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THE OCCURRENCE OF VITAMIN A IN BIOLOGICAL MEMBRANES

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SUMMARY

1. Rats maintained on a low vitamin A intake were intraperitoneally injected with 50.7 μg [11,12-$^3H_2$]retinyl acetate. 15 h later, the animals were killed and liver and kidney plasma membranes, kidney endoplasmic reticulum and erythrocyte ghosts were prepared.

2. The labeled vitamin A content of the membrane preparations varied consistently between 0.003–0.005 μg retinol/mg N, while the level in the homogenate varied from 0.001–0.009 μg retinol/mg N. The labeled vitamin A content of the erythrocyte ghosts was low (max. 0.0001 μg retinol/mg N) probably due to heavy hemoglobin contamination.

3. The radioactive vitamin A compounds were strongly bound to the membranes: *n*-hexane removed only 7 % of the radioactive compounds, whereas chloroform–methanol (2:1, v/v) extraction of the membrane preparations removed 90 % of the radioactive compounds.

4. Thin-layer chromatography of the chloroform–methanol (2:1, v/v) extract of the kidney endoplasmic reticulum, in three different solvent systems, revealed that retinol and retinoic acid were the dominant forms of vitamin A. This was confirmed by combined column and thin-layer chromatography (using a fourth solvent system) of the chloroform–methanol (2:1, v/v) extract of endoplasmic reticulum.

INTRODUCTION

The function of vitamin A in the visual process is well-established, but its general metabolic role in animals remains unknown.

Vitamin A may play a role in maintaining the normal structure and function of cellular membranes: this could explain the function of the vitamin in general metabolism. Roxas *et al.*[1] demonstrated that considerably more ribonuclease was released from a mitochondrial lysosome-rich fraction of the liver of vitamin A-deficient rats than from the pair-fed control. Roels *et al.*[2,3] and Guha and Roels[4] made the same observation for β-glycerophosphate phosphatase and arylsulfatases A and B. These observations were later confirmed by Dingle *et al.*[5] and indicate that vitamin A deficiency labilizes the lysosomal membrane.

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Anderson *et al.*[6] demonstrated that erythrocytes from vitamin A-deficient rats are markedly swollen and distorted compared to those from pair-fed controls. Roels *et al.*[7] found that the rate of release of hemoglobin from erythrocytes of vitamin A-deficient rats is considerably faster than from cells of pair-fed controls, and that ATPase is more closely associated with the membrane of the erythrocyte in control animals than in vitamin A-deficient rats.

Brown's studies of the frog retina showed that rhodopsin molecules are located in the membranes of the stacked disks which form the cylindrical rod outer segments[8]. The site occupied by rhodopsin in this membrane is believed to be highly fluid, suggesting that rhodopsin may be a diffusional ion carrier[9]. Recently Nyquist *et al.*[10] showed that high concentrations of vitamin A compounds, principally retinyl esters, were found in fractions of Golgi apparatus from rat liver. They suggested a specific role of the Golgi apparatus in the mobilization or action, or both, of vitamin A compounds.

We have studied the association of vitamin A compounds with different rat cellular membranes. We found that vitamin A is associated with rat kidney and liver plasma membranes and with rat erythrocyte ghosts and kidney endoplasmic reticulum. The principal forms of the vitamin associated with rat kidney endoplasmic reticulum are retinol and retinoic acid; only trace amounts of retinal and retinyl esters were found in this fraction.

MATERIALS AND METHODS

Rats

21-day-old male albino weanling rats (Sherman strain) were separately caged and fed a vitamin A-deficient diet (Nutritional Biochemical Corp., Cleveland, Ohio) until they ceased to grow (110–120 g). From then on, they were given 8 μg of retinyl acetate in 0.1 ml peanut oil once a week by mouth dropper. When the rats reached weights of 180–225 g, they were used in the experiments. At this stage, no vitamin A could be detected in their livers by the trifluoroacetic acid method, described by Roels *et al.*[11].

Purification and injection of radioactive vitamin A compounds

All-*trans*-[11,12-$^3H_2$]retinyl acetate (specific activity 380 μCi/mg) was a generous gift of Hoffman–La Roche, Inc., Nutley, N. J. It was purified by alumina column chromatography (Huang and Goodman[12]). The rats were injected intraperitoneally with 50.7 μg (19.25 μCi) of the purified retinyl acetate suspended in 0.1 ml aqueous dispersing agent (1 ml of this solution contains 40 mg Cremophore*, 50 mg glycerine, 2.5 mg sodium benzoate, 0.1 mg Nipagen**, 0.8 mg Nipasol**, and distilled water; Hoffman–La Roche, Inc., Nutley, N. J.). Food was removed just prior to the injection and water was given *ad libitum*. 15 h later, the rats were decapitated and exsanguinated and the tissue to be used was removed.

Preparation of cellular membranes

Rat liver plasma membrane was isolated by using the method of Coleman *et al.*[13].

* Cremophore is a surfactant in emulsifying agents, resulting from the reaction of castor oil and ethylene oxide.

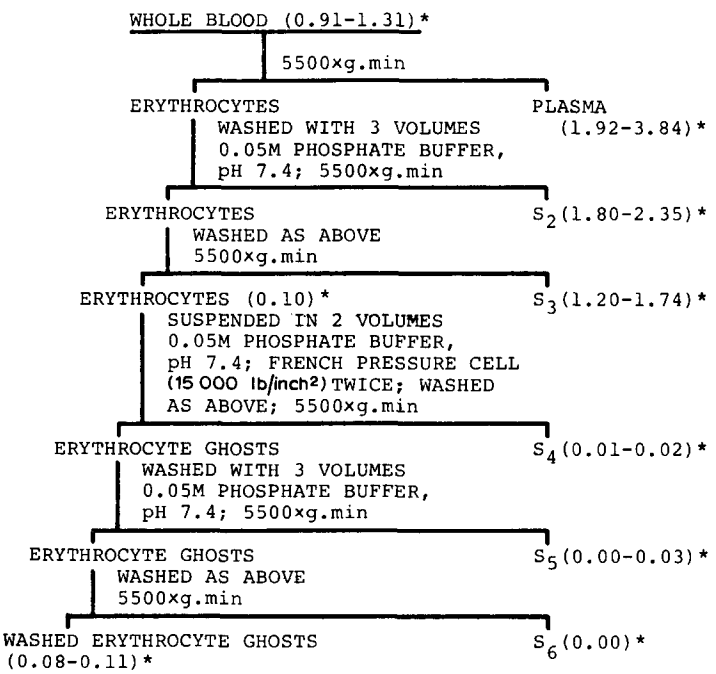
** Nipagen and Nipasol are methyl and ethyl esters of parahydroxybenzoic acid used as preservatives.

Rat kidney plasma membrane was isolated by modifying the technique of Fitzpatrick *et al.*[14]. The crude kidney and liver plasma membrane fractions were resuspended in 1.5 ml of 0.25 M sucrose and loaded onto a discontinuous sucrose gradient of the following composition: ρ = 1.18–1.0 ml; ρ = 1.16–1.5 ml; ρ = 1.13–1.0 ml. The tubes were centrifuged for $12 \cdot 10^6 \times g \cdot \text{min}^*$ in a Beckman SW-39 rotor. The fraction on top of ρ = 1.13, containing the purified liver and kidney plasma membranes, was used in the experiments. Rat kidney endoplasmic reticulum was isolated, following the method of Siekevitz[15].

Blood was collected from the rats in heparin-coated glass centrifuge tubes immediately after the animal was decapitated. The preparation of rat erythrocyte ghosts is outlined in Fig. 1.

Determination of radioactivity in biological material

The kidney and liver fractions were suspended in glass vials in 15 ml toluene–phosphor solution, containing 6.0 g PPO (2,5-diphenyloxazole) and 0.2 g POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene] per l of anhydrous toluene. 2 ml of a general purpose solubilizer (BioSolv III, Beckman Instruments, Inc., Mountainside, N. J.) was added to each vial. The samples were counted in a Beckman LS-100 liquid scintillation counter with fixed-window ISO-Set™. The error selector setting on the



*The range of specific vitamin A content in each fraction from two separate experiments expressed as µg retinol x 10^{-3}/mgN.

Fig. 1. Isolation of rat erythrocyte ghosts under isotonic conditions.

* Centrifugal forces are expressed as time integrals $g \cdot \text{min}$ of the field strength in the center of the tube.

counter provided a standard deviation of ± 2%. Internal standardization with tritiated toluene was used to correct for quenching.

To determine the radioactivity of the fractions prepared from rat erythrocyte ghosts, an aliquot of the sample was pipetted into a glass vial; 0.1 ml H_2O_2 (30%) and 0.1 ml 2 M NaOH were added. The mixture was then heated for 2 h at 80 °C. After cooling, enough acid solubilizer (about 0.2 to 0.5 ml BioSolv II, Beckman Instruments, Inc., Mountainside, N. J.) was added to neutralize the mixture. Liquid scintillation solution and BioSolv III were added as previously explained, and the sample assayed for radioactivity.

Liquid scintillation was also used to assay the radioactivity of the thin-layer chromatograms: a section of the adsorbent was scraped off and placed in a glass vial with 1 g of colloidal silica (Beckman Cab-O-Sil) and 15 ml toluene–phosphor.

Protein determination

Protein was determined by the Lowry method (Lowry *et al.*[16]). Bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Ill.) was used as standard.

Silicic acid column chromatography

Columns (12 mm outer diameter, 10 mm inner diameter, 250 mm length, Bellacour Co., N.Y., with a sintered glass filter and a mini-flow needle valve) were packed by pouring a slurry containing 7 g silicic acid (Unisil, 200–325 mesh, Clarkson Chemical Co., Inc., South Williamsport, Pa.) and 40 ml chloroform onto the column. The column was connected to a fraction collector (LKB Radi-Rac Fraction Collector, Sweden) with a drop counter attachment. Chloroform was used to elute the sample from the column.

Thin-layer chromatography

Glass plates (20 cm × 20 cm, Brinkman Instruments, Westbury, N.Y.) were coated with a 750-μm-thick layer of purified and screened silica gel (Adsorbosil No. 2, Applied Science Laboratories, State College, Pa.).

Solvent systems for thin-layer chromatography

The solvent systems used for the development of the chromatography plates were: (a) cyclohexane–ethyl ether (4:1, v/v; Strohecker and Henning[17]); (b) cyclohexane–ethyl acetate–ethyl ether (7:2:1, v/v/v); (c) ethyl acetate; (d) cyclohexane–benzene–ethyl acetate (5:3:2, v/v/v). Solvent systems b, c, and d were developed in this laboratory for the separation of vitamin A compounds.

Recovery studies of radioactive vitamin A compounds on thin-layer and column chromatography

The recovery of vitamin A compounds on unbuffered silicic acid columms was 76% for retinol, 81% for retinal, 89% for retinoic acid and 86% for retinyl acetate.

The recovery values of vitamin A compounds on unbuffered silicic acid thin-layer chromatograms were 86% for retinol, retinal 93%, retinoic acid 86%, and retinyl acetate 84%.

Electron microscopy

Aliquots of the isolated rat kidney and liver plasma membrane fractions and rat kidney microsomal fraction (endoplasmic reticulum) were fixed for 24 h with 2 % (w/v) glutaraldehyde in 0.125 M cacodylate buffer (pH 7.1) at 3 °C. An aliquot of the membrane fraction was collected on a Millipore filter (Millipore Filter Corp., Bedford, Mass., 13 mm diameter, 0.01 μm mean pore size) following the method of Baudhuin *et al.*[18] to ensure a statistically random sample of the membrane fraction. The Millipore filter containing the membrane fraction was rinsed with cold 0.25 M cacodylate buffer (pH 7.1), and post fixed with 1 % osmium tetroxide, buffered with 0.05 M cacodylate (pH 7.1) for 30 min at 3 °C. The next day, the pellets were infiltrated with a mixture of propylene oxide and Epon-812 (1:1, v/v) and kept at 60 °C for 30 min. The pellets were then embedded in Epon-812 and kept at 60 °C for two days.

Thin sections were prepared with a Porter-Blum MT-2 ultramicrotome fitted with a diamond knife. The sections were collected on carbon-reinforced collodion-covered grids, post-stained with Reynolds' lead citrate (Reynolds[19]), and observed with a Philips EM-200 electron microscope, operating at 60 kV accelerating voltage.

In vitro addition of [11,12-$^3H_2$]retinyl acetate to rat kidney tissue slices

[11,12-$^3H_2$]Retinyl acetate was mixed with ice-cold rat kidney tissue slices just before homogenization at a level comparable to the level that was found in the total homogenate of the *in vivo* experiments (0.1–0.2 μg retinol). After homogenization, the homogenate was separated into three aliquots.

Aliquot I. Aliquot I was extracted three times at zero hours with 10 vol. chloroform–methanol (2:1, v/v). The extract was kept at 0 °C under nitrogen until ready for use.

Aliquot II. Aliquot II was kept at 0 °C under nitrogen until the microsomal fraction of Aliquot III was prepared. At that time (approx. 5 h) it was extracted three times with 10 vol. chloroform–methanol (2:1, v/v).

Aliquot III. The microsomal fraction was prepared from Aliquot III and immediately extracted three times with 10 vol. chloroform–methanol (2:1, v/v).

Vitamin A compounds in the extract of Aliquots I and II were identified by thin-layer chromatography. Vitamin A compounds in Aliquot III were identified by combined silicic acid column chromatography and thin-layer chromatography.

Precautions

(1) We used membrane preparations isolated from healthy rats kept on a low vitamin A intake (8 μg/week) to avoid accumulation of vitamin A in the liver and kidney; (2) we injected a relatively low dose (50.7 μg) of [11,12-$^3H_2$]retinyl acetate per rat; (3) we used isotonic solutions to maintain the membranes as intact as possible; (4) we used nitrogen atmosphere and low light intensity to avoid denaturation of vitamin A compounds during and after isolation of the membrane preparations; and (5) we extracted all membrane preparations immediately after isolation to avoid decomposition of the membrane preparation and the vitamin A compounds.

RESULTS

The presence of vitamin A in cellular membranes

Table I shows the specific vitamin A contents (μg retinol per mg N) of the

tissue homogenate obtained from rats injected with [11,12-$^3H_2$]retinyl acetate and of the purified membrane isolated from it. The labeled vitamin content of the membrane preparations varied between $3.0 \cdot 10^{-3}$ and $5.4 \cdot 10^{-3}$ μg retinol per mg N, while the level in the homogenate varied from $1.4 \cdot 10^{-3}$ to $8.7 \cdot 10^{-3}$ μg retinol per mg N. The labeled vitamin A content of the erythrocyte ghosts was rather low ($1.0 \cdot 10^{-4}$ μg retinol per mg N); however, this level is comparable to that of the red blood cells ($1.0 \cdot 10^{-4}$ μg retinol per mg N). The low retinol content of the ghosts was probably due to the heavy hemoglobin contamination which occurred when we isolated erythrocyte ghosts under isotonic conditions.

The purity of the isolated membrane preparations (kidney microsomal fraction,

TABLE I

VITAMIN A CONTENT IN RAT CELLULAR MEMBRANES

Types of membranes	*Fraction*	*Total nitrogen (mg N)*	*Total vitamin A (cpm)*	*Total vitamin A (μg retinol, × 10⁻³)*	*Specific content (μg retinol, × 10⁻³/mg N)*
Liver plasma membrane					
Expt 1	Homogenate	165.20	159 195	304.00	1.84
	Plasma membrane	0.16	336	0.64	4.05
Expt 2	Homogenate	155.80	114 160	218.00	1.40
	Plasma membrane	0.10	230	0.44	4.40
Kidney plasma membrane					
Expt 1	Homogenate	24.13	110 180	210.40	8.72
	Plasma membrane	0.06	108	0.21	3.50
Expt 2	Homogenate	21.61	50 924	97.25	4.50
	Plasma membrane	0.04	60	0.12	3.00
Kidney endoplasmic reticulum					
Expt 1	Homogenate	43.83	157 760	301.25	6.87
	Endoplasmic reticulum	4.11	11 520	22.00	5.35
Expt 2	Homogenate	39.75	52 053	99.40	2.50
	Endoplasmic reticulum	3.02	6 598	12.60	4.17
Expt 3	Homogenate	20.06	73 313	140.00	6.98
	Endoplasmic reticulum	1.85	4 922	9.40	5.08
Expt 4	Homogenate	21.12	46 606	89.00	4.21
	Endoplasmic reticulum	1.79	3 979	7.60	4.25
Expt 5	Homogenate	29.81	89 100	170.00	5.70
	Endoplasmic reticulum	2.24	4 160	7.94	3.55
Erythrocyte ghost	Twice washed erythrocyte	85.13	4 544	8.66	0.102
	Washed ghost	57.49	3 001	5.72	0.099

kidney plasma membrane, and liver plasma membrane) was examined by electron microscopy. The electron micrographs of the purified membrane preparations are shown in Figs 2a, 2b and 2c.

The nature of the binding of vitamin A to cellular membranes

(1) Saline incubation and chloroform–methanol (2:1, v/v) extraction of rat liver plasma membrane. The saline-soluble protein of the plasma membranes is probably a preparative artifact representing non-membranous protein, whereas the saline-insoluble portion represents the plasma membranes as they appear *in situ* (Benedetti and Emmelot[20]). The procedure outlined in Fig. 3 was used for eliminating the saline-soluble protein from the membrane preparation and for the extraction of the saline residue with chloroform–methanol (2:1, v/v; Folch *et al.*[21]).

Table II shows the distribution of radioactivity and protein nitrogen in the fractions obtained in the procedure outlined in Fig. 3. Table II shows that: (1) no radioactive vitamin A compounds are adsorbed to non-membranous protein attached to the plasma membrane; and (2) the majority (91.5 %) of the radioactive vitamin A compounds associated with the plasma membrane after saline incubation are extracted with chloroform–methanol (2:1, v/v).

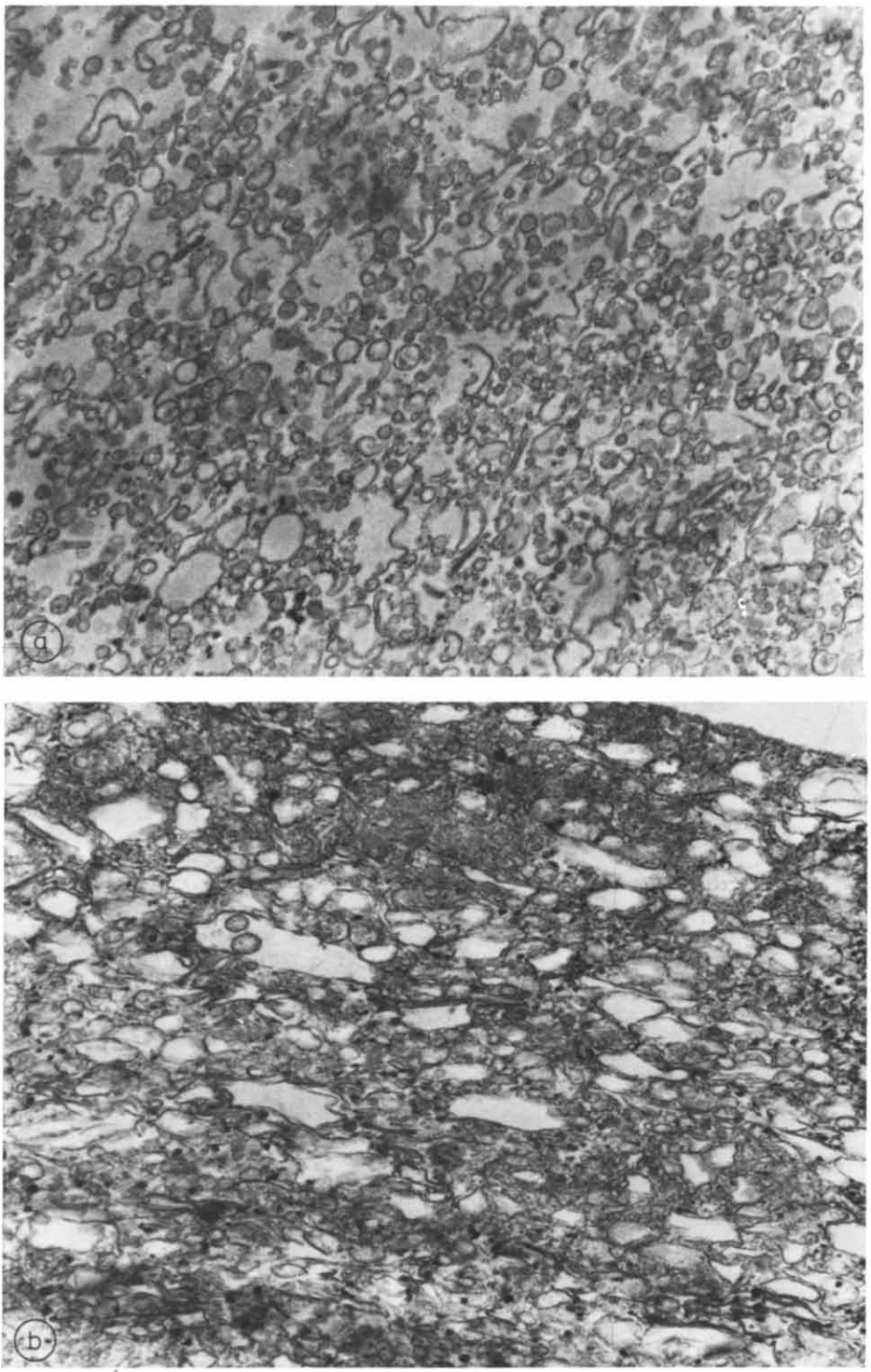
TABLE II

DISTRIBUTION OF RADIOACTIVITY AND PROTEIN NITROGEN IN THE FRACTIONS OF RAT LIVER PLASMA MEMBRANE DESCRIBED IN FIG. 3

Fraction	*N content (mg)*	*Radioactivity (cpm)*	*% Protein*	*% Total radioactivity*
P_1	0.28920	550	100	100
S_1	0.00000	15	0	2.7
S_2	0.00500	0	1.7	0
P_2	0.27900	527	96.5	95.8
		Recovery: 542	98.2	98.5
P_2	0.27900	527	100	100
P_3	—	40		7.5
S_3	—	482	—	91.5
		Recovery: 522	—	99.0

(2) n-Hexane and chloroform-methanol (2:1, v/v) extraction of rat kidney microsomal fraction. Rat kidney microsomal fraction was extracted as shown in Fig. 4. *n*-Hexane removed only 6.6 % of the total radioactivity associated with the fraction, while the more polar solvent, chloroform–methanol (2:1, v/v), removed 89.4 % of the total radioactivity. The residue which was mainly protein retained a small (2.5 %), but possibly significant, percentage of the total radioactivity in the microsomal fraction.

These results indicate that the majority of vitamin A (89.4 %) associated with the rat kidney microsomal fraction is extracted with the polar lipid fraction of the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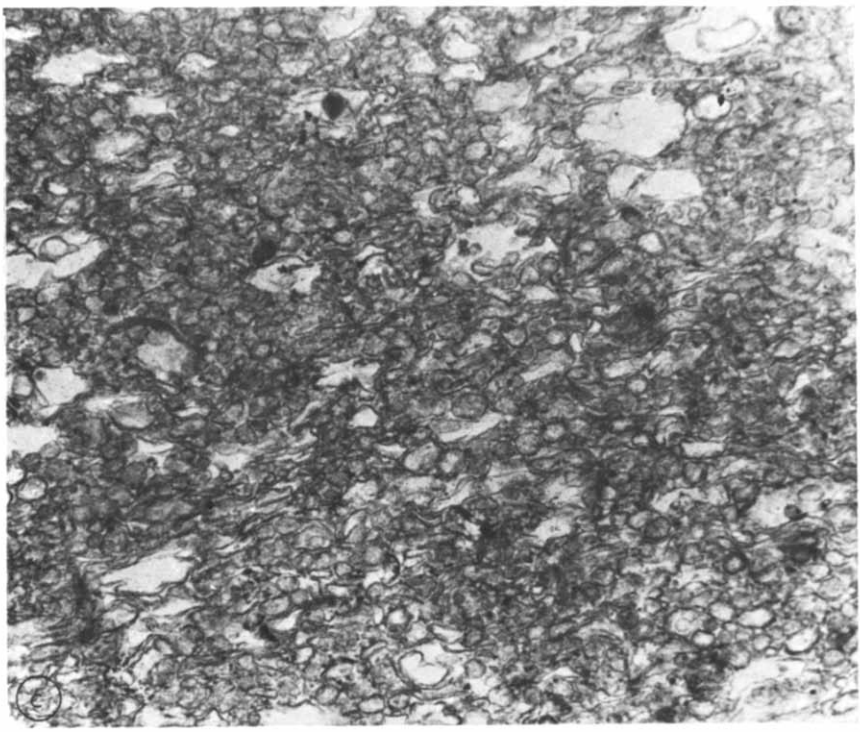

Fig. 2. (a) Electron micrograph of purified rat kidney microsomal fraction. × 27500. (b) Electron rat liver plasma membrane. × 27500. (c) Electron micrograph of rat liver plasma membrane. × 27500.

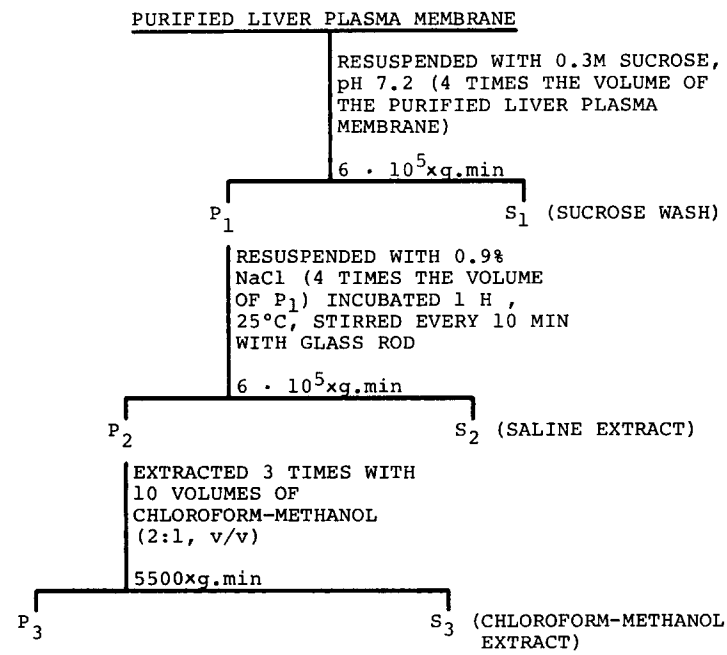

Fig. 3. Saline incubation and chloroform–methanol (2:1, v/v) extraction of rat liver plasma membrane.

membrane. The vitamin A compounds appear to be bound quite firmly to the membrane since a non-polar solvent (*n*-hexane) could only remove 6.6 % of the total radioactive vitamin A compound.

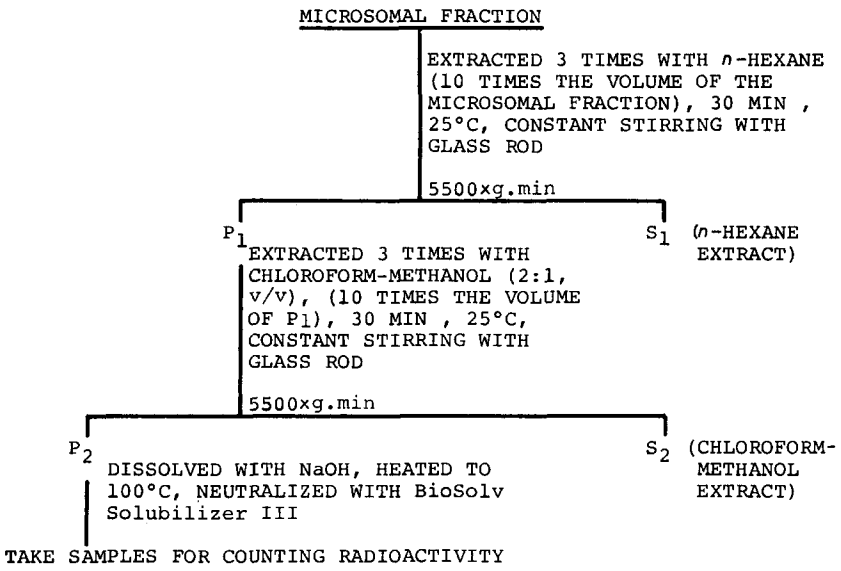

Fig. 4. *n*-Hexane and chloroform–methanol (2:1, v/v) extraction of rat kidney microsomal fraction.

Forms of vitamin A associated with cellular membranes

(*1*) *Thin-layer chromatography of unsaponifiable matter from the kidney microsomal fraction.* The microsomal fraction was saponified with 1 vol. of 1 M ethanolic KOH for 20 min in a water bath at 60 °C. The mixture was rapidly cooled and extracted three times with 10 vol. *n*-hexane and centrifuged for 5500 × *g*·min. The *n*-hexane supernatant was dried down with prepurified nitrogen and applied to a thin-layer chromatogram (Adsorbosil No. 2). Vitamin A standards were applied at the same time. After development, the chromatogram was cut in 1–3-cm sections starting from the origin and proceeding to the solvent front. The Adsorbosil No. 2 was scraped off, placed in glass vials and assayed for radioacitvity. The results are shown in Fig. 5, Expt 1. The major radioactive vitamin A compound has the same R_F as standard all-*trans*-retinol. Trace amounts of radioactivity were found in the same positions as standard all-*trans*-retinal and all-*trans*-retinoic acid.

(*2*) *Thin-layer chromatography of the chloroform–methanol (2:1, v/v) extract of rat kidney microsomal fraction.* The microsomal fraction was extracted three times with 10 vol. of chloroform–methanol (2:1, v/v). The chloroform–methanol extract was centrifuged for 5500 × *g*·min. The supernatant was dried down with prepurified nitrogen and applied to thin-layer chromatograms. Vitamin A standards were applied at the same time. The results are shown in Fig. 5, Expts 2, 3 and 4, using three different solvent systems. The major radioactive vitamin A compounds appeared to be retinol with retinoic acid as the minor radioactive compound in all three experiments.

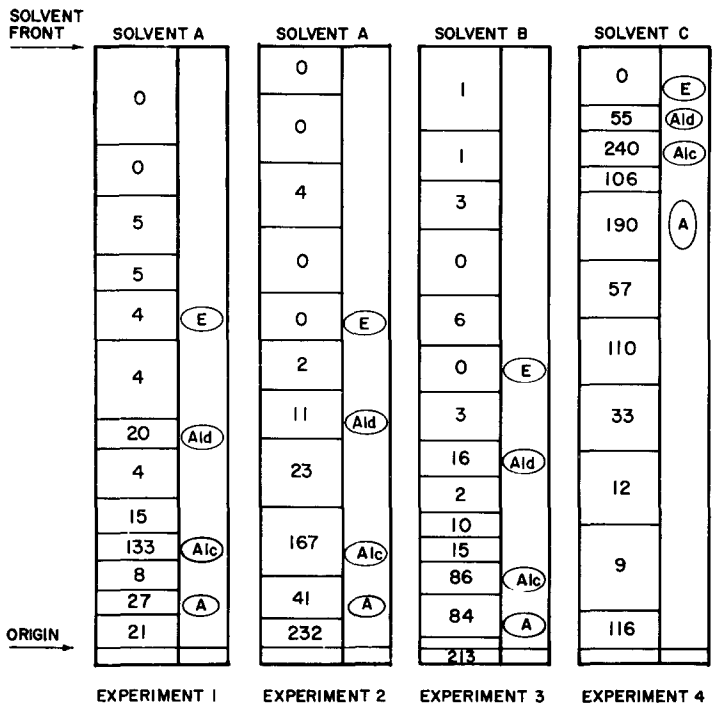

Fig. 5. Thin-layer chromatographic separation of vitamin A compounds from rat kidney microsomal fraction. Numbers represent cpm. Solvent A, cyclohexane–ethyl ether (4:1, v/v); Solvent B, cyclohexane–ethyl acetate–ethyl ether (7:2:1, v/v/v); and Solvent C, ethyl acetate. The non-radioactive vitamin A standards used were: A, all-*trans*-retinoic acid; Alc, all-*trans*-retinol; Ald, all-*trans*-retinal; E, all-*trans*-retinyl acetate.

Trace amounts of retinal appeared in the three experiments. Large amounts of radioactivity were found close to the origin of the chromatogram in Expts 2, 3 and 4.

(3) *Combined column chromatography and thin-layer chromatography.* The rat kidney microsomal fraction was extracted three times with 10 vol. of chloroform–methanol (2:1, v/v) and centrifuged for 5500 × *g*·min. The supernatant was dried down with prepurified nitrogen and reextracted with 20 ml *n*-hexane. The *n*-hexane extract was centrifuged for 5500 × *g*·min. The supernatant was dried down with prepurified nitrogen and the sample dissolved in 2 ml chloroform. To this solution was added 0.1 ml of a solution containing 1 mg all-*trans*-retinol, 1 mg all-*trans*-retinal, 1 mg all-*trans*-retinoic acid and 1 mg all-*trans*-retinyl acetate per 10 ml chloroform. The sample was loaded on a silicic acid column and eluted with chloroform; 1.4-ml fractions were collected. The absorbance of each fraction was monitored at 328 nm (maximal absorption of retinol and retinyl ester) and at 350 nm (maximal absorption of retinoic acid). Fractions 12–16, corresponding to peak 2, were also read at 380 nm (maximal absorption of retinal). The results are shown in Fig. 6. The spectrum of each fraction was also checked in a Beckman DK-2A ratio-recording spectrophotometer. Peak 1 (Fractions 9–11) and Peak 3 (Fractions 19–23) showed the absorption spectrum of retinyl ester and retinol. Peak 2 (Fractions 13–15) showed the absorption

spectrum of retinal. Peak 4 (Fractions 28–31) showed the absorption spectrum of retinoic acid.

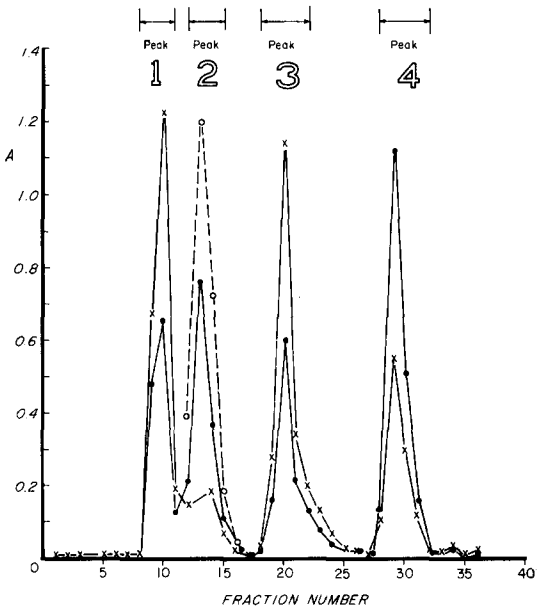

Fig. 6. Separation of vitamin A compounds by silicic acid column chromatography. Fractions were monitored at 328 nm (×—×) and 350 nm (●—●). Fractions in Peak 2 were also read at 380 nm (○—○).

Fractions corresponding to retinyl ester (9–11), retinal (13–15), retinol (19–23), and retinoic acid (28–31) were combined respectively and dried down to 0.1 ml with prepurified nitrogen and applied to thin-layer chromatograms. Vitamin A standards were applied at the same time. The chromatograms were developed and assayed for radioactivity. The results are shown in Fig. 7. Peak 1 showed no radioactive compound migrating with the same R_F as the standard (retinyl acetate), but radioactive vitamin A compounds migrating with the same R_F as standard retinyl palmitate were found. Peak 2 showed insignificant radioactivity at the same R_F as the standard compound retinal. Peak 3 showed a major radioactive compound migrating with the same R_F the standard retinol. Peak 4 showed a major radioactive compound migrating with the same R_F as the standard retinoic acid. It is evident from these results that the major forms of radioactive vitamin A associated with the membrane fraction were retinol and retinoic acid. Trace amounts of retinal and retinyl palmitate were also found.

In vitro binding of vitamin A compounds to kidney endoplasmic reticulum

A control experiment was done to determine the *in vitro* binding of vitamin A to membranes by mixing [11,12-$^3H_2$]retinyl acetate with kidney homogenate. At zero time, the major labeled form of vitamin A in the homogenate was retinyl acetate. 5 h later, the major vitamin A compounds present were retinyl esters (acetate and possibly palmitate). The forms of vitamin A identified after extraction of the endoplasmic

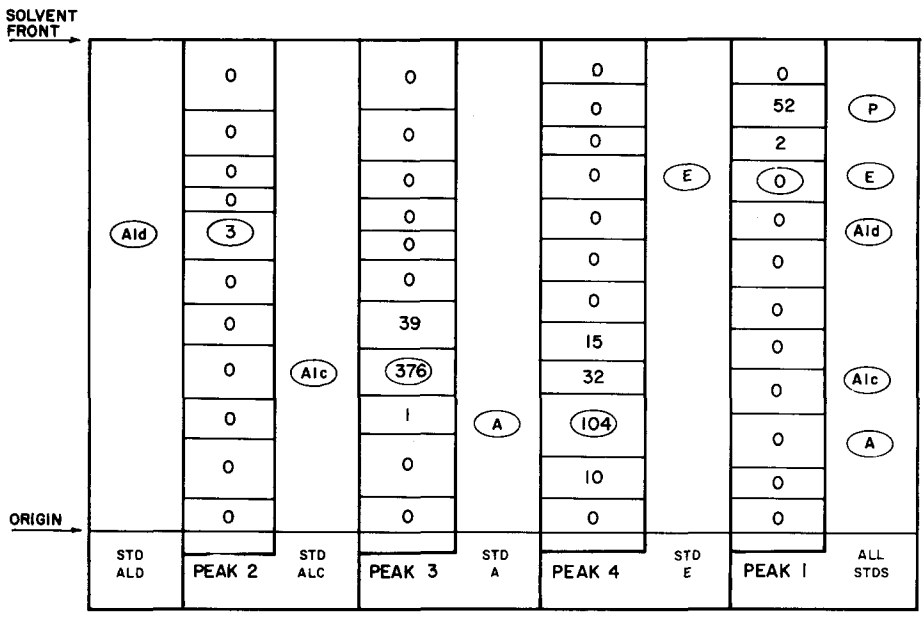

Fig. 7. Identification by thin-layer chromatography of radioactive vitamin A compounds of rat kidney microsomal fraction eluted from silicic acid column. Peaks 1–4 from the silicic acid column (see Fig. 6) were concentrated and applied to a thin-layer chromatographic plate, coated with Adsorbosil No. 2. The solvent system used was cyclohexane–benzene–ethyl acetate (5:3:2, v/v/v). Numbers represent cpm. A, all-*trans*-retinoic acid; Alc, all-*trans*-retinol; Ald, all-*trans*-retinal; E, all-*trans*-retinyl acetate; P, all-*trans*-retinyl palmitate.

reticulum were: 8.9 % retinoic acid, 3.3 % retinal, 19.1 % retinol, 16.3 % retinyl acetate and 23 % retinyl palmitate.

DISCUSSION

The specific function of vitamin A in the visual process is well established, but its general role in maintaining growth, reproduction and survival of animals remains unknown. Lui and Roels[22] reported that liver endoplasmic reticulum, plasma membrane and erythrocyte ghosts from rats maintained on a low level of vitamin A contained radioactivity after intraperitoneal injections of [14,15-$^{14}C_2$]retinol. Since decarboxylation of the terminal carbons (C-14 and C-15) of the vitamin A molecule can occur after injection of [14,15-$^{14}C_2$]retinol in the rat (De Luca and Roberts[23]), this demonstration of radioactivity did not conclusively show that intact vitamin A is incorporated into membranes. We have, therefore, used [11,12-$^{3}H_2$]retinyl acetate to determine whether vitamin A is present in membranes, because *in vivo* incorporation of the label from this molecule in forms other than vitamin A will not occur in 15 h*.

Our results showed that rat liver and kidney plasma membrane preparations and rat kidney endoplasmic reticulum contained about the same specific content of labeled vitamin A (0.00300–0.00535 μg retinol per mg N) while the levels found in the total tissue homogenate varied (0.00140–0.00872 μg retinol per mg N).

The specific vitamin A contents of the membrane fractions reported in this

* Scott W. E. personal communication, Hoffman-La Roche, Inc., Nutley, N. J., 1969.

paper are entirely based on radioactive counts and on the specific activity of the labeled [11,12-$^3H_2$]retinyl acetate we used in our experiments (380 μCi/mg). If our rats, maintained on a marginal vitamin A intake (8 μg/week), had any remaining amounts of vitamin A present in the tissue fractions we examined, then the specific activities we report would err on the low side.

The specific vitamin A contents of rat kidney and liver plasma membranes and rat endoplasmic reticulum from animals on a very low vitamin A intake reported in this paper (0.00300–0.00535 μg retinol per mg N) are comparable to the levels calculated from the data reported for whole, normal rat muscle: 0.0028 μg vitamin A per mg N (Davies and Moore[24]); normal rat brain: 0.00307 μg vitamin A per mg N (Davies and Moore[24]). Normal rat kidney (0.01875 μg vitamin per mg N, Moore[25]) and normal rat blood plasma (0.02330 μg vitamin A per mg N, Moore[26]; Vakil *et al.*[27]) contain higher levels of the vitamin since the kidney serves as a storage organ for vitamin A and blood plasma is used for its transport in animals receiving normal dietary levels of the vitamin.

Recently Nyquist *et al.*[10] found that vitamin A compounds, principally retinyl esters, were concentrated in the Golgi apparatus of normal rat liver. Similar extracts from endoplasmic reticulum or mitochondria revealed none of these compounds on a protein equivalent basis when examined spectrophotometrically at 328 nm. When Nyquist *et al.*[10] examined Golgi apparatus fractions or total homogenates from vitamin A-deficient rat livers by the same spectrophotometric technique or by the Carr–Price method, they could not detect vitamin A. We also could not detect vitamin A when we examined the liver and kidney homogenates of our vitamin A-deficient rats by the trifluoroacetic acid method[11], which has a sensitivity comparable to the methods used by Nyquist *et al.*[10]. The membrane-associated vitamin A compound levels discussed in our work are three orders of magnitude lower than those discussed by Nyquist *et al.*[10] for normal rat liver and could practically only be detected by isotope labeling. On the basis of their results, Nyquist *et al.*[10] have very reasonably suggested a specific role of the Golgi apparatus in the mobilization of retinyl esters from normal rat liver stores.

We found that the dominant forms of vitamin A associated with vitamin-A deficient rat kidney endoplasmic reticulum were retinol and retinoic acid, with only trace amounts of retinyl ester present. This is in agreement with Kleiner–Bössaler and De Luca's[28] report that retinol and retinoic acid are the major forms of vitamin A in the kidney of vitamin A-deficient rats.

The methods we used for the preparation of different membrane fractions have been proved satisfactory by marker enzyme analyses and by electron microscopic observation of the isolated membranes[13–15]. The original method for kidney plasma membrane isolation could not eliminate mitochondrial contamination completely. However, by using discontinuous density gradient centrifugation, we overcame this problem. Our electron micrograph of the isolated membrane fractions show the same type of membrane as was obtained by the authors of the methods we used. We did not see obvious contamination of our membrane fractions with Golgi apparatus. Furthermore, the major vitamin A compounds found in kidney endoplasmic reticulum are retinol and retinoic acid; only trace amounts of retinyl esters were found. This clearly eliminates the likelihood that the vitamin A present in our membrane fractions is due to contamination with Golgi apparatus.

Kleiner-Bössaler and De Luca[28] found that 5 h after intrajugular injection of 10 μg [^{3}H]retinyl acetate to vitamin A-deficient rats, the major radioactive vitamin A compound was retinol with radioactive retinoic acid as a minor form. No retinyl acetate or retinal was detected. They showed that this retinoic acid was formed in the kidney. Our *in vitro* experiments showed that 5 h after mixing 0.1–0.2 μg of [^{3}H]retinyl acetate with kidney homogenate, most of the acetate ester was either transformed to other esters (possibly palmitate) or hydrolyzed to give retinol. Small amounts of retinoic acid and retinal were also formed. The spectrum of vitamin A compounds in the kidney endoplasmic reticulum isolated in our *in vivo* experiments resembles that in kidney homogenate obtained by Kleiner-Bössaler and De Luca[28]. Therefore, although the kidney can hydrolyze retinyl acetate to some extent as indicated in our *in vitro* experiments, the very low levels of retinyl ester found in the kidney of vitamin A-deficient rats indicates that the intraperitoneally or intrajugularly injected retinyl acetate may have been hydrolyzed before reaching the kidney and may have reached this organ in the alcohol form *via* the retinol-binding protein of the plasma[29].

The proteins attached to the plasma membrane which are soluble in physiological saline are generally regarded as preparative artifacts, representing surface-adsorbed, non-membrane protein (Benedetti and Emmelot[20]). Repeated extractions of the membrane preparations with physiological saline did not remove the labeled vitamin A compounds, indicating that the vitamin A compounds were bound to the membrane and not to non-membrane protein.

We then tried to determine how the radioactive vitamin A compounds were bound to the membrane. If the radioactive vitamin A compounds associated with the membrane preparations were bound to the cellular membranes by hydrophobic and weak electrostatic interactions, the radioactivity should easily be extractable into non-polar solvents such as *n*-hexane. Since only 6.6 % of the radioactive vitamin A compounds was extractable with *n*-hexane, the bulk of the vitamin A compounds was firmly bound to the membrane, and not simply adsorbed to the surface. If the vitamin A compounds were bound inside the cellular membrane, the influence of the protein lattice would protect them from extraction by a non-polar solvent such as *n*-hexane.

When the cellular membrane was extracted with *n*-hexane followed by chloroform–methanol (2:1, v/v), 89.4 % of the total radioactive vitamin A was released from the membrane. The vitamin A in this extract could be bound to the membrane through complex polar and/or non-polar interactions.

After chloroform–methanol (2:1, v/v) extraction of the membrane, the protein residue contained 2.5 % of the total radioactivity of the membrane. If vitamin A (retinal) compounds were bound to membrane protein or lipid by a Schiff base linkage, as reported for the binding of retinal in opsin (Bownds[30]; Kimble *et al.*[31]), they would not be extracted with chloroform–methanol (2:1, v/v).

Thin-layer chromatography revealed that the major radioactive vitamin A compounds were retinol and retinoic acid.

Large amounts of radioactivity were found close to the origin of the thin-layer chromatogram; the chemical nature of this substance is not known. Preliminary studies showed that the methanolic solution of the substance had absorption maxima at 396 and 276 nm. The possibility that artifacts were produced during the isolation,

extraction and chromatography processes cannot be eliminated. At present the chemical nature of the unidentified radioactive compounds obtained in these experiments is under investigation.

Combined column and thin-layer chromatography indicated that the forms of vitamin A associated with endoplasmic reticulum were retinol and retinoic acid.

The *in vitro* experiments also showed that all the vitamin A compounds could bind to cellular membranes by direct mixing. This indicates that the same mechanism could occur *in vivo*. The *in vivo* contact of vitamin A compounds with the membrane network in the cytoplasm would cause instant binding of vitamin A to membrane. This binding, in turn, could regulate stability and function of the membrane. Our experiment showed that the affinity of vitamin A for membrane is very strong. The level of vitamin A in membrane remained rather constant even when the total homogenate contained very little vitamin A. All these results suggest that membranes contain constant levels of vitamin A although the membrane is obviously not the storage place for the excess vitamin A in the tissue.

In summary, vitamin A was found associated with rat liver and kidney plasma membranes, rat liver and kidney endoplasmic reticulum, and rat erythrocyte ghosts. The major forms of vitamin A associated with endoplasmic reticulum are retinol and retinoic acid. Trace amounts of retinyl palmitate and retinal were found in the membrane fraction. The majority of the radioactive vitamin A compounds could be extracted with chloroform–methanol (2:1, v/v), thus indicating strong binding of vitamin A to the membrane.

Anderson *et al.*[32] found that the presence of vitamin A in lipid monolayers and bilayers increases the thickness of the hydrophilic layer of the membrane. The presence of vitamin A in cellular membranes could alter the surface charge of the membrane and change the conformation of the membrane-bound enzymes (such as ATPase). This could, in turn, affect the transport of nutrients, ions and water through the membrane and thus influence general cellular metabolism.

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