Metal Ion Interactions and Glutamine Synthetase Activity*

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ABSTRACT: Manganous, cobaltous, and ferrous ions were able to replace magnesium in the activation of sheep brain glutamine synthetase. As the concentration of each of these activating cations was increased, the pH optimum of the enzyme was shifted to lower values. At their respective pH optima, relative activating effects were $Mg^{2+} = Co^{2+} = Mn^{2+} > Fe^{2+}$. Similar displacements of pH optima to more acid values were observed when Mn^{2+}, Co^{2+}, or Fe^{2+} were added to Mg^{2+}-activated glutamine synthetase. In general, the pH optimum for glutamine synthetase varied from 8.5 to 4.8 and was shown to depend on the nature and concentrations of divalent cations individually, their relative concentrations where more than one cation was present, and the ratio of adenosine triphosphate to metal ion. Calcium and barium ions inhibited the magnesium-dependent enzyme at all pH values with no shift in pH optimum. Of the various metals studied, only manganous ions stimulated glutamine synthetase activity in the presence of uridine, inosine, or guanidine triphosphates. Glutamine transferase was activated by Mg^{2+} or Mn^{2+}, but not by other cations. The pH optimum of the transferase depended on the nature, number, and relative concentrations of the activating metals. It is suggested that metal ion interactions may be of significance in the control of cellular metabolism.

Glutamine synthetase catalyzes the synthesis of glutamine from glutamate and ammonia according to the equation:

$$\text{glutamate} + \text{ATP} + NH_3 \overset{E,\ Mg^{2+}}{\rightleftharpoons} \text{glutamine} + P_i + \text{ADP}$$

Like most other ATP[1]-dependent enzymes, it is activated by divalent metal ions (Cohen, 1963; Meister, 1962). Although magnesium, manganous, and cobaltous ions have been reported to stimulate the enzyme, the relative effectiveness of the activating ions on glutamine synthetase activity has not been intensively investigated (Denés, 1954; Levintow *et al.*, 1955). Recent work of Greenberg and Lichtenstein (1959) has shown that the pH optimum of the glutamine synthetase reaction varied with the concentration of magnesium or manganese present. Thus, the relative influence of these metal ions on the enzyme depended on the pH of the system as well as on the nature and concentration of the cation. The present work confirms and extends these studies. It was observed that Mg^{2+}, Mn^{2+}, Co^{2+}, and Fe^{2+} stimulated glutamine synthetase activity. The pH optimum and maximum rate of glutamine synthesis depended on the concentration and nature of the metal ion added and on whether other divalent metal ions were also present.

Materials and Methods

Glutamine synthetase was prepared from sheep or beef brains as described by Pamiljans *et al.* (1962). Purification was taken through step 8 of their procedure. Specific activity of five preparations determined using the assay described by Pamiljans *et al.* ranged from 90 to 117 units/mg of protein. ATP and other nucleotides were purchased from Pabst Laboratories, Milwaukee, Wis. Chromatography of CTP, GTP, and ITP was performed as described in Pabst circular OR-17 (Pabst Laboratories, 1961). Only the expected trinucleotides and traces of the corresponding dinucleotides were detected on paper chromatograms. Alumina C_γ gel was obtained from the Sigma Chemical Co., St. Louis, Mo. L- and D-glutamic acids were purchased from Mann Research Laboratories, Inc. The D-glutamic acid was free of the L isomer as determined by the action of *Clostridium welchii* L-glutamate decarboxylase (Meister *et al.*, 1951). Ammonium sulfate ("Enzyme" grade) was bought from Mann Biochemicals, Inc. Metal salts were reagent grade. Glass-distilled water was prepared using a Corning water distillation apparatus, Model AG-2 (Corning Glass Works, Corning, N. Y.). Acetate buffers were used in the pH range 4–6, and Tris or imidazole buffers in the range 5.5–8.6 (Gomori 1955). Buffers above pH 9 were prepared from glycine. All were 0.2 M with respect to the anion. Net synthesis of glutamine was established by thin layer chromatography of incubation mixtures on glass plates coated with

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[1] Abbreviations used in this work: ATP, UTP, ITP, GTP, adenosine, uridine, inosine, and guanidine triphosphates, respectively.

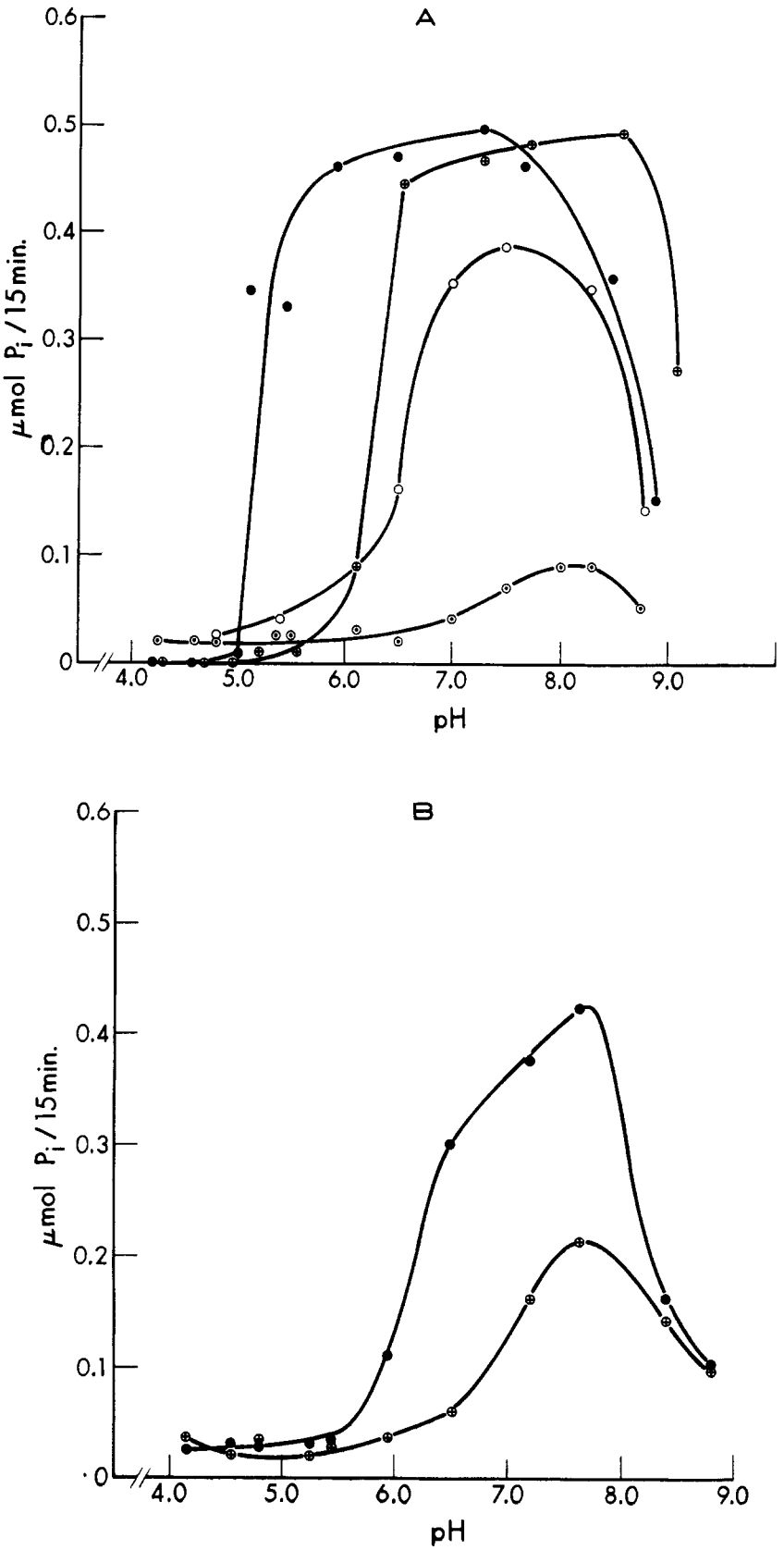

FIGURE 1: Magnesium as activator of glutamine synthetase. (A) L-glutamine synthesis. (B) D-glutamine synthesis. The pH–activity profiles were obtained as described under Materials and Methods. ⊙—⊙, 1 mM Mg^{2+}; O—O, 3 mM Mg^{2+}; ⊕—⊕, 5 mM Mg^{2+}; ●—●, 50 mM Mg^{2+}.

a 0.3-mm-thick layer of cellulose (chromatographic grade, E. Merck A.G., Darmstadt, Germany) using formic acid–water–*t*-butyl alcohol (15:15:70) as developing solvent (Monder and Meister, 1958). R_F values of glutamate and glutamine were 0.45 and 0.32, respectively.

L-Glutamine synthesis was measured in a system containing 50 μmoles of buffer, 0.86 μmole of β-mercaptoethanol, 50 μmoles of L-glutamate, 5 μmoles of ATP, 20 μmoles of ammonium acetate, metal salts as indicated in the text, and 0.5 unit of enzyme in a final volume of 1.0 ml. Glutamate, ATP, and ammonium acetate were prepared at pH 5.5, 6.5, and 7.5 and added to the system in the appropriate pH range in order to avoid major shifts in pH of the complete system. Hydrogen ion activity was measured using Beckman Model G and Model 76 pH meters. The reaction was initiated by the addition of enzyme and incubated in an Eberbach shaking incubator at 37°. Rate of L-glutamine formation was linear with time for at least 15 min, measured colorimetrically by the procedure of Fiske and Subbarow (1925). Addition of sodium chloride to the systems to compensate for variation in ionic strength had no effects on the results obtained, and in most of the experiments described this refinement was omitted.

D-Glutamine synthesis was measured essentially as described above, but because of the slower rate of reaction a 1-hr incubation time was used. Reaction was linear for at least this time interval between pH 5.5 and 8.0 when magnesium was the activating cation. For both L- and D-glutamine systems, controls in which enzyme, metal, glutamic acid, or ATP were separately omitted were employed. Negligible blank values were obtained in all cases except those where metal was left out, when absorbancy values about 2–5% of maximum attained with added metal was observed. Addition of EDTA (10^{-4} M) did not diminish these blank values.

Glutamotransferase was measured in a system composed of 50 μmoles of L-glutamine, 100 μmoles of hydroxylamine adjusted to the pH of the buffer, 0.5 μmole of ADP, 10 μmoles of β-mercaptoethanol, 5 μg of enzyme protein (step 8), 100 μmoles of buffer, and metal ions at various concentrations in a final volume of 1.0 ml.

Glutamohydroxamate synthesis was determined by the ferric hydroxamate technique of Lipmann and Tuttle (1945) after incubation of the mixture for 15 min at 37°. L-Glutamohydroxamate, synthesized chemically by the method of Roper and McIlvain (1948), was used as standard.

Results

Effect of Magnesium Concentration. Figure 1A shows pH–activity curves for L-glutamine synthesis at several Mg^{2+} concentrations. A broad plateau between pH 6 and 8 was observed at 5 mM Mg^{2+}. At 50 mM Mg^{2+} a definite shift in pH optimum to lower values occurred, with no significant change in maximum activity. Measurement of activity with respect to magnesium concentration showed apparent inhibition of enzyme activity at high Mg^{2+} concentrations. At pH 8, for example, enzyme activity increased through 5 mM Mg^{2+}, then decreased as it passed through a leg of the pH–activity curve at 50 mM Mg^{2+}.

In contrast to L-glutamine synthesis, the pH–activity curves for the synthesis of D-glutamine were much less sensitive to metal concentration. Figure 1B shows that between 5 and 50 mM Mg^{2+} no shift in pH optimum was observed.

Effect of Manganous Ion Concentration. A comparison of Figures 1 and 2 illustrates that the effect of Mn^{2+} on the pH–activity curve of glutamine synthetase was more pronounced than that of Mg^{2+}. The broad optimum in the region of pH 6.5 at 0.5 mM Mn^{2+} was shifted to pH 4.8 at 10 mM Mn^{2+} when L-glutamate was a substrate. At 2.5 mM Mn^{2+}, activity equalled that observed with 5 mM Mg^{2+} but was at much lower pH values. Activity at the pH optimum increased up to 2.5 mM Mn^{2+} and decreased at higher concentrations. Between 5 and 10 mM Mn^{2+}, decrease in activity occurred without a corresponding shift in pH optimum. Figure 2B shows that the pH optimum of D-glutamine synthesis was also markedly shifted to lower pH values, but this was somewhat less than for L-glutamine synthesis. The pH optimum of 7.25 at 0.5 mM Mn^{2+} was decreased to 5.7 at 10 mM Mn^{2+} when D-glutamate was the substrate.

Increased ATP levels shifted the pH optimum for L-glutamine synthesis to higher values at constant manganous ion concentrations. The effects were small but significant. Thus, with 2.5 mM Mn^{2+}, the pH optima for 2.5, 5.0, and 10.0 mM ATP were 5.2, 5.4, and 5.6, respectively. It was concluded that the changes in pH optima caused by variations in the concentration of added Mn^{2+} were at least in part due to changes in the ratio of Mn^{2+} to ATP.

Effects of Simultaneous Presence of Mg^{2+} and Mn^{2+}. In preliminary studies on the metal requirements of L-glutamine synthesis, it was found that at pH 7.6 glutamine synthetase activity was much less in the presence of both Mg^{2+} and Mn^{2+} than in the presence of Mg^{2+} alone. Similar observations have been made by Elliot (1953). The explanation of this phenomenon is illustrated in Figures 3A and B. Addition of Mn^{2+} to Mg^{2+}-activated enzyme shifted the entire pH curve to more acid values. The extent of deflection depended on the relative concentration of each cation. When the concentration of Mn^{2+} was 5 mM, equal to that of Mg^{2+}, the pH optimum was 4.8, identical with that obtained with 5 mM Mn^{2+} alone, and the maximum velocity at this pH was the same as that obtained with 5 mM Mg^{2+} at pH 7.6. With 2.5 mM each of Mg^{2+} and Mn^{2+}, a smaller change in pH optimum was seen. In other experiments, a change in optimum pH from 7.6 to 5.5 was observed when Mn^{2+} was added at a level of 0.5 mM to a system containing 5 mM Mg^{2+}. Smaller, but significant effects were observed at a Mn^{2+} level of 0.05 mM. Similar shifts of pH optima with mixtures of Mg^{2+} and Mn^{2+} were seen for D-glutamine synthesis, as indicated in Figure 3B. The changes were smaller,

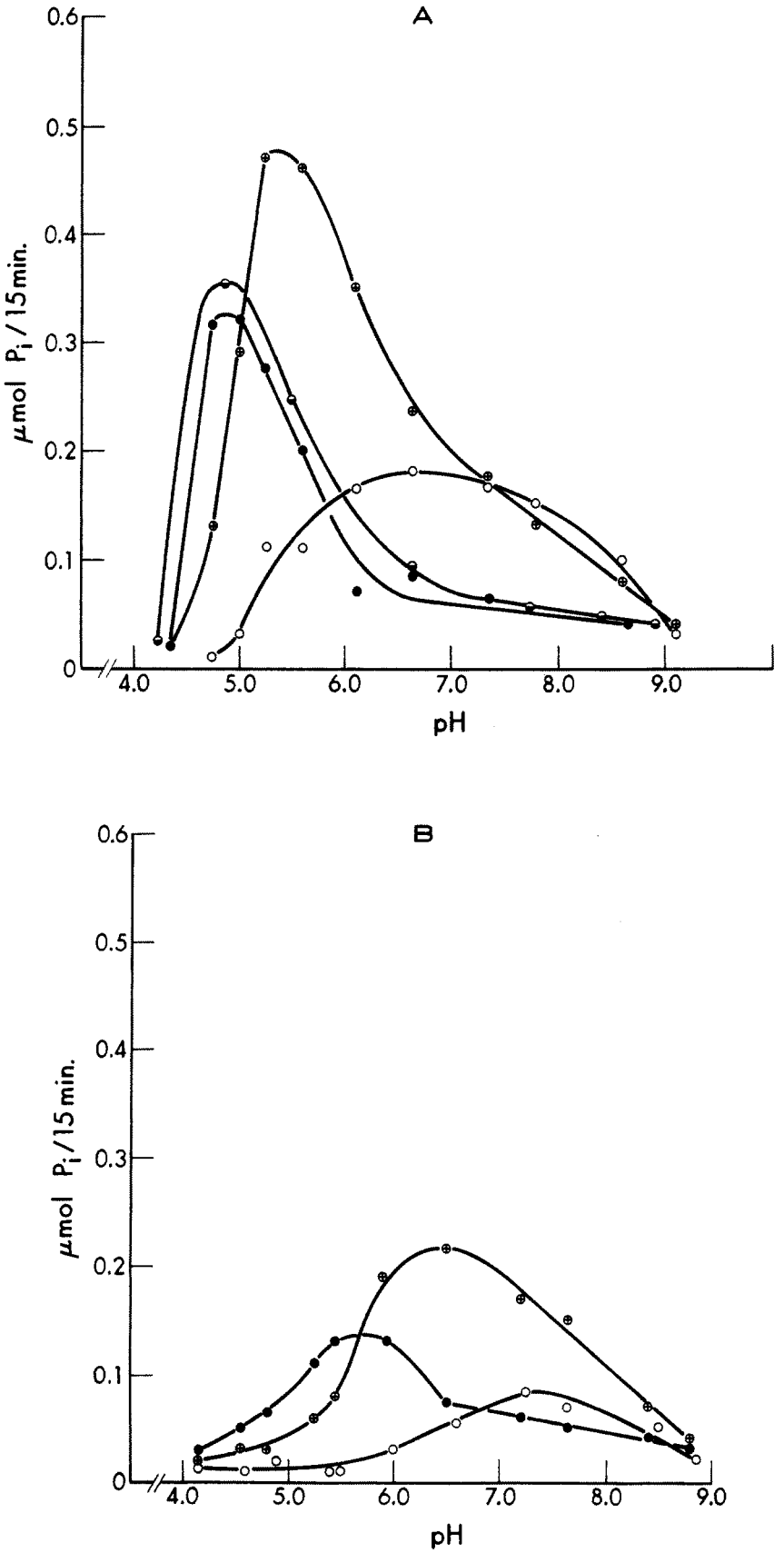

FIGURE 2: Manganese as activator of glutamine synthetase. (A) L-glutamine synthesis. (B) D-glutamine synthesis. ○—○, 0.5 mM Mn^{2+}; ⊕—⊕, 2.5 mM Mn^{2+}; ◒—◒, 5 mM Mn^{2+}; ●—●, 10 mM Mn^{2+}.

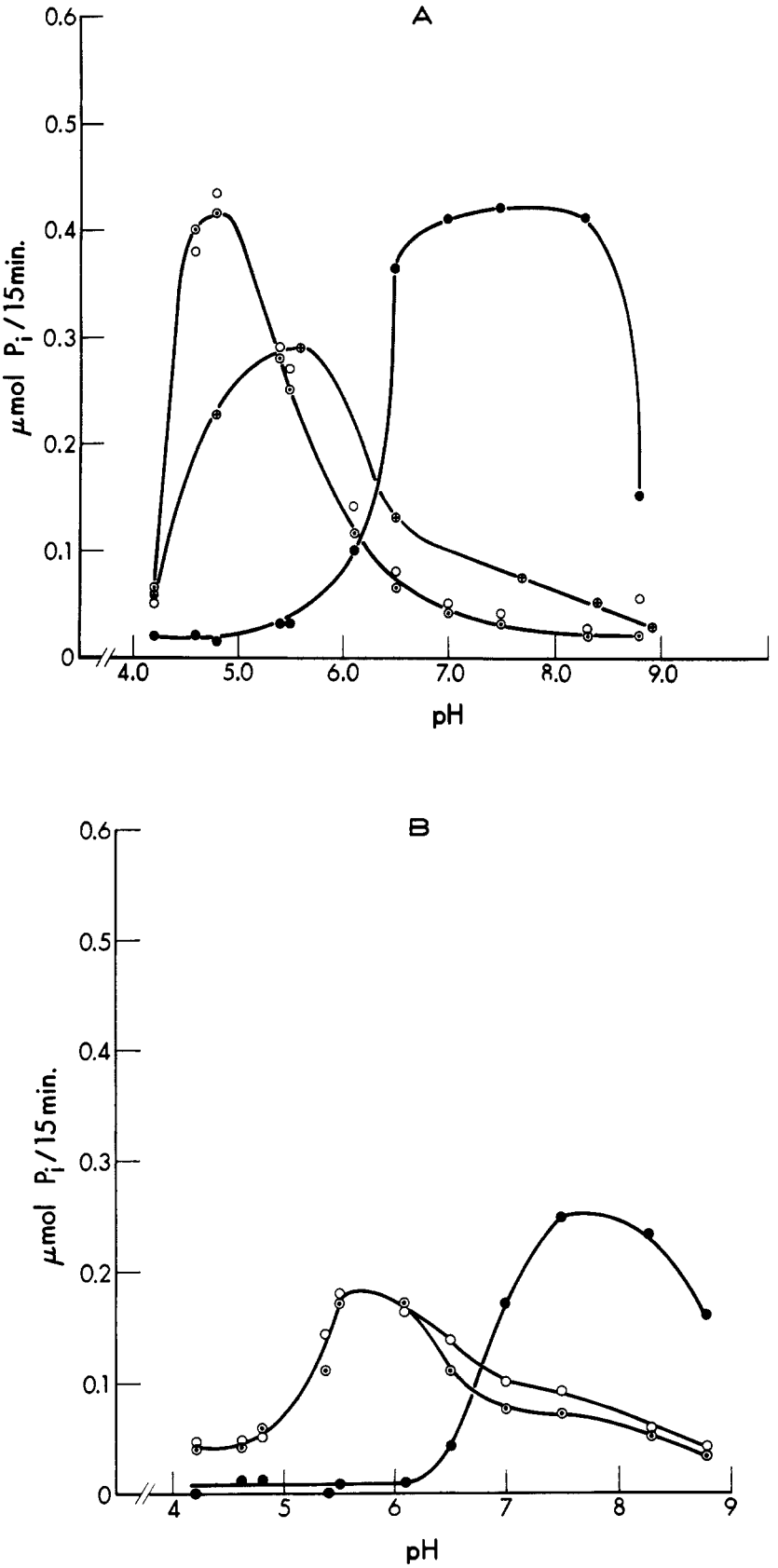

FIGURE 3: Effects of simultaneous presence of magnesium and manganous ions on glutamine synthetase activity. (A) L-glutamine synthesis. (B) D-glutamine synthesis. ●—●, 5 mM Mg^{2+}; ○—○, 5 mM Mn^{2+}; ⊙—⊙, 5 mM Mg^{2+} + 5 mM Mn^{2+}; ⊕—⊕, 2.5 mM Mg^{2+} + 2.5 mM Mn^{2+}.

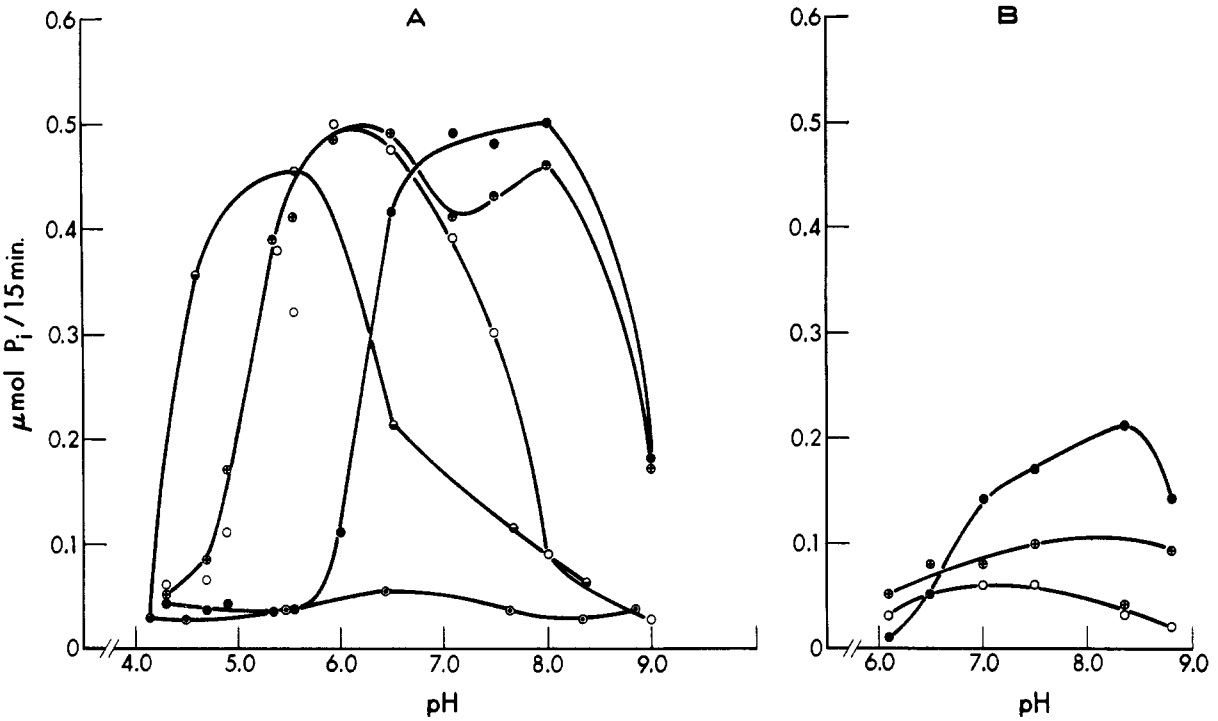

FIGURE 4: Effects of simultaneous presence of magnesium and cobaltous ions on glutamine synthetase activity. (A) L-glutamine synthesis. (B) D-glutamine synthesis. ●—●, 5 mM Mg^{2+}; ⊙—⊙, 1 mM Co^{2+}; ○—○, 5 mM Co^{2+}; ◒—◒ 11 mM Co^{2+}; ⊕—⊕, 5 mM Mg^{2+} + 5 mM Co^{2+}.

and the over-all activity was less than for L-glutamine synthesis.

Activation by Cobaltous Ions. Figure 4A shows that the pH optimum of L-glutamine synthetase with respect to cobaltous ion varied with cation concentration from pH 6.5 at 1 mM Co^{2+} to pH 5.5 at 11 mM Co^{2+}. No further change in the pH profile occurred at 50 mM Co^{2+} (not shown). When 5-mM levels each of Co^{2+} and Mg^{2+} were present in the system a double peaked curve appeared, each maximum corresponding to the maximum for the individual metal ions. It was established that the two maxima were not due to the presence of two enzymes. The ratio of activities remained constant during enzyme purification, and enzyme which was activated maximally by Mg^{2+} was not independently activated further by Co^{2+}. In other experiments, addition of small amounts of Co^{2+} to Mg^{2+}-activated glutamine synthetase (final concentrations 1 mM Co^{2+}, 10 mM Mg^{2+}) caused the pH–activity curves to move from pH 7.6 to 6.2 with no decrease in maximum velocity.

The pH optima of L-glutamine synthesis for mixtures of Mn^{2+} and Co^{2+} varied from 6.0 for Co^{2+} alone to 4.8 for Mn^{2+} alone. The optimum varied between these extremes in proportion to the relative levels of the two metals. Unlike the effects observed with Mg^{2+} and Co^{2+}, a double pH maximum was not observed at any proportion of Mn^{2+} and Co^{2+}.

Figure 4B shows that D-glutamine was synthesized much more slowly than L-glutamine in the cobalt-activated system, consistent with the relative rates of D- and L-glutamine synthesis observed with Mg^{2+} and Mn^{2+}. The pH optimum for D-glutamine synthesis was approximately 7, in contrast with the much lower optimum obtained for L-glutamine synthesis.

Activation by Ferrous Ions. Because the position of ferrous ion falls between those of Mn^{2+} and Co^{2+} in the first transition series of the periodic table, it was of interest to see if Fe^{2+} would also activate glutamine synthetase. Figure 5 shows that Fe^{2+} did indeed catalyze L-glutamine synthesis, but at a much slower rate than did the other metals. In the presence of equimolar Mg^{2+} two pH optima were observed, similar to the effect described above for cobalt. D-Glutamine synthesis was immeasurably small with Fe^{2+} at all pH values.

Effect of Calcium Ions. Unlike the metals discussed above, Ca^{2+} decreased the activity of glutamine synthetase at all pH values without shifting the pH optimum. The effects were similar for L- and D-glutamine synthesis. In confirmation of other studies (Levenbook and Kuhn, 1962; Elliot, 1951) the inhibition could be overcome in part by excess magnesium. However, as indicated in Figure 6, inhibition by Ca^{2+} was not strictly competitive with Mg^{2+}. Inhibition by Ba^{2+} resembled that caused by Ca^{2+}.

Effects of Other Metals. A number of metal ions, including Na^{+}, K^{+}, Cu^{+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+}, Al^{3+}, Ca^{2+}, and Ba^{2+}, were not activators of glutamine synthetase. Ni^{2+} was a potent inhibitor of the Mg^{2+}-catalyzed reaction below pH 8, but was ineffective

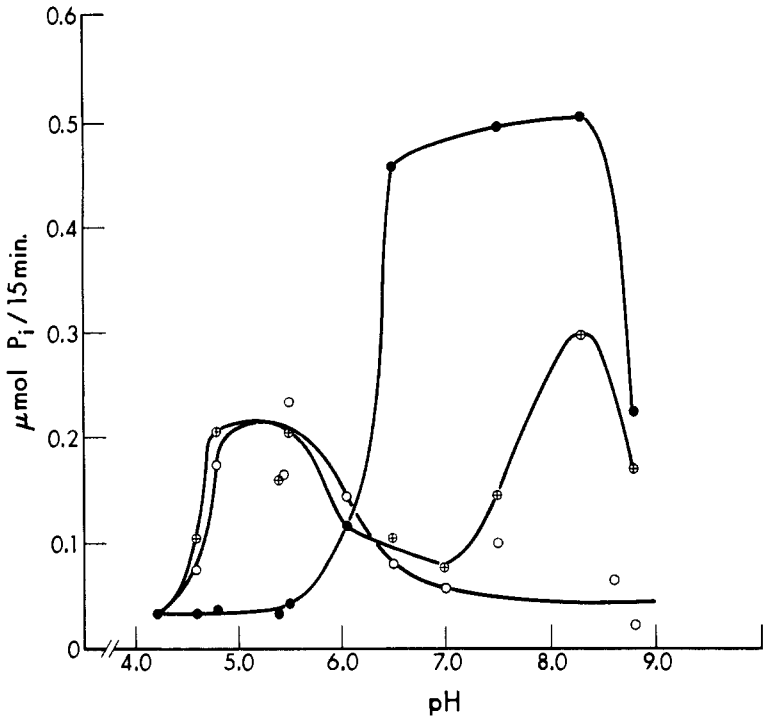

FIGURE 5: Effects of simultaneous presence of magnesium and ferrous ions on glutamine synthetase activity. ●—●, 5 mM Mg^{2+}; ○—○, 5 mM Fe^{2+}; ⊕—⊕, 5 mM Mg^{2+} + 5 mM Fe^{2+}.

above this value. The other metals (except Ca^{2+} and Ba^{2+}) had no effect on Mg^{2+} activation.

Effect of Metal Ions on the Glutamotransferase Reaction. It has been reported that glutamotransferase activity was shifted to lower pH values with increasing manganous ion concentration (Greenberg and Lichtenstein, 1959). Confirmation of these observations is shown in Figure 7. Increasing Mn^{2+} resulted in a shift of the pH optimum to lower values and a decrease in over-all activity. The pH optimum for transferase activity was much higher for Mg^{2+} than for Mn^{2+}. The pH–activity curve corresponded to that of Mn^{2+} alone even in the presence of five times greater excess of Mg^{2+}. However, Fe^{2+}, though not itself an activator, caused the pH optimum of Mn^{2+}-activated enzyme to shift to higher values. Other metals, including Co^{2+}, Ni^{2+}, and Zn^{2+}, had no effect on glutamotransferase under any conditions, alone or in mixtures.

Stimulation of Glutamine Synthetase by Metals and Other Nucleotides. The effects of UTP, ITP, GTP, and ATP on metal activation of glutamine synthetase were compared. The results are summarized in Table I. At comparable metal ion concentrations, only Mn^{2+} was effective in stimulating glutamine synthetase with nucleotides other than ATP. Stimulation by the other metal ions indicated on the table was barely within the limits of detection and therefore not considered significant.

Discussion

The metal requirements for glutamine synthetase have been reported to be satisfied by magnesium, manganese, or cobalt (Denés, 1954; Levintow *et al.*, 1955). In general, the order of activating ability was reported to be $Mg^{2+} > Mn^{2+} > Co^{2+}$, though under some conditions Co^{2+} may be most active (Denés, 1953). Recently, Greenberg and Lichtenstein (1959) demonstrated that under certain conditions the maximum velocity for glutamine synthesis may be greater with Mn^{2+} than Mg^{2+}. Their data suggested that at equimolar concentrations Mg^{2+} and Mn^{2+} may show similar velocities at different pH optima. These results have been confirmed in the present work. In addition, it has been shown that Co^{2+} also catalyzed the synthesis of L-glutamine at the same rate as Mg^{2+}. Ferrous ion activated the enzyme, but at a much lower rate than did the other three cations. Nickel, on the other hand, did not activate the enzyme, although it resembles the cobaltous ion closely with respect to size and charge. The pH–rate profiles illustrating these conclusions for the individual activating metals are summarized in Figure 8. These data show, in addition, how the activation series for these cations varies with hydrogen ion concentration.

Synergistic effects of metals on enzyme systems (Stone and Burton, 1962; Wolff and Resnick, 1963) have been reported. More frequently, mutually antagonistic effects of metal ions have been described, Mg^{2+}–Ca^{2+} and Na^{+}–K^{+} pairs being most studied (Kielley and Meyerhof, 1948; McLeod and Snell, 1948). Dixon and Webb (1958) have compiled a list of other such pairs. The Mg^{2+}–Mn^{2+} pair, in particular, has been reported to inhibit argininosuccinate syn-

TABLE I: Activation of Glutamine Synthetase by Metals and Nucleoside Triphosphates.[a]

Metal Ion	ATP pH Optimum	ATP V	ITP pH Optimum	ITP V	UTP pH Optimum	UTP V	GTP pH Optimum	GTP V
Mg^{2+}	7.6	0.41	8.4	0.03		0.00		0.00
Mn^{2+}	4.8	0.39	4.8	0.10	5.6	0.25	4.8	0.06
Fe^{2+}	5.6	0.05		0.00	6.5	0.02		0.00
Co^{2+}	5.6	0.34	6.5	0.03	6.5	0.04		0.00

[a] The complete systems contained 50 μmoles of buffer, 0.86 μmole of β-mercaptoethanol, 50 μmoles of L-glutamate, 2.5 μmoles of nucleoside triphosphate, 20 μmoles of ammonium acetate, 5 μmoles of the indicated metal ion added as the chloride, and 5 μg of enzyme protein in a final volume of 1.0 ml. V refers to micromoles of P_i released (Fiske and Subbarow, 1925) after 15-min incubation at 37° at the designated pH value.

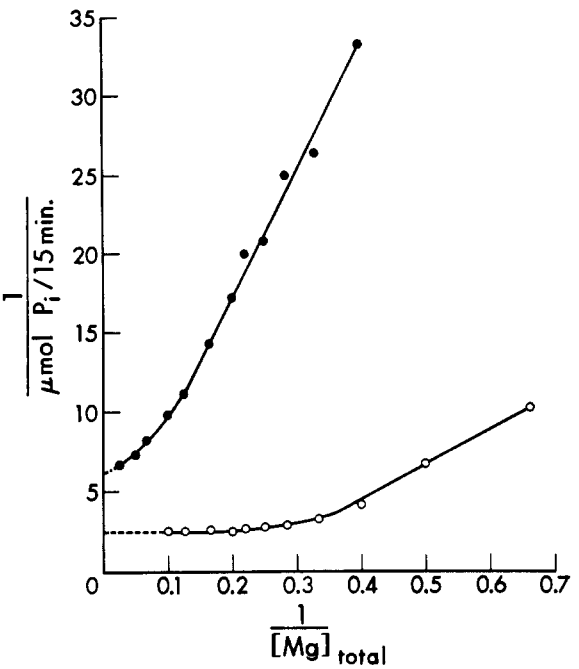

FIGURE 6: Inhibition of Mg^{2+}-activated glutamine synthetase by Ca^{2+}. Double reciprocal plot of the data performed according to the method of Lineweaver and Burk (1934). O—O, Mg^{2+}; ●—●, Mg + 1.5 mM Ca^{2+}. Incubation was performed as described under Materials and Methods.

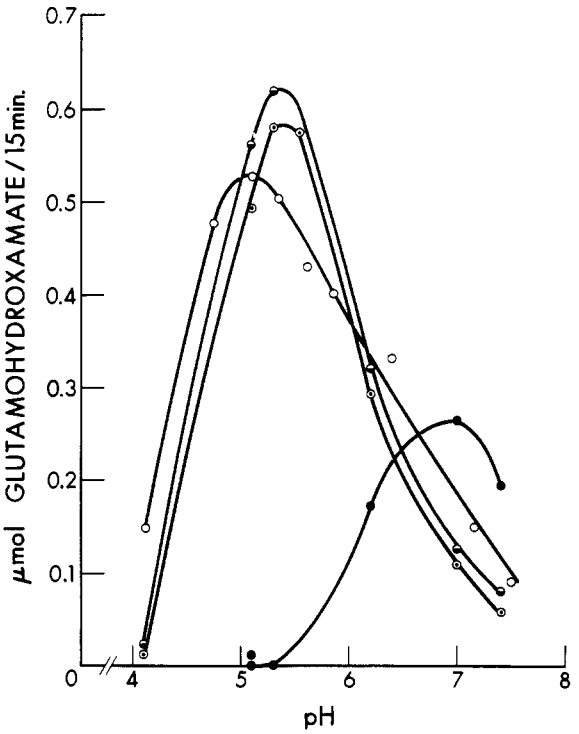

FIGURE 7: Effects of magnesium and manganous ions on glutamotransferase reaction. Procedure is described under Materials and Methods. ●—●, 5 mM Mg^{2+}; ◒—◒, 1 mM Mn^{2+}; O—O, 5 mM Mn^{2+}; ⊙—⊙, 5 mM Mg^{2+} + 1 mM Mn^{2+}.

thetase (Ratner, 1954), glutamine synthetase (Elliot, 1953; Wu, 1964), and micrococcal DNAase (Stone and Burton, 1962). For glutamine synthetase at least, the inhibition by the second metal is partly illusory, being due to a shift in the pH–activity curve rather than to a net decrease in activity. This is true for Co^{2+} and Fe^{2+} as well as for Mn^{2+}. It may be seen that at the magnesium levels used it was possible to obtain inhibition, no effect, or activation by the second cation depending on the pH chosen for study. It is suggested that some competitive or synergistic effects of metal ions on enzyme activation may be due to interactions such as are described in this paper. Similarly, the "inhibitory" effects of excess cation may be explained by shifts in the pH–activity curve, resulting in a displacement of the rate-limiting step to lower pH values. It should be emphasized that these experiments were done at constant levels of ammonia and glutamate. It was assumed that L-glutamate (equal to $20K_m$ at pH 7.6)

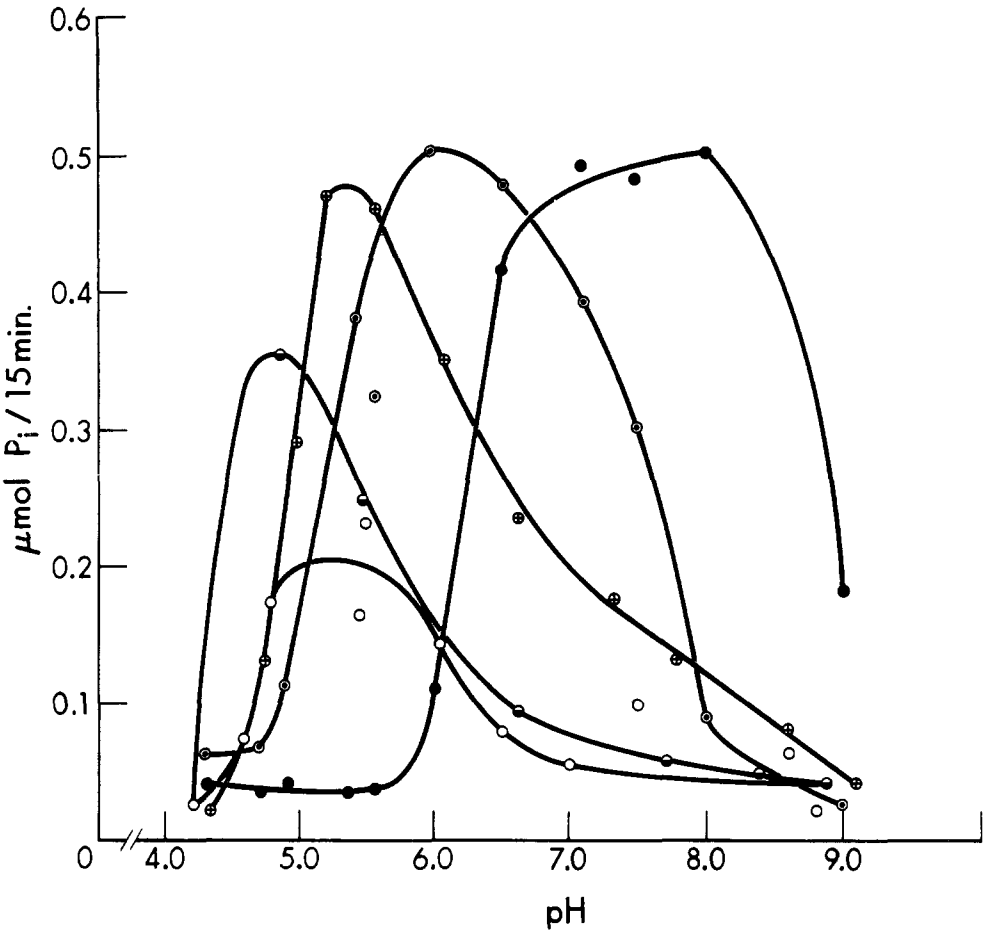

FIGURE 8: Summary of pH–rate profiles of individual metals. ●—●, 5 mM Mg^{2+}; ⊙—⊙, 5 mM Co^{2+}; ⊕—⊕, 2.5 mM Mn^{2+}; ◒—◒, 5 mM Mn^{2+}; ○—○, 5 mM Fe^{2+}.

(Pamiljans *et al.*, 1962) and ammonia (equal to $100K_m$ with respect to L-glutamate at 7.6) (Pamiljans *et al.*, 1962) were sufficiently in excess that these were not limiting at any pH value studied. To establish if this assumption is completely justified will require further investigation. Varying ATP from 2.5 to 10 μM did not influence the results obtained with respect to Mg^{2+}. Similar shifts in pH with Mg^{2+} concentration were observed at all levels of ATP. Small deflections to higher pH values were seen with increasing ATP concentration in the presence of Mn^{2+}. However, even at the highest level of ATP, the shifts were not more than 0.4 pH unit, and were all in the same direction so that the *relative* effects of changes in Mn^{2+} concentration on the pH–activity curve were not affected.

These results suggest that the level of activity of an enzyme such as glutamine synthetase may be governed, at least in part, by the concentrations, distribution, and proportions of metal ions in the environment, as well as the pH. It may be inferred that in the living cell, where such conditions normally exist, variations in these factors may have a profound effect on the rates of synthesis and degradation of key intermediates.

The data presented here allow us to reinterpret some of the observations of Denés (1953, 1954) and Elliot (1953). The inability of Co^{2+} to synthesize D-glutamine in contrast with its ability to synthesize L-glutamine was probably due to a choice of pH outside the optimum for Co^{2+} catalysis where the rate of L-glutamine synthesis was slow and the rate of D-glutamine synthesis was below the limits of detection. The selective inhibition of Mg^{2+}-activated D-glutamine synthesis by Co^{2+}, in contrast with no effect on L-glutamine synthesis described by Denés, is again due to failure to establish conditions where similar effects could be demonstrated for the two stereoisomers. The conclusion that the nature of the metal establishes an absolute stereospecificity of glutamine synthetase is probably not correct since the data presented here show that Mg^{2+}, Mn^{2+}, or Co^{2+} catalyzed the synthesis of both isomers. The data of Leonard *et al.* (1962), who also describe stereospecific effect of metals on glutamine synthetase activity, may be interpreted in a similar way. Demonstration of D-glutamine synthesis by Fe^{2+} may have been prevented by limitations of the analytical techniques used. The evidence presented confirms previous indications that the pH-dependent active intermediate is of low optical specificity (Levintow and Meister,

1953). It probably contains metal ion as well as glutamate, ATP, and enzyme. The structural relations between these components is unclear, though some aspects of this problem are under study (Meister, 1962; Krishnaswamy *et al.*, 1962; Graves and Boyer, 1962).

The data also show that comparisons of ratios of synthetase to transferase activity, in which great variability in ratios between tissues (Trush, 1963) or species (Levintow *et al.*, 1955; DeMars, 1958) may be demonstrated, do not on this basis alone indicate the presence of more than one enzyme. The great sensitivity of these activities to variation in metal ion has been clearly demonstrated here and it may be inferred that, except for the most rigorously defined experimental conditions, such variability must be the rule rather than the exception.

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