

## Reactions between Egg-shell Matrix and Metallic Cations

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### SUMMARY

The interaction between egg-shell matrix and various metal ions has been studied by considering the effect of these ions on the staining of the matrix by toluidine blue. The validity of the procedure and the influence of pH and various anions were tested. The main hypothesis to emerge was that the matrix acts as a chelating agent. Curves obtained when quantities of sodium hydroxide added to matrix were plotted against pH values were found to vary in position according to the absence or presence of cations and with different cations. This also supports the hypothesis that the matrix acts as a chelating agent. The significance of this in relation to the deposition of calcium carbonate as egg-shell is briefly discussed.

### INTRODUCTION

RUBEN and Howard (1950) found that acid mucopolysaccharides occurred at most sites of calcification in the body, whilst Sobel and Burger (1954) have shown that calcium ions can compete with toluidine blue to combine with the organic matrix of rachitic bone. The latter workers then suggested that in the rachitic bone the acid mucopolysaccharide, chondroitin sulphuric acid, was probably the substance for which calcium and toluidine blue were competing. Experiments with other ions led Sobel and Hanok (1952) to state that it was the ionic radius of the various metal ions which caused variations in the degree of inhibition of calcification of the rachitic bone matrix.

Levene and Schubert (1952) approached the problem differently by making a spectrophotometric study of the metachromatism of thiazine dyes produced by chondroitin sulphate. They found that additions of neutral salts of calcium at concentrations of  $10^{-3}$  to  $10^{-1}$  M gave greater losses of metachromatism than did neutral potassium salts.

The organic matrix of hen egg-shell, prepared by the decalcification of egg-shells with ethylenediamine tetra-acetate (EDTA), showed similar properties to rachitic bone, in that calcium ions compete with toluidine blue to combine with it (Simkiss and Tyler, 1957). It was, therefore, decided to investigate the matter further.

### VALIDITY OF THE METHOD

Simkiss and Tyler (1957) used EDTA to decalcify egg-shells, and prepared paraffin sections of formalin-fixed decalcified material on slides in the normal way. Individual preparations were then placed in a series of solutions of 0.01% toluidine blue with different concentrations of calcium ions, and the

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competition between the dye and the cation for the shell matrix was assessed by the intensity of staining.

Before making more extensive use of this method, it was necessary to test the assumption that dye and cation do compete for the shell matrix.

(1) There is the possibility of a reaction between dye and cation. The results obtained with calcium and described in the previous paper might be explained on the grounds that the metallic ions combined with the toluidine blue to produce a non-staining substance and that the matrix itself took no part in the reaction. An experiment was therefore designed to make sure that this was not the case.

Four serial sections of the egg-shell matrix were prepared as above. The first was placed in toluidine blue (0.01%) to show that it stained metachromatically, the second and third were placed in a solution of 250 m. eq. per litre ferric ions (as  $\text{Fe}_2(\text{SO}_4)_3$ ) for 1 h. They were then removed and drained, and whilst the second was put into a 10% solution of the trisodium salt of EDTA to remove the ferric ions, the third was left in glass-distilled water. After 2 h the slide in the EDTA was removed, washed, and left for 1 h in glass-distilled water to wash out any remaining EDTA from the section. Both slides were then placed in 0.01% toluidine blue solution. The slide which had been treated with EDTA stained metachromatically after 5 min, the other showed no staining even after 12 h. The fourth slide was placed in the stain with the ferric ion treated sections. This slide stained metachromatically, showing that the stain itself had not been destroyed.

From this experiment it may be argued that, since the ferric ions and the stain never come together in the free state, the loss of staining must be caused by a combination of the cation and the egg-shell matrix and not by a direct interaction between the ferric ion and the stain. Furthermore, the removal of the ferric ions by means of EDTA regenerates the staining property of the shell matrix.

It may still be suggested that the slide treated with ferric ions fails to stain because the ions come off the section and react with the dye to give a colourless compound, but the result with the fourth slide shows that this cannot be so because the slide stains even though placed in the same solution as that occupied by the slide containing ferric ions. In any event, there is such an excess of toluidine blue over ferric ions that the dye could not all be changed to a non-staining substance. It seems, therefore, that the method is valid in that a combination does actually occur between the shell matrix and cations.

(2) There might be residual EDTA in the sections. EDTA may have combined with the shell matrix during decalcification and therefore it might be the EDTA and not the actual shell matrix which is combining with the cations. To clarify this point, egg-shells were dissolved in hydrochloric acid and washed, and the residue was then dried on to slides. These pieces of shell stained metachromatically with toluidine blue solution and staining could be prevented by using solutions of calcium ions just as it could when EDTA had been used (Simkiss and Tyler, 1957). Thus, acid-decalcified shells showed

the same properties as ones treated with EDTA, and it may, therefore, be concluded that any reaction of the shell matrix with cations is due to the shell matrix itself and not to any EDTA attached to it.

We may thus conclude that the cations do compete with toluidine blue for combination with the matrix. The next stage was to use the method for assessing the competitive value of different cations.

#### EFFECT OF DIFFERENT CATIONS

A series of experiments was set up for each solution, with increasing concentrations of the metal ion in 0.01% toluidine blue solution. Sections were left in these solutions for at least 30 min to reach equilibrium and then the degree of metachromatic staining of the matrix was scored with values between 0 and + + + + as judged by eye. The concentration of cation which produced a marked fall or complete elimination of the staining in the section will be referred to as the 'elimination concentration'.

TABLE I

*Elimination concentrations (m. eq. per litre) for cations used in conjunction with different anions*

<i>Salt</i>	<i>Elimination concentration</i>	<i>pH at elimination concentration</i>
FeCl <sub>3</sub>	1	3.60
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	1	—
AlCl <sub>3</sub>	1	4.34
Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	1	—
BeCl <sub>2</sub> *	5	—
CuCl <sub>2</sub>	5	4.64
CuSO <sub>4</sub>	4	—
NiCl <sub>2</sub>	70	5.06
Ni(NO <sub>3</sub> ) <sub>2</sub>	70	—
ZnCl <sub>2</sub>	100	5.50
Zn(NO <sub>3</sub> ) <sub>2</sub>	100	—
FeCl <sub>2</sub> *	—	3.60
FeSO <sub>4</sub>	100	—
MnCl <sub>2</sub>	160	5.00
MnSO <sub>4</sub>	160	—
CaCl <sub>2</sub>	180	5.10
SrCl <sub>2</sub>	220	4.80
LiCl	325	5.84
NaCl	400	5.50

\* Impure substance.

Water of crystallization not included in formulae.

The results are shown in table I. Each cation was tested along with a chloride ion and then various other anions were used with one or other of the cations. From the table it will be seen that the sulphates and nitrates behaved like the chlorides, the only exception being with ferrous salts. With ferrous sulphate the elimination concentration occurred at 100 m. eq. per litre but with ferrous chloride no staining was obtained with toluidine blue even with

only 50 m. eq. per litre ferrous ions. It may be that this discrepancy is caused by impurities in the ferrous chloride, for it was not possible to obtain this material at analytical reagent standard. Furthermore, this substance readily oxidizes to the ferric state.

#### EFFECT OF pH

No attempt was made to buffer the solutions, since this would lead to complications by adding other cations or competing anions. Measurements of pH were, therefore, made at the elimination concentration. Table 1 shows that the range of pH was from 3.6–5.8 with most of the results between 4.6 and 5.6. Further experiments were carried out to measure the effect these pH differences were having on the staining of the matrix. Solutions of cupric chloride (pH 4.6), calcium chloride (pH 5.1), and sodium chloride (pH 5.5) were brought to a pH of about 3.6 by adding dilute hydrochloric acid, and the results of staining were compared with the untreated solutions as shown in Table 2. Increased acidity results in a fall in staining intensity. The elimination concentration is not altered, however, by this fainter staining. In those

TABLE 2

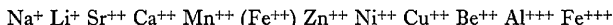
*Effect of pH on staining (O to +++) with toluidine blue in the presence of various concentrations of cation*

Concentration of solution (m. eq. per litre)	Sodium		Calcium		Cupric	
	pH 5.50	pH 3.60	pH 5.10	pH 3.60	pH 4.64	pH 3.60
500	trace	trace	O	O	O	O
400	++	+	O	O	O	O
250	++++	++	O	O	O	O
200	++++	++	+	trace	O	O
150	++++	—	++	+	O	O
100	++++	—	++++	++	O	O
10	++++	—	++++	—	trace	trace
5	++++	—	++++	—	++	+
0	++++	++	++++	++	++++	++

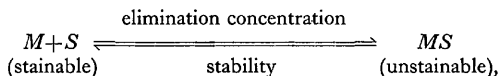
cases where the elimination concentration is shown by a marked fall in staining, the lower general level of staining does not affect the point where the marked fall occurs. Where complete elimination of staining occurs, the additional competition of the hydrogen ions may affect the result slightly. However, this is only likely to affect cupric, beryllium, aluminium, and ferric ions, which, in any event, give a very small value for the elimination concentration. They are also the cations which are most likely to produce the most stable compounds with the matrix and, therefore, would be the least affected by the slight increase in competition due to hydrogen ions. The results with cupric ions at pH 3.6 illustrate this experimentally. From this it is concluded that over the range of values used, the pH affected the intensity of staining but not the elimination concentration.

## ELIMINATION CONCENTRATION AND STABILITY

Ignoring the unreliable ferrous chloride result, it is possible to put the cations in a series of decreasing elimination concentrations as follows:



It seems likely that the elimination concentration for staining gives some measure of the relative affinity of the shell matrix for different ions, a high value indicating little affinity. As the combination of the cation and the shell matrix is presumably in a state of equilibrium, an experiment which measures the stability of the metal-matrix compound should give a similar series to that for the elimination concentration. The relationship of the two may be expressed as follows:



where  $M$  is the cation,  $S$  is the shell matrix, and  $MS$  is the combination of the two.

In measuring elimination concentration we are, presumably, obtaining some measure of the concentration of  $M$  which is necessary to displace the equilibrium so that  $MS$  (unstainable) forms at the expense of  $S$  (stainable). This presumption can be tested by the converse experiment of measuring the stability of  $MS$  when conditions are created for its breakdown.

In an attempt to measure stability the following experiment was tried. Sections of shell matrix were placed in solutions containing 250 m. eq. per litre of the various cations. After 12 h, when it was assumed that the shell matrix and cation would have combined to form the non-staining compound, the slides were drained and transferred to 0.01% toluidine blue solution. They were then observed at regular intervals until metachromatic staining was obtained, i.e. until the reaction produced sufficient of the compound  $S$  to be detected by staining. The time required gave a measure of the stability of  $MS$ .

The results did not enable a series to be established but the general picture strongly supported the evidence based on elimination concentrations. Ferric and aluminium ions gave very stable compounds between cation and shell matrix, for even after 4 days there was no staining with the slides treated with ferric ion and it was only very slight with aluminium. The slide treated with beryllium was stable for about 22 h, cupric ions gave about 5 min stability, and the other cations of Table 1 produced compounds of less than 3 min stability. Even at 500 m. eq. per litre lithium and sodium showed very low stability.

As will be explained in the discussion, this evidence suggested that the shell matrix might contain a chelating agent and therefore it was decided to carry out titration experiments in an effort to obtain more evidence of chelation.

Before leaving this aspect of the subject, it should be stressed that the descriptions of staining with toluidine blue refer to the general state of the section, but that there is considerable evidence to suggest that different layers of the shell stain with a somewhat different intensity.

#### TITRATION EXPERIMENTS

Martell and Calvin (1952) have pointed out that shifts in the titration curves for compounds in the presence and absence of cations are a quick and simple method of testing for chelation, because the process of chelation results in the displacement of protons.

Twelve to fifteen hen egg shells were carefully washed in warm water and then placed in 2N hydrochloric acid for a few seconds. The acid quickly seeped through the organic cuticle and membranes and began to attack the true shell. The cuticle and membranes were thus loosened and the cuticle could then be easily wiped off and the membrane could be peeled off. These shells were then broken into small pieces and dissolved in about 3 l. of N hydrochloric acid, containing 5% formalin to prevent any solution of the organic matter. When all the calcium carbonate of the shell had dissolved, the suspension of organic matter was centrifuged at 2000 r.p.m. for 30 min and the supernatant acid poured away. The gel was then broken up with a jet of water and re-centrifuged, after which it was taken up as a suspension in approximately 50 ml N/100 hydrochloric acid.

It was impossible to obtain exactly the same amount of matrix in each aliquot of the suspension, hence there was no need to ensure that the N/100 acid was absolutely accurate. However, for each batch, the titrations gave consistent results.

Twenty or twenty-five ml portions of the matrix suspension, with or without additions of cations, were titrated with N/50 sodium hydroxide solution and pH readings were taken after each addition. Titrations were made on the following suspensions: (a) suspension alone, (b) suspension plus 0.1 m. eq. copper as cupric chloride ( $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ), (c) suspension plus 0.2 m. eq. copper, (d) suspension plus 0.2 m. eq. nickel as nickel nitrate ( $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ), (e) suspension from shells boiled for 14 hours with 10% sodium hydroxide solution (Tyler and Geake, 1953) before preparing the matrix from them, (f) alkali treated shell plus 0.1 m. eq. copper, and (g) N/100 solution of HCl plus 0.1 m. eq. copper.

Some of the results are shown in fig. 1, but values for additions of alkali below 10 ml have been omitted since they merely represent the neutralization of excess acid and give more or less similar readings for all curves. The suspension of matrix (a) and of treated matrix (e) give smooth curves but they are in quite different positions. The curve for copper in acid (g) shows a step at about pH 6.5, which presumably represents the precipitation of copper as the hydroxide. When the matrix suspension is titrated in the presence of 0.1 m. eq. of copper, the curve (b) gives a small step at about pH 6.5 associated with precipitation, but at much lower pH values, starting about pH 3.5, there

is a considerable deviation of the curve away from curve (a). The curve for treated matrix in the presence of copper (f) gave the step associated with precipitation but little or no deviation, for, until pH 5.7 is reached, this curve is very similar to the curve for treated matrix alone (e). Although the curves (c) and (d) are not presented, it may be stated that with twice the amount of copper present, the step related to precipitation is again from pH 6 to 7, but

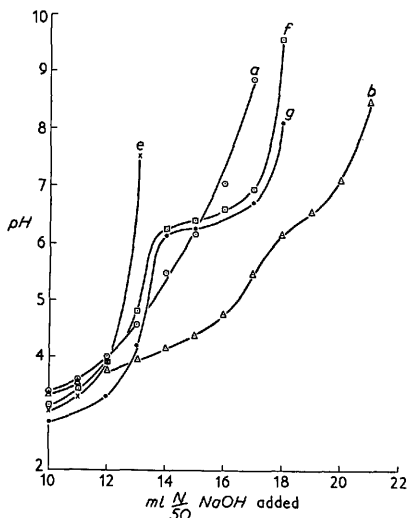


FIG. 1. Titration curves for shell matrix against N/50 sodium hydroxide. a, matrix alone; b, matrix plus copper; c, treated matrix alone; e, treated matrix plus copper; f, treated matrix alone; g, copper alone. Curves c and d are omitted to avoid confusion.

covers a wider range of sodium hydroxide additions; once more, however, there is the deviation starting at about pH 3. The use of nickel gave a step probably associated with precipitation, but also a deviation starting at about pH 4.

These results show that there are steps in the curves when copper and nickel are present and these are associated with precipitation of the hydroxide. In addition, the presence of nickel produces a shift in the curve for the matrix from a normal shell, whilst copper produces an even greater displacement. The shift in these curves shows a displacement of protons, a result typical of chelation. This shift does not occur when matrix from an alkali-treated shell is used, which suggests that the chelating agent in the matrix has been removed by this treatment.

## DISCUSSION

Sobel and Hanok (1952) considered that the organic matrix of rachitic bone combined with various cations according to their ionic radii, but, in the present experiments with decalcified shell matrix, the elimination concentrations and the stability of the metal-matrix compounds do not vary in any direct way either with the ionic radius or, for that matter, with the ionic charge.

Further examination of the results indicates that the series obtained was similar to that for the formation constants of chelation compounds. This

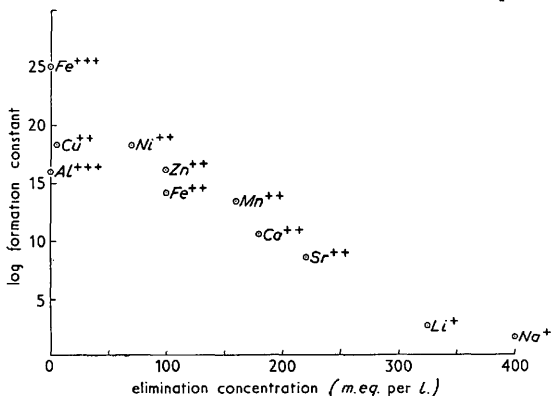


FIG. 2. Graph showing the relationship between the elimination concentration and the logarithm of the formation constant of EDTA chelation compounds for various cations.

relationship is shown in fig. 2, where the elimination concentration has been plotted against the logarithm of the formation constant of EDTA chelation compounds for each cation (Bersworth Chemicals, 1953). Chelation involves a union between the metallic ion and the chelating molecule. The bond arises from dative covalencies, and since in chelation at least two bonds hold the molecule to the metal ion, the structure consists of one or more rings. Different metals have different capacities for co-ordination and there is thus a natural order of metals in all co-ordination problems which is only slightly modified in particular cases by the relative affinity of particular groups for particular metals. In this respect, Irving and Williams (1948) have shown that in the absence of any major spatial limitations in the structure of the molecule, the order of intensity of chelation of metals of the first transition series is independent of the nature of the chelating agent. Thus it would be expected that if there is a chelating agent in the shell matrix, it would not only chelate with metals other than calcium, but would do so in a particular series.

The series of the elimination concentrations for different cations shown here is, in fact, similar to the chelation series of cations, and the variations in stability of the cation-matrix complex (*MS*) with different cations gives some support



to this parallelism. It is known that the strength of chelation will increase with an increase in ionic charge, but in chelation other factors are also involved which complicate a simple relationship with either ionic charge or radius. On the basis of the type of test described by Martell and Calvin (1952), the titration curves obtained show a displacement of protons and thus support the idea that chelation is occurring.

The elimination concentration and stability experiments therefore suggest chelation and the shifts in the titration curve support this concept. Simkiss and Tyler (1957) have shown that the shell matrix contains an acid mucopolysaccharide, probably mucoitin sulphuric acid, and it is now suggested that this is probably the substance which is responsible for the chelation. Mucoitin sulphuric acid contains secondary amino, hydroxyl, carboxyl, and sulphate groups, any of which could take part in the formation of chelate rings, but it must be remembered that some of these groups may be bound to the protein in the matrix and hence it is not yet possible to be more specific as to which groups are involved. In addition, the effect of formalin fixation on protein may alter the picture.

The role played by mucoitin sulphuric acid as a chelating agent in the egg-shell may be of considerable importance to the question of calcification, although the experimental evidence suggests that the chelation compound between calcium and shell matrix is not very stable.

In the hen the calcium is only present in a weak solution in the blood; at the very most 40 mg Ca/100 ml serum, i.e. 20 m. eq. per litre. A chelating agent might act by removing calcium from this weak solution with the formation of a calcium-matrix compound, until carbonate ions can rob it of its calcium ions and form calcium carbonate and free matrix. This mechanism would ensure the precipitation in the shell itself of calcium carbonate, intimately fitted into a definite structure with the matrix, whereas the direct interaction of free calcium and carbonate ions does not seem to offer the same opportunity to build up a structure such as is found in egg shells.

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