similar magnitude and seem to confirm this assumption.

Further species differences are apparent in the responses to hormonal stimuli. The pituitary hormones of peptide structure, corticotropin or growth hormone elicited larger FFA release from the YBM and epididymal fat pad of the guinea pig than from the rat adipose tissue. With catecholamine hormones the converse was true. These species differences are in agreement with those noted by Rudman *et al*(8). The observations on growth hormone effects in guinea pigs, extend those of Fain *et al*(9), who reported that prominent *in vitro* activation of lipolysis by growth hormone in rats may be observed only after a lag period and supplementation with glucocorticoids.

Summary. Yellow bone marrow of guinea pig is capable of triglyceride synthesis from glucose or from free fatty acids and contains a lipolytic system susceptible to activation, particularly upon contact with pituitary hormones. Its metabolic activity is similar to homologous epididymal adipose tissue, although both guinea pig tissues are appreciably less active than the rat epididymal fat pad. The composition and the metabolic characteristics indicate that the yellow bone marrow represents a typical adipose tissue.

1. Shafrir, E., Wertheimer, E., in Adipose Tissue, Handbook of Physiology, A. E. Renold, G. F., Cahill, Jr., ed., Am. Physiol. Soc. publishers, 1965, Chap. 43, Sec. 5, p427.

2. Dole, V. P., J. Clin. Invest., 1956, v35, 150.

3. Kerpel, S., Shafirir, E., Shapiro, B., Biochim. Biophys. Acta, 1961, v46, 495.

4. Gorin, E., Shafrir, E., ibid., 1963, v70, 109.

5. Folch, J., Lees, M., Sloane-Stanley, G. H., J. Randall, R. J., J. Biol. Chem., 1951, v193, 265.

6. Schneider, W. C., Methods in Enzymology, Colowick, S. P., Kaplan, N. O., ed., Academic Press, Inc., publishers, New York, 1957, v3, 680.

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Biol. Chem., 1957, v226, 497.

8. Rudman, D., Brown, S. J., Malkin, M. F., Endocrinology, 1963, v72, 527.

9. Fain, J. N., Kovacev, V. P., Scow, R. O., J. Biol. Chem., 1965, v240, 3522.

Received January 12, 1967. P.S.E.B.M., 1967, v124.

The Binding of Calcium in Mixtures of Phospholipids.* (31984)

RICHARD W. JOOS AND CHARLES W. CARR
Department of Biochemistry, University of Minnesota Medical School, Minneapolis

Recently there has been renewed interest in the interaction of small cations with various phospholipids(1-4). This interest has no doubt been generated to a large extent by certain findings concerning the role of phospholipids in membrane structure and function, by the effects of small cations on membrane permeability, and by new techniques for the separation of the various types of phospholipids in relatively purified form. This recent work on ion-binding indicates that phospholipids bind small cations in a reversible, electrostatic combination, and that even in aqueous micellar suspensions of phospholipids, all of the ionizable groups are readily available. In a study of the binding of calcium in aqueous suspensions of phospholipids, we have confirmed these findings and in addition demonstrated a marked effect of pH on binding. This pH dependence has been shown to be directly related to the nature of the dissociable groups in the various phospholipids.

Methods and materials. The binding measurements were made by using the technique of equilibrium dialysis(5,6). A given amount of desalted phospholipid was dispersed in water either by stirring or evaporation of an organic solution in the presence of water. A known volume of the resulting suspension (usually 10 ml) contained inside a cellophane membrane was placed in an equal volume of 10 m*M* calcium chloride solution and the system ad-

* This investigation was supported by research grant GM 08412 from Nat. Inst. Health, USPHS, Bethesda, Md.

justed to various pHs by addition of saturated $Ca(OH)_2$ or 1 *N* HCl. In some instances, the pH was adjusted with 10 m*M* acetate, Tris, or glycine buffers without affecting the results. The system was then equilibrated by shaking at room temperature for 3 hours. Following equilibration, the pH of the inside solution was measured, and the calcium concentration of both the inside and outside solutions was determined. An analysis of the phospholipid phosphorus was carried out following a sulfuric acid digestion of the sample(7). The calcium was analyzed with a precision of ± 0.2% by an EDTA titration at pH 10.5 with Eriochrome T as the indicator (8). Because phospholipids interfered with the calcium titration, they were precipitated and the bound calcium released in 0.7 *N* HCl before carrying out the titration. The amount of calcium bound was calculated as the difference in concentration between the inside and outside compartment. Because the experiments were carried out under conditions in which the negative binding sites were saturated with calcium, there was no observable Donnan effect. The binding was expressed as the moles of calcium bound per mole of phosphorus in the inside compartment.

In a few instances for some of the purified phospholipids, data were obtained by analyzing only the outside compartment. The amount of calcium in the inside compartment at equilibrium was calculated as the difference between the total calcium initially present and the amount determined in the outside compartment at equilibrium.

Commercial samples of animal cephalin were obtained from Nutritional Biochemicals Corp., and vegetable phospholipid was obtained from Mann Research Labs. Alcohol fractionation was used to obtain dog brain cephalin(9) and beef brain phosphatidylserine (10). Phosphatidylethanolamine and phosphatidylcholine were prepared by silicic acid chromatography of egg yolk phospholipids (11), and monophosphoinositide was obtained from dog liver(12). A phospholipid preparation, inositol phosphatide, was obtained from Nutritional Biochemicals Corp.

Results and discussion. The binding of calcium to commercial samples of animal cephalin at various pHs is presented in Fig. 1. By means of thin-layer chromatography, these cephalin samples were found to be primarily mixtures of phosphatidylethanolamine, phosphatidylserine, and phosphoinositides(13). The free calcium concentration at equilibrium was generally 5.0 ± 0.5 m*M*, a concentration which preliminary experiments had shown to give maximal binding.

The binding of calcium to other phospholipid mixtures is shown in Fig. 2. The mixtures used in this experiment were obtained from egg yolk, dog brain, and a commercial vegetable phospholipid.

That the phospholipid samples did not hydrolyze during the equilibrium was indicated by: (a) Equilibration was very rapid under appropriate conditions and remained constant for several days; (b) The same results were obtained with back titrations in which calcium was added to the cephalin samples at pHs above 10 and the systems shaken for several hours before being adjusted to lower values for the binding measurements; (c) Experiments with egg phospholipids showed essentially no binding below pH 8. If there were hydrolysis of these samples, the hydrolytic products (*i.e.*, free fatty acids and secondary phosphates) would bind calcium well below pH 8.

The different calcium-binding regions in the curves reflect the presence of various types of binding groups in these phospholipid mixtures. This is shown in results obtained with various individual phospholipids. Fig. 3 presents calcum binding to phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine. Half-maximum binding is at pH 2.8 and 8.2 for phosphatidylserine and pH 8.0 for phosphatidylethanolamine. In the above compounds the maximum binding is stoichiometric with respect to the negatively charged groups in the particular lipid. There was no significant binding to phosphatidylcholine below pH 10.0. In similar experiments with the fatty acids, lauric and oleic, the pHs of half-maximum stoichiometric binding were 4.8 and 5.2, respectively(6).

Fig. 4 presents calcium-binding to phosphoinositide preparations. The curve at the left was obtained with the monophosphoino-

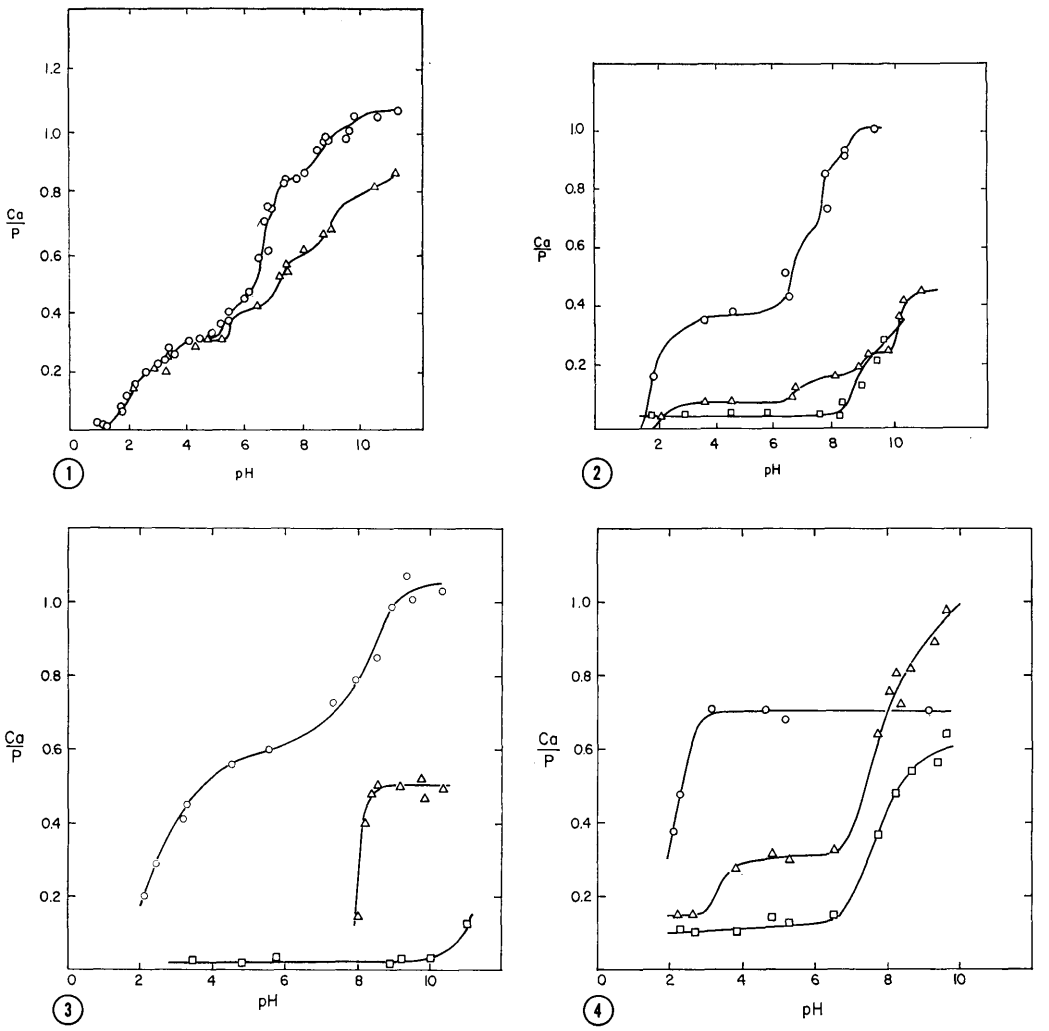

FIG. 1. Binding of calcium with commercial animal cephalin samples. ○ - Sample No. 9746 (5.1 m*M* P); △ - Sample No. 2471 (2.5 m*M* P).

FIG. 2. Binding of calcium with various phospholipid preparations. ○ - Dog brain cephalin (6.9 m*M* P); △ - Vegetable phospholipid (25.0 m*M* P); □ - Egg phospholipids (5.7 m*M* P).

FIG. 3. Binding of calcium with purified phospholipid preparations. ○ - Phosphatidylserine (4.3 m*M* P); △ - Phosphatidylethanolamine (1.3 m*M* P); □ - Phosphatidylcholine (4.1 m*M* P).

FIG. 4. Binding of calcium with purified phospholipid preparations. ○ - monophosphoinositide (0.5 m*M* P); △ - Commercial inositol phosphatide (3.6 m*M* P); □ - Calculated curve, trphosphoinositide; assumption that inositol phosphatide contains 25% phosphatidylserine.

sitide preparation prepared as described by Hanahan and Olley(12). The maximum binding value is 0.7 Ca/P, and the pH for half-maximum binding is 2.0. The value for maximum binding agrees with the value for the equivalent cation composition of this purified fraction as reported by Hanahan and Olley; it is larger than would be expected for stoichiometric binding with monophosphoinositide. Thin layer chromatography revealed our preparation contained two major components, and in view of the binding data which indicated only one binding region as well as a report of major contamination of phosphoinositides by organic sulfates(14), it might be possible that the increased binding was due to a sulfate grouping. Detergent sulfate groups also bind near pH 2(6).

The middle curve of Fig. 4 was obtained with the commercial inositol phosphatide

preparation. Phosphorus analysis of the fractions obtained on thin layer chromatography of this sample indicated 75% of the sample remained near the origin and was considered to be triphosphoinositide(15,16). The remaining 25% was identified as phosphatidylserine. In view of our other data on purified phospholipids, a possible interpretation of the inositol phosphatide binding curve in Fig. 4 is as follows: The region below pH 2 represents binding to the diesterified phosphate in triphosphoinositide. The next binding region near pH 3 comes from phosphatidylserine binding. This corresponds to a binding of 0.17 Ca/P, and an equal amount of binding can be expected for the second negative group in this molecule in the region at pH 8. By using the calcium-binding curve for phosphatidylserine presented in Fig. 3, the experimental curve can be corrected for the contribution of 25% phosphatidylserine. This corrected curve which is the curve at the right in Fig. 4, represents the binding of triphosphoinositide in a phospholipid mixture in which it contributes 75% of the phosphorus. The corrected curve in Fig. 4 is consistent with an equivalent calcium-binding to the diesterified phosphate in the molecule at pH 2 in a manner similar to that for monophosphoinositide. However, the data indicate that the entire binding of the mono-esterified phosphates on the inositol ring is in the pH range 6.5-8.5. On the basis of the currently accepted chemical structure of triphosphoinositide, it appears that mono-esterified phosphate in this molecule will bind calcium only when both protons are dissociated. This binding begins near pH 6.5 and continues until the equivalent amount of calcium is bound to the phosphate group. The observation that mono-esterified phosphate groups will not bind calcium at pH values below the anticipated ionization of the second proton has been reported previously for the phosphoprotein, casein(17, 18).

With this exception of mono-esterified phosphate in triphosphoinositide, the results on purified phospholipids and fatty acids are consistent with an anticipated stoichiometric combination of calcium with the net negative charge on the phospholipids. This net charge is a reflection of the acid dissociation constant for the binding group or the dissociation constant of the cationic partner of a phospholipid zwitterion.

On the basis of the calcium-binding characteristics of the purified preparations, we propose that it is possible to identify the substances in the mixtures which give rise to the complex curves seen in Fig. 1 and 2. In order of increasing pH, the groups that are responsible for the binding regions in Fig. 1 are most likely (a) diesterified phosphate in phosphoinositides, (b) phosphatidylserine carboxyl(4), (c) free fatty acid carboxyl, (d) phosphatidylserine phosphate, and mono-esterified phosphate in phosphoinositides, and (e) phosphatidylethanolamine.† If it is assumed that the magnitude of the binding in a given region is a reflection of the stoichiometric combination of calcium with a particular group, these interpretations are also consistent with the proportions of these substances found in the mixtures with silicic acid chromatography(6).

The pH resolution observed in these experiments is greater than the pH resolution found with hydrogen-ion titration studies. Such a sensitive method offers a useful approach to quantitative investigations of the ionic interactions of phospholipids with many water soluble systems where the complexes are still dispersed in solution. The possibility exists for binding studies on many artificial and natural mixed lipid-protein systems using alkali and alkaline earth cations(19). The marked effect of pH on binding interactions must be considered in studies dealing with electrostatic interactions of phospholipids.

An interesting observation from this binding study is the large amount of calcium-binding groups in the physiological pH range found in different commercial animal cephalins and freshly isolated dog brain cephalin, these groups being contributed by phosphatidylserine, triphosphoinositide, and phosphatidylethanolamine. The slope of the binding in-

† The assignment of phosphatidylethanolamine binding to a slightly higher pH than found with the purified preparation was prompted by the results with egg yolk phospholipids (80% choline phosphatides and 20% phosphatidylethanolamine).

teraction indicates that large variations occur in the concentration of bound ions in the system with comparatively small pH changes. Because may proteins interact with phospholipids through similar reversible, electrostatic combinations as does calcium(10,20,21), some of these protein-phospholipid interactions should have a similar pH sensitivity in the physiological range. It is suggested that a sensitive equilibrium exists between protein, phospholipids and calcium under appropriate conditions. The large effect of pH on these ionic interactions in the physiological range and the ubiquitous distribution of phospholipids invite further speculation on mechanistic models concerning ionic equilibria in cells, active transport, macromolecular transformations of biosystems and other regulating mechanisms of tissue activity, all controlled at least partially by small pH variations affecting binding interactions.

Summary. The binding of calcium in phospholipid mixtures is very dependent on the pH, it being a quantitative reflection of the types of dissociable groups in the phospholipids present. As the pH increases from 6.5 to 8.5, there is a considerable increase in Ca-binding if triphosphoinositide, phosphatidylserine, or phosphatidylethanolamine is present. This effect may be of some consequence in certain physiological processes.

1. Hendrickson, H. S., Fullington, J. G., Biochemistry, 1965, v4, 1599.

2. Rojas, E., Tobias, J. M., Biochem. Biophys. Acta, 1965, v94, 394.

3. Shah, D. O., Schulman, J. H., J. Lipid Res., 1965, v6, 341.

4. Abramson, M. B., Katzman, R., Gregor, H. P., J. Biol. Chem., 1964, v239, 70.

5. Hughes, T. R., Klotz, I. M., Methods of Biochemical Analysis, 1956, v3, 265.

6. Joos, R. W., Ph.D. Thesis, Univ. of Minnesota, 1964.

7. Fiske, C. H., Subbarow, Y., J. Biol. Chem., 1925, v66, 375.

8. Sobel, A. E., Hanok, A., Proc. Soc. Exp. Biol. & Med., 1951, v77, 737.

9. Folch, J., J. Biol. Chem., 1942, v146, 35.

10. ———, ibid., 1948, v174, 439.

11. Hanahan, D. J., Lipide Chemistry, Wiley & Sons, New York, 1960.

12. Hanahan, D. J., Olley, J. M., J. Biol. Chem., 1958, v231, 813.

13. Skipski, V. P., Peterson, R. F., Sanders, J., Barclay, M., J. Lipid Res., 1963 v4, 227.

14. Dittmer, J., Biochem. Biophys. Acta, 1965, v106, 425.

15. Brockerhoff, H., Ballou, C. E., J. Biol. Chem., 1963, v236, 1907.

16. Ellis, R. B., Galliard, T., Hawthorne, J. N., Biochem. J., 1963, v88, 125.

17. Carr, C. W., Arch. Biochem. Biophys., 1953, v46, 424.

18. Zittle, C. A., DellaMonica, E. S., Rudd, R. K., Custer, J. H., ibid., 1958 v76, 342.

19. Carvalho, A. P., Sanui, J., Pace, N., J. Cell. Comp. Physiol., 1963, v62, 311.

20. Chargaff, E., Ziff, M., J. Biol. Chem., 1939, v131, 25.

21. Das, M. L., Crane, F. L., Biochemistry, 1964, v3, 696.

Received July 25, 1966. P.S.E.B.M., 1967, v124.

Gastrointestinal Gas Production in Rats Fed Raw and Heated Navy Beans With or Without Added Antibiotics.* (31985)

M. L. KAKADE AND RAYMOND BORCHERS

Department of Biochemistry and Nutrition, University of Nebraska, Lincoln

An increased production of gastrointestinal gases due to the ingestion of beans has been reported recently in rats(1,2) and humans (3,4). However, few attempts have been made so far to decrease or prevent the increased gastrointestinal gas production in animals fed the bean diet(2). Preliminary experiments indicated that rats fed the heated navy beans produced less gas than was produced by the raw navy beans. The present

* Published with approval of the Director as paper 1963, Journal Series, Nebraska Agri. Exp. Station. Supported in part by USPHS Research Grant no. A-2018.