Thus one explanation for the effect of pressure on the NMR spectra of a polymer of particular chain length is that an increased mobility of the side chain CH_2 residues closest to the helix occurs together with an increased mobility of the α(CH) residue on the helix. There is relatively little effect on the outermost CH_2 residue of the side chain which is much more mobile under all conditions. The sensitivity of the polymer to pressure is greatest when the polymer is tightly coiled, which is the case at lower temperatures, and less when it is loose, which is the case at higher temperatures. Since the random coil form of the polymer would allow greater mobility of the side chains, it would appear that increased pressure favors an uncoiling of the helix, as does increase in temperature, and that, in keeping with le Chatelier's principle, this uncoiling process must be accompanied by a decrease in volume.

Our results, based on a study of a molecule of large molecular weight comparable to that of a protein, thus tend to confirm the direction of change expected from the work on 4-octanone (Kliman, 1969) and to indicate a negative volume change on unfolding of the polymer from a coiled up structure to an unfolded one, with subsequent exposure of hydrophobic groups to the solvent.

The results clearly indicate the feasibility of investigating the pressure dependent properties of biopolymers by high resolution NMR spectroscopy, this being the first such study reported.

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Purification and Some Properties of the Histidyl-tRNA Synthetase from the Cytosol of Rabbit Reticulocytes†

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ABSTRACT: The histidyl-tRNA synthetase of rabbit reticulocyte cytosol has been purified 84 000-fold to apparent homogeneity with a specific activity of 687 nmol of histidyl-tRNA formed per min per mg of protein. Ten to 15% of the enzyme activity is sedimented with the ribosomes while the remainder is in the cytosol. The purified enzyme has a molecular weight of 122 000 as determined by sucrose density gradient centrifugation. Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate suggests that it is composed of two similar subunits with a molecular weight of approximately 64 000. The enzyme has a magnesium optimum of 45 mM; however, this is reduced to 5 mM in the presence of an intracellular potassium concentration (160 mM). The enzyme acylates the two histidine tRNA isoacceptors of rabbit reticulocytes with similar K_m values and at similar rates.

The aminoacyl-tRNA synthetases play a critical role in translating the genetic code because they alone possess the double specificity to recognize both amino acid and tRNA. The His-tRNA[1] synthetase has been purified from *Salmonella typhimurium* (DeLorenzo & Ames, 1970), *Escherichia coli* (Kalousek & Konigsberg, 1974), and *Saccharomyces cerevesiae* (Boguslawski et al., 1974), and the enzyme from each source has been partially characterized. Purification and characterization of this enzyme from animal cells have not, however, been reported previously.

The His-tRNA synthetase has been of particular interest in our investigation of the translational apparatus of rabbit red cell precursors and in the function of the $tRNA^{His}$ isoaccepting species in hemoglobin synthesis (Smith, 1975). Hemoglobin contains relatively more histidine than other proteins, and $tRNA^{His}$ is enriched in reticulocytes as an aspect of special-

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[1] Abbreviations used: $tRNA^{His}$, histidine tRNA; $tRNA^{His}_1$ and $tRNA^{His}_2$ are isoaccepting histidine tRNA species which are eluted from reversed phase chromatographic columns in the order indicated; His-tRNA, histidyl-tRNA; $NaDodSO_4$, sodium dodecyl sulfate.

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ization for hemoglobin synthesis, which constitutes about 90% of the total protein synthesis in these cells (Lodish & Desalu, 1973). Reticulocytes, which are the penultimate stage in red cell differentiation, contain two $tRNA^{His}$ isoaccepting species as resolved by reversed phase chromatography, while rabbit liver contains only one. The two isoacceptors from reticulocytes have similar coding properties (Smith et al., 1974), and each is able to incorporate histidine into all of the histidine containing positions in hemoglobin in a cell free system (McNamara & Smith, submitted for publication). The specificity of the His-tRNA synthetase for each of the isoaccepting $tRNA^{His}$ species and the activity of the enzyme in reticulocytes will be related to the function of His-tRNA in hemoglobin synthesis. Another reason for current interest in animal cell His-tRNA synthetase is that histidinol, a competitive inhibitor of the enzyme, is finding increased use as an inhibitor of protein synthesis (e.g., Hansen et al., 1972; Warrington et al., 1977).

Experimental Procedures

Materials. Nonradioactive amino acids, ATP, Tris, crystalline bovine serum albumin, *p*-hydroxymercuribenzoate, and phosphocellulose (0.93 mequiv/g) were purchased from Sigma Chemical Co. DEAE-cellulose (standard type 70, 0.88 mequiv/g) was obtained from Schleicher and Schuell. Uniformly labeled L-[^{14}C]histidine was purchased from Amersham/Searle. All batches used had specific activities from 300 to 350 mCi/mmol. Fluorescamine (Fluram) was obtained from Roche Diagnostics. Bio-Rad 70 and chemicals for gel electrophoresis were purchased from Bio-Rad Laboratories. Protein standards for molecular weight determinations were purchased from Boehringer-Mannheim.

Phenylhydrazine-Treated Rabbits and Rabbit Reticulocytes. A reticulocytosis of 50–60% was induced in rabbits by injections of phenylhydrazine, and the red cells were collected and washed as described previously (Smith & McNamara, 1972). After the third wash, the packed cells were lysed by the addition of 3 volumes of a solution containing 2.5 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.5. The His-tRNA synthetase was purified from this hemolysate as described below. For most preparations, the reticulocytes from four to six rabbits were used.

tRNA from rabbit liver and reticulocytes was prepared as previously described (Smith & McNamara, 1972). For certain experiments, $tRNA^{His}$ from each of these sources was partially purified by reversed phase column chromatography (RPC-5) (Kelmers & Heatherly, 1971) as previously used in our laboratory (Smith et al., 1974). This chromatographic technique completely resolves the two isoaccepting $tRNA^{His}$ species of reticulocytes. Histidine acceptance by the tRNA was assayed as described previously (Smith & McNamara, 1971a,b) except that the optimal magnesium concentration of 45 mM was used.

Assay of His-tRNA Synthetase. The following were combined in a total volume of 2.10 mL: L-[^{14}C]histidine, 0.67 μM; ATP, 1.2 mM; $MgCl_2$, 45 mM; Tris-HCl, pH 8.5, 12 mM; bovine serum albumin, 0.035 mg/mL; unfractionated rabbit liver tRNA, 1.7 absorbance (A_{260}) units/mL; and 19 unlabeled amino acids not including histidine, 24 μM each. Unfractionated tRNA from liver and reticulocytes containing equivalent amounts of $tRNA^{His}$ provided the same enzyme activity, and, therefore, liver tRNA was used because of its greater availability. The reaction ingredients were kept chilled and a few microliters of the enzyme preparation to be assayed was added. A 400-μL aliquot was taken as a time zero point. The reaction was then incubated at 37 °C, and four additional 400-μL samples were taken at intervals to provide several measurements of the amount of His-tRNA formed over a 5- or 10-min period. Five milliliters of cold 5% trichloroacetic acid was added promptly to each sample, and at least 5 min was allowed for the formation of a precipitate of the macromolecules. The precipitates were collected on 25-mm type HA Millipore filters with 0.45-μm pores, washed three times with cold 5% trichloroacetic acid, dried, and counted. Reaction rates are linear with time up until approximately one-third of the available $tRNA^{His}$ has been aminoacylated. Rates in this linear range were used as initial rates in the determination of enzyme activity.

To locate the enzyme in column and gradient fractions, the assay was modified to conserve tRNA. The reaction mixture was reduced to one-fifth the size described above, the concentration of tRNA was reduced to 0.11 absorbance (A_{260}) unit/mL, and there was a single fixed time of incubation. Once enzyme peaks were located, active fractions were pooled and the five point assay described above was performed.

A unit of enzyme is defined as that amount forming 1 nmol of His-tRNA/min under the conditions of the assay.

Concentrating the Enzyme. A rapid and convenient method was developed for concentrating the enzyme from dilute solutions such as column eluates. This method also permitted removal of the enzyme from solutions containing salt, Tris, and glycerol, which were present during purification but which interfere with gel electrophoresis, protein determinations, and sucrose gradient centrifugation. After the final step in purification, the enzyme is quite labile, and the method was developed because most of the activity was lost in attempts to concentrate it by ultrafiltration and by reverse dialysis using dry Sephadex or concentrated poly(ethylene glycol) solutions.

A small column with a bed volume of 0.250 mL of DEAE-cellulose was prepared. Solutions containing the enzyme and up to a few milligrams of protein were run onto the column. All enzyme was retained even if the input volume was as much as 1000 times the bed volume of the column, provided the concentration of the input solution was less than 0.10 M NaCl. After the input was run onto the column, it was washed five times with several volumes each of a solution containing 10 mM sodium phosphate, pH 7.5, and 4 mM $MgCl_2$. The enzyme was then eluted by the application of several 0.30-mL aliquots of a solution containing 300 mM NaCl, 4 mM $MgCl_2$, and 10 mM sodium phosphate, pH 7.5. The eluate resulting from each aliquot applied was assayed for enzyme activity. All enzyme was eluted in three or four fractions. The recovery of enzyme activity from these columns ranged from 50 to 100%. The enzyme can be concentrated several hundred-fold by this procedure.

Protein Determinations. Protein in concentrations of 0.5 mg/mL or greater was assayed by the method of Lowry et al. (1951). Lower concentrations of protein were determined fluorometrically by the method of Böhlen et al. (1973) using an Aminco-Bowman standard model spectrophotofluorometer. This method permits accurate determinations of protein concentrations as low as 2.0 μg/mL. It cannot be used when amines such as Tris are present. However, this compound can be removed when protein solutions are concentrated as described above. All protein determinations are based on a crystalline bovine serum albumin standard.

Gel Electrophoresis. Electrophoresis on 5% polyacrylamide gels measuring 0.6 × 9.5 cm in the presence of 0.1% $NaDodSO_4$ was done by the method of Maizel (1969). The following protein standards were used in the determination of the molecular weight of the subunit of the His-tRNA synthetase: cytochrome *c*, chymotrypsinogen A, bovine serum albumin,

TABLE I: Purification of His-tRNA Synthetase from Rabbit Reticulocytes.

Step	Protein (mg)	Units	Spec act. (units/mg)	Recovery (%)	Purification
Lysate	27 243	223.45	0.0082	100	1.00
Poststromal supernatant	24 980	208.30	0.0083	93.2	1.13
Postribosomal supernatant	24 180	194.60	0.0081	87.1	0.98
Ribosomal pellet	624	23.45	0.0376	10.5	4.59
$(NH_4)_2SO_4$ precipitate	2 657	155.05	0.0586	69.4	7.15
Dialyzed $(NH_4)_2SO_4$ precipitate	2 111	134.50	0.0637	60.2	7.77
DEAE-cellulose column, pooled fractions	59.2	93.10	1.573	41.7	191.8
Phosphocellulose column, pooled fractions	1.568	72.85	46.5	32.6	5 671
Bio-Rex 70 column, pooled fractions	0.032	22.08	687	9.9	83 780

hen egg albumin, aldolase, and catalase. Samples were heated to 100 °C in 1% $NaDodSO_4$ for 1 min before being applied to the gels.

Sucrose Density Gradient Centrifugation. The method of Martin & Ames (1961) was used for sucrose density gradient centrifugation of the His-tRNA synthetase. A linear gradient from 5 to 20% sucrose buffered at pH 7.5 either with 50 mM Tris-HCl or with 10 mM sodium phosphate was formed in cellulose nitrate tubes measuring 1.2 × 5 cm, and the enzyme or other protein was applied to the top of the gradient in a volume of 0.20 mL or less. Centrifugation was in a Beckman Model L3-50 ultracentrifuge in the SW 50.1 rotor for 7 to 14 h at 50 000 rpm. Samples of 0.16 mL were collected and assayed. Hemoglobin and bovine serum albumin were used as standards to calculate the sedimentation constant and molecular weight of the His-tRNA synthetase by the formulas of Martin & Ames (1961).

Isoelectric Focusing. Isoelectric focusing was performed in the 110-mL column manufactured for that purpose by LKB Instrument Co., using their pH 4–6 ampholyte mixture at a 1% concentration and a gradient from 0 to 75% glycerol. Three days each at 600 V were allowed for formation of the pH gradient and for focusing of the enzyme. Two-milliliter fractions were collected and the fractions were assayed for enzyme activity and their pH was measured for the determination of the P_i of the enzyme.

Results

Enzyme Purification. The His-tRNA synthetase of rabbit reticulocytes was purified by conventional techniques as follows. The osmotic lysate obtained from reticulocytes was centrifuged for 30 min at 16 000*g* to remove cell membranes. Recovery of the enzyme in the supernatant after this procedure was 90 to 95%, and no detectable enzyme activity was sedimented, suggesting that none of the enzyme is bound in a stable association to plasma membranes. The supernatant was then centrifuged for 90 min at 106 000*g* in the Beckman Model L3-50 ultracentrifuge using the Model No. 30 rotor to sediment ribosomes. In the absence of sucrose, glycerol, or other substances which would increase the viscosity of the solution, these forces are sufficient to sediment both ribosomes and ribosomal subunits. Ten to fifteen percent of the enzyme activity was precipitated with the ribosomes, as has been observed by other workers (Irwin & Hardesty, 1972; Ussery et al., 1977). A cold saturated ammonium sulfate solution buffered at pH 7.4 with ammonium carbonate was added slowly to the post ribosomal supernatant with stirring to a final concentration of 70% saturation, and the resulting solution was held for 30 min. The precipitated protein was then sedimented by centrifugation and was washed once with a 70% saturated buffered ammonium sulfate solution. The resulting precipitate was dissolved in a solution of 4 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, and 20% glycerol to a concentration of about 15 mg/mL of protein. The ammonium sulfate precipitation separates the aminoacyl-tRNA synthetases including the His-tRNA synthetase from most of the hemoglobin, which is the major contaminating protein. The dissolved precipitate was dialyzed overnight against 100 volumes of the dissolving solution to remove residual ammonium sulfate.

The dialyzed preparation was run onto a 2.5 × 52 cm column of DEAE-cellulose and eluted with a 2000-mL linear gradient from 0 to 300 mM NaCl with 4.0 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, and 20% glycerol at a flow rate of 2 mL/min. The column had previously been equilibrated with the initial solution of the gradient. The enzyme eluted as a single sharp peak of activity at a salt concentration of approximately 0.16 M NaCl, while the remaining hemoglobin was eluted at the solvent front. The pooled fractions containing the enzyme were immediately placed on a 2.5 × 65 cm column of phosphocellulose and subjected to a 2000-mL linear gradient from 0.20 to 0.40 M NaCl with $MgCl_2$, Tris-HCl, and glycerol as described above. The column had previously been equilibrated with the initial solution of the gradient and the flow rate was approximately 1 mL/min. A large peak of material absorbing at 280 nm was eluted at the solvent front. The enzyme was eluted as a sharp peak of activity by a NaCl concentration of approximately 0.36 M. The above procedures, which required 3 to 4 days from the time the rabbits were bled, were all performed at temperatures of 0 to 4 °C. The active fractions from the phosphocellulose column were pooled and stored at −85 °C for later use.

For a final purification step, the frozen material from the phosphocellulose column was diluted to a conductivity less than that of 0.15 M NaCl, and the resulting solution was applied to a column of BioRex 70 measuring 1 × 15 cm which had been equilibrated over a period of several days by passing the first solution of the eluting gradient through it. The linear eluting gradient of 400 mL was from 0.15 to 0.50 M NaCl and contained $MgCl_2$, Tris-HCl, and glycerol as described above. The column was run at a rate of 0.2 mL/min. Material absorbing at 280 nm was eluted throughout the gradient at a low concentration which changed little. The enzyme was eluted as a sharp peak of activity by approximately 0.26 M NaCl. There was no distinct peak of absorbance at 280 nm corresponding to the enzyme.

A record of a typical purification procedure starting with the reticulocyte rich blood of five rabbits is shown in Table I. The loss of activity associated with ribosomes is reflected in the final value for recovery. Much enrichment of the enzyme is accomplished in relatively few steps, and little protein remains after the procedure. The specific activity of the purified enzyme of 687 units/mg of protein is approximately equal to that of other purified aminoacyl-tRNA synthetases from a variety of eukaryotic cells (for review, see Loftfield, 1972).

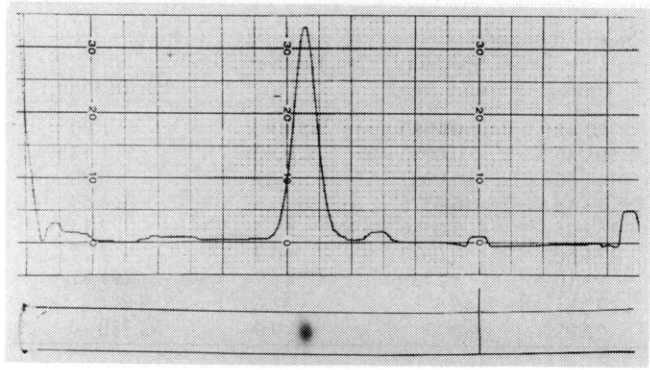

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of the purified His-tRNA synthetase. Purified enzyme (20 μg) was layered on the gel in 0.2 mL of a 20% glycerol solution. Current of 15 mA was applied for 2–3 h. The photograph of the gel shown was taken with polarized light beneath the specimen and this procedure greatly improves the contrast along the side of the gel. The small wire near the bottom of the gel was inserted to mark the migration of a tracking dye, bromophenol blue. Gels, buffers and staining procedures were as described by Maizel (1969). A densitometric scan of the gel is also shown. This scan was performed using a Gilford Model 2400 spectrophotometer with a scanning attachment. Full scale is 1 absorbance unit at 550 nm.

Evidence of Enzyme Purity. Evidence that the enzyme is pure is based on the homogeneity of protein in the preparation and the correspondence of enzyme activity with the protein. Electrophoresis of 20 μg of the enzyme in the presence of 0.10% $NaDodSO_4$ on 5% polyacrylamide gels repeatedly revealed a single band staining with Coomassie Brilliant Blue as shown in Figure 1. The correspondence of the enzyme activity with this protein was demonstrated by sucrose gradient centrifugation of the purified preparation as described above. The peak of enzyme activity and the peak of protein as determined by the sensitive fluorescence technique coincided and no protein was found elsewhere in the gradient. Disc gel electrophoresis was not successful with the purified enzyme because at the necessary concentration, the enzyme did not enter the gel, possibly because of aggregation.

No aminoacyl-tRNA synthetase activity for amino acids other than histidine was detected in the preparation.

Properties of the Enzyme. A. Stability. Stability studies show that the half-life of enzyme preparations through the phosphocellulose column step of the purification procedure is many hours at 37 °C, and it can be stored without loss of activity for many months by freezing at −85 °C. Stability is reduced at protein concentrations of less than 15 μg/mL. The material eluted from the Bio-Rex 70 column is much less stable, with a half-life of 18 min under conditions of the assay and 132 min at 0 to 4 °C. It can, however, be frozen at −85 °C for many weeks without much loss of activity. The enzyme is not stabilized by concentrations of histidine, ATP, or unfractionated tRNA similar to those in the enzyme assay. Glycerol, which has been shown to stabilize some aminoacyl-tRNA synthetases (for review, see Loftfield, 1971) was included during most of the purification steps. It does not appear after careful study, however, to stabilize the purified His-tRNA synthetase.

B. Molecular Weight and Subunits. Centrifugation on sucrose gradients as described above indicates a $s_{20,w}$ value of 6.7 for the purified enzyme. This corresponds to a molecular weight of approximately 122 000 (Martin & Ames, 1961). In contrast, the crude enzyme in the ammonium sulfate precipitate has a molecular weight of approximately 178 000 as determined by the same technique. A likely interpretation of these results is that the crude enzyme binds two tRNA molecules and that these are removed when the enzyme is passed through DEAE-cellulose which has a much greater affinity for tRNA than for the enzyme. A similar decrease in molecular weight of another tRNA enzyme upon purification has been recorded (Deutscher, 1972) and has been attributed to loss of tRNA that is bound to the enzyme in crude cellular extracts. The absorbance of the purified enzyme at 260 and 280 nm indicates that it contains less than 7% nucleic acid (Layne, 1957).

TABLE II: Properties of Rabbit Reticulocyte His-tRNA Synthetase.

Mol wt	122 000
Subunit mol wt	64 000
K_m for ATP	8×10^{-5} M
K_m for L-histidine	5×10^{-7} M
K_m for rabbit liver $tRNA^{His}$ (unfract)	6×10^{-9} M
K_m for rabbit reticulocyte $tRNA^{His}$ (unfract)	6×10^{-9} M
K_m for rabbit reticulocyte $tRNA^{His}_{I}$	6×10^{-9} M
K_m for rabbit reticulocyte $tRNA^{His}_{II}$	4×10^{-9} M
K_m for rabbit liver $tRNA^{His}$ (fract)	4×10^{-9} M
Mg^{2+} optimum (KCl absent)	45 mM
Mg^{2+} optimum (160 mM KCl)	5 mM
pH optimum	8.0–8.5
Isoelectric point	5.1
Turnover no.	84

Electrophoresis on 5% polyacrylamide gels containing 0.1% $NaDodSO_4$ gives, as described above, a single band staining for protein. In comparison with the proteins and subunits used as standards, it can be calculated that the band has a molecular weight of about 64 000. Our interpretation of these data is that the enzyme is composed of two similar subunits. In this respect, it is like the His-tRNA synthetases of *E. coli, S. typhimurium,* and baker's yeast (Kalousek & Konigsberg, 1974; DeLorenzo & Ames, 1970; Boguslawski et al., 1974). While a variety of subunit arrangements has been observed for different aminoacyl-tRNA synthetases, it has been noted (Muench et al., 1975) that the synthetases for any particular amino acid usually have the same subunit arrangement in a variety of organisms.

C. Affinities for Substrates. K_ms for the various substrates of the purified reticulocyte His-tRNA synthetase as determined from Lineweaver & Burk (1934) plots are shown in Table II, which also summarizes other properties of the enzyme. K_ms for each of the two $tRNA^{His}$ species from rabbit reticulocytes as resolved by reversed phase column chromatography and for the single $tRNA^{His}$ species from rabbit liver as partially purified by the same method are indistinguishable. A comparison of activity of the enzyme with the same amounts of the two reticulocyte $tRNA^{His}$ isoacceptors revealed no difference in aminoacylation rates.

D. Cation Effects. A magnesium optimum of 45 mM was found for the purified His-tRNA synthetase. A magnesium optimum previously published for this enzyme using the dissolved ammonium sulfate precipitate indicated that a much lower magnesium concentration gave maximum activity (Smith & McNamara, 1971a,b). These results are readily reconciled because monovalent cations such as the residual ammonium in the crude enzyme preparation can substitute in part for magnesium. In the presence of the intracellular potassium concentration (160 mM) (McCance & Widdowson, 1956), the magnesium optimum is shifted to 5 mM, which is close to the normal intracellular magnesium concentration of 2–3 mM (Herring et al., 1960). While monovalent cations can substitute in part for magnesium, they cannot do so completely, and no His-tRNA is formed in the presence of 160 mM KCl

if magnesium is absent. These results are shown in Figure 2. Manganese salts can be used instead of magnesium with a similar concentration giving maximum enzyme activity; however, reaction rates are slower. Salt concentrations higher than 200 mM have an inhibitory effect on both the rate and the final level of His-tRNA formation (Smith, 1969).

E. Other Properties. Enzyme activity does not vary much with pH in the range from 6.5 to 9.0. Maximum activity is seen between pH 8.0 and 8.5.

An isoelectric point of pH 5.1 was determined by isoelectric focusing. This procedure was also tried as a final purification step; however, the recovery of activity was poor compared with the BioRex column. Moreover, the ampholytes used in the procedure interfere with the determination of protein and are difficult to remove from the enzyme. Therefore, it was impossible to demonstrate a correspondence between protein and enzyme activity in preparations from isoelectric focusing columns.

Enzyme activity is sensitive to sulfhydryl inhibitors. In the presence of 10^{-5} M *p*-hydroxymercuribenzoate, the activity of 3×10^{-4} unit of enzyme was reduced to 48%. In 10^{-4} M inhibitor, activity was reduced to 33%, and in 10^{-3} M no enzyme activity was detectable.

Discussion

A recent study comparing His-tRNA synthetase activity in crude extracts of several kinds of animal cells indicates that the specific activity of this enzyme and of other aminoacyl-tRNA synthetases is lower in reticulocytes than in other cells (Ussery et al., 1977). In retrospect, therefore, reticulocytes are probably not an ideal source of the His-tRNA synthetase. On the other hand, the principal contaminating protein, hemoglobin, is readily separated from the enzyme. The amount of purification required to achieve a homogeneous preparation of the enzyme is comparable to that required for purification of the initiation factors of protein synthesis from reticulocytes (e.g., Merrick & Anderson, 1975; Merrick et al., 1975). The specific activity of the purified reticulocyte His-tRNA synthetase of 687 units/mg of protein is similar to that of several other aminoacyl-tRNA synthetases purified from animal and microbial sources (for review, see Loftfield, 1972). A turnover number of 84 mol of His-tRNA formed per min per mol of enzyme can be calculated. In reticulocytes, the aminoacyl-tRNA synthetases are synthesized at an earlier stage of red cell development (Denton & Arnstein, 1973) and are not renewed continuously as in other kinds of cells. The specific activity of the purified enzyme suggests, however, that degradative changes reducing enzyme activity have not occurred.

Our results confirm the observations of other workers that a small percentage of the His-tRNA synthetase in several kinds of animal cells is attached to ribosomes (Irwin & Hardesty, 1972; Ussery et al., 1977) with which it can be sedimented in solutions of low ionic strength (Roberts & Olson, 1976). In contrast, nearly all of the Phe-tRNA synthetase activity of rat liver and rabbit reticulocytes is attached to ribosomes, from which it can be released by washing with 0.50 M KCl (Tanaka & Hardesty, 1976; Tscherne et al., 1973).

Although some aminoacyl-tRNA synthetases occur in multimolecular complexes with sedimentation constants from about 14 to 25 S, we have never observed reticulocyte His-tRNA synthetase in such a complex, and it has not been observed in complexes in other kinds of cells (Ussery et al., 1977; Bandyopadhyay & Deutscher, 1971; Vennegoor & Bloemendal, 1972; Hampel & Enger, 1973; Som & Hardesty, 1975).

It was originally anticipated that there might be multiple

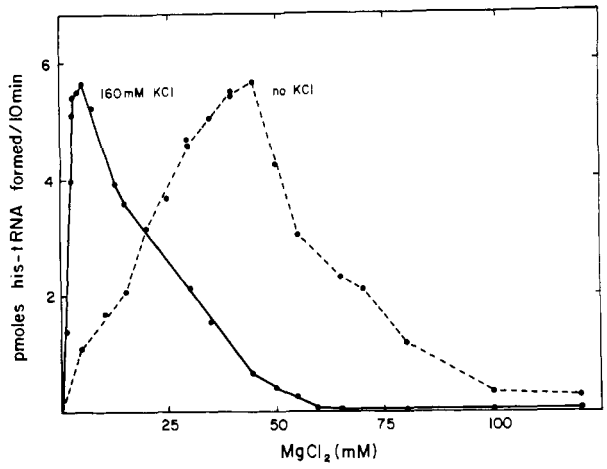

FIGURE 2: Magnesium optimum of the purified His-tRNA synthetase. The activity of 6×10^{-4} unit of purified His-tRNA synthetase at various magnesium concentrations is shown in the presence and absence of 160 mM potassium.

forms of the His-tRNA synthetase in reticulocytes. Such multiple forms have been demonstrated for other aminoacyl-tRNA synthetases (for review, see Loftfield, 1971), and a paper from another laboratory states that chromatographic heterogeneity was observed in His-tRNA synthetase of rat liver (Allende et al., 1966). We have, however, found no evidence that the enzyme is heterogeneous in reticulocytes, even when reticulocyte tRNA, which contains two $tRNA^{His}$ isoaccepting species, was used instead of liver tRNA to assay column fractions. It is possible that some of the His-tRNA synthetase activity observed in liver was derived from mitochondria which, in some cases, contain aminoacyl-tRNA synthetases distinct from those in the cytosol. Heterogeneity of His-tRNA synthetase in yeast is attributable to distinct mitochondrial and cytosol enzymes (Boguslawski et al., 1974). Mitochondria are abundant in liver but scarce in reticulocytes (Bessis & Breton-Gorius, 1964), thus making the detection of a mitochondrial enzyme in the present study unlikely.

The K_m for ATP of the His-tRNA synthetase is similar to that determined for other aminoacyl-tRNA synthetases from both animal and microbial cells. The K_m for histidine is less by a factor of about 10 than the K_ms for amino acid commonly described for aminoacyl-tRNA synthetases, and the K_m for tRNA of about 5×10^{-9} is substantially lower than has been determined for other synthetases. Not only are the K_ms for the two $tRNA^{His}$ isoaccepting species of reticulocytes and the single $tRNA^{His}$ species of rabbit liver indistinguishable, but equal amounts of the two reticulocyte species are aminoacylated at similar rates. A comparable result has been found for the Glu-tRNA synthetase of *E. coli* (LaPointe & Söll, 1972) and its three $tRNA^{Glu}$ isoaccepting species which have indistinguishable K_ms. Although the two $tRNA^{His}$ isoaccepting species of reticulocytes are similar in codon recognition (Smith et al., 1974), consistent with the "Wobble hypothesis" (Crick, 1966), and, although each appears capable of incorporating histidine into all of the histidine containing positions in hemoglobin (McNamara & Smith, submitted for publication) in a cell-free reticulocyte lysate, the isoaccepting species differ in at least two respects. $tRNA^{His}_1$ contains Q base (Kasai et al., 1975; DuBrul & Farkas, 1976; McNamara & Smith, submitted for publication), a modified form of guanine, and $tRNA^{His}_2$ can be enzymatically guanylated (Dubrul & Farkas, 1976). It is of interest that these differences in the $tRNA^{His}$ isoaccepting species appear not to be determinants of their aminoacylation by the enzyme, and that enzyme specificity

cannot provide a basis for preferential utilization of either of the isoaccepting species in hemoglobin synthesis in intact reticulocytes.

Rabbit reticulocytes, produced under the stress of phenylhydrazine treatment, synthesize hemoglobin at a rate of 20 000–30 000 molecules/min (Lingrel & Borsook, 1963; Smith, unpublished results). At least 90% of the protein synthesized by these cells is hemoglobin (Lodish & Desalu, 1973). Since rabbit hemoglobin contains 38 histidine residues per molecule, the reticulocyte must incorporate about 900 000 histidine residues/min to maintain this rate of hemoglobin synthesis. Based on the specific activity of the purified enzyme preparation and the assay of His-tRNA synthetase activity in crude lysates derived from known numbers of reticulocytes, it can be calculated that there are about 3700 molecules of the enzyme/cell. Based on the turnover number of the enzyme, it can be calculated that this number of molecules is capable of synthesizing about 300 000 molecules of His-tRNA per min per cell. This falls short by a factor of about three of the amount of His-tRNA that must be synthesized to maintain the rate of hemoglobin synthesis of the intact cell. Since care was taken to assay the enzyme under optimal conditions, it must be concluded that the lysis of reticulocytes results in loss of some enzyme activity. There is a determination by another laboratory (Ussery et al., 1977) of His-tRNA synthetase specific activity in crude reticulocyte lysates that is three times higher than the value we have repeatedly found (see Table I). In intact reticulocytes, His-tRNA synthetase activity is clearly not limiting in hemoglobin synthesis, as evidenced by the high level of aminoacylation of $tRNA^{His}$ in these cells (Smith & McNamara, 1974).

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