THE SUBCELLULAR LOCALIZATION OF SULFOGALACTOGLYCEROLIPID

Abbey Klugerman

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ABSTRACT

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To my mother and late father

I would like to express my gratitude to my research supervisor, Dr. Mary-Judith Kornblatt, for her guidance and direction in my research endeavors. Discussions with her about the research were not only helpful but intellectually stimulating as well. I would like to thank my research committee for their advice and help, the faculty and technical staff of the Chemistry Department of Concordia University and the biochemistry graduate students. I would also like to add a note of thanks to Franc Rogan for his assistance in doing the fluorescence measurements, Joyce Hodgson for typing the thesis, and to Dr. Jack A. Kornblatt for generally keeping me on my toes.

TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd)

LIST OF FIGURES

LIST OF FIGURES (cont'd)

LIST OF TABLES

LIST OF ABBREVIATIONS

AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
Ci	curie, 2.22×10^{22} disintegrations per minute
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
MES	2-(N-morpholino) ethane sulfonate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometer (wavelength)
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PBS	phosphate buffered saline: 8.0g NaCl, 1.15g Na_2HPO_4, 0.20g KH_2PO_4, 0.20g KCl to 1 liter with water
PBSG	phosphate buffered saline 0.1% (w/v) glucose
SGG	sulfogalactoglycerolipid
Tris	2-amino-2-hydroxymethyl-1-1,3-propane-diol
UDP	uridine-5'-diphosphate

INTRODUCTION

(I-1) GENERAL ASPECTS OF SPERMATOGENESIS

Differentiation is the biological process in which a cell undergoes changes in its structure and biochemistry such that the final cell along this developmental line will be very different from the starting or primary cells. Every cell in the adult of almost all organisms above the procaryotes has differentiated from the primary cells of the embryo. Even in the adult there are cells present that are undergoing the process of differentiation. The continual development of erythrocytes from the primary stem cells in the bone marrow is an example of differentiation. Another example of differentiation is the process known as spermatogenesis.

Spermatogenesis is the differentiation of spermatogonial stem cells that give rise to spermatozoa.[1] It is a continual process in which three principal phases can be distinguished.[2] In the first phase, the spermatogonia proliferate and develop into spermatocytes and at the same time maintain their numbers by renewal.[2] This process starts in the immature animal when the primary gonocytes divide to form type A spermatogonia.[3] The type A spermatogonia divide; some of the daughter cells remain as type A while some differentiate into type B spermatogonia.[1] Type B spermatogonia develop and eventually divide to form primary spermatocytes.[4]

In the second phase of spermatogenesis the spermatocytes go through the development process to become spermatids.[3,6] It is the spermatocytes, during this phase, that undergo meiotic division to reduce the number of chromosomes in the cells from a diploid number (2N) to a haploid number (N).[5] The first type of spermatocyte that arises as a result of the spermatogonial type B division is the prophase I spermatocyte.[6,7] The prophase I spermatocyte then starts to move away from the basal lamina of the seminiferous epithelium of the tubules and develops into the leptotene I spermatocyte.[6] There is no clear distinction between the various stages of the spermatocyte; its development is gradual and therefore it is impossible to histologically determine the exact point when a cell stops being one type of spermatocyte and becomes another. The progress of the differentiation of the spermatocyte continues into the zygotene I spermatocyte where the last of the deoxyribonucleic acid synthesis occurs.[1] These cells develop into metaphase I, then anaphase I and then telophase I.[5] At this point the cells divide and become secondary spermatocytes with twenty-three pairs of identical chromosomes. The secondary spermatocytes go through the quick second phase of meiosis, prophase II, anaphase II and telophase II where the cells divide, splitting the pairs of chromosomes to form spermatids which have twenty-three chromosomes, (N), twenty-two autosomes and one sex chromosome.[5]

The third phase of spermatogenesis, sometimes called spermiogenesis, is the development of the spermatid through a complex series of changes into the spermatozoa.[4] Spermiogenesis can be divided into four main phases in which a number of histological changes take place. The Golgi apparatus becomes associated with the nuclear envelope and the acrosome starts to develop into the "acrosomic" granule.[1,6] The head cap starts to form and eventually both the acrosomic granule and the head cap orientate towards the basement membrane of the tubules. By the time, the acrosomic granule transforms into the characteristic acrosome, the flagella develop and the mitochondria line up along its base. With completion of the differentiation of the "tail" the newly formed spermatozoa is seperated from the other cells in the seminiferous tubule and moves into the vas deferns.[3,4,6] Spermatogenesis is complete.

This progressive view of spermatogenesis in the immature testis is not identical to the waves of spermatogenesis in the adults testis. In the adult, the several steps of the development of the cells are found at different levels in the germinal epithelium, with the spermatogonia at the base and the more differentiated cells, spermatocytes and spermatids, at progressively higher levels.[1,3,4,6] The development of a single spermatocyte into spermatozoa occurs at the same time as other cells in earlier or later stages of development continue to differentiate. The cells of the different phases of

development are not randomly distributed but are associated with a well defined combination of other cells. Spermatids at different phases of development are always associated with spermatocytes and spermatogonia at particular stages of their development. These associations occur in an orderly sequence along the tubule. Thus, in the adult testis there are a series of waves of differentiation along the seminiferous tubule, all in various stages but associated with each other, continuing at the same time.

(I-2) BIOCHEMICAL ASPECTS OF SPERMATOGENESIS

This description of spermatogenesis is a histologically oriented view; there are as well many biochemical changes. These changes may be related to hormones. Hormones have been shown to play a large role in spermatogenesis. An example of such a hormone is that of testosterone. Its exact biochemical action is as yet unknown but it is known that it is required to permit spermatocytes to complete meiosis.[8] The interstitial cells synthesize it[9] and Dorrington and Fritz[10] showed that the spermatocytes metabolize it to dihydrotestosterone. Research on the biochemical changes occurring during spermatogenesis has not always been concerned with hormones and their actions; the roles that enzymes and proteins play in this process have also been examined. Enzymes have been shown to have varying levels during spermatogenesis. These levels may increase or decrease at specific times during the developmental process. The reasons for these variations of the enzyme levels are largely unknown but research in this area is continuing at the present.

The enzyme, 5'-nucleotidase, is an example of such an enzyme that shows a dramatic increase in its specific activity at a particular stage of spermatogenesis. Xuma and Turkington[11] determined that the specific activity of 5'-nucleotidase increases during the

formation of the spermatogonia; it rises steadily from five to twenty-six days and then remains level. The reason for this dramatic increase and its relationship to spermatogonial formation and subsequent differentiation is as yet unknown.

The glycoprotein glycosyltransferases are other enzymes that have been looked at in relationship to spermatogenesis. These enzymes decrease in their specific activities at a specific stage of spermatogenesis. Letts et al[12] looked at this phenomenon by preparing cell suspensions from mouse testes. These cell suspensions were then analysed for the levels of activity of galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylglucosaminide fucosyltransferase and galactoside fucosyltransferase. They found that spermatocytes and early spermatids possessed relatively high levels of the enzymes while late spermatids had lower enzyme levels. They felt that their study suggested that active glycoprotein synthesis is occurring in testis germinal cells during and immediately after meiosis and that this process is turned off in the terminal stages of spermatogenesis. The mechanism whereby the transferase specific activity is decreased in the later spermatogenic stages was unknown but they felt that this correlates with a degeneration of the Golgi apparatus and the formation of the acrosome. Thus, again, there are biochemical changes occurring during the various stages of spermatogenesis that can be correlated to these stages.

Another example of such research is that which has been conducted by Millette and Bellve[13]. They isolated the various spermatogenic cell types of the testis.[2] They then looked for the expression of spermatogenic specific cell surface antigens. They did this by immunofluorescence microscopy, by complement-mediated cytotoxicity and by quantitative measurements of immunoglobulin acceptors on the plasma membrane. They found that the cell surface antigens were detected first in the pachytene primary spermatocytes and expressed in all stages of subsequent differentiation, including mature sperm cells. They suggest then, that there are large changes in the plasma membrane at this stage of spermatogenesis.

Sulfogalactoglycerolipid is another example of a cellular component that is synthesized at a particular stage of spermatogenesis. In 1972, Kornblatt et al[14] and in 1973, Ishizuka et al[15] showed that the major glycolipid of rat and boar testes was a sulfated monoalkyl-monoacyl-glyceryl-monogalactoside. This sulfated glycolipid (sulfogalactoglycerolipid or SGG) has the structure as shown in figure I-1.[14,15,16]

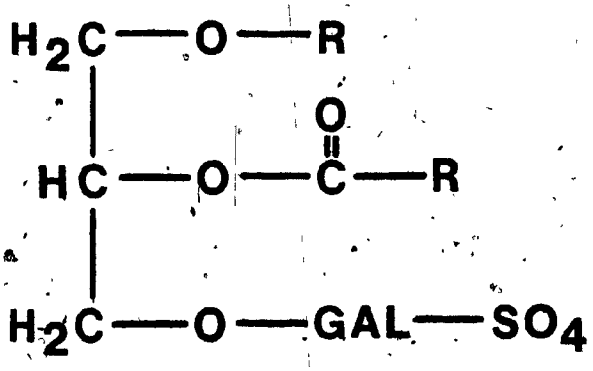
The sulfate group is attached at the 3' position of the galactose.[15,16,17,18] The alkyl and acyl groups were shown to be between eighty and ninety percent C 16:0.[16]

This glycolipid and its non-sulfated form are the major glycolipids of not only rat and boar testes but also of the testes of various other mammalian species such as bull, pig, guinea pig and even man.[14,15] SGG has also been shown to be present in small amounts in the brain.[19,20,21] Compounds similar to SGG have been found in the gastric mucosa and human saliva.[22,23,24] The testes of other species were also studied[17,25]; SGG was not found in the testes of birds and fish but similar compounds were found. The testes of mature ducks were found to contain two principal glycolipids which were determined to be sulfogalactosylceramide and galactosylceramide. The testes of mature trout and salmon also contained two principal glycolipids which were determined to be sulfo-galactosylglucosylceramide and galactosylglucosylceramide. Thus, the presence of SGG and its non-sulfated form, in the testes of the rat, are not unique. The testes of many mammalian and chordate species have also been shown to have a sulfated glycolipid, either SGG or a compound similar to SGG, as its major glycolopid.

The initial studies on SGG showed that it was possible

Figure I-1

SULFOGALACTOGLYCEROLIPID



to label it in vivo with $^{35}S\text{-}SO_4$.[14,26] It was also shown that rat testis homogenate could catalyse the transfer of sulfate from PAPS (3'-phosphoadenosine-5'-phosphosulfate, a sulfate transfering compound in many systems[27]) to chemically desulfated SGG. Other lipids that could serve as an acceptor included those with terminal β-galactosyl residues.[26] This enzyme was termed PAPS:galactolipid sulfotransferase.

The subcellular localization of PAPS:galactolipid sulfotransferase was investigated. The data that were accumulated showed a highly enriched specific activity of the enzyme in a purified Golgi apparatus fraction (as much as a fifty-fold enrichment of Golgi apparatus marker enzymes).[16] Thus, in aggreement with the work done on other systems[27,28,29] the enzyme was determined to be located in the Golgi apparatus.

Kornblatt et al [16] then tried to determine the stage of spermatogenesis in which SGG first appears by measuring the levels of SGG in immature rats. Changes in the levels were correlated with the changes in the cellular composition of the tissue. They found that there were low amounts of both lipid-bound sulfate and lipid-bound galactose. This increase corresponded to the appearance of spermatocytes in the rats' testes (see earlier discussion). The amounts leveled off at twenty-two days and started to decrease, with respect to the total lipid, between twenty-five and thirty days. This corresponds to the appearance

of spermatids (see earlier discussion). These results gave rise to two ideas; one, that the SGG is probably located in the differentiating cells of the testis, and two, that the SGG is sulfated in a cell type earlier than the spermatids.

The activity of the PAPS:galactolipid sulfotransferase in the testis at various points after the birth of the rat was examined. These results agreed with the results of the previous experiment. The activity of the enzyme was very low in the testis until the rats were about ten days old. The activity of the enzyme then rose dramtically until it reached a peak at about fifteen days. The activity plateaued until about twenty-eight days and then decreased to about one-third of the maximal level by fifty-one days after birth. This sudden rise in the activity of the enzyme corresponds to a time in the development of the testis just prior to the appearance of spermatocytes, and the decrease occurs when spermatids begin to appear. These results indicated that the SGG is probably sulfated in the spermatocyte, and not in any cell type earlier than the spermatocyte, that is, spermatogonia or gonocytes. The data also indicates that cell types later than spermatocytes are inactive or are much less active than spermatocytes in synthesizing SGG.

The stage of cell development where the $^{35}SO_4$ is incorporated into SGG was determined in two recent papers. Both Letts et al[30] and Kornblatt (submitted for

publication[31]) agreed that the $^{35}SO_4$ is incorporated into SGG in the early spermatocyte. This was shown two different ways. In the work of Letts et al, the testis of adult rats were injected with $^{35}SO_4$. Twenty four hours later cell suspensions were made of the testis and various cell types were isolated through the use of staput gradients.[32] They found that there was no enrichment of radioactive sulfolipid in either late spermatocytes or spermatids. From this they concluded that incorporation of $^{35}SO_4$ must occur in primary spermatocytes only, probably prior to pachytene or diplotene spermatocytes. Kornblatt injected the testis of rats (adults) with $^{35}SO_4$ and ^{3}H-thymidine and looked for the decrease in the level of radioactivity in the testis and the increase in the level of radioactivity in the vas deferens and the epididymis for a period of six weeks. Knowing that the length of time between the administration of the isotope and its appearance in the epididymis is a function of the stage of spermatogenesis at which the isotope is incorporated into the germinal cells, she found that the ^{35}S appears in the epididymis five to six days before ^{3}H and thus concluded that the synthesis of SGG must stop five to six days after DNA synthesis has stopped. It was then calculated that the incorporation of $^{35}SO_4$ into SGG ends shortly after zygotene spermatocytes become pachytene spermatocytes. Thus, the period of synthesis of SGG is limited to a very discrete stage of spermatogenesis, that of the

early pachytene, zygotene and perhaps even lepotene primary spermatocytes.

In order to determine if, as the location of PAPS: galactolipid sulotransferase indicated, SGG is sulfated in the Golgi apparatus, Schachter[33] injected rats intratesticullarly with $^{35}SO_4$ sacrificed the rats at various times after injection and then measured SGG by counting ^{35}S in the lipid extract from the total testis and from purified Golgi apparatus. Within twenty minutes there was a forty-fold enrichment of specific radioactivity of the SGG in the Golgi apparatus over the total testis. As the time between the injection of the $^{35}SO_4$ and the sacrificing of the rats was increased, the enrichment decreased. By twenty-four hours after injection, there was no more than a three-fold enrichment of the SGG in the Golgi apparatus. This enrichment is low enough that it could be explained by contamination of the Golgi apparatus preparation by SGG in membranes other than Golgi apparatus membranes. Thus, sulfogalactoglycerolipid is sulfated in the Golgi apparatus and once sulfated it leaves the Golgi apparatus and moves to an as yet unidentified cellular compartment. It was the object of the research for this thesis to determine th[illegible] cellular compartment.

(I-3) SUBCELLULAR LOCALIZATION

The determination of a cellular component's subcellular localization can be achieved in a number of ways. It is possible that one could isolate and purify the various cellular compartments and then look for the components' enrichment in any of the compartments. The problem of isolating all the cellular components of the cell is very laborious and time consuming. Another problem with this method is that the results can often be misleading. If a cellular component is shown to be low in concentration in a particular compartment, the question arises as to whether the component is not low in concentration in that compartment but is just a contaminant.

Another method of determining the subcellular localization of a cellular component is to use the approach of Christian de Duve.[34,35,36,37,38] In 1955, he developed a method of following the distribution pattern of the various organelles of the cell.[34] He subjected the homogenized tissue to centrifugations of increasing force and time. He had five fractions, the nuclear fraction, the mitochondria fraction, the light mitochondrial fraction, the microsomal fraction and the supernatant fraction. These fractions were by no means pure but he postulated that a given organelle would be distributed over these fractions in a characteristic manner.[34,38] Any enzyme in that compartment should show this

characteristic distribution. He felt that two enzymes of a single compartment of the cell could not be separated by the centrifugation process.[34] Thus, he proceeded with this fractionation and determined the relative specific activities of various enzymes in the fractions. He presented the distribution patterns of the enzymes over the fractions in the form of histograms. The histograms would then be the distribution patterns for the various enzymes of the cells.[38] He was able to show[34] that quite a number of enzymes with lytic properties, such as acid phosphatase, ribonuclease, deoxyribonuclease, etc., had the same distribution patterns of specific activity on their histograms and thus determined the existence of the lysosomes. He also showed that these enzymes had different distribution patterns than the enzymes of other cellular compartments, such as cytochrome C oxidase, NADPH cytochrome C reductase, glucose-6-phosphatase, etc.

This method would seem to enable the researcher to determine the subcellular localization of a cellular component such as a lipid without having to isolate and purify the various cellular compartments and incur the problems thereof. I felt that I could use this procedure to determine the subcellular localization of sulfogalactoglycerolipid.

(I-4) STATEMENT OF OBJECTIVES

Due to the fact that a very small amount of material was available (the testes of young rats are quite small), I intended to layer homogenates of whole cells on continuous sucrose gradients. The distribution patterns would be similar to the histograms of de Duve. Sharp bands of material on the gradients would not be expected due to the polydispersity of the particles and the heterogeneity of their size, shape and density, even of particles of the same organelles.[38] It would also not be expected that these distribution patterns would yield results which could determine whether or not a component is exclusively in a particular compartment.[38]

The distribution patterns of the various organelles were to be determined by the assaying of various enzyme markers. Since not as much work has been done on the enzymes of the testes as has been done on the liver, not all enzymes to be used have been shown to be specific markers for specific organelles in the cells of the testis. A few have, though, and the enzyme markers for the Golgi apparatus, plasma membrane, mitochondria and the endoplasmic reticilum are some of these. The enzyme marker for the Golgi apparatus was chosen to be UDP-D-galactosyl-transferase which will transfer the galactose from UDP-galactose to a protein acceptor. This enzyme has been shown to be located in the Golgi apparatus of rat

testis.[39,40,41,42] The enzymes chosen for the endoplasmic reticulum were D-glucose-6-phosphatase which has been shown to be located in the endoplasmic reticulum of hamster testis.[43] NADPH cytochrome C reductase has also been shown to be a marker for endoplasmic reticulum.[37] Acid phosphatase, a non specific phosphatase in acid medium has been recognised by de Duves' work[34,38] to be an enzyme marker for lysosomes. The succinate cytochrome C reductase system was chosen as an enzyme marker for mitochondria.[44,45,46] Two enzyme markers were chosen to represent the plasma membrane; 5'-nucleotidase which will hydrolyse the phosphate esters of 5'-nucleotides has been accepted as a plasma membrane marker for quite some time[11,47,48,49] and alkaline phosphatase[37] a non-specific phosphatase in alkaline medium.

Once the patterns for these cellular organelles are determined, if it can be shown that the SGG has the same distribution pattern as the pattern for a particular compartment and is different from the patterns of all the other compartments then it can be said that twenty-four hours after is sulfation in the Golgi apparatus, sulfogalactoglycerolipid moves to that particular cellular compartment.

MATERIALS AND METHODS

(II-1) MATERIALS

[^{35}S]-sodium sulfate (700-1,000 mCi/mMole), [^{14}C]-D-glucose-6-phosphate (52.5 mCi/mMole), uridine diphospho-[^{14}C]-D-galactose (300 mCi/mMole), Aquasol and 2,5-diphenyloxazole were obtained from New England Nuclear-Canada, Ltd. Deoxyribonuclease II, trypsin, soyabean trypsin inhibitor, adenosine-5'-triphosphate, adenosine-5'-monophosphate, nicotinamide adenine dinucleotide phosphate (reduced form), p-nitrophenylphosphate, N-acetylglucosamine, triton X-100, dithiothreitol, sucrose, uridine diphospho-D-galactose, D-glucose-6-phosphate, cytochrome C and 2-(N-morpholino) ethane sulfonate were purchased from the Sigma Chemical Company, St. Louis, Missouri. Fluorescamine was purchased from Hoffmann-LaRoche, Quebec. Cycloheptaamylose was purchased from the Aldrich Chemical Company. All other chemicals used were of reagent grade and were purchased through various commercial supply outlets. All organic solvents used in all experiments were redistilled.

(II-2) ISOTOPE ADMINISTRATION

Male wistar rats, 28 days old, obtained from Canadian Breeding Farms Inc., Quebec, were used in all experiments. The rats were kept under conditions of controlled lighting (approximately 14 hours per day), in a room maintained at 22°C. The rats had free access to food and water.

At the beginning of each experiment, the rats were injected intratesticularly with 20 µCi of [^{35}S]-sodium sulfate per testis. Twenty-four hours later, at approximately 09:30 h, the rats were sacrificed by decapitation and a cell-suspension of testicular tissue was prepared.

(II-3) PREPARATION OF THE CELL SUSPENSION (see figure II-1)

The preparation of the cell suspension, was carried out at room temperature. Normally four rats were used per experiment.

After the rats were sacrificed, their testes were removed and the tunicae were stripped off. The testes were then chopped five times with a mechanical chopper (a battery of fifteen sharp razor blades, spaced 0.8 mm apart). They were suspended in PBSG, by repeated pipettings, twenty times with a pastueur pipette, to a volume ten times the weight of the testis. DNase (1 mg/ml in PBS) was added at a volume one tenth the volume of the suspending buffer. The suspension was allowed to settle for ten minutes and the supernatant was collected. The material that settled was resuspended in PBSG in an equal volume to that used before. DNase was added again, the suspension was allowed to settle for five minutes and the supernatant was collected. The settled material was discarded.

The supernatants were combined and centrifuged in a clinical centrifuge (International Equipment Co., Mass., USA) for five minutes at 300 X g. After centrifugation the pellet was resuspended in PBSG to a volume one-tenth that of the combined supernatants. Trypsin (2.5% w/v in PBS) was added to give a final concentration of trypsin of 0.25%. After thirty minutes, trypsin inhibitor was added

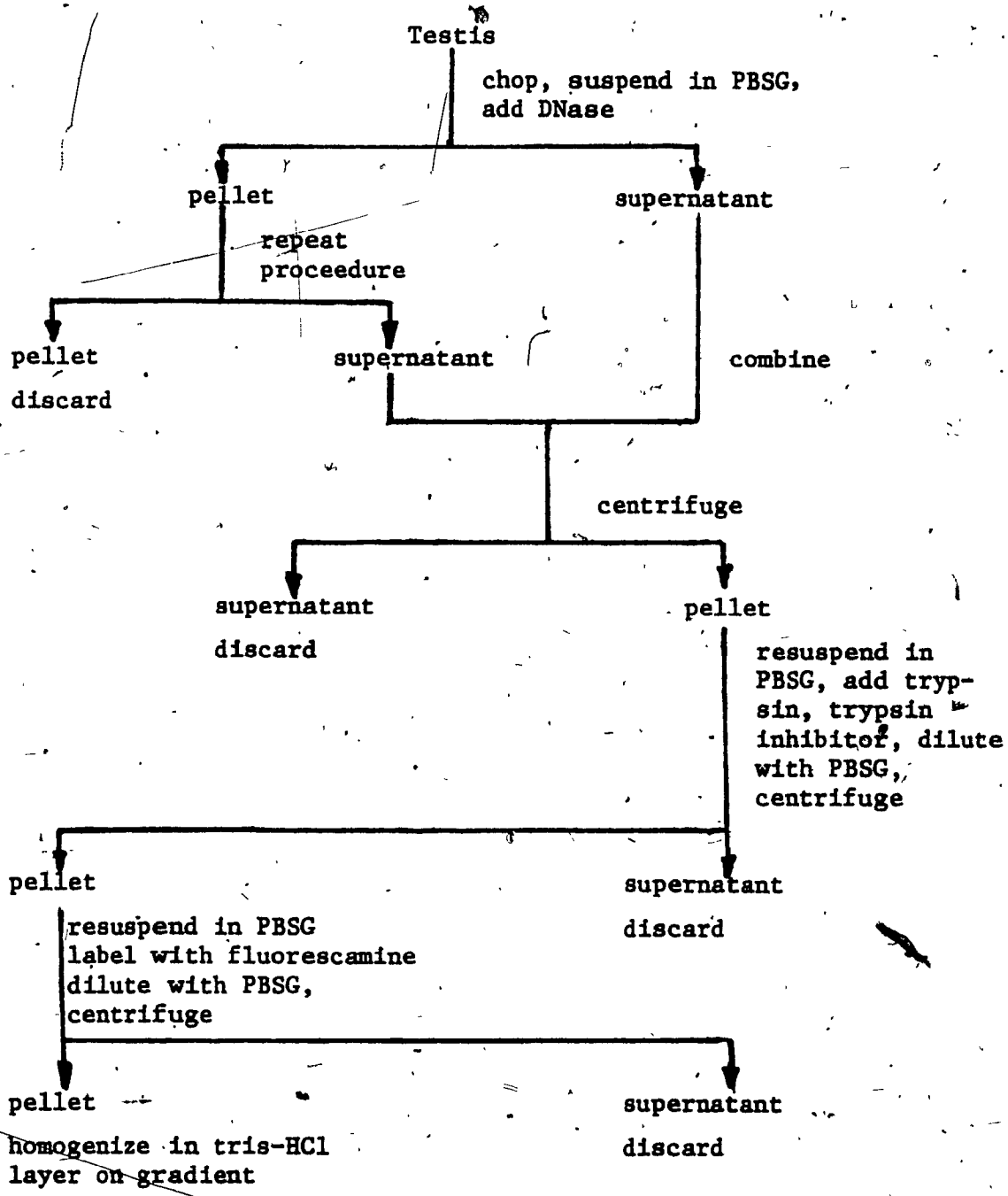
in excess of the trypsin to stop the reaction. The suspension was then diluted by the addition of three volumes of cold PBSG and centrifuged for 5 minutes at 300 X g. The pellet was recovered and was resuspended in PBSG (2.0 ml). It is at this point that the cells were labelled with fluorescamine (see labelling procedures).

After surface labelling, the cells were centrifuged at 300 X g for five minutes and the pellet was resuspended in cold 10 mM tris-HCl buffer, pH 7.4 and homogenized by six strokes of a Potter-Elvehjen homogenizer operating at 500 rpm. The homogenate was centrifuged at 300 X g for three minutes to remove unbroken cells and nuclei. This supernatant was used in further work.

The total number of cells were counted on a haemocytometer with a phase contrast microscope. The amount of lysis and thus the percentage viability throughout the procedure was measured by observing cells' exclusion of trypan blue (1.0% in PBS).[50]

Figure II-1

PREPARATION OF THE CELL SUSPENSION



This figure shows a schematic representation of the cell suspension proceedure, for further details see the section in the Materials and Methods (section II-3).

(II-4) GRADIENT CENTRIFUGATION

After homogenization, sucrose was added to the supernatant prepared from the homogenized cell suspension to bring the mixture to a density equivalent to 10% (w/w) sucrose. this was layered on a 10-50% (w/w) linear continuous sucrose gradient. Gradients were prepared using sucrose, in 10 mM Tris-HCl buffer, pH 7.4, 10 and 50% (w/w).

The gradients were centrifuged in a MSE Superspeed 75 ultracentrifuge, at 100,000 X g for twenty-two hours. When the spins were completed the centrifuge was stopped, the polypropylene centrifuge tubes punctured and eighteen to twenty-two 1.0 ml fractions were collected. These fractions were analysed for their density, fluorescence, protein, the [^{35}S]-SSG and various enzyme markers. Fractions were stored at -20^{o}C.

The density of the fractions were determined using an Abbé refractometer. Numbers obtained were in values equivalent to percentage sucrose (w/w) which were converted to g cm^{-3} [51]

The protein content of the fractions was determined by the method of Lowry et al[52] with optical density read at 600 nm. Bovine serum albumin at a concentration of 4-100 µg was used as a standard.

(II-5) SURFACE LABELLING

Fluorescamine was used to label the surface of the cells.[53]

The cells, normally suspended in 2.0 ml of PBSG, were labelled by the addition of 125 µg of fluorescamine in 5 µl of acetone. Thirty seconds after the addition of the fluorescamine, the suspension was diluted four-fold and immediately centrifuged for five minutes at 300 X g. The cells were then resuspended in PBSG or homogenized in the Tris-HCl buffer. The entire labelling procedure was carried out at room temperature.

Fluorescence was measured using an Aminco-Bowman Spectrophotofluorimeter (American Instrument Company), with Xenon Lamp power, the high voltage at 700 volts, open excitation and emission slits and a sample slit set at #5 for the photomultiplier. The excitation wavelength was 390 nm and the emission wavelength was 475 nm.

Samples for fluorescence measurement were prepared by suspending 0.4 ml aliquots in 2.6 ml of borate buffer, 0.1 M at pH 9.0 which contained 1.0% Triton X-100. Quinine sulfate solutions, from 10.0 to 0.001 µg/ml dissolved in 0.1 N H_2SO_4 were used as standards. Fluorescence of samples are reported in values equivalent to the concentration of quinine sulfate per mg protein.

(II-6) [^{35}S]- LIPID EXTRACTION

The [^{35}S]-SGG was extracted and purified by the method of Kornblatt et al.[16] Homogenates or fractions from gradients were rehomogenized in 20 ml of chloroform: methanol, 2:1, (C:M,2:1) per ml of homogenate. The mixture was filtered directly into a round bottom flask through a glass fiber filter on a scintered glass funnel that was previously chilled. The residue was washed and then rehomogenized in C:M:H_2O (1.2 + 5% H_2O) at the same volume as before. This mixture was also filtered and the filtrate was combined with the previous filtrate.

The combined filtrates were dried in a vacuum and redissolved in 25 ml of C:M (2:1). 5 mls of 0:1 M KCl were added and the mixture was centrifuged at 3500 X g for ten minutes at 4°C. Two phases formed; the upper phase was removed and discarded. The partitioning procedure was repeated two more times with the addition of 12 mls of C:M:H_2O (3:48:47) and 12 mls of C:M:0.1 M KCl (3:48:47). The lower phase was evaporated to dryness and redissolved in 1.0 ml of chloroform.

This lower phase was then layered on a silicic acid (Unicil) column, 100-200 mesh, 1.0 cm in diameter. Four solvents were run through the column and fractions were collected in batch. The solvents were $CHCl_3$, acetone, acetone:methanol (9:1) and methanol. The fractions were evaporated to dryness and redissolved in C:M (2:1).

Aliquots of the redissolved fractions spotted on thin layer chromoatography plates and run with a solvent of C:M:H_2O, (65:25:4). Glycolipids were identified by their reaction with diphenylamine. The [^{35}S]-SGG was found to be exclusively in the acetone fraction.

(II-7) ENZYME MARKER ASSAYS

5'-NUCLEOTIDASE

5'-nucleotidase (EC 3.13.5) hydrolyses nucleotide monophosphates, in this case adenosine-5'-monophosphate to phosphate and adenosine. The assay used was modified from the method of Schachter et al.[54]

The reaction contained, in a final volume of 300 µl, 37.5 µmoles of glycine buffer, pH 8.5, 3.75 µmoles of $MgCl_2.6H_2O$, 2.25 µmoles of 5'-adenosine monophosphate and enzyme. The reaction mixture was incubated at 37°C for thirty minutes. The reaction was stopped by the addition 600 µl of 10% (w/v) trichloroacetic acid. Aliquots were then analysed for the amount of phosphate released by the method of Lowry.[55] 0.5 ml aliquots from the assay mixture were mixed with a solution of 0.5% ammonium molybdate solution (w/v) in 2.0 N H_2SO_4 and 2.0% (w/v) ascorbic acid solution (1:1). This mixture was incubated at 45°C for twenty minutes. The reaction was stopped by chilling the incubation mixture to room temperature. Absorbance was read at 820 nm and compared to a phosphate standard ranging from 10 to 500 µmoles of phosphate. The specific activity of 5'-nucleotidase is expressed as µmoles of phosphate released/hour/mg protein.

ACID PHOSPHATASE

Acid phosphatase (EC 3.1.3.2) catalyses the hydrolysis of p-nitrophenylphosphate to p-nitrophenol and phosphate

in an acid medium.[56] The reaction mixture contained 55 µmoles citric acid buffer, pH 4.8, 6.8 µmoles Na_2-p-nitrophenylphosphate, 5 µl of triton X-100 and enzyme in a final volume of 1.2 mls. The reaction mixture was incubated at 22°C for sixty minutes and the reaction was stopped by the addition of 2.0 mls of 0.1 N NaOH. The absorbance was then read at 405 nm and compared to a standard of p-nitrophenol. Acid phosphatase specific activity was expressed as µmoles p-nitrophenol released/hour/mg protein.

ALKALINE PHOSPHATASE

Alkaline phosphatase (EC 3.13.1) is an enzyme that catalyses the hydrolysis of p-nitrophenylphosphate to p-nitrophenol and phosphate in alkaline medium. The enzyme is assayed by a method adapted from the method of Bergmyer.[56] The reaction mixture contained 50 µmoles of glycinebuffer at pH 10.5, 0.5 µmoles $MgCl_2$ $6H_2O$, 5.5 µmoles Na_2-p-nitrophenylphosphate and enzyme in a final volume of 1.1 mls. The reaction mixture was incubated at 37°C for thirty minutes and the reaction was stopped by the addition of 2.0 mls of 0.1 N NaOH. The absorbance of the solution was measured at 405 nm and compared to a standard solution of p-nitrophenol. The specific activity of alkaline phosphatase is expressed as µmoles p-nitrophenol released/hour/mg protein.

URIDINE DIPHOSPHO-D-GALACTOSYLTRANSFERASE

Uridine diphospho-D-galactosyltransferase (EC 2.4.1.22)

is an enzyme that catalyses the transfer of galactose from uridine diphospho-D-galactose to an acceptor, in this case, N-acetylglucosamine. The reaction mixture contained, in a final volume of 50 µl, 5.0 µmoles N-acetylglucosamine, 0.3 µmoles dithiothreitol, 3.0 µmoles $MnCl_2$, 5.0 µmoles MES buffer at a pH of 5.7, 0.1 µmoles ATP, 0.025 µmoles UDP-[^{14}C]- D-galactose (4 µCi/µmole), triton X-100 and enzyme. The reaction mixture was incubated at 37°C for one hour. The reaction was stopped by adding 5.0 µmoles of EDTA in 20 µl and chilling the reaction tubes by placing them on ice. 50 µl aliquots of the reaction mixture were applied to small columns, 0.7 mm X 20 mm, of Dowex 2X8 in the Cl^- form, and were eluted with 1.5 mls of distilled water. The eluate was collected directly into scintillation vials and then counted for [^{14}C]-N-acetyllactosamine. The specific activity of the enzyme is reported as nmoles N-acetyllactosamine/hour/mg protein. The recipe for the incubation mixture was modified from the method of Schachter et al[54] with ATP added in an attempt to reduce non-specific hydrolysis of UDP-D-galactose according to Fraser and Mookerjea.[57] The method of using the mini-columns to separate the products of the reaction from the substrates was adapted from Bergeron.[58]

D-GLUCOSE-6-PHOSPHATASE

D-glucose-6-phosphatase (EC 3.1.3.9), which catalyses the hydrolysis of D-glucose-6-phosphate to D-glucose and

phosphate, was assayed according to the method of Schachter et al. The method of separation of the products from the reactants was identical to the procedure for uridine diphospho-D-galactosyltransferase as modified from Bergeron.[58] The incubation mixture contained 5.0 µmoles MES buffer at a pH of 6.5, 1.0 µmole [^{14}C]-D-glucose-6-phosphate (400,000 dpm/µmol), 0.06 µl trition X-100 and enzyme at a final volume of 45 µl. The mixture was incubated at 37°C for one hour. At the end of the incubation the reaction was stopped by the addition of 25µl of 10% (w/v) trichloroacetic acid and the assay tubes were then placed on ice. 50 µl aliquots were layered on the mini-columns and washed with 1.5 ml of distilled water, with the eluate collected directly into scintillation vials to be counted. The specific activity of the enzyme is reported as nmoles glucose/hour/mg protein.

NADPH CYTOCHROME C REDUCTASE

NADPH cytochrome C reductase (EC 1.6.2.3) reduces cytochrome C using NADPH as a co-factor. The assay procedure for this enzyme was modified from the procedure of Schachter et al.[54] The incubation mixture contained, in a final volume of 1.2 mls, 250 µmol phosphate buffer at a pH of 7.0, 50 µmol KCN, 1.5 mg cytochrome C and enzyme. The reaction is initiated by the addition of 0.4 umoles of NADPH in 10 µl. The reaction was followed using a Unicam P7000 recording spectrophotometer with the cell compartment thermostated at 37°C. The spectrophotometer was operated

at a constant wavelength of 550 nm and the increase in absorbance per unit time was measured. The absorbance increase was standardized to a reduced solution of cytochrome C. The specific activity of the enzyme is reported as pg cytochrome C reduced/minute/mg protein.

SUCCINATE CYTOCHROME C REDUCTASE

The succinate cytochrome C reductase system (EC 1.3.99.1) catalyses the reduction of cytochrome C using succinate as a substrate. The assay procedure is very similar to that of NADPH cytochrome C reductase and is adapted from the method of Schachter et al.[54] With a final volume of 1.2 ml, the incubation mixture contained 100 µmol phosphate buffer at a pH of 7.0, 50 µmol NaCN, 1.5 mg cytochrome C and enzyme. The reaction is initiated by the addition of 1.0 µmol of succinate in 10 ul and was followed at 37°C using the Unicam P7000 recording spectrophotometer at 550 nm. The specific activity of the enzyme was reported as pg cytochrome C/minute/mg protein.

Linearity, with respect to time and the amount of enzyme was established for all enzyme assays.

The levels of radioactivity of the [^{35}S]-SGG, [^{14}C]-D-glucose and [^{14}C]-N-acetyllactosamine were determined by liquid scintillation counting using a Nuclear Chicago, Unilux II, refrigerated, liquid scintillation counter. The scintillation cocktail for the counting of the [^{35}S]-SGG was prepared according to the method of Kornblatt et al[59] and was a mixture of toluene, methyl cellusolve, and

ethanol at a ratio of 110:88:13 respectively, containing 5.5 gm of 2,5-diphenyloxazole per liter of solution. [^{14}C]-D-glucose and [^{14}C]-N-acetyllactosamine were counted in a commercially available scintillation fluid, Aquasol. Quenched standards were used to prepare quench curves for the conversion of counts per minute to dis integrations per minute. All samples were counted twice for ten minutes.

RESULTS

(III-1) STUDIES ON THE CELL SUSPENSION PROCEDURE

There are many factors or parameters in the procedure of the preparation of the cell suspension (II-3) that are involved in achieving a suspension with as high yield and viability as possible, the method of releasing the cells from the testis, the method of suspension, the buffer, the pH, the method of removing the debris from cells that have been damaged in the original procedure and speeds and times of centrifugation. All of these parameters were varied in order to choose conditions that gave the maximum yield of cells and viability.

Tables III-1 and III-2 are a summary of the results of the experiments that were done in order to determine the proper parameters for the preparation of the cell suspension.

TABLE III-1

STUDIES ON THE SPEEDS AND LENGTHS OF CENTRIFUGATION

Procedure	Total Cells	Live Cells	% Viability
prior to centrifug.	2.24×10^8	1.04×10^8	46.4
800 rpm, 10 min.	1.60×10^8	0.69×10^8	43.1
1100 rpm, 10 min.	1.68×10^8	0.71×10^8	42.3
1400 rpm, 10 min.	1.91×10^8	0.77×10^8	40.3
1700 rpm, 10 min.	2.01×10^8	0.77×10^8	38.3
2000 rpm, 10 min.	2.16×10^8	0.78×10^8	36.1
2750 rpm, 10 min.	2.17×10^8	0.74×10^8	34.1
1400 rpm, 10 min.	1.88×10^8	0.82×10^8	43.6
1400 rpm, 10 min.	1.91×10^8	0.77×10^8	40.3
1400 rpm, 10 min.	1.99×10^8	0.78×10^8	39.2

Times and speeds of centrifugation were examined in a series of eight experiments with each parameter repeated three times. The average values are reported with these values standardized to the wet weight of the testis. The experiments were carried out the with the use of one rat each. Viability of cells in the pellet was measured after the first centrifugation in the cell suspension procedure by trypan blue exclusion.

TABLE III-2

STUDIES ON THE VARIOUS PARAMETERS OF THE PREPARATION

Each experiment in this table used one rat per section; ex.. buffers, temperature, pH, etc.. After each section the best parameter was chosen to be used in the following sections, for example pH 7.4 was then used in the following experiment on the action of the enzymes. Prior to these experiments the cells were chopped with razor blades, suspended through the use of a pipette and then centrifuged at 300 X g for five minutes. The suspending buffer was PBS at pH 7.4 and the procedure was carried out at room temperature.

TABLE III-2

STUDIES ON THE VARIOUS PARAMETERS OF THE PREPARATION

Parameter	Total Cells	Live Cells	% Viability
scalple blades	2.27×10^8	0.91×10^8	40.0
mechanical chopper	2.03×10^8	0.95×10^8	46.8
pipetting (20 times)	2.27×10^8	0.91×10^8	40.0
mechanical shaker	1.78×10^8	0.68×10^8	38.2
PBS	2.12×10^8	0.98×10^8	46.3
Mann's Ringers	2.22×10^8	0.94×10^8	42.3
PBS-EDTA	1.90×10^8	0.78×10^8	41.1
PBS-0.1% glucose	2.05×10^8	1.06×10^8	51.7
0-4 °C	2.56×10^8	0.96×10^8	37.5
22 °C	2.52×10^8	1.32×10^8	52.4
32 °C	1.98×10^8	0.95×10^8	48.0
pH 6.5	2.17×10^8	0.86×10^8	39.6
pH 7.0	1.90×10^8	0.89×10^8	46.8
pH 7.4	2.22×10^8	1.13×10^8	50.9
pH 8.0	1.82×10^8	0.76×10^8	42.0
DNase	1.90×10^8	1.46×10^8	76.8
Trypsin	1.36×10^8	1.34×10^8	98.5

The results of the experiments shown in table III-1 suggest that any centrifugation will kill cells but as the speed and length of centrifugations are increased the number of viable cells recovered increases along with dead cells. Thus, a speed and time of centrifugation where a high yield of cells was recovered along with a small decrease in the viability, was chosen; 1400 rpm or 300 X g for five minutes.

Table III-2 shows the results of experiments where a variety of parameters were examined. Suspending the cells by using a Pasteur pipette increases the yield of both total and live cells and thus, in further experiments, Pasteur pipettes were used. The mechanical chopper did not seem to damage as many cells as chopping the testis with scalpel blades. A variety of buffers were then tested; the PBS-glucose buffer yielded the highest viability and thus it was used for all further experiments. The PBS-EDTA buffer gave a suspension that had many more clumps of cells than what was normally seen. This could be the reason for the drop in the total yield of cells as it is very difficult to count the number of cells in a clump. At temperatures other than room temperature the viability of the cell suspension dramatically decreased and pH 7.4 seemed to be the optimum pH for the survival of the cells.

At this point the viability of the cell suspension was approximately fifty-one percent. Thus, the use of

one or more enzymes was required in order to bring the viability to the required level (greater than ninety-five percent). The use of DNase greatly increased the proportion of live to dead cells. Since spermatocytes have very large nuclei[4] some of the material that I was counting as cells may have in fact been bare nuclei and thus a possible explanation for the dramatic effect on the viability of the cells through the use of DNase. Trypsin increased the percentage viability of the cell suspension to approximately ninety-eight percent. It does so by degrading the dead cells and debris that are then washed away. This is obvious in that there is a dramatic decrease in the total cell count while the viable cell count remains virtually unchanged.

Observations using phase contrast light microscopy showed that the final cell suspension mostly consisted of large round cells without an easily visible nucleous. The rest of the cells in suspension were small; some quite irregularly shaped and some round and smooth.

(III-2) STUDIES ON THE YIELD OF THE LABELLED SGG

One of the most important parameters that is followed in every experiment is the counting of the ^{35}S-isotope (see section II-2). ^{35}S is followed because by twenty-four hours after the injection of ^{35}S-Na_2SO_4 into the testis, the glycolipid has been labelled with the ^{35}S-SO_4 and free sulfate has left the testis. Almost all the ^{35}S is in the SGG and the newly labelled SGG has left the Golgi apparatus and is in its new compartment, thus, in future experiments counting ^{35}S should accurately represent the labelled SGG.

The method of showing that the isotope did infact label the SGG was to follow the distribution of ^{35}S during the extraction and purification of SGG. Table III-3 shows the results of three experiments where the SGG was extracted and purified and then this purified SGG was counted for SGG (see Section II-6).

The results in this table show that approximately ninety percent of the ^{35}S labelled material is SGG. The ten percent of the ^{35}S that was not in the SGG is probably present in other sulfated compounds or due to losses of SGG during the extraction and purification procedure itself.

Table III-4 shows the results of similar experiments in which the SGG was extracted from the final cell suspension rather than from a testicular homogenate.

The results in this table again show that 80 to 95% ninety percent of what is counted as ^{35}S is in fact labelled SGG. Thus in future experiments ^{35}S counting was considered an accurate representation of labelled SGG.

A number of experiments were carried out where the labelled SGG and protein were followed through the procedure of the cell suspension preparation. The results of these experiments are shown in table III-5.

The results shown here are typical of the cell suspension that were normally used in the later experiments. Normally the cell suspension contained about 9,000 to 12,000 DPM of ^{35}S, approximately 10.0 mgs of protein, 1.2 to 1.5 X 10^9 cells with a viability of ninety-six to ninety-eight percent. The specific activity of the labelled SGG in the final cell suspension is approximately equal to the specific activity of the labelled SGG in the initial cell suspension.

The method used to homogenize the cells in suspension was decided upon after a number of parameters were examined.

Table III-3

SGG EXTRACTION AND PURIFICATION FROM TISSUE HOMOGENATES

Experiment #	Fraction	^{35}S (DPM)	% of ^{35}S as SGG
1	total testis	84,551	-
	pure SGG	76,941	91.0
2	total testis	69,122	-
	pure SGG	60,150	87.1
3	total testis	113,124	-
	pure SGG	110,169	97.4

Total ^{35}S counts were determined by counting an aliquot of the starting tissue homogenate from which the extraction was carried out. The quantity of the isotope for the pure lipid was determined by counting the scrapings from TLC plates. These experiments used the testis of one adult rat, injected twenty-four hours prior to their removal from the rat.

Table III-4

SGG EXTRACTION AND PURIFICATION FROM CELL SUSPENSION HOMOGENATES

Experiment #	Fraction	^{35}S (DPM)	% of ^{35}S as SGG
1	total suspension	8,083	-
	pure SGG	6,519	80.7
2	total suspension	11,754	-
	pure SGG	11,166	95.0
3	total suspension	10,433	-
	pure SGG	9,223	88.4

Total ^{35}S counts were determined from an aliquot of the startling homogenate of the cell suspension from which the extraction was carried out. The quantity of the isotope for the pure SGG was determined by counting an aliquot of the acetone batch fraction from the silicic acid column. The experiment used the testis of two, 29 day old rats, injected twenty-four hours prior to their removal from the rats.

Table III-5

LABELLED SGG YIELD IN CELL SUSPENSION

	Initial Suspension	Final Suspension
^{35}S (DPM)	152,580	9,360
protein (mg)	131.2	8.7
yield of ^{35}S (%)	100.0	6.10
yield of protein (%)	100.0	6.1
specific activity (DPM/mg)	1,160	1,080
total cells	2.21×10^9	1.14×10^9
percent viability	52.3	97.8

The results in this table represent one experiment of a number that were done where four rats were used to prepare a cell suspension. Cells were counted and their viability was determined by their exclusion of trypan blue. Aliquots were taken for isotope and protein determinations. The initial suspension is that where the testes are chopped and suspended in buffer. The final suspension is the resultant cell suspension upon which surface labelling is carried out or is homogenized.

(III-3) STUDIES ON THE DISTRIBUTION PATTERNS OF LABELLED SGG VERSUS THE PATTERNS OF THE ENZYME MARKERS

In order to determine the density distribution pattern for the ^{35}S-SGG, normal cell suspensions were prepared, homogenized and layered on linear continuous sucrose gradients. These gradients were centrifuged for varying lengths of time; two, four, eight, twelve, sixteen and twenty-two hours. Fractions from the gradients were analysed for their ^{35}S and protein content. The gradients centrifuged for two, four, eight and twelve hours did not show the ^{35}S-SGG at its equilibrium density. The patterns for the ^{35}S-SGG and protein changed as the length of centrifugation was changed. Centrifugation for sixteen and twenty-two hours gave identical patterns; twenty-two hours was chosen for convenience.

The pattern for the ^{35}S-SGG shown in figure III-1 was typical of all experiments that were done. In all gradients where enzyme markers were assayed, ^{35}S and protein were also determined. Both the SGG and protein are distributed throughout the gradients in an uneven manner. There is a symmetrical, broad peak of the SGG pattern with its density at 1.13 g cm^{-3}. This pattern was very consistent with less than $\pm$ 0.01 g cm^{-3} variation in the peak fraction density over all experiments that were done. The protein pattern is composed of two peaks. There is a large narrow peak at the top of the gradient,

the lower density values towards the top and the higher value towards the bottom, with its density at 1.05 g cm^{-3} and a broad flat peak at 1.18 g cm^{-3}. This and all further experiments were repeated at least three times and the measurements were done in duplicate.

Another experiment was performed to ensure that when counting ^{35}S, I was correctly assuming that I was examining the labelled SGG. In this experiment a gradient was centrifuged with the supernatant of the homogenate of a normally prepared cell suspension layered on top. Twenty, 1.0 ml fractions were collected and the fractions between the densities of 1.10 and 1.15 g cm^{-3} were pooled. The combined fractions had a DPM of 4,120. The testis of one rat, wet weight of 0.46 gms, was added to the mixture to act as a carrier for the small amount of lipid in the fractions from the gradient. The purified SGG (from the acetone fraction of the silicic acid column) had a DPM of ^{35}S of 3,640 which is 88.3 percent of the total counts present in the pooled fractions. Thus, again, the idea that counting ^{35}S is sufficient to examine labelled SGG is validated.

Figures III-2 to III-8 represent the experiments where the supernatants of cell suspension homogenates were prepared and centrifuged in the same manner as in figure III-1 (see Sections II-3 and II-4).

Fractions from the gradients were assayed for the ^{35}S-SGG and protein along with various enzyme markers in

order to determine the equilibrium density distribution patterns of the various cellular compartments. Figure III-2 and figure III-3 represent the distribution patterns for alkaline phosphatase and 5'-nucleotidase respectively. Figure III-4 represents the distribution pattern for acid phosphatase while figure III-5 and figure III-6 represent the equilibrium density distribution pattern of UDP-galactosyltransferase and succinate cytochrome C reductase respectively. Figure III-7 and Figure III-8 represent the density distribution patterns of D-glucose-6-phosphatase and NADPH cytochrome C reductase respectively.

The equilibrium density distribution patterns for alkaline phosphatase and 5'-nucleotidase are well defined, distinct and very similar. They both have one symmetrical peak at 1.13 g cm^{-3}. The density distribution patterns for these two enzymes are identical to the patterns for the ^{35}S-SGG, in their shape and in their average median density of the peaks at 1.13 g cm^{-3}, shown on the same figures which immediately suggests the plasma membrane as the location for the SGG.

The equilibrium density distribution pattern of acid phosphatase has one symmetrical peak at 1.16 g cm^{-3}. The distribution pattern for this enzyme is different from the pattern for the ^{35}S-SGG in that the peak fraction densities of the patterns do not coincide. The pattern is also different from the patterns for the enzymes; alkaline phosphatase and 5'-nucleotidase.

The equilibrium density distribution pattern for UDP-galactosyltransferase has one symmetrical peak at 1.14 g cm^{-3}. This pattern is similar in shape to the pattern of the ^{35}S-SGG but different in that the peak fraction densities of the two patterns are not the same. The pattern for this enzyme is also different from the distribution of the other enzyme markers. An important point to note here is that, though the differences between the distribution patterns of SGG and the Golgi apparatus are small, they are consistent over a number of experiments.

The density distribution pattern of succinate cytochrome C reductase has a density at 1.18 g cm^{-3}. This pattern is different from the distribution patterns for the other enzymes that have been assayed, as well as being different from the distribution pattern for the ^{35}S-SGG and while it corresponds in its peak density to the lower density peak of protein at 1.18 g cm^{-3} the shapes of these two patterns are quite different.

As can be seen, all of the equilibrium density distribution patterns of the enzymes that have been assayed for so far, have distinct and well defined shapes with a narrow and limited range of activity. However, this does not follow for the two endoplasmic reticulum markers, D-glucose-6-phosphatase and NADPH cytochrome C reductase. Figures III-7 and III-8 for these two enzymes respectively, show that there are multiple broad peaks in their patterns. The enzymes' activities are spread throughout the gradients

in a relatively even manner. Their activities in all parts of these gradients are quite low with no more than a two-fold increase over the basal level of activity. Thus, while these patterns are very different from all the other patterns, they correspond well to each other and were therefore taken as accurate representations of the endoplasmic reticulum on these gradients.

Figure III-1

^{35}S-SGG DENSITY DISTRIBUTION PATTERN

In this experiment the supernatant from a homogenized cell suspension was prepared and centrifuged as described in the Material and Methods (section II-3, preparation of the cell suspension). After centrifugation the one ml fraction were assayed for ^{35}S-SGG (●——●) and protein (▲——▲). The results are plotted as specific activities versus density.

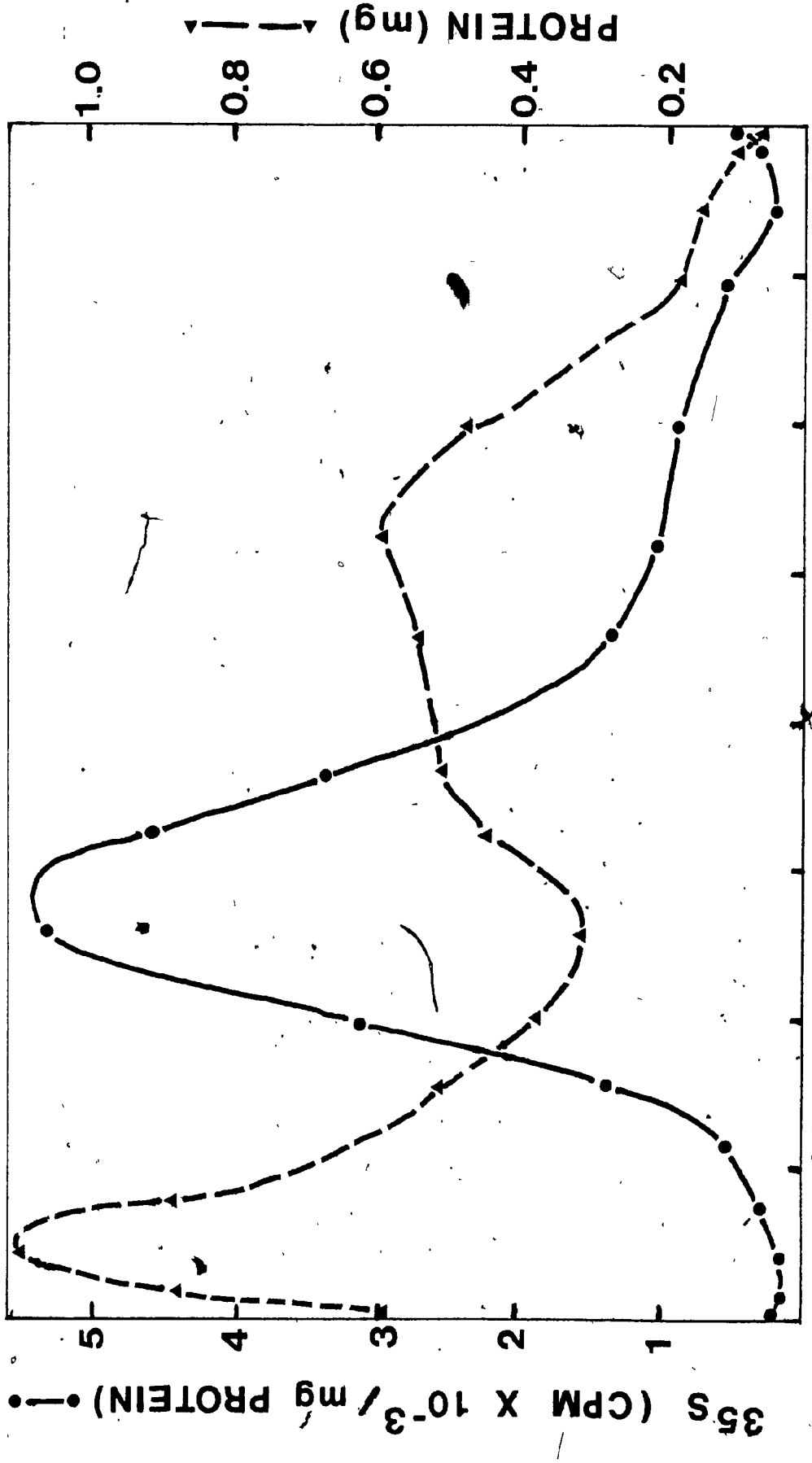


Figure III-1 ^{35}S-SGG DENSITY DISTRIBUTION PATTERN

Figure III-2

DENSITY DISTRIBUTION PATTERN OF ALKALINE PHOSPHATASE

The supernatant from a homogenized cell suspension was layered on a sucrose gradient and centrifuged for 22 hours. Aliquots of fractions from the gradient were assayed for ^{35}S-SGG (●——●) protein, and alkaline phosphatase (▲——▲) in the manner previously described. Results are plotted as specific activities versus density.

Figure III-2 DENSITY DISTRIBUTION PATTERN OF ALKALINE PHOSPHATASE

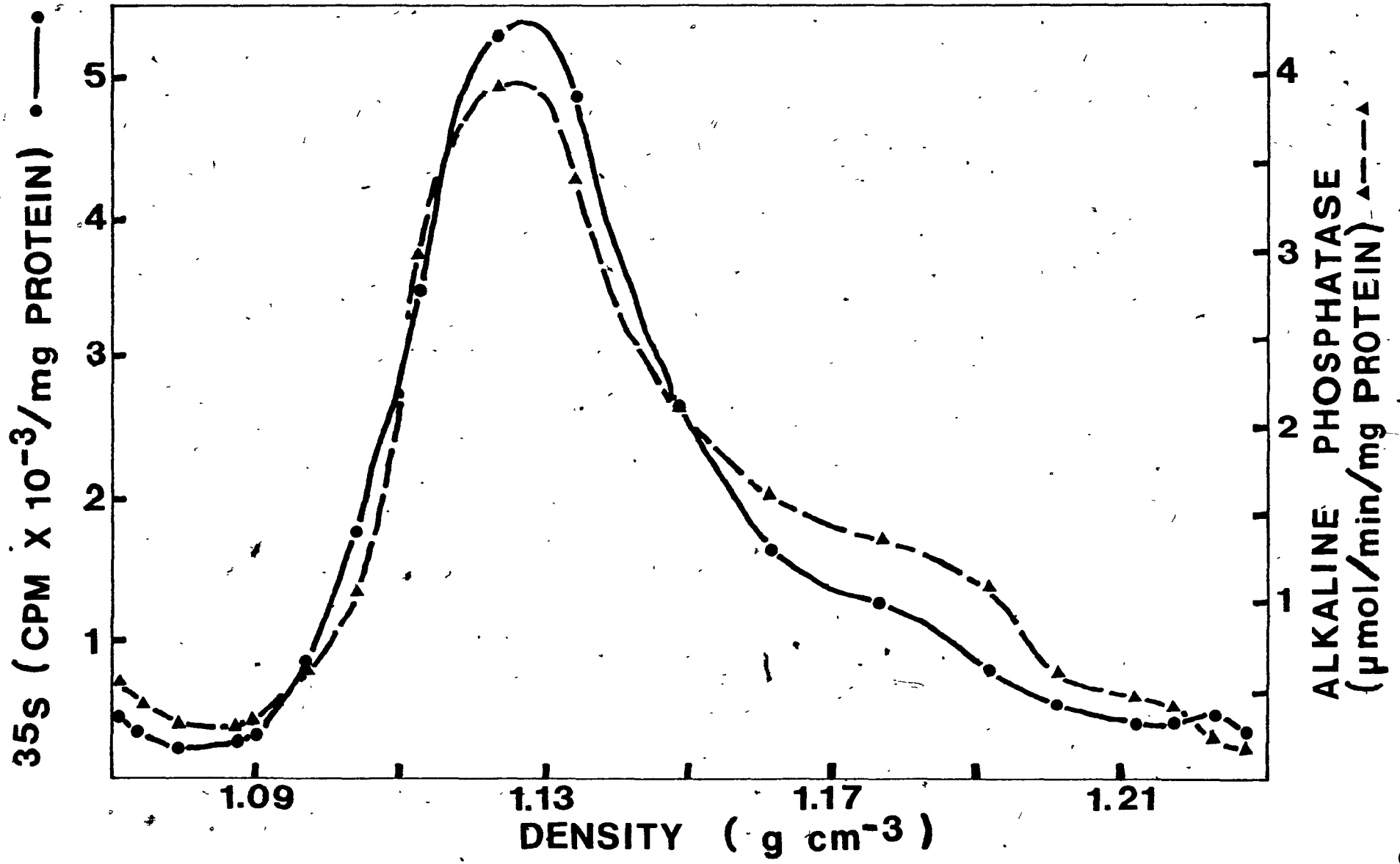


Figure III-3

DENSITY DISTRIBUTION PATTERN OF 5'-NUCLEOTIDASE

The distribution of 5'-nucleotidase (▲——▲) on the same gradient as shown in Figure III-2. The distribution of ^{35}S-SGG (●——●) is shown again. Results are plotted as specific activity versus density.

Figure III-3 DENSITY DISTRIBUTION PATTERN OF 5'-NUCLEOTIDASE

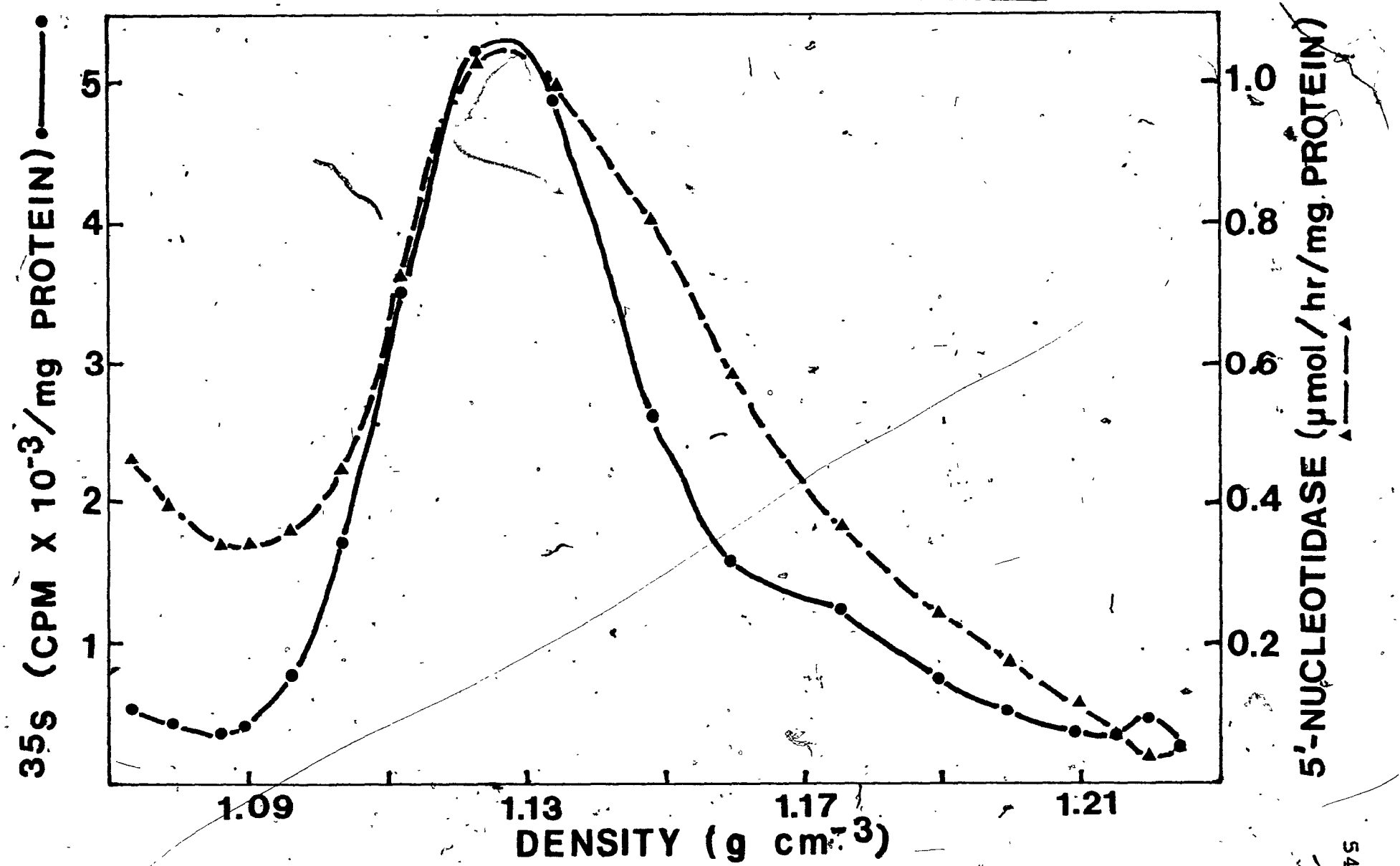


Figure III-4

DENSITY DISTRIBUTION PATTERN FOR ACID PHOSPHATASE

The supernatant from a homogenized cell suspension was prepared and centrifuged as described in Materials and Methods, (preparation of the cell suspension). After centrifugation, the one ml fractions were assayed for protein, ^{35}S-SGG (●——●) and acid phosphatase (▲——▲). The results are plotted as specific activities versus density.

Figure III-4 DENSITY DISTRIBUTION PATTERN OF ACID PHOSPHATASE

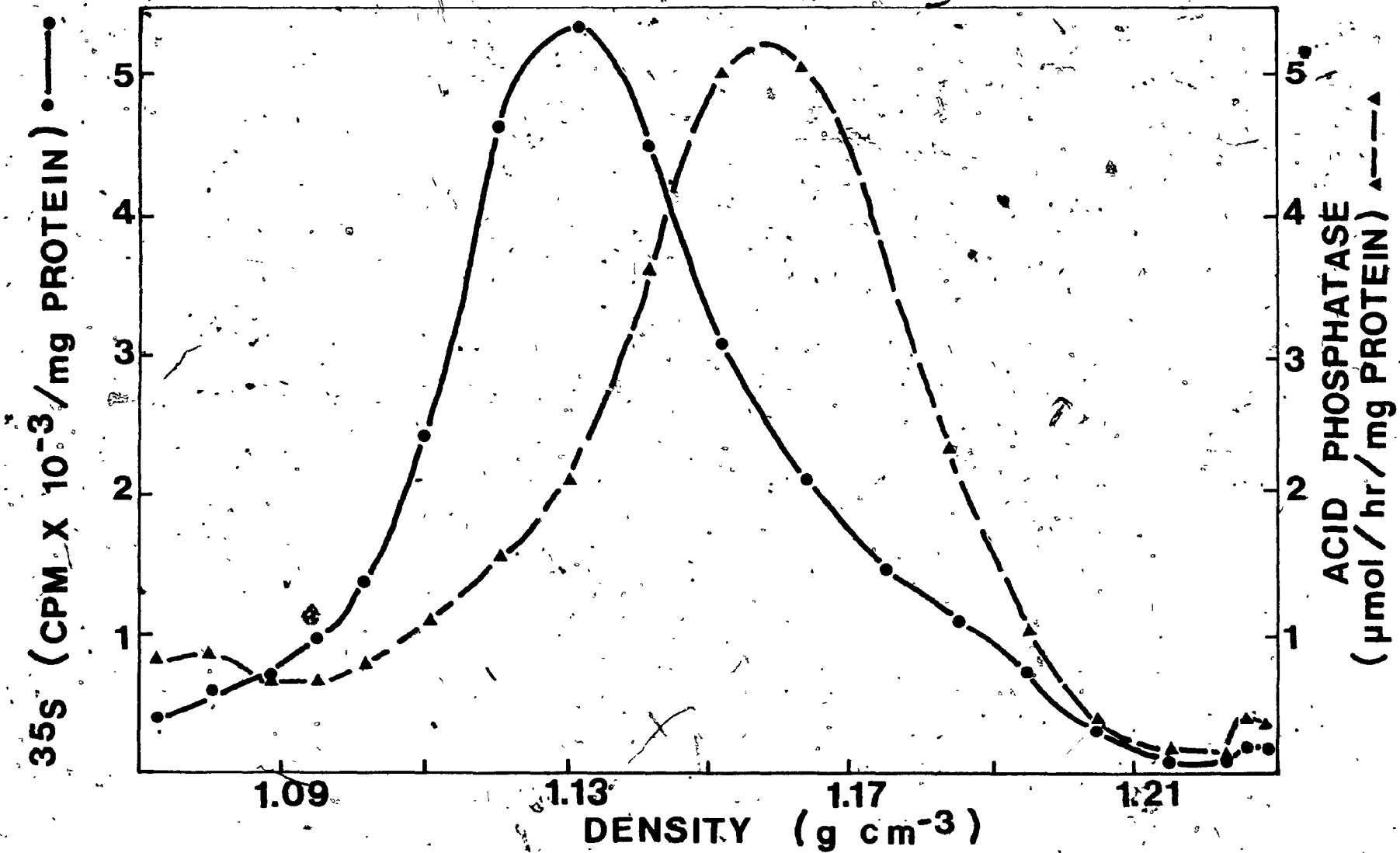


Figure III-5

DENSITY DISTRIBUTION PATTERN OF

UDP-GALACTOSYLTRANSFERASE

The supernatant from a homogenized cell suspension was prepared and centrifuged as described in Materials and Materials. After centrifiguation, the one ml fractions were assayed for protein ^{35}S-SGG (●——●) and UDP-galactosyltransferase (▲——▲). The results are plotted as specific activities versus density.

Figure III-5 DENSITY DISTRIBUTION PATTERN OF UDP-GALACTOSYLTRANSFERASE

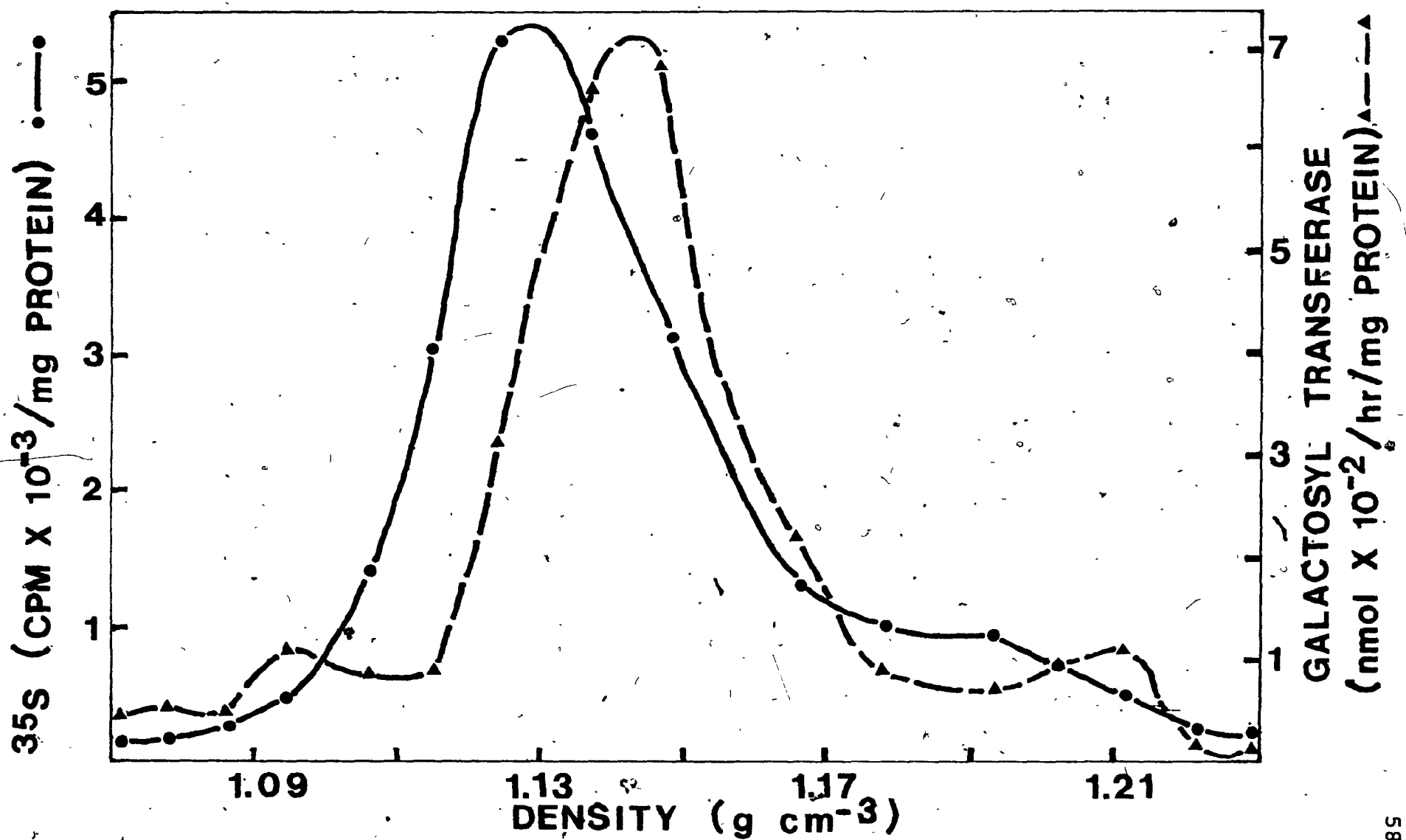


Figure III-6

DENSITY DISTRIBUTION PATTERN OF SUCCINATE CYTOCHROME C REDUCTASE

The supernatant from a homogenized cell suspension was prepared and centrifuged as described in Materials and Methods. After centrifugation, the one ml fractions were assayed for protein ^{35}S-SGG (●——●) and the succinate cytochrome C reductase system (▲——▲). Results are plotted as specific activity versus density.

Figure III-6 DENSITY DISTRIBUTION PATTERN OF SUCCINATE CYTOCHROME C REDUCTASE

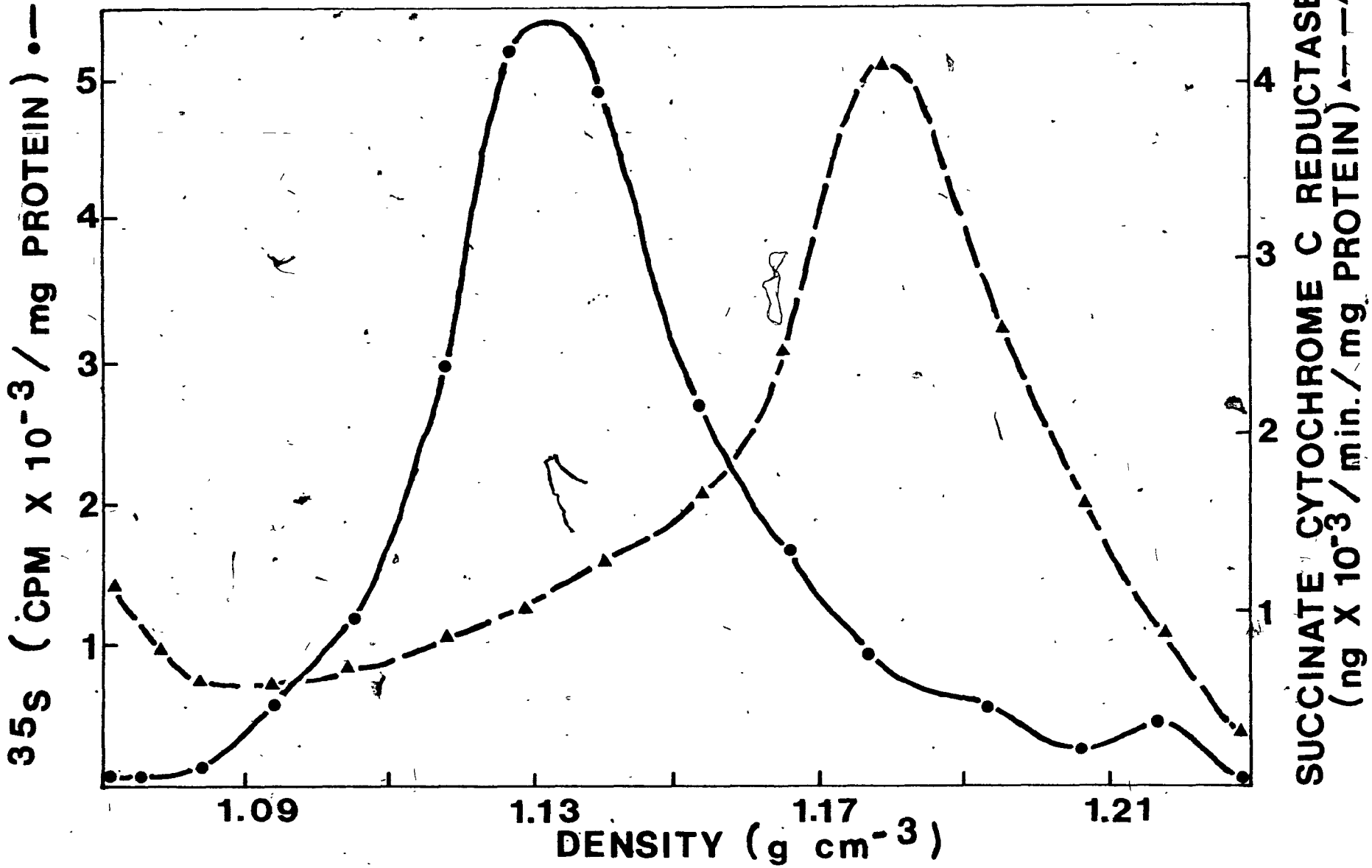


Figure III-7

DENSITY DISTRIBUTION PATTERN OF D-GLUCOSE-6-PHOSPHATASE

The supernatant from a homogenized cell suspension was prepared and centrifuged as described in Materials and Methods. After centrifugation, fractions were assayed for protein, D-glucose-6-phosphatase (▲——▲) and ^{35}S-SGG (●——●). Results are plotted as specific activities versus density.

Figure III-7 DENSITY DISTRIBUTION PATTERN OF D-GLUCOSE-6-PHOSPHATASE

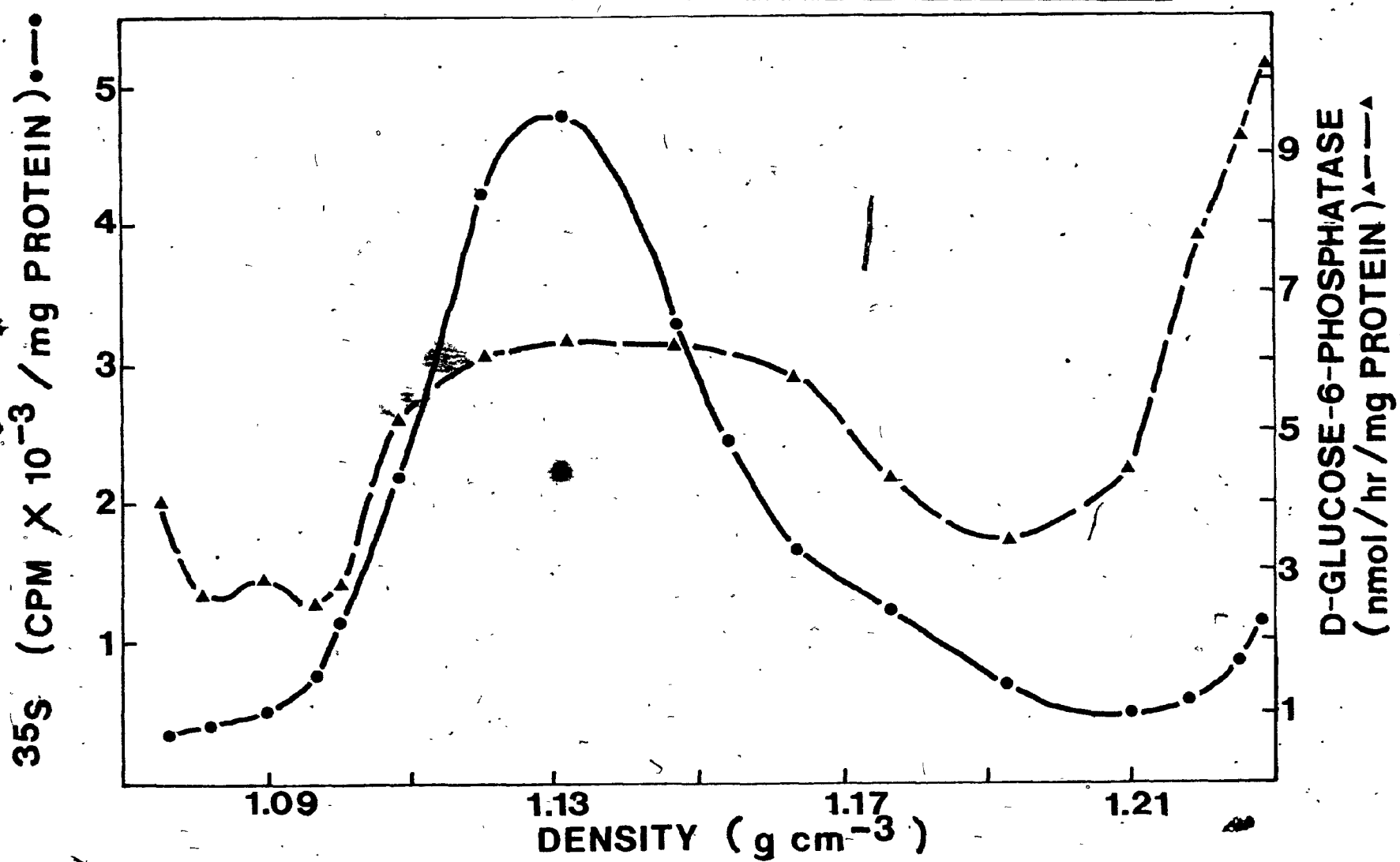
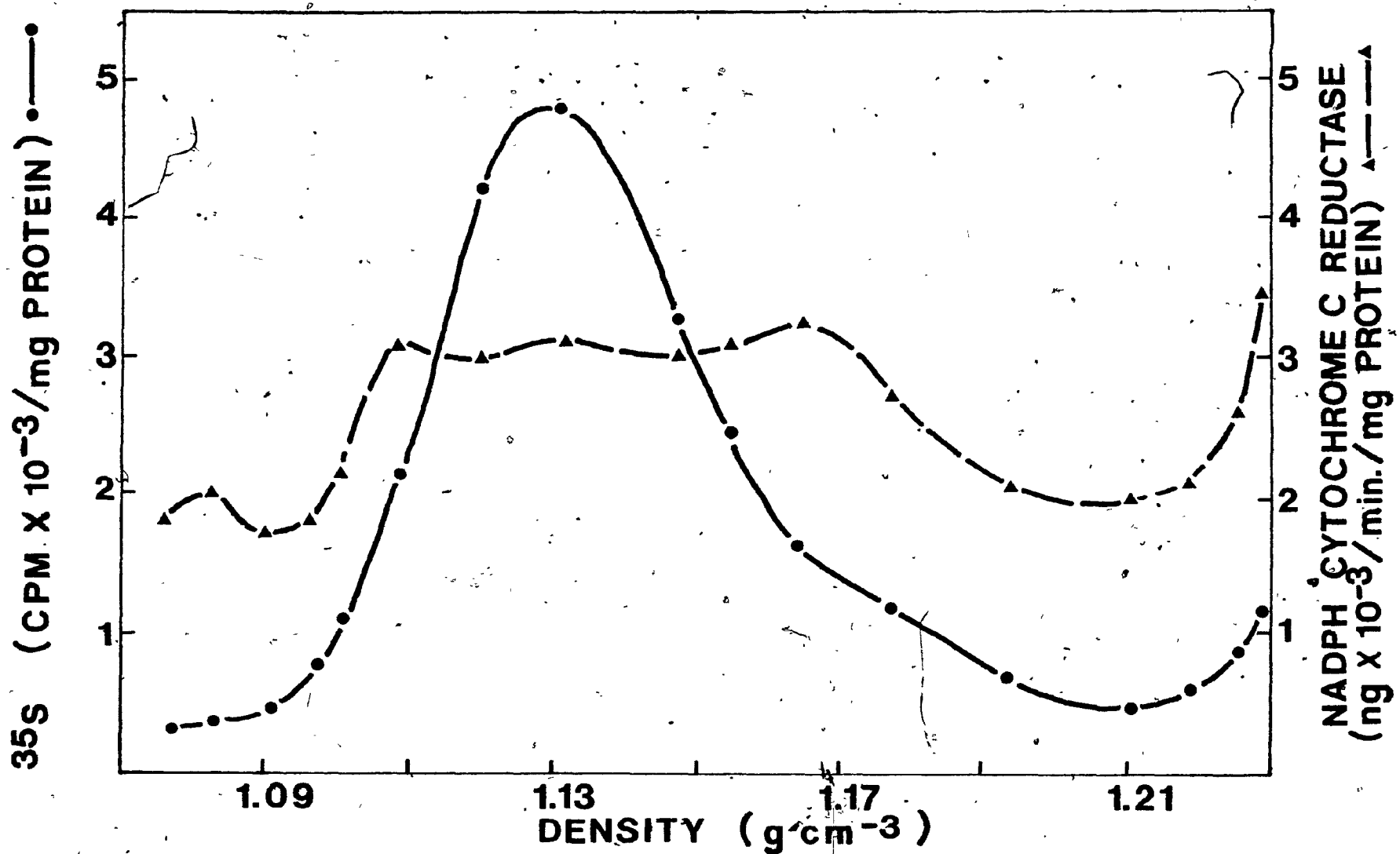


Figure III-8

DENSITY DISTRIBUTION PATTERN OF NADPH CYTOCHROME C REDUCTASE

The supernatant from a homogenized cell suspension was prepared and centrifuged as described in Materials and Methods. After centrifugation, fractions were assayed for protein, NADPH cytochrome C reductase (▲—▲) and ^{35}S-SGG. Results are plotted as specific activities versus density.

Figure III-8 DENSITY DISTRIBUTION PATTERN OF NADPH CYTOCHROME C REDUCTASE



A summary of the results of the equilibrium density distribution patterns of the sucrose gradients can be seen in table III-6.

The results show that the equilibrium density distribution patterns for the ^{35}S-SGG, alkaline phosphatase and 5'-nucleotidase are identical. There are differences in these patterns from the patterns of the other enzyme markers and though these differences, in some cases, may be small, they were consistent over all the experiments that were done. Thus, the data indicate that the location of the ^{35}S-SGG is the plasma membrane.

Table III-6

PEAK DENSITIES FOR THE EQUILIBRIUM DISTRIBUTION PATTERNS

Enzyme or other marker	Peak density ($g\ cm^{-3}$)	Figure #
alkaline phosphatase	1.13	III-2
5'-nucleotidase	1.13	III-3
acid phosphatase	1.16	III-4
gal. transferase	1.14	III-5
succ. cyt. C reductase	1.18	III-6
D-glu.-6-phosphatase	1.11 to 1.17	III-7
NADPH cyt. C reductase	1.11,1.13,1.17	III-8
^{35}S-SGG	1.13	III-1
protein	1.05,1.18	III-1

(III-4) STUDIES ON THE USE OF FLUORESCAMINE AS A SURFACE LABEL

An attempt was made to use 4-phenyl-spirofuran-2(H), 1'-phthalan-3,3'-dione (fluorescamine) as an agent to label the outside surface of the cell in suspension.[53] The experiments entailed the addition of fluorescamine dissolved in acetone to the cell suspension (section II-5). In all experiments the viability of the cell suspension never decreased more than two percent during the labelling procedure.

The labelling of ovalbumin was studied in an effort to standardize the use of fluorescamine. Fluorescamine was added to a series of solutions containing various concentrations of ovalbumin; fluorescence was measured and was found to be linear with protein concentration. One of these samples was then measured for its fluorescence over a period of thirty hours. The intensity decreased with time; after thirty hours there was a fifteen percent reduction of intensity.

A number of other parameters were also examined. The presence of sucrose, tris and triton X-100 had no effect on fluorescent intensity. The addition of a homogenate of a cell suspension caused an increase in fluorescence of labelled ovalbumin but when homogenate was added in the presence of triton X-100 there was no change. For that reason, trition X-100 was added to all samples where

fluorescence intensity was to be measured. Thus, at this point, I felt that I could use fluorescamine in a quantitative manner.

Cells in suspension were labelled with fluorescamine, homogenized and then the supernatant of the homogenate was layered on a sucrose gradient. Figure III-9 shows the results of the equilibrium density distribution pattern established during centrifugation. Fluorescamine shows a well defined, distinct pattern. There are two peaks, one at 1.13 g cm^{-3}, matching the peak densities of ^{35}S, alkaline phosphatase and 5'-nucleotidase and the other at 1.18 g cm^{-3} matching the lower density peak of protein and the peak density of succinate cytochrome C reductase.

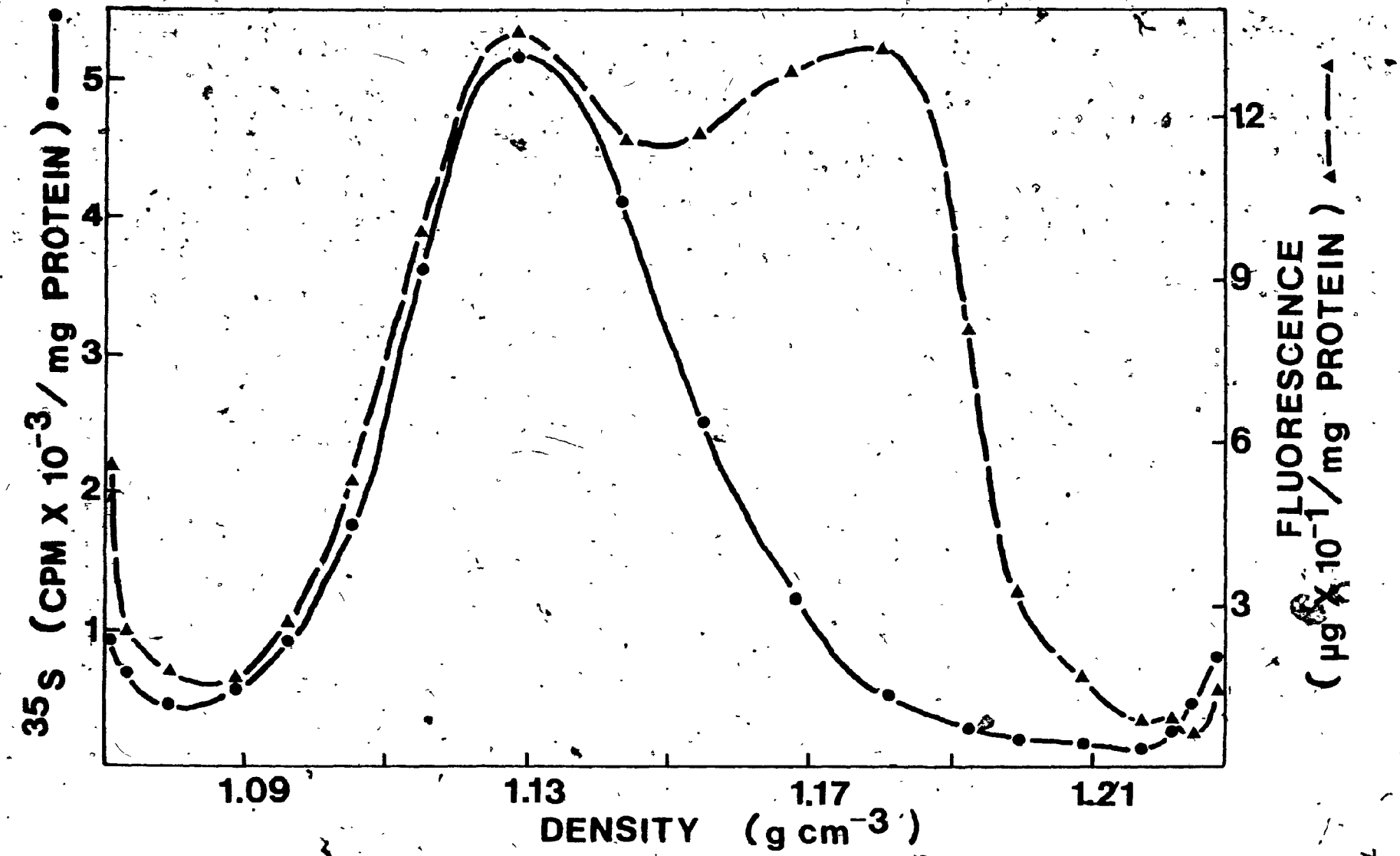
The pattern for the fluorescence was not what was expected as there were two peaks. If the fluorescamine was labelling only the outside surface of the plasma membrane of these cells, it would be expected that only one cellular compartment would be labelled and not two. I felt that by reducing the amount of fluorescamine used in the labelling procedure, the chances of the reaction of fluorescamine with non-specific sites, not on the surface of the cell might be reduced. For this reason, the amount of fluorescamine used in the reaction was reduced from 1,000 µg to 500 µg to 250 µg to 125 µg. Below 125 µg of fluorescamine used, fluorescence intensity was too low to be measured accurately by the spectro-

Figure III-9

DENSITY GRADIENT OF FLUORESCAMINE TREATED CELLS

Cells were labelled with 1000 µg of fluorescamine. The supernatant of this homogenized suspension was then layered on the sucrose gradient. After centrifugation fractions were collected and aliquots of these fractions were analysed for their fluorescence intensity (▲——▲), ^{35}S-SGG (●——●) and protein. Results are plotted as specific activities versus density.

Figure III-9 DENSITY DISTRIBUTION PATTERN FOR FLUORESCAMINE



photofluorimeter. There were no changes in the fluorescence patterns at any of these concentrations. 125 ug of fluorescamine was used in further experiments simply because of the expense of the reagent.

A number of experiments were performed in an attempt to learn more about the two peaks; why there are two and to see if there is any specific labelling of the plasma membrane. In one experiment, a cellular suspension that was not treated with trypsin was reacted with fluorescamine. This was done to see if trypsin was reducing the specific labelling sites on the surfaces of the cell and thus causing more label on inner sites. The cells were homogenized and a supernatant of this homogenate was layered on a linear continuous sucrose gradient. Aliquots of the fractions were analysed in the usual manner. Figure III-10 shows the ^{35}S and protein patterns form this experiment while figure III-11 shows the ^{35}S and fluorescence patterns.

There are a number of changes from the normal that can be observed from the results of this experiment. The shape of the protein pattern remains virtually unchanged though there is a greater amount of protein on the gradient than is usual. This was expected as there was no trypsin present to remove any of the protein. The pattern for the ^{35}S has changed. The peak density has moved from 1.13 to 1.15 $g \cdot cm^{-3}$. Thus, there is a significant change in the density of the compartment in which the SGG is

located when trypsin is used in the preparation of the cell suspension. This change in density of the SGG was shown to be consistent when trypsin was not used to prepare the cell suspension. The fluorescence pattern also changed. The lower density peak shifted along with the SGG from 1.13 to 1.15 g cm^{-3} while the higher density peak at 1.18 g cm^{-3} remains unchanged. These changes are significant because they add more support to the idea that the SGG is located on the plasma membrane and that the lower density fluorescamine peak is a result of some specific labelling of the plasma membrane. The reasons for these conclusions will be explained in the Discussion.

There were quite a number of other experiments that were done in order to learn more about the fluorescamine labelling. In one experiment a cell suspension was layered on Ficoll-Paque (Phamacia) in order to remove the debris and dead cells without the use of trypsin. The viability after such treatment was 95.6 percent. The fluorescence pattern was unchanged from the pattern in figure III-11. In another experiment a normal gradient was prepared from a fluorescamine labelled cell suspension. Fractions between the densities of 1.10 and 1.15 g cm^{-3} were pooled, brought to the equivalent of fifty percent sucrose and used to prepare another gradient. This gradient was centrifuged and aliquots of the fractions analysed for ^{35}S and fluorescamine. The results of this experiment are shown on figure III-12. In another experiment, a

complexed form of fluorescamine was used to label the cells. This form of fluorescamine is a result of complexing fluorescamine with cycloheptaamylose. The result is a water-soluble complex which is supposedly impermeable to the cell. The fluorescence density distribution pattern was identical to the pattern for the uncomplexed fluorescamine.

The results of these experiments were not consistent with the results previously shown. Perhaps the only conclusion that can be drawn from these experiments is that the double fluorescence peak is probably a result of labelling two cellular compartments, at least one of them on the inside of the cell and that this may be a result of either fluorescamine penetration into the cells or labelling of internal sites on debris, free in suspension.

Though the fluorescamine pattern was not what was wanted I felt that it may still have been possible to use this data to support the results of the enzyme markers. Since the higher density peak matches well with the density of the peak for succinate cytochrome C reductase it might have been possible to show that the 1.13 g cm^{-3} peak is a result of some specific labelling of the cell surface. The only ways to answer this question in this system was to do an experiment where cells were labelled before and after they were homogenized. If there was some specificity of surface labelling one would have expected a difference in the two patterns. Cells labelled

before homogenization result in a fluorescence pattern seen in figure III-9, labelling after homogenization should have been different in that there should have been more sites available for labelling. The relative specific fluorescence of the two peaks should have changed with the peak representing the surface labelled material smaller with respect to the other.

Figure III-13 (a) and (b) shows the results of such an experiment. A cell suspension was prepared and then divided in half. One half was treated normally, labelled with fluorescamine, homogenized, layered on a gradient and centrifuged. The results are seen in figure III-13 (a). The other half of the cell suspension was homogenized, then labelled with fluorescamine, layered on a gradient and centrifuged. These results are seen in figure III-13 (b). It is obvious that the two patterns are different with respect to each other. The normally labelled cells show a fluorescence pattern that is typical of all fluorescamine experiments while the cells labelled after homogenization have a typical ^{35}S pattern but a fluorescence pattern that has changed in that the relative heights of the two peaks are different. Before homogenization the relative specific fluorescence of the two peaks is approximately equal while labelling after homogenization causes a lowering of the relative specific fluorescence of the 1.13 $g \cdot cm^{-3}$ peak. Therefore, it was

concluded from this experiment that there is some specific labelling of the surface of the cells.

Figure III-10

^{35}S-SGG DENSITY DISTRIBUTION PATTERN WITHOUT THE USE OF TRYPSIN

After the combined supernatants from the DNase incubations in the cell suspension procedure were centrifuged, the pellet was resuspensed in the homogenizing buffer and the homogenate supernatant layered on a gradient. Trypsin was not used. Fractions from the gradient centrifugation were assayed for the ^{35}S-SGG and protein. (●—●)denotes the ^{35}S-SGG, (▲—▲)denotes protein.

Figure III-10 ^{35}S-SGG DENSITY PATTERN WITHOUT THE USE OF TRYPSIN

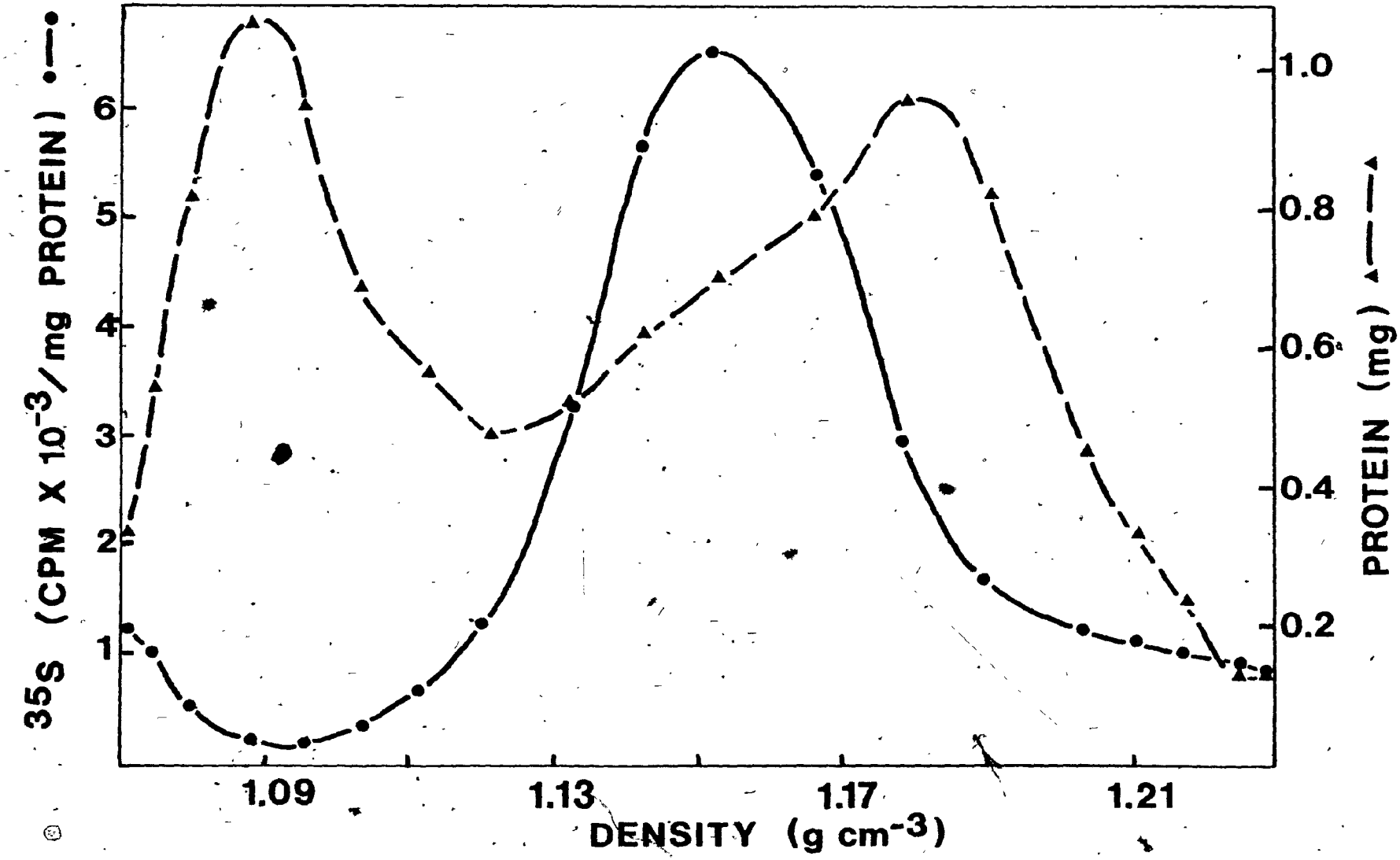


Figure III-11

DENSITY DISTRIBUTION PATTERN OF FLUORESCAMINE TREATED NON-TRYPSINIZED CELLS

In this experiment cells were resuspended and then treated with 125 ug of fluorescamine. The suspension was centrifuged and then homogenized. The homogenate supernatant was layered on a sucrose gradient and centrifuged for 22 hours. Aliquots of fractions from the gradient were analysed for protein, SGG and (●——●) fluorescence intensity (▲——▲). Results are plotted as specific activities versus density.

Figure III-11 DENSITY PATTERN FOR FLUORESCAMINE USING NON-TRYPSINIZED CELLS

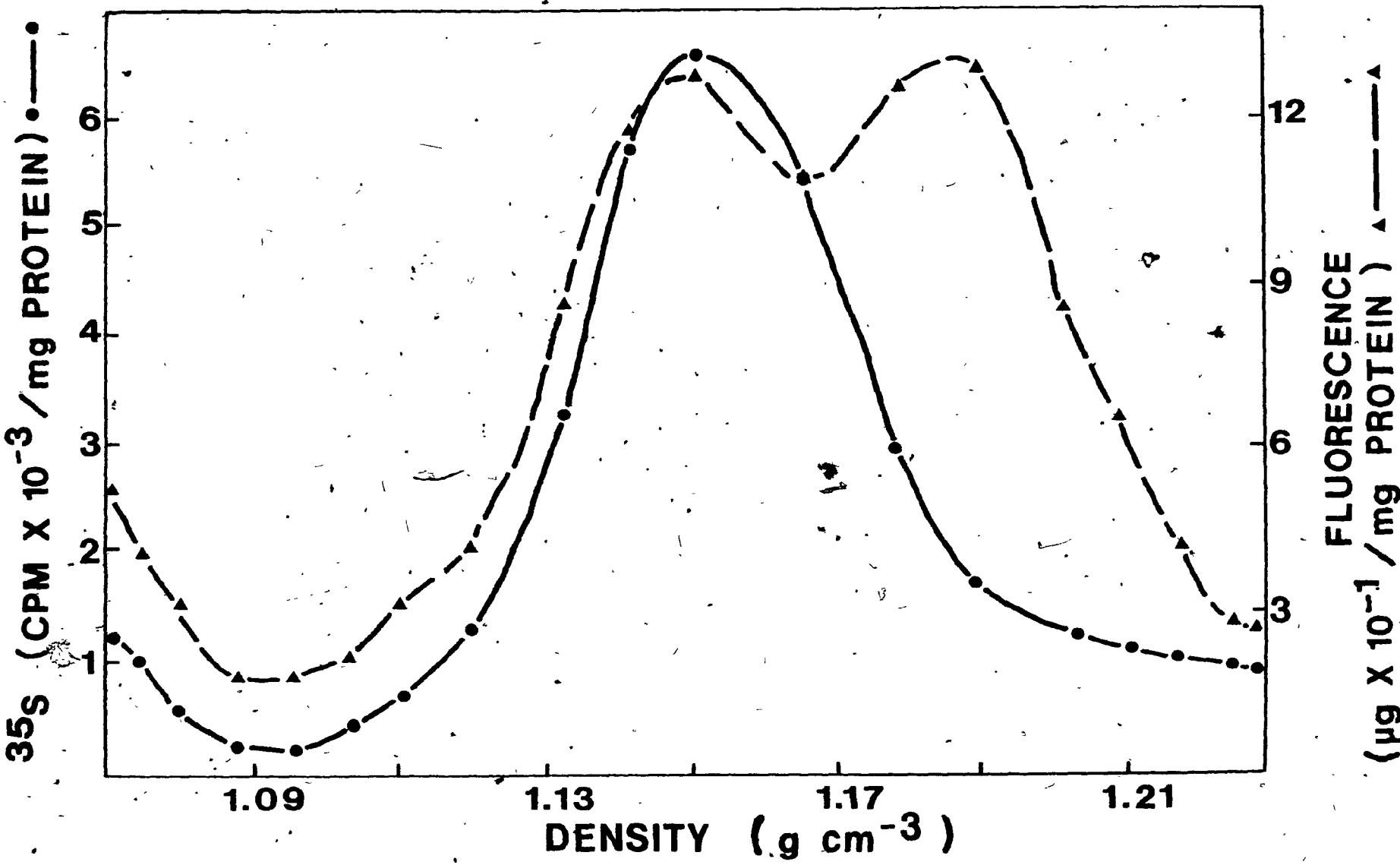


Figure III-12

THE EFFECTS OF RECENTRIFUGATION OF GRADIENT FRACTIONS

In this experiment fractions from a gradient of a fluorescamine labelled cell homogenate were collected and the fractions between 1.11 g cm^{-3} and 1.15 g cm^{-3} were pooled and this mixture was then used to prepare a new gradient. This gradient was centrifuged for 22 hours. Fractions were collected and aliquots from these fractions were assayed for protein, ^{35}S-SGG (●——●) and fluorescence intensity (▲——▲). Results are plotted as specific activities versus density.

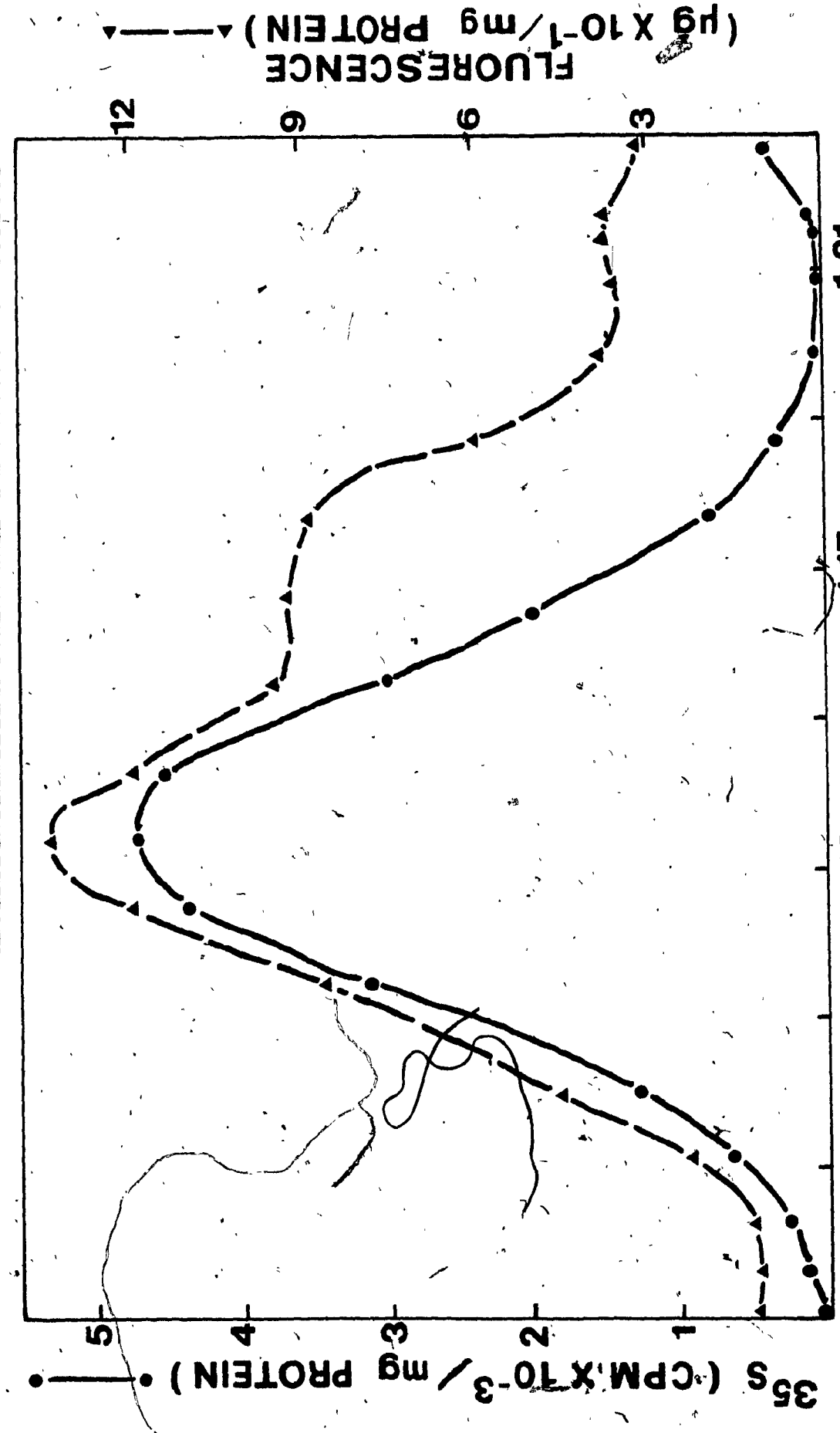


Figure III-12 THE EFFECTS OF RECENTRIFUGATION OF GRADIENT FRACTIONS

Figures III-13 (a) and (b)

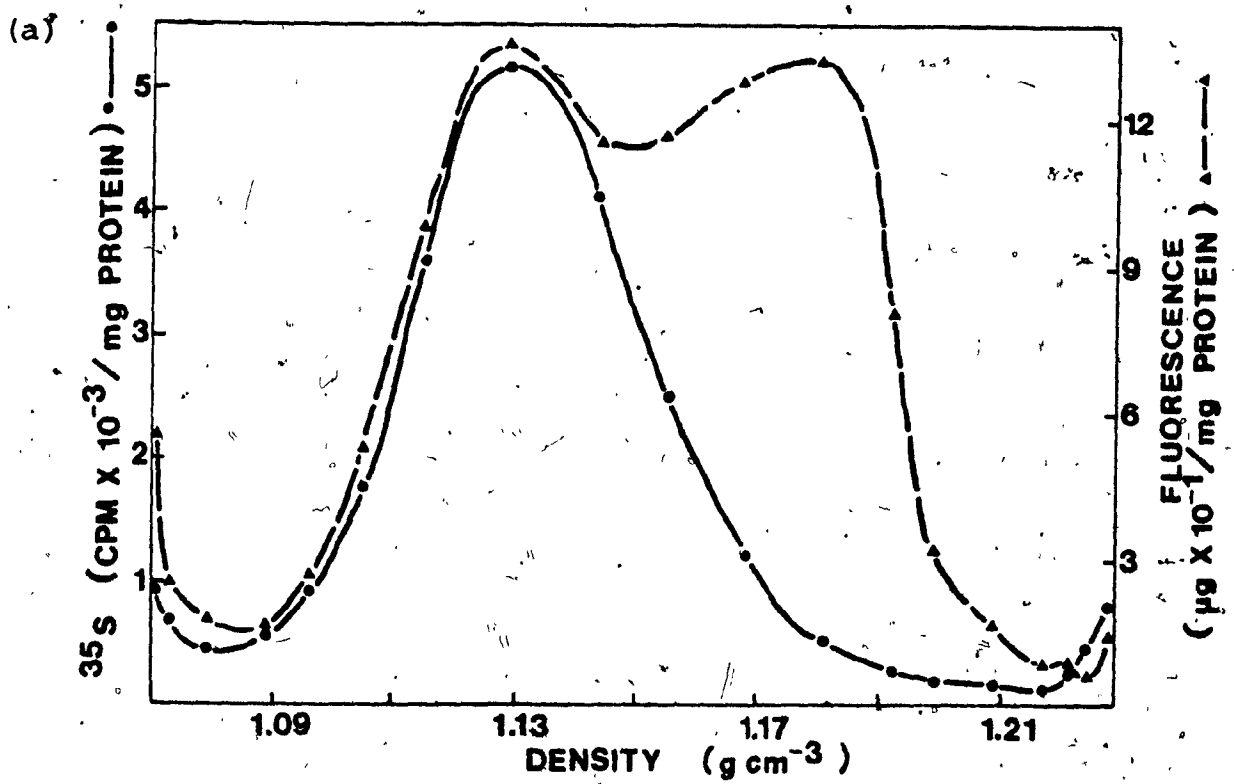
STUDIES ON THE LABELLING OF CELLS BEFORE AND AFTER HOMOGENIZATION

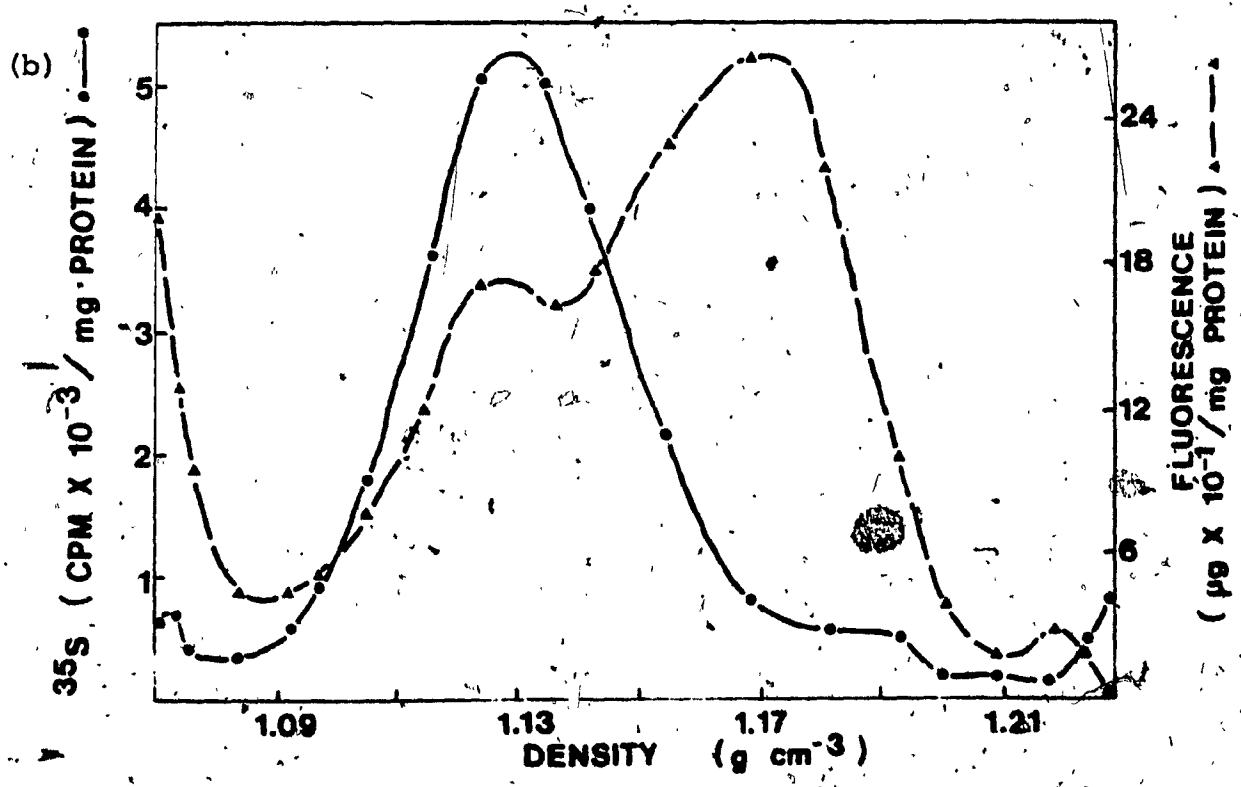
(a) A normally prepared cell suspension was labelled with 125 ug of fluorescamine and layered on a gradient. After centrifugation for twenty-two hours, fractions were collected and assayed for protein, ^{35}S-SGG (●——●) and fluorescence intensity (▲——▲). The results were plotted as specific activities versus density.

(b) This figure represents an experiment that is identical to the experiment in figure III-13 (a) except that the cells were first homogenized and then labelled with fluorescamine. Again (●——●) denotes ^{35}S-SGG and (▲——▲) denotes fluorescence.

Figures III-13 (a) and (b)

STUDIES ON LABELLING BEFORE AND AFTER HOMOGENIZATION





DISCUSSION

The experiments in this thesis were designed in order to determine the identification of the subcellular compartment of sulfogalactoglycerolipid twenty-four hours after its sulfation in the Golgi apparatus. The equilibrium density distribution patterns of the various organelles of the cell and of ^{35}S-SGG were established in the hope that the pattern of one organelle would match that of the SGG and thus establish the location of the SGG. Before any final conclusions can be drawn as to this location, a number of criteria and problems in performing and interpreting the experiments must be examined. Was the starting material heterogenous or homogenous? The original material by nature is heterogeneous but I wanted to work with a specific cell type, the spermatocyte and thus, was it possible that a cell suspension could be prepared that was homogenous with respect to the spermatocyte? Throughout most of the experiments, when counting the ^{35}S, I was assuming that I was looking at the SGG. This criterion had to be established because most of the results of all the experiments were based on the fact that ^{35}S did represent the newly sulfated lipid. In the experiments where the identification of the distribution of the organelles was carried out by the assaying of the marker enzymes, it had to be established that these marker enzymes did represent

those organelles. When surface labelling was used as an additional marker for the plasma membrane it necessitated that the cell suspension be viable such that the label would only come in contact with the surface components of the cells. Once all these criteria were established, then I could draw conclusions as to the subcellular location of SGG.

A cell suspension was prepared but what was this cell suspension made up of? Was the cell suspension homogenous with respect to spermatocytes? Without histological studies I cannot be sure but educated guesses can be made. From the observations using phase contrast light microscopy, it is possible, that the cell suspension contains mostly spermatocytes as they are large, round and have a nucleus that is so large that it is difficult to distinguish it from the rest of the cell. The small cells probably were spermatogonia, erythrocytes and interstitial cells. It is probable that there were also some spermatids but I could not distinguish them from the rest of the cell population. Thus the suspension, heterogenous in nature had the appearance of a homogenous mixture of cells.

It should be realized that I was not the first to prepare a testicular cell suspension; many other workers have done this as well, using a variety of techniques and yielding a variety of results. Most workers prepared cell suspensions in order to isolate and morphologically characterize the different cell types of the testis. Some

workers prepared cell suspensions in order to do metabolic studies or studies such as mine on the spermatogenic cells of the testis. Most workers found that when using immature rats they were able to isolate spermatocyte rich cell suspensions. Using twenty-eight day old rats, Dorrington and Fritz[10] found that eighty-six to ninety-two percent of their cell suspension consisted of spermatocytes. The rest of the suspension was made up of a few spermatogonia, immature spermatids and interstitial cells. They incubated rat tubules in buffer for two hours, constantly shaking to release cells from the tubules; they also used a bovine serum albumin gradient to separate cells from debris. Letts et al[12,30] found that by using a modification of the procedure of Dorrington and Fritz they were able to prepare a cell suspension from twenty-six day old wistar rats that was eighty percent pure in spermatocytes. Thus, it is not unreasonable to say that at this point I had a cell suspension that consisted of anywhere between eighty to ninety percent spermatocytes. Before I could do any of the intended analyses on the fractions from the gradients, it was necessary to be sure that when counting ^{35}S, I was really analysing the newly labelled SGG. The results in tables III-4 and III-5 showed that approximately ninety percent of the ^{35}S that was counted copurified with SGG. There was no evidence for another compound that could account for the remaining ten percent.

This ten percent is probably due to minor sulfated compounds and by incomplete recoveries of the SGG at the various steps of the purification procedure. The experimental error explanation as a source of the ten percent seems reasonable because the value varies quite a bit from experiment to experiment. Thus, at this point in the work, I had a cell suspension of spermatocytes with which I knew I could follow the distribution of newly sulfated SGG as ^{35}S.

One of the interesting results found when determining the recovery of SGG and protein in the cell suspension was that the specific activity of the ^{35}S-SGG in the final cell suspension did not increase over the specific activity in the initial cell suspension (see table III-5). Since the cell type that incorporates the ^{35}S-SO_4 is the spermatocyte and since the suspension was enriched in spermatocytes, the cell suspension should have been enriched in ^{35}S-SGG. The matter was clarified when it was learned that the ^{35}S-SGG is incorporated only into a discrete stage of spermatocyte[30,31] and that this stage probably accounts for only a small portion of the total spermatocyte population. Also, this stage is very early in the development of the spermatocyte and it has been shown that early spermatocytes are poorly released from the tubules[60]. Therefore, even though the cell suspension might be enriched in spermatocytes, it would definitely not be

enriched in that specific stage of spermatocyte and thus, neither would it be enriched in the ^{35}S-SGG.

It can be seen from the first gradient in figure III-1 that I was able to establish the equilibrium density distribution pattern for the ^{35}S-SGG. This pattern is reasonably symmetrical but quite broad. The reason for the broadness is perhaps a result of the polydispersity of the particles. That is, when homogenized, the cell does not break up into a homogenous mixture of equal sized pieces of material. There is a distribution of sizes and densities of particles such that on the continuous isopycnic gradient the particles will distribute in a manner seen for the SGG.[30]

The experiments where the marker enzymes were assayed and their patterns compared to the pattern of SGG were then carried out. The results, seen in figures III-2 through III-8 and table III-6, suggest that the ^{35}S-SGG is located on the plasma membrane. The patterns for both plasma membrane enzyme markers, figures III-2 and III-3 matched the pattern for the newly labelled SGG, while the patterns for the Golgi apparatus, lysosomes, mitochondria and the endoplasmic reticulum, figures III-4 through III-8 respectively, are different. According to deDuve[38] (see introduction) if the distribution pattern for a cellular component matches the pattern for a particular compartment of the cell then component is located in that compartment. "We derive the information not from the properties of

specific fractions believed to approximate a given intracellular component, but from the manner in which properties are distributed over a large number of fractions, which together represent the whole tissue."[39] The results show this to be the case for the SGG and the plamsa membrane.

There are a number of points where the interpretation of the data that have been shown in these figures could be criticized. This critcism is concerned mainly with the enzyme markers themselves. The question arises as to whether they really represent the organelles that I am assuming them to be or are the patterns seen in the figures, artifacts of the gradients and the conditions under which they have been subjected? To answer this question we must be able to compare the data seen in these experiments to the data of other workers.

The data shown for the densities of these marker enzymes match well with the known densities (by the work of other researchers) of the membranes of the organelles which they supposedly represent. The mitochondrial marker succinate cytochrome C reductase in my work had a density of 1.18 g cm^{-3}, this is comparable to the density of 1.20 $\pm$ 0.02 g cm^{-3} that has been established for mitochondria.[61] This difference, though small may it be, could be explained, according to deDuve[34] by the previous history of the mitochondria. That is, the difference could be explained by the differences in the preparation of the tissues.

Microsomal vesicles have been shown to be heterogenous with respect to density according to Rothschild.[61-64] Smooth endoplasmic reticulum vesicles display densities between 1.06 and 1.23 g cm^{-3}. Rough endoplasmic reticulum vesicles have densities that vary between 1.18 and 1.26 g cm^{-3}. It has been debated in the literature whether smooth endoplasmic reticulum is heterogenous with respect to its enzyme profiles as a function of density.[37,65] In the results seen on figures III-7 and III-8 that the material on these gradients are heterogeneous with respect to density. The marker enzymes activities are spread through the gradient and this could only be explained by the heterogeneity of endoplasmic reticulum vesicular density. It is possible that there is a heterogenous mixture of vesicles containing ribosomes to varying degrees.

The densities of the lysosomal and Golgi marker enzymes also match well with the known densities of lysosomes and the Golgi apparatus that have been derived from other tissues. Lysosomes have been shown to have densities in various tissues of 1.16 to 1.18 g cm^{-3} [66], while the Golgi apparatus has been shown to have a density in the range of 1.13 to 1.14 g cm^{-3}.[41,67] As can be seen and as was expected, the density of the Golgi marker is different from the plasma membrane markers, 1.14 as compared to 1.13 g cm^{-3}. It can also be seen that the SGG follows the plasma membrane markers and not the Golgi apparatus marker. Thus, while not proving it conclusively

because of the great overlap of the two profiles, the data support the idea that the SGG, twenty-four hours after its sulfation, is not in the Golgi apparatus.

The plasma membrane enzyme markers shown in figures III-2 and III-3 have densities that could well be compared to the densities known for the plasma membrane. The procedure involved in homogenizing the cells (hypotonic buffer and vigorous homogenizing) probably produces small plasma membrane vesicles. Neville[61] states that small plasma membrane vesicles have densities between 1.12 and 1.16 g cm^{-3}. The densities of the plasma membrane markers are at 1.13 g cm^{-3}. If the trypsin treatment is omitted, the density is 1.15 g cm^{-3}, which is still within the range stated by Neville.[61]

Therefore it is obvious that the values for the densities arrived at from the experiments in this thesis are not unreasonable and thus the problem of comparing these results with the results of other workers is non-existent.

Another problem related with the marker enzymes has to do with the two plasma membrane marker enzymes, 5'-nucleotidase and alkaline phosphatase. Alkaline phosphatase is a non-specific enzyme that hydrolyses monophosphate esters. It will hydrolyse p-nitrophenylphosphate and 5'-adenosine monophosphate (the substrate used for 5'-nucleotidase) at very similar rates.[68,69] Both assays were carried out in glycine buffers at alkaline pH

containing magnesium. The major concern then, was that the activities of the enzymes were not being measured separately. Some preliminary experiments were done in order to establish whether or not this fear was valid. These experiments looked at the activities of the enzymes under varying conditions of metal ion concentrations, buffers and pHs. The results of these experiments showed that the hydrolysis of these two substrates responded differently to the different conditions and thus are most likely due to different enzymes.

In summary, the simplest explanation for the data shown by the results of the enzyme marker distribution patterns is that the new location of the SGG in the spermatocyte after its sulfation is the plasma membrane. There will be some doubt, though, as to the interpretation of this data, because, as was stated in the introduction, not all of the enzymes that were used have been shown to be markers for their particular subcellular locations in the testis. Even though they have been extensively shown to be located in these subcellular compartments of other tissues, it has not been shown that they are located in these compartments in the testis. This is what presents the problem. There will always be the doubt about the intrepretation that the SGG is on the plasma membrane because alkaline phosphatase and 5'-nucleotidase might not be located on the plasma membrane of the testicular germinal cells. For this reason another criterion is

needed for the determination of the plasma membrane, independent of the work done or not done on other tissues.

There are a number of methods other than enzyme assays that can be used for the determination of the distribution of plasma membrane. Cholesterol with its primary location in the cell being that of the plasma membrane[70] has been used on a number of occasions as a marker for the plasma membrane. Another method of following the distribution of the plasma membrane is through the use of a surface label. A surface label is a reagent or compound which will react with the outside surface of viable cells and thus "tag" the plasma membrane. The compound may be either radioactive or fluorescent in nature. One of the advantages of using a method such as this to follow the distribution of the plasma membrane is that it is independent of the tissue or the work done on that tissue. Thus, the use of a surface label was chosen to add an additional marker for the plasma membrane. The surface label used was fluorescamine. Fluorescamine was originally designed as an amino acid label.[71] Upon reaction with free amino groups of amino acids and proteins this compound produces a fluorescent product; it is also hydrolysed producing non-fluorescent by-products. The half time for the production of the fluorescent products is approximately 100 milli-seconds while for the non-fluorescent hydrolysis products it is approximately five seconds.[72] Thus, Hawkes et al felt that fluorescamine

would react with all accessible primary amines on the cell surface and that any fluorescamine passing through the membrane would most likely be hydrolysed to non-fluorescent forms. Thus all fluorescent products should be located on the plasma membrane.[53,73]

A number of conditions must be met before the intended goals of a surface label can be achieved.[74] It must bond covalently with some site on the surface of the cell such that the binding of the reagent is not reversible and that it does not then attach to other sites during further work on the material. The cells in suspension must be whole and viable to the point where the reagent cannot enter the cell. If cells are damaged or there is debris floating in suspension, the reagent might enter the cell and react with these previously unexposed sites. Since there are many more internal sites than external sites labelling results may be confusing; there may seem to be a higher degree of specificity for internal sites than for external sites. The conditions of labelling must be such that they are rapid to prevent the reagents penetrating the cell and mild so as not to damage or destroy more cells and thus expose more internal sites for labelling.

Fluorescamine meets a number of these conditions; it reacts covalently with free amino groups; and, the conditions of labelling are such that the procedure is quick and mild to the point where no more than two percent of

the cells are destroyed during the procedure itself. The need of a highly viable cell suspension was another matter. When the testis were removed from the rat, chopped and suspended in buffer, more than half the cells were damaged or destroyed. A procedure had to be established where a highly viable cell suspension could be prepared. The experiments described in the first part of section III, were such an attempt to develop a procedure for the preparation of a cell suspension that yielded maximum viability. The results show that highly viable cell suspensions could be prepared.

If fluorescamine was useful as a surface label, one would expect a single peak on its density distribution profile, for only one cellular compartment should have been labelled. The shape and peak fraction of the profile should be identical to that of the plasma membrane enzyme markers. It is obvious that the results in figure III-9 were not what were expected. There are two peaks on the pattern at 1.13 and 1.18 g cm^{-3}. The two peaks correspond to those of the plasma membrane and mitochondrial enzyme markers respectively. It can be concluded then that fluorescamine is not specifically labelling the surface of the cells in suspension. Thus, from the data in figure III-9 no conclusions can be drawn to support the previous evidence for the SGG to be located on the plasma membrane.

I have shown that fluorescamine is not a very specific surface label for these rat testicular cells. This

conclusion about the usefulness of fluorescamine as a surface label is supported by Cross and Briggs[75] who showed that fluorescamine is not a specific reagent for the cell surface. They labelled slices of tissue from corn coleoptiles and then fractionated the coleoptiles by differential sedimentation or isopycnic equilibrium sedimentation on sucrose gradients. They found that seventy-five percent of the fluorescence was associated with the soluble proteins of the cell, the specific fluorescence was not very high (only 0.7 times as high as the homogenate). They also found that the distribution of fluorescence was similar to the distribution of a number of intracellular enzyme markers, especially cytochrome C oxidase for mitochondria, as well as being similar to the distribution of a plamsa membrane enzyme marker. Other data that they presented, labelling before and after homogenization of cells, strongly suggested to them that fluorescamine was labelling the endoplasmic reticulum. Thus they felt that fluorescamine was not a specific surface label.

There are a number of reasons why fluorescamine is not a specific surface label in this and other systems. Fluorescamine is a highly lipophilic molecule[53] and as such it would be expected to penetrate membranes quickly and thus label internal sites before non-fluorescent hydrolysis products are formed. There is, as well, an excess of sites to label on the inside of the cell over

the sites on the outside of the cell[76] and thus, this "concentration gradient" would be expected to favor diffusion of fluorescamine into the cell and its reaction with the sites therein.[77]

Though I have shown and given reasons for the non-specificity of fluorescamine it might still be possible to use the data accumulated to support the SGG-plasma membrane evidence. I would have to remove the second 1.18 $g \cdot cm^{-3}$ peak by making the fluorescamine more specific for the surface of the cell, explain why the second peak is labelled to such a highly specific extent and identify more clearly the two peaks I already have. In other words, if I could show that fluorescamine has some selectivity for the surface of the cell then I could probably use the results of these experiments to strengthen the SGG-plasma membrane idea.

In the preparation of the cell suspension trypsin was used. Meistrich[60] claims that trypsin will not have any effect on the live cells in the suspension, that it will degrade only those cells which are dead. This does not seem likely, which will soon be explained, and may account for an even greater "concentration gradient" of sites than was expected and thus lessen the specificity of fluorescamine for the outside surface of the cell. Meistrich assumed that trypsin would not affect the live cells because he only observed a decrease in the population of dead cells. As has already been seen, I have had the same

results. But this alone does not preclude the fact that trypsin might be acting on the membrane of live cells in such a way so as to leave the cells intact. It is possible that trypsin acts on the proteins of the cells' surface but does not degrade them totally as they are imbedded in the lipid bylayer. This would cause a reduction of sites on the surface of the cell for the fluorescamine to react with but at the same time leaving the integrity of the cell intact. Many workers have shown that proteins, both soluble and membrane bound, are affected by the action of trypsin.

When Cuatrecasas[78] incubated adipose tissue cells with 30 ug/ml trypsin for fifteen minutes he found that there was as much as an eighty percent reduction in the binding of insulin to the insulin receptor on the plasma membrane of these cells. Other workers[79] showed that there was a visible degradation of the insulin receptor, as seen on SDS polyacrylamide gels, when cells treated with trypsin at concentrations much less than was used for the preparation of the testicular cell suspensions.[82,60] They found that purified plasma membrane of isolated fat cells on SDS polyacrylamide gel electrophoresis had at least eleven major protein components ranging in apparent molecular weights from 168,000 to 22,000. Of these proteins, when the cells were treated with 10 ug/ml of trypsin for five minutes, the 68,000, 54,000 and 42,000 molecular weight peptides were affected. This was seen as

a reduction in the intensity of the bands of protein. This quantity of trypsin also caused the reduction in the ability of the membranes to bind insulin. Thus, trypsin most definitely has an effect on the proteins in the plasma membrane of cells. Trypsin will reduce the amount of protein, therefore the membranes should be lighter than in non-trypsinized cells. Why then should the cells of the testis be any different?

The question as to the effect of trypsin on the spermatocyte cell suspension was answered by the results of the experiment seen in figure III-10. In this experiment the patterns for the SGG and protein are seen when a cell suspension was obtained without the use of trypsin. Comparing this with the pattern of SGG as a result of trypsinization it can be seen that the patterns are not the same; the non-treated material bands at a higher density, 1.15 as compared to 1.13 g cm^{-3}. Thus, trypsin treatment does affect the cells though they may still be alive and viable.

The effect of trypsin on the banding density of SGG gives another indication as to its location. If the cells are whole and viable, then trypsin should not enter them as its molecular weight is approximately 24,000.[80] Thus, the only effect that trypsin should have, is on the proteins of the outside surface of the plasma membrane. When it is seen that SGG changes in density from 1.15 to 1.13 g cm^{-3} by the treatment with trypsin it suggests that the compartment

in which the SGG is located is affected by the trypsin and thus an indication that the SGG is located on the plasma membrane. This should not be taken as a proof as it was not shown whether or not the other cellular components were affected by the trypsin treatment.

The trypsin treatment might be affecting the specificity of surface labelling and it was therefore logical to attempt labelling with a cell suspension that was not treated with trypsin. The results of this experiment seen in figure III-11, show that the density distribution pattern is very similar to the first one in figure III-9. The heavier peak remains unchanged at 1.18 g cm^{-3} while the lighter peak follows the SGG to 1.15 g cm^{-3}. If, as I have already stated, SGG is on the plasma membrane and that the lighter fluorescence peak is due to labelling the plasma membrane, then it follows that this lighter peak would change its position with the changing SGG as a result of the trypsin treatment. If the heavier peak is due to internal sites being labelled, then its position should not and did not change. But what about the relative intensities of the two peaks? This did not change. If the heavier peak is due to fewer specific sites on the surface of the cell, then, without trypsin there should be more sites and thus, more specific labelling on the cell surface but there is, without trypsin, more intracellular debris in suspension, giving rise to more labelling here. Therefore one would not expect very great differences in

the relative intensities of the two peaks and thus the results were not surprising.

The heavier peak of fluorescence was still an enigma and thus the reason for the experiment where the attempt was made to prepare a cell suspension which was free of internal debris floating in suspension without the use of trypsin. If such a cell suspension could be prepared and I could show that the specific intensity of the heavier peak is not reduced then it would be possible to say that the heavier peak was probably due to penetration of fluorescamine into the cell. If it was found that this heavier peak was reduced significantly then it is probable that the peak was a result of labelling debris in suspension. As was stated in the results section, the specific intensity of the heavier peak relative to the lighter peak did not change and thus it is probable that the heavier peak is due to labelling of internal sites by fluorescamine penetration into the cell.

A confirmation of the hypothesis that the heavier peak is due to fluorescamine penetration into the cells is not given by the results of the experiment where a complexed form of fluorescamine was used to label the cells. This complex form has been shown to be non-penetrating[76,77] and thus if I could show that with this non-penetrating form, there was only one peak for the fluorescence profile then the heavy peak in figure III-9, could be said to be due to labelling internal sites of the

living cell, fluorescamine penetration. The results, a pattern identical to figure III-9, and thus not shown, do not show this to be the case; they indicate that they heavy peak is due to labelling of internal material free in suspension. This seems to be contrary to the results of the ficol-paque experiment. It seems then, that a possible explanation for the heavy peak of fluorescence is due to a number of conditions. In the normal cell preparation there probably is free mitochondria in suspension that the fluorescamine is labelling. The fluorescamine is also probably penetrating the cells and this penetration is probably increased by the use of trypsin in the preparation of the cell suspension. Thus, it seems that the fluorescence caused by the reaction of the cells in suspension with fluorescamine is not specific for the surface of the cells.

There is, though, some specific surface labelling as is seen by the results of the experiment in figure III-13 (a) and (b). When the cells are labelled after they have been homogenized as compared to being labelled before homogenization, the specific labelling of the lower density peak is reduced as compared to the higher density peak. This is what was expected; when cells are homogenized and then labelled there should be more labelling of internal sites (a percent of the total labelling) as compared to the labelling of the plasma membrane. Thus, it seems after all, that there is some specific labelling of the

surface of the cell.

Therefore, while I did not have a good surface labelling pattern, there does seem to be somewhat of a profile for the plasma membrane. The 1.13 g cm^{-3} peak probably represents the plasma membrane and the 1.18 g cm^{-3} peak probably represents mitochondria, however it is labelled. Thus, while fluorescamine labelling did not provide the final piece of evidence for the SGG-plasma membrane idea, it did not detract from the idea and it does give some support to it, however small.

In summary, once sulfated, the SGG leaves the Golgi and is found twenty-four hours later on the plasma membrane. This movement of a glycolcosylated compound from the Golgi apparatus and its subsequent location on the plasma membrane is not unique.

More than eighty percent of all cellular glycolipids are located on the plasma membrane.[81,84] Most glycoproteins are also located on the plasma membrane.[85,86] Much research and work has been done in this area of biochemistry. The backbones of glycoproteins and glycolipids are synthesized on or in the endoplasmic reticulum. Once these backbones have been synthesized some of the sugars are incorporated into them.[33] In fact some of these sugars are added on the proteins while it is still "growing".[33] At this point N-acetylglucosamine and D-mannose residues are incorporated into the protein and lipid backbones.[33,87]

The glycoprotein and glycolipid cores then move to the Golgi apparatus. There are a number of theories on how this is accomplished; the transport might be through a continum of endoplasmic reticulum and the Golgi apparatus or through the use of vesicles.[87,88] However, the transport is accomplished, once in the Golgi apparatus the glycoproteins and glycolipids are completed by the addition of more sugars. Here, L-fucose, D-galactose, sialic acids and some N-acetyl-D-glucosamine are incorporated into the growing sugar chains, one by one.[33]

Once all the sugars have been incorporated onto the proteins and lipids, some of them are sulfated (SGG for example), they move to the plasma membrane, probably accomplished by secretory vesicles. Glycoproteins and glycolipids move through various subcellular organelles during their biosynthesis. The last stages of these syntheses are carried out in the Golgi apparatus and they then move to the plasma membrane.[33] Thus, it can be seen that the sulfation of the SGG in the Golgi apparatus and its subsequent location in the plasma membrane is by no means unusual and is most likely.

What is the purpose of SGG on the plasma membrane? Glycolipids and glycoproteins on the plasma membrane have been shown to have many functions. They have been shown to be involved in intra-cellular communications. For example, glycolipids and glycoproteins have been shown

to be antigenic.[89,90] Having this antigenic property enables cells to identify "foreign" cells in a tissue and thus help to fight off "invasion" by a potentially harmful interloper. By the same token these antigens help in the recognition of cells that are not foreign. This could be a purpose for the SGG. It is possible that SGG has antigenic properties as other sulfatides have been shown to have antigenic properties.

Another possible function for the SGG also lies in the realm of intracellular communication. This has to do specifically with cell-cell interactions. It has been suggested that glycosyltransferases exist on the surface of cells[91,92] and because of this, glycoslyation can occur between cells.[92] The glycosyltransferase present on the surface of one cell could glycosylate glycolipids on the surface of another cell. This may have to do with communication of environmental factors and or cell-cell adhesion. This seems likely for the SGG as during the development of the testis there is the need for much cell-cell interaction and communication because of the interaction of the various cell types during their stages of development.

Another possible function of the SGG on the plasma membrane came to light when the paper by Hansson, Karlsson and Samuelsson was published.[93] Here, they discuss the possibility of the relationship of sulfatide concentration, on the cell surface, to the activity of the sodium-

potassium dependent adenosine triphosphatase. They worked with the sulfatides of the erythrocyte; isolated the sulfatides and then compared these concentrations to the activity of the enzyme. They found that there is indeed a correlation between the concentration of sulfatide and the activity of the enzyme; as it increased in various tissues the activity of the sodium-potassium dependent adenosine triphosphatase increased. They also feel that it is possible for SGG to be involved in the activity of this enzyme and therefore sodium transport. SGG might be acting as a co-factor for the enzyme. Since sulfatides are located on the outside surface of the membrane as most glycolipids and glycoproteins are, they might have a "polar" interaction with the enzyme protein. The sulfate is probably close to the outside of the cationic gate, and thus they feel that "the essential function of a sulfate group (selection of K^+ before Na^+) in close proximity to the gate site is to donate K^+ to a short lived outside site." Thus, we have a possible function of the SGG on the plasma membrane. Some further work in this area could be done to check the relationship of the SGG and the sodium-potassium dependent adenosine triphosphatase through the various stages of spermatogenesis. At this point it would be necessary to show where the SGG is located in the plasma membrane; that is, is the SGG located on the inside of the plasma membrane or on the outside. One possible way to determine this is to incubate cells with aryl sulfatase A.

This enzyme has been shown to remove the sulfate group from sulfatides[94] and if it could be shown that there is activity for the enzyme, then, since the enzyme was not accessible to internal sites, we could say that the SGG is located on the outside surface of the plasma membrane. Of course this does not preclude that SGG could also be located on the inner surface of the plasma membrane.

There are a number of other experiments that could be done in order to add more weight to the argument that SGG is on the plasma membrane. These experiments could include some of the ideas already mentioned, such as, the trypsin experiments where the marker enzymes would be determined before and after trypsin treatment. The use of arylsulfatase A could also help in the determination of the location of the SGG. If we could show activity for ^{35}S-SGG then we could say that SGG is on the plasma membrane, let alone say which half of the bylayer.

Once the location is known, and it now is, SGG can be used as a marker for the plasma membrane because of the fact that it is easily labelled *in vivo*. This fact could be useful in further work on studies of the changes occurring on the plasma membrane during spermatogenesis.

CONCLUSIONS

I have determined, through the use of equilibrium density gradients the patterns for various marker enzymes on those gradients of the cellular organelles of rat spermatocytes. The SGG pattern was the same as the markers of the plasma membrane and different from the markers for other cellular organelles. Therefore, it was concluded that after its sulfation in the Golgi apparatus sulfogalactoglycerolipid moves to the plasma membrane following the same route as most glycolipids and glycoproteins. Thus in agreement with the work of Millette and Belve, it can be shown that at specific times during spermatogenesis, the plasma membrane, which probably plays a major role in the differentiation process and fertilization, changes to a considerable extent.

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