THE EFFECT OF AMINO ACIDS ON THE POLYMORPHIC CRYSTALLIZATION OF CALCIUM CARBONATE

by

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A Thesis

Submitted to the Department of Geology

in Partial Fulfilment of the Requirements

for Geology 4k6

McMaster University

April 1969

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ACKNOWLEDGEMENTS

The author wishes to acknowledge the generous assistance and support given him by Dr. J. R. Kramer in preparing this thesis. The assistance of Mr. F. Tebay and Dr. H. H. Williams is also gratefully acknowledged.

ABSTRACT

The presence of 0.10 moles/l. of glycine, glutamic acid, aspartic acid or leucine in a saturated bicarbonate solution will promote the formation of aragonitic calcium carbonate at 25.2°C. Magnesium ion, with or without an amino acid, also promotes the formation of aragonite. With increased amino acid concentration glutamic acid and alanine tend to promote the formation of more calcitic calcium carbonate, glycine and valine tend to promote the formation of more aragonitic calcium carbonate. Lysine and alanine show an increased tendency to form calcite at higher ionic strengths (.20) whereas glutamic acid shows the reverse. Some correlation with solubility and equilibrium constants for magnesium-amino acid complexes is indicated.

INTRODUCTION

The phenomenon of calcification in biological systems is well documented among the vertebrates and invertebrates (Glimcher, 1960, Wilbur, 1964) although the actual mechanism is not yet properly understood. Investigations into this phenomenon have taken two principal approaches. One, a "synthetic" approach, involves the *in vitro* precipitation of calcium carbonate under controlled conditions to examine the influence of temperature, pH, salinity, presence of other ions and the presence of organic material. The other approach is "analytic" and involves the *in vivo* observation of carbonate shelled organisms with respect to organic and inorganic chemical composition and environment. The majority of previous investigations have been with marine invertebrates using the analytic approach. (Wilbur, 1964; Hare, 1963; Dodd, 1962; Tanaka et. al., 1960; Watabe et. al., 1960; Abelson, 1956.)

From these studies it was evident that the amino acid composition of the protein matrix of the carbonate forming organism was the most significant factor influencing the polymorph of calcium carbonate which occurs. (Simkiss, 1964; Watable et. al., 1960.) The influence of the protein

matrix on the calcium carbonate polymorph is modified, however, by the other factors mentioned above. (Kitano, 1964; Wilbur, 1964; Lowenstam, 1954b; Chave, 1954) This paper will present the results of a preliminary synthetic study of the influence of glycine, alanine, valine, glutamic acid, aspartic acid, leucine and lysine on the polymorph of calcium carbonate precipitated from a bicarbonate solution.

Calcium carbonate in biological systems occurs as low magnesium calcite, high magnesium calcite and aragonite either separately or as mixtures. However, aragonite is unstable at surface temperatures and pressures and normally inverts to calcite. (Fyfe and Bischoff, 1965) Thus, the presence of aragonite in many organisms suggests a factor or combination of factors which tend to stabilize aragonite and permit its formation.

The selection of the amino acids was based on their ubiquitous presence in many carbonate secreting phyla and on their high thermal stability. The latter factor is of importance when considering the preservation of the amino acid composition of fossil organisms. (Abelson, 1956)

The rationale behind the synthetic approach and the reason for performing these experiments are based on the belief that more specific meaningful information can be derived from studies of this kind than from analytical

studies. A synthetic approach permits the control of parameters of a system, simplifying the interpretation of the results and hopefully avoiding multiple or contrary effects of uncontrolled variables. The synthetic approach also gives a better quantitative understanding of the mechanisms in a simple system which can be applied to analytical results in a more complex real system. The success of the synthetic approach can be judged by the degree to which synthetic trends can be fitted to analytic data.

The purpose of this study was to examine the influence of the amino acids, glycine, lysine, alanine, valine, leucine, aspartic acid and glutamic acid on the crystal structure of calcium carbonate homogeneously precipitated from a bicarbonate solution at constant temperature. The influence of amino acid concentration, ionic strength and the presence of magnesium ions was also examined.

EXPERIMENTAL

The method used is a modification of Kitano (1965).

Materials:

Glycine, lysine, alanine, valine, leucine, aspartic acid and glutamic acid. The amino acids were from Fisher Scientific Company.

Sodium chloride, magnesium chloride and calcium carbonate. The inorganic reagents were A.C.S. reagent grade chemicals.

Solutions:

For a set of six samples, a fresh saturated bicarbonate solution was prepared by bubbling carbon dioxide through distilled water containing suspended calcium carbonate for eight hours. The solution was then filtered by vacuum to remove any undissolved calcium carbonate.

Procedure:

Six jacketed flasks were connected to a constant temperature bath which maintained the reactants at 25.2 ± .2°C for the duration of the experiments. The freshly-prepared bicarbonate solution was added in 300 ml aliquots to each of the flasks. To each flask was added

a weighed amount of amino acid, sodium chloride and in half the samples, magnesium chloride. The resulting solutions were then allowed to degas for ten days. They were then filtered and the residue dried and x-rayed. Interpretations were made from diffractometer tracings of the samples.

The first variable considered was the presence of an amino acid (0.10 moles/1.) both with magnesium ion (.015 moles/1.) present and without. This concentration was used because it was in the middle of the range of concentration examined by Kitano for most of his amino acids. The experiments were carried out for all seven amino acids with 0.10 moles/1. of sodium chloride added to raise the ionic strength of the solution to .10. The effect of variations in the ionic strength on the polymorph associated with an amino acid was examined for lysine, glycine, glutamic acid and alanine. The effect of variations in the amino acid concentration on the calcium carbonate were examined for glycine, glutamic acid, valine and alanine both with magnesium ion present and without.

RESULTS

A calibration curve for the x-ray diffraction peaks was prepared using pure calcite and pure aragonite to make six 0.10 g. standard samples of known composition. Pure calcite was obtained from reagent calcium carbonate. Pure aragonite was obtained from the aragonitic shell of a fresh water mollusc, crushed and sieved to 200 mesh. These standard samples were then x-rayed with the experimental samples on the same day using the same x-ray diffractometer settings. The resulting calibration curve (Figure 1) was used to determine per cent calcite for all experimental samples (Table 1). The dominant calcite peak ($2\theta = 29.1°$) was found to have a linear relationship with per cent calcite.

The results of all experiments are given in Table 1 in data form and in Figures 2, 3 and 4 are indicated diagramatically. It should be noted that at 25.2°C a saturated bicarbonate solution will degas in 10 days to give calcium carbonate with only the calcite structure in the absence of magnesium ion. With magnesium ion the same solution will give calcium carbonate with about 30 to 40 per cent calcite structure and 70 to 60 per cent aragonite structure.

The presence of an amino acid, either with or

without magnesium ion, influences the polymorph formed as shown in Figure 2. Without magnesium ion alanine, valine and lysine give a pure calcite structure. Leucine, glutamic acid and aspartic acid result in a mixture of calcite and aragonite with the calcite structure being suppressed, especially for the latter two. Glycine has nearly pure aragonite associated with it. With the addition of magnesium ion the calcite polymorph is strongly suppressed for alanine, valine, leucine and lysine and the calcium carbonate is almost pure aragonite. For glycine the preferred form remains aragonite. However, glutamic acid and aspartic acid tend to prefer a higher per cent calcite form in the presence of magnesium ion.

Figure 3 shows the influence of amino acid concentration on the per cent calcite found in the precipitate for alanine, valine, glycine and glutamic acid. With no magnesium ion present an increase in concentration of glutamic acid increases the per cent calcite formed. However, an increase in glycine concentration has the opposite effect. With magnesium ion present alanine and glutamic acid show increases in per cent calcite with increasing concentration. The opposite effect is observed for glycine and valine. The greatest influence seems to be for glycine with no magnesium ion and for glutamic acid with magnesium ion.

There was no apparent correlation between the properties of the amino acids (Table 2) and the results obtained for the per cent calcite formed.

In summary, (a) magnesium ion depresses the formation of calcite for alanine, valine, leucine and lysine, has no effect on glycine and promotes the formation of calcite for glutamic acid and aspartic acid; (b) leucine, glycine, glutamic acid and aspartic acid suppress the formation of calcite in the absence of magnesium ion whereas alanine, valine and lysine have no effect; (c) an increase in the concentration of glutamic acid and alanine increases the per cent calcite formed. For glutamic acid the effect is reversed and for glycine there is no effect.

DISCUSSION

The correlation of calcium carbonate mineralogy with amino acid composition has been examined by Kitano who found that glycine favoured the formation of aragonite. Agreement with this work is qualitatively good, but quantitatively poor. For example, Kitano found that with 0.05 moles/l. of glycine without magnesium ion the precipitate was 100 per cent calcite, whereas at the same concentration the present work found only 35 per cent calcite. Not until Kitano's solutions reached 0.075 moles/l. was the suppression of calcite noticeable. For glutamic acid there was little or no agreement. Kitano found that glutamic acid, like glycine, suppressed the formation of calcite whereas in the data presented here while the per cent calcite remained low (10 - 30 per cent) it increased with increasing glutamic acid concentration. With alanine, again the results show only qualitative agreement. Kitano's data shows alanine with magnesium ion associated with a 30 per cent calcite precipitate at all concentrations. The present data shows a similar trend but at the 10 per cent calcite level.

There may be several reasons for the observed discrepancies between these sets of data. Kitano makes

no mention of the ionic strength of his solutions and this may have a significant effect (Figure 4). The influence of temperature may be of primary importance here, for as Kitano (1965) and Chave (1954) have shown, the polymorphic precipitation of calcium carbonate is highly temperature sensitive. Kitano's experiments were done at 24 ± 2°C and these experiments at 25.2 ± .2°C and this temperature difference may be sufficient that the results may not be directly comparable. A difference in experimental preparation of the solutions may also have had an effect. Kitano's amino acid solutions were all neutralized to pH 7 but the solutions in this study were not.

Some studies are not directly comparable to this one because they are involved with the mixtures of amino acids found in proteins in living organisms. For example, Hare (1963) found that in the proteins from the shell of *Mytilus californianus* if the ratio of amounts of acidic amino acids (aspartic acid + glutamic acid - ammonia) to basic amino acids (lysine + histidine + arginine) was greater than one, the shell layer was calcite; if less than one, the shell layer was aragonite. If it is assumed that there was magnesium ion present in the environment, then Hare's conclusions are confirmed in this study (Figure 2) but this correlation should not be pressed too far. A logical extension of this study would be to make artificial mixtures

of amino acids and determine the extent of the competition between the various amino acids. This would provide the necessary data for determining the relevance and accuracy of a synthetic study of this kind to observations of the type made by Hare. This has not been done to date.

Glycine deviates from the other amino acids tested in that magnesium ion has no effect upon the polymorph precipitated. This behaviour can be readily explained from Table 2 which shows the high solubility and high equilibrium constant for the magnesium complex with glycine. The high solubility indicates that all the glycine present is in solution (.034 moles) and the equilibrium constant shows that all the magnesium ion (.005 moles) is complexed. The result is that the magnesium ion is effectively removed from the solution and can have no influence on the calcium carbonate polymorph precipitated. Since alanine also has a high solubility, it might be expected to show a similar behaviour. But the equilibrium constant for the magnesium complex is much lower than for glycine and thus some magnesium ion is free to influence the polymorph precipitated. Thus far, it has not been possible to correlate other properties of the amino acids with their behaviour in influencing the crystal structure of calcium carbonate and more work needs to be done in this area. There is still a need for basic data on the amino acids as can be seen

from Table 2.

There were several difficulties encountered in this study. The low solubility of leucine and glutamic acid meant that the amino acid contaminated the filtered precipitate and interpretation of the x-ray diffractometer tracings was very difficult. Another difficulty was that an amino acid was always required to synthesize "pure" aragonite for use as an x-ray standard. The presence of magnesium ion, while promoting aragonite formation, resulted in the precipitation of only a 50 - 70 per cent aragonite-calcite mixture contrary to a 100 per cent aragonite synthesis reported by Kitano et. al. (1962c).

The experiments are quite simple, however, and a strong possibility exists in this synthesis approach of discovering basic relations between the amino acid composition of the protein matrix of an organism and the calcium carbonate polymorph found on the matrix. Further major relationships related to temperature, pH, other ions and ionic strength may also be ascertained.

In conclusion, it was found that the presence of alanine, valine and lysine promoted the formation of calcite, the addition of magnesium ion to them promoted the formation of aragonite, an increase in ionic strength tended to produce more calcite with magnesium ion for lysine and alanine and an increase in amino acid concentration

tended to produce more calcite with alanine and valine with magnesium ion. The presence of leucine, glycine, glutamic acid and aspartic acid tended to promote the formation of aragonite with this effect being enhanced by magnesium ion for leucine, unchanged for glycine and reversed for glutamic acid and aspartic acid. An increase in amino acid concentration increased the promotion of calcite for glutamic acid with magnesium ion strengthening the effect. For glycine a similar increase in concentration promoted the formation of aragonite with magnesium ion present. An increase in ionic strength tended to produce more aragonite with glutamic acid and magnesium ion whereas the promotion of aragonite by glycine was unaffected.

Table 1.

Experimental Results (25.2 ± .2°C)

Amino Acid	Concentration (moles/l)	$MgCl_2$	Ionic Strength	Peak Height ($2\theta = 29.1°$)	% Calcite
Lysine	.10	no	.10	90	100
	.10	yes	.13	1	1
	.10	yes	.20	2	2
Glutamic acid	.10	no	.10	14	17
	.10	yes	.13	25	29
	.05	no	.075	10	12
	.05	yes	.105	11	12
	.10	yes	.20	5	6
Aspartic acid	.10	no	.10	5	6
	.10	yes	.13	30	35

Table 1. (continued)

Experimental Results (25.2 ± .2°C)

Amino Acid	Concentration (moles/l)	$MgCl_2$	Ionic Strength	Peak Height (2θ = 29.1°)	% Calcite
Alanine	.10	no	.10	95	100
	.10	yes	.13	3	3
	.10	yes	.20	11	14
	.20	yes	.18	6	7
Valine	.10	no	.10	120	100
	.10	yes	.13	1	1
	.05	yes	.105	6	7
Leucine	.10	no	.10	36	42
	.10	yes	.13	4	5
Glycine	.10	no	.10	1	1
	.10	Yes	.13	1	1
	.05	no	.075	30	35
	.06	yes	.105	2	2
	.10	yes	.20	1	1

Table 2.
Amino Acid Data

Amino Acid	Formula	Molecular Weight	Solubility (moles/ℓ)	Equilibrium Constant Mg^{++} (pK_{eq})	Ca^{++}
Alanine	$CH_3CH(NH_2)CO_2H$	89.09	1.61	1.96	1.24
Valine	$(CH_3)_2CHCH(NH_2)CO_2H$	117.15	0.57	—	—
Leucine	$(CH_3)_2CHCH_2CH(NH_2)CO_2H$	131.18	0.18	—	—
Glycine	$H_2NCH_2CO_2H$	75.07	2.66	3.45	1.39
Lysine	$H_2N(CH_2)_4CH(NH_2)CO_2H$	182.65	—	—	—
Glutamic acid	$HO_2CCH_2CH_2CH(NH_2)CO_2H$	147.13	0.14	1.90	1.43
Aspartic acid	$HO_2CCH_2CH(NH_2)CO_2H$	133.10	0.54	2.43	1.60

Figure 1.

Calibration Curve

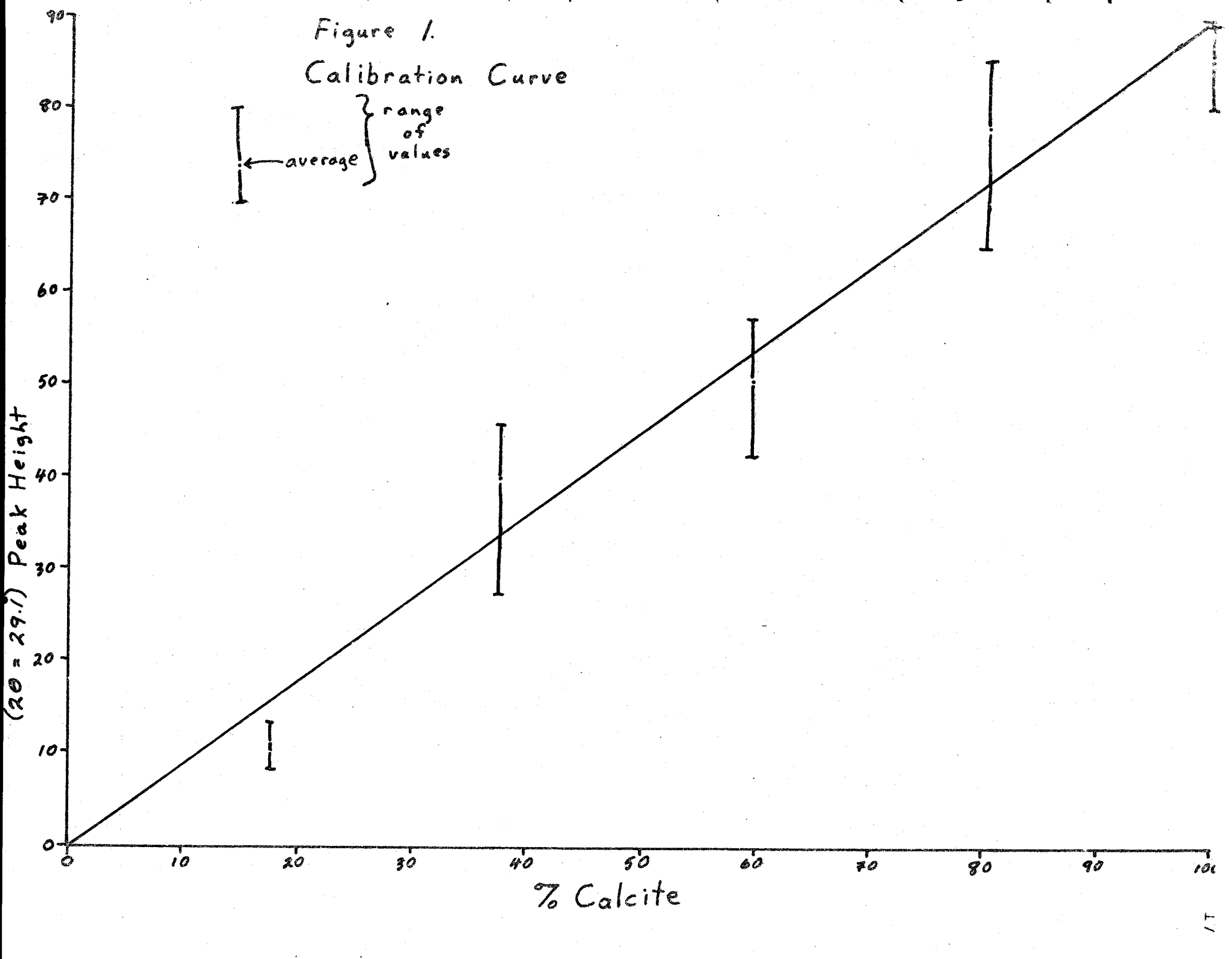


Figure 2.

Influence of Mg^{++}

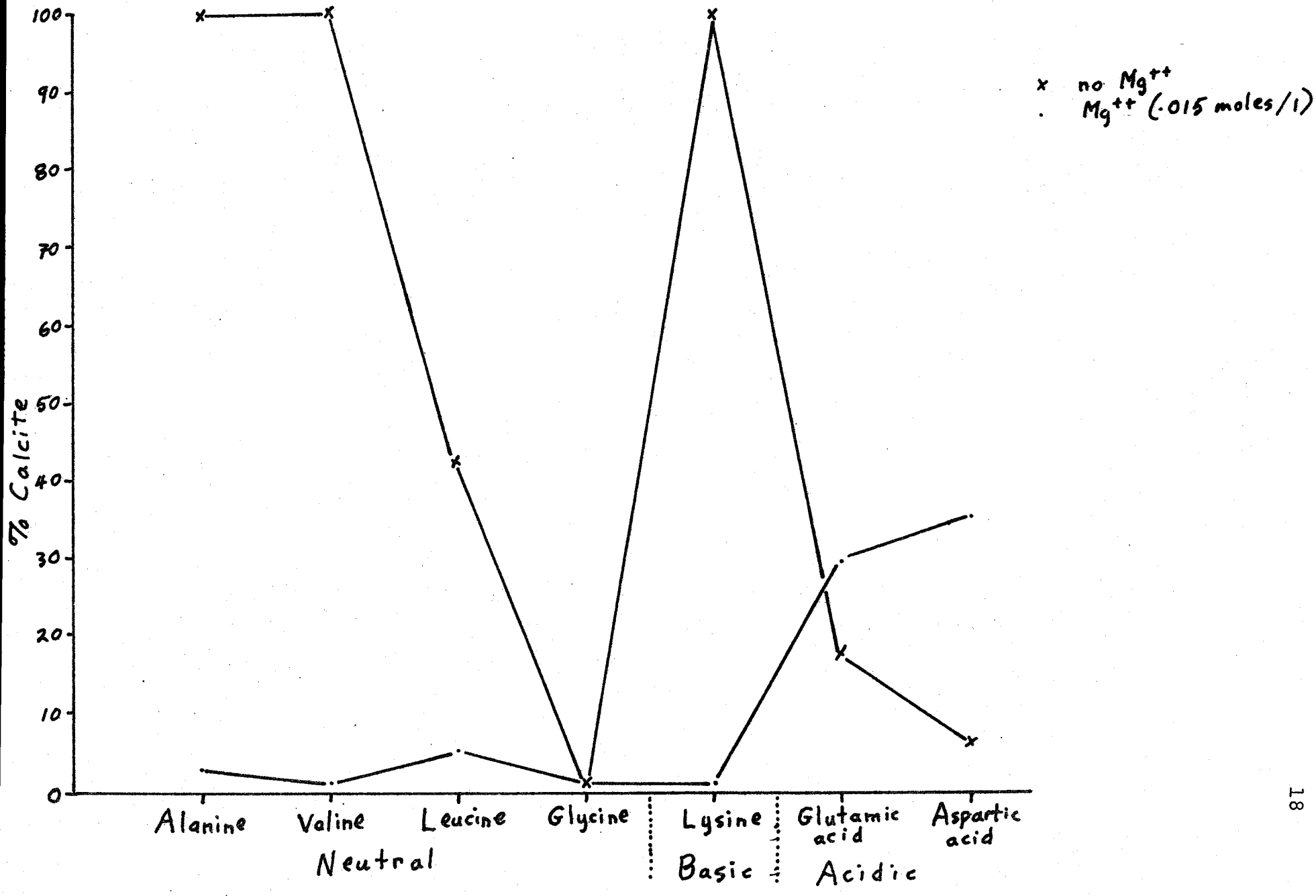


Figure 3.

Influence of Amino Acid Concentration

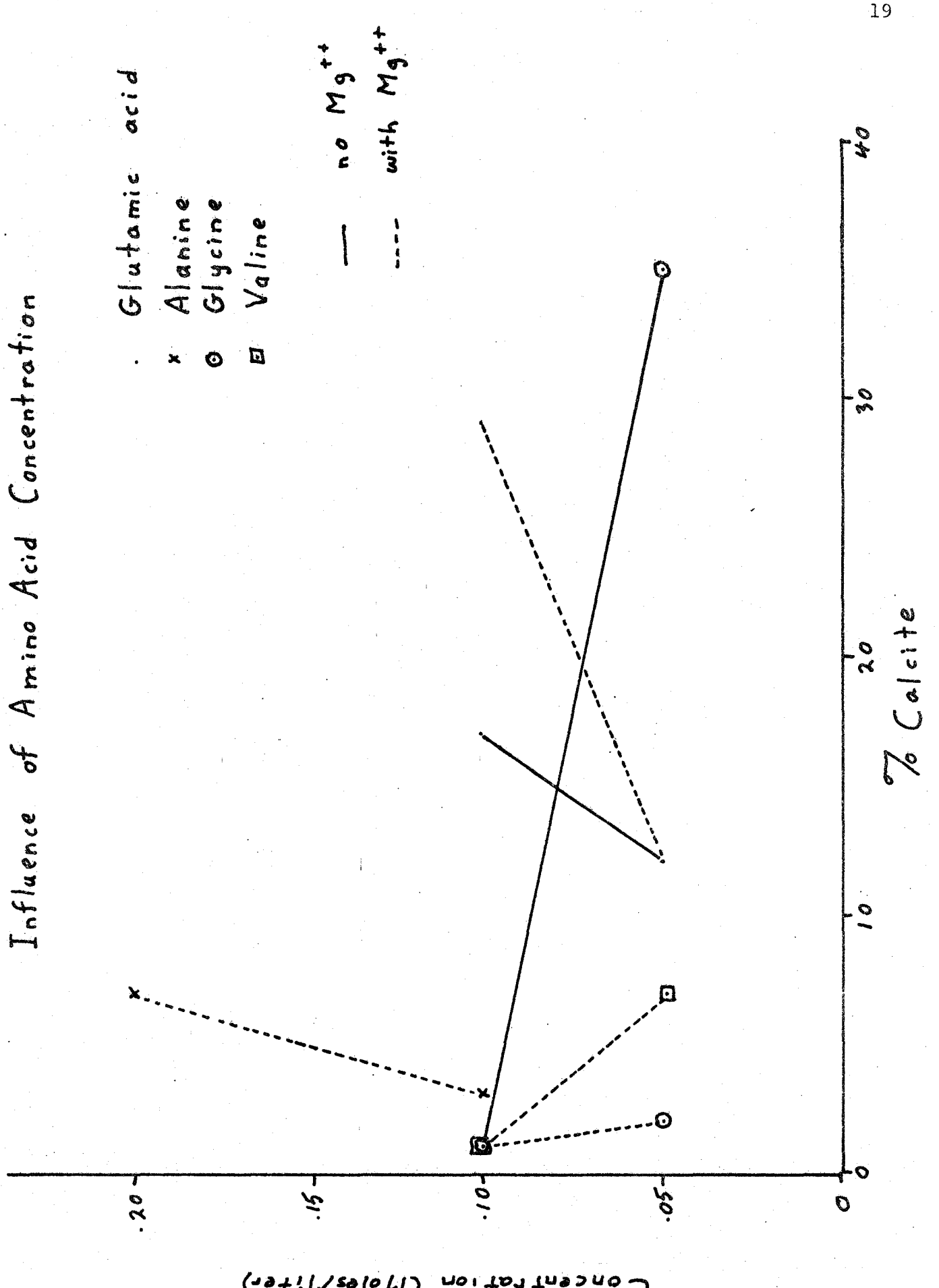
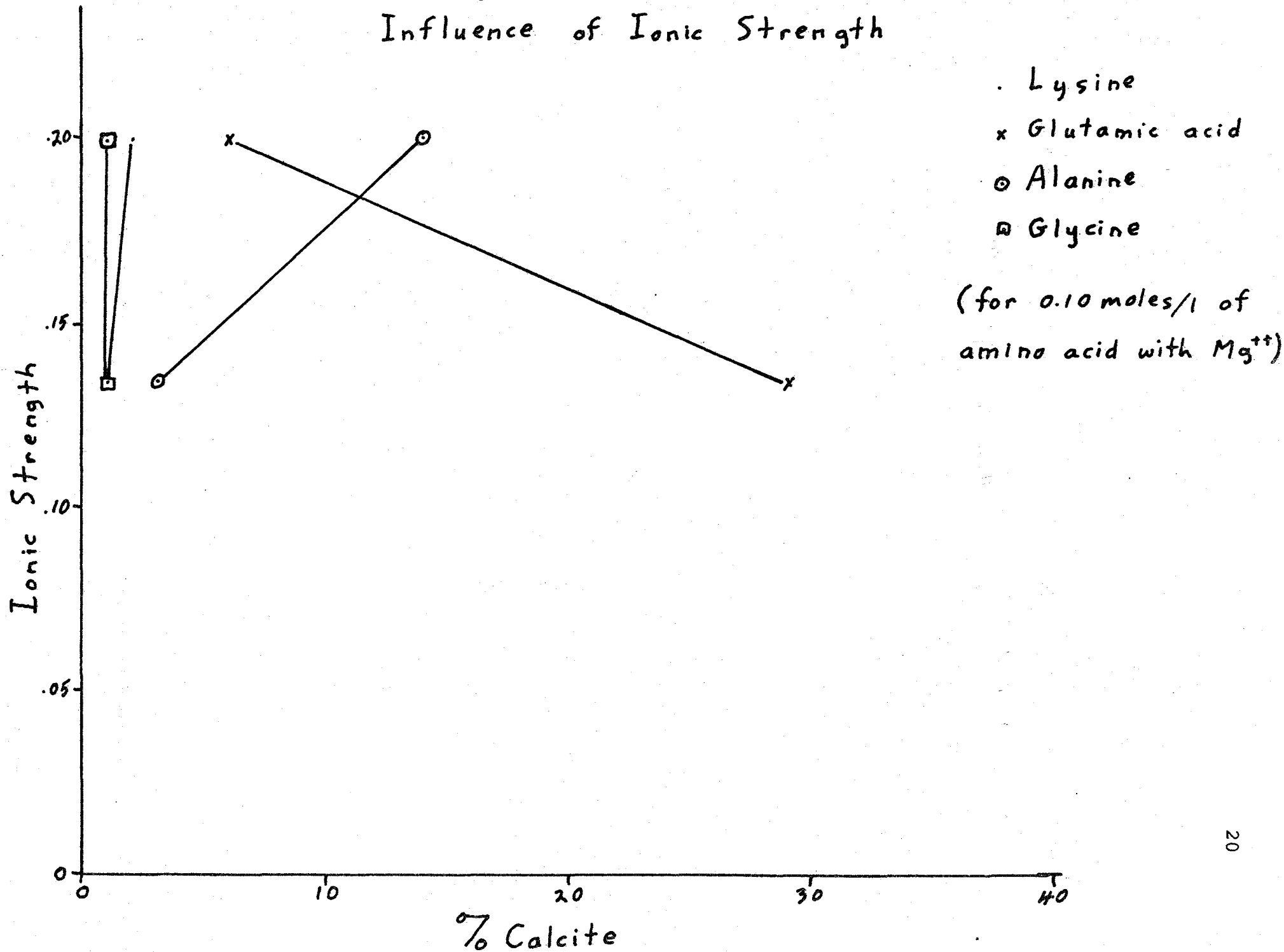


Figure 4.

Influence of Ionic Strength



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