Effect of Chlorpromazine upon Calcification *in vitro*. Interaction with Isolated Epiphyseal Cartilage of Rachitic Rats.* (26807)

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It has been shown that a turbidimetric interaction occurs between chondroitin sulfate (CSA) and chlorpromazine solutions(1). Since CSA is the main component of cartilaginous tissues, and is considered to play a role in the calcification process(2-5), it was considered of interest to determine whether chlorpromazine would interfere with the ossification of epiphyseal cartilage. Therefore, a study was carried out to determine the effects of exposure of epiphyseal cartilage obtained from rachitic rats to various concentrations of chlorpromazine solution, with reference to calcification *in vitro*.

Material and methods. Calcification *in vitro* was studied by simplification of the method described by Sobel, Nobel and Hanok(6). Twenty-one day old weanling rats were raised on U.S.P. Rachitogenic Diet No. 2. Three weeks later they were sacrificed, and the tibia was dissected and sliced longitudinally. The tibial sections were placed in calcifying solution and incubated at 37°C for 20 hours. To observe the presence or absence of calcification, the sections were washed with water, placed in 2% aqueous silver nitrate and exposed to the light of an incandescent lamp for about 10 minutes. Calcification appeared as a dark deposit on the surface of the epiphyseal plate. The extent of this deposit was estimated by using numbers from 0 to 4 for the width of plate covered, and by 0, or 1 to 4 plus signs for the length of plate covered. Using this method the range of values can extend from 0 (0) to 4 (++++). To obtain a figure representing overall calcification for graphic purposes, the plus signs representing length were treated as numbers (0 to 4), and multiplied by the figure representing width. This could give a theoretical maximum value of 4 (4+) = 16.

The calcifying solution used in these experiments contained sodium chloride (0.07M), potassium chloride (0.005M), sodium bicarbonate (0.022M), calcium chloride (0.0025M; 10 mg % calcium) and a combination of NaH_2PO_4 plus Na_2HPO_4 at a molarity of 0.00161 corresponding to 5 mg % phosphorus. The pH was adjusted to 7.3 with CO_2.

Luteocobalti chloride was prepared according to the method of Fernelius(7). All other chemicals used in this study were of reagent grade.†

Measurements of turbidity were made with the Bausch and Lomb Spectronic-20 spectrophotometer, set at a wavelength of 420 mu. Measurements of metachromasy were made with the same instrument, using as an index of metachromasy an Optical Density Ratio (ODR) which is equal to OD 550/OD 635. pH measurements were made with the Beckman Model G, pH meter.

Results. Exposure of tibial sections to various concentrations of chlorpromazine resulted in deposition of the compound, as a white precipitate, over the surface of the epiphyseal plate.

Exposure of tibial sections to a solution containing 1.0% chlorpromazine and 5% luteocobalti chloride (pH 5.81) for 1½ hours, was not followed by the usual deposition of cobalt salt, indicating interference by chlorpromazine. Normally both rachitic and non-ossifiable cartilage would bind luteocobalti chloride(3), forming an orange deposit.

Treatment of tibial sections for one hour with chlorpromazine (*i.e.*, 0.5% or 1.41 × 10^{-2}M) followed by shaking in a solution of toluidine blue O plus calcium chloride (*i.e.*,

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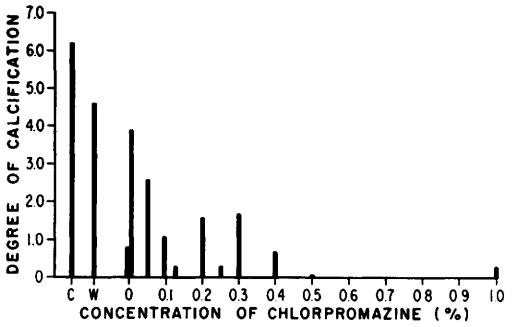

FIG. 1. Inhibition of calcification *in vitro* by prior exposure of sections to chlorpromazine. Sections exposed to concentration of chlorpromazine (as indicated) for one hr, then placed in calcifying solution for 20 hr at 37°C. "C" represents control (N = 22), "W" represents water (N = 18). Total No. of treated sections was 51.

1.06 × 10^{-5}M of dye and 0.125 N of calcium chloride), was not followed by metachromasy. Usually rachitic cartilage will stain metachromatically with solutions of toluidine blue O and calcium chloride. Concomitant exposure to both 0.5% chlorpromazine and the toluidine blue O calcium chloride solution, not only inhibited metachromasy, but caused the deposit of chlorpromazine to become more clearly visible on the epiphyseal plate.

When chlorpromazine was incorporated in the calcifying solution at the pH required for calcification *in vitro* (*i.e.*, 7.3) the process of calcification was inhibited. Shortly after mixing chlorpromazine with the calcifying solution, a turbidimetric interaction occurred, then at the end of incubation period required for calcification, a deposition of needle-like crystals was noticed. To eliminate a possible direct effect of chlorpromazine upon the calcifying solution, sections of rachitic cartilage were exposed to chlorpromazine prior to incubation. The sections were shaken for one hour and then washed with water and incubated in the calcifying solutions for 20 hours at 37°C.

The results (Fig. 1) while manifesting some degree of random variation, indicate that calcification is inhibited by concentrations of chlorpromazine as low as 0.1% (2.8 × 10^{-3}M). Further, degree of calcification appeared inversely related to concentration of chlorpromazine.

Untreated control sections gave an average degree of calcification of 6.2. A total of 27 treated sections were also used as controls to the chlorpromazine experiment. Eighteen sections were treated with distilled water, and 9 were shaken with various concentrations of HCl down to pH 4.3. In both cases degree of calcification did not go below 4.0. In the case of unacidified distilled water, average degree of calcification for the 18 sections was 4.6.

Inhibition of calcification *in vitro* by other agents, *i.e.*, protamine(2,8) and luteocobalti chloride(3) was found to depend on the ratio:

$$\left(\frac{\text{Calcium}}{\text{Inhibitory cation}}\right)$$

meaning that addition of calcium to the solution promoted calcification, preventing inhibition by the inhibitory cations. This reversal of the effect of the inhibitor was also produced by calcium in the case of chlorpromazine as shown in Table I.

Fig. 2 shows that the turbidimetric interaction between chlorpromazine and CSA is a rate phenomenon, the velocity of which steadily decreases over the 35 minute period of study. In the presence of calcium ion (Fig. 2, curve B) rate of growth is initially increased, and a maximum optical density of 1.20 is reached in 10 minutes. From this point curve B descends slowly, while curve A (*i.e.*, without calcium ion) continues to rise, so that by the end of the experiment the 2 curves show tendency to intersect.

When 1.0 N HCl was progressively added to the chlorpromazine-CSA system, no significant change in turbidity occurred until the solution reached pH 2 (Fig. 3). As pH decreased below this point the solution became pink, losing 72% of its turbidity by the time it reached pH 1.0. The pink color was similar to that of chlorpromazine solutions that have been extensively exposed to light.

The chlorpromazine-CSA system was also found to be sensitive to temperature (Fig. 4). With increase in temperature (BC) there was a decrease in turbidity, so that at 70°C the turbidity has been reduced by 88%. Upon recooling the system, turbidity rose again, this time to a higher value than before (CD).

TABLE I. Reversal of Chlorpromazine Inhibition of Calcification *In Vitro* by .5 N $CaCl_2$.

	Sample	Time of exposure, hr	Exp. 1 No. of exp.	Exp. 1 Avg calcification	Exp. 2 No. of exp.	Exp. 2 Avg calcification
A	Control	—	5	4.0	4	3.9
B	Water	1	4	1.8	4	2.6
C	CPZ (1%)	1	4	.5	5	.3
D	"C" followed by .5 N $CaCl_2$	2	4	4.6	5	5.6

Significant changes in turbidity started from a temperature of about 25°C and above.

When chlorpromazine was added to the toluidine blue O-CSA system (*i.e.*, 1.06 × 10^{-5}M toluidine blue-O plus 200 γ of CSA in a total volume of 20 ml) a marked loss of metachromasy was noted. At 25°C and a pH of 6.2, addition of 2 mg % chlorpromazine diminished the metachromasy by 61%.

Discussion. The present study indicates that chlorpromazine interacts with epiphyseal rachitic cartilage, depositing as a white precipitate on the plate surface, preventing binding of luteocobalti chloride, inhibiting metachromasy and finally inhibiting calcification *in vitro*, the latter process being reversed by excess calcium ions. The metachromasy of a solution of toluidine blue O and chondroitin sulfate is also suppressed on titration with chlorpromazine. Further, the interaction between chlorpromazine and CSA has been shown to be a rate phenomenon accelerated by calcium ion. This interaction is relatively stable to acid pH (down to pH 2.0) but is markedly sensitive to temperature changes beyond 25°C. In view of the partial reversibility of the inhibition of calcification *in vitro* by 0.5 N $CaCl_2$, of the demonstrated interaction between CSA and chlorpromazine, and of the competition between chlorpromazine, luteocobalti chloride and toluidine blue O for the binding site on the epiphyseal surface, it is presumed that this binding site is chondroitin sulfate, or the CSA-collagen complex of the cartilage.

Some workers consider that collagen is the essential component of the matrix responsible for nucleation and crystal growth(9), indeed Meyer has suggested that the mucopolysaccharides act as templates for development of

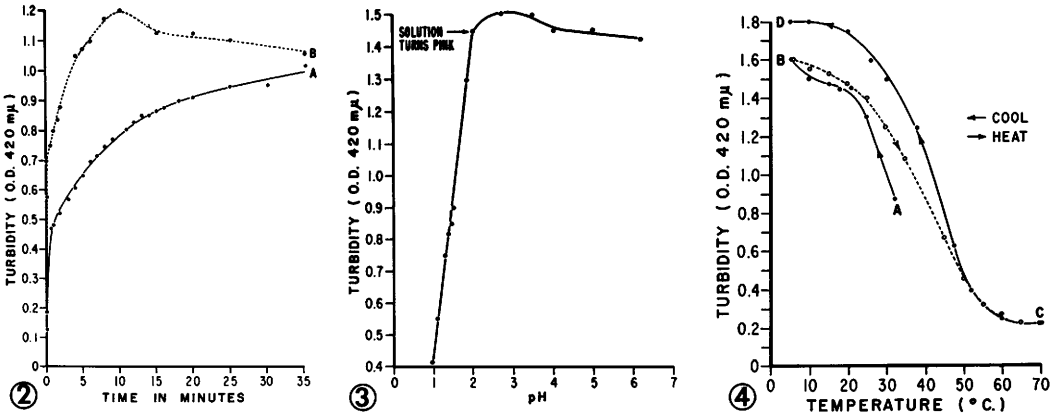

FIG. 2. Turbidimetric interaction of chlorpromazine with chondroitin sulfate as a function of time at room temperature (28°). A) 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml of water. pH 6.2. B) 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml of 0.1 N $CaCl_2$. pH 6.0.

FIG. 3. Effect of pH change (acid region) on interaction between chlorpromazine and chondroitin sulfate. 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml water, titrated with 1.0 N HCl at 25°C.

FIG. 4. Effect of temperature change on interaction between chondroitin sulfate and chlorpromazine. 2 ml of 2% CSA plus 0.55 ml of 0.4% chlorpromazine plus 0.45 ml of water. pH 6.2.

the organized fibrous components of connective tissue(10). Nevertheless, there is a considerable body of evidence indicating that CSA or a CSA-collagen complex functions as the nucleation center. Thus, sulfated mucopolysaccharides exist at sites of both normal and abnormal calcification(11). Rubin and Howard have found an increase in metachromatic staining in regions where active calcification is occurring, which they attribute to an increase in concentration and/or a change in degree of polymerization of CSA(5). Neuman has shown that there is a correlation between the cation binding capacity of cartilage and its sulfate content(4). Miller, Waldman and McLean(12) found that basic dyes inhibit calcification *in vitro* and that this effect can be reversed in presence of calcium and phosphate. Finally it has been demonstrated that protamine(2,8) and luteocobalti chloride(3), both of which precipitate CSA from solution, can cause reversible inhibition of calcification *in vitro*.

The fact that calcification *in vitro* can be accomplished after heating rachitic sections, followed by treatment with calcium chloride, would indicate that enzyme involvement is a minimal factor in the process(11), and that chlorpromazine is not functioning as an enzyme inhibitor in the present instance. It is to be noted, however, that chlorpromazine does function as an inhibitor of a number of enzyme systems. Thus, it suppresses oxidative phosphorylation(13), cytochrome oxidase(13), ATPase(13) and D-amino acid oxidase(14).

This interference with the physical process of calcification will have more pertinent implications for the pharmacology of chlorpromazine, if it can be demonstrated that injection of the drug into the living animal, under both normal and rachitic diets, can either prevent bone formation or potentiate the rachitic state.

Summary. Sections of epiphyseal cartilage from rachitic rats, exposed to solutions of chlorpromazine in water, indicated binding of chlorpromazine to the cartilage surface. In contrast with control, the chlorpromazine pretreated cartilage showed little or no calcification after incubation for 20 hours in a calcifying solution, did not stain metachromatically with a toluidine blue O-calcium chloride solution, and did not bind luteocobalti chloride. Binding of chlorpromazine and/or calcium appeared to be of a competitive nature, excess of one reversing or preventing fixation of the other. Parallel chemical studies support the assumption that chlorpromazine interacts with the chondroitin sulfate of ossifying cartilage.

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