preparation and testing of affinity columns were carried on concurrently and there was considerable interaction between the two lines of study. But for the purposes of pedagogy, the two lines of inquiry have been described separately in the following pages, with cross-references where pertinent.

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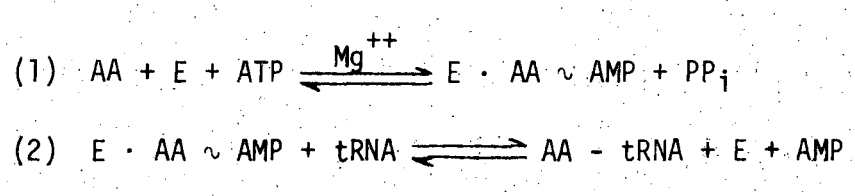
SECTION A: STUDIES ON L-ISOLEUCINE tRNA SYNTHETASE FROM *E. COLI B*

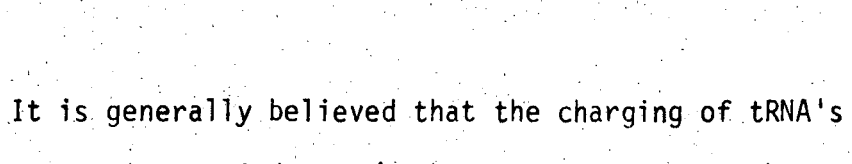
General Properties of Aminoacyl tRNA Synthetases

Aminoacyl tRNA synthetases from the same organism show great variation in structure and are not clustered on the genetic map. It is quite possible that they may have evolved independently of one another. Yet there are certain features which appear common to all of them.

For the synthetases which have been studied, the following generalizations can be made: They are soluble enzymes found in the cell sap, are easily denatured, and (with a few exceptions) are sensitive to anti-sulfhydryl agents. They are capable of carrying out the following reactions:



(1) $AA + E + ATP \xrightleftharpoons{Mg^{++}} E \cdot AA \sim AMP + PP_i$



(2) $E \cdot AA \sim AMP + tRNA \rightleftharpoons AA - tRNA + E + AMP$

It is generally believed that the charging of tRNA's by the synthetases is carried out via the above two-step mechanism, although there is also evidence to indicate that *in vivo* the charging may occur through a concerted rather than sequential pathway.[1]

The synthetases show a high degree of specificity for their specific amino acid and tRNA, and are capable of using only ATP or dATP as an energy source. dATP has shown a higher Km than ATP for all synthetases tested.[1]

Properties of IRS from E. Coli B

The L-isoleucine tRNA synthetase from *Escherichia coli B* was first isolated by Bergmann *et al.* in 1960.[2] It is a single polypeptide chain[3] of molecular weight 112,000[4] with one binding site per molecule for L-isoleucine,[5] ATP,[5] and $tRNA^{ile}$.[6]

The Michaelis constants for L-isoleucine and ATP are 5×10^{-6} moles/l. and 4×10^{-4} moles/l., respectively,[2] and that of $tRNA^{ile}$ is 2×10^{-7} moles/l.[4] The order of binding of the substrates has not been determined.

L-Valine as well as L-isoleucine can be utilized in the pyrophosphate exchange reaction, Eq. (1). The former has a Michaelis constant of 4×10^{-4} moles/l. for this reaction. However, the synthetase will not charge $tRNA^{ile}$ with L-valine. D-isoleucine and naturally occurring L-amino acids other than L-isoleucine and L-valine are not substrates for the enzyme.[2]

IRS begins to denature at pH's less than six[2] or greater than ten.[8] It also becomes denatured in 2.5 M urea[9] and at temperatures greater than 40°C.[1] The binding of substrates serves to protect the enzyme against denaturation by heat[4] or urea.[9]

The enzyme also becomes inactive on standing in air. 2-Mercapto ethanol prevents this inactivation and partially restores activity lost in this manner, suggesting one or more sulfhydryl groups are involved in catalysis. Reaction with antisulfhydryl reagents indicated one substrate-protectable group and eight other titrable groups.[10]

The complex of the synthetase with L-isoleucyl adenylate is very stable in the absence of pyrophosphate or $tRNA^{ile}$ and can be isolated by gel filtration.[11]

Binding Studies

We chose to investigate the geometry of the IRS active site, both as an aid to the synthesis of adsorbents for affinity chromatography, and "because it was there" and deserved to be studied as part of the elucidation of the properties of aminoacyl tRNA synthetases. This investigation was carried out through the determination of inhibition constants or Michaelis constants, and/or dissociation constants of various substrate analogs.

Inhibition constants were determined by inhibition studies using the ATP - [^{32}P] PP_i exchange reaction described by Norris and Berg.[12] Reaction rates (expressed as apparent units/ml) were measured in the presence of a fixed amount of inhibitor and varying amounts of either L-isoleucine or MgATP as appropriate. The inhibitor was at a concentration greater than its inhibition constant, except where this was impossible due to solubility limitations. The substrate not under variance was at saturating concentration. The data were analyzed by the linearization procedure of Eadie[13] using least-square fits. Studies were carried out on IRS purified to homogeneity following the procedure of Norris and Berg[12] and/or on partially purified IRS (C_γ gel eluate, see Section B, p.). The results for studies using either preparation were equivalent.

Michaelis constants were determined by varying the substrate concentration in a series of ATP - [^{32}P] PP_i exchange reactions and plotting the data according to Eadie,[13] except in the cases of α-DL-aminohexanoic and α-D,L-aminoheptanoic acids, which were analyzed by the procedure of Inagami.[14] The procedure is described in Appendix B.

Dissociation constants were measured by Dr. Eggehard Holler using his fluorescence quenching technique.[15] This procedure is also described in Appendix B.

The results of these determinations are summarized in Table 1 for L-isoleucine analogs and in Table 2 for ATP analogs.

Results

The following observations from the tables are particularly worthy of note:

For L-isoleucine analogs:

(1) Modification of the carboxylate yields compounds which are more weakly bound than L-isoleucine. The amount of decrease in binding affinity seems generally correlated with the amount of steric bulk in the modified moiety with the exception of the hexyl ester, which binds slightly better than the ethyl.

(2) The bindings of L-isoleucinol and 2-methyl-1-butylamine are considerably stronger when measured by the pyrophosphate exchange reaction (i.e., in the presence of ATP) than when measured by fluorescence quenching. For all other compounds for which binding was measured by both techniques, the inhibition and dissociation constants are similar.

(3) Modification or removal of the α-amino group results in a complete loss of ability of the analog to bind to IRS. Removal of the α-amino group combined with conversion of the carboxylate to an uncharged moiety gives a weakly binding compound.

Table 1. Binding Constants of L-isoleucine and Analogs to L-isoleucine t-RNA Synthetase

Compound	Method[a]	K_m (moles/ℓ)[b]	K_i (moles/ℓ)[b]	K_s (moles/ℓ)[b]	V_{max} or max quenching[c]
L-isoleucine	F			5.8×10^{-6}	1.0
	P(37°)	5×10^{-6}[d]			1.0
L-isoleucine ethyl ester	P(37°)		3×10^{-4}		1.0
L-isoleucine hexyl ester	P(37°)		4×10^{-5}		1.0
L-isoleucinol	F			5.5×10^{-3}	1.0
	P(37°)		2×10^{-5}		1.0
1-nitromethyl L-isoleucinol	P(37°)		4×10^{-3}		1.0
2-methyl-1-butylamine	F			9×10^{-3}	1.0
	P		10^{-5}		1.0
N-methyl-D,L-isoleucine	F			---	None
	P		$>10^{-2}$		
Guanidino-L-isoleucine	F			---	None
	P		> 0.1		
3-methyl pentanoic acid	F			---	None
	P	>0.5			
3-methyl 1-pentanol	F(10°)			1.6×10^{-3}	1.0
3-methyl 1-pentanal	F(10°)			2.5×10^{-3}	1.0
O-methyl-L-threonine	F			1.2×10^{-4}	1.0
	P	6×10^{-5}			1.6
L-valine	F			1.6×10^{-4}	1.0
	P[d]	4×10^{-4}			0.8
L-norvaline	P[e]	1.5×10^{-3}			0.9
L-alloisoleucine	P[e]			10^{-3}	0.4
α-D,L-aminopentanoic acid	F			2×10^{-3}	1.0
	P	10^{-3}			0.7

Table 1 (continued)

Compound	Method[a]	K_m (moles/ℓ)[b]	K_i (moles/ℓ)[b]	K_s (moles/ℓ)[b]	V_{max} or max quenching[c]
α-D,L-aminohexanoic acid	F			$7x10^{-3}$	1.0
	P	$7x10^{-3}$			0.35
α-D,L-aminoheptanoic acid	P	$2x10^{-2}$			0.35
L-isoleucinol adenylate	F(10°)			10^{-8}	1.0
	P(37°)		$4x10^{-9}$		---[f]
L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate	P(37°)		$2x10^{-8}$		---[f]

[a]P indicates ATP-[^{32}P] PP_i exchange; F indicates fluorimetric titration. Investigations at 25°C unless otherwise noted.

[b]K_m = Michaelis constant; K_i = inhibition constant; K_s = dissociation constant.

[c]Relative to value for L-isoleucine. V_{max} is the rate at extrapolated infinite substrate concentration for those compounds which are substrates and at extrapolated infinite L-isoleucine concentration for those which are inhibitors.

[d]Values from Berg, et al.[16]

[e]Values from Loftfield and Eigner.[17]

[f]Values of V_{max} were dependent on ATP/inhibitor ratio.

Table 2. Binding Constants of ATP and Analogs to L-isoleucine t-RNA Synthetase

Compound	Method[a]	K_m (moles/ℓ)[b]	K_i (moles/ℓ)[b]	K_s (moles/ℓ)[b]	V_{max} or max quenching[c]
ATP	F			2.5×10^{-4}	1.0
	P	4×10^{-4}			1.0
AMP	F			7.5×10^{-4}	0.6
	P		9×10^{-4}		1.0
Adenosine	P		3×10^{-4}		1.0
Adenine	P		3×10^{-2}		1.0
D-Ribose	P		2×10^{-2}[d]		1.0
Pyrophosphate	F			2.6×10^{-4}	1.0
	pe,f	3×10^{-5}			---
Phosphate	P		10^{-2}		1.0
L-isoleucinol adenylate	F(10°)			10^{-8}	1.0
	P		3×10^{-8}		---g
N^6-methyl AMP	P		5×10^{-3}		1.0
L-isoleucinol N^6-methyl adenylate	P		2×10^{-6}		---g
N^6-methyl adenosine			6×10^{-3}		0.9
N^6-(6-aminohexyl) adenosine	P		9×10^{-4}		0.25
N^6-(2-aminoethyl) adenosine	P		6×10^{-4}		0.1
L-isoleucinol N^6-(2-aminoethyl) adenylate	P		5×10^{-7}		---g
$O^{2'},O^{3'}$-Isopropylidene adenosine	P		$>10^{-2}$		1.0

[a] F indicates fluorimetric titration at 25° unless otherwise noted. P indicates ATP-[^{32}P] PP_i exchange at 37°.

[b] K_m=Michaelis constant; K_i=inhibition constant; K_s=dissociation constant.

[c] Relative to value for ATP. V_{max} is the rate at extrapolated infinite concentration for ATP.

[d] Based on the assumptions that only the β-furanose is inhibitory and that this form comprises 18% of the D-ribose in an aqueous solution at equilibrium.

[e] Value from Cole and Schimmel.[18]

[f] PP_i is a substrate for the exchange reaction as well as being a competitive inhibitor of ATP. V_{max} at extrapolated infinite ATP concentration will be dependent on the PP_i concentration.

[g] Value of V_{max} is dependent upon the L-isoleucine/inhibitor ratio.

(4) Removal of a methyl from either branch of the side chain (L-valine and L-norvaline) results in a considerable decrease in binding affinity without greatly affecting catalytic ability as measured by V_{max}. Substitution of a hydrophilic oxygen for a methylene (O-methyl threonine) gives a similar reduction in binding and, interestingly, increases V_{max}.

(5) The effects of extension of either arm of the side chain, as determined by extrapolation from the differences between L-valine and L-alloisoleucine (extension of methyl arm), or among α-D,L-aminopentanoic acid, α-D,L-aminohexanoic acid, and α-D,L-aminoheptanoic acid (extension of ethyl arm), are a moderate reduction in binding as well as a decrease in V_{max}.

(6) The maximum fluorescence quenching was the same, within experimental error, for all compounds which exhibited ability to bind to IRS.

For ATP analogs:

(1) ATP, AMP, adenosine, and pyrophosphate all bind to IRS with similar affinities.

(2) The product of the inhibition constants of adenine and β-D-ribofuranose is approximately equal to that of adenosine.

(3) Alkylation on the 6-amino group is associated with a decrease in competitive binding and the appearance of apparent non-competitive binding. Extension of the alkyl group seems to reduce both types of binding. Addition of an ω-amino group to the alkyl group seems to increase both types of binding.

(4) The results of N^6-alkylation are not always comparable among adenosine, AMP, and L-isoleucinol adenylate.

(5) $O^{2'},O^{3'}$-isopropylidene adenosine shows very weak binding.

(6) The maximum fluorescence quenching produced by ATP and pyrophosphate is the same as that given by L-isoleucine and its analogs. AMP gives a lesser fluorescence quenching.

For L-isoleucyl adenylate analogs:

(1) The inhibition constant of L-isoleucinol adenylate (vs. L-isoleucine) is approximately equal to the product of the inhibition constants of L-isoleucinol and adenosine.

(2) L-isoleucinol adenylate and L-isoleucinol N^6-(2-aminoethyl) adenylate are competitive inhibitors of both L-isoelucine and ATP. Their inhibition constants are lower when measured against L-isoleucine.

(3) The comments on N^6-alkylation above apply likewise here.

(4) The maximum fluorescence quenching of L-isoleucinol adenylate is the same as that for L-isoleucine and its analogs.

Discussion

L-isoleucine tRNA synthetase has been shown to be a monomer[3] with only one active site.[5,6] Our data are consistent with these findings, and have been interpreted on this basis.

Our experiments were all carried out in the absence of $tRNA^{ile}$. It is possible that the formation of an IRS-$tRNA^{ile}$ complex may result in the alteration of the catalytic site and possibly even of the catalytic pathway. Comparison of our results with results obtained in the presence of $tRNA^{ile}$ would be of considerable interest. However, we performed no such experiments. The following discussion applies only to the free enzyme.

The order of binding of the substrates has not been determined. We believe it is random. Both L-isoleucine and ATP can bind to IRS in the absence of the co-ligand, and the strength of this binding is little affected by the presence or absence of the co-ligand, as shown by the very similar values obtained for the Michaelis constants and the dissociation constants.

Moreover, the binding of L-isoleucine, ATP, L-isoleucyl adenylate (formed *in situ* with L-isoleucine and ATP), and L-isoleucinol adenylate all gave the same maximum fluorescence quenching. This suggests that the binding of any substrate induces the same conformational change in the enzyme to give the catalytically active conformation. It is possible that the induction of the same fluorescence quenching may be fortuitous, but this is unlikely. (Further justification for the correlation of fluorescence quenching with conformational changes and for the equation of binding of substrates to the TNS-IRS complex with binding to free IRS may be found in Holler *et al.*[15] or in Appendix B).

This conformation change appears to be induced not only by the binding of the natural substrates, but also by all but one of the substrate analogs tested. With the exception of AMP, all of the compounds which were bound at all gave the same maximum fluorescence quenching.

Similarly, among those analogs which catalyzed the pyrophosphate exchange reaction there was at most a threefold decrease in V_{max} compared to differences of up to 3,000-fold in the K_m values. This implies that the binding of a ligand to the enzyme is rigidly coupled to the induction of the "active" conformation. This behavior is in

marked contrast to that of enzymes such as chymotrypsin, wherein the extent of conformational change and the rate of catalysis is quite sensitive to the geometry of the ligand.[19]

Binding of L-isoleucine. The abolition of binding upon methylation or guanidation of the α-amino group of L-isoleucine can be understood in terms of simple steric hindrance. That binding is also abolished upon removal of the amino group is somewhat more difficult to explain. A clue is offered by the fact that conversion of the carboxylate to a neutral derivative concomitant with removal of the amino group gives analogs to which binding ability has been restored, albeit at a very low level. A possible explanation is that the protonated amino group is paired with a negatively charged group on the enzyme. Removal of the amino group not only eliminates the binding energy provided by ion bridge formation but also allows a repulsive interaction between the unpaired negatively-charged group on the enzyme and the carboxylate. Conversion of the carboxylate to a neutral group eliminates this repulsion and allows the molecule to bind. The binding energy in the latter case is presumably provided by the interaction of the side chain with the enzyme.

When the carboxylate alone is removed to give 2-methyl-1-butylamine, the level of binding as measured by inhibition of the pyrophosphate exchange was reduced by a factor of two, but when measured by fluorescence quenching was reduced almost 2000-fold. The most obvious possible explanation for this discrepancy is that ATP enhances the binding of the analog. The pyrophosphate exchange reaction occurs of necessity in the presence of ATP. When the

dissociation constant of 2-methyl-1-butylamine was determined from the fluorescence quenching in the presence of ATP, it was found to be 4×10^{-6} moles/liter,[8] a value which is lower than that of L-isoleucine itself. Similarly, the K_s was determined to be 3×10^{-5} moles/liter in the presence of pyrophosphate.[8] The value of 10^{-5} moles/liter determined in the pyrophosphate exchange can be viewed as a weighted average of binding constants for the enzyme-ATP complex and for an enzyme-pyrophosphate complex (pyrophosphate is a competitive inhibitor of ATP as well as a substrate for the exchange).

Similar results were obtained for L-isoleucinol, in which the carboxylate of L-isoleucine has been reduced to the uncharged alcohol.

These results, as well as the previous ones, may be interpreted in terms of the following hypothesis: The binding of L-isoleucine is associated with the opening of an ion pair within the active site and the formation of new pairs with the ammonium and carboxylate moieties of the amino acid. Upon formation of L-isoleucyl adenylate the positive component of the active site becomes associated with the phosphate. The absence of either the ammonium or the carboxylate leaves one of the charged enzymic components unpaired and results in corresponding destabilization of the complex and weakening of the binding. The absence of the carboxylate may be compensated for by the presence of ATP, AMP or pyrophosphate. This is quite reasonable in view of the supposition that the positive group involved subsequently becomes associated with the α-phosphate. The pyrophosphate binding site is only proximal to the carboxylate and α-phosphate sites, and pyrophosphate therefore cannot interact as effectively. The enhancement of binding in the presence of pyrophosphate is accordingly considerably less than that seen for ATP or AMP.

If this hypothesis is correct, one should be able to test it by neutralizing one of the components of the ion pair by titration. Basic titration indicated the presence of a group with a pK_a (app.) of 9.4 - 9.5, the neutralization of which was associated with a decrease in binding of L-isoleucine and the induction of fluorescence quenching equal to that induced by the binding of a substrate (see Appendix B). Acid titration was not attempted because the expected pK_a of the negative group was considerably below the pH at which denaturation begins to occur (i.e., pH 6).

When conversion of the carboxylate to a neutral derivative was accompanied by an increase in size of the residue, as in L-isoleucine ethyl ester or 1-nitromethyl L-isoleucinol, the level of binding dropped below that seen for L-isoleucinol or 2-methyl-1-butylamine. This is presumably a simple steric effect. However, on going from the ethyl to the hexyl ester, binding is increased. Any reduction in binding due to steric interference appears to be offset by hydrophobic interactions. Santi et al. have reported a similar effect for the binding of phenylalanyl esters to PRS.[22] Initially we thought the hexyl group might be binding to a part of the adenosine binding site. However, L-isoleucine hexyl ester was not a competitive inhibitor of ATP.

We also determined the effects of shortening or lengthening the branches of the side chain. The removal of a methylene from the ethyl or methyl branches of L-isoleucine gives L-valine and L-norvaline (α-L-aminopentanoic acid), respectively. The values of V_{max} were only slightly decreased and the maximum fluorescence quenching remained unchanged. The values of the binding constants were

significantly increased. The losses in free energy of binding corresponding to the increase in K_s were 3.2 Kcal/mole for removal of the β-methyl and 2.2 Kcal for loss of the γ methyl. These values are considerably higher than the value of 1.1 Kcal/mole for the transfer of a methylene from water to a non-polar solvent.[25] The strength of binding cannot be explained completely on the basis of hydrophobic interactions. This discrepancy was also noted by Loftfield[24] who postulated space-filling effects to account for it. When we determined the binding of O-methyl threonine, an analog in which a methylene has been replaced by a hydrophilic oxygen, the increase in K_m corresponded to a loss of 1.9 Kcal/mole in binding energy. This is also excessive. The difference cannot be explained by space-filling considerations since no "hole" remains in the binding site when this analog is bound. Additional factors must be operative in producing these sorts of effects - effects which are responsible for the very high degree of selectivity shown by the synthetase. At this point, we cannot propose a good explanation for these results. We believe they may be linked to the apparent rigid coupling between the binding of the side chain and the induction of the fixed active conformation.

The effect of elongation of the methyl branch of the side chain was determined using L-alloisoleucine. Because it has an inverted configuration at carbon 3, it is expected that on binding to IRS, the methyl group will occupy the ethyl subsite and vice versa. The changes noted in the parameters will be the result of both elongation of the methyl and shortening of the ethyl. In order to determine the effects of elongation of the methyl only, the data were compared with those for L-valine.

The V_{max} value was approximately halved and the binding was reduced six-fold in going from L-valine to L-alloisoleucine. The effect of extending the methyl group is slight. The results indicate that the methyl binding site is not rigidly closed at the end as is the case for trypsin and chymotrypsin.[20] Were the subsite a closed pocket, the presence of the extra methyl could be expected to push the rest of the analog into a catalytically improper position, with the result of substantial decreases in binding and maximum rate of catalysis.

The effect of lengthening the ethyl branch was determined by comparing the parameters for α-D,L-aminohexanoic acid and α-D,L-aminoheptanoic acid with those for α-D,L-aminopentanoic acid. The results for addition of one methylene were almost identical to those seen for extension of the methyl branch. Addition of a second methylene gave a slight further reduction in binding, but did not affect V_{max}.

The argument for an open-ended methyl site may be invoked regarding the open-endedness of the ethyl binding site as well. In this case, it cannot be completely rigorous. The lack of branching on the side chains of these analogs will allow them to bind to either the methyl or the ethyl subsite. The methyl subsite has already been shown to be open-ended and capable of accomodating the longer chains. The results may reflect binding of the side chain to the methyl site. We can only say that the ethyl subsite may also be open-ended.

Binding of ATP. In studying the binding of the components of ATP, it was found that the product of the inhibition constants of adenine and β-D-ribofuranose was approximately equal to the inhibition

constant of adenosine. Assuming that the inhibition constants are good measures of the dissociation constants, this is equivalent to saying that the free energy of binding of adenosine is approximately the sum of the free energy of binding of its components. Specifically, $\Delta G_{Ade} + \Delta G_{rib} = 2.2$ Kcal + 2.4 Kcal = 4.6 Kcal; $\Delta G_{Ado} = 5.0$ Kcal. The extra 0.4 Kcal of binding energy may be ascribed to a lesser decrease in entropy upon binding the single molecule of adenosine relative to the binding of the two molecules of its components. The binding of adenosine is presumably the result of the independent binding of its components.

One might expect a similar relationship to be found among the binding constants for ATP, AMP, and pyrophosphate. Surprisingly, the dissociation constant for ATP is approximately the same as that of either AMP or pyrophosphate alone. Upon making the appropriate calculations, it is found that the free energy of binding of ATP is 4.3 Kcal/mole less than the sum of the binding energies of AMP and pyrophosphate.

On the basis of kinetic evidence, it has been proposed that the formation of L-isoleucyl adenylate is promoted by approximately 5 Kcal/mole when the substrates are bound to IRS relative to formation in solution.[21] One possible explanation is that upon binding to the enzyme ATP becomes distorted or strained at its reaction site so as to facilitate the reaction. The decrease in free energy of binding of ATP to the enzyme relative to that of its components may be taken as a measure of the strain energy.

The value of 4.3 Kcal/mole for the strain energy does not account for all 5 Kcal/mole by which the reaction is promoted. This

discrepancy can be accounted for by entropy considerations, or it may be the result of the difference in binding of adenosine and AMP. The free energy of binding of AMP is 0.6 Kcal/mole less than that of adenosine. Apparently, as the adenosine and the β,γ-pyrophosphate moieties bind to their subsites, a severe distortion is induced at the α-phosphate. (This is precisely where one would expect distortion to be most effective in promoting the reaction, since it is the residue which undergoes substitution.) The resulting binding is a compromise between maximization of ligand-enzyme interaction and minimization of strain. Upon formation of the L-isoleucyl adenylate, the strain is released and the components achieve maximum interaction with their binding sites.

The effect of N^6-alkylation of ATP analogs was to decrease binding moderately while introducing a decrease in V_{max} which was quite unpredictable. The data are too incomplete to determine any very meaningful structure-activity relationship for the N^6-side chains. (Adenosine derivatives having hydrocarbon chains longer than methyl are too insoluble for testing.) We believe that the reduction in V_{max} may be the result of a non-competitive inhibition. That is, binding of the N^6-alkyl derivatives causes a distortion of the active site which reduces its ability to bind L-isoleucine. This is suggested by the fact that alkylation of the L-isoleucinol adenylate produces a much greater reduction in binding than does the corresponding alkylation of adenosine. Rouget and Chapeville have reported that N^6-(2-hydroxyethyl) ATP is a competitive inhibitor of L-leucine with L-leucine tRNA synthetase.[23] N^6-methyl adenosine shows no competitive inhibition of L-isoleucine, but it also has

little effect on V_{max}. The aminoalkyl adenosines have not been tested.

Modification of the 2' and 3' hydroxyls seems to have fairly drastic effects on the binding. Deoxy-ATP can serve as an energy source for the exchange reaction. Mitra and Mehler studied dATP as a substrate for the tRNA charging reaction and found a reduction in reaction rate as well as a significant increase in K_m.[24] These results are not strictly comparable to our studies, since tRNA was present, but they are indicative. We found no measurable binding with $0^{2'},0^{3'}$-isopropylidene adenosine.

Binding of L-isoleucyl adenylate. L-isoleucyl adenylate is too unstable to be studied directly. Therefore, the stable analog, L-isoleucinol adenylate was used as a model. This compound shows different inhibition constants when measured against L-isoleucine and ATP. This effect presumably is a result of the fact that the Ki(app) will be affected by the concentration of the substrate which is not varied since L-isoleucinol adenylate is a competitive inhibitor of both substrates. The level of the fixed substrate was 2×10^{-3} M in the studies. But since L-isoleucine binds more tightly than ATP, its effect on reducing the binding of the L-isoleucinol adenylate will be more marked and the Ki(app) measured with ATP as the varied substrate will be lower. The dissociation constant, measured in the absence of either substrate, had a value between those of the Ki(app)'s.

Because L-isoleucinol adenylate competes with both substrates, the value of V_{max} will be dependent upon the ratio of the inhibitor to the fixed substrate. Accordingly, values of V_{max} were determined for extrapolated infinite ATP concentration at several concentrations

of L-isoleucine both in the presence and absence of inhibitor. The values of V_{max} were then plotted as functions of the L-isoleucine concentration according to the method of Eadie.[13] This gave a Michaelis constant for L-isoleucine of 6×10^{-6} moles/liter and an inhibition constant for the L-isoleucinol adenylate of 10^{-8} mole/liter. The $(V_{max})_{max}$ values for both plots were identical, indicating true competitive inhibition.

The product of the inhibition constants of adenine and L-isoleucinol is 6×10^{-9} as compared with 10^{-8} for L-isoleucinol adenylate. This indicates that the binding of L-isoleucinol adenylate is primarily due to the independent binding of L-isoleucinol and adenine. The phosphate may possibly cause a slight reduction in the binding. There is little or no decrease in binding due to strain as was found for ATP. We may presume from this that enzyme-bound L-isoleucyl adenylate is also strain-free, as we previously predicted.

The effects of N^6-alkylation have already been discussed in the section on the binding of ATP.

Implications for the preparation of affinity adsorbents. The results of our binding studies indicate that potential affinity adsorbents might be prepared by attaching L-isoleucine to a support via ester or amide formation with the carboxylate or ether formation with the corresponding alcohol, and also by attachment via the side chain.

Attachment via ester, amide or ether formation to a tail attached to the support should be relatively easy and yield a fairly potent adsorbent provided that ATP is present. (These

derivatives will carry no negative charge.)

Attachment via the side chain would yield a somewhat less potent adsorbent. Several problems are apparent. One is that the side chain must retain its branching or binding will be drastically reduced, and other amino acyl synthetases might become bound as well. Retention of branching will necessitate a moderately involved synthesis. One will also be committed to a further fourfold reduction in binding ability, since a synthesis giving proper optical configurations would be prohibitively difficult.

One might also propose attachment of adenosine via the 6-amino group to give an adsorbent. This might adsorb IRS, but would also adsorb the other aminoacyl synthetases as well as numerous other enzymes.

L-isoleucinol adenylate could be attached via any of the sites mentioned above. Ester, amide and ether formation would, of course, not be possible. However, an amino alcohol could be prepared in which the 1-carbon already carried a tail. This amino alcohol would be elaborated into the substituted adenylate, which would then be attached to the support. Since L-isoleucine hexyl ester was not a competitive inhibitor of ATP, it is expected that there would be little steric interference from the appended tail.

Attachment via the side chain would suffer from the drawbacks previously mentioned. Also, the modified amino alcohol would have to be synthesized and suitably protected before it was condensed with AMP.

Attachment via the 6-amino group can be expected to cause a fairly significant decrease in ability to bind the enzyme. However,

the L-isoleucinol adenylate is a sufficiently potent inhibitor that a powerful adsorbent should still result. This route offers the advantage that the tail can be attached after the synthesis of the basic structure, thus reducing the complexity of the synthesis.

Summary

We have presented evidence to indicate that the binding of a substrate induces a conformation change in IRS to what we have called the active conformation. The same conformational change is produced by L-isoleucine, ATP, pyrophosphate, or any of the L-isoleucine analogs which could bind to the enzyme.

The L-isoleucine binding site contains two subsites which bind the methyl and ethyl groups. The methyl site and possibly the ethyl site are open at the end. The strength of binding of the side chain is greater than can be accounted for by hydrophobic interactions alone. This unexpectedly large decrease in binding upon modification of the side chain is one reason for the high degree of selectivity shown by the enzyme.

The binding of the side chain appears to be rigidly coupled to the opening of an ion pair in the active site. New pairs are formed with the charged residues of the L-isoleucine zwitterion. Removal or neutralization of either charged group of the amino acid results in considerable destabilization of the enzyme-ligand complex.

The effect of neutralization or removal of the carboxylate may be compensated for by concomitant binding of ATP, AMP, or pyrophosphate, which show decreasing effectiveness in the order listed. Upon formation of L-isoleucyl adenylate, the positively charged

component of the ion pair becomes associated with the phosphate moiety.

ATP assumes a high energy state upon binding to IRS through induction of strain at the α-phosphate residue. Upon formation of the L-isoleucyl adenylate, the strain is released, and this drives the reaction in the direction of adenylate formation.

Possible affinity adsorbents may be obtained by the attachment of L-isoleucine to a support via a tail on the carboxylate or by attaching L-isoleucinol adenylate via a tail on the 1-carbon of the isoleucyl residue or the 6-amino group of the purine. Poorer adsorbents might be prepared by attaching either ligand via a tail on the side chain. Such derivatives would be less accessible synthetically than those previously mentioned. Attachment of derivatives via the α-amino group or the 2' or 3' hydroxyls would result in adsorbents with little or no ability to bind IRS.

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SECTION B: THE PREPARATION AND TESTING OF AFFINITY ADSORBENTS

Introduction

The technique which has come to be known as affinity chromatography was first successfully applied to the purification of an enzyme by Lerman in 1953 when tyrosinase was purified using azo dyes bound to cellulose,[1] but it was not until five years ago that the technique began to be developed as a general tool for enzyme isolation. Since then the basic methodology has been well developed, primarily through the work of Cuatrecasas and his colleagues,[2] and a number of enzymes and other biological macromolecules have been isolated by the technique.

Basically, affinity chromatography may be applied to the purification of either of any pair of molecules which bind specifically to one another with a strong affinity. Our discussion will be in terms of enzyme-ligand pairs, but the technique is equally applicable to many other pairs, such as antibodies and antigens, or transmitter substances and receptor sites.

The technique consists of attaching a ligand to an insoluble support. When an impure solution of the enzyme is passed through a column of this material, the enzyme will bind to the ligand and be retarded or stopped in its passage through the column, depending upon the strength of the binding interaction, while the impurities pass straight through. If the enzyme is stopped, elution can be effected by changing conditions (e.g., pH or ionic strength) so as to weaken the binding interaction. Or the column may be eluted with a solution of free ligand. The enzyme will partition between the soluble and insoluble ligands and thereby be eluted.

In preparing and using an affinity column, a number of factors must be considered:

A support material should be selected which will allow a maximum number of the bound ligands to be available for interaction with the enzyme, and which does not itself interact significantly with enzyme or impurities. Although there are a number of support materials which might seem suitable, essentially all of the successful purifications which have been effected have used beads of agarose gel (4% w/v) as the support. The key to the success obtained with agarose beads seems to lie in their high porosity (exclusion limit 15,000,000 MW). Highly porous polyacrylamide gels (exclusion limit 400,000 MW) have been used, but these give considerably inferior results compared to agarose gels, despite the fact that the polyacrylamide can carry a considerably higher concentration of ligand.

A ligand should be chosen which exhibits a high binding affinity for the enzyme. A column on which the enzyme is stopped rather than retarded is considerably more desirable, since it can be used to isolate the enzyme from a large volume of solution which may be very dilute in the enzyme. The enzyme must be in rather concentrated form for successful purification on a column which merely retards it.

Agarose gel can carry ligand at a maximum level of about 20 μmoles per milliliter, which is equivalent to a concentration of 0.02 M. To be effective at this concentration, the insolubilized ligand should have a dissociation constant no greater than 10^{-3} moles/liter. Since insolubilization can generally be expected to reduce binding affinity, a ligand with a dissociation of 10^{-3}

moles/liter in the free state could be successfully used only under the most optimal of conditions.

Another complication is that substrates are in general unsuitable as ligands, since they will be converted to products during the purifications. Substrates may be used when the product is bound equally strongly or when conditions can be found which repress catalysis without affecting binding.

The method of attachment should be such as to minimize the decrease in the binding affinity of the ligand upon insolubilization, and should have no adverse effects upon the support structure. It should result in a linkage which is stable to the conditions to which the column will be subjected and should not produce gratuitous functionalities which are reactive or capable of causing non-specific interactions with impurities.

In most cases investigated, direct attachment of the ligand to the support material has proven to be unsuitable, apparently because steric interactions between the enzyme and the polymer backbone of the support have prevented binding to the insolubilized ligand. This problem has been solved by attaching the ligand to the support via some sort of tail. A number of tails have been employed. In the case of a relatively weak inhibitor, a tail of considerable length (14 or more atoms) may be necessary for effective purification.[3] The most frequently used tails have been α,ω-diaminoalkanes or ω-aminocarboxylic acids. They are attached to the gel via the amino group with cyanogen bromide.[4] The ligand may then be attached directly to the bound tail with a water soluble carbodiimide, or the tail may be subjected to further chemical modifications before the ligand is coupled.

Once the affinity adsorbent has been prepared, one must decide on the size of the column to be prepared. If the ligand binds strongly to the enzyme, so that the latter is expected to be stopped rather than retarded on the column, a very short column will suffice. The primary consideration will be that the column not be saturated; that is, that the amount of enzyme placed on the column not exceed the amount of ligand available for binding.

In the case where the enzyme is merely retarded, the same criteria apply as for any other sort of adsorption chromatography.

In loading and washing the column, conditions must be chosen to optimize the enzyme-ligand interaction while minimizing non-specific ones. Of particular importance is selection of the proper ionic strength. A strength too low will encourage non-specific (e.g., ion exchange) interactions between the adsorbent and assorted protein impurities, thus reducing the level of purification. Too high a strength will interfere with the enzyme-ligand binding and may also lead to denaturation of the enzyme. The solution should also be of appropriate pH and contain any cofactors, effectors, etc., which may be necessary for binding of the ligand. It should not contain compounds which bind to the ligand or to the ligand binding site of the enzyme to be purified.

The desorption of a tightly bound enzyme has generally been accomplished in one of two ways. One is to weaken the enzyme-ligand interaction by changing conditions (e.g., pH or ionic strength). In this case, the parameter altered should be altered to a point which will sufficiently weaken the binding to allow the enzyme to be eluted in a sharp band, but not to the point of causing denaturation of the enzyme.

The other method involves eluting the column with a solution of a free ligand (which may or may not be the same as the bound ligand) which will compete with the bound ligand for the enzyme. One drawback to this technique is that the enzyme is frequently eluted in very dilute form. Even though the free ligand may be present in concentration sufficient to cause the enzyme to partition essentially entirely into the mobile phase at equilibrium, the rate at which the enzyme may be eluted from the column will be limited by the rate at which the enzyme can dissociate from the ligand. In the case of a strongly bound ligand, the half-life for dissociation may be many minutes. This problem can be minimized by running the free ligand solution onto the column and then stopping elution. After a sufficient wait to allow equilibration, elution is resumed. Alternatively, the column may be extracted in a batchwise fashion with a solution of free ligand.

One may also combine a change of conditions with introduction of free ligand into the eluent to effect desorption.

These problems as they apply to our systems will be discussed in somewhat greater detail at appropriate places in the description of the research which follows. For a more thorough discussion of these in terms of affinity chromatography in general the reader is referred to several reviews by Cuatrecasas.[2]

Viewed in the light of the above discussion, some of our earlier work may seem somewhat ill-advised. It should be remembered that most of the findings upon which this discussion was based had at that time not yet been found.

Early Work

Repeated losses sustained in the purification of L-isoleucine tRNA synthetase by the procedures of Berg[11] caused us to seek some alternate route for isolating the enzyme. The finding that tyrosine esters and amides were competitive inhibitors of L-tyrosine for the L-tyrosine tRNA synthetase[6] suggested that the same might be true for the L-isoleucine derivatives and, if so, it might be possible to purify IRS by attaching L-isoleucine to some support material via an ester or an amide bond and then passing impure solutions of the synthetase through columns of such material. The synthetase would be selectively retarded and thereby purified.

L-isoleucine ethyl ester was determined to be a competitive inhibitor of L-isoleucine with a Michaelis constant of approximately 3×10^{-4} moles/liter. With this knowledge, we set about to prepare some selective adsorbents. This work was begun in the fall of 1968 and approximately coincided with the publication of Cuatrecasas' first paper on affinity chromatography.[7] However, we did not discover this publication until three months later.

We decided to investigate two possible materials as support structures: beads of Sephadex G-50 and 2% cross-linked polystyrene. The polystyrene was selected for its strength and stability, and the Sephadex for its wetability. With either support, the enzyme should be able to bind only to those L-isoleucine moieties which are attached to the surface of the bead. However, we thought this would be sufficient, granted the very low molar concentrations of the enzyme involved and the fact that a high level of substitution could

be readily obtained. Sephadex G-150 or G-200 would have allowed partial penetration of the IRS into the bead, but results would have been complicated by gel filtration effects.

We realized that it might be necessary to interpose a tail between the support material and the L-isoleucyl moiety to prevent steric interaction between the enzyme and the polymer backbone during binding, and decided to prepare media both with and without tails.

The materials prepared are shown in Figure 1. I was prepared from chloromethylated polystyrene beads by the method of Merrifield.[8] II was prepared by reacting chloromethylated polystyrene with 6-amino-1-hexanol and acetylating with acetic anhydride. After hydrolyzing the acetate ester, the resin was reacted with N-t-BOC-L-isoleucine imidazolide, and the t-BOC group was removed with 1 N hydrochloric acid in acetic acid. III was prepared by reacting Sephadex G-50 with aminoethyl sulfate, followed by aqueous coupling to N-t-BOC-L-isoleucine with N-cyclohexyl N'-morpholinoethyl carbodiimide metho-p-toluenesulfonate. The gel was deblocked in 1 N aqueous hydrochloric acid. IV was prepared by coupling the previously synthesized 6-aminohexyl ester of N-t-BOC-L-isoleucine directly to the Sephadex with cyanogen bromide.[4] It was deblocked as was III. The Sephadex gels were prepared by Dr. Brian Myhr.

In running our test purifications, we decided to use a partially purified enzyme preparation. This was Fraction 2, one of the less pure fractions in the alumina C_γ gel elution step of the Berg procedure. It had a specific activity of ca. 4 units/mg protein as compared with ca. 2 units/mg for the crude extract. Thus the enzyme was not significantly purified except that it was now essentially free of non-protein impurities which could possibly have interfered with the binding.

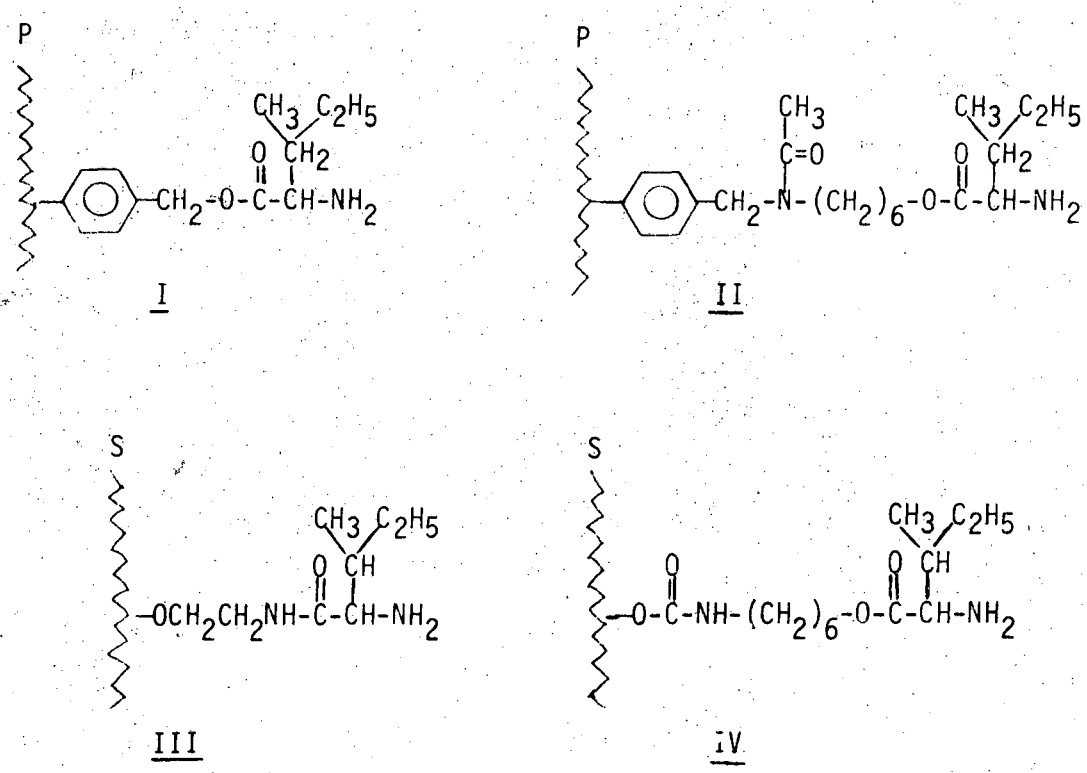

Figure 1. Early affinity adsorbents. The jagged line represents the polymer backbone (P = 2% crosslinked polystyrene beads, 200-400 mesh; S = Sephadex G-50, fine).

This fraction, which will be referred to hereafter as the C_γ gel eluate, was dialyzed against 0.02 M potassium phosphate (pH 7.5) containing 0.01 M 2-mercaptoethanol. It could be stored for long periods of time (i.e., months) at 3°C with only slight loss of activity. However, it was found that during such storage free L-isoleucine was generated, presumably by protein hydrolysis. This necessitated redialyzing the solution before testing a column in order to prevent the L-isoleucine from competing with the bound L-isoleucine for the IRS.

Samples of C_γ gel eluate were chromatographed on columns of the prepared adsorbents using as eluents a buffer of 0.1 M potassium phosphate (pH 7.5) containing 0.01 M 2-mercaptoethanol, or this buffer containing as well 0.01 M magnesium chloride, or 0.005 M ATP, or both. (Since the inhibition constant for L-isoleucine ethyl ester was determined from inhibition studies on the pyrophosphate exchange reaction [Eq. (1), section A], it was not certain whether magnesium and/or ATP might be required for binding. (It was later shown that the binding of L-isoleucine esters was reduced 100-fold in the absence of ATP). The attempted purifications were done at 3°C and at room temperature. None of the absorbents showed the ability to separate enzyme activity from bulk protein under any of the conditions tested. The specific activity of the recovered enzyme was the same, or slightly less, than that of the enzyme applied.

6-Aminohexyl L-isoleucinate Bound to Agarose

At about this point, we found Cuatrecasas' paper describing the purification of staphylococcal nuclease, α-chymotryspin, and carboxypeptidase A using essentially the same basic approach that we were using.[7] A key difference in his procedures was the use of beads of agarose gel as the insoluble support. Agarose is an alternate copolymer of D-galactose and

3,6-anhydro - L-galactose which has the ability to form very stable gels with water at very low concentrations. Hot solutions of agarose can be dispersed in oil and allowed to cool while dispersed to form beads of gel. Such beads are stable indefinitely when kept in aqueous suspension below 30°C. We have found no visual evidence (viewed under a microscope) of degradation after 1 week in either 1 N hydrochloric acid or 1 N sodium hydroxide at room temperature.

Because of the low concentrations of agarose required and because there is no cross-linking (the gel presumably being held together entirely by hydrogen bonds), the effective pore size of the gel is enormous. Beads of 4%(w/v) agarose have a molecular exclusion limit of 15,000,000 MW. This means that most soluble enzymes can freely penetrate into all parts of the gel. Essentially all of the insolubilized ligand thus becomes available for binding. But every silver lining has a cloud and there are a few drawbacks to agarose beads. They have a very low physical strength and are easily broken if subjected to vigorous stirring. Breakage of even some of the beads leads to greatly decreased flow rates. The beads also deform under all but the slightest pressures, again reducing flow rates. Because of the low concentration of agarose in the bead, the amount of ligand which can be attached is rather limited. The gel shrinks irreversibly on drying, freezing or treatment with most solvents. Finally, the material is potentially suseptible to microbial attack. (However, we have never had any problems with this last possibility.)

We decided to prepare an adsorbent using agarose as the support material. The procedure used was essentially the same as the one used to prepare adsorbent IV, Fig. 1. N-carbobenzoxy- 6-amino-1-hexanol was condensed with N-t-BOC-L-isoleucine imidazolide and the product catalytically hydrogenated over 10% palladium on charcoal in the presence of one equivalent of acetic

acid. The protonated 6-amino-1-hexyl N-t-BOC-L-isoleucinate was quickly reacted with cyanogen bromide-activated Bio-Gel A-15M, 100-200 mesh.

Cyanogen bromide-activation is a procedure developed by Porath et al. for the coupling of materials to polysaccharides under mild conditions.[4] The activation involves treating the polysaccharide with cyanogen bromide in aqueous solution at pH 11. The polysaccharide thus activated is capable of coupling primary amines. The mechanism by which this occurs is not known. It is quite likely that cyanate groups are initially formed from the polysaccharide hydroxyls. These then react with the primary amines to give isoureas which hydrolyze to carbamic acid esters. (The coupling is known to proceed with the release of one equivalent of ammonia). An alternate mechanism which has been proposed involves the generation of an iminocarbonate intermediate. However, this requires the presence of cis, vicinal hydroxyls, of which agarose has none. The linkage generated is reported to be quite stable to pH changes, tolerating 10^{-3} M sodium hydroxide and 1 M hydrochloric acid.[11] In our hands, the linkages have proven somewhat less stable.

The gel, now bearing the N-protected ligand at a level of ca. 5 μmoles/ml, was deblocked initially by a 20 minute treatment with anhydrous trifluoroacetic acid. This caused some irreversible shrinkage of the gel and later preparations were deblocked by stirring in 1 N hydrochloric acid for six hours. (There was little difference in the elution behavior of C_γ gel eluate on affinity columns prepared from gels deblocked by either technique.) Only slight losses of bound ligand occurred using either procedure, as determined by ninhydrin assays of gel hydrolysates. To verify that the t-BOC group was being removed by the aqueous hydrochloric acid, a gel was prepared with the insolubilized ligand carrying a ^{14}C-labelled t-BOC group. After a three

hour treatment, 66% of the radioactivity had been lost.

Columns prepared from freshly synthesized adsorbent showed the ability to retard the elution of enzyme activity, (as measured by the ATP - [^{32}P]PP$_i$ exchange assay described by Baldwin and Berg[11]) relative to the elution of protein (as determined by the Lowry technique) when C_γ gel eluate was chromatographed at 3°C using 0.1 M potassium phosphate buffer (pH 7.5) containing 0.01 M each of ATP, 2-mercaptoethanol, and magnesium chloride.

This experiment was repeated several times using columns of freshly prepared adsorbent. The best separation obtained is shown in Fig. 2. The enzyme in the pool of the fractions beyond 40 had been purified approximately sixfold. This result was encouraging, but the column as constituted was not suitable for a preparative scale purification: The protein peak emerged in 1.0 column volume of effluent and the enzyme peak at 1.9 volumes. This requires that the volume of liquid containing the enzyme to be purified be less than the column volume for significant purification to be achieved. Given the fact that sixfold purifications are readily achieved by more classical techniques, the cost and effort required to prepare a column for preparative work would be prohibitive. Moreover, it was found that on reuse a colunm lost part of its ability to retard the enzyme. By the fourth use, it was incapable of effecting any purification. This loss of activity prompted us to determine whether or not the ligand was being removed from the gel. A gel was prepared using ^{14}C-labelled L-isoleucine and employed to chromatograph C_γ gel eluate. By doing radioassays and ninhydrin assays on hydrolysates of portions of the gel after each run, it was determined that ligand was being lost by several paths—by slow continous hydrolysis of both ester and eyanogen bromide-induced linkages, and by a considerably faster hydrolysis of the ester linkage

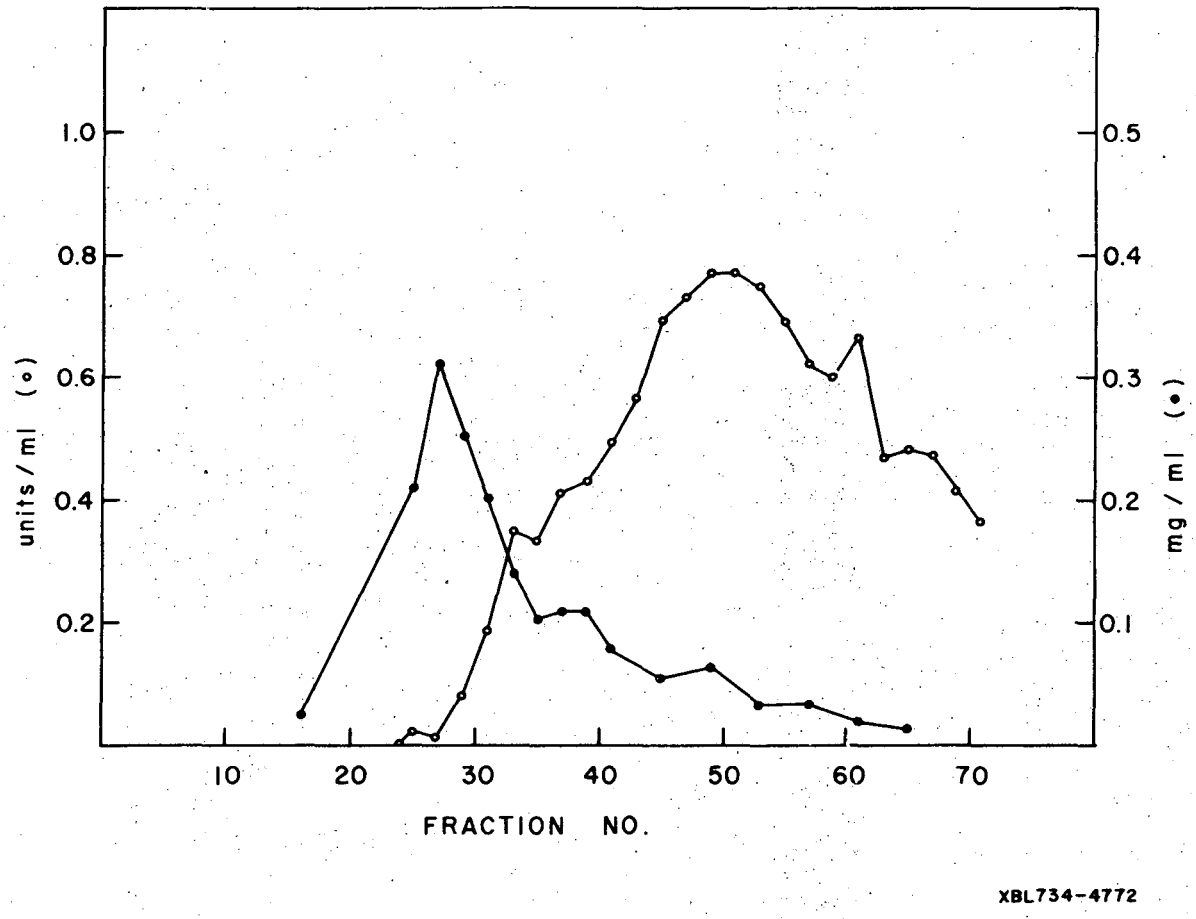

Fig. 2. Chromatographic elution pattern of partially purified IRS on hexyl L-isoleucinate agarose. A ¼" diam. column was filled with 8 ml of the adsorbent equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.01 M each ATP, magnesium chloride, and 2-mercaptoethanol. C_γ gel eluate (0.5 ml) was chromatographed on the column at 3° using the same buffer. Flow rate, 0.1 ml/min; fraction size, 0.3 ml. Protein (filled circles) was measured with the Lowry reagent. Enzyme activity (open circles) was determined from the ATP-[^{32}P]PPi exchange reaction.

during a chromatographic run. The latter is presumably due to the action of esterases in the C_γ gel eluate. Considerable ligand remained bound after four runs, however. Either the remaining ligands were somehow unavailable for binding or some other method of inactivation was operant.

Despite the fact that previously unused adsorbent was required for each test run, we set about trying to improve on the purification we had obtained. Magnesium chloride, ATP, or both were omitted from the elution buffer. Omission of ATP caused precipitation of magnesium phosphate in the column during chromatography. Further investigation indicated that magnesium phosphate precipitation also occurred when ATP was present, although at a much slower rate. These results may be understood in terms of a reduced concentration of magnesium ion in the buffer due to formation of the soluble complex, monomagnesium ATP. Omission of only magnesium chloride from the buffer produced little change in the initial chromatographic elution patterns obtained, but the "inactivation" of the columns on reuse was markedly diminished. It appears that precipitation of magnesium phosphate within the gel was the primary cause of the loss of effectivness of columns on reuse which had been previously noted.

When both magnesium chloride and ATP were omitted from the buffer, a marked loss in enzyme recovery was seen. Since the vanished activity could not be eluted with buffers containing free L-isoleucine, it was presumed that the loss was due to denaturation. ATP is known to afford protection against denaturation.[5,13] Our later finding that the binding affinity of an L-isoleucine analog in which the carboxylate is replaced by a neutral moiety is approximately 100-fold less in the absence of ATP than in its presence makes it unlikely that the loss of activity was due to binding of the enzyme to the column.

We also carried out elutions at room temperature rather than at 3°C

(in these runs, while the column was at room temperature, the fractions were collected in a refrigerated unit at 3°C). This resulted in reduction or elimination of the retardation of the enzyme over bulk protein. Since the enzyme was known to bind L-isoleucine more tightly at 25°C than at 3°C,[13] this behavior led us to suspect that the retardation which we had been observing was entirely due to non-affinity interactions.

To test this hypothesis, we synthesized a gel bearing the 6-amino-1-hexyl ester of L-leucine. Because this amino acid differs from L-isoleucine only in the position of a methyl group, the microenvironment within such a gel should be almost identical to that within gels bearing the L-isoleucine derivative, and the same non-specific interactions, if any, should be seen. L-leucine does not bind to IRS, and no affinity interactions should result.

This gel was almost as effective as the L-isoleucine gel in retarding the elution of enzyme activity, as shown in Fig. 3. (Figure 2 is a best result. Most runs gave elution behavior much closer to that in Fig. 3.) This indicated that the gels bearing 6-amino-1-hexyl L-isoleucinate were not acting as affinity adsorbents.

L-Isoleucine Ester with Polyglycolamine H-163

It was possible that extension of the ester group from an ethyl to a hexyl ester might have impaired the binding of the ligand. L-isoleucine hexyl ester was synthesized and was found to have an inhibition constant of 4×10^{-5} moles/liter, indicating that it was bound more strongly than the ethyl ester ($K_i = 3 \times 10^{-4}$ moles/liter). This left the possiblity that the hexyl tail was not long enough to avoid steric interference to binding of the enzyme by the agarose backbone, or that the wrong kind of

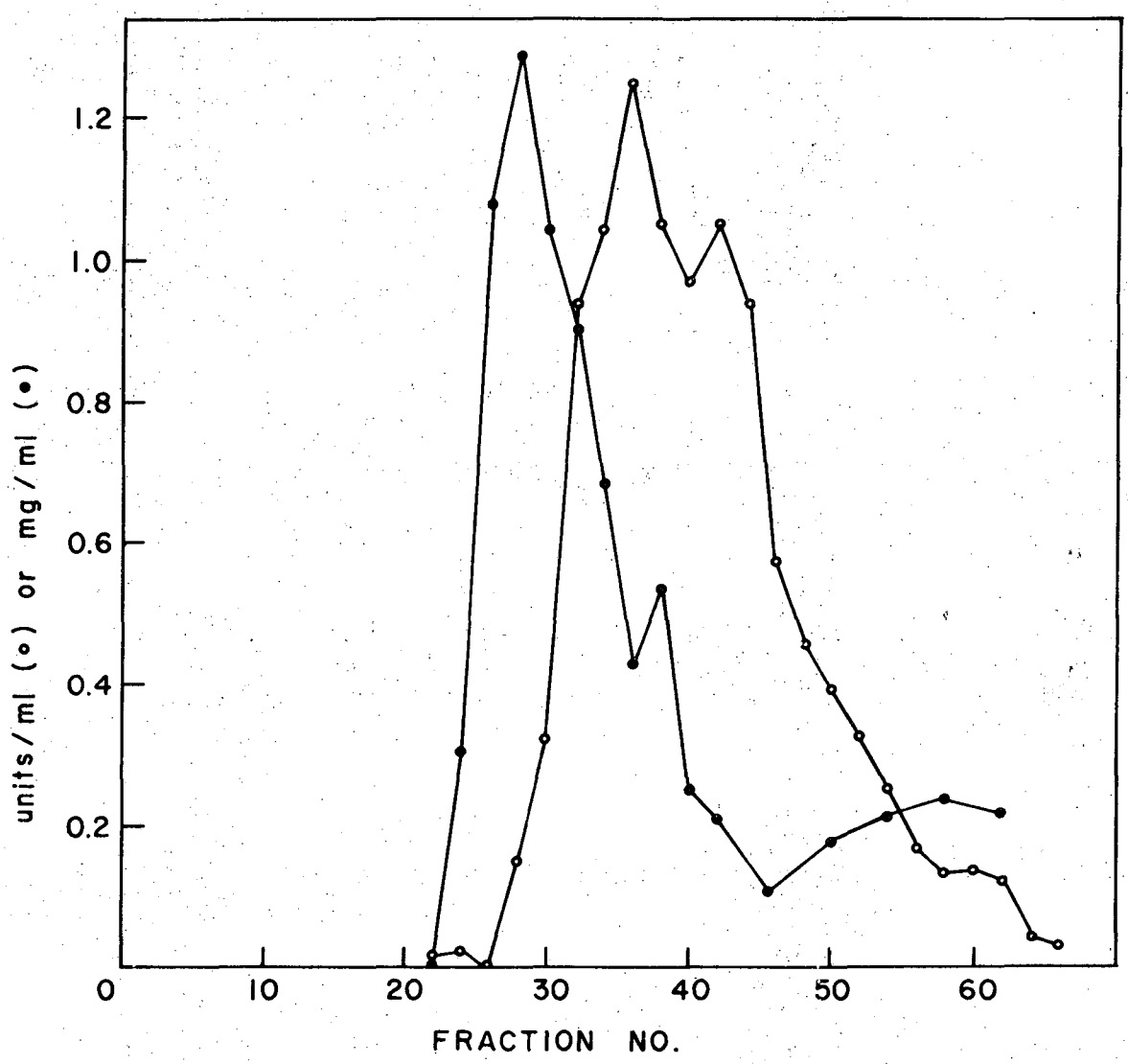

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Fig. 3. Chromatographic elution pattern of partially purified IRS on hexyl L-leucinate agarose. The chromatography was carried out as described under Fig. 2 on a 10 ml column of the adsorbent. Protein is shown by filled circles; enzyme activity is shown by open circles.

tail was being used. The hydrophobic hexyl chain might not be suitable for use in an aqueous system. A tail of polyethylene glycol seemed to be more appropriate. A search revealed the availability of a compound called Polyglycolamine H-163 (from Union Carbide). This has the formula $H_2N\ CH_2CH_2CH_2(OCH_2CH_2)_2OH$ and seemed particularly well suited to our purposes. It was longer than 6-aminohexanol and completely hydrophilic. Gels were prepared from its ester with L-isoleucine in an analogous fashion to the preparation of the gels with an aminohexyl tail.

These gels showed the ability to retard the IRS relative to protein, but not as much as the gels with an aminohexyl tail had done. This indicates that one of the non-specific interactions involved in the enzyme retardation by the gels with an aminohexyl tail was probably hydrophobic bonding.

We also prepared an affinity adsorbent carrying the L-isoleucine ester of Polglycolamine H-163 bound to chloromethylated macroporous polystyrene beads. These beads were created specifically for use in aqueous systems, and consist of highly cross-linked polystyrene permeated by many "macropores." Although the enzyme is expected to be able to bind only to those ligands attached to the surface of the polystyrene, the presence of the macropores increases the surface area to ca. 30 square meters per gram of dry resin. The adsorbent prepared from these beads showed no ability to selectively retard IRS.

Long Tail Adsorbents

We next decided that perhaps it would be necessary to attach the L-isoleucine moiety to the gel with a very long tail. Our resolve was confirmed by the appearance of a report by Steers et al.[2] concerning the

purification of β-galactosidase. The ligand used was p-aminophenyl-β-D-thiogalactopyranoside, a weak inhibitor with a K_i of 5 mM. When this ligand was attached directly to agarose, the resulting adsorbent was unable to effect any purification. Attachment via a short tail (ethylenediamine) gave an adsorbent capable of slightly retarding the enzyme relative to protein. Attachment via a long tail (3-aminosuccinyl 3'-aminodipropylamine) gave an adsorbent which bound the enzyme sufficiently tightly that a change of pH was needed to effect the elution of the enzyme.

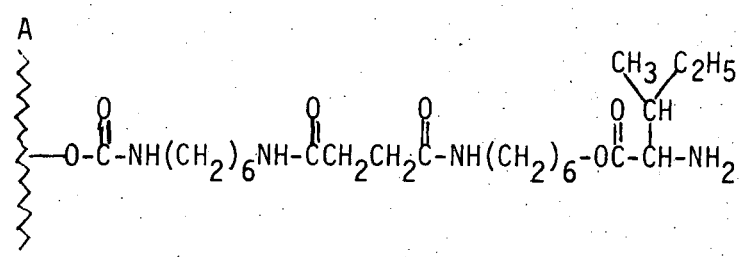
Accordingly, we prepared several adsorbents using very long tails. These are shown in Fig. 4. Adsorbents V and VII showed no ability to separate IRS from protein. When chromatographic runs were done using VI. no enzyme was eluted and the recovery of protein was considerably reduced. Enzyme activity could not be eluted from the column using eluents containing L-isoleucine. The apparent non-specific adsorption of protein which VI showed was never clearly understood.

Possible Alternate Adsorbents

The above results made it fairly clear that insolubilized L-isoleucine esters do not bind to IRS sufficiently strongly to be useful in the preparation of affinity adsorbents. It was necessary to consider systems which would employ ligands which bound the enzyme more strongly.

Three systems appeared serviceable. These involved employing as the insolubilized ligand L-isoleucinol 5'-adenylate, $tRNA^{ile}$ or an antibody against IRS.

L-isoleucinol adenylate is the ester of the amino alcohol L-isoleucinol with adenosine 5'-monophosphate. It is an analog of the enzyme-bound activated amino acid intermediate, L-isoleucyl adenylate. Unlike the



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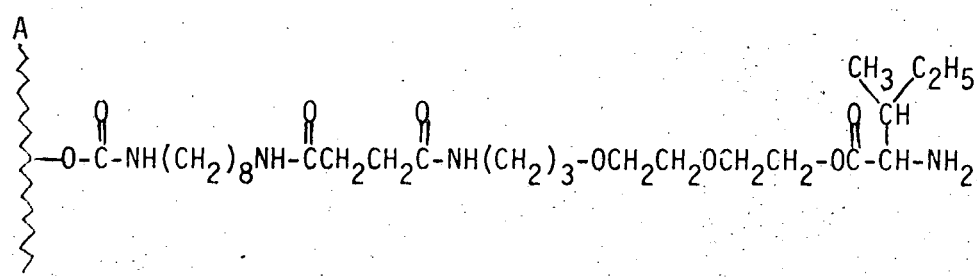

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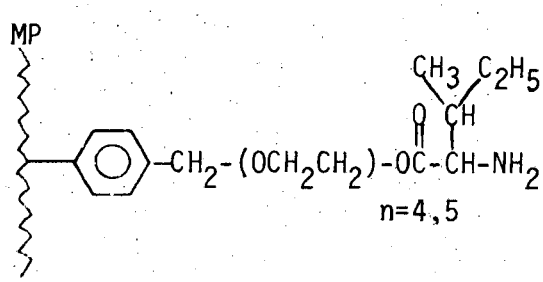


VII

Figure 4. Affinity adsorbents carrying L-isoleucine esters attached by long tails. The jagged line indicates the polymer backbone of the support: A = Biogel A-15M, 100-200 mesh; MP = macroporous polystyrene, 20-40 mesh.

latter, it is completely stable to aqueous hydrolysis under mild conditions. It was reported to have an inhibition constant of 7.4×10^{-9} moles/liter.[14]

The primary problem associated with the use of this ligand would be determination of a method of attaching it which will not severly compromise its binding ability. Once this had been determined and the necessary synthetic procedures worked out, it would be relatively easy to prepare large quantities of adsorbent. There is also the possibility that the ligand might be degraded by a phosphodiesterase in the crude IRS preparations. If this occurs it could probably be inhibited by inclusion of phosphate and/or fluoride in the eluent buffer.

The Michaelis constant of $tRNA^{ile}$ is 2×10^{-7} moles/liter.[5] As a ligand for affinity chromatography, this has the distinction of being more difficult to obtain in pure form than is the IRS which it would be used to purify. Adsorbents prepared from it would be of practical value only if they were capable of being reused many times. The susceptibility of the ligand to degradation by ribonucleases in the impure IRS preparations makes this appear unlikely. (Recent reports have described the purification of $tRNA^{ile}$ by insolubilized IRS[15] and of IRS by insolubilized $tRNA^{ile}$.[16] In the latter case, the $tRNA^{ile}$ was bound as L-ile-$tRNA^{ile}$ via attachment of the α-amino group of the L-isoleucyl moiety to a bromoacetamidobutyl tail attached to agarose. This results in the $tRNA^{ile}$ being attached via a very labile 2'(3') ester bond. Not unexpectedly the authors report that the column rapidly loses its capacity for enzyme retention on reuse.)

To my knowledge, no one had determined a dissociation constant for the binding of IRS to a cognate antibody. Presumably the binding would be quite strong. Immunosorbents have been successfully used for a number of years. The primary drawback to this approach would be the considerable amount of

work necessary to prepare an adsorbent. First, highly purified IRS would be needed to induce antibody formation. Then the antibody itself would require purification. As in the case of using $tRNA^{ile}$ as the insolubilized ligand, this adsorbent would have to be reusable many times to make it a practical tool for the purification of IRS. Since the antibody would be subject to digestion by proteases in the crude IRS preparations, this possibility does not seem likely.

Because adsorbents bearing L-isoleucinol adenylate appeared to be the most readily accessible, the most amenable to production in larger quantities, and, if properly synthesized, the most stable to degradation, we decided to pursue this line of investigation. L-isoleucinol adenylate (L-ile-ol AMP) had been synthesized previously by Cassio et al. from N-carbobenzoxy-L-isoleucinol.[14] Since the carbobenzoxy group cannot be readily removed from an agarose bound ligand, we chose to use N-t-BOC-L-isoleucinol in our synthesis. The Cassio procedure gave very poor yields of the N-t-BOC-isoleucinol 5'-adenylate. Much better success was obtained using the method of Sandrin and Boissonas[17] with minor modifications: 3 mmoles of N-t-BOC-L-isoleucinol were condensed with 1 mmole of N, O, O-triacetyl AMP (prepared according to Khorana[34]) in dry pyridine using 10 mmoles of N, N'-dicyclohexyl carbodiimide for 5 days. The acetyl groups were removed in 9:6 ethanol - concentrated ammonium hydroxide and the t-BOC group with anhydrous trifluoroacetic acid. The L-ile-ol AMP was purified by column chromatography. (This procedure was subsequently improved. The description in the Experimental Section includes the later improvements).

We determined the inhibition constant to be 4×10^{-9} moles/liter when measured against L-isoleucine and 3×10^{-8} moles/liter when measured

against ATP.

We then faced the task of deciding how to attach to the molecule a tail which would subsequently be attached to agarose. The site and method of attachment of the tail to the ligand should be such as to give a stable bond and to cause a minimal decrease in the ability of the ligand to bind to the enzyme. It would also be preferable that the linkage be easily generated.

Our general strategy called for attachment to the L-ile-ol AMP of a tail segment, the free end of which to be terminated by an amino group or a carboxylate. This would then be linked to agarose bearing a tail segment terminated in a carboxylate or amino group, respectively, using a water soluble carbodiimide to generate an amide bond.

The question of where to attach the tail segment to the adenylate would be approached by model studies. We determined the inhibition constant of L-isoleucinol to be 2×10^{-5} moles/liter and that of AMP to be 9×10^{-4} moles/liter. The product of these inhibition constants is 1.8×10^{-8} as compared with an inhibition constant of 3×10^{-8} moles/liter for L-ile-ol AMP. Assuming that the inhibition constants are close approximations of the dissociation constants (later work using fluorescence titration supports this assumption[18]), this result implies that the binding of L-ile-ol AMP is primarily the result of the independent binding of its two components (The free energies of binding calculated from the K_i's are 6.7, 4.3, and 11.0 Kcal/mole for L-isoleucinol, AMP, and L-ile-ol AMP respectively). We believed the effect of introducting a modification into L-isoleucinol or AMP should be almost quantitatively extrapolable to L-ile-ol AMP (later work showed that this assumption was not valid for N^6-alkylated derivatives). We felt that this principle could be extended to modifications

of L-isoleucine and adenosine as well with a high degree of accuracy. Suitable derivatives of the latter two were commercially available.

Because of our greater experience in amino acid chemistry, we initially looked at L-isoleucine derivatives.

N-methyl-D, L-isoleucine, guanidino-L-isoleucine, and 3-methylpentanoic acid (deamino-D, L-isoleucine) gave no measurable binding when measured either by inhibition experiments or by fluorescence titration. This indicates that the presence of an unmodified amino group is absolutely necessary for binding. Attachment of a tail at the amino group would yield a ligand incapable of binding IRS.

A number of molecules were investigated with regard to attachment of the tail to the isobutyl side chain. L-alloisoleucine was used as a model for attachment to the methyl group of the side chain. Presumably it binds to the enzyme with the methyl group in the ethyl binding site and vice versa. The reduction in its binding affinity will be due to two factors: demethylation of the ethyl group and methylation of the methyl group. The decrease in binding due to demethylation of the ethyl alone can be determined from the K_s of L-valine. Therefore, the change in K_s's going from L-valine to L-alloisoleucine may be taken as a measure of the effect of extending the methyl group by one methylene unit. The K_s values for L-valine and L-alloisoleucine are 1.6×10^{-4} moles/liter and 10^{-3} moles/liter, indicating a sixfold reduction in binding.

To determine the effect of extension of the ethyl group, the Michaelis constants of α-D, L-aminopentanoic acid (desmethyl-D, L-isoleucine) α-D, L-aminohexanoic acid, and α-D, L-aminoheptanoic acid were determined. They were 10^{-3}, 7×10^{-3}, and 2×10^{-2} moles/liter, respectively. This indicates 7-fold and 20-fold reductions in binding for extensions of 1 or

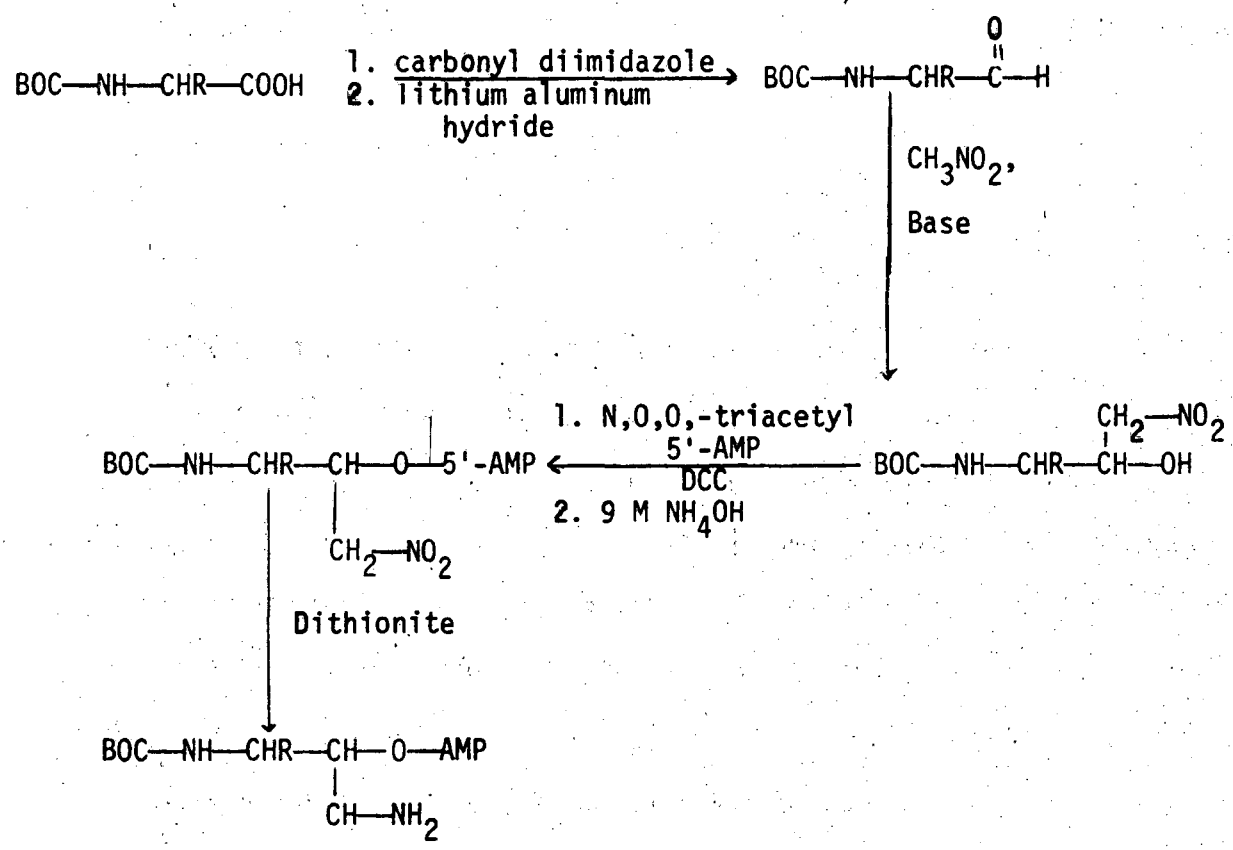
2 methylene units. These results are somewhat ambiguous in that it is impossible to determine whether the side chains of these molecules are binding to the ethyl or methyl binding sites of the enzyme.

The results indicate that a long chain α, ω-diamino alcohol, which would be easily accessible synthetically, would not be suitable for elaboration into the adenylate, since a 10,000-fold reduction in binding affinity could be expected.

A β-ethyl or β-methyl-α, ω-diaminoalcohol would be expected to show much better binding ability, but would probably show at least a 20-fold reduction of binding ability. A synthesis giving proper stereochemistry would be prohibitively difficult so that one would have to accept a further fourfold reduction in binding. Attachment of the tail to the side chain would entail a moderately difficult synthesis and would yield a product with at least an 80-fold decrease in binding ability and quite possibly more.

1-Nitromethyl N-t-BOC-L-isoleucinol 5'-adenylate

Our previous determination of the inhibition constants of L-isoleucine ethyl and hexyl esters indicated that extension of the ester from ethyl to hexyl actually resulted in a slight increase in binding ability. We determined that the hexyl ester was not a competitive inhibitor of ATP. Thus, when it was bound to IRS, the hexyl tail did not occupy the ATP binding site. This suggested that a tail might be readily attached to the hydroxyl-bearing carbon of L-isoleucinol. Moreover, a simple scheme could be proprosed for the synthesis of the substituted L-ile-ol AMP. This is shown below:



N-t-BOC-1-nitromethyl L-isoleucinol was prepared as above. A portion was deblocked in anhydrous trifluoroacetic acid. The resulting 1-nitromethyl L-isoleucinol was found to have an inhibition constant of 4×10^{-3} moles/liter. This corresponds to a 200-fold loss in binding ability relative to L-isoleucinol. This loss was not particularly encouraging, but was tolerable provided no further losses were incurred. It was expected that an increase in binding ability would probably result upon reduction of the nitro group to the less hindered amino function.

Our initial attempt to couple N-t-BOC 1-nitromethyl L-isoleucinol to AMP was unsuccessful. We set about to investigate other coupling procedures using the model compound 1-nitro-2-pentanol, but met with uniform lack of success. At this point we were informed that attempts had been made previously in our laboratory to couple AMP to secondary alcohols and

all were unsuccessful.[19]

We therefore turned to an alternate route to the desired compound, which involved initial phosphorylation of the N-t-BOC- 1-nitromethyl L-isoleucinol and coupling of the resulting compound to a suitably protected adenosine. With this approach the phosphodiester would be formed from a primary rather than a secondary alcohol.

Model phosphorylations were carried out on N-t-BOC-L-isoleucinol using dibenzylphosphorochloridate,[20] β-cyanoethyl phosphate with excess N, N'-dicyclohexylcarbodiimide,[21] and O-benzylphosphorous O'O'-diphenyl phosphoric anhydride.[22] All gave very low yields of the desired product. Since yields were expected to be even worse with the nitromethyl derivative and since there was no guarantee that the subsequent coupling to adenosine would work, this route was abandoned.

We did check to determine whether L-isoleucinol phosphate might be a suitable ligand for affinity adsorbents. A crude preparation was employed to determine a dissociation constant using the fluorescence quenching technique. A value range of 10^{-3} to 10^{-4} moles/liter was obtained. The uncertainty in the value is due to the fact that an impure material was used. The data are of value in that they do serve to set an upper limit on the binding affinity and indicate that phosphorylation of L-isoleucinol reduces binding. The same effect is found when adenosine is phosphorylated (see Section A). L-isoleucinol phosphate is not suitable as a ligand.

L-isoleucinol Adenylate Attached by the Adenosyl Moiety

We then turned to consideration of techniques of attaching the tail through the AMP moiety of the L-ile-ol AMP.

Attachment of the tail by formation of a phosphate triester was

ruled out for two reasons: 1) phosphate triesters are fairly readily hydrolyzed and 2) the ligand would cease to carry a negative charge. Our binding studies had indicated that the binding of L-isoleucine and its analogs was associated with the opeing of an ion pair in the enzyme. Stabilization of the resulting conformation required the presence of both a positively charged and a negatively charged group on the bound ligands (See Section A).

The obvious sites for attaching a tail to the adenosine portion of the molecule are the 6-amino group of the purine and the 2' and 3' hydroxyls of the ribose. Inhibition studies were done on adenosine, N^6-methyl adenosine, and $O^{2'}$, $O^{3'}$-isopropylidene adenosine. The first two had K_i's of $3x10^{-4}$ and $6x10^{-3}$ moles/liter, respectively, while the last showed no measurable binding. These results indicated that ligation via the amino group might be suitable, whereas linkage through either of the hydroxyls probably would not be.

Another approach that was considered was attachment via an azo linkage to the purine system. This approach was initially rejected because of reports that the products of the reaction of AMP with diazonium salts were quite unstable, quickly decomposing to give back AMP.[23, 24] However, we later explored this possibility as a result of a report by Weibel _et al_. that NAD^+ could be stably coupled to glass beads via an azo linkage.[25] Since attack of the positively charged diazonium intermediate on the quaternized pyridinium appears out of the question, linkage must have occured on the adenosine moiety. This prompted us to attempt to couple BOC-ile-ol- AMP to diazotized p-aminobenzamidoalkyl agarose (Fig. 5). The couplings were attempted by the procedure of Weibel[25] and by the procedure of Cuatrecasas.[2a] In both cases essentially none of the ligand was bound.

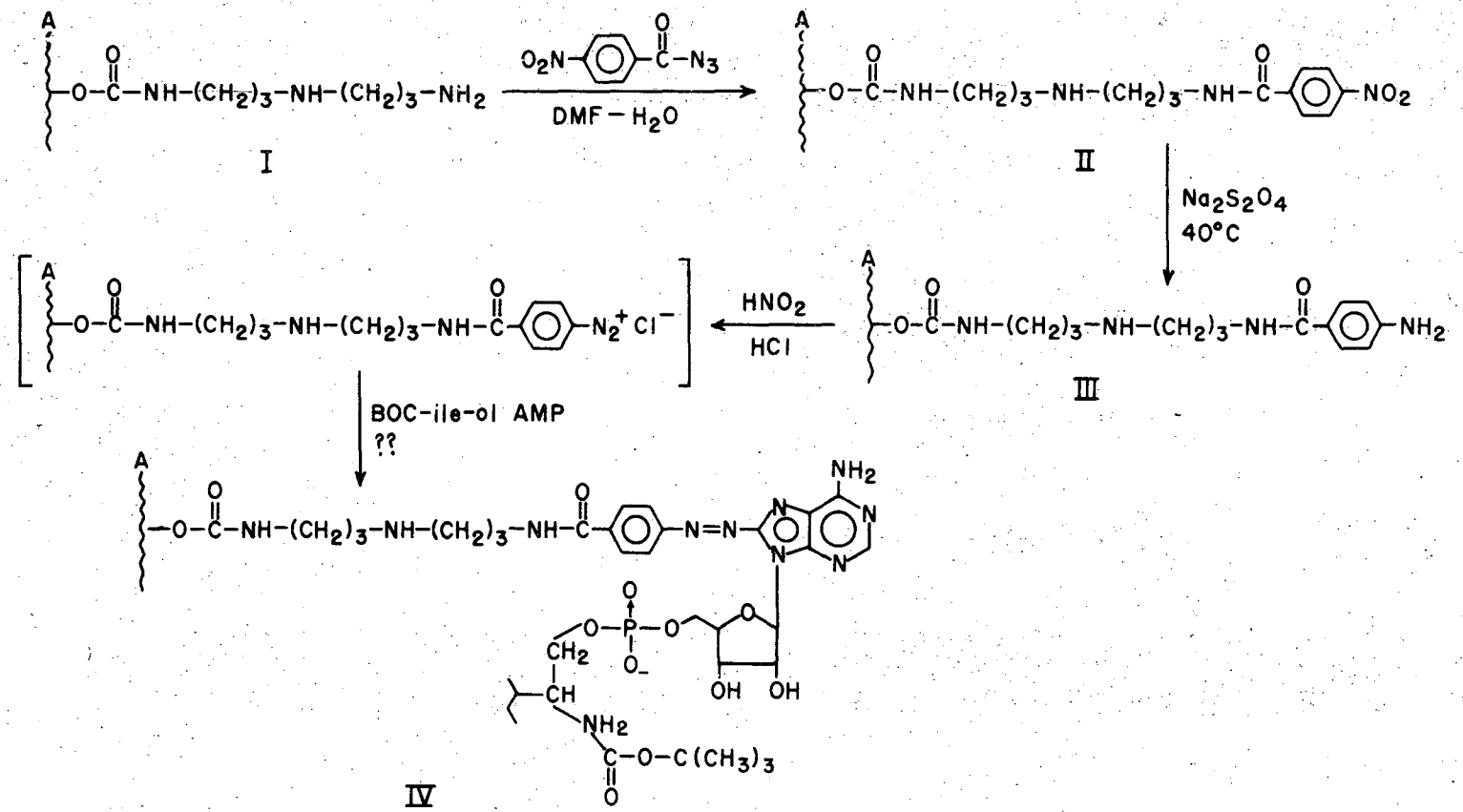


Fig. 5. Possible scheme for attachment of N-t-BOC-isoleucinol 5'-adenylate to agarose via a diazonium coupling.

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Thus, ligation through the 6-amino group seems the only viable approach. To ascertain whether use of substituents longer than methyl would reduce binding further and also to determine the effect of a terminal amino group, N^6-(2-aminoethyl) adenosine and N^6-(6-aminohexyl) adenosine were prepared from 6-chloropurine riboside and the appropriate diamine.

These showed competitive inhibition constants of 6×10^{-4} and 9×10^{-4} moles/liter, respectively, which are only slightly larger than that for unsubstituted adenosine. (That these derivative do indeed bind more strongly than the N-methyl derivative was further verified by the later determination of Ki's for the corresponding substituted L-isoleucinol adenylates: for the methyl derivative, K_i was 2×10^{-6} moles/liter; for the aminoethyl derivative, 5×10^{-7} moles/liter). It would appear that the reduction in binding incurred by introduction of a substituent was approximately offset by an increase in binding due to the terminal amino group.

These derivatives also showed apparent non-competitive inhibition, as did the N^6-methyl derivative, although the latter showed so little that the effect was considered an artifact until larger effects were seen with the other derivatives. The methyl, aminoethyl, and aminohexyl derivative-inhibited reactions showed reductions in V_{max} corresponding to non-competitive inhibition constants of 4×10^{-2}, 4×10^{-4}, and 10^{-3} moles/liter, respectively.

Rouget and Chapeville reported that N^6-hydroxyethyl ATP was a competitive inhibitor of L-leucine as well as ATP for the leucine tRNA synthetase.[26] Such an effect by our derivatives on IRS would explain the apparent non-competitive inhibition we noted. However, the methyl

derivative was shown not to be competitive with L-isoleucine. The cause of this apparent non-competive inhibition remains undetermined.

Two rather simple methods for attachment of BOC-ile-ol AMP to agarose suggested themselves. The first was to try to attach the unmodified BOC-ile-ol AMP to succinylaminoalkyl agarose (a trivial name for an agarose derivative prepared by coupling 3,3'-iminobispropyl amine to agarose and then succinylating the terminal amino functions with succinic anhydride. The procedures are described by Cautrecasas[2a]). A water soluble carbodiimide was used to attempt to form an amide bond between the terminal carboxylate on the gel bound tail and the 6-amino group. The reaction failed. (A later attempt to achieve the coupling in 80% aqueous pyridine using N,N-dicyclohexyl carbodiimide—conditions reported to covalently bind unmodified NAD^+ to a similar agarose derivative—likewise failed).

The second method involved attaching BOC-ile-ol AMP directly to cyanogen bromide-activated agarose. It was expected that the resulting adsorbent would probably be inactive because the inhibitor was attached directly to the polymer backbone. The experiment was nonetheless carried out because of the ease with which the adsorbent could be prepared. After coupling, the ligand was deblocked by stirring in 1 N hydrochloric acid. The resulting material showed ability to retard enzyme activity relative to protein comparable to that seen in Fig. 2. To determine whether this effect was due to affinity interactions, an equivalent adsorbent was prepared using AMP methyl ester. This material gave a superior separation that achieved with the AMP L-isoleucinol ester.

When we attempted to prepare more of the L-ile-ol AMP derivative by this route, we found we were unable to repeat the coupling. Mosbach et al. later reported that NAD^+ could not be coupled to cyanogen bromide-activated

agarose.[27]

It was apparent that at least a portion of the tail would have to be attached to the adenylate before insolubilization could be readily achieved. This could be done most directly by alkylation or acylation of BOC-ile-ol AMP with suitable compounds.

We had previously observed that N, O, O-triacetyl AMP underwent extensive deacylation at all three positions when kept in dilute aqueous solution at 3°C for one month. Futhermore, when N-deacetylation in 1 N hydrochloric acid (deblocking conditions) was followed by the change in the ultraviolet spectrum (λ_{max} N-acetyl AMP 273 nm, λ_{max} AMP 259 nm), the half-life was determined to be five hours. Deblocking of the bound ligand requires six hours. Because of the lability of the N^6-acyl bond, we decided not to investigate the attachment of a tail segment by acylation.

N^6-methyl AMP and N-t-BOC L-isoleucinol N^6-methyl adenylate were prepared as model compounds using dimethyl sulfate in water. The prodedure was a modification of that of Griffin and Reese.[32] Because the 6-amino group is very weakly nucleophilic, initial alkylation occurs at the nitrogen in the 1-position. This can be rearranged in aqueous ammonium hydroxide to give the desired derivative.

The inhibition constants were determined to verify that the results with N^6-methyl adenosine could be extrapolated to the BOC-ile-ol AMP derivative. These were 5×10^{-3} moles/liter for N^6-methyl AMP and 2×10^{-6} moles/liter for N-t-BOC L-isoleucinol N^6-methyl adenylate. Methylation appeared to weaken the binding of BOC-ile-ol AMP considerably more than that of adenosine or AMP. The reduction is still in the tolerable range, however.

L-Isoleucinol N^6-(ω-aminoalkyl)-5'-adenylates

The tail segment we initially decided to attempt to attach was the N-carbobenzoxy-6-amino-1-hexyl moiety. Because alkyl sulfates undergo facile elimination to give alkenes, it is impossible to prepare the higher homologs (> butyl) in useful yields. We therefore chose to attempt our alkylations using the methanesulfonate and toluenesulfonate of N-carbobenzoxy 6-amino-1-hexanol and the model compound, hexyl bromide. Alkylations were attempted on the model compounds adenosine, AMP, and AMP methyl ester. The alkylating agents are almost totally insoluble in water and no reaction could be obtained under conditions similar to those used for methylation. Alkylations were also attempted in dimethyl formamide, pyridine, and a 4:1 mixture of the two. These were done with and without catalytic amounts of sodium iodide; also with and without added silver salts. When the reactions were run at room temperature, 50°C, or 70°C no alkylation was seen, except alkylation of pyridine when possible. At 100°C, the compounds to be alkylated underwent decomposition.

We decided it would be necessary to synthesize a BOC-ile-ol AMP derivative with the tail segment already attached. The route we proposed was as follows: 6-chloropurine riboside (commercially available) would be phosphorylated to give 6-chloropurine riboside 5'-phosphate (6-chloroinosine monophosphate, Cl-IMP). This would be acetylated and the resulting $O^{2'}$, $O^{3'}$-diacetyl compound condensed with N-t-BOC-L-isoleucinol. The product would be treated with a large excess of an α, ω-diamine which would simultaneously displace the chloride and remove the acetyl groups to give the desired N-t-BOC-L-isoleucinol N^6-(ω-aminoalkyl) adenylate (See Fig. 6). By deferring the displacement of the chloride to the final step, it can be coupled with the deacetylation and one also avoids the necessity

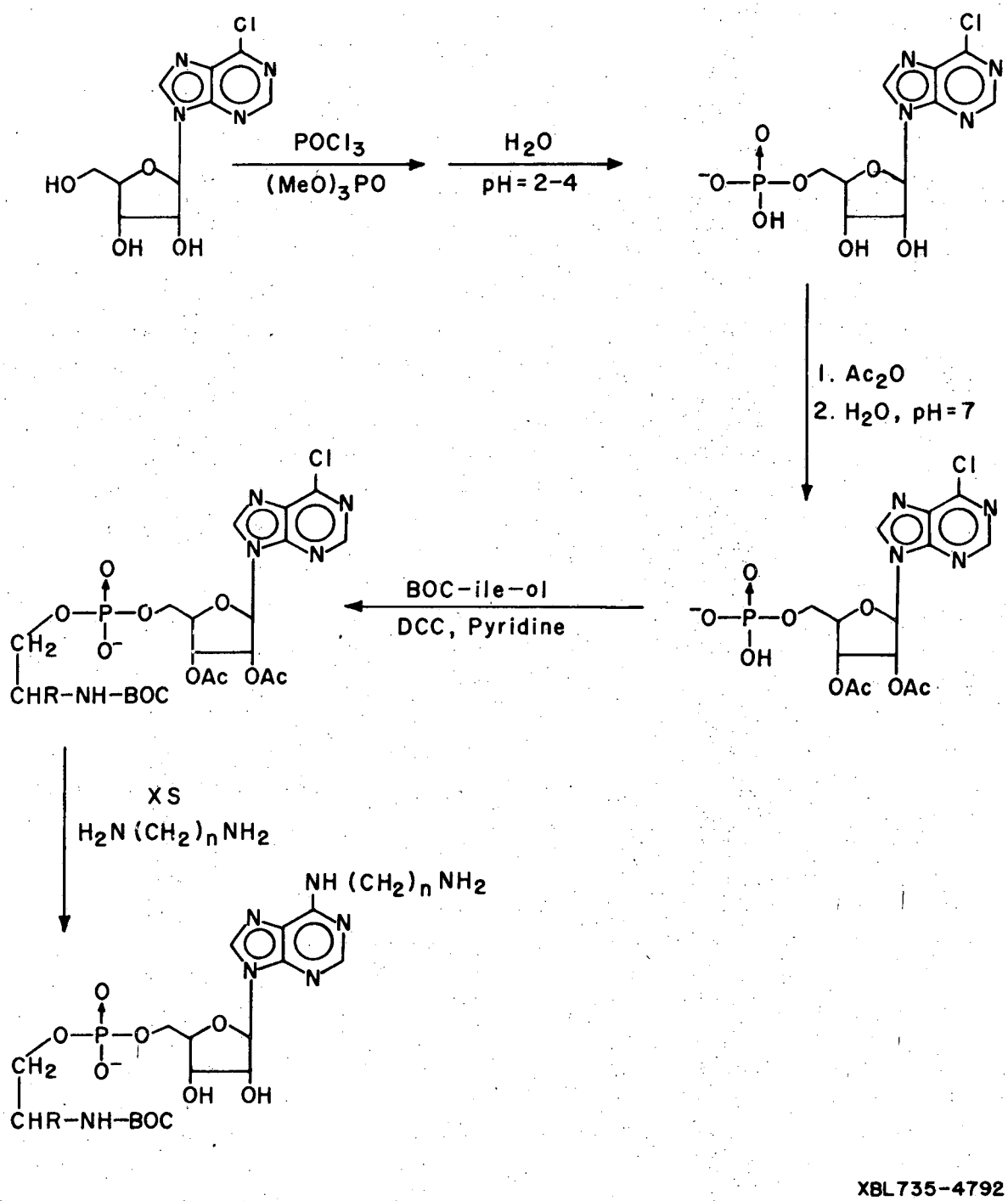

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Figure 6. Synthesis of N-t-BOC-L-isoleucinol N^6-(ω-aminoalkyl)-5'-adenylates.

of protecting and deprotecting the ω-amino group. This gives a 50% reduction in the number of steps involved compared with the more obvious synthesis beginning with the preparation of N^6-(ω-aminoalkyl) adenosine.

A preparation for Cl-IMP was reported in the literature by Hampton and Maguire.[29] This procedure is quite long and tedious and gives a yield of only 17%. We felt that this could be considerably improved upon and ultimately achieved a one-day synthesis with a 70-75% yield. The procedure was based upon a phosphorylation technique developed by Yoshikawa et al.[30] One equivalent of 6-chloropurine riboside was phosphorylated using a solution of three equivalents of phosphoryl chloride and one equivalent of water in trimethyl phosphate. These conditions reduce phosphorylation at the 2' or 3' positions to trace amounts. The resulting phosphorodichloridate was then hydrolyzed at pH 2-4, and the Cl-IMP isolated as the barium salt.

This was converted to the monopyridinium salt with pyridinium bisulfate; then it was acetylated and condensed with N-t-BOC-(U) ^{14}C-L-isoleucinol (0.5μc/mmole), following the procedures used for the preparation of BOC-ile-ol AMP. Radioactively labelled L-isoleucinol was employed to facilitate determination of the amount of the ligand bound to the agarose after coupling. The resulting N-t-BOC-(U) ^{14}C -L-isoleucinol ester with $O^{2'}$, $O^{3'}$-diacetyl 6-chloroinosine monophosphate was treated with a 25-fold excess of ethylene diamine to give N-t-BOC-(U) ^{14}C-L-isoleucinol N^6-(2-aminoethyl)-5'-adenylate (BOC-ile*-ol AE-AMP) which was isolated by chromatography on silica gel using 7:3 acetone-water.

A portion of the compound was deblocked in anhydrous trifluoroacetic acid and the resulting L-ile*-ol AE-AMP was purified by column chromatography. Inhibition studies indicated that it had inhibition constants of

5×10^{-7} moles/liter (vs. ATP) and 2×10^{-8} moles/liter (vs. L-isoleucine). It showed apparent non-competitive inhibition in both determinations; however, this was merely a reflection of the fact that it is a competive inhibitor of both substrates. (V_{max} values were determined against ATP at several L-isoleucine concentrations. When these were plotted against V_{max}/(L-ile), an inhibition constant of 10^{-8} moles/liter was obtained.)

Several problems were encountered in the synthesis of BOC-ile*-ol AE-AMP. They were: 1) the final product is contaminated with acetate; an unknown material which appears to be an acetone condensation product produced during the chromatography (it is found at the same concentration in all fractions); and an apparently inorganic impurity (it cannot be detected on TLC using a variety of visualizing agents nor does it give a signal above noise level on NMR); 2) the reaction solvent, pyridine, slowly reacts with the 6-chloropurine derivatives to give quaternized pyridinium salts. These are insufficiently reactive, under the conditions used, to be displaced by ethylene diamine to give the desired products; and 3) the displacement of the chloro group by ethylene diamine is accompanied by extensive dealkylation to yield the unsubstituted BOC-ile*-ol AMP.

The most likely source of the extraneous acetate is the acetylation reaction of 6-chloroinosine monophosphate. Acetic acid (or acetate) may be somehow adsorbed to the product. It was found that the $O^{2'}$, $O^{3'}$-diacetyl 6-chloroinosine monophosphate (diAc Cl-IMP) could be purified by dissolving in a minimum of chloroform and then adding five volumes of isopropanol. The resulting precipitate is discarded. When BOC-ile-ol AE-AMP was prepared from material purified in this manner, it was free of acetate and was purer than any other we have prepared. However, the purification results in a loss of about 20% of the diAC Cl-IMP, with a

concomitant loss in the final procuct yield. We then discovered that only slightly poorer results (in terms of final product purity) could be obtained simply by repeating the lyophilization of the diAc Cl-IMP twice. No extra losses in yield were incurred by this procedure.

The acetone condensation product could be removed by washing with acetone.

The existence of the other impurity(s) was inferred from radioassay of BOC-ile*-ol AE-AMP which was homogenous to TLC and showed no extraneous protons on NMR. Samples of the compound gave fewer counts than would be required for 100% pure material.

Since anticipated procedures for coupling the ligand to agarose required a primary amino group and this impurity had none as judged by its failure to give a reaction with ninhydrin, we felt that the coupling procedure would serve to effect final purification and we made no further attempts to remove the impurity.

In order to prevent the reaction of pyridine with the 6-chloropurine moiety, we decided to run the reaction in 2, 6-dimethylpyridine (lutidine). However, the diAc Cl-IMP was almost totally insoluble in this solvent. The fact that this slight change in solvents had such a major effect suggested that finding another suitable solvent might prove extremely difficult. Instead we decided to try to reduce the reaction time and hence the contact time with pyridine.

The preparation of BOC-ile-ol AMP was used as a model and was allowed to run 5 days as usual.[17] Aliquots were withdrawn at 1 and 3 days and worked up through heptane extration. All three were then deprotected simultaneously and the workups completed. The (percentage) crude yield from the 1-day reaction was greater than that of the others and the

crude product was also somewhat purer. Thus it appears unnecessary and undesireable to run this type of coupling for longer periods of time.

During these studies it occurred to us that the acetylation step might be dispensed with. If so, there would be no problem with acetate contamination and formation of the pyridinium salts would be reduced (the acetylation is run in pyridine). In the procedure[17] upon which ours is based, acetylation was presumably done to protect the hydrosyls against phosphorylation. Our work on the 1-nitromethyl L-isoleucinol adenylate (q.v.) had convinced us that secondary alcohols could not be phosphorylated under the conditions which were being employed.

We decided to do a model reaction first, coupling N-t-BOC-L-isoleucinol to AMP, but found that AMP was totally insoluble in anhydrous pyridine. We therefore prepared the mono (tetrapropylammonium) salt of AMP in hopes that this would be somewhat more soluble. Slight solubility is all that is necessary, since formation of the soluble product will eventually bring all the starting material into solution. The tetrapropyl ammonium salt also showed no significant solubility, but we attempted the coupling anyway, based on the above premise. After 5 days, there was no visible diminution in the undissolved salt and no product could be detected.

Although the bis-(tetrapropylammonium) salt might show better solubility, it was felt that the reaction mixture would then be too basic for coupling to occur.[31] It appears that acetylation will remain a necessary step simply in order to confer solubility on the reactant.

The major problem with the synthesis was the dealkylation side reaction. The ratio of BOC-ile*-ol AMP to BOC-ile*-ol AE-AMP in the initial preparation was approximately two to one. Prevention of dealkylation

would have increased the yield from 10% to 30%, which is approaching the 35-40% yield obtained in the normal preparation of BOC-ile-ol AMP.

In studying the dealkylation, it was found that it occurred more extensively when the displacement - deacetylation reaction was carried out in refluxing methanol than when done at room temperature. When done at 3°C, only a slight decrease in dealkylation was noted and the time for completion of the reaction was considerably prolonged.

Since dealkylation did not occur in the preparation of N^6-(2-aminoethyl) adenosine, we dicided to see whether it would occur in the preparation of N^6-(2-aminoethyl) AMP. It did.

Although we did not understand the mechanism of the dealkylation, we felt that it might be linked to the potential for aziridine elimination inherent in the aminoethyl group. Were this the case, an aminohexyl chain should be relatively stable to dealkylation. Accordingly, we prepared some N^6-(6-aminohexyl) AMP and found that the reaction proceeded without dealkylation. However, when N-t-BOC-(U) ^{14}C-L-isoleucinol N^6-(6-aminohexyl) adenylate (BOC-ile*-ol AH-AMP) was prepared, dealkylation did occur and the ratio of dealkylated to alkylated material was again ca. 2:1.

These results did not clarify the mechanism, but did suggest that BOC-ile*-ol AH-AMP might be prepared in better yield from AH-AMP. This would require the protection and deprotection fo the ω-amino group, steps which the original synthesis had been designed to avoid, but might eliminate the necessity for acetylation, since the addition of an N-carbobenzoxy 6-amino hexyl group might make the derivative sufficiently soluble without acetylation.

Another possibility is suggested by the recent report of Guilford

et al. on the preparation of AH-AMP from Cl-IMP.[32] The reaction was achieved using neutral, aqueous hexamethylene diamine hydrochloride. Since the dealkylation is very likely linked to some sort of nucleophilic attack, the ability to use neutral conditions could minimize or eliminate it.

Certain problems can be anticipated. BOC-ile-ol diAc Cl-IMP is quite insoluble in water and could be expected to be even less so in ionic aqueous solutions. Our reactions have normally been run in ethanolic solution. Neutral hexamethylene diamine (or ethylene diamine) hydrochloride is only slightly soluble in ethanol, which is unacceptable considering the large excess which is required. The reaction can probably be achieved at elevated temperatures.

Both of these possibilities are worthy of investigation. However, we had obtained sufficient quantities of the ligands to begin to prepare adsorbents. We felt that there was no point in spending too much time improving the synthesis until we had shown that adsorbents which could be successfully used for affinity chromatography could be prepared from the ligands.

Coupling Reactions

Initially, we attempted to couple the BOC-ile*-ol AE-AMP and BOC-ile*-ol AH-AMP to succinylaminoalkyl agarose using either 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in aqueous media. Using a 50% excess of the appropriate adenylate and a 10-fold excess of carbodiimide (based on the number of tail segments bound to the gel), the ligands were bound at a level of less than 1 μmole per milliliter of gel.

Only about 5% of the agarose bound tail segments were ligated. We also reacted the ligands directly with cyanogen bromide activated agarose at pH 8.5 and pH 10, achieving couplings of 0.5 μmoles/ml and 1.5 μmoles/ml respectively. The coupling of 3,3'-iminobispropylamine at pH 10 gives a level of substitution of 15-20 μmoles/ml. In all cases, the BOC-ile*-ol AE-AMP was coupled slightly more efficiently than was the BOC-ile*-ol AH-AMP.

We hypothesized that the reason for the poor efficiency of these reactions was the sequestration of the ω-amino group in an ion pair with the phosphate moiety. This would keep the amino group in a sterically crowded environment as well as raising its pKa by stabilizing the protonated form. This should result in considerably lowered reactivity of the amino group and would allow hydrolysis of the carbodiimide carboxylate complex to compete favorably with the coupling of the amine.

Molecular models indicated that such ion pairs could readily be formed with either compound. The ion paired form of the aminoethyl derivative was quite rigid, whereas that of the aminohexyl compound was fairly flexible. This suggests that the ion paired conformation of the aminoethyl compound would be somewhat less stable than that of the aminohexyl compound due to a greater decrease in entropy upon formation. This would explain the greater reactivity of the aminoethyl derivative despite the fact that its amino group has intrinsically more steric hindrance.

To determine the relative rates of hydrolysis versus coupling, some of the aminoethyl derivative was reacted with a 4-fold excess of acetic acid and a 10-fold excess of N-ethyl N'-(3-dimethylaminopropyl) carbodiimide hydrochloride at concentrations comparable to a coupling reaction. The reaction was followed by TLC. During the first 24 hours the

UV- and ninhydrin-positive BOC-ile*-ol AE-AMP spot decreased and a second spot, UV-positive and ninhydrin-negative with higher Rf, appeared and increased. This was presumably the acetamidoethyl derivative. After 24 hours, at which time spots were of approximately equal intensity by visual estimation, no further change was noted in the ratios. Thus, it appears that even in this case, wherein steric considerations were minimal, hydorlysis occurs at a rate approximately 20 times that of coupling.

Raising the pH to increase the reactivity of the amino group is not acceptable for either coupling technique. Carbodiimide couplings cannot be carried out at pH's much above 7, because the reactive species in the coupling is the protonated adduct of the acid with the carbodiimide. At higher pH's, there is very little of this species in solution and acylurea formation predominates.[31] Cyanogen bromide couplings cannot be efficiently carried out at pH > 10 because the activated intermediate is quickly decomposed under such conditions.[2a]

In order to increase the amount of coupling, it would be necessary to increase the amount of carbodiimide used or decrease the rate of hydrolysis. Mosbach et al. had just published a paper describing the coupling of NAD^+ to a modified agarose using N,N'-dicyclohexyl carbodiimide (DCC) in 80% aqueous pyridine.[27] This system appeared very attractive. DCC is highly soluble in the solvent so that very large excesses could be employed. The concentration of water was reduced fivefold. Because DCC is quite hydrophobic, the concentration of water in its solvation shell (or that of its adduct with the carboxylic acid) could be expected to be considerably lower yet. The technique had the drawback that it could be expected to cause irreversible shrinkage of the agarose beads (the procedure includes washing with n-butanol to remove

N,N'-dicyclohexylurea. However, Mosbach et al. had successfully employed the adsorbent which they had prepared by this technique for affinity chromatography. Whatever shrinkage occurred was apparently not overly detrimental.

We were prompted to try coupling our derivatives. Test couplings were run for 8 days on both the aminoethyl and aminohexyl derivatives, as well as unsubstituted BOC-ile*-ol AMP. The latter was done to ascertain that any coupling of the aminoalkyl derivatives which might be obtained would be via the ω-amino group. The gels were then washed as prescribed,[27] followed by washing with large volumes of 0.01 M phosphate (pH 7.5) and water.

Radioassay showed that the BOC-ile*-ol AE-AMP had been coupled at a level of 2.4 μmoles/ml; the BOC-ile*-ol AH-AMP, 1.2 μmoles/ml; and the unsubstituted BOC-ile*-ol AMP, 0.08 μmoles/ml. The coupling appears to proceed in reasonable yield and to occur primarily via amide formation with ω-amino group. The greater coupling of the aminoethyl derivative may be understood in terms of its forming a less stable ion pair with the phosphate, as discussed previously. After deblocking, the aminoethyl derivative was retained at a level of 1.9 μmoles/ml, and the aminohexyl derivative, 0.4 μmoles/ml.

The deblocking procedure used above and in subsequent adsorbent preparations differed slightly from the previously used procedure of stirring in 1 N aqueous hydrochloric acid for 6 hours at room temperature. Instead 1 N hydrochloric acid in 1:1 ethylene glycol-water was used. We switched to the latter solvent because considerable hydrolysis of the cyanogen bromide-induced linkage had been occurring during deblocking. The rationale for the change was that the rate-determining step for the

removal of the t-BOC group was dissociation of the t-butyl carbonium ion. This reaction should be independent of the concentration of water. Whereas acid hydrolysis of the presumed carbamate ester involves nucleophilic attack by water on the protonated carbonyl and is sensitive to water concentration. (Ethylene glycol is expected to react solvolytically at a much lower rate). Possible loss of ligand due to hydrolysis of the purine-sugar bond may also be reduced in this solvent. We hoped to reduce the rate of ligand loss without affecting the rate of deblocking. A less aqueous medium was not employed because of the possibility of irreversible shrinkage of the gel. A study on BOC-ile-ol AMP showed that it wad deblocked in this medium with a half-life of 130 min. This is comparable to that seen in aqueous acid. Loss of ligand from deblocked gels was reduced but the results were quite erratic and could not be readily quantitated.

We next carried out a large-scale coupling of the BOC-ile*-ol AE-AMP to succinylaminoalkyl agarose. After 8 days, the gel was collected by filtration, washed free of precipitated DCU, and returned to the filtrate with additional DCC. The reaction proceeded 8 days more. The gel was again collected and washed free of DCU. This was followed by copious washing with 0.01 M phosphate (pH 7.5) and with water. The gel was found to have undergone some irreversible shrinkage, from 20 ml to 16 ml in total volume. Radioassay indicated a level of coupling of 3.4 μmoles/ml, and after deblocking, 2.6 μmoles/ml. The deblocked material was tested with trinitrobenzene sulfonate,[2a] giving a medium-orange color which indicates the presence of primary amines. The gel before deblocking gave a negative test. This gel was used to make affinity columns, the employment of which is described in the next section.

The success of this coupling procedure led us to investigate couplings

in other partially aqueous media, in hopes of finding a procedure which would require less time and would not lead to irreversible shrinkage of the agarose gel.

Test couplings of 5 days' duration were carried out on BOC-ile*-ol AE-AMP in 1:1 dimethylformamide-water (A), 1:1:1 dimethylformamide-dioxane-water (B), 3:1 dioxane-water (C), and 1:1 ethylene glycol-water (D), after verifying that these solvent systems did not cause irreversible shrinkage of the agarose gel. N-ethyl N'-(3-dimethylaminopropyl) carbodiimide hydrochloride was used as the coupling agent. The following levels of coupling were found: A, 2.3 μmoles/ml; B, 4.1 μmoles/ml; C, 5.4 μmoles/ml; and D, 0.9 μmoles/ml. After deblocking, the retention levels were: A, 0.8 μmoles/ml; B, 1.57 μmoles/ml; C, 2.56 μmoles/ml; and D, 0.5 μmoles/ml. The coupling in 3:1 dioxane-water looks very promising. None of the adsorbents prepared by these latter couplings have been tested as affinity agents.

Buffer Studies

Before carrying out any separations on the affinity adsorbent, we decided to determine which of the available buffers would be most suitable to use in the eluent. Previously, we had used phosphate buffer because of its stabilizing effect on the enzyme. Since phosphate is an inhibitor of ATP, we felt it might also compete with the insolubilized ligand for IRS, thus reducing the efficiency of the column.

Accordingly, we decided to screen a number of alternate buffers. The primary characteristics we looked for were: 1) little or no color development with the Lowry reagent, and 2) little or no inhibition of the ATP-(^{32}P)PP_i exchange reaction.

The following buffers were checked for color development with the Lowry reagent: borate, cacodylate, tris(hydroxymethyl)amino methane (Tris), N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES), N- tris(hydroxymethyl)methyl glycine (Tricine), N,N-bis(hydroxyethyl) glycine (Bicine), N-tris(hydroxymethyl)methyl aminoethane sulfonate (TES), N,N-bis(hydroxyethyl)aminoethane sulfonate (BES), and triethanolamine. Ten microliter aliquots of 1 M solutions of the buffers were treated with the Lowry reagent under assay conditions and the following optical densities at 750 nm were found: borate, 0.0; cacodylate, 0.0; Tris, 0.09; HEPES, 1.60; Tricine 0.02; Bicine, 3.5; TES, 0.005; BES, 0.57; and triethanolamine, 3.4.

On the basis of these results, borate, cacodylate, Tris, Tricine, and TES were selected to be tested for inhibition on the pyrophosphate exchange reaction. The exchange reaction was carried out on a fixed amount of IRS under standard assay conditions, except that the usual Tris buffer was replaced by the test buffer so as to give a final concentration of 0.05, 0.10, or 0.15 M. Borate was found to be highly inhibitory. The others, except TES, were found to be slightly inhibitory. TES was shown to stimulate the exchange slightly. That is, the rate of exchange rose slightly with increasing buffer concentration, whereas with all other buffers, the exchange rate dropped slightly with increasing buffer concentration.

TES was chosen as the most suitable buffer. We decided to use the following solution as the column eluent for testing the affinity gel: 0.02 M TES (pH 7.8) containing 0.01 M 2-mercaptoethanol, 0.005 M magnesium chloride, and 0.05 M potassium chloride. This we will refer to as Elution Buffer 1.

Affinity Columns

The gel prepared by coupling BOC-ile*-ol AE-AMP to succinylaminoalkyl agarose using DCC in 80% aqueous pyridine, hereinafter referred to as Affinity Gel I, was used to prepare columns and these were tested for their ability to purify L-isoleucine t-RNA synthetase.

A portion of C_γ gel eluate was chromatographed on a column of the adsorbent using Elution Buffer 1. The results may be seen in Fig. 6A. An equivalent run was done on unsubstituted agarose. These results are shown in Fig. 6B. A run on succinylaminoalkyl agarose gave an elution pattern which differed little from that seen on unsubstitited agarose.

It may be seen that the elution behavior of the bulk protein is almost identical on both columns. This indicates that the affinity gel is not giving non-specific interactions with the proteins and that the shrinkage which occurred during coupling did not have an untoward effect on pore size. Ninety nine percent of the protein was recovered from the affinity column and 85% of the protein from the unsubstituted agarose.

On the agarose column, IRS eluted slightly ahead of the protein peak, indicating a slight gel permeation effect. One-hundred and sixteen per cent of the enzyme activity was recovered. On the affinity gel, a small enzyme peak emerges concurrently with the protein peak, containing only 14% of the applied enzyme activity. This behavior occurs frequently in affinity chromatography[2c] and should not be construed as indicating saturation of the column. (The column has a theoretical capacity of 39 μmoles. Seventy-five picomoles of IRS were chromatographed in the run shown.) The rest of the enzyme was presumably bound to the column (or denatured).

Elution of a bound enzyme is normally accomplished through a change of pH, an increase in ionic strength, or inclusion of a soluble ligand in

Figure 6. Chromatographic elution patterns of partially purified L-isoleucine t-RNA synthetase on (A) Affinity Gel 1 and (B) unsubstituted agarose. A 1/4" diameter column was poured using 15 ml of the appropriate gel and equilibrated with Elution Buffer 1 (0.02 M N-Tris(hydroxymethyl) methylaminoethyl sulfonate, pH 7.8; 0.01 M 2-mercaptoethanol; 0.005 M magnesium chloride; 0.05 M potassium chloride). 0.5 ml of C_γ gel eluate (6.76 mg/ml protein; 10.1 units/ml IRS) which had been dialyzed against the buffer was run on and then eluted with the buffer. The columns were run at 20°C with a flow rate of 0.25 ml/min. Fractions of 1.3 ml were collected in a refrigerated chamber at 5°C. Protein (filled circles) was measured with the Lowry reagent. Enzyme activity (open circles) was determined by the ATP-[^{32}P] PP_i exchange reaction.

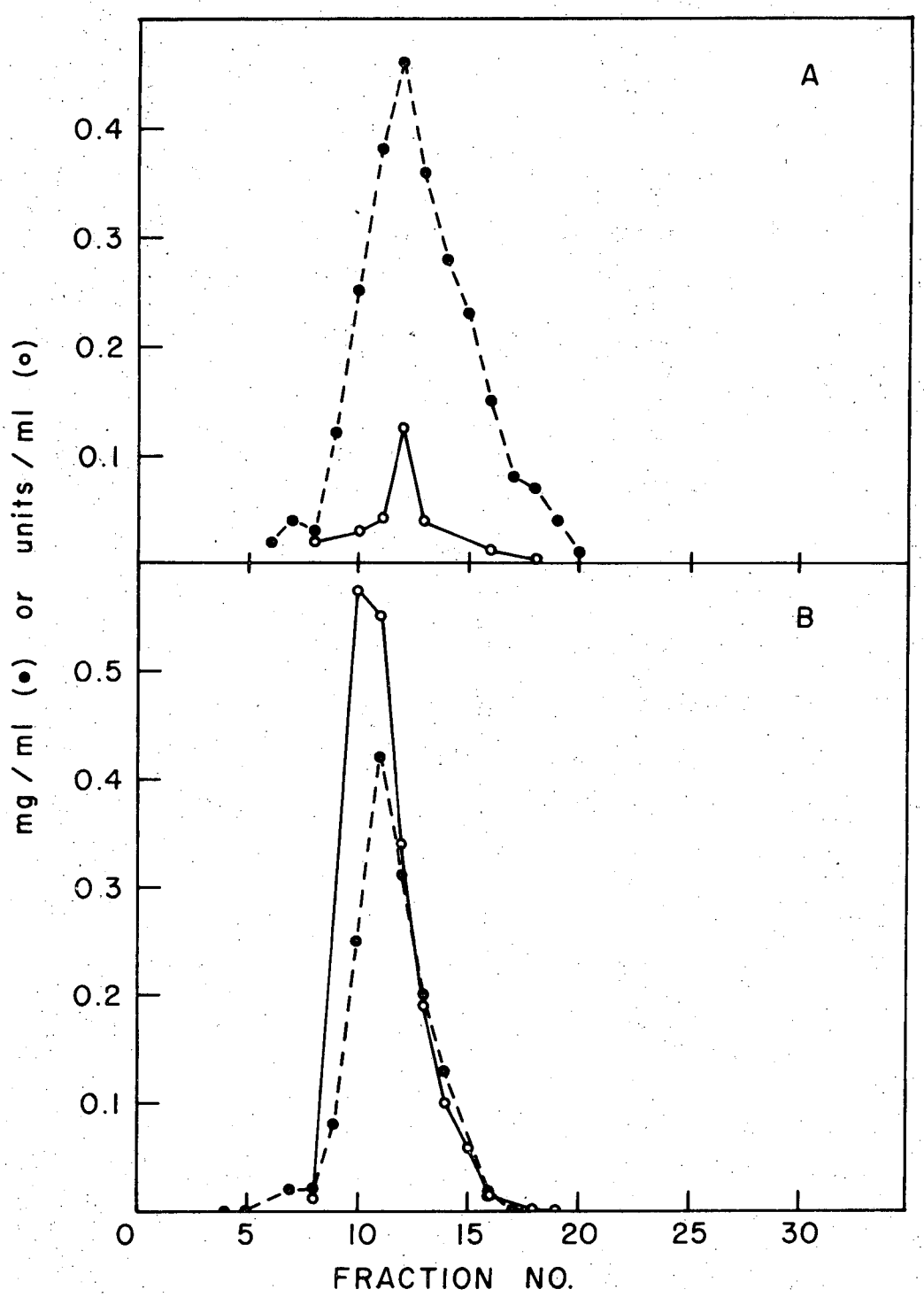

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Fig. 6

the eluent buffer. Since IRS is denatured at pH's greater than 10[18] or less than 6[33] and is also denatured in 2.5 M urea,[13] we decided to attempt elution using soluble ligands.

Portions of C_γ gel eluate were placed on a column of Affinity Gel 1 and the column was washed with Elution Buffer 1 until the bulk protein peak had been completely eluted. The elution buffer was then changed to one which contained in addition to the components of Elution Buffer 1 0.01 M L-isoleucine, or a combination of 0.01 M L-isoleucine and 0.01 M ATP. The latter mixture would presumably form the aminoacyl adenylate in situ.

These techniques resulted in the slow elution of enzyme at very low levels of activity. Much of the enzyme activity applied was not recovered. A typical run is seen in Fig. 7.

The slow removal of the enzyme was attributed to a slow rate constant for the dissociation of the enzyme from the insolubilized ligand. In order to circumvent this problem, after removal of the protein peak with Elution Buffer 1, one column volume of the buffer containing the free ligand(s) was passed into the column and elution was halted for one or eighteen hours to allow equilibration of the IRS between the soluble and insolubilized ligands. Then elution was continued with the second buffer. This resulted in an initial slight increase in eluted IRS activity followed by a precipitous drop to elution of no activity (Fig. 8).

Batchwise extraction of IRS was also attempted. After placing some C_γ gel eluate on a column and washing off the bulk protein, the adsorbent was removed from the column and extracted overnight with five volumes of Elution Buffer 1 containing also either 0.1 M L-isoleucine or 10^{-3} M L-ile-ol AMP. In the latter cases the extract was dialyzed to remove

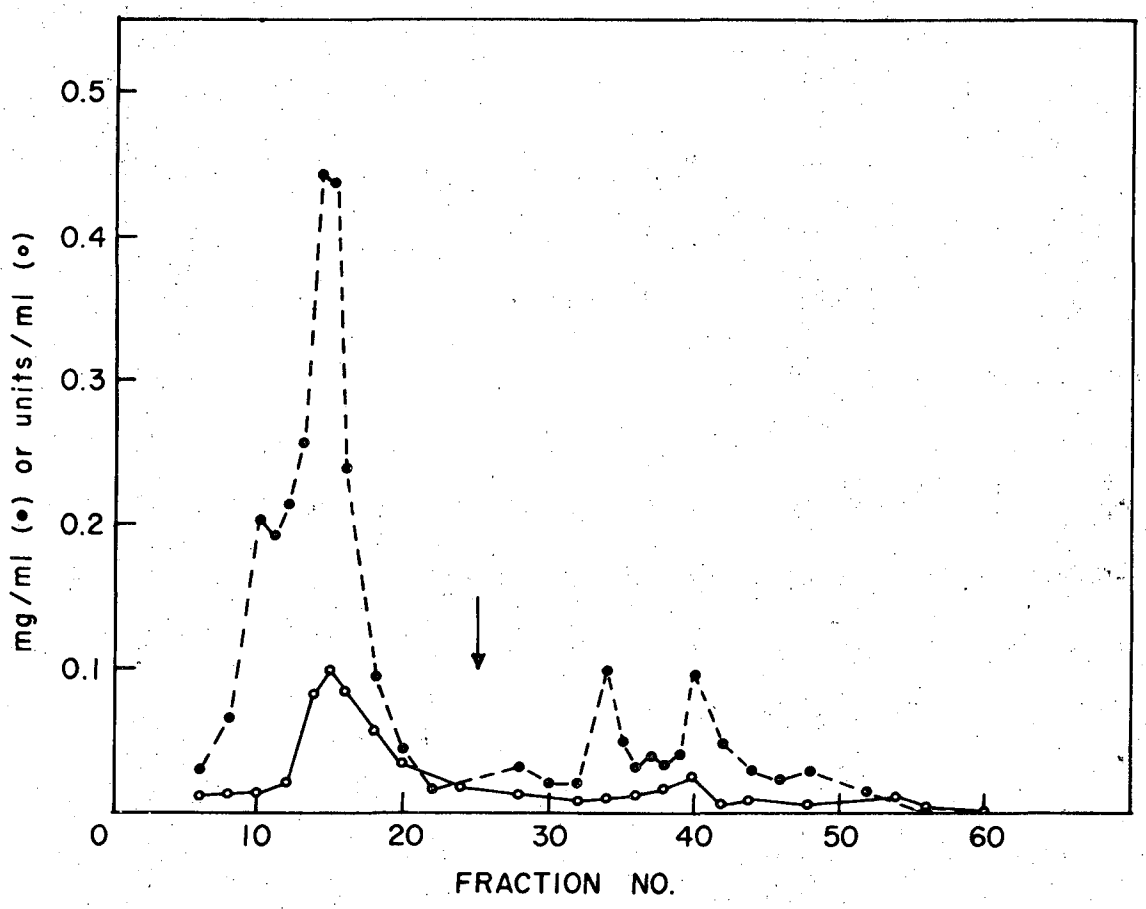

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Figure 7. Chromatographic elution pattern of partially purified IRS on Affinity Gel 1. Chromatography was run as described for Fig. 6 (A), except that a flow rate of 0.2 ml/min was used and fractions of 1.0 ml were collected. After elution of fraction No. 25 (arrow), the elution buffer was changed to one which contained in addition 0.01 M L-isoleucine and elution was continued. Protein (filled circles) was measured with the Lowry reagent. Enzyme activity (open circles) was determined by the ATP-[^{32}P] PP_i exchange reaction.

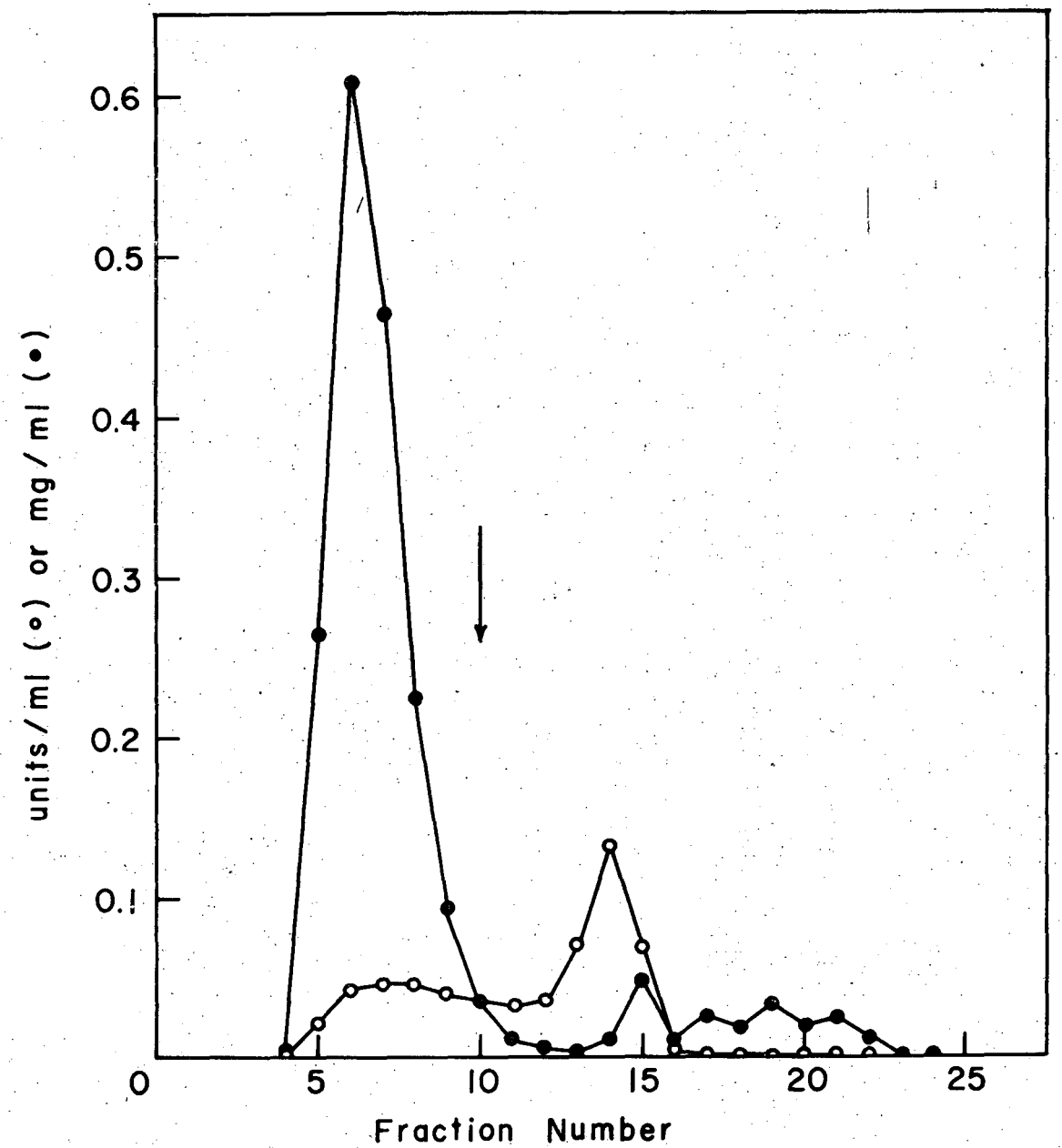


Figure 8. Chromatographic elution patter of partially purified IRS on Affinity Gel I. A 1/4" column was filled with 2 ml of the gel equilibrated with Elution Buffer 1. C_γ gel eluate (0.3 ml, containing 2.0 mg protein and 2.25 units of IRS) was placed on the column and chromatographed with the buffer at a flow rate of 0.08 ml/min. Fractions of 1.1 ml were collected. After ten fractions were collected, 2 ml of Elution Buffer 1 containing 0.01 M each L-isoleucine and MgATP were run onto the column. The column stood overnight at 20° and elution was resumed as before except that the latter buffer was now employed. Protein (filled circles) was measured with the Lowry reagent using the appropriate buffer as a blank. Enzyme activity (open circles) was determined from the ATP-(^{32}P)PP_i exchange reaction.

the inhibitor before the IRS assay was carried out. Only slight amounts of IRS were recovered in this manner (ca. 10% with L-isoleucine extraction and ca. 2% with BOC-ile-ol AMP extraction).

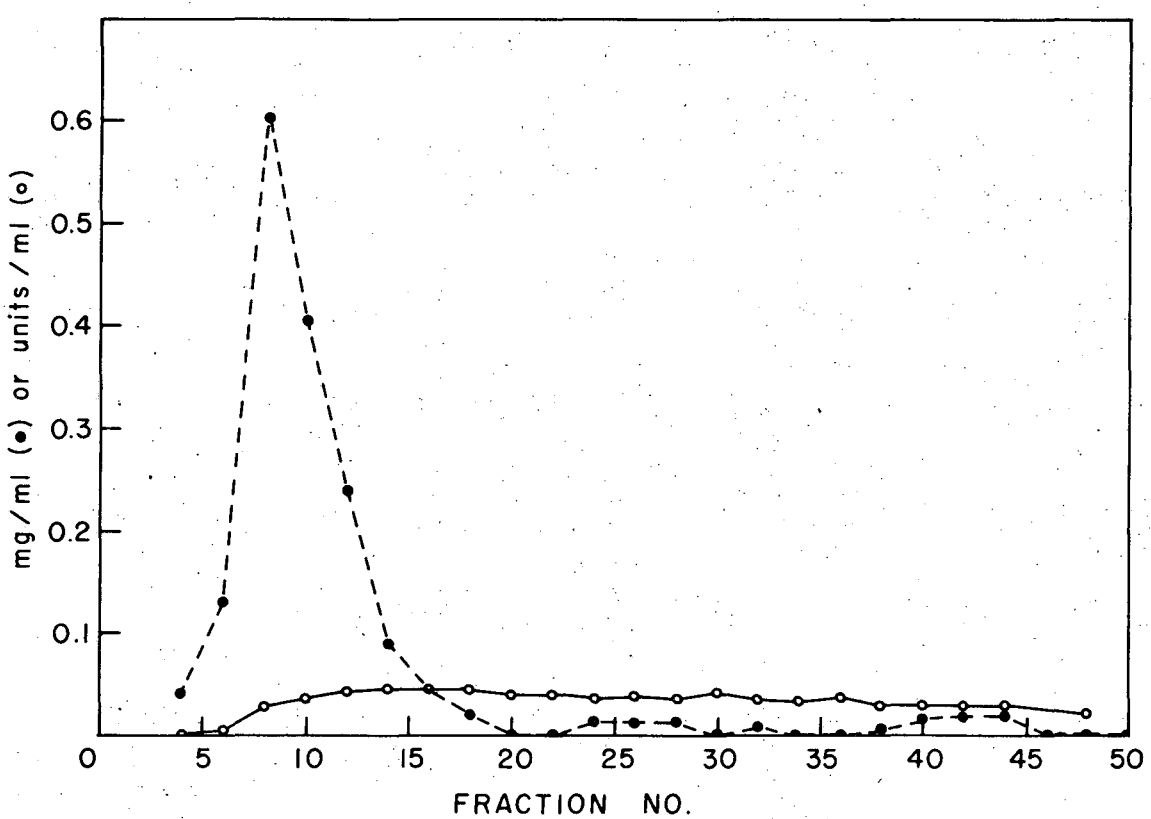
These results made it seem quite likely that the enzyme was denatured after adsorption onto the gel. This denaturation was apparently induced by the bound ligand, since no denaturation was seen when unsubstituted agarose was employed.

To test this hypothesis, a portion of C_γ gel eluate was dialysed against Elution Buffer 1 containing 0.01 M L-isoleucine and chromatographed using the same buffer. Based on the K_m of L-isoleucine and the K_i of L-ile-ol AMP, one can calculate a partition ratio of 5:1 between the stationary and mobile phases in this system, as compared with a ratio of greater than 10,000:1 in the absence of L-isoleucine. The IRS should be retarded under these conditions rather than retained. While L-isoleucine can normally protect IRS against denaturation, it cannot protect it against denaturation induced by binding to the insolubilized ligand since the two cannot be simultaneously bound. If such denaturation is occurring, the elution pattern should not differ much from that seen with the L-isoleucine-free eluent.

The pattern seen on running the experiment (Fig. 9) is what one would expect for a partitioning between the phases with incomplete equilibration. Enzyme activity recovery was ca. 50%, and suggests that much of the applied enzyme activity can be recovered from the columns. The enzyme in fractions 20-50 was purified three- to fourfold.

The results were encouraging. However, this technique is not suitable for preparative scale work because the IRS is only retarded.

Since elution with soluble ligands did not appear practicable, we



XBL733-4734

Figure 9. Chromatographic elution pattern of partially purified IRS on Affinity Gel 1 while continuously in the presence of 0.01 M L-isoleucine. A 1/4" diameter column was poured from 2 ml of Affinity Gel 1 and equilibrated with Elution Buffer 2 (0.02 M TES, pH 7.8; 0.02 M 2-mercaptoethanol; 0.005 M magnesium chloride; 0.05M potassium chloride; 0.01 M L-isoleucine), 0.3 ml of C_γ gel eluate (7.1 mg/ml protein, 11.7 units/ml IRS) previously dialyzed against the buffer was chromatographed on the column with the buffer at 20°C, flow rate 0.1 ml/min. Fractions 1-29 were 0.6 ml each; fractions 30-50 were 0.8 ml each. Fractions were collected at 5°C. Protein (filled circles) was measured with the Lowry reagent. Enzyme activity (open circles) was determined from the ATP-[32P] PP_i exchange reaction.

then tried to elute the bound enzyme by changing the ionic strength of the eluent. The previous studies had indicated that the IRS was bound quite tightly to the insolubilized ligand. We felt it would be possible to use a phosphate buffer without significantly reducing the level of binding of IRS to the adsorbent. Use of a phosphate buffer would be desirable because phosphate was considerably superior to TES (or any other buffer tested) in minimizing denaturation of IRS during storage. It might exert a similar effect in minimizing denaturation during chromatography.

A portion of C_γ gel eluate was placed on a column of Affinity Gel I equilibrated with 0.02 M phosphate (pH 7.5) containing 0.02 M 2-mercaptoethanol and the column was washed with the buffer until the bulk protein was completely eluted. Then the column was eluted with a gradient of phosphate to 1 M final concentration. The gradient was 0.02 M in 2-mercaptoethanol and the fractions were collected in vial containing 0.02 M aqueous 2-mercaptoethanol to dilute the eluent and prevent denaturation by prolonged contact of the enzyme with the high molarity solution. Thirteen percent of the applied enzyme activity was collected in the peak emerging concurrently with the protein peak. Only trace amounts of activity were eluted with the phosphate gradient.

In addition to showing that the enzyme activity could not be eluted by changing the ionic strength of the medium this experiment showed that the use of phosphate buffer had no untoward effect on the binding of the enzyme to the column: the elution pattern during elution with 0.02 M phosphate was almost identical to that seen in other runs using Elution Buffer 1 (e.g., Fig. 1A).

We then turned to pH shift elution. Since IRS is somewhat less sensitive to basic conditions, a shift to basic pH was tried first.

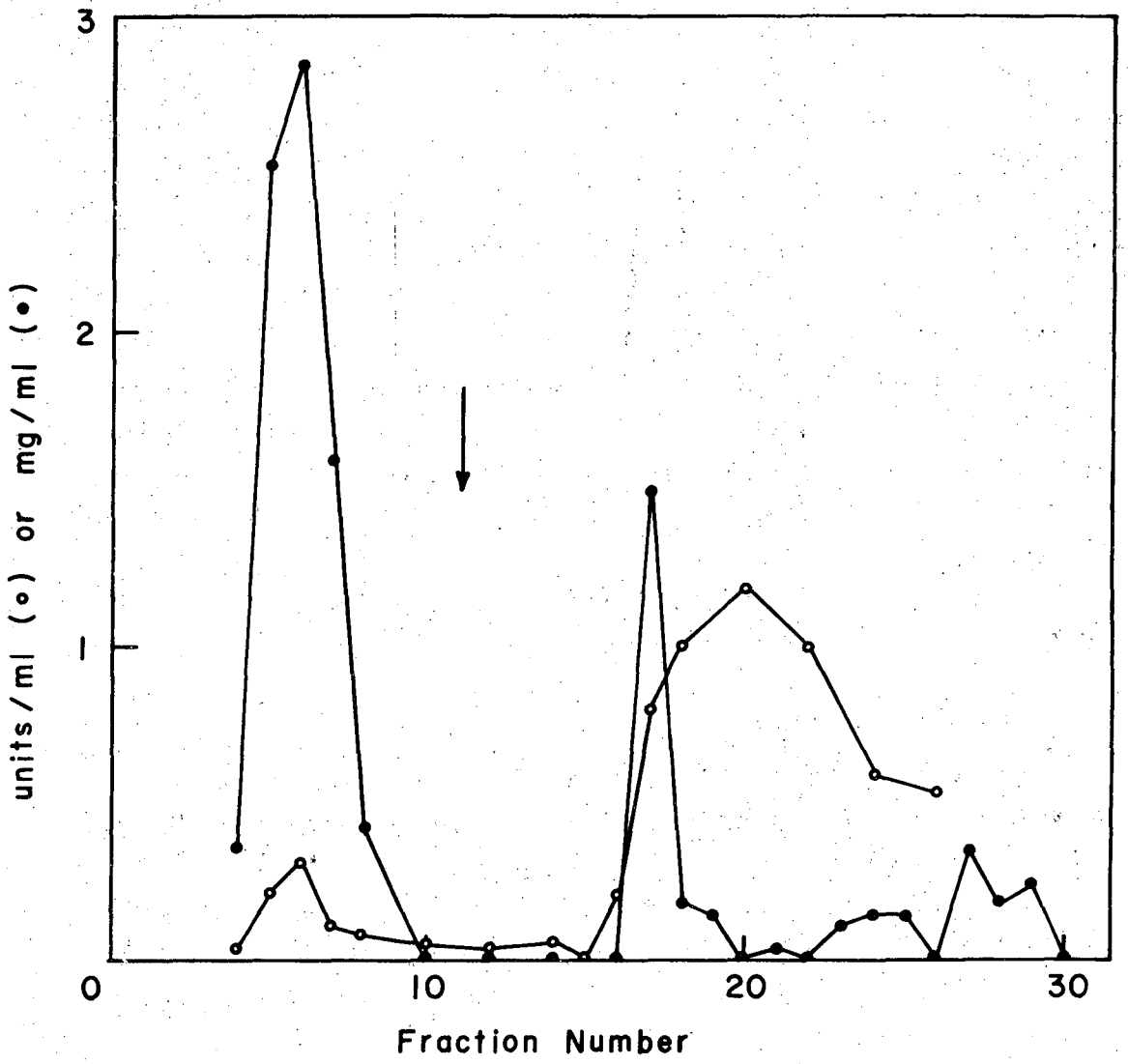
A portion of C_γ gel eluate was placed on a column of Affinity Gel 1 and washed with 0.02 M phosphate (pH 7.5) containing 0.02 M 2-mercaptoethanol until the bulk protein was completely removed. Then the column was eluted with 0.02 M phosphate (pH gradient: pH 7.5 to pH 11.6) containing 0.02 M 2-mercaptoethanol and 0.01 M L-isoleucine. This combined the pH shift technique with the soluble ligand technique. The fractions were collected in vials containing 0.1 M phosphate buffer at pH 6.25 to reduce the pH of the effluent upon collection and minimize denaturation. The pH gradient in the fractions after collection was 6.7 to 7.4

This procedure caused the elution of a second peak of enzyme activity. Maximum activity was eluted at pH ≈ 9.5. Not surprisingly, this is the apparent pKa of an amino acid in the active site of IRS, the titration of which causes a conformation change in the enzyme and reduces its ability to bind L-isoleucine (see Appendix B).

Fifteen percent of the applied activity was recovered in the second peak. It is probable that a considerable amount of IRS was denatured when the pH gradient exceeded 10.

We repeated the experiment, except that a buffer of constant pH 9.9 was substituted for the gradient. The results are shown in Fig. 10.

This may be considered our first successful purification of IRS by affinity chromatography. Forty-one percent of the applied activity was recovered and activity was still being eluted at a significant level at the point where the collection of fractions was stopped. The increase in enzyme purity in the second peak is only 4.5-fold. However, it is expected that the column was saturated with non-specifically bound protein during the chromatography, whereas it had the theoretical capacity to bind 40,000 times as much IRS as was placed on it. Thus, on a



XBL734-4766

Fig. 10. Chromatographic elution pattern of partially purified IRS on Affinity Gel I. A 1/4" diam. column was filled with 2 ml of the gel equilibrated in 0.02 M phosphate (pH 7.5) containing 0.02 M 2-mercaptoethanol. C_γ gel eluate (0.3 ml, containing 1.21 mg. protein and 3.06 units of IRS) was chromatographed at 20° using this buffer. Flow rate was 0.1 ml/min. Ten fractions of 1.1 ml were collected. At this point (arrow) the buffer was changed to 0.02 M phosphate (pH 9.9) containing 0.02 M 2-mercaptoethanol and 0.01 M L-isoleucine. Elution was continued as before. Fractions of 0.9 ml were collected in tubes to which 0.3 ml of 0.1 M phosphate (pH 6.25) containing 0.02 M 2-mercaptoethanol had been added. Protein (filled circles) was measured with the Lowry reagent. Enzyme activity (open circles) was determined from the ATP-^{32}P)PP_i exchange reaction.

preparative scale run, the level of enzyme activity eluted in the second peak could easily be 100-fold as great or more, while the level of protein eluted would only be increased by the extra amount of bound IRS. The result would be a quite dramatic purification.

There remains only the adaptation of the technique to preparative scale. This work is in progress.

It is anticipated that this procedure can be readily extended to the purification of the other aminoacyl tRNA synthetases.

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EXPERIMENTAL

Materials

3-Methyl-1-pentanal, 3-methyl-1-pentanol, 2',3'-isopropylidene adenosine, cyanogen bromide, 6-amino-1-hexanol, 1,8-diaminooctane, 3,3'-iminobispropyl-amine, N,N'-carbonyldiimidazole, N,N'-dicyclohexylcarbodiimide, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate, trifluoroacetic acid, t-butylazidoformate, and 2-mercaptoethanol were purchased from Aldrich Chemical Co.,; L-isoleucine, N-t-BOC-L-isoleucine, N-t-BOC-L-leucine, ATP, ninhydrin, BES, TES, HEPES, Bicine, Tricine, and triethanolamine from Nutritional Biochemicals Co.; N-methyl-D,L-isoleucine, O-methyl threonine, AMP, adenosine, D-ribose, and L-valine from Calbiochem; 3-methyl pentanoic acid, 2-methyl-1-butylamine, and hexamethylene diamine from K & K Laboratories; α-amino-D,L-aminopentanoic acid, α-D,L-aminohexanoic acid, α-D,L-aminoheptanoic acid, thionyl chloride, phosphoryl chloride, methanesulfonyl chloride, nitromethane, dimethyl sulfate, sodium dithionite, p-nitrobenzoyl azide, hexyl bromide, and ethylenediamine from Eastman Organic Chemicals; adenine, and (U) ^{14}C-L-isoleucine, from Schwarz/Mann; carbobenzoxy chloride from Mann Research Laboratories; N^6-methyl adenosine and 6-chloropurine riboside from P-L Laboratories; BioGel A-15 and BioBeads S-X2 from BioRad Laboratories; 1-ethyl 3-dimethylaminopropyl carbodiimide hydrochloride from Cyclo Chemical Co.; CNBr-Activated Sepharose from Pharmacia; Tris from Sigma Chemical Co.; Sodium ^{32}P-pyrophosphate from International Chemical and Nuclear Corp.; Polyethylene Glycol 200 from Baker Chemical Co.; Biosolv BBS-3

from Beckman Instrument Co.; Fluor Concentrate II from Research Supplies Laboratory; Cabosil from Cabot Corp.; Lithium Aluminum Hydride from Alpha/Ventron Inorganics; Polyglycolamine H-163 from Union Carbide; p-toluenesulfonyl chloride and 10% palladium on charcoal from Matheson, Coleman and Bell; 2,4,6-trinitrobenzene-sulfonic acid from Pierce Chemical Co.; and E. coli. B cells from Miles Laboratories. Macroporous polystyrene was a gift from Dow Chemical Co. All other chemicals were reagent grade.

3-Methyl-1-pentanal, 3-methyl-1-pentanol, 3-methyl pentanoic acid, 2-methyl-1-butylamine, ethylene diamine, phosphoryl chloride, methanesulfonyl chloride, thionyl chloride, nitromethane, and di-methylsulfate were distilled before use.

Analytical Procedures

Protein assay (Lowry test).[1] The Lowry reagent was prepared by combining 20 ml of a 10% solution of sodium carbonate in 0.5 N sodium hydroxide and 2 ml of a solution of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate and diluting to 100 ml with distilled water. Fifty microliters of the solution to be tested was added to 5 ml of the above solution, mixed, and allowed to stand for 10 min. Then 0.5 ml of a solution prepared by diluting 10 ml of phosphomolybdotungstate reagent with 7 ml of water was added to the test solution and mixed immediately. Color was allowed to develop for 1 hour and then the absorbance at 750 nm was measured and the amount of protein determined from a standard curve prepared using beef serum albumin.

IRS Assay was carried out using the ATP-(^{32}P)PP_i exchange assay described by Norris and Berg.[2] Five to fifty microliters of the solution to be tested was added to 1.0 ml of a solution containing 0.1 M Tris · HCl (pH 8.0), 10 mM KF, 10 mM 2-mercaptoethanol, 3 mM $MgCl_2$, 2mM L-isoleucine, 2 mM MgATP, and 2 mM sodium (^{32}P) pyrophosphate (10^4-10^5 counts min^{-1} $\mu mole^{-1}$). The solution was incubated 15 min at 37°, and the reaction stopped by addition of 0.25 ml 14% percholoric acid and 0.3 ml of 12% acid-washed Norit. The solution was shaken briefly and chilled in an ice bath for 5 min. The Norit was collected by centrifugation and washed with 3×3 ml of ice water. It was then suspended in 2.0 ml of 2% conc. ammonium hydroxide in 50% aqueous ethanol. A 0.5 ml portion of the suspension was dried in a planchet and counted in a Nuclear Chicago end window counter. The assay was linear in the range 0-0.15 unit, where one unit is defined as the

incorporation of 1 μmole of (^{32}P)PP_i into ATP under assay conditions.

Michaelis constants were determined by carrying out the above assay on a fixed amount of IRS (ca. 0.1 unit). Either L-isoleucine or MgATP, as appropriate, was omitted from the assay solution and varying amounts of the substrate to be tested were added to the solution. The velocity of the reaction was determined as apparent units and was plotted against the velocity divided by the concentration of the substrate. A straight line was fitted to the points by the least squares method and the Michaelis constant was determined from the slope of the line.

The Michaelis constants of α-D, L-aminoheptanoic acid and α-D, L-aminohexanoic acid were determined as described in Appendix B.

Inhibition constants were determined from the apparent Michaelis constant (Km') of either L-isoleucine or MgATP using the relationship

$$Km' = (1 + [I]/K_i)\, Km, \text{ where } [I] = \text{inhibitor concentration.}$$

The apparent Michaelis constants were determined in the presence of a fixed amount of inhibitor as described above. The concentration of the inhibitor was greater than its inhibition constant unless prohibited by limited solubility.

L-isoleucine ethyl and hexyl esters contained trace amounts of L-isoleucine which led to incorrect inhibition constants. The amount of L-isoleucine was estimated by determining the rate of exchange in the presence of the ester without added L-isoleucine. This was done using the relationship

$$[S] = K_m \frac{1 + [I]/K_i}{(V_{max}/V)-1},$$

where [S] = L-isoleucine concentration,

[I] = ester concentration,

V = rate of exchange,

V_{max} = extrapolated rate at infinite L-isoleucine concentration.

The apparent value of K_i was used. Upon determination of [S], this value was used to correct the concentration values of L-isoleucine in the original inhibition study and a new K_i was determined. This value was then introduced in the above equation to obtain a more accurate value of [S]. The process was repeated until no further changes were obtained in the values of K_i and [S].

Dissociation constants were determined from fluorescence titrations as described in Appendix B.

Ninhydrin assays were used to determine the amount of ligands bound to adsorbents. The adsorbent was hydrolysed overnight at 100° in 1 N sodium hydroxide. Nitrogen gas was bubbled through the hot solution for 30 min to remove ammonia. An aliquot of the hydrolysate was acidified (pH 1-5) with conc. HCl and diluted to an estimated concentration of 0.2 to 1.0 mM in primary amine. To 0.5 ml of this solution was added 1 ml each of 2×10^{-4} M KCN in pyridine, and 80% phenol. The mixture was heated 10 min in boiling water and 0.5 ml of 5% ninhydrin in ethanol was added. After mixing, the solution was heated 5 min further and diluted to 10 ml with 60% ethanol. The optical density at 570 nm was measured and the amount of ligand determined from a standard curve. An optical density of 1.0 corresponded to 0.473 mmoles of L-isoleucine.

Radioassays. A solution of the substance to be counted in 0.5 ml of ethanol or water was dissolved in 18 ml of a scintillation solution prepared by mixing 825 ml of toluene, 125 ml of Bio-Solv BBS-3 and 50 ml of Fluor Concentrate II. In the case of agarose adsorbents, 0.5 ml (settled volume) of the gel was added to the scintillation fluid and allowed to become solvated. The solvated beads are almost completely transparent in the medium. They were suspended in the fluid with the aid of colloidal silica (Cabosil). The solutions or suspensions were counted in a Packard Tri-Carb instrument.

Thin layer chromatography was done on 10 cm strips cut from Eastman Chromagram Sheets of silica gel or cellulose impregnated with fluorescent indicator using the solvent systems indicated. Visualization was done routinely with ultraviolet light, iodine, and 0.5% ninhydrin in 49:1 ethanol-acetic acid.

The 2, 4, 6-Trinitrobenzenesulfonate Test was performed as described by Cuatrecasas and Anfinsen.[9]

Synthetic Procedures

L-isoleucine ethyl ester hydrochloride. L-isoleucine (6.5 g, 50 mmole) was suspended in 50 ml of dry ethanol and the mixture chilled to -10°C. Thionyl chloride (7.5 ml, 104 mmole) was added in three portions at 5 min intervals to the stirred suspension. The mixture was allowed to warm to room temperature and then heated to reflux under a drying tube. After 1 1/2 hours the solution was cooled and ethanol was removed under reduced pressure to give a thick oil. This was dissolved in 50 ml of ether and placed in a freezer overnight. The precipitated L-isoleucine ethyl ester hydrochloride was recrystallized

twice by dissolving in 5 ml of ethanol, adding 50 ml of ether and chilling. This yielded 2.3 g (24%) of white crystals, M. P. (uncorr.) 92-3°C. Analysis—Calculated for $C_8H_{18}ClNO_2$: C, 49.09; H, 9.27; Cl, 18.14; N, 7.15. Found C, 49.23; H, 9.25; Cl, 18.20; N, 7.36. Enzymatic assay with IRS indicated that the ester was contaminated with 0.5% L-isoleucine.

L-isoleucine hexyl ester. Thionyl chloride (1.0 ml, 13.8 mmole) was dissolved in 12.5 ml of dry hexanol and the solution chilled to -10°C. L-isoleucine (1.64 g, 12.5 mmole) was added to the stirred solution which was protected by a drying tube and the mixture was slowly heated to 45°C and maintained there until a homogenous solution resulted. It was then stirred overnight at r. t. while nitrogen was bubbled through to remove SO_2 and HCl. The solution was diluted with 15 ml of heptane and extracted with 2×15 ml of water followed by 2×15 ml of 1 N citric acid. The combined extracts were brought to pH 12.5 with 1 N potassium hydroxide and were extracted with 2×25 ml of ether. The combined ethereal extracts were washed with 2×25 ml of 5% sodium bicarbonate, dried over sodium sulfate, filtered, and saturated with hydrogen chloride. The solution was evaporated under a nitrogen stream and the residue dissolved in 25 ml methylene chloride. The solvent was removed under reduced pressure. Solution and evaporation were repeated twice more and the resulting oil dried under a nitrogen stream for 3 days. The resulting amorphous waxy solid could not be recrystallized. It was soluble in both heptane and water, and had no well-defined melting point. It was homogeneous to TLC on silica gel using 1:1 acetone-water (R_f 0.70) or 9:1 chloroform-triethylamine (R_f 0.69). Analysis—Calculated for $C_{12}H_{26}ClNO_2$: C, 57.20; H, 10.40; Cl, 14.10; N, 5.60. Found.

C, 57 0; H, 10.5; Cl, 13.9; N, 5.5. Enzymatic assay with IRS indicated that it was contamined with 0.4% L-isoleucine.

L-isoleucinol. Small portions of lithium aluminum hydride were added to 250 ml of peroxide-free tetrahydrofuran until no bubbling was noted on addition of more $LiAlH_4$. The solution was placed in a 500 ml round-bottomed flask with a sidearm, which was fitted with a condenser topped with a drying tube; the system was then purged with N_2. $LiAlH_4$ (9.5 g, 0.25 mole) was added, and the suspension was chilled in an ice bath. L-isoleucine (13.1 g, 0.1 mole) was added in small portions to the magnetically stirred mixture, allowing bubbling to subside between additions. The mixture was then stirred for 1 hour at 0.5°C and 1 hour at room temperature. It was refluxed for 1 hour and then distilled for 30 min (this removed about 100 ml of THF). The remaining mixture was allowed to cool and 250 ml of ether was added. (Note: either was freshly opened "anhydrous", but considerable evolution of hydrogen was noted). The mixture was chilled and water saturated-ether followed by small portions of water were added to the cold stirred mixture. After excess water had been added, the mixture was stirred for 2 hours at 0-5°C followed by stirring for 1 hour at room temperature. Aluminum hydroxide was filtered off and washed with hot methanol (2×50 ml) and ether (50 ml). The combined filtrate and washings were evaporated under reduced pressure to give an aqueous solution which was extracted with 200 ml of ether, saturated with NaCl, and extracted with 100 ml of ether. The combined extracts were washed with 50 ml of saturated aq. NaCl and dried over sodium sulfate for 1 hour. They were filtered and evaporated under reduced pressure to give a yellow

oil which was distilled in vacuo to yield a white syrup, BP 76-8° (3 mm Hg) which spontaneously crystallized to needles on standing. Recrystallization from ether afforded 6.6 g (56%) of white needles of MP 41.5-3°, $(\alpha)D = 4.64°$ (c 0.127 M).

1-nitromethyl N-t-BOC-L-isoleucinol

N-t-BOC-L-isoleucine hemihydrate (4.1 g, 17.1 mmole) was dissolved in chloroform and dried over magnesium sulfate. The solution was filtered and evaporated to a gum which was dissolved in 40 ml 1:1 ether-tetrahydrofuran. This was reacted for 30 min with 2.78 g (17.1 mmole) N,N'-carbonyl diimidazole to yield the imidazolide. The solution was placed under suction briefly to remove carbon dioxide and chilled in an ice-acetone bath.

Clarified ethereal lithium aluminum hydride (LAH) was prepared by refluxing 5 g of LAH in 250 ml of ether for 3 hours, cooling, filtering through glass wool into a graduated cylinder, stoppering, and allowing to settle for at least one day. Just before use, the solution was standardized by pipetting 5 ml into 20 ml of ice water. Ten ml of 1 N HCl was added and the mixture heated on a steam bath until all the precipitate was dissolved. The solution was cooled and back-titrated with 1 N KOH to pH 3.7. Solutions were between 1.2 and 1.5 N.

A volume of this solution containing 34.2 meq. of LAH was added over 10 min to the stirred imidazolide solution. The solution was stirred 30 min further in the ice-acetone bath. Then 5 ml 95% ethanol was added, followed by 5 ml of water. The mixture was stirred 10 min at room temperature and filtered. The residue was washed with 10 ml portions of methanol, ether, chloroform and methanol again.

The combined filtrate and washings were evaporated under reduced pressure to ca. 15 ml. Fifty ml of chloroform were added, the aqueous phase was separated and discarded, and the organic phase washed with 20 ml portions of 1 N citric acid (2X), water, 5% sodium carbonate, water, and saturated aqueous sodium chloride. The solution was dried over magnesium sulfate, filtered, and evaporated under reduced pressure to yield an oil.

Gas chromatography of this oil on a 5'×1/4" column of SE-30 at 200° with a flow rate of 50 ml/min showed peaks with retention times (min.) of 3:15 (a), 4:15 (b), 4:35 (c), and 5:15 (d). Peak (a) compromised about 50% of the material and was identified as an aldehyde. Peak (c) was identified as N-t-BOC-L-isoleucinol. The other two peaks remain unidentified. The aldehyde could be precipitated as the 2,4-dinitrophenyl hydrazone, MP 165-7°. Analysis Calculated for $C_{17}H_{24}N_5O_6$: C, 51.64; H, 6.37; N, 17.71. Found: C, 51.49; H, 6.40; N, 17.65.

To the oil was added 20 ml of nitromethane and 5 drops of triethylamine. The solution was held at 3° for two weeks.

The product mix was chromatographed on an 8.5×2.2 cm column of silica gel with 50 ml of chloroform, 100 ml of a gradient of 0-10% methanol in chloroform and 50 ml of 10% methanol in chloroform. The 1-nitromethyl N-t-BOC-L-isoleucinol emerged from 105-160 ml.

The material was rechromatographed on a 6.6×2.2 cm column of silica gel using acetone. Various fractions of the eluate were gas chromatographed under the conditions described above. The 1-nitromethyl N-t-BOC-L-isoleucinol (retention time, 12:00 min) was contaminated with ca. 4% of an impurity of retention time 2:50. This impurity had remained at this level throughout the purification and had co-chromatographed

exactly with the product in both systems. This behavior suggests that it may be a decomposition product produced in the injector assembly during gas chromatography.

The appropriate fractions were combined and evaporated under reduced pressure. The residue was dissolved in carbon tetrachloride and the solution allowed to evaporate slowly. This gave 1.73 g (36.6%) of a white waxy solid without a well defined melting point. This also contained 4% impurity by GLC.

NMR (CCl_4): δ, 5.03 (d, J = 10, 1H); 4.38 (s, 1H); 4.24 (d, J = 3, 2H); 4.4-4.0 (m, 1H); 3.55-3.15 (m, 1H); 2.0-1.0 (m, 3H); 1.42 (s, 9H); 1.10-0.67 (m, 6H).

<u>N-t-BOC-(U)^{14}C-L-isoleucinol.</u> (U)^{14}C-l-isoleucinol (1.94 g, 16.5 mmole, 0.5 μC/mmole) was dissolved in 30 ml of pyridine. To this was added 5 ml (35 mmole) of t-butyl azidoformate dissolved in 45 ml of ethyl acetate. The solution was protected with a drying tube and kept 3 days at room temperature. The solution was evaporated under a nitrogen stream in a hood to give an oil which was dissolved in 50 ml of ethyl acetate. This was washed with 30 ml portions of 1N citric acid (2X), water, 5% sodium bicarbonate (2X), water, and saturated sodium chloride. It was dried over magnesium sulfate for 1 hour, filtered, and evaporated under reduced pressure to an oil.

This oil was further dried under a nitrogen stream for 3 days to yield 3.3 g of material. Radioassay indicated the material emitted only 96 ± 1% of the radioactivity expected for pure product. The impurity is presumably unevaporated ethyl acetate. When a thin film of material was dried under vacuum, a purity of 99% was obtained. The corrected

yield was 3.1 g (86%). TLC on silica gel using 9:1 chloroform methanol gave a single spot(R_f 0.70). NMR(CCl_4): δ, 5.03 (d, J = 10, 1H); 3.67-3.17 (m, 4H); 2.0-1.0 (m, 3H); 1.45 (5, 9H); 1.09-0.70 (m, 6H).

N-t-BOC-(U)^{14}C-L-isoleucinol 5'-adenylate

N,O,O-triacetyl AMP (0.48 g, 1 mmole), prepared according to Rammler and Khorana,[3] was dissolved in 10 ml of pyridine and 0.67 g (3 mmole) of 96% pure N-t-BOC-(U) ^{14}C-L-isoleucinol (0.5 μC/mmole) was added, followed by 2.06 g (10 mmole) of N,N'-dicyclohexyl carbodiimide dissolved in 2 ml of pyridine. The solution was stirred for 24 hours at r.t. in the dark. The mixture was filtered and the precipitated N,N'-dicyclohexylurea was washed with 2×2 ml of pyridine. The combined filtrate and washings were evaporated under a nitrogen stream to a thick gum which was washed with 3×20 ml of heptane by trituration and decantation. The gum was dissolved in 20 ml of 1:1 pyridine-water and allowed to stand at r.t. for 24 hr. The mixture was filtered and evaporated under a nitrogen stream to yield a gum which was dissolved in 9 ml of absolute ethanol. Concentrated ammonium hydroxide (6 ml) was added and the solution allowed to stand 2 days at r.t. It was evaporated and the residue was washed with 3×20 ml of ether by trituration and decantation. Then it was dissolved in methanol and precipitated by addition of ether. The precipitate was collected and dried over P_2O_5 in vacuo for 18 hr. This yielded 0.28 g of an off-white powder which showed trace impurities on TLC using 7:3 acetone-water on silica gel (R_f 0.79). Radioassay indicated it was 94±2% pure. Material at this level of purity was used in some experiments. Completely pure material was prepared by column chromatography on silica gel using 7:3

acetone-water. Fractions containing UV-absorbent material were combined and the acetone was removed under reduced pressure. The aqueous solution was filtered and lyophilized. The lyophilizate was suspended in a small amount of hot methanol and the suspension allowed to cool. The methanol was removed by centrifugation. The extraction was repeated once more. The residue was dried over P_2O_5 in vacuo for 18 hr. The resulting white powder was homogeneous to TLC on silica gel using 7:3 acetone-water (R_f 0.84) and on cellulose using 4:15 n-butanol-acetic acid-water (R_f 0.58) with MP 214-5° (d). Radioassay indicated it was 100 ± 1% N-t-BOC-L-isoleucinol 5'-adenylate monohydrate.

Unlabeled N-t-BOC-L-isoleucinol 5'-adenylate was prepared in the same manner.

L-isoleucinol 5'-adenylate. N-t-BOC-L-isoleucinol 5'-adenylate (200 mg, 0.35 mmole) containing trace impurities (see above) was dissolved in anhydrous trifluoroacetic acid. After 10 min the solution was quickly evaporated under a nitrogen stream and the residue dissolved in 2 ml of methanol. This solution was evaporated and the residue again taken up in methanol. Ten volumes of ether were added and the precipitate was collected by suction filtration. It was chromatographed on a column (2.2 cm diam.) of 30 g of silica gel using 3:1 methanol-water. Fractions containing UV-absorbent material were combined and methanol was removed under reduced pressure. The aqueous solution was lyophilized. The lyophilizate was dissolved in methanol and precipitated with 10 volumes of ether. The precipitate was collected, washed with ether, and dried over P_2O_5 in vacuo at 100° overnight. This gave 123 mg (79 %) of a white powder, MP 169-75° (reported MP 175° [4]) which was homogeneous to TLC on silica gel using 1:1 acetone-water (R_f 0.76).

UV (0.01 M potassium phosphate, pH 7.5): max 259 nm (ϵ 12, 100); min 227 nm. Analysis—Calculated for $C_{16}H_{22}N_6O_7P \cdot 0.3H_2O$: C, 42.5; H, 6.2; N, 18.6; P, 6.9. Found: C, 42.6; H, 6.2; N, 18.5; P, 6.9. NMR(D_2O): δ, 8.50(s, 1H); 8.25(s, 1H); 5.98(d, J = 6, 1H); 5.0-4.4 (m, partially covered by HOD peak, 3H); 4.27-4.04(m, 2H); 3.82 (d, J = 5, 2H); 3.34(t, J = 5, 1H); 2.0-1.0(m, 3H); 1.0-0.5(m, 6H).

N^6-(2-aminoethyl)adenosine. A suspension of 194 mg (0.68 mmole) of 6-chloropurine riboside in 0.5 ml (7.45 mmole) of ethylene diamine and 7 ml of dry ethanol was refluxed for 3 hr under a drying tube. The resulting solution was cooled and kept overnight at 3°. The resulting precipitate was recrystallized from 4 ml of dry ethanol. The white crystals were collected, washed with 10 ml of ethanol, and dried over P_2O_5 in vacuo for 18 hr at 65°. The dry crystals weighed 160 mg (76% yield) and had a MP of 198-201°C. They were homogeneous to TLC on silica gel using 7:3 acetone-water (R_f 0.24). UV (0.01 M potassium phosphate, pH 7.5) max 266 nm (ϵ 16, 200); min 229 nm.

NMR (as the deuterochloride in D_2O): δ, 8.29 (s, 1H); 8.25 (s, 1H); 6.02(d, J = 5, 1H); 5.0-4.2 (m, partially covered by HOD peak, 3H); 4.2-3.8 (m, 4H); 3.38 (t, J = 6, 2H).

N^6-(6-aminohexyl)adenosine was prepared in the same manner using hexamethylene diamine. The resulting cream-colored crystals weighed 220 mg (86% yield). They were contaminated with 14% of the di-substituted hexamethylene diamine derivative. This could not be removed, nor significantly reduced, by recrystallization. In the inhibition studies with this compound, it was assumed that a molecule of the di-substituted compound bound identically as a mono-substituted molecule. UV (0.01 M

potassium phosphate, pH 7.5): max 268; min 231. NMR (as the deutero-chloride in D_2O): 8.30 (s, 1H); 8.18 (s, 1H); 6.0 (d, J = 6, 1H); 4.8-4.0 (m, partially covered by HOD peak, 3H); 4.0-3.75 (m, 2H); 3.75-3.4 (t, poorly defined, 2H); 3.2-2.8 (t, poorly defined, 2H); 2.2-1.2 (m, 8H).

N^6-methyl adenosine 5'-phosphate was prepared by the method of Griffin and Reese[5] with minor modifications. Analysis—Calculated for $C_{11}H_{15}N_5O_7PNa \cdot 3H_2O$: C, 29.9; H, 4.9; N, 16.0; P, 7.2 Found: C, 30.2; H, 4.84; N, 16.0; P, 7.1. UV (0.01 M potassium phosphate, pH 7.5): max 267 nm (ϵ 16,200); min 230 nm.

N-t-BOC-L-isoleucinol N^6-methyl 5'-adenylate. N-t-BOC-L-isoleucinol adenylate (200 mg, 366 μmole) was dissolved in 1.5 ml of water, and 0.25 ml of dimethyl sulfate was added in 10 portions at 5 min intervals to the stirred solution. The mixture was stirred 2 hr further. The pH was maintained between 4.5 and 6.5 throughout the reaction by addition of 4 N NaOH. The unreacted dimethyl sulfate was extracted with ethyl acetate (1 ml) and ether (2 ml). The aqueous solution was placed on a column of AG1-X8 (Cl^- Form) (8×0.8 cm) and eluted with water. The UV-absorbent fractions were combined and evaporated to dryness under a nitrogen stream.

The resulting N-t-BOC-L-isoleucinol 5'(1-methyl adenylate) was rearranged in aqueous ammonium hydroxide (pH 11) at 37°C for 2 days. The solution was evaporated to dryness under a nitrogen stream. The residue was dissolved in 1:1 acetone-water, and chromatographed on a 4.4×2.2 cm column of silica gel using the same solvent with a flow rate of 1 ml/min. Fractions of 3 ml were collected. The product was found in fractions 10-14. These were combined and evaporated to dryness.

The residue was dissolved in a minimum of ethanol, filtered, and precipitated with ether. The precipitate was collected and air dried. It was dissolved in water. The solution was filtered and lyophilized. This gave 127 mg (62%) of a white powder which was homogeneous to TLC on silica gel using 1:1 acetone-water (R_f 0.78).

L-isoleucinol N^6-methyl 5'-adenylate. N-t-BOC-L-isoleucinol N^6-methyl 5'-adenylate (127 mg, 226 μmole) was dissolved in 2 ml of anhydrous trifluoroacetic acid. After 5 min, the solution was evaporated under a nitrogen stream. The residue was triturated with 10 ml of ether and collected by centrifugation. It was dissolved in 1 ml of methanol and reprecipitated with 10 ml of ether. The precipitate was collected, air dried and dissolved in 3 ml of water. The solution was lyophilized. The lyophilizate was chromatographed on a column (5×3 cm) of silica gel using 7:3 acetone-water with flow rate of ca. 1 ml/min. Fractions of 5 ml were collected. The product was found in fractions 8-12. These were combined and the acetone removed under reduced pressure. The aqueous solution was filtered and lyophilized. This gave 87 mg (84%) of a cream colored powder which was homogeneous to TLC on silica gel using 7:3 acetone-water (R_f 0.58) and on cellulose using 4:1:5 n-butanol-acetic acid-water (R_f 0.43).

NMR(D_2O): δ, 8.30 (s, 1H); 8.08 (s, 1H); 6.07 (d, J = 5, 1H); 5.0-4.2 (m, partially covered by HOD peak, 3H); 4.23-4.0 (m, 2H); 3.60 (d, J = 5, 2H); 3.23 (d, J = 5, 1H); 3.05 (s, 3H); 1.0-0.5 (m, 6H). UV(0.01 M potassium phosphate, pH 7.5): max 267 nm (ε 16,100); min 231 nm.

N-t-BOC-(U)^{14}C-L-isoleucine was prepared according to Schwyzer et al.[12] with minor modifications using (U)^{14}C-L-isoleucine (0.5 μC/mmole). Thereby was obtained an oil which was 84% pure by radioassay. It was homogeneous and chromatographically identical to authentic N-t-BOC-L-isoleucine on TLC in several systems. The impurity is presumably unevaporated chloroform in solution. The corrected yield was 67%.

Adsorbent I (Fig. 1, Sec. B). Bio Beads S-X2, chloromethylated (2.95% by weight chlorine, 8.31 meg/g) were condensed with N-t-BOC-L-isoleucine and the resulting resin deblocked, all according to Merrifield.[6] Ninhydrin assay of the hydrolysate from a portion of the resin indicated a coupling level of 190 mmoles of L-isoleucine per gram of resin (23% efficiency).

Adsorbent II (Fig. 1, Sec. B) was prepared by dissolving 2.0 g (17.1 mmole) of 6-amino-1-hexanol in 25 ml of 4:1 dimethoxyethane-ethanol and suspending 2.0 g (1.66 meq. Cl) of BioBeads SX-2 chloromethylated, in the solution which was then stirred at reflux for 48 hr. The resin was then washed with ethanol (3×10 ml), methanol (3×10 ml), and ether (2×10ml), and dried over P_2O_5 in vacuo. A Cl analysis showed 9% of the chlorine still unreacted.

The resin was suspended in 20 ml of dimethoxyethane and 5 ml of acetic anhydride was added. The mixture was stirred at room temperature overnight, and washed with ethanol (2×10 ml), water (2×10 ml), methanol (2×10 ml), and ether (2×10 ml), and dried over P_2O_5 in vacuo. IR showed both ester (1720 cm^{-1}) and amide (1650 cm^{-1}) peaks.

The resin was then saponified in 1 N NaOH in water-ethanol-methanol-dioxane (1:1:1:1) for 3 hr at ca. 60°, and washed with water (2×10 ml), methanol (2×10 ml), and ether (2×10 ml), and dried over P_2O_5 in vacuo. IR showed no ester peak; the amide peak remained. The resin was coupled with N-t-BOC-L-isoleucine imidazolide as follows: N-t-BOC-L-isoleucine hemihydrate (0.480 g, 2 mmole) was dissolved in 5 ml of methylene chloride and dried over magnesium sulfate for 60 min. It was filtered and N,N'-carbonyldiimidazole (0.324 g, 2 mmole) was added. Effervescence ensued. The solution was stirred under a drying tube for 1 hr and then added to a suspension of the N-(6-hydroxy-hexyl)-N-acetyl poly (p-vinyl benzyl amine) resin (1.14 g, 0.86 meq) in 10 ml of methylene chloride to which 20 microliters of 1 N sodium ethoxide in ethanol had been added. The mixture was stirred under a drying tube for 2 days at room temperature and for 16 hr at ca. 50°C. The resin was collected by suction and washed with methylene chloride (2×10 ml) and acetic acid (2×10 ml). It was stirred in 1 N hydrogen chloride in acetic acid for 30 min. It was then washed with acetic acid (2×10 ml), methylene chloride (2×10 ml), ethanol (2×10 ml), methanol (2×10 ml), and ether (2×10 ml). It was subsequently dried over P_2O_5 in vacuo overnight. Amino acid analysis indicated that 0.262 mmole of amino acid per gram of resin was incorporated. This corresponds to 35% of the hydroxyls being esterified.

Adsorbent III (Fig. 1, Sec. B) was prepared by Dr. Brian Myhr.[7]

Adsorbent IV (Fig. 1, Sec. B) was prepared by Dr. Brian Myhr.[8]

N-Carbobenzoxy-6-amino-1-hexanol. 6-Amino-1-hexanol (4.68 g, 40 mmole) was dissolved in 50 ml of 1 N aqueous sodium hydroxide,

and the solution chilled in an ice bath. Carbobenzoxy chloride (6.8 g, 40 mmole) and 30 ml of 2 N NaOH were added in 6 equal portions at 10 min intervals to the stirred solution. The solution was stirred 30 min further at 0-5°C. Approximately 20 ml of chloroform was added to the mixture to make the resulting gum more fluid, and the mixture was stirred rapidly with a propeller stirrer at room temperature overnight. The emulsion was transferred to a separatory funnel and acidified to pH 2 with concentrated hydrochloric acid. It was extracted with chloroform (3×50 ml) and ether (50 ml). The combined extracts were dried over magnesium sulfate for 60 min and filtered. The filtrate was cooled to 0° and the resulting precipitate collected, and dried over P_2O_5 in vacuo overnight. The filtrate was then evaporated in a rotary evaporator under vacuum and the resulting residue recrystallized from chloroform-ether. The collected crystals were dried over P_2O_5 in vacuo overnight. Both crops melted at 82 - 83.5°C and weighed 6.91 g (69% yield) combined. NMR($CDCl_3$): δ, 7.33 (s, 4H); 5.10 (s, 2H); 3.60 (t, J = 6, 2H); 3.37-3.0 (m, 2H); 1.67-1.27 (m, 8H).

N-Carbobenzoxy-6-amino-1-hexyl N-t-BOC-L-isoleucinate. N-t-BOC-L-isoleucine hemihydrate (2.4 g, 10 mmole) was dissolved in 10 ml of methylene chloride. The solution was dried over $MgSO_4$ for 1 hr and filtered. To this was added 1.62 g (10 mmole) of N,N'-carbonyl diimidazole. The solution was stirred magnetically under a drying tube for 1 hr. N-carbobenzoxy 6-amino-1-hexanol (2.51 g, 10 mmole) was added with sufficient methylene chloride to give complete solution. Two drops of 1 N sodium ethoxide in ethanol were added and the solution stirred under a drying tube for 2 days. Fifty ml of

chloroform was added and the solution was washed with 20 ml each of water, 1% ammonium sulfate, water, 5% sodium bicarbonate, water, and saturated sodium chloride. It was dried over magnesium sulfate for 1 hr, filtered, and evaporated in vacuo to yield 4.8 g (103%) of a slightly cloudy oil showing trace impurities on TLC on silica gel using chloroform (R_f 0.58). NMR($CDCl_3$): δ, 7.30 (s, 5H); 5.35-4.90 (broad peak, including a singlet at 5.08, 4H); 4.37-4.00 (m, including apparent doublet at 4.20, J = 6, 3H); 3.38-2.92 (m, 2H); 2.3-1.1 (m, including singlet at 1.45, 20H); 1.10-0.67 (m, 6H).

N-Carbobenzoxy-6-amino-1-hexanol N-t-BOC-L-leucinate was prepared in an identical manner to the L-isoleucine analog. This gave a thick oil, homogeneous to TLC (R_f 0.57), in an apparent yield of 109%. NMR($CDCl_3$): δ, 7.28 (s, 5H); 5.35-4.90 (broad peak, including a singlet at 5.07, 4H); 4.37-3.90 (m, including apparent triplet at 4.08, J = 6, 3H); 3.38-2.92 (m, 2H); 2.2-1.1 (m, including singlet at 1.46, 20H); 1.10-0.67 (m, 6H).

N-Carbobenzoxy-aminopropoxyethoxyethanol was prepared from Polyglycolamine H-163 in the same manner as N-carbobenzoxy-6-amino-1-hexanol except that the addition of chloroform was unnecessary. This gave a 94% yield of a pale yellow oil homogeneous to TLC on silica gel using 9:1 chloroform-methanol (R_f 0.62). NMR($CDCl_3$): δ, 7.30 (s, 5H); 5.77-5.30 (broad s, 1H); 5.05 (s, 2H); 3.80-2.93 (m, including singlet at 3.57, 13H); 1.73 (distorted quintet, J = 6, 2H).

N-Carbobenzoxy-aminopropoxyethoxyethyl N-t-BOC-L-isoleucinate was prepared in the same manner as the N-carbobenzoxy-6-amino-1-hexyl ester. It was further purified by column chromatography on

silica gel using chloroform. This gave a 50% yield of an oil showing trace impurities on TLC on silica gel using chloroform (R_f 0.30). NMR($CDCl_3$): δ, 7.31 (s, 5H); 5.57-5.23 (broad s, 1H); 5.07 (s, 2H); 4.38-4.00 (m, including doublet at 4.20, J = 6, 3H); 3.76-3.05 (m, including singlet at 3.58, 10H); 2.05-1.05 (m, including distorted quintet, at 1.75, J = 6, and singlet at 1.45, 12H); 1.05-0.67 (m, 6H).

Removal of N-carbobenzoxy group. The N-carbobenzoxy compound was dissolved in methanol (5 ml/mmole) in a flask equipped with a bubbler and oil seal. To this was added a suspension of 10% palladium on charcoal (0.3 g/mmole) in cold methanol (1 ml/g) and one equivalent of acetic acid. The system was purged with nitrogen and hydrogen was then bubbled through the mixture at a sufficient rate to provide good stirring for 2 hrs. The mixture was filtered and the charcoal was washed with methanol (2×5 ml/mmole). The ammonium group was converted to the free amine with NaOMe and the solution was used immediately in a coupling reaction with cyanogen bromide-activated agarose.

Coupling to agarose. To a suspension of BioGel A-15 in an equal volume of water was added finely ground cyanogen bromide (150 mg/ml of gel). The pH of the well-stirred suspension was adjusted and maintained at 10.5-11.5 by addition of 4 N NaOH. The temperature was maintained at ca. 5° by addition of ice. After 12 min, a large quantity of ice was added. The suspension was quickly filtered with suction and washed with 4 volumes of cold 0.1 N $NaHCO_3$, followed by 2 volumes of cold 0.1 N $NaHCO_3$ in 1:1 water-dioxane. The gel was suspended in one volume of the latter and the methanolic solution of deblocked ω-amino ester (0.1 mmole/ml of gel) was added. The suspension was stirred

1-2 days at 3°. It was washed with two volumes each of 0.1 N $NaHCO_3$ in 1:1 water-dioxane, 0.1 N $NaHCO_3$, water, 1 N acetic acid, water, and 0.02 M potassium phosphate (pH 7.5). The gel was stored in the latter at 3°.

Removal of N-t-BOC group. The gel was washed well with 1 N HCl and then stirred in two volumes of the same for 6 hrs at r.t. It was then washed with twenty volumes each of water and 0.02 M potassium phosphate (pH 7.5) and stored in the latter at 3°. Ninhydrin assays of hydrolyzed portions of the gels indicated the following levels of coupling: hexyl L-isoleucinate agarose, 4.7 μmoles/ml; hexyl L-leucinate agarose, 4.8 μmoles/ml; and propoxyethoxyethyl L-isoleucinate agarose, 8.6 μmoles/ml.

Chloromethylated macroporous polystyrene. Macroporous polystyrene, 20% cross-linked, 18-20 mesh (20 g) was suspended in 60 ml of chloroform and the suspension chilled in an ice bath. To the stirred suspension was added a cold solution of 2.5 ml of anhydrous $SnCl_4$ in 10 ml of methyl chloromethyl ether. The suspension became pink. After 30 min, the beads were collected by filtration and washed with 50 ml portions of 3:1 dioxane-water (4X), 3 N HCl in 3:1 dioxane-water (3X), water(2X), dioxane (2X), methanol (2X) and ether (2X). They were dried over P_2O_5 *in vacuo* for 18 hr. Combustion analysis indicated the beads were 3.26% chlorine, (1.84 meq./g) corresponding to chloromethylation of 10% of the benzene rings.

Propoxyethoxyethyl L-isoleucinate macroporous polystyrene. To 5 g of chloromethylated macroporous polystyrene was added a methanolic solution of aminopropoxyethoxyethyl N-t-BOC-L-isoleucinate prepared

by deblocking 5.1 g (10 mmole) of the N'-carbobenzoxy compound. The suspension was stirred for 7 days. The beads were washed with 3×20 ml of methanol and 2×20 ml of 1 N HCl in acetic acid and then stirred in the latter for 20 min at r. t. The beads were then washed with 3×20 ml portions of acetic acid, water, 0.02 M potassium phosphate (pH 7.5), water, methanol, and ether. The beads were dried over P_2O_5 in vacuo for 18 hr. The resin was found to have bound 0.54 mmole of ligand per gram of resin.

Aminohexyl, aminooctyl, and aminopropylaminopropyl agarose were prepared by the procedure of Cuatrecasas and Anfinsen[9] for aminoethyl agarose using BioGel A-15 and hexamethylene diamine, octamethylene diamine, or 3,3'-iminobispropyl amine, respectively. The resulting gels carried 12, 10, and 14 μmoles of tail per ml of gel, repsectively.

Succinylaminohexyl, succinylaminooctyl, and succinylaminoalkyl (succinylaminopropylaminopropy) agarose were prepared by the procedure of Cuatrecasas and Anfinsen[9] for succinylaminoethyl agarose. The gels so prepared gave pale yellow colors in the trinitrobenzenesulfonate test, which indicated that the terminal amino groups had all been succinylated.

Adsorbent V (Fig. 4, Sec. B). N-carbobenzoxy-6-amino-1-hexyl N-t-BOC-L-isoleucinate (2.40 g, 5.15 mmole) was decarbobenzoxylated as described previously, except that the methanolic solution of the acetate was not neutralized, but was evaporated under reduced pressure to give an oil which was dissolved in 40 ml of dimethylformamide. This was added to 40 ml of succinylaminohexyl agarose, followed by 6.35 g (15 mmole) of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide

metho-p-toluenesulfonate. The mixture was stirred 3 days at r.t. The gel was then washed with 2×40 ml of 1:1 water-dioxane, followed by 60 ml portions of water (2×), 1 N citric acid (2×), water, 5% $NaHCO_3$, and water (3×). It was deblocked by stirring in 1 N HCl for 6 hr at r.t. and washed with 500 ml each of water and 0.01 M potassium phosphate (pH 7.5). It was stored in the latter at 3°. The gel was found to carry 8 μmoles/ml of L-isoleucine.

Adsorbent VI (Fig. 4, Sec. B) was prepared similarly to Adsorbent V using N-carbobenzoxy-aminopropoxyethoxyethyl N-t-BOC-L-isoleucinate and succinylaminooctyl agarose. The resulting gel carried 9 μmoles/ml of L-isoleucine.

PEG polystyrene. Sodium (0.8 g, 35 mmole) was dissolved in 50 ml of warm polyethylene glycol 200, a mixture of primarily tetra- and pentaethylene glycol. This solution was added slowly to a suspension of 14 g of chloromethylated macroporous polystyrene (0.92 meq. Cl/g) in 100 ml of 1:1 dioxane-polyethylene glycol 200. The reaction was stirred under a drying tube at 50° for 5 days. The beads were washed with 50 ml portions of dioxane (2×), water (2×), methanol (2×), methylene chloride, 1:1 methylene chloride-ether, and ether (3×). They were dried over P_2O_5 *in vacuo* for 18 hrs. Combustion analysis showed only 0.31 meq. Cl/g left on the resin. IR: 3300-3500 cm^{-1} (shoulder, ν O-H); 1110 cm^{-1} (νC-O). CMR($CDCl_3$): δ 64.75.

Adsorbent VII (Fig. 4, Sec. B). N-t-BOC-L-isoleucine hemihydrate (1.0 g, 4.17 mmole) was dissolved in 10 ml of methylene chloride and dried over magnesium sulfate for 1 hr. The solution was filtered and 0.675 g (4.17 mmole) of N,N'-carbonyldiimidazole was added. After

stirring for 1 hr under a drying tube, 7.75 g of PEG polystyrene was added and stirring was continued for one week. Then the beads were washed with 30 ml portions of chloroform (2X), ethanol (2X), water (3X), methanol (2X) and ether (2X). The beads were stirred in 1 N HCl in acetic acid for 1 hr. They were then washed with 50 ml portions of water (5X), 0.01 M potassium phosphate (pH 7.5), water (2X), methanol, ethanol, and ether (2X). They were dried over P_2O_5 in vacuo for 18 hr. L-isoleucine was found to be bound to the resin at a level of 112 μmoles/g.

p-Aminobenzamidoalkyl agarose (III, Fig. 5, Sec. B) was prepared by the procedure of Cuatrecasas and Anfinsen[9] for p-aminobenzamidoethyl agarose using aminopropylaminopropyl agarose in place of aminoethyl agarose. The intermediate conversion to the p-nitro derivative was 100% complete as indicated by the development of only a pale yellow color with trinitrobenzenesulfonate. The extent of conversion to the amino derivative was not quantitated, but was extensive, as indicated by the development of a deep red-orange color with trinitrobenzenesulfonate.

Diazonium Couplings. (A) According to Cuatrecases[9]: p-Aminobenzamidoalkyl agarose (5ml) was washed with and suspended in 1 N hydrochloric acid to give a total volume of 10 ml. The solution was chilled in an icebath, and 10 ml of cold 0.2 M sodium nitrite was added. The solution was stirred magnetically 7 min under suction. It was quickly suction filtered, at which time the beads turned yellow and became tightly adherent to one another. It was washed with 20 ml of cold, saturated sodium borate, and added to a solution of 5 mg (0.1 mole) of N-t-BOC-(U)-^{14}C-L-isoleucinol 5'-adenylate (0.2 μC/mmole) in 3 ml of the same buffer. The suspension was stirred 16 hr at 4°C.

The gel was then washed with 500 ml each of 0.05 M phosphate (pH 7.5) and water. Radio assay indicated 0.15 μmoles of inhibitor bound per ml of gel. After deblocking in 1 N HCl in 1:1 ethylene-glycol-water for 6 hrs, followed by thorough washing, only 0.03 μmoles/ml remained.

(B) According to Weibel et al.[10]: p-Aminobenzamidoalkyl agarose (5ml) was thoroughly washed with and suspended in 4 NHCl to give 10 ml total volume. The suspension was stirred in an ice bath and 10 ml of cold 5% sodium nitrite was added. Stirring was continued for 20 min under suction. The gel was gravity filtered on a coarse fritted-glass funnel and 40 ml of cold 1% sulfamic acid was percolated through to destroy excess nitrous acid. The gel was then suction filtered, again turning yellow and clumping. It was washed with 0.1 M Tris (pH 8.5) and suspended in a solution of 5 mg of N-t-BOC-(U)-^{14}C-L-isoleucinol 5'-adenylate (0.2 μC/mmole) in 5 ml of the same buffer and shaken for 1 hr at ambient temperature. It was washed as in (A) above. Radio assay indicated only 0.04 μmoles of inhibitor bound per ml of gel.

N^6-Agarosyl L-isoleucinol 5'-adenylate. BioGel A-15 (40 ml) was activated with 8 g of cyanogen bromide according to Cuatrecasas and Anfinsen[9] and added to a solution of 0.36 g (660 μmole) of N-t-BOC-L-isoleucinol adenylate in 40 ml of 0.1 N $NaHCO_3$ (pH 8.5). The suspension was stirred at 3° for 3 days. The gel was then washed with 0.1 M potassium phosphate (pH 7.5) until effluent had no absobance at 259 nm. The gel was stirred in 1 N HCl for 6 hr at r. t. It was washed with 500 ml each of water and 0.01 M potassium phosphate (pH 7.5) and stored in the latter at 3°. L-isoleucinol adenylate was found bound to the gel at a level of 2.4 μmoles/ml.

Methyl 5'-adenylate was prepared by the method of Khorana[11] in 88% yield. The material was homogeneous to TLC on silica gel using 7:3 acetone-water (R_f 0.58).

N^6-agarosyl methyl 5'-adenylate was prepared in the same manner as the L-isoleucinol equivalent. Methyl adenylate was bound to the gel at a level of 1.5 μmoles/ml.

Attempted coupling of N-t-BOC-L-isoleucinol 5'-adenylate with succinylaminoalkyl agarose. Succinylaminoalkyl agarose (3 ml) was suspended in 2 ml of water in which 53 mg (93 μmoles) of N-t-BOC-L-isoleucinol 5'-adenylate was dissolved. To this was added 126 mg (300 μmoles) of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate and the mixture was shaken for 2 days at r.t. The gel was washed with water until the eluent was free from absorbance at 259 nm. The gel was found to have bound less than 0.1 μmoles of adenylate per ml of gel.

Another coupling attempt was made following the procedure used for the preparation of Affinity Gel I (see below) with a total reaction time of 8 days. N-t-BOC-(U)-^{14}C-L-isoleucinol 5'-adenylate (2 μC/mmole) was employed. Radioassay indicated a level of binding of 0.08 μmole/ml.

N-carbobenzoxy-6-aminohexyl-1-tosylate. N-Carbobenzoxy-6-amino-1-hexanol (1.0 g, 4 mmole) was dissolved in 15 ml of pyridine and the solution chilled in an ice bath. To this was added 1.52 g (8 mmole) of toluenesulfonyl chloride. The solution was protected with a drying tube and kept at 3° for 18 hr. It was decanted from the precipitated pyridine hydrochloride into 150 ml of ice water which resulted in the precipitation of an oil. After 2 hr, the mixture was extracted with 3×50 ml of ether.

The combined extracts were washed with 2×60 ml of cold 1 N HCl, 100 ml of cold water, and 50 ml of saturated aqueous sodium chloride, and were dried over potassium carbonate for 2 hr at 3°. The mixture was filtered and the filtrate evaporated under reduced pressure to yield a pale yellow oil. This was dried over P_2O_5 in vacuo for 18 hr, during which time it crystallized. The crystals were cream-colored and weighed 1.22 g (76% yield). They showed a trace impurity to TLC on silica gel using chloroform (main spot, R_f 0.47). NMR ($CDCl_3$): δ, 7.77 (d, J = 8, 2H); 7.5-7.16 (m, 7H); 5.08 (s, 2H); 4.0 (t, J = 7, 2H); 3.33-2.93 (m, 2H); 2.42 (s, 3H); 2.0-1.1 (m, 8H).

N-carbobenzoxy-6-aminohexyl-1-mesylate. The preparation was begun in the same manner as that of the tosylate, but using methanesulfonyl chloride (BP 34°, 10 torr). Upon standing in the ice water, the oil partially crystallized. The water was decanted and the residue was washed with 5×50 ml each of ice water and then petroleum ether by trituration and decantation. It was dried over P_2O_5 in vacuo for 18 hr. This gave 0.88 g (67% yield) of yellowish crystals with MP 34-36.5. They were homogeneous to TLC on silica gel using chloroform (R_f 0.47). NMR ($CDCl_3$): δ, 7.33 (s, 5H); 5.22-4.80 (broad peak overlapping singlet at 5.05, 3H); 4.17 (t, J = 6, 2H); 3.33-3.0 (m, 2H); 2.95 (s, 3H); 2.0-1.1 (m, 8H).

Attempted N^6-alkylations. The following reactions were followed by TLC on silica gel using 7:3 acetone water. When UV-adsorbent spots other than starting material were seen, these were cut out and extracted into 2 ml of 0.01 M potassium phosphate (pH 7.5) and the UV spectrum of the extract taken to determine if the material contained an N^6-alkylated purine moiety. The latter have a maximum adsorbance at 266 nm.

In water: The reaction was carried out as in the preparation of N-t-BOC-L-isoleucinol N^6-methyl 5'-adenylate, but using methyl adenylate and N-carbobenzoxy-6-aminohexyl tosylate or mesylate. The reaction was allowed to run for 1 day. No reaction could be detected. The alkylating agents appeared to be totally insoluable in water.

In dimethylformamide: A solution of 0.14 M methyl adenylate in dimethylformamide was prepared. To 1 ml portions of this solution were added one equivalent of either the tosylate, the mesylate, or hexyl bromide; or, one equivalent of the alkylating agent plus a trace of sodium iodide; or, one equilvanet of the agent plus one equivalent of silver acetate or silver nitrate. The reactions were kept in the dark for one week, being checked occasionally with TLC. No new UV absorbent spots were seen. The reactions were held at 50° for 24 hr. No evidence of alkylation was found. Similar result was obtained after 18 hr at 70°. When the reactions were held at 100° for 90 min, considerable decomposition occured to give multiple UV-absorbent spots. The same pattern of spots was given by the control, which was simply the solution of methyl adenylate without added alkylating agents. This indicated that all the new spots were decomposition products. This was verified by the determination of their spectra.

In 4:1 dimethylformamide-pyridine or in pyridine: To 0.1 ml portions of solutions of 0.113 M methyl adenylate in 4:1 dimethylformamide-pyridine or 0.1 M N-t-BOC-L-isoleucinol adenylate in pyridine were added 20 equivalents of either the tosylate, the mesylate, or hexyl bromide. The reactions were allowed to proceed one week in the dark and were followed by TLC. During this time, a second UV-absorbent

spot appeared and grew in each reaction. In each case, the new spot had a UV spectrum suggesting an alkylated pyridine (peak at 258 nm with shoulders at 253 nm and 264 nm). The level of adenylate appeared to be unchanged throughout each reaction.

6-Chloropurine Riboside 5'-phosphate. A solution of 0.56 ml (6 mmole) of phosphoryl chloride in 5 ml of trimethyl phosphate was chilled to -10°C and 36 μl. (2 mmole) of water was added. After stirring under a drying tube for 20 min, 0.57 g (2 mmole) of 6-chloropurine riboside was added. After stirring 30 min further at -10°C, the temperature was raised to 3°C and the reaction stirred for 3 hr more.

Then 10 ml of ice water was added and hydrolysis of the phosphorchloridate was allowed to proceed for 30 min. During this time pH was maintained at 2-4 by addition of 4 N sodium hydroxide. The pH was brought to 6 and the solution extracted with 3×25 ml of chloroform to remove trimethyl phosphate. Then 8.1 ml of 1 M barium acetate was added. The precipitated barium phosphate was filtered off and washed with 10 ml of water. The combined filtrate and washing were lyophilized. The lyophilizate was suspended in 100 ml of 90% of ethanol and filtered. The residue was dissolved in 50 ml of water and the insoluble material removed by filtration. The filtrate was diluted with 100 ml of absolute ethanol and the precipitated barium 6-chloropurine riboside 5'-phosphate was collected by centrifugation. It was washed with 100 ml of ethanol followed by 50 ml of ether. After drying, the material was redissolved in water, filtered, precipitated, and washed as above. This cycle was repeated once more so as to remove all barium phosphate. The white solid was then dried over P_2O_5 in vacuo yielding 0.768 g (71%) of white

powder, MP: blackens at 233-5°C. Analysis—Calculated for $C_{10}H_9N_4O_7PClBa \cdot 2H_2O$: C, 22.4; H, 2.44; N, 10.4; P, 5.8; Cl, 6.6. Found: C, 22.5; H, 2.6; N, 10.2; P, 5.9; Cl, 6.6. TLC on cellulose using 4:1:5 n-butanol-acetic acid- water gave a main spot at $R_f = 0.35$ and a minor spot at $R_f = 0.06$ corresponding to inosinic acid. Elution of the spots and quantitation by UV spectrometry indicated inosinic acid comprised ca. 1% of the product. TLC on polyethylene imine cellulose using 0.1 M NaCl indicated the presence of a trace of diphosphate.

N-t-BOC-(U)-^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate

Barium 6-chloropurine riboside 5'-phosphate (1.008g, 2 mmole) was dissolved in 100 ml of water and 4 ml of 0.5 M pyridinium bisulfate was added. Barium sulfate was removed by centrifugation and the solution lyophilized. The resulting white solid was dissolved in 20 ml of pyridine and 12 ml of acetic anhydride and kept in the dark 18 hr at ambient temperature. The solution was evaporated under a nitrogen stream to a thick oil which was dissolved in 100 ml of water and washed with 3×10 ml of 1:1 carbon tetrachloride-methylene chloride followed by 20 ml of ether. The solution was then lyophilized. The lyophilizate was redissolved in 300 ml of water and relyophilized. The resulting tan material was dissolved in 13 ml of dry pyridine and 1.12 g (5.2 mmole) of N-t-BOC-(U)^{14}C-L-isoleucinol (0.4 μC/mmole) was added. The brown solution turned purple. N,N'-Dicyclohexylcarbodiimide (4 g, 19.5 mmole) was added, and the mixture was sealed and stirred for 24 hr. The precipitated N,N'-dicyclohexylurea was removed by filtration and washed with 3×3 ml of dry pyridine. The combined filtrate and washes were evaporated under a nitrogen stream to a gum which was washed

with 3×40 ml of heptane by trituration and decantation. The gummy solid was then dissolved in 10 ml of absolute ethanol and 10 ml of ethylene diamine (BP 89.5°C) was added. The purple solution turned red-brown. The solution was sealed and kept for 48 hr at 3°C followed by 24 hr at ambient temperature. During all manipulations through this point, the materials were protected from light insofar as was conveniently possible. The solution was evaporated under a nitrogen stream and the resulting gum was washed with 3×40 ml of ether by trituration and decantation. The resulting material was extracted with 2×50 ml of water and the extracts were lyophilized. The water insoluble material was dissolved in 3 ml absolute ethanol and 2 ml of ethylene diamine was added. After 2 days in the dark, this solution was worked up as above to yield a "second crop." The two crops were combined, dissolved in a minimum of water, and decanted from a small amount of insoluble material. The solution was lyophilized. The lyophilizate was dissolved in 8 ml of 7:3 acetone-water and chromatographed on a column (3 cm diameter) of 110 g of silica gel using the same solvent with a flow rate of 2 ml/min. 20 ml fractions were collected. The composition of the fractions was determined by TLC (7:3 acetone-water on silica gel) and fractions 21-35 and 36-45 were pooled. The pools were evaporated *in vacuo* to remove acetone, and the aqueous solutions filtered and lyophilized. The lyophilizates were washed with acetone, dissolved in minimum volumes of water, and small amounts of insoluble material were removed by centrifugation. The solutions were again lyophilized to give 170 mg (14%) and 18 mg (2%) respectively of material which was homogeneous to TLC using 7:3 acetone-water on silica gel (R_f 0.63)

and 3:2 ethanol-1 M ammonium acetate (pH 7.5) on cellulose (R_f 0.94); but showed slight traces of impurity using methanol on silica gel (R_f 0.25) or 4:1:5 n-butanol-acetic acid-water (R_f 0.71). Spots were visualized by UV, iodine, and ninhydrin.

Radioassay indicated ^{14}C-L-isoleucinol present at a level equivalent to 82 ± 2% N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate for 21-35 and 68 ± 4% for 36-45. The corrected total yield was 12%.

<u>N-t-BOC-(U)^{14}C-L-isoleucinol N^6(6-aminohexyl) 5'-adenylate</u> was obtained by an analogous procedure employing hexamethylene diamine in place of ethylene diamine. Radioassay indicated the material was 70% pure. Corrected yield was 11%.

<u>(U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate.</u> N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate (50 mg of 83% pure material, 71 mmoles) was dissolved in 1 ml of anhydrous trifluoroacetic acid. After 15 min, the solution was quickly evaporated under a nitrogen stream. The residue was dissolved in 1 ml of methanol and precipitated with 5 ml of ether. The precipitate was collected, washed with ether, and dissolved in 5 ml methanol. The solution was evaporated under a nitrogen stream. The residue was chromatographed on a column (22×2.2 cm) of silica gel using 1:1 acetone-water, flow rate 1 ml/min. Fraction size was 15 ml. The product was found in tubes 14-28. These were combined and acetone was removed under reduced pressure. The cloudy aqueous solution was centrifuged for 30 min at 9000 × g. and then lyophilized. The lyophilizate was washed with acetone and dissolved in 10 ml of water, centrifuged as before, and relyophilized. This gave 15 mg of a pale yellow powder. Radioassay

indicated that the material contained a total of 14.1 mmoles (20% yield) of labeled material. This corresponds to a purity of only 46%. However, the only impurity was a trace of the acetone condensation product produced routinely in chromatography with acetone-water, as seen on TLC on silica gel using 1:1 acetone-water (R_f 0.22) or on cellulose using 4:1:5 n-butanol-acetic acid-water (R_f 0.09), and the NMR spectrum showed no extraneous peaks. Inhibition studies were done using this material with the concentration determined from the radioassay.

NMR(D_2O): δ, 8.40 (s, 1H); 8.28 (s, 1H); 6.10 (d, J = 5, 1H); 5.1-4.3 (m, partially covered by HOD peak, 3H); 4.3-4.0 (m, 2H); 4.0-3.62 (m, 4H). 3.6-3.0 (m, 3H); 2.0-1.0 (m, 3H); 1.10-0.6 (m, 6H).

<u>N^6-(ω-Aminoalkyl) AMP's</u> were prepared by the procedure for the N^6-(ω-aminoalkyl) adenosines using the monopyridinium salt of 6-chloropurine riboside 5'-phosphate in place of 6-chloropurine riboside. The precipitates were dissolved in water and one equivalent of barium chloride was added. The barium salt was precipitated by the addition of two volumes of ethanol. The precipitates were subjected to TLC on cellulose using 70:2:19 saturated ammonium sulfate-isopropanol-water. The reaction with ethylene diamine gave two spots at R_f 0.30 and R_f 0.60. The less mobile spot was identified by its R_f and UV spectrum as AMP. The other spot had the spectrum of an N^6-alkylated adenine derivative and is presumably N^6-aminoethyl AMP. The ratio of N^6-aminoethyl AMP to AMP was 2:1. The reaction with hexamethylene diamine gave a single spot at R_f 0.36 with the spectrum of an N^6-alkylated adenine derivative. It should be noted that a small amount of AMP (< 5%) might be hidden in the spot since the spot overlapped the R_f 0.30 position slightly.

This level of impurity would not be detected in the UV spectrum. No further attempt to characterize these compounds was made.

Coupling of N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(ω-aminoalkyl) 5'-adenylates to cyanogen bromide-activated agarose. "CNBr-Activated Sepharose" (0.5 g) was washed over 15 min with 100 ml of 0.001 N HCl, followed by a quick wash with 0.1 N $NaHCO_3$ containing 0.5 M NaCl. The gel was split into two portions which were added to solutions of either 50 mg of 30% pure N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) adenylate or 30 mg of 70% pure aminohexyl derivative dissolved in 2.5 ml of the cold buffer. The pH of the suspensions was adjusted to 8.5 with 1 N KOH and they were shaken overnight at 3°. They were then washed with 100 ml each water and 0.01 M potassium phosphate (pH 7.5). Radioassay indicated binding levels of 0.6 μmoles/ml for the aminoethyl derivative and 0.5 μmoles/ml for the aminohexyl derivative.

Similar reactions were carried out at pH 10. The levels of substitution were 1.6 μmoles/ml for the aminoethyl derivative and 1.3 μmoles/ml for the aminohexyl derivative.

Affinity Gel I. Succinylaminoalkyl agarose (20 ml) was washed with 300 ml of water using suction, followed by 4 × 30 ml of 80% aqueous pyridine. It was suspended in the same and allowed to settle. The excess solvent was decanted. To this was added 74 mg of 87% pure N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate (0.3 μC/mmole) dissolved in 2 ml of water, followed by 4 g of N,N'-dicyclohexylcarbodiimide dissolved in 8 ml of pyridine. The reaction was shaken for 8 days at ambient temperature. The gel was collected by suction filtration and washed with 10 ml of 80% aqueous

pyridine. The combined filtrate and washing were retained. The gel was further washed by percolation with 80% aqueous pyridine (3×15 ml), water (3×20 ml), absolute ethanol (2×40 ml), n-butanol at 37°C (2×40 ml), absolute ethanol (2×40 ml), water (2×40 ml), and 80% aqueous pyridine (3×40 ml). The gel was returned to the combined filtrate and first washing, 4 g of DCC were added, and the reaction was shaken 8 days further. The washing procedure was repeated, except that the final washes with 80% aqueous pyridine were replaced by washing with 1 liter of 0.01 M phosphate (pH 7.5). Radio assay indicated 3.4 μmoles of inhibitor coupled per ml of gel. 16 ml of gel were obtained. A test with trinitrobenzene sulfonate was negative.

The gel was then deblocked by shaking for 6 hours in 1 N HCl in 1:1 ethylene glycol-water. It was washed with 1 liter of water followed by 1 liter of 0.01 M phosphate (pH 7.5). Radioassay indicated 2.6 μmoles/ml of inhibitor were retained on the gel. The trinitrobenzenesulfonate test gave a medium orange color, indicating that the bound inhibitor had been deprotected.

<u>Test couplings of N-t-BOC-(U)^{14}C-L-isoleucinol 5'-adenylate to succinylaminoalkyl agarose using mixed solvents.</u> 10 mg of 82% pure N-t-BOC-(U)^{14}C-L-isoleucinol N^{6}-(2-aminoethyl) 5'-adenylate (0.4 μC/mmole) were dissolved in 2 ml of the appropriate solvent. 2 ml of succinylaminoalkyl agarose, thoroughly washed with the solvent, were added, followed by 100 mg of N-ethyl N'-(dimethylaminopropyl) carbodiimide hydrochloride. The reactions were shaken at room temperature. Two more 100 mg portions of the carbodiimide were added at 1-day intervals followed by two further days of shaking. The gels were

collected and washed with 20 ml of 5% acetic acid followed by 40 ml of water. They were deblocked by shaking for 6 hours in 1 N HCl in 1:1 water-ethylene glycol and washed with 20 ml portions of water, 0.1 M phosphate (pH 7.5) and water.

The following solutions were tested (bound radioactive substrate in μmoles/ml, before and after deblocking, are given in parenthesis): 1:1 dimethylformamide-water (2.3, 0.8); 1.1 ethylene glycol-water (0.9, 0.5); 1:1:1 dimethylformamide-dioxane-water (4.1, 1.6); and 3:1 dioxane-water (5.4, 2.6).

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PART II: THE ATTEMPTED PURIFICATION OF RNA-INSTRUCTED DNA POLYMERASE BY AFFINITY CHROMATOGRAPHY

Introduction

RNA-instructed DNA polymerase (RDP) is an enzymatic function with the capability of catalyzing DNA synthesis on an RNA template. RDP activity was first demonstrated independently by Baltimore[1] and by Temin and Mizutani[2] in 1970. Since then, RDP activity has been detected in every infectious oncogenic virus which has been tested, whereas non-oncogenic viruses seem to lack this activity.

This finding of an apparent correlation between the presence of RDP activity and oncogenicity has led to intensive investigation of RDP in many laboratories, including ours. These studies have all been done on crude preparations, and much of the data has therefore been rather ambiguous. The availability of "pure" RDP would allow studies of much greater scope and rigor.

However, RDP has proven quite resistant to significant purification, primarily because of the ease with which it is inactivated. Attempts to purify the material have quickly resulted in complete loss of activity. We felt that affinity chromatography might be especially suited to the purification of RDP, since it is a one-step procedure which can be carried out under very mild conditions. Moreover, Allan Tischler had already prepared a number of quite potent rifamycin-based inhibitors of RDP.[3]

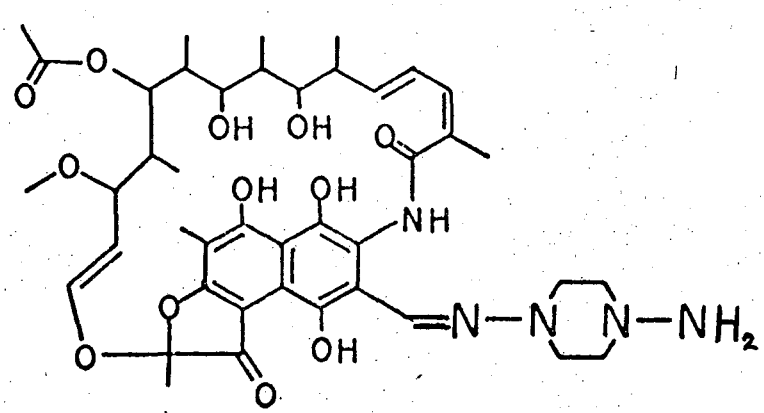
Other nucleotide polymerases are known to bind to rifamycin derivatives, and it was quite likely that they would interact with the adsorbent. We hoped that the binding properties would be sufficiently different that we could effect selective or sequential desorption of those materials which became adsorbed.

At present, no definite link has been established between the ability of the rifamycin derivatives to inhibit RDP and their ability to inhibit focus formation other than a correlation between the effectiveness of the derivatives in producing the two effects. If inhibition of focus formation is mediated by effects besides that of RDP inhibition, it may be possible to isolate other enzymes (e.g., other nucleotide polymerases) which may be involved.

We therefore undertook the preparation and testing of adsorbents carrying insolubilized rifamycin derivatives. This work was carried out in collaboration with Allan Tischler and Frances Thompson.

Preparation of the Adsorbent

None of the prepared inhibitors was suitable for attachment to agarose without modification, but an analog of rifampicin, N-amino-N-desmethyl rifampicin (I), was, and could be readily synthesized by the condensation of rifaldehyde with a large excess of 1,4-diaminopiperazine. This was prepared and found to have an inhibition constant of 6.7×10^{-5} moles/l.



I

It was expected that the affinity of this ligand for RDP would increase upon insolubilization, since in homologous series of rifaldehyde derivatives inhibition constants generally decreased with increasing steric bulk. N-heptylimino-N-desmethyl rifampicin, prepared by condensing N-amino-N-desmethyl rifampicin with heptanal, has an inhibition constant of 2.4×10^{-5} moles/1.

The N-amino-N-desmethyl rifampicin was coupled to succinylaminoalkyl agarose using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate in 1:1 dimethylformamide-water. The resulting gel was deep orange in color. Considerable material had been adsorbed and copious washing with 1:1 dimethylformamide-water and 1:1 ethylene glycol-water was needed before the effluent from the washed material was free of color.

Affinity Chromatography

A column was poured from the gel and washed with a buffer of 0.05 M Tris (pH 7.8) containing 0.1 M potassium chloride, 0.001 M dithiothreitol, 0.005% Triton X-100, and 20% glycerol. The effluent from the column was orange indicating either that all of the adsorbed ligand had not been removed, or that the bound ligand was being hydrolyzed. Prolonged washing reduced the color intensity of the effluent to a very low level, but never completely eliminated it. The level of the free ligand in the effluent, judged by the color intensity, was too low to compete significantly with the bound ligand.

A portion of crude RDP preparation was chromatographed on the column using the same buffer. The results are shown in Fig. 1. The fact that two peaks are obtained indicates that some sort of interaction

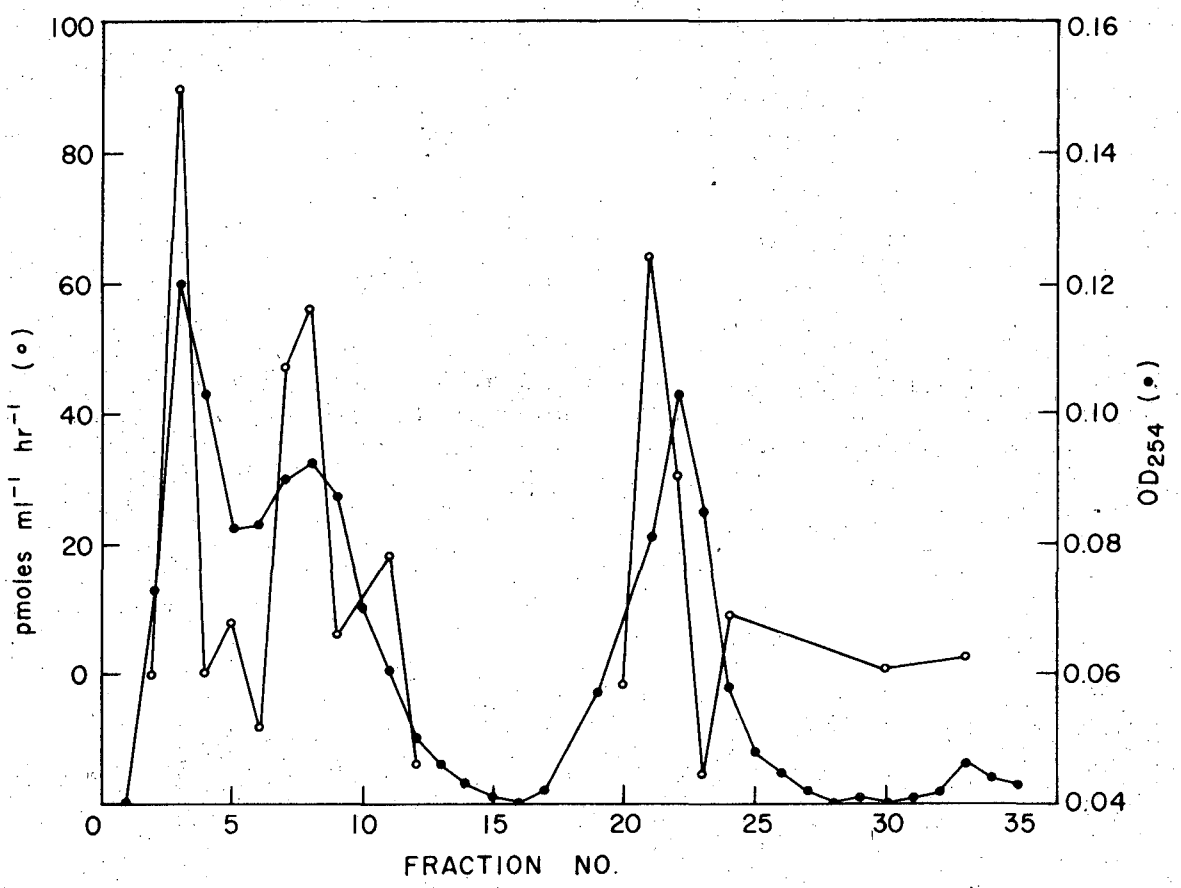


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Fig. 1. Chromatographic elution pattern of crude RDP on agarose-bound N-amino-N-desmethylrifampicin. A $\frac{1}{2}$" diam. column was filled with 25 ml of the adsorbent equilibrated with 0.05 M Tris (pH 7.8) containing 0.1 M KCl, 0.001 M dithiothreitol, 0.005% Triton X-100, and 20% glycerol. A crude preparation of RDP (480 μl, containing 2250 units (pmole/hr) of RDP was chromatographed on the column at 3° using the same buffer. Flow rate, 3 ml/min; fraction size, 2 ml. Protein (filled circles) was measured by the optical density at 254 nm. The residual adsorbance (0.04 units) was due to the continual elution of low level of N-amino-N-desmethylrifampicin. RDP (open circles) was measured by the incorporation of ^{3}H-dTTP into DNA under assay conditions.

is occurring although it is not clear what sort. Only 20% of the applied activity was recovered. The column was subsequently eluted with gradients of 0.1-0.5 M potassium chloride and of 0.005-0.10% of Triton X-100, the other components of the buffer being held constant. Neither procedure effected the elution of significant amounts of more material.

The results at this point are much too incomplete to draw any conclusions. The work is being continued by Allan Tischler and Frances Thompson.

EXPERIMENTAL

N-amino-N-desmethyl-rifampicin. The preparation is described in Tischler *et al.*[3]

Coupling of N-amino-N-desmethyl rifampicin to succinylaminoalkyl agarose. To 25 ml of succinylaminoalkyl agarose (prepared as described in Part I) was added 240 mg of 76% pure N-amino-N-desmethyl rifampicin (220 μmoles) in 20 ml of dimethylformamide. (The other 24% is rifamazine, the compound produced when two molecules of rifaldehyde condense with one molecule of 1,4-diaminopiperazine. It lacks a free amino group and will not be coupled.) One gram (2360 μmoles) of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate was added and the mixture shaken for 18 hr at r.t. The deep orange gel was washed with 1:1 dimethylformamide-water until the effluent was free of color (*ca.* 2 liters), followed by 1:1 ethylene glycol-water until the effluent was again free of color (*ca.* 1 liter). Then it was washed with 1 liter of water. A portion of the gel was hydrolyzed and the amount of rifamycin chromophore in the hydrolysate determined spectrometrically. This indicated that the gel had been substituted at a level of 6.3 μmoles/ml.

Crude RDP. RDP activity was extracted from UCl-B tissue culture cells which had been transformed by Moloney leukemia virus. The procedure has been described by Thompson et al.[4]

RDP Activity was assayed by the procedure of Thompson et al.[4] Triton X-100 was used at a concentration of 0.005%.

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PART III: ACTIVE INSOLUBILIZATION OF PREBIOLOGICAL COMPOUNDS-- THE CLAY-MEDIATED POLYMERIZATION OF AMINOACYL ADENYLATES

INTRODUCTION

Since Miller's classic experiment demonstrating the production of amino acids under possible primitive earth conditions,[1] there has been more than ample demonstration that amino acids would have been spontaneously formed in considerable quantities on the prebiotic earth. Possible pathways for the abiogenic creation of purines, pyrimidines, and sugars,[2] as well as nucleosides and nucleotides,[3] have also been demonstrated.

The next step in the chemical evolution leading to a living system is the polymerization of these monomers into polypeptides and polynucleotides. The various schemes which have been suggested for such polymerizations require relatively high concentrations of monomers to produce polymers of meaningful size. It is highly unlikely that such concentrations would have been achieved in the primitive sea. This problem has led Bernal to propose that clay deposits might have served to concentrate primitive organic molecules by adsorption.[4]

Paecht-Horowitz *et al.* have demonstrated that montmorillonite clay will adsorb aminoacyl adenylates and protect them from hydrolysis, thus allowing them to polymerize to sizable peptides in very high yield.[5] As a model for prebiological polymerizations, this system has much to recommend it: (1) It allows for a high degree of polymerization under dilute conditions at neutral pH without the use of elevated temperature; *i.e.*, under the conditions which were most probably found in the primitive sea. (2) the aminoacyl adenylate is the intermediate through which biosynthesis of protein occurs in contemporary organisms.

(3) The adenylate can also serve as an intermediate for the formation of polynucleotides, since the phosphate as well as the carboxylate is activated by the phosphoanhydride bond (Fig. 1). As Eigen has elegantly demonstrated, the cooperative interaction of polypeptides and polynucleotides would be mandatory for any self-reproducing system capable of evolution.[5] Which is to say, that polypeptides and polynucleotides must have evolved concurrently and not sequentially. Thus the spontaneous polymerization of both would be necessary for the initiation of chemical evolution.

We believe that this system could well serve as a model for the study of protein-nucleotide interaction under prebiotic conditions. Accordingly, we proposed to study the reaction rates and product distribution of aminoacyl adenylates and to compare them with the results for the corresponding aminoacyl guanylates, cytidylates, and uridylates. Initially, we hoped to determine whether there are chemical reasons which would make the adenylate the preferred method of activation. We envisioned the work as being possibly extendable to the elucidation of a chemical basis for the CpCpA terminus of tRNA and ultimately for the genetic code.

We also hoped to develop conditions which would favor the formation of polynucleotides from these activated intermediates and to use this system as a model for the study of nucleotide polymerizations.

Properties of Montmorillonite

Before proceeding with the description of our experimental work, it would be well to describe the properties of montmorillonite.

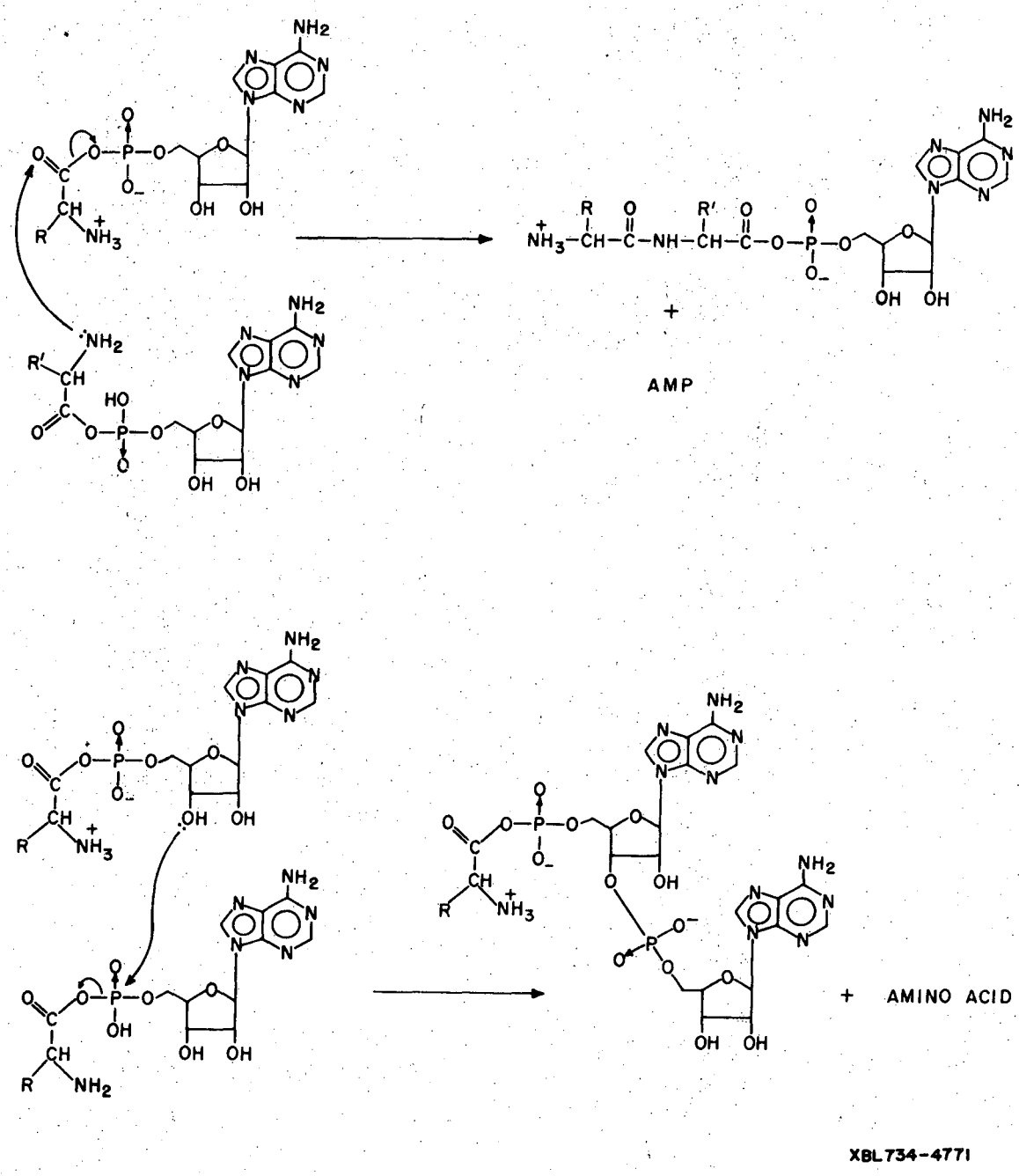

Figure 1: Possible routes of polymerization of aminoacyl adenylates: A, to give polypeptides; B, to give polyadenylic acid.

Montmorillonite occurs in flat irregular sheets which are 10 Å thick and of varying length and width. An idealized unit cell is shown in Figure 2. Actually, no montmorillonite exists homogeneously in this form. In actual samples of montmorillonite, some of the Al^{3+} ions in the central layer are replaced by Mg^{++} or Fe^{3+}, and some of the Si^{4+} in the tetrahedral layer is replaced by Al^{3+}. The replacement of Al^{3+} by Mg^{++} or Si^{4+} by Al^{3+} results in a net negative charge which is balanced by exchangeable cations which are adsorbed to specific sites on the face. In general, the affinity of the clay for various cations increases with the charge of the cation.

It is possible to convert montmorillonites into forms in which all the exchangeable cations are the same, e.g., sodium montmorillonite. The montmorillonite used in these studies was Montmorillonite No. 25 from the John C. Lane Tract in Upton, Wyoming. The sodium form has the formula

$$(Si_{7.84}\ Al_{0.16})(Al_{3.10}\ Fe_{0.30}\ Mg_{0.66})(OH)_4\ O_{20}$$

$$\underbrace{\hspace{6em}}_{Na_{0.82}}$$

Because of the dissociation of the exchangeable cations, the faces carry a net negative charge, while along the edges where the cationic core is exposed is a slight positive charge. As a result, montmorillonites which have only monovalent exchangeable cations (e.g., Na^+ or H^+) tend to form monodispersed face to edge orientations in aqueous solutions. The resulting gel-like structure causes even rather dilute solutions to be quite viscous. Polyvalent exchangeable cations allow bridging between the faces (i.e., the cation is linked to anionic sites

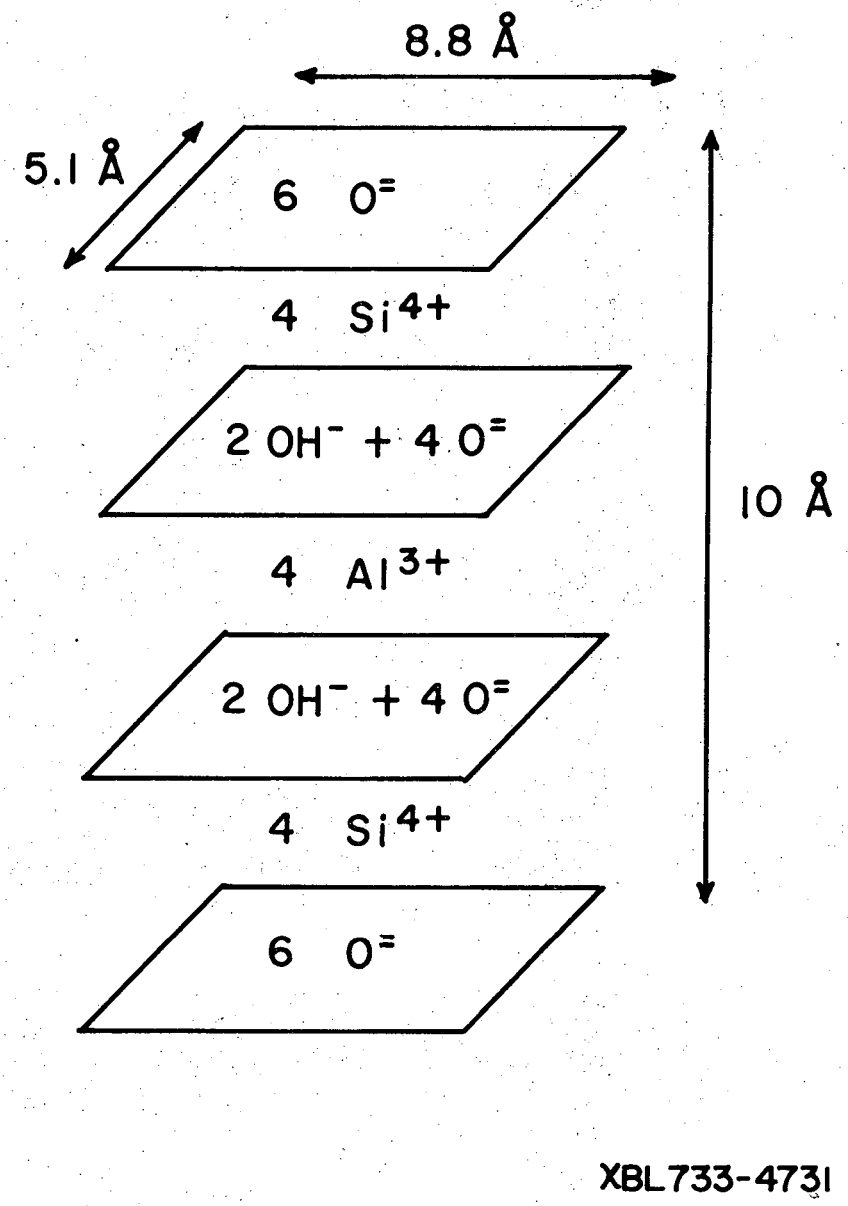

Figure 2: Idealized unit cell of montmorillonite.

on two different plates). This results in the formation of face to face aggregates which may be many plates in depth. Such montmorillonites tend to clump into larger particles, or domains. This clumping reduces the effective surface area of the montmorillonite considerably, but opens up the possibility of intercalation between the layers. Thus, variations in adsorption properties are not readily predictable from the knowledge of the exchangeable cation composition.

We decided to study sodium, calcium, and aluminum montmorillonite as representative of clays carrying monovalent, divalent, and trivalent exchangeable cations, respectively. The sodium form was prepared according to Posner.[7] It was then centrifuged as a dilute solution (∿1% w/v) and the portion remaining suspended after 60 min at 9000 x g was retained. This suspension was dialyzed against water for 24 hrs to remove chloride ion, lyophilized, and ground to a fine powder in a small ball mill. The calcium and aluminum forms were prepared by treating a suspension of the sodium form (after centrifugal fractionation) with a 2 M solution of the appropriate salt for 24 hrs. (The polyvalent cations readily displace the sodium.) This was followed by dialysis, lyophilization, and grinding as before..

Suspensions of the three forms differed considerably in their properties. The sodium form gave a translucent, apparently monodisperse suspension which did not settle upon standing and became quite viscous at concentrations >3%. The calcium form gave an opaque suspension which settled on prolonged standing. The aluminum form gave what was clearly a suspension of aggregates which settled very quickly.

Electron micrographs were made of the various clays, after shadowing with chromium (Figure 3). All the forms showed some tendency

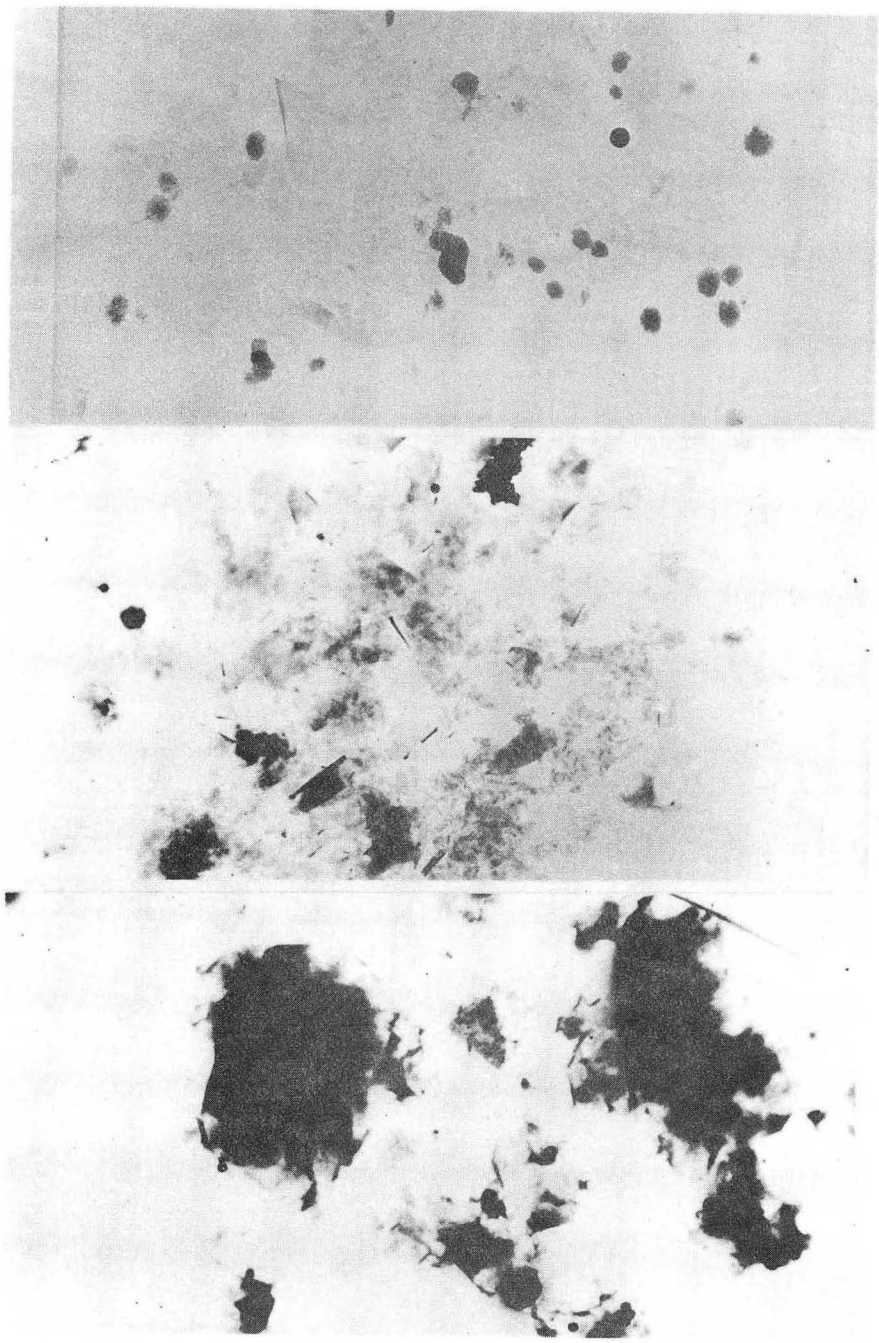

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Figure 3: A) Particles of sodium montmorillonite (x 20,000).
B) Particles of calcium montmorillonite (x 9,400).
C) Particles of aluminum montmorillonite (x 9,400).

to aggregate when prepared for electron microscopy. The degree of aggregation was slight for the sodium form, but considerable for the forms with polyvalent cations. The aluminum form again showed the most intense aggregations. The size of the individual flakes, as determined from micrographs of the sodium clay, ranged downward from 2 microns, with the bulk of material being less than 1 micron. Since centrifugation occurred before ion replacement, the same range of sizes of individual flakes should be found in all three preparations.

Binding Studies

In order to better interpret future results, we decided to determine the binding of L-alanine, poly-D,L-alanine (av. MW ∿1600), AMP, and polyadenylic acid (av. MW ∿3400) to the various forms of montmorillonite.

The mechanisms by which organic compounds become adsorbed to clays have been discussed thoroughly by Mortland.[8] Several mechanisms may be operant in the studies we undertook. Amino acids become bound to clays primarily via ion exchange reactions at pH's below the isoelectric point. Purine derivatives may also bind in the protonated form by ion exchange; however, conditions must be quite acidic. Anionic compounds in general are repelled by the negatively charged clay particles. But adsorption can occur with polyvalent cations through cation bridging; that is, the cation serves as a counter ion for both a clay anionic side and the organic anion. We expected to see considerable binding of AMP and polyadenylic acid to aluminum montmorillonite by this mechanism. Other effects which will be operative are Van der Waal's forces, hydrogen bonding between the adsorbed

compound and the silicate oxygens, and for the polymers, an entropy effect, wherein adsorption of one molecule of polymer results in the desorption of many water molecules.

Our adsorption studies were carried out in the pH range 0.7-10. A solution of the material whose adsorption was to be studied was adjusted to the appropriate pH with 1 N HCl or NaOH. A portion of clay was added and the suspensions stirred for 24 hrs. The clay was removed by centrifugation and the supernatant checked for loss of the adsorbate. The clay was washed with 0.1 M sodium chloride, which should displace any adsorbed material, and the wash was assayed for adsorbate. ^{14}C-L-alanine was measured radiometrically and with ninhydrin; poly-D,L-alanine was measured with the Lowry reagent,[9] and AMP and polyadenylic acid were assayed spectrophotometrically.

For all compounds and for all three forms of the clay, adsorption was zero within experimental error throughout the pH range studied (it should be noted that AMP and polyadenylic acid precipitated from the solutions at acid pH).

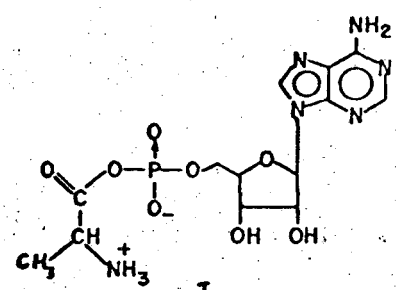
Greenland _et al._[10] have studied the binding of glycine and its oligomers to sodium and calcium montmorillonite using much larger quantities of clay than were employed by us. When his values (_e.g._, 15 mg/g for glycine on sodium montmorillonite) are extrapolated to the scale we operated on, the amount of material adsorbed becomes too small to have been detected by our techniques. This tends to support our findings for alanine and polyalanine.

The fact that aluminum montmorillonite failed to bind any polyadenylic acid was quite surprising, in view of a report that phosphates are strongly adsorbed by aluminum mica.[11]

Paecht-Horowitz et al. report that L-alanyl 5'-adenylate adsorbs at up to 400 mg/g of sodium montmorillonite, but do not indicate how this value was arrived at. Due to the extreme reactivity of the compound, the value is rather questionable. This could probably best be tested by measuring the binding of the stable amino alcohol analog, L-alaninol 5'-adenylate. This has not yet been done.

Polymerization Studies

I decided to initiate my polymerization studies by repeating the work of Paecht-Horowitz et al., using L-alanyl adenylate (I).[5]



I

I initially attempted to prepare L-alanyl adenylate by the method of Berg,[12] but could not obtain a product of more than 30% purity. I then switched to the more elaborate procedure of Moldave et al.[13] By employing a modification of this technique, I was able to obtain purities of up to 87%. Because of the extreme lability of the product, this is probably close to the limiting value for purity. Routine yields were about 70%.

The purity was determined by reacting the product with neutral hydroxylamine. The resulting hydroxamate was measured spectrometrically as the iron(III) complex using synthetic alanyl hydroxamate hydrochloride as the standard.

The first attempt to carry out a clay mediated polymerization was done as follows: 75 mg of sodium montmorillonite was suspended in 60 ml of water at ambient temperature in a chamber which was continuously purged with nitrogen. Portions of alanyl adenylate (∿20 mg) were added at 10-min intervals. The pH was maintained at 8.1 by titration with 4 N sodium hydroxide using a pH-stat. The reaction was followed by the addition of titrant as a function of time. A typical portion of the resulting trace is shown in Figure 4.

It will be noted that titrant addition was necessary whenever a new portion of alanyl adenylate was added and was necessary only at such times. This behavior is not at all what was expected. The expected behavior is also shown in Figure 4. The intact alanyl adenylate is expected to be in the zwitterionic form shown in I. The phosphate is sufficiently acidic that it will not remain protonated on lyophiliztion from acetic acid when the ammonium group is present as the counter ion. The zwitterion should have no appreciable acidity at pH = 8.1. However, the alanyl adenylate preparations always contain some hydrolyzed material, i.e., alanine adenylate, $\overset{+}{N}H_3CHRCOOH$ $HAMP^-$, which will be acidic. Thus, when a portion of alanyl adenylate is added to the reaction chamber, titrant will be required to neutralize the hydrolyzed portion. Then, as the intact alanyl adenylate is either hydrolyzed or polymerized, more acid will be generated, requiring further additions of titrant before the addition of another portion of adenylate. This behavior would be expected even if no polymerization is occurring, since hydrolysis is not instantaneous (the half-life at pH 7.2, 37°C, is 5 min[14]).

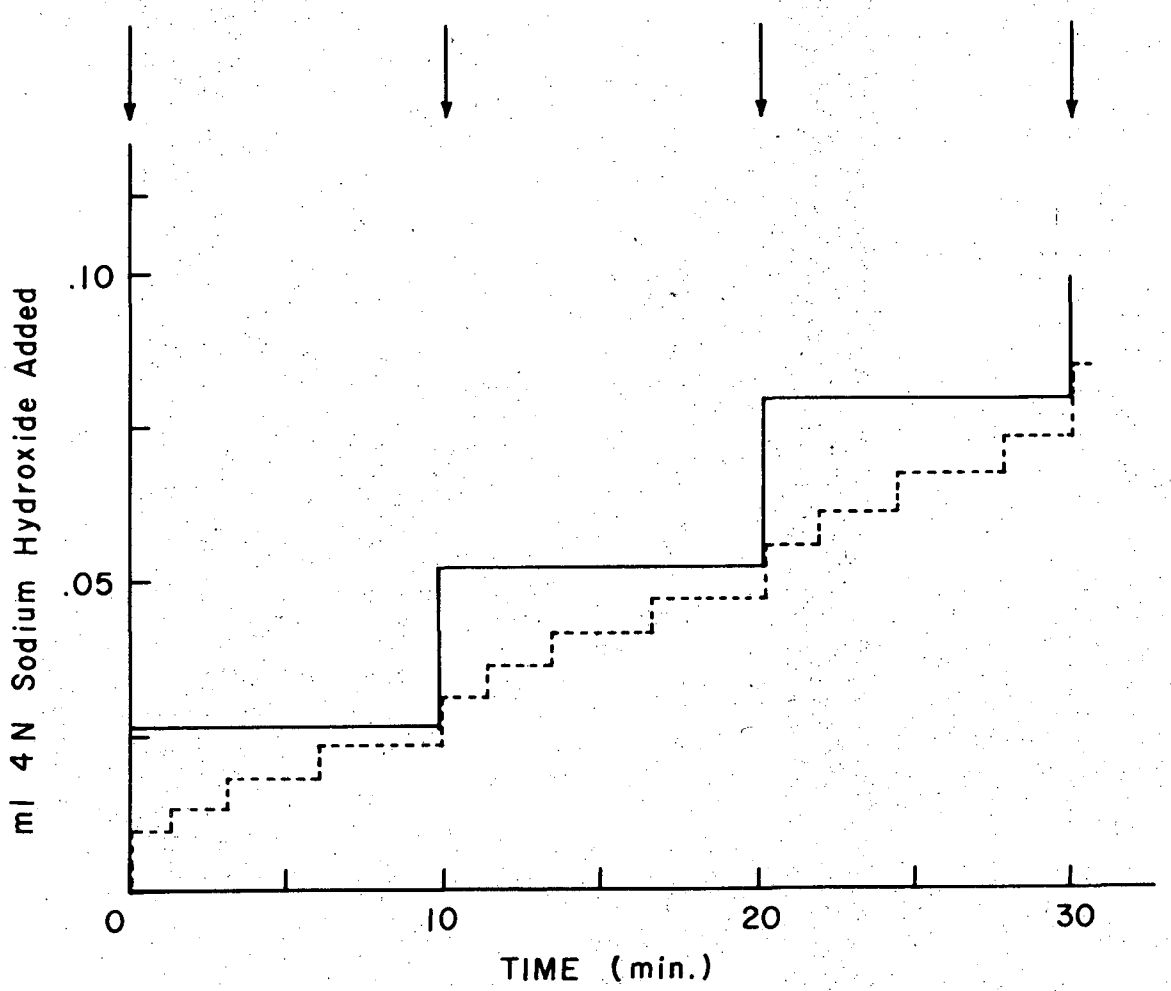


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Figure 4: Titrimetric behavior during clay mediated polymerization of L-alanyl adenylate. Behavior expected is shown with a broken line. Behavior seen is shown with a solid line. Arrows indicate the addition of a portion of L-alanyl adenylate.

The apparent behavior of the system may have been the result of poor operation by the pH-stat. The instrument was "homemade" from components and was quite old. It was actually only capable of maintaining the pH within about ± 0.1 unit of the specified pH. Moreover, the titrant addition speed was fixed. It is likely that overtitration may have occurred, resulting in the presence of excess base sufficient to neutralize the acid generated before the next addition of alanyl adenylate. On addition of further alanyl adenylate, the pH would become sufficiently lowered to initiate titrant addition which would be continued for too long due to poor response characteristics, and excess base would again be present..

After the last portion of alanyl adenylate had been added, the reaction was allowed to continue for 24 hrs more, during which time only three insignificant titrant additions occurred.

The clay was removed by centrifugation, suspended in 20 ml of water, sonicated, and again centrifuged. The combined supernatants were then passed through a Diaflo PM-30 membrane (cutoff size: MW ~30,000) to remove any clay which might remain.

The solution was assayed for alanine with ninhydrin[15] and for polyalanine with the Lowry reagent.[9] The assays indicated that the solution contained 36.6 mg of free alanine and 4.4 mg of polyalanine. The alanyl adenylate used in the experiment contained 112 mg of alanine. Recently, I learned from Dr. Paecht-Horowitz that only the D,L-isomer polymerizes to give sizable peptides. When D- or L-alanyl adenylate is employed, the polymerization is terminated after four to five steps by the precipitation of the oligopeptide.[17] Presumably, the missing

L-alanine in my experiment was precipitated in such a manner. This could be determined by hydrolysis of the clay residue. Unfortunately, it has been discarded.

It was discovered that AMP interfered with the Lowry assay. Therefore, the solution was passed through a column of Dowex 1-X8 (Cl^-) to free it from AMP. A Lowry assay on the resulting solution indicated a total of only 0.6 mg of polyalanine now remained. Perhaps the rest had been retained as the peptide 2'(3') ester with AMP. Paecht-Horowitz et al. have indicated that the peptides which they obtained from this procedure were so esterified.[5]

Because of the poor response characteristics of the pH-stat used in this experiment and the peculiar titrimetric behavior observed, it wass decided to obtain a new pH-stat before repeating this rather equivocal experiment. This required about six months. During this wait I decided to devote full time to the affinity chromatography project. These studies were turned over to Dr. John McCullough.

EXPERIMENTAL

Materials

Montmorillonite #25 from the John C. Lane Tract, Upton, Wyoming, was purchased from Ward's Natural Science Establishment, Inc.; N,N-dicyclohexylcarbodiimide, L-alanine, poly-D,L-alanine (av. MW 1600), and polyadenylic acid (av. MW 3400) from Nutritional Biochemical Co.; AMP and ninhydrin from Calbiochem; palladium chloride and 10% palladium on charcoal from Matheson, Coleman, and Bell; and hydroxylamine hydrochloride from Baker and Adamson. All other compounds used were of reagent grade.

Binding Studies

A solution of the compound whose binding was to be studied was brought to the appropriate pH with either 1 N HCl or 1 N NaOH and the volume brought to 10 ml. Final concentrations were: L-alanine 0.1 M; poly-D,L-alanine 0.5 mg/ml; AMP 0.1 M; and polyadenylic acid 0.1 mg/ml.

Five milligrams of the appropriate montmorillonite, finely ground, was added to the solution, which was capped and stirred for 24 hrs at ambient temperature. The solutions were then centrifuged for 60 min x 15,000 g and the supernatants decanted. The precipitated clay was resuspended in 10 ml of 0.1 M sodium chloride and again centrifuged after 24 hrs.

The supernatant and wash were each assayed for the presence of the compound under study with the appropriate method. L-alanine was assayed with ninhydrin.[15] U-^{14}C-L-alanine was determined by scintillation counting in a Packard Tri Carb instrument. Poly-D,L-alanine was assayed with the Lowry reagent as described previously.[15] AMP and polyadenylic acid were quantitated from the absorbance at 259 nm. In all cases, no loss (within experimental error) of any substrate was found in the supernatant, nor was any substrate found in the salt wash.

N-Carbobenzoxy-L-alanine

N-Carbobenzoxy-L-alanine was prepared following a procedure suggested by Greenstein and Winitz:[16] L-Alanine (25 g, 0.28 mole) and sodium bicarbonate (59 g, 0.56 mole) were suspended in 400 ml of water, which was vigorously stirred with a propeller stirrer. Carbobenzoxy chloride (52.3 g, 0.31 mole) was added in 6 portions at 12-min

intervals. The mixture was stirred overnight and brought to pH 10 with 1 N NaOH. The solution was extracted with 100 ml of ether, and then brought to pH 3 with 1 N HCl. It was extracted with 3 x 60 ml of ethyl acetate and the combined extractss were dried over magnesium sulfate for 60 min, filtered, and evaporated in vacuo to give a white residue. This was recrystallized from ether-petroleum ether. The crystals were collected with suction and dried over P_2O_5 in vacuo overnight. The resulting white crystals weighed 31.5 g (50%) with MP 85.5-86.5°C (reported 87°C[16]). A second crop of 13.9 g (22%), MP 83-5°C, was also obtained.

N-Carbobenzoxy-L-alanyl 5'-adenylate

All manipulations were carried out in a cold room at 3°C unless otherwise indicated. AMP·H_2O (2.2 g, 6 mmole) and N-carbobenzoxy-L-alanine (1.34 g, 6 mmole) were dissolved in 25 ml of 3:1 pyridine-water. To this solution was added N,N'-dicyclohexyl carbodiimide (24 g, 116 mmole) dissolved in 24 ml of pyridine. The resulting mixture was stirred 3-1/2 hrs and then filtered. The precipitated N,N'-dicyclohexyl urea was washed with 5 ml of 3:1 pyridine-water and the wash added to the filtrate. The combined filtrate and wash were allowed to separate into two phases (∿5 min) and the bottom layer was drained into 350 ml of acetone at -15°C. This was stirred briefly and centrifuged at -20°C by bringing the centrifuge to 6000 x g and immediately shutting off. After decantation of the supernatant the precipitate was washed with 200 ml of acetone at -20°C by shaking in the sealed centrifuge bottle and recentrifuging as before. It was then suspended in 60 ml of acetone at -15°C and

filtered in a cold (3°C) dry box with a medium sintered glass filter funnel. It was then washed with 2 portions of ether (3°C), in the dry box and dried over P_2O_5 *in vacuo* overnight at 3°C.

It is expected that the resulting material is not pure N-carbobenzoxy-L-alanyl 5'-adenylate. However, purity was not determined until after removal of the carbobenzoxy group. The crude yield was 2.11 g (64%). It was stored at -15°C.

Palladium black

Palladium chloride (3 g) was dissolved in 20 ml of 6 N HCl by heating on a steam bath for 2 hrs. This solution was poured into 500 ml of boiling, glass-distilled water. Three milliliters of 88% formic acid was added followed by 4 N potassium hydroxide (carefully) until a pH of 9 was reached. The mixture was boiled 10 min and the pH brought to 7 with 88% formic acid. After cooling, the precipitated palladium black was washed several times with glass-distilled water by trituration and decantation. It was stored under glass-distilled water.

L-Alanyl 5'-adenylate

N-Carbobenzoxy-L-alanyl 5'-adenylate (800 mg, 1.45 mmole) was dissolved in 15 ml of 90% acetic acid in 25 ml pear flask with side arm through which a gas bubbler was introduced. The solution was chilled in an ice bath and *ca.* 3 g (drained wet weight) of palladium black was added. The flask was capped with an oil-filled vapor seal and purged with nitrogen. Hydrogen was bubbled through the solution for 20 min at a rate sufficient to give good stirring. The vessel was again purged with nitrogen. The palladium black was filtered

off in a cold (3°C) dry box and washed with 6 ml of glacial acetic acid. The combined filtrate and washing were immediately frozen and lyophilized. The lyophilizatee weighed 440 mg (73%). It was determined by the hydroxamate assay, described below, to be 87% L-alanyl 5'-adenylate.

Hydroxamate Assay

One to four milligrams of the alanyl adenylate preparation were dissolved in 50 μl of neutral 2 M hydroxylamine, prepared fresh by mixing equal portions of 4 M hydroxylamine hydrochloride and 3.15 M sodium hydroxide. After 3 min the solution was diluted with 1 ml of water followed by 1 ml of a solution of 10% ferric chloride and 3.3% trichloroacetic acid in 0.66 N hydrochloric acid. The solution was shaken rapidly and the absorbance at 540 nm was measured against a blank to which no alanyl adenylate had been added. The complex with synthetic alanyl hydroxamate hydrochloride was used as a standard. This gave a molar extinction coefficient of ε = 212,000.

Clay-mediated Polymerization

Sodium montmorillonite (75 mg) was suspended in 60 ml of water in a closed chamber continuously purged with nitrogen. Potassium dihydrogen phosphate (100 mg) was added and the pH was adjusted to 8.1. Portions of L-alanyl 5'-adenylate (∿20 mg) were added at 10-min intervals. pH was maintained at 8.1 with 4 N NaOH using a pH-stat. In this manner 504 mg of 60% pure alanyl adenylate was added, followed by 285 mg of 78% pure alanyl adenylate. Reaction was allowed to continue after addition of the adenylate for 24 hrs further, with pH still maintained at 8.1.

The solution was centrifuged for 60 min at 11,000 x g. The clay was then suspended in 20 ml of water, sonicated, and recentrifuged. The combined supernatants were then ultrafiltered through a Diaflo PM-30 membrane (cutoff size: MW ∿30,000) to remove any remaining clay. Assays with ninhydrin[15] and the Lowry reagent[9] indicated that the solution contained 36.6 mg of free alanine and 4.4 mg of polyalanine out of 112 mg alanine introduced. Poly-D,L-alanine (av. MW 1600) was used as the standard for the Lowry assays.

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Finally, I would like to thank my parents, who got me started and made it all possible.

And now a word from our sponsor: This thesis has been brought to you by the National Science Foundation and the U. S. Atomic Energy Commission.

APPENDIX A

ABBREVIATIONS

In addition to the usual abbreviations (e.g., g, ml, etc.), the following abbreviations and trivial names were used in this work:

AA: an amino acid

AA-tRNA: a 2'(3')-aminoacylated transfer ribonucleic acid

AE-AMP: N^6-(2-aminoethyl)-adenosine 5'-phosphate

Affinity Gel I: an adsorbent for affinity chromatography prepared by coupling BOC-ile*-ol AMP to succinylaminoalkyl agarose and deblocking.

AH-AMP: N^6-(6-aminohexyl)-adenosine 5'-phosphate

Aminobenzamidoalkyl agarose: a gel consisting of p-aminobenzamidopropylaminopropylamine coupled to cyanogen bromide activated agarose

AMP: adenosine 5'-phosphate

ATP: adenosine 5'-triphosphate

Bicine: N,N-bis(hydroxyethyl)glycine

BES: N,N-bis(hydroxyethyl)-2-aminoethylsulfonic acid

BOC-ile-ol AMP: N-t-BOC-L-isoleucinol 5'-adenylate

BOC-ile*-ol AMP: N-t-BOC-(U)^{14}C-L-isoleucinol 5'-adenylate

BOC-ile*-ol AE-AMP: N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate

BOC-ile*-ol AH-AMP: N-t-BOC-(U)^{14}C-L-isoleucinol N^6-(6-aminohexyl) 5'-adenylate

BP: boiling point

Cγ gel eluate: Partially purified L-isoleucine tRNA synthetase (see p. 38)

Cl-IMP: 6-chloropurine riboside 5'-phosphate (6-chloroinosine monophosphate)

CMR: carbon-13 nuclear magnetic resonance (spectrum)

d: doublet

dATP: 2'-deoxy adenosine 5'-triphosphate

DCC: N,N'-dicyclohexyl carbodiimide

DCU: N,N'-dicyclohexyl urea

diAc Cl-IMP: 6-chloropurine 2',3'-diacetyl riboside 5'-phosphate (2',3'-diacetyl 6-chloroinosine monophosphate)

E: an aminoacyl tRNA synthetase

E·AA-AMP: complex of an aminoacyl tRNA synthetase with an aminoacyl adenylate

Elution Buffer 1: a buffer of 0.02 M TES (pH 7.8) containing 0.01M 2-mercaptoethanol, 0.005 M magnesium chloride, and 0.05 M potassium chloride.

GC: gas-liquid chromatography

HEPES: N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid

IR: infrared (spectrum)

IRS: L-isoleucine tRNA synthetase

K_i: an inhibition constant

K_m: a Michaelis constant

K_s: a dissociation constant

L-ile-ol AMP: L-isoleucinol 5'-adenylate

L-ile*-ol AMP: (U)^{14}C-L-isoleucinol 5'-adenylate

L-ile*-ol AE-AMP: (U)^{14}C-L-isoleucinol N^6-(2-aminoethyl) 5'-adenylate

L-ile*-ol AH-AMP: (U)^{14}C-L-isoleucinol N^6-(6-aminohexyl) 5'-adenylate

L-ile-tRNA^{ile}: 2'(3') ester of L-isoleucine with the terminal adenosine of tRNA^{ile}

m: multiplet

Mg ATP: monomagnesium salt of ATP

MP: melting point (uncorrected)

NAD^+: nicotinamide adenine dinucleotide

NMR: (proton) nuclear magnetic resonance (spectrum)

PPi: pyrophosphate

PRS: phenylalanyl tRNA synthetase

RDP: RNA-instructed DNA polymerase

r. t.: room temperature

s: singlet

succinylaminoalkylagarose: a gel prepared by coupling 3, 3'-iminobis-propylamine to cyanogen bromide-activated agarose and then succinylating the free terminal amine with succinic anhydride

t: triplet

t-BOC: the tert-butyloxycarbonyl group

TES: N-tris(hydroxymethyl)methylaminoethane sulfonic acid

TLC: thin-layer chromatography

TNS: 2-p-toluidinylnaphthalene-6-sulfonate

Tricine: N-tris(hydroxymethyl)methyl glycine

Tris: tris(hydroxymethyl)aminomethane

tRNA: transfer ribonucleic acid

$tRNA^{ile}$: transfer ribonucleic acid specific for L-isoleucine

UV: ultraviolet (spectrum)

V_{max}: velocity of an enzymatic reaction extrapolated to infinite substrate concentration.

APPENDIX B

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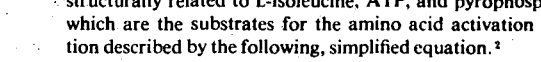
On the Active Site Topography of Isoleucyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* B†

Eggehard Holler,‡ Petrie Rainey,§ Ann Orme, Edward L. Bennett, and Melvin Calvin*

ABSTRACT: The topography of the active site of Ile-tRNA synthetase has been investigated by using structural analogs for L-isoleucine and ATP. Radioactive ATP-[^{32}P]PP$_i$ exchange methods and fluorescence titration methods were applied for determination of binding properties of substrates and inhibitors. The results are consistent with a hydrophobic region at the L-isoleucine specific site to accommodate the aliphatic side chain. The α-amino group is electrostatically bound to an anionic site. It is believed that this site is part of an ion pair in the free enzyme. The ion pair can be opened by either L-isoleucine and related compounds or by ATP, AMP, and pyrophosphate. Opening is coupled to the interaction of the hydrophobic side chain. When the electrostatic interaction is sterically prevented, as in case of methylation or guanidation of the α-amino group, hydrophobic interaction is also prohibited. The ion pair can be easily opened by the intact substrate, when both the α-amino group and carboxylic group are present. Compounds lacking the carboxylic group are good ligands only when ATP or AMP is simultaneously bound to the enzyme. This observation has an important bearing on the use of α-amino alcohols as inhibitors for elucidation of the catalytic pathway. Free-energy considerations indicate that the affinity for binding of ATP to Ile-tRNA synthetase is 4 kcal less than anticipated from the binding affinities measured for its structural components. AMP and pyrophosphate. Furthermore, the standard free energy of formation of enzyme-bound L-isoleucine adenylate and pyrophosphate from enzyme-bound substrates is favored by 5 kcal as compared with the reaction in the free solute state. We conclude that ATP when enzymically bound is in a high free-energy state, thus facilitating the formation of enzyme-bound L-isoleucine adenylate.

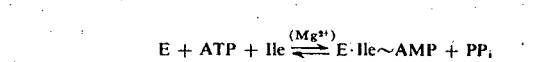
Probing of the active site of enzymes has been accomplished by binding experiments using a structural variety of substrates and inhibitors as, for instance, in the case of chymotrypsin (Blow,[1] 1971) and trypsin (Keil,[1] 1971). This method has not been rigorously applied to the family of aminoacyl-tRNA synthetases except for its recent use on Phe-tRNA synthetase (Santi *et al.*, 1971), on Val-tRNA synthetase (Owens and Bell, 1968), and on Pro-tRNA synthetase (Papas and Mehler, 1970).

In this article we report results obtained for probing of the active site of Ile-tRNA synthetase using compounds structurally related to L-isoleucine, ATP, and pyrophosphate, which are the substrates for the amino acid activation reaction described by the following, simplified equation.[2]



$$E + ATP + Ile \overset{(Mg^{2+})}{\rightleftharpoons} E\cdot Ile\sim AMP + PP_i$$

It is believed that the enzyme-bound intermediate, L-isoleucyl adenylate, reacts in a second step with the cognate tRNA^{Ile} to form Ile-tRNA^{Ile} (Berg, 1958) according to the following equation.



$$E\cdot Ile\sim AMP + tRNA^{Ile} \rightleftharpoons Ile\text{-}tRNA^{Ile} + E + AMP$$

Possibly, formation of the Ile-tRNA synthetase-tRNA^{Ile} complex is associated with some rearrangement at the active site. With this in mind, we must distinguish between probing of the active site of free enzyme and probing of tRNA^{Ile}-bound enzyme. From comparison of the results the extent and the consequences of the tRNA^{Ile}-induced rearrangement will be evident.

The investigation reported in this paper is restricted to the probing of the free enzyme only. L-Isoleucine related compounds lacking either the α-amino group or the carboxylic group, or both, were used as probes. Further, the effects of methylation and guanidation of the α-amino group and the effect of chain elongation of the L-isoleucyl side chain on binding properties, catalytic properties, and fluorescence properties were evaluated. Similarly, we have measured the extent of binding of the structural components of ATP, *i.e.*, adenine, D-ribose, adenosine, AMP, and pyrophosphate. In part, we have examined the interactions of inhibitors in the presence of co-ligands. Part of this investigation has been published recently (Holler *et al.*, 1971).

Materials and Methods

Ile-tRNA synthetase was obtained as a 300- to 350-fold purified preparation from *Escherichia coli* B cells (Miles Laboratory) following a method described by Baldwin and Berg (1966). Protein was determined by the method of Lowry *et al.*, (1951). The specific activity of the freshly purified enzyme was 650-750 units/mg, where one unit is defined as the formation of 1 μmol of [^{32}P]ATP from [^{32}P]PP$_i$ in 15 min at 37° under standard conditions (Baldwin and Berg, 1966). The published value of the molecular weight (112,000, Baldwin and Berg, 1966) was used to calculate the enzyme

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[1] Reference is made to review articles.

[2] Abbreviations used are: E, isoleucyl transfer ribonucleic acid (Ile-tRNA) synthetase; E·Ile·ATP, Ile-tRNA synthetase-L-isoleucine-adenosine triphosphate complex; Ile∼AMP, L-isoleucyl adenylate; E·Ile∼AMP·PP$_i$, Ile-tRNA synthetase-L-isoleucyl adenylate-pyrophosphate complex.

concentration. Enzyme was stored at −15° in 0.02 M sodium phosphate buffer, pH 7.5, which contained 0.1 mM glutathione.

tRNA was purified from *E. coli* B cells (Schwarz BioResearch) according to the derivation method described by Gilliam *et al.* (1967, 1968). Finally, the preparation was purified over a Bio-Gel P20 column (57 cm × 1 cm) by elution with 0.02 M phosphate buffer, pH 8.0, at 4°. The preparation was 20% pure $tRNA^{Ile}$ (in terms of A_{260} units). No distinction has been made between different isoaccepting forms of $tRNA^{Ile}$. The concentration of total tRNA was estimated spectrophotometrically on the assumption that 21.4 A_{260} units are equivalent to 1 mg/ml of tRNA (Stephenson and Zamecnik, 1961).

$[^{32}P]PP_i$ was prepared from $[^{32}P]P_i$ (International Chemical and Nuclear Corp.) as described by Berg (1958). Guanidino-L-isoleucine was prepared from L-isoleucine and 5-methylthioisourea following the method described by Fasold *et al.* (1961) and from L-isoleucine and 1-guanyl-3,5-dimethylpyrazole nitrate as described by Habeeb (1960). Both preparations gave identical results; the material was negative against ninhydrin, soluble in water, and melted at 228° with decomposition (the closely related compounds for leucine and valine melt with decomposition at 242 and 240°, respectively, Prager and Jacobson, 1944). 3-Methylpentanoic acid, 3-methyl-1-pentanol, and 2-methyl-1-butanol were freshly redistilled before use. Amino acids and amines were found to be homogeneous by thin-layer chromatography (Eastman Chromagram, 6060 Silica Gel). Solvent mixtures were *n*-butyl alcohol saturated with 20% acetic acid, and benzene-pyridine-acetic acid (80:20:5, v/v/v).

The L-isoleucine ethyl and hexyl esters were prepared as the hydrochlorides with thionyl chloride in the appropriate alcohol by the method of Brenner and Huber (1953) with minor modifications. No attempt was made to distill the esters. L-Isoleucine ethyl ester hydrochloride had a mp of 92–93°. *Anal.* Calcd for $C_8H_{18}ClNO_2$: C, 49.09; H, 9.27; Cl, 18.14; N, 7.15. Found: C, 49.23; H, 9.25; Cl, 18.20; N, 7.36.

The hexyl ester hydrochloride could not be crystallized and was obtained as an amorphous, waxy solid. *Anal.* Calcd for $C_{12}H_{26}ClNO_2$: C, 57.20; H, 10.40; Cl, 14.10; N, 5.60. Found: C, 57.0; H, 10.5; Cl, 13.9; N, 5.5.

Both compounds were homogeneous to thin-layer chromatography on silica gel using 7:3 acetone-water or 9:1 chloroform-triethylamine.

L-Isoleucinyl adenylate was synthesized by the procedure of Sandrin and Boissonnas (1966) and Cassio *et al.* (1967) with minor modifications. The product was homogeneous to thin-layer chromatography on silica gel using methanol or 7:3 acetone-water: ultraviolet (uv) (0.01 M potassium phosphate, pH 7.5) max 259 nm (ε 12,100); min 227 nm. *Anal.* Calcd for $C_{16}H_{27}N_6O_7P \cdot 0.3H_2O$: C, 42.5; H, 6.2; N, 18.6; P, 6.9. Found: C, 42.6; H, 6.2; N, 18.5; P, 6.9.

3-Methyl-1-pentanol and 3-methylpentanal were obtained from Aldrich, 2-methyl-1-butylamine and 3-methylpentanoic acid from K&K Laboratories, and 2-methyl-1-butanol, α-DL-aminopentanoic acid, α-DL-aminohexanoic acid, and α-DL-aminoheptanoic acid from Eastman Organic Chemicals. *O*-Methyl-L-threonine, *N*-methyl-DL-isoleucine, valine, adenosine, and AMP were purchased from Calbiochem, L-isoleucine methyl ester, potassium 2-*p*-toluidinylnaphthalene-6-sulfonate, and *S*-methylisothiourea sulfate from Sigma, L-isoleucine, L-isoleucinol, and ATP from Nutritional Biochemical Corp., and 1-guanyl-3,5-dimethylpyrazole and adenine from Schwarz/Mann. All other chemicals were reagent grade and purchased from Baker Chemical Co. Deionized and distilled water was used which had been boiled and cooled under nitrogen to remove oxygen and carbon dioxide.

Radioactive ATP-$[^{32}P]PP_i$ Exchange Reaction. The method used was essentially the method described by Baldwin and Berg (1966), at pH 8.0, 25°, in 0.05 M Tris-HCl buffer and 0.01 M 2-mercaptoethanol. Radioactivity was measured as counted by a Nuclear-Chicago end-window Geiger counter. The amino acid specific exchange reaction was measured as a function of varying concentrations of amino acid. Numbers of counts per minute were plotted as a function of concentration of substrate according to the method of Eadie (1942) or Lineweaver and Burk (1934). The value for the Michaelis-Menten constant, K_m(app), and its value in the presence of an inhibitor at a fixed concentration, K_m^I(app), were determined from the slope of the linear plots. The inhibition constant for competitive inhibition was calculated according to $K_i = [I]_0/[(K_m^I(\text{app})/K_m(\text{app})) - 1]$ where the symbol $[I]_0$ refers to the initial concentration of the inhibitor. For α-DL-aminohexanoic acid and α-DL-aminoheptanoic acid, the Michaelis-Menten constant was evaluated from a set of data where the concentration of a competitive inhibitor, 2-methyl-1-butylamine, was varied and the concentration of the substrate was kept constant. Evaluation is accomplished by use of the equation

$$v = v_0 - v[I]_0K_m(\text{app})/[K_i(K_m(\text{app}) + [S]_0)] \qquad (1)$$

which is derived as follows. The Michaelis-Menten equations for the absence and presence of a competitive inhibitor are

$$v_0 = V_{max}\frac{[S]_0}{K_m(\text{app}) + [S]_0}$$

and

$$v = V_{max}\frac{[S]_0}{K_m(\text{app})[1 + [I]_0/K_i] + [S]_0}$$

respectively. The symbols refer to v_0, the rate in the absence of inhibitor; v, the rate in the presence of inhibitor; V_{max}, the maximum rate; $[S]_0$ and $[I]_0$, initial concentration of substrate and inhibitor, respectively; K_m(app) and K_i, the apparent Michaelis-Menten constant and inhibition constant, respectively. Dividing the first equation by the second and rearranging, eq 1 is obtained. The data in Figure 1 were plotted according to this equation and the value for K_m(app) was calculated from the slope using the value $K_i = 10$ μM. The procedure is generally applicable as long as substrate and inhibitor are in excess of enzyme. The same procedure has been previously described by Inagami (1964). Maximum exchange rates for these two analogs were determined from the Michaelis-Menten equation, using the rate of exchange as measured in the absence of the inhibitor. Initial concentrations of the reaction mixtures were 0.5-1.5 nM Ile-tRNA synthetase, 2 mM ATP, 2 mM sodium pyrophosphate (4×10^4-8×10^4 counts min^{-1} μmol^{-1}), 5 mM $MgCl_2$, 10 mM KF, 0.1 M Tris-HCl, 10 mM 2-mercaptoethanol, 3 μM to 0.6 mM L-isoleucine, or 3 μM to 0.6 mM *O*-methyl-L-threonine, or up to 1.2 mM *N*-methyl-DL-isoleucine or guanidino-L-isoleucine, or up to 27 mM α-DL-aminopentanoic acid, or up to 29.8 mM α-DL-aminohexanoic acid, or up to 6.9 mM α-DL-aminoheptanoic acid. Inhibitors were used at concentrations higher

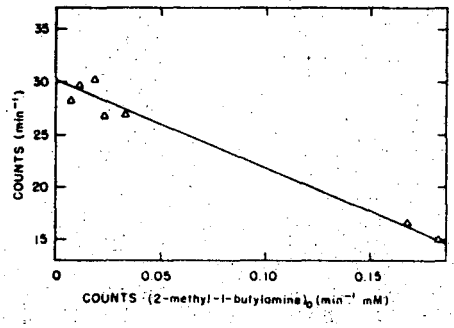


FIGURE 1: Determination of the Michaelis-Menten constant for ATP-[^{32}P]PP$_i$ exchange maintained in the presence of α-DL-aminoheptanoic acid, at pH 8.0, 25°. The rate of exchange is measured as a function of various concentrations of 2-methyl-1-butylamine, a competitive inhibitor. Evaluation is based on the equation $v = v_0 - v[I]_0K_m(\text{app})/[K_i(K_m(\text{app}) + (S)_0]$, where the symbols refer to v_0, the rate of exchange when no inhibitor is present, $[I]_0$ and $[S]_0$, the initial concentrations of inhibitor and substrate, respectively. Initial concentrations were 5 nM Ile-tRNA synthetase, 3.45 mM α-DL-aminoheptanoic acid, 2 mM ATP, 2 mM sodium pyrophosphate (6×10^4 cpm μmol^{-1}), 5 mM $MgCl_2$, 10 mM KF, 0.1 M Tris-HCl, and 0.01 M 2-mercaptoethanol.

than indicated by the inhibition constant. 2-Methyl-1-butylamine was varied from 0.12 to 12.3 μM in the presence of α-DL-aminohexanoic acid at 29.8 mM or α-DL-aminoheptanoic acid at 3.45 mM. Exchange reactions for ATP and related compounds were conducted at 37°, pH 8.0. Initial concentrations of the reaction mixtures were 12 nM Ile-tRNA synthetase, 2 mM L-isoleucine, 2 mM sodium pyrophosphate [1×10^4–2×10^4 counts min^{-1} μmol^{-1}), 10 mM KF, 10 mM 2-mercaptoethanol, 0.1 M Tris-HCl, and 0.05–5 mM ATP. The $MgCl_2$ concentration was varied concomitantly with ATP to provide a 1 mM excess of $MgCl_2$ over the total concentration of ATP plus pyrophosphate. Inhibitor concentrations were: AMP, 2 mM; adenosine, 5.5 mM; and adenine, 10 mM.

tRNA-Charging Reaction. Potassium 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) was tested for inhibition of the tRNA charging reaction using a procedure similar to that described by Muench and Berg (1966).

The amount of incubation mixture used was 800 μl. Initial concentrations were 1 mM ATP, 3 mM $MgCl_2$, 0.025 mM L-[^{14}C]isoleucine (specific activity 25 μCi/μmol), 0.2 mM TNS, 0.04–0.4 260-nm absorbance units of tRNA, 0.4 M Tris-HCl buffer, 1 mM 2-mercaptoethanol, 5 mM KCl, and 0.005 μM Ile-tRNA synthetase. TNS was omitted in control experiments. The mixture was incubated at 24° and 100-μl samples were withdrawn at 5-min intervals. The samples were immediately mixed with 200 μl of an ice-cold solution of 20 mg/ml of RNA from Torula (Calbiochem, lot 44585) and precipitated upon addition of 2 ml of 2 N HCl. The precipitate was allowed to stand on ice for 5 min and was then filtered through a glass fiber filter (Whatman GF/C). The filter was washed several times with 2 N HCl and 45% ethanol, dried, and finally counted in a Packard Tri-Carb scintillation counter.

Fluorescence Titration. The amount of complex formation of a ligand with Ile-tRNA synthetase was measured as the degree of quenching of the fluorescence of TNS which was reversibly bound to the enzyme. The method has been previously described (Holler *et al.*, 1971; Holler and Calvin, 1972). Fluorescence was measured with a Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A. Excitation at 290 nm of the enzyme tryptophan residues as well as excitation at 366 nm of TNS and emission at 470 nm were used. As described previously (Holler *et al.*, 1971), the degree of quenching was higher when the 290-nm excitation was used. Slits were 10 nm for both excitation and emission light path. The sample compartment was thermostated within ±0.5° and was flushed with nitrogen.

In a typical titration experiment aliquots of 0.5 or 1 μl of a solution containing the ligand under study were added from a syringe to 700 μl of buffer containing enzyme and TNS. The mixture was carefully stirred with the needle and 3 min were allowed for the temperature to equilibrate. Radiation was admitted only for the period of fluorescence measurement (about 3 sec) in order to prevent possible photodecomposition. Titration curves were corrected for dilution as measured separately using distilled water instead of titrant. Concentrations of enzyme and TNS were 0.05–0.15 μM, and 5–20 μM, respectively. Buffers were 0.05 M Tris-HCl and 0.02 M sodium phosphate; all solutions contained 0.01 M 2-mercaptoethanol unless otherwise stated.

Under all conditions except for L-isoleucinyl adenylate, ligand was in excess of enzyme and the linearization procedure described by Eadie (1942) was used for evaluation of the dissociation constant and of the maximum quenching at (extrapolated) infinite concentration of ligand. The concentration of L-isoleucinyl adenylate was comparable with the concentration of enzyme and the degree of fluorescence quenching was evaluated according to the method of Dahlquist *et al.* (1966).

Titrations were mostly done at pH 8.0; when pH was varied, sufficient NaCl was added to provide a constant ionic strength of 0.05 M. Errors were calculated as mean deviations for two or more determinations.

Kinetic Measurement. The kinetics of binding of L-isoleucine and 2-methylbutylamine to Ile-tRNA synthetase were measured on a Durrum-Gibson stopped-flow spectrophotometer which had been modified so as to observe the TNS fluorescence as previously described (Holler and Calvin, 1972). Reactions were initiated by rapid mixing of a solution containing enzyme and TNS with an equal volume of a solution containing TNS and the ligands under study. Solutions were made in 0.05 M Tris-HCl buffer, pH 8.0. An excitation wavelength of 290 nm was used and emission was observed through a cut-off filter, Corning No. 373 (35% transmission at 416–436 nm and 80% at 511 nm). The rate constants of the observed reactions were determined from the slopes of the first-order plots and evaluated on the basis of an apparent bimolecular reaction mechanism (Holler and Calvin, 1972) according to the expression

$$k_{obsd} = k_{21} + k_{12}[\text{ligand}]_0 \quad (2)$$

The symbols k_{12} and k_{21} refer to the rate constants for the formation and dissociation, respectively, of enzyme-ligand complexes. The subscript zero indicates initial concentrations ($\gg[E]_0$).

Results

Effect of TNS on the tRNA Charging Reaction. Figure 2 shows the degree of charging as function of time for three different initial concentrations of tRNA. The solution was saturated with TNS which gave a sufficient concentration to complex most of the enzyme (K_s = 0.07 mM; Holler *et al.*,

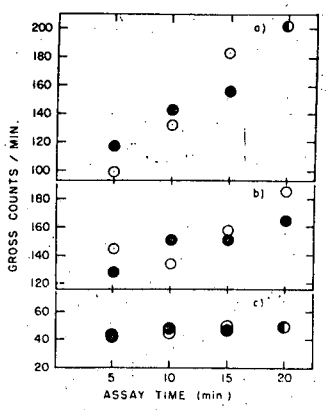


FIGURE 2: $tRNA^{Ile}$-charging reaction catalyzed by Ile-tRNA synthetase in the presence of TNS at pH 8.0, 25°. The extent of amino acylation of $tRNA^{Ile}$ was measured as a function of time in the presence of 0.2 mM TNS (●) and in the absence of TNS (○). Initial concentrations were 1 mM ATP, 3 mM $MgCl_2$, 0.025 mM L-isoleucine, 5 nM Ile-tRNA synthetase, 0.4 M Tris-HCl buffer, 1 mM 2-mercaptoethanol, 5 mM KCl and (a) 0.15 μM, (b) 0.09 μM, and (c) 0.02 μM $tRNA^{Ile}$. Activities are not corrected for background activities and losses due to adsorption effects. It is seen that the extent of charging as a function of time is the same, within experimental error, whether or not TNS is added to the reaction mixture.

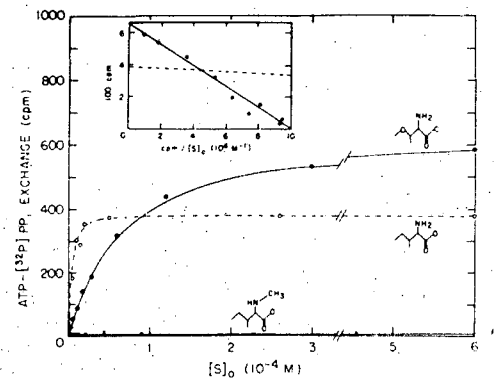


FIGURE 3: ATP-[^{32}P]PP_i exchange for L-isoleucine (○) and *O*-methyl-L-threonine (●), at pH 8.0, 25°. Plot in the inset according to linearization procedure of Eadie (1942) for evaluation of maximum exchange rate (from intercept) and Michaelis-Menten constant (from slope). The broken line has been calculated from parameters, which have been determined from a separate Eadie plot for L-isoleucine. Exchange is not observed for *N*-methyl-DL-isoleucine (bottom). Initial concentrations were 1 nM Ile-tRNA synthetase, 2 mM ATP, 2 mM sodium pyrophosphate (8×10^4 cpm μmol^{-1}), 5 mM $MgCl_2$, 10 mM KF, 0.1 Tris-HCl, and 0.01 M 2-mercaptoethanol.

1971). $tRNA^{Ile}$ and L-isoleucine were at concentrations close to their dissociation constants, 0.2 μM (Baldwin and Berg, 1966) and 5 μM (Cole and Schimmel, 1970; Holler *et al.*, 1971), respectively, so as to provide sensitivity against TNS-induced changes of kinetic and equilibrium parameters. Data are not corrected for background activities. Rates were expected to be inexact because of adsorption effects at the low concentrations of reactants applied (Demushkin *et al.*, 1971). However, if TNS does not impair the enzyme activity, we should find the same activities in the absence and presence of the dye. According to Figure 2, there is agreement within the experimental error suggesting that TNS, at 0.2 mM, has little or no effect on the enzyme activity. The same conclusion has been reached with respect to the amino acid activation reaction (Holler *et al.*, 1971).

L-Isoleucine and Related Compounds. The interaction between Ile-tRNA synthetase and various compounds which are structurally related to L-isoleucine was investigated in terms of Michaelis-Menten constants or inhibition constants and maximum ATP-[^{32}P]PP_i exchange rates and in terms of dissociation constants and maximum quenching of fluorescence intensity as measured in the titration experiments. The results are listed in Table I. The kinetic parameters of the exchange reactions were evaluated directly from Eadie plots (Eadie, 1942) except for α-DL-aminohexanoic acid and α-DL-aminoheptanoic acid. Since the level of exchange for these was low we found it easier to determine the values for K_m(app) from inhibition experiments, as described under Materials and Methods.

We have determined the type of inhibition with respect to L-isoleucine for 2-methyl-1-butylamine, α-DL-aminohexanoic acid, α-DL-aminoheptanoic acid, and *O*-methyl-L-threonine. The inhibition was found to be competitive.

Finally, we have determined the values for the maximum rate of exchange and for the maximum quenching of the fluorescence. The values listed in Table I refer to those for L-isoleucine as unity. Experimental errors determined as standard deviations are between 10 and 15% of the values listed. Maximum quenching as compared to fluorescence in the absence of substrate or inhibitors was about 40% for L-isoleucine, ATP, or pyrophosphate (Holler *et al.*, 1971).

From Table I the following observations were made. (1) in addition to L-isoleucine, radioactive exchange was maintained in the presence of *O*-methyl-L-threonine (Figure 3), L-valine, α-DL-aminopentanoic acid, α-DL-aminohexanoic acid, and α-DL-aminoheptanoic acid. The rate of exchange decreased in the order of listing. *O*-Methyl-L-threonine had a 50% higher exchange rate than L-isoleucine. (2) There is no obvious correlation between the values of the maximum rate of ATP-[^{32}P]PP_i exchange and the value for the Michaelis-Menten constant. The apparent decrease of the maximum rate for the racemic substrates is presumably due to inhibition by the unreactive D enantiomer (Mehler, 1970). Similarly, we found that the degree of maximum quenching of the fluorescence was not a function of the dissociation constant, but was the same, within experimental error, for all substrates tested. (3) Values for K_m(app) and K_i are almost identical, indicating that binding of co-ligands (as in the ATP-[^{32}P]PP_i exchange) had little, if any, effect on the dissociation constant as measured by fluorescence titration. (4) Modification of the amino group of L-isoleucine by either methylation or guanidation was associated with the loss of binding. (5) When the 3-methyl group or the terminal methyl group was removed from the side chain, the stability of the enzyme-amino acid complex was considerably reduced. Elongation as measured for α-DL-aminohexanoic acid and α-DL-aminoheptanoic acid had little effect on the maximum rate of exchange and only a modest effect on the value of the Michaelis-Menten constant. (6) Blocking of the carboxylic group as in L-isoleucine methyl, ethyl, and hexyl ester reduced the affinity for complex formation and, of course, abolished the catalytic reaction. Interestingly, the affinity increased as the length of the alcohol component increased. A similar observation has been reported by Santi *et al.*

TABLE I: Interaction of L-Isoleucine and Related Compounds with Ile-tRNA Synthetase at pH 8.0.

Compound[a]	Method[b]	K_i[c] (μM)	K_m[d] (μM)	K_s[e] (μM)	Max Quenching or Max Exchange Rate[f]
L-Isoleucine	F			5.8 ± 0.8	1.0[i]
	P			4.0[g]	
	P		5[h]		1.0
O-Methyl-L-threonine	F			(1.2 ± 0.2) × 10^3	1.0
	P		60 ± 10		1.6
L-Valine	F			(1.6 ± 0.5) × 10^3	
	P		3.9 × 10^2[h]		~0.5
	P		8 × 10^2[i]		0.8
α-DL-Aminopentanoic acid	F			(2.2 ± 0.7) × 10^3	1.0
	P		(1.15 ± 0.1) × 10^3		0.7
α-L-Aminopentanoic acid	P		1.5 × 10^3[i]		0.9
α-DL-Aminohexanoic acid	F			(7 ± 2) × 10^3	1.0
	P		7 × 10^2[j]		0.35
α-DL-Aminoheptanoic acid	P		1.8 × 10^4[j]		0.35
N-Methyl-DL-isoleucine	F				None
	P	>10^4			
Guanidino-L-isoleucine	F				None
	P	>10^5			
L-Isoleucine methyl ester	F			(5.9 ± 0.8) × 10^2	1.0
L-Isoleucine ethyl ester	P (37°)	2.7 × 10^2[j]			
L-Isoleucine *n*-hexyl ester	P (37°)	40[j]			
3-Methylpentanoic acid	F			>1 × 10^5	None
	P	>5 × 10^5			
L-Isoleucinol	F (10°)			(5.5 ± 1) × 10^3	1.0 (excitn 290 nm)
1.5 mM PP_i + 2 mM $MgCl_2$	F (10°)			~2 × 10^3	
1 mM PP_i + 2 mM $MgCl_2$	F			(1.4 × 0.3) × 10^3	1.0 (excitn 290 nm)
	P	23[k]			
L-Isoleucinyl adenylate	F (10°)			(1.3 ± 0.3) × 10^{-2}	
	P	7 × 10^{-3}[k]			
3-Methyl-1-pentanol	F			(2.5 ± 1) × 10^3	(excitn 290 nm)
	F (10°)			(1.6 ± 0.3) × 10^3	1.0
3-Methylpentanal	F (10°)			(2.5 ± 1) × 10^3	
2-Methyl-1-butanol	F			(1.7 ± 0.3) × 10^4	1.0
	P	>5 × 10^5			
2-Methyl-1-butylamine	F			(9 ± 1) × 10^3	1.0
	F (12°)			(5.1 ± 1.0) × 10^3	1.0
	P	10 ± 2			
2 mM PP_i	F			(9 ± 2) × 10^3	
1 mM PP_i + 1.5 mM $MgCl_2$	F			(1.7 ± 0.6) × 10^3	
1 mM ATP	F			3.8 ± 1.0	
1 mM ATP + 2 mM $MgCl_2$	F			4.5 ± 1.0	
1 mM ATP, 1 mM PP_i + 1.5 mM $MgCl_2$	F			2.5 ± 0.5	
4.6 mM AMP	F			27 ± 2	
1 mM ATP	Kinetically			2.6[j]	

[a] Investigated at 25° unless otherwise stated. [b] Method of investigation: P, ATP-[^{32}P]PP_i exchange; F, fluorimetric titration. Excitation wavelength 366 nm, emission wavelength 470 nm. [c] Inhibition constant from ATP-[^{32}P]PP_i exchange measurements. [d] Michaelis-Menten constant from ATP-[^{32}P]PP_i exchange measurements. [e] Dissociation constant from titration. [f] With reference to values for L-isoleucine, which were arbitrarily set equal to 1.0. [g] Cole and Schimmel, 1970. [h] Berg *et al.*, 1961. [i] Loftfield and Eigner, 1966. [j] Experimental error was not calculated. [k] Cassio *et al.*, 1967. [l] Standard deviations are 10–15%.

(1971) for Phe-tRNA synthetase from *E. coli*. (7) When the α-amino group was removed, as in 3-methylpentanoic acid, an inert compound was obtained. (8) Removal of the α-amino group and substitution of the carboxylic group by an alcohol or an aldehyde group, as in 3-methyl-1-pentanol or in 3-methylpentanal, reduced binding considerably but did not abolish it. (9) When the α-amino group was not removed and the carboxylic group was substituted by hydrogen or an alcohol group as in 2-methyl-1-butylamine and L-isoleucinol, respectively, the modified compounds were potent inhibitors in the radioactive exchange reaction but poor ligands for the fluorimetric titration in absence of co-ligands.

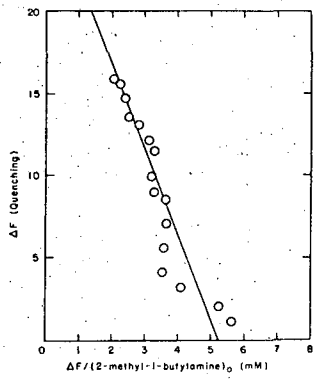


FIGURE 4: Titration of Ile-tRNA synthetase–TNS with 2-methyl-1-butylamine at pH 8.0, 12°. All other ligands were omitted from the reaction mixture. Initial concentrations were 0.19 μM enzyme, 12 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Concentration of 2-methyl-1-butylamine was varied between 0.19 and 7.7 mM, excitation wavelength, 366 nm; emission wavelength, 470 nm. Light scatter was reduced by placing a 430-nm cutoff filter in the emission path.

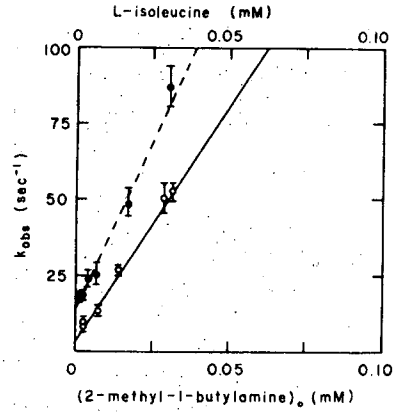


FIGURE 5: Kinetics of fluorescence quenching following rapid mixing of a solution containing enzyme with a solution containing ATP plus 2-methyl-1-butylamine (open circles) or L-isoleucine (filled circles), at pH 8.0, 25°. Experiments were accomplished with a modified Durrum-Gibson stopped-flow spectrofluorimeter. Initial concentrations were 0.075 μM Ile-tRNA synthetase, 43 μM TNS, 1 mM ATP, 0.05 M Tris-HCl, and 0.01 M 2-mercaptoethanol.

Binding of 2-Methyl-1-butylamine under Various Conditions. In the absence of co-ligands, 2-methyl-1-butylamine interacts only weakly with Ile-tRNA synthetase as measured by titration, Figure 4. Addition of pyrophosphate, AMP, or ATP at saturating concentrations was associated with a strong decrease of the value for the dissociation constant. ATP produced a decrease about 1000 times greater than that produced by pyrophosphate. The coupling was not sensitive to magnesium ions (<2 mM). In the presence of ATP or Mg-ATP^{2-} 2-methyl-1-butylamine binds almost as strongly as L-isoleucine, as evidenced from titration experiments. The value for the inhibition constant from the ATP-[^{32}P]PP$_i$ exchange reaction was found to be higher than expected for ATP as the only co-ligand. Our interpretation of this is that part of the enzyme is bound to pyrophosphate instead of to ATP. Since 2-methyl-1-butylamine binds more weakly when pyrophosphate is the co-ligand, the inhibition constant of the exchange reaction would indeed be expected to have a higher value.

Kinetics of Binding of 2-Methyl-1-butylamine in the Presence of ATP. The value of the dissociation constant for binding of 2-methyl-1-butylamine to Ile-tRNA synthetase–ATP is comparable with the value for L-isoleucine. It was of interest to see whether the kinetics were also similar. Fluorescence was measured as a function of time following the rapid mixing of a solution containing enzyme plus TNS with a solution containing ATP, TNS, and 2-methyl-1-butylamine at various concentrations. Only one process was observed which did not appear when the solutions containing the reactants separately were mixed with an enzyme solution. The observed rate constants were plotted as shown in Figure 5 as a function of the concentration. The linear concentration dependence is consistent with the apparent bimolecular reaction

$$E + I \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EI$$

and the values for the rate constants, k_{12} and k_{21}, were determined from eq 2 to be $k_{12} = 1.5 \times 10^6$ M^{-1} sec^{-1} and $k_{21} = 4$ sec^{-1}. The value for the kinetically defined dissociation constant, $K_s = k_{21}/k_{12} = 2.6$ μM is in agreement with the value from the titration experiments. The kinetic constants were found to be indeed similar to those observed for L-isoleucine, namely, $k_{12} = 2.2 \times 10^6$ M^{-1} sec^{-1} and $k_{21} = 15$ sec^{-1} (Holler and Calvin, 1972).

pH Dependence for L-Isoleucine Binding and Fluorescence Quenching. The following investigation was designed to collect information about ionizing groups which are somehow involved in substrate binding and fluorescence quenching. The dissociation constant for the binding of L-isoleucine and the fluorescence intensities of the enzyme–TNS complex and of the enzyme–TNS–L-isoleucine complex were measured over the pH range 7–10 at a constant ionic strength of 0.05 M (Figure 6). We believe that no pH-dependent denaturation occurs in this range for the following reasons. (1) Fluorescence of the protein when excited at 280 nm has been reported to be sensitive to denaturation (Penzer *et al.*, 1971). Thus, protein fluorescence may be used to determine when denaturation occurs. Upon increasing pH, we found that fluorescence became unstable only when pH 10 was exceeded. (2) The degree of fluorescence at saturating concentrations of L-isoleucine was found to be invariant against pH. This observation would hardly be consistent with a pH-induced denaturation.

It is seen from Figure 6 that fluorescence of the enzyme–TNS complex begins to decrease when pH 8.5 is exceeded. Similarly, the stability of the enzyme–L-isoleucine complex, as reflected by the value of K_{Ile}, remains constant up to pH 8.5, and then decreases. The fluorescence of the enzyme–TNS–L-isoleucine is invariant against pH and apparently coincides with fluorescence of the enzyme–TNS complex at pH 10. The observations are consistent with a pK(app) = 9.3–9.5 for an ionization of a group involved in the L-isoleucine-induced fluorescence quenching. The pH profile for the dissociation constant is too incomplete to decide whether it is determined by the same pK(app).

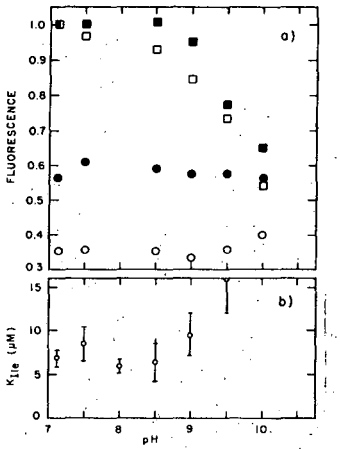


FIGURE 6: Fluorescence intensity of Ile-tRNA synthetase–TNS and of the Ile-tRNA synthetase–TNS–L-isoleucine complex (a) and the stability of the Ile-tRNA synthetase–TNS–L-isoleucine complex (b) as a function of the pH of the reaction mixture at 25°. Squares refer to the enzyme–TNS complex and circles to the enzyme–TNS–L-isoleucine complex. Excitation at 290 nm is indicated by open symbols and excitation at 366 nm by filled symbols. Emission was observed at 470 nm. The values for the fluorescence intensity are normalized to those at pH 7.25. Initial concentrations were 0.18 μM enzyme, 9 μM TNS, 4 μM to 2.4 mM L-isoleucine, 0.05 M Tris-HCl, and sufficient NaCl to provide a constant ionic strength of 0.05 M. Note the stronger quenching for excitation at 290 nm, giving evidence for a substrate-induced conformation change (Holler *et al.*, 1971).

TABLE II: Interaction of ATP and Related Compounds with Ile-tRNA Synthetase at pH 8.0.[a]

Compound	Method	K_i (mM)	K_m (mM)	K_s (mM)	Max Quenching[b]
ATP	F			0.25 ± 0.03	1.0
	P		0.15		
	F (37°)			0.52[d]	
	P (37°)		0.42[d]		
AMP	F			0.75 ± 1	0.6
	P (37°)	0.9[d]			
Adenosine	P (37°)	0.25[d]			
Adenine	P (37°)	30[d]			
D-Ribose	P (37°)	17[d,e]			
Pyrophosphate	F			0.26 ± 0.07	1.0
	P[c]		0.03		

[a] Investigation at 25° unless otherwise stated. The same comments apply as for Table I. [b] Standard deviations are 10–15%. [c] Cole and Schimmel, 1970. [d] Experimental error was not calculated. [e] Based on assumptions that only the β-furanose form is inhibitory and that this form comprises 18% of the D-ribose in an aqueous solution at equilibrium (Angyal and Pickles, 1967).

ATP and Related Compounds. The interaction of ATP and related compounds with Ile-tRNA synthetase was investigated in a fashion similar to that for L-isoleucine and related compounds. The results from ATP-[^{32}P]PP$_i$ exchange and titration experiments are summarized in Table II. Inhibition constants were measured against ATP. AMP, adenosine, adenine, and D-ribose were found to be competitive inhibitors. A typical Lineweaver–Burk plot (Lineweaver and Burk, 1934) is shown for ATP, adenosine, and adenine in Figure 7. Some of the observations for the L-isoleucine related compounds were repeated here. (1) The values for the Michaelis–Menten constants are similar to the values of the dissociation constants as determined from fluorescence titration. (2) The degree of fluorescence quenching is the same for L-isoleucine, ATP, and pyrophosphate.

When the inhibition constant of adenosine is compared with those of its components, adenine and D-ribose, we find that the K_i(app) for adenosine is approximately equal to the product of the K_i(app) values for the latter two compounds, indicating the likelihood that the adenyl and ribosyl moieties of adenosine bind essentially independently of one another.

When we compare the binding of ATP with its components, AMP and pyrophosphate, however, we discover that all three bind with roughly equivalent affinities. Thus the dissociation constant of ATP is much greater than would have been predicted from the dissociation constants of the subunits.

Discussion

Ile-tRNA synthetase very likely exists as a monomer in aqueous solutions under conditions similar to those used in our experiments (Baldwin and Berg, 1966; Arndt and Berg, 1970; Berthelot and Yaniv, 1970). One molecule of L-isoleucine (Berthelot and Yaniv, 1970) or one molecule of L-isoleucyl adenylate (Baldwin and Berg, 1966) was found to combine with one molecule of enzyme, suggesting one active site per protein molecule. This conclusion is in accord with our finding that kinetic and equilibrium investigations are interpretable on basis of a single site for each ligand under study.

Structural Components of the L-Isoleucine Specific Site. Loftfield and Eigner (1966) have discussed the involvement of hydrophobic forces in the specific enzyme–L-isoleucine interaction. Further evidence has been reported by Holler and Calvin (1972) on the basis of thermodynamic properties. Particular information is gained by comparison of the values for the dissociation and Michaelis–Menten constants of compounds with varying structures of the side chain. Removal of the 3-methyl group of L-isoleucine as in α-DL-aminopentanoic acid is associated with a 400-fold increase of the value for the dissociation constant, equivalent with the loss of approximately 3.6 kcal for the standard free energy of complex formation. (No correction is made for the D enantiomer. If the D enantiomer does not bind, the actual loss is smaller by 0.4 kcal.)

Similarly, when the methyl group of the longer tail of the L-isoleucine side chain is removed, generating L-valine, 2.2 kcal is lost. We conclude that both the methyl and the ethyl groups of L-isoleucine bind to subsites which are both hydrophobic. Consequently, when the methylene group of the ethylene subsite is replaced by oxygen, the extent of hydrophobic interaction is decreased as indicated by the loss of 1.9 kcal of standard free energy as evaluated for *O*-methylthreonine. The increments for hydrophobic binding exceed by far what is known from analysis of enzyme–ligand interactions. Heidberg *et al.* (1967) have investigated in detail the hydrophobic interaction between 1-*n*-alkylamines and the active site of trypsin. They have determined the incre-

ments to be 1.5 kcal for the methyl group and 1.1 kcal for the methylene group, the same values as have been found for the transfer of hydrocarbons from water to nonpolar solvents (Kauzmann, 1959; Nemethy and Scheraga, 1962). A similar conclusion has been put forward by Loftfield and Eigner (1966).

It was of further interest to determine whether the two hydrophobic subsites, which may be considered as pockets or slots, were closed at their ends, as is the case for trypsin and chymotrypsin (Steitz *et al.*, 1969). When the *n*-alkyl side chain of the α-aminopentanoic acid is elongated we expect one of two alternative responses. Either the elongated tail extends beyond the end of the slot if it is open, or the α-amino group and the carboxylic group are pushed out of their places into catalytically improper positions if the end of the slot is closed. For the first alternative, we would expect that the elongated substrate maintains the same level of ATP-[^{32}P]PP$_i$ exchange as observed for α-aminopentanoic acid and that the Michaelis-Menten constant would be almost invariant. For the second alternative, a substantial decrease in the rate of exchange and binding affinity would be expected. Comparison of the data for α-DL-aminopentanoic acid, α-DL-aminohexanoic acid, and α-DL-aminoheptanoic acid indicates that both parameters were only slightly changed, suggesting that one, if not both, ends of the subsites are not rigidly closed.

The results of the removal of the α-amino or carboxylic group are intriguing. When they were both removed and the carboxylic group was replaced by an alcohol or aldehyde group the affinity for forming complexes with Ile-tRNA synthetase dropped by 3.7 kcal for the 3-methylpentyl-1-derivatives and by 4.8 kcal for 2-methyl-1-butanol. The different values indicate a weak interaction for the alcohol or aldehyde group of the 3-methylpentyl-1 derivatives. However, when this group becomes a carboxylic group, interaction is abolished. It is possible that the alcohol or (hydrated) aldehyde group interacts *via* a hydrogen bond with the enzyme and that the abolishing effect of a carboxylic group is an electrostatic repulsion directed from a negatively charged group at the active site. Possibly this negative charge could interact favorably with the α-ammonium group of the amino acids, contributing the 3.7 kcal missing for the neutral 3-methylpentyl-1 derivatives. However, we learn from the dissociation constants for L-isoleucinol and 2-methyl-1-butylamine (in the absence of co-ligands) that our model is incomplete or incorrect. Comparing L-isoleucine with L-isoleucinol and 2-methyl-1-butylamine (in the absence of co-ligands) we find that stability of the enzyme-ligand complexes is decreased by 4.1 and 4.5 kcal, respectively. Thus, incorporation of the α-amino group alone does not enhance the binding affinity. Yet, in the specific substrate L-isoleucine the α-amino group must contribute at least 4.5 kcal, the only difference being that the carboxylic group is present. Apparently, both groups must be involved simultaneously in order to provide a maximum of interaction. A sensible approach to mimic the electrostatic effect of the carboxylic group seemed to us to provide an additional external negative charge, for instance, by binding ATP, AMP, or pyrophosphate to their specific sites. The outcome of the experiments is in accord with our assumption. When ATP is present as a co-ligand, 2-methyl-1-butylamine binds as strongly as L-isoleucine. The effects for AMP and pyrophosphate are gradually weaker. Similarly, L-isoleucinol binds 60 times more strongly under conditions of ATP [^{32}P]PP$_i$ exchange than in the absence of co-ligands. The similarity of the interactions of 2-methyl-1-butylamine and L-isoleucine

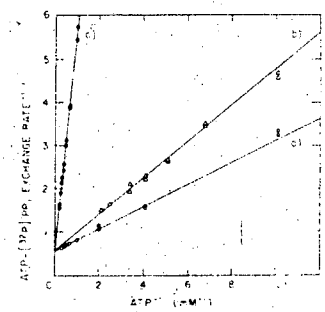


FIGURE 7: Lineweaver-Burk plot for the rate of ATP-[^{32}P]PP$_i$ exchange as a function of the ATP concentration in the presence of inhibitors, 37°, pH 8.0. Initial concentrations were 1.2 nM Ile-tRNA synthetase, 2 mM L-isoleucine, 2 mM sodium pyrophosphate, 10 mM KF, 0.1 M Tris-HCl, 0.01 M 2-mercaptoethanol, 0.05-5 mM ATP. The reaction mixture contained (a) no inhibitor, (b) 10 mM adenine, and (c) 5.5 mM adenosine. The concentration of $MgCl_2$ was varied concomitantly with the concentration of ATP to provide a 1 mM excess of magnesium over the total concentration of ATP plus pyrophosphate.

with Ile-tRNA synthetase includes also the kinetic constants, as is indicated in Figure 5, and presumably also the type of mechanisms, which was found to be a two-step process for L-isoleucine (Holler and Calvin, 1972).

The observation that L-isoleucine-related compounds, lacking either the α-ammonium group or the carboxylic group, are poor inhibitors, if at all, together with the finding that ATP, AMP, and pyrophosphate promote the interaction between the active site and an inhibitor lacking the carboxylic group, can be understood on the basis of the following tentative model. One of the structural components of the active site is an ion pair. Binding of L-isoleucine is associated with an opening of the pair and formation of new pairs at the α-ammonium group and at the carboxylic group, respectively, of the substrate. The positive charge paired with the substrate carboxylic group may simultaneously interact with the oligophosphate group of ATP. Since we have found that binding of L-isoleucine and ATP is independent (Holler *et al.*, 1971), this positively charged group must have a similar position in both enzyme ligand complexes. The opening of the ion pair could be the initiation of the conformation change which was observed to follow the attachment of L-isoleucine to the enzyme (Yarus and Berg, 1969; Holler *et al.*, 1971; Holler and Calvin, 1972). The ion pair is also opened when, for instance, ATP binds to its specific site. The rate of the opening (and the conformation change) varies with the extent of the side chains of both the L-isoleucine-related compounds and the ATP-related compounds. In the case when the carboxylic group is lacking, as for L-isoleucinol and 2-methyl-1-butylamine, the open ion pair is poorly stabilized, presumably because positively charged groups approach each other too closely. However, when the open conformation has already been stabilized by ATP or related compounds, attachment of the inhibitor is easy.

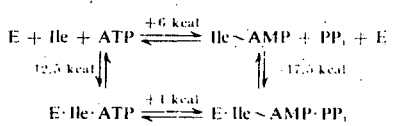
The extent of the conformation change is reflected in the degree of fluorescence quenching induced by binding of the various ligands. From Tables I and II we have evidence that the extent is invariant against the structure of the compound in the binary enzyme ligand complex. Thus, neutral L-iso-

leucine-related compounds induce the same degree of quenching as do L-isoleucine, ATP, AMP, and pyrophosphate. Apparently, the hydrophobic binding is rigidly coupled to the opening of the ion pair. Moreover, the rigidity of the substrate specific site is indicated by only a threefold decrease in the maximum rate of ATP-[^{32}P]PP$_i$ exchange as compared with a 3000-fold difference between the values for K_m(app) when L-isoleucine and α-DL-aminoheptanoic acid are compared (Table I). Furthermore, rigidity may be the reason why methylation or guanidation of the α-amino group is associated with a complete loss of binding ability. Apparently, binding of the modified ammonium group is prohibited by steric hindrance, thus concomitantly preventing binding at the rigidly coupled hydrophobic site. As has been pointed out by Loftfield and Eigner (1966), this rigidity is in severe contrast to the sensitivity of the rate of hydrolysis catalyzed by chymotrypsin and trypsin as a function of the stability of the enzyme–substrate complexes.

From the description of our model an easy test of the ion-pair hypothesis is suggested. It must be possible to open the ion pair simply by pH titration, by neutralizing the positive component. We have done the experiment, Figure 6, following the fluorescence properties of the enzyme-TNS and the enzyme-TNS-L-isoleucine complex. In accord with our hypothesis, we found that fluorescence quenching is produced on increasing pH, ultimately reaching that produced by the binding of a substrate. The apparent pK of 9.5 is in agreement with the ionization of an amino group.

In summary, we have presented evidence for the existence of a hydrophobic binding site for L-isoleucine consisting of two subsites to take up the methyl group and the ethyl group, respectively, of the aliphatic side chain. One or both subsites may be open at the ends. The strength of binding to these subsites cannot be accounted for on the basis of hydrophobic interactions alone but presumably reflects a coupling to secondary interactions. It is supposed that the hydrophobic binding is rigidly linked to the opening of an ion pair. The opening is easily accomplished with L-isoleucine, ATP, or AMP, but appears to be difficult with L-isoleucinol, 2-methyl-1-butylamine, or 3-methylpentanoic acid, presumably because of electrostatic repulsion. The strong inhibition observed for L-isoleucinol and 2-methyl-1-butylamine as observed in the ATP-[^{32}P]PP$_i$ exchange reaction is coupled to the binding of ATP, whereas the binding of L-isoleucine apparently is not. We think that care has to be taken when the binding of an amino alcohol analog of an amino acid is used as a tool to investigate the kinetic pathway of the catalytic reaction as has been done in the case of the leucyl activating enzyme from *E. coli* (Rouget and Chapeville, 1971).

Free-Energy Considerations for the Ile-tRNA Synthetase ATP Complex. We have recently presented evidence (Holler and Calvin, 1972) that formation of L-isoleucyl adenylate from L-isoleucine and ATP is promoted by approximately 5 kcal when substrates and products are bound to Ile-tRNA synthetase as compared to the reaction in the free solute state. The relation between the standard free energy for the reaction of L-isoleucine and ATP and the standard free energy of the reaction when they are bound to the enzyme is described by the following equation



It is seen that the L-isoleucyl adenylate formation is promoted by complex formation with the reactants because the standard free energy of binding both substrates is overcompensated by the standard free energy of binding both products. One possible explanation is that ATP takes up free energy upon binding to the enzyme resulting in partial distortion or strain of those parts of the substrate which have to undergo reaction. As indicated in Table II, each of the structural components of ATP (*i.e.*, AMP and pyrophosphate) binds separately to the enzyme with an affinity comparable to that of ATP itself. Linked together as ATP, these components tend to come as close as possible into contact with their particular binding sites in order to achieve a maximum of interaction. This tendency apparently forces the ATP into a strained configuration. The final result is a compromise between the intensity of ligand-enzyme interaction and the degree of distortion or "strain" of the ATP molecule. As an estimate of the free energy taken up in the distortion, we may consider the difference between the free energy of binding of ATP and the sum of the binding energies of AMP and pyrophosphate as determined from the dissociation constants. Thus, $\Delta G_{dist} = \Delta G_{ATP} - (\Delta G_{AMP} + \Delta G_{PP_i}) \approx -5$ kcal + 4.3 kcal + 5 kcal = 4.3 kcal. Of course, this calculation can only be regarded as a crude approximation. Nevertheless, the value of 4.3 kcal for ΔG_{dist} is in agreement with the estimated 5 kcal by which the enzyme-mediated L-isoleucine activation is favored over the non enzymic reaction.

We found that adenosine is bound somewhat more strongly than AMP. A tight binding of moieties on both sides of the residue undergoing reaction is exactly what one would anticipate for the effectiveness of a distortion in enhancing reactivity. Since it is the α-phosphate of ATP which undergoes nucleophilic substitution in the reaction, we would have indeed expected the adenosine and the β,γ-pyrophosphate to be bound quite strongly to the enzyme.

We have tentatively proposed that ATP assumes a high energy state when bound to the enzyme. A high energy state of the substrate has been proposed for the hydrolysis of chitohexose by lysozyme on the basis of crystallographic and chemical evidence (Blake *et al.*, 1967; Rupley and Gates, 1967; Rupley *et al.*, 1967). The hypothesis may be of general interest. Santi *et al.* (1971) have reported values of the Michaelis Menten and inhibition constants for the interaction of ATP, AMP, adenosine, and pyrophosphate with Phe-tRNA synthetase from *E. coli* which are consistent with the hypothesis.

The ATP [^{32}P]PP$_i$ exchange reaction of the L-isoleucine system requires magnesium ions. We believe that the function of magnesium may be understood mainly in terms of orientation of the pyrophosphate moiety within the Michaelis-Menten complexes and possibly as a stabilizer of the transition state, as has been discussed by Santi *et al.* (1971). Experiments are underway to elucidate this requirement and to add support to our hypothesis.

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APPENDIX C: A PROPOSAL FOR THE ACTIVE INSOLUBILIZATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE

A major obstacle to the use of insolubilized enzymes for the large-scale synthesis of various materials has been the requirement of coenzymes for many of the possible reactions. In the current state of the art, it has been necessary to provide the coenzymes in the reaction broth. This necessitates the subsequent separation of the coenzyme from the product and its disposal or recycling. Either approach is economically unfeasible.

A solution to this problem would be the attachment of the coenzyme to the matrix to which the enzyme is bound. The coenxyme must be attached in such a way that it may interact freely with the enzyme during the reaction and then diffuse away to be regenerated either by cheap, soluble reagents in the broth which can be readily separated from the product, or, where appropriate, by a second enzyme bound to the matrix.

We believe that N^6-substituted NAD^+ or NADP could be synthesized and insolubilized using techniques similar to those developed for the insolubilization of L-isoleucinol adenylate.

There have been several reports in the literature of the insolubilization of NAD^+. It has reportedly been attached to supports by reaction with cyanogen bromide-activated agarose,[1] by coupling with a carbodiimide,[1,2] or by diazonium coupling.[1,3] All of the reactions were carried out on unmodified NAD^+, and in all cases the coupling was postulated to have occurred on the adenosine moiety. We attempted to couple N-t-BOC-L-isoleucinol adenylate to agarose using all these techniques and met with a uniform lack of success. It seems unlikely that the adenosine moiety of the latter should differ significantly in reactivity from that of the former. We feel that the reported couplings may be questionable.

Moreover, the covalent linkages which should have been produced by these procedures can be expected to be quite labile and readily hydrolyzed in neutral solution (see Part I, Sec. B). Attachment via N^6-alkyl derivatives should yield a quite stable material.*

Our procedures can also be readily adapted to attachment of NAD^+ by a polyethylene glycol tail, which we believe would be most suitable for the proposed system. Specifically, Polyglycolamine H-163 could be reacted with 6-chloropurine riboside 5'-phosphate and the resulting molecule elaborated into a substituted NAD^+. Another molecule of Polyglycolamine H-163 could be attached to the support (This could be done concurrently with the insolubilization of the enzyme). The two free hydroxyl ends of the derivatives could be selectively joined with divinyl sulfone.[4]

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*We realize that NAD^+ itself will be slowly degraded, even in cold, neutral water. However, it should be possible to prepare the matrix in such a way that the NAD^+ is normally in a complex with the enzyme. This should result in considerable stabilization of the coenzyme (and should help stabilize the enzyme as well).

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