

Interaction of Cromolyn Sodium in Aqueous Solution with Magnesium Ions

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Abstract □ The interaction of the antiasthmatic drug cromolyn sodium with Mg^{2+} ions in solution was studied. Selective ion electrode and torsion viscometer measurements were used to establish the degree of interaction. At the concentrations studied, the cromolyn ion had no more affinity for Mg^{2+} than SO_4^{2-} . However, thixotropic gels were formed between $4 \times 10^{-2} M$ cromolyn sodium and $4 \times 10^{-4} M Mg^{2+}$. At lower concentrations of Mg^{2+} , viscosity still increased significantly. However, when the cromolyn concentration was lowered, the increase in viscosity was much less marked.

Keyphrases □ Cromolyn sodium—interaction with magnesium ions in solution □ Magnesium ions—interaction with cromolyn sodium in solution □ Antiasthmatic agents—cromolyn sodium, interaction with magnesium ions in solution □ Metals—magnesium ions, interaction with cromolyn sodium in solution

Cromolyn sodium¹ represents a new pharmacological approach to allergic disease, particularly asthma (1). As is often the case, new and useful drugs are in widespread use before there is any clear understanding of their mechanism of action; this situation is particularly true of cromolyn sodium. However, it seems well accepted that cromolyn sodium does not interfere with antigen-antibody reactions and that it probably acts on the biochemical events in the mast cell that follow the antigen-antibody interaction on the membrane (2).

BACKGROUND

Several investigators (3, 4) explained its mechanism of action on the basis of the role of bivalent ions, especially calcium. However, until recently, these hypotheses arose more from the knowledge that calcium and magnesium play a key role in antigen-induced secretion of histamine from mast cells rather than any direct studies on the interaction of calcium and magnesium ions on cromolyn. By using calcium ionophores (substances that transport calcium across biological membranes and other organic phases), further understanding has been gained of the complex biochemical nature of histamine release from mast cells (5-7).

It seems that the primary event after union of antigen with mast cell-fixed antibody may be an increase in the mast cell membrane permeability to calcium, which promotes entry of calcium into the cell. The movement of calcium ions from the extracellular to the intracellular compartment of mast cells is sufficient to stimulate metabolically intact cells to secrete histamine. The calcium ionophore provides an alternative route for calcium to enter into mast cells, by passing the calcium-gating mechanism operated by the antigen-antibody reaction.

Although a preliminary study (8) suggested that the ionophore-induced release of histamine from rat mast cells could be inhibited by cromolyn, more extensive experiments did not demonstrate any effect (9). Cromolyn probably acts by blocking the antigen-induced transport of calcium either directly or indirectly. Binding of cromolyn to Ca^{2+} could not be demonstrated by various sensitive physical techniques (10); the effect in calcium influx is probably indirect.

As part of a study of the cromolyn ion, various metal-ion salts were prepared, including cromolyn magnesium. This salt was selected following difficulties in the preparation of a clear 2% (approximately $4 \times 10^{-2} M$) cromolyn sodium solution for clinical investigation of rhinitis. In spite of the use of purified water BP for clinical trial supplies, faintly opalescent solutions were obtained frequently. In subsequent investigations in-

volving analysis of purified water BP for heavy metals, the level of Mg^{2+} was found to be important. At low concentrations of Mg^{2+} (10^{-5} - $10^{-6} M$), opalescent solutions were obtained; the highest Mg^{2+} concentration studied ($4 \times 10^{-4} M$) in a $4 \times 10^{-2} M$ cromolyn sodium solution caused the solution to gel after 2 or 3 hr at room temperature, so that a 1.27-cm diameter tube of solution could be laid on its side before the gel slid down the tube. At this point, there was no sign of visible precipitate and it took about 48 hr before it was detectable. As either the cromolyn or Mg^{2+} concentration was reduced, the "gel" was more fluid. However, if the temperature was reduced, the gel consistency could be increased.

It was decided to follow up these initial interesting observations since the Mg^{2+} -cromolyn interaction might be of some biological significance. The purpose of this study was to investigate the gelling of cromolyn sodium solutions by Mg^{2+} . This interaction was investigated directly by comparative results between binding effects of sulfate and cromolyn for magnesium ions (selective ion electrode measurements) and apparent viscosity measurements of cromolyn sodium solutions in the presence of varying amounts of magnesium acetate (torsion viscometry).

EXPERIMENTAL

Selective Ion Electrode Measurements—A divalent metal electrode was used to detect Mg^{2+} . Magnesium acetate (10^{-1} - $10^{-4} M$, depending on the experiment) was added to sodium sulfate solutions (10^{-2} - $10^{-3} M$) to standardize the ion scale on the pH meter². After calibration, magnesium acetate (10^{-1} or $10^{-2} M$) was titrated into sodium sulfate (10^{-2} or $10^{-3} M$) to confirm the meter settings, followed by replicate titrations of magnesium acetate into cromolyn solutions.

The meter was calibrated to read directly the known magnesium concentrations of the sulfate standards. This step assumed that all magnesium ions were free, not bound to sulfate ions, which was correct for the lower concentrations ($10^{-4} M$). At higher concentrations ($10^{-3} M$), sulfate did bind a small amount of magnesium. Thus, all results for the cromolyn ion are comparative between the binding effects of sulfate and cromolyn for magnesium ions.

Only water that complied with the requirements of the BP for purified water was used; it could not contain more than 0.04 ppm of Mg^{2+} . It was freshly distilled from a neutral glass still fitted with an efficient device to prevent the entrainment of droplets and was used within 4 hr.

The first series of experiments (Series A) used dilute ($10^{-3} M Na_2SO_4$ - $10^{-3} M$ cromolyn) solutions, and the electrode responded to the H^+ concentration formed from carbonic acid, arising from dissolved carbon dioxide in the distilled water. To avoid this problem, $10^{-4} M$ sodium carbonate was used to maintain the pH close to or slightly above 7.

Series A calibration solutions were $10^{-4} M Mg(CH_3COO)_2$ - $10^{-4} M Na_2CO_3$ - $10^{-3} M Na_2SO_4$ and $10^{-3} M Mg(CH_3COO)_2$ - $10^{-4} M Na_2CO_3$ - $10^{-3} M Na_2SO_4$. Series A titrations were $10^{-2} M Mg(CH_3COO)_2$ into $10^{-3} M Na_2SO_4$ - $10^{-4} M Na_2CO_3$ and $10^{-2} M Mg(CH_3COO)_2$ into $10^{-3} M$ cromolyn- $10^{-4} M Na_2CO_3$.

The second set of experiments used a more concentrated solution ($10^{-2} M$), which approached the $4 \times 10^{-4} M Mg^{2+}$ - $4 \times 10^{-2} M$ cromolyn solutions in which gelling had been found. To avoid severe Na^+ interference, the cromolyn concentration was $10^{-2} M$ (~0.5%). Even at this concentration, there was some Na^+ interference, and it was not possible to calibrate the meter to read the free Mg^{2+} concentration directly. Nevertheless, it was possible to obtain comparative results between the binding of Mg^{2+} by cromolyn and by sulfate. In these experiments, since carbonic acid was not a problem, it was not necessary to use a dilute sodium carbonate solution.

Series B calibration solutions were $10^{-3} M Mg(CH_3COO)_2$ - $10^{-2} M Na_2SO_4$ and $10^{-2} M Mg(CH_3COO)_2$ - $10^{-2} M Na_2SO_4$. Series B titrations

¹ Disodium cromoglycate, Intal, Fisons.

² Model 7030, Electronic Instruments Ltd., Chertsey, Surrey, England.

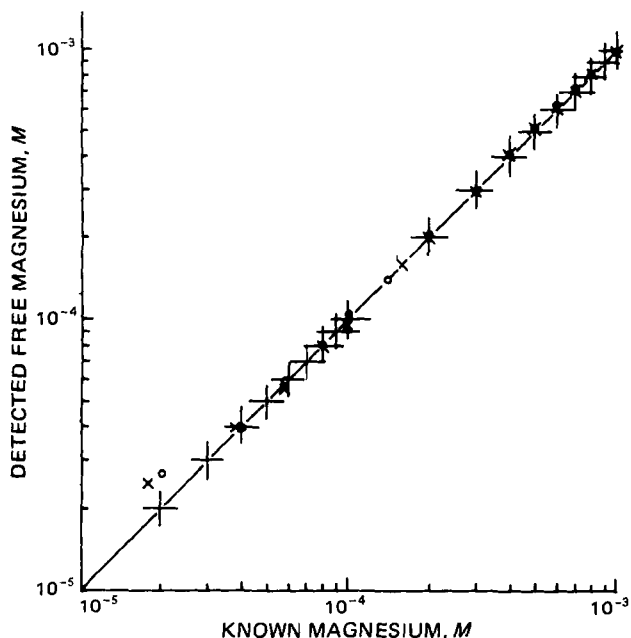


Figure 1—Titration of 10^{-2} M magnesium acetate with 10^{-4} M sodium sulfate (X) or 10^{-3} M cromolyn (O) (Series A).

were 10^{-1} M $\text{Mg}(\text{CH}_3\text{COO})_2$ into 10^{-2} M Na_2SO_4 and 10^{-1} M $\text{Mg}(\text{CH}_3\text{COO})_2$ into 10^{-2} M cromolyn. A volume of 50 ml of solution was used in these experiments, and the solution was stirred continuously to ensure homogeneity. All experiments were replicated.

A final series (C and D) of selective ion electrode measurements was made on fixed, more concentrated solution; the Mg^{2+} concentration was monitored as a function of time. These experiments covered the concentration at which gelling had previously been observed, i.e., 4×10^{-4} M Mg^{2+} – 4×10^{-2} M cromolyn.

The fixed composition experiments, where the free magnesium concentration was monitored with time, were: Series C, 4×10^{-4} M $\text{Mg}(\text{CH}_3\text{COO})_2$ – 10^{-2} M Na_2SO_4 and 4×10^{-4} M $\text{Mg}(\text{CH}_3\text{COO})_2$ – 10^{-2} M cromolyn; and Series D, 4×10^{-4} M $\text{Mg}(\text{CH}_3\text{COO})_2$ – 4×10^{-2} M Na_2SO_4 and 4×10^{-4} M $\text{Mg}(\text{CH}_3\text{COO})_2$ – 4×10^{-2} M cromolyn. The concentration of 4×10^{-4} M $(\text{CH}_3\text{COO})_2$ closely corresponds with 10 ppm of Mg^{2+} , and 4×10^{-2} M cromolyn is close to 2%. At these concentrations, the solution was known to gel.

Series C and D experiments were performed in quadruplicate because the reproducibility was poor.

Viscosity Measurements—Since the solutions were known to be thixotropic, a torsion viscometer³ was used that had an additional scale at the top of the torsion wire. This scale enabled the torsion to be applied to the wire without disturbing the solution prior to measurement and, therefore, avoided a reduction in the solution's apparent viscosity. The wire (38 cm; 36 swg⁴) and cylinder (41.5 mm diameter) were chosen to give maximum sensitivity in the range of 15–300 cps. The cylinder was covered by approximately 1 cm of solution, and the temperature was controlled at 21° by a water jacket fed from a thermostated bath.

To operate the viscometer, the top of the torsion wire was rotated 180° while the flywheel on the lower end, a few centimeters above the cylinder, was clamped. The flywheel was released, and the overswing (swing exceeding the offset 180°) was noted. The viscosity was derived from the overswing by calibrating the viscometer with water–glycerol mixtures.

For most experiments, the viscosity was measured 1 min after inserting the freshly prepared solution into the viscometer. After 5 min, the viscosity was remeasured, the solution was stirred thoroughly, and a further measurement was taken. The solution was left undisturbed for a fixed time, and the 5-min procedure was repeated. This reading was the time since the thixotropic solution was last stirred but the actual elapsed time was longer. This procedure was repeated at intervals. A few experiments were undertaken with calcium ions present to see if they caused a similar effect.

³ Laboratory model VS-010, A. Gallenkamp & Co., London EC2 PER, England.

⁴ Standard wire gauge, British Standards Institute.

Table I—Variation in Apparent Free Magnesium-Ion Concentration with Time^a

| Series C ^b | | Series D ^c | |
|-----------------------|-------------------------------------|-----------------------|-------------------------------------|
| Time | $\times 10^{-4}$ M Mg^{2+} | Time | $\times 10^{-4}$ M Mg^{2+} |
| 1 min | 4.0 (4.0–4.1) | 1 min | 4.0 (3.9–4.1) |
| 10 min | 4.0 (3.9–4.0) | 10 min | 4.0 (3.9–4.0) |
| 2 hr | 3.1 (3.0–3.2) | 6 hr | 2.9 (2.4–3.2) |
| 24 hr | 3.1 (2.9–3.2) | 24 hr | 2.6 (2.1–3.0) |
| 2 days | 2.7 (2.4–2.9) | 2 days | 2.4 (1.9–2.9) |

^a Calculation of the two correlation coefficients on a semilogarithmic scale for linearization gives $r = -0.94$ for C and $r = -0.97$ for D. ^b Using 4×10^{-4} M $\text{Mg}(\text{CH}_3\text{COO})_2$ – 10^{-2} M cromolyn. ^c Using 4×10^{-4} M $\text{Mg}(\text{CH}_3\text{COO})_2$ – 4×10^{-2} M cromolyn.

RESULTS

Interaction between Cromolyn and Magnesium Ions—The results of titrating 10^{-2} M $\text{Mg}(\text{CH}_3\text{COO})_2$ into 10^{-3} M Na_2SO_4 – 10^{-4} M Na_2CO_3 and the corresponding titration into 10^{-3} M cromolyn– 10^{-4} M Na_2CO_3 are shown in Fig. 1. The results down to 4×10^{-5} M closely follow a 45° slope, which indicates that the actual magnesium-ion concentration and the measured free magnesium-ion concentration as measured at 2×10^{-5} M actual Mg^{2+} may result from a low level of divalent metal impurity (the electrode detected all divalent metals). There is no difference between the cromolyn and sulfate lines in Fig. 1 and, therefore, cromolyn has no more affinity for magnesium than sulfate at these concentrations.

Figure 2 shows the titration of 10^{-2} M $\text{Mg}(\text{CH}_3\text{COO})_2$ into 10^{-2} M Na_2SO_4 or 10^{-2} M cromolyn. The instrument was calibrated at the 10^{-2} M Na_2SO_4 point, so the sodium sulfate line passes through this point. It does not pass through the 10^{-3} M point because the interference from sodium ions could not be entirely compensated by instrumental settings. The significant fact is that the cromolyn points show little deviation from the sulfate points. If anything, more magnesium is detected with cromolyn than with sulfate, probably because sulfate ions bind a small amount of magnesium. Above 10^{-3} M, sulfate binds magnesium to a small extent. It can be concluded that, at these concentrations, cromolyn ions bind magnesium equal to or less than sulfate.

The fixed composition results are shown in Table I. All results were normalized to equivalent sulfate solutions measured throughout the experiments at the same time as the cromolyn solutions. Table I shows that the free magnesium-ion concentration fell with time and that this change was detected long before any precipitate was seen. Means of four values are quoted because the reproducibility was poor; $\pm 20\%$ was the order of variation about the mean for the extreme results of the four. A reduction in free magnesium-ion concentration is to be expected as soon as precipitation occurs.

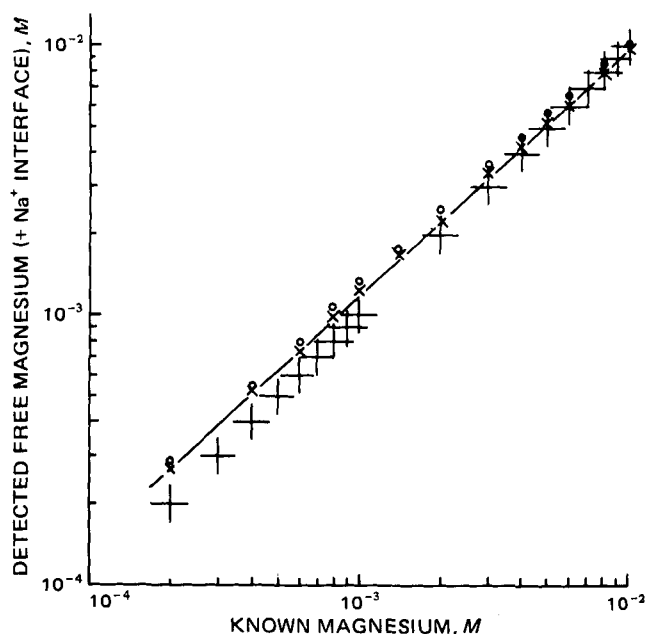


Figure 2—Titration of 10^{-2} M magnesium acetate with 10^{-2} M sodium sulfate (X) or cromolyn (O) (series B).

Table II—Change of Viscosity with Time

| Stirred-Unstirred | $4 \times 10^{-2} M$ Cromolyn | | $4 \times 10^{-4} M$ Mg^{2+} | | $1.2 \times 10^{-4} M$ Mg^{2+} | | $2.4 \times 10^{-4} M$ Mg^{2+} | | $4 \times 10^{-4} M$ Mg^{2+} | | $2 \times 10^{-4} M$ Mg^{2+} | | $4 \times 10^{-4} M$ Mg^{2+} | |
|-------------------|-------------------------------|----|--------------------------------|----------------|----------------------------------|-----|----------------------------------|-----|--------------------------------|----|--|----|--|---|
| | U ^a | S | U | S ^b | U | S | U | S | U | S | Together with $1.2 \times 10^{-4} M$ Ca^{2+} | | $2.5 \times 10^{-4} M$ Ca^{2+} Alone | |
| Time (t) | Viscosity, cps | | | | | | | | | | | | | |
| 1 min | <8 | <8 | <8 | <8 | <8 | <8 | 14 | <8 | <8 | <8 | | | | |
| 5 min | 8 | 8 | 8 | 8 | 8 | 19 | 26 | 15 | 10 | 8 | | | | |
| 15 min | 8 | 8 | 9.5 | 11 | 10 | 240 | 20 | 106 | 22 | 1 | 14 | 8 | 8 | 8 |
| 40 min | 10 | 8 | 15 | 12 | 21 | 10 | 500 | 46 | 260 | 29 | 24 | 16 | 8 | 8 |
| 60 min | 10 | 8 | 19 | 14 | 24 | 10 | 500 | 34 | 500 | 34 | 39 | 18 | 8 | 8 |
| 16 hr | 13 | 8 | 38 | 14 | 250 | 28 | | | | | 150 | 31 | 8 | 8 |
| 48 hr | 16 | 8 | 61 | 14 | 500 | 130 | | | | | 280 | 33 | 8 | 8 |

^a Unstirred for time *t*. ^b These solutions were stirred for 1 min immediately after the unstirred measurement and the viscosity was remeasured.

Viscosity Measurements—The viscosity measurements and assessments are shown in Tables II and III. The viscosity of $4 \times 10^{-2} M$ cromolyn solutions at room temperature changed little in 48 hr, but the addition of $1.2 \times 10^{-4} M$ Mg^{2+} produced a noticeable effect. With $4 \times 10^{-4} M$ Mg^{2+} , the viscosity increased rapidly; after 1 hr, the solution had quite definitely gelled. A reduction in the cromolyn concentration markedly reduced the increase in viscosity; $10^{-2} M$ cromolyn- $4 \times 10^{-4} M$ Mg^{2+} only slightly increased in viscosity after 18 hr.

However, an increase in the magnesium-ion concentration in the more dilute cromolyn solutions did increase the viscosity; e.g., with $10^{-2} M$ cromolyn- $9.6 \times 10^{-4} M$ Mg^{2+} , the viscosity noticeably increased. If part of the magnesium was replaced by calcium, the solutions behaved as though only magnesium at the reduced concentration was present; the calcium ions had no effect. All solutions were thixotropic and showed much lower viscosity after stirring (Table II).

DISCUSSION

The specific ion electrode measurements in which Mg^{2+} was titrated into cromolyn sodium solutions showed that cromolyn ions had no more tendency to bind magnesium than did sulfate during the first 20 min after mixing, the time taken for a single titration. The fixed composition experiments showed that the free magnesium-ion concentration did fall with time, and this effect was detectable up to 48 hr before any precipitate of cromolyn magnesium was seen.

The viscosity experiments clearly showed that $4 \times 10^{-2} M$ cromolyn with 1.2×10^{-4} , 2.4×10^{-4} , and $4 \times 10^{-4} M$ Mg^{2+} increased in viscosity; at $4 \times 10^{-4} M$ Mg^{2+} , the effect was sufficiently marked to describe the

solution as gelled. The time for gelling to be detected was only 10 min for $4 \times 10^{-4} M$ Mg^{2+} and up to 1 hr for $1.2 \times 10^{-4} M$ Mg^{2+} . When the concentration of cromolyn was lowered, the increase in viscosity was much less marked. With $10^{-2} M$ cromolyn and $4 \times 10^{-4} M$ Mg^{2+} at room temperature, it took 18 hr for a change in viscosity to be noticed. Even then, the increase was slight in contrast to the gelling with $4 \times 10^{-2} M$ cromolyn. At 5°, the $10^{-2} M$ cromolyn- $4 \times 10^{-4} M$ Mg^{2+} solution did gel after 48 hr.

A $4 \times 10^{-2} M$ cromolyn solution with $4 \times 10^{-4} M$ Mg^{2+} was supersaturated with respect to cromolyn magnesium, and a faint precipitate of this salt was seen after 48 hr. When observed microscopically, the crystals of cromolyn magnesium were exceptionally elongated and appeared to be branched. These extremely thin, thread-like crystals interwove, and the branches appeared to interconnect at several points. Prior to this precipitate being detected either by slight cloudiness of the solution or microscopically with crossed polars, the nuclei of the salt must be forming and, as they grow, must take up this thread-like, interwoven structure. The growth of such a fine structure would increase the solution viscosity.

However, the rapidity of the gelling and slowness of the precipitate to form suggest that a simple, very fine thread-like network is not the only mechanism involved in the first stage of gelling. It seems unlikely that if it could form in a matter of minutes, it would take 48 hr to grow to a microscopically detectable size. The sharp dependence of gelling on the cromolyn concentration, even though it is in large excess over magnesium, strongly suggests that these ions, in excess of those needed to bond with Mg^{2+} , are involved in gelling.

REFERENCES

- (1) J. S. G. Cox, *Nature (London)*, **216**, 1328 (1967).
- (2) J. C. Foreman and L. G. Garland, *Br. Med. J.*, **1**, 820 (1976).
- (3) J. W. Kerr, M. Govindaraj, and K. R. Patel, *ibid.*, **2**, 139 (1970).
- (4) T. S. C. Orr, D. E. Hall, and A. C. Allison, *Nature (London)*, **236**, 350 (1972).
- (5) J. C. Foreman, J. L. Mongar, and B. D. Gomperts, *ibid.*, **245**, 249 (1973).
- (6) D. E. Cochrane and W. W. Douglas, *Proc. Natl. Acad. Sci. USA*, **71**, 408 (1974).
- (7) R. P. Siraganian, A. Kulczycki, G. Mendoza, and H. Metzger, *J. Immunol.*, **115**, 1599 (1975).
- (8) H. G. Johnson and M. K. Bach, *ibid.*, **114**, 514 (1975).
- (9) L. C. Garland and J. L. Mongar, *Int. Arch. Allergy Appl. Immunol.*, **50**, 27 (1976).
- (10) A. C. Spataro and H. B. Bosmann, *Biochem. Pharmacol.*, **25**, 505 (1976).

Table III—Change of Viscosity with Time for Solutions Stored at Room Temperature

| Hours since Solution Prepared | $4 \times 10^{-2} M$ Cromolyn | | | $4 \times 10^{-4} M$ Mg^{2+} | | |
|-------------------------------|-----------------------------------|------------|----------------------------|--------------------------------|-----|--|
| | 1 | 4 | 1 | 4 | 9.6 | |
| | Viscosity Assessment | | | | | |
| 1 | NCD ^a | Slight gel | NCD | | | |
| 2 | NCD | Slight gel | NCD | | | |
| 18 | Very slight increase in viscosity | Stiff gel | Gelled | | | |
| 42 | Very slight increase in viscosity | Stiff gel | Less gelled, precipitating | | | |

^a No change detected.