SOME PROPERTIES OF GLYCYL-RNA SYNTHETASE OF RAT LIVER

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ABSTRACT

Some Properties of Glycyl-RNA Synthetase of Rat Liver

The time course, effect of enzyme concentration and effect of concentration of substrates on glycine-dependent ATP-^{32}PP exchange have been determined. The apparent Km values for glycine, ATP and PP are 0.6 mM, 0.076 mM and 0.21 mM respectively. The Mg^{++} optimum for the glycine-dependent ATP-^{32}PP exchange catalyzed by the 50-60% (saturation) $(NH_4)_2SO_4$ fraction was 2 mM. This was not changed by the addition of $(NH_4)_2SO_4$ or SRNA.

The apparent Km for glycine (4 µM), effect of enzyme concentration and Mg^{++} optimum (10 mM) were determined for glycyl-RNA formation catalyzed by the 50-60% (saturation) $(NH_4)_2SO_4$ fraction from rat liver.

The effect of pH on the rates of glycine-dependent ATP-^{32}PP exchange and of glycyl-RNA formation has been determined. Maximal activity was found in the pH range 7.0 to 8.0. The results indicated that there may be an ionisable group in the active center of glycyl-RNA synthetase which has a pK of 6.2 to 6.7. This corresponds to the secondary nitrogen of a histidine residue. Further evidence that might indicate the presence of histidine in the active center was obtained by photo-oxidation of histidine residues in the enzyme with methylene blue. It was found that glycine, but not other substrates (ATP, SRNA) protected the enzyme from photo-oxidation. This protection was specific because alanine

did not protect the enzyme from photo-oxidation with methylene blue.

Previously, it was found that when RNA was removed from the protein during purification, the enzyme became unstable and sensitive to activation by mercaptoethanol or inhibition by p-chloromercuribenzoate. These findings indicate the presence of a cysteine side-chain in the active center. It was found that SRNA but not other substrates (glycine, ATP) protected glycyl-RNA synthetase against inactivation by p-chloromercuribenzoate. It appears that this effect of SRNA is a specific one because ribosomal RNA does not give this protection.

The effect of glycine analogs which act as competitive and noncompetitive inhibitors on glycine-dependent ATP-^{32}PP exchange activity are reported in this work. Methylamine and betaine were found to be competitive inhibitors with K_Is of 6.3 mM and 26 mM respectively. These results may indicate that glycine is bound to the enzyme at an anionic site. Another analog of glycine, glycinamide, was found to be a strong inhibitor of the glycine-dependent ATP-^{32}PP exchange. This inhibition was noncompetitive with respect to glycine.

A possible mechanism for glycyl-RNA formation is proposed, based on the above findings.

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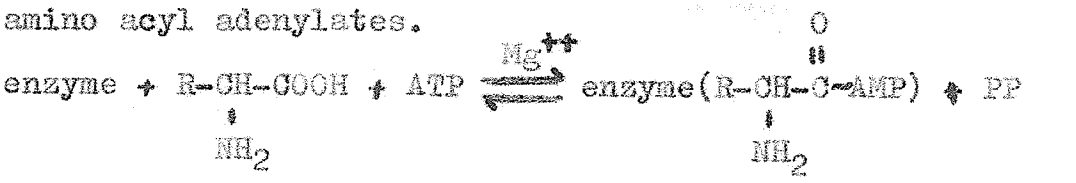
INTRODUCTION

(A) General

All proteins are composed of amino acids bound to each other through peptide linkages which are formed by joining a carboxyl group of one amino acid to the amino group of another amino acid. Only within the last nine years has real progress in understanding the formation of these bonds been made. It has now been found that protein biosynthesis occurs in three steps (1):

(a) activation of amino acids through the carboxyl groups with ATP by specific activating enzymes to form enzyme-bound amino acyl adenylates.



(b) acceptance of the amino acyl moiety of the enzyme-bound amino acyl adenylate by specific acceptor RNA (SRNA) to form amino acyl RNA esters with the release of AMP and enzyme.

(c) transfer of the activated amino acids from the amino acyl RNA esters to ribosomal particles where peptide linkages are formed with release of the SRNA. Messenger RNA, attached to the ribosomes, acts as a template for protein biosynthesis (1). The messenger RNA is synthesized in the

nucleus of the cell under DNA control (1).

A large amount of experimental evidence supports the above mechanism. The enzymatic formation of enzyme-bound amino acyl adenylates from adenosine triphosphate and amino acid has been recognized for several years (2-8). Hoagland (9) in 1955 with a rat liver supernatant showed that radioactive inorganic pyrophosphate (^{32}PP) of the reaction medium would exchange with PP of ATP upon addition of small amounts of rat liver supernatant to ATP, ^{32}PP, Mg^{++} and amino acids. This exchange was termed "amino acid-dependent ATP-^{32}PP exchange". Hisman et al (4) in 1957 showed that any one of the twenty naturally occurring amino acids would satisfy the amino acid requirement for an ATP-^{32}PP exchange. Evidence has also been shown that an ATP-^{32}PP exchange can be obtained with the extracts of many different tissues (10-15). The amino-dependent ATP-^{32}PP exchange is one method of measuring amino acid activation. The activation step may also be measured by "trapping out" the amino acid of the enzyme-bound adenylates by a non-enzymatic reaction in the presence of high concentrations (1-3 M) of hydroxylamine (NH_2OH) to form stable amino acyl hydroxamates. The hydroxamates will form a brown-coloured complex with Fe^{+++} in acid solution which may be measured colorimetrically (3,8,9). Hoagland's hypothesis that an intermediate enzyme-bound adenylate, formed during activation, was verified when Karasek et al (6) isolated tryptophanyl adenylate from an incubation mixture containing the purified tryptophan activating enzyme, tryptophan, ATP and Mg^{++}. Synthetically prepared amino acyl adenylates (16-18) have been shown to take part in the reversal of reaction (a) and to take part in the formation of

amino acyl-RNA esters in reaction (b) (17,18).

The same enzyme that activates the amino acid also catalyzes the transfer of the amino acyl moiety from the amino acyl adenylate to a specific type of ribonucleic acid (SRNA) (7,19-23,31). Thus, this class of enzyme has been designated "amino acyl-RNA synthetase" and the enzyme specific for the activation of a single amino acid, i.e. glycine, as glycyl-RNA synthetase. In the new nomenclature of the International Union of Biochemistry these enzymes are called "amino acyl-RNA ligases(AMP)" and the one specific for the activation of glycine would be called glycine-RNA-ligase(AMP).

The equilibrium constants for amino acyl-RNA formation have been found to be approximately 1.0 (7) indicating the linkage between the SRNA and amino acid to be a "high energy" linkage.

The amino acyl-RNA synthetases which catalyze amino acid-dependent ATP-^{32}PP exchanges also catalyze ATP-^{14}C-AMP exchanges (20,24) which is sensitive to ribonuclease and dependent on amino acids.

From the additivity of amino acid-dependent ATP-^{32}PP exchange for different amino acids and from partial purification of various activating enzymes from tissue extracts, it is known that each amino acid is activated by a specific amino acid activating enzyme (3,7,8,11,25-29). Each enzyme is stereospecific, activating only the L-amino acid.

Only a few cases have been cited where amino acid analogs, very closely related in structure to the naturally occurring amino acids, have been found to be activated (17). This accounts for the incorporation of unnatural substances like ethionine, selenomethionine, p-flurophenylalanine, azatryptophan and tryptozan into proteins (17). The purified isoleucyl-RNA synthetase from E. coli (23) catalyze the formation of valyl adenylate as well as isoleucyl adenylate. The K_m for the valine is about 100-fold higher than that for isoleucine. However, isoleucyl-RNA synthetase will not catalyze formation of valyl-RNA (7). It seems reasonable to suppose, therefore, that in vivo there is probably little competition between amino acids for any single amino acid activating enzyme. Amino acid analogs may act as competitive and noncompetitive inhibitors even though they are not activated (17,31).

Each amino acid activating enzyme is also specific for ATP and SRNA. The ATP cannot be replaced by GTP, UTP, CTP, dATP or AMP (7). Ribosomal RNA or synthetic polynucleotide prepared with polynucleotide phosphorylase do not function as amino acid acceptors (7). Mg^{++} is required for the amino acid-dependent ATP-^{32}PP exchange and for the amino acyl-RNA formation (2,19). In the case of glycine activating enzyme, Mn^{++} can replace Mg^{++} (30).

The enzymes involved in protein synthesis have been shown to exhibit some degree of species specificity.

Activating enzyme from rat liver or yeast will esterify leucine to transfer RNA from rat liver or yeast but not to E. coli transfer RNA (32). Thus, the SRNA and activating enzyme from rat liver or yeast probably differ in structure from SRNA and activating enzyme from E. coli.

The inhibitory effect of PCMB on amino acid activation and the restoration of activity by addition of reduced glutathione, cysteine or mercaptoethanol indicated that amino acid activating enzymes require -SH groups for their activity (8,15,22,33,34).

Soluble RNA isolated from mammalian liver (35), yeast (13,36) and E. coli (37) have been found to exhibit similar physical and chemical features. Molecular weight determination of SRNA indicates approximately 24,000 with a chain length of about 70 nucleotides (37-40). Structural studies have shown that the SRNA is single stranded with one end terminated by the trinucleotide sequence cytidylic acid- cytidylic acid- adenylic acid (41-43) and the other end by a nucleoside diphosphate (pGp) (44). Only one nucleotide diphosphate (pGp) and one nucleoside (A) are released per mole on alkaline digestion. Berg et al (45) have shown that 69% of the SRNA molecules contain adenine in the fourth position (from the adenylic end), 17% contain guanine in the fourth position and 94% contain a pyrimidine in the fifth position. Studies on specific SRNA for leucine and isoleucine (46) show

terminal sequences of:

Isoleucine ——pGpCp(UpC)pApCpCpA

Leucine 1 ————————pGpCpApCpCpA

Leucine 2 ————————pGpUpApCpCpA

This indicates that specific SRNA for the amino acids differ from each other by different nucleotide sequences between the pGp end and the CpCpA end.

The amino acids are linked to the acceptor RNAs through ester bonds between the carboxyl groups of the amino acids and the 2' or 3' hydroxyl groups of the terminal adenylic acid of the SRNAs (10).

Unfractionated SRNAs of many species have a high guanine and cytosine content as well as a near equivalence of complementary bases (11,13,41,47). This indicates that base pairing may occur between guanine and cytosine, adenine and uracil. From hypochromic effect studies, approximately 80% of the bases appear to be paired (48). X-Ray diffraction studies also indicate the SRNA molecule has a "hair pin" structure (38,39), not found in other types of RNA. In the "loop" region the bases would be unpaired.

SRNA contains "odd" nucleotides, pseudouridylic acid and various methylated derivatives of the four common ribonucleotides, including ribothymidylate, not found to any appreciable extent in other types of RNA (49). These methylated bases appear to be situated around the center of

the SRNA molecule (50,51). The methylation of the bases and formation of pseudouridylic acid occurs after formation of the polynucleotide chain (52,53). The unpaired bases in this region may be involved in the association of SRNA with the ribosomal template.

Chapeville et al (54) have shown that cysteine, attached to its normal acceptor RNA may be converted, while still attached, to alanine, by reduction with H_2 in the presence of Raney nickel catalyst. The peptide bond formation , using alanine attached in this way to cysteine acceptor RNA, is stimulated by poly UG which normally stimulates the incorporation of cysteine only. Also, alanine in this form was incorporated into cysteine places in hemoglobin, synthesised by rabbit reticulocyte ribosomes (54). Thus an amino acid, once attached to SRNA, no longer participates in coding, indicating that the code is embodied in the structure of the SRNA and this in turn is specific for each amino acid activating enzyme.

SRNA, thus, plays a central role in protein biosynthesis, accepting activated amino acids and transferring them to the ribosomal particles. There are probably three specificity sites on the SRNA molecule: (a) the site where the amino acid activating enzyme interacts (b) the site where the template interacts ("coding region") (c) a third site which must be invoked to explain species specificity of the interaction of the specific activating enzyme with the specific acceptor RNA. All sites must be characterised by

a unique nucleotide sequence (17).

From genetic experiments (55), it is believed that a minimum of three nucleotides in the messenger RNA is required to "code" one amino acid. When synthetic polyuridylic acid performed the role of messenger RNA, polyphenylalanine was formed (56). The code word for phenylalanine would be UUU. The coding region in the specific phenylalanyl RNA would have the complementary sequence in the "loop" region of AAA. The code words for all twenty amino acids have been worked out using other synthetic templates (57,58). Evidence for degeneracy of code words was discovered when two leucine acceptor SRNA were partially purified by countercurrent distribution (59). Two acceptor SRNA have also been found for threonine (60), lysine (61) and methionine (7). Recently, Von Ehrenstein and Dais (62) reported the presence of 3 leucine acceptor SRNA. Goldstein et al (63), assaying for amino acid acceptor activity, employing 16 amino acids, revealed the presence of 29 specific SRNAs, 3 of which were proline acceptor SRNAs, 4 were leucine acceptor SRNAs, and one was glycine acceptor SRNA.

(B) Glycine Activation

Pigeon pancreas (64a), rat mammary glands (64b) and E. coli (4,65) were examined for glycine activation by ATP-^{32}PP exchange method but very little glycine-dependent ATP-^{32}PP exchange occurred. Cormier and Novelli (66) partially purified a glycine activating enzyme from extracts of Photobacterium fischeri that catalyzed a reaction between ATP, glycine, and hydroxylamine, of which the products were glycyl hydroxamate, ADP and inorganic phosphate. Subsequently, Cormier, Stulberg, and Novelli (25) obtained results which indicated the formation of an intermediate glycyl phosphate during glycine activation. This intermediate contains an anhydride linkage between the carboxyl group of glycine and the phosphate group. However, this enzyme appears to be involved in purine biosynthesis rather than taking part in protein biosynthesis (30). Glycine has also been found to be a precursor of several other naturally-occurring compounds: glutathione (67,68), hippuric acid (69), creatine phosphate (70) and porphyrins (71). During the biosynthesis of these compounds, the glycine molecule is not activated but rather is used as an acceptor molecule, reacting with other activated molecules.

Fraser found that a fraction which precipitated at pH 5 from the 105,000 x g supernatant of rat liver catalyzed glycine-dependent ATP-^{32}PP exchange (30) and the formation of glycyl-RNA (72). This enzyme differs from the glycine

activating enzyme discovered by Novelli et al (25,66) and is probably involved in protein biosynthesis.

Fraser (30) demonstrated that glycyl-RNA synthetase activity could be obtained from 105,000 x g supernatant of a homogenate of rat liver in high yield (30%) with fairly good purification (40-fold) in three simple steps: precipitation of the enzyme from the 105,000 x g supernatant at pH 5, heating of the pH 5 fraction in the presence of ATP, and one $(NH_4)_2SO_4$ fractionation step. At a certain stage in purification, sudden changes in the properties of the glycyl-RNA synthetase enzyme occur. These changes in the properties of the enzyme are observed when the enzyme in the 50% $(NH_4)_2SO_4$ supernatant fraction (50% saturation with respect to $(NH_4)_2SO_4$) is precipitated by 60% $(NH_4)_2SO_4$ (60% saturation). The stability of the enzyme decreased considerably. Glycyl-RNA formation and glycine-dependent ATP-^{32}PP exchange activity became sensitive to sulfhydryl agents. 10-20 mM Mercaptoethanol, cysteine or reduced glutathione stimulated (18-fold) the glycine-dependent exchange catalyzed by the "50-60% $(NH_4)_2SO_4$" fraction. Mercaptoethanol (10 mM) stimulated glycyl-RNA synthetase activity in this fraction by 4-fold. The requirement of Mg^{++} for the exchange activity was lowered from 20 mM for the enzyme in "40% $(NH_4)_2SO_4$ supernatant" fraction to 2 mM for the enzyme in "50-60% $(NH_4)_2SO_4$" fraction. The "50-60% $(NH_4)_2SO_4$" fraction was found to contain a relatively small amount of RNA. Fraser (30) suggested the possibility that the changes in the properties of the enzyme may be

attributed to a dissociation from the enzyme of ribonucleic acid, probably the specific glycine acceptor RNA, with an unmasking of a sulfhydryl group. Hele (33,74-76) has obtained evidence that SRNA stabilises the leucine and isoleucine activating enzymes in "RNA-low" fractions from rat liver.

The present work reports the effect of glycine analogs which act as competitive and noncompetitive inhibitors of glycine-dependent ATP-^{32}PP exchange. From measurements of K_Is for competitive inhibitors, it is possible by making certain assumptions to make some tentative deductions about the nature of the glycine binding site of glycyl-RNA synthetase. The effect of pH on glycine-dependent ATP-^{32}PP exchange and glycyl-RNA formation has been determined. The results indicated that there may be an ionizable group in the active center of glycyl-RNA synthetase which has a pK of 6. This could correspond to the secondary nitrogen of a histidine residue. Further evidence for the presence of histidine in the active center was obtained by photo-oxidation of histidine residues in the enzyme with methylene blue. It is not known if cysteine and methionine (both known to be photo-oxidised in RNase (82-84)) are photo-oxidized in glycyl-RNA synthetase. Glycine protected the enzyme from photo-oxidation whereas alanine did not. Evidence has also been obtained for the presence of a sulfhydryl group in the active center of glycyl-RNA synthetase. It was found that SRNA protected the enzyme against inactivation by p-chloromercuribenzoate. It appears

this effect of SRNA is a specific one because rRNA does not give this protection. A possible mechanism for glycyl-RNA formation is proposed based on these findings.

MATERIALS AND METHODS

Radioisotopes

Two samples of l-^{14}C-glycine used in this work were obtained from Merck, Sharp and Dohme of Canada Limited, P. O. Box 899, Montreal 3, Quebec. One had a specific activity of 5.0 μc/mg; 1 c.p.m. was equivalent to 0.86 μμ mole glycine. The other had a specific activity of 5.0 μc/mg; 1 c.p.m. was equivalent to 0.91 μμ mole glycine.

Radioactive inorganic pyrophosphate solution was prepared by roasting $K_2H^{32}PO_4$ for 16 hours at 540°C, dissolving up the residue in water, and adjusting the pH to 7.4. The radioisotope was obtained from the Commercial Products Division of Atomic Energy of Canada, Ltd., P. O. Box 93, Ottawa, Ontario.

Chemicals

Crystalline disodium ATP and ADP were obtained from the Sigma Chemical Co., 3500 DeKalb St., St. Louis 18, Mo., U.S.A. It was dissolved in water and KOH added to bring the pH of the solution to 7.4.

Unlabelled glycine was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The analogs of glycine were obtained from Mann Research Laboratories, Inc., New York 6, N.Y. They were dissolved in water and the pH carefully adjusted to 7.4.

All other chemicals were "Analar" grade, obtained from British Drug Houses (Canada) Ltd., Barclay Ave., Queensway,

Toronto 14, Ont.

rRNA was obtained from Dr. L.H. Cohen, Biochemistry Dept., Medical College, University of Manitoba.

Tissue Fractions

Fresh livers were obtained from 150- to 200-g male Holtzman albino rats (Holtzman Co., 421 Holtzman Rd., Madison, Wis., U.S.A.) that had been stunned by a blow on the head and quickly exsanguinated. The livers were removed quickly and plunged into ice-cold 0.35 M sucrose solution. After the connective tissues had been removed and the livers patted dry, weighed and minced with scissors, a volume of "liver medium A" equal to 2.3 times the weight of the livers in grams was added (30% weight/volume homogenate). The "liver medium A" contained 0.35 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, and 0.017 M K_2HPO_4. The $MgCl_2$ was not added until the pH of the solution had been adjusted to 7.4 with a small addition of 2N HCl. The preparation was homogenized quickly by making 7 or 8 passes with a Teflon pestle in a Potter homogenizer. The "pH 5 enzymes" fraction was prepared in a similar manner to that of Keller and Zamecnik (79). The rat liver homogenate was centrifuged at 10,000 x g for 10 minutes in a Servall-SS-2 refrigerated centrifuge. The supernatant from this spin was collected and respun at 105,000 x g for one hour in a refrigerated Model L Spinco

ultracentrifuge. The supernatant from this spin (known as "105,000 x g supernatant") was carefully adjusted to pH 5.0 with 1M acetic acid and allowed to stand in ice from 5 to 10 minutes to allow complete precipitation. It was then centrifuged at 10,000 x g for 10 minutes in a refrigerated Servall-RC-2 centrifuge and the supernatant discarded. The precipitate was collected and suspended in a volume of "liver medium A" (see page 14) equal to the original weight of the rat liver in grams from which the pH 5 enzyme fraction was prepared. This enzyme preparation is known as the "pH 5.0 enzymes". In all further purifications 20 mls. of the "pH 5 enzymes" were used. It was found that the "pH 5 enzymes" could be frozen at -15°C and stored in this manner for 4 weeks with little loss of glycyl-RNA symthetase activity.

In subsequent steps in the purification (30), the "pH 5 enzymes" were made 1.0 mM in ATP (adding 0.40 mls of 0.05 M Na_2K_2ATP to the 20.0 mls of enzyme solution) heated at 55°C for 3.0 minutes and then cooled in an ice bath stirring with the thermometer all the while. The heating and cooling procedures were carried out rapidly (within 10 minutes). The "heated pH 5 fraction" was spun at 10,000 x g in a refrigerated Servall-RC-2 centrifuge for 10 minutes and the precipitate was discarded. The supernatant was then made 50% in $(NH_4)_2SO_4$ at 0°C by adding solid salt without adjusting the pH. After gentle stirring for 15 minutes, the

residue, "0-50% $(NH_4)_2SO_4$" fraction, was spun at 10,000 x g for 15 minutes in a refrigerated Servall-RC-2 centrifuge. The supernatant from this fraction was made 60% saturated in $(NH_4)_2SO_4$ at 0°C by adding solid salt without adjusting the pH. After gentle stirring for 15 minutes, the enzyme solution was spun at 10,000 x g for 15 minutes in a refrigerated Servall-RC-2 centrifuge and the precipitate was dissolved in the desired amount of 0.1 M Tris buffer. This fraction was termed the "50-60% $(NH_4)_2SO_4$" fraction. In the "50-60% $(NH_4)_2SO_4$" fraction only 6% yield with a 25-fold purification was obtained (73). When any of the $(NH_4)_2SO_4$ fractions were stored frozen at -15°C overnight, all activity was lost (30). Under the same conditions, the heated pH 5 fractions lost 60-75% activity but the glycine activating enzyme in the "105,000 x g supernatant" fraction and the "pH 5" fraction remained stable. In fact, the "pH 5" fraction could be stored frozen for one week with no loss of activity.

Measurement of Glycyl-tRNA Synthetase Activity

Amino acid activation may be measured in three ways:

(a) amino acid-dependent ^{32}PP incorporation into ATP

(b) ^{14}C-amino acid incorporation into SRNA

(c) formation of amino acid hydroxamate

Only the first two methods (a and b) were used in this work. The first method known as "amino acid-dependent ATP-^{32}PP exchange" was measured essentially as described by Hoagland (9).

The final 1.0 ml of incubation mixtures were 1.0 mM in both ATP and $^{32}PP_i$, 2 mM in Mg^{++}, 10 mM in mercaptoethanol and 100 mM in Tris buffer, pH 7.5. The incubations were carried out for 15 minutes at 37°C in the presence of enzyme and in the absence and presence of 1.0 mM glycine. The rate of glycine-dependent ATP-$^{32}PP_i$ exchange was linear for 25 minutes (see Results) after which no further exchange occurred The reaction was terminated by addition of 4.0 mls of 5.6% cold TCA. The A$T^{32}P$ was separated by charcoal adsorption according to the method of Crane and Lipmann (80). The mixture was filtered into 100 mg of acid washed charcoal, stirred and allowed to stand for 10 minutes. It was then washed 6 times on filter paper with a 0.01 M tetrasodium phosphate solution, pH 7.5, to act as a carrier to remove the $^{32}PP_i$. The charcoal, after being dried in a Canleb oven and transferred to a test tube with a small spatula, was heated with 5 ml of 1N HCl for 20 minutes in a boiling water bath. The eluted radioactive material was separated from the charcoal by filtration. An aliquot (0.5 ml) of the solution was plated on stainless steel planchets and the radioactivity determined. In this method of measuring amino acid activation, enzyme fractions must be nearly free of endogenous amino acids or the blank in which no amino acid is added is found to be very large. Also, pyrophosphatase may be present in the enzyme preparation and hydrolyze the $^{32}PP_i$. This would lessen the incorporation of $^{32}PP_i$ into ATP. Some pyrophosphatase enzymes

are inhibited by KF. Thus, KF could be used to increase the amino acid-dependent ATP-^{32}PP exchange.

The second method known as "labelling of specific acceptor RNA" was developed by Berg and Bergmann (7) and modified by Fraser (72). The method involved measurement of the effect of the enzyme on the rate of labelling of the SRNA from a heated (75°C for 3.0 minutes) rat liver "pH 5 enzymes" fraction by a ^{14}C-labelled amino acid. The heated fraction contained no glycyl-RNA synthetase activity but the glycine acceptor RNA remained intact since the addition of a small amount of active enzyme ("50-60% $(NH_4)_2SO_4$" fraction) fully restored the incorporation of l-^{14}C-glycine into SRNA. The rate of incorporation of amino acid into SRNA was directly proportional to the amount of added enzyme solution until an excess of enzyme was added (72). The amount of enzyme used was adjusted so that the rate of labelling was linear for 10 minutes (72) after which no further labelling occurs (the amount of SRNA present became "saturated" with amino acid). The rate of formation of glycyl-SRNA was also proportional to the substrate (glycine) concentration when the substrate concentration was limiting (72). Tests were always run without added enzyme (blank) to insure that the activity of the heated pH 5 enzymes fraction, used as a source of SRNA, was destroyed and in the presence of excess enzyme (control) to insure that the saturation level of SRNA had not been attained. The final 1.0 ml incubation mixtures were 5.0 mM in ATP, 10mM in Mg^{++},

10 mM in mercaptoethanol, 100 mM Tris buffer, pH 7.5, 0.5 ml heated (75-77°C for 5 minutes) pH 5 fraction (final concentration 5.8 mg/ml protein, 0.2 mg/ml SRNA) and l-^{14}C-glycine as indicated (see Results). The incubation was carried out for 10 minutes at 37°C in the absence and presence of "50-60% $(NH_4)_2SO_4$ enzymes" fraction. The reaction was terminated by addition of 1.0 ml of 22.5% cold TCA containing 2.0% glycine carrier. The protein-RNA precipitate was spun down in an international clinical centrifuge for 3 minutes. The precipitate was washed in the cold, twice with 3 mls of 5.6% TCA containing 2% glycine carrier, once with 3 mls of acetone and once with 3 mls of ether. The precipitate was finally suspended in 1 ml of ether and transferred to stainless steel planchets and the radioactivity determined. This method is very sensitive in measuring amino acid activation. It is possible to detect activation of amino acids in less than 10 µg of "pH 5 enzymes" protein without further purification (72).

Paper Strip Electrophoresis of Nucleotides

Paper strip electrophoresis (81) was used to determine whether or not ATP was lost from the reaction mixture (glycine-dependent ATP-^{32}PP exchange) by the action of enzymes other than glycyl-RNA synthetase present in the pH 5 fraction. Prior to the electrophoresis toward ATP, 1.0 ml of reaction mixture containing 0.01 M ATP, 100 mM Tris buffer,

pH 7.5, 1.0 mM PP, 2.0 mM $MgCl_2$, 10 mM mercaptoethanol, 0.3 ml "pH 5" fraction and H_2O was incubated for different times (see Results). The reaction was terminated by the addition of 0.1 volume (0.1 ml) of cold 1N perchloric acid to the samples in an ice-bath. The precipitate was centrifuged, and the cold extract was neutralised immediately with 1N KOH to pH 6 to 7 using 0.04% brom thymol blue as an internal indicator (0.01 ml). The mixture was allowed to stand for 5 minutes to bring about the precipitation of potassium perchlorate which was removed by centrifuging in an international clinical centrifuge for 3 minutes. The supernatant solution was immediately submitted to paper strip electrophoresis by applying 10 λ of solution with a sample stripper applicator to 30.5 x 3 cm Beckman-Spinco filter paper strips #32046, previously moistened with buffer solution (0.035 M citric acid and 0.0148 M sodium citrate) at pH 3.8. 400 Volts were applied on the strips by a Beckman-Spinco Model R paper electrophoresis system. After 3 hours, the strips were dried and observed under ultra-violet light. The positions corresponding to ATP and ADP were eluted with 4.0 mls of 1.0N HCl (boiled for 30 minutes). The samples were read directly on a Beckman Spectrophotometer and the amounts of ATP and ADP determined (see Results).

Photo-oxidation of Glycyl-tRNA Synthetase in the Presence of Methylene Blue

Photo-oxidation of proteins in the presence of a low concentration of methylene blue will affect, mainly, the "reactive" histidine molecules and at a much slower rate, the "reactive" methionine and cysteine molecules (82-84). The photochemical action of methylene blue on ribonuclease resulted in a complete inactivation when the only chemical change observed was the photo-oxidation of 3 moles of histidine (82). Thus, the photo-oxidation procedure was performed as described by Weil et al (82) except that high mercaptoethanol concentration (33 mM) was added to the enzyme solution, prior to photo-oxidation, to prevent any photo-oxidation of cysteine.

The entire operation was carried out in a dark room at 3°C. The purified enzyme solution (see Results for enzyme solutions used) in 0.1 M Tris buffer, pH 7.5, contained in a small beaker, was made 0.01% with methylene blue and 33 mM with mercaptoethanol. Prior to photo-oxidation, the temperature of the solutions was 3°C. Visible light from a 150-W "spotlight" lamp placed at a distance of 30 cm served as a light source. The temperature change during photo-oxidation was from 3°C to 22°C. After the desired time of photo-oxidation, the methylene blue was removed (photo-oxidation terminated) by mixing gently with 50 mg of charcoal. The enzyme solution was filtered and the "photo-oxidized" protein was assayed (see Results) for glycine-dependent ATP-^{32}PP exchange activity.

Isolation of SRNA

Equal volumes of "pH 5 enzymes" fraction and 90% (by weight) phenol were shaken together, mechanically, for one hour. The mixture was spun at 10,000 x g for 20 minutes in a refrigerated Servall-RC-2 centrifuge. The aqueous top layer was carefully removed with a Pasteur pipette and extracted twice with an equal volume of ether to remove phenol. One-tenth volume of a 20% potassium acetate solution was added. 2.5 Volumes of cold (-20°C) ethanol was then added to this solution to precipitate the SRNA. The mixture was allowed to stand at -15°C for at least 16 hours and then spun at 10,000 x g for 20 minutes as above. The precipitate was dissolved in water at the desired concentration and dialyzed overnight with 0.015 M EDTA. The solution was then dialyzed overnight again against a large volume of distilled water. Both dialyses were performed at 10°C. The SRNA was prepared only once and the final concentration of the SRNA solution was 0.67 mg/ml.

Analytical Determinations

The methods of paper chromatography which were used here for analysis of impurities in analogs of glycine was described by Fraser (72). The solvent system used was: secondary butanol: concentrated formic acid: water (100:15:25). 5.0 γ of the analog solution was spotted on #1 Whatman paper. The chromatogram was run 16 hours in the solvent system in one direction. After drying, the papers were sprayed with a

ninhydrin solution (Sigma Chemical Co., St. Louis 18, Mo., U.S.A.) and the positions of the spots noted.

Protein and SRNA of the resuspended "50-60% $(NH_4)_2SO_4$" precipitate fraction were estimated from the 260 mμ and 280 mμ absorbances of the enzyme solution by the method of Warburg and Christian (85).

The "40% $(NH_4)_2SO_4$ supernatant" and "pH 5 enzymes" fractions contain a large amount of SRNA which will give a large absorbance when measured by the UV spectrophotometer. Thus, the protein estimation of the "40% $(NH_4)_2SO_4$" and the "pH 5 enzymes" fractions were made by the method of Lowry et al (86) using 5 times crystallized egg albumin (Sigma Chemical Co.) as a standard. Both the Warburg and Christian and the Lowry method were in good agreement. SRNA was measured spectrophotometrically (72) using an extinction coefficient of 34.2 mg^{-1} cm^2 (87).

Determination of Radioactivity

All radioactive samples (^{14}C and ^{32}P) were plated on stainless steel planchets by evaporating to dryness under a 150-W infrared lamp and counted on a Nuclear Chicago micromil window gas-flow counter. No correction for self absorption was necessary because the determination of radioactivity was made at infinite thinness. For each set of samples a background (empty planchet) was counted.

RESULTS

Measurement of Glycine-Dependent ATP-^{32}PP Exchange

(i) Time Course

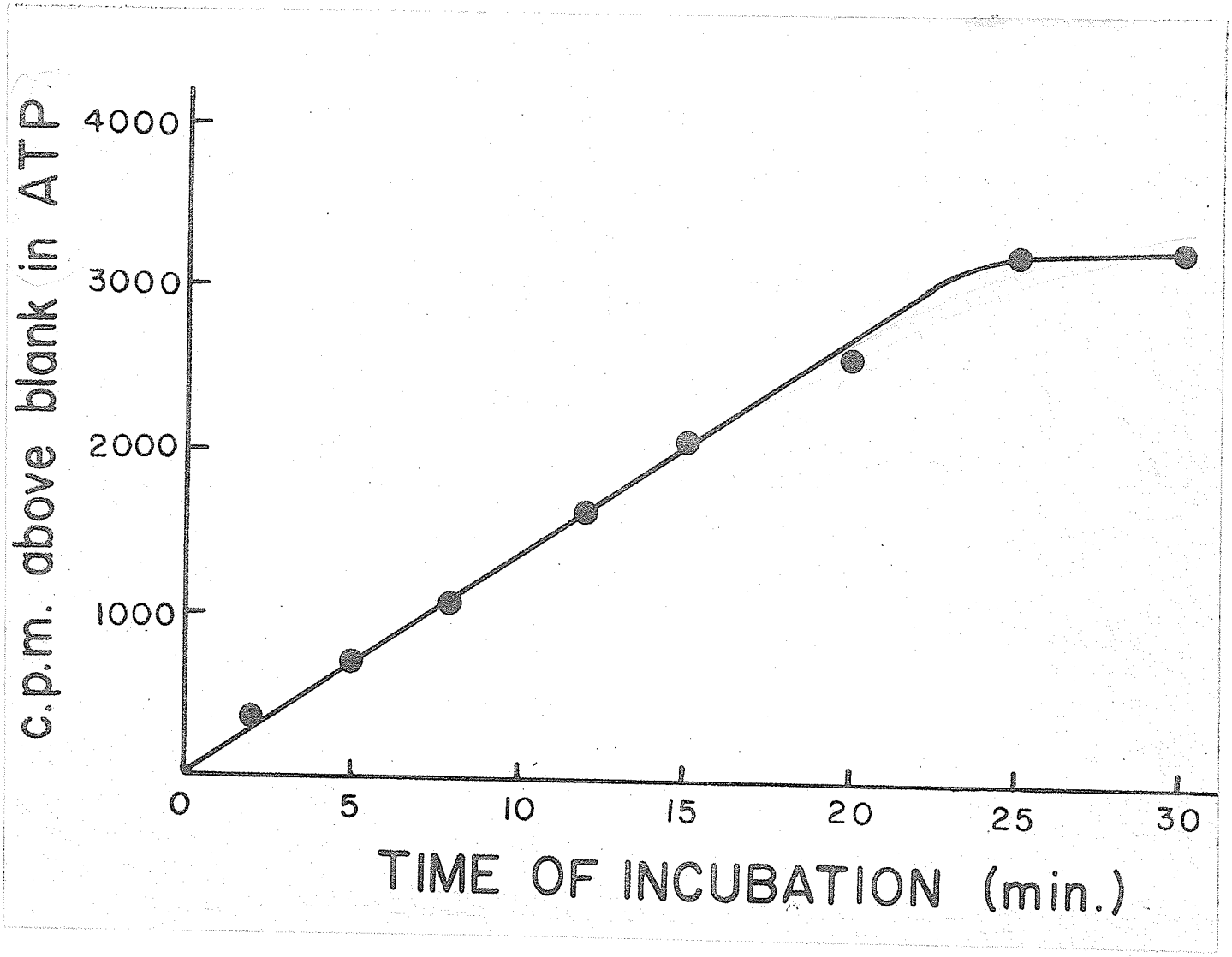
It may be seen in figure (1) that the time course of glycine-dependent ATP-^{32}PP exchange is linear up to 25 minutes. After 25 minutes the rate decreased. The concentrations of glycine, ATP and ^{32}PP in the reaction mixture were each 1.0 mM and the enzyme fraction used was the 50-60% $(NH_4)_2SO_4$ fraction. The blank incorporations increased with time also but each point recorded was corrected for the respective blank.

(ii) Enzyme Concentration

The effect of enzyme concentration on glycine-dependent ATP-^{32}PP exchange was measured with a relatively impure preparation, "pH 5 enzymes" fraction, and a more purified fraction, 50-60% $(NH_4)_2SO_4$ fraction. It may be seen in figure 2B. that at low concentrations of the cruder enzyme fraction, the exchange activity was directly proportional to the enzyme concentration. At higher enzyme concentrations, the activity was not proportional to the enzyme concentration and in other experiments even decreased to zero. In the presence of 50 mM KF, the activity was proportional to enzyme concentration over a wider range of enzyme concentration (fig. 2A.). In this case alone, at higher enzyme concentrations, the activity was not proportional to enzyme concentration. In the presence of KF the activities were much higher. With a purer enzyme fraction, 50-60% $(NH_4)_2SO_4$ fraction, the activity was proportional to

FIGURE 1.

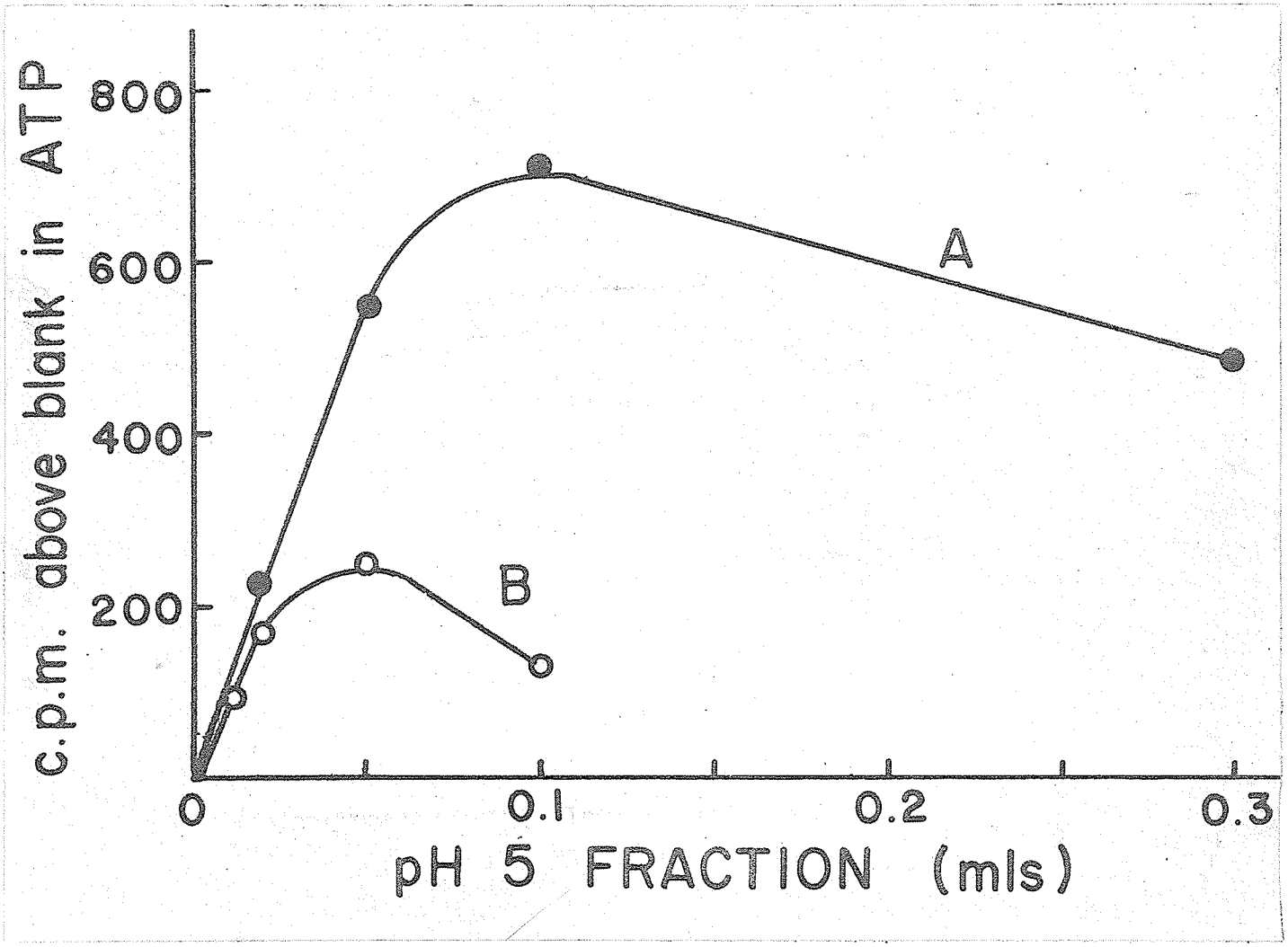
Time Course of Glycine-Dependent ATP-^{32}PP Exchange



Note: Assays were carried out according to the procedure described in Materials and Methods except that each point was obtained by incubating the reaction mixture with 0.3 mls 50-60% $(NH_4)_2SO_4$ fraction for various times. Blanks with no added glycine were run for each time tested.

FIGURE 2A. and 2B.

Effect of Enzyme Concentration on Glycine-Dependent ATP-^{32}PP Exchange: pH 5 Fraction



Note: Curve B. Assays were carried out according to the procedure described in Materials and Methods using the "pH 5 enzyme" fraction. The concentration of protein in the pH 5 fraction was 5.8 mg/ml. For each amount of enzyme tested a blank with no added glycine was determined. Curve A. Each point was obtained as in B. except that the 1.0 ml of reaction mixture contained 50 mM KF in addition.

enzyme concentration over a wide range (fig. 3). It did not decrease at high enzyme concentrations.

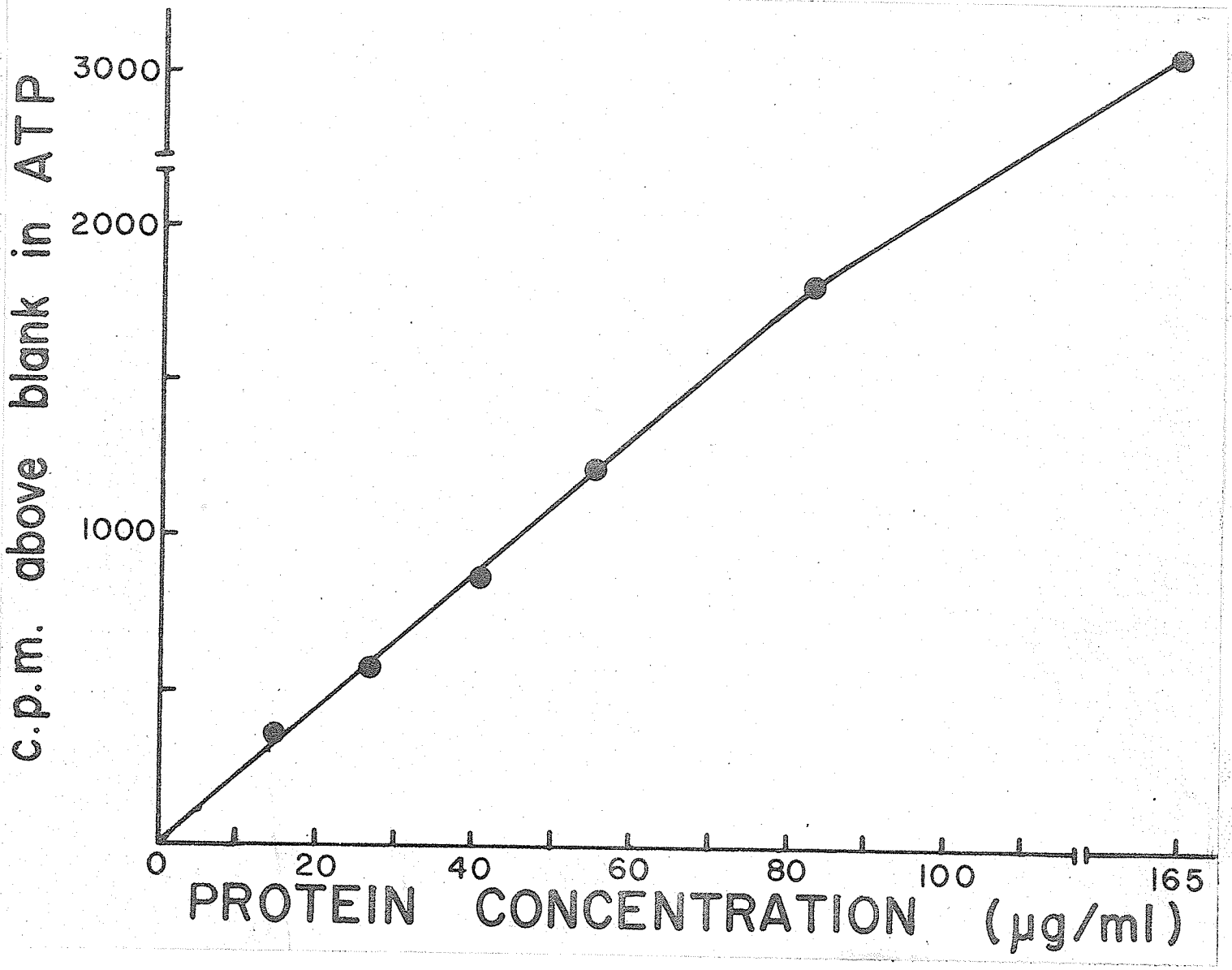
An experiment was performed to determine the stability of ATP in the reaction mixture containing the "pH 5 enzymes" fraction. The incubations were carried out under the same conditions as those used for glycine-dependent ATP-^{32}PP exchange except that glycine was not present. After termination of the reaction at different times, samples of the reaction mixtures were analyzed by paper strip electrophoresis for nucleotide composition. It may be seen from results presented in Table 1. that no appreciable loss of ATP from the incubation mixture occurs in one hour. Also, no appreciable amounts of AMP or ADP were detected during the hour incubation.

(iii) Substrate Concentration

The rate of ^{32}PP incorporation into ATP during the 15 minute incubation period was proportional to the concentration of glycine over the range 0 to 1.0 mM and reached a maximum at 1.2 mM (fig. 4A.). From the Lineweaver-Burk plot (see Appendix), the Km for glycine was calculated to be 0.6 mM (fig. 4B.). The rate of exchange activity was proportional to the addition of ATP over the range 0 to 0.1 mM and reached a maximum at 0.15 mM (fig. 4A.). From the Lineweaver-Burk plot, the Km for ATP was calculated to be 0.076 mM (fig. 4B.). The rate of exchange was proportional to addition of ^{32}PP over the range 0 to 0.4 mM and reached a maximum at 0.65 mM (fig. 4A.). From the Lineweaver-Burk plot the Km for ^{32}PP was calculated to be 0.21 mM (fig. 4B.).

FIGURE 3.

Effect of Enzyme Concentration on Glycine-Dependent ATP-^{32}PP Exchange: 50-60% $(NH_4)_2SO_4$ Fraction



Note: Assays were carried out according to the procedure described in Materials and Methods except that each point had a different amount of 50-60% $(NH_4)_2SO_4$ fraction added. Protein concentration was determined as outlined in Materials and Methods. Blanks with no added glycine were run for each protein concentration tested. The broken portions of abscissa and ordinate have exactly the same scale as the unbroken portions of abscissa and ordinate.

TABLE I

Stability of ATP in the Reaction Mixture During Measurement of Glycine-Dependent ATP-^{32}PP Exchange

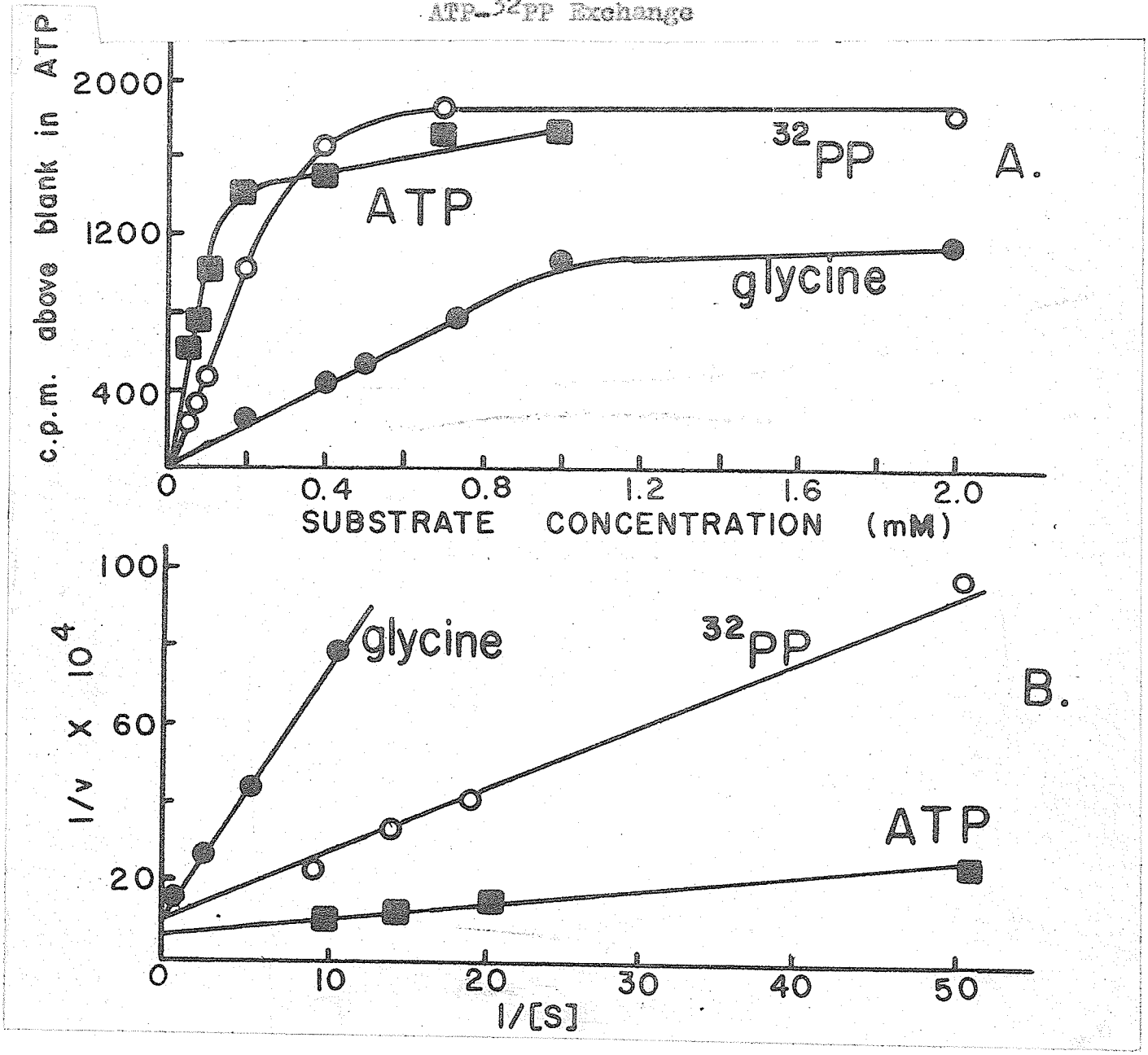
Time of Incubation (minutes)	Optical Density (Bands eluted from paper electrophoresis strip in 4 ml of 1N HCl)	
	ATP	ADP
0	0.430	0.015
5	0.410	0.00
15	0.425	0.00
30	0.430	0.010
60	0.435	0.00

Note: Assays were carried out according to the procedure described in Materials and Methods except the final concentration of ATP was 10 mM and glycine was not present. 0.3 mls of "pH 5 enzymes" fraction was used.

When 0.1 μM each of standard ADP and ATP were subjected to paper strip electrophoresis and eluted from the papers under identical conditions, optical density of 0.410 and 0.430 respectively were obtained.

FIGURE 4A. and 4B.

Effect of Substrate Concentration on Glycine-Dependent ATP-^{32}PP Exchange



Note: Assays were carried out according to the procedure described in Materials and Methods except each point was obtained by incubating the reaction mixture with a different amount of added substrate. The 50-60% $(NH_4)_2SO_4$ fraction was used in these assays. In B. the results are presented in the form of a Lineweaver-Burk plot, where v is c.p.m. above blank and [S] is the substrate concentration (mM).

(iv) Magnesium Ion Requirement

The effect of Mg^{++} concentration on glycine-dependent ATP-^{32}PP exchange catalyzed by the 50-60% $(NH_4)_2SO_4$ fraction is shown in figure 5. Maximal exchange activity was found at 2.0 mM Mg^{++} (curve B.). Above 4 mM Mg^{++}, the rate of exchange dropped off slowly. In the presence of SRNA the response of the enzyme activity to changes in Mg^{++} concentration was the same, except that the activities are higher, especially at low Mg^{++} concentrations. The concentration of added SRNA was equivalent to the amount found in the 40% $(NH_4)_2SO_4$ supernatant fraction (30). The Mg^{++} optimum is not changed in the presence of added $(NH_4)_2SO_4$ (up to 40% of saturation).

Measurement of Glycyl-RNA Formation

(i) Enzyme Concentration

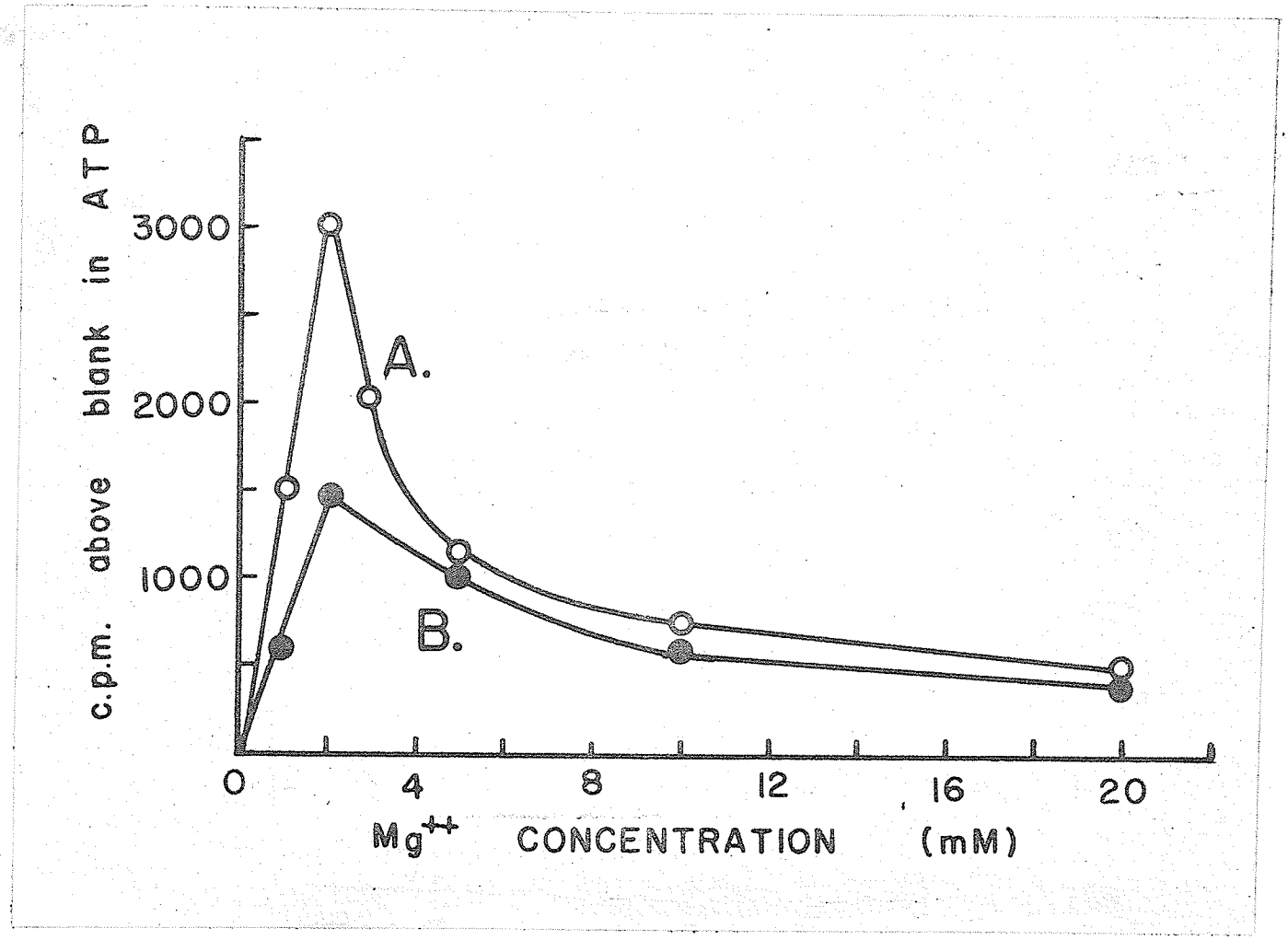
In figure 6, the rate of glycyl-RNA formation (measured by glycine incorporation during a 10 minute incubation period) was proportional to the concentration of added 50-60% $(NH_4)_2SO_4$ fraction over the range 0 to 20 µg/ml protein. At higher enzyme concentrations the SRNA became saturated during this time. Controls were always run to determine the levels of saturation of SRNA by using excess enzyme.

(ii) Glycine Concentration

In the presence of a low concentration of enzyme (16 µg/ml), the rate of glycyl-RNA formation was proportional to the glycine concentration over the range 0 to 8 µM (fig. 7).

FIGURE 5.

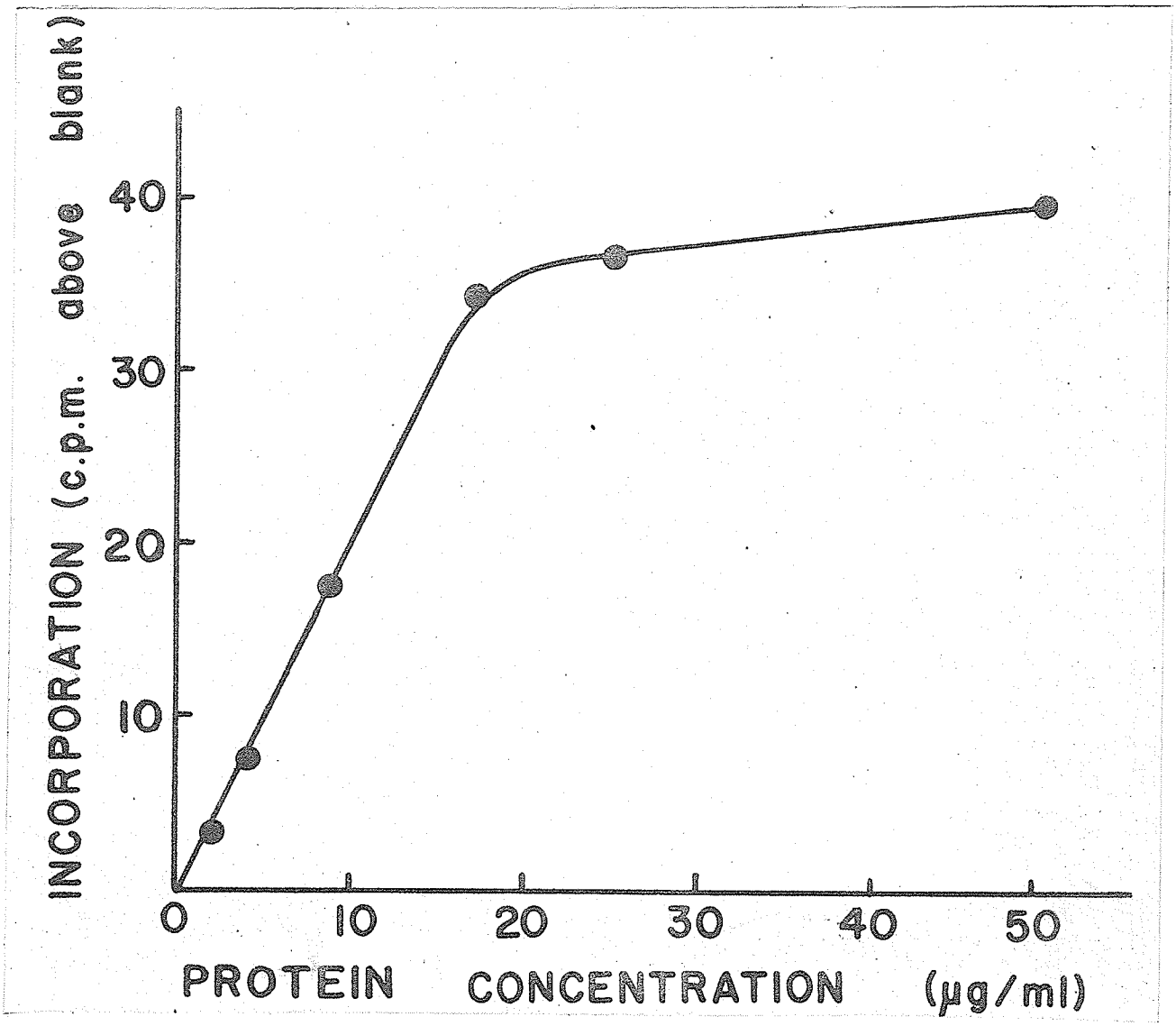
Effect of Mg^{++} Concentration on Glycine-Dependent ATP-^{32}PP Exchange in the Absence and Presence of Added SRNA



Note: Curve B. Effect of Mg^{++} concentration. Assays were carried out according to the procedure described in Materials and Methods except each point was obtained by incubating the reaction mixture with different amounts of added Mg^{++}. Blanks with no added glycine were run for each Mg^{++} concentration tested. The 50-60% $(NH_4)_2SO_4$ fraction was used in these assays. Curve A. Effect of Mg^{++} concentration in the presence of SRNA. The SRNA was prepared as described and dialyzed against EDTA and then water (see Materials and Methods). Each point in the figure was obtained as above except the final concentration of SRNA in the incubation mixture was 40 µg/ml.

FIGURE 6.

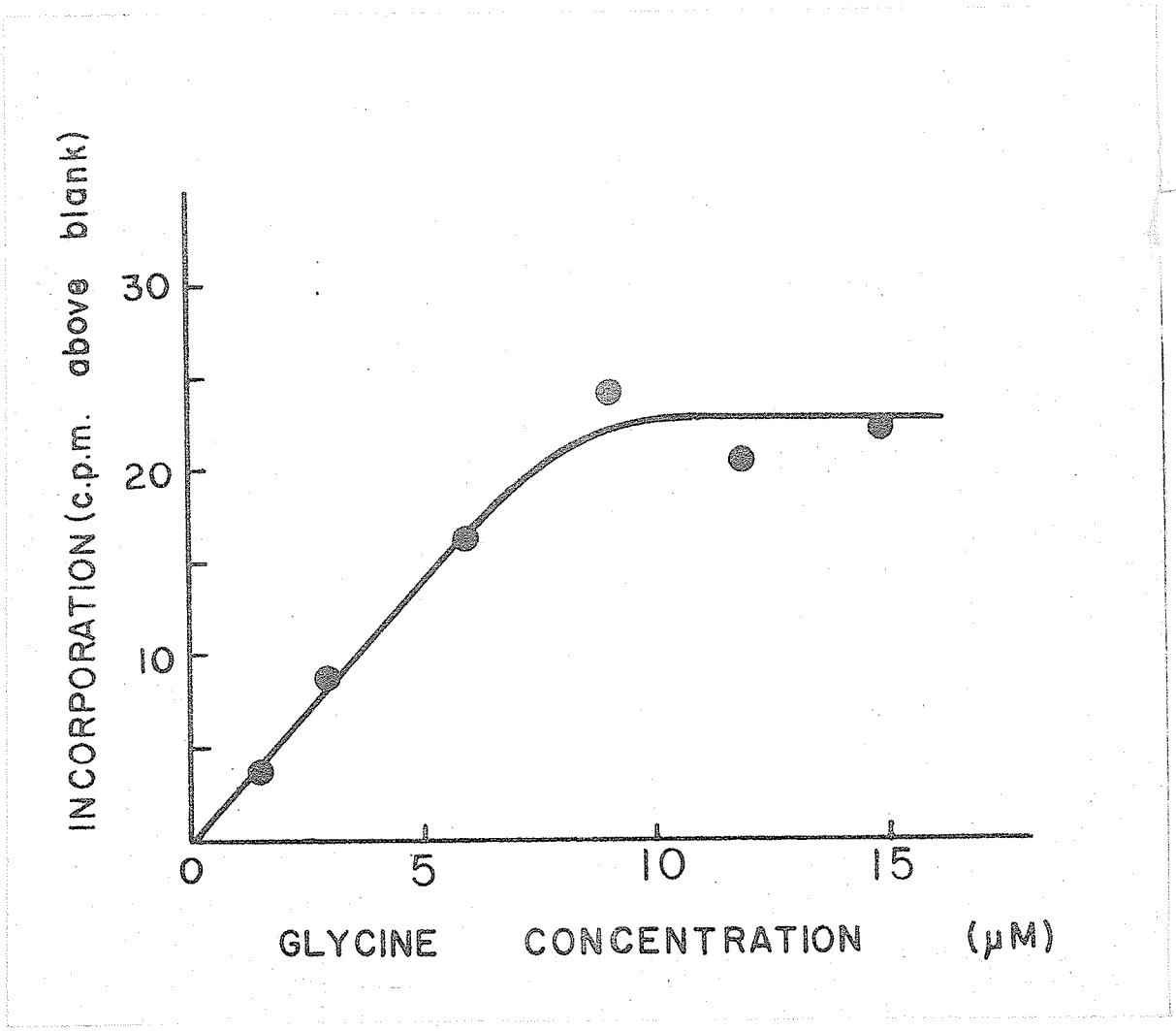
Effect of Enzyme Concentration on the Rate of Glycyl-RNA Formation



Note: Assays were carried out according to the procedure described in Materials and Methods except that the amount of 50-60% $(NH_4)_2SO_4$ fraction added was varied. The reaction mixture contained 11.88 µM l-^{14}C-glycine (14,000 c.p.m.). For each point, a blank with no added enzyme was determined. A control run with excess enzyme (290 µg/ml pH 5 fraction) gave 43 c.p.m. above blank. Background in this experiment was 1.5 c.p.m. Counting was done on a Nuclear Chicago micromil window gas-flow counter with a coincidence circuit for low background counting.

FIGURE 7.

Effect of Glycine Concentration on the Rate of Glycyl-RNA Formation



Note: Assays were carried out according to the procedure described in Materials and Methods. The amount of glycine added was varied. 0.1 ml 50-60% $(NH_4)_2SO_4$ fraction (16 μg protein) catalyzed the reaction. Blanks and control were determined as in figure 6. A control run with excess enzyme gave 130 c.p.m. above blank. Background was 1.8 c.p.m.

Above 10 μM glycine concentration, the enzyme in the reaction mixture appeared to be saturated. From a Lineweaver-Burk plot of these data the apparent Km for glycine was found to be approximately 4 μM.

(iii) Magnesium Ion Requirement

The results presented in figure 8 show the effects of Mg^{++} concentration on labelling of SRNA from heated rat liver pH 5 fraction with l-^{14}C-glycine. Maximal rate of glycyl-RNA formation occurred at 10 mM Mg^{++} concentration. There was very little activity below 5 mM Mg^{++} concentration or above 20 mM Mg^{++} concentration.

Effect of pH on Glycine Activation

(i) Effect of pH on Glycine-Dependent ATP-^{32}PP Exchange

The effect of pH on glycine-dependent ATP-^{32}PP exchange is demonstrated in figure 9A. Over the pH range 6.0 to 7.0, the rate of exchange activity steadily increased. From pH 7.0 to pH 8.0, maximal exchange activity was observed. Half-maximal activity occurred at pH 6.7.

(ii) Effect of pH on Glycyl-RNA Formation

The effect of pH on labelling of SRNA in heated rat liver pH 5 fraction with l-^{14}C-glycine is demonstrated in figure 9B. Over the pH range 5.0 to 7.0 the rate of incorporation steadily increased. From pH 7.0 to pH 8.5 maximal activity was observed. Half-maximal incorporation activity occurred at pH 6.2.

Photo-oxidation of Glycyl-RNA Synthetase

(i) Time Course of Photo-oxidation

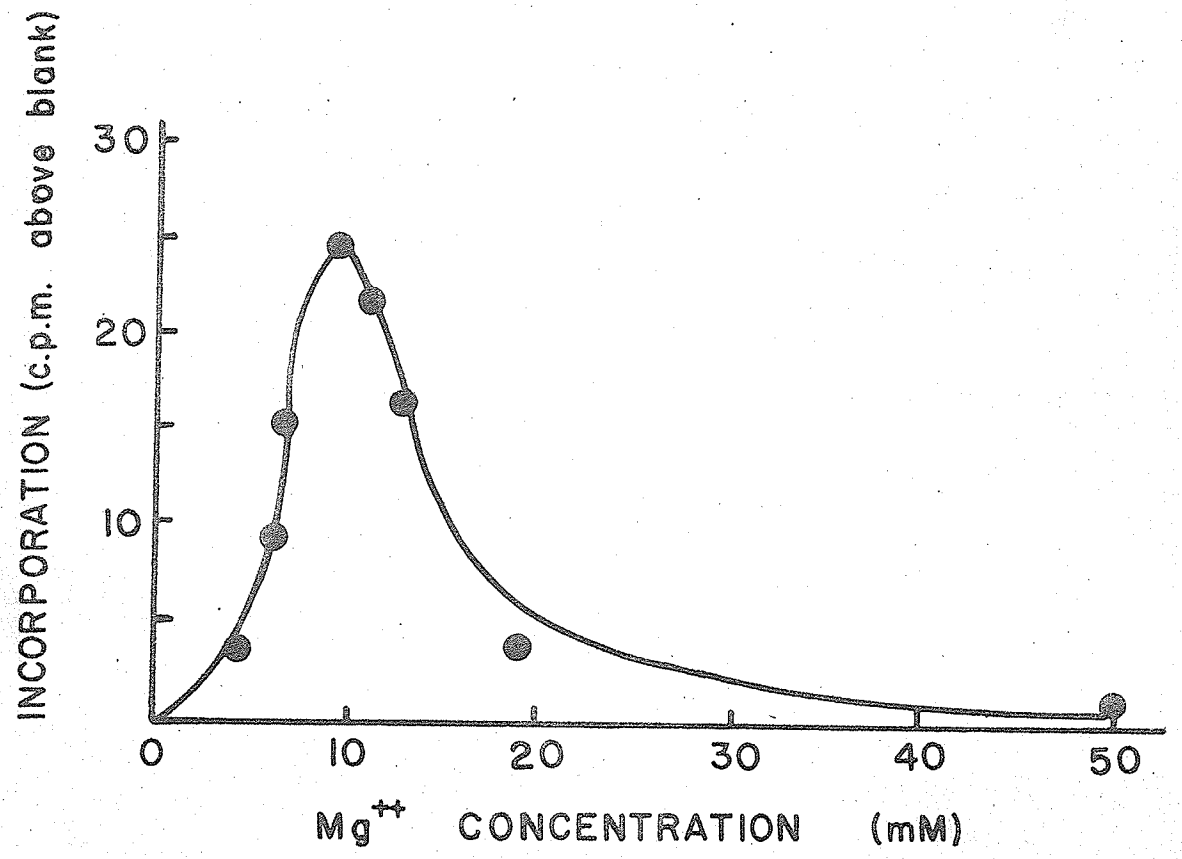
Photo-oxidation of glycyl-RNA synthetase was carried out by incubating the enzyme in the presence of methylene blue under a bright light. At the end of the photo-oxidation period, the methylene blue was removed by adsorption on charcoal. Assays were carried out on the filtrate of the filtered enzyme solutions. Two controls were run for this assay. In the first control, the enzyme solution containing methylene blue was not exposed to light. In the second control, the enzyme solution alone, was treated with charcoal. It was found that the glycyl-RNA synthetase of the 50-60% $(NH_4)_2SO_4$ fraction was inactivated by the treatment of charcoal alone. However, the 40% $(NH_4)_2SO_4$ supernatant fraction (30) was not inactivated by either the treatment with methylene blue in the dark or by the treatment of charcoal. Therefore, this fraction was used to study the photo-inactivation of glycyl-RNA synthetase. The time course for photo-oxidation of glycyl-RNA synthetase of the 40% $(NH_4)_2SO_4$ fraction is shown in figure 10. The glycine-dependent ATP-^{32}PP exchange activity of this fraction was lost on photo-oxidation in an exponential manner with time. The percent loss of activity after 30 minutes was 85%.

(ii) Effects of Substrates on the Photo-oxidation Process

The results in Table II show that photo-oxidation of the enzyme in the 40% $(NH_4)_2SO_4$ fraction produced an approximate 70% decrease of glycine-dependent ATP-^{32}PP exchange activity, while in the presence of 3.3 mM glycine, the corresponding loss of activity

FIGURE 8.

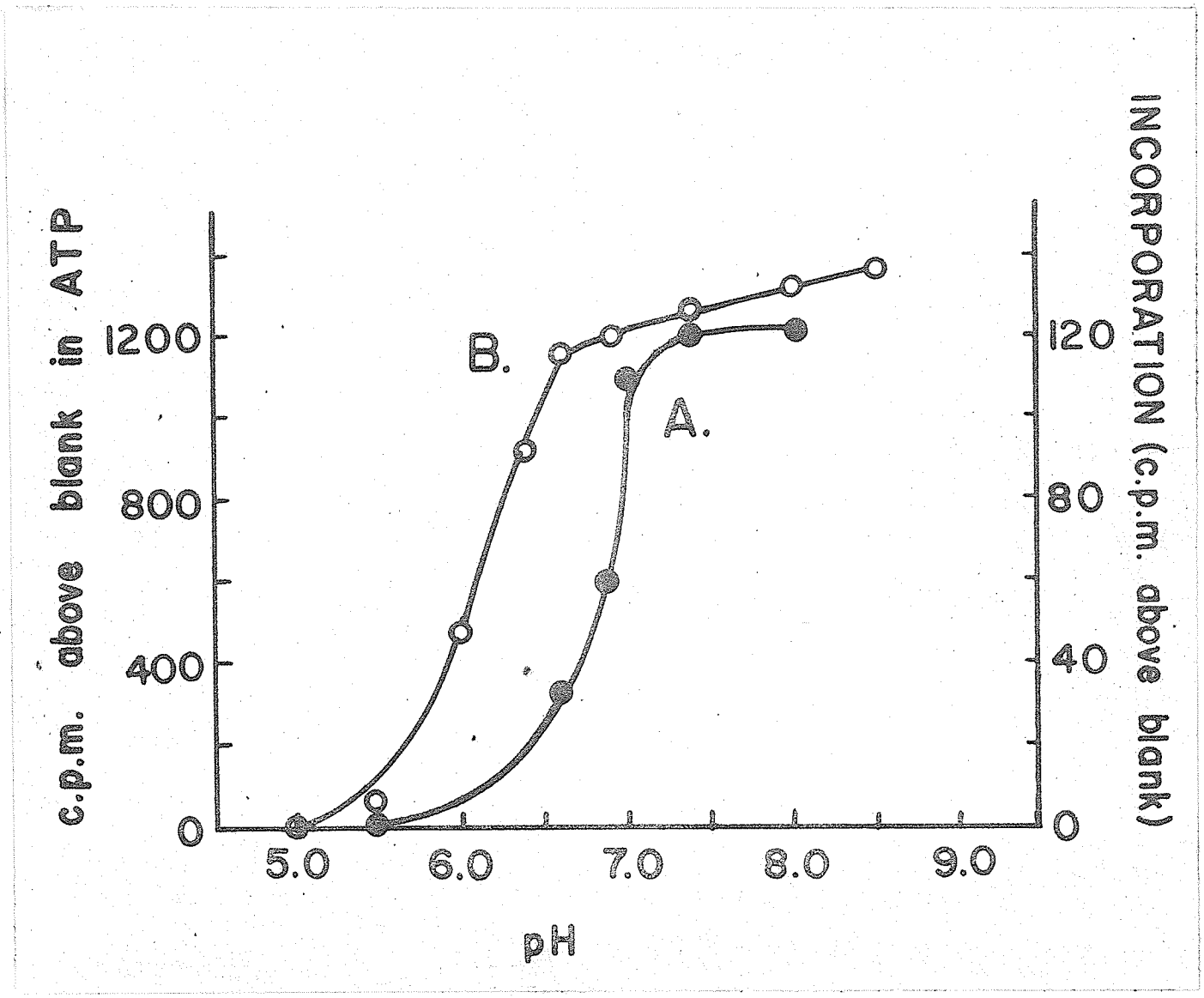
Effect of Mg^{++} Concentration on the Rate of Glycyl-RNA Formation



Note: Assays were carried out according to the procedure described in Materials and Methods except that the amount of Mg^{++} added was varied. The reaction mixture contained 25.8 µM 1-^{14}C-glycine (28,000 c.p.m.). 0.1 ml 50-60% $(NH_4)_2SO_4$ fraction (10 µg protein) catalysed the reaction. Blanks and control were determined as in figure 6. A control run with excess enzyme gave 61 c.p.m. above blank. Background was 1.8 c.p.m.

FIGURE 9.

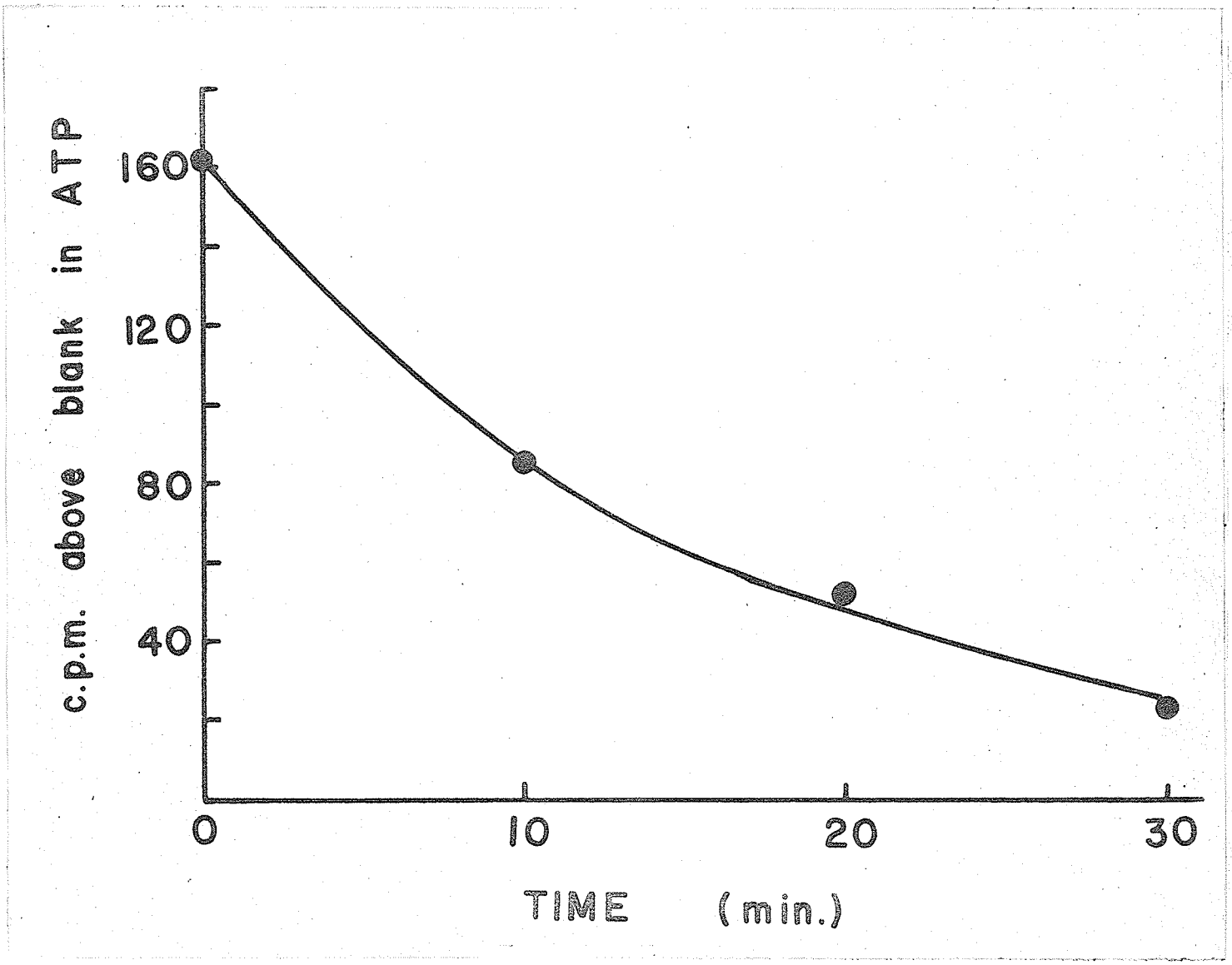
Effect of pH on Glycine Activation



Note: Assays were carried out according to the procedure described in Materials and Methods except that the pH was varied with Tris maleate buffer (88). Curve A. Effect of pH on glycine-dependent ATP-^{32}PP exchange. Blanks with no added glycine were run for each pH. The 50-60% $(NH_4)_2SO_4$ fraction (0.3 mls) catalyzed the reaction. Curve B. Effect of pH on glycyl-RNA formation. The reaction mixture contained 148.5 μM l-^{14}C-glycine (173,000 c.p.m.) and the reaction catalyzed by 0.1 ml 50-60% $(NH_4)_2SO_4$ fraction. For each pH a blank with no added enzyme was determined. A control run with excess enzyme gave 550 c.p.m.

FIGURE 10.

Time Course of Photoinactivation of Glycyl-RNA Synthetase



Note: The "photo-oxidation" was allowed to proceed for the times indicated. 33 mM Mercaptoethanol was added to the enzyme fraction prior to photo-oxidation. The photoinactivation and assay (0.3 mls of enzyme solution) of glycyl-RNA synthetase in the 40% $(NH_4)_2SO_4$ fraction were performed as outlined in Materials and Methods.

TABLE II

Photoinactivation of Glycyl-RNA Synthetase in the Presence of Substrates

Substrates Preincubated with Enzyme	Incorporation of ^{32}PP into ATP	
	expt. I (c.p.m. above blank)	expt. II (c.p.m. above blank)
none (control, in dark)	161	147
none (test, in light)	52	43
glycine (3.3 mM)	134	113
ATP (3.3 mM)	52	42
glycine plus ATP (3.3 mM each as above)	51	40
SRNA (67 µg/ml)	53	43
alanine (3.3 mM)	50	46

Note: The enzyme solutions were incubated with glycine or ATP or SRNA at the concentrations indicated in the table with 33 mM mercaptoethanol for 20 minutes in an ice bath before the addition of methylene blue. After the addition of the dye, a period of 20 minutes was allowed for photo-oxidation. During this period, the temperature of the enzyme solutions rose from 3°C to 25°C. At the end of this time, the charcoal was added to adsorb the methylene blue and the samples filtered. The filtrates were assayed for enzyme activity according to the procedure described in Materials and Methods.

was only 30%. Neither ATP, glycine plus ATP, SRNA or alanine provided this protection against the loss of activity by photo-oxidation.

The Effect of Sulfhydryl Agents on Glycine Activation

(i) Effect of PCMB on Glycine-Dependent ATP-^{32}PP Exchange

The results presented in figure 11 show the effects of different concentrations of PCMB on glycine-dependent ATP-^{32}PP exchange. No inhibition was observed at 1×10^{-6} M but concentrations of 1×10^{-5} M or above gave 100% inhibition. 50% Inhibition occurred at approximately 5×10^{-6} M.

(ii) Effect of Substrates on PCMB Inhibition

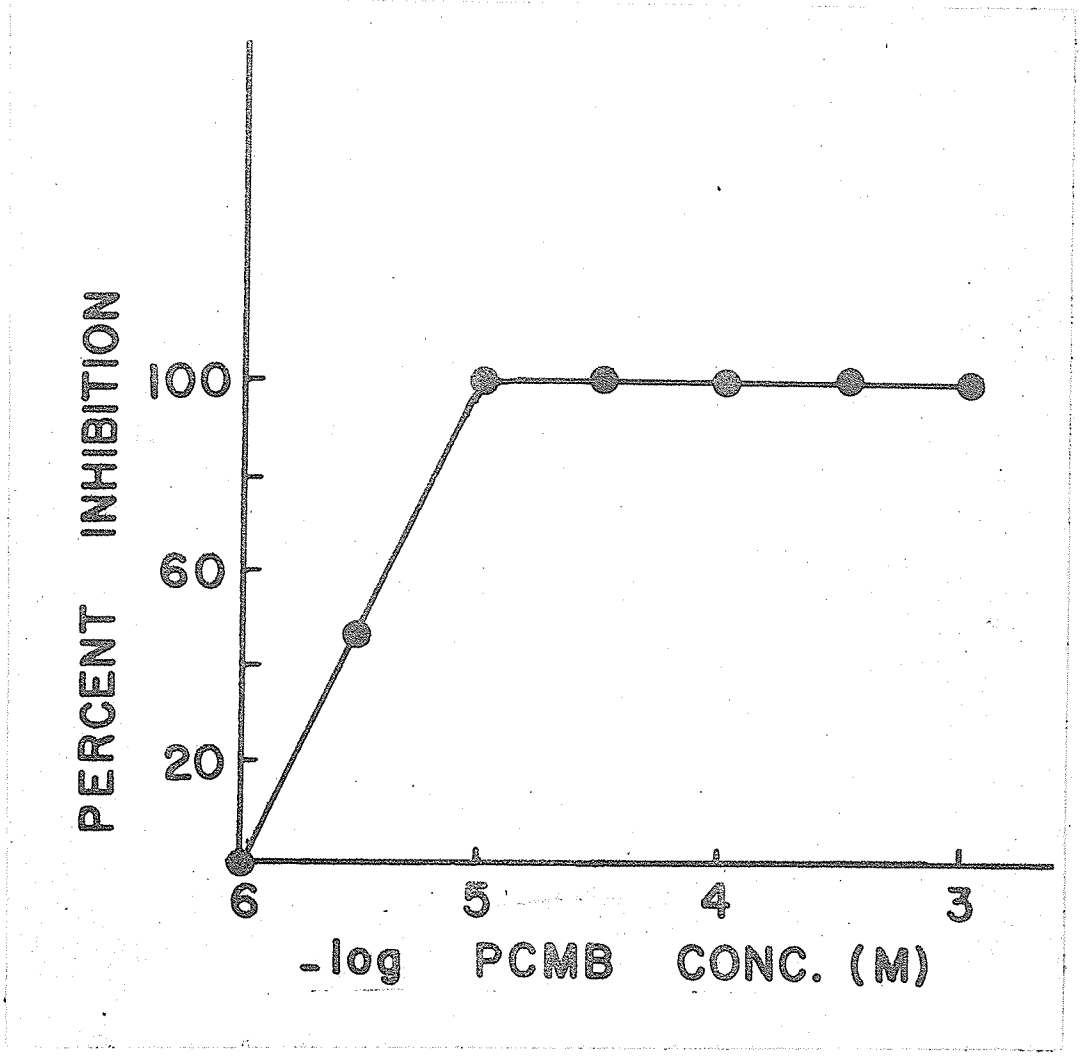
The results presented in Table III show that when 8.57×10^{-6} M PCMB had been added to the incubation mixture about 90% inhibition of glycine-dependent ATP-^{32}PP exchange occurs. This value agrees with the value reported in figure 11. When the enzyme was preincubated with SRNA, only approximately 63% inhibition occurred. This protection was not afforded with other substrates, glycine or ATP or by preincubating the enzyme solution with an equivalent amount of rRNA.

Effect of Ionic Strength on Glycine-Dependent ATP-^{32}PP Exchange

The results presented in figure 12 show the effect of added KCl concentration on glycine-dependent ATP-^{32}PP exchange. No inhibition occurred until concentration of added KCl was above 60 mM. Above 60 mM KCl added, a rapid inhibition occurred until the concentration of added KCl was 100 mM. As the KCl concentration became greater, the rate of increase of

FIGURE 11.

Effect of PCMB Concentration on Glycine-Dependent ATP-^{32}PP Exchange



Note: The PCMB was added to the incubation mixtures at 3°C just prior to the addition of 50-60% $(NH_4)_2SO_4$ fraction (59 µg/ml protein). The incubations were carried out immediately for 15 minutes at 37°C. For each point, a blank with no added glycine was determined. The reaction was terminated and samples prepared for counting as outlined in Materials and Methods.

Note: No mercaptoethanol was added in this experiment.

TABLE III

Effect of Substrate on PCMB Inhibition of Glycyl-RNA Synthetase

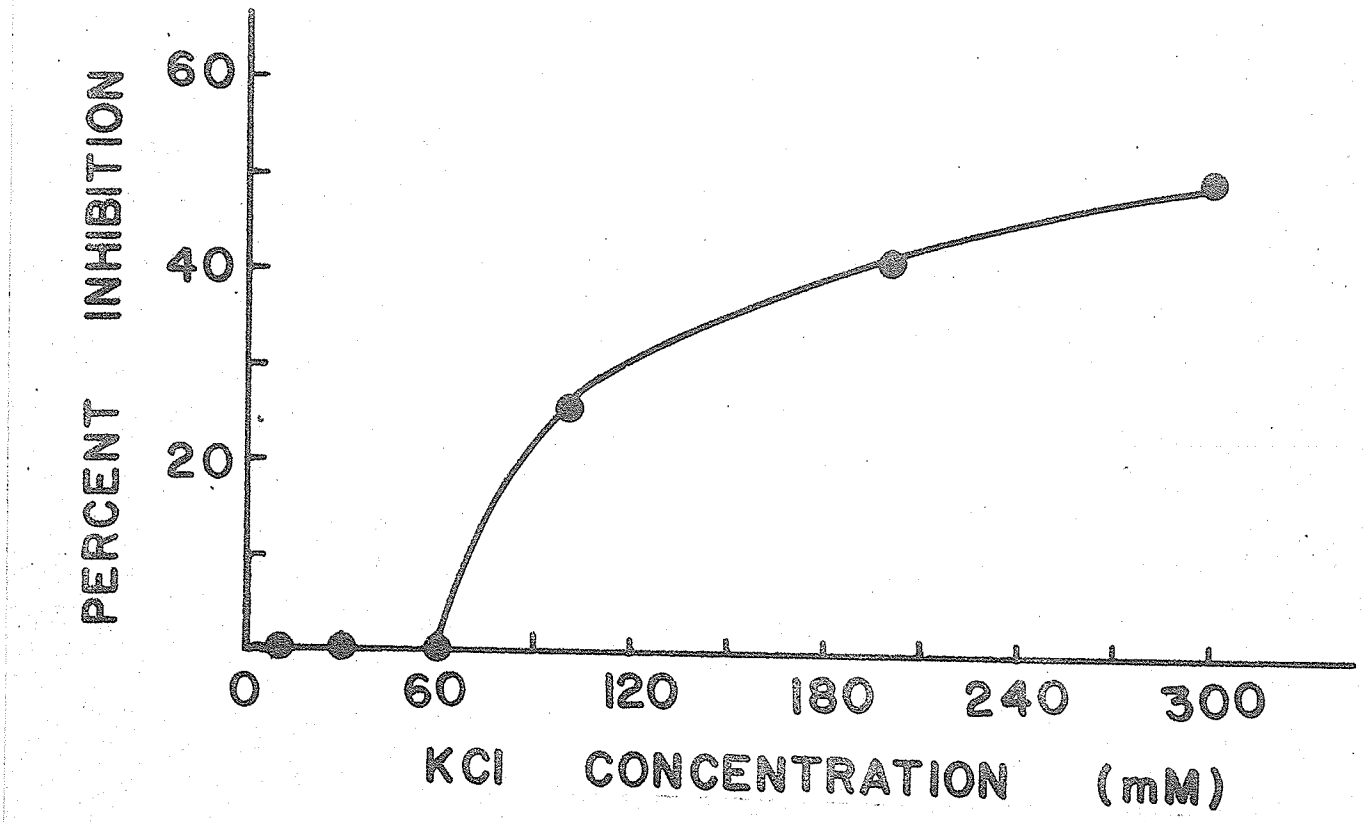
Substrates Preincubated with Enzyme	Incorporation of ^{32}PP into ATP	
	expt. I (c.p.m. above blank)	expt. II (c.p.m. above blank)
none (control, no PCMB added)	134	164
none (test, PCMB added)	14	22
glycine (3.3 mM)	15	19
ATP (3.3 mM)	13	23
SRNA (67 µg/ml)	43	70
ribosomal RNA (67 µg/ml)	14	23

Note: The preincubations of the enzyme with either glycine or ATP or SRNA or rRNA at the concentrations indicated in the table were carried out for 10 minutes at 30°C. The enzyme solution containing the substrates were then added to the incubation mixtures so that the final concentrations of substrates were those usually employed in assay of exchange activity (see Materials and Methods). Assays were carried out according to the procedure in Materials and Methods.

Note: No mercaptoethanol was added in this experiment.

FIGURE 12.

Effect of KCl Concentration on Glycine-Dependent ATP-^{32}PP Exchange



Note: Assays were carried out according to the method described in Materials and Methods except varying amounts of KCl were added.

inhibition was slower. In two other experiments performed, similar curves were obtained except that the inhibition continued to increase above 100 mM KCl to a somewhat greater extent. The percent inhibition at 300 mM KCl was 60% and 65%.

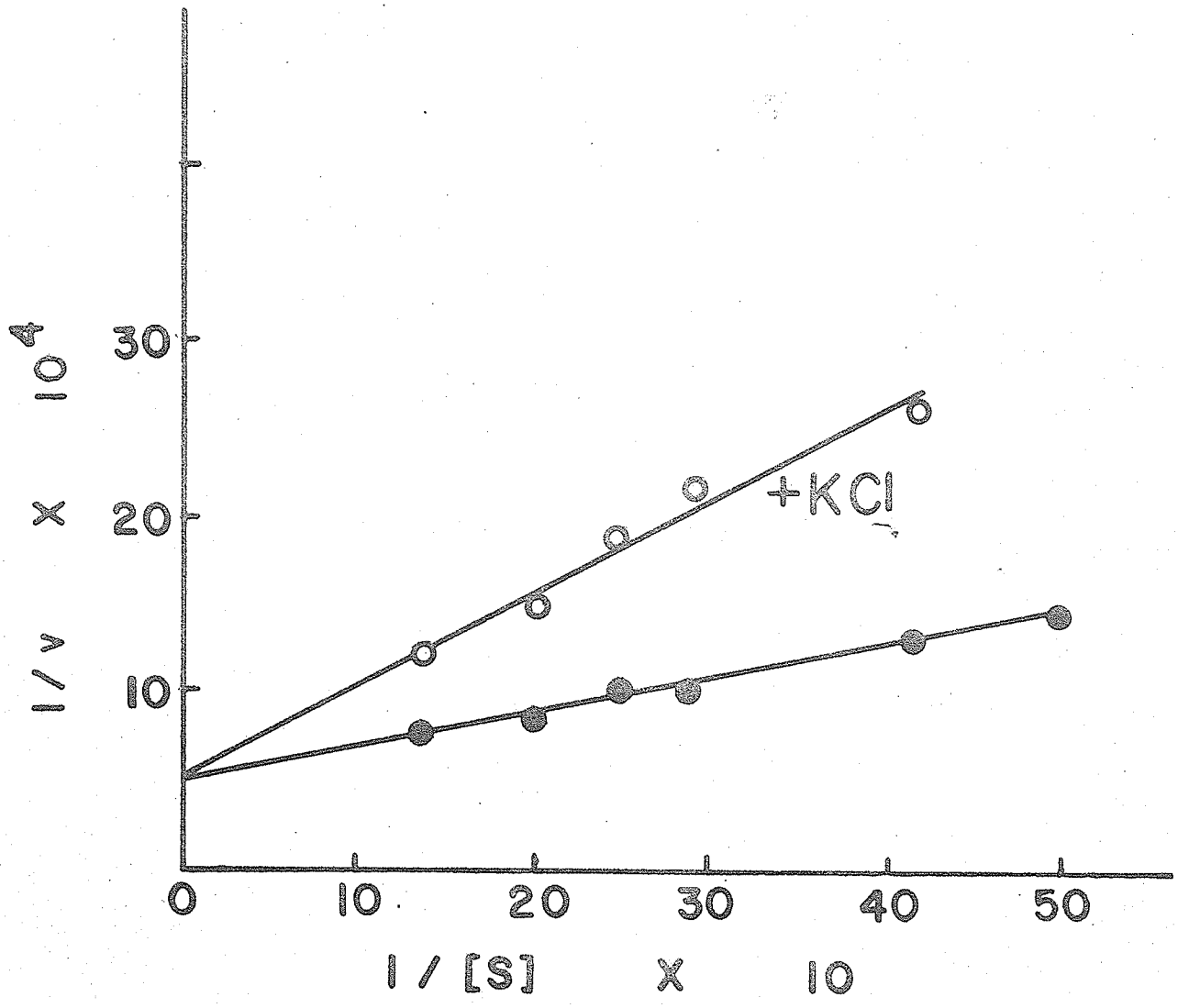
The effect of glycine concentration on glycine-dependent ATP-^{32}PP exchange was measured in the absence and presence of 0.1 M KCl. A Lineweaver-Burk plot of these data, shown in figure 13, indicates that the inhibition of glycine activation shows competitive kinetics with an apparent K_I for KCl of 87 mM.

Effects of Glycine Analogs on Glycine-Dependent ATP-^{32}PP Exchange

Several types of glycine analogs were used in this work. They were amino acids and glycine derivatives, α-carboxyl and α-amino substituted glycine analogs. Preliminary experiments were performed to determine what concentration of each glycine analog inhibited glycine-dependent ATP-^{32}PP exchange. The purity of each analog was examined by one-dimensional chromatography (see Materials and Methods). All analogs used in these experiments were chromatographically pure. Three types of inhibition were found as seen from Lineweaver-Burk plots (see Appendix) of data obtained from experiments in which the exchange rate was measured at different glycine concentrations in the absence and presence of inhibitor. These were (i) competitive inhibition in which the two lines met on the ordinate (ii) non-competitive inhibition in which the two lines met on the negative

FIGURE 13.

Lineweaver-Burk Plot Showing the Variation of Glycine-Dependent ATP-$^{32}PP_i$ Exchange Activity With Glycine Concentration in the Absence and Presence of 0.1 M KCl

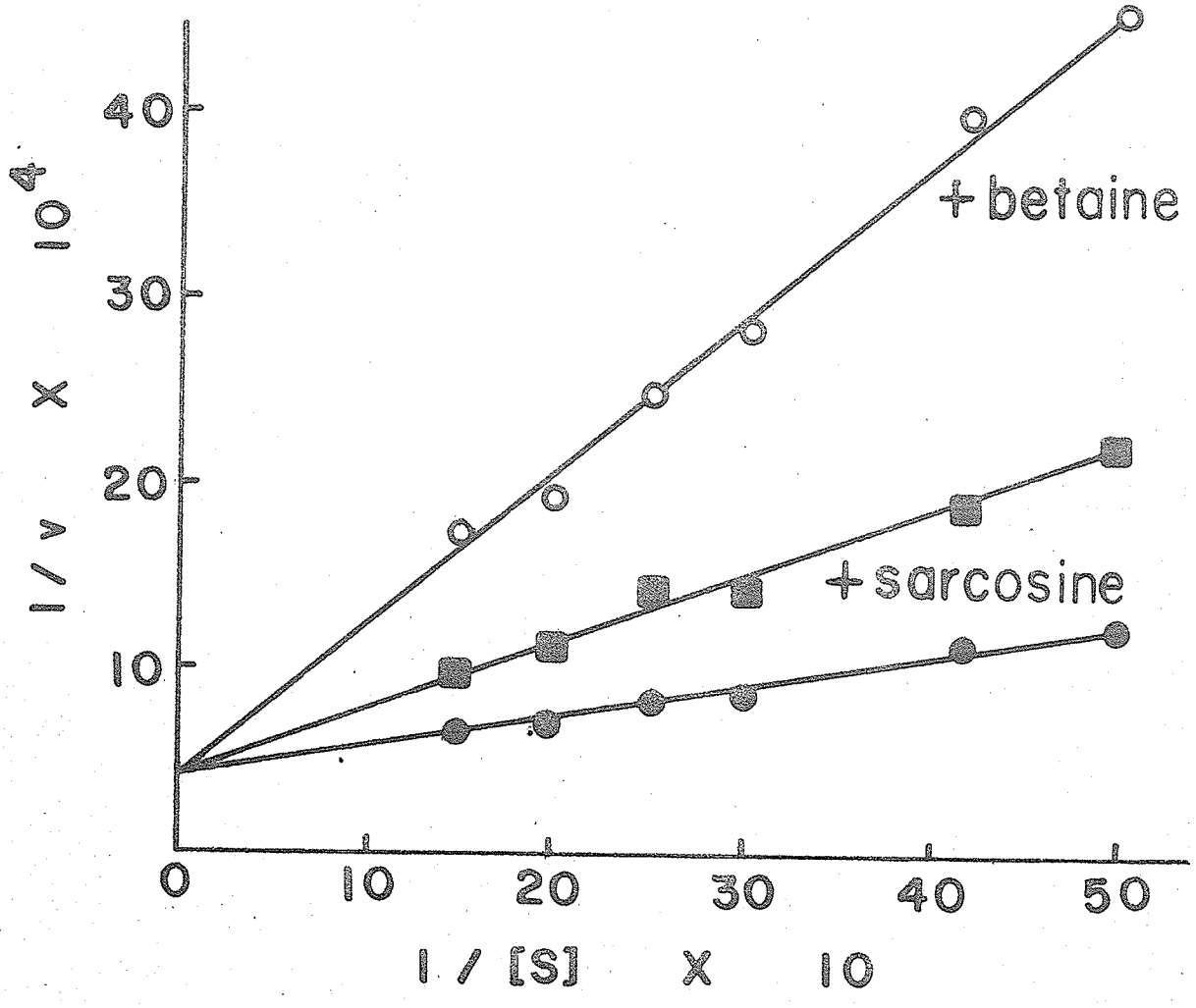


Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentration of KCl in the reaction mixture was 0.1 M.

abscissa (iii) uncompetitive inhibition in which the two lines were parallel. Five analogs of glycine showed competitive inhibition kinetics (see figures 14 and 15). From the data presented in Table IV, it may be seen that the apparent K_Is for methylamine, betaine, sodium acetate, L-prolyl glycine and sarcosine are respectively 6.3 mM, 26 mM, 80 mM, 114 mM and 128 mM. Representative Lineweaver-Burk plots for noncompetitive and uncompetitive analogs of glycine are presented in figures 16 to 20. The α-nitrogens in methylamine, betaine and sarcosine are positively charged under conditions of assay, but in compounds in which the α-nitrogen of glycine are in amide linkages the α-N is uncharged. These compounds were found to be noncompetitive with respect to glycine except for L-prolyl glycine. The reason for this competitive kinetics is not understood, but they were identical in two experiments. In Table V it can be seen that relatively low K_Is were found for hippuric acid and N-substituted glycine dipeptides. Those analogs with hydrophobic side-chains appeared to be better inhibitors (see Table V). Carboxyl substituted glycine analogs were found also to be non-competitive except for glycyl L-phenylalanine. This analog showed uncompetitive kinetics (see figure 20). One other analog of glycine which showed uncompetitive kinetics was L-alanine (see figure 18). At concentrations of 10 mM and 100 mM for glycyl L-phenylalanine and for L-alanine respectively, inhibitions of 42% and 44% were obtained. The carboxyl substituted glycine analogs with hydrophobic side-chains were better inhibitors (see Table V). Serine was found to be a relatively poor noncompetitive

FIGURE 14.

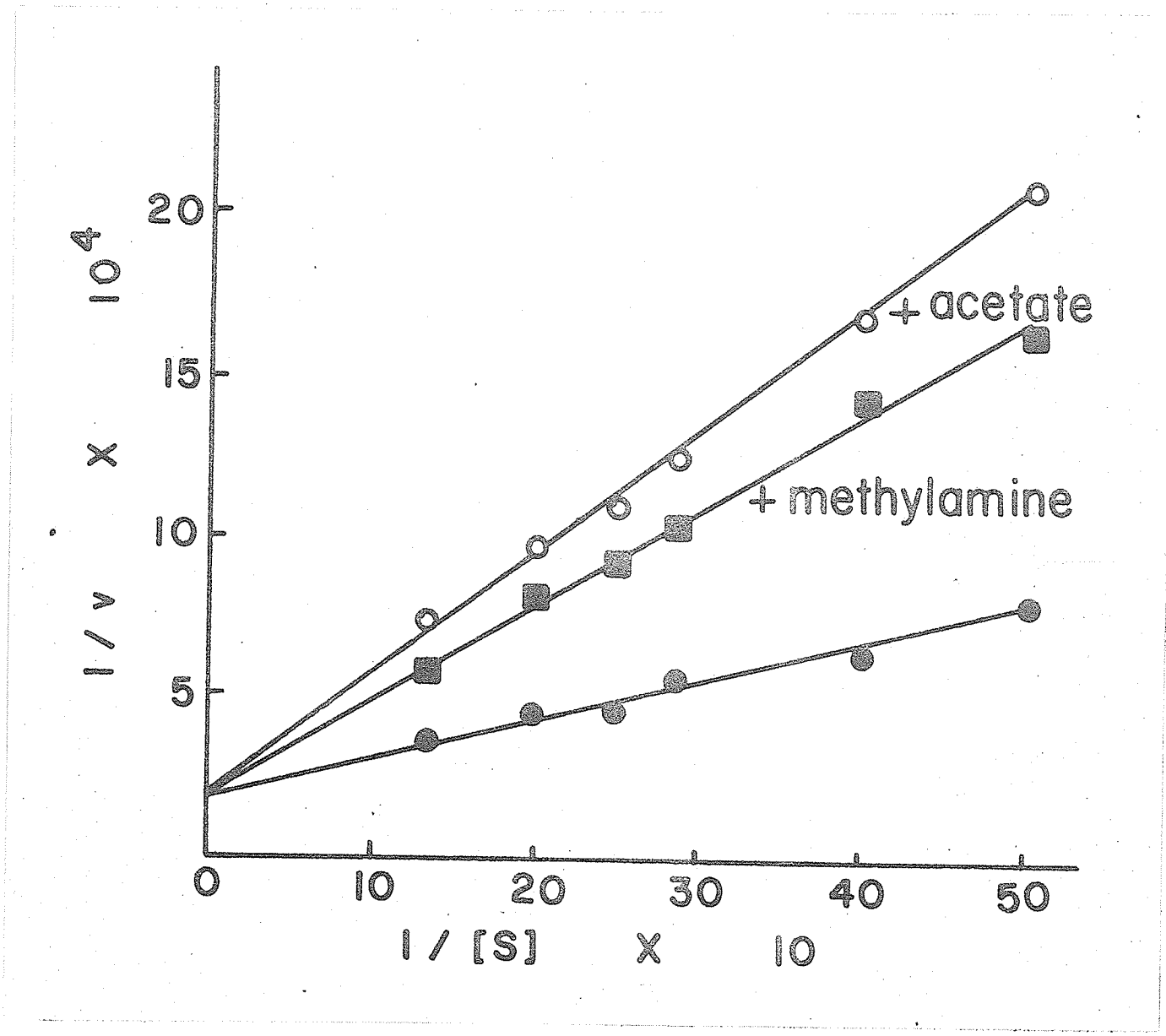
Lineweaver-Burk Plots Showing the Variation of Glycine-Dependent ATP-^{32}PP Exchange Activity With Glycine Concentration in the Absence and Presence of 0.1 M Betaine and 0.1 M Sarcosine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentrations in the incubation mixture of Betaine and Sarcosine were both 0.1 M.

FIGURE 15.

Lineweaver-Burk Plots Showing the Variation of Glycine-Dependent ATP-^{32}PP Exchange Activity With Glycine Concentration in the Absence and Presence of 0.1 M Acetate and 0.01 M Methyl Amine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentration in the incubation mixture of acetate and methyl amine were 0.1 M and 0.01 M respectively.

TABLE IV

Effect of Glycine Analogs on Glycine-Dependent ATP-^{32}PP Exchange

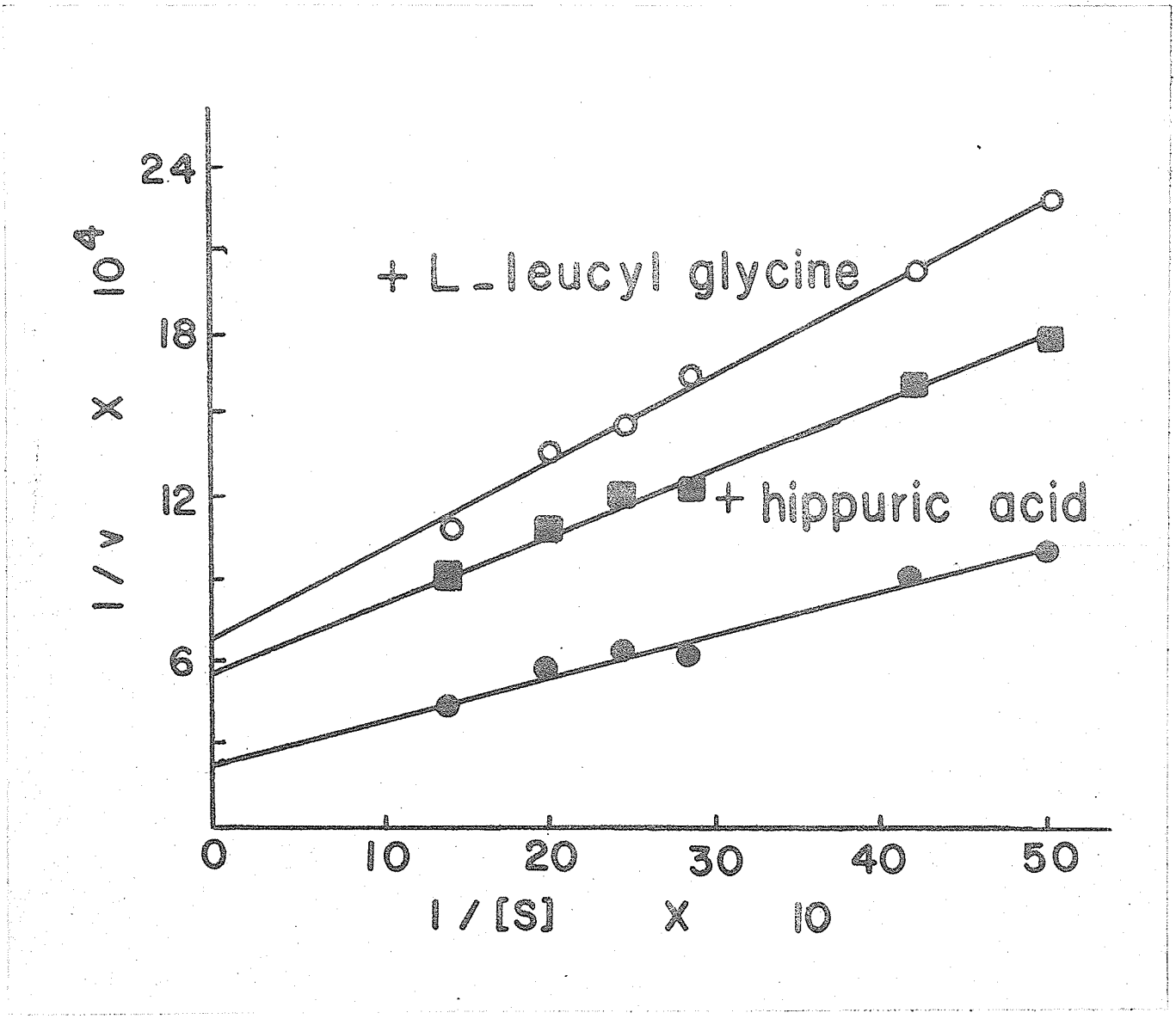
A. Competitive Inhibitors:

Analog	Concentration used (mM)	Km for glycine (mM)		K_I for Analog (mM)	
		expt.I	expt.II	expt.I	expt.II
methyl amine	10	0.59	0.58	6.5	6.1
acetate	100	0.60	0.70	73.5	85.7
sarcosine	100	0.60	0.60	132.0	120.0
betaine	100	0.60	0.60	25.0	27.2
L-prolyl glycine	100	0.62	0.59	107.8	119.0

Note: Assays were carried out according to the procedure described in Materials and Methods. Stock solutions of the inhibitors were carefully adjusted to pH 7.5 with 1N KOH or 1N HCl before addition to the reaction mixtures.

FIGURE 16.

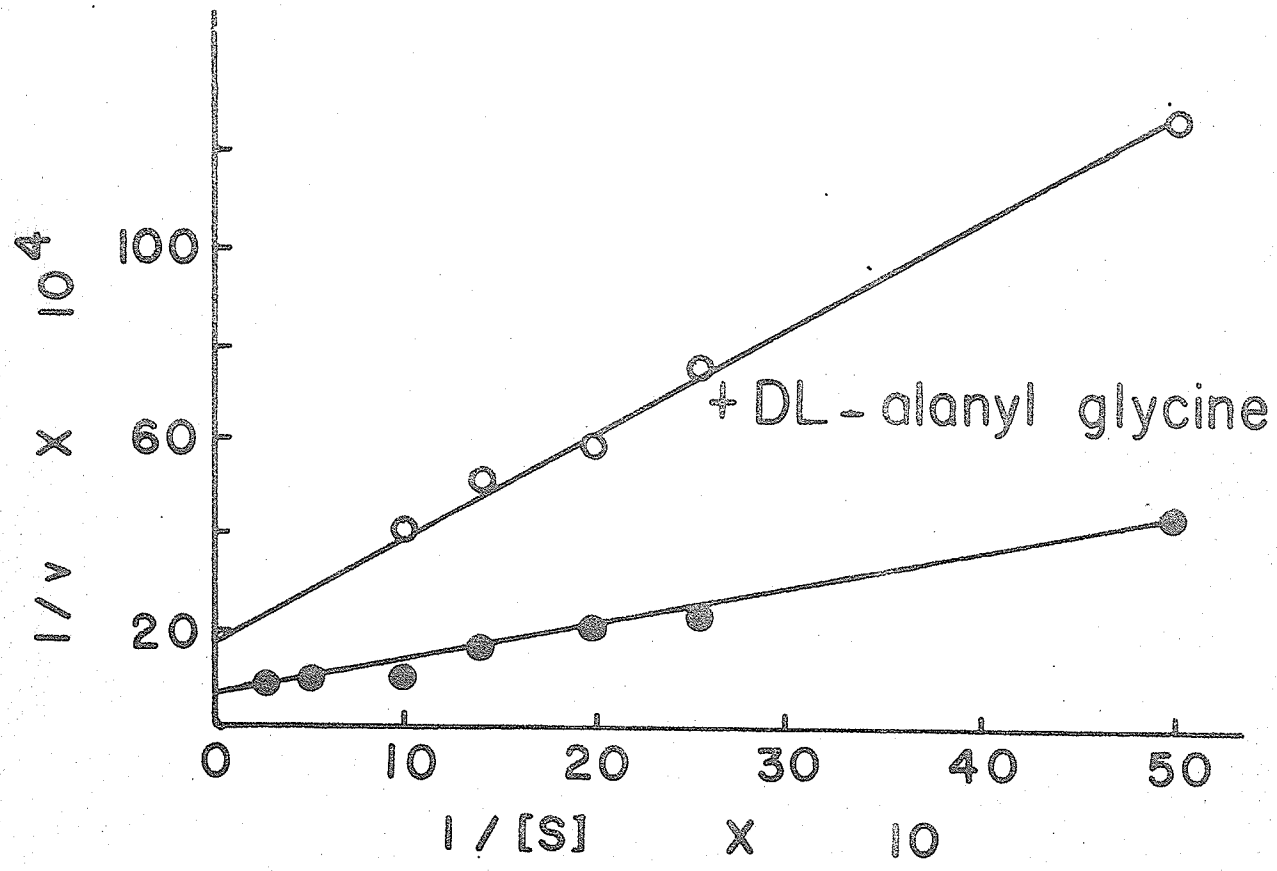
Lineweaver-Burk Plots Showing the Variation of Glycine-Dependent ATP-^{32}PP Exchange Activity With Glycine Concentration in the Absence and Presence of 0.01 M Hippuric Acid and 0.05 M L-Leucyl Glycine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentrations in the incubation mixture of hippuric acid and L-leucyl glycine were 0.01 M and 0.05 M respectively.

FIGURE 17.

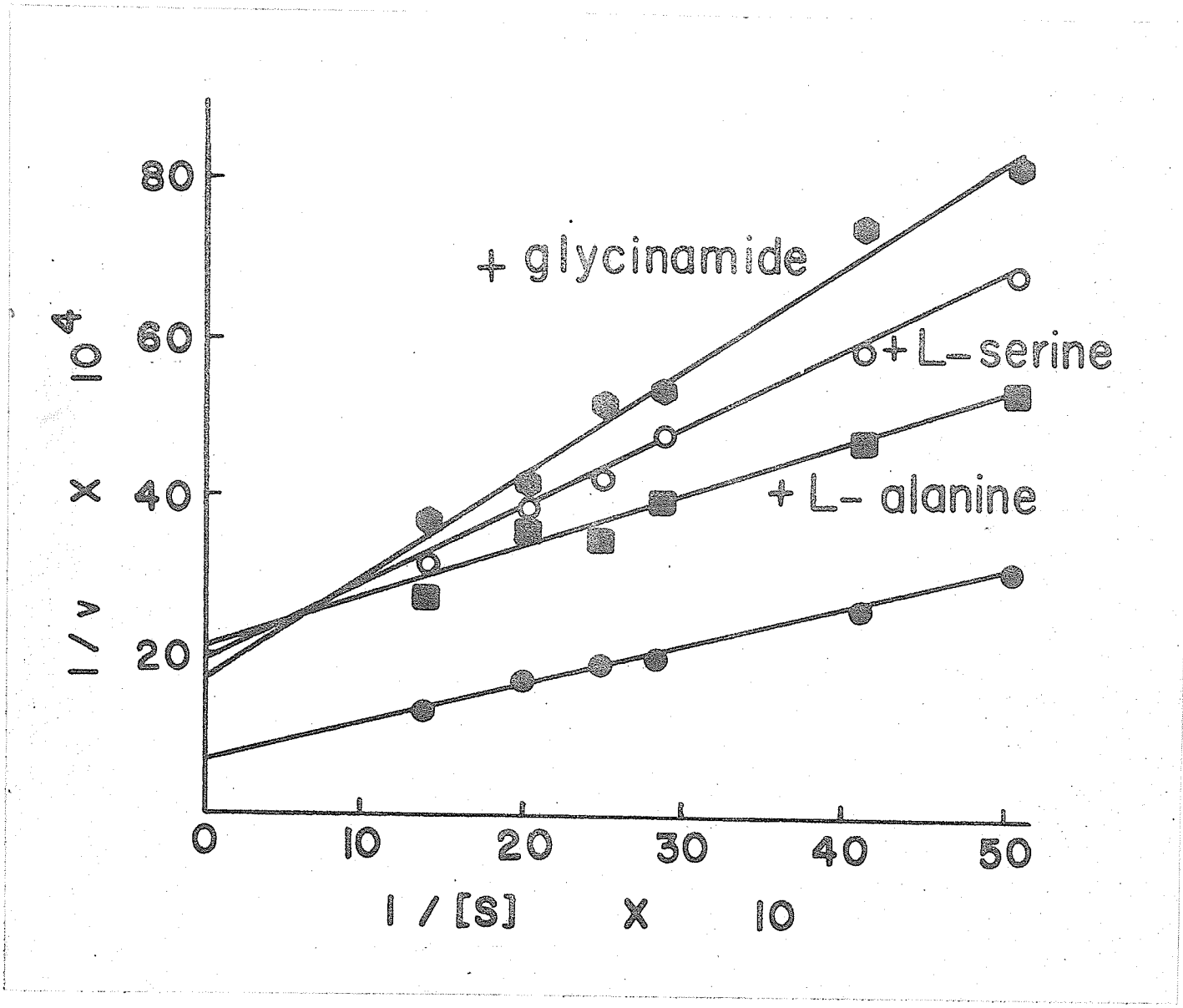
Lineweaver-Burk Plot Showing the Variation of Glycine-Dependent ATP-^{32}PP Exchange Activity With Glycine Concentration in the Absence and Presence of 0.025 M DL-Alanyl Glycine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentration of DL-alanyl glycine in the incubation mixture was 0.025 M.

FIGURE 18.

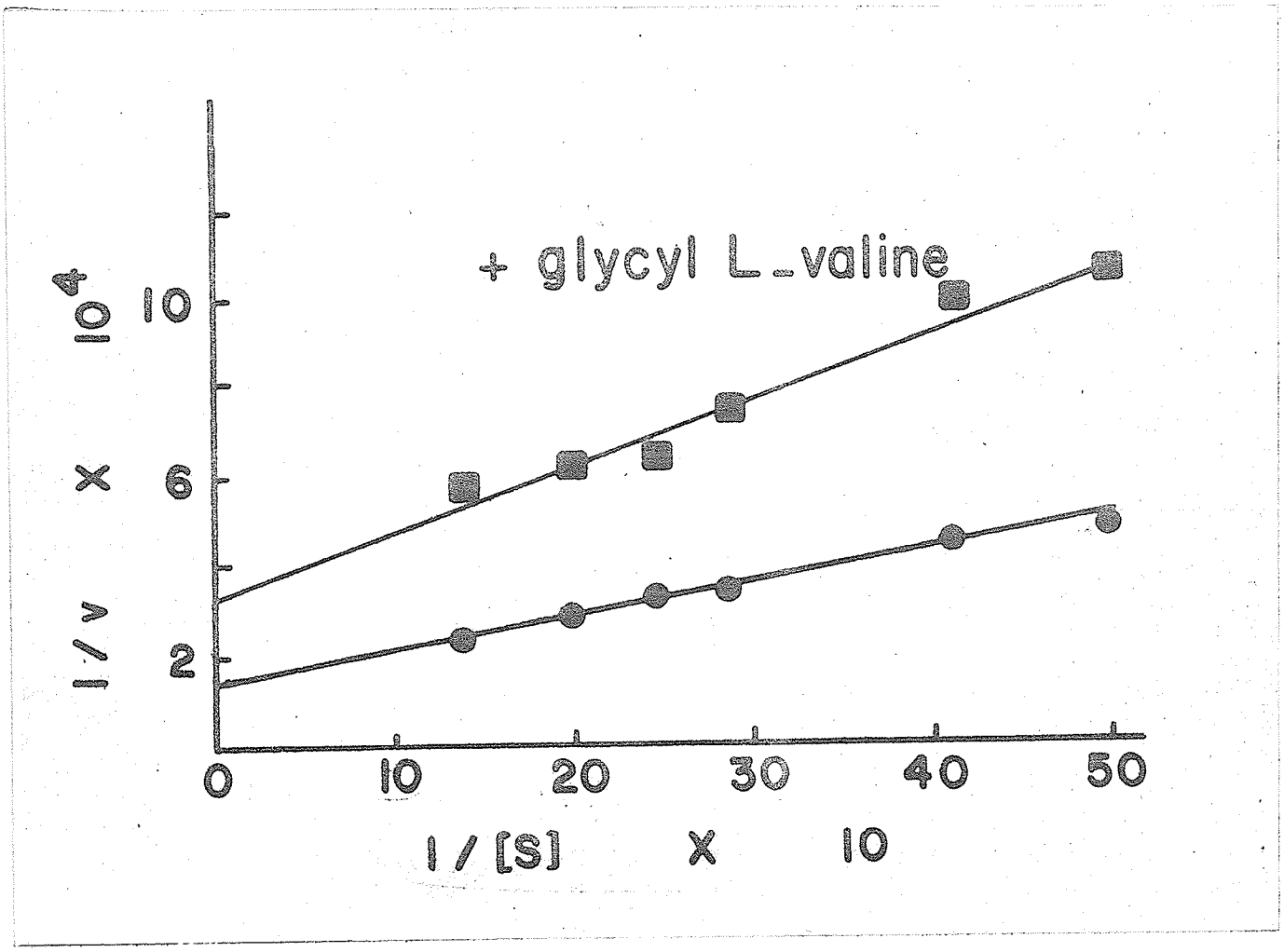
Lineweaver-Burk Plots Showing the Variation of Glycine-Dependent ATP-$^{32}PP_i$ Exchange Activity With Glycine Concentration in the Absence and Presence of 0.0025 M Glycinamide, 0.063 M L-Serine and 0.1 M L-Alanine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentrations of glycinamide, L-serine and L-alanine in the incubation mixture were 0.0025 M, 0.063 M and 0.1 M respectively.

FIGURE 19.

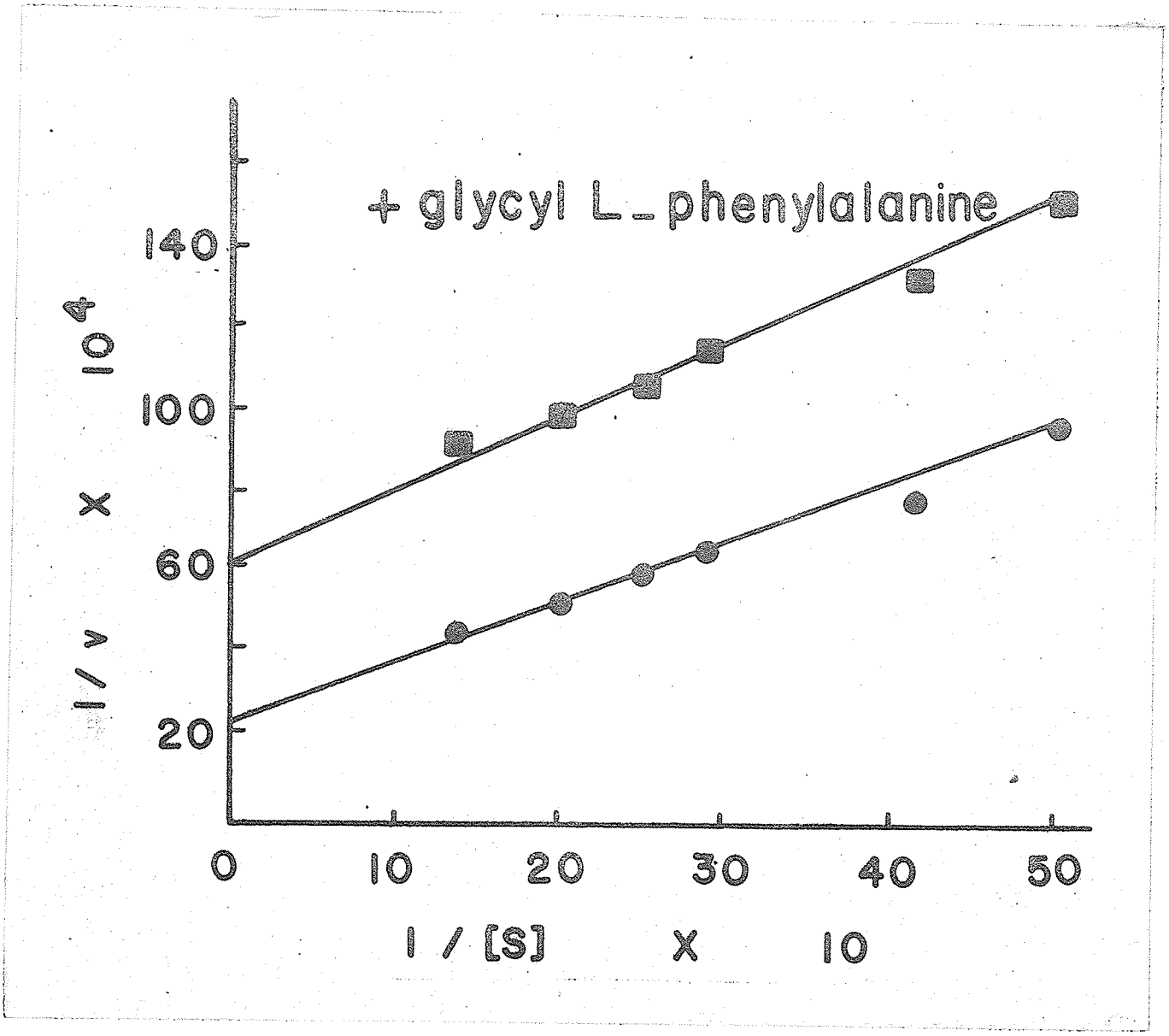
Lineweaver-Burk Plot Showing the Variation of Glycine-Dependent ATP-^{32}PP Exchange Activity With Glycine Concentration in the Absence and Presence of 0.1 M Glycyl L-Valine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentration in the reaction mixture of glycyl L-valine was 0.1 M.

FIGURE 20.

Lineweaver-Burk Plot Showing the Variation of Glycine-Dependent ATP-^{32}PP Exchange Activity With Glycine Concentration in the Absence and Presence of 0.01 M Glycyl L-Phenylalanine



Note: Assays were carried out according to the procedure described in Materials and Methods. The final concentration in the reaction mixture of glycyl L-phenylalanine was 0.01 M.

TABLE V

Effect of Glycine Analogs on Glycine-Dependent ATP-^{32}PP Exchange

B. Noncompetitive and Uncompetitive Inhibitors:

Analog	Type of Inhibition*	Km for glycine (mM)		K_I for analog (mM)	
		expt.I	expt.II	expt.I	expt.II
N-Substitutions					
N-acetyl glycine	N	0.61	0.60	62.5	66.6
hippuric acid	N	0.61	0.57	4.7	14.1
B-alanyl glycine	N	0.62		100.0	
L-alanyl glycine	N	0.62		16.7	
DL-alanyl glycine	N	0.60		8.3	
L-valyl glycine	N	0.62		19.3	
L-leucyl glycine	N	0.62	0.57	19.3	34.2
L-phenylalanyl glycine	N	0.59		33.2	
C-Substitutions					
glycinamide	N	0.61	0.59	2.0	1.3
glycyl glycine	N	0.60		15.8	
glycyl glycyl glycine	N	0.60	0.60	20.8	27.7
glycyl L-alanine	N	0.59		83.4	
glycyl L-serine	N	0.57		21.5	
glycyl L-valine	N	0.59	0.57	80.0	87.7
glycyl L-leucine	N	0.60	0.59	11.1	8.9

* Type of Inhibition: N, noncompetitive; U, uncompetitive

TABLE V Continued

Analog	Type of Inhibition*	Km for glycine (mM)		K_I for analog (mM)	
		expt.I	expt.II	expt.I	expt.II
C-Substitutions					
glycyl L-phonyl-alenine	U	0.62		-	
Amino Acids					
L-alanine	U	0.59	0.59	-	-
L-serine	N	0.60	0.59	41.5	63.5

* Type of Inhibition: N, noncompetitive; U, uncompetitive

Note: Experiments in Table V were carried out exactly as in Table IV.

inhibitor (see figure 18). The strongest noncompetitive inhibitor of glycine-dependent ATP-^{32}PP exchange may be seen in Table V to be glycinamide. Also shown in Table V are the values obtained from the Lineweaver-Burk plots for the apparent Km for glycine. The average value for this Km was calculated from these data and was 0.60 $\pm$ 0.02 mM.

DISCUSSION OF RESULTS

From the time course for glycine-dependent ATP-^{32}PP exchange (see figure 1), it can be seen that the initial rate of the reaction is measured when the samples are incubated for 15 minutes. The decrease in the activity after 20 minutes might be accounted for either by the depletion of one or more substrates in the reaction or by the exchange reaction approaching equilibrium.

The decrease of activity, shown in the enzyme concentration curve (see figure 2), obtained with the pH 5 fraction, may be due to the depletion of one or more substrates in the incubation mixture by enzymes other than glycyl-RNA synthetase. It is unlikely that ATP is depleted, because experiments showed that ATP was not degraded appreciably in the reaction mixture (see Table I). It is possible that appreciable PP is degraded during the incubation since PPase activity is present in the pH 5 fraction (30). It was also previously found that KF would inhibit PPase activity (9). It may be seen from figure 2 that KF stimulated the glycine-dependent ATP-^{32}PP exchange catalyzed by the pH 5 fraction. The results show then, that at higher enzyme concentrations the decreases in the exchange activity might be due, at least in part, to the presence of PPase activity. In more purified enzyme preparations this phenomena was not observed (see figure 3), indicating that the enzyme or enzymes responsible for this decrease in activity must have

been removed in the purification procedure. The more purified preparations are known to contain much less PPase activity (30).

Assuming that the Km for each substrate is a measure of a dissociation constant (Michaelis-Menten assumption) of an enzyme-substrate complex, then the affinities of the enzyme for each substrate (glycine, ATP and PP) may be compared. This enzyme-substrate complex involves glycine, but the actual complex is unknown. The complex may also involve ATP and PP or both substrates in some form or other. The Kms found for glycine, ATP and PP were 0.60 mM, 0.076 mM and 0.21 mM respectively (see figure 4). Thus, glycyl-tRNA synthetase probably has the greatest affinity for ATP, less affinity for PP and the least affinity for glycine.

The metal ion, Mg^{++}, has been shown to form with ATP and PP, Mg^{++}-ATP and Mg^{++}-PP complexes which may be the true substrates of the enzyme (89). Such complexes may also be the true substrates for glycyl-tRNA synthetase. The glycine-dependent ATP-^{32}PP exchange catalyzed by the 50-60% $(NH_4)_2SO_4$ fraction exhibited a Mg^{++} optimum of 2 mM in confirmation of previous findings (30). At low Mg^{++} concentrations (below 2 mM) the Mg^{++} may be involved in forming Mg^{++}-ATP and Mg^{++}-PP complexes. Above 2 mM Mg^{++}, the excess metal ion may associate with anionic sites in the active center of the enzyme, possibly with the anionic site which binds glycine (see later discussion). However, the Mg^{++} optimum for glycine-dependent ATP-^{32}PP exchange catalyzed by the 40% $(NH_4)_2SO_4$ supernatant fraction was found by Fraser (30)

to be 20 mM. The concentration of SRNA in the 40% $(NH_4)_2SO_4$ supernatant fraction (0.4 mg/ml) is high in comparison to the concentration of SRNA in the 50-60% $(NH_4)_2SO_4$ fraction (0.02 mg/ml). Since RNA can bind Mg^{++} (90), the effect of added SRNA on glycine-dependent ATP-^{32}PP exchange in the "RNA-low" fraction was tested. It may be seen in figure 5 that no increase of Mg^{++} optimum was observed. It is apparent then, that the difference in the Mg^{++} optima of the two enzyme fractions cannot be accounted for by the difference in SRNA concentration. These results are unlike those found by Hele (33) for the leucine and isoleucine activating enzymes. Hele has shown that the addition of SRNA to "RNA-low" enzyme fractions will increase the Mg^{++} optima to those observed with the "pH 5 enzymes" fraction for leucine- and isoleucine-dependent ATP-^{32}PP exchange. Hele (33) postulated that the pyrophosphate exchange might then occur by two mechanisms, one requiring a low Mg^{++} concentration for fractions low in SRNA concentration, and the other requiring a high Mg^{++} concentration for fractions high in SRNA concentration. The present results in this work does not support Hele's conclusion at least for glycine activating enzyme. The 40% $(NH_4)_2SO_4$ supernatant fraction has a high salt concentration compared to the 50-60% $(NH_4)_2SO_4$ fraction. The possibility that the difference in $(NH_4)_2SO_4$ concentration could account for the different Mg^{++} optima was tested. When $(NH_4)_2SO_4$ was added to the 50-60% $(NH_4)_2SO_4$ fraction to the level of 40% of saturation, no change in the Mg^{++} optimum was observed. Thus, it can be

concluded that the difference in $(NH_4)_2SO_4$ concentration is not responsible for the different Mg^{++} optima of the two enzyme fractions. The reason for the different Mg^{++} optima is not yet clear. One possibility may be that there are more than one glycine activating enzyme involved in protein biosynthesis and one or more of the enzymes may have been removed during purification.

The characteristics of glycyl-RNA formation were determined previously by Fraser (72) with the pH 5 fraction. The formation of glycyl-RNA was found to be dependent on glycine, ATP, Mg^{++}, and was sensitive to ribonuclease. Fraser found that the glycyl-RNA synthetase activity could be assayed by making the enzyme concentration limiting so as not to saturate all the SRNA with glycine in the time allowed for the assay. In this work, the rate of glycyl-RNA formation was also found to be proportional to the enzyme concentration up to 20 µg/ml of protein with the 50-60% $(NH_4)_2SO_4$ fraction (see figure 6). With high concentrations of protein, the SRNA became saturated with glycine during the time allowed for assay. The apparent Km for glycine for glycyl-RNA formation catalyzed by the 50-60% $(NH_4)_2SO_4$ fraction was found in this work to be 4 µM (see figure 7). Fraser (30) previously found the Km for glycine to be 3 µM with the pH 5 fraction. The Mg^{++} optimum for glycyl-RNA formation catalyzed by the 50-60% $(NH_4)_2SO_4$ fraction was found in this work to be 10 mM (see figure 8) in agreement with that found by Fraser (72) for the 40% $(NH_4)_2SO_4$ supernatant fraction. Thus, the Mg^{++} optimum for glycyl-RNA formation is the same in the 40% $(NH_4)_2SO_4$ supernatant and the 50-60% $(NH_4)_2SO_4$ fractions in contrast to the results found for glycine-dependent ATP-^{32}PP exchange.

The reason for the difference in response of the two enzyme fractions to varying Mg^{++} concentration for the glycine-dependent ATP-^{32}PP exchange reaction and for glycyl-RNA formation is not clear.

The effects of pH on glycyl-RNA formation and glycine-dependent ATP-^{32}PP exchange are quite similar (see figure 9). Since these velocities were determined at the same concentration of substrates throughout the pH range, it cannot be stated with certainty whether this curve represents an effect upon the enzyme or upon a substrate. If it represents the titration of a group of the enzyme, then this group has a pK of about 6.2 to 6.7. This pK is approximately that of the imidazole group of free histidine (6.0) (92), considering possible neighboring group effects in the enzyme. The histidine would be in the uncharged form in order for glycine activation to occur. Similar effects of pH on tryptophane (8), tyrosine (8) and proline (91) activating enzymes have been observed. Further evidence indicating that histidine may be in the active center was obtained from photo-oxidation experiments. Histidine residues in proteins are preferentially photo-oxidized in the presence of methylene blue (93,94). Cysteine and methionine may be oxidized as well, but at a much slower rate. Since the sulphydryl group of a cysteine side-chain is required for the activation of glycine (see below), mercaptoethanol was included during the photo-oxidation of glycyl-RNA synthetase in the hope that it would keep the sulphydryl group of a cysteine side-chain in the reduced form. It was found that glycine, but not other substrates (ATP and SRNA) protected the enzyme

against photoinactivation (see Table II). This effect was specific because alanine did not protect the enzyme from photo-oxidation. This specific protection by glycine suggests that the sensitive group is contained in the active center in the region where glycine associates with the enzyme. The fact that there was no protection against photo-oxidation by the adenylate (glycine plus ATP) (see Table II) may indicate that when the adenylate is formed the glycine may move away from this group. The nature of the photo sensitive group cannot be deduced with certainty, but the high sensitivity of histidine to photo-oxidation, and the results of the pH activity study suggests that it may be a histidine group.

Recently, Fraser (30) demonstrated that the enzyme in the 50-60% $(NH_4)_2SO_4$ fraction was sensitive to sulphydryl agents in contrast to the enzyme in the 40% $(NH_4)_2SO_4$ supernatant fraction. Since the former fraction contains a low amount of SRNA, it was suggested by Fraser, that the specific glycine acceptor RNA might dissociate from the glycyl-RNA synthetase on purification with a simultaneous unmasking of a sulphydryl group. Further experimental evidence presented in this work also suggests this possibility. It was found that SRNA, but not other substrates (glycine and ATP), when preincubated with the enzyme, protected the enzyme from inactivation by PCMB (see Table III). This protection was specific since ribosomal RNA did not provide this protection. This specific protection of the sulphydryl group by SRNA suggests that the sulphydryl group is located in the active center of the enzyme and is "masked" by the SRNA. Hele (33) has shown evidence for the presence of a sulphydryl

group in the active centers of leucine and isoleucine activating enzymes of rat liver also. Hele found that PCMB inhibits and glutathione activates the amino acid-dependent ATP-^{32}PP exchange catalyzed by the leucine and isoleucine activating enzyme with "SRNA-low" fractions of rat liver. However, Hele has shown that preincubating the leucine activating enzyme with SRNA does <u>not</u> protect the enzyme against inactivation by PCMB. Allen et al (22) have shown that leucine and ATP together protected the leucine activating enzyme of guinea pig liver against PCMB inhibition. Slight protections were afforded by leucine and by ATP alone. These suggest that the sulphydryl group is in the region of the adenylate intermediate complex. Although most investigators appear to agree that there are sulphydryl groups in the active centers of amino acid activating enzymes, there appears to be considerable doubt as to its location and the role it plays in the activation process. Hele (33) has postulated that the transfer of the activated amino acid to the terminal adenosyl unit of SRNA might involve a thioester intermediate along the lines suggested by Ingraham and Green (95) for the activation of acetate. The present work does not rule out the possibility that the sulphydryl group is in region of the enzyme associated with the adenylate.

Concentrations of KCl above 60 mM were found to inhibit glycine-dependent ATP-^{32}PP exchange competitively (see figures 12 and 13). Evidence has been obtained for an anionic site in the active center of glycyl-SRNA synthetase which binds

glycine (see later discussion). It is possible that high concentrations of K^+ could compete with glycine for this site. Another possible explanation for the competitive inhibition by KCl is that KCl breaks a sensitive salt bridge not involving the anionic binding site but one which is perhaps in or near the active center. When the salt bridge is broken, the shape of the active center would change and the enzyme thus be inactivated.

Two analogs of glycine, methylamine (see figure 15) and betaine (see figure 14) showed competitive kinetics with apparent K_Is of 6.3 mM and 26 mM respectively. The low K_I for methylamine, which is much like glycine in structure except for the absence of a carboxyl group, suggests an anionic binding site for glycine in the active center of the activating enzyme. This site would be a point of attachment of glycine, through the positively charged amino group of glycine, to the enzyme. Betaine has a higher K_I. It is possible that it also competes with glycine for this anionic site of the enzyme. The results on the competitive inhibition of glycine-dependent ATP-^{32}PP exchange by sarcosine and sodium acetate are difficult to interpret since the concentrations of the inhibitors used indicate that the inhibition might be due to the "salt effect" discussed above (see Table IV). The reason why L-prolyl glycine showed competitive kinetics unlike the other N-substituted dipeptides is not understood. The low K_Is for hippuric acid and N-substituted glycine dipeptides as compared to the high K_I for

N-acetyl glycine suggests that hydrophobic bonds may play a role in the inhibition of the exchange by these analogs. The binding of these analogs to the enzyme may occur at the "glycine site" or at some other site. These two possibilities cannot be distinguished in the present work. If it is assumed that the binding of the N-substituted glycine analogs to the enzyme is at the "glycine site", then the difference in K_Is for β-alanyl glycine and L-alanyl glycine might be explained. The positive amino group in β-alanyl glycine may be located further away from the anionic site in the enzyme associated with glycine and thus the enzyme would have less affinity for β-alanyl glycine than for L-alanyl glycine. Glycinamide (see figure 18) was a very effective inhibitor indicating a strong association with the enzyme. The amide group of glycinamide may play a role in this strong association as well as the positively charged amino group, by making the carbonyl carbon more electropositive. It is possible that this would allow a stronger interaction with a nucleophilic site on the enzyme involving histidine (see below). Other C-substituted analogs of glycine (see Table V) indicate that hydrophobic groups may play a role in binding these glycine analogs to the enzyme. This binding may also occur at the "glycine site" or at some other site on the enzyme. Two uncompetitive inhibitors were found in this work, glycyl L-phenylalanine and L-alanine. The interpretation of these results is difficult. Another amino acid, L-serine, which is also similar in structure to glycine, showed noncompetitive kinetics with

respect to glycine activation. It did not appreciably inhibit glycine activation.

It is apparent from all of the above results that glycyl-RNA synthetase is a highly specific amino acid activating enzyme. In summary, it may be concluded that the active center of this enzyme probably contains an anionic site at which glycine is bound as well as histidine and cysteine side-chains which take part in the activation process. The possible roles played by these groups are discussed below.

Every amino acid activating enzyme which has been studied to date is also specific for ATP and for the acceptor RNA (SRNA). The ATP cannot be replaced by GTP, UTP, CTP, dATP or AMP (7). From this specificity, it may be suggested that the ATP may interact with the enzyme via the 6-amino group, the ribose and the phosphates. It is believed that there is at least one specific acceptor RNA for each kind of amino acid incorporated

into protein (17). Since the nucleotide sequences at the amino acid acceptor ends of these molecules are common (pCpCpA), the different activating enzymes must interact with other internal sequences of nucleotides very specifically as well (see Introduction).

Some experiments suggest that glycyl-RNA synthetase is a flexible molecule. During the purification, it was found that ATP stabilized the enzyme against heat denaturation (30). It was previously found also, that removal of SRNA from the enzyme at one stage of the purification, greatly reduced its stability (30). Figure 2 also indicates that SRNA stabilizes the enzyme since the addition of SRNA to the "RNA-low" enzyme fraction increases the activity. Similar results indicating that SRNA stabilizes amino acid activating enzymes were obtained by Hele (33,74-76) with the leucine and isoleucine activating enzymes.

A possible mechanism for glycyl-RNA formation based on the findings in this work may be proposed as follows (see figure 21):

(a) initial adsorption of glycine to the enzyme at an anionic binding site

(b) reaction of glycine with a histidine side-chain by a nucleophilic attack on the carbonyl carbon of glycine leading to the formation of an unstable intermediate

(c) transfer of glycine from the histidine region of the enzyme to the ATP site through a nucleophilic attack of the carboxyl oxygen atom of glycine on the α-phosphorus

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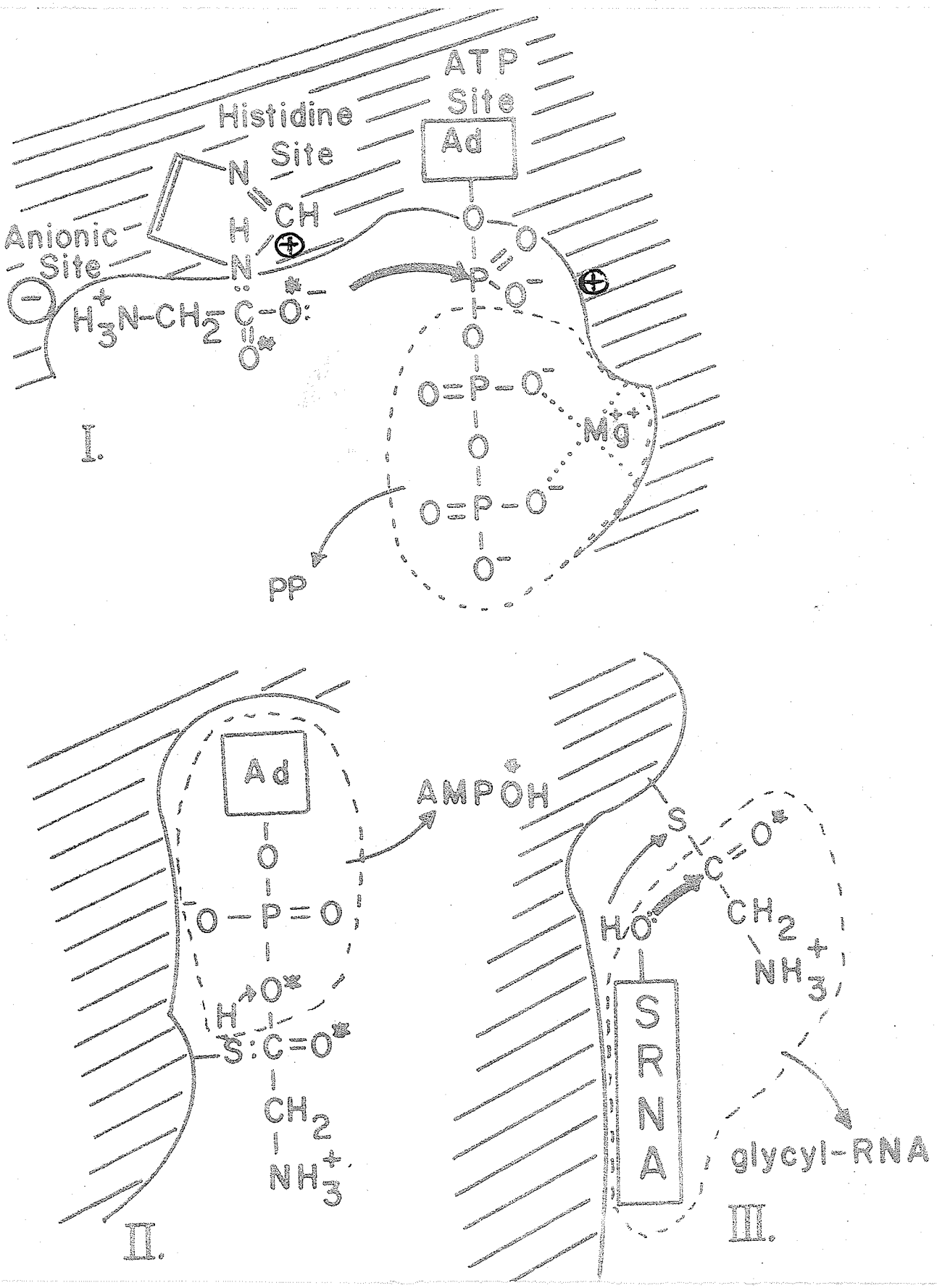
FIGURE 21.

Diagramatic Representation of a Possible Mechanism of Amino Acid Activation



atom of ATP, forming glycyl~AMP and splitting out PP

(d) nucleophilic attack of the sulphydryl group on the carbonyl carbon of glycine in the glycyl~AMP with the formation of an intermediate thioester, releasing AMP

(e) reaction of the amino acyl thioester intermediate with SRNA to form amino acyl-RNA

The initial adsorption of glycine to the enzyme probably occurs through a salt linkage between the positive amino group of glycine and the anionic site on the enzyme. The glycine is positioned so that the nitrogen of the uncharged imidazole group of histidine in the enzyme can perform a nucleophilic attack on the carboxyl carbon of glycine.* The nitrogen of the imidazole group of histidine has been postulated by other workers to perform a nucleophilic attack on carbonyl carbons of substrates, peptides, esters and amides, for the hydrolytic enzymes trypsin (92) and chymotrypsin (96). Here the result of this attack is to make the oxygens of the carboxyl group of glycine more reactive toward the α-phosphate atom of ATP. When this nucleophilic attack of the carboxyl oxygen on the α-phosphate atom of ATP takes place, pyrophosphate is split out and glycyl~AMP forms. This reaction would result in the transfer of the amino acyl moiety from the histidine region of the enzyme to the ATP site. The carbonyl carbon of glycine in the adenylate could then associate with the sulphydryl group which could perform a nucleophilic attack on the carbonyl carbon

* Two cationic sites on the glycyl-RNA synthetase or a divalent metal ion might be involved to facilitate both the nucleophilic attack of the histidine on the glycine atom and the nucleophilic attack of the carboxyl group of glycine on the α-phosphate atom of ATP (see figure 21).

forming an amino acyl thioester and splitting out AMP. Tracer studies with O^{18}-labelled amino acids (97) have shown that one of the oxygen atoms is found in AMP on amino acyl-RNA formation. Both labelled oxygens must appear in the intermediate amino acyl adenylate. The final step in amino acyl-RNA formation would be the reaction of SRNA with the thioester intermediate to form amino acyl-RNA with the release of the free enzyme. Thus, this mechanism postulates that the sequence of events consists of the splitting out of PP from ATP followed by the splitting out of AMP from the amino acyl adenylate. The histidine is associated with glycine and the sulphydryl group is associated with the glycyl adenylate in the formation of glycyl thioester.

Glycine-dependent ATP-^{32}PP exchange has been found to be dependent on the presence of a sulphydryl group (see figure 11). The role of the sulphydryl group in the exchange reaction can be postulated in the above mechanism. If it is assumed that the adenylate is associated with the sulphydryl group, then this association would probably make the glycine carboxyl oxygen-phosphorus bond more susceptible to attack by pyrophosphate. Pyrophosphate could then react with the adenylate to form ATP and split out amino acid. It can be predicted from this mechanism that the glycyl adenylate would protect the enzyme from PCMB inhibition. Allen et al (22) suggested that the sulphydryl group is in the region of the adenylate intermediate complex in the leucine activating enzyme of guinea pig liver.

They have shown that leucine and ATP together protected the enzyme against PCMB inhibition whereas each substrate separately gave only slight protection. It is possible that the splitting out of PP and the formation of glycyl adenylate would not be sensitive to PCMB inhibition. This could perhaps be tested by following the cleavage of ATP labelled in the two terminal phosphates, either in the presence of pyrophosphatase or in the presence of high concentration of NH_2OH to "trap" the adenylate.

Novelli and Stulberg (see 93) have suggested a different mechanism for amino acid activation. They visualized a "concerted" reaction in which no intermediate adenylate was formed. In this mechanism, amino acid, ATP and SRNA are bound to the enzyme with the amino acid located on the surface of the enzyme between ATP and SRNA in contrast to the present mechanism where the linear sequence of substrates is amino acid, ATP and SRNA. The negative oxygen of the amino acid performs a nucleophilic attack on the α-phosphorus atom of ATP with the splitting out of PP and at the same time the oxygen of the 2' or 3' hydroxyl group of the terminal adenosyl unit of SRNA performs a nucleophilic attack on the carbonyl carbon of the amino acid. The result is that amino acyl-RNA, PP and AMP forms simultaneously. This mechanism would require the attack of only one electrophilic group on the

carbonyl carbon of glycine. In this mechanism, the amino acyl moiety is not "transported" on the surface of the enzyme. In the mechanism proposed in this work, the glycine is "transported" from the histidine to the sulphydryl region of the enzyme. The present mechanism for glycine activation differs from all other mechanisms of amino acid activation previously discussed (see 98) in including a role for the imidazole side-chain of a histidine residue. This is probably not the only conceivable mechanism for glycyl-RNA formation.

ABBREVIATIONS

The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; PP, inorganic pyrophosphate; ^{32}PP, radioactive inorganic pyrophosphate; rRNA, ribosomal ribonucleic acid; SRNA, soluble ribonucleic acid or acceptor RNA or transfer RNA; RNase, ribonuclease; TCA, trichloroacetic acid; Tris buffer, tris (hydroxymethyl) amino methane; PCMB, p-chloromercuribenzoate; c.p.m., counts per minute; µM, micromolar; mM, millimolar; M, molar; µg, microgram; mg, milligram; ml, milliliter; O.D., optical density; λ, wavelength.

APPENDIX

Enzyme Kinetics

The rate of an enzyme catalyzed reaction increases progressively, with increase in substrate concentration, in a hyperbolic manner to reach a level of maximal activity beyond which the rate is dependent only on the enzyme concentration. The Michaelis-Menten equation (77), $v = Vm[S]/([S] + Km)$, gives the expected hyperbolic curve when v is plotted against [S].

[S] is the substrate concentration.

v is the rate of reaction.

Vm is the maximal rate at high enzyme-saturating substrate concentration under specified experimental conditions.

Km is commonly termed the "Michaelis constant". It is a measure of the dissociation constant for the ES complex and an inverse measure of the affinity of the enzyme for the substrate.

There are seven basic assumptions involved in the formulation and interpretation of the Michaelis-Menten equation (99). These are: (a) an enzyme-substrate complex forms (b) Km is the dissociation constant of the complex (c) the free substrate concentration is equivalent to the concentration of substrate added (d) the products are released from the enzyme rapidly (e) the over-all reaction is irrevereible (f) one substrate molecule is bound at one enzyme site (g) concentrations of reactants may be used instead of activities.

The Km derived from the Lineweaver-Burk method (78) is a measure of a dissociation constant for an enzyme-substrate complex. This enzyme-substrate complex for glycyl-RNA synthetase involves glycine, ATP and PP in some form or other but the actual complex is unknown. It is not known if the Kms for ATP, glycine and PP calculated by the Lineweaver-Burk method are actually a measure of the dissociation constants of the respective enzyme-substrate complexes.

According to Lineweaver and Burk (78), Vm and Km for a particular enzyme may be determined graphically by plotting $1/v$ against $1/[S]$, since the reciprocal form of the Michaelis-Menten equation, $1/v = 1/(Vm) + Km/(Vm[S])$, (Lineweaver-Burk form), requires that such a plot will result in a straight line of slope Km/Vm and will intersect the $1/v$ axis at $1/Vm$. The value of Km may be found directly by extending the plot to the intersection with the $1/[S]$ axis, this being $-1/Km$. Km may be determined directly from the slope of the straight line which is Km/Vm.

Similarly, the rate equation for completely competitive inhibition, $v = Vm[S]/([S] + Km\left[1 + [I]/K_I\right])$, may be rearranged into a reciprocal form for useful plotting. The transformed equation, $1/v = 1/Vm + (Km/Vm[S])(1 + [I]/K_I)$, was outlined by Lineweaver and Burk (78) and is frequently referred to as a "Lineweaver-Burk equation".

v, Km, $[S]$ and Vm are exactly as outlined for the Michaelis-Menten equation.

K_I, inhibitor dissociation constant, is a measure of the dissociation constant for EI complex and an inverse measure of the affinity of the enzyme for the inhibitor.

[I] is the inhibitor concentration.

K_I may be directly determined from the slope of the straight line which is $(Km/Vm)(1 + [I]/K_I)$.

The usual meaning attached to competitive inhibition is that the inhibitor reacts reversibly with a site on the enzyme where a complex is formed between the enzyme and substrate. The degree of competitive inhibition will thus depend on Km and [S]. Noncompetitive inhibition is assumed to involve reaction of the inhibitor with a region other than the active center so that combination of the substrate with the substrate site is unaffected but the breakdown of the ES complex is prevented. The degree of noncompetitive inhibition will not depend on [S] or Km but only on [I] and K_I. Some inhibitions are only partially competitive, partially noncompetitive or of a mixed type.

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