Complexes of Calcium and Magnesium with Oxytetracycline.* (27339)

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Tetracycline and related compounds that form complexes with calcium and other metal ions are deposited in all newly calcifying tissue and can be used to label the growing skeleton(1). The antibiotic action of tetracyclines also may depend upon metal ion complexes(2). The chemistry of some of these complexes has been studied and stability constants derived(3-7). Neither this information nor the stoichiometry is available for calcium ions. Reactions of oxytetracycline with magnesium and calcium, the most abundant multivalent metal ions in animal body fluids, were investigated in order to elucidate the mechanisms of deposition of oxytetracycline in bone. With calcium it was possible to employ the McLean-Hastings frog heart method, and this will be reported in the following article. Spectrophotometry, as described below, proved to be a convenient way to assay the affinity of oxytetracycline for both metal ions; pH, ionic strength, and oxytetracycline concentration were held within physiological ranges. The calcium was raised to high levels and the reactions to hypothetical changes in concentration between serum and bone were observed under controlled conditions. This revealed a step-wise addition of metal to oxytetracycline. Affinity constants were calculated for a 1:1 and 2:1 calcium or magnesium oxytetracycline complex.

Materials and methods. Oxytetracycline (Terramycin, Pfizer) was purified by adjusting the pH of an aqueous solution to 5.0. The resulting precipitate was dissolved in $CaCl_2$ saturated methanol and reprecipitated as described by Regna *et al.*(6). This product was again recrystallized from water. The resulting dihydrate was identified by chromatography in both solvent systems of Kelly and Buyske(9), by its decomposition point and by its ultraviolet spectra. No contamination could be detected.

Optical density measurements were made on a Beckman Model DU Spectrophotometer; high purity distilled water was used. Ionic strength was 0.15 and pH was determined after each experiment. Room temperature was employed. One hour was allowed for equilibration.

Results. Metals induced a bathochromic shift in solutions of oxytetracycline (Table I), but did not absorb themselves at these wave lengths. Previously, Goldman(10) demonstrated a difference spectra for magnesium chelates of oxytetracycline, while Ishidate and Sakaguchi(7) have shown metal ions alter the absorption spectra of chlortetracycline.

The difference spectrum enables one to apply Job's principle of continuous variations (11). Results of typical experiments employing calcium and copper are shown in Fig. 1. At these concentrations, one mole of oxytetracycline complexes with one mole calcium, but with 2 moles of copper.

A variation of Job's principle is illustrated in Fig. 2. Here the concentration of oxytetracycline plus calcium was held constant and mole fraction calcium varied as conventionally done, but rather than plotting optical density differences, pH change was recorded. The curve was asymetrical because of the buffer capacity of oxytetracycline. Such use of Job's principle may be valuable when spectral changes are non-existent.

Increasing concentrations of calcium or magnesium ions added to a constant amount of oxytetracycline increased optical density at 375-380 mμ until a plateau was reached (Fig. 3). If the quantity of metal was further raised optical density again increased until a second plateau was reached (Fig. 3). As metal ion to antibiotic ratio was further

* This work was aided by grants from U.S.P.H.S., Squibb Inst. for Medical Research, and Southern California Chapter of Arthritis and Rheumatism Foundation.

TABLE I. Absorption Properties of Some Oxytetracycline Complexes.

Metallic ion	Range of metal to ligand ratio*	pH†	Absorption maximum, mμ	Molar extinction coefficient & wave length for calculations
None	—	7.41	365	12,300 at 380 mμ
Ca^{++}	Up to 100:1	7.41	375	16,700 " " "
Ca^{++}	150:1 to 1000:1	7.41	375	18,500 " " "
Ca^{++}	Greater than 2,400:1	7.41	375?	—
None	—	7.32	360	11,600 at 380 mμ
Mg^{++}	Up to 35:1	7.32	370	17,400 " " "
Mg^{++}	50:1 to 200:1	7.32	370	19,500 " " "
Mg^{++}	Greater than 600:1	7.32	375	—
None	—	7.32	360	8,300 at 395 mμ
Cu^{++}	Up to 0.8:1	7.32	375	14,600 " " " & 13,900 at 415 mμ
Cu^{++}	1.1:1 to 6:1	7.32	415	17,100 at 415 mμ

* Oxytetracycline concentration was about 22 μM in all cases.
† The spectra are pH sensitive.

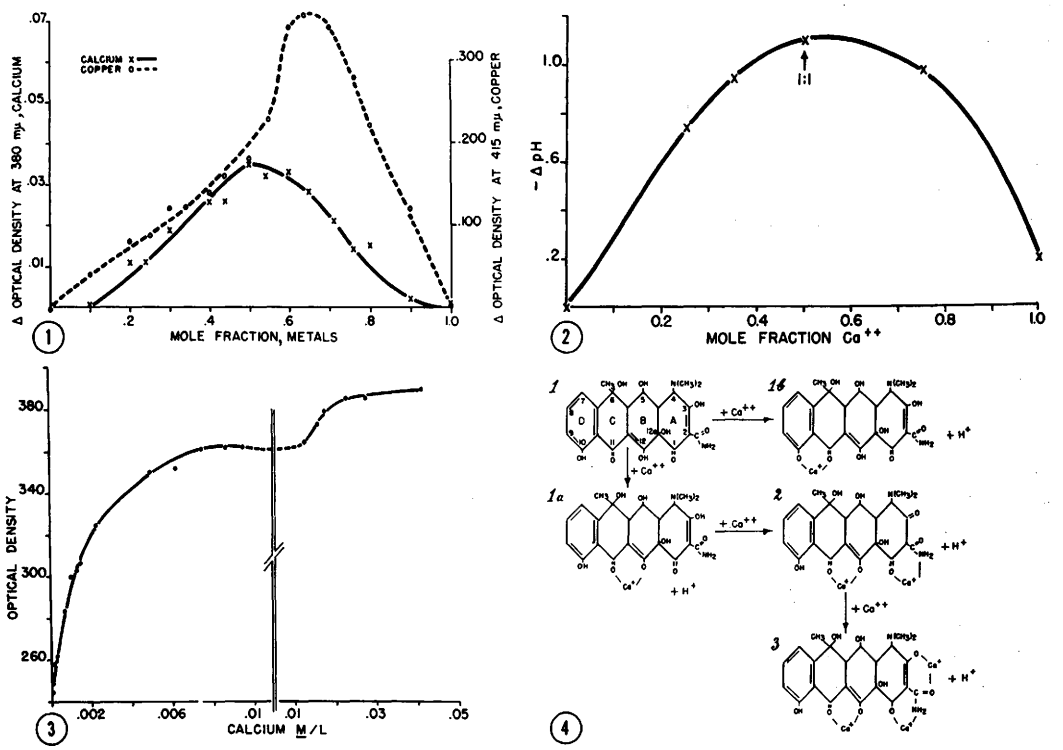

FIG. 1. Spectrophotometric demonstration of the stoichiometry of calcium and cupric ion reacting with oxytetracycline, using Job's method of continuous variations(11). Total concentration of oxytetracycline plus metal was 12.5 μM. The pH was held at 7.35 ± .02 with 5 mM barbital buffer.

FIG. 2. Demonstration of the metal:ligand stoichiometry using change in pH rather than change in optical density. Concentration of oxytetracycline plus calcium was 2.50 mM. The stock solution of $CaCl_2$ and oxytetracycline was adjusted to pH 7.4 before final dilution.

FIG. 3. Typical example of effect of increasing calcium concentration upon optical density at 380 mμ. Oxytetracycline concentration was 20.9 μmolar and the pH 7.37. Tris buffer was used. When magnesium was employed in place of calcium the curves obtained were similar but further displaced to the left (Table I).

FIG. 4. Proposed reaction sequence of oxytetracycline with metal ions.

TABLE II. Stability Constants for the Metal Complexes of Oxytetracycline.

Metallic ion	1:1 complex: No. determinations	Avg association constant K_1	±S.D.	Avg log K_1	2:1 (metal ligand) complex: No. determinations	Avg association constant K_2	±S.D.	Avg log K_2
Cu^{++}	7	—	—	6.5–8.0*	4	1.28×10^5	3.4×10^4	5.1
Mg^{++}	16	3,640	750	3.6†	4	250	37	2.4
Ca^{++}	10	1,250	310	3.1	6	103	23	2.0
Ca^{++}‡	15	950	22	3.0	8	78	26	1.9

* Relatively large error due to the exceedingly low concentration of unassociated cupric ion. Value had been reported as 7.2(3).

† Previously reported as 3.8(4).

‡ Determined in Tris buffer pH 7.38. The other values were determined in barbital pH 7.32-7.41.

elevated optical density appeared only slightly affected. However, concomitantly buffer capacity was exceeded and pH dropped. Copper induced a step-wise shift in absorption maxima, from 360 mμ to 375 mμ and then to 415 mμ (Table I).

The data could be explained by assuming formation of a series of different molecular species. When oxytetracycline (OTC) concentration was maintained near 20 μM and the Ca:OTC ratio was increased to 100:1, the reaction may be written as $Ca^{++} + OTC \rightleftharpoons CaOTC$. When the calcium to oxytetracycline ratio was greater than 150:1 but less than 1000:1 a second reaction, $Ca^{++} + CaOTC \rightleftharpoons Ca_2OTC$, also occurs. A similar series of reactions may be postulated to occur for magnesium and copper. Magnesium saturated the first site at a 35:1 ratio and the second site at a ratio of about 200:1. The first site was filled when the copper to oxytetracycline ratio was about 1:1 and the second site was full when the ratio of copper to oxytetracycline was 6:1 (Table I).

By using optical density values of the plateau region to obtain extinction coefficients it is possible to calculate concentrations of each of both species of oxytetracycline in solution,† and to derive the affinity constants.‡ These are given in Table II.

The existence of a tetracycline calcium diethylbarbiturate (barbital) complex has been proposed(12). To test possible effects of buffer, affinity constants for calcium complexes were determined using tris (hydroxymethyl) aminomethane (Tris) as well as barbital buffer (Table II). The difference in log K is not significant.

When the product of the concentrations of oxytetracycline and metal was plotted against the calculated value of oxytetracycline metal complex a straight line was obtained. The slope of this line was equivalent to calculated affinity constants. Thus, linearity could be and was used as a criteria of validity of the extinction coefficients.

Under some conditions significant quantities of oxytetracycline may be bound while insignificant quantities of calcium are bound. By choosing such experiments at different oxytetracycline levels, as illustrated in Table III, Experiment 1, it is possible to solve the following equation for x since terms containing Ca^{++} cancel:

$$\frac{[OTC_1]^x \times [Ca_1^{++}]^y}{[CaOTC_1]} = \frac{[OTC_2]^x \times [Ca_2^{++}]^y}{[CaOTC_2]}$$

A value of one is obtained for x. When this value is used in order to calculate y, we find that y also equals one. (Table III, Experiment 2). A similar set of calculations can be made (with less accuracy) for the reaction $Ca^{++} + CaOTC \rightleftharpoons Ca_2$ OTC; again, a 1:1 stoichiometry appears confirmed.

If one mole of metal displaces one $[H^+]$

† The following formula was used: $x(E_1) + (\text{Total OTC} - x)E_2 =$ the observed optical density. Where $x =$ the concentration of species one, while E_1 and E_2 are the extinction coefficients of the two molecular species involved. Concentration of species 2 = Total OTC − x.

‡ $K_1 = \dfrac{[\text{Ca OTC}]}{[Ca^{++}][\text{OTC}]}$; $K_2 = \dfrac{[Ca_2\,\text{OTC}]}{[Ca^{++}][\text{Ca OTC}]}$

Where [OTC], [Ca OTC] and [Ca_2 OTC] are derived spectrophotometrically and $[Ca^{++}]$ = Total calcium − [Ca OTC] − 2 [Ca_2 OTC].

TABLE III. Direct Calculation of Exponential Terms in Equilibrium Reaction for Calcium Oxytetracycline Formation.

Exp.	Total oxytetracycline, M/l	Free oxytetracycline, M/l	Calcium oxytetracycline, M/l	Total calcium	Calculated exponent
1 a	2.5×10^{-5}	7.0×10^{-6}	1.8×10^{-5}	1.25×10^{-3}	$1.0 = x$
b	5.0×10^{-6}	1.4×10^{-6}	3.6×10^{-6}	1.25×10^{-3}	$1.0 = x$
2 a	2.0×10^{-5}	8.7×10^{-6}	1.13×10^{-5}	6.25×10^{-4}	$1.0 = y$
b	2.0×10^{-5}	5.4×10^{-6}	1.46×10^{-5}	1.25×10^{-3}	$1.0 = y$

one can compare theoretical liberation of H^+ with experimental values. Using derived affinity constants to calculate the theoretical $[H^+]$ liberated one obtains good correlation when this value is compared to experimental values (Fig. 2) which have been corrected for buffering capacity of oxytetracycline.

Discussion. The validity of the conclusions drawn appears to be confirmed for the following reasons: (a) values of affinity constants of the 1:1 copper and magnesium complexes agree with those obtained titrimetrically (Table II); (b) when the product of the concentration of metal and oxytetracycline is plotted *vs* calculated concentrations of calcium oxytetracycline complex, a straight line ensues only when proper extinction coefficients are employed; (c) when values obtained for the concentrations are employed to calculate exponentials of the equilibrium reaction in a manner using no assumptions, the values obtained equal one, showing that two 1:1 reactions do occur in sequence (Table III); (d) values obtained for the calcium oxytetracycline complex using heart perfusion technics for calcium ion analysis correspond to those reported herein(13); (e) calculated values of H^+ liberated are equivalent to values obtained experimentally; (f) that 1:1 complexes cause the maximum shift in wave length agrees with Conover's(14) conclusion that the most important complexing site is responsible for the bathochromic shift in the long ultraviolet region.

Conversely, since this bathochromic shift has been demonstrated to be associated with the 11:12β-dicarbonyl system(14), it must be concluded that this is the site of the 1:1 complex (Fig. 4). The concomitant decrease in pH (Fig. 2) is caused by H^+ displacement. As the addition of the second atom of metal is associated with similar but smaller spectral changes the same chromophoric center must be affected. As another atom of metal could not be placed in the system itself (*i.e.*, associated with the 10:11 oxygens), this second atom is possibly associated with the 1 ketone as shown in Fig. 4 reaction 2. That increasing the metal level still higher causes only little spectral change but decreases the pH, suggests addition of another metal atom at a point further isolated from the 11:12β-dicarbonyl chromophore system. Assuming the chelation centers are limited to those proposed by Conover(14), this metal probably is added *via* reaction 3. Although it is highly improbable that metals could be bound to the 10:11 and 11:12β-carbonyl systems simultaneously, possibly the 1:1 complex is a mixture of *1a* and *1b*. According to Conover (14) *1a* should be most important.

Although 1:1 complexes previously have been reported to exist(3,4), higher than 1:1 metal to ligand complexes have not been reported for divalent metals. However, Sakaguchi and coworkers(7,15) have found 2:1 complexes of Fe^{3+} and Zr^{4+}. Data contained herein show different metals do not necessarily form different type complexes but rather form 1:1 or 2:1 complexes at different concentrations. Thus under conditions employed by Sakaguchi's group relatively strongly bound metals showed spectral properties indicative of a 2:1 complex as did copper in our studies and more weakly bound metals showed properties of a 1:1 complex—as did calcium under conditions employed here.

The existence of 1:2 metal-oxytetracycline complexes has been reported(3,4,6,10). Although an effort was made, no spectral evidence for the existence of this complex could be found. It is suggested such 1:2 complexes occur only at higher pH's, as Albert(3) observed 1:2 metal-ligand complexes when pH "rose", and Goldman(10) found that at pH's

above 7.6 magnesium induces both a 380 mμ difference maximum and a 417 mμ difference minimum in the oxytetracycline spectrum. The difference minimum did not exist below pH 7.6 and increased with alkalinity. Perhaps, ionization of phenolic hydrogen permits formation of 1:2 metal-oxytetracycline chelates.

When 1:1 or higher calcium to oxytetracycline complexes are formed, it is unlikely that all of the coordinating positions of calcium are satisfied. Thus apatite surface calcium could react with oxytetracycline and permit accumulation of this fluorophore in bone. Possibly, spatial configuration of apatite surface calcium allows 2 or more calcium atoms to simultaneously bind one molecule of tetracycline, enhancing the force of attraction.

Summary. Calcium and magnesium binding by oxytetracycline, observed spectrophotometrically, occurs in a step-wise fashion. A variant of Job's principle applies to these reactions. The log K_{CaOTC} was found to be 3.0 and the log K_{Ca_2OTC} 2.0. The log K_{MgOTC} equaled 3.6; while log K_{Mg_2OTC} was 2.4. Still higher metal to ligand complexes may exist, but the 1:1 and 2:1 oxytetracycline complexes are postulated to be the chief molecular species occurring *in vivo*.

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Received November 15, 1961. P.S.E.B.M., 1962, v109.

Calcium Oxytetracycline Complexes.* New Apparatus for Frog Heart Method of Estimation of Calcium Ion Concentration. (27340)

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Chelation of calcium by oxytetracycline in solutions of physiologic composition can be determined by the frog heart method of McLean and Hastings(1). The experimental design that Hastings *et al.*(2) used to measure calcium binding by citrate, is clearly applicable to oxytetracycline. The technic of the original method is difficult, tedious, and discouraging to some investigators, but it is generally regarded as more valid than any other as a detector of free calcium ions. With the aid of new electronic devices described below, the procedure is rapid, relatively simple and as accurate for calcium as spectrophotometric methods employed by Ibsen and Urist(3).

* This work was aided by grants from U.S.P.H.S., Squibb Inst. for Medical Research, and Southern California Chapter of Arthritis and Rheumatism Foundation.