CELL MEMBRANE CHARACTERISTICS OF GLUCOCORTICOID RESPONSIVE LYMPHOID TUMORS

by

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ABSTRACT

Ultrastructural, histochemical and biochemical characteristics of cell surface structures were compared in corticoid-sensitive (CS) and -resistant (CR) mouse lymphomas.

Histochemical techniques applied to electron microscopy involved the use of the electron-absorbing, cationic dyes Alcian blue 8GX and ruthenium red and the metal salt, lanthanum nitrate. The studies were carried out on lymphosarcoma P1798-CS and -CR. Alcian blue binding was also tested on 6C3HED-CS and -CR lymphoma cells. Examination by electron microscopy revealed Alcian blue complexes on cell surfaces of CS P1798 and 6C3HED cells. Reaction product could be observed as an electron-dense coat of variable thickness on the outer leaflet of the cell membrane, indicating the presence of anionic sites. A small number of cells in the CS population of both tumors lacked coat material. Cells from CR P1798 and 6C3HED tumors showed no or few scattered electron-opaque dye deposits on their surfaces, with the exception of a small number of cells that had a continuous cell coat. Ruthenium red and lanthanum nitrate binding was essentially negative on both P1798-CS and -CR cell surfaces; reaction product when present was barely discernable.

In the intact tumor tissue as well as in cell suspensions, P1798-CR cells were frequently observed to adhere to each other by cytoplasmic bridges or junction-like complexes, whereas CS cells generally showed a looser arrangement with

fewer contact sites.

Intact cells, examined by scanning electron microscopy, showed smooth and moderately villous surfaces in both CS and CR cell preparations. Approximately 45% of CR cells revealed a prominent single protrusion, resulting in a pear- or mushroom shape. Relatively few (11%) of such irregular patterns were seen in P1798-CS cell preparations.

Cell membrane glycopeptides of P1798-CS and -CR were studied by differential labeling in vivo with L-fucose-^{14}C and -^{3}H. Separation of pronase-digested glycopeptides by gel filtration on Bio-Gel P-10 showed markedly increased amounts of high molecular weight and reduced amounts of low molecular weight fractions in P1798-CR as compared to -CS. Removal of glycopeptide-bound sialic acid (SA) by neuraminidase resulted in an overall shift of both CS and CR membrane glycopeptides to a lower molecular weight. Differentially labeled fractions showed altered ratios and a greater uniformity of major peak fractions. In vivo steroid treatment (6 hours) produced an additional low molecular weight peak in the elution profile of desialylated glycopeptides. A 7 hour in vivo exposure to glucocorticoids resulted in a greater density of both P1798-CS and -CR isolated plasma membranes. The chromatographic pattern of glycopeptides derived from these membranes showed a general shift to a higher molecular weight for both CS and CR samples. Incubation with neuraminidase produced a new low molecular weight peak (not seen in untreated preparations), similar to that seen

6 hours after steroid application but more prominent.

These results indicate that exposed cell surfaces of P1798-CS and -CR differ in charge but show that cell membrane-derived glycopeptides of both tumors contain negatively charged SA residues. The difference in CS and CR L-fucose-containing sialoglycopeptides suggests that the absence of anionic sites on CR cell surfaces is related to structural properties of CR carrier glycoproteins. The described observations further imply that the outcome of steroid-induced alterations (cell death or survival) is influenced by cell membrane characteristics involving carbohydrate components.

DEDICATION

To Dr. Vincent P. Hollander whose guidance and generous support made this work possible. For his kindness and understanding I shall always be grateful.

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INTRODUCTION

Objective and Approach of Study

The purpose of this investigation was to elucidate the role of the plasma membrane in the cytolytic process induced by glucocorticoid hormones in lymphoid tumors.

Two thymus-derived lymphomas, known as P1798 and 6C3HED, were selected for an ultrastructural study of the cell surface. Lymphosarcoma P1798 was also used for a biochemical analysis of plasma membrane-derived glycopeptides. This tumor is the object of study in several laboratories, and subcellular changes related to the steroid-mediated lytic process are partially known. 6C3HED is not routinely used as an experimental model in the analysis of corticosteroid action.

Both P1798 and 6C3HED are available as corticoid-sensitive (CS) and -resistant (CR) tumors thus providing an ideal system for correlating biochemical changes resulting from glucocorticoid action.

The cell surface was investigated by histochemical electron microscopic techniques which permit a visual detection of exposed sites on the cell membrane. Such sites involve the carbohydrate portion of the cell coat which is known to be of diverse nature (Spiro, 1970; Winzler, 1970; Rapin and Burger, 1974) and may play an important role in biological function.

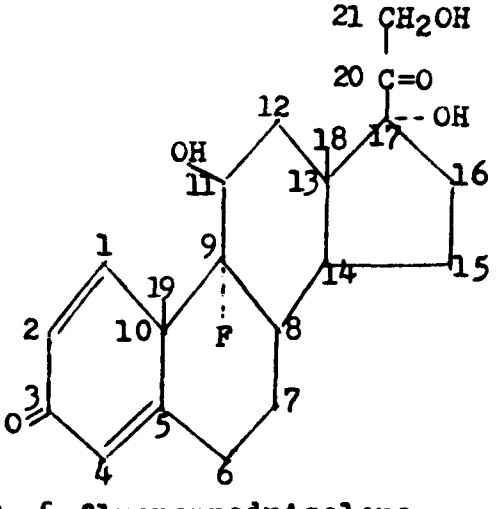
The biochemical studies involved a limited analysis of isolated plasma membranes and the separation of cell membrane-

derived, *in vivo* L-fucose-labeled glycopeptides by column chromatography.

Known Mechanisms of Action of Glucocorticoids and Historical Background

Glucocorticoids and their analogues are known for their cytolytic action in certain lymphoid tissues. This effect constitutes the basis for the use of these hormones as therapeutic agents in malignancies involving the lymphoid system.

The cytotoxic effectiveness of corticosteroids is directly related to their chemical structures. The importance of the 11β-hydroxyl group in cortisol has been demonstrated by the failure of its 11-deoxy analogue (cortexolone) to show glucocorticoid activity (Harris, 1970), although specific binding to receptor proteins is not impaired (Kaiser et al, 1972). The presence of a double bond in the 1:2 position and the addition of halogens, such as fluoride, in specific positions on the cyclopentano-perhydro-phenanthrene nucleus of steroid hormones can increase the lethal action up to 100-fold. It is for this reason that synthetic analogues (see diagram), such as prednisolone (double bond between carbons 1 and 2), 9-α-fluoroprednisolone (9-FP) (1-2 double bond and fluoride on carbon 9) and others are preferred therapeutic drugs.



9-α-fluoroprednisolone

The first evidence of a steroid-induced cytotoxic ac-

tion in lymphoid tumors was given by Heilman and Kendall in 1944. Their studies showed that lymphomas in mice regress almost completely after the administration of pharmacological doses of corticosterone. In 1945, Dougherty and White described steroid-mediated morphological changes in lymphoid tissues. Subcellular molecular events leading to cell death have been studied for the last two decades, however, the exact sequence of biochemical reactions is still imprecisely known.

Metabolic changes that occur in P1798-CS and other responsive lymphoid tissues as a result of glucocorticoid action involve a decrease in the oxidation of free fatty acids (Turnell et al, 1973); inhibition of transport of essential nutrients such as glucose (Morita and Munck, 1964; Munck, 1968; Rosen et al, 1970a, 1972), amino acids (Rosen et al, 1972), and nucleosides (Stevens et al, 1973); inhibition of precursor incorporation into nucleic acids and protein (Rosen et al, 1970b, 1972; Morita and Munck, 1964; Makman et al, 1971; Stevens et al, 1971) and an increase in ribonuclease activity (Ambellan and Hollander, 1966). These alterations are preceded by the formation of specific hormone receptor complexes and by one or more steps involving macromolecular synthesis (Baxter et al, 1972; Munck, 1971). A working hypothesis for the sequence of events leading to cell death was proposed by Munck (1971) and includes the following steps: Glucocorticoids form a complex with specific cytoplasmic receptors. The stable hormone receptor complex is then trans-

located into the nucleus by a temperature-sensitive step and then attaches to specific chromosomal sites. Presumably the synthesis of a protein is induced which involves an irreversible, temperature-sensitive, actinomycin D-, cycloheximide- and puromycin-sensitive step. This anabolic process is followed by inhibition of glucose uptake resulting in decreased availability of ATP which is required for the synthesis of macromolecules such as DNA, RNA and protein. Thus the lack of this high energy compound then induces catabolic events leading to cell lysis.

In P1798-CS lymphocytes, glucose deprivation in the presence of cortisol in vitro has been reported to result in marked suppression of nucleotide and amino acid incorporation (Rosen et al, 1972). These results were challenged by Stevens et al (1974), who found no correlation between nucleoside uptake and incorporation by P1798-CS cells (in vitro) and glucose transport. Studies by other authors also indicate that the need for glucose as a key factor in the catabolic action of corticosteroids in lymphoma cells (Gabourel and Aronow, 1962) and thymocytes (Drews and Wagner, 1970; Makman et al, 1967) is questionable.

The first step in physiological effects of glucocorticoids is believed to be binding of the hormone to specific cytoplasmic receptor proteins (Gehring et al, 1972). Munck and Brinck-Johnson (1968) reported that in the rat thymus, this specific binding is dependent on the presence of either oxygen and ATP or glucose and ATP; however, for cortisol to

elicit its physiological effect, oxygen, glucose, and ATP must be present. Studies with inhibitors of receptor binding indicate that receptor proteins are absent from the cell surface (Rousseau and Tomkins, 1972), but this does not preclude association with the cytoplasmic side of the cell membrane. The detailed biochemical events beyond glucocorticoid receptor binding are obscure. Antibiotic-sensitive synthetic steps already described suggest that transcription and translation of RNA play a role in the catabolic process that follows these events.

Steroid-induced loss of nuclear structure in rat thymocytes requires both respiration and extracellular inorganic phosphate and involves the formation of a nuclear phosphoprotein (Whitfield et al, 1968). Corticoid responsiveness of lymphoid cells is not related to the cell generation cycle. The cytotoxic effects of glucocorticoids may become evident either before or after the cell loses its capacity to divide (Harris, 1970). Studies by Story and Melnykovych (1973b) have shown that prednisolone decreases the rate of cell division in mouse lymphoma cells and suggest that exposure to the steroid must occur during the cell cycle preceding the one during which the increase in generation time becomes obvious. In contrast to pharmacological doses, low concentrations of cortisol (10^{-8} M) do not induce cell lysis but stimulate proliferation of thymocytes maintained <u>in vitro</u> (Whitfield et al, 1970).

There is no direct evidence that the primary site of

action of glucocorticoids is the cell membrane. The rapid distribution of steroids throughout target cells does not suggest a membrane-active mechanism (Munck, 1971). However, steroid-induced functional changes in responsive lymphoid cells, such as impaired uptake of certain nutrients, suggest that structural alterations of cell membrane components do occur. Evidence for such modifications was given by Story et al (1973a,b) who reported an early reduction of choline incorporation into the lipid cell fraction and phospholipids of the cell membrane of cultured L5178Y mouse lymphoma cells. Dell' Orco and Melnycovych (1970a,b) reported similar in vitro effects of prednisolone in human embryonic Henle cells and HeLa S3 cells using ^{32}P-phosphoric acid as label. Suppression of ^{32}P uptake was evident as early as 10 minutes after addition of the steroid and preceded all other alterations brought about by the hormone. Both ^{32}P and choline are constituents of the phospholipid fraction which makes up approximately 50% of the lipid content of cell membranes (Bosman et al, 1969). A change in the phospholipid/protein ratio could be reflected in profound changes in cell metabolism. It has been shown that by modifying the lipid composition of the cell membrane it is possible to alter membrane structure as well as membrane permeability (Willmer, 1961). Turnell et al (1973) found that cortisol increases the free fatty acid pool in thymocytes and in P1798-CS cells in vitro. Based on their observation that substitution of cortisol by free fatty acids in the incubation medium had the same effect

as the steroid, they proposed that the accumulation of free fatty acids leads to nuclear damage followed by cell lysis.

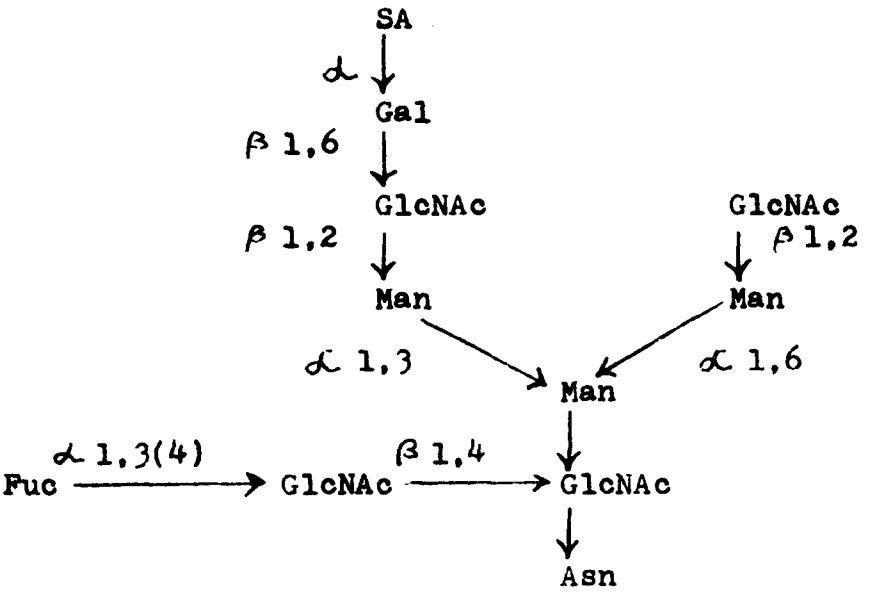
In hepatoma cells, glucocorticoids have been shown to alter electrophoretic, antigenic and adhesive properties of the cell surface (Ballard and Tomkins, 1970). The induction of these changes required synthetic steps sensitive to actinomycin D, cycloheximide, pH and temperature, suggesting that de novo protein synthesis is involved in steroid-mediated membrane changes that involve the oligosaccharide chains of surface glycopeptides.

Cell Surface Glycopeptides and their Role in Cell Metabolism

Evidence is accumulating that the carbohydrate portion of cell membrane glycoproteins is associated with a variety of biological phenomena. Different cells have a unique glycoprotein composition which could explain a number of diverse physiological events. This subject has been reviewed in detail by Spiro (1970), Winzler (1970), Kraemer (1971), and Hughes (1973), and only information pertinent to this study is discussed.

In glycoproteins, one or several oligosaccharide chains are covalently linked to the polypeptide. Carbon 1 of the reducing terminal sugar may be linked to: (a) the amide group of asparagine, (b) the hydroxyl groups of serine, threonine, hydroxy-lysine or hydroxy-proline residues, or (c) to sulfur in cysteine residues. N-acetylglucosamine was found to always be linked to asparagine. An example is the carbohydrate chain found on a myeloma immunoglobulin heavy chain seen in

the diagram below:

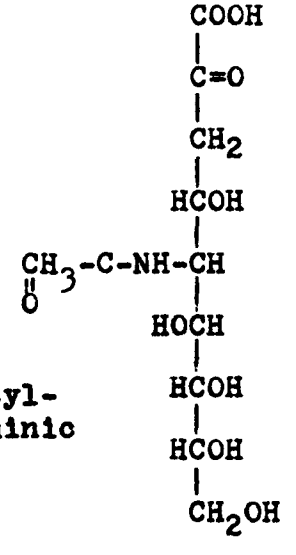


Copied from Hughes (1973), pg.196

There is strong evidence that most of the carbohydrates of the cell membrane are exposed on the external surface and can be removed as glycopeptides by proteolytic enzymes (Javaid and Winzler, 1974; Winzler, 1970). The oligosaccharide chains are made up of a limited number of sugars including D-galactose, D-mannose, D-glucose (rarely), N-acetylglucosamine, N-acetylgalactosamine, L-fucose and sialic acid. Both fucose and sialic acid are terminal sugars and are usually linked to galactose residues (sialic acid may also be linked to N-acetylgalactosamine and fucose to N-acetylglucosamine) (Hughes, 1973). There is some evidence that, at least in the liver, sialyl and fucosyl transferases compete at random for the same galactose terminals (Hudgin and Schachter, 1971). The activated forms of these sugars that are recognized by

specific transferases are GDP-fucose (Bekesi and Winzler, 1967) and CMP-sialic acid (Pricer and Ashwell, 1971). The glycosyltransferases that catalyse the assembly of glycoprotein associated oligosaccharides have been found in the endoplasmic reticulum and in the Golgi complex (Schachter et al, 1970; Hughes, 1973). The sequence of sugar residues in the carbohydrate chain apparently is determined by the specificities of the different enzymes for the sugar nucleotide (GDP, CMP and UDP) and the acceptor (Spiro, 1970).

Of interest in this investigation are the sialic acids which make up a group of neuraminic acids that differ in their side groups (Gottschalk, 1960b). The 9 carbon backbone of sialic acid is derived from pyruvic acid and mannosamine, and the known neuraminic acids of animal cells are N-acyl and N-acyl-O-acyl derivatives. The structure of N-acetylneuraminic acid, which is frequently found in animal cell membranes, is shown below. Presumably most of the sialic acid is attached to protein rather than to lipid (Weed and Reed, 1966). The closely spaced, negatively charged carboxyl groups of this sugar are believed to give rigidity to the cell membrane through mutual electrostatic repulsion (Gottschalk, 1960a). The biological function of the various sialic



N-acetyl-neuraminic acid

acids and other surface sugar components, which may exist in many different combinations and permutations, seem completely obscure. Neuraminic acid plays a role in the homing of lymphocytes. The removal of this sugar by neuraminidase results in temporary migration of thoracic duct lymphocytes to the liver rather than to the spleen and lymphnodes (Gesner and Woodruff, 1969). L-fucose and N-acetyl-D-galactosamine are also involved in proper homing of the cells (Gesner and Ginsberg, 1964). Surface sialic acids have been shown to promote implantation of the ovum (Gasic and Gasic, 1970), and sialic acid-rich chorionic gonadotrophins, produced by trophoblasts, promote luteal function. Neuraminidase treatment of tumor cells apparently results in alterations of tumor histocompatibility antigens, thereby reducing transplantability (Sanford, 1967; Cormack, 1970). Galactose is involved in intercellular adhesive specificity which enables like cells to recognize each other (Roth et al, 1971). Cox and Gesner (1968) demonstrated that certain naturally occurring sugars, such as L-fucose and D-mannose, can profoundly alter cell growth and metabolism. These effects appeared to vary with different cell lines and could be selectively altered, depending on the type of sugar added to the medium. These observations raise the possibility that other agents, such as glucocorticoid hormones, which affect cell function and metabolism, may do so by rearranging the microstructure of surface glycopeptides. Examination of this possibility was the aim of this investigation.

MATERIALS AND METHODS

Animals and Tumors

CS and CR cell lines of lymphosarcoma P1798 and 6C3HED, carried in female Balb/c (NIH Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, Bethesda, Md.) and C3H mice (C3H/He J, The Jackson Laboratory, Bar Harbor, Maine), respectively, were used in this study. Both tumors were maintained by subcutaneous transplantation of small tissue pieces (ca. 1 cmm) into the right flank as described by Stevens et al (1971).

CS and CR tumors were tested in vivo for corticoid sensitivity with pharmacological doses (25 mg/kg) of 9-FP. Criteria for corticoid sensitivity were shrinkage of tumor mass in CS strains and continued tumor growth in CR strains (Stevens et al, 1974).

The P1798-CR and the semiresistant -CR1 and -CR2 strains were initially produced from the CS strain by chronic steroid treatment as described by McCain Lampkin and Potter (1958). A P1798-CS strain (referred to as P1798-AR) that had been made asparaginase resistant (by frequent, increasingly larger doses of E.Coli asparaginase through several transplant generations) had become CR for unknown reasons.

ELECTRON MICROSCOPY

Preparation of P1798 Lymphosarcoma for Morphological Study after Steroid Treatment

Tumor-bearing (P1798-CS and -CR) mice were injected i.p. with 9-FP (25 mg/kg). Control animals received injections of

steroid vehicle. At intervals of 3, 6, 7, 8, 12 and 18 hours after treatment, 2 animals each of treated and control groups were sacrificed by cervical dislocation. With the aid of 2 small spatulas, cells were teased out of tumor slices into cold fixative, a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde, 1% osmic acid, and saturated lead citrate (1.5:1.5:2:1) (Simionescu et al, 1972) in 0.1 M sodium cacodylate, pH 7.4. The remaining tissue was removed by filtering the cell suspension through 4 layers of surgical gauze. 30 minutes after fixation at 4° C, the cell pellet was overlaid with 2% aqueous uranyl acetate for 20 minutes at room temperature, followed by dehydration in a graded series of ethanol, cleared in propylene oxide and embedded in Epon 812 as described by Luft (1961).

Thick sections (0.5 μ) were prepared with glass knives and subsequently stained with Azure B (0.02% in 1% sodium borate) on a hotplate at 60° C. The morphology of cells was examined by electron microscopy (on thin sections) and the percentage of damaged cells (cells showing pyknosis and visible focal damage) in a field of 1000 cells was determined by light microscopy (on thick sections).

Reaction with Alcian Blue, Ruthenium Red and Lanthanum Nitrate

Cells (P1798-CS and -CR and 6C3HED-CS and -CR) of at least 2 different tumor transplant generations were teased out of the tissue in Hank's balanced salt solution (Hank'sBSS), pH 7.4, at room temperature. After gentle centrifugation (150 x g) for 5 minutes, portions of the cell pellet were processed for high resolution histochemistry of the cell surface.

All fixatives were buffered with 0.1 M sodium cacodylate and the pH varied with the procedure. The cells were suspended in:

(a) 2% glutaraldehyde (pH 6.5) containing 1% Alcian blue 8GX (Allied Chemical Corp., Morristown, N.J.) and fixed for 1 hour at room temperature. The material was postfixed in 1% OsO_4 (pH 7.2) for 1 hour at 0° C (Behnke and Zelander, 1970).

(b) 2% glutaraldehyde (pH 7.2), fixed for 1 hour at room temperature and postfixed in 1% OsO_4 (pH 7.2) for 1 hour at 0° C. Both fixatives contained 50 mg% ruthenium red (K & K, Inc., Plainview, N.Y.) (Dermer et al, 1974).

(c) 2.5% glutaraldehyde (pH 6.9) containing 1% lanthanum nitrate, fixed at 0° C for 1 hour and postfixed in 1% OsO_4 (pH 6.9), also containing 1% lanthanum nitrate, for 1 hour at the same temperature (Overton, 1969).

Procedures (b) and (c) were used for P1798-CS and -CR only. Control cells were processed in the absence of Alcian blue, ruthenium red and lanthanum nitrate, respectively. After rinsing in 0.1 M cacodylate buffer, all samples (experimental and controls) were dehydrated and embedded as described before.

Fixation in the presence of Alcian blue was also performed on P1798-CS and -CR cells 3, 6, 8, and 15 hours after treatment (in vivo) with 9-FP (25 mg/kg body weight).

Fixation of Solid P1798 and 6C3HED Tumors

Small pieces of tumor tissue (P1798-CS and -CR and

6C3HED-CS and -CR), with a diameter of approximately 0.5 mm, were fixed in 2.5% glutaraldehyde in Millonig's phosphate buffer (pH 7.2) and postfixed in 1% OsO_4 (in the same buffer) for 30 minutes each at room temperature (Hyat and Giaquinta, 1970). To enhance membrane contrast, the fixed tissue was stained en bloc with 1% aqueous uranyl acetate for 20 minutes. To prevent the formation of a precipitate (uranyl acetate reacts with phosphate), the material was briefly rinsed with distilled water before staining. After dehydration in a series of graded acetone (25%, 75%, 95%, and 100%), the tissue was embedded in Epon 812 (Luft, 1961).

Thin sections of all samples for electron microscopy were cut either on an Mt-2 Porter-Blum or an LKB ultramicrotome and stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Electron micrographs were taken with a Phillips 300 electron microscope at 80 kV.

Preparation of P1798-CS and -CR Cells for Scanning Electron Microscopy

Two different transplant generations each of P1798-CS and -CR were processed as follows: The tumor cells were removed from the tissue in Hank's BSS as described before (each sample consisted of pooled cells from 3 tumors). After 3 washes (in Hank's BSS), a monolayer of cells was prepared by aspiration-filtration of "Flotronic" silver membranes (Flotronic Inc., Spring House, Pennsylvania) as described by de Harven et al (1973). The hydrated monolayers were immediately fixed for at least 48 hours (at 4° C) with 1% glutaraldehyde (pH 7.3, 320 mOsm.). After two rinses in buffer

and dehydration in a graded series of ethanol, the samples were prepared for "critical point" drying (Polliack et al, 1973). This involved further dehydration by a graded series of amyl acetate/absolute ethanol and then absolute amyl acetate. The specimens were dried in a Denton critical point apparatus (Model DCP-1) using CO_2 gas at a pressure of approximately 1500 psi. The dried samples were coated with a thin layer of carbon and gold on a rotary stage (Denton Vacuum Apparatus, DV-502) at an angle of 15°. Scanning electron micrographs were taken with a Cambridge S4 scanning electron microscope at 20 kV.

Counting of Irregular Cells

The percentage of irregularly-shaped cells (pear- or mushroom shape) in P1798 and 6C3HED cell populations was determined by light microscopy in a hemocytometer.

Removal of Dead Cells and Red Blood Cells by Centrifugation on Hypaque-Ficoll

Hypaque-Ficoll was prepared by mixing 10 parts of 33.9% Hypaque (Winthrop Laboratories, New York, N.Y.) and 24 parts of 11% Ficoll (polysucrose; Pharmacia Fine Chemicals, Piscataway, N.J.) with a final density of 1.082 at 23° C.

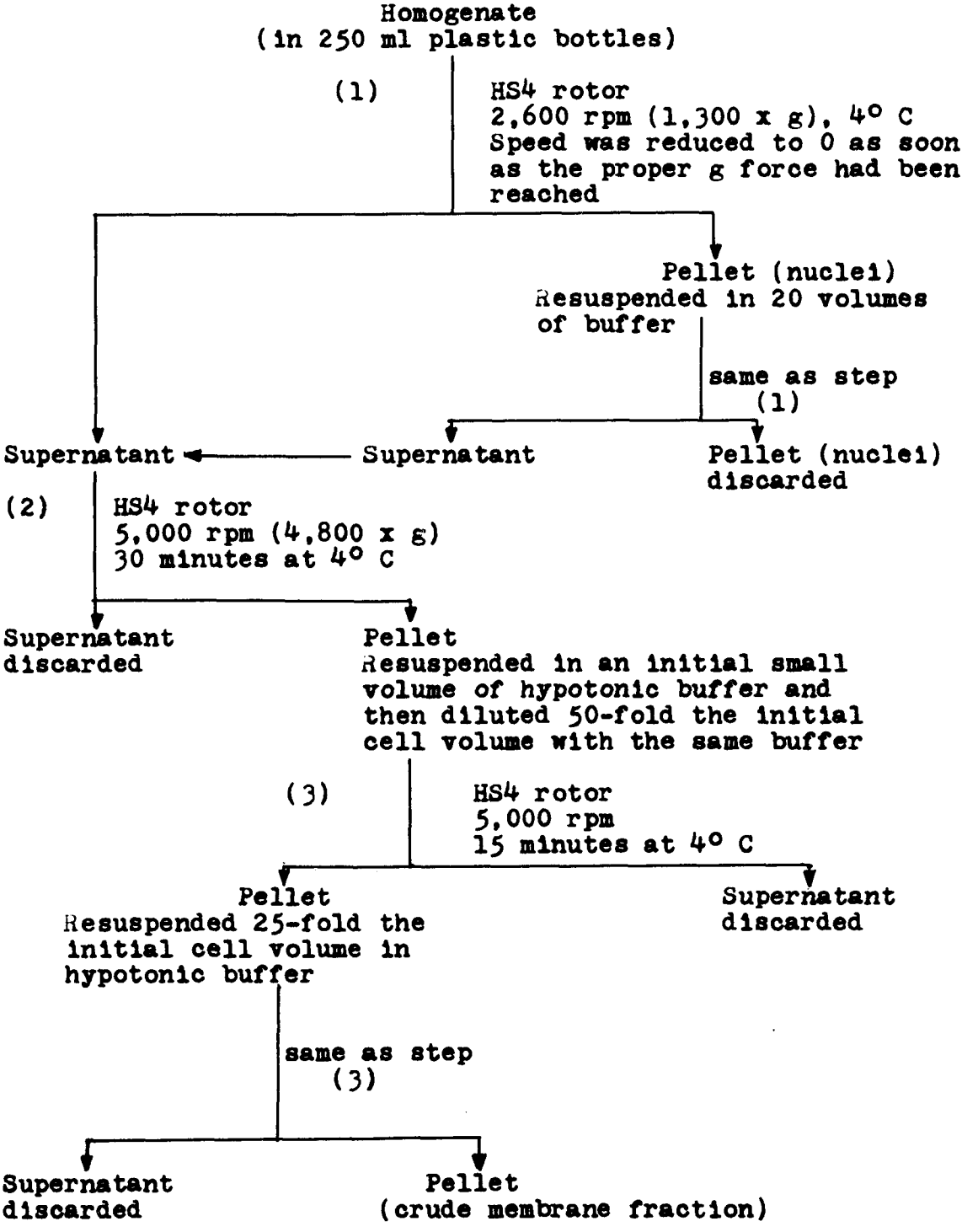
P1798-CS and -CR cell suspensions (10^8 cells in 1 ml of Hank's BSS, pH 7.4) were layered on 2 ml of the above Hypaque-Ficoll mixture and centrifuged at 1000 x g for 5 minutes. The cell layer at the Hypaque-Ficoll/Hank's BSS interface was aspirated with a Pasteur pipette and washed in 10 ml of Hank's BSS. Dead cells and red blood cells could be collected at the bottom of the Hypaque-Ficoll layer. The washed viable

cells (as judged by trypan blue exclusion) were used for fixation in the presence of Alcian blue by the procedure already described.

ISOLATION OF PLASMA MEMBRANES

Animals, carrying tumors of 1 to 2 gm in size, were sacrificed by cervical dislocation. Tumors were removed, freed of surface fat and necrotic tissue and then processed for the isolation of plasma membranes under ice-cold conditions. The tissue was minced in Hank's BSS, pH 7.4, and filtered through 4 layers of surgical gauze. A packed cell pellet was obtained by centrifugation of the cell suspension at 150 x g for 5 minutes. For the removal of red blood cells, the pellet was resuspended in a mixture of 1 volume of Hank's BSS and 1 volume of 0.85% ammonium chloride, pH 7.4 (pH was adjusted with 1 M Tris). The suspension was again centrifuged for 5 minutes (at 150 x g) and the packed cell volume recorded. The supernatant, which contained hemoglobin and red blood cell ghosts, was removed, and the remaining tumor cells were processed for the isolation of cell membranes as described by Ray (1970) with some modifications. The packed cell volume was resuspended in 2 volumes of hypotonic buffer (1 mM $NaHCO_3$ and 2 mM $CaCl_2$, pH 7.5). Cells were allowed to swell for 10 minutes and then homogenized in a Dounce homogenizer (pestle B) by 20 to 30 gentle downstrokes. Breakage (at least 95%) of cells was monitored by phase microscopy. The homogenate was diluted 100-fold the initial cell volume with hypotonic buffer and left standing for 10 minutes. For

subsequent steps, the following flow sheet was used:



The crude membrane pellet was resuspended in a small volume of hypotonic buffer and homogenized in a 7 ml Dounce homogenizer (pestle A) by 1 to 2 gentle downstrokes to break up small clumps. The suspension was diluted with 60% sucrose to a final concentration of 48% sucrose. Portions of 7.5 ml of the membrane suspension were placed in Beckman cellulose nitrate tubes (1" diameter X 3 1/2") and carefully overlaid with 7 ml of 45%, 12 ml of 41% and 8 ml of 37% sucrose. All sucrose solutions were made up in 5 mM Tris containing 5 mM $CaCl_2$ (pH 7.4). The tubes were centrifuged for 2 hours at 25,000 rpm in an SW27 rotor at 4° C. Membrane fractions appeared at the interfaces between 37% and 41% (L1), 41% and 45% (L2) as well as between 45% and 48% sucrose (L3) in form of a white, compact sheet. The membrane layers were removed with a Pasteur pipette, washed free of sucrose by repeated suspension and centrifugation in 50 ml of .85% sodium chloride. Each membrane fraction was tested for enzyme activity and the morphology examined by electron microscopy. The samples were fixed with 1% OsO_4 (in 0.1 M sodium cacodylate, pH 7.3) for 30 minutes at 0° C, dehydrated in ethanol and embedded as described before.

CHEMICAL ASSAYS

For protein determinations, the samples (whole cells, homogenates and membrane fractions) were hydrolyzed with 1 N NaOH at 60° C for 2 hours or over night. Protein was then assayed by the method of Lowry et al (1951) using bovine serum albumin as the standard. This method is based on the for-

mation of a reduced phosphomolybdate-phosphotungstate complex which is measured as a blue chromophore at 700 mμ. The reducing agents are tyrosine, tryptophan, and cysteine residues of protein, and thus the intensity of the color developed (in 30 minutes at room temperature) depends on the proportion of these amino acids present.

Succinate dehydrogenase was assayed as described by Reid (1972). The assay mixture (containing 125 μmoles sodium phosphate, 62.5 mg 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazoliumchloride (INT), 75 nmoles sodium succinate, and 50 μg protein, pH 7.4, in a total volume of 1 ml) was incubated for 15 minutes at 37° C in a gyratory shaker. The reaction was stopped with cold 10% trichloroacetic acid (TCA) and the chromogen (reduced tetrazolium) extracted with 4 ml ethyl acetate. Absorption was read at 420 mμ, and the concentration of reduced INT was calculated from the extinction coefficient of formazan (reduced INT).

Glucose-6-phosphatase was assayed in 1 ml of 0.1 M sodium acetate containing 5 μmoles glucose-6-phosphate (sodium salt), and 100 to 200 μg protein at pH 6.1 (Hay, 1970). The mixture was incubated at 37° C in a gyratory shaker. After 30 minutes, the reaction was stopped with 1 ml of cold 6% TCA. The inorganic phosphate released was determined by the method of Chen et al (1956) which involves the reduction of a phosphomolybdate complex by ascorbic acid. The resulting blue chromophore was developed in a waterbath at 45° C for 20 minutes. The method is sensitive to nano mole

amounts of inorganic phosphate, and readings at 820 mμ are proportional to phosphate concentrations to an optical density of at least 1.8.

Mg^{2+}-activated ATPase was assayed at pH 7.5 using ATP as substrate (Ray, 1970). The incubation mixture consisted of 10 μmoles $MgCl_2$, 10 to 20 μg protein and 10 μmoles ATP in a total volume of 1 ml. After incubation for 30 minutes at 37° C, the reaction was stopped by adding 1 ml of cold 6% TCA. Inorganic phosphate released was determined as described above for glucose-6-phosphatase with the exception that color was developed at room temperature (for 10 minutes) to keep the absorption of ATP blanks low.

P-nitrophenyl phosphatase was assayed at pH 9.0 in a total volume of 1 ml containing 100 μmoles Tris, 2.5 μmoles $MgCl_2$, 1 μmole p-nitrophenyl phosphate, 0.1% Triton X-100 and 100 to 150 μg protein (Ray, 1970). The mixture was incubated for 30 minutes at 37° C in a gyratory shaker. Since protein was not removed, separate sample blanks without substrate were run and readings at 420 mμ corrected for blanks. P-nitrophenol, which has a yellow color in alkaline solution, was used as the standard.

Sialic acid was determined by the method of Jourdian (1971), using N-acetylneuraminic acid as standard. Contrary to the most widely used periodate-thiobarbituric acid method (Warren, 1959), this procedure does not require acid hydrolysis of sialic acid glycosides and is not affected by lipids, amino acids, or sugars. Sialic acids contained in 300 to

500 μg of sample protein in a volume of 0.5 ml were reacted with 0.1 ml of 0.04 M periodic acid at 0° C for 30 minutes. The oxidized, glycosidically bound and free sialic acids were then heated (for 15 minutes at 100° C) with 1.25 ml of Svennerholm resorcinol reagent. After cooling, the brown chromophore was extracted with 1.25 ml of isoamylalcohol and absorbances determined at 630 mμ.

SEPARATION OF CELL MEMBRANE GLYCOPEPTIDES BY COLUMN CHROMATOGRAPHY

Labeling and Treatment with 9-FP

Fractionation of plasma membrane glycopeptides was performed on 9-FP-treated and untreated lymphosarcoma P1798-CS and -CR. Groups of 10 to 12 each of CS and CR tumor bearing animals were selected for differential labeling in vivo with L-fucose-^{14}C and -^{3}H. Each animal of an experimental group was injected intraperitoneally with either 12.5 μCi of L-fucose-1-^{14}C or 25 μCi of L-fucose-(1,5,6,-^{3}H). The isotopes were suspended in 0.5 ml of 0.85% sodium chloride. L-fucose-^{14}C (56.2 mCi/mmole) and L-fucose-^{3}H (3.73 Ci/mmole) were obtained from New England Nuclear (Boston, Mass.).

Four hours after labeling, the animals were sacrificed, the tumors (ranging from 1-1.5 g in size) removed and cell membranes isolated as described above. For labeling studies 6 and 7 hours after steroid treatment, animals received intraperitoneal injections of 9-FP (25 mg/kg bodyweight) 2 and 3 hours, respectively, before the application of radioactive fucose.

Preparation of Labeled Plasma Membranes for Gel Filtration

Appropriate portions of differentially labeled P1798-CS and -CR cell membranes (pooled L1 and L2 fractions) were solubilized in 1 ml of 1% dodecyl sodium sulfate (SDS) at 60° C for 45 minutes to 1 hour. The solubilized membrane proteins were diluted to a total volume of 15.5 ml with 0.2 M phosphate buffer, pH 7.8, containing 1.5 mM $CaCl_2$ and 10 mg pronase (K & K, Inc., Plainview, N.Y.) and further processed by the method of van Beek et al (1973). Digestion was carried out for 5 days at 37° C on a gyratory shaker. Bacterial growth was prevented by the addition of a few drops of toluene. Fresh pronase (10 mg) was added at 24 hour intervals. At the end of the incubation period, the denatured enzyme was removed by centrifugation at 48,000 x g for 20 minutes. The clear supernatant was dialyzed for 24 hours in cellulose tubing at 6° C against 1 liter of 0.1 M NaCl and for an additional 24 hours against 1 liter of twice-distilled water. The non-dialyzable material was lyophilized and then dissolved in 10 ml of 0.05 M sodium acetate buffer, pH 5.5, containing 6.8 mM $CaCl_2$. The mixture was divided into 2 equal portions. To one of the samples, 50 units of Vibrio cholerae neuraminidase (General Biochemicals, Chagrin Falls, Ohio) were added; the other portion served as the control. Both samples were incubated for 2 hours at 37° C on a gyratory shaker. The preparations were then lyophilized and stored at -20° C until fractionation by chromatography.

Gel Filtration

The lyophilized material was dissolved in 1 ml eluent buffer (0.1 M Tris-acetate, pH 9, containing 0.1% SDS, 0.01% EDTA, and 0.1% mercaptoethanol). Blue dextran 2000 (Pharmacia Fine Chemicals, Piscataway, N.J.) and phenol red were added to serve as high and low molecular weight markers, respectively. The samples were fractionated on Bio-Gel P-10, 200-400 mesh (Bio-Rad Laboratories, Richmond, California). The column measured 100 x 11 mm, and the samples were eluted in 0.8 ml fractions at a rate of 3 ml per hour. ^{3}H and ^{14}C radioactivity was counted in Aquasol (New England Nuclear, Boston, Mass.) with either a Packard Tri-Carb Model 3380 or an Intertechnique SL-30 liquid scintillation spectrometer. All fractionation experiments were repeated with reversed isotope labeling.

Testing for Purity of Radioisotopes

The purity of fucose-^{14}C and -^{3}H was confirmed by thin layer chromatography on 20 x 5 cm cellulose plates (J.T. Baker Chemical Co., Phillipsburg, N.J.) which were developed in n-propanol-H_2O-ethylacetate (7:2:1) and in butanol-ethanol-H_2O (10:1:2). The position of the isotopes was determined with a Packard radiochromatogram scanner (model 7201) and compared with the Rf of non-radioactive fucose.

RESULTS

Reaction of P1798 and 6C3HED Cells with Alcian Blue

Examination of Alcian blue-treated P1798 lymphoma cells by electron microscopy showed electron-dense aggregates, referred to as cell coat, on surfaces of the large majority of cells from the CS tumor. Dye complexes could be observed without counterstaining of sectioned material with heavy metals; however, the density of cell coats was much augmented by contrasting sections with uranyl acetate and lead citrate. The thickness and continuity of coat material along the cell surface was variable as shown in figures 1 to 3. In all fields examined, only a relatively small number of cells lacked electron-opaque Alcian blue complexes. By contrast, cells from the CR tumor showed a conspicious absence of (fig. 4) or only rare, scattered deposits of coat material on their surfaces. A few cells within a CR population showed a definite positive surface reaction with the dye. In control preparations, no electron-dense material was observed on the external leaflet of the cell membrane.

In the light of these observations, it was of interest to investigate CR and CS cell lines of another lymphoid tumor that would answer the question of whether the described surface charge differences in CS and CR P1798 are peculiar to a particular lymphoma or are a more general phenomenon. 6C3HED-AS and -AR tumor lines, which are carried in C3H mice (as compared to P1798, carried in Balb/c mice), proved to be CS and CR, respectively. Examination of Alcian blue-treated

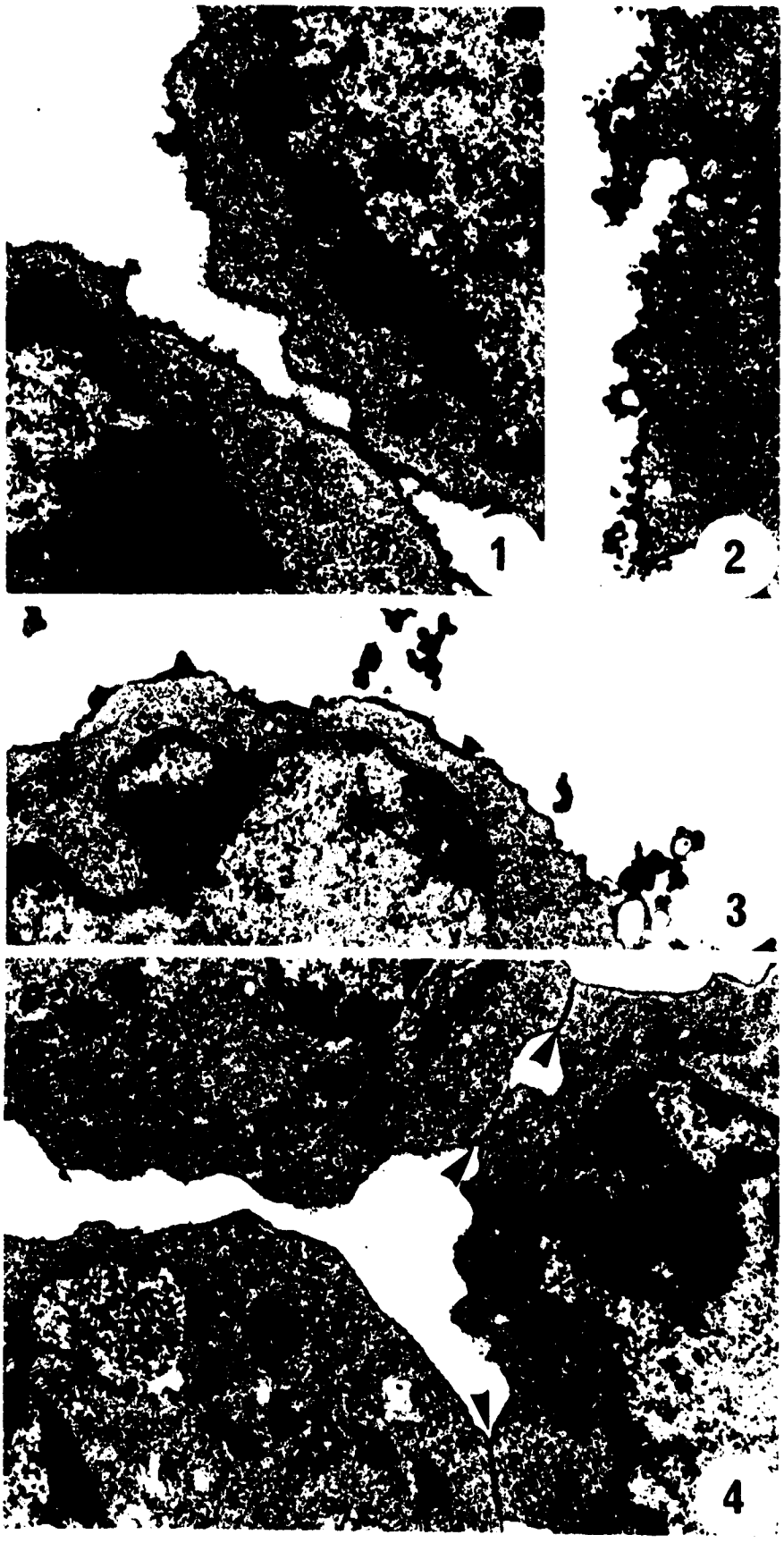
Figs. 1 - 4. P1798-CS/CR cells fixed in the presence of Alcian blue 8GX.

Fig. 1. P1798-CS cells. Cell surfaces show a continuous dense coat. X 25,000.

Fig. 2. P1798-CS cell. The irregular cell surface shows densely packed, fuzzy coat material. X 25,000.

Fig. 3. P1798-CS cell. The electron-opaque cell surface deposits show a discontinuous, patchy distribution. X 25,000.

Fig. 4. P1798-CR cells. Cell surfaces lack electron-dense coat. Several cell-to-cell contact sites can be seen (arrows). X 25,000.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6C3HED-CS and -CR cells revealed that the pattern of dye binding was similar to that described for P1798 cell preparations. Figures 5 and 6 demonstrate cell coats of CS cells resembling those observed on P1798-CS cell surfaces (figs. 1 to 3). Figure 7 shows the plasma membranes of 2 adhering CR cells that lack electron-dense material on their surfaces. Similar to P1798 strains, 6C3HED-CS and -CR cell populations were not completely homogeneous with respect to the presence or absence of a cell coat.

To strengthen these observations, 2 additional P1798-CR cell lines (P1798-CR1 and -CR2) were produced from the CS strain by chronic steroid treatment. The resulting semi-CR strains showed that induction of corticosteroid resistance is associated with a loss of surface coat material. Another P1798-CS strain, made resistant to asparaginase, was found to have become CR. No explanation can be given for this change, but the new CR line (P1798-AR) lacked a cell coat. Although P1798-CR1, -CR2, and -AR fail to regress after treatment with 9-FP (25 mg/Kg bodyweight, a dose that is maximally effective in the parent strain), they do not continue to grow. This semiresistance to corticoids may be reflected in differences in relative ratios of uncoated and coated cells between CR and semi-CR cell lines. However, a quantitative evaluation of large numbers of micrographs of each strain would be necessary to test this possibility.

Cell Adhesion in CS and CR Tumors

In solid tumors of P1798-CR, cells were frequently ob-

Figs. 5 - 7. 6C3HED-CS/CR cells fixed in the presence of Alcian blue 8GX.

Fig. 5. 6C3HED-CS cell. The cell surface is irregular and shows a more or less continuous densely-stained coat. X 25,000.

Fig. 6. 6C3HED-CS cell. Electron-dense, flaky material covers the cell surface. X 25,000.

Fig. 7. 6C3HED-CR cells. Cell membranes show a broad cell-to-cell contact site (arrows). Alcian blue aggregates appear to be absent from the cell surfaces. X 25,000.


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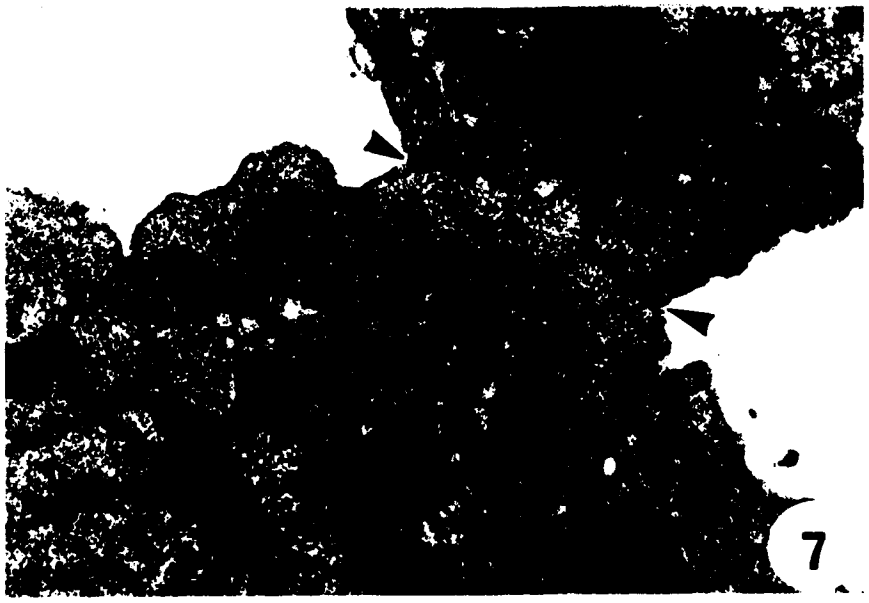
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served to show cell-to-cell contact sites (figs. 8 and 9). Junction-like complexes were also seen between CR cells that had been removed from tumor slices by gentle teasing with small spatulas. This can be seen in the Alcian blue-treated cells of figure 4 (arrows). P1798-CS solid tumors generally showed a looser arrangement of cells with few contact sites (fig. 10). Electron-lucid cells, as seen in figure 10, were more frequently encountered in P1798-CS than in -CR tumors.

The capacity for cell adhesion was also observed in cell populations (prepared for histochemistry with Alcian blue) of the 6C3HED-CR tumor (figs. 7 and 11). In cell suspensions of both types of CS lymphoma (P1798 and 6C3HED), adhering cells were rarely observed. An attempt was made to examine differences in cell adhesion in CS and CR 6C3HED solid tumors, but extensive necrosis in all processed materials made an accurate evaluation impossible. Differences in firmness between 6C3HED-CS and -CR tumors, as observed in P1798-CS and -CR lymphomas, were not obvious, perhaps due to the presence of an extensive network of muscle fibers (observed by electron microscopy) in both tumors.

Three-Dimensional and Surface Morphology of P1798-CS and CR Cells

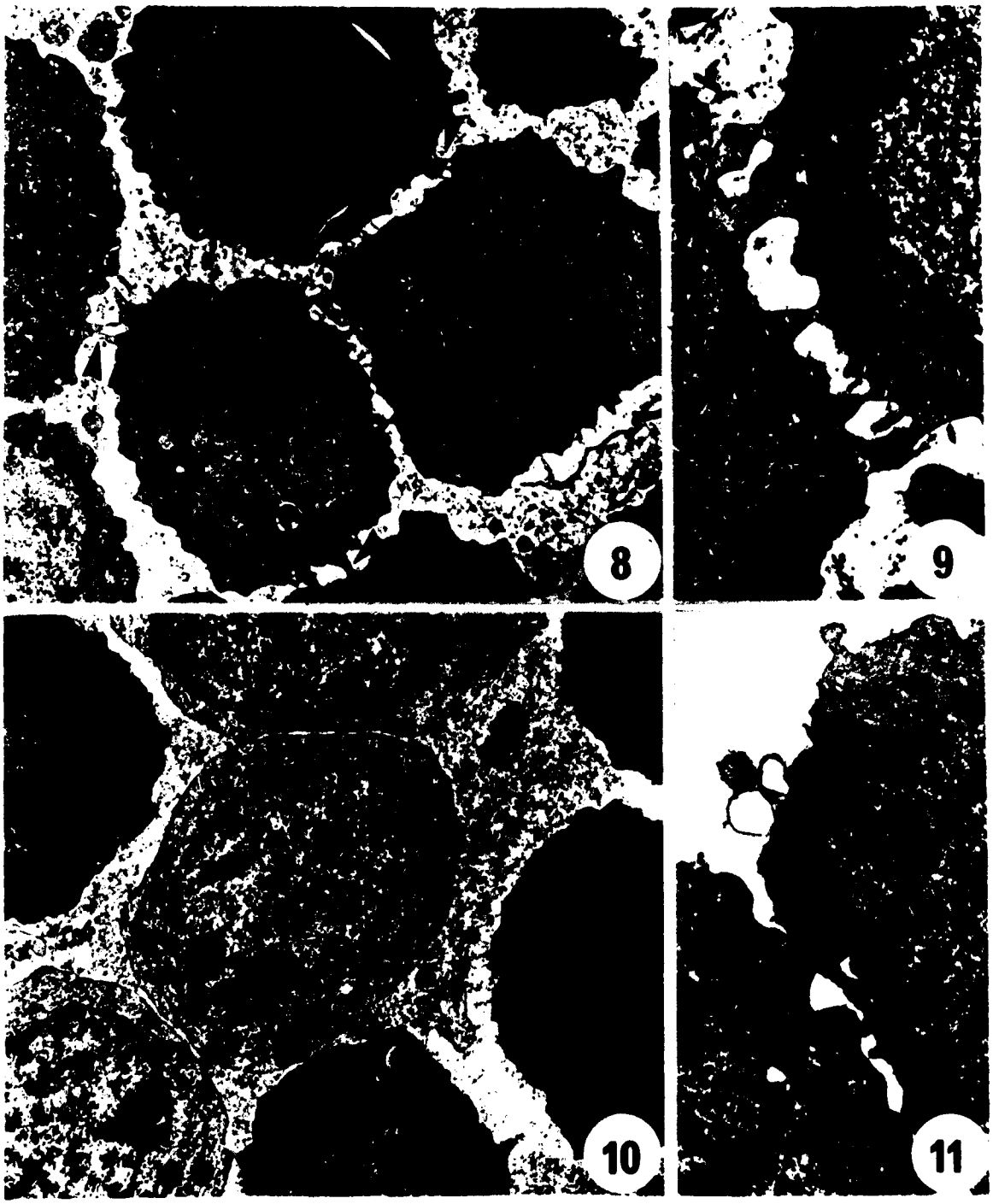
In general, cells of both P1798-CS and -CR tumors showed smooth and moderately villous surfaces (figs. 12 and 13), corresponding to a pattern that has been described for human thymocytes (T-cells) (Polliack et al, 1973). In the CR preparation, a significant number of cells, which amounted to 45% when counted by light microscopy, was observed to show a

Fig. 8. P1798-CR solid tumor. Several cells are attached to each other by small contact sites between cytoplasmic protrusions. X 6,000.

Fig. 9. P1798-CR solid tumor. Two cells are attached to each other by cell-to-cell contact sites. X 19,500.

Fig. 10. P1798-CS solid tumor. Electron-dense and electron-lucid cells lacking contact sites. X 6,000.

Fig. 11. 6C3HED-CR cells (fixed in the presence of Alcian blue). Cell membranes of 2 cells adhere to each other by short cell-to-cell contact sites (arrows). X 13,400.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single prominent bleb, resulting in a pear- or mushroom shape (fig. 13). Such irregular shapes were also observed in sectioned material (figs. 14 and 15), and since they could be counted in unfixed cell suspensions (by light microscopy), the possibility of an artifact due to fixation is unlikely. Similar cell shapes were observed in 6C3HED lymphoma cell suspensions by light microscopy; however, the relative numbers of these irregular cells in CR (16%) and CS (6%) cell populations were lower than in the P1798 lymphosarcoma.

Binding of Ruthenium Red and Lanthanum Nitrate to Cell Surfaces of Lymphosarcoma P1798

In general, fixation of both P1798-CS and -CR cells in the presence of ruthenium red resulted in sparsely distributed electron-opaque deposits on the cell surface. Relatively few cells showed a slightly thicker coat (figs. 16 and 17), comparable to that produced by Alcian blue on surfaces of CS cells.

Binding of lanthanum nitrate to cell membrane components of both P1798-CS and -CR cells was found to be insignificant. As seen in figures 18 and 19, electron-dense coat material is barely visible.

Effect of Hypaque-Ficoll on Alcian Blue-Stained Surface Coat of P1798 Cells

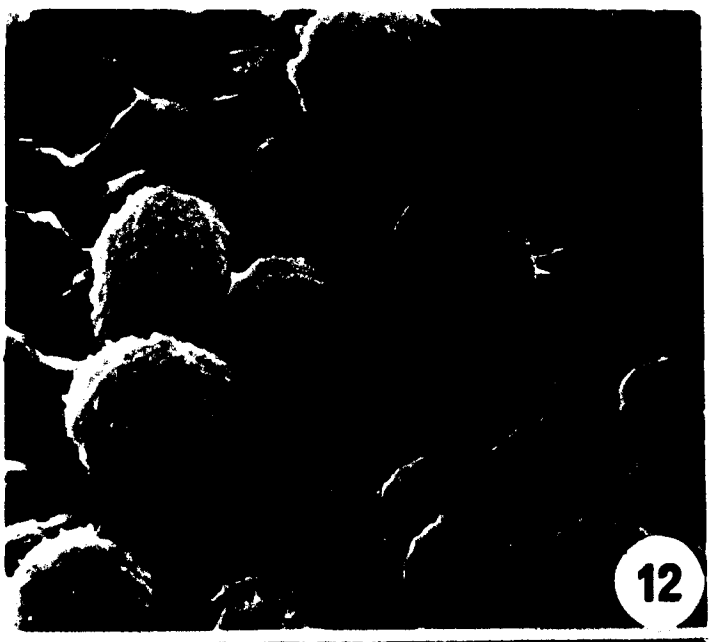
Centrifugation on a Hypaque-Ficoll cushin, followed by fixation in the presence of Alcian blue, resulted in a loosening of stained and unstained membrane material from CS cell surfaces (figs. 20 and 21). The remaining cell membrane either lacked or showed few dye complexes. In contrast,

Fig. 12. Scanning electron micrograph of P1798-CS cell preparation. Cells of variable diameter show smooth or moderately villous surfaces. X 3,300.

Fig. 13. Scanning electron micrograph of P1798-CR cell preparation. Cells with a surface texture resembling that in fig. 12. Several cells show a pear- or mushroom shape (arrow). X 3,300.

Fig. 14. Section of pear-shaped, Alcian blue-treated P1798-CR cell with an eccentric nucleus. The cytoplasmic protrusion shows membranous vesicles at the cell surface (arrow). X 10,200.

Fig. 15. Section of mushroom-shaped, Alcian blue-treated P1798-CR cell with membranous material on the surface of the protruding cytoplasmic bleb (arrow). X 6,300.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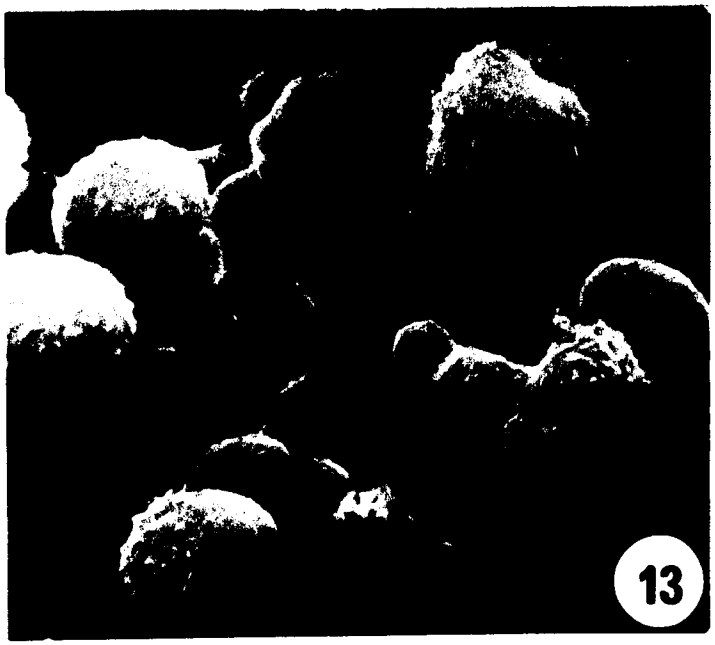
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Fig. 16. P1798-CS cells stained with ruthenium red. Cell surfaces show an electron-dense coat. X 25,000.

Fig. 17. Ruthenium red-stained P1798-CR cell. Cell surface shows a thin, electron-dense coat. X 25,000.

Fig. 18. Lanthanum nitrate-stained P1798-CS cell. Cell surface shows barely visible electron-dense deposits. X 25,000.

Fig. 19. Lanthanum nitrate-stained P1798-CR cell. As in fig. 18, the cell surface shows barely visible coat material. X 25,000.


16


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18


19

the detachment of membranous material from the surface of CR cells exposed a thin electron-dense layer on the cell membrane. This can be seen in figure 22 which shows a magnified area of the pear-shaped cell in figure 23.

Reaction with Alcian Blue of P1798 Cells after 9-FP Treatment In Vivo

A visible cell coat, resulting from Alcian blue binding, was observed on CS cell membranes up to 6 hours after 9-FP treatment (fig. 24). The morphological appearance of dye complexes on cell surfaces was not significantly different from that described previously in untreated cell preparations (figs.1 to 3). 8 and 15 hours after treatment, hardly any reaction with Alcian blue could be observed (fig. 25). Steroid-treated CR cells (3, 6, 8, and 15 hours after 9-FP application) essentially did not differ from untreated control cells (fig. 4) in their capacity to bind the dye.

Morphological Changes in P1798 Lymphosarcoma Following 9-FP Treatment In Vivo

No significant morphological changes could be detected in P1798-CR cells at any time after steroid treatment. Due to localized necrotic processes, a general phenomenon in tumor tissues, a relatively small number of cells, showing various degrees of damage, was observed in untreated tumor cell preparations.

P1798-CS and -CR cell populations were examined at 0, 3, 6, 8, 12, and 18 hours after steroid treatment. In CS cell sections, only a certain percentage of cells, that increased

Figs. 20 - 23. P1798-CS/CR cells that were centrifuged on a cushion of Hypaque-Ficoll (to remove dead cells) and subsequently fixed in the presence of Alcian blue 8GX.

Fig. 20, 21. P1798-CS cells. Stained and unstained membrane material appears to loosen from the cell surface. X 25,700.

Fig. 22. P1798-CR cell. Membrane material, mostly unstained, is loosening form the surface. The remaining cell membrane shows a thin, electron-dense coat. X 14,000.

Fig. 23. Low magnification of fig. 22 showing the whole, mushroom-shaped cell. X 5,600.

20

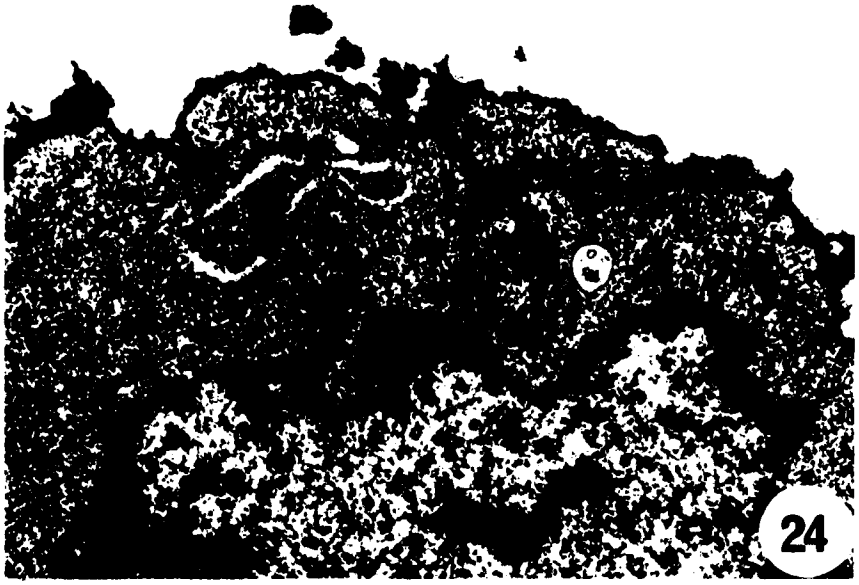
21

22

23

Fig. 24. Alcian blue-stained P1798-CS cell 6 hours after treatment (*in vivo*) with 9-FP. The cell surface shows a flaky, continuous coat. X 25,700.

Fig. 25. Alcian blue-stained P1798-CS cell 8 hours after treatment (*in vivo*) with 9-FP. The cell surface lacks coat material. X 25,000.



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with time after 9-FP application was seen to be in various stages of lysis at all time intervals examined. The character of these changes did not differ from those normally observed in a small number of cells that have been freed from tumor tissue by gentle teasing. These observations, in addition to the asynchrony of response to corticosteroid action, made it difficult to establish criteria for 9-FP-specific cell damage as well as for the sequence of degradative events.

Phenomena that were observed in dying cells of untreated and treated tumors included: a decrease in size of the nucleolus; dissociation of polysomal complexes and endoplasmic reticulum-associated ribosomes; shrinking of Golgi vesicles; appearance of lysosomal bodies and autophagic vesicles in various stages of digestion. Cells in more advanced stages of degenerative changes showed a breakdown of nuclear, cytoplasmic and plasma membranes; a decrease in euchromatin-heterochromatin ratio, followed by complete condensation of nuclear and cytoplasmic remnants, resulting in pyknosis. To assess the degree of damage at different time intervals after steroid treatment, only pyknotic cells and cells showing visible (at a magnification of 1000 X) cytoplasmic damage were counted by light microscopy. Affected cells, such as seen in figures 26 and 27, could easily be recognized in 0.5 to 1 micron-thick, Azure B-stained sections.

Chart 1 shows that in sections of CR cells, the number of dying cells remained more or less stable after various times of treatment. In CS cell preparations, an insigni-

ficant increase in the percentage of damaged cells occurred up to 6 hours of steroid action. Between 6 and 8 hours after treatment, the number of affected cells increased from 11 to 45%. After this time, the effectiveness of the steroid tapered off. By 18 hours following 9-FP application, the number of damaged cells had increased to only 58%.

Fig. 26. P1798-CS cells 12 hours after treatment (in vivo) with 9-FP (Alcian blue-stained). 3 pyknotic cells (arrows) with condensed chromatin material are seen in the area. No coat material is present on the cell surfaces. X 11,500.

Fig. 27. P1798-CS cell 6 hours after (in vivo) treatment with 9-FP (Alcian blue-stained). The cytoplasm shows a large lysosomal body (L) and focal damage in the cytoplasm (arrows). Alcian blue aggregates are seen on the cell surface. X 16,300.


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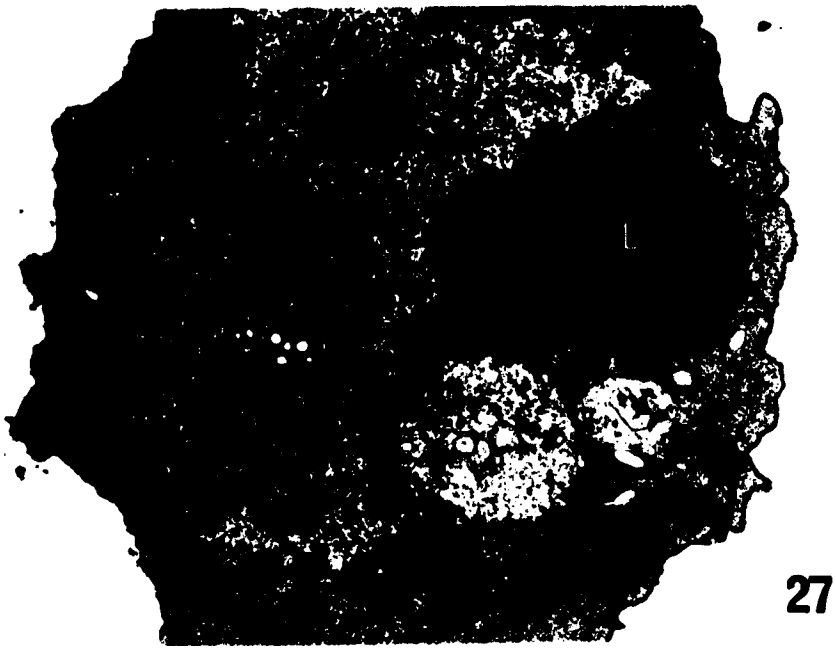
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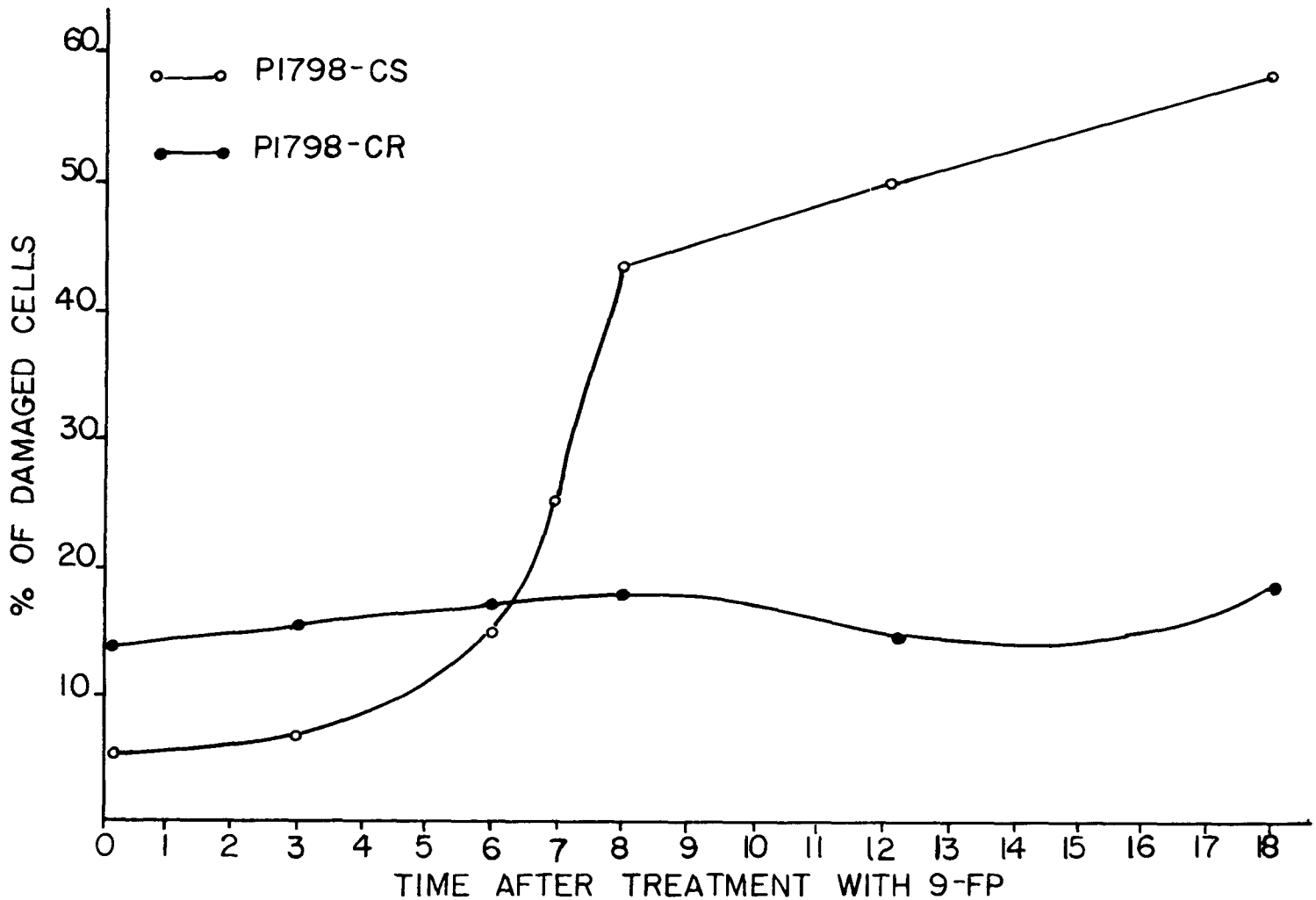
Chart 1.

P1798-CS and -CR tumors were treated *in vivo* with 9-FP. The number of damaged cells (including cells showing pyknosis or visible focal damage) at different times after steroid treatment is reported as percent of total number of cells (1000) counted by light microscopy.


P1798-CS
P1798-CR
% OF DAMAGED CELLS
60
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
TIME AFTER TREATMENT WITH 9-FP

Isolation of Plasma Membranes from Lymphosarcoma P1798

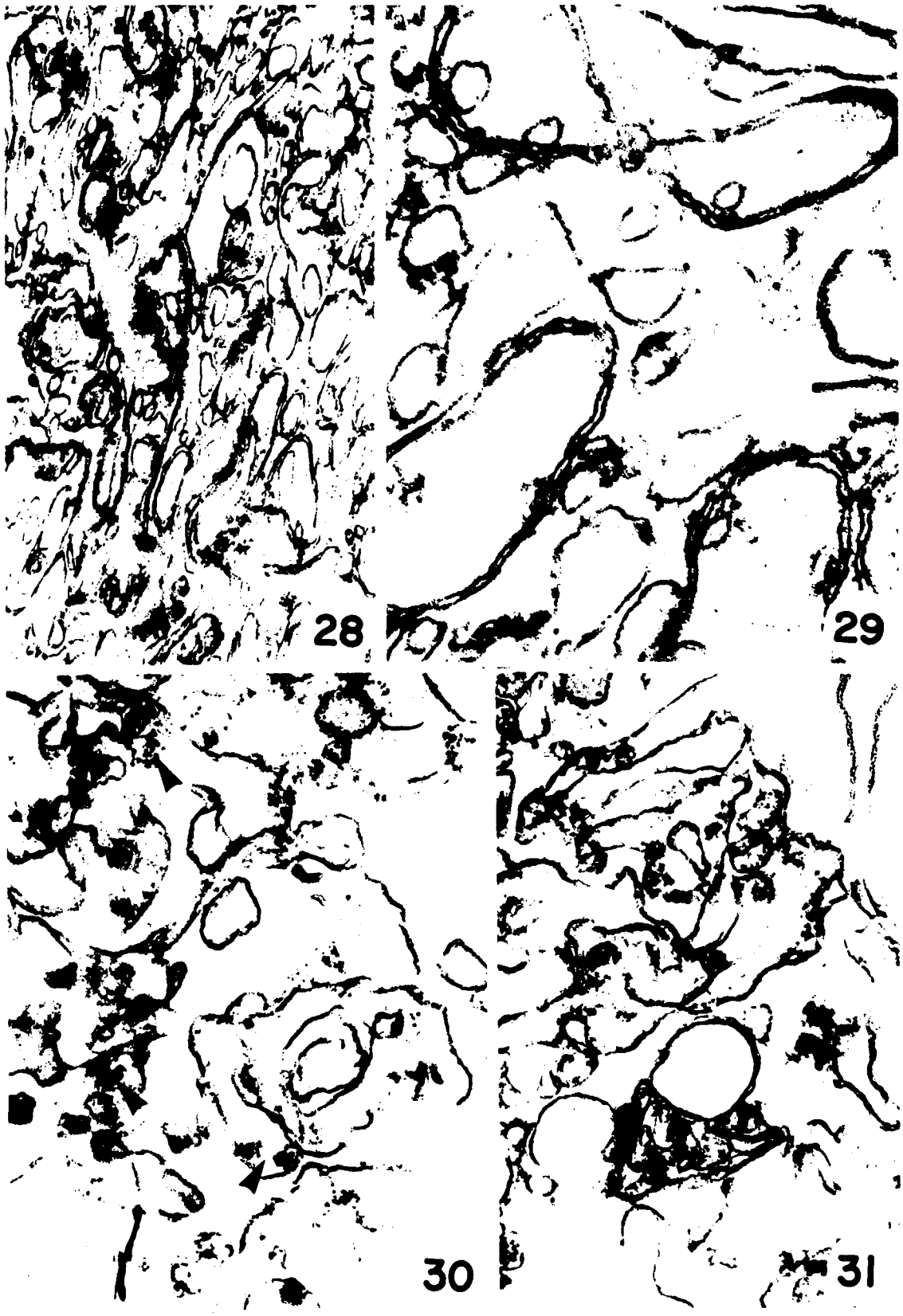
Considerable time was necessary to develop suitable conditions for the isolation of a relatively pure plasma membrane fraction from lymphosarcoma P1798. Various methods, involving flotation of membrane fractions through a sucrose step gradient (Boone et al, 1969), separation by charge in a two-phase polymer system (Brunette and Till, 1971; Hourani et al, 1973), and filtration through glass beads (Warley and Cook, 1973) proved to be unsuccessful for this system. As observed by light- and electron microscopy, P1798-CS and -CR cell suspensions contain large numbers of free nuclei which presented a problem during homogenization. In the absence of calcium ions in the hypotonic medium, breakage of nuclear membranes caused the formation of an intractable nucleoprotein gel. Finally, the isolation procedure described by Ray (1970) for rat liver plasma membranes, involving homogenization in the presence of 0.5 mM $CaCl_2$, extreme dilution of the homogenate, centrifugation of the crude membrane fraction, and purification by flotation through a sucrose step gradient, was modified considerably for P1798 to give satisfactory results. The calcium ion concentration of the hypotonic medium had to be increased to 2 mM to prevent leakage of DNA from nuclei. A concentration of 1 mM was satisfactory but caused the removal of the outer nuclear membrane. Only in the presence of the higher calcium concentration, did nuclei retain a double membrane. Before precipitation of the crude membrane fraction from the diluted homogenate, the intact

nuclei were removed by a very brief initial centrifugation (a step not included in the Ray procedure). Centrifugal speeds used by Ray to sediment the membrane ghost fraction, had to be increased for P1798 to prevent loss of most plasma membranes with the supernatant.

As stated by Hinton (1972), in addition to stabilizing membranes, the presence of calcium ions in the homogenization medium prevents an overlap of mitochondrial and plasma membrane bands, and the inclusion of magnesium ions to the sucrose gradient (as in this procedure) increases the difference in density between plasma membrane-derived and endoplasmic reticulum-derived vesicles. After flotation through the sucrose step gradient, relatively pure plasma membrane sheets were obtained from layers appearing at the interfaces between 37% and 41% (L1) and between 41% and 45% sucrose (L2). Figures 28 and 29 are electron micrographs of L1 material. No mitochondria or other cytoplasmic contaminants are visible. The inset of figure 29 shows the trilaminar structure that is typical of plasma membranes. Figures 30 and 31 show sections of L2 membranes. Although a few ribosomes are present (arrows), no mitochondria can be seen, and the membrane sheets show the unit membrane structure (inset of fig. 31). A thin, fluffy sheet of material, containing mostly membranes of the endoplasmic reticulum, adhering ribosomes and virus particles (the latter are known to be present in P1798 - Turnell et al, 1973) was found between the 45% and 48% sucrose layers. Mitochondria were present only in the pellet.

Figs. 28, 29. Isolated plasma membranes of L1 fraction (37%/41% sucrose interface layer). No mitochondria or other contaminating material are visible. Fig. 28. X 41,000; fig. 29. X 80,000. The inset of fig. 29 shows the trilaminar structure that is characteristic of cell membranes. X 190,000.

Figs. 30, 31. Isolated plasma membranes of L2 fraction (41%/45% sucrose interface layer). No mitochondria but a few ribosomes can be seen (arrows). Figs. 30 and 31. X 51,300. Inset of fig. 31 shows the trilaminar structure of the cell membbrane. X 93,000.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In addition to morphology, marker enzymes were used as criteria for membrane purity. Results of enzyme activity are given in table 1.

5'-nucleotidase and Na^+- and K^+-activated, Mg^{2+}-dependent ATPase are generally accepted marker enzymes of the plasma membrane for most systems (Hinton, 1972). In this investigation, recovery of these enzymes in both L1 and L2 layers (CS and CR) was too low to be accurately measured. P-nitrophenyl phosphatase (another plasma membrane marker) also showed low, but measurable activity in all L1 and L2 fractions. The only enzyme (of those tested) that appeared to be most concentrated in cell membranes of both P1798-CS and -CR as compared to the homogenate was Mg^{2+}-activated ATPase. The activity of this enzyme in CS membranes was higher in the L2 as compared to the L1 fraction. No significant difference in activity was found in CR L1 and L2 layers. The mitochondrial enzyme, succinate dehydrogenase, appeared to be absent, and glucose-6-phosphatase (a marker enzyme of the endoplasmic reticulum) showed low activity in all L1 and L2 samples. As compared to reports by other authors (van Blitterswijk et al, 1973; Allan and Crumpton, 1970; and Hinton, 1972), glucose-6-phosphatase activities were within the acceptable range.

As demonstrated in table 2, total recoveries of cell membrane protein (L1 and L2) ranged from approximately 3 to 5% of starting protein. L2 fractions showed a comparatively low recovery (less than 1% of protein present in the

Table 1

Specific Activities of Selected Enzymes in Homogenates and Plasma Membrane Fractions of Lymphosarcoma P1798

Preparation	Mg^{2+}-dependent ATPase	%*	p-nitrophenyl phosphatase	%*
Homogenate-CS	2.36 ± .63 (3)		0.12 ± .03 (5)	
Homogenate-CR	4.07 ± .39 (3)		0.18 ± .04 (5)	
CS-L1	10.67 ± .53 (3)	7.4	0.24 ± .06 (5)	5.7
CR-L1	19.17 ± .18 (3)	7.8	0.19 ± .03 (5)	3.4
CS-L2	23.10 ± .52 (3)	0.9	1.38 ± .35 (5)	1.2
CR-L2	18.13 ± .47 (3)	1.6	1.30 ± .27 (5)	1.5
Preparation	**Glucose-6-phosphatase**	**%***	**Succinate dehydrogenase**	**%***
Homogenate-CS	0.28 ± .05 (3)		0.68 ± .17 (5)	
Homogenate-CR	0.36 ± .04 (3)		0.64 ± .09 (5)	
CS-L1	0.41 ± .07 (3)	3.6	0 (5)	0
CR-L1	0.50 ± .19 (3)	4.0	0 (5)	0
CS-L2	0.34 ± .14 (3)	1.0	0 (5)	0
CR-L2	0.50 ± .15 (3)	1.5	0 (5)	0

*The % distribution of the plasma membrane enzymes present in the homogenate (100%).
All enzyme activities are expressed in μmoles of product produced/mg protein/hour.
Results are given as means ± S.E. The numbers in parenthesis refer to the number of experiments.

homogenate).

The concentration of sialic acid in all cell membrane fractions was found to be quite variable in both P1798-CS and -CR. However, average values of 6 determinations were lower for CR homogenates, L1 and L2 fractions than for the same CS preparations (table 3). L2 fractions of both CS and CR tumors contained comparatively low amounts of sialic acid.

Table 2

% Recovery of Plasma Membrane Protein
Protein content of homogenate = 100 %.

CS - L1	4.01 ± 0.79	(6)*	CS - L2	0.56 ± 0.28	(6)
CR - L1	3.73 ± 0.42	(6)	CR - L2	0.71 ± 0.38	(6)

* Number of determinations.
Results are given as means ± S.E.

Table 3

nMoles Sialic Acid per mg Protein

Homogenate - CS	19.2 ± 1.7 (6)*
Homogenate - CR	17.2 ± 3.3 (6)
CS - L1	46.7 ± 7.5 (6)
CR - L1	36.3 ± 1.0 (6)
CS - L2	19.4 ± 5.2 (4)
CR - L2	13.4 ± 6.2 (4)

* Number of determinations
Results are given as means ± S.E.

Glycopeptides of P1798-CS and -CR Cell Membranes before and after Treatment with 9-FP

The following experiment was initially designed to separate (*in vitro* fucose-labeled) cell surface glycopeptides derived from trypsinates of both P1798-CS and -CR cells; however, this approach was abandoned for the following reasons: P1798-CS and -CR lymphoma cells, that are removed from tissue slices by gentle teasing or mincing, contain a considerable number of free nuclei and dead cells. Trypsin was found to digest non-viable cells as well as free nuclei within a relatively short time, resulting in contamination of cell surface trypsinates with undesirable cell constituents. An attempt was made to obtain a population of viable cells (as judged by trypan blue exclusion) by centrifugation of tumor cells on a cushion of Hypaque-Ficoll. As described earlier, this procedure removed cell membrane material from both CS and CR cells. In addition to these observations by electron microscopy, preliminary incubation studies with these cells in Hank's BSS resulted in the release of considerable amounts of protein as well as sialic acid into the medium. These phenomena were not observed when CS and CR cells were incubated under similar conditions without prior centrifugation on Hypaque-Ficoll. Possibly membrane changes, involving the activation of specific enzymes, are induced by Hypaque-Ficoll. To avoid artifacts, the following investigation was carried out on isolated cell membranes of lymphosarcoma P1798-CS and -CR.

In vivo steroid-treated and -untreated differentially

labeled P1798-CS and -CR tumors were processed for the isolation of cell membranes, as described in detail in "Materials and Methods." Pooled L1 and L2 (plasma membrane) fractions of CS and CR preparations were combined, solubilized with SDS and digested extensively with pronase. The resulting oligosaccharide chains of glycoproteins, which could be assumed to be attached to only a few amino acids, were separated into 2 equal portions. One of these fractions was incubated with neuraminidase and the other served as the control. Subsequently the 2 samples (neuraminidase-treated and untreated) were eluted separately from a column of Bio-Gel P-10 (an agarose gel). Gel chromatography separates molecules primarily according to differences in molecular dimensions and is therefore a useful technique for separating complex mixtures of glycopeptides. The exclusion limit of Bio-Gel P-10 is 10,000 (molecular weight). Molecules of this size and larger do not penetrate the pores of this gel matrix and are eluted with the void volume.

Radioactivity incorporated into the cell membrane fraction within 4 hours after injection of L-fucose-^{14}C and -^{3}H was found to be sufficient for this study. In an <u>in vivo</u> system, ratios of amounts of radioactivity and number of cells cannot be controlled, and specific activities (dpm/mg protein), which varied considerably from experiment to experiment are therefore of no value for this study.

Approximately 13.5 to 15% of both ^{14}C- and ^{3}H-labeled material was lost during dialysis after pronase digestion.

Recovery of radioactivity from the column was high and ranged from 90 to almost 100% for all samples eluted.

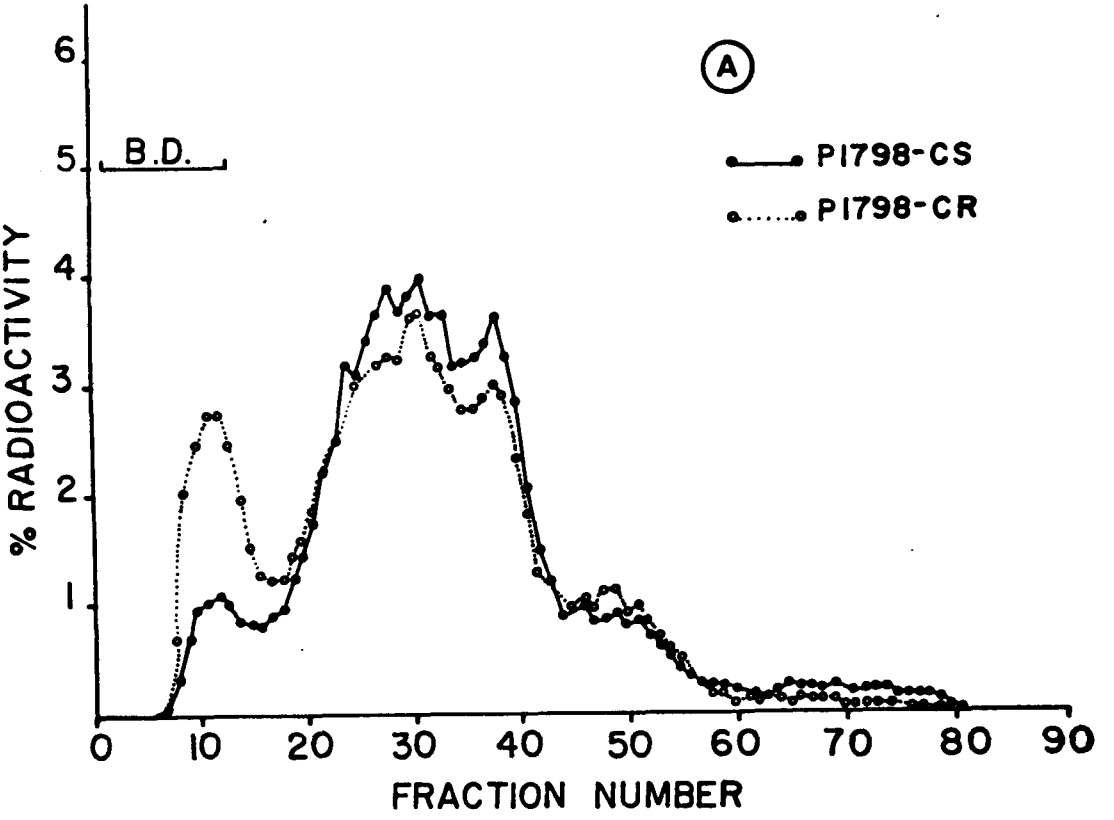
Chart 2-A shows the elution profile of differentially labeled P1798-CS and -CR samples that had not been exposed to *in vivo* steroid treatment. Both tumor preparations contained early eluting, high molecular weight fractions which accounted for 18 to 20% of total radioactivity in CR as compared to only 7 to 9% in CS samples. Fractions of smaller molecular size, making up the major portion of fucose-containing glycopeptides, were present in lower amounts in CR relative to CS preparations. A third low molecular weight peak is barely discernable in both CS and CR elution profiles.

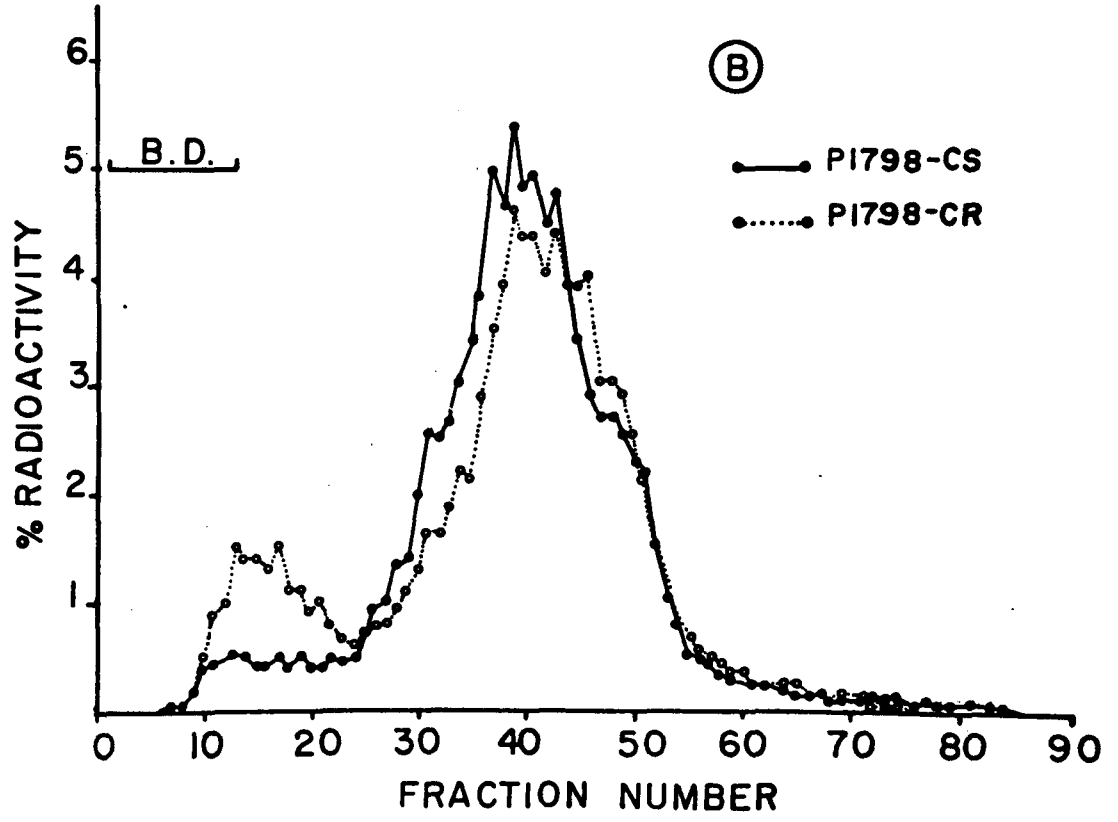
Removal of glycopeptide-bound sialic acid by *Vibrio cholerae* neuraminidase resulted in an overall shift of both CS- and -CR-derived fractions to an apparently lower molecular weight (chart 2-B). Differentially labeled glycopeptides showed altered ratios that were increased or decreased in particular fractions of either tumor. This was reflected in differences in the degree of shift of CS and CR desialylated fractions, indicating a greater sialic acid density in early eluting and most major peak CR as compared to CS glycopeptides. Major peak fractions were reduced in number and showed a greater uniformity in molecular size as those seen in chart 2-A. The small third peak between fractions 44 and 55 (chart 2-A) had disappeared. Elution patterns similar to those described were reproduced in 2

Chart 2.

Elution profiles of L-fucose-containing glycopeptides of SDS-solubilized and pronase-digested cell membranes from differentially labeled P1798-CS and -CR, following filtration through Bio-Gel P-10 before (A) and after treatment with neuraminidase (B).

The radioactivity of the fractions is reported as percentage of total radioactivity eluted from the column. B.D. = blue dextran.


A
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6


B
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
6

additional experiments.

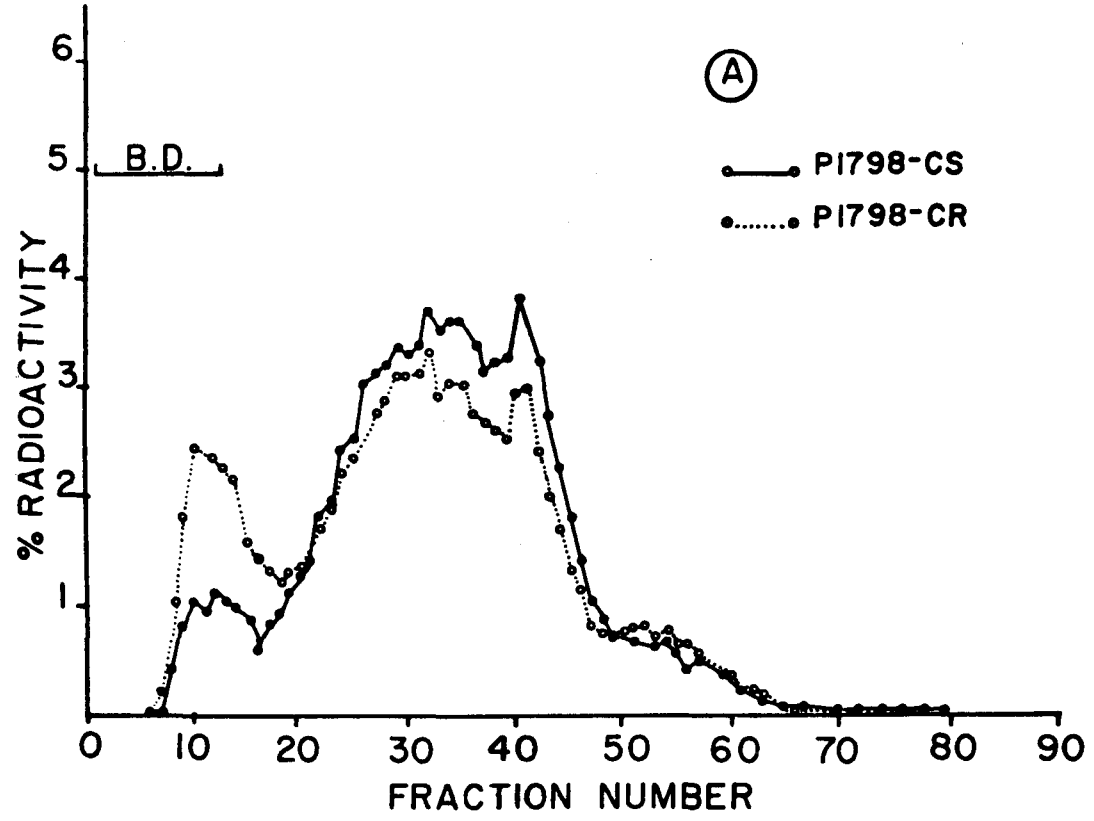
No significant change in glycopeptide fractions was observed 6 hours after steroid treatment (chart 3-A) with the exception that incubation with neuraminidase produced a new low molecular weight peak (chart 3-B) which is barely visible in chart 3-A. Reversed isotope labeling showed essentially the same pattern.

The same experiment 7 hours after 9-FP treatment showed a shift to the left in the elution profile, apparently due to an increase in molecular weight or size (chart 4-A). Major peak fractions were reduced in number (as compared to untreated samples), thereby producing a narrower, higher peak. Neuraminidase treatment induced the appearance of an additional low molecular weight peak similar to but more prominent than that found 6 hours after exposure to 9-FP (chart 3-B). Contrary to all other glycopeptide preparations so far described, the removal of sialic acid from this sample (7 hours after 9-FP treatment <u>in vivo</u>) did not significantly change the fractional distribution of radioactivity in most major fraction peaks (chart 4-B). Reversed isotope labeling essentially produced the same changes described above (chart 5-A); however, the distribution of the 2 low molecular weight fractions in the neuraminidase-treated sample revealed a somewhat different pattern (chart 5-B). Major peak glycopeptides appeared to contribute less to the total radioactivity than did those produced by the removal of sialic acid moieties (reversed in the previously described sample). Differences in

Chart 3.

Elution profiles of L-fucose-containing glycopeptides of SDS-solubilized and pronase digested cell membranes from differentially labeled P1798-CS and -CR 6 hours after treatment *in vivo* with 9-FP. Samples were filtered through Bio-Gel P-10 before (A) and after treatment with neuraminidase (B).

The radioactivity of the fractions is reported as percentage of total radioactivity eluted from the column. B.D. = blue dextran.


A
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

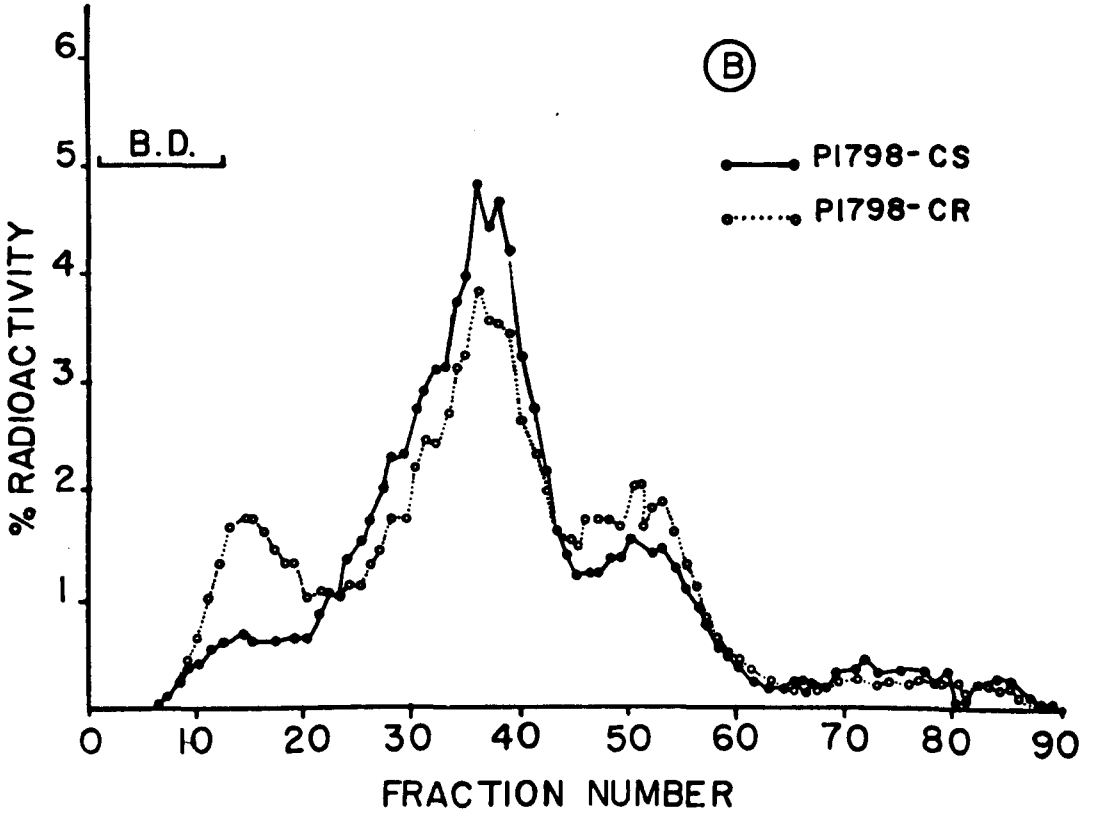
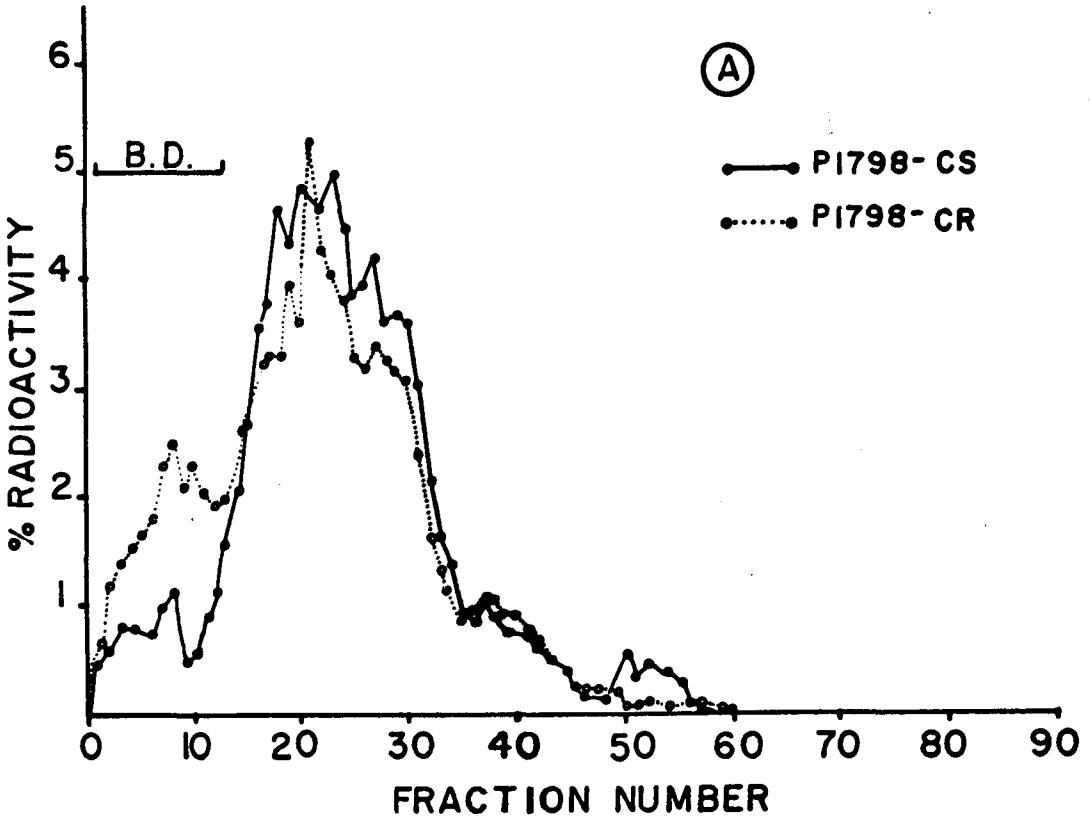

B
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Chart 4.

Elution profiles of L-fucose-containing glycopeptides of SDS-solubilized and pronase-digested cell membranes from differentially labeled P1798-CS and -CR 7 hours after *in vivo* treatment with 9-FP. Samples were filtered through Bio-Gel P-10 before (A) and after treatment with neuraminidase (B).

The radioactivity of the fractions is reported as percentage of total radioactivity eluted from the column. B.D. = blue dextran.


A
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
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6

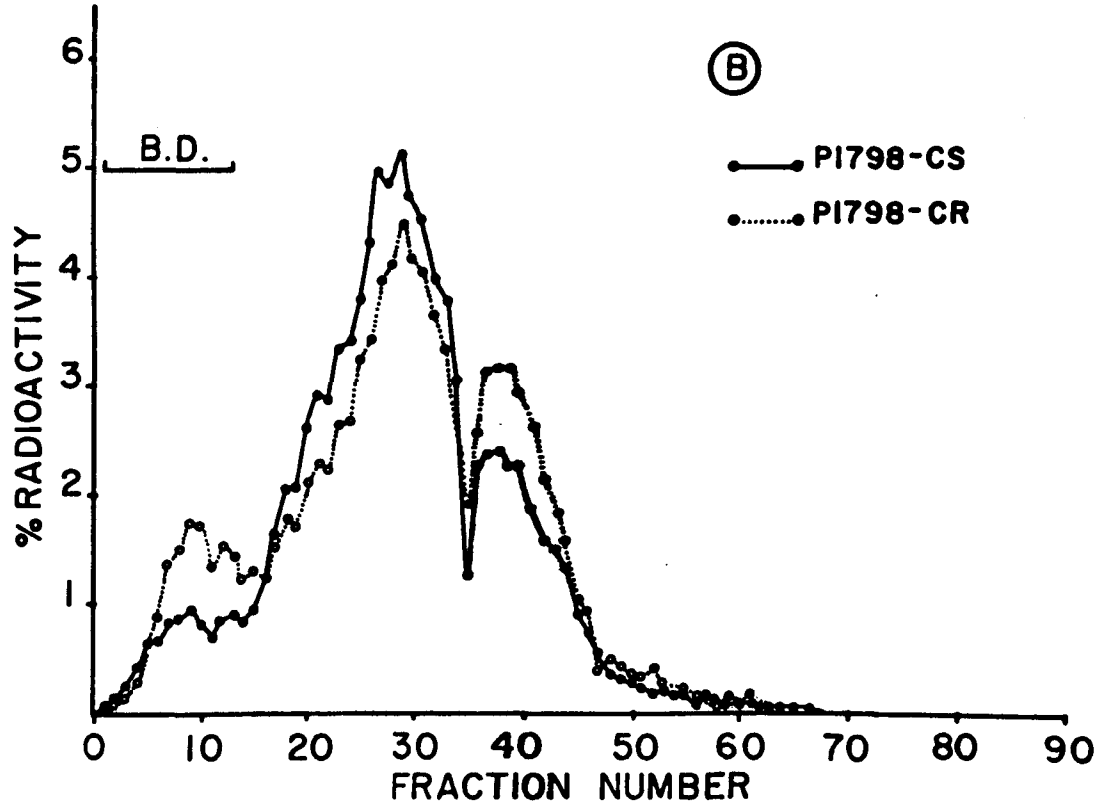
B
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
FRACTION NUMBER
0
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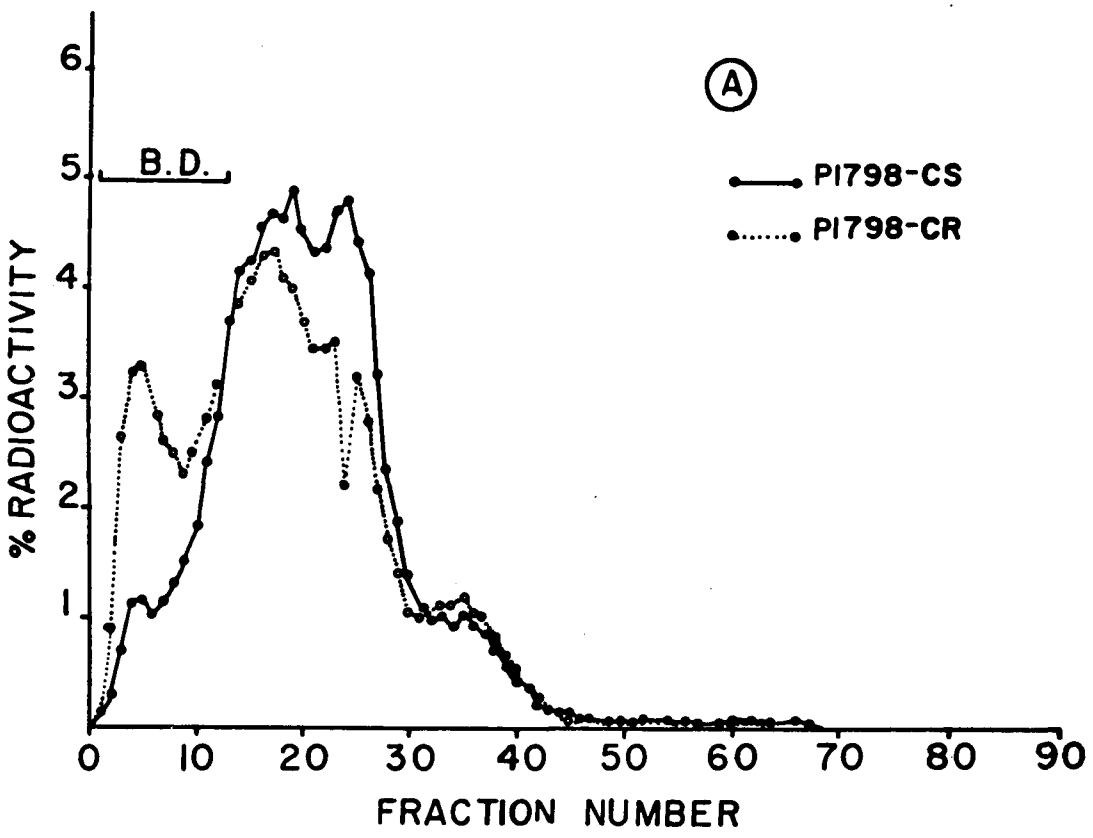
Chart 5.

Elution profiles of L-fucose-containing glycopeptides of SDS-solubilized and pronase-digested cell membranes from differentially labeled P1798-CS and -CR 7 hours after treatment *in vivo* with 9-FP. Samples were filtered through Bio-Gel P-10 before (A) and after treatment with neuraminidase (B).

The radioactivity of the fractions is reported as percentage of total radioactivity eluted from the column. B.D. = blue dextran.


A
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
1
2
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4
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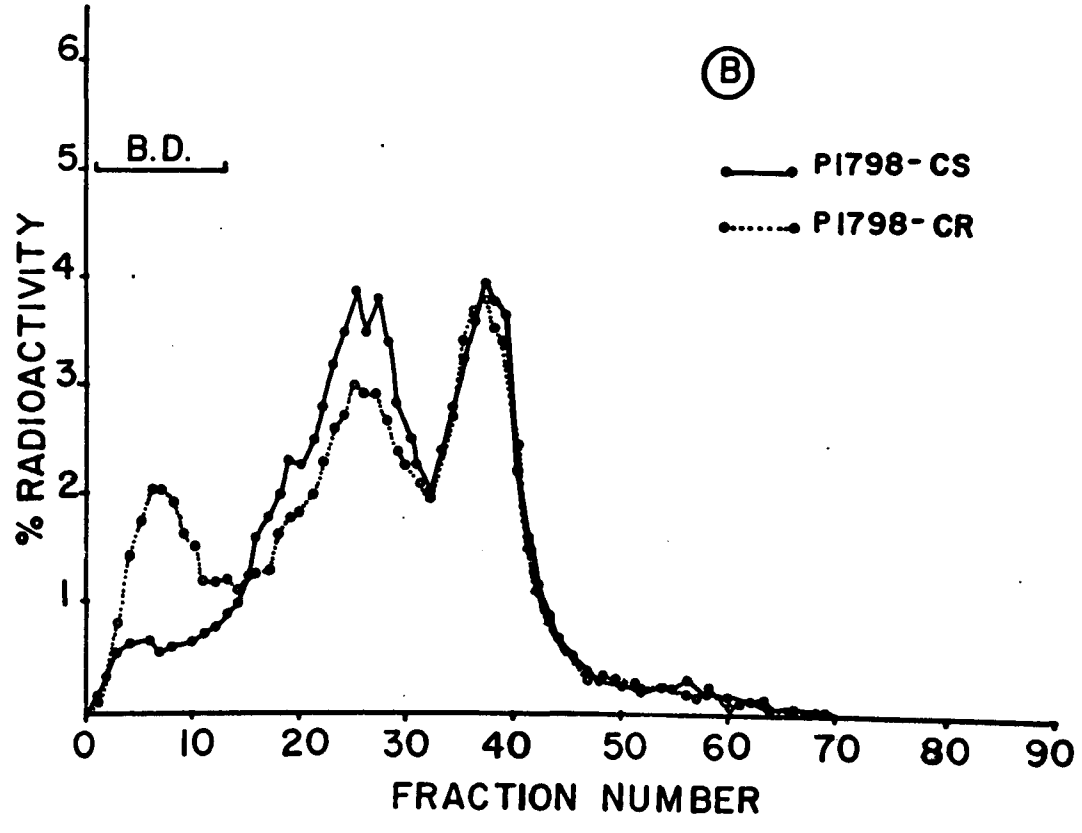
6


B
B.D.
P1798-CS
P1798-CR
% RADIOACTIVITY
FRACTION NUMBER
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sialic acid density in all treated CS and CR samples (6 and 7 hours) were less pronounced than in untreated controls. This is indicated by the position of desialylated glycopeptides in charts 3, 4 and 5-B relative to those in 3, 4 and 5-A.

P1798-CS and -CR plasma membranes, which were isolated 7 hours after exposure <u>in vivo</u> to 9-FP for the purpose of chromatographic studies on pronase-digested L-fucose-labeled glycopeptides, showed a changed banding pattern of both CS and CR samples in the sucrose gradient. As compared to untreated controls, a significant portion of L1 fractions (approximately 50%), usually found at the 37%/41% interface region, had shifted to the 41%/45% sucrose interface.

DISCUSSION

Ultrastructural, histochemical and biochemical differences involving the cell membrane exist between lymphosarcoma P1798-CS and -CR. Histochemical studies also gave evidence for surface membrane differences between lymphoma 6C3HED-CS and -CR. Both P1798 and 6C3HED tumors exhibited acidic groups on surfaces of CS cells when fixed in the presence of the cationic dye Alcian blue. The absence of such anionic sites on the exposed surface of the majority of CR cells in both lymphomas suggests that external negatively charged molecules influence corticosteroid-induced cytolysis. Both P1798 and 6C3HED initially arose in the thymus (Mc Cain Lampkin and Potter, 1958; Gardner et al, 1944), and their T-cell origin has been confirmed immunologically by the detection of theta antigens (Shevack et al, 1972; Bianco and Nussenzweig, 1971). More than 90% of normal thymocytes have been reported to be corticoid-sensitive (Nordling et al, 1972; Wioland et al, 1972) and were found to have cell coats of variable thickness (Bona and Anteunis, 1973) similar to those observed in this investigation on surfaces of CS lymphoma cells. Variations in lymphoid cell coat thickness correlate with differences in electrophoretic mobility (Zeiller et al, 1972; Wioland et al, 1972; Nordling et al, 1972) and appear to have functional significance.

As shown in this study, the response to steroid treatment is asynchronous. The number of cells affected increases

with time after exposure to glucocorticoid hormones, and all stages of the degradative process can be observed in the dying cell population. The responsiveness of CS cells to corticosteroids appears to be random and not cycle-stage-specific (Harris, 1970). Perhaps variations in negative charge concentration and distribution can be directly related to the effectiveness and speed of the cytolytic process in individual cells.

In the thymus, a small population of medullary T-cells (thymocytes) is known to be corticoid-resistant (Ishidate and Metcalf, 1963). However, no information on the presence or absence of an acidic cell coat (as determined by Alcian blue-specific binding) could be found in the literature. The correlation between corticoid sensitivity of thymocytes and Alcian blue staining remains to be determined.

All CS strains tested show almost complete shrinkage of tumor mass following treatment with pharmacological doses of glucocorticoids. If corticoid resistance is related to a lack of negative surface charge, the survival of the small number of cells lacking a cell coat within a CS population could be responsible for the ultimate ineffectiveness of steroid treatment. This possibility is supported by the observation that CR tumors (P1798-CR1 and -CR2), derived from the established CS line, consisted mainly of cells lacking coat material. The presence of a small number of coated cells in the semi-CR (P1798-CR1, -CR2 and -AR) tumors could then account for the incomplete corticoid resistance of these

cell lines.

The mechanism responsible for steroid-induced corticoid resistance as a stable, heritable trait is unknown. Sibley and Tomkins (1974a,b) suggested a mutational origin for insensitivity to adrenal hormones, although no such evidence has been established. CR lymphoid cells are present in a normal animal (Ishidate and Metcalf, 1963) and, as suggested by Kaiser et al (1974), lymphosarcoma P1798 may also consist of a mixed (CS and CR) cell population. The possibility that a small number of CR cells is present in a CS tumor offers an alternative explanation for the process involved in the transition from steroid sensitivity to steroid resistance. The cytotoxic action of glucocorticoids may enable surviving CR cells to grow unhindered by a larger population of CS cells. In untreated CS tumors, attraction of opposite charges between CS and CR cells could be a restraining factor for growth of CR cells in a predominantly CS population, and the reverse situation might exist for CS cells in a prevalent CR population. It has not been established whether CR cell surfaces are electrically neutral or carry a positive charge. If cationic sites are present, the resulting attractive forces between CR and CS cells (in a heterogeneous tumor) might reduce the invasive capacity typical of malignant cells, resulting in a regional inhibition of mitosis, especially of minority populations (CS or CR). The described situation is merely a possibility and not fact.

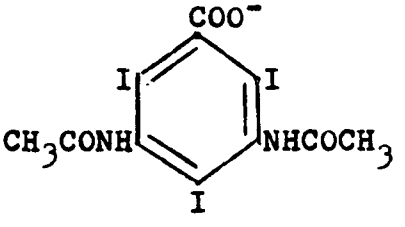
The cell-to-cell contact sites between CR cells may

be related to the deficiency in negative surface charge. Neutralization of anionic sites on cell surfaces by polymeric cations has been shown to induce membrane-to-membrane interactions leading to cell fusion, loss of membrane rigidity, and increase in membrane permeability (Quinton and Philpott, 1973; Vollet and Roth, 1974). Since CR thymocytes are immunologically different from CS thymocytes (Gleichman et al, 1974), the contact sites between CR P1798 and 6C3HED cells may be of functional significance. For example, electrotonic coupling, i.e., the flow of ions between cells, which may be a means of signal exchange through low resistance cell-to-cell junctions, has been reported in lymphocytes (Hulser and Peters, 1972). One might assume that a lack or deficiency of negative surface charge on CR cells of P1798 and 6C3HED tumors is associated with a decreased transmembrane potential which affects membrane resistance and ionic permeability. Metabolic variations of this kind in different types of lymphocytes would necessitate differences in cell membrane components that might be expected to influence the response to corticosteroids.

Weiss (1965) reported that treatment of Sarcoma 37 and Ehrlich murine ascites tumor cells with neuraminidase induces deformability of surface membranes and enables cells to form a low radius of curvature. The prevalence of irregular cell shapes in CR cell lines may thus be due to the absence of stabilizing anionic sites which, as suggested by Quinton and Philpott (1973), maintain the structural integrity of the

cell membrane. The lack of negatively charged groups on CR cell surfaces does not imply their absence in the cell membrane. Anionic molecules could be located at deeper sites due to conformational properties of carrier glycoproteins. The detection of acidic groups (as demonstrated by Alcian blue binding) exposed after centrifugation of CR cell suspensions on a cushion of Hypaque-Ficoll supports this possibility. An explanation for the loosening of membrane material from both CS and CR cells by this procedure cannot be given without a more thorough investigation of this phenomenon.

Whereas Ficoll is an inert, nontoxic polysucrose (Yu et al, 1973), Hypaque is a highly charged, aromatic compound as shown in the diagram below. The compound is known as an



X-ray contrast medium and can occasionally cause adverse reactions in patients, including transient proteinuria (description pamphlet, Winthrop Laboratories, New York, N.Y.). The exposure of cell suspensions to a relatively high concentration of this chemical could then explain the observed effects on the cell membrane (denuding of surface membranes and release of protein and sialic acid into the incubation medium). The Hypaque-Ficoll density gradient method (Boyum, 1968) is routinely used in research laboratories to separate different cell types found in peripheral blood. The accidental discovery by this author that this procedure may induce membrane changes points

to the need for caution in the interpretation of experimental results whenever cells have been exposed to such treatment. For this study, the artificially induced removal of cell surface material offered an explanation for the discrepancy between the presence of sialic acid moieties in both CS and CR (P1798) cell membranes (as determined biochemically) and the absence of negative surface charge in CR cell populations.

Sialic acid probably contributes most of the anionic sites on CS (P1798 and 6C3HED) cell surfaces just as it does in leukemic lymphocytes (Cook and Jacobson, 1968; Anteunis, 1974). Enzymatic removal of sialic acid was attempted by incubating P1798-CS and -CR cells in the presence of neuraminidase; however, insignificant amounts of this aminosugar were released into the medium. The optimum pH for this enzyme is 6.5 (Rosenberg and Einstein, 1972), but the cells survived only at pH 7.0. Apparently a considerable amount of sialic acid escapes removal by neuraminidase from many cells, since certain sialosphingolipids and 4-O-N-diacetylneuraminic acid in glycoproteins are resistant to conventionally used sialidases (Winzler, 1970). There is evidence that RNA may contribute to the net surface negativity in normal, rapidly proliferating mouse lymphocytes (Bennett et al, 1969); however, it has been shown that in lymphoid cell populations, including thymocytes, ionized carboxyl groups of sialic acid contribute a much greater percentage of peripheral anionic sites than does RNA (Mayhew and Weiss, 1968).

In addition to Alcian blue 8GX, 2 other electronab-

sorbing stains, ruthenium red and the metal salt lanthanum nitrate were used to reveal surface layers of P1798-CS and -CR cells by electron microscopy. All of these chemicals react with polysaccharide-containing structures, collectively known as the "glycocalyx" (Bennett, 1963), on the external surface of cells, but they show different affinities.

Alcian blue is a copper phthalocyanin complex with up to 4 positively charged isothiouronium groups that presumably bind indiscriminately to negatively charged groups (including sialomucins) by electrostatic interaction (Scott, 1972; Scott et al, 1964), thus forming insoluble, osmiophilic complexes (Behnke and Zelander, 1970). The presence of a copper atom in the Alcian blue molecule makes this dye suitable as an electron-absorbing stain.

Ruthenium red, a small polyvalent cation (proposed structural formula: $\left[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5\right]^{6+}$), apparently stains lipids, connective tissue components (such as hyaluronic acid and chondroitin sulfate), some amino acids and RNA (Luft, 1971a,b; Martinez-Palomo, 1970) when used in the presence of osmic acid. As reported by Howell (1974), a high density of negative charges appears necessary for morphologically visible ruthenium red binding. Generally the plasma membrane stains poorly (Luft, 1971b), possibly because the dye does not complex with sialic acid (Luft, 1971a; Morgan, 1968; Dermer et al, 1974). Morgan (1968) reported binding of this chemical to hyalurnidase-susceptible surface material of sarcoma cells. Santer et al (1973), who success-

fully reacted surface coats of different classes of murine lymphocytes (including thymocytes) with ruthenium red, reported that trypsin or neuraminidase removed the coat. Although trypsin might be expected to have such an effect, the prevention of ruthenium red binding by neuraminidase is in contradiction to the results of extensive studies by Luft (1971a) and reports by other authors (Morgan, 1968; Dermer et al, 1974). Santer et al used a crude enzyme preparation and included 50% fetal calf serum (known to contain glycoproteins) in the dye-containing fixative. Perhaps contaminating enzymes removed ruthenium red-binding molecules (in addition to sialic acid) which might have been (at least in part) serum-derived components adhering to the surfaces of cells.

This investigation showed very little ruthenium red binding to surfaces of either P1798-CS or -CR cells. Only a small number of cells had a visible coat (figs. 16 and 17) comparable to that obtained with Alcian blue. Thus it can be assumed that this dye reacts with components different from those binding to Alcian blue. RNA, which presumably complexes with ruthenium red, apparently does not contribute significantly to the negative surface charge of P1798 cells.

Lanthanum nitrate-positive surface layers have been reported to be susceptible to phospholipase but resistant to proteolytic enzymes, EDTA, DNase, and several mucolytic enzymes (Lesseps, 1967). Both P1798-CS and -CR cells showed little if any lanthanum-positive material on their surfaces (similar to most ruthenium red-stained cells of these tumors).

These results are in agreement with those of Bona and Anteunis (1973) who reported lanthanum-negative cell coats in the majority of mouse thymocytes. They observed that after sialidase and sulfatase treatment, all cells were stainable with lanthanum. In contrast to thymocytes, mouse bone marrow lymphocytes (B-cells) apparently had a thick coat that was susceptibe to β-glucosidase or pronase treatment. These results suggest that lanthanum binds to certain neutral sugar components at the cell surface.

Bona and Anteunis (1973) also reacted mouse bone marrow cells (B-cells) and thymocytes (T-cells) with phosphotungstic acid at low pH which produced a positive surface reaction in T-cells and a negative reaction in B-cells (as observed by electron microscopy). Phosphotungstic acid at low pH bound specifically to sialoglycoproteins as Rambourg (1968) had reported earlier. The phosphotungstic acid-negative, immature B-cells, located within the bone marrow itself, are resistant to high doses of hydrocortisone (Levine and Claman,1970). P1798 cells that do not bind Alcian blue are also corticoid-resistant. This dye is known to stain sialoglycoproteins, although not specifically (Scott et al, 1964). Assuming that sialic acid accounts for most of the surface negativity in P1798-CS cells (and possibly 6C3HED cells), the results of this study are in agreement with those of Bona and Anteunis (1973). This strengthens the possibility that surface electronegativity is a contributing factor for corticoid sensitivity.

The inaccessibility of anionic sites (Alcian blue positive) within the cell membrane of CR cells may be a decisive factor in the failure of these cells to respond to the lethal action of corticosteroids. Cryptic sites are known to influence cellular regulatory mechanisms. Surface membranes of neoplastic cells, for example, differ from normal cells in that they can bind certain mitogenic agents such as concanavalin A and wheat germ agglutinin. The carbohydrate components involved in this binding are α-methyl-D-glucopyranoside and N-acetyl-glucosamine, respectively. These sugars are exposed in nontransformed cells only during mitosis (Glick and Buck, 1973; Fox et al, 1971; Burger, 1969; Shohan and Sachs, 1974). Treatment of normal nonmitotic cells with trypsin can unmask these hidden sites and temporarily induce neoplastic behavior such as loss of contact inhibition (Poste, 1972; Inbar and Sachs, 1969; Burger, 1973). These observations illustrate that the configuration of cell surface structures plays an important role in cell function.

The relatively easy denuding of both CS and CR (P1798) cell surfaces by the Hypaque-Ficoll procedure implies that cell coat components (which presumably differ in CR and CS cells) are loosely attached to the rest of the cell membrane, possibly by divalent ions. Evidence supporting such a possibility has been presented by several investigators. Codington et al (1970), Culp and Black (1972), Culp (1974), and Terry (1974) were able to remove surface proteins from cancer cells with specific chelating agents such as EDTA and

EGTA. Studies by Humphreys (1967) indicate that released surface glycoproteins may play a role as specific cell ligands, since reaggregation of dissociated homologous cells was accelerated in their presence. Exposure of P1798-CS and -CR cells to mild chelators in an in vitro system could give further clues to the role of surface electronegativity in steroid-induced cell lysis.

Dissimilarities in surface-exposed sites on CS and CR lymphoma cells, detected by electron microscopy, were substantiated by demonstrated biochemical differences of isolated P1798-CS and -CR cell membranes and cell membrane-derived L-fucose-labeled sialoglycopeptides.

The recovered cell protein in the plasma membrane samples of both CS and CR tumors (approximately 3-5% of total cell protein) corresponds to an amount expected to be found in this fraction (de Duve, 1971). The densities of L1 and L2 preparations, which differed slightly in CS and CR samples, suggest that ratios of protein to lipid are higher in CR cell membranes as compared to those of CS cells. The mean sialic acid content based on membrane protein was lower in CR than in CS samples, although amounts present in individual samples showed considerable variation. Glick et al (1970) reported similar variations in sialic acid content of mouse fibroblast membranes and suggested that such differences may be related to growth rate or changes in protein content of the cell. Perhaps the lower average sialic acid content and the greater density of CR relative to CS cell

membranes relate to a faster growth of P1798-CR tumors (own observations) and a higher protein content per cell (CS: 5.5-5.6 mg protein/10^8 cells; CR: 7.2-7.4 mg protein/10^8 cells) as compared to CS tumors. This is reflected in differences in electron density (figs. 8 and 9) between CS and CR cells. Kinetics of sialyl transferring and cleaving enzymes may differ in the two tumors. Dissimilar positions of sialic acid residues in CS and CR cell membranes support such a possibility.

The plasma membrane marker enzymes 5'-nucleotidase and Na^+, K^+-activated, Mg^{2+}-dependent ATPase did not follow the distribution of cell membrane ghosts in either P1798-CS or -CR preparations. Similar observations were made by other authors for HeLa cells (Johnson et al, 1974) and for leukemic mouse lymphocytes (Warley and Cook, 1973). 5'-nucleotidase activity has been shown to be much lower or undetectable in cell membranes from human chronic lymphocytic leukemia cells than in normal lymphocytes (Quagliata et al, 1974). P-nitrophenylphosphatase in both P1798-CS and -CR preparations showed higher activity in the denser L2 as compared to L1 membrane fractions. It is known that plasma membranes are composed of distinct areas with different enzyme activities (de Duve, 1971). Vesicles of fragmented membranes (or whole membrane ghosts of cells differing in density and enzyme activity) may be expected to have separated out in the L1 and L2 layers. Mg^{2+}-dependent ATPase also showed a higher specific activity in CS-L2 as compared to CS-L1 fractions;

however, in CR-L1 and -L2 samples, specific activities of this enzyme did not differ significantly. Since L1 fractions contained the largest portion of recovered cell membranes, the higher Mg^{2+}-dependent ATPase activity in CR-L1 as compared to CS-L1 cell membranes could be an influential factor in corticoid resistance.

L-fucose was chosen as a marker for in vivo labeling of surface glycoproteins, since it has been reported to be the most specific cell membrane label (Atkinson and Summers, 1971). This methylpentose is incorporated directly into macromolecules and is not a precursor of other sugars and amino acids (Kaufman and Ginsberg, 1968; Herscovics, 1970). Fucose is attached to preformed polypeptides (Herscovics, 1970), and all fucose not incorporated into glycoproteins is excreted through the kidney (Coffey et al, 1964). Studies by Bekesi and Winzler (1967) suggest that utilization of this sugar involves the following steps: fucose $\longrightarrow$ fucose-1-P $\longrightarrow$ GDP-fucose $\longrightarrow$ glycoprotein. Autoradiographic studies of various cell types (in the rat), including lymphocytes, have shown that fucose label appears over Golgi vesicles, the subcellular site of membrane glycoprotein and glycolipid synthesis (Bosman et al, 1969), within a few minutes after administration of the isotope. From the Golgi complex the label migrates mostly to the plasma membrane and to lysosomes in some cells (many lysosomal hydrolases are glycoproteins) (Bennett et al, 1974; Bennett and Leblond, 1970, 1971). The pattern obtained with radioactive D-glucosamine,

a precursor of sialic acid, is more complex and less clear than that described for fucose, since this sugar is also incorporated into N-acetylglucosamine and N-acetylgalactosamine (Buck et al, 1970). Most sialoglycopeptides, that are of major interest in this study, contain both fucose and sialic acid in variable amounts (Warren et al, 1972; Katzman and Eylar, 1968) as terminal residues in their carbohydrate sidechains (Hughes, 1970; Gottschalk, 1960b; Lloyd et al, 1968). Therefore, the greater specificity of L-fucose as compared to D-glucosamine (a precursor of sialic acid) makes this pentose more suitable to facilitate the isolation and separation of cell membrane sialoglycopeptides.

The chromatographic patterns obtained in this investigation indicate that fucose-labeled glycopeptides derived from cell membranes of P1798-CS and -CR after extensive proteolytic digestion, differ in molecular weight, size and sialic acid density. The differences in the neuraminidase-induced shift in the elution patterns of untreated CS and CR preparations suggest that most CR fractions have a higher sialic acid content than CS fractions. This is no indication of higher total amounts of this sugar in P1798-CR cell membranes (than in P1798-CS cell membranes), since only fucose-containing sialoglycopeptides are accounted for. In addition, neuraminic acids in CS and CR oligosaccharides may differ in their side groups that increase the asymmetry and size of glycopeptides. Thus the influence of these negatively-charged molecules on the Stoke's radius (radius of ellipsoids

of revolution) may vary in degree in the two samples. For asymmetric molecules, such as sialoglycopeptides, the molecular weight and the Stoke's radius are codeterminants of the chromatographic profile (Tanford, 1961).

The pattern of differentially labeled CS and CR glycopeptides shown in chart 2-A is strikingly similar to that reported by van Beek et al (1973) for malignant and nonmalignant lymphoblasts (initially derived from a spontaneous mouse lymphosarcoma). These authors fractionated fucose-labeled sialoglycopeptides obtained from trypsinates of the cultured lymphoblasts by a procedure identical to that described for this study. Their samples from the malignant cell line showed an elution profile resembling that of P1798-CS and glycopeptides derived from their nontransplantable strain exhibited a pattern corresponding to that observed for P1798-CR glycopeptides. No information as to corticoid sensitivity of the two cell lines was given.

<u>In vivo</u> steroid treatment for 6 hours did not change the elution profiles of P1798-CS and -CR glycopeptides (note the similarity of charts 2-A and 3-A). However, the formation of a new low molecular weight peak in neuraminidase-treated samples (both CS and CR) implies a change in structure and/or conformation of sialoglycopeptides. The chromatographic patterns obtained 7 hours after steroid action <u>in vivo</u> differ from those seen in untreated P1798-CS and -CR samples. All glycopeptide fractions derived from both CS and CR cell membranes eluted faster which suggests an in-

crease in molecular weight or size. A new prominent low molecular weight peak, that appeared after the removal of sialic acids by neuraminidase, suggests that these sugar residues have increased in number on certain early eluting and major peak fractions. Such changes may involve the activation of specific sialidases as well as sialic acid transferases and possibly other enzymes involved in the metabolism of surface carbohydrates.

The somewhat different elution patterns of the two steroid-treated (7hours) desialylated P1798-CS and -CH glycopeptide preparations cannot be readily explained. The variations in sialic acid concentration per mg protein (mentioned earlier) or differences in degree of cytolysis in the two transplant generations used could have been contributing factors. Considerable variations in hormone receptor binding in P1798 tumors have been reported by Kaiser et al (1974). The extent of changes resulting from steroid action may thus be influenced by the metabolic condition of cells.

The described glucocorticoid-induced alterations of both P1798-CS and -CH glycopeptides were reflected in a changed density of the isolated plasma membranes that were used for pronase digestion after exposure <u>in vivo</u> to 9-FP for 7 hours. The increase in quantity of L2 membrane fractions in the sucrose gradient indicates a decrease in the lipid/protein ratio and apparently an increase in molecular weight of glycoproteins. Glucocorticoid-induced inhibition of choline incorporation into the membrane phospholipid frac-

tion of mouse lymphoma cells has been reported by Story et al (1973). Prednisolone has also been shown to inhibit cholesterol and phospholipid metabolism in HeLa cells (Dell' Orco and Melnykovych, 1970).

Alcian blue staining demonstrated a loss of exposed anionic sites from surfaces of CS cells between 6 and 8 hours after exposure to 9-FP (figs. 24 and 25). A similar glucocorticoid-induced reduction in cell surface charge was reported by Ballard and Tomkins (1970). They observed a decrease in electrophoretic mobility of hepatoma cells after exposure to corticosteroids. Neither electrophoresis nor lack of Alcian blue binding can distinguish between the loss of anionic sites, addition of cationic groups, or relocation of charged molecules. In this study, a significant loss of negatively charged sialic acid residues is not reflected in the chromatographic patterns (charts 4 and 5) of treated P1798-CS and -CR preparations. Presumably exposure to steroid hormones results in reshuffling of sugar residues in cell membrane glycopeptides. Such alterations may allow oligosaccharide chains to unfold and assume larger molecular dimensions. This could then explain the shift to the left in the elution profile. In the remolding process, sialic acid moieties on CS cell surfaces may become inaccessible to Alcian blue or become deionized by binding to oppositely charged sites on the cell membrane. Apparently the cell surface changes resulting from steroid action differ in P1798-CS and -CR. This is indicated by the absence of visible Al-

cian blue binding on CR surface membranes before and after exposure to glucocorticoids.

The specificity of many biological events resides in particular carbohydrate units and in their sequence in oligosaccharide chains of plasma membrane glycoproteins. If the lack or deficiency of anionic sites on CR cell surfaces and their presence on CS cell surfaces is universal, one could postulate that a functional relationship between electronegativity on the external cell membrane and steroid-mediated cytoplasmic events determines the therapeutic effectiveness of glucocorticoid hormones. Impairment of transport phenomena in corticoid responsive lymphoid tissues (Rosen et al, 1970; Stevens et al, 1973; Munck, 1970), which has not been observed in CR lymphocytes, strongly suggests involvement of the cell membrane in the cytotoxic process. It has been proposed that defective cytoplasmic hormone-receptor binding (Gehring et al,1971; Hackney et al, 1970; Rosenau et al, 1972) is related to steroid resistance; however, not all CR cell lines show evidence of altered receptors. Studies by Lippman and Thomson (1973) indicate that qualitative differences between specific cytoplasmic glucocorticoid receptors exist which can be recognized by nuclear binding sites. This evidence offers new explanations for steroid resistance in some systems but fails to do so in others.

This study has demonstrated that steroid-induced alterations in cell surface glycopeptides, containing both L-fucose and sialic acid, occur not only in P1798-CS but

also in P1798-CR. These observations suggest that the microstructure of surface glycoprotein oligosaccharides, which was shown to differ in the two tumors, is a codeterminant in the final outcome of metabolic events initiated by glucocorticoids.

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