PURIFICATION AND PROPERTIES OF SOME HALOPHILIC ENZYMES

STUDIES ON PURIFICATION AND PROPERTIES OF SOME HALOPHILIC ENZYMES

By

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ABSTRACT

The methods of affinity chromatography, heat treatment, and chromatography on agarose with $(NH_4)_2SO_4$ were used in the presence of high salt concentrations to purify halophilic enzymes. The emphasis was on the purification of tryptophanase and tryptophanyl-tRNA ligase.

Tryptophanyl-tRNA ligase was purified to homogeneity and its molecular weight and amino acid composition was determined. Its kinetic properties were examined. The properties of this halophilic enzyme were compared to the properties reported for non-halophilic tryptophanyl-tRNA ligases.

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LIST OF ABBREVIATIONS

AHT	Agarose-hexamethylenediamine-tryptophan.
A_x	The absorbance at x nm. An A_x unit has an absorbance of 1 in a 1 cm pathlength.
BSA	Bovine serum albumin.
K_D	Dissociation constant.
K_m	Michaelis constant.
MDH	Malate dehydrogenase.
ME	2-mercaptoethanol.
PAGE	Polyacrylamide gel electrophoresis.
PLP	Pyridoxal 5'-phosphate, cofactor for tryptophanase.
PRS	L-Phenylalanine:tRNA ligase (AMP) (E.C. 6.1.1.9).
SDS	Sodium dodecyl sulfate.
Solution D	3 M KCl, 0.1 M Mg $(C_2H_3O_2)_2$, 20 mM Tris-HCl, pH 7.7, 0.8 mM ME.
Solution D'	3 M KCl, 0.1 M Mg $(C_2H_3O_2)_2$, (magnesium acetate), 10 mM Tris-HCl, pH 7.7, 8 mM ME.
Solution E	5 M NaCl, 0.1 M Mg $(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 7.5, 8 mM ME.
TCA	Trichloroacetic acid.
Tris	Tris (hydroxymethyl) aminomethane.
tRNA	Transfer ribonucleic acid.
Trp	Tryptophan.
TRS	L-Tryptophan:tRNA ligase (AMP) (E.C. 6.1.1.2), also referred to in the literature as tryptophanyl-tRNA synthetase.
UV	Ultraviolet light.

1. INTRODUCTION

1.1. Halophilic bacteria and their proteins

Extremely halophilic bacteria are adapted to growing in 20-30% salt and the genus *Halobacterium* can only tolerate NaCl concentrations as low as 12-15%. In adapting to such a highly ionic environment, the internal concentration of monovalent cations in these bacteria has become very high. Christian and Waltho (1962) reported that the intracellular K^+ concentration was 4.6 moles/kg cell water and the Na^+ concentration was 1.4 moles/kg cell water for *Halobacterium salinarium* which was grown in 4 M NaCl + 0.03 M KCl.

The dependence of halobacteria on concentrated salt appears to be attributable to properties of their proteins. Thus lysis in dilute salt is due not to osmotic shock but to the disintegration of the cell envelope. This has been suggested to be due to the mutual repulsion of acidic envelope proteins (Kushner and Onishi, 1966; Stoekenius and Kunau, 1968; Kushner, 1968). Ribosomes of *Halobacterium cutirubrum* are similar to the ribosomes of non-halophilic bacteria but rely on high ionic strength for their stability. At low ionic strength many of the ribosomal proteins dissociate (Bayley and Kushner, 1964; Bayley, 1966). This dissociation has been attributed to the acidity (Bayley, 1966) and

polarity (Lanyi, 1974) of the proteins. Furthermore, many enzymes from halophilic bacteria require concentrated salt for their stability and activity (for review see Lanyi, 1974). By contrast, studies on tRNA's from *H. cutirubrum* and *E. coli* in heterologous cell-free protein synthesis systems (White and Bayley, 1972) suggest that nucleic acids are not critically dependent on salt concentrations for their functioning.

A study of the proteins of halophilic bacteria should therefore provide an important approach to understanding how the molecular biology of these bacteria has adapted to an environment of saturated salt. In particular, enzymes offer a convenient means for investigating salt-protein interactions since, as they are biochemical catalysts, their activities and stabilities can be easily monitored.

1.2. Purification of halophilic enzymes

The major difficulty of studies on halophilic bacteria is that many of the enzymes are inactive and unstable at the low ionic strengths employed by conventional purification procedures such as ion exchange and electrophoresis.

For studies of enzyme kinetics, purification may be necessary because in most cases crude mixtures of enzymes contain other enzymes which can act on substrates, products and the enzyme of interest and the mixtures also may contain substrates, products and interfering compounds. Also the determination of molecular activity and amino acid composition

requires pure enzymes free of contaminating proteins.

To purify halophilic malate dehydrogenase, Holmes and Halvorson (1965) dialyzed cell extracts against low ionic strength buffers containing stabilizing substrate, purified the enzyme by conventional techniques and then reactivated the enzyme by dialysis against high ionic strength buffers. Yield was less than 1%. Similar procedures were used to purify or partially purify a number of other halophilic enzymes including isocitrate dehydrogenase, citrate synthetase and aspartate transcarbamylase. Recoveries of enzyme activity by these types of procedures were generally quite low which may indicate that the final products were contaminated with inactive enzyme molecules. This method often irreversibly inactivated halophilic enzymes completely, while enzymes that were reactivated often required less than 1 M salt for maximum activity.

Some halophilic enzymes are able to tolerate high and low levels of salt (salt-tolerant) while others depend on high levels of salt (salt-dependent). This may be related to the nature of the reactions they catalyze. For example, MDH and catalase bind substrates quite well in high and low levels of salt (Lanyi and Stevenson, 1969); aminoacyl-tRNA ligases could not reversibly bind tRNA if removed from their normal salt concentrations because

protein-nucleic acid interactions are salt-sensitive (Loftfield, 1972). To purify salt-dependent enzymes special techniques are required.

Ornithine transcarbamylase could not be reactivated from lower ionic strength solutions but was purified in the presence of 4.3 M NaCl and 0.1 M ornithine by acetone fractionation, gel filtration, sucrose gradient centrifugation and calcium phosphate gel chromatography (Dundas, 1970). The enzyme was 50-fold purified and showed mainly one band on polyacrylamide gels.

Louis and Fitt (1971a, 1971b) describe the purification of RNA- and DNA-dependent RNA polymerases and Peterkin and Fitt (1971) describe the partial purification of polynucleotide phosphorylase by a combination of pH 4 precipitation, $(NH_4)_2\ SO_4$ precipitation, dextran gel chromatography and hydroxyapatite chromatography. High salt solutions were used throughout. Purifications were extensive and homogenous protein preparations were achieved on the first two enzymes but a great deal of the purification depended on the very unusually small size (11,000-18,000 daltons) of these enzymes and separation from nucleic acids was not shown.

Thus, although a number of techniques have been used to purify particular halophilic enzymes, in some cases to homogeneity, some of these techniques apply only to salt-tolerant enzymes while other techniques are not adequate to extensively purify most halophilic enzymes

which are present in small amounts.

In this thesis a variety of purification techniques were adapted or developed to help separate halophilic enzymes. Most of these techniques rely on adsorption chromatography in the presence of high salt concentrations. Some techniques could be applied to all halophile enzymes while others were more specific in application. All avoided the use of low ionic strengths.

1.3. Properties of halophilic enzymes

Many non-halophilic enzymes require small amounts of salt for stability (von Hippel and Schleich, 1969) and activity (Suelter, 1970); halophilic enzymes generally require larger amounts of salts. Studies of halophilic enzymes have concentrated on the effects of ions to learn how these proteins are able to function in such an unusual environment and to help to understand protein-ion interactions in general. That halophilic proteins would need to be different from non-halophilic proteins has been indicated by Jencks (1969). He noted that high concentrations of monovalent salts had deleterious effects on non-halophilic proteins largely due to the unfolding of the tertiary and quaternary structures normally maintained by ionic forces.

Indeed, the physical properties, activity and stability of halophilic enzymes may respond differently to ionic strength when compared to those of enzymes from

non-halophilic organisms. Holmes and Halvorson (1965) found that the sedimentation coefficient of halophilic MDH was much reduced in low salt concentrations while Hubbard and Miller (1969) found that halophilic isocitrate dehydrogenase in lower salt concentrations was inactivated, sedimented more slowly on density gradients and eluted more rapidly from dextran gel columns. These observations on the effect of low ionic strength on halophilic enzymes are similar to the results of Tanford (1968) on exposing non-halophilic proteins to guanidinium chloride. He suggests that low salt concentrations also denature the protein by opening up its tightly-folded native structure.

Evidence for the influence of salts on proteins comes also from the work of Holmes (1964). In the appendix of his thesis he gives data indicating that while bovine serum albumin and crude protein extracts from *Bacillus cereus* showed a 2-fold and a 10-fold increase, respectively, in viscosity when the concentration of NaCl was increased from 0.17 M to 4.3 M, a crude protein extract from *H. salinarium* showed only a 30% decrease in viscosity. This data suggests that salts dissociate groups and subunits of non-halophilic proteins while neutral monovalent salts give halophilic proteins a more tightly-folded conformation as suggested by Holmes and Halvorson (1965). Whether salts are chaotrophic (i.e.,those that disrupt the secondary, tertiary and quaternary structures of the proteins) or antichaotrophic

thus depends on the nature of the proteins as well as on the nature of the salts.

Three features common to many halophilic enzymes are related to their unusual physical properties and salt requirements. One is that they are often more acidic than their non-halophilic counterparts because they contain more acidic and/or fewer basic amino acid residues (Lanyi, 1974). In low ionic strength solutions negatively charged groups would repel each other and would tend to unfold the protein structure (Baxter, 1959). The protection of halophilic enzymes by salts or "charge-shielding" should be complete at less than 1 M salt, whereas many halophilic proteins require much higher concentrations for stability.

Another feature of halophilic enzymes is decreased hydrophobicity (Lanyi, 1974). Proteins are generally built up around a core of hydrophobic amino acid residues. This configuration is probably the most stable because when hydrophobic amino acid residues are exposed to water they form clathrates which are relatively rigid structures. This decreases the entropy of the protein solution. The formation of a hydrophobic core stabilizes enzymes in the specific conformation which allows catalysis by highly specific arrangement of amino acid residues. Halophilic enzymes appear to contain fewer hydrophobic amino acid residues which do not allow the formation of a compact structure in a low ionic strength environment. Therefore,

these enzymes need to be "salted-out" to allow a stable, active conformation. Salt-stabilization may also involve the decreased "solubility" of the amide backbone (peptide bonds) of the protein and/or the effects of salts on "H_2O-structure" (Jencks, 1969).

A third feature,which is shared by non-halophilic and halophilic proteins, is interactions with specific ions. Binding of ions to proteins probably requires a specific configuration of amino acid residues as was observed for Na^+ binding to myosin (Saroff, 1967). The activation of non-halophilic enzymes by monovalent cations has been reviewed by Suelter (1970) and Evans and Sorger (1966). Most of these non-halophilic enzymes were activated specifically by Na^+ or K^+ and Rb^+ while NH_4^+ often substituted K^+.

Halophilic enzymes usually are activated and/or stabilized to various degrees by different monovalent and divalent ions. Lanyi (1974) surveys a number of enzymes and finds that some enzymes from halophilic bacteria do and others do not require multimolar salt concentrations for activity and/or stability. He observed that the ratio of the maximum activities with K^+ versus Na^+ varies from less than 2 to much greater than 5. Higa *et al.* (1974) examined the effect of different salts and salt concentrations on the activity of 7 halophilic enzymes and found that

maximal activities were at 0.4 M $CaCl_2$, 0.2 M $CaCl_2$, 0.1 M NaCl and 3 M NaCl. When stability was examined for 1 M monovalent and 0.1 M divalent cations, five enzymes were most stable in NaCl while the other two were most stable in KCl and $MgCl_2$. The salt giving maximum stability and activity was the same in only one case. Specific ions effects have also been observed in enzyme kinetics with specific ions acting as competitive or non-competitive inhibitors for substrates or allosteric effectors (Lanyi, 1974).

1.4. Tryptophanyl-tRNA ligase and tryptophanase

L-Tryptophan: tRNA ligase (AMP) (E.C.6.1.1.2), referred to as TRS was chosen for detailed study in this thesis. In addition to the information that may be gained on the interaction of monovalent cations with the protein itself, this class of enzymes serves as a model system for protein-nucleic acid interactions (Kisselev and Favorova, 1974) as they contain no nucleotide cofactors. For halophilic ligases the influence of salt on these tRNA-ligase interactions can be studied. TRS catalyzes the following reaction:

$$\text{L-Trp} + \text{ATP} + \text{tRNA}^{\text{Trp}} \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \text{L-tryptophanyl-tRNA}^{\text{Trp}} + \text{AMP} + PP_i.$$

TRS is present in all organisms and is one of 20 enzymes which "activate" amino acids as a first step in the synthesis

of proteins. These enzymes react with three different substrates and produce three products. Also, as is necessary for the fidelity of protein synthesis, they discriminate among 20 amino acids and at least 60 tRNA species in this very important step of the genetic determination of protein primary structure. For these reasons, these enzymes have been extensively studied in non-halophilic organisms.

In spite of gross changes in the physiological response to ions, the protein synthesizing system of extremely halophilic bacteria has not lost its fidelity (Bayley and Griffiths, 1967; Bayley and Griffiths, 1968; White and Bayley, 1972). Thus the influence of salts on the structure and function of TRS and especially the interactions between TRS and $tRNA^{Trp}$ in the presence of high salt concentrations are of interest. Loftfield and Eigner (1967) and Loftfield (1972) have found that high concentration of salt reduce the binding of tRNA to non-halophilic aminoacyl-tRNA ligases but such a result is not obtained for halophilic enzymes. An analysis of the results of Griffiths and Bayley (1969) led Loftfield (1972) to the conclusion that the rate-limiting step for halophilic tRNA ligases in low salt concentrations is the dissociation of tRNA from the enzyme.

The tRNA molecule is a strongly charged polyanion which, it has been suggested (Loftfield, 1972), interacts

electrostatically with the aminoacyl-tRNA ligase. If these forces are of primary importance then halophilic TRS must compensate the weakening of these forces by salts.

An examination of purified *H. cutirubrum* TRS activity and stability was conducted with an emphasis on the effects of salts. It was hoped this would further the understanding of halophilic protein structure and function and also increase the understanding of the aminoacyl-tRNA ligase reaction. Specifically, how does salt affect the kinetics of this important and highly specific reaction which usually requires the tight binding of tRNA and enzyme (Kisselev and Favorova, 1974)?

An examination of purification methods for L-tryptophan indole ligase (deaminating) (E.C. 4.1.99.1), or tryptophanase (originally identified as a contaminant in the purification of TRS) was also conducted in this thesis. Tryptophanase is found only in bacteria and almost always in bacteria of the animal intestinal tract (DeMoss and Moser, 1969). The role of tryptophanase appears to be to regulate, in conjunction with tryptophan synthetase, the level of tryptophan within the bacterial cells (Snell, 1975). While tryptophanase from different bacteria have significantly different amino acid compositions, their structure and enzymology appear quite similar (London and Goldberg, 1972; Hoch *et al.*, 1966; Cowell *et al.*, 1973; Yoshida *et al.*, 1974). The enzymes are tetrameric molecules with molecular weights

slightly greater than 200,000 daltons. They show transitions between tetrameric, dimeric and monomeric forms which suggest that hydrophobic and ionic forces stabilize the tetrameric structure. Tryptophanases are activated by NH_4^+, K^+ or Rb^+ and are inhibited by Na^+ or Li^+. Ions have been shown to affect the association of subunits and of the single pyridoxal 5'-phosphate (PLP) cofactor bound to each subunit. The reaction mechanism of tryptophanase has been studied in considerable detail and appears to be similar to that of other PLP enzymes which catalyze by an α,β-elimination reaction.

Tryptophanase from *H. cutirubrum* is induceable and stable and represents a protein which is present in relatively high amounts and is easy to work with. Its requirement for saturated KCl for maximum activity and stability distinguishes it from other tryptophanases. The effects of salt on the interactions of the subunits and PLP cofactor, activity and stability of halophilic tryptophanase could increase our understanding of this and similar enzymes.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals and reagents were reagent grade and obtained from commercial sources.

Singly or uniformly labelled L-amino acids, toluene-^{14}C, and sodium pyrophosphate-^{32}P were obtained from New England Nuclear Corp. Deoxyribonuclease I was obtained from Worthington Biochemicals. Sephadex, Sepharose 4B, Blue Dextran 2000, aldolase, ribonuclease, chymotrypsinogen and ovalbumin were obtained from Pharmacia Fine Chemicals.

Halobacterium cutirubrum strain 9, from the National Research Council in Ottawa, were used in all the studies reported in this thesis.

2.2 Growth of bacteria

H. cutirubrum were grown in complex medium using a bench fermentor and harvested with a Sharples centrifuge as described by Bayley (1971).

When tryptophanase was to be induced, 1.5 mM L-Trp was added to the sterilized medium. The medium contained about 0.04 mM Trp added with casamino acids. Logarithmically growing cells to be used as a source for enzyme were grown to a final optical density at 650 nm of 0.65-0.80 before harvesting. Cells to be used for their tRNA were grown to

late log phase to a final A_{650} of 1.5-2.0. Medium which had been used to prepare cells for their enzymes could be reused to provide cells for tRNA. Cells to be extracted for tRNA either were used immediately or were stored frozen at -50°C following the washing of the cells.

2.3. Preparation of biological extracts

Note : all procedures were performed at 0-4°C unless otherwise specified. Buffers containing multimolar salt were filtered through 0.45 micron nitrocellulose filters.

2.3.1. S-150 extract

Freshly harvested, washed cells were homogenized for 20 seconds at full power in a Virtis omnimixer with 1 ml of solution D [3 M KCl, 20 mM Tris-HCl pH 7.6, 0.1 M Mg $(C_2H_3O_2)_2$ and 0.8 mM 2-mercaptoethanol (ME)] or D' [3 M KCl, 0.1 M Mg $(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 7.6, and 8 mM ME] per gram wet weight of cells. One mg of DNase per 30 ml of solution D or D' was added and homogenization was continued for a further 20 seconds. The homogenate was then centrifuged at 150,000 x g for 2.5 hr. The clear supernatant was removed and dialyzed against 400 volumes of solution D or D'. This preparation, the S-150 extract, was then either used directly or frozen with liquid N_2 in 0.1-3 ml volumes in nitrocellulose tubes and stored at -50°C (at which temperature it was stable for several months) until they were quick-thawed in a 37°C water bath before use.

2.3.2. Transfer RNA

The method of preparation was modified from that of von Ehrenstein (1968). The cells were homogenized in 10 ml of 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 1 mM ME and 10 ml of 88% phenol per gram of cells in a Waring blender at high speed for 1 min. The aqueous and phenolic phases were separated by centrifuging, the upper, aqueous phase was removed and the phenolic phase was reextracted with an equal volume of aqueous buffer. The pooled aqueous phase was then precipitated by ethanol and fractionated by isopropanol (von Ehrenstein, 1968). The final precipitate was washed with 95% ethanol and then with anhydrous ether; it was dried in a vacuum and stored at -20°C.

The tRNA prepared as described above was discharged of amino acids by dissolving it in 2 M Tris-HCl,pH 9 and incubating at 37°C for 1 hr. Precipitation was achieved by adding 0.1 volume of 20% $KC_2H_3O_2$,pH 5 and 2 volumes of ethanol, cooling to -15°C for 10 hr and then centrifuging at 6000 g for 15 min. The precipitated tRNA was further purified by the method of Kelmers *et al.* (1965). The tRNA was dissolved in starting buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and I mM ME). It was charged onto a column of DEAE-cellulose equilibrated in the starting buffer and was then washed with 7 column volumes of the same buffer and then with 12 column volumes of starting buffer containing 0.3 M NaCl. The tRNA was eluted by starting buffer containing 1 M NaCl and precipitated

as described above, washed with 95% ethanol and freeze dried. This crude tRNA was used routinely for aminoacylation assays and contained 19 pmole $tRNA^{Trp}$ per A_{260} unit.

2.4. Preparation of agarose-hexamethylenediamine-Trp

The procedure was modified from that described by Robert-Gero and Waller (1972) and is summarized in Figure 1. In the hexamethylene substitution of agarose (Sepharose 4B) the pH was maintained at 10.5-11.5 by adding 4 M KOH dropwise. The degree of substitution was estimated qualitatively by the colour test of Inman and Dintzis (1971).

The preparation of o-nitrophenylsulphenyl derivatives of amino acids was as described by Zervas *et al.* (1963) with the following alterations: To the reaction mixture was added 100-300 cpm of L-Trp-3-^{14}C per μmole of L-Trp in order to determine the purity of products and the amount of amino-acyl substitution of the agarose. The purity of the two o-nitrophenylsulphenyl derivatives of Trp (Fig. 1) was 95-105%, based on the cpm per mg, when counting efficiency was calculated using toluene ^{14}C as an internal standard. After the o-nitrophenylsulphenyl-Trp esters had been synthesized, filtered, neutralized and washed extensively with H_2O, they were freeze-dried for 3-9 hr to a constant weight.

The activation and coupling of o-nitrophenysulphenyl-Trp (Figure 1) were as described by Robert-Gero and Waller (1972) for methionine. Some preparations required a repetition of the $Na_2S_2O_3$ reduction to remove the protecting

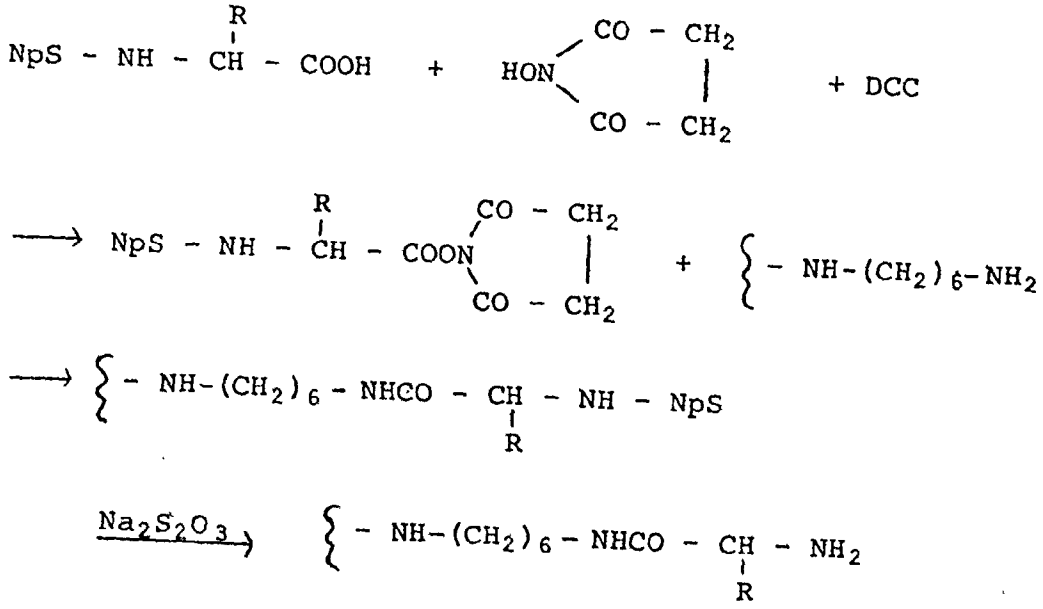
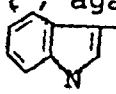


Figure 1: Summary for the preparation of the affinity column

Abbreviations: NpS, o-nitrophenylsulphenyl; DCC, dicyclohexylcarbodiimide; {, agarose or Sepharose 4B; and the R-group for L-Trp is 

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group, as a residual yellow colour was observed. The amount of aminoacyl substitution was controlled by adding different amounts of o-nitrophenylsulphenyl-Trp and ranged from 3.9 to 12 μmoles Trp per g wet weight of substituted agarose.

Derivatized agarose could be reused after chromatography of S-150 extracts by washing extensively with H_2O, several volumes of 1 M urea, 0.5 M sodium dodecyl sulfate (SDS) followed by H_2O and then starting buffer for the next chromatography.

2.5. Enzyme assays

2.5.1. Aminoacyl-tRNA ligases

Assays were done in 10 x 75 mm tubes and contained in 0.25 ml or 0.13 ml each of the following: 1.2 mM ATP (neutralized), 23 mM Tris-HCl, 4.8 KCl, 1 M NaCl, 40-300 pmoles of *H. cutirubrum* tRNA (2-15 A_{260} units, prepared as described in Section 2.6.), 25 mM Mg $(C_2H_3O_2)_2$ and 10 or 20 μl enzyme, usually in solution D'.

Assays for the phenylalanyl-tRNA ligase were at pH 7.9 and contained 2.7 nmole of 470 μcurie per μmole L-Phe-^{14}C and 2.9 mM ME. Assays for the Tyr enzyme were at pH 7.9 and contained 2.6 nmoles of 460 μcurie per μmole L-Tyr-^{14}C and 2.9 mM ME. Assays for the Trp enzyme (TRS) were at pH 7.5 and contained 0.4 - 1 nmoles of 45-53 μcurie per μmole L-Trp-^{14}C (purified as described in Section 2.6.) and 0.1 mM ME. Unless otherwise specified, kinetic and stability studies of TRS were in 0.13 ml volumes and

contained 9.5 mM $Mg(C_2H_3O_2)_2$, 78 mM Tris, 150-350 pmoles of tRNA, 3.5 mM ATP and 7.4 μM L-Trp-^{14}C. Control assays contained buffer instead of enzyme solution.

To measure the amount of tRNA in a sample assays contained the tRNA sample of interest in place of crude tRNA and an excess of S-150 extract. A control to measure endogenous tRNA contained no added tRNA.

Assays were preceded by a 5 min preincubation at 37°C during which rapid shaking was used to dissolve KCl added to the assay as a dry solid. The enzyme was added and the shaking incubation was continued for 30 min. The reaction was terminated by the addition of 0.4 mg BSA and 5 ml of 5% TCA at 0°C. The mixture was shaken, allowed to stand for 10 min and centrifuged for 10 min at 1000 x g. The precipitate was resuspended in 1 ml of 5% TCA and filtered on 0.45 micron nitrocellulose filters, washed with 5 ml of 5% TCA, dried at 90°C for 20 min and counted to determine the amount of ^{14}C-labelled amino acid which had been charged onto the TCA-precipitated tRNA. With each set of assays control assays lacking enzyme were included and their radioactivity was subtracted from the others.

2.5.2. Tryptophanase

Assays were done in 15 x 150 mm tubes and consisted of 0.8 ml solution D' containing 4.4 mM L-Trp, 0.4 mM pyridoxal-5'-phosphate (PLP) and 0.13 mg bovine serum

albumin; 0.8 ml of toluene was added to remove the product, indole, from the reaction mixture. Enzyme was added to the assay last and the assay was incubated at 37°C for 60 min using a shaking frequency of 130 cycles per min. The production of indole was determined by gently vortexing the assay, carefully removing 0.2 ml of the toluene layer and assaying for indole by adding colour reagent and measuring the A_{570} as described by Yanofsky (1955). Control assays without enzyme and with various amounts of indole were used to determine standard curves.

2.5.3. Assays for other halophilic enzymes

The assay for isocitrate dehydrogenase was described by Aitken *et al.* (1970) and that for catalase was described by Lanyi and Stevenson (1969). The assays for malate dehydrogenase (MDH) contained, in 1.2 ml of solution D', 0.42 mM cis-oxaloacetate, 0.17 mM NADH and enzyme. The A_{340} was recorded continuously and the recording of the initial 30 seconds was used to determine MDH activity. The assay for aldehyde dehydrogenase (ADH) was analogous to that for MDH except that no cis-oxaloacetate was included (the 0.1 M Mg $(C_2H_3O_2)_2$ in solution D' served as substrate). MDH assays were corrected by subtracting ADH activity.

2.6. Purification of L-Trp-3-^{14}C

The labelled compound as supplied commercially was found to give a very high background in control assays, probably due to radiolytic decomposition. To remove

impurities, the substrate was chromatographed on Whatman 3MM paper using H_2O as the solvent in the ascending manner. There were 5 UV-absorbing or -fluorescing bands. The most strongly UV-absorbing band migrated with the R_F of L-Trp and gave a UV spectrum of Trp. This band was cut out and eluted with H_2O in a descending fashion. Impurities accounted for approximately 10% of the radioactivity and were associated predominantly with the UV-absorbing and -fluorescing bands.

2.7. Analytical techniques

2.7.1. Protein concentration determined by the method of Lowry *et al.* (1951).

The method was scaled down to give a final volume of 1.3 ml and the volume of the enzyme solution varied from 5-200 µl. BSA standards of 20 or 40 µg were used for each assay series. The enzyme was usually in high concentrations of salts and ME which necessitated the restriction of volumes to 40 µl or less. Control assays contained equal volumes of the same buffer and when the enzyme had been concentrated and dialyzed to remove L-Trp, an equal volume of 20 mM L-Trp buffer was also concentrated, dialyzed and assayed as a control.

2.7.2. SDS-polyacrylamide gel electrophoresis

Electrophoresis of proteins in the presence of SDS dissociates proteins into their subunits which then migrate according to molecular weight. The method of Weber and Osborn (1969) was used. Halophile proteins were dialyzed

against H_2O first to remove salts and only 1/3 of Weber and Osborn's amount of $(NH_4)_2\ S_2O_8$ was used. Ribonuclease, catalase, and ovalbumin were used as molecular weight markers. Gels were fixed for 15 min in 10% acetic acid at 23°C and stained for 30 min in 10% acetic acid, 25% methanol and 0.25% Coomassie Blue at 37°C and then were destained in several changes of 10% acetic acid in 25% methanol at 37°C. Gels were stored in 10% acetic acid. The electrophoretic mobility relative to bromophenol blue was calculated and was graphed as a function of the log of the molecular weight as described by Weber and Osborn (1969).

2.7.3. Molecular weight determination on dextran gels

Molecular weights of halophilic enzymes were estimated by dextran gel chromatography using Sephadex G-100 Superfine.

A 1.25 x 28 cm column of dextran gel was equilibrated in solution D'. The following samples were applied to and eluted from the column with solution D': 1 ml containing S-150 extract (25 mg protein) and 0.33 mg of Blue Dextran 2000 (the latter was used to determine the column void volume); 0.5 ml containing 0.43 mg Blue Dextran, 3.2 mg aldolase and 2.8 mg chymotrypsinogen; and 0.5 ml containing 0.43 mg Blue Dextran, 3.4 mg ribonuclease and 3.1 mg ovalbumin. Fraction volumes were approximately 1.1 ml and were determined by weighing the tubes empty and full. An estimate of molecular weight was determined by calculating

the K_{AV} and plotting the K_{AV} against the log of the molecular weight (see Section 4 below).

2.7.4. Amino acid analysis

The amino acid composition of TRS was determined by the methods described by Blackburn (1968) based on the work of Moore and Stein (1963) and Spackman *et al.* (1958). TRS and a control sample of 20 mM L-Trp in solution D', prepared as described in Section 3.1.1. was dialyzed against water and dried under reduced pressure in 5 ml ampules. Redistilled 6 M HCl was added to the ampules. They were evaculated, flushed with N_2 and sealed. The samples were heated to 100°C for 25 hr, dried under reduced pressure and resuspended in buffer. The samples were analysed in a Beckman 120C or 119 amino acid analyzer and the amount of each amino acid was determined. Corrections were made for the reduced recoveries of Thr, Ser and Cys (Blackburn, 1968).

The Trp content was calculated from the UV spectrum of TRS and control samples dialyzed against water. BSA was used as a standard.

3. PURIFICATION PROCEDURES FOR HALOPHILIC ENZYMES

Three main procedures of protein purification are presented in this section:

a) Affinity chromatography (3.1.) for the purification of enzymes for which tryptophan is a substrate was the first method. This was designed originally for the purification of TRS (Trp-tRNA ligase). Some of the TRS preparations were found to contain appreciable amounts of tryptophanase. Chromatography conditions were therefore examined and a method was found to separate these two enzymes and to purify TRS to homogeneity and tryptophanase to near-homogeneity when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

b) Tryptophanase was also purified by the simpler method of heat treatment followed by $(NH_4)_2SO_4$ fractionation (3.2.). The purification achieved by this method was compared to that obtained by affinity chromatography.

c) A new method of adsorption chromatography involving the binding of nucleic acids and proteins to agarose in the presence of multimolar $(NH_4)_2SO_4$ and their elution with a gradient of decreasing $(NH_4)_2SO_4$ concentration has been devised (3.3.). It results in the effective separation of

halophilic enzymes and tRNAs from a crude cell lysate, as judged by results with ADH, MDH, TRS, tryptophanase and $tRNA^{Phe}$. An effective purification of tryptophanase after heat treatment was also achieved by this method.

3.1. Affinity chromatography

In the last 5-10 years, methods of protein purification, which have traditionally been very time-consuming, tedious and uncertain, have been assisted by affinity chromatography (Cuatrecasas *et al.*,1968). Affinity chromatography is based on the unique ability of biologically active materials to bind specifically and reversibly (bio-affinity) other materials which are called ligands. Usually ligands are attached covalently to solid supports such as agarose, solutions containing the biologically active materials are passed over column material containing the specific ligand and, if conditions are appropriate,the biologically active material remains selectively bound to the ligand. Then the biologically active material is eluted, preferably by the ligand in solution, but in many cases inhibitors, pH changes, ionic strength changes or dissociating agents have been used.

If an enzyme is to be purified by affinity chromatography it is important to consider that derivatization of the ligand,which is usually the substrate, may change its enzyme binding properties. Also the length and chemical nature of the spacer arm, which is the molecular covalent

link of the ligand to the solid support, may affect the accessibility of the ligand to the binding site of the enzyme. Principles and methods of affinity chromatography have been extensively reviewed (Cuatrecasas, 1970; Turkova, 1974; Guilford, 1973; May and Zaborsky, 1974).

O'Carra *et al.* (1974) discuss nonbiospecific interferences possible on "affinity columns". Ion exchange and hydrophobic chromatography due to interactions of charges and hydrophobic groups on the ligand and spacer arm are the most common. Salt-sensitive interactions possibly due to van der Waals or electrostatic interactions are also observed and this type of interference should not exist in high salt concentration. Another interaction O'Carra describes as "ligand-dependent nonbiospecific adsorption" is due to interactions of enzymes with hydrophobic spacer arms after binding to the ligand; this interference is characterized by a progressive loss of enzyme recovery with chronological delay of elution. A last interaction described by O'Carra *et al.* (1974) is "compound affinity" in which weak bioaffinity is supported by nonbiospecific interactions and biospecific elution can still be used.

If affinity or biospecific chromatography is the sole interaction occurring then the retardation (R_{bio}) of the enzyme, which is the elution minus void volume, divided by the column volume, can be described by the equation:

$$R_{bio} = \frac{[Im]}{K_s} \qquad (1)$$

where [Im] is the molar concentration of the covalently bound ligand and K_s is the molar dissociation constant for the covalently bound ligand. In the presence of the bioeluting ligand in the same purely bioaffinity chromatography system the bioelution retardation, R_{bio}, can be defined by the equation:

$$R_{bio} = \frac{[Im]}{K_s} \left(\frac{K_c}{K_c + [C]} \right) \qquad (2)$$

where K_c and [C] are the dissociation constant and concentration, respectively, of the bioeluting ligand.

Purification of aminoacyl-tRNA synthetases by affinity chromatography has been reported many times within the last 5 years. However, the enzymes were either eluted without the use of substrate-containing solutions (Forrester and Hancock, 1972; Robert-Gero and Walker, 1972) or were eluted with salt gradients (Bartkowiak and Pawelkiewicz, 1972; Remy *et al.*, 1972; Hayashi, 1973; Joyce and Knowles, 1974). This suggests that their purification was based mainly on ion exchange chromatography. The chromatography of halophilic enzymes on ligand-containing columns in high salt concentrations precludes ion exchange chromatography.

3.1.1. Affinity chromatography of TRS

Agarose-hexamethylenediamine-Trp (AHT) columns were highly satisfactory for the chromatographic purification of halophilic TRS in high salt buffers. The enzyme could be eluted readily by L-Trp dissolved in concentrated salt solutions. Before an effective procedure could be developed,

however, conditions influencing chromatography had to be optimized.

Small columns were used to examine the effect of salt concentration and cation specificity on the chromatography of TRS on AHT. Much more TRS was bound when 5 M NaCl buffer was used rather than 3 M NaCl buffer (Table 1). The 3 M KCl buffer system gave greater TRS binding than the 5 M NaCl buffer. This demonstrates that both ionic strength and specific ion effects are important to binding. Flow rate had no effect.

Small analytical columns were also used to examine the effect of various levels of substitution of agarose-hexamethylenediamine with L-Trp. The relative elution pattern of TRS on these columns was examined (Figures 2 and 3). Chromatography employed S-150 extract on 6 columns of AHT containing 0-8.9 µmole L-Trp/g of drained, wet column material. The agarose-hexamethylenediamine column (Figure 2a) did not retard TRS relative to the protein peak. TRS was retained when columns containing 4.3 µmole or more of Trp/g AHT were used. Figures 2 and 3 show that the sharpness of the elution profile decreased with increasing amounts of L-Trp on the agarose and that the recovery of input TRS decreased from 100 to 4%. Small differences in AHT preparation might account for the differences in elution from the 4.3 and 4.7 µmole AHT.

It is apparent that these data do not fit the situation described by equation (2) (Section 3.1.).

Table 1

Affinity Chromatography[1] of TRS and Tryptophanase on 5 μmole/g AHT with 3 M and 5 M NaCl and 3 M KCl buffers

Salt concentration	3 M NaCl	5 M NaCl	3 M KCl
% TRS bound	2	19	35
Total recovery of TRS (% input)	94	81	51
% tryptophanase bound	0	12	0
Total recovery of tryptophanase (% input)	95	89	93

[1] To the 1.6 ml, 7.2 cm high columns were added 25 mg S-150 extract. Flow rate was 400 ml/hr/cm^2 of column surface area. Chromatography buffers contained 0.1 M $Mg(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 7.7 and 8 mM ME. A 25 ml wash with this buffer was followed by a 15 ml elution with this buffer plus 16 mM L-Trp.

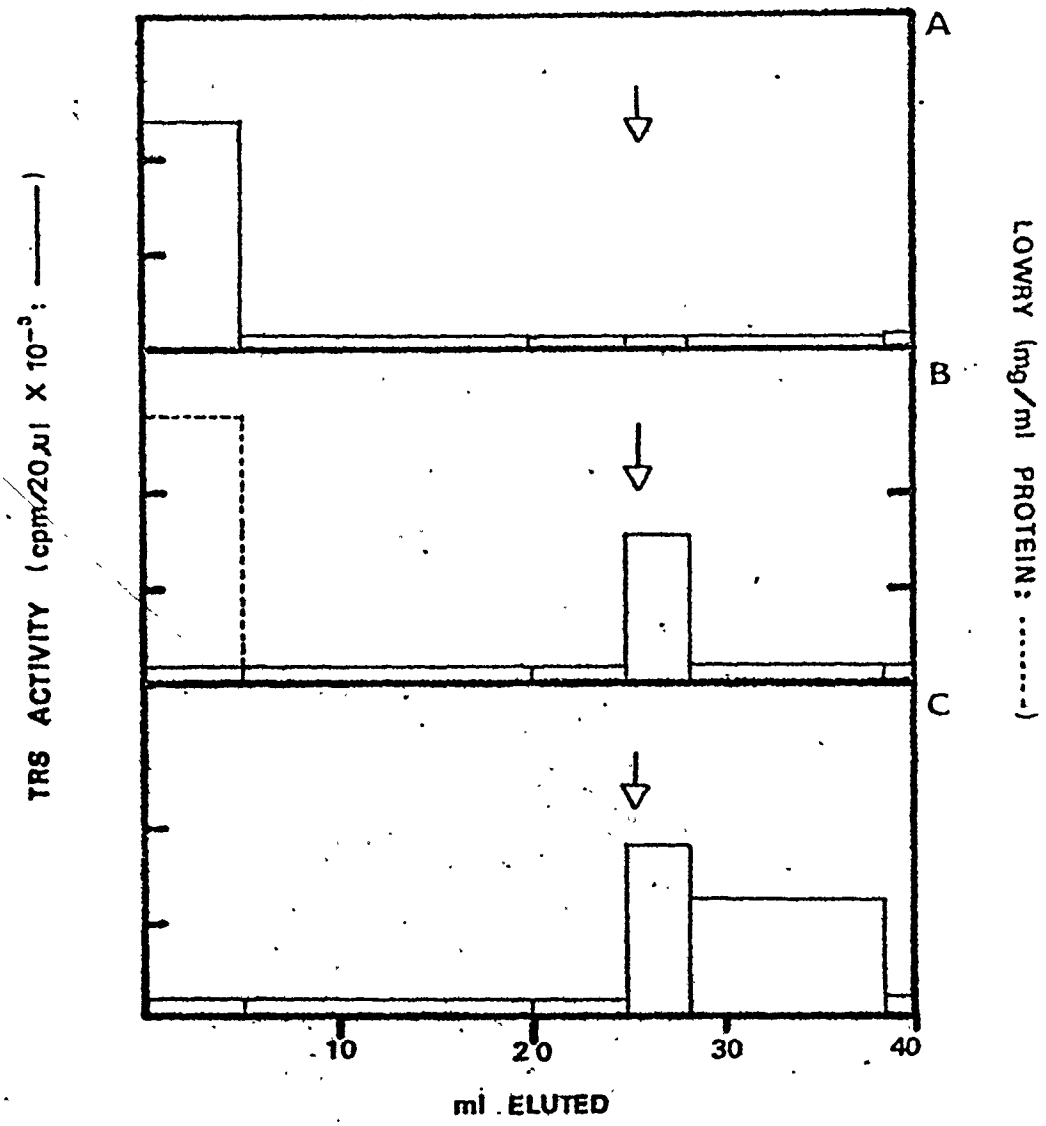


Figure 2: Affinity chromatography of TRS on analytical columns

To three 1.6 ml or 0.53 x 7.2 cm columns of AHT substituted with a) zero; b) 4.3 and c) 4.7 µmole Trp per g AHT were added 24 mg protein of S-150 extract. With flow rates of 400 ml/hr/cm^2 of column surface, fraction volumes of 5,15 and 5 ml were eluted with solution D' followed by 3,10 and 2 ml of 20 mM L-Trp in solution D'. The arrows indicate the addition of 20 mM L-Trp. Recoveries of input TRS were 100, 32 and 41%, respectively.

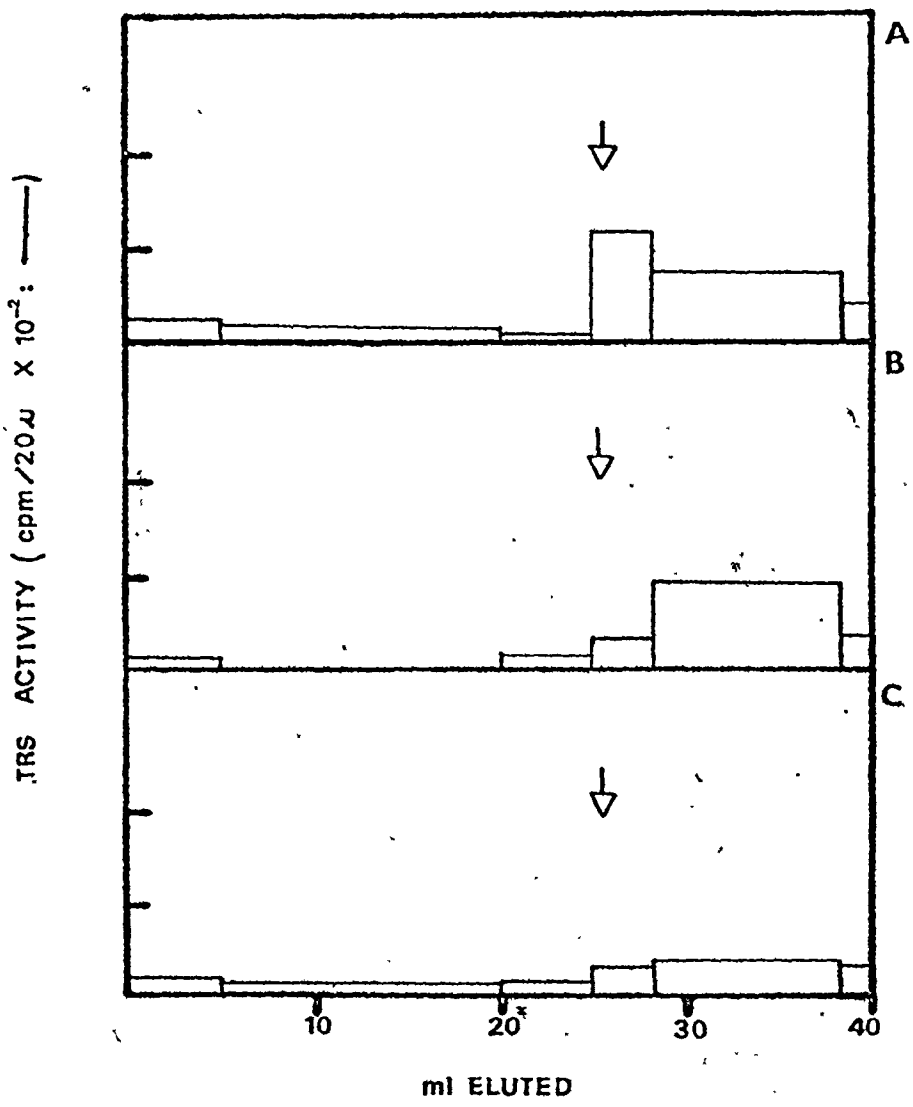


Figure 3: Affinity chromatography of TRS on analytical columns containing more Trp.

Columns of AHT substituted with a) 5.6; b) 6.0 and c) 8.9 µmole Trp per g AHT were used. Other conditions were the same as in Figure 2. Recoveries of input TRS were 14, 12 and 4%, respectively.

For example, increases in Im from 4.3 to 6.0 increased R_{bio} from 1 to 5. It can be concluded that non-specific factors affect elution of TRS. Salt-sensitive and ionic interactions can be ruled out because solution D' contains 3 M KCl. Ligand-dependent, non-biospecific adsorption is not possible because recoveries in Figure 3 where chromatography took 0.45 hr were not less than for Figure 5 where chromatography took 13 hr. If hydrophobic chromatography were the sole interaction responsible for adsorption then biospecific elution should not occur and the separation of TRS from other proteins binding by hydrophobic interactions should not be possible. Thus the results observed might be explained by compound affinity in which affinity chromatography and hydrophobic chromatography are both occurring.

Another interpretation of the data in Figures 2 and 3 is that the immobilized Trp on AHT does not have uniform affinity for TRS. The levels of Trp on AHT were several orders of magnitude greater than the levels of TRS retained during affinity chromatography. Therefore, progressive Trp substitution of AHT might more than proportionally increase the TRS binding sites available.

To prepare TRS for kinetic analysis and amino acid analysis larger columns and more S-150 extract were employed. For preparative chromatography on a large column, AHT with 5.2 μmole Trp/g was unsatisfactory (Figure 4).

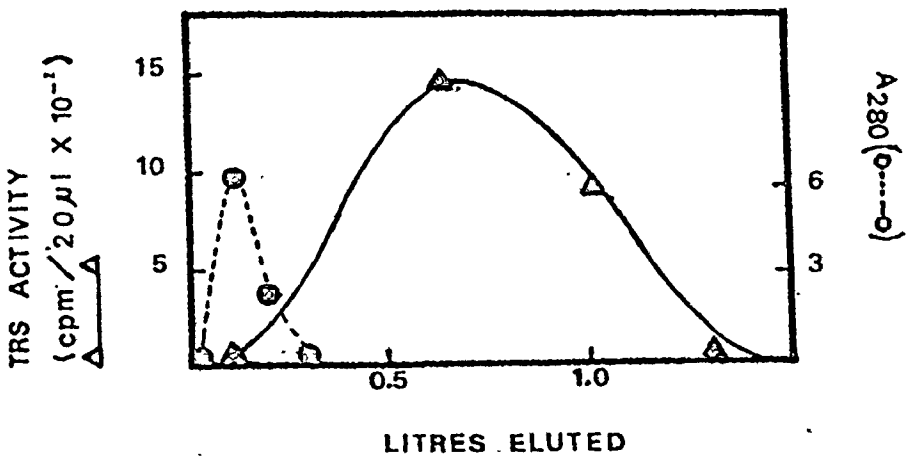


Figure 4: Preparative affinity chromatography of TRS on AHT containing 5.2 µmole Trp/g AHT

TRS in 825 mg S-150 extract was applied to a 33 ml 1.2 x 22 cm column. Elution was with solution D' at 400 ml/hr/ cm^2 of column surface area.

Although not shown by the A_{280} plot in Figure 4, there was still A_{280}-absorbing material eluting until the column had been washed with 80-100 column volumes of solution D'. But this extensive wash eluted almost all of the TRS applied. Using half as much S-150 extract did not solve this problem.

However, the TRS in up to 50 mg protein in S-150 extract per ml AHT could be bound tightly to 6 μmole Trp/g AHT columns and L-Trp-induced elution volume was small (Figure 5). Recovery of input TRS was 40%. When similar column chromatography was performed using solution D' the results were almost identical to Figure 5.

When the eluted TRS peak from the Figure 5 column was analyzed by SDS-PAGE the gels showed several protein bands (Figure 6A). The enzyme could be further purified by a second passage through the same affinity column after washing (Section 2.4.). The TRS-binding capacity of the column remained unchanged (3 m units or 20-50 μg of TRS/ml of column material of which 30-40% was recovered). When TRS from this second chromatography was analyzed by SDS-PAGE only one protein band was observed (Figure 6B). Purification for this preparation of TRS was approximately 1000-fold. This preparation was used in the studies of Section 5 of this thesis and is referred to as "purified TRS".

Purification data for several preparations of TRS is summarized in Table 2. The purification factors and

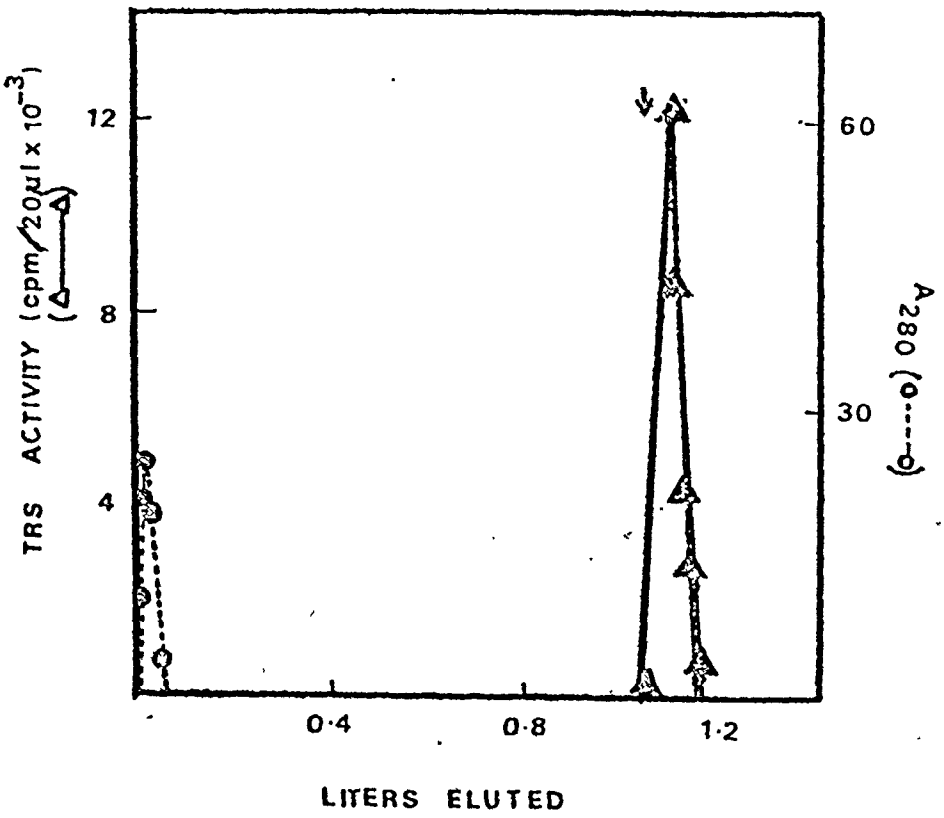


Figure 5: Preparative affinity chromatography of TRS on AHT with 6 µmole Trp/g.

Two hundred and thirty six mg of S-150 extract protein was applied to a 10 ml 117 cm high column of AHT. Buffers contained solution D. The arrow indicates the point at which 20 mM L-Trp was added. Of the input TRS, 40% was recovered in the Trp-eluted peak.

Figure 6: SDS-polyacrylamide gel electrophoresis of purified and crude TRS and tryptophanase

A: 11 µg of partially purified TRS prepared by one affinity column passage. B and I: One and 1.3 µg, respectively, of purified TRS prepared by two affinity column passages. C,D,E and F: 0.5, 48,37 and 32 µg, respectively of tryptophanase purified by affinity chromatography. G: 4.1 µg of partially purified tryptophanase prepared by heat treatment and $(NH_4)_2SO_4$ fractionation. H: 20 µg of partially purified tryptophanase prepared by heat treatment and agarose-$(NH_4)_2SO_4$ chromatography. The sharp lower bands in gels A,D,E and F correspond to the position of bromophenol blue.

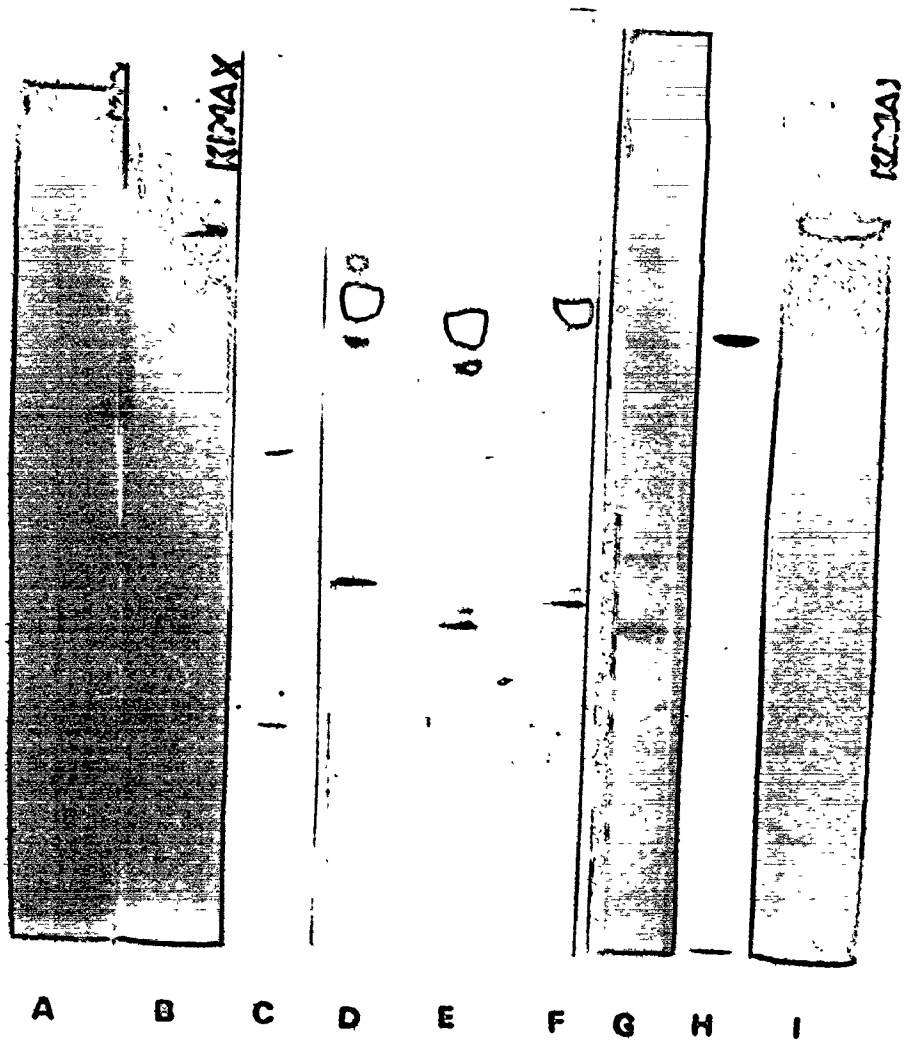

KMAX
KMAX
A
B
C
D
E
F
G
H
I

Table 2

Summary of TRS Affinity Chromatography Purification and Recovery Data for Several Preparations

Purification factor	% recovery	μmole Trp/g AHT
400[1]	-	5.4
340[1]	37	5.6
400[1]	40	5.6
-	40	6
1000	9	6;4.7[3]
980	4[2]	6;6[3]

[1] Specific activity was measured for peak fractions only.

[2] In the second chromatography the column was overloaded and hence recovery was low.

[3] Second column passage.

purity of the product were greater than any of the previously reported methods of affinity chromatography of aminoacyl-tRNA ligases. In addition, the recovery of TRS was reasonably high.

3.1.2. Affinity chromatography of tryptophanase

In the initial purification of TRS on affinity columns containing Trp, some preparations were contaminated with appreciable amounts of tryptophanase. The possibility of using affinity chromatography for the purification of this enzyme was also investigated.

In the preparation of TRS using 3 M KCl in solution D', the chromatography of tryptophanase showed a slight retardation relative to the protein peak (Figure 7). However, an insignificant amount of tryptophanase was retained on the column and eluted with L-Trp (see also Table 1). When chromatography buffers contained 5 M NaCl (solution E) in place of 3 M KCl in solution D' (Figure 8 and Table 1) appreciable amounts of the tryptophanase were bound and were eluted only with a wash containing L-Trp Chromatography using 3 M NaCl instead of 5 M NaCl prevented the binding of tryptophanase (Table 1). It is possible that ionic strength is critical since both 3 M NaCl and 3 M KCl buffers are unsatisfactory as chromatography buffers.

There was no correlation between ionic conditions required for binding to AHT and those for maximum enzyme

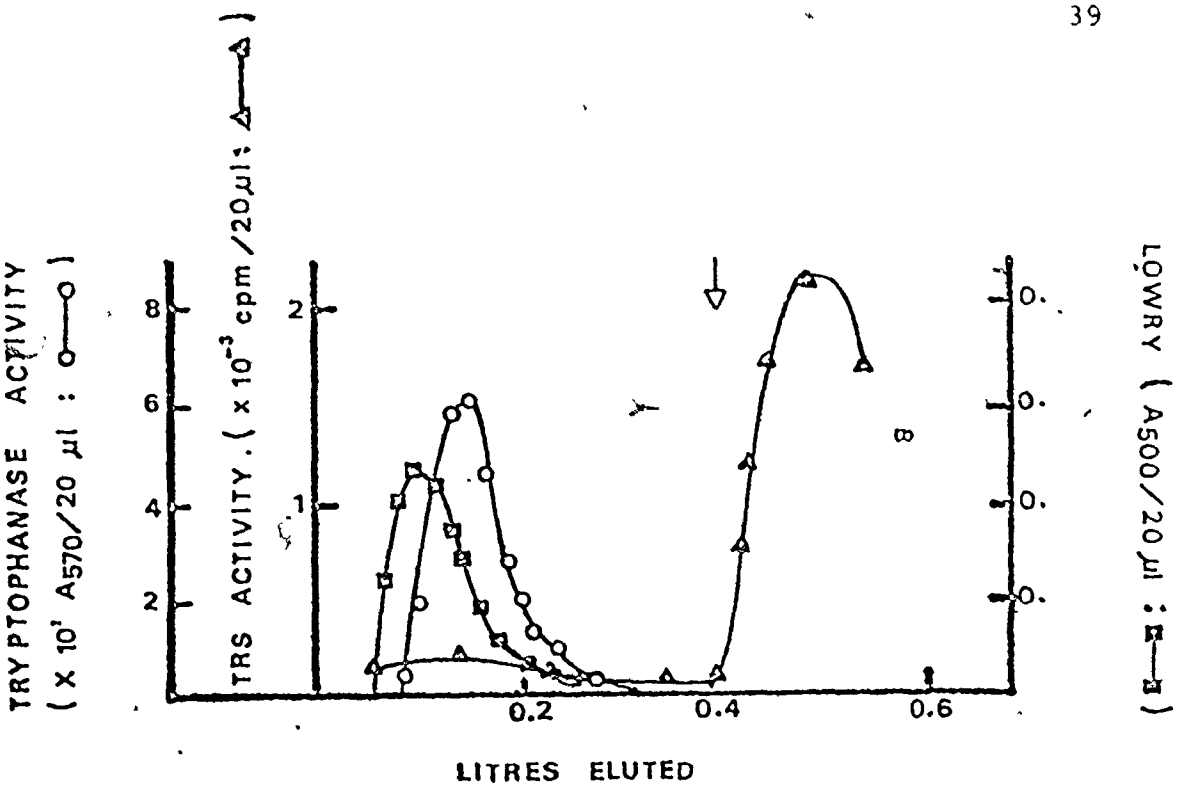


Figure 7: Affinity chromatography of TRS and tryptophanase on an AHT column containing 6 µmole Trp/g using buffers containing solution D' (3 M KCl)

S-150 extract (610 mg protein) was chromatographed on a 12.8 ml (15 cm high) column. The arrow indicates the point at which 20 mM L-Trp was added.

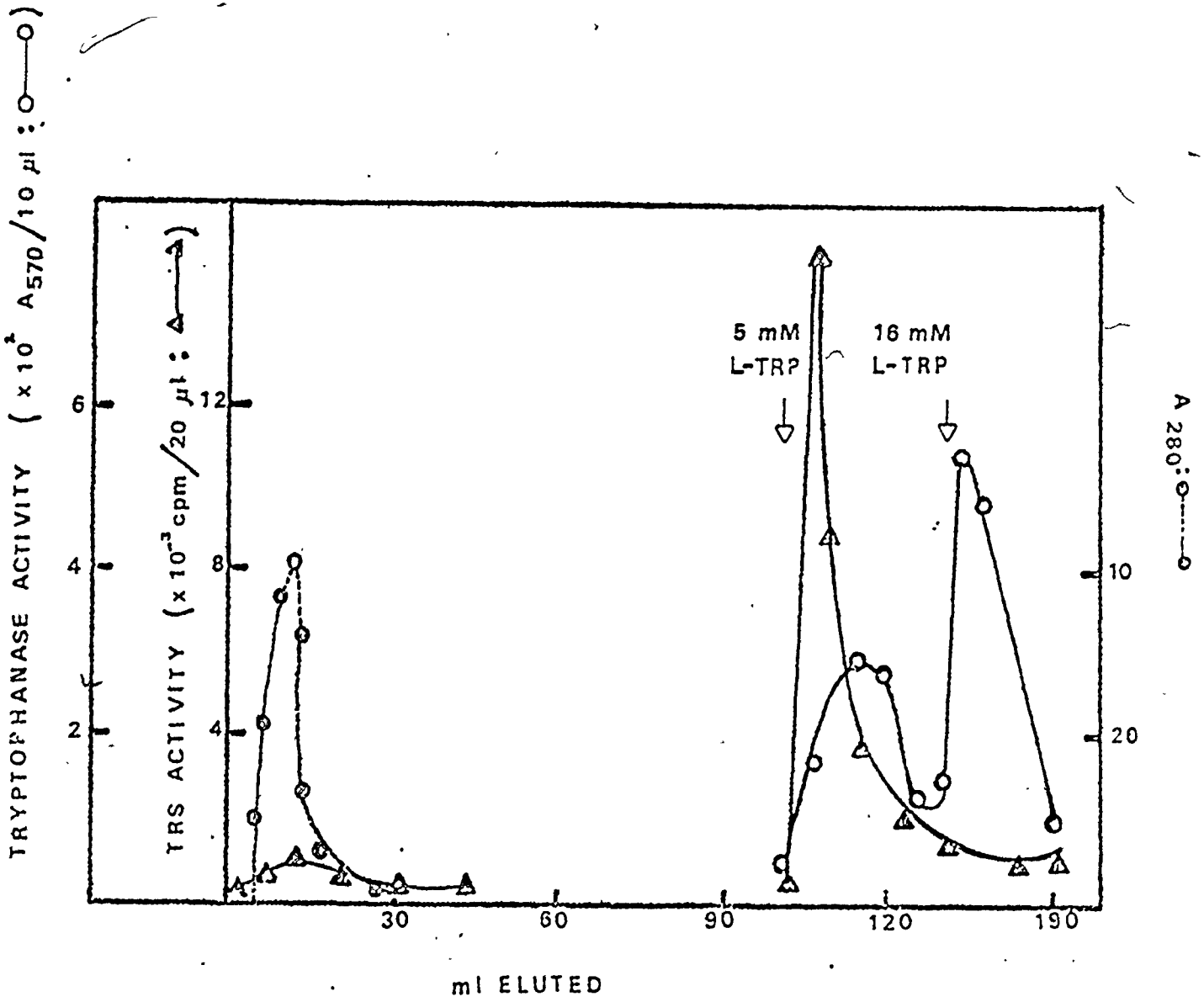


Figure 8: Affinity chromatography of TRS and tryptophanase on a column of 5.6 µmole Trp/g AHT using solution E minus PLP (5 M NaCl).

S-150 extract (225 mg protein) was chromatographed on a 7.3 ml, 8.6 cm high column.

activity. Tryptophanase activity increased 20% when 5 M instead of 3 M KCl was used but the use of 5 M NaCl instead of 3 M KCl reduced tryptophanase activity by 85%. It may be that the ionic strength is important for the binding of tryptophanase to Trp but that K^+ is required for catalytic activity, as it is for other bacterial tryptophanases.

The effect of the level of Trp immobilized on AHT on the affinity chromatography of tryptophanase in 5 M NaCl was examined on small columns (Figures 9 and 10). Neither the agarose-hexamethylenediamine column (Figure 9a) nor the 4.3 μmole Trp/g AHT column retained tryptophanase although the enzyme was slightly more retarded in the latter. When the μmole Trp/g AHT was increased from 4.7 to 8.9 the retention of tryptophanase increased slightly. Elution volumes also increased with the amount of Trp immobilized on the gel. As for TRS, equation (2) does not describe the relative elution behaviours. The 6 μmole Trp/g AHT had an R_{bio} approximately 70% larger than that of the 5.6 μmole Trp/g AHT.

On 5.6 μmole/g Trp/g AHT columns (Figure 8), tryptophanase was retarded more than TRS, whereas on 4.3 μmole/g columns tryptophanase is retained very much less than TRS (Figures 9 and 2, respectively). One possibility is that hydrophobic chromatography is a more important factor for tryptophanase than for TRS. Thus, while the retardation due to bioaffinity is less, the hydrophobic

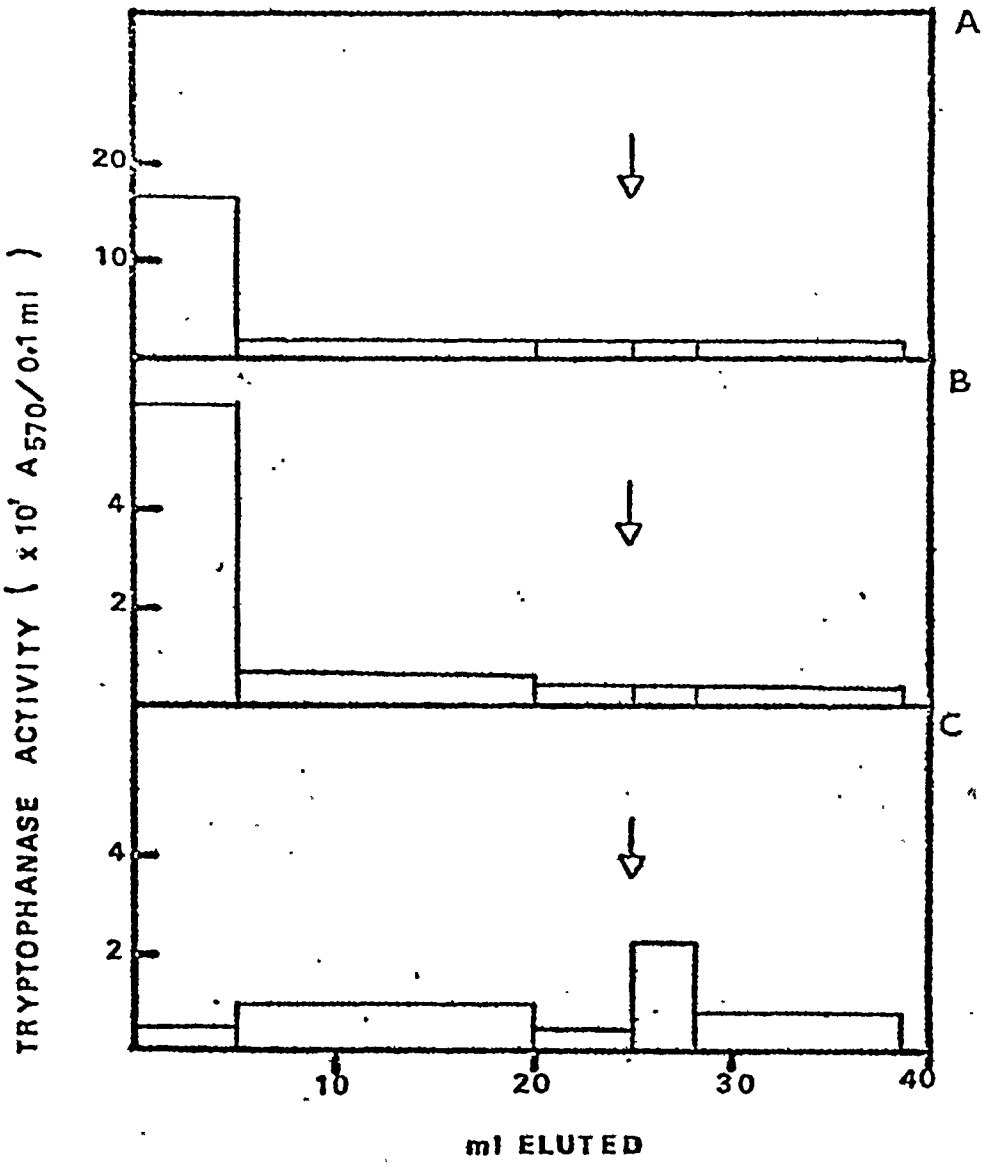


Figure 9: Affinity chromatography of tryptophanase on analytical columns.

The same columns as in Figure 2 were used, containing AHT substituted with a) zero; b) 4.3; and c) 4.7 µmole Trp per g AHT. In (a), 26 mg S-150 extract was applied; for the others (including Figure 10), 11 mg extract was used. This extract was prepared from *H. cutirubrum* cells which had been induced to produce more than the basal level of tryptophanase by the addition of L-Trp to the growth medium. The arrows indicate the addition of 16 mM L-Trp in solution D'. Recoveries were 90,95 and 70% of input, respectively.

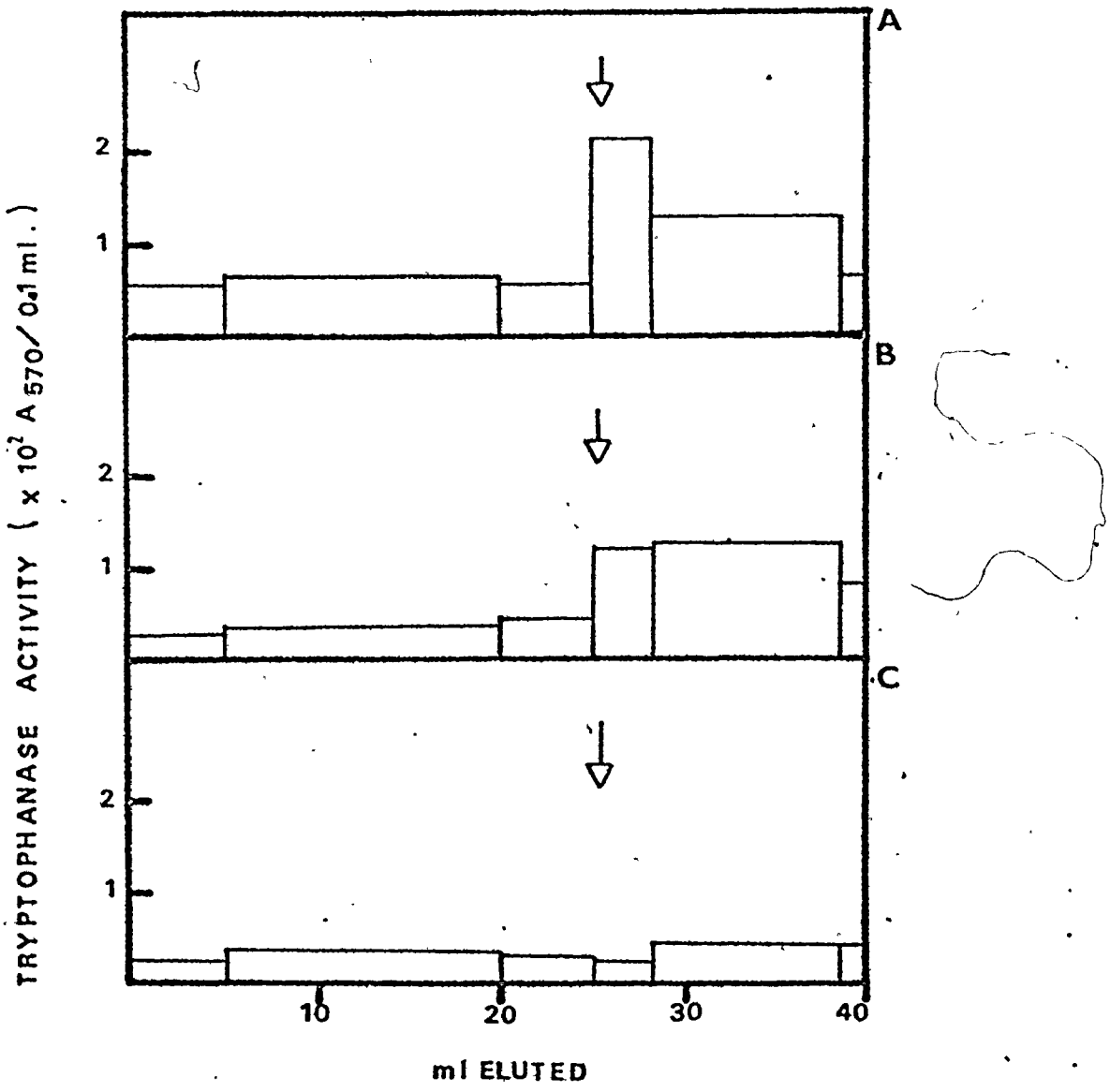


Figure 10: Affinity chromatography of tryptophanase on analytical columns.

The same columns as in Figure 3 were used, containing AHT substituted with a) 5.6; b) 6.0 and c) 8.9 µmole L-Trp per g AHT. Other conditions were the same as in Figure 9. Recoveries were 59,42 and 16% of input, respectively.

interactions are probably stronger for tryptophanase. The covalent modification of L-Trp in the preparation of AHT may produce more strongly binding derivatives of Trp than free Trp.

The presence of PLP in solution E increased retention of tryptophanase about 3-fold. This suggests that the holoenzyme but not the apoenzyme binds to AHT columns and subsequently PLP was added to most buffers when binding to AHT was required.

For larger scale purification of tryptophanase by affinity chromatography it was necessary first to chromatograph the S-150 extract on AHT using solution D' to remove some cochromatographing proteins. As well as TRS (see Figure 8), several other proteins also bound to AHT; attempts to identify them as other Trp metabolism enzymes such as Trp oxygenase were unsuccessful. A second chromatography of the tryptophanase peak with chromatography conditions similar to those of Figure 8 yielded preparations containing mainly one band on SDS-PAGE.

Two such preparations of tryptophanase will be described. In the first, 415 mg of S-150 extract was passed through a 30 ml column of AHT with 10.2 μmole Trp per g using 3 M KCl buffer. The tryptophanase peak (as in Figure 7) was then purified by affinity chromatography using solution E with no PLP on a 10 ml column of AHT with 5.6 μmole per g. After a 50 ml wash, tryptophanase was eluted with

16 mM L-Trp. The results were similar to the 16 mM L-Trp elution shown in Figure 8 for tryptophanase. However, no detectable TRS and little protein was seen in this second column. The L-Trp-eluted peak was analyzed on SDS-PAGE and the gel is shown in Figure 6C.

In the second preparation, 540 mg of S-150 extract, a 23 ml column of 5.6 μmole Trp/g AHT using solution D', and a 54 ml column of the same AHT in solution E were employed. After a 3 liters wash in the second chromatography the tryptophanase was eluted, concentrated and analyzed by SDS-PAGE. The result is shown in Figures 6D, E and F for 3 successive L-Trp eluted fractions. Purification for the three fractions was calculated to be 19-fold, 22-fold and 10-fold respectively. Because concentrated samples of L-Trp solutions were not used as blanks for the Lowry test these purification factors are low; based on purity of tryptophanase on SDS-PAGE and of the purification factors for tryptophanase of equivalent purity on SDS-PAGE these fractions should be approximately 80 to 200-fold purified.

This purification is extensive but only small amounts of tryptophanase can be purified by this method. About 3 m units tryptophanase is bound per g AHT containing 5.6 μmole Trp per g. This is 15-30 μg of pure tryptophanase or 25-100% as many tryptophanase as TRS molecules which are retained.

3.2. Heat treatment and $(NH_4)_2SO_4$ fraction of tryptophanase

A classical and simple method for enzyme purification which can be applied to heat stable halophilic enzymes is heat treatment. A preparation containing the enzyme of interest is put into a solution in which the enzyme is quite stable. The pH, salt concentration and protein concentration can be quite important. Also the inclusion of substrates, cofactors and inhibitors may further stabilize the enzyme. The temperature and time are determined at which most of the enzyme remains active and in solution while other proteins are inactivated and precipitated. Inactivated proteins are centrifuged out or filtered and a simple rapid purification is achieved. Fractionation by $(NH_4)_2SO_4$ precipitation is also a classical simple method which should be applicable to most halophilic enzymes because they are stable in solutions of this salt. These two methods were combined for the purification of tryptophanase.

The crude enzyme containing the stabilizing cofactor, PLP, was heat-treated at 76°C for 10 min. This treatment resulted in a 4.7-fold purification and a 94% recovery of tryptophanase (Table 3). The enzyme was further purified by $(NH_4)_2SO_4$ fractionation. Precipitation of tryptophanase from 2.46 M to 3.28 M $(NH_4)_2SO_4$ yielded 14.4-fold purification with 88% recovery of S-150 extract activity (Table 3). A second precipitation from 2.75 to 2.92 M $(NH_4)_2SO_4$ resulted in a net 40-fold purification with 51% recovery. This preparation was analyzed by SDS-PAGE and the result

Table 3

Purification of Tryptophanase by Heat Treatment and $(NH_4)_2 SO_4$ Fractionation

Treatment	Untreated S-150	Heat at 76°C	2.46-3.28 M $(NH_4)_2SO_4$ ppt	2.75-2.92 M $(NH_4)_2SO_4$ ppt
Purification factor	1.0	4.7	14.4	40
% recovery	100	94	88	51

S-150 extract, obtained from induced bacteria, contained 48 mg/ml protein and 15 munits/ml tryptophanase. This enzyme, in solution D' plus 0.4 mg/ml PLP, was heated to 76°C in less than 2 min, maintained at 76°C for 8 more min and then was cooled to less than 25°C in 1 min. The enzyme was filtered through Whatman 3 MM paper and dialyzed against 60% saturated or 2.46 M $(NH_4)_2SO_4$, 8 MM Tris-HCl, pH 6.7, 1.6 mM ME (2.46 $(NH_4)_2SO_4$ buffer). It was then centrifuged at 12000 g for 10 min to remove insoluble material. The supernatant was adjusted to 3.28 M $(NH_4)_2SO_4$ by the addition of solid salt. The precipitate was dissolved in 2.46 M $(NH_4)_2SO_4$ buffer. The $(NH_4)_2SO_4$ fractionation procedure was repeated to obtain tryptophanase precipitating from 2.75 M to 2.92 M $(NH_4)_2SO_4$. More than 20% of the tryptophanase activity was recovered in fractions not shown in the table.

is shown in Figure 6G. The subunit molecular weight of the most darkly stained band (54,000) corresponds to that for Figure 6C, suggesting that the major band in both was tryptophanase.

The same purification to the one described above but without added PLP gave only 86% recovery for the heat-treated fraction and smaller percent recoveries and purifications for subsequent fractions. Fractions precipitated by $(NH_4)_2SO_4$ were treated with 1 mM D, L-penicillamine to remove endogenous PLP (Morino and Snell, 1967) and the precipitates consisted mostly of distinct crystals. This result suggests that *H. cutirubrum* tryptophanase does not crystallize if it contains PLP, as is the case for *E. coli* tryptophanase (Morino and Snell, 1967).

3.3. Agarose-$(NH_4)_2SO_4$ chromatography

Recently, techniques have been developed for the separation of proteins (Rimerman and Hatfield, 1973; Porath *et al.*, 1973) and polysaccharides and glycoproteins (Bussey *et al.*, 1975) on agarose gels substituted by aliphatic amino acids or cross-linked by benzyl groups. Separation was reportedly due to the size and hydrophobicity (Bussey *et al.*, 1975), solubility in $(NH_4)_2SO_4$ (Rimerman and Hatfield, 1973), and "salting-out" adsorption (Porath *et al.*, 1973) of the macromolecules. Binding was achieved in high concentrations of phosphates or sulfates and was shown to be dependent on substitution of the agarose

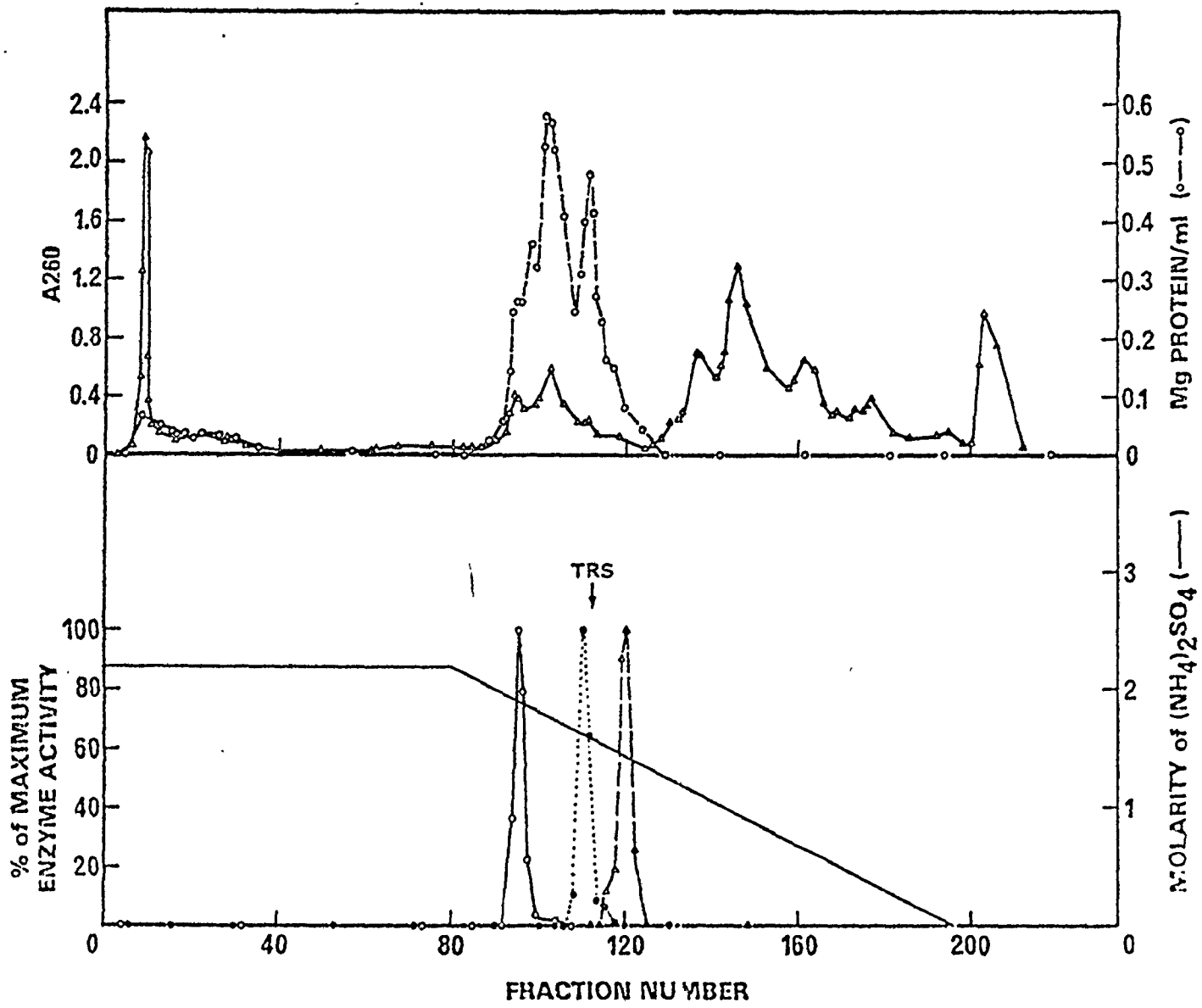
(Rimerman and Hatfield, 1973; Bussey *et al.*, 1975). The high capacity of gels and the general stability of proteins in high concentrations of these antichaotrophic ions suggest that the method would be useful in studies of halophilic proteins since these proteins are unstable in low salt concentrations.

For the purification of halophilic enzymes a new technique has been developed which does not require the agarose to be substituted but which does require high concentrations of $(NH_4)_2SO_4$.

When an S-150 extract of *H. cutirubrum* was added to buffer, (10 mM $Mg(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 6.7, 8 mM ME) containing 2.4 M $(NH_4)_2SO_4$, to give a protein concentration of 3 mg/ml and was then centrifuged at 10,000 for 10 min., 95% of the malate dehydrogenase (MDH), aldehyde dehydrogenase (ADH), tryptophanase and TRS remained in the supernatant. The clear red-brown supernatant was then applied to a column of agarose (Sepharose 4B). None of the 4 enzymes were detected in an extensive 2.4 M $(NH_4)_2SO_4$ buffer wash (Figure 11) but they were eluted by a gradient of $(NH_4)_2SO_4$ from 2.4 M to 0 in a pH 7.5 buffer of the same composition as before. ADH, MDH and tryptophanase were eluted as sharp peaks while TRS eluted as a broader peak in the main protein-containing fractions, 90-120. Recoveries were 85-93% for all four enzymes with purifications of 8-fold, 10-fold, 36-fold and 4-fold, respectively for tryptophanase, MDH, ADH and TRS. For clarity the

Figure 11: Fractionation of *H. cutirubrum* proteins and nucleic acids on agarose

The column was 1.5 x 25.5 cm with a volume of 45 ml. The S-150 supernatant contained 127 mg protein and 8% nucleic acids in 90 ml of 2.38 M $(NH_4)_2SO_4$ buffer. After the S-150 supernatant was applied the column was washed with 2.38 M $(NH_4)_2SO_4$ buffer and eluted with a linear gradient using 400 g or 350 ml 2.38 M $(NH_4)_2SO_4$ buffer in the mixing chamber and 400 g or 400 ml 0 M buffer in the reservoir. Fractions were 6.3 ml and protein was determined by the Lowry method. The maximum activities were 200 units/ml for ADH; 660 units/nl for MDH and 6.4 nmole/min/ml at 37°C for tryptophanase. Recovery of protein was 90% and of A_{260}-absorbing material was 95%. The arrow indicates the position of peak TRS activity.


A260
Mg PROTEIN/ml (o—o)
% of MAXIMUM ENZYME ACTIVITY
MOLARITY of $(NH_4)_2SO_4$ (——)
TRS
FRACTION NUMBER
0
40
80
120
160
200
2.4
2.0
1.6
1.2
0.8
0.4
0.6
0.5
0.4
0.3
0.2
0.1
100
80
60
40
20
3
2
1

profile of enzyme activity of TRS was not shown in Figure 11.

During application of S-150 extract and the 2.4 M $(NH_4)_2SO_4$ buffer wash a red-brown material was observed within the top 4 cm of the column. It was not a film or precipitate on the column surface and therefore was binding to agarose by adsorption and not due to precipitation. This material eluted in fractions 102-106. Assuming that the volume of the column to which other proteins are bound is the same as for the red-brown material, the capacity of the agarose exceeded 20 mg protein/ml. Based on the retention of the four enzymes on the whole column in Figure 11 a conservative estimate of agarose capacity would be 2 mg protein/ml.

The A_{280}/A_{260} ratio for fractions 90-125 varied from 1.3 - 1.75, indicating that the content of nucleic acids should be less than 1% of the protein content (Warburg and Christian, 1941). The method, however, depends on a standard curve obtained with RNA and BSA. Protein A_{260} and A_{280} is dependent on the content of Trp, Phe and Tyr.

Nucleic acids were present in fractions 7-12 and fractions 125-210. The material in fractions 125-210 contained no proteins based on the A_{280}/A_{260} ratio of 0.55 and the Lowry test for protein (Figure 11). The ratio of the A_{672} for the test for ribose to the A_{260} for the peak A_{260} containing fractions from 125-210 was the same as for the yeast RNA standard (Table 4). This indicates

Table 4

Assays of Peak Nucleic Acid-Containing Fractions from Figure 11 for RNA and $tRNA^{Phe}$

Fraction number	8	136	146	162	177	203	yeast RNA
A_{672} orcinol/ A_{260} ratio[1]	0.02	0.075	0.076	0.075	0.075	0.076	0.076
Cpm L-Phe ^{14}C charged/10 µl	0	5	128	9	15	15	-

[1] Fractions were dialyzed against distilled H_2O to remove $(NH_4)_2SO_4$ and assayed for RNA by the orcinol test for ribose (A_{672}) and this was divided by the A_{260} of the dialyzed fractions.

the presence of RNA free of DNA and proteins. Of the peak RNA-containing fractions, only fraction 146 contained $tRNA^{Phe}$ (Table 4). The charging capacity for Phe of this fraction was 12 μ moles/A_{260}, which was 40% of that for halophile tRNA prepared by the method of von Ehrenstein (1968). This lower acceptance suggests either that the peak for $tRNA^{Phe}$ occurred some fractions away from tube 146, or more probably that RNA species other than tRNA were also eluted in these fractions. The material in fractions 8-12 of Figure 11 had an A_{280}/A_{260} ratio of 0.53-0.59 and was probably mostly small DNA fragments formed by deoxyribonuclease digestion in the preparation of S-150 extracts because 80% of the A_{260} of these fractions was lost during dialysis. The 20% of the A_{260} which remained from fraction 8 after dialysis represented, in part, protein, as observed from the Lowry test in Figure 11, and, in part, RNA, as the A_{672}/A_{260} ratio was greater than 0 (Table 4).

Tryptophanase was purified by a combination of heat treatment and agarose-$(NH_4)_2SO_4$ chromatography. When S-150 extract was heated to 72°C for 10 min in solution D' plus 0.4 mg/ml PLP, purification was 4.1-fold. The heated extract was filtered through Whatman 3 MM paper, dialyzed against 2.38 M $(NH_4)_2SO_4$ buffer, and centrifuged. The supernatant was 5-fold purified over the S-150 extract. The supernatant was subjected to agarose-$(NH_4)_2SO_4$ chromatography and the results are shown in Figure 12. When fractions 135-146 containing tryptophanase activity were pooled and concentrated, the specific activity of the enzyme

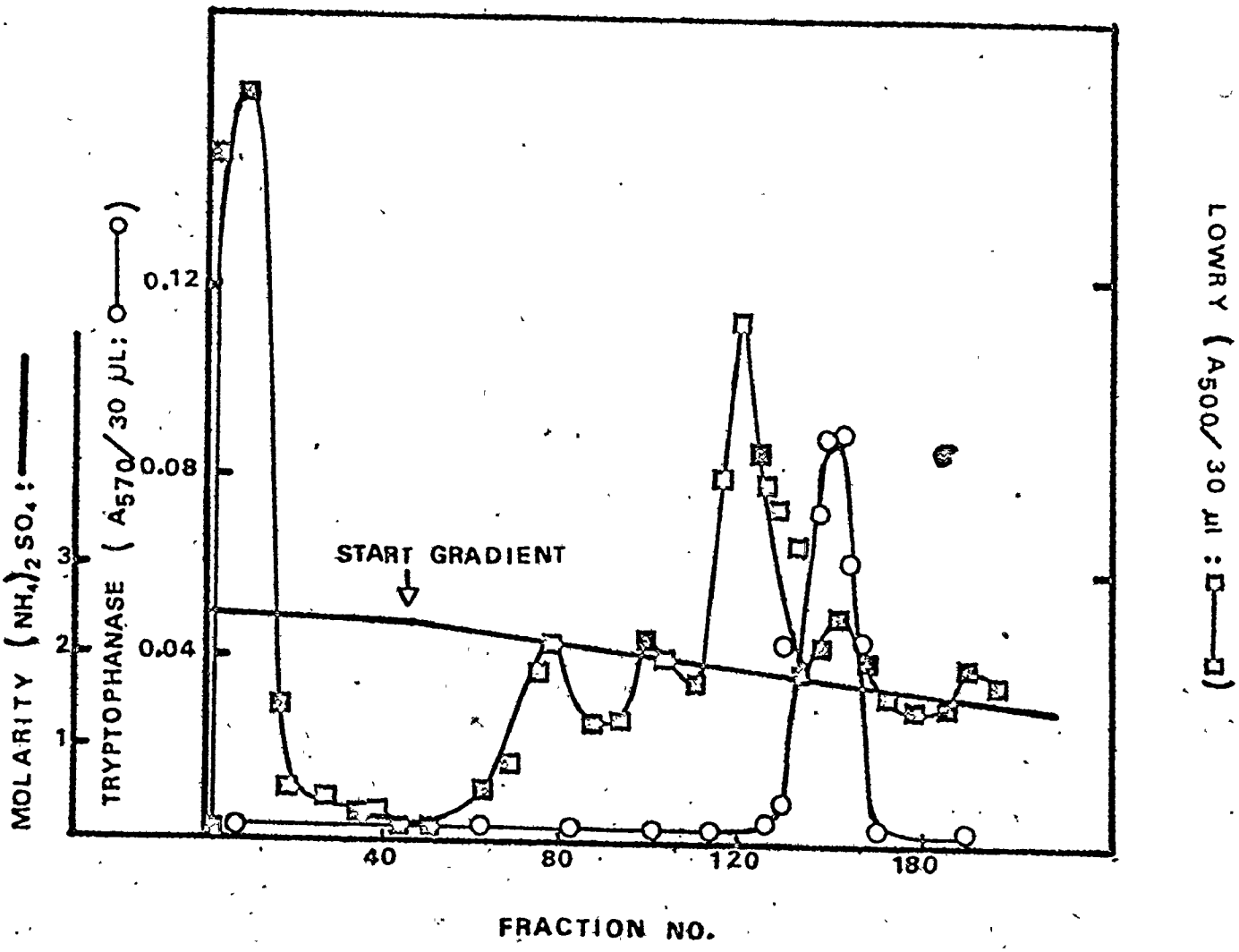


Figure 12: Agarose-$(NH_4)_2SO_4$ chromatography of tryptophanase.

An 8.5 ml 17 cm high column of agarose (Sephadex 4B) equilibrated in 2.38 M $(NH_4)_2SO_4$ buffer was used. Heat-treated enzyme was added to the column; the column was washed with 110 ml of 2.38 M $(NH_4)_2SO_4$ buffer followed by a 500 ml gradient of 2.38 $(NH_4)_2SO_4$ to 1.4 M $(NH_4)_2SO_4$ buffer.

had increased 15-fold (compared to an 8-fold increase in Figure 11) to a level 90-fold greater than that of the S-150 extract. The tryptophanase peak from Figure 12 was again chromatographed in the same column chromatography procedure. The second tryptophanase peak was concentrated. It was 200-fold purified over crude extracts and was nearly homogeneous when analyzed by SDS-PAGE (Figure 6H). The main band had a molecular weight similar to that for tryptophanase in other preparations (Figures 6C,D,E,F and G).

3.4. Discussion of purification procedures

Enzymes from halophilic bacteria are generally unstable in low salt concentrations. Three methods have been devised to purify such enzymes in the presence of high concentrations of salt. Heat treatment combined with $(NH_4)_2SO_4$ fractionation and agarose-$(NH_4)_2SO_4$ chromatography were found to be relatively quick and effective general methods for purifying these enzymes. Tryptophanase was found to be particularly amenable to purification by such procedures. These fractionation procedures were modified from those used to purify non-halophilic enzymes.

A new fractionation procedure has been developed for the purification of halophilic enzymes. The chromatography on unsubstituted agarose in high concentrations of $(NH_4)_2SO_4$ was found to be a single-step, high yield and effective method of fractionating proteins and nucleic acids. The basis for separation is not known at this time. The mechanism

may involve a partial salting-out of the macromolecules to a degree where interactions between the macromolecules are not strong enough to effect a precipitation from solution but where the interactions between the polysaccharides of the gel and the macromolecules in solution are sufficiently strong to adsorb the macromolecules. Decreasing the salt concentration would then solubilize the macromolecules in a form of partition or adsorption chromatography. This purification method should prove to be particularly useful for halophilic systems in which many enzymes are unstable when antichaotrophic salts are removed. The separation of nucleic acids is important in protein purification also, particularly if nucleic acids are substrates.

The most effective method for fractionating halophilic enzymes was affinity chromatography. The preparation of an affinity column, AHT, has been described and its application to the purification of TRS and tryptophanase has been demonstrated.

TRS was purified to homogeneity on SDS-PAGE by two successive passages through AHT with biospecific elution with L-Trp. As TRS is an enzyme which irreversibly loses its activity in low salt concentrations, chromatography was performed using 3 M KCl and 0.1 M $Mg(C_2H_3O_2)_2$ in all buffers. The 10% yield and 1000-fold purification were both very high considering the exceptionally refractory nature of most halophilic enzymes.

The affinity chromatography purification of halophilic TRS was much more extensive than for comparable methods for non-halophilic aminoacyl-tRNA ligases (Robert-Gero and Waller, 1972; Forrester and Hancock, 1972; Remy *et al.*, 1972). For these authors bioelution was not found to be practical. In many instances, enzymes were eluted with salt gradients so that the procedures were probably ion exchange chromatography. Even when 0.2 M phosphate buffer was used half of the protein was non-specifically adsorbed onto the columns of Rebert-Gero and Waller (1972). Using higher concentrations of salt would probably interfere with enzyme binding.

Elution of halophilic TRS required a relatively small volume of 20 mM L-Trp because no retention due to ionic binding occurred. Thus, the lack of ionic binding and other salt-sensitive interactions allows a much more effective purification.

Affinity chromatography of tryptophanase has also been successful on the same AHT columns. Tryptophanase prepared by 2 column chromatographies was shown to have one main band on SDS-PAGE which was of the same molecular weight as the main protein bands on gels for tryptophanase purified by two different techniques. Selective tryptophanase binding was found in 5 M NaCl buffer as opposed to 3 M NaCl or 3 M KCl buffers. This might be construed to indicate that hydrophobic chromatography is the sole mechanism of fractionation since hydrophobic forces are enhanced by higher ionic strength solutions. However, the separation

of tryptophanase from other proteins which are probably also bound due to hydrophobic interactions should not then occur.

The effect of salt concentration and PLP on tryptophanase binding suggests an approach which might assist various halophilic enzyme purification schemes. For the various fractionation procedures available an initial purification with one buffer may be followed by another purification with a buffer of different pH and containing different ingredients such as substrates, cofactors, products and any of the antichaotrophic salts. By the appropriate manipulation of the second buffer a significant further purification might be achieved. Such an approach was used in the affinity chromatography of tryptophanase. The approach could be used for other techniques. For example, isocitrate dehydrogenase has twice the molecular weight in 3 M NaCl than it has in 3 M KCl (Aitken and Brown, 1972). This property could be used to purify the enzyme on dextran gels.

4. MOLECULAR WEIGHT ESTIMATION OF HALOPHILIC ENZYMES

Estimates of the molecular weights of halophilic enzymes were made by chromatography on Sephadex gel and by electrophoresis under denaturing conditions on SDS-polyacrylamide gels.

For proteins chromatographed on Sephadex G100, the partition coefficient between the liquid and gel phases, K_{AV}, is linearly proportional to the logarithm of molecular weight in the range from 4000 to about 100,000 daltons, provided the proteins are of low asymmetry and contain no carbohydrate (Andrews,1964; 1965). The partition coefficient K_{AV} was calculated from the formula

$$K_{AV} = \frac{Ve-Vo}{Vt-Vo}$$

where Ve is the elution volume (peak enzyme activity or A_{280} for standard proteins), Vo is the void volume or the volume between the gel grains in the column bed (taken to be the peak A_{630} of dextran blue 2000 which is completely excluded from the gel), and Vt is the total volume of the gel bed or volume of water that fills the column to the same height.

A column of Sephadex G-100 (Superfine grade) was equilibrated in solution D' and four standard proteins and an S-150 extract were chromatographed in the same buffer. The calibration curve obtained with the standards is shown

in Figure 13, and the molecular weights estimated from this curve for several halophilic enzymes are given in Table 5.

These estimates are limited by the following considerations:

1. The calibration curve departs increasingly from linearity above 100,000 daltons (Andrew, 1964). This means that estimates from Figure 13 are low for molecular weights above this value and are high for molecular weights below it.
2. These proteins are assumed to meet the requirements stated earlier in being nearly spherical and in lacking carbohydrate.
3. It is assumed that the same relationship between K_{AV} and log molecular weight exists in the concentrated salt solutions used here as in normal buffers. Results with two enzymes suggest that this assumption may not lead to gross errors. For catalase, the present estimate in concentrated salt is similar to that of 240,000 obtained by Lanyi and Stevenson (1969) with dextran gel chromatography in 0.2 M NaCl. Molecular weight data on isocitrate dehydrogenase has been reported by Aitken *et al.* (1970). These authors found in dextran gel filtration experiments that the molecular weight depended on buffer composition. One of the values was 71,000, which is similar to that in Table 5.

The molecular weights of purified TRS (Figure 6B) and of purified tryptophanase (Figures 6C, E and H) were also estimated by the SDS-PAGE method of Weber and Osborn (1969). The calibration curve and the estimates of molecular

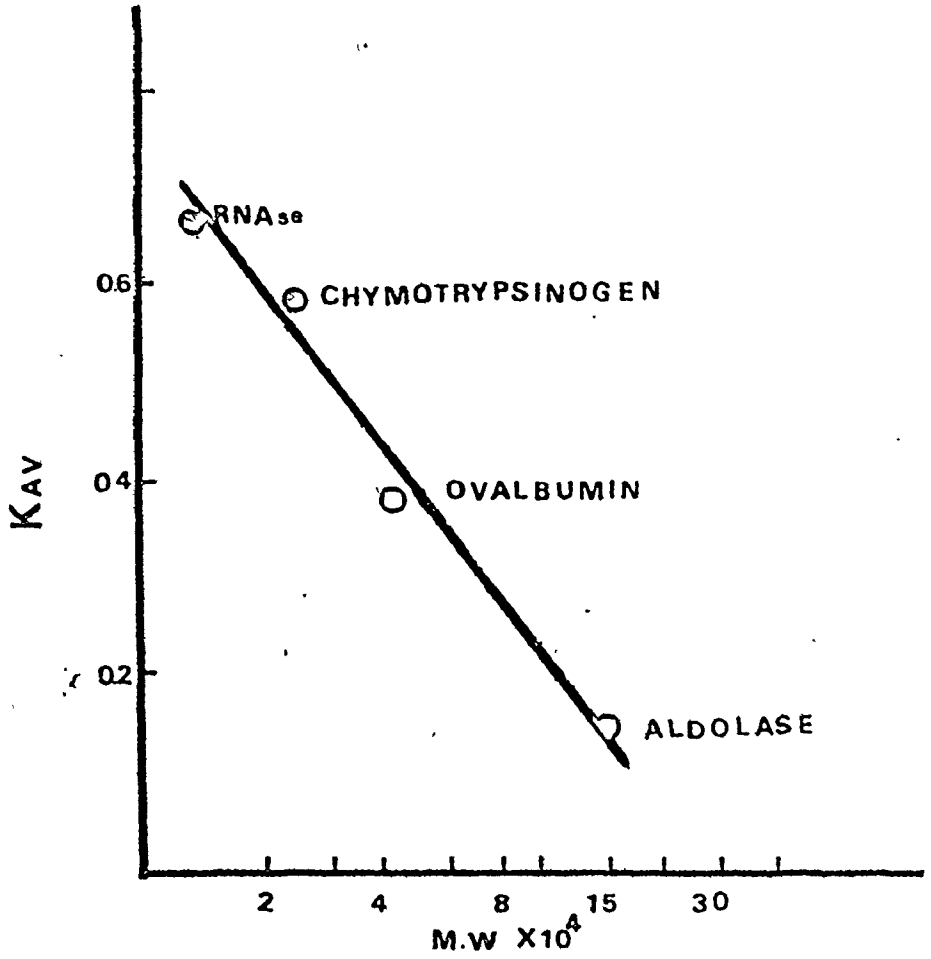


Figure 13: Dextran gel molecular weight calibration curve

A 49 ml column of Sephadex G100 Superfine was used. Procedures are described in Section 2.7.3 and calculations are described in the text.

Table 5

The Molecular Weights of Halophilic Enzymes Estimated by Gel Filtration

Halophilic enzyme	Estimated molecular weight
TRS	120,000
PRS	240,000
Tyrosinyl-tRNA ligase	17,000
Catalase	210,000
Isocitrate dehydrogenase	60,000
Tryptophanase	190,000

weight are shown in Figure 14. Estimates by this method are reliable only if the proteins contain no carbohydrate residues or phosphate groups.

The molecular weight of TRS estimated by gel filtration was about 120,000 daltons (Table 5), while under denaturing conditions, SDS-PAGE gave in three separate experiments values between 63,000 and 65,000 daltons. Although these estimates are approximate, it may be tentatively suggested that the enzyme contains two subunits and is of the α_2 type. Three TRS enzymes from other systems have been reported of the α_2 type. The molecular weight of *E. coli* TRS is 74,000 on dextran gels and 37,000 daltons on SDS-PAGE (Joseph and Muench, 1971). Human placental TRS SDS-PAGE molecular weight is 58,000 while the molecular weights on sucrose gradients and dextran gels are 100,000 and 125,000 respectively (Penneys and Muench, 1974). For yeast TRS, the SDS-PAGE molecular weight is 50,000 daltons while the dextran chromatography molecular weight is 110,000 daltons (Hossain and Kallenbach, 1974).

Within the limitations of the estimates reported here, therefore, halophilic TRS appears comparable in size and subunit structure to the corresponding enzymes in several other systems.

Tryptophanase gave a molecular weight by gel filtration of about 190,000 and by gel electrophoresis (Figure 6C, E and H) of between 53,000 and 56,000 daltons.

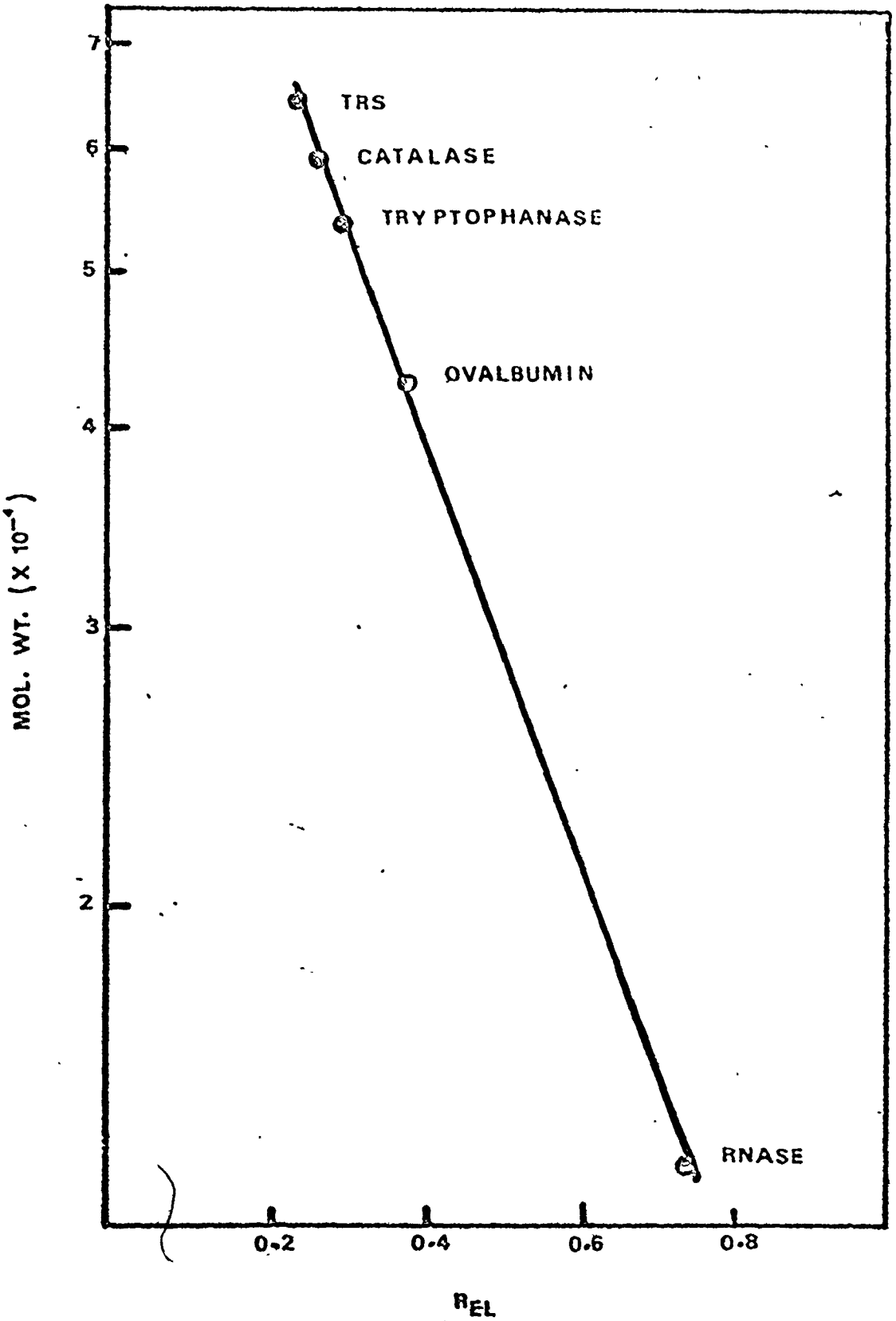


Figure 14: Estimations of molecular weight by SDS-PAGE

Standard proteins, TRS and tryptophanase were electrophoresed on gels and their electrophoretic mobilities relative to Bromophenol blue (R_{EL}) are plotted against the log of their molecular weights.

For the reasons discussed earlier, the value from gel filtration is probably grossly underestimated. It is possible therefore that this enzyme is of the α_4 type with a molecular weight of over 200,000 daltons. This would make it similar in size to the tryptophanases of several other bacteria. That of *E. coli* K12 is 223,000 daltons (London and Goldberg, 1972) and of *E. coli* B, 220,000 daltons with 55,000 dalton subunits (Morino and Snell, 1967); *Bacillus alvei* tryptophanase is 208,000 daltons (Hoch *et al.*, 1966); *Proteus rettgeri* tryptophanase is 210,000 daltons; and *S. funduliformis* tryptophanase is 245,000 daltons.

Thus tryptophanase appears to have a similar molecular form in all of the bacteria studied, including *H. cutirubrum*. Tryptophanase from non-halophilic bacteria are fully active below 0.1 M K^+ but are not inhibited at higher concentrations. The halophilic enzyme, with 20% more activity at 5 M KCl, therefore has an exaggerated but not unusual requirement for K^+.

Molecular weight estimates for two other halophilic aromatic aminoacyl-tRNA ligases are also included in Table 5. That for PRS (240,000 daltons) is undoubtedly low but is the same order of magnitude as those reported for this enzyme in other organisms viz. 180,000 to 290,000 (Kisselev and Favorova, 1974). Halophilic Tyr-tRNA ligase appears to have an exceptionally low molecular weight (17,000).

The shape of its elution profile from dextran gels was skewed toward the void volume whereas those of other enzymes were not. This suggests the subunits were being dissociated and subunit interactions were occurring during chromatography. Even so, the molecular weight of this Tyr ligase is low. While for non-halophilic systems the Tyr-tRNA ligases generally have the lowest molecular weights, the lowest reported is 40,000 daltons and the lowest molecular weight reported for any aminoacyl-tRNA ligase subunits is 31,000 daltons (Kisselev and Favorova, 1974). On this basis, halophilic Tyr-tRNA ligase therefore appears to deserve further study.

5. PROPERTIES OF PURIFIED HALOPHILIC TRYPTOPHANYL-tRNA LIGASE

Initial experiments were performed to determine enzyme and substrate concentrations necessary to give linear enzyme activity up to 30 min.

5.1. The effect of pH on TRS activity

Tryptophanyl-tRNA ligase (TRS) which had been purified as described in Section 3.1.1 (Figure 6B) was examined for the effect of pH on the maximum velocity. The optimal TRS activity was at pH 7.5 and the activity was limited to a relatively small pH range from 6.4 to about 9 (Figure 15).

The shape of a pH curve can be determined by three factors (Dixon and Webb, 1964). It may be the result of a true reversible effect on the maximum velocity, or a decrease in the affinity for substrates so that velocity is less than maximum, or an instability of the enzyme at pH extremes. The shape of the pH curve for TRS probably is the result of a true reversible effect on the maximum velocity, since excess substrates were used and the pH curve was quite sharp (Fig. 15).

5.2. The effects of NaCl, KCl and Tris concentrations on TRS activity and stability

The amount of $tRNA^{Trp}$ charged in a 30 min period increased linearly when NaCl concentration increased from 0 to 1.7 M (Figure 16). A clearer illustration of the effects of ionic strength and NaCl and KCl concentrations can be made by taking the following data from Table 6:

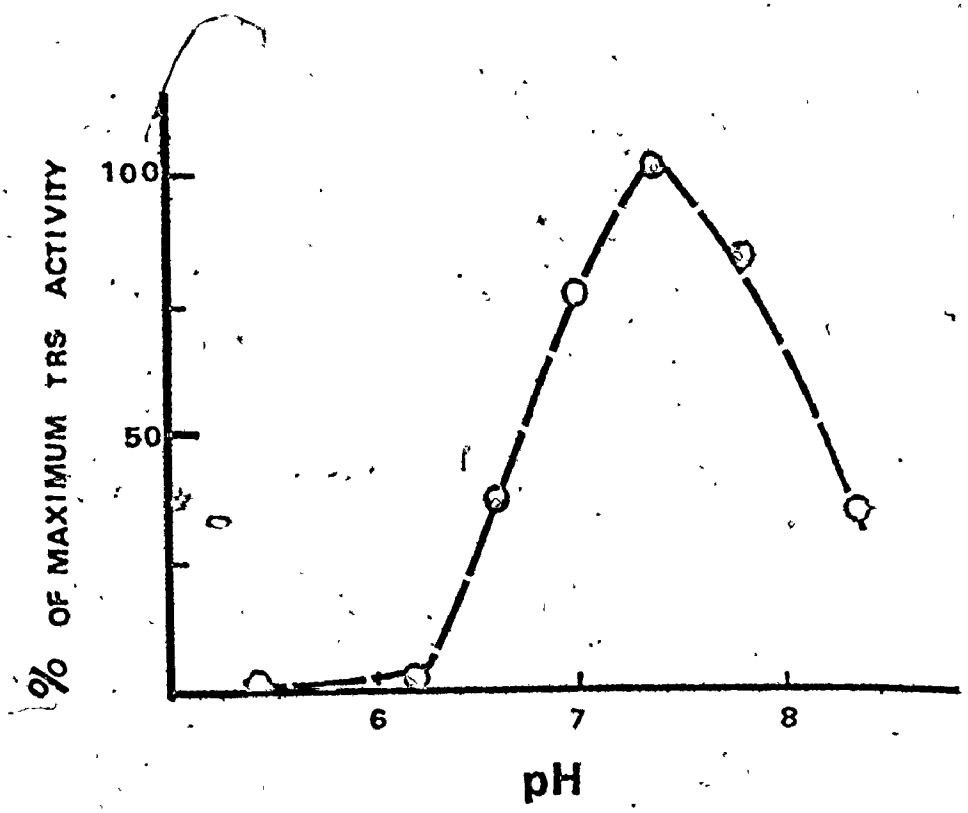


Figure 15: The effect of pH on purified TRS

Assays were in 0.13 ml containing 0.96 M NaCl, 4.8 M KCl, 7.44 μM L-Trp-^{14}C, 1.15 mM ATP (neutralized), 19.1 mM Mg $(C_2H_3O_2)_2$, 1.73 μM $tRNA^{Trp}$, 0.16 mM ME and 0.15 M Tris-maleate. The maximum cpm per assay was 3180 and the variation of duplicates from the average value was not greater than 6%.

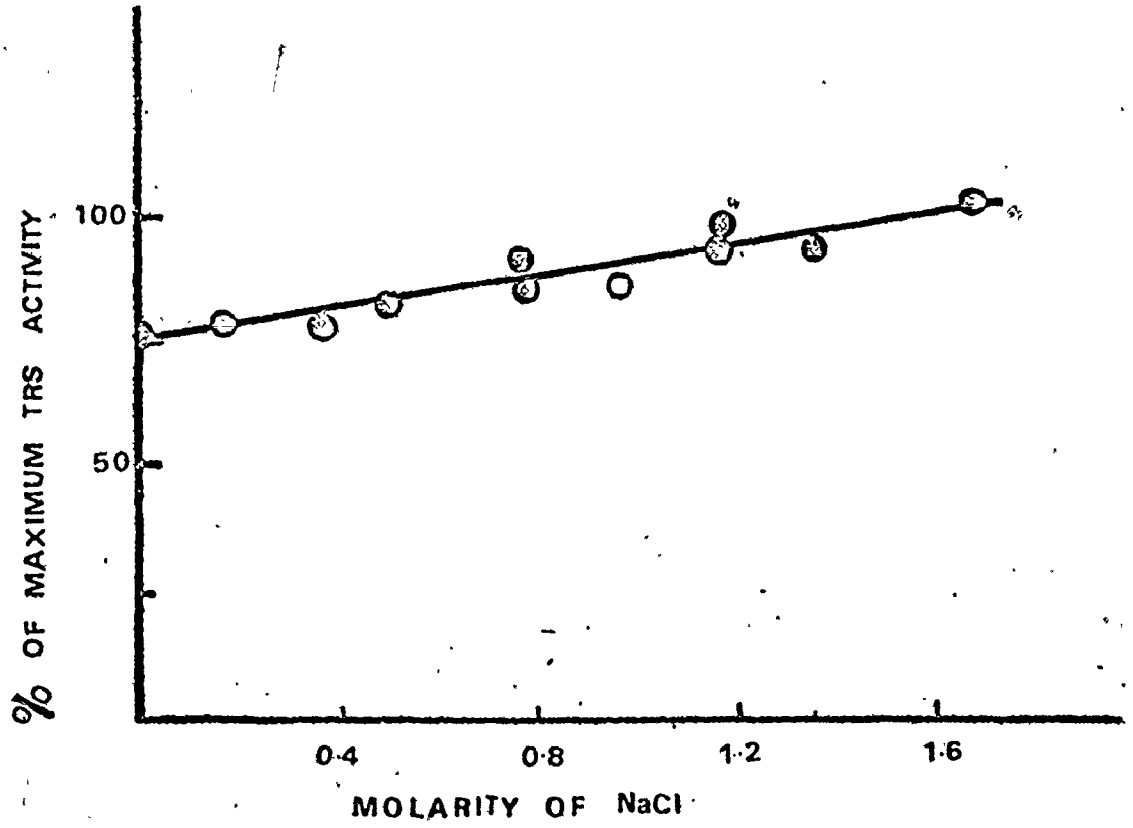


Figure 16: The effect of NaCl concentration on purified TRS activity

Assay conditions were as described in Figure 15 except that 4.45 mM ATP, 10.5 mM Mg $(C_2H_3O_2)_2$, 78 mM Tris-HCl, pH 7.5 and the indicated amounts of NaCl were used. The maximum cpm per assay was 1580. The KCl concentration was saturating and varied from 5.3 M at 0 M NaCl to 4.5 at 1.7 M NaCl. The points represent single assays.

Salt concentration	4.8 M KCl 1.2 M NaCl	4.3 M KCl 1.2 M NaCl	5.3 M KCl No NaCl	3.8 M KCl 3.5 M NaCl
% change of activity	0	-3	-20	0
% change of stability	0	-20	-6	-54

This data shows that decreasing NaCl concentration from 1.2 to 0 M caused a loss of activity (amount of tRNA charged during a 30 min assay) while TRS stability (% loss of activity during a 30 min preincubation) changed little. When the KCl concentration is lowered from 4.8 M to 4.3 M a loss of TRS stability occurred while TRS activity was not significantly changed. The above data show no correlation of TRS stability and activity to either the ionic strength or Cl^- concentration. Thus, near the optimal enzyme assay conditions K^+ is important for TRS stability and Na^+ is important for TRS activity.

An increase from 8 to 78 mM Tris-HCl increased the stability of TRS 20% but Tris-HCl had no stimulatory effect (Table 6). Tris is known to accelerate aminoacylation of non-halophilic enzymes (Eigner and Loftfield, 1974), possibly by participating in the reaction as a general base. The use of a buffer which does not affect the rate of the aminoacylation reaction is desirable.

5.3. The effects of sulfhydryl-group protecting and blocking reagents on tryptophanyl-tRNA ligase

Experiments were performed to examine the importance of sulfhydryl-groups (SH-groups) on TRS activity. Similar

Table 6

The Effects of Various Assay Conditions on Purified TRS Activity and Stability[1]

Assay condition[2]	TRS activity[3]	TRS stability[4]
No TRS	-	-7
No tRNA	-	-100
No ATP	-	-5
No Trp	-	-11
4.3 M KCl 1.2 M NaCl	-3	-20
No NaCl 5.3 M KCl	-20	-6
3.8 M KCl * 3.5 M NaCl	0	-54
8 mM Tris-HCl	-2	-20
0.25 mM Mg^{++}	-65	+14
20 mM $KC_2H_3O_2$	0	-2
2.1 mM * ME	+3	-1
0.8 mg/ml * BSA	+2	-8

Table 6

[1] The effects are expressed as a % change from the standard assay.

[2] Standard assay conditions were 1.2 M NaCl, 4.8 M KCl, 7.44 µM L-Trp-^{14}C, 4.5 mM ATP, 9.2 mM Mg $(C_2H_3O_2)_2$, 78 mM Tris-HCl, pH 7.5, 1.28 µM tRNA and 0.6 mM ME.

[3] Corrections were made for changes in the stability of TRS by the various assay conditions. Standard assays gave 9,720 cpm.

[4] TRS stability was determined by preincubating the complete assay minus TRS, tRNA, ATP or L-Trp, respectively, for the first four assays at 37°C for 30 min. These assays were then started by the addition of TRS or the missing substrate and continued as usual and the numbers express the % change in TRS activity relative to the standard non-preincubated assay giving 9,720 cpm. The other eight assays were preincubated with the indicated assay conditions but without L-Trp for 30 min. Then the assays were adjusted to the standard assay conditions except in three cases (*) and the normal 30 min assay was completed. For these last eight assays, the TRS activity remaining after preincubation of the "no Trp" assay was the standard assay. Variation of duplicates from the average value was less than 10% for all numbers in the table.

experiments with halophilic phenylalanyl-tRNA ligase (PRS) were done for comparison. The activities of the two enzymes in S-150 extracts as a function of the concentrations of two SH-protecting reagents, ME and dithiothreitol, are plotted (Figure 17). While TRS shows only a slight inhibition by both reagents, PRS is protected by both with inhibition only at higher concentrations. Activities were higher in the presence of dithiothreitol than ME for both TRS and PRS.

The effects of SH-blocking reagents on TRS and PRS activities is shown in Table 7 and are consistent with the data of Figure 17. In the experiments with S-150 extracts both enzymes were treated with reagents in the same tubes. With DTNB or NEM at 10^{-4} M TRS was not inhibited but PRS was inhibited 100% and 20%, respectively. The 10^{-3} M concentrations only slightly inhibited TRS. $HgCl_2$ inactivated both TRS and PRS, even at 10^{-4} M, possibly due to precipitation of the enzymes after reacting with both essential and non-essential SH-groups.

Purified TRS is slightly more inhibited than was S-150 extract (Table 7). This may be related to the TRS stabilizing effect of endogenous tRNA (see Section 5.5.3.) or of other macromolecules in the S-150 extract.

The effects of ME on the stability of TRS in S-150 extracts and purified TRS can be seen in Table 8. Adding 8.2 mM instead of 0.16 mM ME to purified TRS in 3 M KCl, 10 mM Tris-KCl, 20 mM $Mg(C_2H_3O_2)_2$ buffer increased the half-life ($T_{\frac{1}{2}}$) from 0.7 hr to 1.8 hr. However, the $T_{\frac{1}{2}}$ of S-150

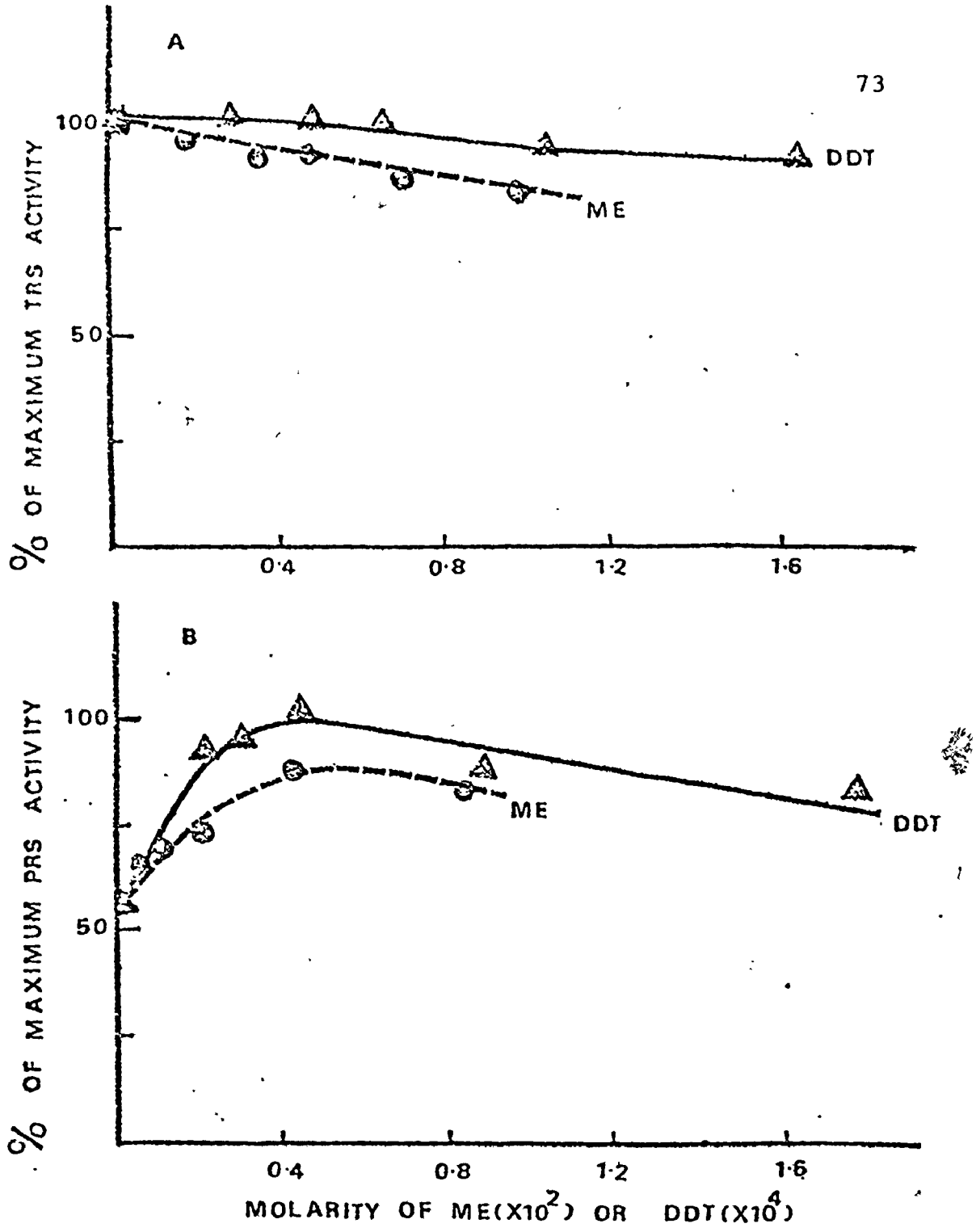


Figure 17: The effect of concentration of ME or dithiothreitol (DTT) on (a) TRS and (b) PRS activities

S-150 extract had been dialyzed against 3 M KCl, 0.1 M $Mg(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 7.7 before the assay. Maximum activities were 1520 cpm/1.0 µl for TRS and 207000 cpm/10 µl for PRS.

Table 7

The Effects of SH-Group Blocking Reagents on TRS and PRS Activities

Enzyme[1]		TRS			PRS		
S-150 extract	Inhibitor concentration	0	10^{-4} M NEM[2]	10^{-3} M NEM	0	10^{-4} M NEM	10^{-3} M NEM
	% of control[4] cpm	100	107	83	100	80	21
S-150 extract	Inhibitor concentration	0	10^{-4} M DTNB[3]	10^{-3} M DTNB	0	10^{-4} M DTNB	10^{-3} M DTNB
	% of control[5] cpm	100	107	92	100	0	0
S-150 extract	Inhibitor concentration	0	10^{-4} M $HgCl_2$	10^{-3} M $HgCl_2$	0	10^{-4} M $HgCl_2$	10^{-3} M $HgCl_2$
	% of control[5] cpm	100	0.3	1.6	100	3.4	3.3
Purified TRS	Inhibitor concentration	0	10^{-4} M NEM	10^{-3} M NEM			
	% of control[6] cpm	100	90	66			
Purified TRS	Inhibitor concentration	0	10^{-4} M DTNB	10^{-3} M DTNB			
	% of control[6] cpm	100	92	79			

Table 7

[1] The enzyme was dialyzed against solution D and adjusted to the indicated concentrations of SH-group blocking reagents and kept on ice for 15 min. It was then assayed in the presence of 0.17 mM ME to protect tRNA from oxidation.

[2] N-ethylmaleimide.

[3] 5,5'-dithiobis(2-nitrobenzoic acid).

[4] 5920 cpm and 26,100 cpm for TRS and PRS, respectively.

[5] 3380 cpm and 66,400 for TRS and PRS, respectively.

[6] 1480 cpm.

Table 8

Stability Studies on TRS in Various Buffers and at Two Temperatures

Buffer	TRS	Temperature	$T_{\frac{1}{2}}$
3 M KCl, 10 mM Tris-HCl, pH 7.6, 20 mM $Mg(C_2H_3O_2)_2$, 0.16 mM ME	purified, 2.0×10^{-4} units/ml	30°C	0.7 hr
As above, + 7.2×10^{-4} M ATP	purified, 2.0×10^{-4} units/ml	30°C	0.7 hr
As on top line, + 8.2 mM ME	purified, 2.0×10^{-4} units/ml	30°C	1.8 hr
As on top line, + 8 mg/ml tRNA	purified, 2.0×10^{-4} units/ml	30°C	6.2 hr
3 M KCl, 0.1 M $Mg(C_2H_3O_2)_2$, 20 mM Tris-HCl, pH 7.5	purified, 4.4×10^{-4} units/ml	30°C	2.5 hr
3 M KCl, 0.1 M $Mg(C_2H_3O_2)_2$, 20 mM Tris-HCl, pH 7.5	purified, 3.1×10^{-4} units/ml	30°C	2.5 hr
3 M KCl, 0.1 M $Mg(C_2H_3O_2)_2$, 20 mM Tris-HCl, pH 7.7, 0.8 mM ME	S-150 extract 8.7×10^{-5} units/ml	4°C	28 days

3 M KCl, 0.1 M $Mg(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 7.7, 8 mM ME	S-150 extract 1.1×10^{-4} units/ml	$4^{\circ}C$	3.4 days
5 M NaCl, 0.1 M $Mg(C_2H_3O_2)_2$, 10 mM Tris-HCl, pH 7.7, 8 mM ME	S-150 extract 1.1×10^{-4} units/ml	$4^{\circ}C$	6.4 days

[1] The half-life of TRS, determined from semi-log plots of the activity remaining after incubation of TRS under the designated conditions of temperature and buffer until at least 50% of the TRS activity was lost.

extract was decreased from 28 days to 3.4 days at 4°C when 8 mM instead of 0.8 mM ME was used. Thus the effects of different concentrations of SH-group blocking and protecting reagents on purified TRS are the reverse of those on TRS in S-150 extracts.

Changing the concentration of ME from 0.6 mM to 2.1 mM had no significant effect on the activity and stability of purified TRS (Table 6), suggesting that neither the enzyme nor the cognate tRNA contain essential SH-groups which are readily oxidized.

5.4. The effect of Mg^{++} concentration on TRS activity and stability

For TRS the optimum concentration of Mg^{++} is approximately 10 mM and the activity is highly sensitive to changes in the concentration of Mg^{++} (Figure 18). The stability of TRS decreased 14% when the concentration of Mg^{++} was increased from 0.25 to 9.2 mM (Table 6). The optimum Mg^{++} concentration in Figure 18 should therefore be 7% greater and some of the decline in activity observed at higher Mg^{++} concentrations may be attributable to decreased TRS stability.

TRS stability increased 360% when the Mg^{++} concentration was increased from 20 to 100 mM in absence of tRNA and ATP (Table 8). However, TRS stability decreased when Mg^{++} concentration was increased from 0.25 to 9.2 mM in the presence of tRNA and ATP (Table 6). This suggests that TRS

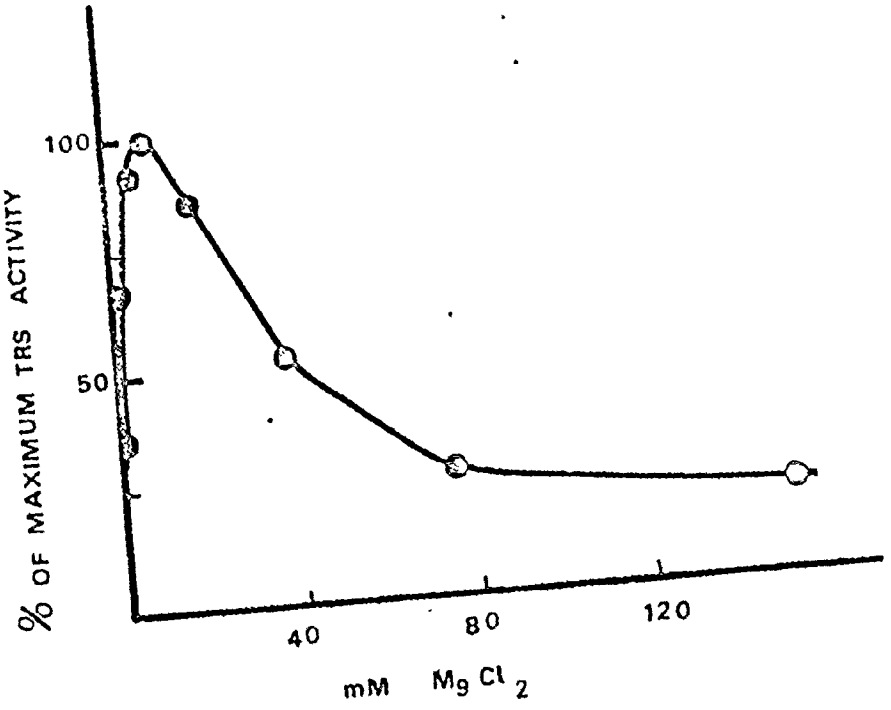


Figure 18: The effect of the concentration of Mg^{++} on purified TRS activity

Assay conditions were as described for Figure 16 except that 0.96 M NaCl and the indicated amounts of $MgCl_2$ were used. The maximum cpm per assay was 1320 and the difference of duplicates from average cpm was not greater than 8%.

stability is not decreased by the binding of Mg^{++} to TRS in the latter case but probably by interacting or competing with ATP or tRNA or with TRS-ATP or TRS-tRNA complexes. As only tRNA has a large influence on TRS stability, tRNA is more likely to be involved.

An assay in which 20 mM $KC_2H_3O_2$ was added (Table 6) shows that none of the above results were due to the acetate ion ($C_2H_3O_2^-$) which usually accompanies Mg^{++}.

5.5. The effects of substrate concentration on TRS activity and stability

5.5.1. L-Tryptophan

Michaelis-Menten kinetics were observed for the effect of L-Trp on purified TRS activity (Figure 19). Little increase in TRS activity occurred above 7 µM Trp and therefore this concentration was used in all TRS assays. The Lineweaver-Burk plot for this data is shown in the bottom of Figure 19. From the plot the K_m was calculated to be 1.3×10^{-6} M.

The stabilization of TRS by Trp appeared to be minimal. The absence of Trp from the preincubation mixture for 30 min at 37°C (Table 6) resulted in a loss of 11% of the TRS activity when compared to a control which was not preincubated. However, even preincubation of the 3 substrates of TRS without the enzyme resulted in a comparable reduction in the amount of tRNA charged.

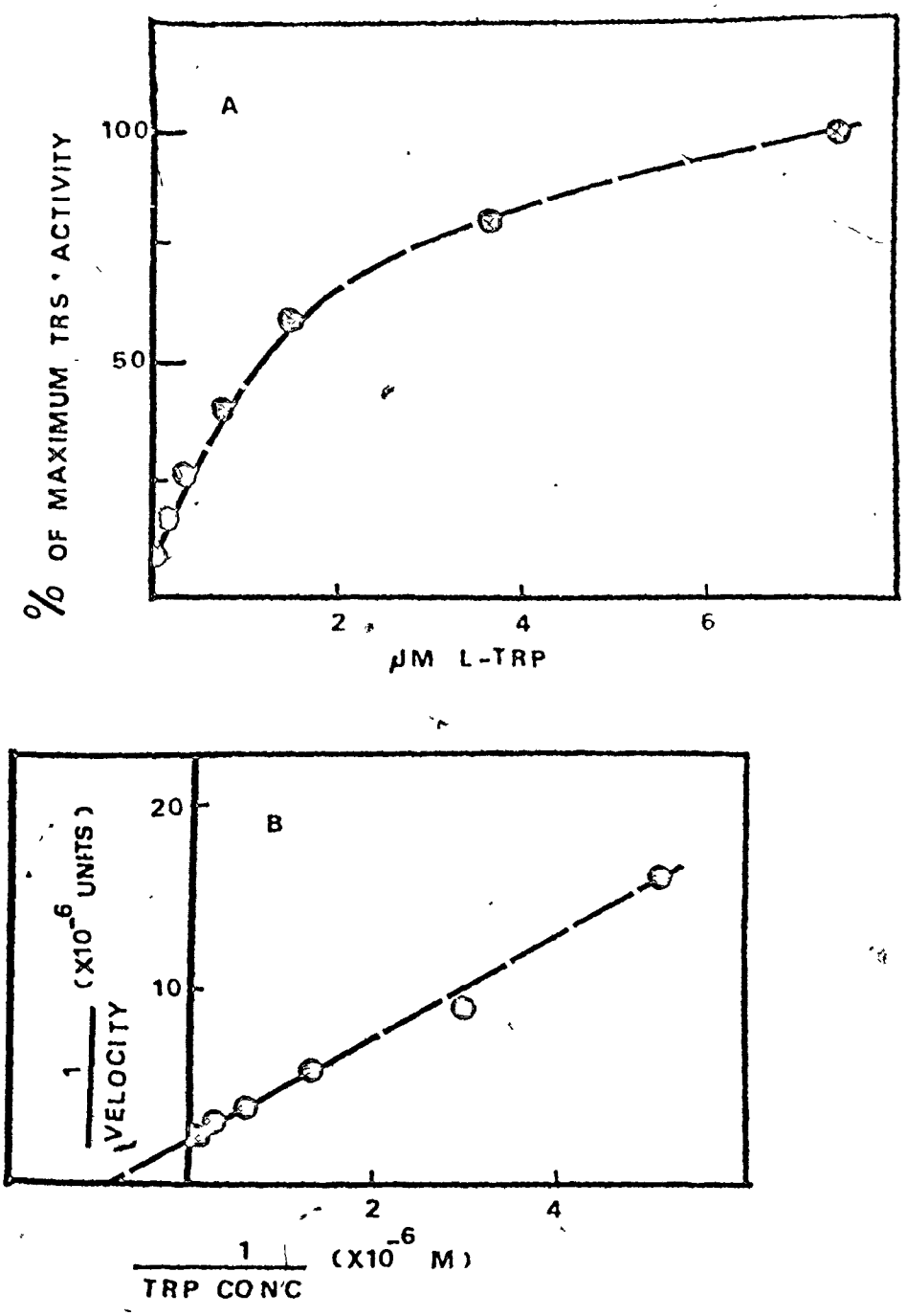


Figure 19: The effect of L-Trp-^{14}C concentration on purified TRS activity

Assay conditions were the same as for Figure 15 except that 81 mM Tris-HCl, pH 7.5 was used. Control incubations containing no enzyme were done for each concentration of L-Trp used. The 100% value was 1310 cpm and the difference of duplicates from average values was not greater than 3%.

5.5.2. Adenosine triphosphate

The Mg^{++} and ATP concentration optima for aminoacyl-tRNA ligases can depend on the ATP/Mg^{++} ratio and/or the amount of ATP-Mg^{++} complex. Therefore, when the effect of ATP on purified TRS was examined (Figure 20) the optimum Mg^{++} concentration of Figure 18 was used. The top graph of Figure 20 shows a hyperbolic increase in TRS activity until the ATP level is about 4 mM. Above this concentration TRS is inhibited. The bottom graph of Figure 20 shows the Lineweaver-Burk plot which gives a K_m value for ATP of 3.1 x 10^{-4} M.

Data on TRS stability shows that ATP has very little, if any, effect. The absence of ATP from an otherwise complete assay during a 30 min preincubation led to a loss of TRS activity of only 5% which is comparable to preincubating the assay without TRS (Table 6). Furthermore, the stability of TRS at 30°C in a buffer containing 3 M KCl, 10 mM Tris-HCl, pH 7.6, 0.16 mM ME and twice the Figure 18 optimum for Mg^{++} was not changed by the addition of twice the Km value or 0.72 mM ATP (Table 8).

5.5.3. Transfer RNA

The kinetics of TRS are of special interest as the high ionic strength of aminoacylation may affect the tRNA-TRS interactions. In Figure 21, the plot of TRS activity versus tRNA concentration gave a sigmoidal curve (top graph). The Lineweaver-Burk plot (bottom graph of Figure 21) gave

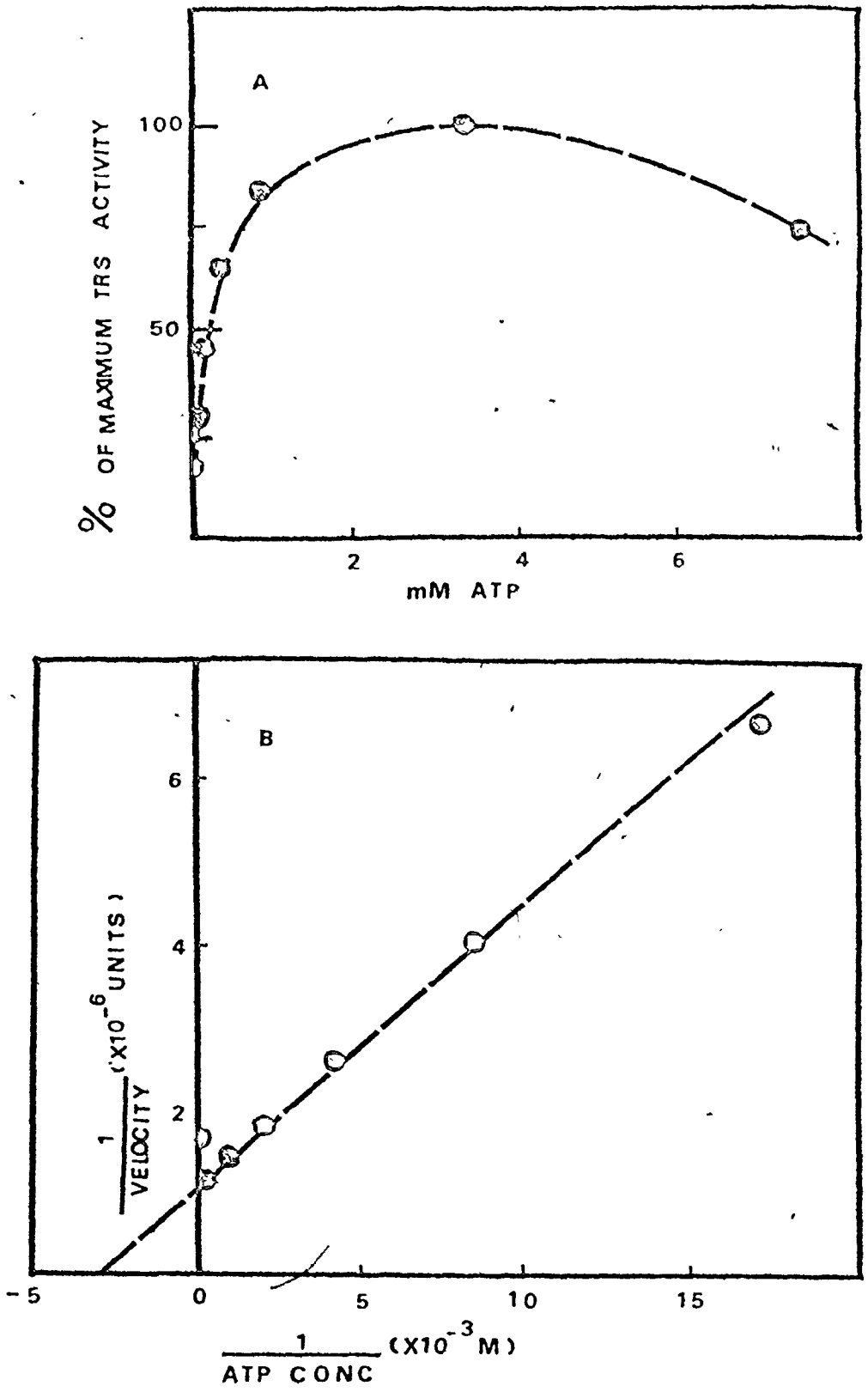


Figure 20: The effect of ATP concentration on purified TRS activity

Assay conditions were as described for Figure 18, using 9.7 mM Mg $(C_2H_3O_2)_2$ and variable amounts of ATP. The maximum cpm was 2700 and variation of duplicates from average values was less than 9%.

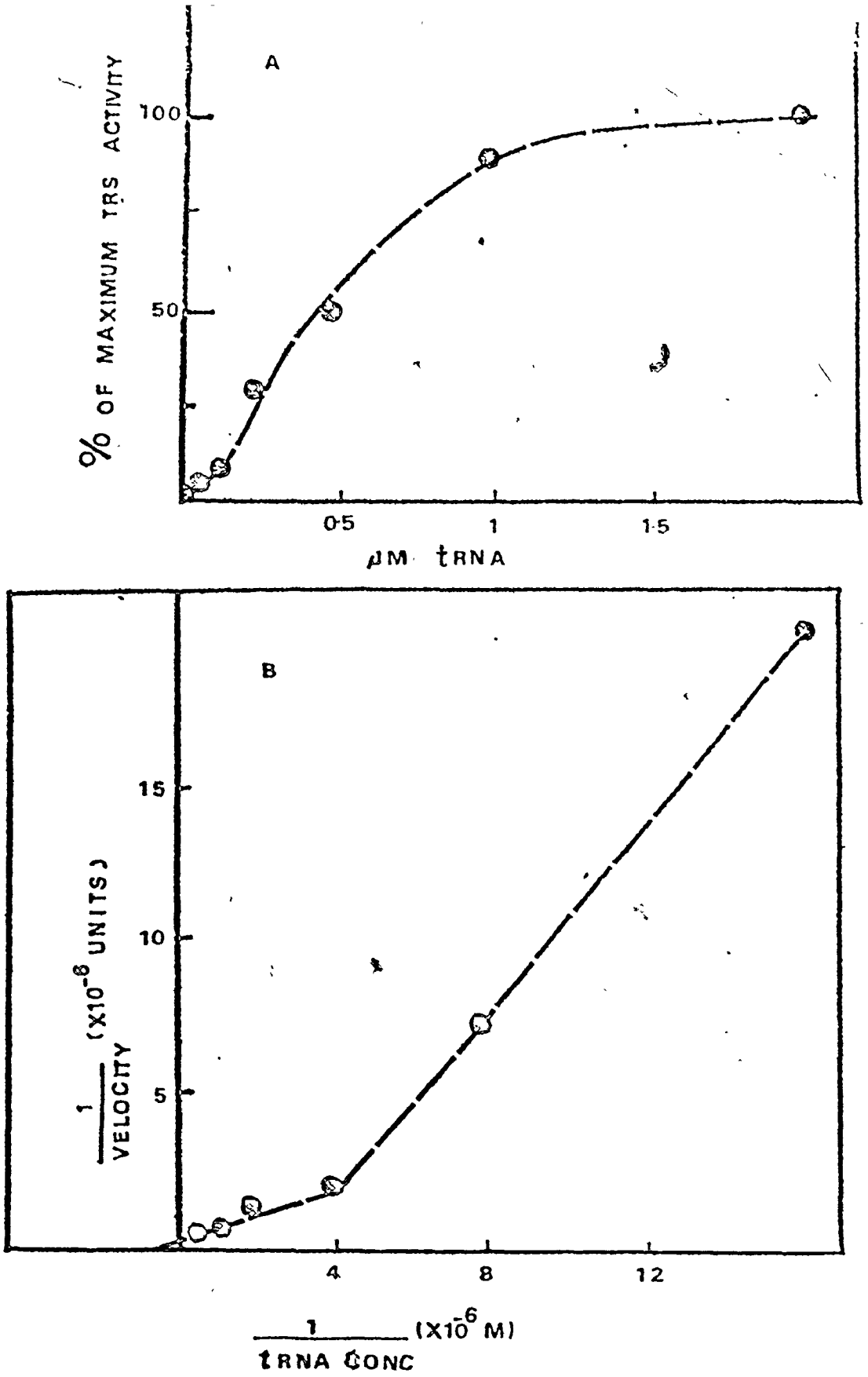


Figure 21: The effect of tRNA concentration on purified TRS activity

Assay conditions were the same for Figure 19 except that 7.4 μM Trp was used. Control incubations were done for each concentration of tRNA used. The 100% value was 6160 cpm and the variance of duplicates from average values was not greater

a straight line and a K_m of 1.3×10^{-6} M only for assays done with not less than 0.25 μM $tRNA^{Trp}$. Similar results were obtained in a second experiment using up to 1.7 μM $tRNA^{Trp}$. The graph of the tRNA concentration was sigmoidal and the Lineweaver-Burk plot gave a K_m value of 1.2×10^{-6} M for higher tRNA concentrations.

The sigmoidal curve of halophilic TRS activity as a function of tRNA concentration can be explained in terms of enzyme inactivation at low substrate concentrations. A complete TRS assay lacking only tRNA lost all enzyme activity in 30 min at 37°C (Table 6). In six similar assays the percent of TRS remaining varied from -1 to ± 1.1% with an average value of -0.2%. In Table 8 the $T_{\frac{1}{2}}$ of TRS in 3 M KCl buffer at 30°C increased more than 8-fold after the addition of 8 mg/ml or 2.2 μM $tRNA^{Trp}$ from *H. cutirubrum*.

Another method of examining $tRNA^{Trp}$-TRS interactions is the analysis of the effect of $tRNA^{Trp}$ on TRS stability. This can be used to determine the dissociation constant. According to the method of Sŭdi (1970), the dissociation constant represents the constant, K_D in the reaction:

$$tRNA^{Trp} + TRS \underset{}{\overset{K_D}{\rightleftharpoons}} TRS - tRNA^{Trp}$$

$$TRS \xrightarrow{k_o} D \qquad TRS - tRNA^{Trp} \xrightarrow{k_{tRNA^{Trp}}} D$$

D is the inactive enzyme and k_o and $k_{tRNATrp}$ are the first order rate constants for the inactivation of TRS and

TRS-$tRNA^{Trp}$, respectively. Then K_D can be expressed in the following formula

$$\frac{1}{\Delta k_i} = \frac{1}{k_o - k_{tRNA^{Trp}}} + \frac{K_D}{k_o - k_{tRNA^{Trp}}} \quad \frac{1}{[tRNA^{Trp}]}$$

where Δk_i is the difference between the apparent rate constant for inactivation of TRS in the presence and absence of some concentration of $tRNA^{Trp}$. This equation is valid if it is assumed that the rate of inactivation is too slow to interfere with the maintainance of equilibrium concentrations and the concentration of free $tRNA^{Trp}$ remains constant during the inactivation procedure. As the rate of inactivation of TRS-$tRNA^{Trp}$ was very small relative to k_o ($T_{\frac{1}{2}}$ was $\geq$ 6 hrs vs < 2 min, respectively) the $k_{tRNA^{Trp}}$ term can be neglected. Then an examination can be made of the binding of TRS to $tRNA^{Trp}$ by measuring the amount of TRS activity remaining after a 30 min preincubation with various levels of $tRNA^{Trp}$.

The effect of $tRNA^{Trp}$ concentration on TRS stability during a 30 min preincubation at 37°C is seen in Figure 22. In the bottom of Figure 22 there is a non-linear plot of the reciprocal of the amount of TRS protected by $tRNA^{Trp}$ versus the reciprocal of the $tRNA^{Trp}$ concentration. A tangent to the curve gives a value for the dissociation constant (K_D) which is the same as the K_m, 1.3×10^{-6} M.

The effect of various polyribonucleotides on the activity and stability of TRS was examined (Table 9). The amounts of polyribonucleotides correspond to 1.1-1.4 μM

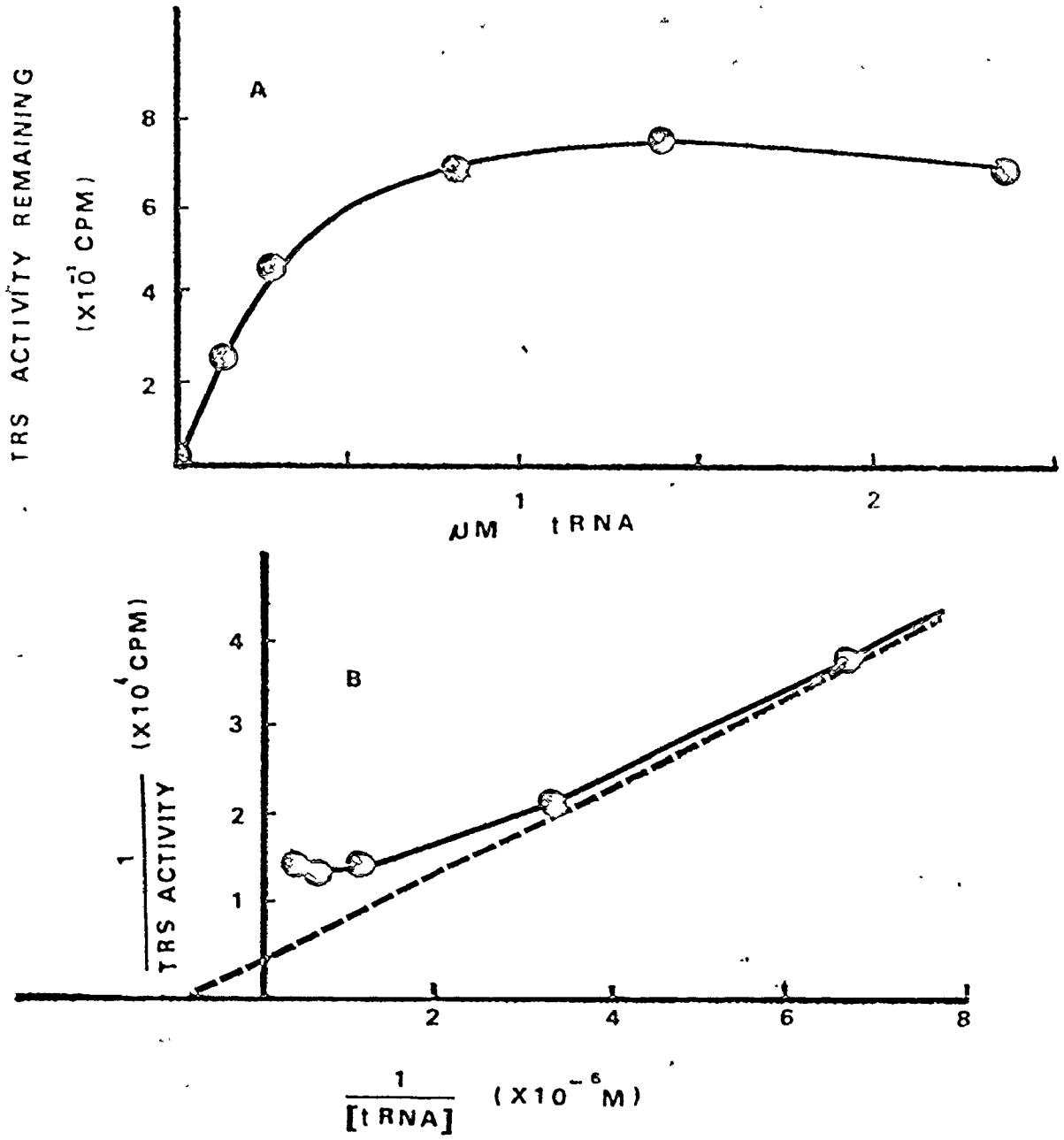


Figure 22: The effect of tRNA concentration on the stability of purified TRS

TRS in 0.11 ml assays containing 4.8 M KCl, 1.1 M NaCl, 91 mM Tris-HCl, pH 7.5, 11 mM Mg^{++}, 0.75 mM ME and 4.1 mM ATP was preincubated at 37°C for 30 min with various amounts of tRNA. Then 8.8 mM L-Trp-^{14}C and tRNA to 2.4 μM were added and a normal 30 min incubation was continued. Duplicate assays did not vary more than 7% from the averages.

Table 9

The Effects of Polyribonucleotides on TRS Activity and Stability

Polyribonucleotides[1]	0	*E. coli* tRNA	Yeast tRNA	$tRNA^{-Trp}$	Polyuridylic acid	Polycytidylic acid	Polyinosinic acid
Inhibition[2]	0	17.5	84.8	21	6	74.5	-6
Stability ($T_{1/2}$ in min)[3]	1.65	5.9	3.4	1.9	1.7	3.5	2.25

[1] The amounts of polyribonucleotides were 0.5 mg = 8.7 A_{260} *E. coli* tRNA; 0.5 mg = 7.6 A_{260} yeast RNA; 1 mg = 8.9 A_{260} *H. cutirubrum* tRNA lacking Phe, Tyr and Trp tRNAs ($tRNA^{-Trp}$); 0.5 mg polycytidylic and polyuridylic acid and 0.2 mg polyinosinic acid.

[2] The inhibition of TRS is expressed as the percent activity lost due to the presence of polyribonucleotides relative to assays with none (100% = 13,100 cpm). Assays contained in 0.17 ml, 4.9 M KCl, 0.74 M NaCl, 60 mM Tris-HCl, pH 7.5, 0.6 mM ME, 5.6 µM Trp, 9.7 mM Mg $(C_2H_3O_2)_2$, 2.6 mM ATP and 13.7 A_{260} = 1.55 µM $tRNA^{Trp}$.

[3] The stability of TRS was determined by preincubating the assay described above for 2 min at 37°C without $tRNA^{Trp}$ and then adding the $tRNA^{Trp}$ and continuing the assay for 30 min. The percent of the activity of similar but not preincubated assays which remains after the 2 min preincubation was determined. The $T_{1/2}$ was estimated from a semi-log plot.

in Figure 22 if the amount of polyribonucleotides is equated to that of $tRNA^{Trp}$. The polyribonucleotides gave little protection compared to $tRNA^{Trp}$, which protected more than 90% of the TRS over 30 min (Table 6). *E. coli* tRNA increased the $T_{\frac{1}{2}}$ of TRS more than 3-fold while yeast tRNA and polycytidylic acid increased it more than 2-fold. The stabilizing effects of polyribonucleotides were not proportional to their inhibition of TRS activity (Table 9).

5.6. Amino acid analysis of TRS

Both the activity and stability of TRS molecules and of proteins in general depend on the spatial arrangements of amino acid residues. Such arrangements are stabilized by hydrogen bonding, ionic bonding, van der Waals forces and interactions between hydrophobic amino acids. Hydrophobic amino acids tend to form a stable center of the protein while polar amino acids interact with the aqueous environment of the protein molecule. For such structures to exist in halophilic TRS in the presence of multimolar salts, a different amino acid composition may be necessary. The amino acid composition of halophilic TRS was determined and characterized to examine this possibility.

Purified TRS for which the polyacrylamide gel is shown in Figure 6C was analyzed for its amino acid composition and the results are presented in Table 10. Also, the percent composition of each amino acid was calculated as were the percent composition of *E. coli* and bovine TRS.

Table 10

Amino Acid Analysis[1] of Purified TRS

Amino acid	Approximate no. of residues per subunit molecular weight[2]	Mole % composition of TRS		
		H. cutirubrum	*E. coli*[3]	Bovine[4]
Asx	79.3	13.4	11.4	11.6
Thr	37.5	6.3	4.1	5.2
Ser	30.8	5.2	6.1	5.9
Glx	75.8	12.8	12.5	10.9
Pro	22.5	3.8	5.3	4.0
Gly	46.1	7.8	7.1	6.7
Ala	92.0	15.6	9.9	7.2
Val	46.5	7.9	7.9	5.2
Cys	6.6	1.1	0	1.7
Met	3.3	0.55	2.0	2.3
Ile	18.1	3.1	4.4	6.0
Leu	38.9	6.6	8.2	7.4
Tyr	14.4	2.4	2.6	3.8
Phe	13.7	2.3	2.6	6.3
Lys	12.9	2.2	8.5	7.1
His	15.1	2.6	2.6	2.3
Arg	35.4	6.0	4.4	4.8
Trp	0.9	0.15	0.6	0.9
NH_4^+	46.2	7.8	-	-

[1] Method described in Section 2.7.4.

[2] The subunit molecular weight of 64,000 daltons (Section 3.1.1.) was used.

[3] Data calculated from results of Joseph and Muench (1971).

[4] Data calculated from results of Lemaire *et al.* (1969).

This allows an easier comparison of the amino acid content of the halophilic enzyme with the smaller procaryotic TRS and the eucaryotic TRS which has a similar molecular weight to *H. cutirubrum* TRS. The precent composition of TRS for *H. cutirubrum* was significantly higher in Ala than the values for *E. coli* and bovine TRS. Lower percent compositions for *H. cutirubrum* TRS were seen for Met, Lys, Trp, Pro and Ile. Thus the *H. cutirubrum* TRS was lower in the hydrophobic and basic amino acid residues and was higher in Ala content.

The relative hydrophobicity of TRS can be estimated from the hydrophobicity index (Bigelow, 1967). This index uses the ratio of the solubilities of amino acid side chains in ethanol and water to calculate the free energy of transfer. The free energies of transfer for different amino acid side chains averaged over the mole % amino acid composition of a protein yield the average free energy change per residue, or the hydrophobicity index of a protein. For *H. cutirubrum*, *E. coli* and bovine TRS, values of 0.86, 1.04 and 1.14 Kcal/residue respectively were calculated (Table 11).

5.7. Discussion of TRS properties

5.7.1. Kinetic studies of TRS

The experiments on the kinetics of TRS from *H. cutirubrum* show that the enzyme is greatly affected by changes in the salt environment. This protein may be

Table 11

A Comparison of the Properties of Four Tryptophanyl-tRNA Ligases

	H. cutirubrum	*E. coli*[1]	Bovine[2]	Human[3]
Molecular weight	128,000	74,000	108,000	116,000
Subunit molecular weight	64,000	37,000	54,000	58,000
K_m for L-Trp[4]	1.3×10^{-6}	10^{-5}	2.5×10^{-6}	2.8×10^{-6}
K_m for ATP[4]	3×10^{-4}	9×10^{-5}	1.5×10^{-4}	1.6×10^{-4}
K_m for $tRNA^{Trp}$[4]	1.3×10^{-6}	5×10^{-7}	1.5×10^{-7}	1.1×10^{-7}*
Molecular activity[5]	34	1200	240	200
Isoelectric point	-	6.2	-	5.2
pH optimum	7.5	8.8	8.5	8.0
Hydrophobicity index[6]	0.86	1.04	1.14	-

[1] Data and calculations from the data of Joseph and Muench (1971).

[2] Data and calculations from the data of Dorizzi (1972) and Lemaire *et al.* (1969).

[3] Data from Penneys and Muench (1974).

[4] Units for K_m are moles per liter.

[5] Molecular activity is expressed as the μmoles tRNA charged with Trp per min per molecule of enzyme.

[6] Hydrophobicity index is expressed as kcal/residue and was calculated according to Bigelow (1967).

* For this value only the K_m is for non-homologous tRNA, which was from yeast.

described as being extremely halophilic; the salt concentration required for the expression and maintenance of enzymic activity is much greater than the concentrations normally associated with enzyme cofactors.

Salts are thought to participate in ion binding and charge screening of halophilic proteins (Lanyi, 1974). Both of these may affect the pH requirements of the enzyme. Cation binding could lower the pK of certain groups on amino acids at the active site and necessitate a lower pH for catalytic activity. Conversely, a group with a high pK may require cation binding to lower the pK to physiological pH. The alkali side of the pH curve for succinyl papain has been shown to be lowered by cation binding (Sluyterman and de Graaf, 1972). Similar cation binding effects may occur for *H. cutirubrum* TRS since its pH optimum was lower than those of *E. coli*, bovine and human TRS (Table 11).

Also, for enzyme stability and activity, similar charges may need to be in close proximity. This could be facilitated by the screening of charges on amino acids by ions. Lanyi (1974) argues that such charge screening by high concentrations of NaCl or KCl produce a broader pH profile for four halophilic enzymes than at lower salt concentrations. However, many cases of sharper pH profiles for halophilic enzymes have been obtained. Lanyi states that for these enzymes charge-dependent effects predominate despite the highly ionic environment of the protein molecules.

Not only was the pH activity profile of TRS much sharper than those of other halophilic enzymes but it was also considerably narrower than that for *E. coli* TRS (Joseph and Muench, 1971).

Further studies on the effect of pH on Km values is necessary before concluding whether the ionizations which dictate the shape of the pH curve affect the maximum velocity or Km of the TRS reaction. For example, a change in the Km for tRNA in response to pH which is as sharp as that observed for *E. coli* Arg-tRNA ligase (Mitra *et al.*, 1970) could also restrict the pH curve for TRS.

Halophilic TRS functioned optimally in high levels of NaCl and KCl, corresponding to *in vivo* concentrations. Near the optimal levels of these salts, changing Na^+ affected primarily the activity while changing K^+ affected the stability of TRS. The salt concentration required by TRS is larger than for most halophilic enzymes for which comparable data is available in the literature. TRS is indeed halophilic and not merely salt-tolerant.

Requirements of non-halophilic enzymes for monovalent salts for the activation (Suelter, 1970) and stabilization (von Hippel and Schleich, 1969) is not unusual although the concentrations range from 0.01 to 0.1 M. Further studies on TRS could clarify how the enzymic mechanism has been changed by differences in the enzyme and by its salty environment. If the mechanism were little altered from non-halophilic enzymes then the halophile enzyme

would be a useful model system for understanding the effects of salt on the TRS reaction mechanism and on enzyme reactions generally. Also, the effect of ion binding on the physical properties of the protein and on the levels of binding remaining after specific amino acid residues have been chemically modified may help to understand protein-salt interactions. Since these interactions should be greater in magnitude for halophilic proteins, the halophile system may be the best system for such studies.

The SH-group of Cys is one of the ionizable groups often found at the active sites of enzymes and its oxidation often leads to a loss of enzymic activity. All the aminoacyl-tRNA ligases were once thought to be sulfhydryl-group-dependent enzymes; there is recent evidence that SH-groups are not always necessary for catalysis by these enzymes. The Lys charging enzyme from *E. coli* was found to have no essential SH-groups (Stern *et al.*, 1966) although $tRNA^{Lys}$ was oxidized by SH-group blocking reagents. For bovine TRS, SH-groups were important only because they were necessary for subunit association (Iborra *et al.*, 1973).

For halophilic TRS, SH-group reagents had little effect. SH-protecting reagents were weak inhibitors (Figure 17) while SH-blocking reagents at low concentrations increased slightly the activity of TRS in crude extracts (Table 7). However, Table 7 suggests a small negative effect of SH-blocking reagents and Table 6 could suggest a small positive effect of SH-protecting reagents for

the activity of the purified enzyme. Endogenous RNA in the crude S-150 extracts may be responsible for this difference of the crude and purified enzymes. The possible mechanisms of SH-group reagents is therefore discussed later.

The resistance of TRS to SH-blocking reagents explains how this enzyme is protected from the 900 moles of Pb^{++} and other high amounts of heavy metals contaminating KCl that must pass over each mole of TRS in the affinity chromatography procedure of Section 3.1.1.

An important factor in the purification of TRS by affinity chromatography was the inclusion of 0.1 M Mg $(C_2H_3O_2)_2$ in all chromatography buffers because Mg^{++} increases the stability of purified TRS. Some non-halophilic aminoacyl-tRNA ligases are also protected from inactivation by Mg^{++} (Mitra *et al.*, 1970; Lapointe and Soll, 1972) while others are little affected (Tscherne *et al.*, 1973).

Mg^{++} may affect the stability and activity of TRS by binding to free TRS molecules. However, several other mechanisms involving TRS and/or tRNA and/or ATP are possible. These mechanisms are discussed later.

The level of Mg^{++} necessary for maximum TRS activity is not unusually high relative to non-halophilic systems. This suggests either that high concentrations of KCl and NaCl do not significantly change Mg^{++} functions or that TRS has been modified to give normal kinetics in high salt concentrations. K^{+}-ATP complexes are also possible at high KCl concentrations.

The response of enzyme activity to Mg^{++} concentrations for different aminoacyl-tRNA ligases may, but need not, show an optimal Mg^{++} level. Griffiths and Bayley (1969) reported that *H. cuturibrum* Leu-tRNA ligase did not decline in activity up to 80 mM Mg^{++}. Yarus (1972) reported that *E. coli* Arg-tRNA ligase had a distinct Mg^{++} optimal concentration.

Whether an optimum Mg^{++} concentration is observed or not, the concentration of ATP is always important. This has been attributed to an $(ATP\text{-}Mg)^{=}$ concentration or an $ATP:Mg^{++}$ ratio at which the reaction velocity is maximum.

The ATP concentration versus TRS activity profile (Figure 20) showed substrate inhibition at high ATP concentrations. This could be due to the classical type of substrate inhibition where molecules "crowd" the active site, due to a competition of ATP with another substrate, or due to an effect similar to that described for inorganic pyrophosphatase (Bailey and Webb, 1944). They suggest that an excess of ATP over Mg^{++} could deplete Mg^{++} necessary for the other components of the TRS reaction and/or an excess ATP not complexed with Mg^{++} could act as an inhibitor. That excess ATP is inhibitory is supported by a Lineweaver-Burk plot which gave a straight line for all points except that for the highest ATP concentration (Figure 20, bottom). A curved line for most points would be obtained for classical substrate inhibition (Cleland, 1970).

The purported substrate, $[ATP\text{-}Mg]^{=}$, is polyanionic and may bind monovalent ions or may form ionic bonds to groups on TRS which are charge-screened in the presence of high monovalent salt concentrations. Either should interfere with and decrease the binding of ATP to TRS. While the Km is defined as the substrate concentration at half maximal velocity, it under some circumstances equals the dissociation constant of the enzyme-substrate complex. The dissociation constant is an index of tightness of binding of the enzyme and substrate. The K_m for ATP of halophilic TRS was the largest of the four enzymes in Table 11. To decide conclusively if the halophilic TRS binds ATP weakly, more sophisticated methods as described by Kisselev and Favorova, 1974, are required.

$(ATP\text{-}Mg)^{=}$ did not affect the stability of TRS (Table 8). ATP not complexed with Mg^{++} was not tested. Reports on non-halophilic systems indicate ATP and $(ATP\text{-}Mg)^{=}$ can have no effect, can stabilize, or can destabilize aminoacyl-tRNA ligases (Norris and Fowden, 1973; Tscherne *et al.*, 1973).

Trp is a zwitter ion at neutral pH and is the most hydrophobic of the amino acids. Therefore, Trp probably does not bind ions and binds to TRS without the salt effects which might affect the binding of $[ATP\text{-}Mg]^{=}$ and tRNA. The K_m for Trp of halophile TRS was slightly smaller than those for the three non-halophilic TRS's (Table 11).

The presence of Trp increased the $T_{\frac{1}{2}}$ of TRS approximately 2-fold. This stabilization is comparable to that seen for plant aminoacyl-tRNA ligases (Norris and Fowden, 1973).

The presence of $tRNA^{Trp}$ increased the $T_{\frac{1}{2}}$ of TRS more than 200-fold. This value is exceptionally high. In a literature survey, six examples of aminoacyl-tRNA ligase protection by tRNA were found but all were much smaller than for TRS. Lapointe and Soll (1972) found that *E. coli* Glu-tRNA ligase had a 6-fold greater $T_{\frac{1}{2}}$ in the presence of cognate tRNA. Two *ts* mutants of *E. coli* Val-tRNA ligase had 10- and 12-fold greater $T_{\frac{1}{2}}$ in the presence of tRNA while the wild-type enzyme was not stabilized (Yaniv and Gros, 1969). Rat Phe-tRNA ligase had only a 1.3-fold increase in $T_{\frac{1}{2}}$ while *E. coli* Arg-tRNA ligase showed a 4-fold increase in $T_{\frac{1}{2}}$ in the presence of tRNA.

The stabilization of halophilic TRS by tRNA was dependent on the presence of the cognate $tRNA^{Trp}$ species (Tables 6 and 9). Also the inhibition of TRS by polyribonucleotides (Table 9) was not proportional to their effect on TRS stability. Therefore, some feature of halophilic $tRNA^{Trp}$, which is probably shared to a lesser degree by *E. coli* and yeast tRNA and polycytidylic acid, is important for TRS stabilization but not for activity. While *E. coli* and yeast tRNA and polycytidylic acid stabilized TRS they were not charged with Trp by TRS. Possibly the conformation of the polyribonucleotides but

not their binding to TRS is critical.

The K_m for tRNA was greater for *H. cutirubrum* TRS enzyme than for the *E. coli*, bovine and human enzymes (Table 11). Generally, aminoacyl-tRNA ligases have K_m values for tRNA which are quite small and close to 10^{-7} M (Novelli, 1967).

The best index of affinity of an enzyme for a substrate is the dissociation constant (K_D). The K_D for halophilic TRS was estimated from the effect of tRNA concentration on TRS thermal inactivation (Figure 22). The curve in Figure 22 was not hyperbolic. A similar protection curve for crude tRNA was obtained for the *E. coli* Arg ligase by Mitra *et al.* (1970). Lapointe and Soll (1972) used purified $tRNA_3^{Glu}$ and obtained a hyperbolic curve for *E. coli* Glu-tRNA ligase.

The slight decline in TRS stabilization at the highest concentration (Figure 22) might be due to tRNA complexing of Mg^{++} or Trp or the inhibition of $tRNA^{Trp}$ binding by non-cognate tRNAs present in the crude preparation of tRNA. The latter suggestion is supported by the 21% inhibition of TRS charging by non-cognate *H. cutirubrum* tRNAs (Table 9). A more reliable estimation of K_D could be made with higher TRS and lower tRNA concentrations, when the problems discussed above should not occur.

The K_D for halophile TRS was approximately 1.3×10^{-6} M for the binding of $tRNA^{Trp}$. This is larger

than any of the 22 values given for the binding of 10 different species of tRNA to their cognate aminoacyl-tRNA ligases in the review by Kisselev and Favorova (1974). These values varied from 10^{-6} to 10^{-9} but most were between 10^{-7} M and 10^{-8} M.

The K_D values for acylated and non-acylated tRNAs has been the subject of scientific interest recently with respect to the mechanism(s) by which aminoacyl-tRNA ligases catalyze their reactions. Earlier theories had involved ping pong or sequential mechanisms which were supported by data obtained by an ATP-PP_i exchange and the rate of this reaction is generally 10- to 100-fold greater than the rate of tRNA charging. Furthermore, the intermediate, aminoacyl-AMP, was found bound to a number of the enzymes, suggesting that the "activation" of the amino acid was the first step in a ping pong or sequential reaction mechanism.

Loftfield (1972) put forward a concerted reaction mechanism. In his paper, he also suggested that salts decrease the rate of non-halophilic tRNA-enzyme association which is the rate-limiting step of the concerted reaction. He proposed that for halophilic aminoacyl-tRNA ligases the rate-limiting step which requires high salt concentration was the dissociation of aminoacylated tRNA from the enzyme.

But data contradicting Loftfield's theories have been found. Yarus and Berg (1969) studying an *E. coli* enzyme and Hélène and Brun (1971) studying *E. coli* Val-tRNA ligase concluded that the rate-limiting steps

were the dissociation of charged tRNA from the enzymes. Also, Yarus (1972) found that a change of tRNA conformation and not electrostatic interactions were responsible for tRNA-enzyme binding. The emerging consensus appears to be that different aminoacyl-tRNA ligases from various organisms may have different mechanisms or different rate-limiting reactions.

The halophile charging system may help the understanding of the involvement of electrostatic forces in tRNA-enzyme binding. White and Bayley (1972a) found that many tRNA species from *H. cutirubrum* could be charged by *E. coli* enzymes and vice versa. Thus the tRNA species have not been fundamentally changed in an adaptation to high salt concentrations. If the tRNA is little changed and the enzyme mechanism is not dissimilar to those of non-halophilic ligases the molecular activity may reflect how efficient the reaction is in the presence of salt. The molecular activity of TRS was 6-fold and 35-fold less than those of the three TRS species in Table 11. Other aminoacyl-tRNA ligases have given molecular activities from 8-640 μ mole/min/molecule (Joseph and Muench, 1971). Thus halophile TRS does not catalyze the aminoacyl charging of tRNA more slowly than all other animoacyl-tRNA ligases, but the rate is significantly slower than for beef, human or *E. coli* TRS.

The TRS reaction is very complex. It involves Mg^{++}, Na^{+}, K^{+}, three substrates and three products.

Therefore, it is not surprising to find that some of the molecules and ions influence the effects of others on the activity and stability of TRS.

Mg^{++} decreases the tRNA-induced stabilization of TRS. Raising the Mg^{++} concentration decreased TRS stability in the presence of tRNA (Table 6). If tRNA was absent Mg^{++} stabilized TRS (Table 8). Further evidence of Mg^{++} interference with tRNA stabilization of TRS can be found. In Table 8, tRNA increased the $T_{\frac{1}{2}}$ of TRS only 8-fold when 20 mM Mg^{++} was present in the buffer. However, a much stronger protection was seen in the presence of 9 mM Mg^{++} (Table 6).

The effects of Mg^{++} on tRNA-induced stabilization of TRS may involve interactions of Mg^{++} with TRS and/or $tRNA^{Trp}$ and/or ATP. The active forms of both substrates are usually complexed with Mg^{++}. Mg^{++} has been shown to affect the binding constants of ATP and tRNA for aminoacyl-tRNA ligases (Lam and Schimmel, 1975) as well as maximum velocities for their reactions. Mg^{++} could also compete with $tRNA^{Trp}$ for a binding site on TRS.

Mg^{++} has been shown to have various effects on aminoacyl-tRNA ligase stability by different authors. Lapointe and Soll (1972) found that Mg^{++} and an Mg^{++}-tRNA complex but not tRNA alone stabilized Glu-tRNA ligase from *E. coli*. Norris and Fowden (1973) found that $(ATP\text{-}Mg)^{=}$ but not ATP was important for the stabilization of many mung bean ligases. The thermal inactivation of

rat Phe-tRNA ligase was decreased in the presence of Mg^{++} plus Phe and tRNA ± Mg^{++}. However, it was increased in the presence of ATP, and, to a lesser extent, in the presence of $(ATP\text{-}Mg)^{=}$. Subunit dissociation was responsible for the inactivation of the rat enzyme.

Mg^{++}, ME and $tRNA^{Trp}$ stabilize halophilic TRS. However, Mg^{++} and ME appear to decrease the effectiveness of $tRNA^{Trp}$ stabilization. Therefore, all three may be competing for the same site on TRS or they may affect a more general phenomenon such as subunit dissociation. Iborra *et al.* (1973) found that subunit dissociation inactivated beef TRS. They also found that, as is true for most enzymes inactivated by such dissociation, inactivation rates were concentration dependent.

Halophilic TRS was quite stable in standard assay conditions but at low levels of Mg^{++} and ME and in the absence of $tRNA^{Trp}$ inactivation was faster. If fast inactivation were shown to be TRS concentration-dependent then $tRNA^{Trp}$ or ME or Mg^{++} might reduce or eliminate the effect of TRS dilution on stability. Such an analysis might further link the mechanism of TRS stabilization by the Mg^{++}, ME and tRNA and suggest the mechanism involves subunit dissociation. Physical evidence for such a mechanism could be sought by dextran gel filtration although inactivated enzyme would have to be detected by a non-enzymatic method such as fluorimetry.

Many sophisticated techniques for examining the

interaction of components in the aminoacyl-tRNA ligase reaction have been described (Kisselev and Favorova, 1974). The techniques include thermal inactivation, adsorption onto nitrocellulose filters, equilibrium dialysis and fluorimetry. These could be used to study combination effects of the TRS reaction components. Also, an examination of the influence of ionic environment on the enzyme system would be possible.

As stated in the Introduction, halophilic enzymes are often more acidic and less hydrophobic than their non-halophilic counterparts. Therefore, the amino acid composition of TRS was of special interest.

Figure 23, based on Bigelow (1967), shows the hydrophobicity index for a range of globular proteins plotted against molecular weight. There appears to be a lower limit to the hydrophobicity and this can be readily explained if a globular protein is represented as a shell of predominantly polar residues surrounding a core of predominantly non-polar residues. Clearly the average hydrophobicity/residue for the whole protein will depend on the relative volumes of shell and core and these in turn will depend on the size and molecular weight of the protein. The larger a protein becomes, the larger will be the core relative to the shell. The protein will then contain relatively more non-polar residues within this core and the average hydrophobicity will increase.

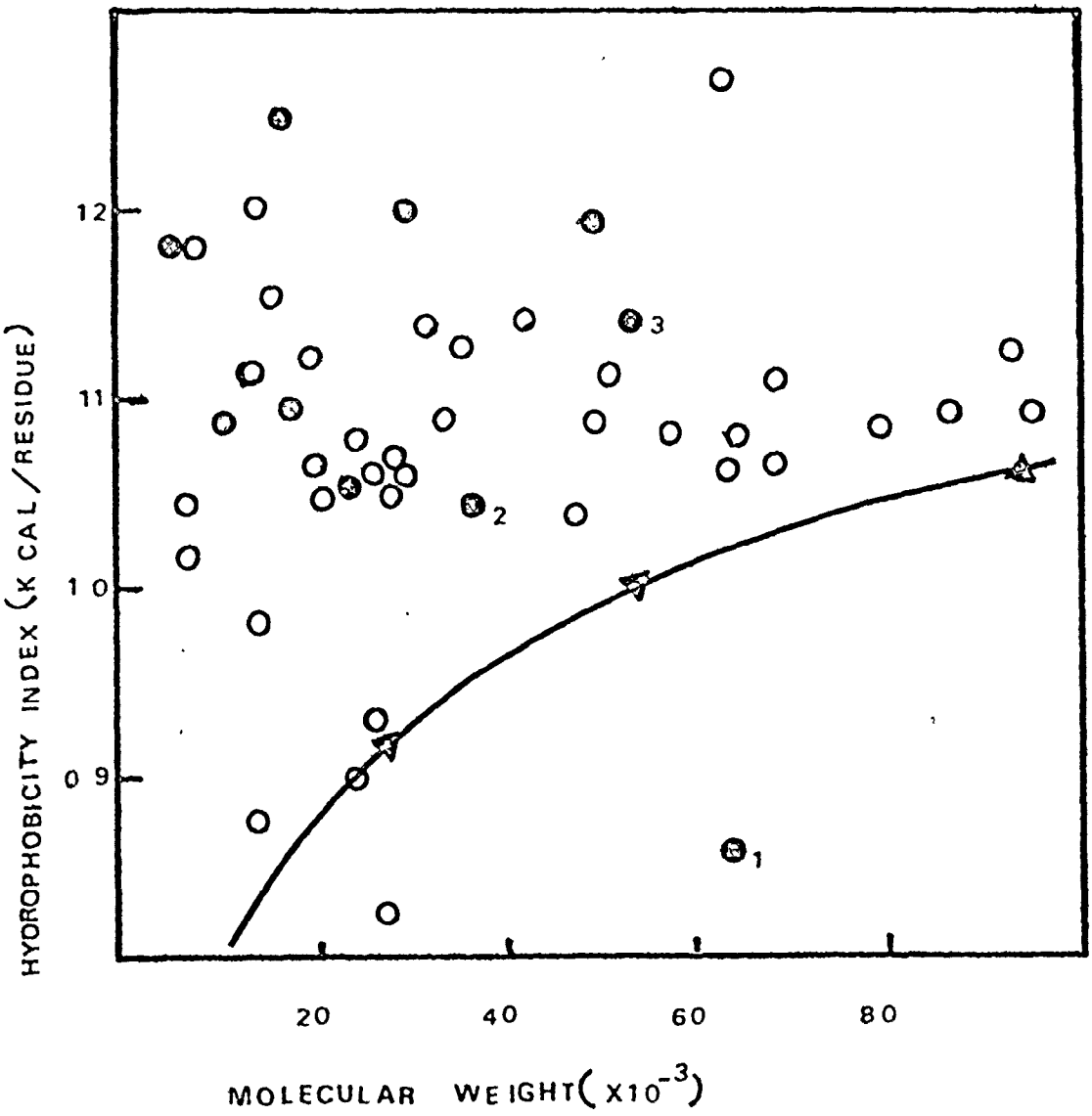


Figure 23: Average hydrophobicities of globular proteins plotted against their molecular weights (or the subunit molecular weights in the case of associated proteins).

Positions are indicated for *H. cutirubrum*, *E. coli* and bovine TRS by 1,2 and 3, respectively. This figure is based on Bigelow (1967). Filled circles represent associated proteins and open circles represent non-associated proteins. The curve and filled triangles represent the theoretical lower limit to the hydrophobicity index for globular proteins (Bigelow, 1967).

By choosing suitable values for the thickness of the shell and for the average hydrophobicities in the shell and core, Bigelow (1967) obtained the curve shown in Figure 23, which follows the lower limit quite closely.

When the hydrophobicity indices for the three TRS in Table 11 are plotted on Figure 23, those for *E. coli* and bovine TRS fall among the values for globular proteins of similar size, whereas that for halophilic TRS is 230 cal/residue below the lower limit. (Note that even if the molecular weight estimate for the subunit were in serious error, the hydrophobicity index would still fall below this limit.) This low hydrophobicity is consistent with the results of calculations carried out on a range of halophilic proteins by Lanyi (1974) and may be explained by an increased hydrophobicity of amino acid side chains in concentrated salt solutions. The hydrophobicity as used by Bigelow is based on the relative solubilities of the side chains in water and ehtanol but the salt solution:ethanol solubility ratio may be greater. The general effect of the salting-out salts will be a decreased solubility of the amino acid residues within the protein as discussed by Jencks (1969). Hydrophobic amino acids are less soluble in water and this allows a stable structure within the proteins. The less hydrophobic amino acid residues of TRS form a stable structure within the protein only because of their decreased solubility in salt.

Specific effects of NaCl and KCl may be dependent on the amino acid composition of halophilic proteins. For example, the 7.3 mole percent excess acidic over basic amino acids in TRS may be related to Na^+, K^+ or Mg^{++} binding as well as the pH profile. However, at the present time, the role of Na^+ and K^+ (Suelter, 1970) and other ions in the activation of enzymes is a frequently occurring but poorly understood phenomenon.

Finally, the unusually strong dependence of halophile TRS on $tRNA^{Trp}$ for stability may be related to its amino acid composition and high salt environment. That the TRS reaction is dependent on charge effects is suggested by the sharp pH curve. In order to bind tRNA in high salt concentrations halophilic TRS may need to be more polar to allow sufficiently strong electrostatic interactions between the two molecules.

Once TRS is bound in a TRS-$tRNA^{Trp}$ complex, it would be even less hydrophobic and more acidic than free TRS. Thus the complex would have even more halophilic characteristics than the free TRS.

6. SUMMARY

Three methods of purifying halophilic proteins while they remain in high salt concentrations have been developed.

TRS has been purified by two passages over agarose-hexamethylenediamine-Trp affinity columns. The enzyme was about 1000-fold purified, was homogeneous on SDS gel electrophoresis (SDS-PAGE) and 10% of the initial enzyme activity was recovered. On the same columns tryptophanase could be resolved from TRS because it was not retained when 3 M KCl buffers were used. But it was retained when 5 M NaCl buffers were used and retention was enhanced in the presence of the cofactor, PLP. In this way, tryptophanase was purified to near-homogeneity as shown by SDS-PAGE.

Tryptophanase was also purified to near-homogeneity by heat treatment followed by $(NH_4)_2\ SO_4$ fractionation. The heat treatment could also be followed by a new method of fractionation in which proteins and nucleic acids were resolved by chromatography on agarose in the presence of multimolar $(NH_4)_2SO_4$. A decreasing concentration of $(NH_4)_2SO_4$ was used to elute proteins and nucleic acids bound when added to the column in 2.4 M $(NH_4)_2SO_4$ solution. The two methods purified tryptophanase 40-fold and 200-fold, respectively.

TRS was found to have a subunit molecular weight on SDS-PAGE of about 64,000. From dextran gel filtration it is tentatively suggested that TRS is a dimer with an α_2-structure. Tryptophanase had subunit molecular weight of about 54,000 may be a molecule with an α_4 structure and a molecular weight of over 200,000. The molecular forms of TRS and tryptophanase appear to be similar to those reported for non-halophilic proteins.

The properties of TRS were studied and compared to non-halophilic aminoacyl-tRNA ligases. The effect of Trp concentration on TRS activity was comparable to that for non-halophilic ligases. TRS activity was typically dependent on Mg^{++} concentration. The ability of tRNA to stabilize TRS was reduced by Mg^{++}.

Halophilic TRS activity was not significantly affected by either SH-group protecting or blocking reagents. This is in contrast to results for most aminoacyl-tRNA ligases.

Near the optimum salt concentrations for TRS, Na^+ activated TRS while K^+ stabilized it. Optimum assay conditions were approximately 4.8 M KCl and 1 M NaCl.

The amino acid composition of TRS showed an excess of acidic over basic amino acids. TRS also contains relatively more polar and non-hydrophobic residues. This is characteristic of many halophilic proteins but is markedly different from non-halophilic ligases, from non-halophilic globular subunit proteins, and from all

non-halophilic globular proteins of comparable molecular weight.

The K_m of TRS for ATP was higher than those for the three non-halophilic TRS's used for comparison. The pH curve for TRS was narrower than those of other halophilic enzymes and sharper than that of *E. coli* TRS.

The kinetics of TRS with respect to tRNA were of the most interest and also the most unusual. Its K_m was larger than any of the three found for non-halophilic TRS. Its K_D was larger than any values reported for non-halophilic ligases. In addition, halophile TRS showed an exceptionally strong dependence on $tRNA^{Trp}$ for its stability.

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