THE EFFECT OF DIETARY VITAMIN A ON THE MORPHOLOGY AND A MAJOR MEMBRANE GLYCOPROTEIN OF THE 13762 RAT MAMMARY ADENOCARCINOMA ASCITES CELLS

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NOMENCLATURE

ASGP-1	-	ascites sialoglycoprotein-1
dpm	-	disintegrations per minute
fuc	-	fucose
galNacOH	-	N-acetylgalactosaminitol
gal	-	galactose
gln	-	glucosamine
glcNac	-	N-acetylglucosamine
g	-	acceleration due to gravity
Gdn HCl	-	guanidine hydrochloride
MAT	-	mammary ascites tumor
NANA	-	N-acetylneuraminic acid
SA	-	sialic acid
SDS	-	sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

The essential role of vitamin A in visual processes has been known for decades and is well understood (1). It is also important in some undefined manner, for epithelial differentiation, maintenance of reproductive capacity, and for the growth and general health of the normal animal (2-8). The parent substance, all-trans-retinol (vitamin A alcohol), can be chemically modified in many ways (9, 10) to give a large number of analogs collectively called retinoids (11). Some of these analogs showed less toxicity and better target organ specificity than retinol, while others were inactive (9, 11, 12).

The term "vitamin A" is used to refer to those analogs which can restore normal growth in animals maintained on a vitamin A-deficient diet (7, 11). These include its naturally occurring oxidation products, all-trans-retinal (vitamin A aldehyde) and all-trans-retinoic acid (vitamin A acid), and a storage form, all-trans-retinyl palmitate.

Retinoids and Neoplasia

The possible relationship between vitamin A and cancer has attracted the attention of researchers since 1926, when Fujimaki (13) observed the development of stomach carcinomas in rats fed a vitamin A-deficient diet. It was also found that vitamin A deficiency leads

to metaplastic changes in the epithelial tissues of the gastrointestinal, respiratory and urogenital tracts (2, 14-16). These changes may be considered to be among the first steps in the transformation from normal to neoplastic cells. Further support for the idea of a relationship between vitamin A and cancer came from studies which demonstrated that vitamin A deficiency enhanced the susceptibility of many epithelial tissues to carcinogenesis (17). In addition, several retinoids were shown to possess anti-tumor and/or anti-carcinogenic activities both in vivo and in vitro (6, 9, 11, 17).

In a study (18) using epidermal cells cultured on collagen, it was found that vitamin A favored cells' spreading, accelerated confluence, and reduced the size of the multilayered cell colonies. The mode of shedding of cells into the medium was also altered. The effects of vitamin A on the markers of differentiation were heterogeneous as evidenced by its inhibition of keratinization in every case, and its induction of pre-keratinization, particularly at low concentrations.

Felix et al. (19) showed that both the incidence and the growth of S91 melanoma in allogeneic mice was inhibited upon oral or intraperitoneal administration of retinyl palmitate two weeks before and after subcutaneous tumor inoculation. In another study, Rettura et al. (20) used a syngeneic system in which the C3HBA mammary adenocarcinoma was inoculated subcutaneously in the nipple area of C3H/Hej female mice. They found that feeding retinyl palmitate (beginning on the day of tumor transplantation) slowed the tumor growth markedly. The treatment prolonged the survival of the animals, but did not decrease tumor incidence. Heilman and Swarm (21) found

that 13-cis-retinoic acid prevented the development of tumor transplants, and arrested tumor growth in rats bearing established tumors. Inhibition of tumor growth, and tumor regression by the trimethylmethoxyphenyl analogs of retinoic acid were also noted by Trown et al. (22). In other studies retinoic acid caused a regression of up to 51.4% of chemically induced papillomas (23). Chemically induced skin carcinomas also regressed to some degree (24, 25). Studies by Edwards et al. (26) on the differentiation of a teratocarcinoma cell line suggested that the routes of differentiation in embryogenesis may be determined, in part, by concentration gradients of retinoids. At low concentrations (10^{-9}M) of retinoic acid, the in vitro growth of S91 and B16 cells was inhibited by 55 and 30%, respectively (27). Lotan and Nicolson (28) studied the inhibitory effects of retinoic acid or retinyl acetate on the growth of untransformed, transformed, and tumor cells in vitro. The growth of all mammary tumor cell lines tested was inhibited. Human breast carcinoma $H_5$0578 was the least sensitive, whereas the growth of the rat mammary tumor cell line 13762 NF was dramatically inhibited.

Vitamin A was also shown to inhibit the growth and development of some virally induced benign and malignant tumors (29-31). Retinyl palmitate, in non-toxic amounts, inhibited the growth and enhanced the regression of both pox lesions and Murine Sarcoma Virus-induced established tumors (30, 31). Atrophy and inhibition of growth of papillomas was achieved by administration of toxic doses of either retinol or retinyl palmitate to rabbits inoculated with Shope rabbit papilloma virus (29).

Mode of Action of Vitamin A

Several hypotheses have been proposed to explain the effects of vitamin A and/or its analogs on cells, and as anti-tumor and anti-carcinogenic agents: (1) The effects of retinoids on cell proliferation and differentiation are similar to the actions of several hormones. Steroid hormones are thought to affect gene expression by affecting the amounts of specific cytoplasmic mRNAs and/or ribosomal and transfer RNAs. They were also shown to induce alterations in nuclear enzymes, chromatin, and the synthesis of proteins (32). Several of these effects have been observed in cells or tissues treated with vitamin A. The similarities between the actions of retinoids and steroid hormones suggested comparable mechanisms of action. This suggestion was supported by the finding of specific intracellular retinoid binding proteins, reminiscent of steroid binding proteins, in many vitamin A affected tissues (33, 34). Thus, it was proposed that following entry of the retinoid into target cells, a complex is formed between it and its binding protein. The complex can then be translocated to the cell nucleus and can modulate gene expression (33, 34).

(2) Another possible mechanism of action of retinoids is through an effect on glycolipid and glycoprotein synthesis. De Luca (6) suggested that phosphorylated derivatives of retinoids may be involved in the glycosylation of membrane glycoproteins by acting as carriers of monosaccharides across the hydrophobic lipid bilayers. This may cause alterations in the glycosylation patterns of certain glycoproteins or glycolipids which may, in turn, affect the genome or alter

the cellular functions by epigenetic mechanisms (35-37).

(3) Stimulation of humoral and/or cellular immunity was another postulated mechanism for the action of retinoids. Although retinol is in itself non-antigenic, it was shown to induce, in mice, the formation of antibodies to bovine gamma globulin by acting as an adjuvant (38). The same effect was seen in rats following administration of retinol, retinal or retinoic acid (39), while retinyl acetate and retinyl palmitate were less effective. Retinyl palmitate induced a several fold stimulation of antibody production against other soluble proteins such as bovine serum albumin (40, 41). These studies demonstrated an enhancement of humoral responses to antigens by vitamin A. Parallel to these findings, it was also found that retinyl palmitate caused a significant reduction in rejection time if administered before or soon after grafting C57BL/6 mice with skin from isologous mice (42). Retinyl palmitate and retinoic acid also shortened the survival time of CBA/H mouse homograft in CBH mice by one half (43). These latter studies illustrated the activity of vitamin A as a stimulator of cell-mediated immune responses.

13762 Rat Mammary Adenocarcinoma

In the present study, the 13762 rat mammary adenocarcinoma was used as a model to determine the effects of vitamin A and related retinoids on tumors because its glycoproteins, cell surface and some enzymes have been well characterized (44-55). Moreover, its growth is known to be inhibited by retinoids (28).

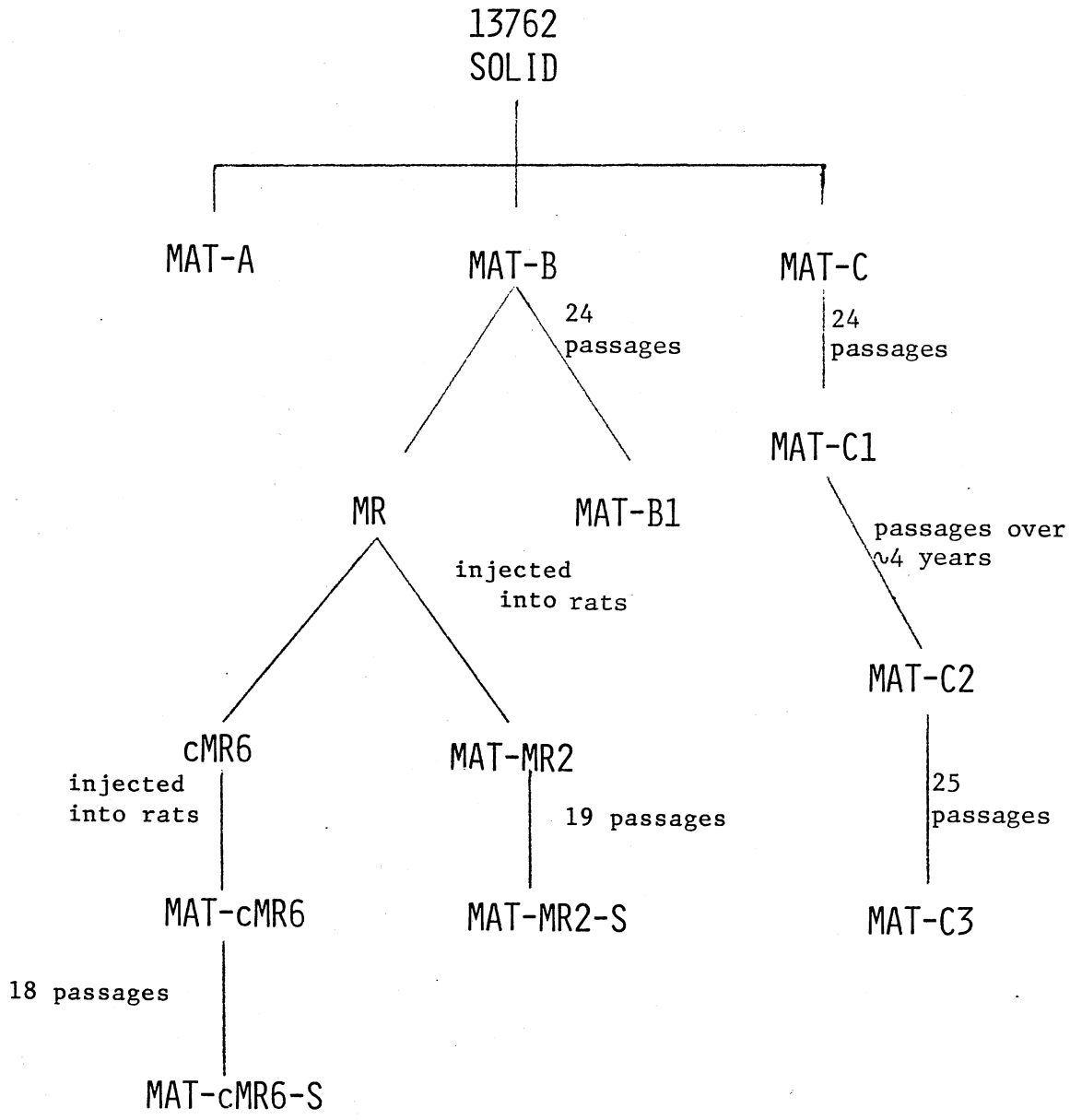
The 13762 rat mammary adenocarcinoma is a dimethylbenzanthrene-induced solid tumor (56). It was adapted for ascitic growth and

three morphologically distinct ascites sublines, MAT-A, MAT-B, and MAT-C were derived (57). After passage in vivo in the laboratory of K. L. Carraway (45), two additional sublines, MAT-B1 and MAT-C1, were obtained from MAT-B and MAT-C, respectively (Figure 1). MAT-A, MAT-B, and MAT-B1 cells could not be readily distinguished morphologically. They had unbranched microvilli, ruffles, ridges and blebs associated with the cell surface. The MAT-C1 cells had a highly irregular surface and extensively branched microvilli with few ridges, ruffles and blebs (45). The MAT-C subline had an intermediate morphology. Most of the cells had numerous short, straight unbranched microvilli and few ruffles, ridges or blebs. About 10% of the population had branched microvilli (45). Two of the five sublines (MAT-C and MAT-C1) could be transplanted in ascitic form, into mice. The MAT-A, MAT-B, and MAT-B1 sublines were non-xenotransplantable (47).

The MAT-B1 and MAT-C1 sublines have been extensively studied in the laboratory of Dr. K. L. Carraway. Both sublines have large amounts of a major high molecular weight cell surface sialoglycoprotein (ASGP-1) identified by chemical, enzymatic and metabolic labeling (47, 49-51). The two sublines differed markedly in concanavalin A receptor redistributions, and agglutinability by concanavalin A (45, 52). The two sublines also differed with respect to membrane sialoglycoprotein and sialic acid content, and the oligosaccharide composition and sulfation of ASGP-1 (47-51). The physical properties of the MAT-B1 and MAT-C1 ASGP-1 were very similar. The amino acid compositions of both were essentially the same (46).

In addition to the previously described 13762 rat mammary adenocarcinoma ascites sublines, new lines were obtained (Figure 1). New

Figure 1. Lineage of the 13762 Mammary Adenocarcinoma Sublines.



MAT - ASCITES
MR - CULTURED
c - CLONED
S - SIALOGLYCOPROTEIN

sublines were derived from a variant (MR) of MAT-B adapted to cell culture by Dr. Erling Jensen, Mason Research Institute. Uncloned MR cells were injected into rats to give the MAT-MR2 ascites sublines. These latter cells were passaged until ASGP-1 appeared, after about 19 passages, to give the MAT-MR2-S (51) subline. In addition, one of the clones of MR cells (50), named cMR6, was injected into rats to give an ascites tumor line (MAT-cMR6). This was passaged for about 18 passages before ASGP-1 appeared abruptly to give the MAT-cMR6-S (50). The MR cells (cloned or uncloned) had fewer prominent microvilli than the ascites cells derived from them (54). However, the most dramatic morphological difference occurred at the stage of appearance of ASGP-1. The MAT-cMR6-S cells had branched microvilli, while the MAT-MR2-S did not (54). Both lines had limited receptor mobility upon treatment with fluorescent concanavalin A. The MAT-cMR6-S subline was xenotransplantable into mice whereas the MAT-MR2-S was not. The oligosaccharide composition of ASGP-1 from the MAT-cMR6-S (54) was more similar to that of the MAT-B1 ASGP-1 than to that of the MAT-C1 ASGP-1 (54). The MAT-MR2-S ASGP-1 oligosaccharide pattern was simpler than any studied previously (54).

Additional new sublines were obtained from the MAT-C1 subline. The MAT-C1 cells were passaged for more than four years during which one instance of spontaneous change was noted. The new variant was named MAT-C2. Concomitant with the change to the MAT-C2 form, a bacterial infection was discovered in the animal colony used for passage. About 25 passages of the MAT-C2 cells in healthy animals gave rise to what was named MAT-C3 cells (54). The MAT-C2 cells had

a predominant morphology similar to that of the MAT-MR2-S, not exhibiting the extensively branched microvilli of the MAT-C1 cells (47). The MAT-C2 oligosaccharide pattern also resembled that of the MAT-MR2-S with a slight shift, and the cells were not xenotransplantable (54). The MAT-C3 cells were not xenotransplantable and few of them had branched microvilli. The oligosaccharide pattern, on Bio-Gel P-4, of their ASGP-1 reverted back to one resembling that of MAT-C1 cells (54).

The present study was designed to evaluate the therapeutic potential of all-trans-retinoic acid against the 13762 rat mammary adenocarcinoma sublines. How retinoids alter the cells was the critical question. In an attempt to understand the mechanism of action, several parameters were measured: 1) tumor growth, xenotransplantability and survival of the tumor bearing animals; 2) cell morphology; 3) the density, molecular weight and oligosaccharide pattern of the major membrane glycoprotein (ASGP-1). The first two parameters are expressions of tumor behavior. The third is an expression of the effects of vitamin A on glycoprotein production.

Any correlation between the effects of retinoids on tumor growth and the effects on these various cell parameters would be particularly significant.

CHAPTER II

THE EFFECT OF VITAMIN A ON THE MAJOR CELL SURFACE SIALOGLYCOPROTEIN OF THE 13762 RAT MAMMARY ADENOCARCINOMA

Introduction

Glycoproteins play important roles in a number of cell surface phenomena (58, 59) including the escape of malignant tumor cells from immune surveillance by the host (60). One postulated mechanism is that the shedding of glycoproteins and cell surface antigens from the tumor may block the destruction of the tumor cells by the immune system (61-63). Another mechanism by which glycoproteins protect the tumor from immune surveillance is by masking the cell surface antigens (64). For example, two sublines of the mouse TA3 mammary adenocarcinoma have been studied and found to differ in morphology, agglutinability by concanavalin A, and transplantability across strain or species histocompatibility barriers (65). The allo- and xenotransplantable TA3-Ha ascites cells had large amounts of epiglycanin, a major sialoglycoprotein. The strain specific TA3-St ascites cells had little or no epiglycanin (66, 67). It was postulated that epiglycanin protects the tumor cells either by "masking" the histocompatibility antigen (64, 65) or by being released from the cell surface (68) to block immune destruction of the tumor (61, 69).

The 13762 tumor sublines used in this study have large amounts of a major cell surface sialoglycoprotein (ASGP-1) which has been characterized by chemical enzymatic and metabolic labeling (44, 46, 49-51, 54). ASGP-1 contains > 70% of the [^{3}H]glucosamine label incorporated into trichloroacetic acid-precipitable radioactivity in both MAT-B1 and MAT-C1 cells. The abundance (43, 44, 55) and organization (44) of ASGP-1 at the cell surface suggest that it plays a role in cell surface properties which are important to tumor survival.

The effects of retinoids on the biosynthesis of carbohydrate-containing macromolecules are well documented. Studies revealed that the synthesis of specific glycoproteins in the epithelial cells of intestine, trachea and cornea decreased in vitamin A deficient animals (6, 70, 71). Administration of vitamin A to depleted animals stimulated the synthesis. In a study using pre-chondrogenic mouse embryo limb bud, it was shown that retinoic acid inhibited the in vitro differentiation of mesenchymal cells to chondrocytes which form cartiloginous nodules (72). The control mesenchymal cells differentiated and synthesized cartilage proteoglycan. Those cultures exposed to retinoic acid continued to produce proteoglycans characteristic of the prechondrogenic mesenchyme (72). In addition, the expression of two cell-surface proteins was also affected (73-75). Plotkin et al. (76) showed that vitamin A deficiency greatly depressed galactosyl transferase activity in microsomes of rat tracheal epithelium. Galactosyl transferase activity was restored by adding retinol to the microsomes or by dosing the deficient animals with retinyl acetate.

De Pavia et al. (74) studied the effect of 1 to 5 doses of 15,000 I.U. of vitamin A on N-acetyl neuraminic acid (NANA) concentrations in

gingival tissues from rats. An increase in sialic acid concentration was observed in the animals that received up to 4 doses of vitamin A, while a decrease in concentration was observed in the group given 5 doses. De Luca et al. (78) demonstrated the presence of retinyl phosphate in mammalian liver, and that vitamin A deficiency decreased the pool of retinyl phosphate. Creek et al. (78) found that rat liver endoplasmic reticulum membranes catalyzed significant transfer of mannose to endogenous acceptor proteins in the presence of exogenous retinyl phosphate. This suggested the presence of GDP-mannose:retinyl phosphate mannosyl-transfer activity acting on glycoproteins at the postprocessing level. In another study, Comley et al. (80) demonstrated the synthesis of retinyl phosphate mannose from GDP-mannose and exogenous retinyl palmitate in the B. pahani adult filarial nematode microsomes, and suggested that retinyl phosphate may function as an intermediate carrier in filarial glycoprotein synthesis. Sasak et al. (81) showed an increase in the synthesis of higher molecular weight glycopeptides (MW 2100) and a corresponding decrease in the lower molecular weight fraction (MW 1500) of Balb/c 3T12-3 cell surface glycoprotein, upon retinoid treatment. Ferrari et al. (82) studied the effect of retinol on macromolecular synthesis in the SKMel 28 and HeLa cultured cell lines. Vitamin A caused a 60% inhibition of DNA synthesis when added at 50 μM concentration. This inhibition was reversible. In addition, the synthesis of cytoplasmic proteins, acid-soluble nuclear proteins and of non-histone chromosomal proteins was also inhibited by the vitamin.

In studies on the use of retinoids in glycoprotein production, Chou (83) showed that retinoic acid induced the synthesis of low levels

of immunoreactive pregnancy-specific β1-glycoprotein (PSβG). Synergistic induction of PSβG synthesis was achieved when retinoic acid was used simultaneously with either 8-bromo-cAMP, cholera toxin, methyl isobutylxanthine, and 5-bromo-2'-deoxyuridine. Clark et al. (84) showed that retinyl acetate increased the uptake of [^{3}H]glucosamine and [^{14}C]serine into secreted mucins, in a concentration dependent manner. The studies also indicated that vitamin A has a greater effect on the synthesis of the carbohydrate moiety of the mucins, than on the protein moiety.

Another type of retinoid-induced changes in surface glycoproteins has been reported. Borek et al. (85) observed that short exposure of rat liver cell cultures to retinol increased their agglutinability with plant lectins. In addition, retinol caused an increase in the proteolytic enzymes in the extracellular medium, and such enzymes are known to enhance cell agglutination. In another study (86) retinol treatment decreased the thickness of the surface Ruthenium red-staining material. The binding of positively charged colloidal iron particles was enhanced while negatively-charged colloidal particle binding was reduced, in L1210 Leukemia cells in vitro. These observations were similar to those on cell surfaces after neuraminidase treatment. It was thus suggested that retinol may cause the release of lysosomal neuraminidase. With human lymphocytes and mouse Landschutz ascites tumor cells, direct measurement of released and cell-bound sialic acid showed that in retinol-treated cells, sialic acid was released by cleavage of terminal residues, rather than by proteolytic cleavage of sialoglycopeptides (87).

The previous examples and many others are demonstrations of the vitamin A-mediated modifications of cellular glycoproteins, glycolipids, glycosaminoglycans, proteoglycans, and their biosynthesis, in vivo and in vitro. Two types of changes in cell surface glycoconjugates have been described: An effect due to the release of lysosomal hydrolases (85-87), and a more specific effect, presumably a result of modulations in the glycosylation patterns of discrete glycoconjugates (6, 70-84). Retinoids could affect the rate of glycosylation, and possibly the type and amount of sugar moieties that comprise the carbohydrate portion of a glycoprotein. As discussed previously, glycoproteins were implicated in several membrane and cell surface properties. The changes in the glycosylation of certain glycoproteins may affect the genome, and the cellular functions and differentiation by epigenetic mechanisms (35). For example, the expression of some genomes may be altered at or beyond the trascriptional level. In addition, changes in glycoprotein or other glycoconjugate production may alter membrane or cell surface properties, thus altering, for example, intercellular recognition, adhesion and aggregation processes which are involved in differentiation (36, 37). The alterations in the membrane glycoproteins themselves, or in other cellular functions and cell surface properties could affect the survival of tumor cells. Therefore, the objective of this research was to determine the effects of vitamin A on the 13762 rat mammary adenocarcinoma cells and their major cell surface glycoprotein (ASGP-1). This glycoprotein has been implicated in roles essential for the tumor survival. The results of our studies on ASGP-1 are presented in some detail later in

this chapter.

Materials and Methods

Materials

D-[1-^{14}C]Glucosamine (50 to 60 mCi/mmol), D-[1-^{3}H]glucosamine (2 to 6 Ci/mmol) and D[-1-^{3}H]galactose (9-11 Ci/mmol) were obtained from Amersham; Instagel was from Packard; guanidine hydrochloride (grade 1) was from Sigma; CsCl (99.99%) was purchased from Varlacoid. All-trans-retinoic acid was a gift from Hoffmann-La Roche, Inc., and was in the form of gelatinized beadlets containing 10.3% retinoic acid by weight.

Vitamin A deficient purified diet (Table I) was prepared for us by and purchased from Teklad (Madison, WI). Wayne certified Lab-Blox, a non-purified closed formula diet was purchased from Wayne Feed Division, Continental Grain Company (Chicago, IL).

F-344 female rats, 28 days old, were obtained from Charles River Breeding Laboratories (Wilmington, MA).

Tumor Sublines and Cells. Initially, a preliminary study was attempted on the effects of "vitamin A" on the MAT-C1 and the MAT-B1 cells, since these sublines have been thoroughly studied, more than any others, in the laboratory of Dr. K. Carraway. However, the maintenance of the MAT-C1 tumor line could not be achieved in our laboratory, as initially evidenced by electron microscopic studies on cell morphologies. Studies on the ASGP-1 oligosaccharide pattern indicated that they have a pattern resembling that shown for MAT-C1 and MAT-C3 cells (46, 54). The morphology mainly resembled that of

TABLE I

VITAMIN A DEFICIENT PURIFIED DIET FOR RATS

Ingredient	g/Kg
Casein, "Vitamin-Free" Test	193.0
DL-Methionine	3.0
Corn Starch	665.1343
Cottonseed Oil	50.0
Fiber (Cellulose)	50.0
Mineral Mix, AIN-76[1]	35.0
Biotin	0.0004
Vitamin B_{12} (0.1% Trituration in Mannitol	0.0297
Calcium Pantothenate	0.0661
Choline Dihydrogen Citrate	3.4969
Folic Acid	0.002
Menadione Sodium Bisulfite Complex	0.05
Niacin	0.0991
Pyridoxine HCl	0.022
Riboflavin	0.022
Thiamin HCl	0.022
DL-Alpha Tocopheryl Acetate (100 U/g)	0.05
Vitamin D in Corn Oil (400,000 U/g)	0.0055

[1]AIN-76. J. Nutrition 107, 1340-1348, 1977.

the MAT-C cells, with short stubby unbranched microvilli. It was decided that the cells were of the MAT-C3 line. Both before and while the preliminary studies were carried out, the two laboratories exchanged tumor bearing rats. It was during that time that the changes from MAT-C1 to MAT-C2 to MAT-C3 were observed in the laboratory of Dr. K. Carraway (54). In the preliminary studies, the morphology and oligosaccharide pattern of the MAT-B1 cell line were as reported previously (47).

In another attempt to study the effects of "vitamin A" on the MAT-B1 and MAT-C1 sublines, tumor bearing rats were obtained from Dr. K. Carraway. Again, the attempts to maintain these close to homogeneous lines in our laboratory failed. Based on the morphological and biochemical studies it was decided that we had a heterogeneous MAT-B population termed "MAT-BH" and a heterogeneous MAT-C population termed "MAT-CH". In the present study, the MAT-B1, MAT-C3, MAT-BH and MAT-CH lines were used. These sublines were maintained by routine weekly passage as described previously (47). After recovery from the peritoneal cavity, the cells were washed three times with cold Dulbecco's phosphate buffered saline without calcium or magnesium, by centrifugation at 100 x g in an SS-34 rotor for 3 min.

Methods

Cell Labeling. Metabolic labeling with [^{3}H]glucosamine (70-100 μCi) or [^{14}C]glucosamine (30 μCi) was accomplished by injection of the compound in 0.2 ml of 0.9% NaCl into the peritoneal cavity of a tumor-bearing rat, approximately 16 h prior to killing the animals

and recovery of the cells.

Measurement of Radioactivity. Aliquots of gradient or column fractions were mixed with 4.5 ml of scintillation fluid and counted in a model PL-1 Prias liquid scintillation counter (Packard). The ^{3}H- and ^{14}C-counts were corrected for efficiency and overlap using the external standard channels ratio when both isotopes were used.

Membrane Preparation. Membrane vesicles were obtained as described by Sherblom et al. (46) and Huggins et al. (44): The washed cells were suspended in 10 volumes of 10 mM Tris, pH 8.0 and after 2 minutes on ice were centrifuged at 600 x g in a Sorvall SS-34 rotor for 2 min. The swollen cell pellet was suspended in 10 volumes of 10 mM Tris, pH 8.0 and homogenized by 5 to 6 strokes of a Dounce homogenizer with a tight pestle. Breaking of the cells was monitored by phase contrast microscopy and was stopped when 70-80% of the cells were broken and the nuclei appeared distended. To stabilize the nuclei, 0.1 volume of 100 mM NaCl, 30 mM MgCl was added. Centrifugation of the homogenate at 1000 x g for 1 min pelleted the nuclei which were discarded. The resulting supernatant solution was centrifuged at 10,000 x g for 10 min (SS-34 rotor). The supernatant solution from this step was then, in turn, centrifuged at 100,000 x g for 105 min (Beckman SW 27 or SW 28 rotor). The resulting pellet (crude membrane fraction) was suspended in 4 M guanidine HCl using a small Dounce homogenizer.

Purification and Density Determination of ASGP-1. Aliquots of crude membrane fractions from the cells were mixed with those from

their corresponding controls in different cell combinations. In some cases, groups of cells from one or more supplemented rats were mixed with cells from each of two different control rats, separately. In other cases, the cells from one control rat were mixed with each of two different cell groups from separate supplemented rats. The type of combination used depended on the availability of sample. Each mixture was layered onto preformed CsCl gradients (46) (density, 1.3 to 1.5 g/ml, in 4 M guanidine HCl) which were centrifuged in a Beckman Ti 75 rotor at 4°C, 270,000 x g for 22-24 h. Fractions were collected from the bottom of the gradients. Densities of selected fractions from the CsCl gradients were determined by the difference in dry and filled weight of a 50 µl pipet calibrated with distilled water. Fractions representing the major radioactive peak were pooled as ASGP-1. CsCl and Gdn HCl were removed from these fractions by dialysis/concentration into distilled water using a collodion bag apparatus (Schleicher and Schuell). The dialysate concentrate contained ASGP-1 and was dissolved in approximately 1.0 ml of distilled water.

Gel Filtration with 1% SDS. An aliquot of experimental and control ASGP-1 mixture, which had been dialyzed against distilled water, was solubilized with 1% SDS, 50 mM Tris, pH 8.0 and applied to a Sepharose CL-2B column (1 x 110 cm) equilibrated and eluted with the same buffer. A fraction collector was used to collect the 80 drop fractions. Aliquots (100 µl) were analyzed for radioactivity.

Alkaline Borohydride Treatment. An aliquot of the ASGP-1 samples

that had been purified on CsCl gradients, dialyzed and concentrated, was incubated at 45°C with 1.0 M $NaBH_4$, 0.05 M NaOH for 16 h and then neutralized with glacial acetic acid. The treated sample was then applied to a Bio-Gel P-4 (200-400 mesh) column (1 x 110 cm) equilibrated and eluted with 0.1 M pyridine acetate, pH 6.0. The 25 drop fractions were collected using a fraction collector. Aliquots (100 µl) of the eluting fractions were analyzed for radioactivity. The column was calibrated with dextran and galactose.

Statistical Analysis. The Wald-Wolfowitz two-sample runs test was used to detect significant peak shifts at the 0.05 significance level ($P < .05$). Data were normalized on a percentage basis before the test was applied.

Experimental Design

MAT-B1 and MAT-C3 Sublines. Upon arrival, the female rats were processed through routine quarantine procedures for 10 days. They were fed the non-purified diet until the animals were required for experimentation. Each experimental period started by dividing the rats, which belonged to the same age group, into a supplemented and a control group. In the case of the MAT-B1 subline, four experiments (I, II, III and IV) were performed over four different time periods. In each experiment, there was a supplemented group and a respective control group, each group consisting of 3 to 5 rats. In experiment I, the rats of the supplemented group (group I) were fed the vitamin A-deficient purified diet supplemented with 12 mg of retinoic acid/kg diet. In experiments II, III and IV, the corresponding supplemented

groups II, III and IV received 60, 90 and 120 mg retinoic acid/kg diet, respectively. As mentioned earlier, each experiment had its own control group, and all the control groups were continued on the closed formula non-purified diet. The same was the case for the MAT-C3 subline. However, only three experiments were performed. In experiment I, group I was fed the vitamin A-deficient purified diet supplemented with 12 mg retinoic acid/kg diet. For experiments II and III, groups II and III were fed 60 and 120 mg retinoic acid/kg diet. The controls from all the experiments were fed the non-purified diet (Table II).

The control and the retinoic acid supplemented animals were kept on their respective diets for three weeks. However, at the beginning of the third week, the animals (60-90 days old) received intraperitoneal injections of either MAT-B1 ($2.8\text{-}3 \times 10^6$) or MAT-C3 ($1.2\text{-}1.4 \times 10^6$) tumor cells. At the end of the third week, the animals were killed and the tumor cells were recovered from the peritoneal cavity.

MAT-BH and MAT-CH Sublines. For each of the sublines used in this experiment, the animals, which belonged to the same age group, were divided into a control group and three different supplemented groups, I, II, and III. Each group consisted of 4 to 6 rats. Group I received approximately 4.5 mg retinyl palmitate/kg purified, vitamin A deficient diet. Groups II and III were fed the vitamin A-deficient purified diet supplemented with 60 and 120 mg retinoic acid/kg diet, respectively (Table III). Other experimental conditions were the same as for the MAT-B1 and MAT-C3 sublines. The 4.5 mg retinyl palmitate/kg purified diet is considered an adequate dietary vitamin A supplement. Also, the supplemented rats were not vitamin

TABLE II

THE MAT-B1 AND MAT-C3 DIET GROUPS

Subline	Group	Diet and Supplement
MAT-B1	Controls (C)	Non-purified Diet[1]
	I	12 mg RA[2]/kg Purified Diet
	II	60 mg RA/kg Purified Diet
	III	90 mg RA/kg Purified Diet
	IV	120 mg RA/kg Purified Diet
MAT-C3	Controls (C)	Non-purified Diet
	I	12 mg RA/kg Purified Diet
	II	60 mg RA/kg Purified Diet
	III	120 mg RA/kg Purified Diet

[1] 2 mg carotene, 4.5 mg retinyl palmitate/kg diet.

[2] All-*trans*-retinoic acid.

TABLE III

THE MAT-BH AND MAT-CH DIET GROUPS

Tumor Line	Group	Diet and Supplement
MAT-BH	Controls (C)	Non-purified Diet[1]
	I	4.5 mg RP[2]/kg Purified Diet
	II	60 mg RA[3]/kg Purified Diet
	III	120 mg RA/kg Purified Diet
MAT-CH	Controls (C)	
	I	4.5 mg RP/kg Purified Diet
	II	60 mg RA/kg Purified Diet
	III	120 mg RA/kg Purified Diet

[1] 2 mg carotene, 4.5 mg retinyl palmitate/kg diet.

[2] All-trans-retinyl palmitate (Rovimix A-325, Hoffmann-La Roche-Nutley, New Jersey).

[3] All-trans-retinoic acid.

A deficient. They had a normal retinol status, but were supplemented with pharmacological doses of all-trans-retinoic acid, or with adequate doses of retinyl palmitate. The 60 mg retinoic acid/kg diet is equivalent to 23 x vitamin A levels in vitamin A sufficient diets. The 120 mg retinoic acid/kg diet is equivalent to 46 x vitamin A levels in vitamin A sufficient diets.

Results

Density Gradient Centrifugation

Control ASGP-1 was copurified with ASGP-1 from each of the experimental groups, using preformed CsCl gradients in 4 M Gdn HCl as explained in the methods. Inspection of the density gradient profiles for MAT-B1 membrane vesicles revealed no apparent density differences between glycoprotein from group I, III (Figure 2), or IV and that from control. ASGP-1 from group II seemed less dense or slightly less dense than control ASGP-1. The density profiles for the MAT-C3 revealed no density differences between group II (Figure 3) or group III glycoprotein and control MAT-C3 ASGP-1. However, group I ASGP-1 appeared to be less dense than control ASGP-1 (Figure 4).

Density gradient profiles for membrane vesicles from MAT-BH and MAT-CH cells indicated that MAT-BH ASGP-1 from group I cells had a higher density than control MAT-BH ASGP-1 (Figure 5). ASGP-1 from group II MAT-BH cells was as dense or slightly less dense than control ASGP-1. ASGP-1 from group III cells was as dense as control MAT-BH ASGP-1 (Figure 6).

In the case of the MAT-CH line, the results varied from one

Figure 2. Density Gradient Centrifugation of MAT-B1 Membrane Vesicles from Control and Group III in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group III (90 mg retinoic acid/kg diet) MAT-B1 cells (△) mixed with membrane vesicles from [^{14}C]-glucosamine-labeled control MAT-B1 cells (■). Density curves (✱).

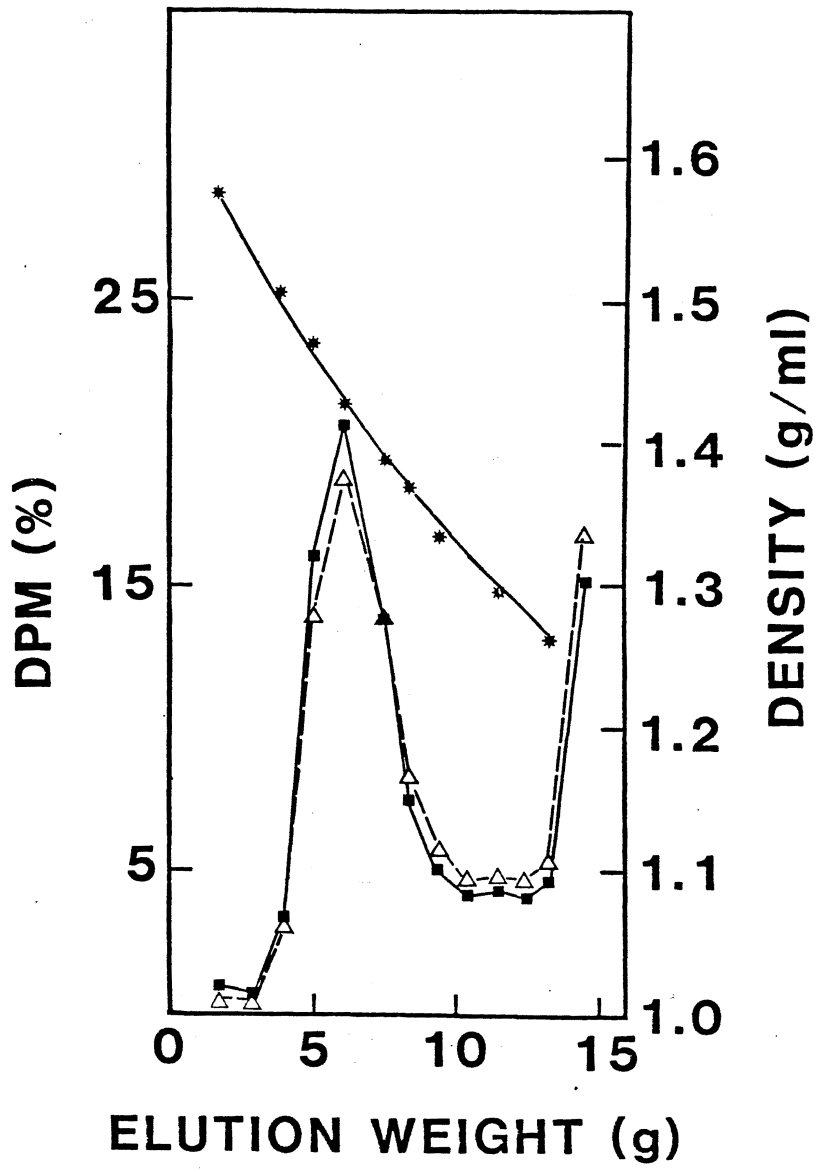

DPM (%)
25
15
5
0
5
10
15
ELUTION WEIGHT (g)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DENSITY (g/ml)

Figure 3. Density Gradient Centrifugation of MAT-C3 Membrane Vesicles from Control and Group II in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group II (60 mg retinoic acid/kg diet) MAT-C3 cells (△) mixed with [^{14}C]glucosamine-labeled control MAT-C3 cells (■). Density curve (✱).

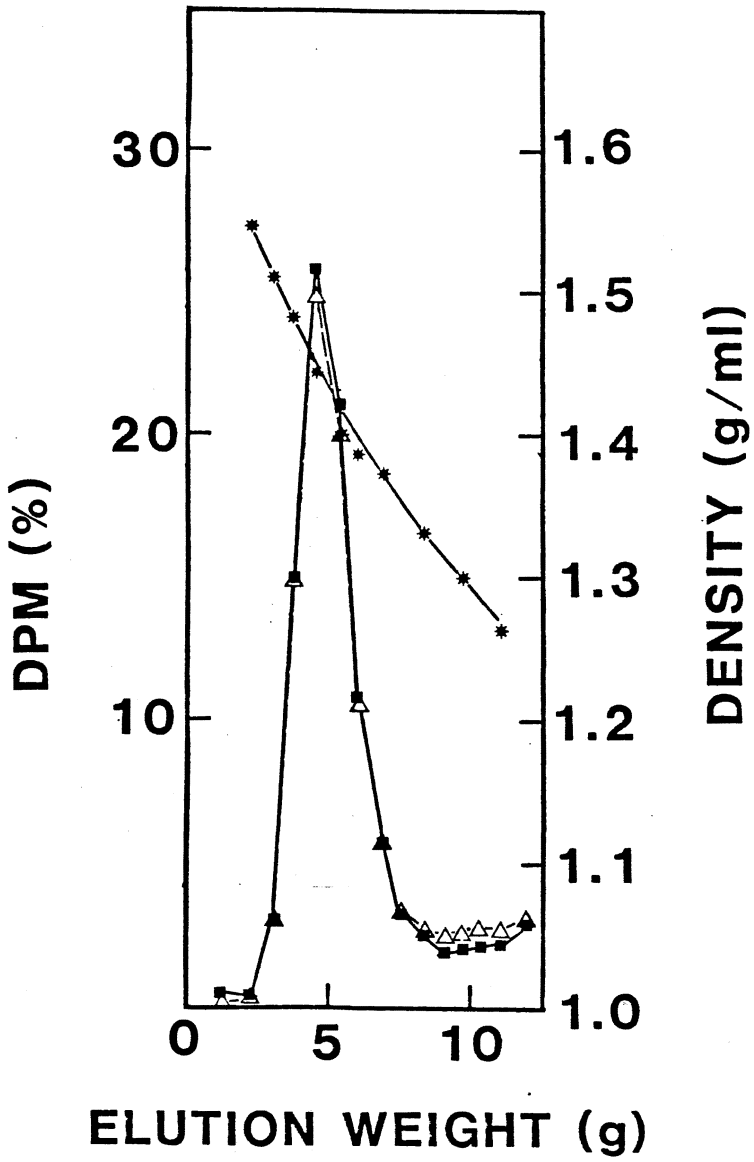

30
20
10
0
5
10
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DPM (%)
DENSITY (g/ml)
ELUTION WEIGHT (g)

Figure 4. Density Gradient Centrifugation of MAT-C3 Membrane Vesicles from Control and Group I in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group I (12 mg retinoic acid/kg diet) MAT-C3 cells (△) mixed with membrane vesicles from [^{14}C]-glucosamine-labeled control MAT-C3 cells (■). Density curve (✱).

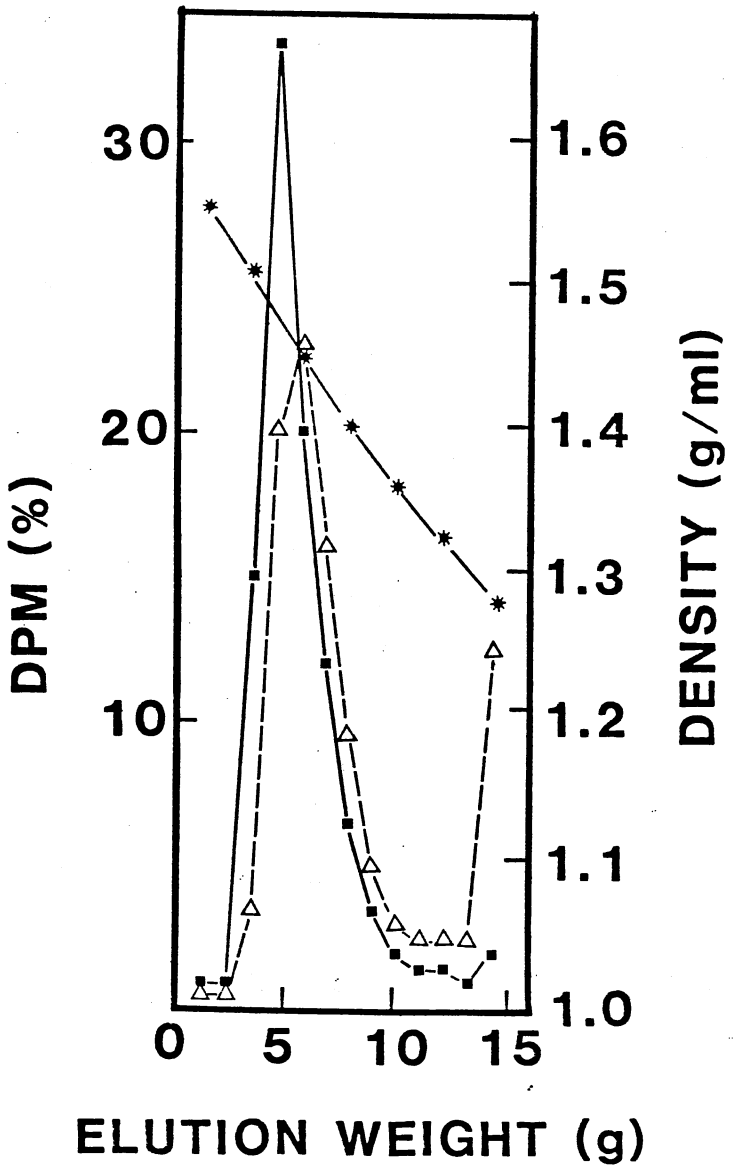

30
20
10
DPM (%)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DENSITY (g/ml)
0
5
10
15
ELUTION WEIGHT (g)

Figure 5. Density Gradient Centrifugation of MAT-BH Membrane Vesicles from Control and Group I in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{14}C]glucosamine-labeled group I (4.5 mg retinyl palmitate/kg diet) MAT-BH cells (■) mixed with membrane vesicles from [^{3}H]glucosamine-labeled control MAT-BH cells (△). Density curve (✱).

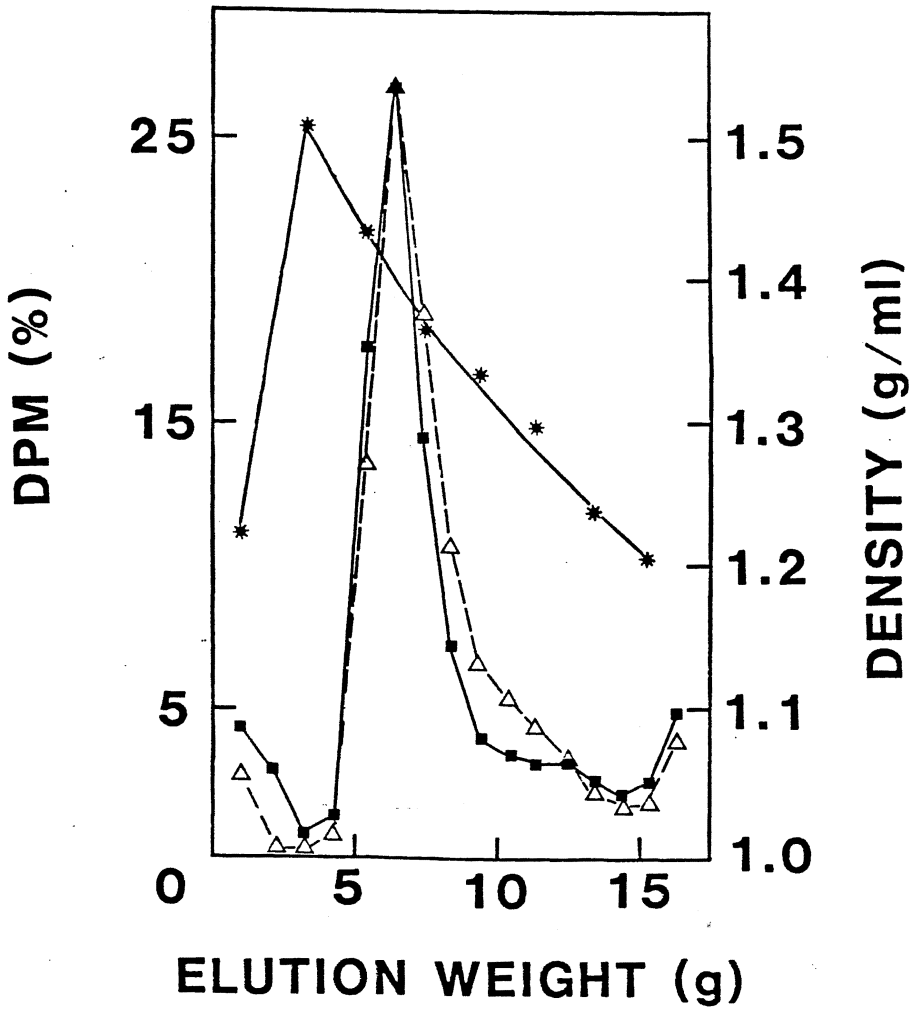
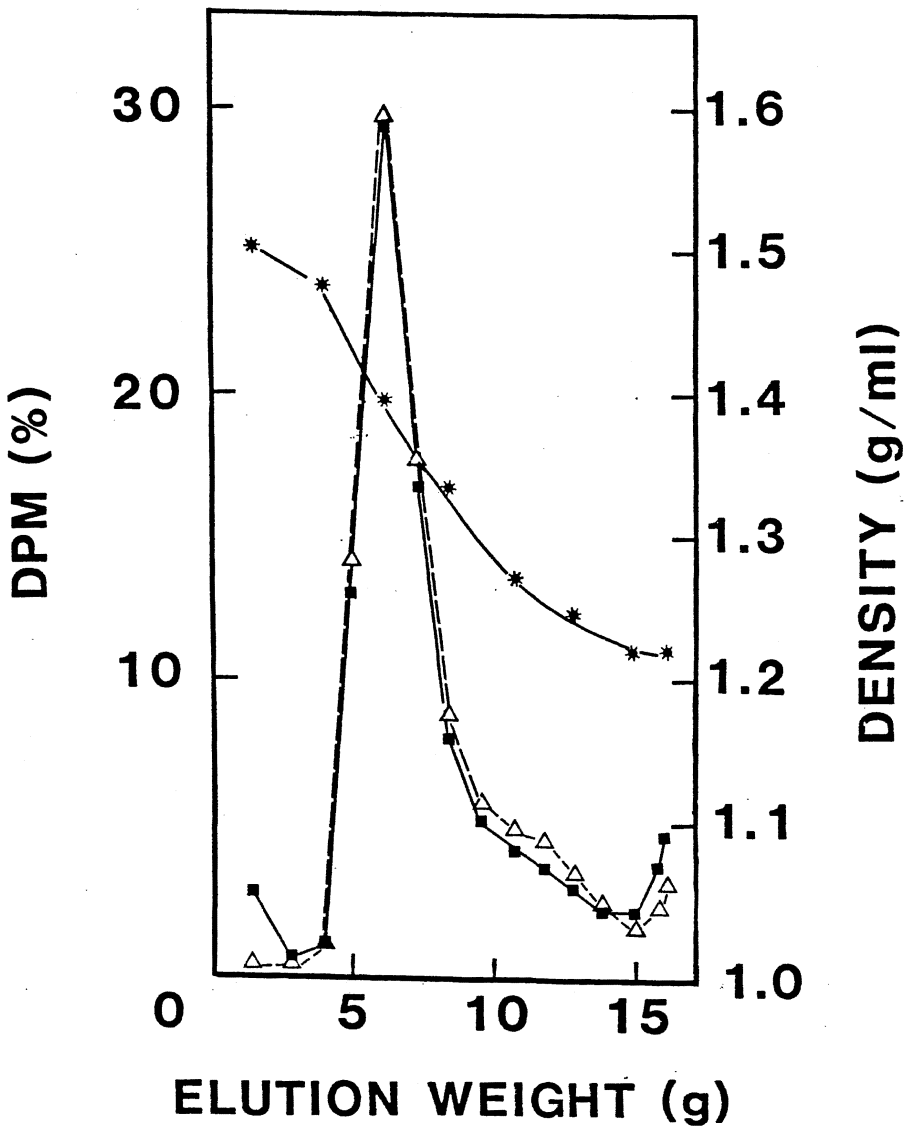

25
15
5
0
DPM (%)
1.5
1.4
1.3
1.2
1.1
1.0
DENSITY (g/ml)
5
10
15
ELUTION WEIGHT (g)

Figure 6. Density Gradient Centrifugation of MAT-BH Membrane Vesicles from Control and Group III in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group III (120 mg retinoic acid/kg diet) MAT-BH cells (△) mixed with membrane vesicles from [^{14}C]-glucosamine-labeled control MAT-BH cells (■). Density curve (✱).


30
20
10
0
5
10
15
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DPM (%)
DENSITY (g/ml)
ELUTION WEIGHT (g)

group to another. Upon comparing group I ASGP-1 with control, group I ASGP-1 appeared more dense than control MAT-CH ASGP-1 in one cell combination (Figure 7), and less dense in another. Group II ASGP-1 appeared either as dense or slightly less dense than control ASGP-1 (Figure 8). ASGP-1 from group III was less dense than control in the two combinations tested (Figure 9).

Characterization of ASGP-1 by Gel Chromatography on Sepharose CL-2B

As described in the Materials and Methods, glycoproteins from different combinations of ASGP-1 from supplemented groups and their respective controls were copurified. Aliquots from each of the purified combinations were applied to a Sepharose CL-2B column. Gel chromatography profiles for the MAT-B1 subline revealed no significant differences between group I or group II or group IV (Figure 10) glycoproteins and control. However, group III glycoprotein was significantly higher ($P < .05$) in molecular weight than control MAT-B1 glycoprotein. MAT-C3 glycoproteins from all the supplemented groups were significantly ($P < .05$) lower in molecular weight than control MAT-C3 ASGP-1 (Figure 11 and 12).

Gel filtration data for the MAT-BH subline indicated that group I ASGP-1 was either larger ($P < .05$), in one cell combination (Figure 13), or as large as control MAT-BH ASGP-1 in another. Group II ASGP-1 was smaller than control MAT-BH ASGP-1 ($P < .05$) in both combinations tested, and might be less heterogeneous. ASGP-1 from group III MAT-BH cells was consistently smaller ($P < .05$) than control ASGP-1 (Figure 14). MAT-CH glycoprotein from group I was larger ($P < .05$) than control in both combinations studied (Figure 15). ASGP-1 from groups II and III were

Figure 7. Density Gradient Centrifugation of MAT-CH Membrane Vesicles from Control and Group I in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group I (4.5 mg retinyl palmitate/kg diet) MAT-CH cells (△) mixed with membrane vesicles from [^{14}C]-glucosamine-labeled control MAT-CH cells (■). Density curve (✱).

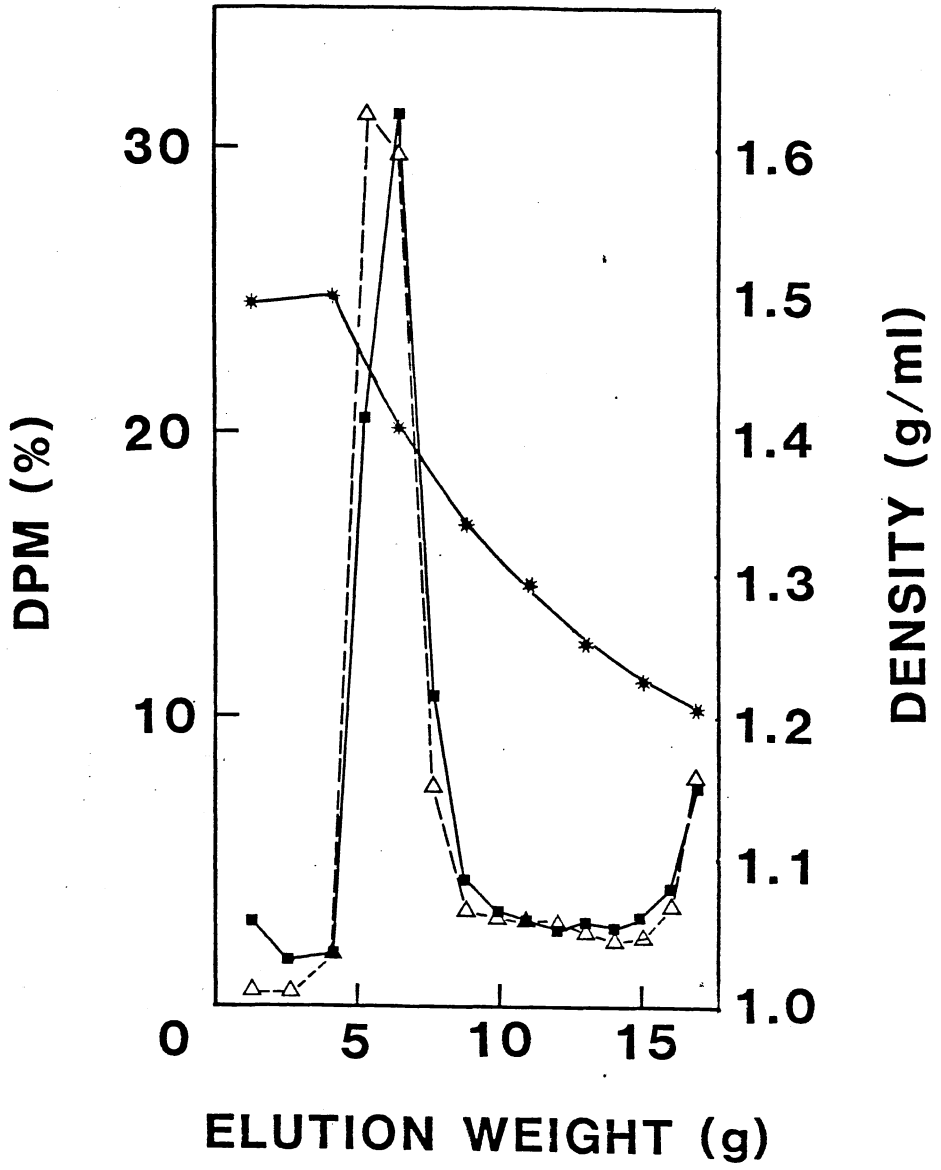

30
20
10
0
DPM (%)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DENSITY (g/ml)
5
10
15
ELUTION WEIGHT (g)

Figure 8. Density Gradient Centrifugation of MAT-CH Membrane Vesicles from Control and Group II in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group II (60 mg retinoic acid/kg diet) MAT-CH (△) cells were mixed with membrane vesicles from [^{14}C]-glucosamine-labeled control MAT-CH cells (■). Density curve (✱).

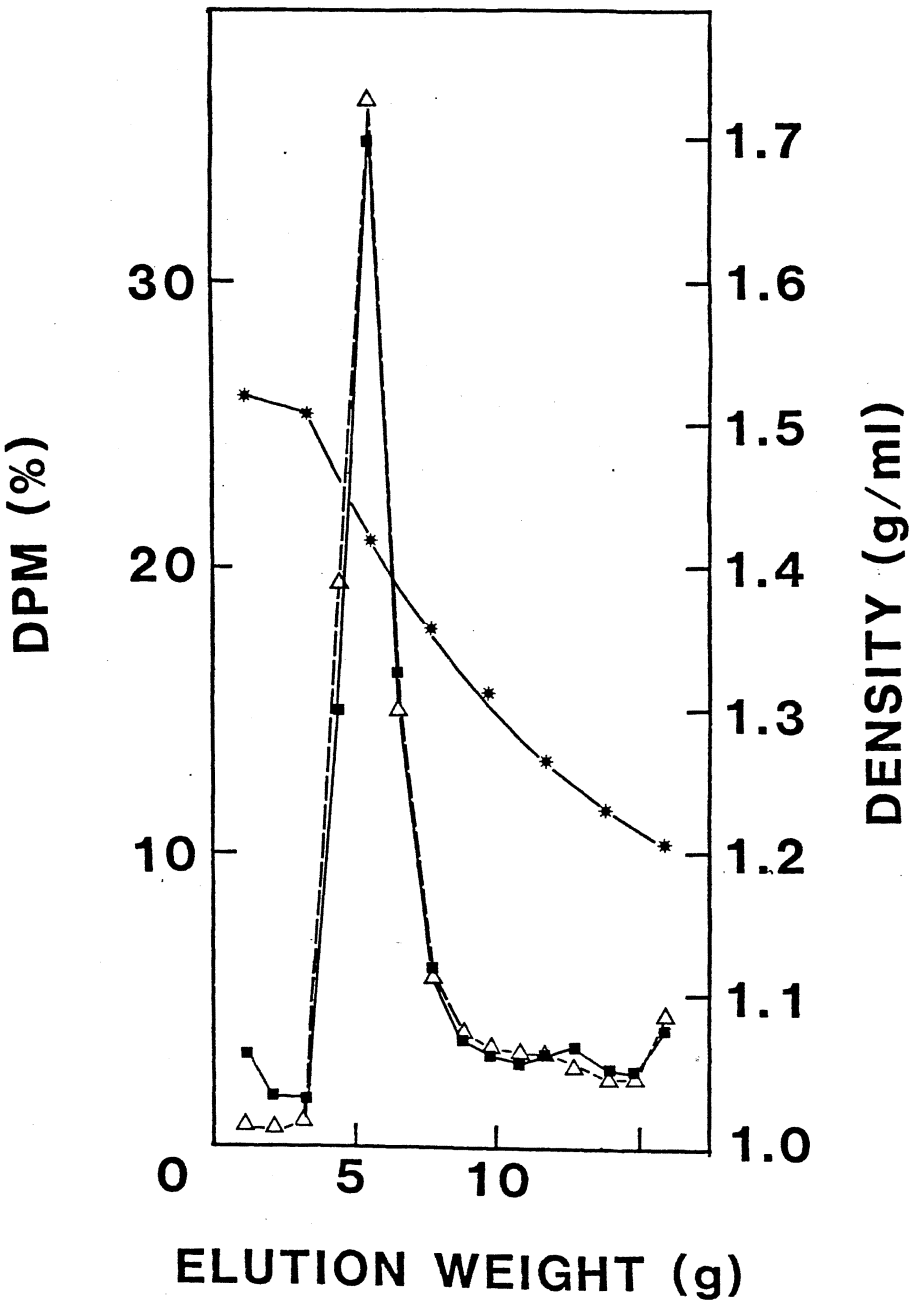

DPM (%)
30
20
10
0
5
10
ELUTION WEIGHT (g)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DENSITY (g/ml)

Figure 9. Density Gradient Centrifugation of MAT-CH Membrane Vesicles from Control and Group III in CsCl Gradients Containing 4 M Gdn HCl. Membrane vesicles from [^{3}H]glucosamine-labeled group III (120 mg retinoic acid/kg diet) MAT-CH cells (△) mixed with membrane vesicles from [^{14}C]-glucosamine-labeled control MAT-CH cells (■). Density curve (✱).

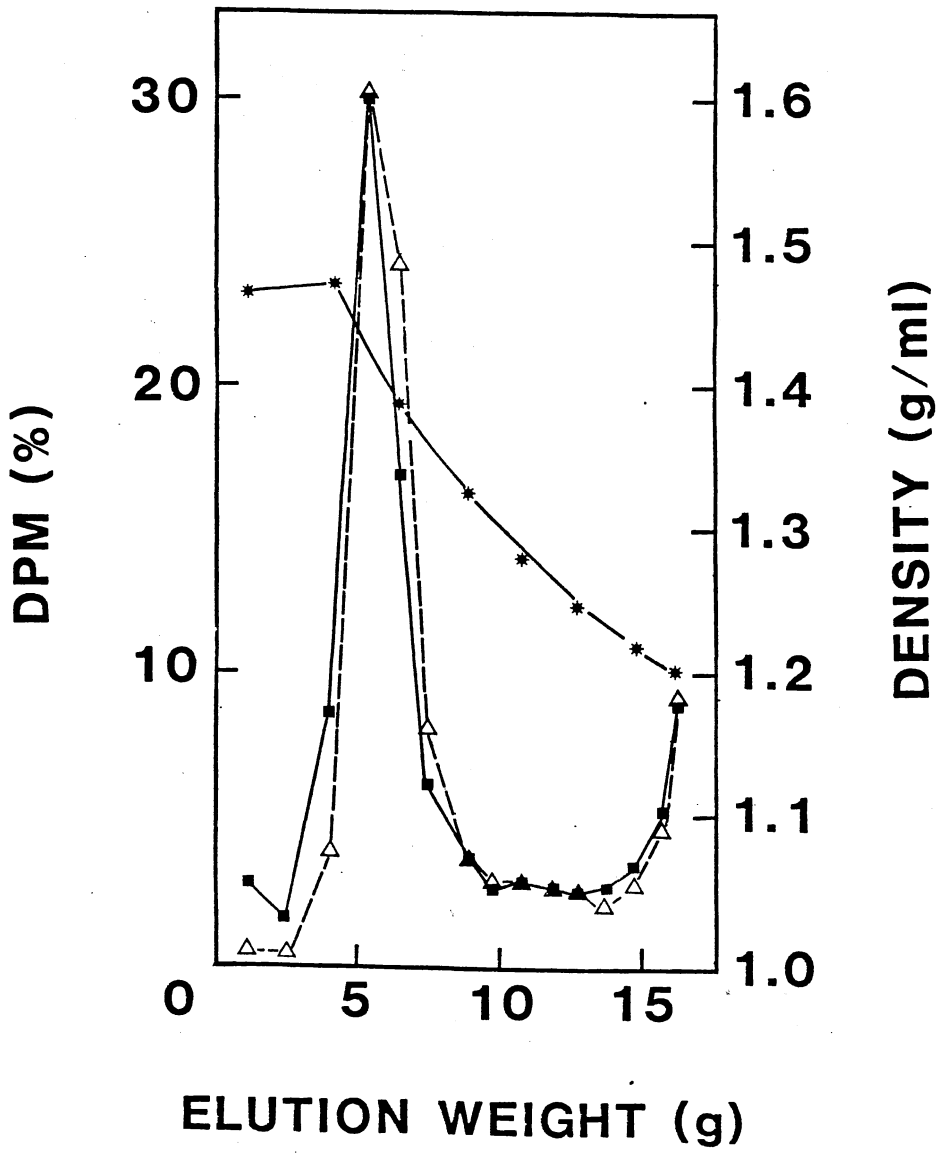
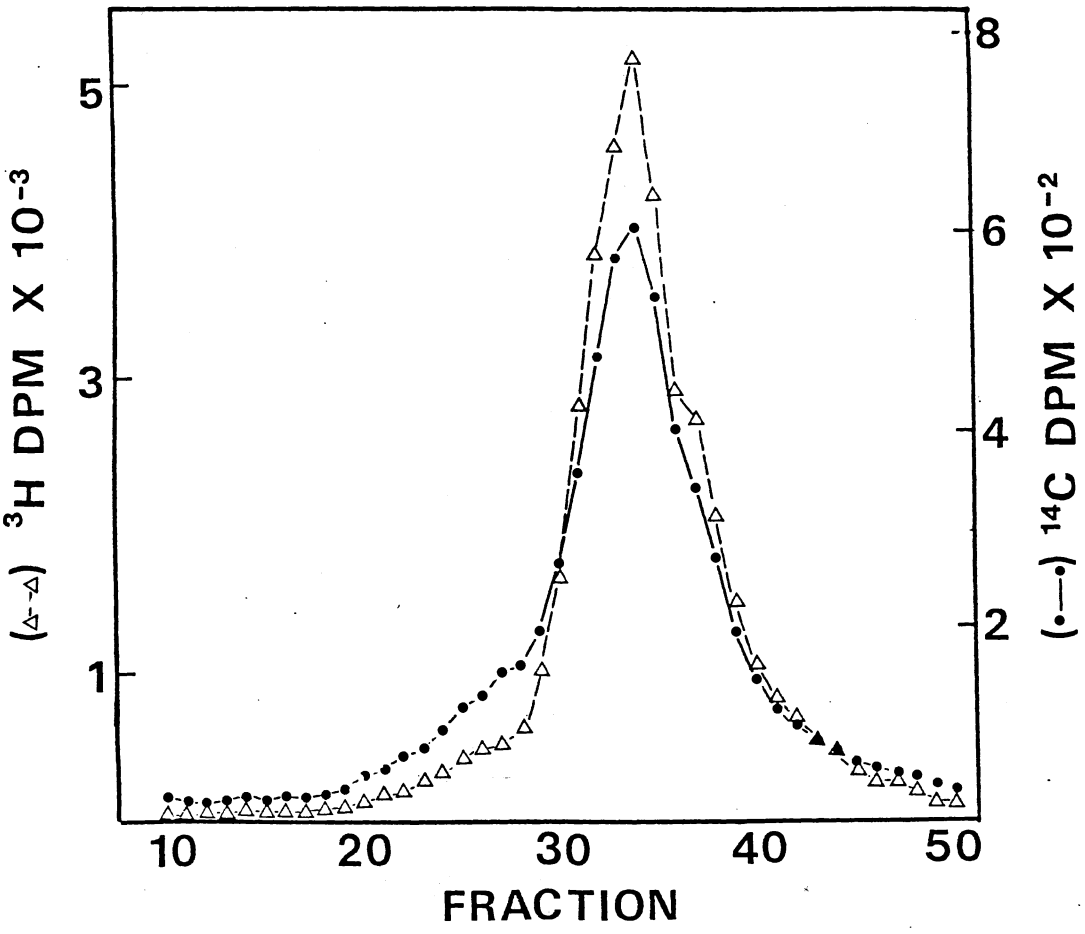

30
20
10
0
5
10
15
1.6
1.5
1.4
1.3
1.2
1.1
1.0
DPM (%)
DENSITY (g/ml)
ELUTION WEIGHT (g)

Figure 10. Gel Filtration of Purified MAT-B1 ASGP-1 from Control and Group IV on Sepharose CL-2B with 1% SDS. [^{3}H]glucosamine-labeled MAT-B1 ASGP-1 from group IV (120 mg retinoic acid/kg diet) (△) mixed with [^{14}C]glucosamine-labeled control MAT-B1 ASGP-1 (●).


(△-△) ³H DPM X 10⁻³
(•—•) ¹⁴C DPM X 10⁻²
5
3
1
8
6
4
2
10
20
30
40
50
FRACTION

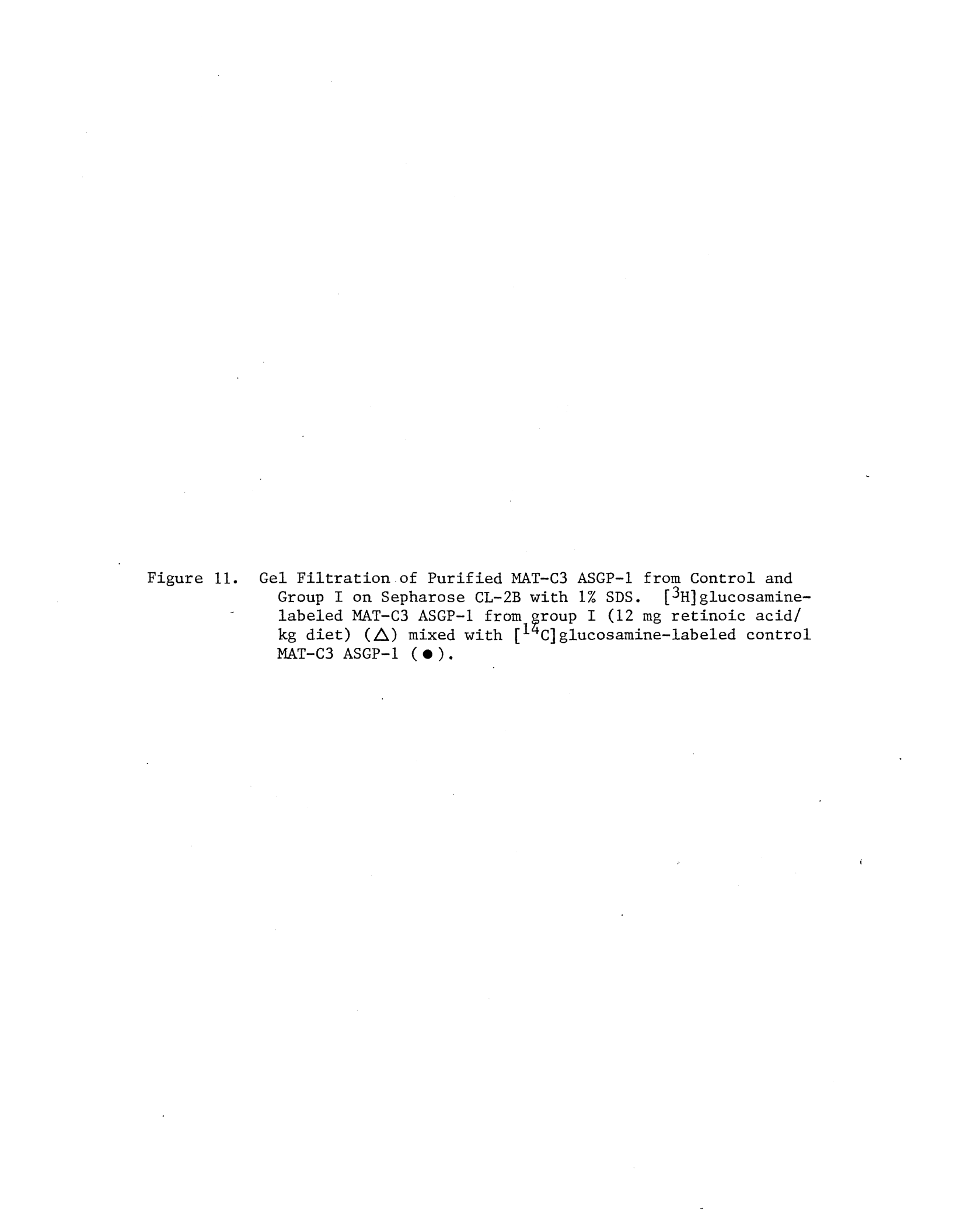


Figure 11. Gel Filtration of Purified MAT-C3 ASGP-1 from Control and Group I on Sepharose CL-2B with 1% SDS. [^{3}H]glucosamine-labeled MAT-C3 ASGP-1 from group I (12 mg retinoic acid/kg diet) (△) mixed with [^{14}C]glucosamine-labeled control MAT-C3 ASGP-1 (●).

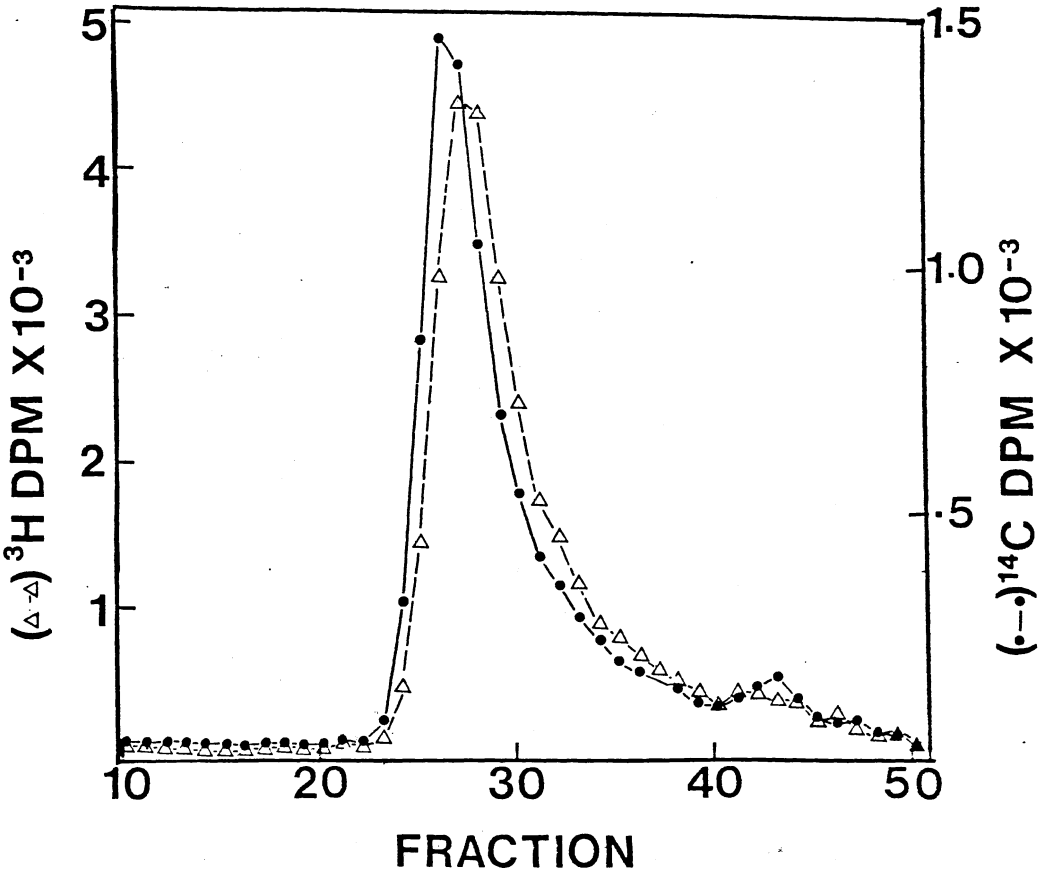

5
4
3
2
1
1.5
1.0
.5
10
20
30
40
50
FRACTION
(△-△) ^{3}H DPM X 10^{-3}
(•—•) ^{14}C DPM X 10^{-3}

Figure 12. Gel Filtration of Purified MAT-C3 ASGP-1 from Control and Group III on Sepharose CL-2B with 1% SDS. [^{3}H]-glucosamine-labeled MAT-C3 ASGP-1 from group III (120 mg retinoic acid/kg diet) (△) mixed with [^{14}C]glucosamine-labeled control MAT-C3 ASGP-1 (●).

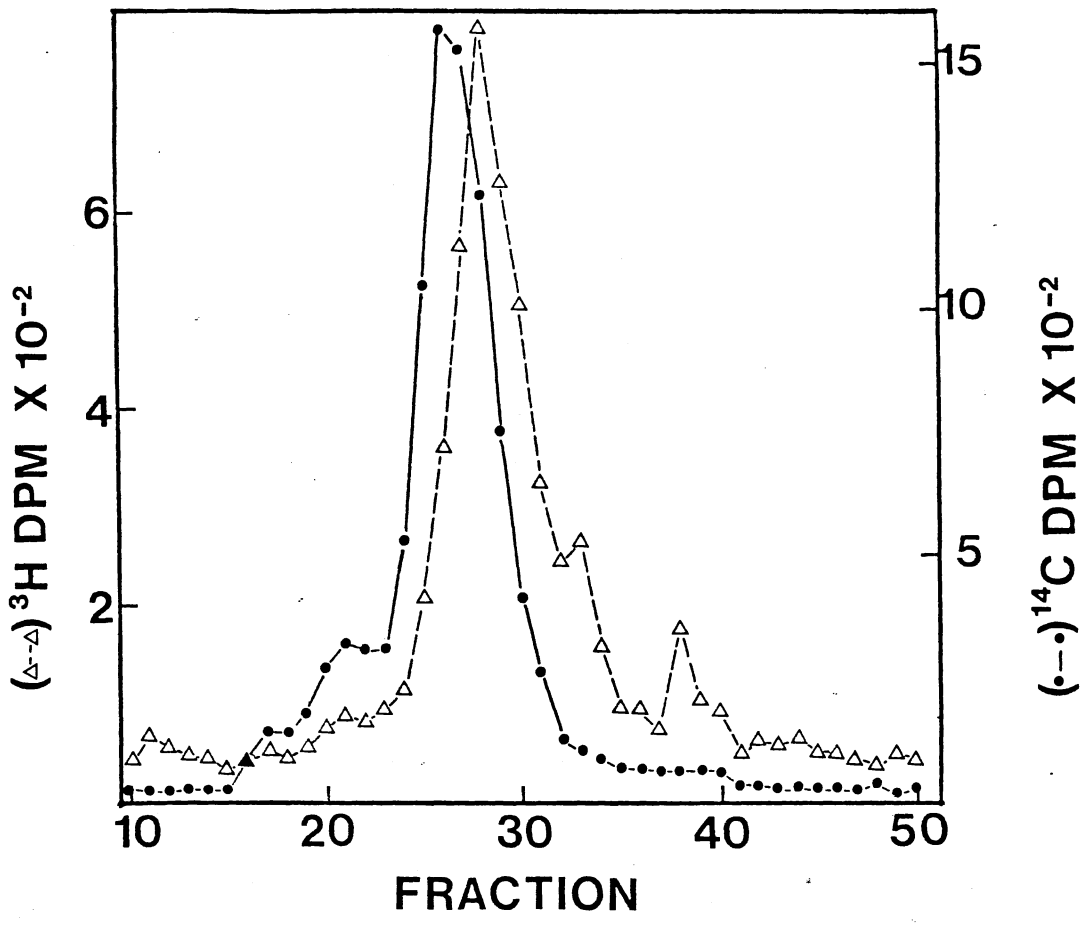

(△-△) ^{3}H DPM X 10^{-2}
(•—•) ^{14}C DPM X 10^{-2}
FRACTION
2
4
6
5
10
15
10
20
30
40
50

Figure 13. Gel Filtration of Purified MAT-BH ASGP-1 from Control and Group I on Sepharose CL-2B with 1% SDS. [^{14}C]glucosamine-labeled MAT-BH ASGP-1 from group I (4.5 mg retinyl palmitate/kg diet) (●) mixed with [^{3}H]glucosamine-labeled control MAT-BH ASGP-1 (△).

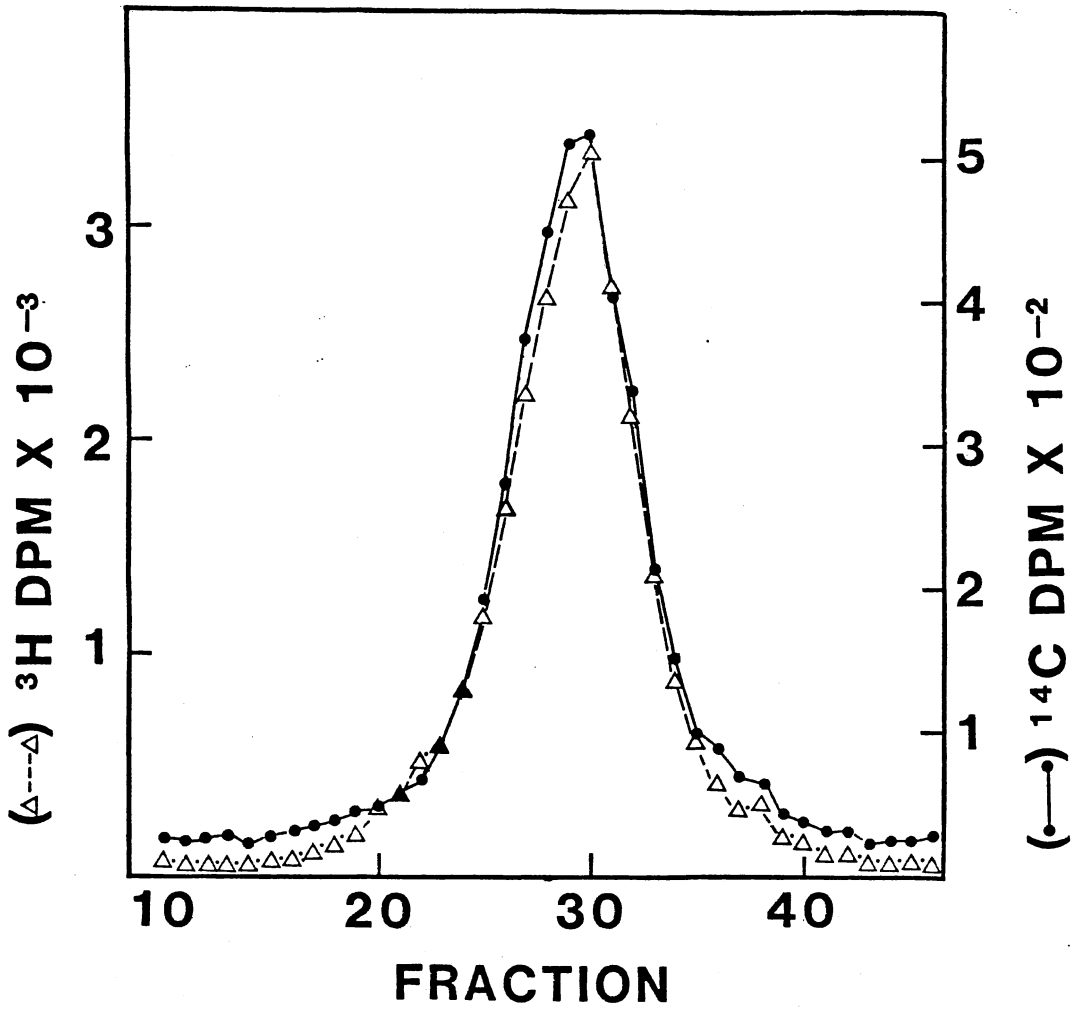

(△---△) ³H DPM X 10⁻³
(●—●) ¹⁴C DPM X 10⁻²
1
2
3
1
2
3
4
5
10
20
30
40
FRACTION

Figure 14. Gel Filtration of Purified MAT-BH ASGP-1 from Control and Group III on Sepharose CL-2B with 1% SDS. [^{3}H]glucosamine-labeled MAT-BH ASGP-1 from group III (120 mg retinoic acid/kg diet) (△) mixed with [^{14}C]glucosamine-labeled control MAT- BH ASGP-1 (●).

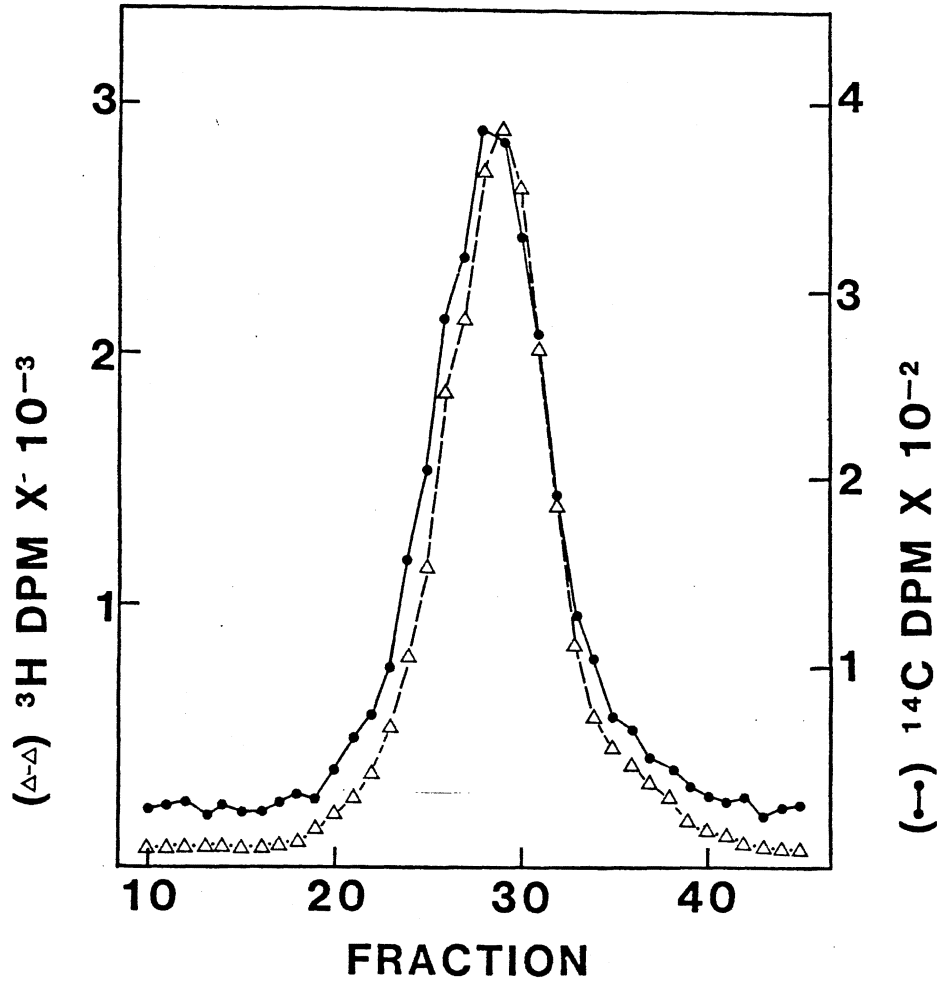
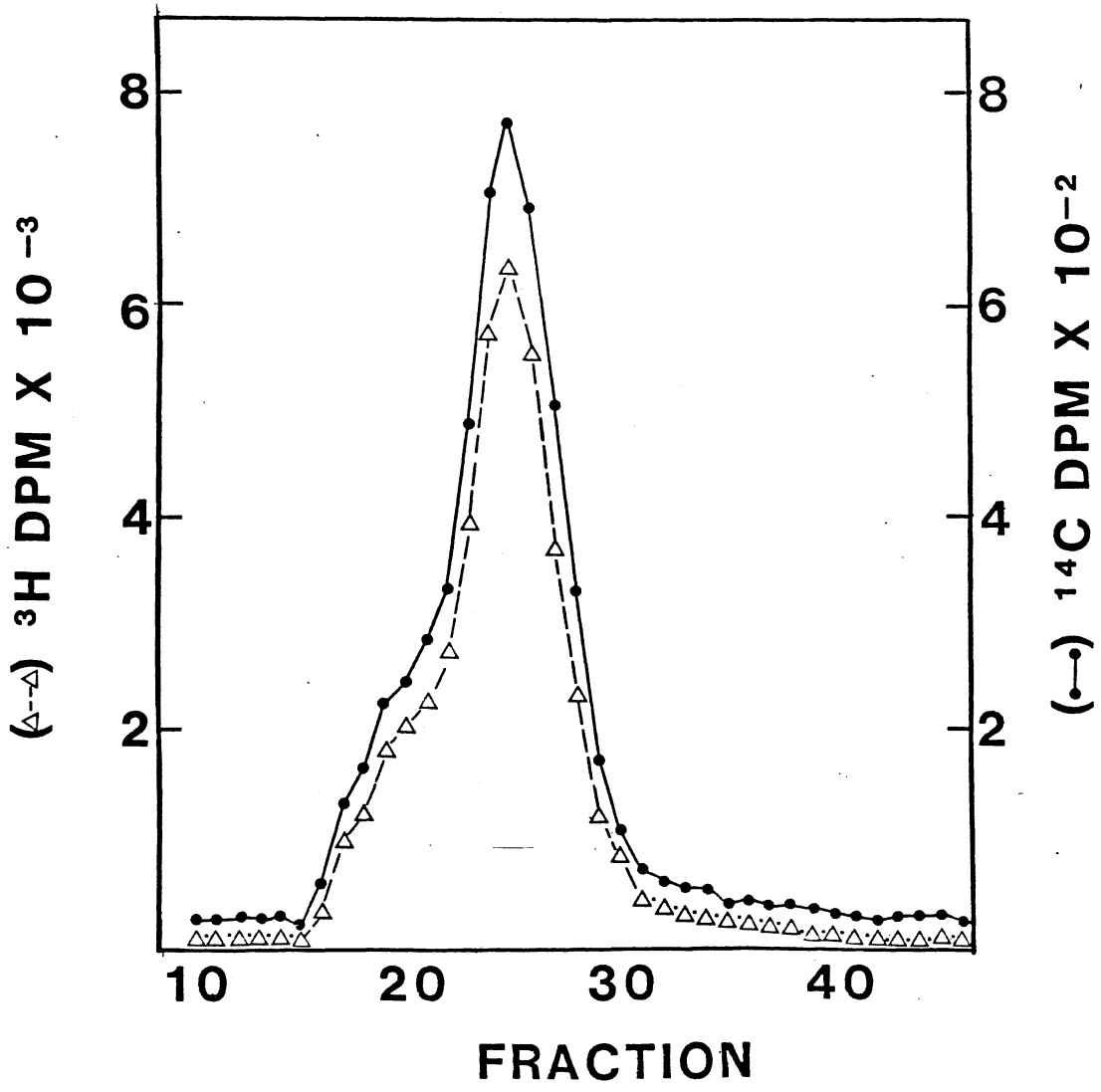

(△-△) ³H DPM X 10⁻³
(●-●) ¹⁴C DPM X 10⁻²
FRACTION
10
20
30
40
1
2
3
4

Figure 15. Gel Filtration of Purified MAT-CH ASGP-1 from Control and Group I on Sepharose CL-2B with 1% SDS. [^{3}H]glucosamine-labeled MAT-CH ASGP-1 from group I (4.5 mg retinyl palmitate/kg diet) (△) mixed with [^{14}C]glucosamine-labeled control MAT-CH ASGP-1 (●).

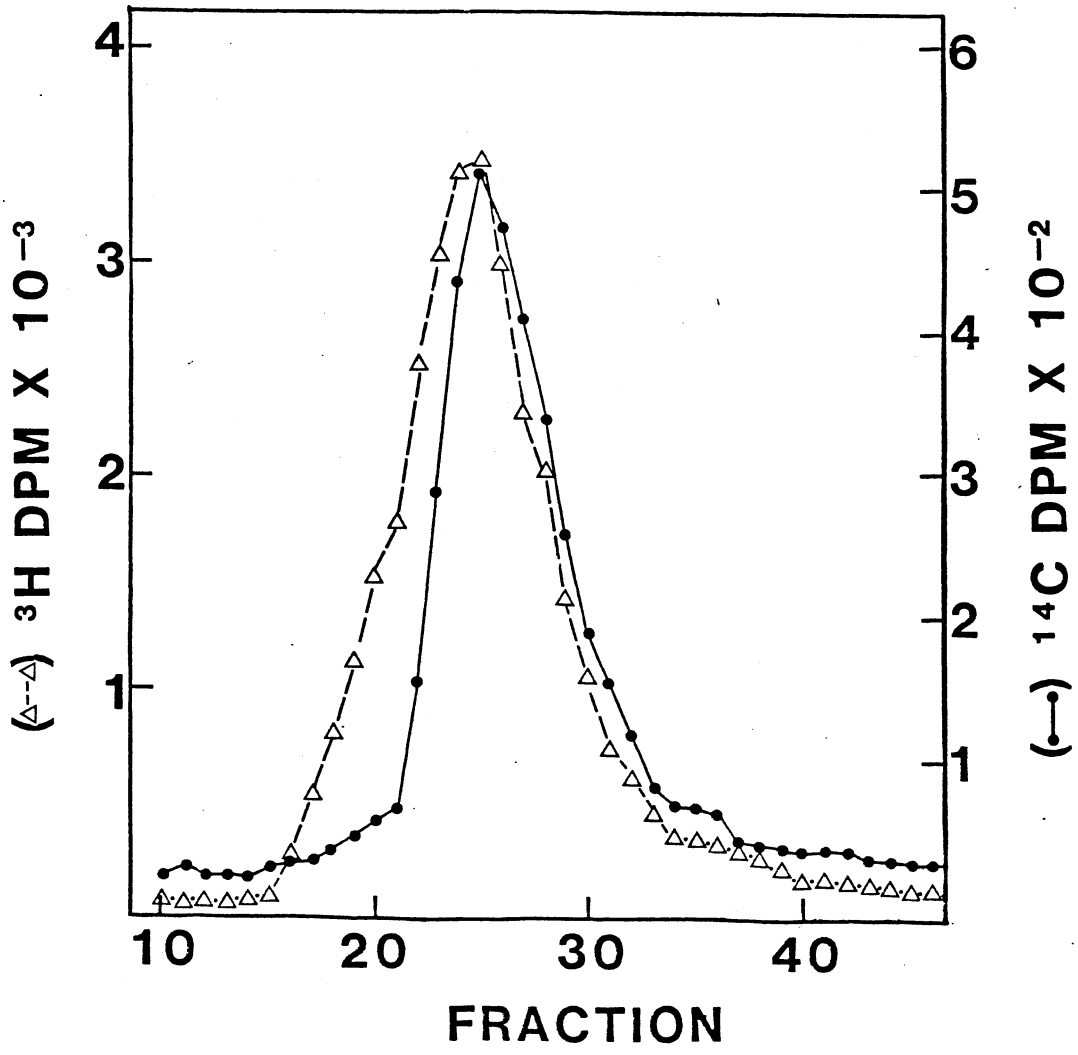

(△-△) ³H DPM X 10⁻³
(●—●) ¹⁴C DPM X 10⁻²
8
6
4
2
10
20
30
40
FRACTION

consistently significantly larger ($P < .05$) than control MAT-CH ASGP-1 (Figure 16).

Oligosaccharides Released by Alkaline Borohydride Treatment

Release of the O-linked oligosaccharides of ASGP-1 was accomplished by alkaline borohydride β-elimination (88). Bio-Gel P-4 chromatography of the alkaline borohydride digest from MAT-B1 cells resulted in the separation of three major glucosamine containing oligosaccharides. The labeling studies revealed that the majority of the MAT-B1 oligosaccharides were found in peaks III and IV as shown previously for the MAT-B1 subline by Sherblom et al. (46). No significant differences in the oligosaccharide patterns of control and each of group II, III, or IV (Figure 17 and 18) MAT-B1 ASGP-1 were observed. In the case of group I, there was not enough sample to run the oligosaccharide test. Labeling studies revealed no differences in the oligosaccharides released by alkaline borohydride treatment of copurified group I and control MAT-C3 ASGP-1 (Figure 19). For both glycoproteins, the oligosaccharides appeared predominantly in peaks II and III upon gel filtration on a Bio-Gel P-4 column as shown previously for MAT-C3 ASGP-1 (54). On the other hand there were distinct differences ($P < .05$) in the released oligosaccharides when MAT-C3 ASGP-1 from group III and control cells were compared (Figure 20). The ^{14}C and ^{3}H peaks were not coincident at peak II. This suggested that the control and group III oligosaccharides that comprise this peak might not be the same. The ^{14}C and ^{3}H peaks coincide at peak III. The shoulder to the side of the

Figure 16. Gel Filtration of Purified MAT-CH ASGP-1 from Control and Group III on Sepharose CL-2B with 1% SDS. [^{3}H]glucosamine-labeled MAT-CH ASGP-1 from group III (120 mg retinoic acid/kg diet) (△) mixed with [^{14}C]glucosamine-labeled control MAT-CH ASGP-1 (●).


(△--△) ³H DPM X 10⁻³
(●—●) ¹⁴C DPM X 10⁻²
FRACTION
10
20
30
40
1
2
3
4
1
2
3
4
5
6

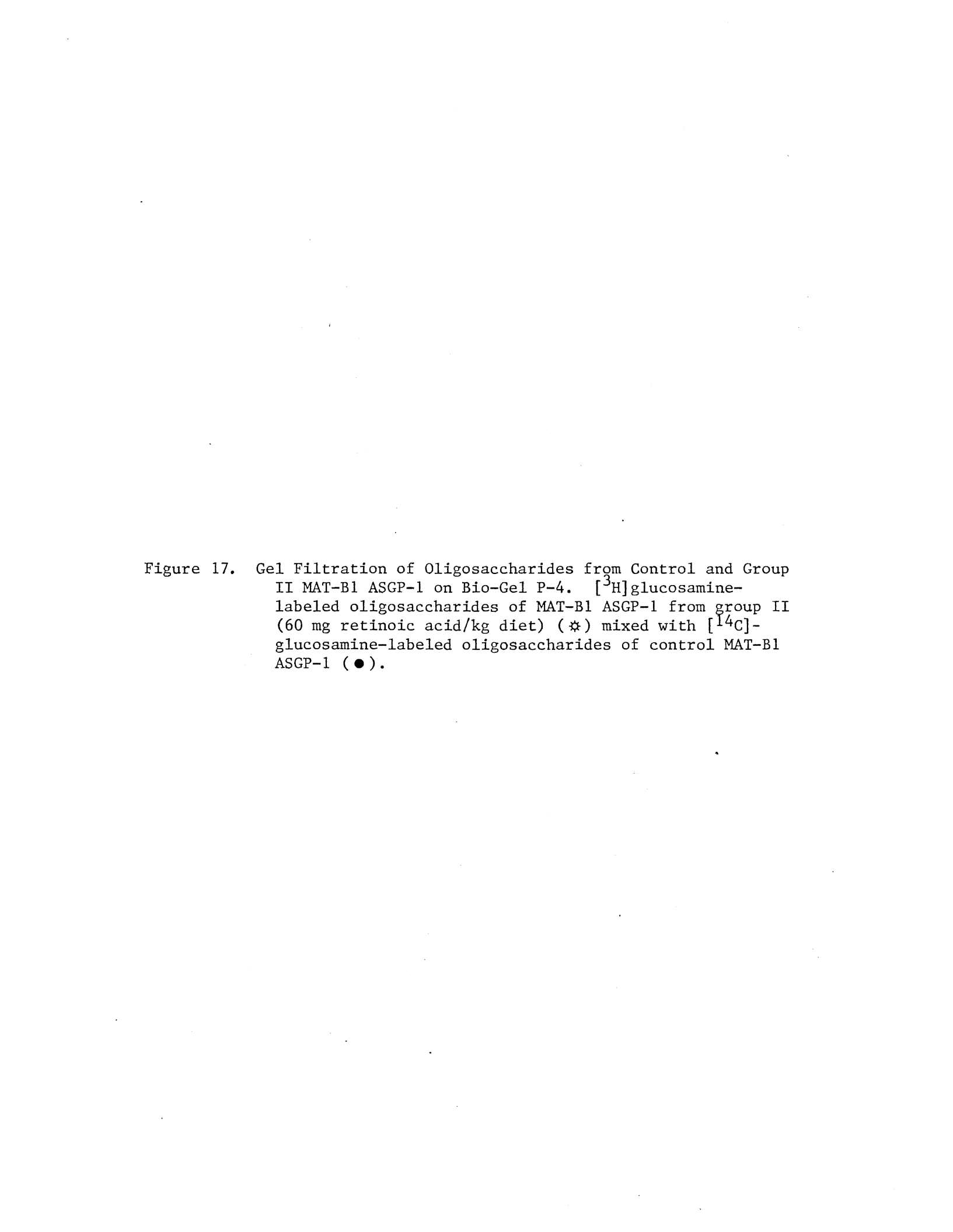
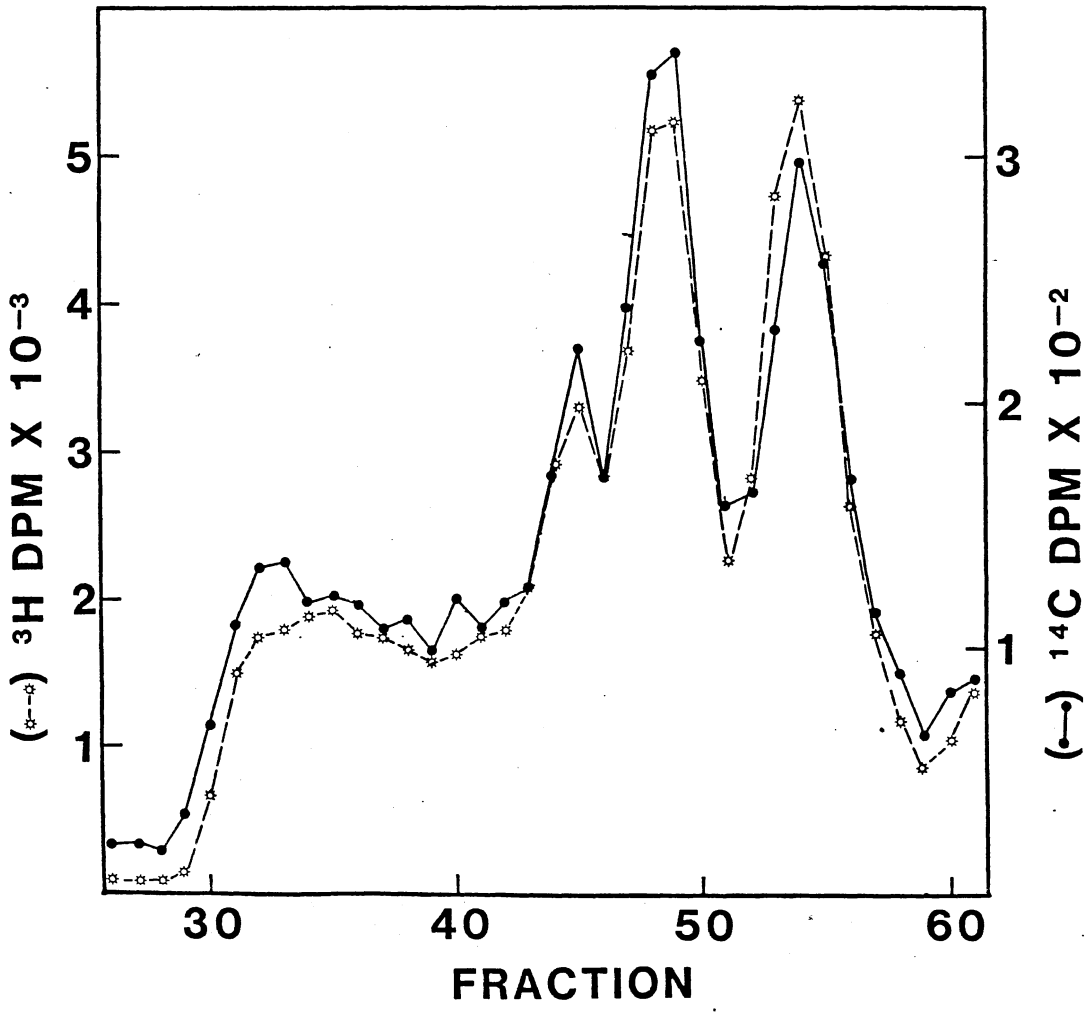


Figure 17. Gel Filtration of Oligosaccharides from Control and Group II MAT-B1 ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-B1 ASGP-1 from group II (60 mg retinoic acid/kg diet) (✡) mixed with [^{14}C]-glucosamine-labeled oligosaccharides of control MAT-B1 ASGP-1 (●).


(--✡--) ³H DPM X 10⁻³
(—●—) ¹⁴C DPM X 10⁻²
1
2
3
4
5
1
2
3
30
40
50
60
FRACTION

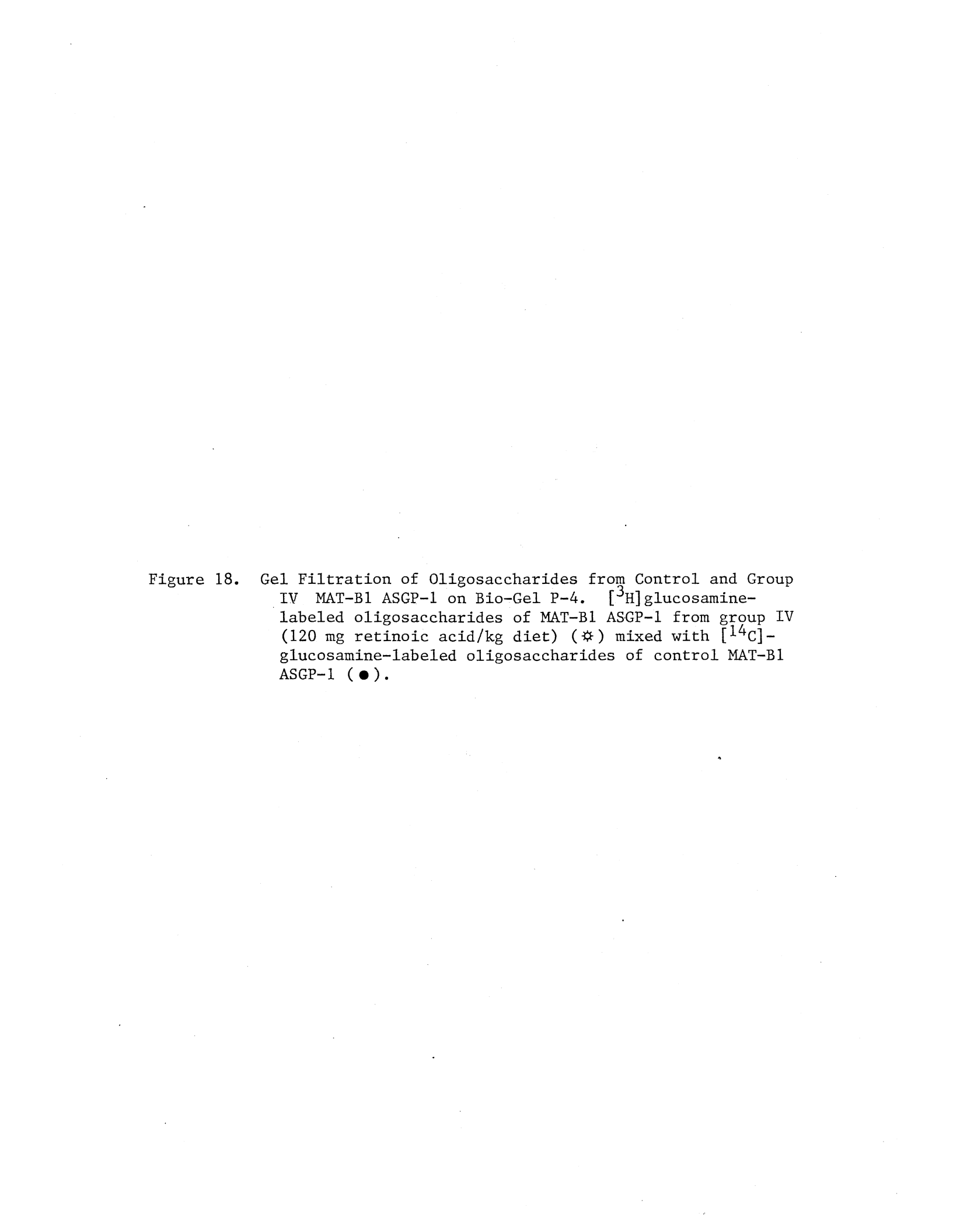


Figure 18. Gel Filtration of Oligosaccharides from Control and Group IV MAT-B1 ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-B1 ASGP-1 from group IV (120 mg retinoic acid/kg diet) (✲) mixed with [^{14}C]-glucosamine-labeled oligosaccharides of control MAT-B1 ASGP-1 (●).

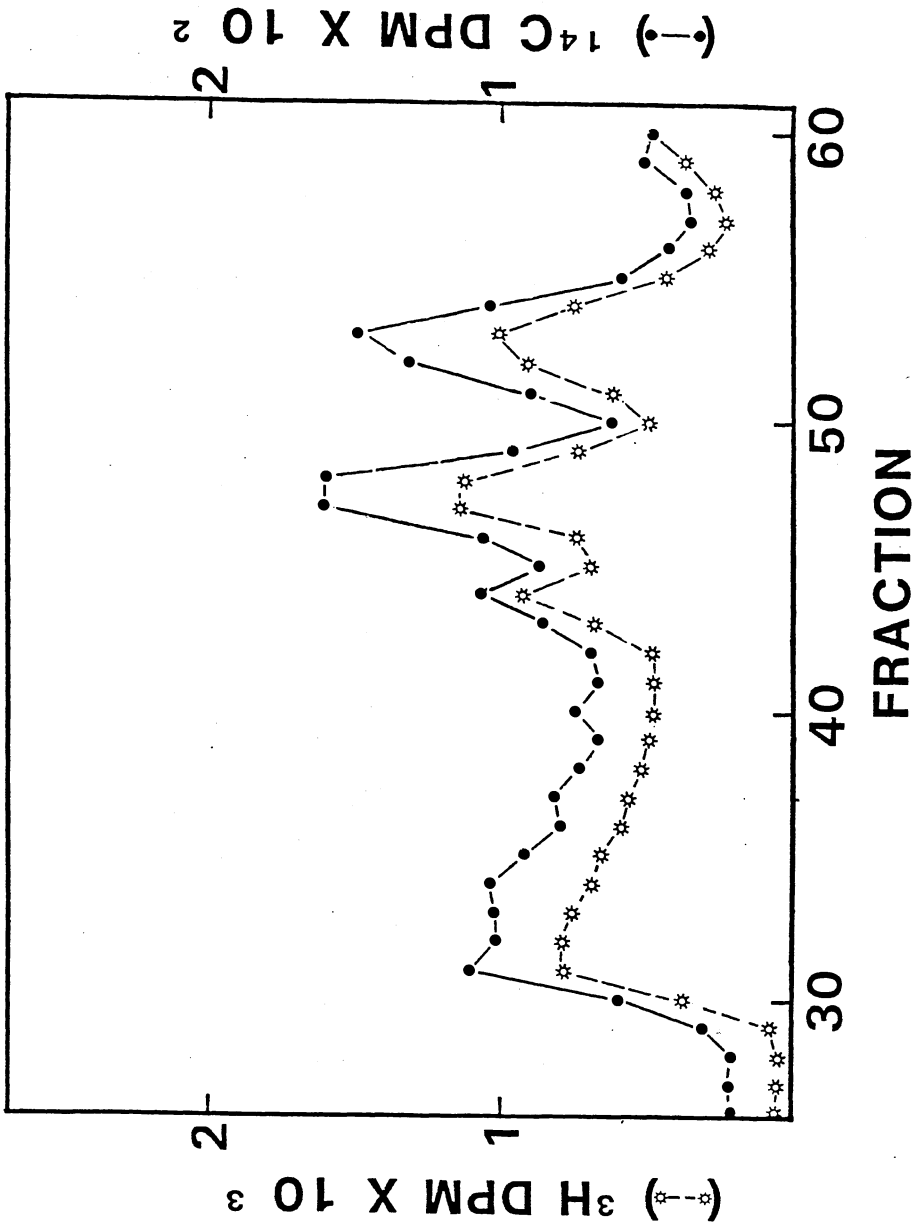

(•—•) 14C DPM X 10 2
2
1
60
50
40
30
FRACTION
2
1
(✲--✲) 3H DPM X 10 3

Figure 19. Gel Filtration of Oligosaccharides from Control and Group I MAT-C3 ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-C3 ASGP-1 from group I (12 mg retinoic acid/kg diet) (✲) mixed with [^{14}C]glucosamine-labeled oligosaccharides of control MAT-C3 ASGP-1 (●).

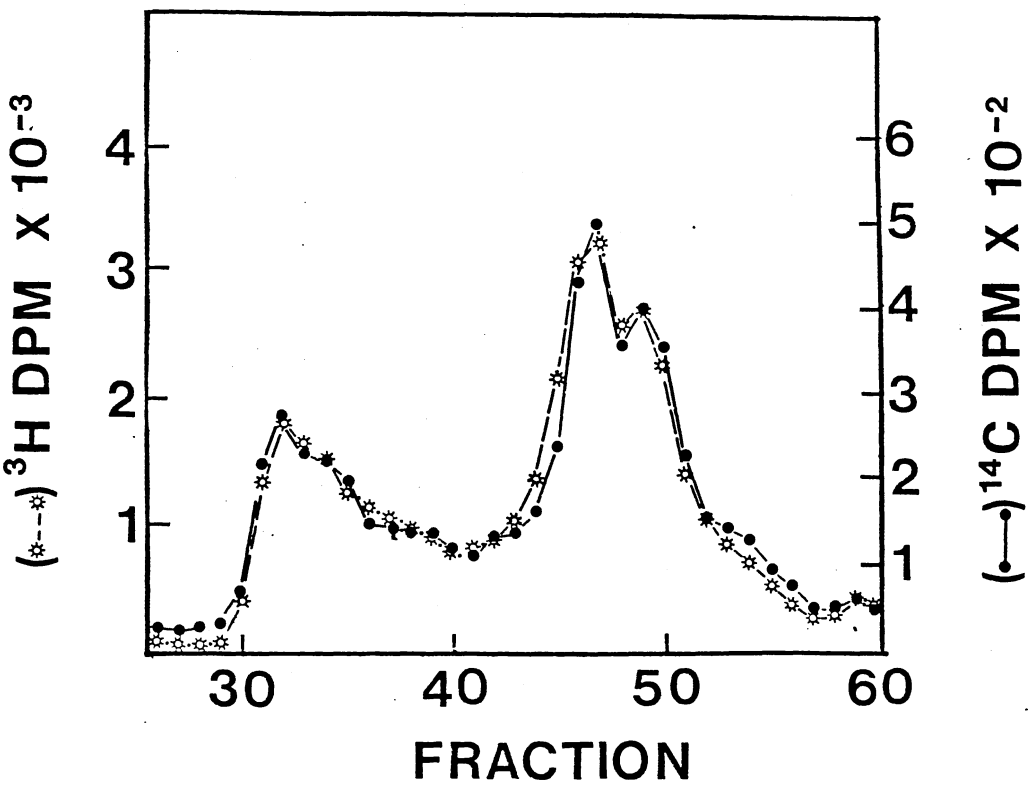
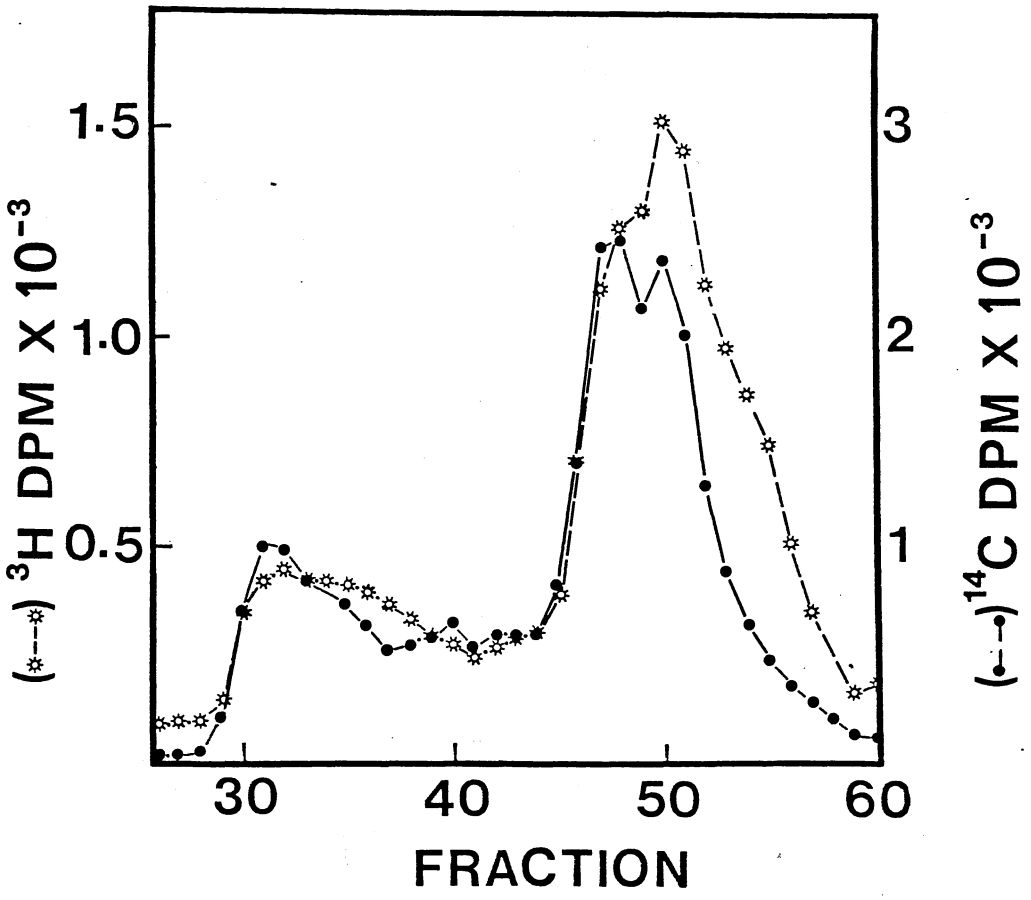

(---)^{3}H DPM X 10^{-3}
(—)^{14}C DPM X 10^{-2}
1
2
3
4
1
2
3
4
5
6
30
40
50
60
FRACTION

Figure 20. Gel Filtration of Oligosaccharides from Control and Group III MAT-C3 ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-C3 ASGP-1 from group III (120 mg retinoic acid/kg diet) (✡) mixed with [^{14}C]-glucosamine-labeled oligosaccharides of control MAT-C3 ASGP-1 (●).


1.5
1.0
0.5
(✲--✲) ^{3}H DPM X 10^{-3}
3
2
1
(•--•)^{14}C DPM X 10^{-3}
30
40
50
60
FRACTION

^{3}H peak suggested a greater heterogeneity of oligosaccharide with a shift towards the lower molecular weight oligosaccharides. With respect to MAT-C3 group II, there was not enough sample to obtain the oligosaccharide pattern.

The results of oligosaccharide studies on the MAT-BH and MAT-CH sublines were variable. When MAT-BH ASGP-1 from group I was compared with control MAT-BH ASGP-1, their oligosaccharide patterns coincided (Figure 21). However, in one of the cell combinations, there appeared to be a slight increase in the relative amount of peak II oligosaccharides of group I (^{14}C labeled). When MAT-BH ASGP-1 from groups II and III (Figure 22) was compared to the control, the ^{3}H and ^{14}C peaks were not coincident, mainly at peaks III and IV. The oligosaccharides comprising the ^{3}H peaks (group III) appeared to be larger in the average molecular weight than those of the ^{14}C peaks (control). In addition, there seemed to be a slight decrease in the relative amount of peak II as compared to control, in all combinations studied. However, a large apparent increase in the relative amount of peak IV from group III was observed in one of the cases where group III and control glycoproteins were compared (Figure 22).

The MAT-CH oligosaccharide pattern (Figures 23 and 24) was different from that of the MAT-BH (Figures 21 and 22). The MAT-CH oligosaccharides was predominantly found in peaks II and III as shown for the MAT-C1 subline by Sherblom et al. (46). When control MAT-CH ASGP-1 was compared to ASGP-1 from the other groups, the main differences appeared to be between control and group I ASGP-1 (Figure 23). In this case, the ^{3}H and ^{14}C peaks did not coincide

Figure 21. Gel Filtration of Oligosaccharides from Control and Group I MAT-BH ASGP-1 on Bio-Gel P-4. [^{14}C]glucosamine-labeled oligosaccharides of MAT-BH ASGP-1 from group I (4.5 mg retinyl palmitate/kg diet) (●) mixed with [^{3}H]glucosamine-labeled oligosaccharides of control MAT-BH ASGP-1 (✲).

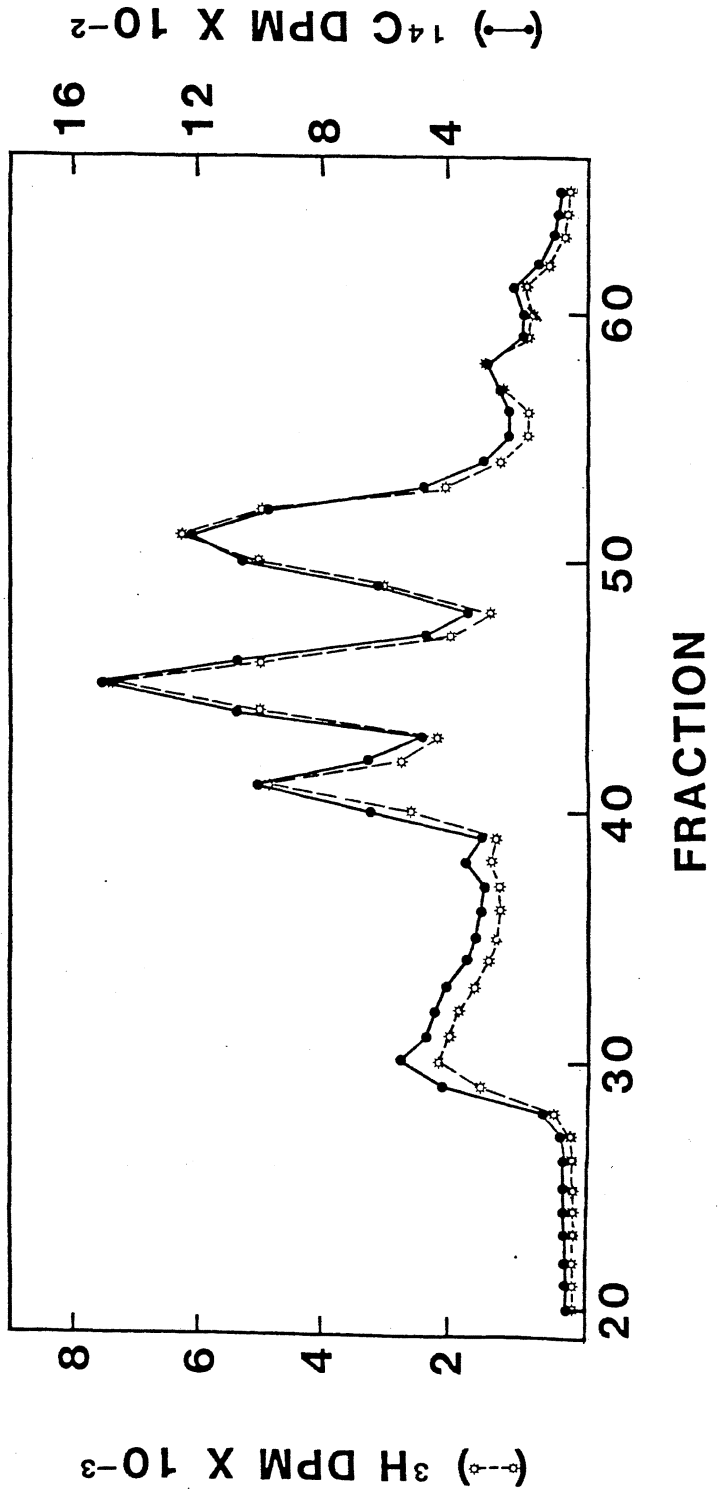

(⊶) 14C DPM X 10-2
16
12
8
4
60
50
40
30
20
FRACTION
8
6
4
2
(⊷) 3H DPM X 10-3

Figure 22. Gel Filtration of Oligosaccharides from Control and Group III MAT-BH ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-BH ASGP-1 from group III (120 mg retinoic acid/kg diet) (✲) mixed with [^{14}C]-glucosamine-labeled oligosaccharides of control MAT-BH ASGP-1 (●).

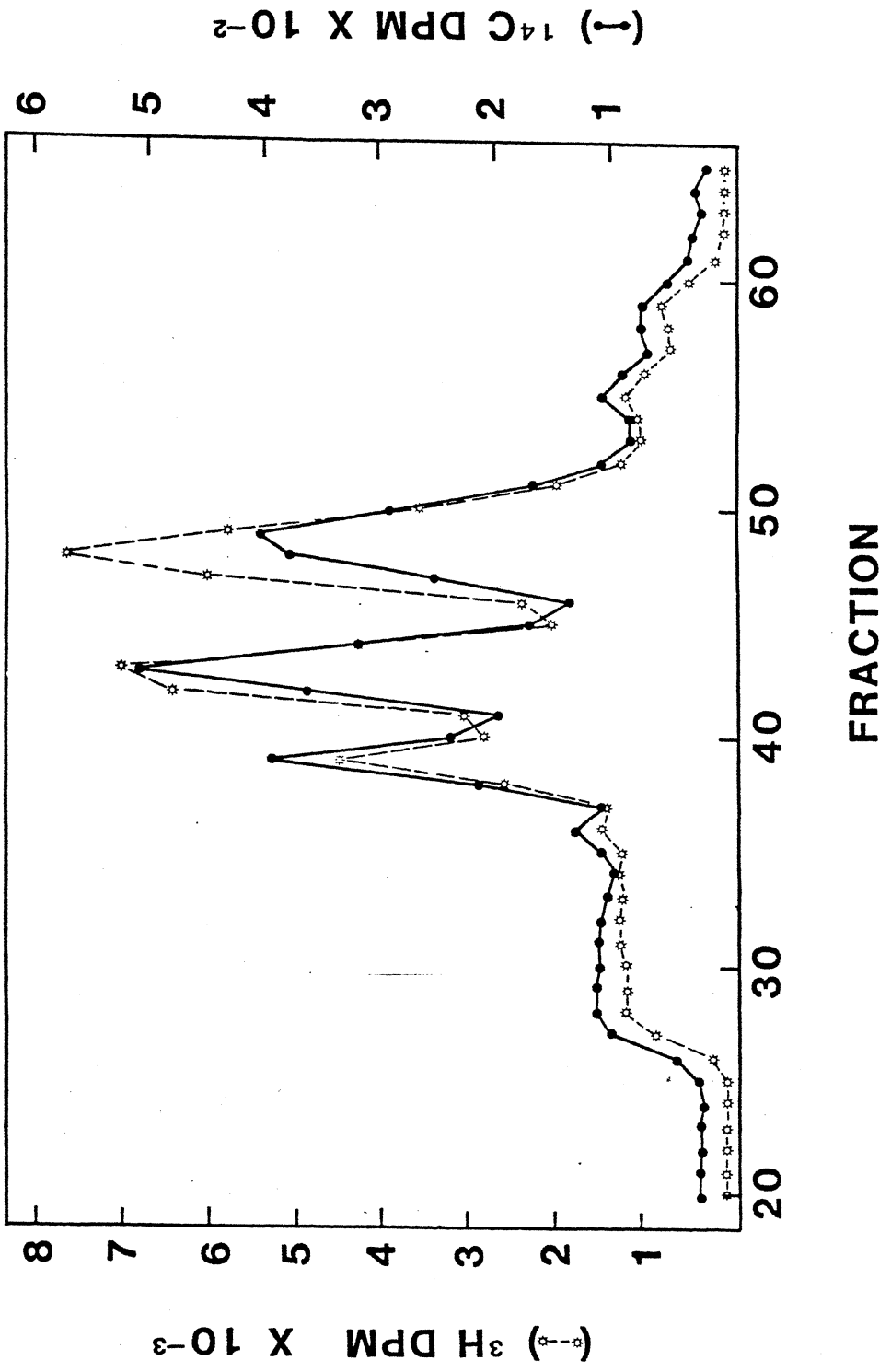

(—) 14C DPM X 10-2
6
5
4
3
2
1
20
30
40
50
60
FRACTION
8
7
6
5
4
3
2
1
(--) 3H DPM X 10-3

Figure 23. Gel Filtration of Oligosaccharides from Control and Group I MAT-CH ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-CH ASGP-1 from group I (4.5 mg retinyl palmitate/kg diet) (✲) mixed with [^{14}C]glucosamine-labeled oligosaccharides of control MAT-CH ASGP-1 (●).

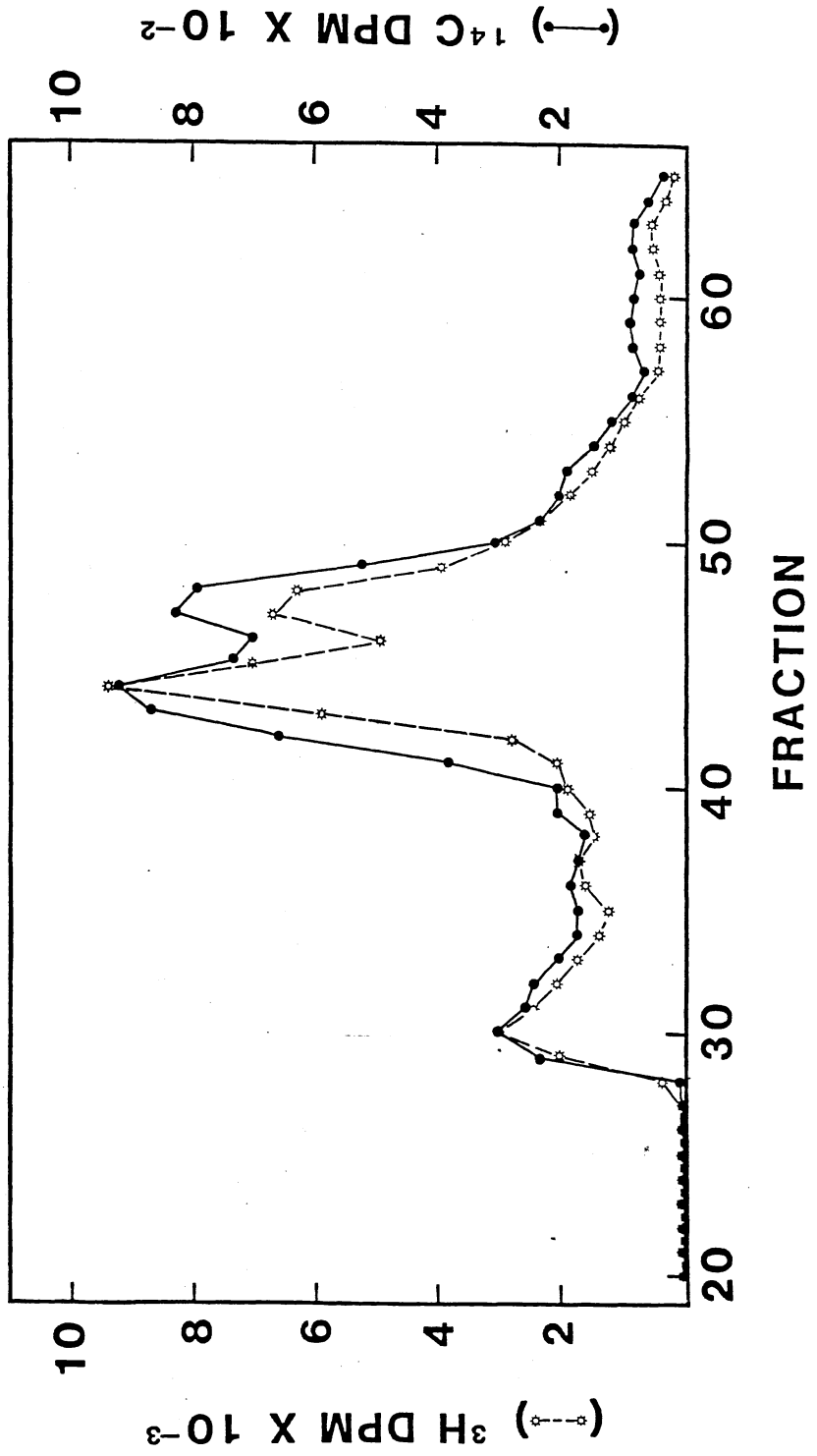
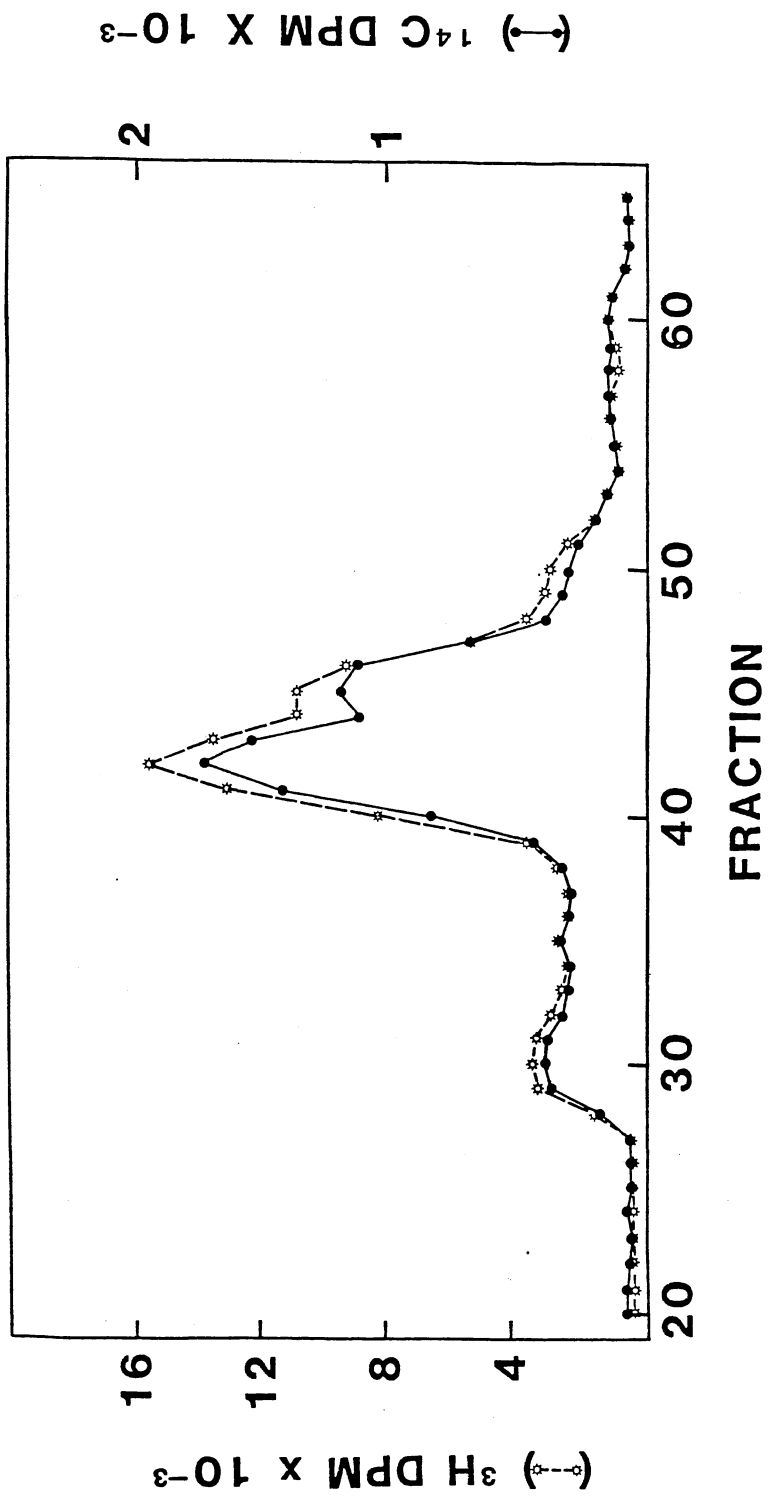

(—•—) 14C DPM X 10-2
10
8
6
4
2
20
30
40
50
60
FRACTION
10
8
6
4
2
(---☼---) 3H DPM X 10-3

Figure 24. Gel Filtration of Oligosaccharides from Control and Group III MAT-CH ASGP-1 on Bio-Gel P-4. [^{3}H]glucosamine-labeled oligosaccharides of MAT-CH ASGP-1 from group III (120 mg retinoic acid/kg diet) (✲) mixed with [^{14}C]glucosamine-labeled oligosaccharides of control MAT-CH ASGP-1 (●).


(⊷) 14C DPM X 10-3
2
1
60
50
40
30
20
FRACTION
16
12
8
4
(⊶) 3H DPM x 10-3

at peak II. The peak II oligosaccharides of group I appeared to be less heterogeneous than that for control. The whole oligosaccharide pattern was significantly ($P < .05$) shifted to the right from control in one of the cell combinations of I vs. control. The oligosaccharide patterns of groups II and III seemed to match that of the control (Figure 24) except for, in some instances, a very slight decrease in the relative amount or an increase in heterogeneity of the oligosaccharides of peak III.

Discussion

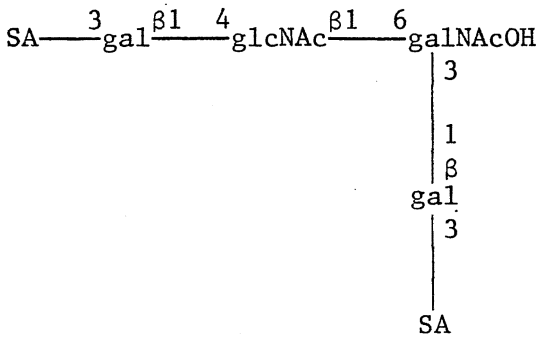
In this chapter, the effects of dietary retinyl palmitate and retinoic acid on the density, molecular weight and oligosaccharide patterns of the 13762 rat mammary adenocarcinoma cell membrane major glycoprotein ASGP-1 were investigated. The effects varied with the ascites tumor sublines used and/or with the type and concentration of retinoid used. The observations on the density of the glycoprotein varied from no effects (MAT-B1 groups I, III and IV; MAT-C3 groups II and III; MAT-BH groups II and III) to an increase [MAT-BH group I; MAT-CH group I (one cell combination)], or a decrease [MAT-B1 group II; MAT-C3 group I; MAT-CH groups I (one cell combination), II or III] in the density of the glycoprotein from supplemented rats as compared to control. The densities of glycoproteins depend largely on the carbohydrate/protein ratio and also on the type of carbohydrate residues present. An increase in density suggests a higher carbohydrate/protein ratio and/or a higher percentage of acidic residues, such as sialic acid, which bind Cs^+, and tend to have lower partial specific volumes. On the other hand, a decrease in density suggests

a lower carbohydrate/protein ratio and/or a decrease in the percentage of acidic residues.

The glycoproteins' molecular weights also varied. The molecular weights of ASGP-1 from MAT-B1 groups I, II or IV were not affected by dietary retinoic acid. The molecular weights of the glycoproteins from MAT-C3 groups I, II or III, and MAT-BH groups II and III were lower than those of controls. ASGP-1 from MAT-B1 group III, MAT-BH group I (one replica), or from MAT-CH groups I, II or III was larger than ASGP-1 from the respective controls. A change in the number and/or type of carbohydrate and/or amino acid residues would affect the molecular weight of a glycoprotein.

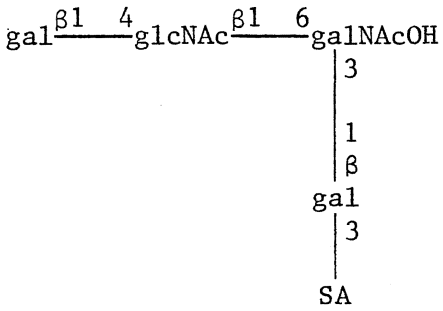
The oligosaccharide patterns of the MAT-B1 and the MAT-BH sublines were as shown previously for the MAT-B1 subline (46). The majority of oligosaccharides were in peaks III and IV. In the case of the MAT-C3 and MAT-CH sublines, the oligosaccharide patterns resembled those previously shown for the MAT-C3 subline (54). Previous studies (54, 89) showed that MAT-B1 peak II was homogeneous and disialylated with the following structure:

II-3:



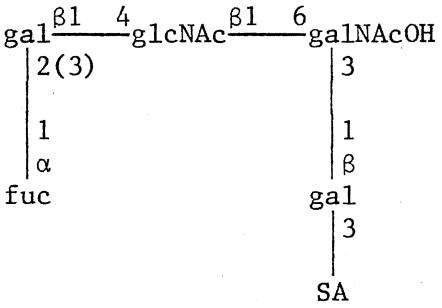
MAT-B1 peak III was found to be heterogeneous with respect to its oligosaccharide composition. It was deduced to be a mixture of three oligosaccharitols as follows (89):

III-2a:



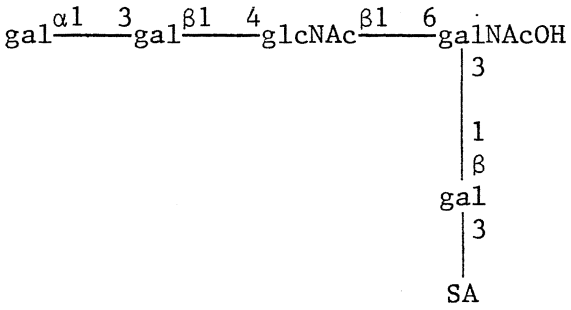
50%

III-2b:



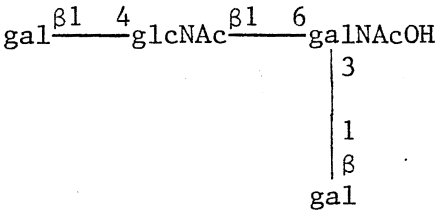
10%

III-2c:



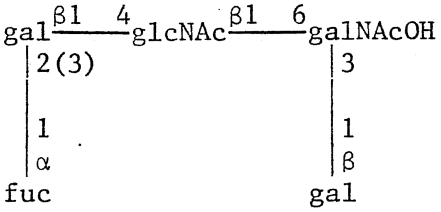
MAT-B1 peak IV was also found to be heterogeneous and neutral. It consisted of 2 fucosylated and two non-fucosylated oligosaccharitol chains with the following proposed structures (89):

IV-1a:



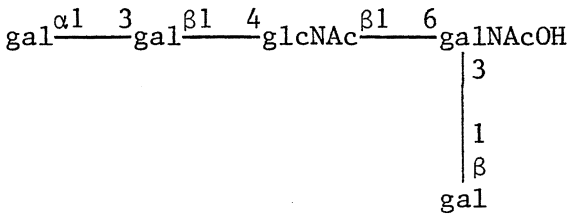
35%

IV-1b:



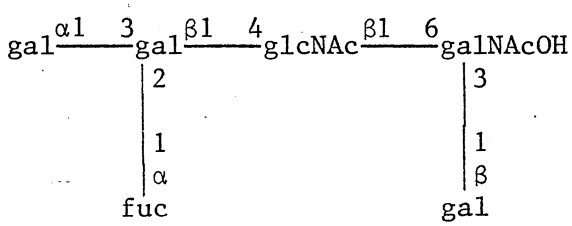
13%

IV-1c:



33%

IV-1d:



18%

The MAT-C3 and MAT-CH oligosaccharide patterns resembled those shown for MAT-C3 (54, 89). The MAT-C3 pattern itself resembled that of MAT-C1 shown by Sherblom et al. (46). The oligosaccharide compositions of MAT-B1 and MAT-C1 ASGP-1 were found to be only quantitatively different, as shown in Table IV [taken from Hull, S. R., 1982 (89)].

The observations that the ^{3}H and ^{14}C peaks of oligosaccharide patterns did not coincide, in some cases, suggested that they differ in oligosaccharide composition. The difference in oligosaccharide composition, in turn, can be due to changes in the number of carbohydrate residues of the oligosaccharides present in a peak, or in the relative amounts of the different oligosaccharides comprising a Bio-Gel P-4 peak. For example, in the case of the MAT-BH subline, group III (Figure 22), the ^{3}H and ^{14}C, did not coincide. Group III ASGP-1 (^{3}H) had peaks III and IV shifted to the left as compared to those from control ASGP-1. This suggested that group III peaks had a higher relative amount of the larger oligosaccharides comprising the peak. The number of carbohydrate residues/oligosaccharide does not seem to be the only determinant of the chromatographic profile

TABLE IV

RECOVERY OF GLUCOSAMINE LABEL IN THE OLIGOSACCHARITOL FRACTIONS OF MAT-B1 AND MAT-C1 ASGP-1[1]

Fraction	% Glucosamine Recovered[o]	
	MAT-B1	MAT-C1
I	8.7	16.3
II	15.6	39.3
III	40.5	34.5
IV	35.0	9.8

[1]Taken from Hull, S. R. 1982 (89).

[o]Radioactive counts were quantitated for each fraction and compared to the total (all fractions) recovered.

and position on Bio-Gel P-4. For example, as shown by Hull (89), MAT-B1 peak II, peak III-2b and III-2c all have 6 sugar residues. However, they chromatograph differently on Bio-Gel P-4. Thus, differences in the types of sugar residues present in the oligosaccharides of a peak, and/or in the number of residues/oligosaccharide could account for the non-coincident peaks.

There are several possible explanations for the changes observed. An increase in the carbohydrate/protein ratio, which leads to an increase in density, might be, for example, due to hydrolytic release of a carbohydrate-poor portion of ASGP-1. A decrease in the carbohydrate/protein ratio would lead to a decrease in density, and could be due to hydrolytic release of carbohydrate rich portions of the glycoprotein. As will be indicated in Chapter III, lysosomes were not observed in the cells examined by electron microscopy. However, it is possible that extracellular hydrolases, secreted by other cells into the peritoneal cavity, could act on ASGP-1. The association of ASGP-1 to another glycoprotein (ASGP-2) at the cell surface (90) makes it accessible to the possible action of extracellular hydrolases. Increases in extracellular hydrolases, upon retinoid treatment, were reported previously (85 - 87). Another factor that could cause changes in carbohydrate composition would be a change in the relative amount or the type of sugars added to, or cleaved off the glycoprotein at the posttranslational level. Several studies have demonstrated the involvement of retinoids in the glycosylation of glycoproteins. Some of these studies have been reviewed in the introduction of this chapter (76-82). Posttranscriptional or posttranslational modifications of the protein portion could also be modulated by retinoids,

resulting in a larger or smaller polypeptide. Either the portion of the respective RNA transcript translated (through differential splicing or alteration of readthrough of termination codons), or the posttranslational processing of the polypeptide could be affected. Several investigators have attempted to relate vitamin A status to RNA metabolism. For example, Tsai and Chytil (91) demonstrated a decreased elongation rate of RNA synthesis, and a decrease in the number of nacent RNA chains in the nuclei of liver cells isolated from vitamin A-deficient animals. Studies by Reese et al. (92) suggested that retinoic acid treatment of rat prostate cells resulted in the induction of alkaline phosphatase activity. Another study by Russell et al. (93) indicated that the antiproliferative effects of retinoids on Chinese hamster ovary cells were related to the retinoids' ability to inhibit the G_1-specific expression of ornithine decarboxylase in those cells. The authors suggested that the inhibition might be due to posttranslational modification of the enzyme by transglutamase (TGase), which would lead to loss of decarboxylase activity. They also suggested that the modified protein might be the one that acts as an initiation factor for ribosomal RNA synthesis (94).

With respect to the studies presented in this chapter, some of the effects on ASGP-1 could be explained by one or a combination of the previously mentioned possible mechanisms. For example, ASGP-1 from group III MAT-C3 cells was as dense, but lower in molecular weight than control MAT-C3 ASGP-1. In addition, that glycoprotein was richer in the lower molecular weight oligosaccharides than control MAT-C3 ASGP-1. This effect could be due to hydrolytic release of both protein and carbohydrate portions of the glycoprotein, in such a

manner that the carbohydrate/protein ratio, and thus the density of the glycoprotein would not be changed. In the case of the MAT-CH ASGP-1 from group III, there was a decrease in the density and an increase in the molecular weight of the glycoprotein as compared to control MAT-CH ASGP-1. The oligosaccharide patterns from the two glycoproteins coincided. The lower density of group III MAT-CH ASGP-1 indicates a lower carbohydrate/protein ratio. Since the molecular weight was higher than that of control, it seemed possible that the hydrolytic posttranslational modification of the protein portion of the glycoprotein was depressed, leading to a larger protein moiety and a lower carbohydrate/protein ratio. Alternatively, the increased density and molecular weight of MAT-BH group I ASGP-1, as compared to MAT-BH control ASGP-1, suggested a greater extent of glycosylation of the MAT-BH group I glycoprotein.

This study and several others demonstrate the involvement of retinoids in glycoprotein production and modification. It appeared that retinoids affect both the protein and the carbohydrate moieties of glycoproteins.

CHAPTER III

EFFECT OF VITAMIN A ON THE MORPHOLOGY AND XENOTRANSPLANTABILITY OF THE 13762 RAT MAMMARY ADENOCARCINOMA SUBLINES

Introduction

Studies have shown that vitamin A and its analogs can modulate the differentiation patterns of several cell types *in vitro* and *in vivo*. Several of these studies were reviewed in Chapters I and II. Differentiation itself is defined by several biochemical as well as morphological parameters that may or may not be affected by retinoids. In an attempt to have a better understanding of the diverse effects of "vitamin A" status on different systems, investigations were directed to the cellular and subcellular level. In fact, several of the different effects of vitamin A on cells have been confirmed by studying the direct action of vitamin A on isolated cell organelles.

It has been shown that vitamin A alters cell surface characteristics in a variety of cell lines. Vitamin A restored contact inhibition of growth in both Balb/c 3T12-3 mouse fibroblasts (95) and L cells (96). In another study, Patt et al. (97) reported that the removal of retinol from the growth medium of 3T3 fibroblast cells slightly increased their saturation density, and that its addition reduced it in a dose dependent manner. In a contradictory report

(98), neither the 3T3 nor the L929 fibroblastic cells showed a significant change in growth rate or saturation density upon treatment with free or RBP-bound[1] retinol.

Studies on cell adhesiveness indicated that retinol and retinoic acid increased the adhesion of Balb/c 3T12-3 transformed mouse fibroblasts to the substratum (99). In another study using Ag8-1 Syrian hamster kidney fibroblasts (100), retinoic acid caused an increase in cell-substratum adhesiveness and a decrease in cell-to-cell cohesiveness. Hassell et al. (75) showed that chondrogenesis was inhibited by vitamin A. During this process, the cell shape was affected and the cellular accumulation of fibronectin was altered. Ogiso et al. (101) observed that retinoic acid treatment of a pluripotent teratocarcinoma cell line resulted in a decrease in the F9 antigens, an enhanced secretion of plasminogen activator and the disappearance of peanut agglutinin receptors. Studies by Lotan (102) on the susceptibility of different human melanoma and breast carcinoma cell lines to retinoic acid growth inhibition indicated that the SK-BR-3 and 734B carcinoma cell lines were inhibited by 83 and 50%, respectively. Both cell types spread and assumed a flattened morphology after 72 hr exposure to retinoic acid. Studies on the erythrocyte membrane indicated that the addition of vitamin A to erythrocytes in vitro induced cytoplasmic vacuolation followed by hemolysis (103). Distortion of red blood cell membrane by vitamin A was evidenced by the presence of cup shaped cells or those with dimpling of the cell membrane after 1 min exposure to 20 μg of vitamin

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[1] Retinol binding protein.

A/ml of red cell suspension (104). Distention of the endoplasmic reticulum, the formation of lysosomes and the swelling of mitochondrial and Golgi apparatus vesicles were observed after the addition of vitamin A to fibroblasts in vitro (105). Matter and Bollag (106) observed a marked regression of mouse skin papillomas upon a single injection of the aromatic retinoid Ro 10-9359. Electron microscopic studies indicated that the drug caused an increase in the production of mucopolysaccharides and their secretion into the extracellular space. Vacuolization and loss of cytoplasmic constituents into the extracellular space following labilization of the plasma membrane were also observed. In addition, there was an increase in formation of lysosomes and necrotic cells. In another study on the keratoanthoma, topical applications of retinoic acid caused an increase in the number of Golgi cisternae and vesicles, and in the rough entoplasmic reticulum. This was accompanied by the formation of gap junctions (107). Brown et al. (108) studied the effect of vitamin A on the morphology of differentiating keratinocytes in vitro. High retinoid concentration in the medium resulted in the loss of the ability to stratify and keratinize. The cells were "oddly shaped", contained many vacuoles and developed elongated microvilli. Vitamin A also caused mitochondrial swelling (109), and labilization of lysosomal membranes (110, 111).

In several of the previously reviewed studies, it was suggested that retinoids exert most of their effects on cellular morphology via their actions on the different cellular membranes. It has been shown that once incorporated into membranes, retinol can be readily removed with chloroform/methanol, but is difficult to extract with hexane

(112). This observation and others on the effects of retinoids on membranes suggest a retinoid-lipoprotein interaction. However, there were studies on protein-free lipid monolayers (113, 114) and bilayers (115-118) which demonstrated that retinoids affect several physical parameters of the lipid bilayer component of membranes. All the previously reviewed studies, and others, demonstrate the potential of retinoids in affecting cellular growth and differentiation in several systems. Retinoids exert their actions on these processes via their effects, at the subcellular level, on the different cellular membranes and organelles. Based on the results of these many investigations, the effects of vitamin A on the morphology, growth and xenotransplantability of the 13762 rat mammary adenocarcinoma sublines were studied. The results are presented in this chapter.

Materials and Methods

The animals, diets, experimental design and cell isolation procedures were as described in Chapter II. The samples examined from each dietary group consisted of a collection of cells from all the different rats of that group.

Scanning Electron Microscopy (SEM)

Cells (2-5 x 10^7/ml saline (0.9% w/v NaCl)) were washed once to three times with saline and centrifuged at 100 x g. They were added to ∿10 volumes of 1% glutaraldehyde in 0.1 M cacodylate, 0.1 M sucrose buffer, pH 7.4, and fixed overnight at 4^{o}C. In the case of the MAT-B1 and MAT-C3 sublines, the cells (while still in fixative) were applied

to serum coated coverslips prepared by incubating sterile coverslips in 10% calf serum McCoys medium for 24 hrs. The cells were allowed to settle on the coverslips overnight at $4^{o}C$. In the case of the MAT-BH and MAT-CH sublines, the cells were washed three times in buffer, applied to polylysine-coated coverslips and allowed to settle for 15 min. The samples (after three washings in buffer in the case of MAT-B1 and MAT-C3 cells) were then treated with 1% osmium tetroxide in 0.1 M cacodylate, 0.1 M sucrose buffer for 30 min. They were washed one to three times in buffer and then dehydrated with a graded series (50%-100%) of aqueous ethanol solutions. The samples were dried at critical point using liquid CO_2. The coverslips were mounted on aluminum stubs and coated with 150 Å (MAT-B1 and MAT-C3) to 200 Å (MAT-BH and MAT-CH) of gold palladium. Samples were viewed with a Jeol JSM-35 scanning electron microscope operated at 15 kv. Micrographs were recorded on Polaroid film, type 55.

Transmission Electron Microscopy (TEM)

Cells (2-5 x 10^{7}/ml saline) were washed one to three times with saline and centrifuged at 100 x g. They were added to ∿10 volumes of 1% glutaraldehyde in 0.1 M cacodylate, 0.1 M sucrose buffer, pH 7.4, and fixed overnight at $4^{o}C$. The samples were washed three times in buffer and fixed secondarily with 2% osmium tetroxide in buffer for two hours. The cells were washed thoroughly in H_2O (MAT-B1 and MAT-C3) or in buffer (MAT-BH and MAT-CH). In the case of the MAT-B1 and MAT-C3 sublines, the samples were stained in 1% aqueous uranyl acetate overnight at room temperature, and then washed once in water. (Note: The MAT-BH and MAT-CH samples were not stained at this stage.)

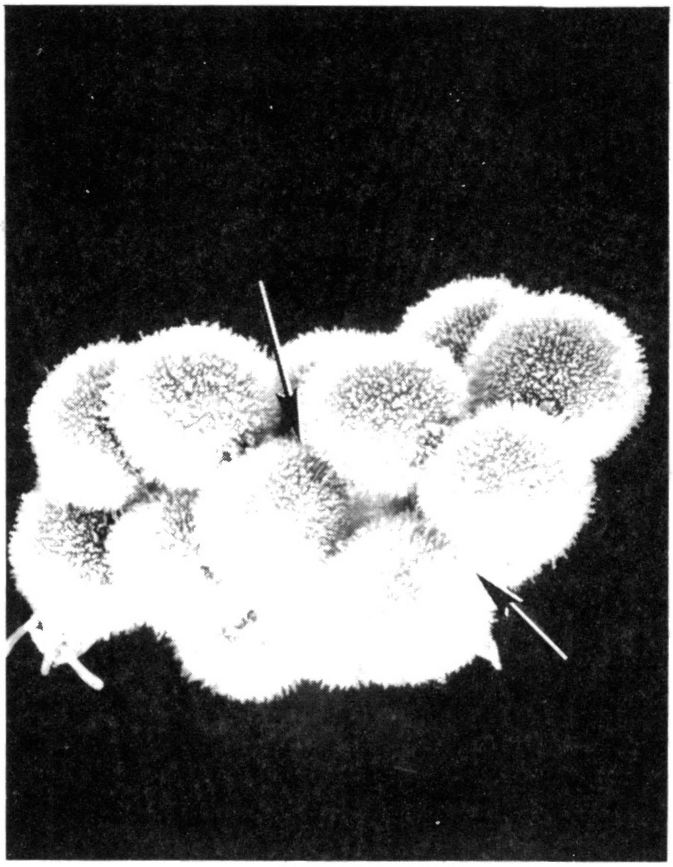
The samples were dehydrated with a graded series (50-100%) of ethanol and then washed three times in propylene oxide. The samples were infiltrated with a 1:1 propylene oxide and polybed and were capped either overnight (MAT-B1 and MAT-C3) or for 48 hrs (MAT-BH and MAT-CH). The samples were then left uncapped for 7 hrs under vacuum. The cells were then imbedded in 100% polybed and cured at 60°C for 48 hrs. Silver sections were obtained using a diamond knife. Staining of the sections was performed using uranyl/acetate and lead citrate. Sections were viewed in a Philips EM 200 electron microscope.

Parameters Studied by SEM and TEM

Scanning electron microscopy was used to characterize the surface morphologies of the 13762 rat mammary adenocarcinoma. Besides the general morphology, two main parameters were evaluated: (1) number and degree of branching of the microvilli and (2) the frequency of contact between the tumor cells to form clusters of two or more cells (Figure 25). Both the number of branched microvilli per cell, and the number of branches/microvillus contribute to the degree of branching and were not separated in this study. The observed morphologies were categorized as previously defined (47, 54) where possible. The rest were termed "uncategorized".

Transmission electron microscopy was used to study the fine structure of the cells: (1) Cell viability was determined following the general fine structures of the cytoplasm and nucleus. Necrotic cells were defined by their disintegrating cytoplasm and their nuclei

Figure 25. Scanning Electron Micrograph Illustrating a Cluster of Cells. MAT-C3 cells from a rat fed the purified diet supplemented with 12 mg retinoic acid/kg diet (MAT-C3 group I). Note microvilli linking the cells (arrows). X 2500



with marginated chromatin[2]; (2) the shape of the cell was also determined, e.g. spherical, oval, irregular, etc.; (3) the frequency and type of contact between cells. In microvillar contact (Figure 26A), microvilli from one cell touched the microvilli or bodies of other cells. In cytoplasmic contact cytoplasm from different cells was continuous forming cytoplasmic bridges (Figure 27). If the cells were involved in both types of contact, they were categorized under 'cytoplasmic contact'. (4) Nucleoli were characterized by their position within the nucleus (119) (Figure 28). A nucleolus was considered marginated when it was located against the inner membrane of the nuclear envelope (Figure 28B). It was considered non-marginated when it was located towards the central region of the nucleus away from the nuclear envelope (Figure 28A). The number of nucleoli per cell was also noted. Nucleolar margination and the increase in the number of nucleoli per cell were associated with increased protein synthesis, as reviewed by Ghadially (119). Cells were categorized according to number of nuclei per cell and the position of the nucleolus within the nucleus. (5) Mitochondrial configuration was determined according to Ghadially (119). Normal looking mitochondria were referred to as showing an orthodox configuration 'high energy form' (Figure 29A), and mitochondria with dense matrix and swollen cristae as showing the condensed configuration, 'low energy form' (Figure 29B). Cells were categorized into those having mitochondria in the orthodox configuration, mitochondria in the condensed

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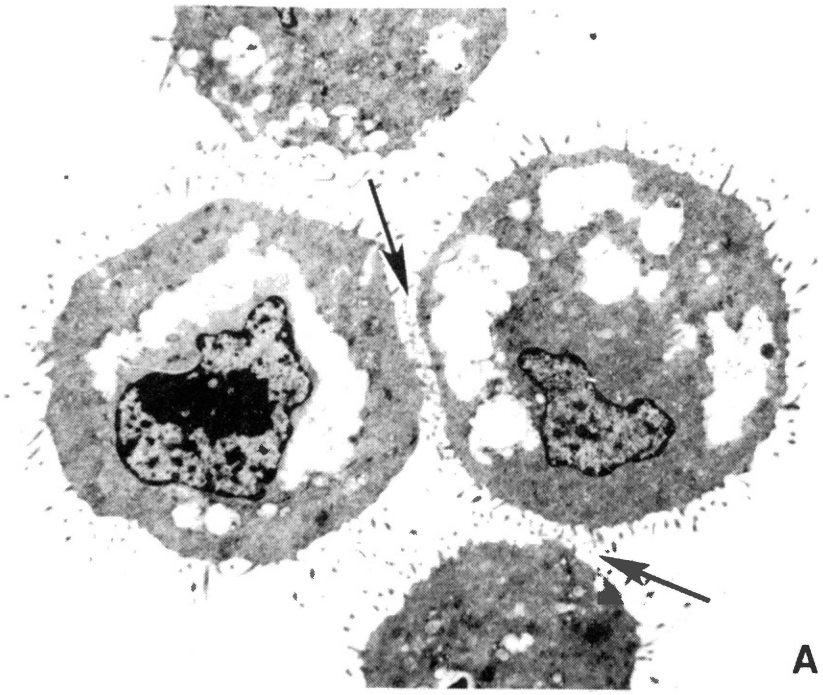
[2]A condensation of chromatin occurs along or adjacent to the inner nuclear membrane, while chromatin disappears from other parts of the nucleus (119).

Figure 26. Transmission Electron Micrographs of MAT-BH Cells Illustrating the Criteria Used for Evaluating Contact.

A. MAT-BH cells from a rat fed the purified diet supplemented with 4.5 mg retinyl palmitate/kg diet (MAT-BH group I). Note microvillar contact between cells (arrows); the microvilli of a cell are touching the microvilli or the bodies of other cells. X 5000

B. MAT-BH cells from a rat fed the purified diet supplemented with 120 mg retinoic acid/kg diet (MAT-BH group III). There is no contact between the cells (arrow). X 5000


A

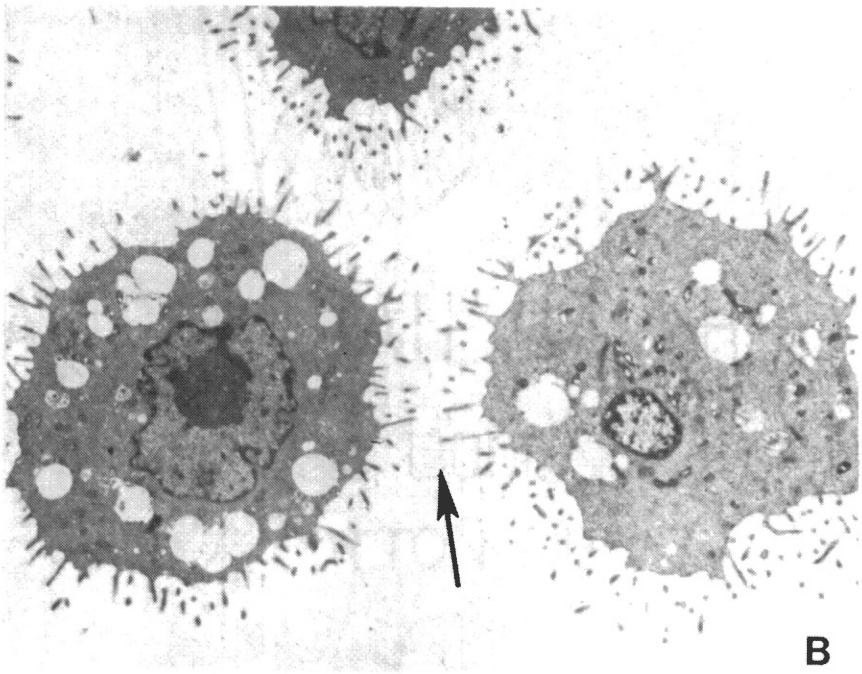

B

Figure 27. Transmission Electron Micrograph of MAT-CH Cells Illustrating the Criterion for Defining Cytoplasmic Contact. MAT-CH cells from a rat fed the purified diet supplemented with 120 mg retinoic acid/kg diet (MAT-CH group III). Note cytoplasmic bridges and continuity of cytoplasm from different cells (arrows). X 5500

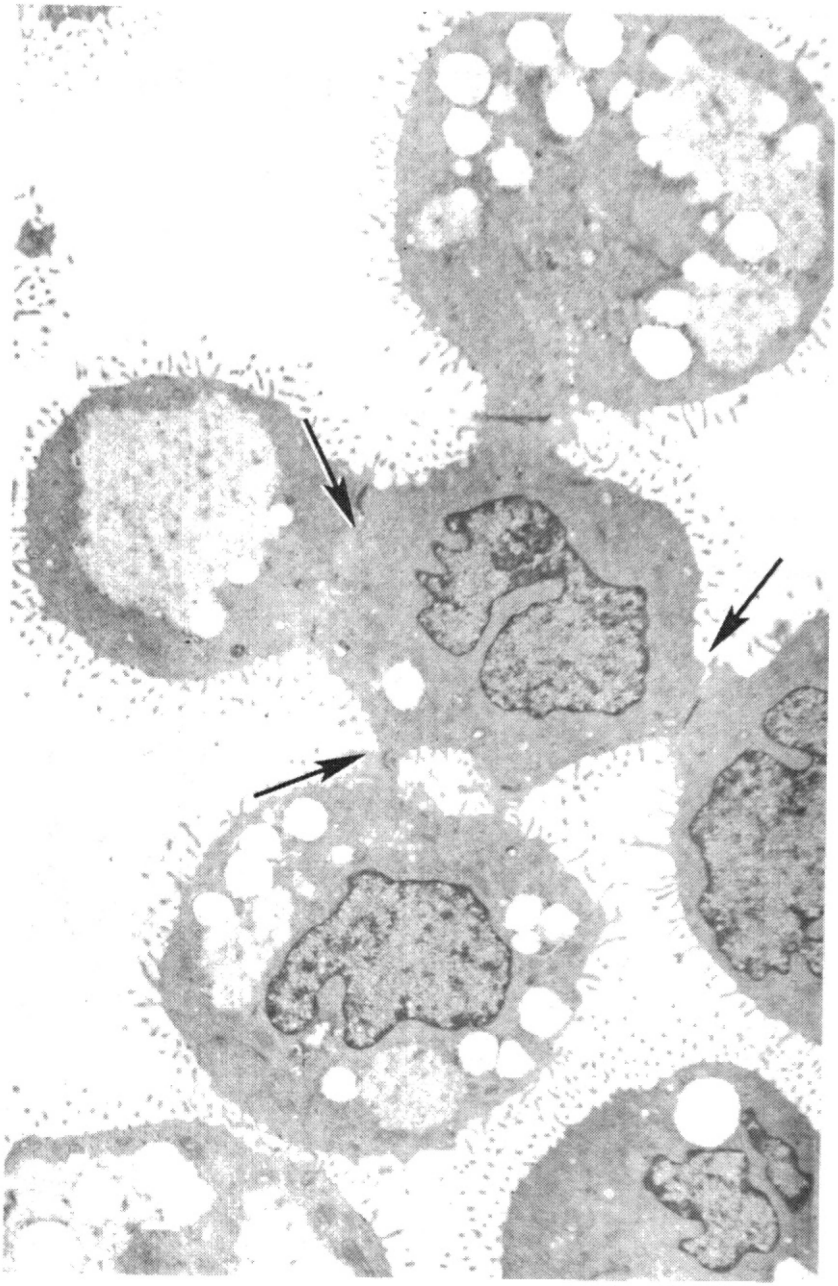
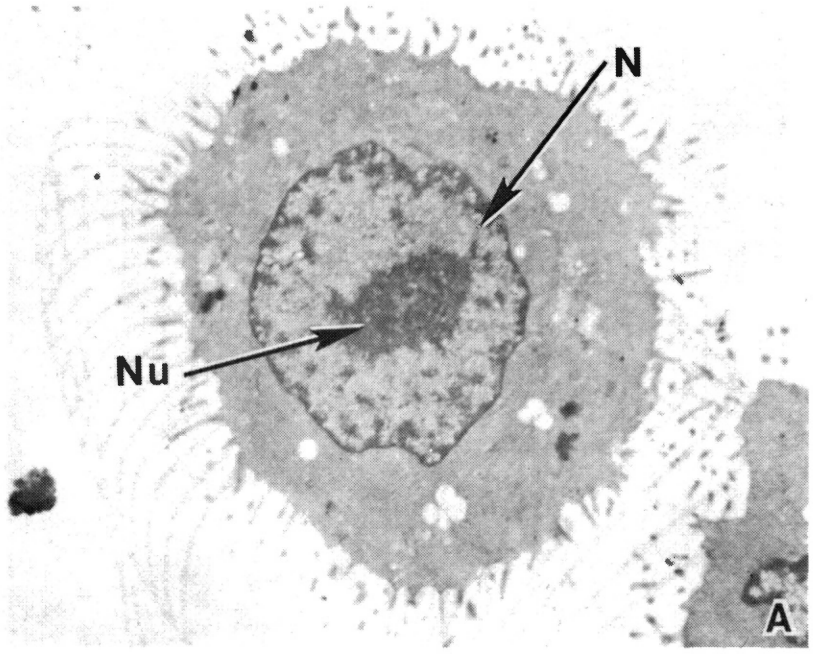


Figure 28. Transmission Electron Micrographs of 13762 Rat Mammary Adenocarcinoma Cells Illustrating the Criteria Used for Defining the Position of the Nucleolus Within the Nucleus.

A. MAT-BH cell from a rat fed the purified diet supplemented with 120 mg retinoic acid/kg diet (MAT-BH group III). The cell has a non-marginated nucleolus (Nu) which is located towards the central region of the nucleus (N) away from the nuclear envelope. X 6500

B. MAT-CH cell from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-CH group II). The cell has a marginated nucleolus (Nu) which is located against the inner membrane of the nucleus (N). X 6500


N
Nu
A

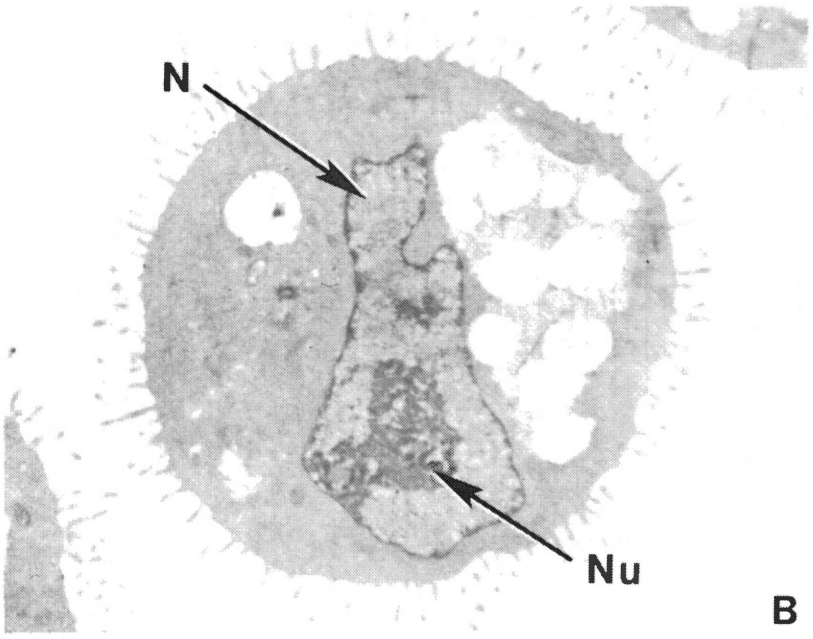
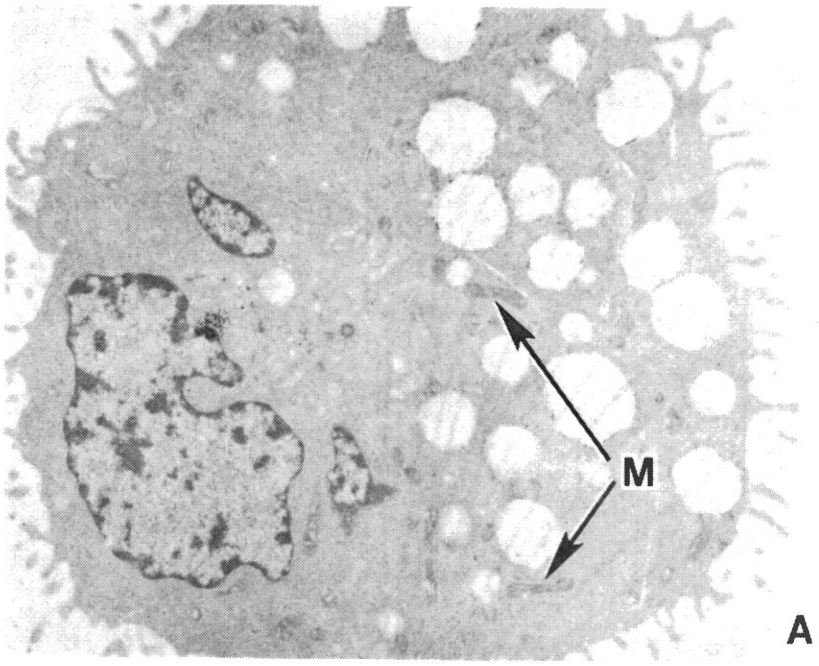
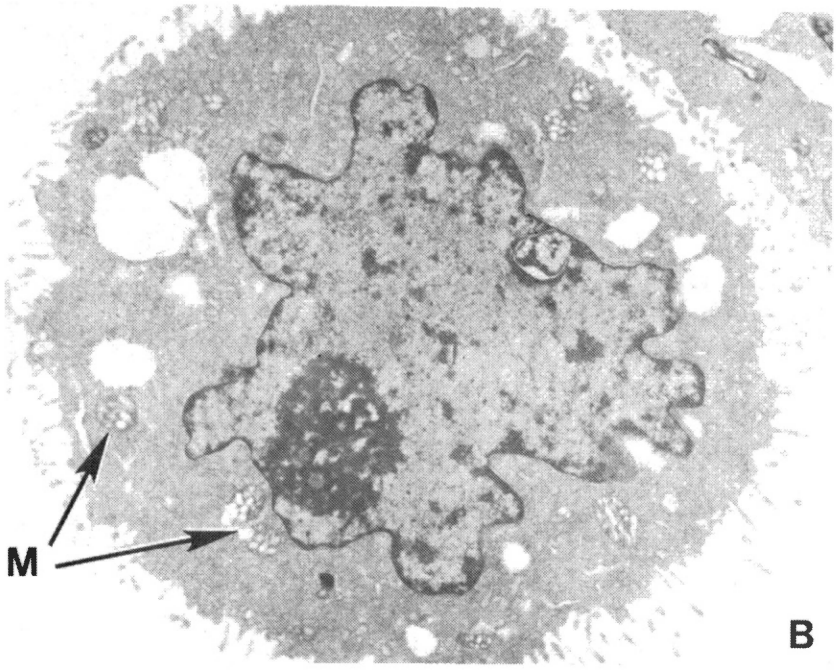

N
Nu
B

Figure 29. Transmission Electron Micrographs of 13762 Rat Mammary Adenocarcinoma Cells Illustrating the Criteria Used for Defining Mitochondrial Configurations.

A. MAT-BH cell from a rat fed the purified diet supplemented with 120 mg retinoic acid/kg diet (MAT-BH group III). The mitochondria (M) are in the orthodox configuration, "high energy form". X 8000

B. MAT-CH cell from a control rat fed the non-purified diet. The mitochondria (M) of this cell are in the condensed configuration, "low energy form". Note markedly ballooned mitochondrial cristae and dense mitochondrial matrix. X 8000


M
A


M
B

configuration, or a mixture of both.

(6) Glycogen appeared as lucent or faintly stained areas (glycogen lakes) (119) within the cytoplasm. The glycogen content was estimated and cells were categorized into four different categories: "0", no glycogen; "+", small amount of glycogen; "++", moderate amount of glycogen; "+++", large amount of glycogen (Figure 30). (7) Lipid appeared as electron-lucent to moderately electron-dense droplets (119). The small droplets were usually spherical in shape. Large droplets were usually distorted or irregular in shape (Figure 30A). Cells were categorized according to the number of lipid droplets per cell.

Tumor Growth and Xenotransplantability

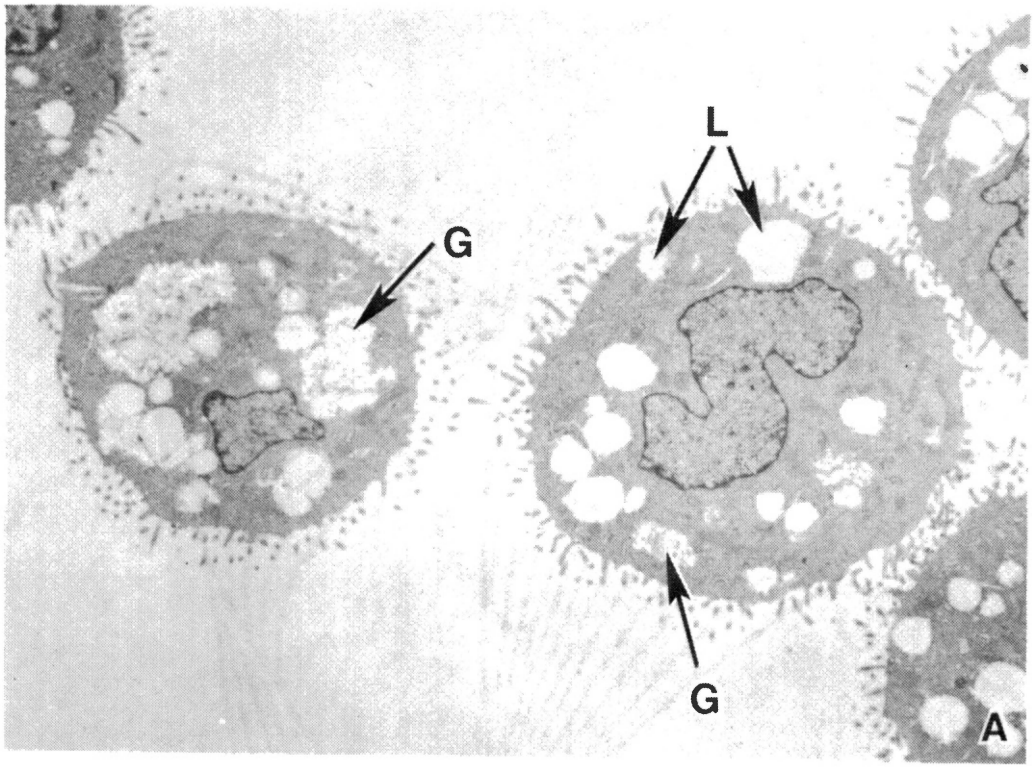
The effect of vitamin A on ascitic tumor growth and xenotransplantability was investigated: (1) The incidence of the MAT-BH and MAT-CH tumor development in the rats of the different dietary groups were recorded (Table V). (2) Transplantation of 13762 ascites cells into C57BL/6j mice on different vitamin A diets was performed as shown in Tables VI and VII.

The mice (weanling) were processed through routine quarantine procedures. In the first experiment, in which the effect of dietary retinoic acid on xenotransplantability was studied, the mice were fed the closed formula non-purified diet until they were required for the experiment. The experiment started by dividing the mice into 3 major dietary groups (Table VI): (1) A control group in which the mice were kept on the non-purified diet; (2) a group fed the vitamin A-deficient purified diet supplemented with 4.5 mg retinyl palmitate/kg diet;

Figure 30. Transmission Electron Micrographs of 13762 Rat Mammary Adenocarcinoma Cells Illustrating the Criteria Used for Estimating the Glycogen Content, and for Defining Lipid.

A. Two MAT-CH cells from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-CH group II). The cell on the right has a "+" glycogen (G) content. The one on the left has a "++" glycogen content (G). L, lipid droplets. X 5000

B. Two MAT-CH cells from a rat fed the purified diet supplemented with 120 mg retinoic acid/kg diet (MAT-CH group III). The cell on the right has no "0" glycogen. The cell on the left has a "+++" glycogen (G) content. X 5000


L
G
G
A

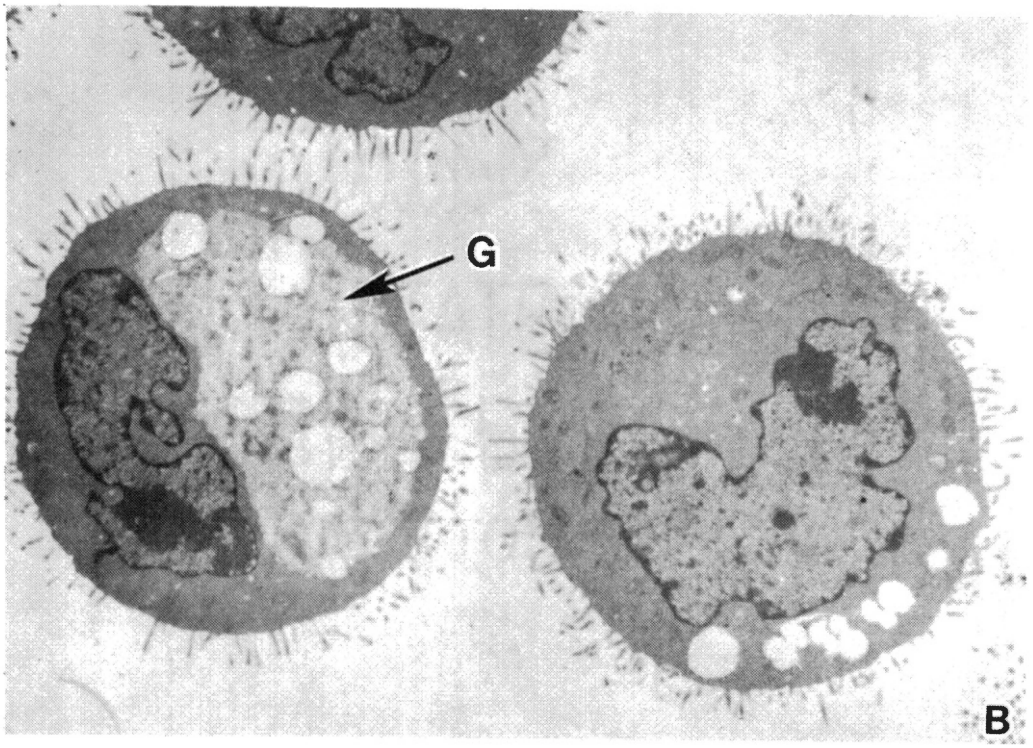

G
B

TABLE V

EFFECT OF VITAMIN A ON TUMOR GROWTH

Tumor Line	Group	No. of Rats	% Incidence[1]	
MAT-BH	C	5	40	(2/5)
	I	6	50	(3/6)
	II	6	100	(6/6)
	III	6	100	(6/6)
MAT-CH	C	5	40	(2/5)
	I	4	100	(4/4)
	II	5	80	(4/5)
	III	5	100	(5/5)

[1]Incidence: Number of animals with tumors/total number of animals.

TABLE VI

XENOTRANSPLANTABILITY OF MAT-BH AND MAT-CH IN MICE

Injection	Total No. of Mice	Diet	No. of Mice Dead
MAT-BH	8	Non-purified Diet	2 Died[1]
	6	4.5 mg RP[3]/kg Diet	3 Killed[2]
	10	120 mg RA[4]/kg Diet	2 Killed
MAT-CH	8	Non-purified Diet	1 Died
	6	4.5 mg RP/kg Diet	2 Killed
	10	120 mg RA/kg Diet	0
Saline	3	Non-purified Diet	0
	2	4.5 mg RP/kg Diet	0
	8	120 mg RA/kg Diet	0

[1]Mice died, spontaneously, of tumor growth.

[2]Mice appeared very ill, and were killed.

[3]Retinyl palmitate.

[4]Retinoic acid.

TABLE VII

XENOTRANSPLANTABILITY OF MAT-BH AND MAT-CH IN MICE FED VITAMIN A-DEFICIENT DIET

Injection	Total No. of Mice	Diet	No. of Mice with Tumors[1]	No. of Mice Dead	Average Serum Retinol Conc. µg/dl
MAT-BH	13	Vitamin A-Deficient Purified Diet	9	3	21[2]
	5	Non-purified Diet	3	3	16[3]
MAT-CH	12	Vitamin A-Deficient Purified Diet	9	0	21[4]
	5	Non-purified Diet	0	0	21[5]
Saline	12	Vitamin A-Deficient Diet	0	0	19[6]

[1]As judged by the physical symptoms of tumor growth as discussed in text.

[2,4,6]Each value was obtained from the average of three determinations on three out of each group.

[3,5]Each value is obtained from the average of two determinations on two out of five mice in each group.

[3]The low value might be due to the low amount of serum obtained from one of the mice tested.

(3) a group fed the vitamin A-deficient purified diet supplemented with 120 mg retinoic acid/kg diet. The mice (∿70 days old) were injected with 1 x 10^6 MAT-BH or MAT-CH tumor cells, or with 0.9% NaCl, at the beginning of the third week of the experimental period. Tumor growth was monitored for 11 weeks after tumor injection.

In the second experiment, the effect of vitamin A deficiency on xenotransplantability was studied. The weanling mice were started on the non-supplemented vitamin A-deficient purified diet upon arrival. A respective control group was fed the non-purified diet. The groups were kept on their respective diets for 16 weeks, after which the mice were injected with MAT-BH or MAT-CH tumor cells, or with 0.9% NaCl. The mice were monitored periodically for 8 weeks. The mice were then killed and the serum retinol levels were determined for sample mice from each group, using the method of Bieri et al. (120). As shown in the Results section, mice fed the vitamin A-deficient diet appeared to have normal serum retinol levels.

Statistical Analysis

The Chi square test was used to detect significant effects of diet on the different parameters studied. Significance levels were denoted between parenthesis w-en a statistically significant effect was observed.

Results

Scanning Electron Microscopy

The MAT-B1 Subline. The control MAT-B1 cells resembled the

typical MAT-B1 cells which have been maintained as a stable line over several years (47). They were spherical in shape and had unbranched microvilli. Some had more microvilli than others. Ruffles, ridges and blebs were often associated with the cell surface (Figure 31). There were few free cells, the majority being in clusters or aggregates of two or more with a low to moderate amount of contact between cells. The characteristics of the MAT-B1 cells from group I and II did not differ much from those of control; however, there might have been an increase in the surface density of microvilli with increased retinoic acid concentration in the diet.

The MAT-C3 Subline. The control MAT-C3 cells had a mixed morphology. Most of the cells resembled the MAT-C cells (47) and some resembled the MAT-MR2-S morphology (54) (Figure 32). The control MAT-C3 cells had numerous unbranched microvilli and exhibited occasional blebs. Cells with branched microvilli were not seen in the samples examined. Some of the cells were present in clusters of two or more while others were free of contact to other cells. There were usually quite a few contact points between one cell and another in a clump. The predominant morphology of the MAT-C3 cells from group I was similar to that shown by control MAT-C3 cells. However, some of the cells had some ridged microvilli and/or indentations on the cell surface (Figure 33A). Branched microvilli occurred very rarely. Both cell clumps and free cells were seen. The MAT-C3 cells derived from group II (60 mg RA/kg diet) appeared to be highly adhesive and clumped. While the control MAT-C3 cells appeared spherical, most of the MAT-C3 cells from group II were not. Some

Figure 31. Scanning Electron Micrograph of MAT-B1 Cell from a Control Rat Fed the Non-purified Diet. Note that the microvilli are not branched and often curved. There are areas free of microvilli. X 11,000

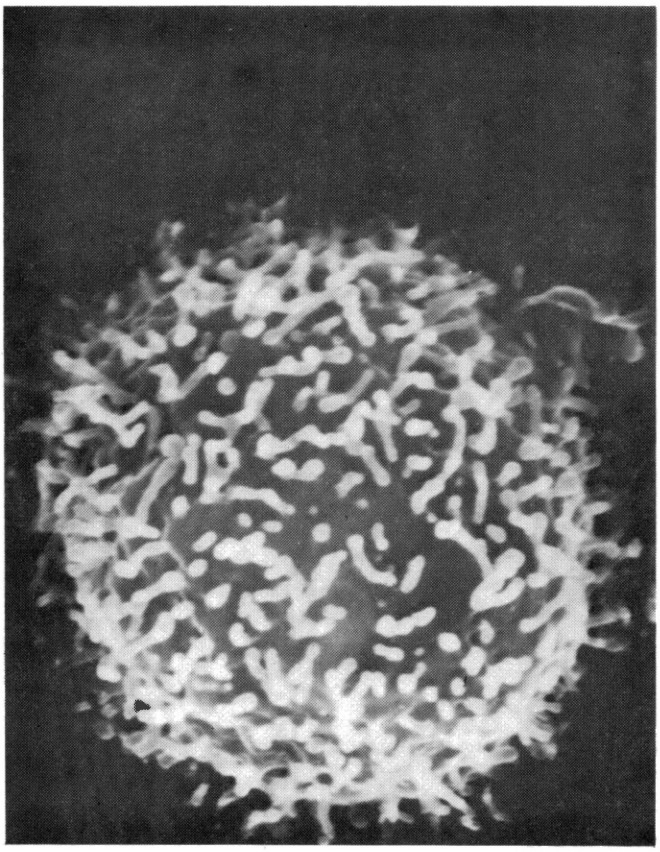
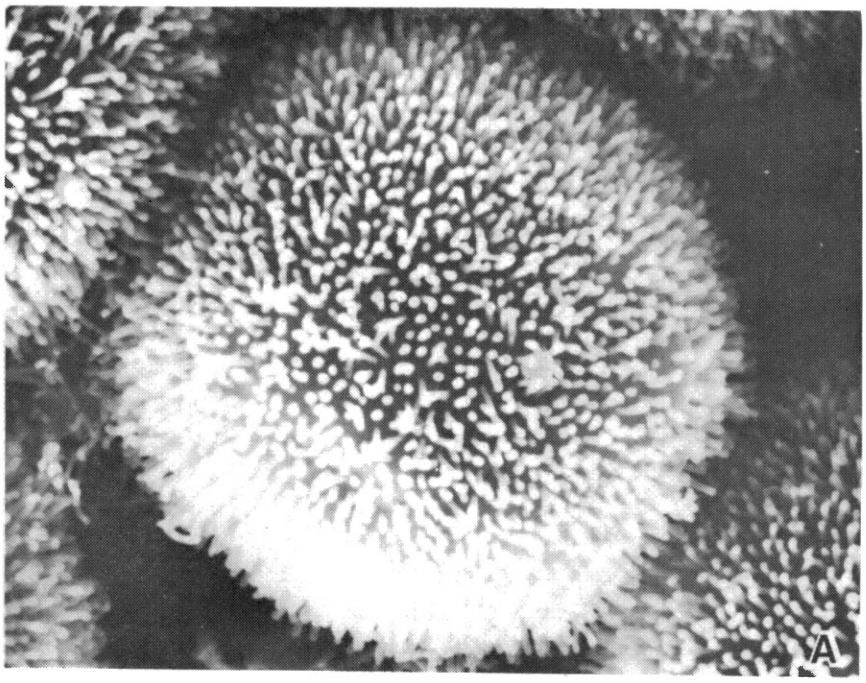


Figure 32. Scanning Electron Micrographs Illustrating the Different Morphologies of MAT-C3 Cells from Control Rats Fed the Non-purified Diet.

A. A MAT-C3 cell resembling the MAT-C (45) morphology with numerous short, stubby and unbranched microvilli extending straight from the cell body. X 9000

B. A MAT-C3 cell resembling the MAT-MR2-S morphology (54) with unbranched microvilli. X 8500


A

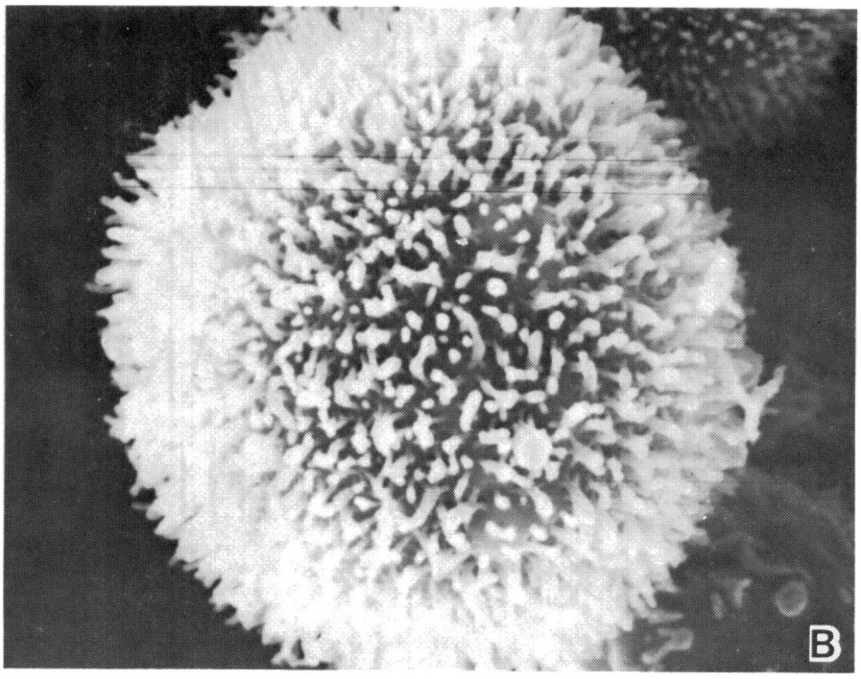
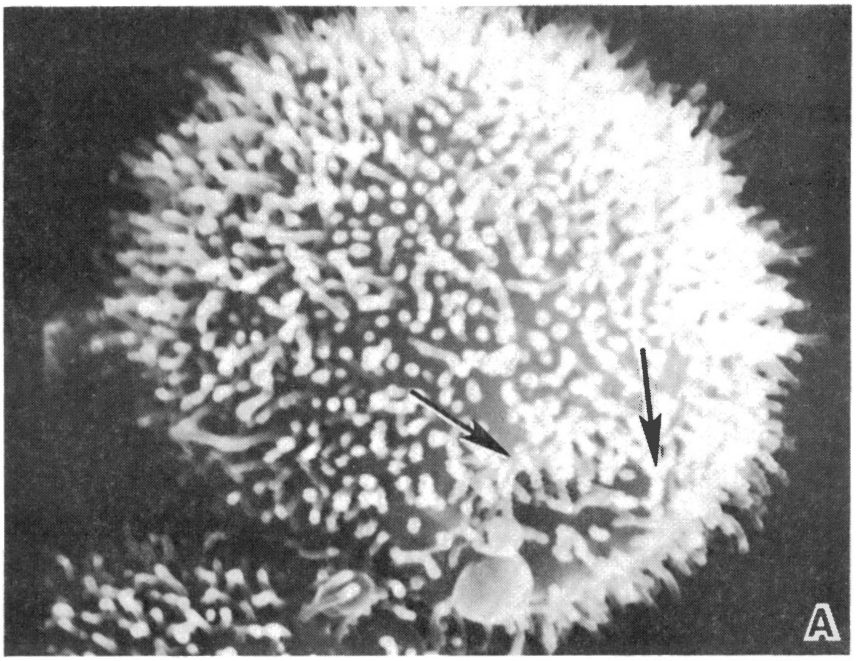
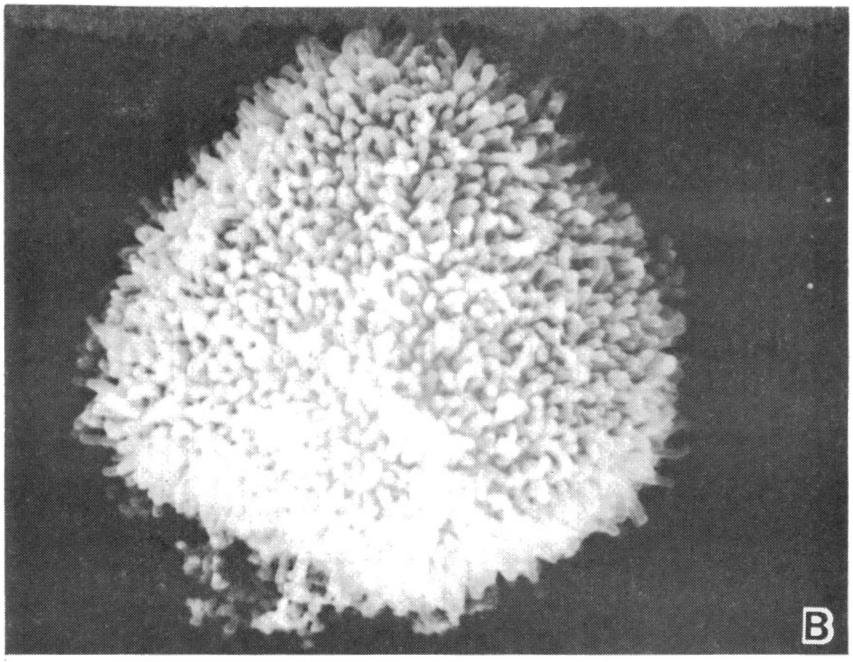

B

Figure 33. Scanning Electron Micrographs of MAT-C3 cells from Groups I and II.

A. Scanning electron micrograph of MAT-C3 cell from a rat fed the purified diet supplemented with 12 mg retinoic acid/kg diet (MAT-C3 group I). Note the ridged microvilli (arrows) and the indentation of the cell surface. X 12,000

B. Scanning electron micrograph of MAT-C3 cell from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-C3 group II). Note the increase in the number and length of microvilli, and the distortion of shape as compared to Figure 32. X 8000


A


B

of the cells had an oval or elongated shape while others had irregular surfaces (Figure 33B). Indentations of the cell surface were also observed. The microvilli were still unbranched but appeared longer than those observed in the case of control and group I MAT-C3 cells. Several long microvilli appeared to link the cells of a clump (Figure 34).

The MAT-BH Subline. The MAT-BH subline appeared to be a heterogeneous population as examined by scanning electron microscopy and illustrated in Figures 35 and 36. The majority of the population from each of the diet groups was categorized into several previously described morphologies (47). Some cells had the MAT-B1 morphology (Figure 35B), while others looked like the MAT-B cells (Figure 35A). In addition, some had the MAT-C1 morphology with branched microvilli (Figure 36B) while another group resembled the MAT-C cells (Figure 36A). The rest of the population was a mixture of morphologies that could not be categorized into any of the previously identified morphologies. Besides the general morphology, two main parameters, the frequency of contact between cells, and the degree of microvillar branching, were examined, as shown in Tables VIII and IX, respectively. There was a significant ($P < .05$) effect of diet on the frequency of contact, the significant difference being between group I and the control ($P < .005$). The fraction of cells in group I that were involved in contact ($\sim$98%) was larger than that of control ($\sim$80%). Of groups II and III, $\sim$80% and $\sim$85% of the cells were involved in contact, respectively, and were not significantly different from control. The number of cells with branched microvilli was also

Figure 34. Scanning Electron Micrograph of MAT-C3 Cells from Group II. MAT-C3 cells from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-C3 group II). Note long tangled microvilli linking the cells in a cluster. X 11,500

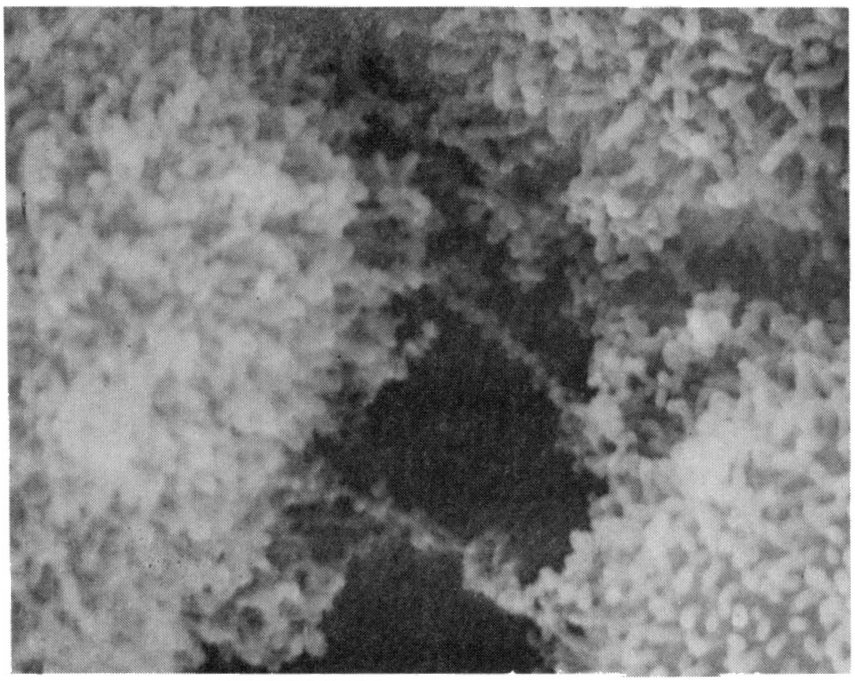
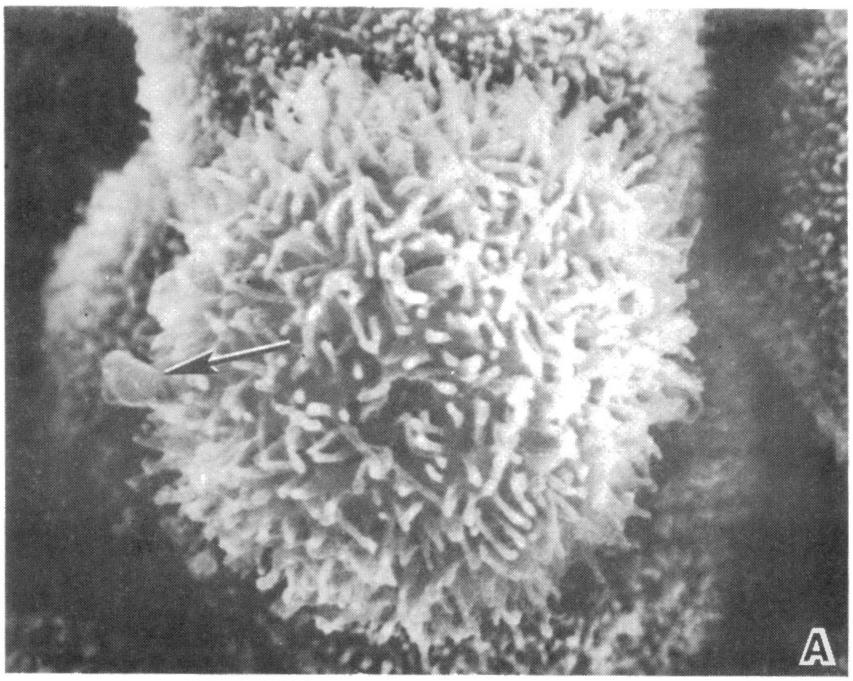


Figure 35. Scanning Electron Micrographs Illustrating the Different Morphologies of the Heterogeneous MAT-BH Subline.

A. MAT-BH cell from a control rat fed the non-purified diet. The morphology resembles that of the MAT-B cells (45). Note ruffled surface, occasional blebs (arrow) and unbranched microvilli. X 7500

B. Scanning electron micrograph of a MAT-BH cell from a rat fed the purified diet supplemented with 4.5 mg retinyl palmitate/kg diet (MAT-BH group I). The morphology resembles that of MAT-B1 cells (45). Note long curved microvilli, some of which adhere closely to an irregular cell surface. X 7000


A

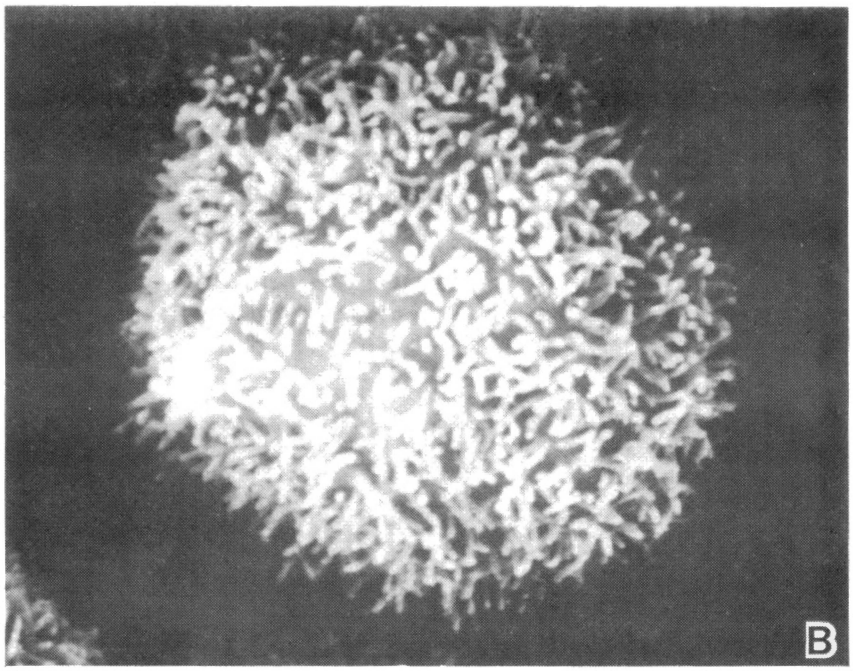
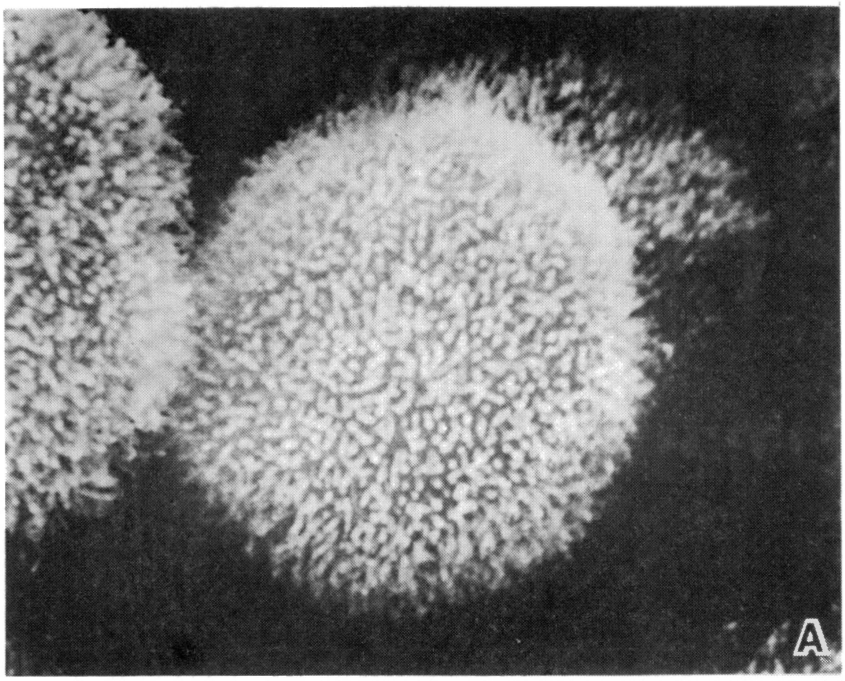

B

Figure 36. Scanning Electron Micrographs Illustrating the Different Morphologies of the Heterogeneous MAT-BH Subline.

A. MAT-BH cell from a control rat fed the non-purified diet. The morphology resembles that of MAT-C cells (45). Note numerous short, stubby microvilli extending straight from the cell surface. X 6000

B. MAT-BH cell from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-BH group II). The morphology resembles that of MAT-C1 morphology (45). Note irregular surface, covered with microvilli, several of which are extensively branched (arrows). Microvilli appear clustered and branching is not obvious when viewed straight on. Branching is more readily observed at the periphery. X 6000


A

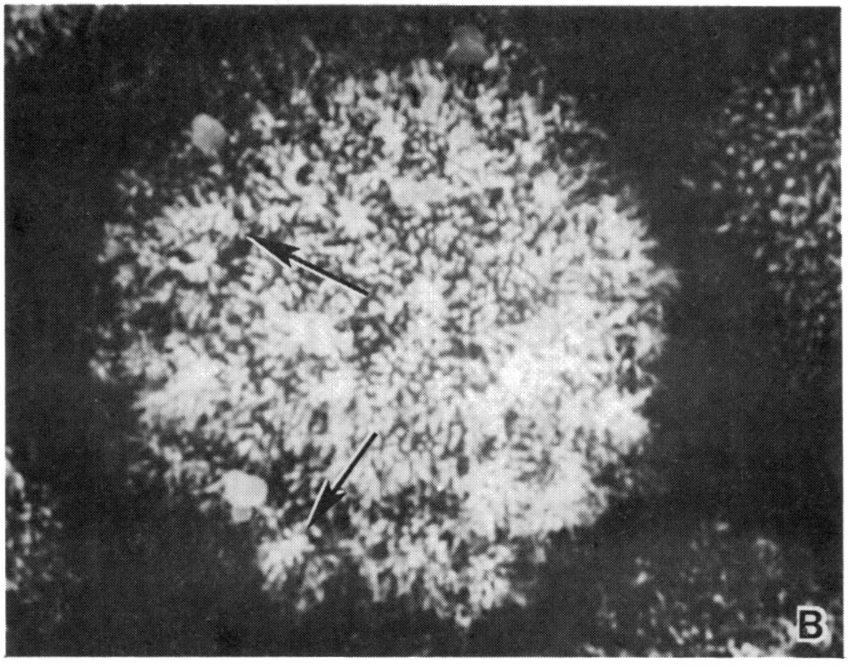

B

TABLE VIII

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE FREQUENCY OF CONTACT BETWEEN THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY SCANNING ELECTRON MICROSCOPY

Diet	No Contact[1]	Contact[2]
	No. of Cells	
C	61	239
I	5	230
II	86	348
III	66	361

[1]The cell was free of contact with other cells.

[2]The cell had contact with at least one other cell.

TABLE IX

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE MICROVILLAR BRANCHING OF THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY SCANNING ELECTRON MICROSCOPY

Diet	Degree of Microvillar Branching[1]		
	0[2]	+[3]	++[4]
	No. of Cells		
C	266	24	18
I	188	9	1
II	402	30	15
III	342	23	14

[1]Both the number of branched microvilli per cell and the numbers of branches of each microvillus contribute to what was termed "Degree of branching".

[2]No branched microvilli/cell.

[3]Moderate degree of branching/cell.

[4]High degree of branching/cell.

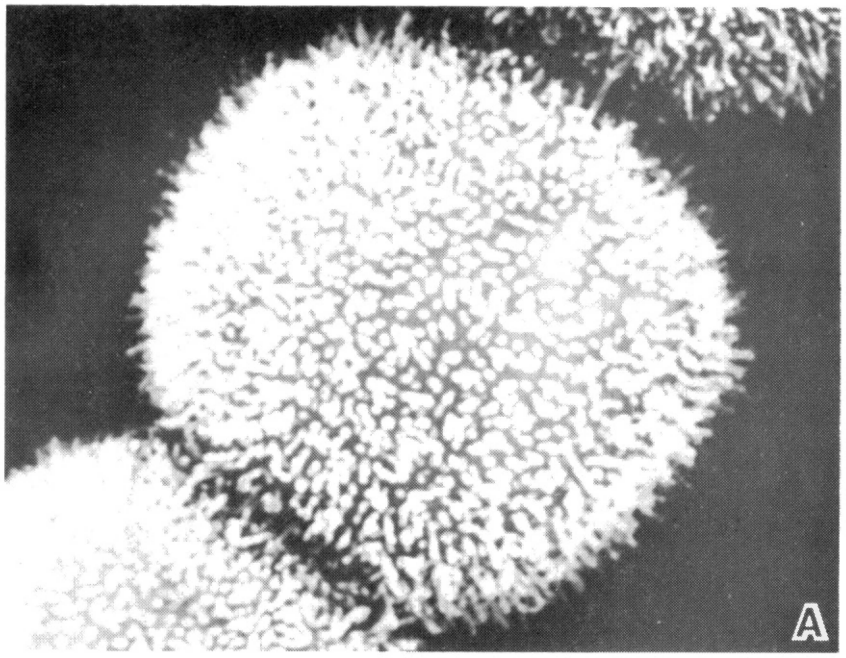
significantly affected by diet, the difference being between group I and the control ($P < .01$). In group I, only 5% of the cells had branched microvilli as compared to ∿14% for the control. There was no significant effect of diet on the degree of branching (+ vs ++).

The MAT-CH Subline. The MAT-CH cell population from each of the diet groups was also heterogeneous (Figure 37). A minority ranging from ∿5-12% had branched microvilli and resembled the MAT-C1 morphology (Figure 37B). Part of the remaining majority of cells had the MAT-C morphology with short, straight unbranched microvilli (Figure 37A). However, a good portion of the cells in each group could not be categorized into any previously determined morphology. These cells had a lot of microvilli that did not seem branched, but were present in clusters distributed over the cell surface. There was a significant difference between group I and control in the fraction of cells having branched microvilli which was ∿5% and ∿12% for group I and control, respectively (Table X). Groups II and III did not differ significantly from control in that respect; however, each of these groups was significantly different from control with respect to the degree of branching ($P < .025$). The samples from retinoic acid supplemented rats had a larger proportion of cells with a high degree of branching, relative to the control (Table X). The majority of the MAT-CH cells from each of the diet groups were involved in cell to cell contacts. However, group II had a higher ($P < .05$) fraction of cells (90.4%) involved in contacts, as compared to control (84.9%) (Table XI).

Figure 37. Scanning Electron Micrographs Illustrating the Different Identified Morphologies of the Heterogeneous MAT-CH Subline.

A. MAT-CH cell from a rat fed the purified diet supplemented with 4.5 mg retinyl palmitate/kg diet (MAT-CH group I). The morphology resembles that of MAT-C cells (45) with numerous short, stubby, unbranched microvilli. X 7500

B. MAT-CH cell from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-CH group II). The morphology resembles that of MAT-C1 cells (45). Note extensive branching of most microvilli (arrows). Branching is more readily observed at the periphery. When viewed straight on, microvilli appear clustered and branching is hard to observe. Also note cells in background with MAT-C morphology. X 5000


A

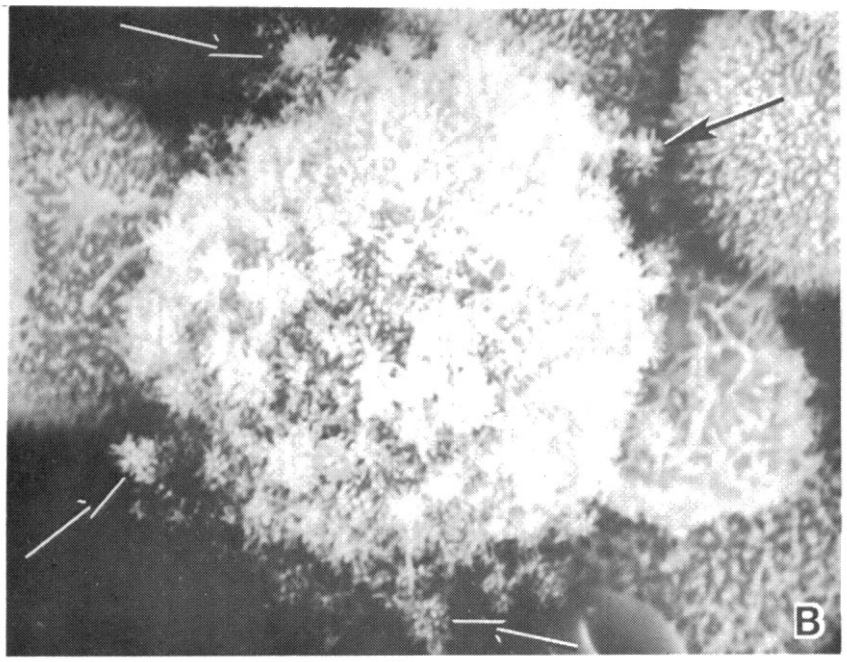

B

TABLE X

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE MICROVILLAR BRANCHING OF THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY SCANNING ELECTRON MICROSCOPY

Diet	Degree of Microvillar Branching[1]		
	0[2]	+[3]	++[4]
	No. of cells		
C	291	33	6
I	247	8	5
II	251	17	12
III	301	16	12

[1] Both the number of branched microvilli per cell and the number of branches of each microvillus contribute to what is termed "Degree of branching".

[2] No branched microvilli/cell.

[3] Moderate degree of branching/cell.

[4] High degree of branching/cell.

TABLE XI

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE FREQUENCY OF CONTACT BETWEEN THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY SCANNING ELECTRON MICROSCOPY

Diet	No Contact[1]	Contact[2]
	No. of Cells	
C	53	299
I	47	213
II	28	265
III	60	294

[1]The cell was free of contact with other cells.

[2]The cell had contact with at least one other cell.

Transmission Electron Microscopy

Sample cells from each of the different groups were analyzed for general morphology, frequency and type of contact between cells, glycogen and lipid content per cell, and the characteristics of the mitochondria and nucleoli, as described in the experimental procedures. The criteria used were as defined in the materials and methods section and in Figures 26-30.

The MAT-B1 Subline. The control MAT-B1 cells were either spherical or irregular in shape with curved unbranched microvilli. Some cells seemed to have more microvilli than others (Figure 38). A large fraction of the cells (64.3%) were involved in microvillar or cytoplasmic contact or both (Table XII). The glycogen content per cell ranged from no glycogen observed to large amounts of glycogen as illustrated in Table XIII. The lipid content per cell ranged from 0-11 droplets (Table XIV). The mitochondrial populations of ∿93% of the control MAT-B1 cells were in the orthodox configuration, 'high energy state'. Only ∿7% of the control MAT-B1 cells had a mixture of orthodox and condensed 'low energy state' mitochondria (Table XV). Nucleoli were observed in some cells and not others, and ∿20% of the cells with nuclei had more than one nucleolus. Some of the nucleoli were marginated and some were not (Table XVI). All the control MAT-B1 cells examined appeared to be normal cells with a normal chromatin pattern (Figure 38). Most of group I MAT-B1 cells and some of those of group II were irregular in shape, and appeared to have more microvilli than control cells. There was a significantly ($P < .01$) higher frequency of contact between group I cells than between control cells

Figure 38. Transmission Electron Micrograph of MAT-B1 Cells from Control Rats Fed the Non-purified Diet. X 6000

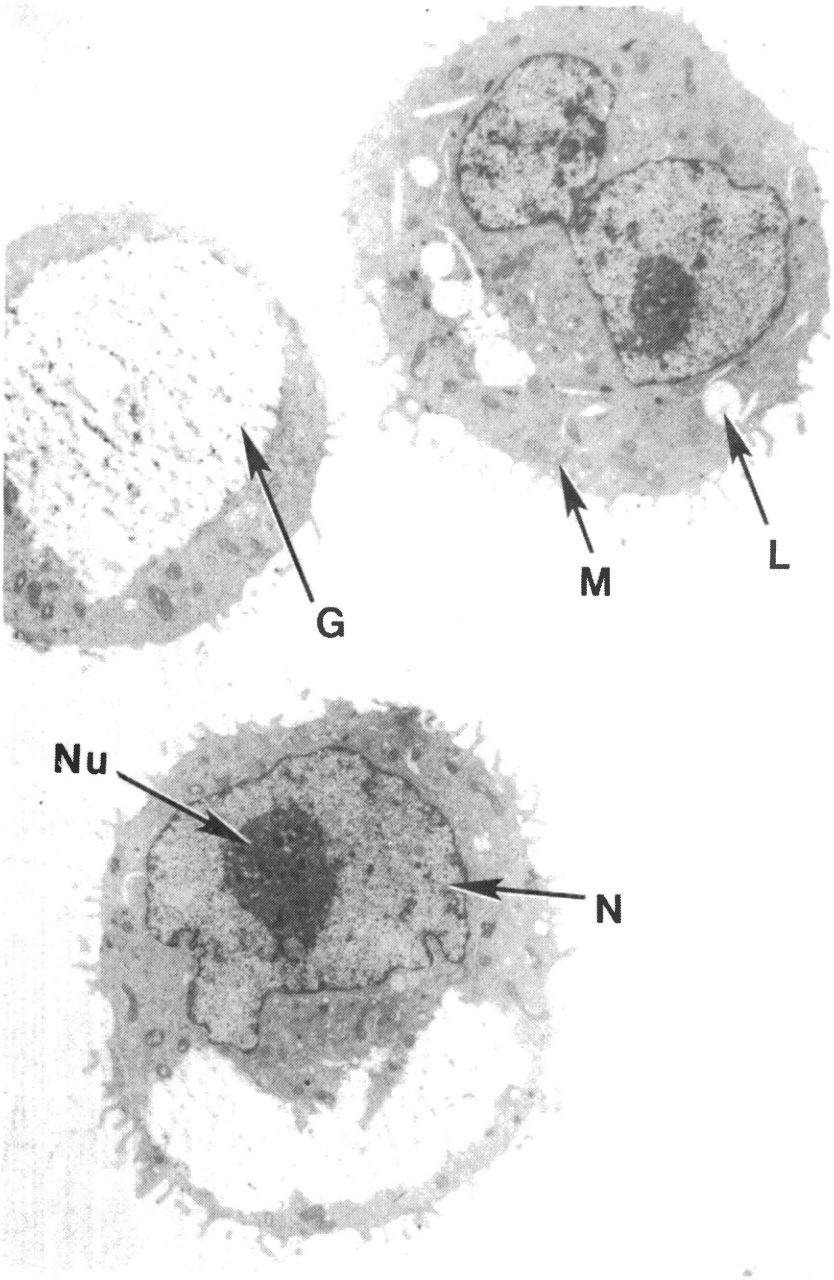

G
M
L
Nu
N

TABLE XII

THE EFFECT OF RETINOIC ACID ON THE FREQUENCY AND TYPE OF CONTACT BETWEEN THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY TRANSMISSION ELECTRON MICROSCOPY

Diet	Contact		No Contact
	Microvillar[1]	Cytoplasmic[2]	
	No. of Cells		
C	7	11	10
I	21	9	2
II	17	2	8

[1]Microvilli of cells are touching the microvilli or the cell body of one or more other cells.

[2]Cell bodies are in contact, and cytoplasmic bridges are formed. Type 1 contact can sometimes be seen in this category.

TABLE XIII

THE EFFECT OF RETINOIC ACID ON THE GLYCOGEN CONTENT OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Glycogen Content/Cell			
	0[1]	+[2]	++[3]	+++[4]
	No. of Cells			
C	5	9	8	4
I	20	4	3	0
II	3	8	4	1

[1]No glycogen present.

[2]Small amount of glycogen.

[3]Moderate amount of glycogen.

[4]Large amount of glycogen.

TABLE XIV

THE EFFECT OF RETINOIC ACID ON THE LIPID CONTENT OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Lipid Droplets/Cell		
	0	≤5	6-11
	No. of Cells		
C	3	18	5
I	5	14	8
II	0	9	6

TABLE XV

THE EFFECT OF RETINOIC ACID ON THE MITOCHONDRIAL CONFIGURATION OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Mitochondrial Configuration		
	Orthodox[1]	Condensed[2]	Mixed[1]
	No. of Cells		
C	28	0	2
I	27	0	1
II	16	13	7

[1]Mitochondria in the orthodox configuration 'high energy form'.

[2]Mitochondria in the condensed configuration 'low energy form'.

[3]Both type 1 and type 2 mitochondria are present.

TABLE XVI

THE EFFECT OF RETINOIC ACID ON THE NUCLEOLI OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Nucleoli/Cell			Positions in Nucleus	
	0	1	>1[1]	Marginated[2]	Non-Marginated[3]
	Number of Cells				
C	7	8	2	7	5
I	8	12	2	9	7
II	5	7	2	8	4

[1] 2 to 3 nucleoli per cell.

[2] The nucleolus is located at the nuclear envelope.

[3] The nucleolus is located towards the central region of the nucleus away from the nuclear envelope.

(Table XII). The frequency of occurrence of cytoplasmic bridges, however, was significantly lower in groups I ($P < .01$) and II ($P < .05$) than that of the control (Table XII). Most of group I cells did not seem to have any apparent glycogen lakes and the rest of the cells had little to moderate amounts of glycogen. This observation was significantly different ($P < .005$) from that for control in that respect (Table XIII). There was no significant difference between control MAT-B1 cells and MAT-B1 cells from group I with respect to lipid content, nucleolar characteristics or chromatin as shown in Tables XIV, XVI, and XVII, respectively. No comparisons between control and group II MAT-B1 cells with respect to the former three parameters were made due to reasons that will be discussed below. Concerning mitochondrial configuration, again as shown for control, almost all group I cells examined had mitochondria in the orthodox form. However, there was a significant increase ($P < .005$) in the relative number of group II cells having mitochondria in the condensed (36%) or mixed (19%) forms (Table XV). In addition, there was a significant difference ($P < .005$) between group II and control MAT-B1 cells with respect to the chromatin pattern. Of the cells that had a visible nucleus, 50% had marginated chromatin (Table XVII). In conclusion, as judged by mitochondrial configuration, chromatin pattern and the general state of the cells, control and group I cells examined were all normal. However, a significant increase ($P < .005$) in cell necrosis was noted for group II MAT-B1 cells (Table XVIII, Figure 39). This latter observation led to the inability to analyze glycogen and lipid content, and nuclear characteristics for this group.

TABLE XVII

THE EFFECT OF RETINOIC ACID ON THE CHROMATIN PATTERN OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Chromatin Pattern	
	Normal	Marginated[1]
	Number of Cells	
C	20	0
I	21	0
II	13	13

[1] 'A condensation of chromatin occurs along or adjacent to the inner membrane of the nuclear envelope, while chromatin disappears from other parts of the nucleus' (119).

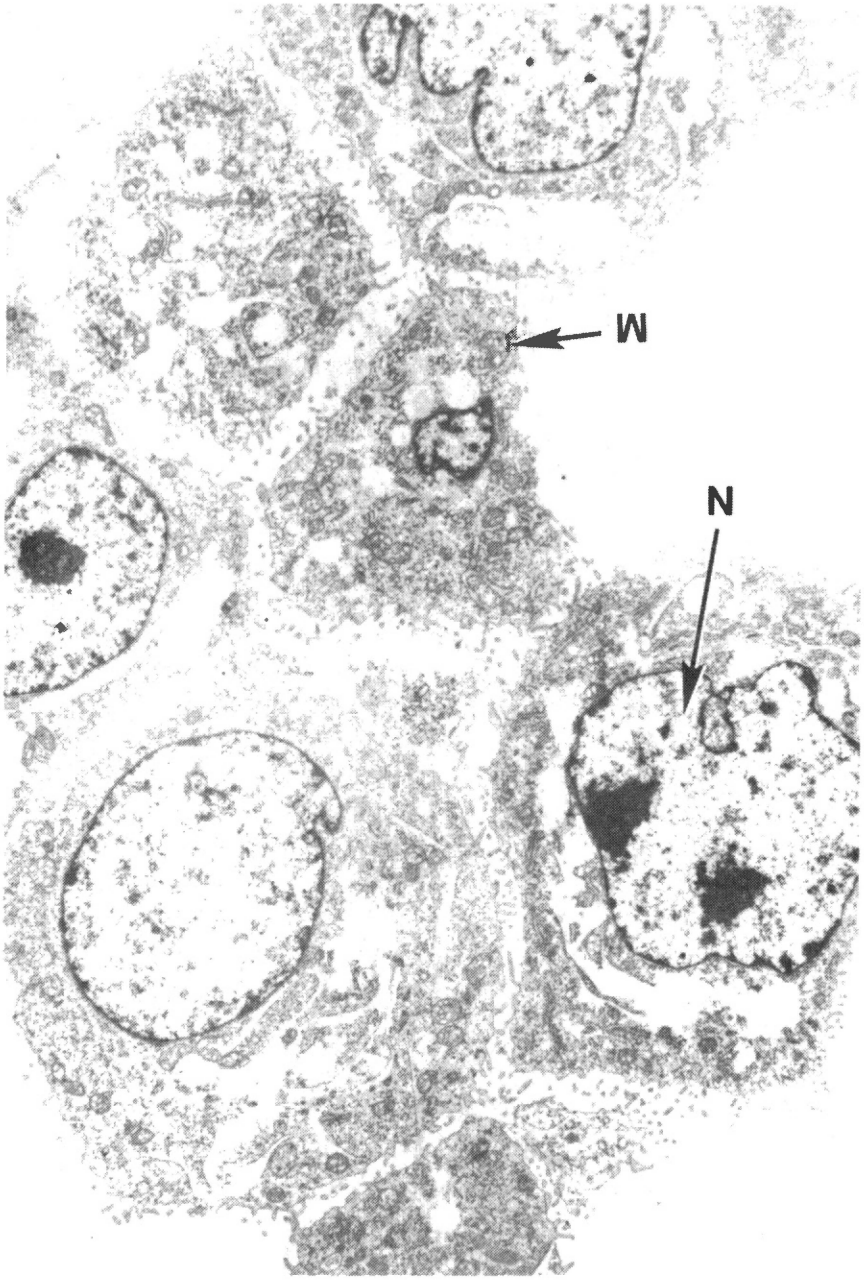
TABLE XVIII

THE EFFECT OF RETINOIC ACID ON THE VIABILITY OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY TRANSMISSION ELECTRON MICROSCOPY

Diet	General Fine Structure	
	Normal	Necrotic[1]
	Number of Cells	
C	28	0
I	31	0
II	18	18

[1]The cells exhibited disintegrating cytoplasms, and necrotic nuclei with marginated chromatin patterns.

Figure 39. Transmission Electron Micrograph of MAT-B1 Cells from Group II. MAT-B1 cells from rats fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-B1 group II). These cells are necrotic as indicated by the disintegrating cytoplasm, and nuclei with marginated chromatin patterns. The mitochondria of these cells are in the condensed configuration "low energy form". Compare to control cells with normal fine structure (in Figure 38). M, mitochondria; N, nucleus. X 5000


W
N

The MAT-C3 Subline. The control MAT-C3 cells were mostly spherical with moderate quantities of short to medium straight unbranched microvilli (Figure 40). The majority of the cells (91%) were involved in microvillar and/or cytoplasmic contacts with other cells (Table XIX). The majority of the cells lacked glycogen (Table XX) and the rest had little or negligible amounts. The lipid content ranged form 0-20 droplets per cell, as shown in Table XXI. The mitochondria were all in the orthodox form, Table XXII. The number of apparent nucleoli per cell ranged from 0-3 (Table XXIII), and 69% of them were marginated. All the cells with nuclei had normal chromatin patterns, and appeared to be normal looking cells (Figure 40).

The MAT-C3 cells from group I were usually spherical with short to medium length microvilli. There was a significant decrease ($P < .005$) in the fraction of cells involved in contacts (Table XIX), as compared to control MAT-C3 cells. In addition, there was a significant increase ($P < .01$) in the number of cells exhibiting glycogen lakes (Table XX). MAT-C3 cells from group I looked normal and did not differ significantly from control MAT-C3 cells with respect to lipid content, and nucleolar, mitochondrial (Tables XXI-XXIII) and chromatin characteristics.

The MAT-C3 cells of group II had an apparent increase in shape irregularity (Figure 41) as compared to control (Figure 40). They seemed to have an increased number of microvilli, some of which seemed longer and more curved than seen in the case of control. There was a significant difference ($P < .005$) between group II cells and control cells with respect to the presence or absence of glycogen

Figure 40. Transmission Electron Micrograph of MAT-C3 Cells from Control Rats Fed the Non-purified Diet. L, lipid; M, mitochondrion; N, nucleus; Nu, nucleolus. X 9000

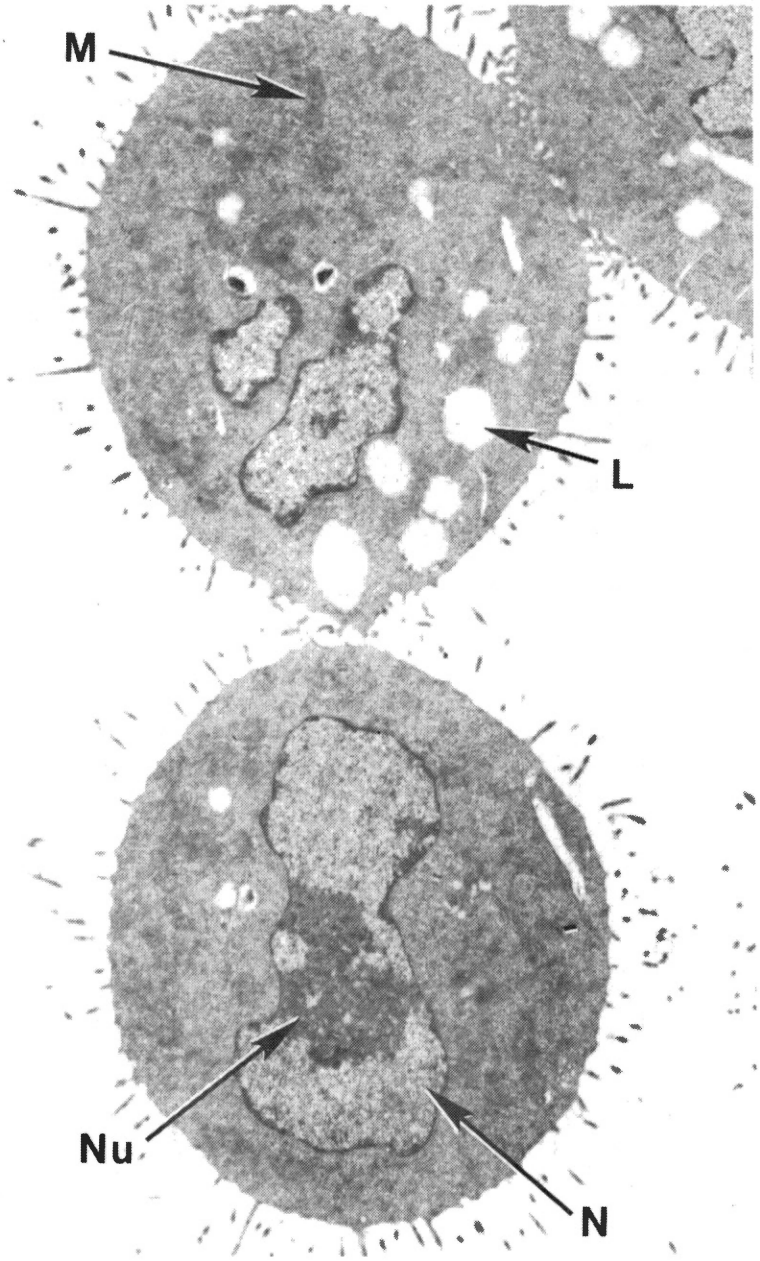

M
L
Nu
N

TABLE XIX

THE EFFECT OF RETINOIC ACID ON THE FREQUENCY AND TYPE OF CONTACT BETWEEN THE 13762 MAT-C3 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY TRANSMISSION ELECTRON MICROSCOPY

Diet	Contact		No Contact
	Microvillar[1]	Cytoplasmic[2]	
	No. of Cells		
C	18	12	3
I	9	3	9
II	19	10	0

[1]Microvilli of cells are touching the microvilli or the cell body of one or more other cells.

[2]Cell bodies are in contact, and cytoplasmic bridges are formed. Type 1 contact can sometimes be seen in this category.

TABLE XX

THE EFFECT OF RETINOIC ACID ON THE GLYCOGEN CONTENT OF THE 13762 MAT-C3 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Glycogen Content/Cell			
	0[1]	+[2]	++[3]	+++[4]
	No. of Cells			
C	31	3	0	0
I	13	8	0	0
II	11	10	5	3

[1]No glycogen present.

[2]Small amount of glycogen.

[3]Moderate amount of glycogen.

[4]Large amount of glycogen.

TABLE XXI

THE EFFECT OF RETINOIC ACID ON THE LIPID CONTENT OF THE 13762 MAT-C3 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Lipid Droplets/Cell				
	0	≤ 5	6-10	11-20	>20
	No. of Cells				
C	3	14	11	6	0
I	0	6	9	7	1
II	0	9	9	5	4

TABLE XXII

THE EFFECT OF RETINOIC ACID ON THE MITOCHONDRIAL CONFIGURATION OF THE 13762 MAT-C3 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Mitochondrial Configuration		
	Orthodox[1]	Condensed[2]	Mixed[3]
	No. of Cells		
C	39	0	0
I	20	0	1
II	29	0	2

[1]Mitochondria in the orthodox configuration 'high energy form'.

[2]Mitochondria in the condensed configuration 'low energy form'.

[3]Both type 1 and type 2 mitochondria are present.

TABLE XXIII

THE EFFECT OF RETINOIC ACID ON THE NUCLEOLI OF THE 13762 MAT-C3 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

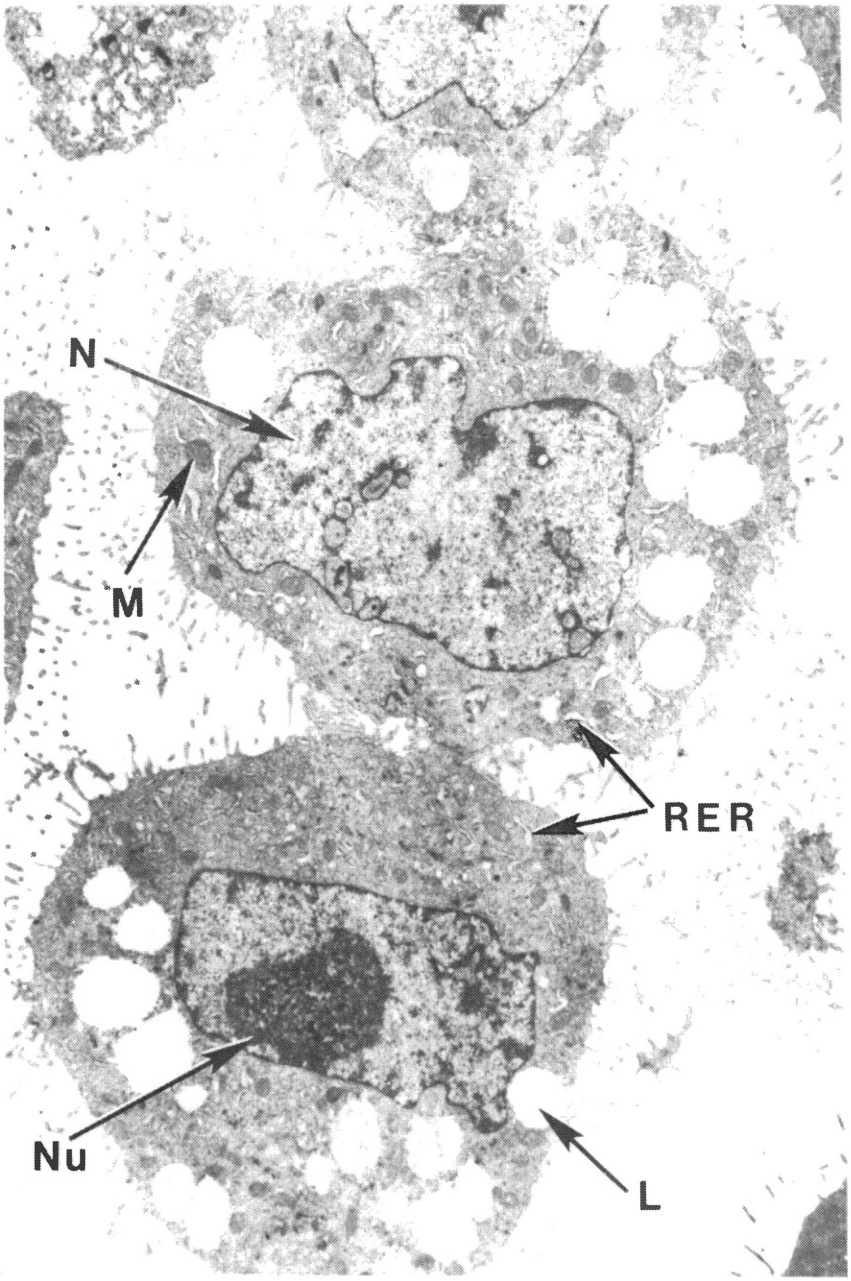
Diet	No. of Nucleoli/Cell			Positions in Nucleus	
	0	1	>1[1]	Marginated[2]	Non-Marginated[3]
	Number of Cells				
C	8	14	7	20	9
I	7	6	3	11	2
II	3	11	7	17	8

[1] 2 to 3 nucleoli per cell.

[2] The nucleolus is located at the nuclear envelope.

[3] The nucleolus is located towards the central region of the nucleus away from the nuclear envelope.

Figure 41. Transmission Electron Micrograph of MAT-C3 Cells from Group II. MAT-C3 cells from a rat fed the purified diet supplemented with 60 mg retinoic acid/kg diet (MAT-C3 group II). Note the increased activity of the cells as indicated by the marked hypertrophy of the rough endoplasmic reticulum (RER) as compared to control cells in Figure 40. Dilatation and vesiculation of the rough endoplasmic reticulum is also observed. Also note the irregularity in shape as compared to control cell in Figure 40. L, lipid; M, mitochondrion; N, nucleus; Nu, nucleolus. X 5500


N
M
RER
Nu
L

lakes, and an apparent difference in the amount of glycogen present, as shown in Table XX. MAT-C3 cells from group II did not differ from control with respect to lipid content, or to the characteristics of the mitochondria, nucleoli (Tables XXI-XXIII) and chromatin. All the cells examined looked normal (Figure 41). However, it is important to mention that going from control to group I to group II, there was an apparently significant increase in the synthesizing activity of the MAT-C3, as evidenced by the hypertrophy of the rough endoplasmic reticulum and the active cytoplasm (Figure 41).

The MAT-BH Subline. The control MAT-BH cells were usually semi-spherical and sometimes irregular in shape. The microvilli were usually unbranched and moderate in number. Branched microvilli were sometimes seen on some of the cells. Almost all the cells were free of contact with others (Table XXIV). Almost half the number of cells examined (∿44%) were devoid of glycogen (Table XXV). When present, glycogen lakes were usually low to moderate amounts. The lipid content varied between 0 and >20 droplets per cell (Table XXVI). A major fraction of the cells (∿62%) had a mitochondrial population in the orthodox configuration. The rest of the cells had condensed mitochondria or a mixture of both mitochondrial configurations (Table XXVII). Most of the cells (71%) had at least one nucleolus, and in 67% of the cases the nucleolus was marginated (Table XXVIII). Almost all the cells with nuclei (96%) had normal chromatin pattern and 97% of all cells had a normal general cytology.

Group I MAT-BH cells (for example, the ones in Figure 26A) resembled control MAT-BH cells in the general morphology. A signifi-

TABLE XXIV

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE FREQUENCY AND TYPE OF CONTACT BETWEEN THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY TRANSMISSION ELECTRON MICROSCOPY

Diet	Contact		No Contact
	Microvillar[1]	Cytoplasmic[2]	
	No. of Cells		
C	0	2	67
I	11	4	38
II	81	9	61
III	6	13	81

[1] Microvilli of cells are touching the microvilli or the cell body of one or more other cells.

[2] Cell bodies are in contact, and cytoplasmic bridges are formed. Type 1 contact can sometimes be seen in this category.

TABLE XXV

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE GLYCOGEN CONTENT OF THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Glycogen Content/Cell			
	0[1]	+[2]	++[3]	+++[4]
	No. of Cells			
C	35	20	15	9
I	25	20	12	5
II	70	41	34	5
III	49	31	16	9

[1]No glycogen present.

[2]Small amount of glycogen.

[3]Moderate amount of glycogen.

[4]Large amount of glycogen.

TABLE XXVI

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE LIPID CONTENT OF THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Lipid Droplets/Cell				
	0	≤5	6-10	11-20	>20
	No. of Cells				
C	9	21	24	16	1
I	1	21	29	12	4
II	5	41	41	40	9
III	0	38	30	27	6

TABLE XXVII

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE MITOCHONDRIAL CONFIGURATION OF THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Mitochondrial Configuration		
	Orthodox[1]	Condensed[2]	Mixed[3]
	No. of Cells		
C	48	11	19
I	36	11	9
II	101	9	21
III	73	8	18

[1]Mitochondria in the orthodox configuration 'high energy form'.

[2]Mitochondria in the condensed configuration 'low energy form'.

[3]Both type 1 and type 2 mitochondria are present.

TABLE XXVIII

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE NUCLEOLI OF THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Nucleoli/Cell			Positions in Nucleus	
	0	1	>1[1]	Marginated[2]	Non-Marginated[3]
	Number of Cells				
C	15	30	6	28	14
I	14	22	2	14	12
II	34	42	17	54	22
III	29	43	10	34	29

[1] 2 to 3 nucleoli per cell.

[2] The nucleolus is located at the nuclear envelope.

[3] The nucleolus is located towards the central region of the nucleus away from the nuclear envelope.

cantly larger ($P < .005$) fraction of cells (28%) were involved in some type of contact with other cells, as compared to 3% for control (Table XXIV). A significantly higher ($P < .05$) incidence of cytoplasmic bridges was noted, as compared to control (Table XXIV). Group I cells were not significantly different from control with respect to glycogen content (Table XXV), mitochondrial configuration (Table XXVII) or the nucleolar characteristics (Table XXVIII). However, a significant increase ($P < .025$) in the number of cells containing lipid was noted, as compared to controls (Table XXVI). Most of the cells with apparent nuclei had normal chromatin (92%) and 95% of all the cells looked normal.

The MAT-BH cells from group II resembled those from control except that in some cases the microvilli seemed more curved and increased in number. There was a significant increase ($P < .005$) in the relative number of cells ($\sim$60%) involved in cell to cell contact, as compared to control (Table XXIV). In addition, the incidence of cytoplasmic bridges was significantly higher ($P < .005$) than that for control (Table XXIV). A significant increase ($P < .025$) in the fraction of cells with lipid (Table XXVI), and decrease ($P < .025$) in the fraction of cells with at least some condensed mitochondria (Table XXVII) was noted, as compared with control. Group II MAT-BH cells did not differ from control with respect to glycogen content (Table XXV) or the characteristics of the nucleoli (Table XXVIII). All the cells with apparent nuclei had normal chromatin patterns and all the cells examined appeared to be normal.

The MAT-BH cells from group III (for example, the cells in Figure 26B) resembled control MAT-BH in the general morphology.

There was a significant increase ($P < .005$), with respect to control, in the relative number of cells involved in intercellular contact and those containing lipid (Tables XXIV and XXVI). MAT-BH cells from group III did not differ from control cells with respect to glycogen content (Table XXV), distribution of mitochondrial configurations (Table XXVII), or the characteristics of the nucleoli (Table XXVIII). Almost all the cells had a normal appearance, and the ones with visible nuclei had normal chromatin patterns.

The MAT-CH Subline. Most of the control MAT-CH cells were usually spherical in shape with straight, short to medium microvilli, mostly unbranched. A good fraction of the cells (40%) were involved in intercellular contacts (Table XXIX). The glycogen and lipid contents are illustrated in Tables XXX and XXXI, respectively. As shown in Table XXXII, 39% of the cells contain mitochondria in the condensed configuration. Nucleoli were apparent in 71% of the cells examined (Table XXXIII) and the majority of them were marginated. The chromatin patterns and the general cell cytology appeared to be normal for those cells.

The MAT-CH cells from groups I, II and III resembled control cells in general appearance and microvillar characteristics. There were no significant differences between cells from these groups and those from control with respect to incidence and type of intercellular contact (Table XXIX), lipid and glycogen content (Tables XXX and XXXI) or the characteristics of the nucleoli (Table XXXIII). Cells from each of group I, II and III differed significantly ($P < .005$) from control with respect to mitochondrial configuration. As shown

TABLE XXIX

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE FREQUENCY AND TYPE OF CONTACT BETWEEN THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS EXAMINED BY TRANSMISSION ELECTRON MICROSCOPY

Diet	Contact		No Contact
	Microvillar[1]	Cytoplasmic[2]	
	No. of Cells		
C	8	4	18
I	12	11	51
II	22	15	35
III	30	25	50

[1]Microvilli of cells are touching the microvilli or the cell body of one or more other cells.

[2]Cell bodies are in contact, and cytoplasmic bridges are formed. Type 1 contact can sometimes be seen in this category.

TABLE XXX

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE GLYCOGEN CONTENT OF THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Glycogen Content/Cell			
	0[1]	+[2]	++[3]	+++[4]
	No. of Cells			
C	11	7	6	5
I	41	19	14	6
II	27	25	8	14
III	49	30	12	13

[1]No glycogen present.

[2]Small amount of glycogen.

[3]Moderate amount of glycogen.

[4]Large amount of glycogen.

TABLE XXXI

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE LIPID CONTENT OF THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Lipid Droplets/Cell				
	0	≤5	6-10	11-20	>20
	No. of Cells				
C	0	7	5	12	5
I	2	13	28	33	3
II	1	12	16	34	12
III	0	19	29	46	9

TABLE XXXII

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE MITOCHONDRIAL CONFIGURATION OF THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	Mitochondrial Configuration		
	Orthodox[1]	Condensed[2]	Mixed[3]
	No. of Cells		
C	19	8	4
I	73	1	5
II	65	4	4
III	109	0	0

[1]Mitochondria in the orthodox configuration 'high energy form'.

[2]Mitochondria in the condensed configuration 'low energy form'.

[3]Both type 1 and type 2 mitochondria are present.

TABLE XXXIII

THE EFFECT OF RETINYL PALMITATE AND RETINOIC ACID ON THE NUCLEOLI OF THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS

Diet	No. of Nucleoli/Cell			Position in Nucleus	
	0	1	>1[1]	Marginated[2]	Non-marginated[3]
	Number of Cells				
C	6	13	2	15	2
I	19	33	6	38	7
II	18	33	6	39	6
III	30	40	9	43	15

[1] 2 to 3 nucleoli per cell.

[2] The nucleolus is located at the nuclear envelope.

[3] The nucleolus is located towards the central region of the nucleus away from the nuclear envelope.

in Table XXXII, most of the cells from each of these groups had mitochondria exclusively in the orthodox 'high energy form' configuration, as compared to only ∿61% of the control MAT-CH cells. The chromatin patterns and general cell cytology appeared to be normal in all cases.

Effects on Tumor Growth

The incidences of ascites tumor development in the different dietary groups were compared. As shown in Table V, 100% incidence of MAT-BH ascites tumor line occurred in the all-trans-retinoic acid treated animals, while animals injected with MAT-BH tumor cells and fed the non-purified diet or the retinyl palmitate supplemented diet had 40% and 50% incidence, respectively. Thus, it appeared that excess doses of all-trans-retinoic acid did not decrease, but on the contrary, increased the incidence of the MAT-BH tumors in the rats. The effects of retinyl palmitate and retinoic acid on the MAT-CH tumor growth were also studied. As shown in Table V, control rats fed the non-purified diet had a 40% incidence of the MAT-CH ascites line. An increase to 80-100% tumor incidence occurred in the animals fed the retinyl palmitate or the all-trans-retinoic acid diets. Data on incidence of tumor growth in the case of MAT-B1 and MAT-C3 sublines were not obtained.

Xenotransplantability

Xenotransplantability of the different sublines of the 13762 rat mammary adenocarcinoma was studied previously (47, 54). In the case of the xenotransplantable sublines, the tumor-bearing mice increased in weight, and had very swollen abdomens and arched spines. Eventually,

the mice died. In the case of the non-xenotransplantable sublines, the tumor cells grew in the peritoneal cavities of the injected mice. The mice also manifested the external symptoms of tumor growth, as in the case of the xenotransplantable sublines. However, by the end of the 30 or 90 day observation period, the mice recovered. As shown in Table VI, there was no drastic effect of diet on the survival of mice fed the retinoic acid supplemented diet and injected with the MAT-BH and MAT-CH sublines, as compared to controls. Data on the external manifestations of tumor growth were not obtained.

In the second experiment, mice were fed the vitamin A-deficient purified diet for 16 weeks, as described under materials and methods. However, the average serum retinol levels of these mice did not differ significantly from those of control mice (Table VII). The mice were monitored periodically from the time of tumor injection until the time of killing, and external signs of tumor growth were noted. Based on the number of animals manifesting tumor growth symptoms, and on the number of mice that died of the tumors, it appeared that the incidence of the MAT-BH tumor in mice fed the vitamin A-deficient diet (9/13 mice with tumors) did not differ from that of the control mice (3/5 mice with tumors). However, the fraction of animals that were fed the vitamin A-deficient diet and that died of the tumor (3/13) was lower than that for controls (3/5). Thus, feeding the vitamin A-deficient diet enhanced the survival of the animals (Table VII). In the case of the MAT-CH subline, all mice survived until the end of the experimental period. However, the mice on the vitamin A-deficient diet had a higher incidence of tumor (9/12 mice) than that for controls (0/5 mice) as judged by the external manifestations of

tumor growth.

Discussion

The objectives of the work presented in this chapter were to study the effects of dietary retinoic acid on the incidence, xeno-transplantability, morphology and viability of the 13762 mammary adenocarcinoma sublines. The effects of retinoic acid on the homogeneous tumor cell populations, MAT-B1 and MAT-C3, were more conclusive than those on the heterogeneous populations, MAT-BH and MAT-CH. High concentrations of retinoic acid in the diet (60 mg/kg diet) enhanced mecrosis in the MAT-B1 subline. This was judged by the disintegrating cytoplasms and necrotic nuclei of a good portion of the MAT-B1 cells examined. Cell necrosis was accompanied by a significant increase in the condensed mitochondrial population. Cellular necrosis upon retinoid treatment was demonstrated previously in studies where retinol and retinyl acetate (121) or the aromatic retinoid Ro-9359 (106) were shown to have inhibitory effects on the incidence and development of skin papillomas. In the first study, retinoid treatment caused an increase in lysosomal enzyme activities of the pre-malignant cells, which lead to cellular necrosis (121). In the second study, retinoid treated skin papillomas had an increased number of lysosomes, as compared with normal epithelium. In the retinoid treated cells, labilization of the plasma membrane was accompanied by vacuolization and loss of cytoplasmic constituents and finally necrosis (106).

In our studies, cellular lysosomes were rarely seen. However, an increase in the secretion of extracellular hydrolases was demonstrated previously in other systems (85-87). Thus, it was possible

that hydrolases secreted, by other cells, into the peritoneal cavity could labilize the plasma membrane and/or the cellular organelles of the tumor cells to cause necrosis. Another possibility is that retinoic acid, through its surfactant and 'membrane seeking' activities (113-118) might labilize the plasma membrane leading to ingress of extracellular fluids, leakage of cytoplasmic constituents and finally necrosis, without the need for hydrolases. A third possibility is that retinoic acid, through adjuvant activities, might enhance the contribution of the host immune response to tumor regression. Retinoids stimulated both humoral and cell mediated immune responses in different systems (38-43). Some studies indicated that enhancement of antibody formation, to bovine serum albumin and to sheep red blood cells in mice, by retinol, required thymus-dependent lumphocytes, bone marrow-derived lymphocytes, and macrophages (41, 122). In other systems, antibody production could be stimulated by retinoid, not requiring the production of lymphocytes or macrophages (123). Vitamin A alone, or in combination with other treatments, was shown to stimulate the host anti-tumor immune response in several systems (19, 124, 125). Cell-mediated immune response to the tumor could be achieved by activation of macrophages and/or cytotoxic T-cells, which recognize and lyse the tumor cells (126-128). In the case of the MAT-B1 cells used in this study, there is a possibility that high doses of retinoic acid in the diet could have enhanced the activation of macrophages and/or lymphocytes which could gain access to the peritoneal cavity and lyse the MAT-B1 cells. Killing of tumor cells can also be achieved by a combination of the action of hydrolases and the immune response.

The effect of retinoic acid on the MAT-C3, MAT-BH and MAT-CH sublines were different from those on the MAT-B1 cells. The vitamin did not seem to affect the viability of these cells, as viewed by electron microscopy. In fact, 60 mg of retinoic acid/kg diet seemed to activate the MAT-C3 cells, as evidenced by the increased activity of the endoplasmic reticulum, and the cytoplasm in general. In contrast to the studies illustrating the anti-carcinogenic and anti-tumor activities of retinoids, there are others demonstrating that the induction of malignant growth at some sites, such as the colon (129), mammary gland (130), and bladder (131) was not affected by retinoids. Interestingly enough, retinoids were shown to even enhance the induction or the growth of some tumors such as estrogen or progesterone induced mammary tumors in mice (132), or MNU induced tracheal cancer in hamsters (133). In both cases, retinoid was administered in the diet. The increased synthetic activity in the MAT-C3 cells could be due to direct effects of retinoic acid on the amount of mRNAs transcribed and/or translated. Another possibility is that retinoic acid could affect glycoproteins which in turn could affect cellular functions by epigenetic mechanisms. These possibilities were discussed in more detail in Chapter II. The observation that MAT-C3 cells became irregular in shape upon retinoic acid administration could be explained by the surfactant action of retinoids, discussed previously. Similar shape changes have been observed in other systems (104, 108).

Several cell organelles were examined: Nuclei, nucleoli, chromatin, mitochondria, and endoplasmic reticulum. In addition to cell viability, other parameters such as glycogen and lipid content,

microvillar characteristics and cell-to-cell cohesiveness were also studied. Morphological studies indicated that in most of the cases where cell-to-cell cohesiveness was significantly affected, retinoid treatment enhanced cell-to-cell contact (MAT-B1 group I, MAT-BH group I, II and III, and MAT-CH group II).

The effects on contact could be due to modulation, by the retinoids, of cellular properties which are involved in cell-to-cell recognition and cohesiveness. Among these properties are the production of fibronectin and/or its receptors, and the cell surface glycoconjugates. The effects of retinoids on these parameters were demonstrated in the previously reviewed studies. For example, morphological and biochemical studies on rat islets revealed that 13-cis-retinoic acid increased islet cell-to-cell cohesiveness, and at high concentrations, caused clumping of the cells (134). Cells in a clump were intact and gap junction linked the cells. These effects were accompanied by an increase in insulin release.

In the present studies, retinyl palmitate (MAT-CH group I) or retinoic acid (MAT-BH group II, and MAT-CH groups I, II and III) in the diet appeared to reduce the occurrence of mitochondria in the condensed configuration 'low energy form'. This suggests enhanced respiratory activities of the cells, which require coupled mitochondria in the orthodox configuration 'high energy form'. In the case of the MAT-B1 group II cells, there was an increase in the mitochondrial population having the condensed configuration, especially in the necrotic cell population. The effects of retinoids on mitochondrial morphologies and configurations were demonstrated previously (105, 109). For example, uncoupling and swelling of mitochondria

was observed upon addition of excess retinol to normal mitochondria (105, 109, 135). These observations were associated with increases in permeability to the dye Nitroblue Tetrazolium (136) and to malate dehydrogenase and β-glucoronidase (137). Addition of retinol restored normal shape and function in abnormally shaped mitochondria isolated from hypovitaminosis A rats (138). Mitochondria isolated from livers of either hyper- or hypovitaminosis A rats displayed diminished P/O ratios and enhanced respiration (139, 140). It was suggested that retinol may function by stabilizing membranes and maintaining proper control of respiration and oxidative phosphorylation (141). Retinoic acid was shown to promote some mitochondrial swelling, but could not restore normal function to vitamin A poisoned rat mitochondria (135). However, Stillwell et al. (142) studied the effect of retinol and retinoic acid on the P/O ratios of coupled mitochondria. Both retinoids were shown to uncouple mitochondrial oxidative phosphorylation. The fact that the effects observed were not site specific suggested that vitamin A acts as a general membrane bilayer disrupting agent (113-118).

It was shown (143, 144) that electron transport is required for the condensed-to-orthodox ultrastructural transformation. This transformation occurs in mitochondria under state IV conditions where exogeneous substrate is present and ADP is deficient. The reverse transformation, from orthodox to condensed configuration, was shown to parallel suppression of acceptor control and oxidative phosphorylation.

Nucleolar characteristics were examined, since an increase in the number of nucleoli per cell, and their margination were associated

with increased protein synthetic activities (119). In our experiments, there was no significant effect of retinyl palmitate or retinoic acid on these parameters.

The lipid content of the tumor cells was evaluated. In some of the cases, retinyl palmitate (MAT-BH group I) or retinoic acid (MAT-BH groups II and III) caused an increase in the lipid content of the cells. In each of these cases, this observation was correlated with increased cell-to-cell cohesiveness. Lipid droplets represent a potential source of energy and provide short carbon chains which can be used in the synthesis of cellular membranes and other cellular lipid containing material (119). While the presence of a few lipid droplets in the cytoplasm is considered normal, gross lipid deposition in some cells is considered pathological, and constitutes fatty degeneration, as reviewed by Ghadially (119).

The glycogen content of tumor cells was also studied. In all cases studied, there was no significant effect of retinyl palmitate or retinoic acid on the amount of glycogen present. However, when the mere presence or absence of glycogen was studied, it was shown that retinoic acid enhanced the appearance of glycogen in some cases (MAT-C3 groups I and II) and depressed it in others (MAT-B1 group I). Cells store glycogen as a metabolic fuel, and thus it might be assumed that its abundance is an indicator of increased cellular metabolic activity. This assumption, however, was proven to be wrong. In many instances, as reviewed by Ghadially (119), increased glycogen deposits in the cytoplasm indicated diminished usage rather than increased metabolic activity.

Studies on the incidence and xenotransplantability of the ascites

sublines of the tumor indicated that in some cases (MAT-BH group II and III in rats, MAT-CH groups I, II and III in rats, MAT-CH in mice fed deficient diet) tumor incidence increased as compared to controls. In other cases (MAT-BH in mice fed the vitamin A-deficient diet) the tumor incidence was not affected, but the potential of the tumor to kill the animals was inhibited, as judged by observations on the survival of the tumor-bearing animals. My interest in the xenotransplantability was based on the evidence for effects of retinoids on the immune responses, as discussed previously (38-43).

The observation that, in some of the cases of this study, tumor incidence was enhanced upon retinoid treatment is supported by similar observations in other systems, as reviewed previously in this chapter (132, 133). In another recent study on the relation of serum vitamin A and E and carotenoids on the risk of cancer in humans (145), it was found that the subjects with subsequent lung cancer had a higher mean base-line retinol level than that in their matched controls. Narisawa et al. (146) showed that animals with adequate intake of vitamin A develop significantly more tumors than animals deprived of vitamin A. It is possible that some tumors are retinoid dependent.

CHAPTER IV

SUMMARY

The objective of this study was to determine the effect of dietary vitamin A on the 13762 rat mammary adenocarcinoma ascites sublines: MAT-B1, MAT-C3, and two heterogeneous cell lines, MAT-BH and MAT-CH. Parameters measured were: 1) Tumor growth incidence; 2) cell morphology; 3) the density, molecular weight and oligosaccharide pattern of the major membrane glycoprotein (ASGP-1); 4) xenotransplantability.

Rats were fed either a non-purified, closed formula, vitamin A-adequate diet (controls), a vitamin A-deficient purified diet supplemented with 4.5 mg all-*trans*-retinyl palmitate/kg diet (2.4 mg retinol equivalent/kg diet), or a vitamin A-deficient diet supplemented with 12, 60, 90, or 120 mg all-*trans*-retinoic acid/kg diet. In the xenotransplantability studies, mice were fed either a non-purified, closed formula, vitamin A-adequate diet (controls), a vitamin A-deficient purified diet, or a vitamin A-deficient purified diet supplemented with 120 mg all-*trans*-retinoic acid/kg diet. Tumor passage was achieved by intraperitoneal injection of the animals with 1-3 x 10^6 tumor cells.

The effects of retinoic acid and retinyl palmitate on the different cell parameters of the four sublines are summarized in Tables XXXIV, XXXV, XXXVI and XXXVII.

TABLE XXXIV

EFFECT OF DIETARY ALL-TRANS-RETINOIC ACID (RA) ON THE MORPHOLOGY, AND MAJOR MEMBRANE GLYCOPROTEIN (ASGP-1) OF THE 13762 MAT-B1 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS COMPARED TO CONTROL[1,2]

Parameters	Group I	Group II	Group III	Group IV
Retinoid and Supplement	12 mg RA/ kg diet	60 mg RA/ kg diet	90 mg RA/ kg diet	120 mg RA/ kg diet
ASGP-1				
Density	NE	↓	NE	NE
Molecular weight	NE	NE	↑	NE
Oligosaccharide pattern				
Peak II	-	NE	NE	NE
Peak III	-	NE	NE	NE
Peak IV	-	NE	NE	NE
Contact (TEM)[4]				
General	↑	NE	-	-
Microvillar	↑	NE	-	-
Cytoplasmic	↓	↓		
Chromatin pattern (as compared to 100% normal in control)	NE	↑ in margination	-	-
Nucleoli				
No./cell	NE	NE	-	-
Margination	NE	NE	-	-
Mitochondria	NE	↑ occurrence of condensed configuration	-	-
Glycogen				
No. of cells with glycogen	↓	-	-	-
Amount of glycogen/cell	NE	-	-	-
Lipid				
No. of cells with lipid	NE	-	-	-
Amount of lipid/cell	NE	-	-	-

TABLE XXXIV (Continued)

Parameters[3]	Group I	Group II	Group III	Group IV
Retinoid and Supplement	12 mg RA/ kg diet	60 mg RA/ kg diet	90 mg RA/ kg diet	120 mg RA/ kg diet
Cell viability (as compared to 100% in control)	100%	50%	-	-
Shape	NE	NE	-	-

[1]Controls were fed a closed formula vitamin A-adequate, non-purified diet.

[2]NE, no effect; ↑, increase; ↓, decrease; -, not examined.

[3]Parameters were previously defined in Chapters II and III.

[4]As examined by Transmission Electron Microscopy.

TABLE XXXV

EFFECT OF DIETARY ALL-TRANS-RETINOIC ACID (RA) ON THE MORPHOLOGY, AND MAJOR MEMBRANE GLYCOPROTEIN (ASGP-1) OF THE 13762 MAT-C3 RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS COMPARED TO CONTROL[1,2]

Parameter[3]	Group I	Group II	Group III
Retinoid and Supplement	12 mg RA/ kg diet	60 mg RA/ kg diet	120 mg RA/ kg diet
ASGP-1			
Density	↓	NE	NE
Molecular weight	↓	↓	↓ ↑ low MW[4] oligosaccharides
Oligosaccharide pattern			
Peak II	NE	-	↓ MW
Peak III	NE	-	shoulder to the right of peak III
Contact (TEM)[5]	↓	NE	-
Chromatin pattern (as compared to 100% normal in control)	NE	NE	-
Nucleoli			
No./cell	NE	NE	-
Margination	NE	NE	-
Mitochondria	NE	NE	-
Glycogen			
No. of cells with glycogen	↑	↑	-
Amount of glycogen/cell	NE	NE	-
Lipid			
No. of cells with lipid	NE	NE	-
Amount of lipid/cell	NE	NE	-

TABLE XXXV (Continued)

Parameter[3]	Group I	Group II	Group III
Retinoid and Supplement	12 mg RA/ kg diet	60 mg RA/ kg diet	120 mg RA/ kg diet
Cell viability (as compared to 100% in control)	NE	NE on viability, ↑ synthesizing activity	-
Cell shape	NE	Distorted (Irregular)	-

[1]Controls fed a closed formula vitamin A-adequate non-purified diet.

[2]NE, no effect; ↑, increase; ↓, decrease; -, not studied.

[3]Parameters are as defined in Chapters II and III.

[4]Molecular weight as indicated by elution volume from Bio-Gel P-4.

[5]As studied by Transmission Electron Microscopy.

TABLE XXXVI

EFFECTS OF DIETARY ALL-TRANS-RETINOIC ACID (RA) AND ALL-TRANS-RETINYL PALMITATE (RP) ON THE INCIDENCE, MORPHOLOGY AND MAJOR MEMBRANE GLYCOPROTEIN (ASGP-1) OF THE 13762 MAT-BH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS COMPARED TO CONTROL[1,2]

Parameter[3]	Group I	Group II	Group III
Retinoid and Supplement	4.5 mg RP/ kg diet	60 mg RA/ kg diet	120 mg RA/ kg diet
ASGP-1			
Density	↑	NE or ↓	NE
Molecular weight	NE or ↑	↓	↓
Oligosaccharide pattern			
Peak II	NE, ↑ amount	↓ amount	↓ amount
Peak III	NE	↑ MW[4]	↑ MW
Peak IV	NE	↑ MW	↑ MW and amount
Contact			
SEM[5]	↑	NE	NE
TEM[5]			
General	↑	↑	↑
Microvillar	↑	↑	NE
Branching of Microvilli			
No. of cells with branched microvilli	↓	NE	NE
Degree of branching	NE	NE	NE
Chromatin pattern (as compared to 100% normal in control)	92% Normal	100% Normal	100% Normal
Nucleoli			
No./cells	NE	NE	NE
Margination	NE	NE	NE
Mitochondria	NE	↓ occurrence of condensed configuration	NE

TABLE XXXVI (Continued)

Parameter[3]	Group I	Group II	Group III
Retinoid and Supplement	4.5 mg RP/ kg diet	60 mg RA/ kg diet	120 mg RA/ kg diet
Glycogen			
No. of cells with glycogen	NE	NE	NE
Amount of glycogen/cell	NE	NE	NE
Lipid			
No. of cells with lipids	↑	↑	↑
Amount of lipid/cell	NE	NE	↑
Cell Viability (as compared to 97% in controls)	95%	100%	100%
Cell Shape	NE	NE	NE
Incidence of tumor in rats (as compared to 40% in controls)	50%	100%	100%

[1]Controls were fed a closed formula vitamin A-adequate, non-purified diet.

[2]NE, no effect; ↑, increase; ↓, decrease; -, not examined; if contradictory effects were recorded, they represent different cell combinations (replicates).

[3]Parameters were previously defined in Chapters II and III.

[4]Average molecular weight as indicated by elution volume from Bio-Gel P-4.

[5]As examined by Scanning Electron Microscopy.

[6]As examined by Transmission Electron Microscopy.

TABLE XXXVII

EFFECTS OF DIETARY ALL-TRANS-RETINOIC ACID (RA) AND ALL-TRANS-RETINYL PALMITATE (RP) ON THE INCIDENCE, MORPHOLOGY AND MAJOR MEMBRANE GLYCOPROTEIN (ASGP-1) OF THE 13762 MAT-CH RAT ASCITES MAMMARY ADENOCARCINOMA CELLS AS COMPARED TO CONTROL[1,2]

Parameter[3]	Group I	Group II	Group III
Retinoid and Supplement	4.5 mg RP/ kg diet	60 mg RA/ kg diet	120 mg RA/ kg diet
ASGP-1			
Density	↓ or ↑	NE or ↓	↓
Molecular Weight	↑	↑	↑
Oligosaccharide Pattern			
Peak II	less heterogeneous, ↓ MW[4]	NE	NE
Peak III	NE or ↓ MW	NE	NE
Contact			
SEM[5]	NE	↑	NE
TEM[6]	NE	NE	NE
Branching of Microvilli			
No. of cells with Branched Microvilli	↓	NE	NE
Degree of Branching	NE	↑	↑
Chromatin Pattern (As compared to 100% normal for control)	NE	NE	NE
Nucleoli			
No./cell	NE	NE	NE
Margination	NE	NE	NE
Mitochondria	↓ occurrence of condensed configuration	↓ occurrence of condensed configuration	↓ occurrence of condensed configuration

TABLE XXXVII (Continued)

Parameter[3]	Group I	Group II	Group III
Retinoid and Supplement	4.5 mg RP/ kg diet	60 mg RA/ kg diet	120 mg RA/ kg diet
Glycogen			
No. of cells with glycogen	NE	NE	NE
Amount of glycogen/cell	NE	NE	NE
Lipid			
No. of cells with lipids	NE	NE	NE
Amount of lipid/cell	NE	NE	NE
Cell Viability (as compared to 100% in control)	NE	NE	NE
Cell Shape	NE	NE	NE
Incidence of tumor in rats (as compared to 40% in control)	100%	80%	100%

[1]Controls were fed a closed formula vitamin A-adequate, non-purified diet.

[2]NE, no effect; ↑, increase; ↓, decrease; -, not examined; if contradictory effects were recorded, they represent different cell combination (replicates).

[3]Parameters were previously defined in Chapters II and III.

[4]Average molecular weight as indicated by elution volume on Bio-Gel P-4.

[5]As examined by Scanning Electron Microscopy.

[6]As examined by Transmission Electron Microscopy.

A 100% growth incidence of MAT-BH ascites tumor line in rats occurred in the groups supplemented with either 60 or 120 mg retinoic acid/kg diet. Groups fed the control diet or the retinyl palmitate supplemented diet had 40% and 50% incidence of MAT-BH ascitic growth, respectively. Control rats had a 40% incidence of ascitic MAT-CH growth. An increase to 80-100% MAT-CH ascitic growth incidence occurred in the animals fed the retinyl palmitate or the retinoic acid supplemented diets.

The effects of dietary vitamin A on cell morphology were more conclusive for the homogeneous cell lines, MAT-B1 and MAT-C3, than for the heterogeneous cell lines MAT-BH and MAT-CH. The Chi square test was used to detect significant effects of diet on the different parameters studied.

High retinoic acid concentrations in the diet (60 mg retinoic acid/kg diet) induced necrosis in the MAT-B1 subline, and enhanced the synthetic activity of the MAT-C3 line as indicated by hypertrophy of the rough endoplasmic reticulum and active cytoplasm. Both retinyl palmitate and retinoic acid, in the diet of MAT-BH tumor bearing rats, tended to enhance MAT-BH cell-to-cell cohesiveness and the occurrence of lipid deposits in the cell. In the MAT-CH subline, a decrease in the occurrence of mitochondria in the condensed configuration (low energy form) was observed upon dietary supplementation with retinyl palmitate or retinoic acid.

Both retinoic acid and retinyl palmitate affected the density, molecular weight, and the oligosaccharide pattern of ASGP-1. The effects varied with the tumor subline, and with the type and concentration of retinoid used. The vitamin A analogs appeared to affect

both the carbohydrate and protein moieties of the glycoprotein. The Wald-Wolfowitz two-sample runs test (147) was used to detect significant changes ($P < .05$) in the molecular weight of ASGP-1. The test could not be applied to the density or oligosaccharide profiles, since the peaks consisted of fewer than 9 points each.

The observation that, in some cases, replicate experiments on the MAT-BH and MAT-CH did not give consistent results might be due to the heterogeneity of these cell lines.

Compared to controls (fed a closed formula non-purified vitamin A-adequate diet), there was no effect of diet on the survival of mice injected with MAT-BH or MAT-CH cells and fed a purified vitamin A-deficient diet supplemented with 120 mg retinoic acid/kg diet. Mice fed a vitamin A-deficient diet for 16 weeks were not retinol-deficient. MAT-BH tumor incidence in these mice did not differ from that of control mice; however, there was an enhanced survival of the tumor bearing mice. In the case of MAT-CH subline, all of the mice survived. However, the mice fed the vitamin A-deficient diet had a higher incidence of MAT-CH ascitic growth as compared to controls.

Further study will be required to identify the structural changes in ASGP-1, to determine more specifically the mechanisms behind the changes observed, and to study in more detail the effect of excess retinoic acid on other parameters of the tumor cells such as membrane fluidity and membrane enzyme content, cell surface receptor mobility, and agglutinability by concanavalin A.

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