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PURIFICATION AND CHARACTERIZATION OF CHICK INTESTINE BRUSH BORDER MEMBRANE

EFFECTS OF 1α(OH) VITAMIN D_3 TREATMENT

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Summary

A new technique has been developed for the isolation of membrane vesicles from the vitamin D-deficient and vitamin D-treated chick intestinal brush border membrane. The technique involves removal of nuclei from a low speed pellet by discontinuous sucrose gradient centrifugation. The resulting intact brush borders are then homogenized in 0.5 M Tris and the membrane fragments purified on a glycerol gradient. This preparation represents a 20-fold purification of the brush border marker sucrase. After 1α-hydroxyvitamin D_3 treatment there is a significant increase in membrane phospholipid phosphorous, an alteration in the fatty acid composition of the phosphatidylcholine fraction of membrane phospholipid, and a decrease in sucrase specific activity.

Introduction

The chick is frequently used as a model for investigating both the metabolism and action of vitamin D_3 [1]. One of the major actions of 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), the active vitamin metabolite is enhancing calcium transport in the intestine. The precise cellular site or sites of $1,24(OH)_2D_3$ actions have not been established, but present evidence indicates that the microvillar membrane of the brush border of the intestinal epithelium

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Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 1α(OH)D_3, 1α-hydroxyvitamin D_3; $1,25(OH)_2D_3$; 1,25-dihydroxyvitamin D_3; SDS, sodium dodecyl sulfate.

is one of these sites. This evidence includes the fact that prior administration of vitamin D_3, 25 hydroxyvitamin D_3 ($25(OH)D_3$), or $1,25(OH_2D_3$ to vitamin D-deficient chicks leads to changes in the biochemical properties of subcellular fractions enriched in brush border membrane [2—6]. However, the techniques used for isolating the chick intestinal brush border have been adapted, with only minor modification, from methods developed by Forstner et al. [7] for isolation of this organelle from the intestinal mucosa of the vitamin D-replete rat. In none of these reports was there documentation of the adequacy of these methods for preparing highly purified brush borders or brush border membranes from chicks. When we undertook such an analysis of our own preparation, isolated by either the method of Forstner et al. [7] or by more recently developed techniques [8,9], we found that these techniques, when applied to the intestine of the vitamin D-deficient chick, were unsatisfactory in terms of both yield and purification of brush border enzyme markers. Our interest in obtaining purified membranes from chick intestine was heightened by the reports of Hopfer et al. [10,11] that the homologous membranes isolated from rat intestinal brush borders form osmotically active vesicles, and the transfer of solutes into these vesicles bears many similarities to transport across the intact intestinal epithelium. These studies, which have greatly clarified our understanding of glucose absorption across the intestine, suggested to us that new insights into the mechanism of action of vitamin D on the intestine might be elucidated if such vesicle preparations could be made from intestinal mucosa of vitamin D-deficient and vitamin D-replete chicks. Consequently, we have developed a reproducible technique for isolating membrane vesicles from vitamin D-deficient and vitamin D-replete chick intestinal mucosa brush border and have characterized the effect of vitamin D treatment on membrane lipid and protein composition.

Materials and Methods

Animals. White Leghorn cockerels obtained (Moyers Chicks, Quakertown, Pa.) one day after hatching were immediately placed on a vitamin D-deficient diet [12]. They were raised in a darkened room and used for experiments at 25—35 days of age (serum calcium <6.5 mg per 100 ml). All animals were starved, but allowed water ad libitum 24 h prior to being killed. Nearly all the work devoted to the development of the membrane isolation method was carried out in vitamin D-deficient chicks. However, once the method was perfected, it was also employed in the isolation of membrane vesicles from the intestinal mucosa of vitamin D-replete animals. These animals were prepared by the administration of 1 μg of 1α-hydroxyvitamin D_3 ($1\alpha(OH)D_3$) by crop intubation 18 h prior to killing. By this time, the rate of transmucosal calcium transport has returned to normal values [1].

Preparation of brush border membranes. Four chicks were used in each preparation. Each chick was killed by decapitation, the duodenum was quickly removed and chilled by immersion in ice-cold 0.15 M NaCl. The segment was then flushed with the same medium (about 10 ml), slit open onto a paper towel, blotted, and the mucosa scraped with a Teflon-coated spatula. The scrapings from all four animals were collected on a piece of tared aluminum foil

chilled on ice and then weighed (yield approx. 0.6—0.9 g mucosa per chick). The scrapings were mixed with the spatula and transferred to a beaker containing ice-cold buffer (2.5 mM sodium EGTA 2 mM sodium HEPES, pH 7.4), final volume 100 × tissue weight. The scrapings were suspended by suction into a syringe fitted with a blunted 18 g needle and then homogenized by six passes in a 250 ml glass-Teflon homogenizer (Glenco) at 1800 rev./min. The homogenate was centrifuged in a Sorvall RC2 centrifuge for 20 min at 400 × *g*, 4° C. The supernatant was carefully removed. The pellet (Fraction P) was then resuspended in 10 ml ice-cold buffer (5 mM $MgCl_2$ in the EGTA/HEPES buffer described above) by 8 cycles of suction-expulsion through an 18 g needle. This suspension was layered over a gradient of 10 ml 50% (w/v) sucrose in $MgCl_2$/EGTA/HEPES buffer (refractive index, 1.405) and 10 ml 63% sucrose (refractive index, 1.423). The gradient was centrifuged for 75 min at 26 000 rev./min (average force 90 000 × *g*). Material at the lower interface, containing the brush borders, was suspended in 34 ml of buffer and collected by centrifugation for 15 min at 4° C in a Sorvall RC2 centrifuge at 27 000 × *g* (Fraction I). This fraction was composed almost entirely of intact brush borders from intestinal mucosal cells. When the rest of the gradient was required for recovery analysis, the entire contents of the tube above the lower interface was suspended in $MgCl_2$/EGTA/HEPES buffer and centrifuged in the same manner, and similarly that below the interface was collected by centrifugation (Fractions AI and BI). When a portion of the purified brush border fraction was to be saved for assay, an aliquot of the resuspended interface material was centrifuged for 30 min at 30 000 rev./min in the SW 50.1 rotor.

To prepare brush border membrane fragments the pellet from the preceding centrifugation was suspended by syringe suction in 20 ml 0.5 M Tris · HCl, pH 7.2. The suspension was homogenized for two 1-min periods in a Waring Blendor at top speed in a chilled semi-micro jar (Thomas No. 3392-GO5). The resulting homogenate was layered over a gradient composed of 4-ml portions of 37% glycerol (w/w) in 50 mM $MgCl_2$ (refractive index, 1.382), 45% glycerol in 50 mM $MgCl_2$ (refractive index, 1.392) and 60% glycerol in 50 mM $MgCl_2$ (refractive index, 1.415), and then centrifuged in the Beckman L5-50 centrifuge, SW 27 rotor, at 21 000 rev./min (average force 58 500 × *g*) for 10 min plus the unbraked deceleration time. The milky bands within the gradient were pooled, diluted with ice-cold deionized water and centrifuged for 20 min at 4° C in a Sorvall RC2 centrifuge (27 000 × *g*), yielding an opalescent pellet designated brush border membranes (Fraction PB). When required for recovery analysis, the material above and below these bands was similarly suspended and centrifuged (Fractions AB and GF). The membrane pellet was generally resuspended in 1 ml for assays and other experiments.

Protein was determined using the Folin-Ciocalteu reagent as described by Layne [13] except that standards (bovine serum albumin) were adjusted to contain amounts of buffer salts identical to those present in the samples, and all standards and samples (0.2 ml) were made 0.4% in SDS before assay.

Marker enzymes. Enzyme assays for all fractions in the purification were performed after adjustment of sample buffer salts to identical concentrations. The enzymes were assayed under conditions of linearity with respect to time and enzyme concentration (except for cytochrome oxidase, see below).

Sucrase was assayed by a modification of the glucose oxidase method described by Messer and Dahlquist [14]. Duplicate 250-μl samples were incubated for 30 min at 37° C with an equal volume of 100 mM soidum maleate, pH 5.8, 56 mM sucrose. After the reaction was stopped by boiling, 0.500 ml of "Tris-Glucose Oxidase" reagent (Worthington Glucostat Special) was added and the tubes were incubated at 37° C for at least 30 min. The reaction was terminated by addition of 1 ml 66% H_2SO_4 and the absorbance at 560 nm determined. Glucose production was calculated after subtraction of a boiled enzyme blank on the basis of a glucose standard curve incubated with the Tris-Glucose Oxidase reagent.

β-Glucuronidase was assayed with *p*-nitrophenyl β-glucuronide (Sigma) as described by Palmieri and Koldovsky [15] except that the incubation contained 0.1% Triton X-100.

NADPH-cytochrome *c* reductase was assayed spectrophotometrically by following the reduction of cytochrome *c* (Sigma) at 550 nm [16].

Monoamine oxidase was assayed by following the rate of conversion of [2-^{14}C]serotonin (New England Nuclear) into an ethyl acetate-extractable product [17]. Aliquots of the extract were mixed with a scintillation fluid consisting of 84 ml Spectrafluor (Amersham-Searle), 2.0 l toluene and 1.2 l methylcellosolve, and the radioactivity determined in a Packard TriCarb scintillation counter Model 3385.

Cytochrome *c* oxidase was assayed spectrophotometrically by following the oxidation of reduced cytochrome *c* at 550 nm. The preparation of reduced cytochrome *c* and assay were as described by Yonetani and Elliott [18], except that the first-order rate constant was used as an index of activity; this value was proportional to enzyme concentration in the range used for assay.

Electron microscopy of the isolated fractions was performed as described by Mooseker and Tilney [19]. Membrane preparations were fixed in 1% glutaraldehyde, 0.1 M phosphate buffer, pH 7.0, for 30 min, then postfixed in 1% OsO_4, 0.1 M phosphate, pH 6.0, for 45 min, and stained with uranyl acetate before dehydration and embedding in Araldite. Thin sections stained with uranyl acetate and lead citrate were examined in a Philips 200 electron microscope. The processing of the other fractions were similar; cacodylate buffer rather than phosphate buffer, however, was employed.

Lipid extraction. In preparation for lipid analysis α-tocopherol was added at the homogenate step in an effort to minimize oxidation. The presence of this compound in the homogenate, however, interfered with both the sucrase and protein assays necessitating the following procedure. The suspension of mucosal scrapings was given an initial homogenization by a single pass of the glass-Teflon homogenizer. After removal of appropriate aliquots for sucrase and protein assays, 200 ml of the remaining homogenate was mixed with 1.5 g of α-tocopherol (Sigma) and homogenization was resumed. Because the α-tocopherol coated the pestle, diminishing the clearance and presumably leading to more vigorous homogenization, only three further passes were used. To remove the bulk of large α-tocopherol droplets, the homogenate was filtered through two layers of cheese-cloth before the first centrifugation. The remainder of the preparation was as described above. The different homogenization did not appear to affect the distribution of protein or sucrase in the fractions separated during the isolation.

The final membrane preparation was extracted at 4°C using the method of Bligh and Dyer [20]. All solvents were redistilled and bubbled with argon for 30 min before use. To control for losses during subsequent analysis and correct for volume changes due to evaporation of volatile solvents, [1-^{14}C]oleic acid (Amersham-Searle) (about 10^6 cpm) was added to each extract.

Lipid dry weight was determined by pipetting aliquots of extract into small pledgets of aluminum foil which had been maintained under vacuum for 12 h before taring. After evaporation of visible solvent the pledgets were kept in a vacuum dessicator for 24 h and weighed on a Mettler Model MS-S/A microbalance; further maintenance under vacuum did not change the weights. In some experiments the pledgets were then dropped into scintillation vials for determination of [1-^{14}C]oleate recovery; this was always within 5% of the expected value.

Thin-layer chromatography. For routine phospholipid separations the method of Skipski et al. [21] was employed. Glass plates 8 × 10 inches were coated with a slurry of 15 g silica H (Brinkmann) in 32 ml 1 mM Na_2CO_3 to yield a layer about 0.25 mm thick. Before use the plates were activated at 110°C for 1 h. Standards (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, lysophosphatidylcholine and lysophosphatidylinositol) were obtained from Sigma. Standards and samples were spotted under an argon atmosphere and solvent tanks were flushed with argon before equilibration with the solvent system chloroform/methanol/acetic acid/water (50 : 30 : 8 : 4, v/v). After development in this system the plates were dried under a nitrogen atmosphere. Spots were visualized in specific lanes by blowing air through a Pasteur pipet filled with iodine crystals; lanes destined for fatty acid analysis were avoided. After the latter lanes were scraped, the spots remaining on the plate could be visualized by fluorescamine (Roche) for phosphatidylserine, phosphatidylethanolamine and lysophosphatidylinositol; or by a char reagent (3 ml 37% formaldehyde and 97 ml concentrated H_2SO_4). Identification of phospholipids in the extract was made on the basis of R_F in the above system, reactivity to fluorescamine, and chromatography on silica H in the following two-dimensional system: chloroform/methanol/28% NH_4OH (65 : 25 : 5, v/v) followed by chloroform/acetone/methanol/acetic acid/water (3 : 4 : 1 : 10 : 5, v/v).

Lipid phosphorus analysis was carried out by the method of Bartlett [23] modified for the use of smaller volumes. When analysis of spots from a one-dimensional thin-layer chromatography was necessary, an adjacent lane was scraped at corresponding distances from the origin as a silica gel blank.

Cholesterol was determined by the method of Zlatkis et al. [24] except that a 1 ml final volume was used to increase sensitivity. To verfify that the color yielding material was cholesterol, the extract was chromatographed on silica H in hexane/diethyl ether/acetic acid (55 : 45 : 1, v/v). The entire sample lane was scraped in intervals and the cholesterol assay performed in the presence of the silica gel; color development occurred only at the position corresponding to the R_F of standard cholesterol.

Fatty acid composition of separated phospholipid classes was determined by gas-liquid chromatography of the methyl esters. Appropriate zones from thin-layer plates were scraped (within 10 min after completion of chromatography)

into methanolysis tubes [25]. Methanolic HCl (5%, w/v) was added and the tubes were sealed after flushing with argon. After overnight incubation at 60°C the methyl esters were extracted into hexane, concentrated and injected into a Varian Aerograph Model 2100 gas-liquid chromatograph equipped with flame ionization detectors. A 6 ft × 4 mm internal diameter glass U column packed with 10% EGSS-X on 100—120 mesh Gas Chrom P (Supelco) was employed for all analyses. Injector and column temperatures were maintained at 190°C. The mass response was quantitated by triangulation. Identification of the various peaks was made on the basis of (1) plots of log retention time vs. carbon number, (2) relative retention times given in the literature [26], and (3) chromatography with esters of known structure. Estimates based on duplicate injections differed by no more than about 5%.

Polyacrylamide slab gel electrophoresis was carried out by a technique combining the features of the discontinuous buffer system described by Laemmli [27] and the linear gradient of acrylamide (7—11%) described by Margolis and Kenrick [28]. Gels (3 mm thick) were poured in a model SE 500 Hoefer Scientific apparatus. Protein samples (75 μg) were prepared as described by Laemmli [27]. The electrophoresis was run at 30—50 mA, constant current. The gels were stained overnight in 0.5% Coomassie Blue in 25% isopropanol, 10% acetic acid, and destained in one wash of 25% isopropanol, 10% acetic acid, followed by several washes of 10% acetic acid. The molecular weight markers, phosphorylase *A*, bovine serum albumin, and cytochrome *c* were obtained from Sigma. Actin and myosin standards were a gift from Dr. Mark Mooseker.

Results

Isolation of brush border and brush border membrane vesicles

In our initial efforts to isolate intact brush borders from the chick intestinal mucosa we employed the method of Forstner et al. [7]. However, we were unable to obtain purified chick brush borders using this procedure, which had been developed for preparing rat intestinal brush borders. Consequently, we introduced several modifications. When scrapings from chick duodenal mucosa were homogenized in 100 vol. of sodium EDTA, pH 7.4, and the homogenate subjected to low speed centrifugation, a highly variable yield (10—85%) of the sucrase activity was found in the pellet. Three changes were introduced to improve consistency and extent of recovered marker. First, it was found that in the initial homogenization, if a Teflon-glass homogenizer was used rather than a Waring Blendor, recovery was improved; second, a large part of the variability and poor recovery could be eliminated if the chicks were starved for 24 h before their intestines were removed and third, substitution of the sodium EDTA buffer with 2 mM HEPES, 2.5 mM sodium EGTA, pH 7.4 [10] improved yield.

After preparation of an initial low-speed pellet the method of Forstner et al. [7] involves several washes of this pellet and a NaCl extraction followed by a glass wool filtration to remove nuclear contamination. This procedure was not successful in completely removing nuclei from our chick-derived material. Moreover, repeated washing led to a reduction of sucrase specific activity. Although sucrose density gradient centrifugation was reportedly ineffective in

removing nuclei in the rat preparation [7], we were able to develop a satisfactory sucrose gradient method for removing nuclei in the chick preparation. Addition of 5 mM $MgCl_2$ to the EGTA/HEPES buffer kept the nuclei compact, reduced fragmentation and facilitated sedimentation of the nuclei through the densest part of the gradient. Of a variety of gradient compositions examined, the best yield of brush borders virtually free of nuclei, was obtained with a two step gradient containing 50% (w/v) sucrose layered over 63% sucrose. When the low speed pellet, resuspended in $MgCl_2$/EGTA/ HEPES and layered over this gradient, is centrifuged for 75 min at 26 000 rev./min (SW 27 rotor), the brush border marker is found at the 50—63% sucrose interface. This material, collected by resuspension and recentrifugation, is a highly purified fraction of intact brush borders essentially free of nuclei and contains almost 50% of the starting sucrase activity, with a total sucrase purification of about 10-fold with respect to protein, or about 2.4-fold over the low-speed pellet material (Table I).

The isolated brush borders prepared by this technique were largely intact and retained their microvillar structures. In order to extract core protein from the microvilli and obtain a membrane vesicles fraction from the purified brush borders, an adaption of the method of Eichholz and Crane [29] was employed. The procedure finally adopted consisted of: (1) resuspending the brush borders in 0.5 M Tris · HCl, pH 7.4, (2) homogenization of this material for two 1-min periods in a Waring Blendor; and (3) application of this homogenate to a discontinuous glycerol gradient. A flow sheet outlining the entire isolation procedure finally developed is shown in Fig. 1. The recovery of total protein, sucrase activity and marker enzymes in a set of typical experiments are summarized in Table I, and an electron micrograph of a typical field from the final washed vesicles which are largely devoid of core material. On the outer side of most of the vesicles there is an amorphous appearing material which resembles the glycocalyx found on the external surface of intact microvilli. Recovery of sucrase activity in the final fraction (PB) was approx. 36% of the original activity or 75% of the activity in the purified brush border fraction (I). There were very small amounts of β-glucuronidase activity, monoamine oxidase, NADPH-cytochrome *c* reductase, and cytochrome oxidase in the final purified membrane fraction.

Characterization of purified membrane vesicles

The purified membranes were first characterized in terms of their protein content. Fig. 3 illustrates the pattern of proteins obtained when successive membrane preparations from vitamin D-deficient and $1\alpha(OH)D_3$-treated chicks were extracted, and then subjected to SDS-polyacrylamide gel electrophoresis. As can be seen, the pattern is highly reproducible from one preparation to the next. The electrophoretic mobility of the molecular weight markers indicated in the figure was determined from purified standards run on an identical gel. The membrane preparation contained a strong presumptive actin band which comigrates with the purified actin standard. Prior administration of $1\alpha(OH)D_3$ did not alter the distribution of protein and marker enzyme in the different fractions of the isolation procedure. Consequently the control and $1\alpha(OH)D_3$ membrane preparations were sufficiently similar to be meaningfully compared.

TABLE I

ANALYSIS OF FRACTIONS ENCOUNTERED IN PURIFICATION OF BRUSH ORDER BORDER MEMBRANES FROM CHICK INTESTINAL MUCOSA

Values are expressed as the mean ± S.D. The number of independent measurements of each parameter is shown in parenthesis.

Total value in homogenate fraction	Protein (mg/ml) 1.53 ±0.19 (%)	Sucrase (nmol/min per ml) 37.5 ± 6.2 (20)		β-Glucuronidase (nmol/min per ml) 4.2 ± 1.2 (4)		Monoamine oxidase (nmol/min per ml) 7.8 ± 2.1 (4)		NADPH-cytochrome *c* reductase 24.6 ± 16.3 (7)		Cytochrome *c* oxidase (K (min^{-1})/ml) 14 ± 9 (2)	
		(%) *	Purification	(%)	Purification	(%)	Purification	(%)	Purification	(%)	Purification
Homogenate (H)	100	100	1.0	100	1.0	100	1.0	100	1.0	100	1.0
Low speed supernatant (S_1)	78	17.7	0.23	92.9	1.12	97.3	1.2	84.5	1.1	103	1.3
	±8	±4.1	±0.04	±0.2	±0.02	±5.7	±0.3	±6.6	±0.2	±4	±0
	(13)	(12)	(12)	(4)	(4)	(4)	(4)	(7)	(7)	(2)	(2)
Low speed pellet (P_1)	19.6	80.1	4.2	11.2	0.54	12.3	0.6	8.1	0.4	5.8	0.28
	±3.8	±11.3	±0.5	±5.7	±0.31	±2.8	±0.2	±3.8	±0.2	±0.6	±0.04
	(20)	(19)	(19)	(4)	(4)	(4)	(4)	(7)	(7)	(2)	(2)
above interface (AI)	3.5	7.8	2.7	3.2	0.78	5.1	1.7	4.4	1.5	3.6	0.94
	±1.2	±3.9	±1.7	±1.2	±0.13	±1.9	±0.5	±1.5	±0.6	±1.2	±0.0
	(14)	(13)	(13)	(4)	(4)	(4)	(4)	(7)	(4)	(2)	(2)
Interface (I)	4.9	48.9	10.1	1.5	0.30	2.5	0.55	2.2	0.5	1.9	0.22
	±0.8	±8.1	±1.5	±0.3	±0.97	±0.5	±0.09	±0.7	±0.2	±1.0	±0.01
	(21)	(20)	(20)	(4)	(4)		(4)	(7)	(7)	(2)	(2)
Below interface (BI)	7.5	8.4	1.1	2.3	0.27	1.9	0.27	4.0	0.6	1.3	0.14
	±1.3	±4.0	±0.5	±0.5	±0.06	±0.8	±0.10	±1.2	±0.3	(1)	(1)
	(13)	(13)	(12)	(4)	(3)	(4)	(4)	(4)	(4)		
Above bands (AB)	0.13	2.2	13.0	0.01	0.08	0.1	0.7	0.03	0.2	0.02	0.1
	±0.04	±0.9	±3.1	±0.00	±0.03	±0.1	±0.7	±0.03	±0.2	(1)	(1)
	(3)	(3)	(3)	(2)	(2)	(2)	(2)	(2)	(2)		
Pooled bands (PB)	1.6	35.8	22.8	0.07	0.05	0.2	0.14	0.09	0.06	0.06	0.04
	±0.4	±7.07	±6.2	±0.06	±0.04	±0.2	±0.12	±0.08	±0.04	(1)	(1)
	(7)	(7)	(1)	(2)	(2)	(2)	(2)	(5)	(5)		
Glycerol pellet (GP)	0.8	7.3	8.1	0.2	0.12	0.93	0.7	0.3	0.45	0.50	0.3
	±0.5	±6.2	±2.4	±0.1	±0.05	±0.45	±0.4	±0.18	±0.18	(1)	(1)
	(5)	(5)	(5)	(2)	(2)		(2)	(3)	(3)		

* Percentage yield and purification are expressed relative to the original homogenate.

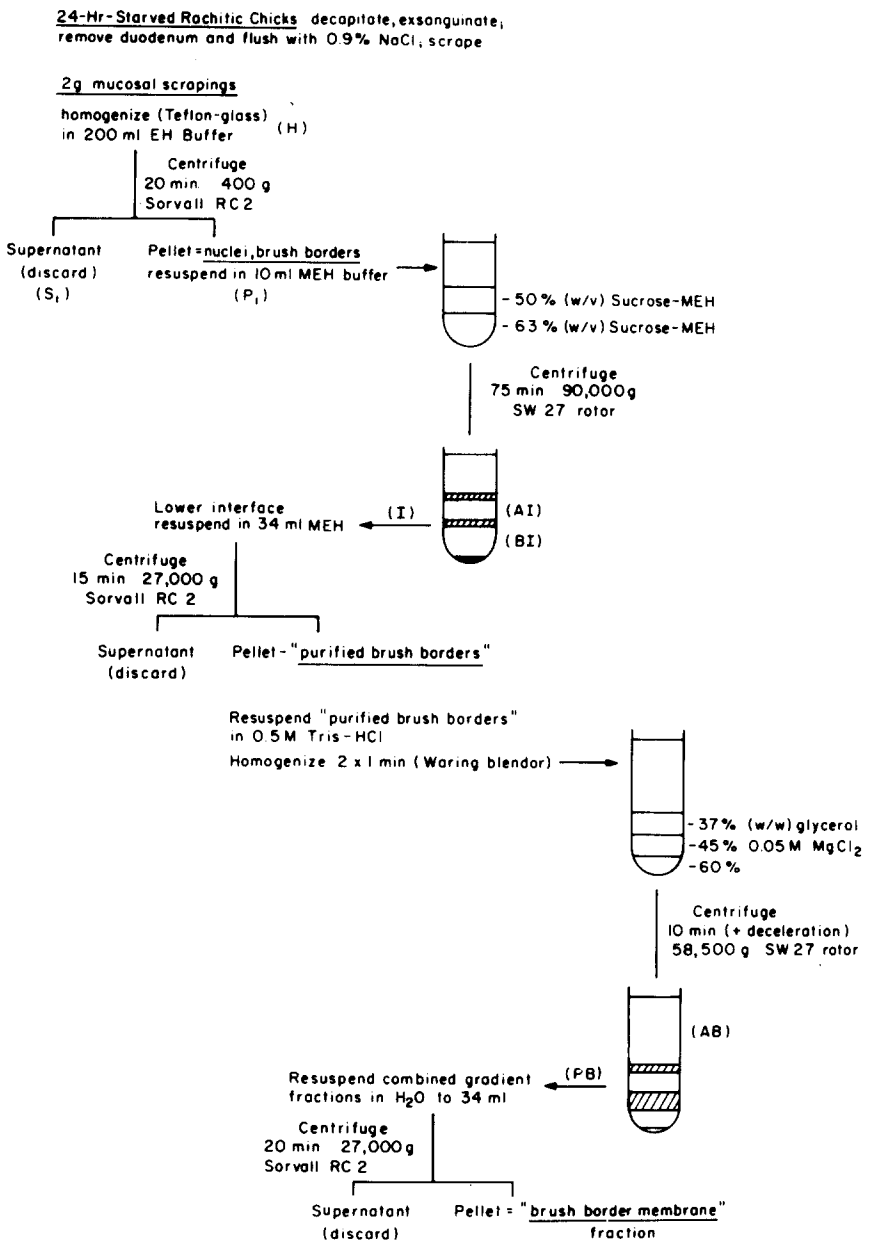

Fig. 1. Purification scheme for chick duodenal brush border membrane. Details of procedure are described in the text.

When membrane preparations from vitamin D-deficient control and $1\alpha(OH)D_3$-treated chicks were compared, there were no gross changes in protein patterns seen on the stained gel except for a consistently greater peak at one region of the gel representing a protein with a molecular weight in the range of 90 000 (Fig. 3). It is noteworthy that there was practically no detect-

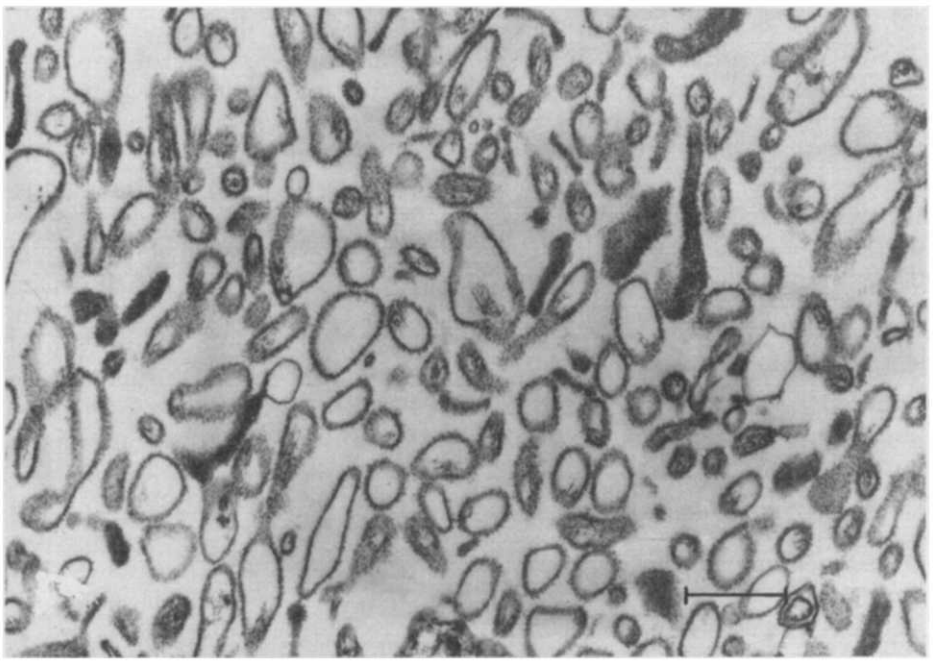

Fig. 2. Isolated brush border membrane vesicles. A typical field contains predominantly closed vesicles. Occasionally an intact microvillus is seen (bar 0.5 μm, ×120 000).

able protein in the region of the gel in which the calcium binding protein of Wasserman and Corradino [30] would be expected to appear, i.e. 28 000 molecular weight (see Discussion). Unexpectedly, 1α(OH)D_3 treatment led to a fall in the specific activity of sucrase in the final membrane fraction (Table II). This reflects a true change and not an artifact of the isolation since the specific activity of sucrase in the original homogenates showed a similar decrease after 1α(OH)D_3 treatment (0.044 ± 0.005 μmol product formed/mg protein per min

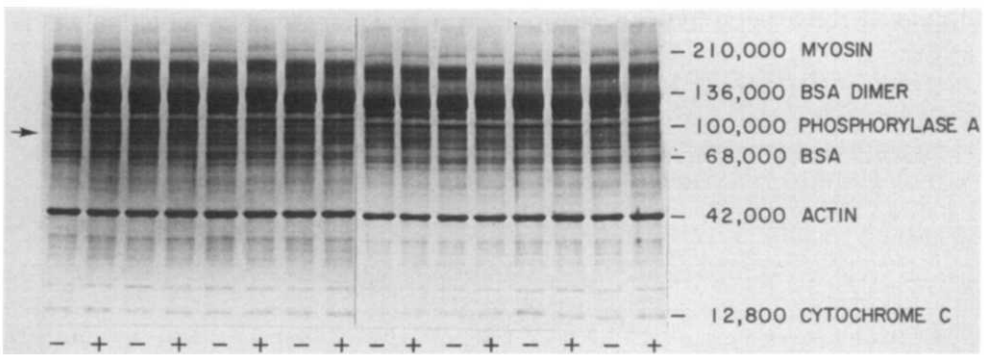

Fig. 3. SDS-polyacrylamide gel electrophoresis of chick duodenal brush border membrane from rachitic- (—) or 1α(OH)D_3-treated (+) animals. Samples from eight successive preparations were run. The arrow at the left points to the band which shows consistently greater intensity in the preparations from 1α(OH)D_3-treated animals. The mobility of the molecular weight standards is shown at the right border. BSA, bovine serum albumin.

vs. 0.030 ± 0.007; control vs. 1α(OH)D_3 treated; $n = 9, P < 0.005$).

The contents of total lipid, total phosphorous, and cholesterol, are summarized in Table II. In addition, the lipid extract was examined by thin-layer chromatography to determine the phospholipid classes present. In the one-dimensional system described by Skipski et al. [21] three major spots were visualized by iodine vapor, corresponding to the positions of standard phosphatidylethanolamine, phosphatidylcholine, and the comigrating phosphatidylserine and phosphatidylinositol. In a two-dimensional system (see Materials and Methods) four major spots were found, owing to the separation of the latter two phospholipids. To confirm the identity of the phospholipid species identified in the two systems, the spots tentatively identified in the one-dimensional system were scraped, eluted and rerun in the two-dimensional system. These spots migrated as would be expected from the identification made in the one-dimensional system. The one-dimensional system was used routinely for analysis and provided the results shown in Table III. In a single analysis on the two-dimensional system the ratio of phosphatidylinositol to phosphatidylserine was 2 : 3 (by phosphorus content) suggesting estimates of 9 and 14% of each phospholipid, respectively, in the membrane preparation. Again the reproducibility of the isolation procedure is illustrated by the highly consistent lipid composition observed in successive preparations (Tables II and III).

Treatment of vitamin D-deficient chicks with 1α(OH)D_3 18 h prior to killing had no effect on total lipid content nor cholesterol content of the subsequently isolated brush border membranes (Table II). However, 1α(OH)D_3 treatment resulted in a significant increase in the content of lipid phosphorus, from 6.1 ± 0.5 to 6.9 ± 0.4 ($P < 0.005$) per mg of membrane protein. This increase was a consistent finding in the nine consecutive experiments. However, as shown in Table III, prior treatment with 1α(OH)D_3 did not significantly alter the relative proportions of the different phospholipid classes.

A second significant change in lipid composition was observed in the fatty acid composition of the phosphatidylcholine. When the phospholipids extracted from the membranes isolated from vitamin D-deficient and 1α(OH)D_3-treated chicks were separated by one-dimensional thin-layer chromatography, and the fatty acid compositions of the major classes determined by gas-liquid chromatography, the results shown in Table IV were obtained. It is

TABLE II

CHARACTERIZATION OF BRUSH BORDER MEMBRANE LIPID AND SUCRASE ACTIVITY: EFFECT OF 1α(OH)D_3 TREATMENT

Values are the mean ± S.D. of n separate determinations. Statistical significance is based on analysis by paired t-test.

	Control	1 (OH)D_3 —	n	P
Total lipid (mg/mg protein)	0.49 ± 0.03	0.47 ± 0.07	4	n.s.
Cholesterol (μg/mg protein)	121 ± 21	132 ± 14	9	n.s.
Lipid phosphorus (μg/mg protein)	6.1 ± 0.5	6.9 ± 0.4	9	<0.005
Sucrase specific activity (μmol product/mg protein per min)	0.80 ± 0.12	0.64 ± 0.13	11	<0.005

n.s., not significant.

TABLE III

PHOSPHOLIPID COMPOSITION OF BRUSH BORDER MEMBRANES FROM RACHITIC AND 1α(OH)-D_3-TREATED CHICKS

Results are expressed as percent of total lipid phosphorus. Values are expressed as the mean ± S.D. of six separate preparations.

Phosphatidylethanolamine	46.7 ± 4.4	46.7 ± 3.8
Phosphatidylseine/phosphatidyl inositol	23.5 ± 1.8	25.5 ± 1.9
Phosphatidylcholine	12.2 ± 2.3	14.0 ± 2.1
Sphingomyelin	4.8 ± 1.9	5.2 ± 1.3
Lysophosphatidylethanolamine	3.2 ± 0.4	3.2 ± 1.0
Lysophosphatidylcholine	2.3 ± 1.5	2.0 ± 0.9
Other including origin	7.2 ± 2.6	3.7 ± 2.7

noteworthy that the fatty acid composition of each phospholipid class was distinct. Also, pretreatment of vitamin D-deficient chicks with 1α(OH)D_3 caused no change in the fatty acid composition of either phosphatidylethanolamine or the combined phosphatidylserine-phosphatidylinositol fraction. However, there were distinct changes in the phosphatidylcholine fraction. There were increases in the weight percentage of 18 : 2, and 20 : 4, and decreases in 22 : 0 and in two unidentified peaks designated X and Y induced by prior 1α(OH)D_3 treatment. Peaks X and Y had retention times corresponding to a saturated carbon chain length of 21 and 23 carbons, respectively.

Alternative methods of preparation

Recently, Kessler et al. [9] have described a rapid method for the preparation of isolated brush border membrane vesicles from several mammalian species. This technique consists of initial homogenization in a Waring Blendor fol-

TABLE IV

FATTY ACID COMPOSITION OF BRUSH BORDER MEMBRANES: EFFECT OF 1α(OH)D_3 TREATMENT

Values are expressed as a weight percentage of the total fatty acid composition. Each value represents the mean ± S.D. of six separate preparations. Statistical analysis is based on analysis by paired *t*-test.

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine-phosphatidylinositol	
	Control	1α(OH)D_3	Control	1α(OH)D_3	Control	1α(OH)D_3
16	39.5 ± 3.9	37.9 ± 3.9	8.8 ± 0.9	8.5 ± 0.8	5.7 ± 1.0	5.4 ± 0.5
16 : 1	0.8 ± 0.1	0.8 ± 0.1	1.5 ± 0.3	1.6 ± 0.4	0.6 ± 0.1	0.7 ± 0.1
18 : 0	25.8 ± 1.2	15.0 ± 1.7	44.7 ± 3.5	44.0 ± 2.4	60.8 ± 3.3	55.5 ± 4.4
18 : 1	6.0 ± 1.8	6.6 ± 1.9	5.8 ± 0.9	5.4 ± 1.3	5.4 ± 1.2	5.6 ± 1.4
18 : 2	15.8 ± 3.2	19.5 ± 3.7 *	23.9 ± 7.2	27.0 ± 1.9	22.1 ± 2.8	23.9 ± 2.9
20 : 0	—	—	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20 : 1	—	—	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
X	3.1 ± 1.8	2.4 ± 1.5 *	—	—	—	—
22 : 0	3.0 ± 0.7	1.9 ± 0.3 *	—	—	—	—
20 : 4	2.4 ± 0.9	3.0 ± 1.0 *	8.8 ± 2.3	9.3 ± 1.6	3.0 ± 0.1	3.0 ± 0.7
Y	1.9 ± 0.7	1.2 ± 0.3 *	—	—	—	—
22 : 6	—	—	2.1 ± 0.8	2.5 ± 0.7	—	—

* $P < 0.05$.

lowed by addition of either calcium or magnesium salts and then differential centrifugation to recover the membrane vesicles. Because this method is considerably more rapid than the method we developed, we explored the possibility of employing this technique for the preparation of membrane vesicles from the chick intestine. However, we had difficulty adapting the procedure of Kessler et al. [9] to the chick intestine. Purified vesicles could be prepared by a modification of this method. These vesicles were similar in composition and purity to those described, but the yield of the final material was only 25–40% of that obtained by the presently described methods, and the modifications greatly increased the total preparation time. Hence, this alternative method did not prove suitable for isolation of membrane vesicles from chick intestine.

Discussion

The present study describes a simple, reproducible method for the preparation of highly purified membrane vesicles from the isolated brush border of intestinal mucosal cells of the chick. Our total purification as assessed by the increase in specific activity of sucrase is somewhat less than that reported for similar preparations in the rat. Under optimal conditions, we obtained an approximate 20-fold purification of sucrase activity whereas Hopfer et al. [10] reported a 25–30-fold purification in the rat preparation.

One possible source of contaminating protein which could reduce the calculated sucrase purification is the microvillar core material. Regularly spaced cross-bridges have been observed by electron microscopy connecting the core filaments with the microvillar membrane in chick intestinal microvilli [19]; consequently, it is possible that such cross-bridges bind core to membrane more tightly in chick than in mammalian species, making the membrane more difficult to free from this core material. In fact, our polyacrylamide gel electrophoresis patterns (Fig. 3) reveal a strong band at the position of actin, which is known to be the major protein of the core filaments [19]. However, in view of the tight association of actin with a variety of cell membranes, some of the actin present may be a true membrane component. Brush border membrane preparations from other species have also contained presumptive actin when examined by gel electrophoresis [31,32]. Consequently, with the data presently at hand one cannot determine how the actin in our preparation compares quantitatively to that in preparations from other species or to what extent our somewhat lower sucrase purification can be explained by core protein contamination.

Another possible source of contaminant protein is membrane derived from other organelles. The analysis of contaminant marker enzymes suggests that other potential membrane contaminants have been reduced to very low levels (Table I), but it is not possible to calculate from these data what percentage of the protein in our final preparation arose from other membranes because we have no estimate of the amounts of these membranes that are present in the intact cell relative to brush border membrane. Almost all of the membrane fragments observed in electron micrographs of our preparation show a coating of glycocalyx "fuzz" (Fig. 2) similar to that observed coating the microvillar membranes in intact tissue. On the other hand, of the membrane fragments observed

in adherent cytoplasm in high power electron micrographs of our intact brush border preparation, none could be seen bearing this glycocalyx marker. Thus, unless such cytoplasmic membranes can acquire such "fuzz" during the final membrane purification steps (which seems highly unlikely), then the electron micrographs suggest minimal contamination by such cytoplasmic membranes.

Although we have considered possible contamination of our brush border membrane preparation with core material and other membrane fragments, the lower sucrase purification of our preparation does not necessarily mean that our membrane preparation is less pure than similar preparations from other species; the different in sucrase purification could simply represent species difference. In the rat intestine the sucrase activity is known to decrease from villus tip to villus base [33]. If a somewhat steeper gradient existed in the chick, then a greater percentage of pure brush borders would be relatively unmarked by sucrase; a situation which would limit the maximal possible sucrase purification attainable in a mixed population of brush borders. Alternatively, if the brush border membrane in chick represented a greater fraction of the total cellular protein than in rat, this situation would also reduce the maximum attainable sucrase purification.

Two features of the lipid composition of our chick brush border membrane preparation are atypical for plasma membranes, the high cholesterol/phospholipid ratio and the predominance of phosphatidylethanolamine relative to phosphatidylcholine [34]. It is of interest that the same features are characteristic of similar membrane preparations from mammalian species [35,36]. In rat [35] and mouse [36] independent preparations of the baso-lateral membranes of the epithelial cells show lower cholesterol/phospholipid ratios and predominance of phosphatidylcholine relative to phosphatidylethanolamine. That these unusual features of brush border membrane lipid composition have survived in this differentiated region of the epithelial cell membrane throughout the independent evolution of birds and mammals suggests that these features are of functional significance to the brush border.

The present results demonstrate that prior treatment of vitamin D-deficient chicks with $1\alpha(OH)D_3$ produces changes in the structure of subsequently purified membrane vesicles from the brush borders of intestinal mucosal cells. These changes include: a change in protein content (Fig. 3), sucrase content, lipid phosphorous content (Table II), the fatty acid content of the phosphatidylcholine fraction (Table III) and, as previously reported [37], an increase in rat of calcium uptake.

At present we cannot relate the difference in protein staining of a band in the 90 000 dalton region with any functional activity. However, it is of interest that Wilson and Lawson [38] have reported that treatment of vitamin D-deficient chicks with $1,25(OH)_2D_3$ leads to an incrase in rate of labeling of a 90 000 dalton protein. Because of its size, it is clearly not the 28 000 dalton calcium-binding protein which Wasserman and Corradino [30] report is increased by vitamin D treatment. In fact the 28 000 dalton region of the gels contained no major protein band, consistent with the complete separation of this soluble protein from the particulate membrane vesicle preparation.

An unexpected finding was the decrease in sucrase specific activity following $1\alpha(OH)D_3$ treatment (Table II). Conceivably this could be explained if the

$1\alpha(OH)D_3$ caused a proliferation of cells near the base of the villi, since cells in this region are known to be relatively poor in sucrase [33] but would be expected to contribute brush border membrane protein. Vitamin D_3 treatment has been reported to increase [^{3}H]thymidine incorporation into deoxyribonucleic acid in rat intestine [39] and to increase the average villus length in chick [40], but it is doubtful whether these findings can explain the observed decrease in sucrase activity. Thus, the mechanism and significance of this effect cannot be assessed at present.

The two major changes in lipid composition induced by $1\alpha(OH)D_3$ treatment were an increase in the content of lipid phosphorous per mg of membrane protein, and a specific change in fatty acid profile found in the phosphatidylcholine fraction. These results differ from those previously reported from this laboratory [6]. However, it is clear that the brush border fraction analyzed in the earlier study was much less pure than the membrane fraction employed in the present study. Hence, a detailed discussion of the difference between the results of the two studies appears unwarranted.

The significance of these changes in lipid structure remain to be established. On the basis of previous work [41,42] concerned with the mechanism of action of aldosterone on transcellular Na^+ transport in the amphibian urinary bladder, we concluded that this steroid hormone probably exerted a primary effect upon membrane lipid turnover, and we demonstrated that inhibition of the aldosterone-induced turnover of fatty acids in membrane phospholipids led to an inhibition of the aldosterone-mediated increase in transcellular Na^+ transport. From these results, we were led to consider the possibility that certain steroid hormone might regulate cell metabolism by altering membrane lipid structure, and thereby the function of membrane proteins. The present results extent this possibility to the action of $1,25(OH_2D_3$ upon intestinal calcium transport.

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References

1 Omdahl, J.L. and DeLuca, H.F. (1973) Physiol. Rev. 53, 327—372
2 Haussler, M.R., Nagode, L. and Rasmussen, H. (1970) Nature 228, 1199—1201
3 Norman, A.W., Mircheff, A.K., Adams, R.H. and Spielvogel, A. (1970) Biochim. Biophys. Acta 215, 348—359
4 Moriuchi, S. and DeLuca, H.F. (1976) Arch. Biochem. Biophys. 174, 367—372
5 Melancon, M.J. and DeLuca, H.F. (1970) Biochemistry 9, 1658—1664
6 Goodman, D.B.P., Haussler, M.R. and Rasmussen, H. (1972) Biochem. Biophys. Res. Commun. 46, 80—86
7 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381—390
8 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98—112
9 Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136—154

10 Hopfer, V., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25—32
11 Hopfer, V., Sigrist-Nelson, K. and Morer, H. (1975) Ann. N.Y. Acad. Sci. 264, 414—427
12 Norman, A.W. (1966) Am. J. Physiol. 211, 829—834
13 Layne, E. (1957) Meth. Enzymol. 3, 447—454
14 Messer, M. and Dahlquist, A. (1966) Anal. Biochem. 14, 376 -392
15 Palmieri, M.J. and Koldovsky, O. (1972) Biochem. J. 127, 795—800
16 Benufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J. (1974) J. Cell Biol. 61, 188—200
17 McCaman, R.E., McCaman, M.W., Hunt, J.M. and Smith, M.S. (1965) J. Neurochem. 12, 15—23
18 Yonetani, T. and Elliott, W.B. (1966) Biochem. Prep. 11, 14—20
19 Mooseker, M. and Tilney, L. (1975) J. Cell Biol. 67, 725—743
20 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
21 Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374—378
22 Skidmore, W.D. and Entemann, C. (1962) J. Lipid Res. 3, 471, 275
23 Bartlett, G.F. (1959) J. Biol. Chem. 234, 466—471
24 Zlatkis, A., Zak, B. and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486—492
25 Kishimoto, Y. and Radin, N. (1965) J. Lipid Res. 6, 435—436
26 Ackman, R.G. and Burgher, R.D. (1963) Can. J. Biochem. Physiol. 41, 2501—2505
27 Laemmli, U.K. (1970) Nature 227, 680—685
28 Margolis, J. and Kenrick, K.G. (1968) Anal. Biochem. 25, 347—362
29 Eichholz, A. and Crane, R. (1965) J. Cell Biol. 26, 687—691
30 Wasserman, R.H. and Corradino, R.A. (1973) Vitam. Horm. 31, 43—103
31 Fujita, M., Kawai, K., Asano, S. and Nakao, M. (1973) Biochim. Biophys. Acta 307, 141—151
32 Maestracci, D., Schmitz, J., Preiser, H. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 113—124
33 Weiser, M. (1973) J. Biol. Chem. 248, 2536—2541
34 McMurray, W.C. (1973) in Form and Function of Phospholipids (Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C., eds.), Chapt. 9, pp. 205—251, Elsevier/North-Holland, Amsterdam
35 Douglas, A., Kerley, R. and Isselbacher, K.J. (1972) Biochem. J. 128, 1329—1338
36 Kawai, K., Fujita, M. and Nakao, M. (1974) Biochim. Biophys. Acta 369, 222—233
37 Rasmussen, H., Max, E.E. and Goodman, D.B.P. (1977) in Vitamin D: Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism (Norman, A.W., ed.), pp. 913—925, de Gruyter, Berlin
38 Wilson, P.W. and Lawson, D.E.M. (1977) Biochim. Biophys. Acta 497, 805—811
39 Birge, S.J. and Alpers, D.H. (1973) Gastroenterology 64, 977—982
40 Spielvogel, A.M., Farley, R.D. and Norman, A.W. (1972) Exp. Cell. Res. 74, 359—366
41 Goodman, D.B.P., Wong, M. and Rasmussen, H. (1975) Biochemistry 14, 2803—2809
42 Lien, E.L., Goodman, D.B.P. and Rasmussen, H. (1975) Biochemistry 14, 2749—2754