References

CARROLL, K. K., J. Lipid Res. **1**, 171 (1960). — COLEMAN, D. L., W. W. WELLS, and C. A. BAUMANN, Arch. Biochem. Biophys. **60**, 412 (1956). — JONES, R. J., S. C. KRAFT, S. HUFFMAN, E. L. BALTER, and R. B. Gordon, Circulation Res. **1**, 530 (1953). — JONES, R. J., O. K. REISS, and M. F. GOLDEN, Proc. of the Symposium on Drugs Affecting Lipid Metabolism, Milano 1960, ed. by S. GARATTINI and R. PAOLETTI, p. 212 (Amsterdam 1961). — PRANGE, I., A. SNOG-KJAER, G. KOFOED NIELSEN, and H. DAM, Acta Pathol. Microbiol. Scand. **42**, 29 (1958). — ROSENHEIM, O. and T. A. WEBSTER, Biochem. J. **35**, 920 (1941). — SCHÖNHEIMER, R., Z. f. Physiol. Chemie **192**, 77 (1930). — SNOG-KJAER, A., I. PRANGE, F. CHRISTENSEN, and H. DAM, Z. f. Ernährungswiss. **4**, 14 (1963). — WAGNER, H., L. HÖRHAMMER and P. WOLFF, Biochem. Z. **334**, 175 (1961).

Authors' address
c/o Professor HENRIK DAM, Afdelingen for Biokemi og Ernaering Danmarks Tekniske Højskole, Østervoldgade 10 L II, København K, (Danmark)

From the Department of Dairy Science, University of Alberta, Edmonton, Alberta (Canada)

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

By S. C. BATRA**) and J. M. DE MAN

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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**) Present address: Faculty of Pharmacy, University of Alberta.

It is desirable to increase our knowledge of the properties of these compounds as many of the phosphates are used as additives in a wide variety of food products and a large number of these applications undoubtedly depend on the calcium complexing ability of the phosphates.

Materials and Methods

Sodium hexametaphosphate and sodium tetraphosphate were commercial products generously supplied by Rumford Chemical Works, Rumford, R. I. Sodium tripolyphosphate (Food grade) was a gift of Calgon, Inc., Pittsburgh, Pa. All other chemicals were reagent grade.

The concentration of free calcium ions was determined by a colorimetric murexide method described elsewhere (7). Freezing point depressions were measured with a Fiske Model I milk cryoscope.

Solutions containing 5, 10 or 20 mmoles of P and solutions containing 5, 10 or 20 mmoles of citrate were made up and adjusted to 0.16 M ionic strength with either sodium chloride or tetraethylammonium bromide. The latter chemical was used because it has been claimed (4, 5, 8) that the tetraethylammonium ion does not form complexes with phosphates as might be the case with sodium ions.

The dissociation constant K was calculated from

$$\frac{[Ca^{++}] \cdot ([\text{Total anion}] - [\text{Total Ca}] + [Ca^{++}])}{[\text{Total Ca}] - [Ca^{++}]} = K$$

on the assumption that the inactivated calcium is combined reversibly and stoichiometrically, forming a 1 : 1 complex.

Results and Discussion

To determine the calcium complexing ability of the various phosphates and citrate, 2 ml of each solution containing 5, 10 or 20 mmoles of these compounds were mixed with 2 ml of a solution containing 5 mmoles of calcium ions and 2 ml of Tris buffer of p_H 6.8 (7). Free calcium ion determinations in these mixtures

Table 1. Free calcium ion concentration and pK values of solutions of calcium chloride in the presence of polyphosphates and citrate, at 25° C, ionic strength. 16 M and p_H 6.8

Complexing agent	P mmoles	Ionic strength made up with					
		Sodium chloride			Tetraethylammonium bromide		
		Ca^{++} mmoles	Ca compl. mmoles	pK	Ca^{++} mmoles	Ca compl. mmoles	pK
Sodium hexametaphosphate	5	3.75	1.25	2·92	3.4	1.6	2.97
	10	2.5	2.5	2.62	1.6	3.4	2.96
	20	1.0	4.0	2.89	0.7	4.3	3.09
Sodium tripolyphosphate	5	4.0	1.0	2.89	3.75	1.25	2.92
	10	2.75	2.25	2.53	2.2	2.8	2.74
	20	1.5	3.5	2.87	0.8	4.2	3.02
Sodium tetraphosphate	5	3.8	1.2	2.92	3.25	1.75	2.99
	10	2.4	2.6	2.66	1.5	3.5	3.05
	20	1.1	3.9	2.87	0.7	4.3	3.09
Sodium citrate	5	2.6	2.4	2.89	3.75	1.25	2.92
	10	1.5	3.5	2.53	2.2	2.8	2.74
	20	0.8	4.2	2.87	0.8	4.2	3.02

indicated that primary sodium phosphate (NaH_2PO_4) and secondary sodium phosphate (Na_2HPO_4) did not show any complexing activity at these concentrations, in all cases the calcium ion concentration of the mixture was 5 mmoles. Sodium pyrophosphate could not be included in this series of experiments because it formed precipitates with calcium ions in the presence of the Tris buffer, due to its high pH in solution.

Results obtained with sodium hexametaphosphate, sodium tripolyphosphate, sodium tetraphosphate and sodium citrate are listed in Table 1. Mean pK values of the calcium complexes of these compounds are given in Table 2, and are only slightly different from one another. The citrate complex had a higher pK value than the polyphosphate complexes. In all cases the pK value was higher in the presence of tetramethylammonium bromide than in the presence of sodium chloride, indicating some complexing of the sodium ion as well. The pK value of the citrate complex in the presence of sodium chloride was slightly lower (3.03) than the figures reported by JOSEPH (1) and by HASTINGS et al. (2) who found values of 3.17 and 3.22 respectively.

Table 2. Mean pK values of the calcium complexes of polyphosphates and citrate; at 25° C, ionic strength. 16 M and pH 6.8

Complexing agent	Formula	Mean pK in sodium chloride	Mean pK in tetraethylammonium bromide
Sodium hexametaphosphate	$(NaPO_3)_6$	2.88	3.00
Sodium tripolyphosphate	$Na_5P_3O_{10}$	2.76	2.89
Sodium tetraphosphate	$Na_6P_4O_{16}$	2.81	3.04
Sodium citrate	$Na_3C_6H_5O_7$	3.03	3.10

The absence of calcium complexing ability of orthophosphate could also be demonstrated with the freezing point depression method. Isotonic solutions of calcium and sodium chloride, primary potassium phosphate and citrate were prepared and equal quantities mixed in the combinations listed in Table 3.

Table 3. Freezing point depressions of solutions of calcium and sodium chloride, primary potassium phosphate and citrate and their mixtures

Solution	Freezing point
$CaCl_2$	0.572
NaCl	0.573
KH_2PO_4	0.571
K_3Citr	0.567
NaCl + KH_2PO_4	0.573
NaCl + K_3Citr	0.570
$CaCl_2$ + KH_2PO_4	0.571
$CaCl_2$ + K_3Citr	0.540

There was no interaction between sodium and phosphate or citrate ions and between calcium and orthophosphate ions. The combination of calcium and citrate ions resulted in a considerable lowering of the freezing point depression indicating the formation of complex ions.

It was shown by DE MAN and BATRA (9) that orthophosphates have little or no effect on the stability of milk proteins and that citrate and polyphosphates have an important stabilizing action on the casein micelles. Although it is frequently stated that calcium can be bound in the form of complex ions by orthophosphates (10), no evidence for such action was obtained in the present work. If orthophosphate under certain conditions does have an effect on the stability of the caseinate micelles it is most likely through a different mechanism than citrate which is an effective calcium ion complexing agent.

Evidence for the lack of calcium complexing ability of orthophosphates was provided earlier by GOSSELIN et al. (11), who showed that administration of these phosphates in rats did not cause hypocalcemia, whereas pyro, tripoly and hexametaphosphates caused severe hypocalcemia, which was attributed to the complexing of calcium by these salts.

Summary

The calcium ion complexing ability of orthophosphates, polyphosphates and citrate was determined by measuring free calcium ions in mixtures of these salts and calcium chloride. Primary and secondary orthophosphates showed no calcium complexing ability; sodium citrate, sodium hexametaphosphate, sodium tripolyphosphate and sodium tetra phosphate were effective calcium complexing agents and the pK values in the presence of sodium chloride and of tetraethylammonium bromide were determined. All pK values were higher in the absence of sodium ions indicating a competitive effect of the sodium ion. The lack of calcium complexing ability of ortho-phosphate could also be demonstrated by a cryoscopic technique.

References

1. JOSEPH, N. R., J. Biol. Chem. **164**, 529 (1946). — 2. HASTINGS, A. B., MCLEAN, F. C. EICHELBERGER, L., HALL, J. L., and DE COSTA, E., J. Biol. Chem. **107**, 351 (1934). — 3. WAZER, J. R. V. and CALLIS, C. F., J. Am. Chem. Soc. **78**, 2376 (1956). — 4. GREENWALD, I., REDISH, J. and KIBRICK, A., J. Biol. Chem. **135**, 65 (1940). — 5. GOSSELIN, R. E. and COGHLAN, E. R., Arch. Biochem. Biophys. **45**, 301 (1953). — 6. SMITH, R. M. and ALBERTY, R. A., J. Am. Chem. Soc. **78**, 2376 (1956). — 7. BATRA, S. C. and deMAN, J. M., Milchwissenschaft. **42**, 473 (1964). — 8. SMITH, R. M. and ALBERTY, R. A., J. phys. Chem. **60**, 180 (1956). — 9. DEMAN, J. M. and BATRA, S. C., J. Dairy Sci. **47**, 954 (1964). — 10. JENNESS, R. and PATTON, S. Principles of Dairy Chemistry (New York 1959). — 11. GOSSELIN, R. E., TIDBALL, C. S., MEGIRIAN, R., Maynard, E. A., DOWNS, W. L., and HODGE, H. C., J. Pharmacol. Exptl. Therap. **108**, 117 (1953).

Authors' address:
J. M. DE MAN, PH. ASSOCIATE Professor of Dairy and Food Chemistry, S. CBATRA, M. SC RESEARCH ASSOCIATE, Universiti of Alberta, Edmonton, Canada