CALCIUM-BORO-SACCHARATE-GLUCONATE: STUDIES ON PREPARATION, TOXICOLOGY AND EFFECT ON SERUM CALCIUM LEVEL IN COWS

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A Thesis Submitted to the Graduate Committee

For the Degree of

MASTER OF SCIENCE

in

BIOLOGY

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1942

For friendly advice, practical criticism and generous aid, I acknowledge my indebtedness to:

Dr. R. D. HATCH

Dr. I. D. WILSON

Dr. A. D. PRATT

Prof. J. B. LUCAS

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INTRODUCTION

"Milk fever" of cows is the popular name for the clinical syndrome evinced by lactating cows suffering from hypocalcemia. It includes weakness, paralysis, cessation of lactation and a characteristic posture of the head and neck. The hypocalcemic condition is brought on by the secretion of calcium salts in the milk in excess of the calcium made available to the animal in its feed or by the calcium stores of the body. According to Little and Wright (12), milk fever usually occurs when the serum calcium falls to about 4.1 mg. percent, the normal level, as reported by Newton (14), being around 10 mg. percent.

The rational therapeutics of this condition is the administration of easily utilizable calcium salts to the animal. These are made available most rapidly through the agency of intravenous injection of sterile solutions of these salts. The action of calcium salts in causing the rapid recovery encountered in many cases of milk fever is not understood. Glaser (8) holds that "The beneficial effect of calcium therapy is analogous to that produced by rest in psychiatric cases. In both rest and calcium therapy, there is a shift of calcium from the blood to the tissue cells."

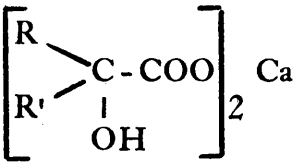
The calcium salts most widely used by veterinarians at present are calcium chloride, calcium lactate and calcium gluconate (7,18). Calcium lactate is the most toxic of these and is used less commonly than the others. Taking the minimum lethal dose of calcium lactate as 1.00, the comparative lethal dosages of calcium chloride and calcium gluconate are, according to the work of the Baldacci (2) on rabbits, 1.14 and 1.85 respectively. Carratala (3) reports that, in dogs and rabbits, when an injection rate of one cc. per minute is used the comparative lethal dosages of these salts are 1:1.17:-1.82 respectively, while when an injection rate of two cc. per minute is used the comparative lethal dosages are 1:1.44:2.11. Carratala explains this dosage differential as being due to different amounts of calcium in identical concentrations of the various salts. One aim of this investigation will be to show that this explanation is erroneous.

It is a logical assumption that the therapeutic agent which will make available to the animal more calcium per unit volume administered will be the more efficient one. Since the clinical efficacy and comparative non-toxicity of calcium gluconate have been established by experiment and experience, a search was instituted for a compound closely related to calcium gluconate but containing more calcium per unit weight. The compound chosen was calcium d-saccharate. This is the calcium salt of d-saccharic acid, and a study of the structural formulae of this acid and of d-gluconic acid (Figs. Ia, Ib) shows that theoretically *one* saccharate ion should be able to combine

with one calcium ion while *two* gluconate ions are necessary for combination with one calcium ion. That this is the case can be seen by a study of the formulae in Figures IIa and IIb.* One unit weight of calcium d-saccharate contains 39% more calcium than does the same weight of calcium d-gluconate.

Before the physiological activity and toxicity of the calcium d-saccharate could be evaluated, the necessity arose of preparing a stable solution of the compound. In this respect, the difficulty of insolubility was encountered; the compound is very insoluble in water. The work of Austin (1), Dryerre and Greig (6), Pasternack and Giles (15) and De Carli (5) was utilized to overcome this difficulty. Austin found that the presence of boron compounds materially increases the solubility of calcium gluconate in water. De Carli states that the addition of 3.67% boric acid increases the solubility of calcium gluconate in water from 3.3% to 39%, while Dryerre and Greig utilized boric acid in the preparation of a 20% solution of calcium gluconate. These three authors believe that a complex of the type calcium gluconate-boric acid-water is formed which accounts for the action in increasing the solubility. Pasternack and Giles claim that calcium gluconate and calcium saccharate in water manifest a mutual solubility-increasing effect.

After this investigation was begun, a patent was issued to R. O. Roblin (17) by the U. S. Patent Office covering "therapeutically useful compositions containing available calcium." The claims are made for the preparation of therapeutic compositions containing 1% to 5% boric acid and at least 16% calcium alpha-hydroxyisobutyrate or other calcium salts having the structural formula



Wherein R represents hydrogen or a methyl, ethyl or propyl radical, and R' represents a methyl, ethyl, or propyl radical, and where R and R' together contain at least two carbon atoms. A study of figure IIb will show that neither calcium gluconate nor calcium saccharate falls in this family of compounds. The determination of the effect of boric acid on the solubility of calcium d-saccharate and the preparation of a solution containing boric acid, calcium d-gluconate and calcium d-saccharate are included in this investigation.

The preparation of this solution was followed by a study of its effect on the serum

* These formulae were obtained from the chemistry laboratories of Chas. Pfizer & Co., Inc., N. Y.

calcium level in cows. Calcium-Boro-Gluconate (Jen-Sal), since it is one of the therapeutic agents in wide use at present and since it also contains boric acid, was taken as the basis of comparison. Godden and Duckworth (9), working with a 10% solution of calcium gluconate, report that "only about 15% of the calcium injected could be found in the serum 12 minutes after injection." Glaser (8) also states that calcium salts injected into the animal system soon disappear from the blood. This investigation undertakes to test the accuracy of these statements and also to follow the course of the level of calcium in the serum when administered to cows as the salts of both d-gluconic and d-saccharic acids.

The determination of serum calcium involves the selection of a suitable technic. The principle of most of the reported technics includes the precipitation of calcium as calcium oxalate (4, 10, 11, 13, 16, 19, 20). In most cases where the calcium is precipitated as oxalate, the mixture is centrifuged so that the precipitate is collected at the bottom of a test tube. During the course of a determination, the idea presented itself that if the precipitate could be collected in a small graduated tube the volume could be read directly and might thereby offer a means for a simplified and more rapid determination.

EXPERIMENTAL PROCEDURE AND RESULTS

Determination of solubility:

Since it is known that the presence of boric acid increases the solubility of calcium gluconate in water, a study was initiated to determine the effect of boric acid on the solubility of calcium d-saccharate in water. A concentrated solution of boric acid was prepared by adding a large, but undetermined, amount of boric acid powder to boiling distilled water. Five samples of ten cc. each were evaporated to dryness and weighed in order to determine the amount of boric acid present. The details of this procedure are as follows: five uncovered petri dishes were cleaned thoroughly with bichromate cleaning solution, dried in an oven and weighed on a microbalance. Ten cc. of the boric acid solution were run into each dish from a burette graduated in tenths of a cc., and the dishes were placed in an incubator at 37 degrees C. for three days. At the end of this time the evaporation was complete, and the samples were allowed to stand in the laboratory for two days for the purpose of having them reach stability in regard to water of crystallization. The dishes containing the samples were then weighed and the dish weight subtracted from the total weight to give the sample weight. The weights and results are shown in Table I.

The boric acid solution (0.4700 moles per liter) was kept tightly stoppered to prevent evaporation of the water, and this solution was used as the stock solution from which various concentrations were prepared for utilization as solvents. Boric acid solutions of the following concentrations were used:

0.0000 molar
0.0500 molar
0.1000 molar
0.1500 molar
0.2000 molar
0.2092 molar
0.2209 molar
0.2268 molar
0.2500 molar
0.3000 molar

The data on the preparation of these various concentrations from the stock solution are shown in Table II.

The determination of the solubility of calcium d-saccharate in each of these solvents was made as follows: an excess of calcium d-saccharate was placed in a 100 cc. Erlenmeyer flask containing 40 cc. of the solvent. The mixture was shaken vigorously

for two minutes and allowed to stand at room temperature for 24-48 hours with intermittent shaking. The flasks were then placed in a refrigerator until the temperature of the mixture reached 19 degrees C. The mixture was shaken again to preclude any supersaturation and then filtered through Whatman No. 1 filter paper. During

Table I
Determination of Boric Acid in Stock Solvent Solution

Plate no.	Amt. Sol'n (cc.)	Weight (gms.)		Net wt./10 cc. (gms.)	Ave. wt./10 cc. (gms.)	Moles/liter
		empty	after evap'n			
5	10	52.4920	52.7884	0.2964		
7	10	47.9106	48.2078	0.2972		
8	10	46.9584	47.2559	0.2974	0.2969	0.4700
9	10	48.7274	49.0245	0.2971		
11	10	48.9158	49.2123	0.2965		

filtration, the temperature rose an average of two degrees C., giving an average filtration temperature of 20 degrees C. The filtrate was transferred to a burette, and ten cc. quantities were measured into three clean, weighed petri dishes. Evaporation, weighing and calculation were carried out as in the standardization of the stock solution. The results of these determinations are listed in Table III and graphically represented in Figure 3. These show that an increase in the concentration of boric acid

Table II
Preparation of Various Concentrations of Solvent from Stock Solution

Stock Solution	Water (distilled)	Total Volume	Moles per Liter
0.00 cc.	40.00 cc.	40.00 cc.	0.0000
4.20 cc.	35.80 cc.	40.00 cc.	0.0500
8.40 cc.	31.60 cc.	40.00 cc.	0.1000
12.60 cc.	27.40 cc.	40.00 cc.	0.1500
16.80 cc.	23.20 cc.	40.00 cc.	0.2000
17.80 cc.	22.20 cc.	40.00 cc.	0.2092
18.80 cc.	21.20 cc.	40.00 cc.	0.2209
19.30 cc.	20. 70 cc.	40.00 cc.	0.2268
21.00 cc.	19.00 cc.	40.00 cc.	0.2500
25.20 cc.	14.80 cc.	40.00 cc.	0.3000

Table III
Solubility of Calcium d-Saccharate in Various Concentrations of Acid Solution

Plate No.	Boric Acid		Wt. of Plates (gms.)		Wt. Residue (gms.)	Wt. Boric Acid (gms.)	Saccharate Dissolved	
	Molarity	Volume	empty	after evap'n			Net wt. (gms.)	Moles/liter
2			49.4057	49.4108	0.0051		0.0051	
3			51.1904	51.1950	0.0046		0.0046	
4	0.0000	10 cc.	54.4000	54.4049	0.0049	0.0000	0.0049	0.0015
11			48.9156	48.9206	0.0050		0.0050	
12			51.8684	51.8733	0.0049		0.0049	
4			54.3981	54.4385	0.0400		0.0099	
6	0.0500	10 cc.	47.1155	47.1565	0.0410	0.0305	0.0105	0.0032
9			48.7274	48.7677	0.0403		0.0098	
2			49.4052	49.4815	0.0763		0.0153	
11	0.1000	10 cc.	48.9158	48.9935	0.0777	0.0610	0.0167	0.0052
12			51.8678	51.9467	0.0789		0.0179	
5			52.4920	52.6089	0.1169		0.0253	
7	0.1500	10 cc.	47.9136	48.0272	0.1136	0.0916	0.0220	0.0073
8			46.9584	47.0725	0.1141		0.0225	
7			47.9136	48.0684	0.1548		0.0327	
9	0.2000	10 cc.	48.7274	48.8806	0.1532	0.1221	0.0311	0.0101
11			48.9158	49.0709	0.1551		0.0330	
5			52.4920	52.6600	0.1680		0.0387	
11	0.2092	10 cc.	48.9158	49.0825	0.1667	0.1293	0.0374	0.0120
12			51.8678	51.8678	0.1687		0.0394	
2			49.4052	49.5809	0.1757		0.0391	
6	0.2209	10 cc.	47.1124	47.2885	0.1761	0.1366	0.0395	0.0122
7			47.9106	48.0859	0.1753		0.0387	
4			54.3981	54.5780	0.1799		0.0397	
8	0.2268	10 cc.	46.9584	47.1388	0.1804	0.1402	0.0402	0.0125
9			48.7274	48.9077	0.1803		0.0401	
5			52.4920	52.6885	0.1965		0.0439	
8	0.2500	10 cc.	46.9584	47.1539	0.1955	0.1526	0.0429	0.0136
12			51.8678	52.0645	0.1967		0.0441	
2			49.4054	49.6452	0.2398		0.0567	
4	0.3000	10 cc.	54.3981	54.6387	0.2406	0.1831	0.0575	0.0180
6			47.1155	47.3574	0.2450		0.0588	

in the solvent causes an increased solubility of the saccharate. However, the saccharate is not sufficiently soluble in boric acid solution at ordinary temperatures for the latter to be used as sole solvent in the preparation of a therapeutically efficient calcium d-saccharate solution.

Since the use of calcium d-saccharate as a therapeutically efficient calcium carrier was one of the aims of this investigation, it was decided to utilize the combined dissolving powers of boric acid and calcium d-gluconate. The following formula was used:

Calcium d-Gluconate (U.S.P.)	100.0 g.
Boric Acid (U.S.P.)	10.0 g.
Calcium d-Saccharate	37.5 g.
Distilled Water	500.0 cc.

The gluconate, boric acid and water were mixed and boiled until solution was complete. To the boiling solution, the calcium d-saccharate was added slowly until 37.5 grams had been added. The addition was done very slowly, because the saccharate, as it comes in contact with the boiling solution, causes foaming and spattering. After all the saccharate had been added and dissolved, the solution was filtered through cotton.

Determination of stability:

One-hundred cc. of the above solution were placed in each of three bottles. Two of these were stoppered after having been autoclaved for 15 minutes at 12 pounds steam pressure. The third was allowed to cool while left open in the laboratory before being stoppered. This was done to determine the physical stability of the solution and also its resistance to fungus contamination, no preservative having been added. The solution is not resistant to fungus contamination, growth being evident within a week in the unstoppered bottle. Physically, however, the solution is very stable; after six months there was no evidence of precipitation or crystallization.

Determination of toxicity:

Since the boric acid-saccharate-gluconate mixture was to be used ultimately in a study of its effect on the serum calcium level of cows, it was deemed advisable to determine its toxicity as compared with that of a commercial mixture containing boric acid and calcium gluconate. For this comparison, Calcium Boro-Gluconate, product of a veterinary pharmaceutical concern*, was chosen, primarily because of its boric

* Jensen-Salsbery Laboratories, Inc., Kansas City, Mo.

acid content. Intravenous administration to rabbits was used.

Albino rabbits, ten weeks old, were procured and were kept in wire-bottomed cages. They were fed Purina Rabbit Checkers in order to insure a constant diet. The toxicity determinations were made when the rabbits were seven months old. The animals were restrained in a specially built stock (Figure IV), and the solutions were injected into the external auricular vein through a 22-gauge needle. Rabbits 5, 10 and 18 received Calcium Boro-Gluconate (C-B-G); rabbits 7, 8 and 12 received Calcium-Boro-Saccharate Gluconate (C-B-S-G), rabbit 9 received physiological saline solution. The purpose of the saline solution was to determine whether the death of the other rabbits was due to the toxic action of the substance injected or to shock brought on by increased blood volume.

All solutions were injected at the rate of two cc. per minute with the following exception: the physiological saline solution was injected at the rate of two cc. per minute until 200 cc. had been injected; after this the rate was increased to from four to five cc. per minute. This increase was instituted to prove that the two cc. per minute rate was not inducive to shock and also to strengthen the hypothesis that the death of the rabbits receiving the calcium solutions was a manifestation of qualitative rather than quantitative effects. The results of this section of the experiment are listed in Table IV.

Effect on serum calcium level in cows:

Since the C-B-S-G solution was found to be non-toxic in comparison with the C-B-G solution, it was felt that it would be safe to inject the C-B-S-G into cows. As this solution contains 6.7 grams, or 32.58%, more calcium than the C-B-G solution, an investigation was undertaken to determine whether or not this difference would manifest itself in the effects of the two solutions on the serum calcium level of cows. Three cows were used for this investigation, each animal receiving 300 cc. of the solutions under test. The schedule of injections and blood withdrawals is shown in Table V.

The solutions were injected slowly into the jugular vein, a Simplex intravenous outfit being used for the injections and 50 cc. Erlenmeyer flasks for the collection of blood. The volume of blood collected ranged from 25 cc. to 40 cc. in each case. Sample no. 1 was withdrawn through the injection needle (14 gauge) just prior to the start of injection; sample no. 2 was withdrawn through the same needle after 150 cc. of solution had been injected. Before this sample was taken, blood was allowed to flow through the needle for about 20 seconds to wash out any calcium solution which

Table IV
Comparative Toxicity of C-B-G and C-B-S-G Solutions for Rabbits

Rabbit No.	Weight (grams)	Sex	Solution	Rate of Injection	Lethal Dose		Comments
					Net	Average	
5	3170	M	C-B-G	2 cc./min.	98 cc.	0.031 cc./gm.	urinated once
10	2558	F	C-B-G	2 cc./min.	100 cc.	0.039 cc./gm.	urinated twice
18†	2520	F	C-B-G	2 cc./min.	69 cc.	0.027 cc./gm.	no urination
7	3230	M	C-B-S-G	2 cc./min.	127 cc.	0.039 cc./gm.	urinated once
8	2882	F	C-B-S-G	2 cc./min.	123 cc.	0.042 cc./gm.	urinated three times
12	2895	F	C-B-S-G	2 cc./min.	114 cc.	0.039 cc./gm.	no urination
9	3042	M	0.85% NaCl	variable*	272 cc.	0.090 cc./gm.	no urination

† Severely infested with ear-mites.
* 2 cc. per minute until 200 cc. had been injected; 4-5 cc. per minute thereafter.

might influence the accuracy of the determination. Sample no. 3 was withdrawn through the same needle after 300 cc. of solution had been injected. With this sample, as with sample no. 2, the needle was first washed out with blood. The remainder of the samples were withdrawn through 14-gauge California bleeding needles according to the schedule shown in column 4 of Table V.

Each sample was allowed to stand at room temperature for about one hour before being placed in a refrigerator at five degrees C. After three to four hours in the refrigerator, the clots were broken away from the sides of the flasks and the samples then returned to the refrigerator. The sera used in the determinations were separated from the clots 18-20 hours after the samples had been collected. Calcium determinations were run in duplicate on the serum from each sample. In these determinations, the Kramer-Tisdall technic (11), utilizing 2 cc. of serum, was employed. The results of these determinations are listed in Figures V and VI and Table V.

The injections caused an initial rise of varying degree in the serum calcium level. In all cases but one, there followed a rapid decline in this level, but in no case did the level return to normal before the end of an hour. In the one exception mentioned above, following an injection of C-B-S-G Solution, the serum calcium started to fall off slowly, but rose again after about five minutes to a level above the initial peak. After about 25 minutes it fell off rapidly. Figure V shows the course of the serum calcium level during each trial, based on the pre-injection level as zero. Figure VI shows the average course of the three trials in which C-B-S-G Solution was used and the two trials in which C-B-G Solution was used.

Determination of efficacy of centrifuge tubes:

In an attempt to simplify the technic for serum calcium determination, specifications for a special centrifuge tube were drawn up and submitted to the Corning Glass Works, Corning, N. Y. These specifications are shown in Figure VII. Four tubes suitable for evaluation were received. The determinations were carried out as follows: a standard calcium solution was prepared by dissolving 0.495 grams of dried calcium chloride in 1000 cc. of water. This resulted in a solution containing 0.1788 mgm. calcium per cc. Three series of tests were run on each tube using one cc., two cc. and three cc. each of standard calcium solution and 3% ammonium oxalate solution in each tube. After these solutions had been mixed, the tubes were placed in the refrigerator for ten minutes for more rapid precipitation of the calcium oxalate. They were then centrifuged at 1400 r.p.m. for ten minutes. Readings were made on the column of precipitate in the graduated capillary portion of the tubes. The results

Table V

Variation in Serum Calcium Level Following Injections of C-B-G and C-B-S-G Solutions.

Trial No.	Cow	Solution Injected	Time Bled	Sample No.	Mgm. Ca per 100 cc. Serum	Base** Reading	Difference mgm./100 cc.
1	Veeman Belle	C-B-S-G	B.I.*	1	11.3	11.3 mgm. per 100 cc. serum	0.0
			150 cc. I.C.†	2	14.5		3.2
			300 cc. I.C.	3	15.0		3.7
			3 min. A.I.‡	4	14.6		3.3
			8 " "	5	13.9		2.6
			13 " "	6	12.8		1.5
			23 " "	7	12.7		1.4
			33 " "	8	13.8		2.5
			48 " "	9	13.0		1.7
2	Veeman Burke Belle	C-B-G	B.I.	1	9.8	9.8 mgm. per 100 cc. serum	0.0
			150 cc. I.C.	2	11.1		2.4
			300 cc. I.C.	3	13.3		5.3
			3 min A.I.	4	12.0		4.9
			8 " "	5	11.3		5.1
			17 " "	6	10.6		5.5
			23 " "	7	10.5		6.1
			28 " "	8	10.6		3.9
			33 " "	9	10.8		3.7
			38 " "	10	10.4		3.5
			43 " "	11	10.2		3.1
			60 " "	12	9.7		3.1
3	Veeman Belle	C-B-G	B.I.	1	10.2	10.2 mgm. per 100 cc. serum	0.0
			150 cc. I.C.	2	13.2		1.6
			300 cc. I.C.	3	14.8		5.4
			3 min A.I.	4	12.4		3.4
			8 " "	5	12.1		3.6
			13 " "	6	11.7		2.5
			18 " "	7	11.6		2.2
			23 " "	8	11.5		2.9
			28 " "	9	11.3		2.2
			33 " "	10	11.5		1.0
			43 " "	11	11.4		0.9
			58 " "	12	11.5		0.8
4	Sensation Veeman	C-B-S-G	B.I.	1	10.1	10.1 mgm. per serum 100 cc.	0.0
			150 cc. I.C.	2	11.7		3.0
			300 cc. I.C.	3	15.5		4.6
			5 min A.I.	4	13.5		2.2
			10 " "	5	13.7		1.9
			15 " "	6	12.6		1.5
			21 " "	7	12.3		1.4
			26 " "	8	13.0		1.3
			32 " "	9	12.3		1.1
			41 " "	10	11.1		1.3
			51 " "	11	11.0		1.2
			62 " "	12	10.9		1.3
5	Sensation Veeman	C-B-S-G	B.I.	1	9.9	9.9 mgm. per 100 cc. serum	0.0
			150 cc. I.C.	2	12.3		1.3
			300 cc. I.C.	3	15.2		3.5
			7 min. A.I.	4	14.8		2.2
			12 " "	5	15.0		1.5
			18 " "	6	15.4		0.8
			25 " "	7	16.0		0.7
			29 " "	8	13.8		0.8
			39 " "	9	13.6		1.0
			50 " "	10	13.4		0.6
			59 " "	11	13.0		0.4
			71 " "	12	13.0		-0.1

* Before Injection; this determines the normal level.
† Injection Complete; blood drawn after this volume of solution had been injected.
‡ After Injection; this is the elapsed time between the completion of injections and the drawing of samples.
** The normal, or pre-injection, reading taken as zero in order to determine effect of solutions on serum calcium level.

of these tests are shown in Table VI. During the course of this investigation, one unsuccessful set of determinations was made on sheep serum.

Since the capillary portion of the centrifuge tube is too small to be cleaned easily, a method was devised to facilitate this procedure. Two wash-bottles were prepared, each having an outlet composed of glass tubing drawn out to a diameter and length which allowed it to be inserted loosely into the capillary portion of the centrifuge tube. One bottle was filled with distilled water and the other with 50% hydrochloric acid. The acid was used to dissolve the caked precipitate at the bottom of the tube, and the water was used to wash out the resultant acid solution. Suction applied to the water bottle accomplished the withdrawal of any liquid remaining in the capillary portion of the tube.

DISCUSSION

The determination of the solubility of calcium d-saccharate in various concentrations of boric acid shows that the solubility increases steadily as the boric acid solution becomes more concentrated. In view of the findings by Dryerre and Greig (6) on the solubility of calcium gluconate in boric acid, this is not unexpected. Of special interest, however, is the definite break occurring in the solubility curve (Fig. III) in the region between 0.20 and 0.25 molar boric acid solutions. The points in and around this region were checked carefully, and it is the author's belief that experimental error plays no part in the apparent discrepancy in the smoothness of the curve.

The significance of this break asserts itself in the light of the following statement by Dryerre and Greig:

> "When calcium gluconate is dissolved in a solution of boric acid, we have reason to believe that a new chemical compound is formed, for which, in the absence of precise information regarding its structure, we suggest the name 'calcium boro-gluconate.' "

It is a common occurrence in plotting curves in solubility tests, conductance tests, freezing-point change determinations, etc., to find a break in an otherwise smooth curve at the point, or in a region, where a new compound is formed. In view of this, and in view of the very close chemical relationship of calcium saccharate to calcium gluconate, it is not untenable to assume at the break in the saccharate-boric acid curve a new compound, calcium boro-saccharate, is formed. Based on this assumption, the mixture prepared in the course of this investigation containing calcium gluconate, calcium saccharate and boric acid has been called Calcium-Boro-Saccharate-Gluconate, the final solution being known as C-B-S-G Solution.

Pasternack and Giles (15) state: "A (calcium gluconate) solution of 20% concentration requires one part of calcium saccharate to 20 parts of calcium gluconate (for the stabilizing of the gluconate solution). For solutions of higher concentration, calcium saccharate should be used up to the limit of solubility of approximately 19 parts to 100 parts of calcium gluconate."

This limit of solubility holds only when no other agent, such as boric acid, is present. As can be seen from the data on the preparation of C-B-S-G solution, the presence of 10 parts of boric acid allows as much as 37.5 parts of calcium saccharate to be dissolved in the solution. This calcium saccharate, beside stabilizing the solution of calcium gluconate, adds materially to the concentration of calcium in the solution as a whole.

Fungus contamination of the solution can be controlled easily and safely by steam sterilization of the container and contents or by the addition to the solution of small amounts of preservative such as phenol (0.5%).

The tests for the determination of the comparative toxicities of C-B-G and C-B-S-G Solutions show that the ratio of toxicity is approximately 1.00:1.25 respectively. Taking the figures reported by Carratala (3) for the two cc. per min. injection rate into rabbits, the comparative lethal dosages of calcium lactate, calcium chloride, calcium gluconate and C-B-S-G Solution are 1.00:1.44:2.11:2.64 respectively. The C-B-S-G Solution is the least toxic for rabbits, and it may be assumed that this holds true in the case of cows, since reports on experimental work with dogs, rabbits and mice do not indicate any species idiosyncrasy toward either the calcium ion, the gluconic acid radical or the lactic acid radical. During the present investigation, cows receiving 300 cc. of the C-B-S-G Solution intravenously showed no evidence that this solution was in any way toxic as compared with cows receiving the same amount of C-B-G Solution.

The dosage differential as reported by Carratala is explained by him as being due to different amounts of calcium in identical concentrations of the various salts. Since calcium gluconate contains 9.3% calcium and calcium lactate contains 18.3%, this explanation holds for these two compounds. It does not hold, however, in the case of calcium chloride which contains more than three times as much calcium (56.5%) per unit weight as does calcium gluconate.

The difference in calcium content may be part of the explanation, but it is evidently not the complete one; the other ion of each compound must be taken into account. In view of the fact that lactic acid is constantly being elaborated by the musculature of the body, it would seem that the lactate radical from the calcium lactate should not result in any unusual deleterious effect. However, since the serum calcium level rises so suddenly following the injection of calcium salts, the indication is that the calcium is rapidly split from the molecule. In the case of calcium lactate, this rapid evolution of lactate radical in the blood stream may account for the greater toxicity of this salt as compared with calcium chloride, assuming, with reason, that the toxicity of the chloride ion is low. Extending this explanation to the place of calcium gluconate in the dosage series, the gluconic acid radical may be reduced to glucose, and the toxicity of glucose is nil when present in the amounts encountered here. The reason for the position of the C-B-S-G Solution in the series will be discussed fully later.

Table VI
Determination of Accuracy of Centrifuge Tubes

Tube No.	2			3			4			5		
Det'n No.	Standard Calcium Solution	3% Oxalate Solution	Reading (mm.)	Standard Calcium Solution	3% Oxalate Solution	Reading (mm.)	Standard Calcium Solution	3% Oxalate Solution	Reading (mm.)	Standard Calcium Solution	3% Oxalate Solution	Reading (mm.)
1	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00
2	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00
3	1 cc.	1 cc.	1.50	1 cc.	1 cc.	1.50	1 cc.	1 cc.	1.50	1 cc.	1 cc.	1.50
4	1 cc.	1 cc.	2.50	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.50	1 cc.	1 cc.	2.00
5	1 cc.	1 cc.	1.75	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.00	1 cc.	1 cc.	2.25
6	1 cc.	1 cc.	2.75	1 cc.	1 cc.	1.50	1 cc.	1 cc.	1.50	1 cc.	1 cc.	1.50
7	2 cc.	2 cc.	5.00	2 cc.	2 cc.	3.50	2 cc.	2 cc.	3.00	2 cc.	2 cc.	2.50
8	2 cc.	2 cc.	6.00	2 cc.	2 cc.	4.00	2 cc.	2 cc.	6.00	2 cc.	2 cc.	6.00
9	2 cc.	2 cc.	4.00	2 cc.	2 cc.	3.00	2 cc.	2 cc.	4.00	2 cc.	2 cc.	4.00
10	2 cc.	2 cc.	4.00	2 cc.	2 cc.	2.50	2 cc.	2 cc.	3.50	2 cc.	2 cc.	4.00
11	2 cc.	2 cc.	3.50	2 cc.	2 cc.	4.00	2 cc.	2 cc.	2.75	2 cc.	2 cc.	4.50
12	2 cc.	2 cc.	4.50	2 cc.	2 cc.	3.00	2 cc.	2 cc.	4.50	2 cc.	2 cc.	4.00
13	3 cc.	3 cc.	5.50	3 cc.	3 cc.	6.00	3 cc.	3 cc.	6.00	3 cc.	3 cc.	6.50
14	3 cc.	3 cc.	6.50	3 cc.	3 cc.	6.50	3 cc.	3 cc.	5.50	3 cc.	3 cc.	5.00
15	3 cc.	3 cc.	4.50	3 cc.	3 cc.	6.50	3 cc.	3 cc.	5.75	3 cc.	3 cc.	6.00
16	3 cc.	3 cc.	6.50	3 cc.	3 cc.	5.75	3 cc.	3 cc.	6.50	3 cc.	3 cc.	6.50
17	3 cc.	3 cc.	6.00	3 cc.	3 cc.	5.50	3 cc.	3 cc.	7.00	3 cc.	3 cc.	5.75
18	3 cc.	3 cc.	5.50	3 cc.	3 cc.	5.00	3 cc.	3 cc.	6.00	3 cc.	3 cc.	6.50

The above explanations are based mainly on assumption. An attempt at a definite explanation brings forth an outline of work which must be done. In order to come closer to the correct explanation, it would be necessary to:

(1) determine the lactic acid content of the blood before and after the injection of calcium lactate solution.

(2) determine the glucose content of the blood before and after the injection of calcium gluconate solution.

(3) determine the effect of the pH of the blood on the rate of decomposition of calcium salts injected intravenously.

(4) using catheterization, determine the rate of excretion of the various calcium salts following intravenous administration.

These latter two points stem from the facts that rate of liberation of free calcium ions may explain the dosage differential, and varying sustenance of the increased calcium level due to varying rates of renal secretion may also help in formulating a definite explanation.

The fact that rabbit no. 9 was able to survive the injection of a quantity of physiological saline solution more than twice that of any of the other solutions shows definitely that the death of the rabbits receiving C-B-G and C-B-S-G Solutions was due primarily to the qualitative effects of the substances injected and not merely to quantitative effects. The question of a correlation between lethal dose and frequency of urination arises, since, in the trials on the C-B-G Solution, the ascending order of frequency of urination corresponds to that of lethal dose. In the trials on C-B-S-G Solution, rabbit no. 12, which did not urinate, survived the smallest dose, whereas rabbit no. 8, which urinated three times, was not able to survive a dose four cc. less than rabbit no. 7 which urinated twice. The volume of urine voided by rabbit no. 8 may have been less than that voided by rabbit no. 7, and the difference of four cc. in the lethal dose may be due to natural variation. However, these correlations and suppositions do not bear weight when viewed in the light of the fact that rabbit no. 9 survived the injection of over 270 cc. of fluid without urinating at all. The outstanding conclusion to be drawn here is that in these trials the determinations have been of the lethal doses of the salts from a qualitative angle rather than the determination of the resistance of rabbits to the injection of measured volumes of fluid.

In preparing a schedule to be followed in the drawing of blood samples for various determinations, particularly, as in this investigation, when no anesthetic or sedative is used, it is not advisable to set down the exact times at which these samples are to be drawn. The struggles of the animal against restraint or during the insertion of the

needle often cause a variation in the time of actual drawing as much as two or three minutes after the time proposed in the schedule. It is best merely to list the tentative times and then record the exact time after each sample has been drawn.

A study of Figure V shows that the statement by Godden and Duckworth (9) that "only about 15% of the calcium injected could be found in the serum 12 minutes after injection" has been corroborated in this investigation. These authors used a 10% solution of calcium gluconate, while in the present trials more highly concentrated calcium solutions were used. The serum calcium level falls very rapidly after the injection of calcium salts. Figure VI shows graphs computed from three trials with C-B-S-G Solution and two trials with C-B-G Solution. Since the individual trials vary so greatly (see Fig. V), trends, rather than exact figures, will be used in this discussion.

With both solutions, the serum calcium level rises rapidly during injection and falls rapidly following injection, but it rises higher, falls more slowly and does not fall so far after the injection of C-B-S-G Solution as it does following the injection of C-B-G Solution. Of interest and importance is the fact that, following the use of C-B-S-G Solution, there is a secondary peak in the serum calcium curve about one-half hour after injection. This phenomenon is of importance since it may be a starting point in the determination of the actual fate of injected calcium salts in the animal body. The difference in the initial calcium level peaks following the injection of C-B-G and C-B-S-G Solutions is small, and it would not be illogical to assume that this rise is due to the calcium from both the calcium gluconate and the calcium saccharate, but mainly from the calcium gluconate. This leaves the secondary peak which occurs after C-B-S-G injection to be accounted for.

This secondary peak may be explained theoretically as follows: The calcium ion is more tightly bound in calcium saccharate than is the calcium in calcium gluconate. This assumption is borne out by the difference in solubility, calcium gluconate being readily soluble to the extent of about 10% in water while calcium saccharate is virtually insoluble in water. This leads to further theorizing: As the C-B-S-G Solution enters the blood stream, both the calcium gluconate and calcium saccharate begin dissolving in the serum. Since the calcium gluconate is more highly soluble than is the calcium saccharate, the calcium from the former is predominantly instrumental in bringing about a condition of "calcium saturation" in the serum. At this point, most of the saccharate is as yet undissolved, and the calcium ion has not yet been liberated. When the serum calcium level falls to a certain point, there is a level of calcium ion

concentration set up at which the calcium from the saccharate can enter into solution. When this occurs, the secondary peak appears. This delayed break-down of the calcium saccharate would account for the fact that the increased serum calcium level is sustained longer and at a higher value following C-B-S-G injection than following C-B-G injection.

Also to be considered is the following theory: The serum calcium, as determined by its precipitation as oxalate, is the sum of the free calcium ions in the serum and the calcium still tied up as gluconate or saccharate, since both calcium gluconate and calcium saccharate react with ammonium oxalate solution *in vitro* to precipitate calcium oxalate. On this basis, the reason for the serum calcium not falling to its original level following the first peak is that, although the free calcium is rapidly removed from the blood stream, there is still some calcium gluconate or calcium saccharate, or both, to react with the ammonium oxalate. Repeating one of the assumptions of the first theory, most of the decomposition of the saccharate takes place after the calcium from the calcium gluconate has almost completely disappeared from the blood stream. It is known that the injection of glucose intravenously causes a temporary rise in the serum calcium level. The sustained rise of serum calcium following the injection of either calcium solution may be due in part to the formation of glucose by the reduction of the gluconate or saccharate radicals. If such a reduction takes place, the delayed decomposition of calcium saccharate would result in a delayed production of glucose. As this glucose is formed, the calcium level rises and thus the secondary peak is accounted for.

These explanations are purely theoretical and suggest further work which should be done. In order to prove or disprove these theories, or to formulate a more plausible theory, a series of tests similar to those carried out in this investigation should be performed. In addition to determining changes in the serum calcium level, determinations should also be made on the serum glucose levels. Recently, Anderson and Essex reported on "Studies on Barbiturates Especially Their Cyclic Disappearance from and Reappearance in the Blood Following Intravenous Injection."* It may be that if serum calcium determinations following the injection of C-B-S-G Solution were carried out to cover a period of several hours it would be found that a similar cyclic disappearance and reappearance occurs.

During this investigation, cows suffering from milk fever, or hypocalcemia, were

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* Proc. Staff Meet. Mayo Clin., XVII, 337, June 3, 1942.

not available, so the determinations were made on normal cows. However, there is no reason to doubt that C-B-S-G Solution would be more efficient than C-B-G Solution in elevating and maintaining the serum calcium level in hypocalcemic cows as it is in normal cows. This is another problem suitable for investigation.

The tests of the accuracy and efficacy of the special centrifuge tubes in the determination of serum calcium showed that they are unreliable when used as they were in this investigation. This can be seen by noting the extreme variation obtained in the readings listed in table VI. With the use of a calcium solution of known concentration, successful results would be evidenced by consistent and predictable readings; the tubes under tests here showed no such consistency.

The determination of the calcium content of serum by reading, against a graduated scale, the volume of calcium precipitated as oxalate is a sound principle. Various factors which would influence the results of such determinations can be controlled. Some of the factors in this class are temperature, speed of centrifugalization, period of time over which centrifugalization is carried out and time allowed for the precipitate to form before centrifugalization. There are, however, two obstacles which must be overcome before these factors can be standardized: interference by serum protein and adherence of some of the precipitate to the sides of the tube. This adherence occurs when serum is used and also when a calcium solution is used.

Although no actual coagulation of protein is evident, the presence of calcium oxalate crystals and serum in the capillary portion of the tube during centrifugalization results in a packing and cohesion so that the precipitated mass packs irregularly at the bottom of the tube and gives rise to inaccurate and variable readings. This difficulty should be capable of removal by the precipitation of the serum protein before the addition of the ammonium oxalate. During centrifugalization, varying amounts of precipitate adhere to the walls of the tubes, principally at the point where the wall of the upper portion of the tube curves toward the capillary portion. This adhesion does not occur uniformly around the circumference but is concentrated in an area on one side — that side which faces down when the arms of the centrifuge are horizontal. There are two methods by which this factor of adhesion can probably be eliminated: (1) by the use of the newer type of centrifuge wherein the arms are not allowed to swing to a horizontal position but are fixed at an angle of about 40 degrees from the vertical axis of the machine, and (2) by modifying the design of the tube so that the wide portion tapers gradually into the capillary portion rather than curving sharply (Fig. VII).

CONCLUSIONS

From the results of the previously described experiments, the following conclusions were drawn:

1. Boric acid increases the solubility of calcium d-saccharate in water. An increase in the concentration of boric acid results in an increase in the solubility of the saccharate.

2. Boric acid in solution reacts with calcium d-saccharate to form a new compound to which the name calcium boro-saccharate has been given.

3. A solution containing 10 parts acid, 37.5 parts calcium d-saccharate and 100 parts calcium gluconate in 500 parts distilled water is stable and is less toxic for rabbits and no more toxic for cows, when injected intravenously, than is the commercial product, Calcium-Boro-Gluconate.

4. The intravenous injection of either of these solutions into cows causes an immediate, marked rise in the serum calcium level. This rise is followed by a rapid drop, and, in the case of C-B-S-G Solution, there is a second rise in about one-half hour after injection.

5. The special centrifuge tubes for the determination of serum calcium are not reliable. Changes in contruction and technic are necessary before the efficacy of such tubes can be improved.

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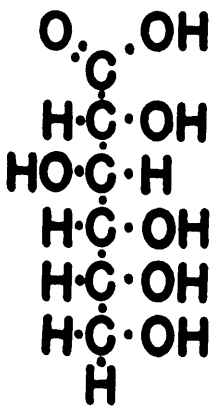

FIG. I A
D-GLUCONIC ACID

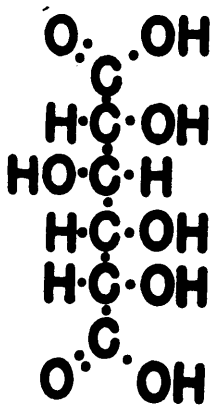

FIG. I B
D-SACCHARIC ACID

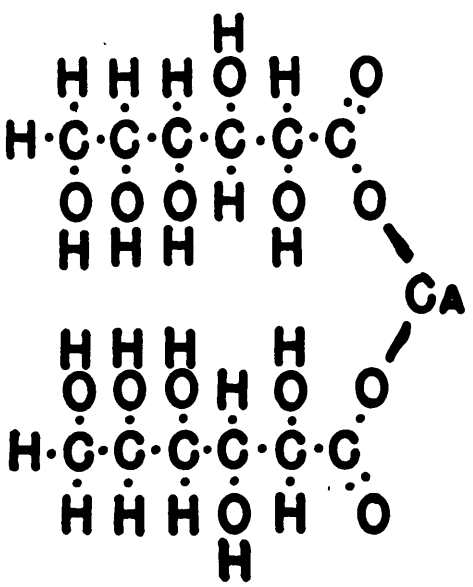

FIG. II A
CALCIUM GLUCONATE

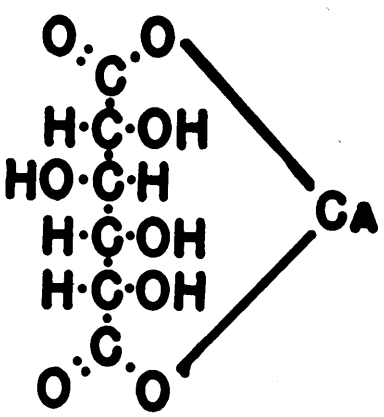

FIG. II B
CALCIUM SACCHARATE

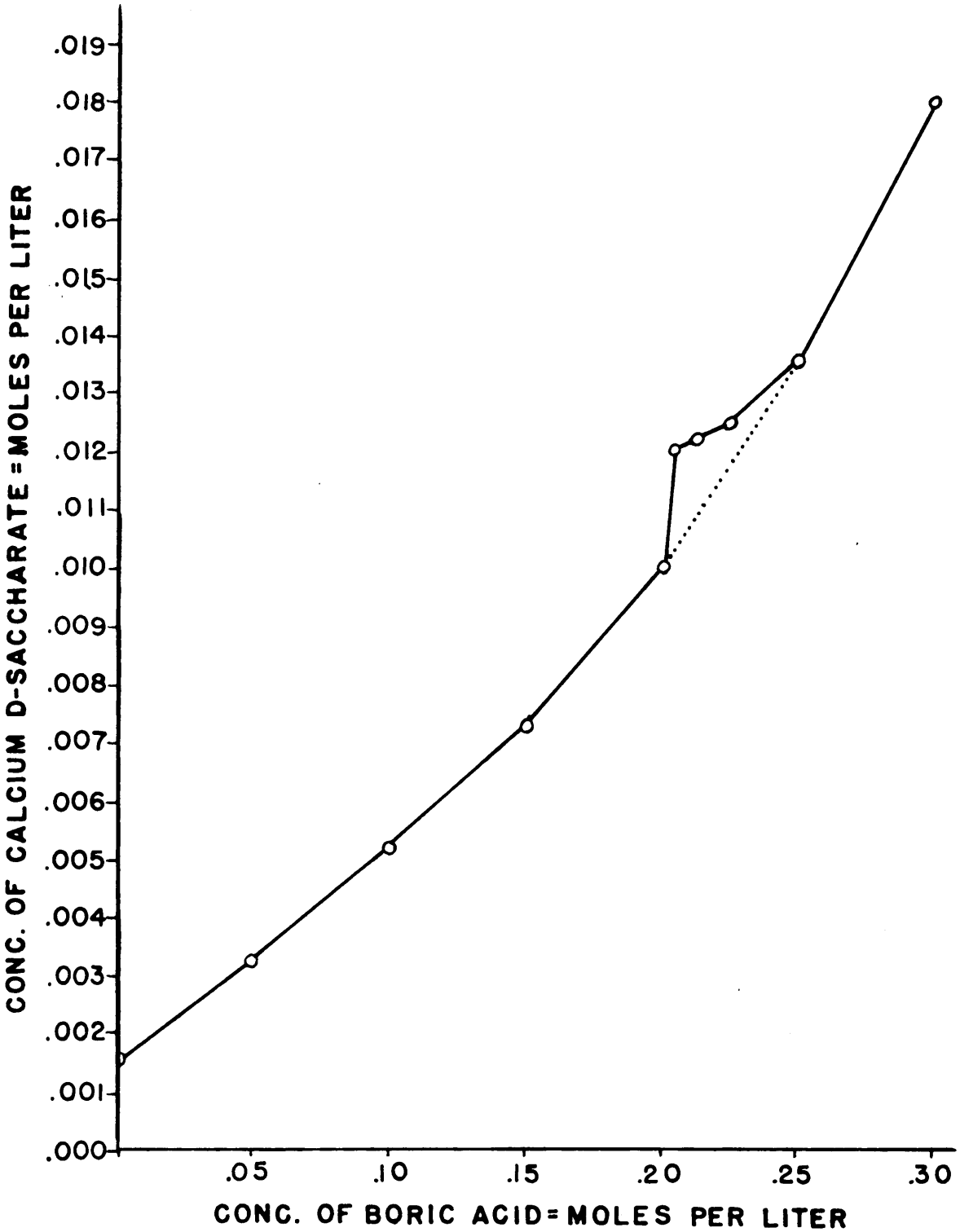

FIG. III - SOLUBILITY OF CALCIUM D-SACCHARATE IN BORIC ACID SOLUTION

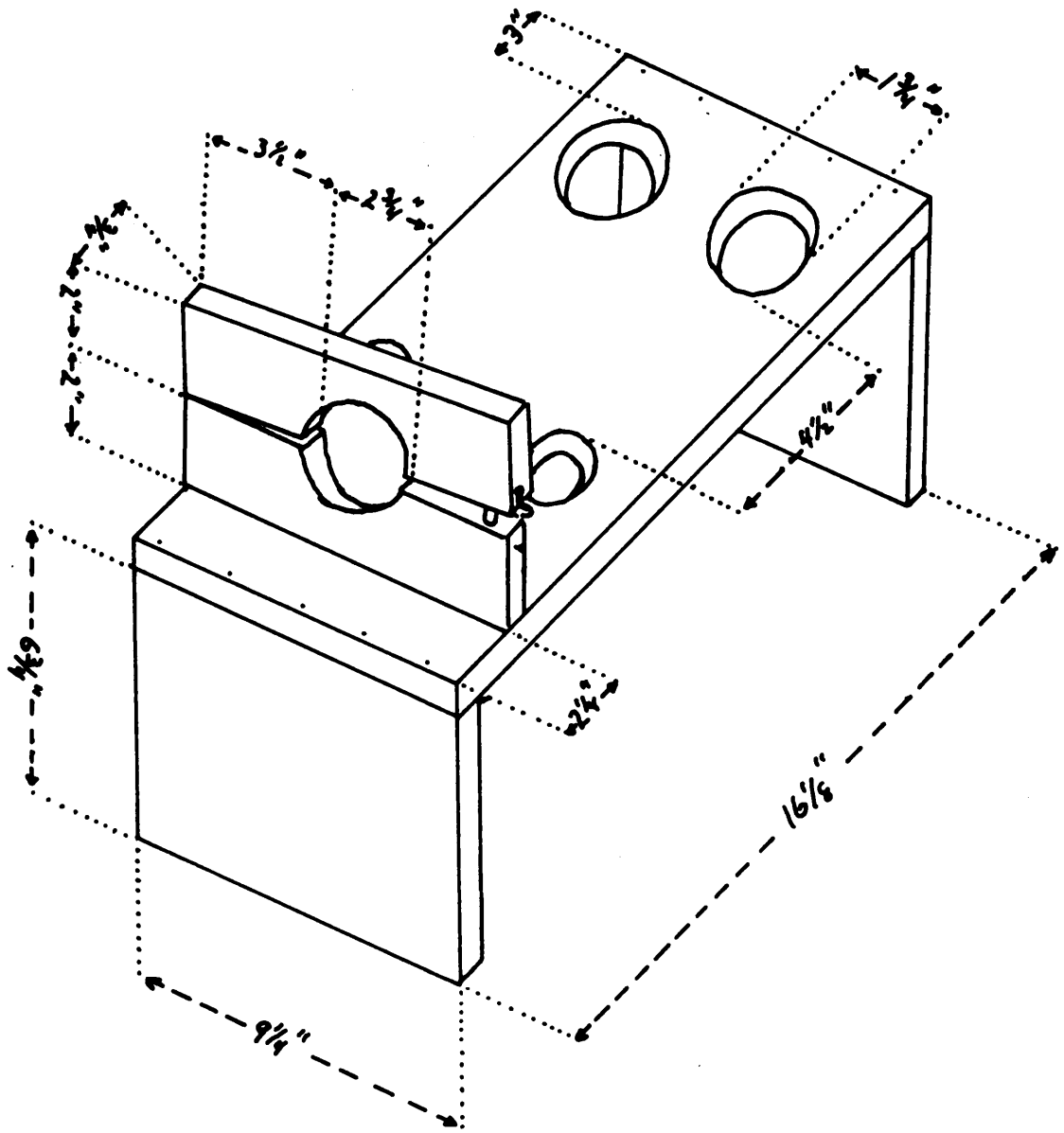

FIG. IV - SPECIAL STOCK FOR RESTRAINING RABBITS

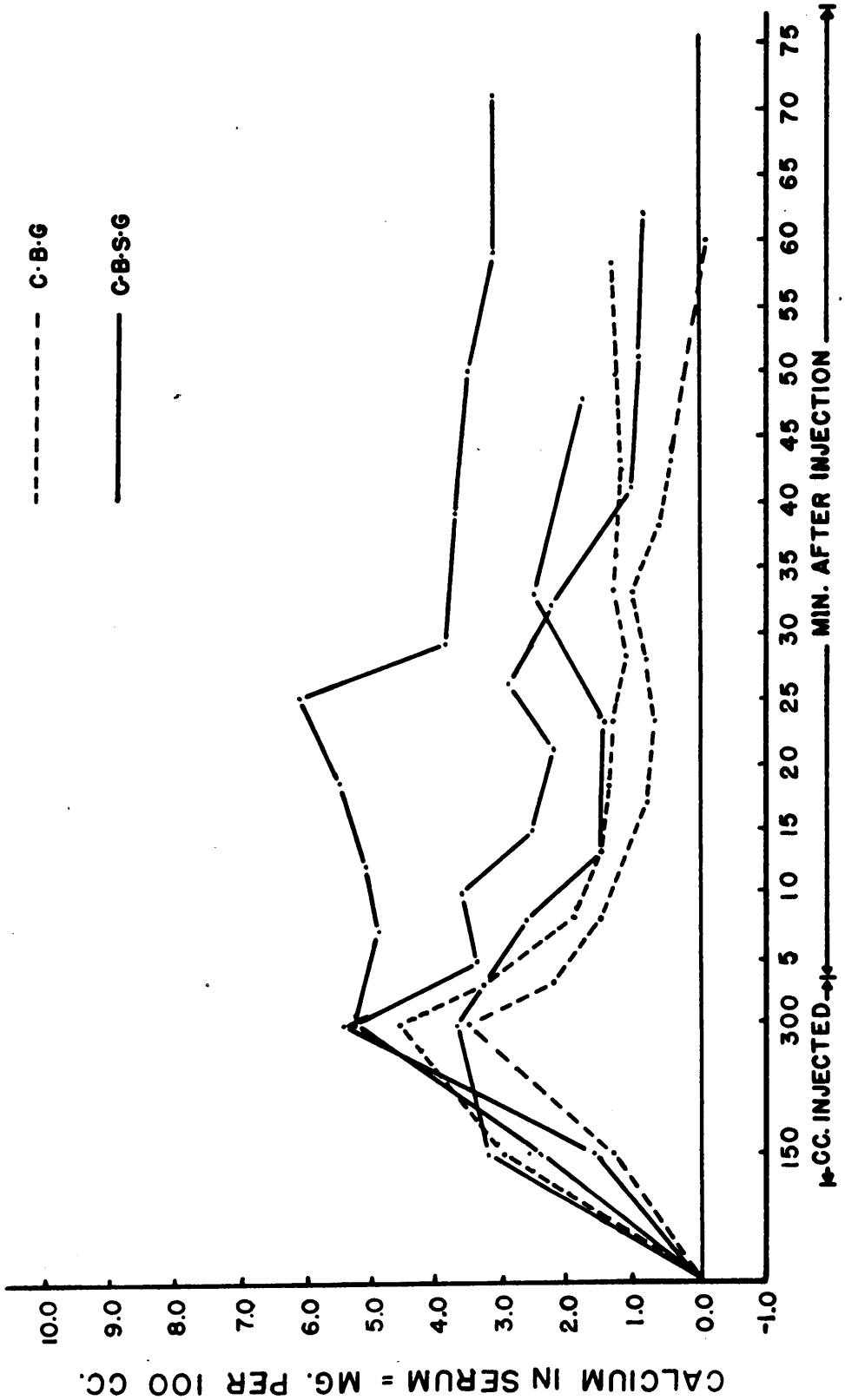

FIGURE V- COURSE OF SERUM CALCIUM LEVEL FOLLOWING C-B-G AND C-B-S-G INJECTIONS.

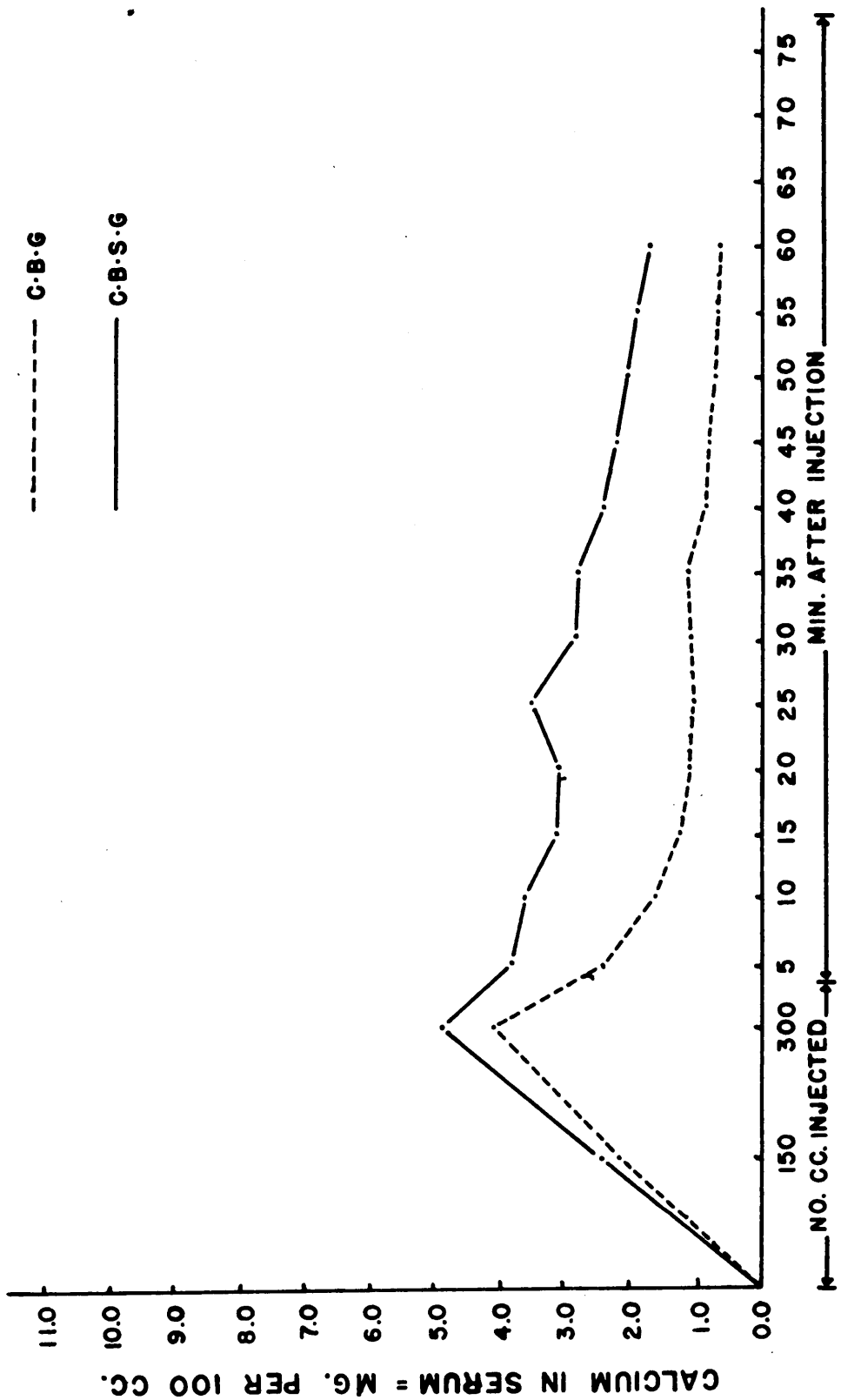

FIGURE VI- COURSE OF SERUM CALCIUM LEVEL FOLLOWING C-B-G AND C-B-S-G INJECTIONS [AVERAGE]

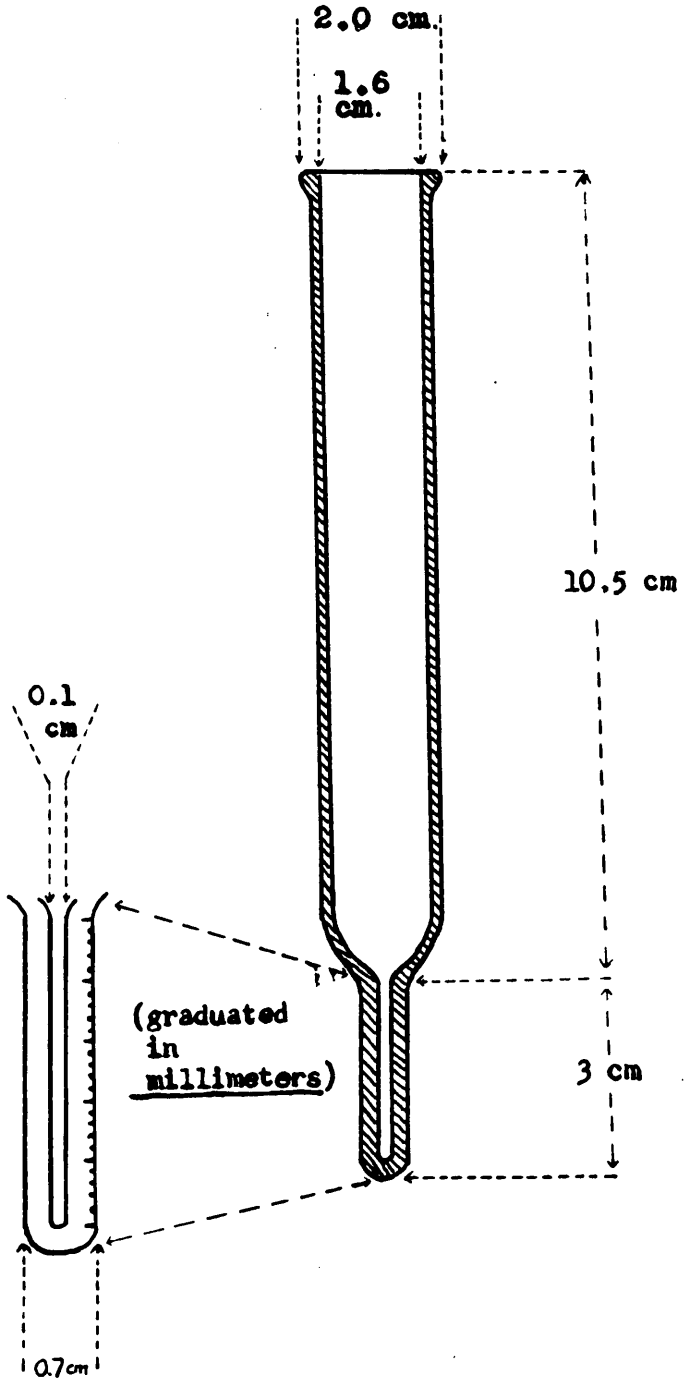

FIG. VII - CENTRIFUGE TUBE
FOR SERUM CALCIUM DETERMINATION