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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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).

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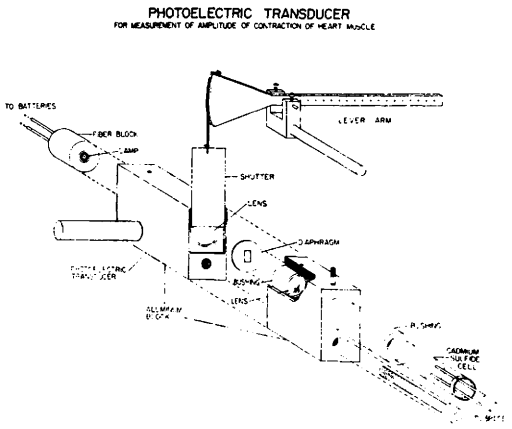

FIG. 1. Mechanical drawings showing construction of photoelectric transducer cardiogram for frog heart analysis for calcium ions.

Material and methods. Physiologic salt solutions were prepared at temperature of 20°C, and maintained at pH 7.4 by tris (hydroxymethyl) aminomethane [Tris] buffer as follows: NaCl, 155.4; KCl, 2.4; glucose, 111.0; Tris, 10.0 mM/l. Calcium chloride was added as required from standard solutions. Oxytetracycline was purified by recrystallization.

Twenty frog hearts (*Rana pipiens* and *Rana catesbiana*) were prepared by cannulating the aorta and perfusing the ventricle as described by McLean and Hastings(1). The sinus venosus and pulmonary vessels were ligated to avoid leakage. A thread tied to the tip of the ventricle was used to transmit the contraction of the heart to a balanced lever arm.

The precision of the frog heart technic was improved by interposing a transducer between the heart and a recording device. Heart contractions activated a photoelectric cell system and produced impulses for relay to a Sanborn recording system (Model No. 299); this transformed mechanical energy to electrical energy. A bridge circuit was employed to convert small changes in resistance to large changes in voltage that were fed into the recording system (Fig. 1).

The following steps were essential for reliable performance of a frog heart preparation: (i) perfusion of the ventricle for at least 20 minutes to allow extirpated heart muscles to adapt to experimental solutions; (ii) filling of the heart with constant volume of solution to insure a constant work load for muscle; (iii) constant moistening of the exterior of the heart with solutions of reference; (iv) repeated bracketing of unknowns or experimental solutions between standard solutions of reference until the results are consistent, rinsing 3 times with every change of solution; (v) allowing a 30-minute period of rest in the refrigerator at 10°C in a constant humidity chamber to revive the heart when the amplitude of contraction appears to be diminishing; (vi) room temperature of 25° or below was essential to maintain the heart in good working condition. When the technic has been mastered, one preparation can perform properly for more than 12 hours.

Calculations of affinity constants were based on an assumption that the calcium oxytetracycline complex forms in a 1:1 ratio at physiologic concentrations of oxytetracycline (3). Total concentrations of the oxytetracycline components were determined initially by gravimetric methods and were verified by spectrophotometric analysis.

Calculations were made with the use of the following equations:

$$K_{CaOTC} = \frac{CaOTC}{Ca^{++}\,OTC} = \frac{\text{Total Ca} - Ca^{++}}{Ca^{++}\,(\text{total OTC} - \text{total Ca} + Ca^{++})}$$

Total concentrations of calcium and oxytetracycline were measured chemically; calcium ion concentrations were determined by the frog heart.

Magnesium forms complexes with oxytetracycline and a special method of calculation is necessary for experimental solutions containing Mg^{++} as well as Ca^{++}; for this reason, magnesium was omitted from the solutions. We have prepared solutions containing magnesium and made mathematical corrections using the affinity constant of magnesium-oxytetracycline, 3.64×10^3,(3) and found no significant alteration in mean value for the affinity constant of calcium oxytetracycline.

Results. The apparatus used in these experiments confirmed: (i) the accuracy of McLean and Hastings determinations of cal-

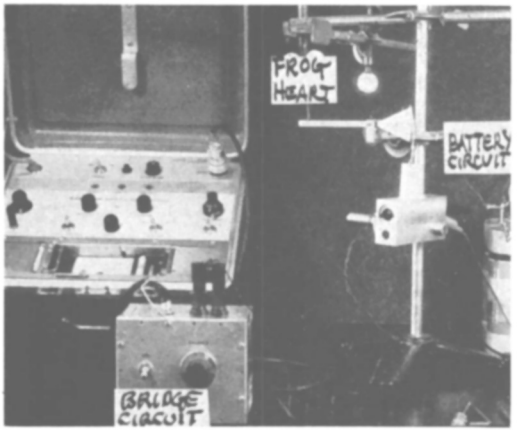

FIG. 2. Photograph showing scale and arrangement of equipment illustrated in Fig. 1, and use of an electronic recording system for the frog heart method.

cium ion concentration in standard physiologic salt solutions, and (ii) Hastings *et al.* (2) analyses of calcium in solutions of citrate. The electronic devices illustrated in Fig. 1 improved the accuracy, sensitivity, and graphic records. Oxytetracycline was used in present experiments in concentrations higher than necessary for labelling of bones, but this was only 25% of the levels used by Hastings *et al.*(2) to observe effects of citrate on calcium. In the range of concentration between 0.20 to 0.25 m*M*/l K_{CaOTC} was calculated for 13 solutions and the log of the mean K was 3.04 (Table I). The precision of the method was of the order of magnitude of ± 0.2 m*M*/l.

Experimental solutions were also prepared and tested with bicarbonate buffer, as employed by McLean and Hastings(1). The results were identical with Tris buffered solutions. Thus, Tris had no adverse effects either upon the heart or calcium binding by oxytetracycline.

Log K of calcium citrate was 3.19 (Table II). These values are comparable to those obtained with the frog heart nearly 30 years ago by Hastings *et al.*(2). The affinity of citrate for calcium ions was therefore slightly greater than that exhibited by oxytetracycline; the mean deviation was ± 0.010 m*M*/l.

Discussion. Oxytetracycline is now commonly used for experimental and clinical investigation of calcium and bone metabolism. This amphoteric substance, at pH of blood, binds polyvalent cations in heterocyclic rings by the mechanism described as chelation. Reactions with calcium, the most abundant element in vertebrate animals, occur in fluids of the body and in actively growing surfaces of the skeleton.

In our solutions, the proportion of calcium to oxytetracycline was approximately 4:1. Calcium ion concentration had to be maintained within the range to which the heart was sensitive. Increases in oxytetracycline concentration above 0.40 m*M*/l, with or without corresponding increases in calcium ion concentration, produced bizarre contractions of the heart muscle. Because the heart tolerated only a narrow range of concentra-

TABLE I. Observations and Calculations of the Affinity Constant of Calcium-Oxytetracycline Complexes.

Solution No.	Total Ca	Total OTC	Calculated Ca^{++}	Observed Ca^{++}	Difference	CaOTC	OTC	K	Log K
	mM/l		mM/l			mM/l			
1	.90	.25	.784	.78	−.004	.12	.13	1182	3.07
2	.90	.25	.784	.78	−.004	.12	.13	1182	3.07
3	.85	.25	.739	.75	+.011	.10	.15	889	2.95
4	.85	.25	.739	.74	+.001	.11	.14	1062	3.03
5	.95	.22	.844	.83	−.014	.12	.10	1447	3.16
6	.95	.22	.844	.84	−.004	.11	.11	1191	3.08
7	.90	.22	.804	.80	−.004	.10	.12	1042	3.02
8	.95	.25	.830	.84	+.010	.11	.14	936	2.97
9	.90	.25	.784	.80	+.016	.10	.15	834	2.92
10	.90	.25	.784	.80	+.016	.10	.15	834	2.92
11	.90	.25	.784	.82	+.036	.80	.17	574	2.76
12	.90	.25	.784	.75	−.034	.15	.10	2000	3.30
13	.80	.25	.692	.69	−.002	.11	.14	1139	3.06

Mean K = 1.10×10^3. Log mean K = 3.04.

TABLE II. Observations and Calculations of the Affinity Constant of Calcium-Citrate Complexes.

Solution No.	Total Ca	Total CIT	Calculated Ca^{++}	Observed Ca^{++}	Difference	CaOTC	OTC	Log K
1	2.00	1.64	1.003	1.00	−.003	1.00	.64	3.19
2	2.00	1.64	1.003	1.00	−.003	1.00	.64	3.19
3	2.00	1.64	1.003	1.03	+.027	.97	.67	3.15
4	2.00	1.64	1.003	1.00	−.003	1.00	.64	3.19
5	2.00	1.64	1.003	.99	−.013	1.01	.63	3.21

Mean K = 1.543×10^3. Log mean K = 3.19.

tions of calcium and oxytetracycline, the stoichiometry of the complex had to be determined by the spectrophotometric method(3). When 1:1 complexes were assumed, the log of the affinity constant was 3.04 and very nearly the same as –3.0 to 3.1– found spectrophotometrically. Thus while the frog heart measured calcium ions, and the spectrophotometric method measured oxytetracycline, the two methods were in remarkable agreement.

Calcium has a lower affinity for oxytetracycline than magnesium, manganese, cobalt, or any other divalent cation in blood. The amount of this substance (1.0 g day) that a man tolerates without effects produces serum concentration of approximately 10 γ per ml; approximately 75% is bound to protein and only 25% is free in solution to complex with metal ions(4). Under these conditions, calcium and oxytetracycline formed complexes chiefly in a 1:1 ratio, but in bone matrix where calcium ion concentration may be higher, the proportions might increase to 2 to 1 or as high as 3 to 1. Because of the high concentration of calcium ions, in relation to other polyvalent ions in blood, the mass law operates in favor of calcium complex formation, and this effect is localized and enhanced in calcifying tissues. The mechanism and stoichiometry of combinations of tetracycline with calcium and bone salts are under investigation and will be presented later.

Summary. Calcium complexes of oxytetracycline were analyzed by the use of the frog heart method with an improved apparatus employing a vertical shutter, photoelectric transducer, bridge circuit, and recording system. This method was readily applicable to complex biologic solutions and produced results comparable to those in the preceding article obtained by spectrophotometry on pure solutions. The log K of the 1:1 calcium oxytetracycline complex was 3.04, compared with 3.19 for the 1:1 complex of citric acid and calcium.

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Impurity in RNA Preparations which Inactivates RNase Inhibitor and Increases Alkaline RNase Activity.* (27341)

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Following the demonstration of both acid and alkaline RNases in rat liver homogenate (1,2), de Lamirande and Allard(3) have pointed out that different laboratories have found different ratios of alkaline to acid

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Abbreviations: RNase, ribonuclease; RNA, ribonucleic acid; CMS, p-chloromercuriphenylsulfonic acid; TCA, trichloroacetic acid.