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The effect of orally ingested cerebroside on the excretion of coprostanol in cholesterol-fed rats

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With 4 tables

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ROSENHEIM and WEBSTER (1941) showed that in rats fed cholesterol (e.g., 138–200 mg per rat per day), the excretion of coprostanol was greater when cholesterol was ingested together with dried cholesterol-free brain (1.0–1.6 g)

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or with the cerebroside phrenosine (300 or 330 mg) than when cholesterol was fed together with dried cholesterol-free tissues such as pancreas, kidney, liver or horse flesh.

CARROLL (1960) examined the effect of cerebroside preparations on sterol excretion in rats fed diets without added cholesterol and found that the excretion of fecal LIEBERMANN-BURCHARD-positive sterols (mainly cholesterol) was greater when cerebrosides were ingested. The fatty acid esters obtained by hydrolyzing the cerebrosides had a similar but less marked effect on the excretion of LB-positive sterols.

JONES et al. (1953) found that ingestion of a lipid-poor and cholesterol-free residue of brain mitigated hypercholesterolemia and atherosclerosis in cholesterol-fed chicks. Further, JONES et al. (1961) found that the rate of disappearance of ^{14}C-total activity of serum cholesterol in two patients after receiving 1-^{14}C sodium acetate was increased by ingestion of cerebroside. They ascribed these effects to the ability of cerebrosides to bind fecal sterols and bile acids sufficiently to prevent their reabsorption and promote their fecal excretion.

Pure cerebrosides are not easy to prepare in substantial quantity. However, through the courtesy of Professor E. KLENK, Physiologisch-Chemisches Institut der Universität, Köln, we have received a quantity of sterol-free cerebroside prepared in his laboratory from brain. Thereby we have been able to repeat ROSENHEIM and WEBSTER's experiments in a slightly modified form, and to provide more comprehensive evidence for the assumption that adsorption of cholesterol to cerebroside is the explanation of the effect of ingested cerebroside on coprostanol excretion.

Experimental

Young female rats were kept on an ordinary stock diet until they reached a body weight of about 119 g. They were then distributed into 7 groups of 10. At time intervals of a few days the various groups were started on the basal diets indicated in table 1.

Three groups (nos. 20, 21, and 22) received the diet with rice starch, two groups (nos. 23 and 24) received the diet with glucose, and two groups (nos. 25 and 26) received the diet with lactose.

After two weeks, 1% cholesterol was incorporated into the diets of groups 21, 24 and 26, whereas 1% cholesterol and 3% cerebroside were incorporated into the diet of group 22, at the expense of the corresponding amount of carbohydrate.

Immediately thereafter, feces were collected (in ethanol) from all groups during three consecutive periods of three days each, whereafter the rats were killed with chloroform.

The content of the colon was taken out for determination of pH (semi-micro glass electrode) and for bacteriological studies as described below.

The fecal sterols were extracted in the following way: Portions representing the excretion during three days for each group were ground in a mortar with ethanol (50 ml) and extracted in Soxhlet (3–4 hours) with diethylether-ethanol (10:1 v/v) (prolonged extraction did not further increase the amount of sterol in the extract). The extracts were evaporated to dryness and saponified with 10–15 ml methanolic KOH (4n), in the presence of 100 mg pyrogallol as antioxidant, on steam bath under reflux for 1 hour. After addition of an equal volume of water, the non-saponifiable fraction was extracted with 3×30 ml diethyl ether, washed with water and dried with anhydrous sodium sulfate, evaporated, and the residue dissolved in chloroform (25 or 50 ml according to the quantities).

The components of the non-saponifiable fraction were separated by thin-layer chromatography on silicagel G (Merck), moving phase petroleum ether : diethyl ether (70:30, v/v) or, in some cases, diethyl ether : petroleum ether : glacial acetic acid (70:30:1) in a jar

Table 1. Basal diets for the various groups

	Groups 20, 21, 22	Groups 23, 24	Groups 25, 26
Casein, crude[1])	20 g	20 g	20 g
Rice starch[2])	59.3 g		
Glucose		59.3 g	
Lactose			59.3 g
Lard	15 g	15 g	15 g
Salt mixture[3])	5 g	5 g	5 g
Vitamin mixture[4])	0.5 g	0.5 g	0.5 g
Choline chloride	0.2 g	0.2 g	0.2 g

saturated with solvent vapor. The spots were developed by spraying with sulfuric acid 50% (v/v) and heating on an electric hot plate, 300° C. The density of the charred spots was measured semi-quantitatively on Densicord with integrator (Photovolt Corporation, New York, N.Y.) and compared with standards of pure sterols, all determinations being made in triplicate.

Vitamins A and D were given as an aqueous solution containing 2000 I.U. vitamin A and 200 I.U. vitamin D_3 per ml (gift from Ferrosan, Ltd., Copenhagen). 0.1 ml of this solution was given twice a week to each animal, corresponding to 57 I.U. vitamin A and 5.7. I.U. vitamin D_3 per animal per day.

The four sterols used as standards are listed below in the order of increasing mobility:

Δ^7-cholestenol,
Cholesterol and cholestanol (moving at the same rate),
Coprostanol.

They showed the following characteristic colors after being sprayed with sulfuric acid but before being charred:

Δ^7-cholestenol: red-orange
Cholesterol: red-violet
} after a few minutes' heating.

Cholestanol: yellow-brown
Coprostanol: yellow-brown
} after being heated somewhat longer.

In some cases the amount of total and saturated sterols were also determined by digitonin precipitation with and without bromination according to SCHÖNHEIMER (1930).

p_H was determined in the colonic content of all the animals with exception of three animals fed the glucose diet.

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[1]) "Dairinex" from Danish Dairy Industry and Export Company, Ltd., Stege, Denmark.

[2]) Amylum oryzae, Ph. Dan. 1948, from Northern Drug and Chemical Company, Ltd., Copenhagen.

[3]) MCCOLLUM and SIMMONDS' No. 185 (1918), supplemented with Cu, Mn and I: NaCl, 0.2335 g; $MgSO_4$, 0.3595 g; NaH_2PO_4, H_2O, 0.469 g; K_2HPO_4, 1.289 g; $Ca(H_2PO_4)_2$, H_2O, 0.730 g; Ca-lactate, $5H_2O$, 1.758 g; ferric citrate, 0.1595 g; $CuSO_4$, $5H_2O$, 6.5 mg; $MnSO_4$, H_2O, 19.7 mg; KI, 0.65 mg.

[4]) Biotin, 0.05 mg; folic acid, 0.05 mg; thiamine hydrochloride, 5 mg; riboflavin, 5 mg; pyridoxine hydrochloride, 5 mg; calcium pantothenate, 5 mg; nicotinic acid, 8 mg; p-aminobenzoic acid, 35 mg; inositol, 15 mg; ascorbic acid, 5 mg; vitamin K substitute (dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester, "Synkavit", Roche), 1 mg; d,l-α-tocopheryl acetate ("Ephynal", Roche), 10 mg; and sucrose to make 500 mg.

Bacteriological examination of the colonic content was carried out in all the groups, viz. from six animals in each of the groups fed rice starch diets containing cholesterol (group 21) or rice starch diets containing cholesterol plus cerebroside (group 22), and from a few of the animals in each of the other groups.

The total number of bacteria per g colonic content was determined by spreading in AC-medium (Difco) and tomato agar containing sodium azide (SNOG-KJAER et al., 1963). The number of colonies obtained with the two media did not differ to any great extent; the highest figure was considered as representing the total count of bacteria.

Simultaneously, inoculations were made with 1 ml of different dilutions of the colonic content (10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}) in 10 ml of a substrate suitable for growth of coprostanol producing bacteria, prepared in the following way:

40 g beef brain was suspended into 500 ml water, adjusted to pH 7.2 with KOH, and autoclaved at 120° C for 1 hour on two successive days, whereafter the following ingredients were added:

0.8 g NaH_2PO_4, H_2O

0.8 g K_2HPO_4

0.1 g soluble starch

0.1 g ascorbic acid

0.05 g cysteine hydrochloride.

The p_H was adjusted to 7.4–7.6, and the autoclaving repeated (for $^1/_2$ hour).

Growth in this medium was continued for at least six days.

Formation of coprostanol during the incubation was examined by applying a small quantity of the mixture, by means of an inoculation loop, on the thin-layer plate and running the chromatogram as already described.

Results and Discussion

The thin-layer chromatograms of the non-saponifiable matter from feces showed complete separation of cholesterol from the faster moving coprostanol, and the spots corresponding to these two sterols showed the earlier mentioned characteristic colors with sulfuric acid. A spot moving slower than cholesterol showed the color with sulfuric acid characteristic of Δ^7-cholestenol, and, probably was identical with that sterol. In addition to these one more spot was found, viz. one moving faster than coprostanol. This last mentioned spot might represent the "sterol D" described by COLEMAN et al. (1956).

The spots presumably representing Δ^7-cholestenol and "sterol D" were marked only in the cases where the rats had not received cholesterol in the diet because the non-saponifiable matter from these rats had to be applied to the plates in quantities representing a much larger amount of feces than was necessary in the cases where the rats had received dietary cholesterol. Apparently the substances represented by these spots did not originate from cholesterol.

As shown in table 2 (last vertical column), some coprostanol was present in the feces from all the groups. In the groups not receiving dietary cholesterol, the ratio coprostanol/cholesterol was highest in the glucose group and lowest in the lactose group. When cholesterol was given (without cerebroside), the ratio coprostanol/cholesterol was lower than in the corresponding groups without dietary cholesterol. The highest ratio coprostanol/cholesterol occurred in the rice starch group receiving cholesterol and cerebroside; (the amount of cerebroside available was not sufficient for supplementing the diets of the other groups with this substance under the circumstances of the present experiment).

Table 2. Data for fecal sterols

Group no.	Diet characteristics	Section of experimental period[1])	Wet weight of feces g	Sterols determined by digitonin precipitation without and with preceding bromination			Ratio coprostanol / cholesterol[2])
				Total sterol mg	Saturated sterol mg	Ratio saturat. / unsaturat.	
20	Rice starch	II	10	111			1.34
		III	9	115			1.41
21	Rice starch, cholesterol	II	10	712	231	0.48	0.58
		III	8	789	304	0.63	0.61
22	Rice starch, cholesterol, cerebroside	I	20	1010	564	1.21	1.3
		II	20	1272	851	2.02	2.2
		III	20	1300	920	2.42	2.4
23	Glucose	I	10	116			1.72
		II	11	117			1.82
		III	10	113			1.87
24	Glucose, cholesterol	I	10	954	316	0.49	0.55
		II	11	1360	420	0.45	0.82
		III	13	1238	376	0.44	0.47
25	Lactose	I	13	93			1.03
		II	14	101			1.08
		III	15	112			0.69
26	Lactose, cholesterol	I	15	230			0.20
		II	15	566			0.18
		III	18	569			0.11

[1]) I = first third of the 9-day experimental period.
II = second third of the 9-day experimental period.
III = last third of the 9-day experimental period.

[2]) Determined by thin-layer chromatography and measured on Densicord.

In the cases where the ratio saturated/unsaturated sterols was calculated from determinations of total and saturated sterols by digitonin precipitation without and with preceding bromination, this ratio corresponded fairly well with the ratio coprostanol/cholesterol determined by thin-layer chromatography.

The figures for total fecal sterols were higher in the groups receiving dietary cholesterol than in the corresponding groups without dietary cholesterol. In the groups receiving dietary cholesterol (but without cerebroside), the highest figures for total fecal sterols occurred in group 24 (glucose), and the lowest in group 26 (lactose). In the group receiving the rice starch diet with cholesterol and cerebroside (group 22), the amount of total fecal sterol was as high as in the glucose group with cholesterol.

The amount of saturated fecal sterols was two to three times as high in the group receiving cholesterol and cerebroside (group 22) as in the corresponding group receiving cholesterol without cerebroside (group 21), whereas the amount of unsaturated sterols was about the same in both groups.

The wet weight of feces was highest in the group receiving cerebroside, and second highest in the lactose group. The reason for this is, apparently, that the ingested cerebroside (approximately 9 g for 10 rats in three days) is excreted, and the feces from the lactose fed animals contain more water than the feces

Table 3. Bacteriological examinations of colonic content of rats on the various diets[1])

Group no.	Diet characteristics	Coprostanol formation as determined by TLC			Total number of bacteria × 10^9 per g colonic content
		Dilution 10^{-4}	Dilution 10^{-5}	Dilution 10^{-6}	
20	Rice starch	+	+	+	1
		+	+	0	2
		+	0	0	1.6
21	Rice starch, cholesterol	+	0	0	1
		+	+	0	6
		+	+	0	4
		+	0	0	2
		+	0	0	1
		+	0	0	7
22	Rice starch, cholesterol, cerebroside	+	+	0	2
		+	0	0	3
		+	+	0	6
		+	0	0	4
		+	+	0	3
		+	+	0	0.7
23	Glucose	+	0	0	1.1
		+	0	0	1.8
24	Glucose, cholesterol	+	+	0	2
		+	+	0	1
25	Lactose	+	0	0	40
		+	0	0	23
26	Lactose, cholesterol	+	0	0	100
		0	0	0	60

[1]) The figures in each horizontal line refer to the colonic content of one animal.

from the other groups. (The food consumption during the 9-day experimental period was 10 g per day per rat in the rice starch groups nos. 20, 21, and 22).

The conclusion to be drawn from the above mentioned results is that ingestion of cerebroside causes an increase of the conversion of ingested cholesterol to coprostanol, in accordance with the observations of ROSENHEIM and WEBSTER (1941).

It is obvious to explain this phenomenon in accordance with JONES et al. (1953) by assuming that the cerebroside carries cholesterol through the entire length of the intestine whereby more cholesterol becomes exposed to the action of the intestinal flora.

This mode of explanation is supported by the fact that more sterol is excreted with the feces when cholesterol is fed together with cerebroside (table 2).

As seen from table 3, the limit for conversion of cholesterol to coprostanol by incubation with colonic content *in vitro* is found at the same dilution (10^{-6}) for groups 21 and 22.

The total count of bacteria is approximately the same in all the groups except the lactose groups where the count is much higher, apparently due to abundant growth of lactic acid bacteria.

As seen from table 4, great variations of p_H of the colonic content do not occur. However, the "average p_H" of the colonic content in all the groups

receiving dietary cholesterol is slightly lower than that in the corresponding group without dietary cholesterol. Further, in the group receiving cerebroside and cholesterol, the "average p_H" is slightly lower than in the corresponding group receiving cholesterol only. The cause of these apparent variations is not clear.

Table 4. pH of colonic content of rats on the various diets

Group no.	Diet characteristics	pH of colonic content from individual rats						average[1])
20	Rice starch	6.4	6.4	6.7	6.8	6.8	6.9	6.77
		7.0	7.0	7.4	7.5			
21	Rice starch, cholesterol	6.2	6.3	6.3	6.3	6.4	6.6	6.46
		6.6	6.7	6.8	7.0			
22	Rice starch, cholesterol, cerebroside	5.8	5.8	6.1	6.4	6.5	6.6	6.25
		6.7	6.7	6.8	7.0			
23	Glucose	6.4	6.6	6.7	6.8	6.8	6.8	6.68
		6.9						
24	Glucose, cholesterol	6.1	6.3	6.4	6.5	6.5	6.5	6.51
		6.8	6.9	7.0	7.2			
25	Lactose	5.5	5.6	5.7	6.4	6.5	6.6	6.04
		6.6	6.8	6.9	7.3			
26	Lactose, cholesterol	5.7	5.7	5.8	5.9	6.0	6.0	5.95
		6.1	6.4	6.4	6.5			

[1]) "Average p_H" is the negative logarithm of the average hydrogen ion concentration.

The adsorption of cholesterol to cerebroside was demonstrated directly, by shaking 2 ml of a transparent colloidal solution of cholesterol in 48 millimolar sodium deoxycholate with 60 mg cerebroside, separation of the cerebroside from the solution by high speed centrifugation and determination of the cholesterol concentration of the supernatant and in a similarly treated solution without cerebroside. Cholesterol was extracted from 0.5 ml supernatant by addition of 0.1 ml absolute ethanol and shaking 3 times with 2 ml diethyl ether; after evaporation cholesterol was determined by the LIEBERMANN-BURCHARD reaction.

Three such experiments gave the following results:

	Cholesterol microMol/ml		Cholesterol microMol/ml	
Exp. no. 1:	Without cerebroside treatment	1.11	After cerebroside treatment	0.57
Exp. no. 2:	Without cerebroside treatment	1.08	After cerebroside treatment	0.56
Exp. no. 3:	Without cerebroside treatment	1.06	After cerebroside treatment	0.57

The cerebroside precipitates from the above mentioned adsorption experiments were dried in N_2-stream and extracted 3 times with 3 ml ethyl ether which was separated from the precipitate by centrifugation each time. The following amounts of cholesterol (determined by the LB-reaction) were recovered:

Exp. no. 1: 0.35 microMol from the cerebroside precipitate from 1 ml
Exp. no. 2: 0.35 microMol from the cerebroside precipitate from 1 ml
Exp. no. 3: 0.27 microMol from the cerebroside precipitate from 1 ml

Thus, the recovery was not quantitative.

The difficulty involved in quantitative extraction of cholesterol from cerebroside containing matter has previously been reported by JONES et al. (1961).

Since the publication of our experiments on bacterial conversion of cholesterol into coprostanol independent of cerebrosides in the medium (PRANGE et al., 1958), thin-layer chromatography has been introduced whereby it is possible to detect impurities in the medium more accurately than before.

We have, therefore, repeated these *in vitro* incubation experiments with the modification that a solution of cholesterol in chromatographically purified egg lecithin was used instead of a solution of cholesterol in soybean lecithin and tween 80. The absence of cerebroside from the purified egg lecithin was verified by thin-layer chromatography according to WAGNER et al. (1961). The cholesterol solution had the following composition: 5 mg cholesterol, 10 mg lecithin in 5 ml of water. It was sterilized by autoclaving at 115 °C 15 min. and added to 5 ml of the similarly sterilized medium described in our previous publication (PRANGE et al., 1958). A bacterial culture from feces of rats in group 20 which had been reinoculated twice through a medium of the above mentioned composition was used as inoculum. After incubation at 37° C for 6 days, conversion of cholesterol to coprostanol was demonstrated by thin-layer chromatography as described in the foregoing section. It is therefore certain that the bacterial conversion does not require the presence of cerebroside in the incubation medium.

The following tests showed that the cerebroside preparation used in this study was free from cholesterol and contained no phospholipids except a trace of shingomyelin.

By thin-layer chromatography on Silicagel G (Merck) with petroleum ether:diethyl ether:glacial acetic acid (80:20:1) in saturated vessel and comparison with standards of cholesterol (R_f = 0.13), cholesterol palmitate (R_f = 0.86) and tripalmitin (R_f = 0.63), no impurity migrating in this system could be found.

Thin-layer chromatography on silicagel G with chloroform: methanol:water (65:25:4) in saturated vessel showed spots with the following R_f values:

cerebrosides	0.84 0.79
cerebroside-sulfuric acid esters	0.52 0.50
sphingomyelins	0.41 0.39
trace of ganglioside	0.18

The identification of the spots was based on the R_f values indicated by WAGNER et al. (1961).

The heaviest spot was that with R_f = 0.79, which is assumed to represent phrenosine (cerebron, containing the saturated C_{24}-hydroxy acid cerebronic acid).

Since lecithin, if present, would migrate as the cerebroside sulfuric acid esters, the chromatogram was sprayed with DRAGENDORFF's reagent (mixture of basic bismuth nitrate in acetic acid, and aqueous potassium iodide), which yields an orange-red color with choline phosphatides. The only region which gave a positive reaction was that corresponding to sphingomyelins, and here the reaction was very weak. The R_f value 0.50 for lecithins in the system used was verified by standards.

Summary

The influence of orally ingested cerebroside on the conversion of cholesterol into coprostanol in the intestine of rats has been reexamined.

Ingestion of cerebroside to rats receiving a diet containing cholesterol increased the fecal excretion of total and saturated sterols, the ratio saturated to unsaturated sterols determined by digitonin precipitation with and without preceding bromination, and the ratio coprostanol to cholesterol determined by thin-layer chromatography.

Ingestion of cerebroside did not change the total count of bacteria per g of colonic content of the rats.

By incubation of a brain substrate with a series of dilutions of colonic content from rats receiving the cholesterol containing diet with and without cerebroside, the limit for conversion of cholesterol to coprostanol was found at dilutions of the same order of magnitude.

Cholesterol held in solution by chromatographically purified lecithin, shown by thin-layer chromatography to be free from cerebroside, could be converted into coprostanol by incubation *in vitro* with a culture of bacteria. Presence of cerebroside, therefore, is not a condition for formation of coprostanol.

Approximately half of the cholesterol present in an aqueous transparent solution of cholesterol in sodium deoxycholate could be adsorbed to cerebroside merely by being shaken with it.

These results confirm the assumption that the effect of ingested cerebroside on the excretion of coprosterol is due to adsorption of a considerable part of the cholesterol present in the intestine on the insoluble cerebroside, whereby an increased amount of cholesterol becomes exposed to the action of bacteria in the coecum and large intestine.

Zusammenfassung

Der Einfluß von oral verabreichtem Cerebrosid auf die Umbildung von Cholesterin in Coprosterin im Darmtractus der Ratte wurde von neuem untersucht.

Verabreichung von Cerebrosid an Ratten, die mit einer cholesterinhaltigen Nahrung gefüttert wurden, erhöhte die Exkretion von Gesamt-Sterin und gesättigtem Sterin, das Verhältnis von gesättigtem zu ungesättigtem Sterin (ermittelt durch Digitoninfällung mit und ohne Brombehandlung), sowie das Verhältnis von Coprosterin zu Cholesterin (durch Dünnschicht-Chromatographie bestimmt).

Verabreichung von Cerebrosid veränderte nicht die Gesamtzahl der Bakterien pro Gramm Coloninhalt der Ratten.

Bei Inkubation von einem Hirnsubstrat mit verschiedenen Verdünnungen von Coloninhalt von Ratten, die mit einer cholesterinhaltigen Nahrung mit und ohne Cerebrosid gefüttert waren, wurde die Grenze für Coprosterinbildung bei Verdünnungen von derselben Größenordnung gefunden.

Cholesterin, gelöst in einer wässerigen kolloiden Lösung von auf chromatographischem Wege gereinigtem Lecithin, das sich durch Dünnschicht-Chromatographie als cerebrosidfrei erwies, konnte durch Inkubation mit einer Bakterienkultur in Coprosterin umgebildet werden. Anwesenheit von Cerebrosid ist deshalb keine Bedingung für die Coprosterinbildung.

Wenn eine transparente wässerige Lösung von Cholesterin in Natriumdesoxycholat mit Cerebrosid geschüttelt wurde, adsorbierte das Cerebrosid ungefähr die Hälfte des Cholesterins.

Die vorliegenden Ergebnisse bestätigen die Annahme, daß die Wirkung des Cerebrosids auf die Exkretion von Coprosterin dadurch zustandekommt, daß ein beträchtlicher Teil des sich im Darmtraktus befindlichen Cholesterin auf das unlösliche Cerebrosid adsorbiert, und dadurch der Resorption entzogen und der Wirkung der Bakterien im Dickdarm und Coecum ausgesetzt wird.

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The complexing of calcium by citrate, ortho- and polyphosphates *)

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With 3 tables

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Introduction

There is substantial evidence that divalent cations have a strong tendency to form partially dissociated complexes in solution with the anions of some organic acids. In a number of cases this effect has been studied quantitatively and expressed in terms of well defined dissociation constants (1, 2). Some reports indicate that calcium and orthophosphates may form similar complexes and the pK values of these complexes, calculated with the aid of some assumptions (3), have been reported (4, 5, 6).

Polyphosphates are well known as excellent complexing agents for alkaline earth metals (6) and for some of these the stability of their calcium complexes has been described (4).

One of the major difficulties encountered in this field has been the lack of a reliable and simple method for the determination of calcium ions in solution. Since such a method is now available (7) it was decided to study the formation of calcium complexes with citrate and phosphates as they are of considerable importance in biological systems such as milk, blood, and muscle tissue. Polyphosphates were included in this study because of their known complexing ability and possible usefulness in practical applications of this effect.

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