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An Investigation Of The Subcellular Distribution Of The Glycolipids Of Dog Small Intestinal Mucosa.

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AN INVESTIGATION OF THE SUBCELLULAR DISTRIBUTION OF THE GLYCOLIPIDS OF DOG SMALL INTESTINAL MUCOSA

by

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PREFACE

Earlier studies had shown that there were at least eight glycolipid types in dog small intestine. The original objective of this investigation was to isolate these glycolipids from the subcellular fractions of the mucosa of dog small intestine and to compare them on a molar basis. It was thought that such a comparison might show some unique distribution of these glycolipids in the subcellular fractions or organelles of this tissue. The methodology which was necessary to accomplish this objective, however, was lacking both in our laboratory and in the literature. Therefore, much of the effort expended in the present investigation was directed toward the development of this methodology. Specifically, the methods investigated concerned: (1) a hydrolysis technique which would conveniently allow both the analyses of small amounts of tissue and large numbers of tissue samples, (2) the cell fractionation of the mucosa of dog small intestine, and (3) the thin layer chromatographic separation of glycolipids of the small intestine containing more than three sugar residues per molecule.

PART I: REVIEW OF LITERATURE

CHAPTER I. TERMINOLOGY AND ABBREVIATIONS

Animal glycolipids can be divided into two classes. Those compounds which contain sialic acid are known as the gangliosides. Those which do not contain sialic acid can be referred to as the asialoglycolipids, ceramide hexosides, or oligohexosides. Ceramide glycosides is a general term which can be used to refer to both gangliosides and asialoglycolipids. Gangliosides may contain from one to three sialic acid residues per molecule. The number of sialic acid residues is indicated by a prefix, i.e., trisialoganglioside. Two types of sialic acid are known, N-acetylneuraminic acid and N-glycolylneuraminic acid. Asialoglycolipid is a general term which includes glycolipids having one or more sugar residues per molecule. The terms hexoside or glycoside are qualified by a prefix denoting the number of sugar residues in the glycolipid, i.e., monohexoside or pentaglycoside. Since all known avian and mammalian glycolipids contain a ceramide moiety, this latter term is frequently omitted. Thus, a "ceramide hexaglycoside" becomes simply "hexaglycoside". The term oligohexoside indicates a glycolipid containing three or more sugar residues per molecule.

When the specific carbohydrate sequence of a glycolipid is shown, a short hand method is used. Thus, "N-acetylgalactosaminyl-N-acetylgalactosaminimylgalactosylgalactosylglucosylceramide" becomes "galNAc-galNAc-gal-gal-glc-Cer". The following abbreviations are used: glc - glucosyl; gal - galactosyl; galNAc - N-acetylgalactosaminyl; glcNAc - N-acetylglucosaminyl; NANA - N-acetylneuraminic acid; NGNA - N-glycolylneuraminic acid; Cer - ceramide.

Other terms which are commonly used are presented below. A cerebroside is a monohexoside. A prefix is used to denote the type of sugar residue in the molecule, i.e., glucocerebroside or galactocerebroside. Sulfatide is a term used to describe a glycolipid which has a sulfate residue esterified to a galactose moiety. These compounds are mono- or diglycosides. A hematoside is a monosialoganglioside, which contains one residue each of glucose and galactose per molecule (NGNA- or NANA-gal-glc-Cer). Tetraglycosides having the carbohydrate sequence "galNAc-gal-gal-glc-Cer" are also called aminoglycolipid or globoside. Tay-Sachs' ganglioside is a tetraglycoside having the carbohydrate sequence "galNAc-gal-glc-Cer". Hexaglycosides

NANA

D-1 and D-2 are both mixtures of oligohexosides having six or more glycose residues per molecule. They were isolated from pooled dog whole intestine. Their carbohydrate sequence is unknown. Hexaglycosides D-1 and D-2 were combined and separated into two components using thin layer chromatography. The faster moving of these components is Hexaglycoside I, the slower, Hexaglycoside II. Their composition and carbohydrate sequence are unknown.

CHAPTER II. STRUCTURE OF GLYCOLIPIDS

Animal glycolipids are compounds which are made up of three component parts: (1) a carbohydrate portion, (2) a fatty acid portion and (3) a long chain amine portion. As will be seen in the following discussion, there is some heterogeneity in the structure of each of these three parts. The carbohydrate portion is linked through C-1 of glucose or galactose to the primary hydroxyl group of sphingosine (or a similar long chain amine). The fatty acid is connected by an amide linkage to the C-2 amino group of sphingosine.

1. The Carbohydrate Portion.

Certain sugars are characteristic of mammalian tissue glycolipids: D-glucose, D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose, and the N-acetyl and N-glycolyl derivatives of neuraminic acid. Interestingly enough, these same sugars are found in the glycoprotein blood group substances (Watkins, 1966), with the exception of glucose. These sugars are linked together into straight or branched chains ranging in size from one to seven glycose units.

a. Carbohydrate stems.

A survey of glycolipid structure in a number of tissues from various mammalian species indicates that there are possibly only five basic carbohydrate stems, and all the glycolipids known at the present time can be considered to be either precursors or products of these stems. As will be seen, glycolipids of several different stems may co-exist in the same tissue. The five stems are characterized by their sequence of glycose units and are as follows:

(i) glcNAc-gal-glc-Cer

(ii) galNAc-gal-glc-Cer

(iii) gal-gal-glc-Cer

(iv) NANA- or NGNA-gal-glc-Cer

(v) gal-Cer

(i) glcNAc-gal-glc-Cer.

At the present time, there are two tissues which are known to have glycolipids with this stem: human adenocarcinoma (Hakomori, 1967), rabbit erythrocytes and reticulocytes (Eto et al., 1968), and bovine erythrocytes (Kuhn and Wiegandt, 1964). The linkages of this stem are: glcNAc-(1→3)-gal(1→4)-glc-(1→1)-Cer.

(ii) galNAc-gal-glc-Cer.

This stem is unique to brain tissue. It should be noted that glucosamine has not been reported in brain glycolipids (Ledeen, 1966). This unique glycolipid stem is extremely interesting in light of the fact that brain malignancies have never been known to metastasize. Perhaps malignant cells from the brain would be easily recognized in other tissues and "inactivated". The linkages in this stem are: galNAc-(1→4)-gal-(1→4)-glc-(1→1)-Cer (Kuhn and Wiegandt, 1963; Ledeen and Salsman, 1965).

(iii) gal-gal-glc-Cer.

A larger number of tissues is characterized by having glycolipids with this stem: human kidney (Makita, 1964; Makita et al., 1964; Rapport et al., 1964; Martensson, 1966), human spleen (Svennerholm, 1963b; Wagner, 1964), erythrocyte stroma of humans (Yamakawa et al., 1962a,b; 1963; 1965; Vance and Sweeley, 1967), rabbits (Eto et al., 1968) and hog (Miyatake et al., 1968), human blood plasma (Svennerholm and Svennerholm, 1963a,b; Vance and Sweeley, 1967), human blood serum (Svennerholm and Svennerholm, 1963a), dog whole intestine (Vance et al.,

1966; McKibbin, 1969), human liver (Svennerholm and Svennerholm, 1963b), human placenta (Svennerholm, 1965), pig lung (Gallai-Hatchard and Gray, 1966), and mouse ascites sarcoma cells (Gray, 1965). Rabbit erythrocytes are interesting in that a glycolipid with the glcNAc-gal-glc-Cer stem has also been described (Eto et al., 1968). Linkages have not been studied in all the tissues known to have this stem, but in human erythrocytes (Yamakawa et al., 1962a,b; 1965), hog erythrocytes (Miyatake et al., 1968) and human kidney (Makita et al., 1964; Makita and Yamakawa, 1964), they are as follows: gal-(1→4)-gal(1→4)-glc(1→1)-Cer.

(iv). NANA- or NGNA-gal-glc-Cer (hematoside).

This stem is found in a number of tissues and is the major ganglioside in the following tissues: human spleen (Svennerholm, 1963b), adrenal medulla, where both NANA, NGNA occur (Ledeen et al., 1968b), human liver (Eeg-Olofsson et al., 1966), horse erythrocytes (Klenk and Padberg, 1962; Klenk et al., 1962; Handa and Yamakawa, 1964) which have only NGNA, dog erythrocytes, which have both NANA and NGNA (Handa and Yamakawa, 1964) and pig lung (Gallai-Hatchard and Gray, 1966). This stem also occurs in human brain (Penick et al., 1966; Ledeen et al., 1968a; Eeg-Olofsson et al., 1966), human placenta (Svennerholm, 1965), and mouse ascites sarcoma (Gray, 1965). It may also occur in lens of the human eye (Feldman et al., 1966). In cat erythrocytes, this stem, with an additional NGNA residue, has been described (Handa and Handa, 1965). The stem is known to co-exist with the galNAc-gal-glc-Cer stem of brain, the gal-gal-glc-Cer stem of human placenta, mouse ascites sarcoma cell and human spleen.

The linkages of the stem are NANA- or NGNA-(2→3)-gal-(1→4)-glc-(1→1)-Cer.

(v). gal-Cer.

This stem occurs as a major component of normal human brain tissue (Eeg-Olofsson et al., 1966). It also occurs, usually as a minor component, in human kidney (Makita, 1964; Makita and Yamakawa, 1964; Martensson, 1966), human leukocytes (Miras et al., 1966) and blood serum (Svennerholm and Svennerholm, 1963a). A possible derivation of this compound, in which a fatty acid is esterified to the C-6 hydroxyl group of galactose, has been described in pig brain grey matter, (Kishimoto et al, 1968).

b. Carbohydrate linkages.

The following generalizations can be made concerning the chemical linkages of glycolipid carbohydrates. Only carbons 1 and 4 are substituted in glucose and carbons 1, 3, and 4 in galactose, galactosamine and glucosamine. Additionally, galactose may be esterified with a fatty acid at carbon 6. Only fucose and neuraminic acid form side chains. Only one fucose linkage has been described: fucose-glcNAc (1→4) (Hakomori, 1967). NAGA and NANA are linked only to galactose (2→3) or to another NAGA or NANA (2→8).

A summary of the linkages of various glycose units in glycolipids is given in Table I with pertinent references.

2. The Fatty Acid Portion.

The information concerning fatty acid composition of glycolipids in mammalian tissues is abundant. Table II lists a number of publications which deal with glycolipid fatty acid composition by tissue and species.

In general, in any particular type of glycolipid, fatty acids occur as a spectrum, and fatty acids having from 14 to 26 carbon atoms may be present. Some of these fatty acids may be present in only trace amounts. The fatty acids which often contribute 20 per cent or more of the fatty acid spectrum in a glycolipid molecule are most commonly C16:0, C18:0, C20:0, C22:0, C22:1, C24:0 and C24:1.

Fatty acids with a hydroxyl group on carbon 2 have been described in brain of humans (Menkes et al., 1966; Svennerholm and Stallberg-Stenhagen, 1968), pigs (Kishimoto et al., 1968), rats (Kishimoto and Radin, 1966), human kidney (Makita, 1964; Martensson, 1966), human blood serum, spleen and liver (Svennerholm and Svennerholm, 1963b), in mouse ascites sarcoma (Gray, 1965), in pig brain in the acylgalactocerebrosides (Kishimoto et al., 1968), and in trace amounts in pig lung (Gallai-Hatchard and Gray, 1966).

In some tissue glycolipids, a relatively few fatty acids may predominate. For instance, brain tissue gangliosides are characterized by high stearic acid content (often over 70-90%) (Sambasivarao and McCluer, 1964; Ledeen and Salsman, 1965; Dain et al., 1962; Ledeen et al., 1968a; Stanacev and Chargaff, 1965). In contrast, in erythrocyte glycolipids of humans, cats, dogs, hogs, and horses, the longer chain fatty acids predominate (C22 and C24, both saturated and unsaturated). Only in dog erythrocyte glycolipids does stearic acid make a significant contribution.

A most interesting speculation is that fatty acids may play some part in determining the metabolic pathways that a glycolipid precursor molecule may enter. This could be accomplished either directly or indirectly. Directly, enzymes which determine which glycose moiety is added to a glycolipid precursor molecule may have some substrate specificity depending on which fatty acid is present in this molecule. This is difficult to visualize, however if one assumes that the fatty acid and the long chain amine are both embedded in the cell membrane, as has been suggested (Vandenheuvel, 1963; O'Brien, 1965). It must be observed, though, that the position of any molecule within a cell membrane is, at this time, completely unknown. Indirectly, the fatty acid could determine the type of membrane with which a glycolipid would be associated. For instance, myelin is characterized by saturated fatty acids, and mitochondrial membranes by unsaturated fatty acids (O'Brien, 1967). A certain percentage of a particular fatty acid, then, might assure that a certain necessary complement of a particular glycolipid will be associated with a particular subcellular organelle. This localization, in turn, might vary enzyme availability due to cell compartmentation.

At any rate, circumstantial evidence that fatty acids may indeed have some role in metabolic pathway selection in glycolipid biosynthesis exists in the literature, and is presented below.

In one case of subacute sclerosing leukoencephalitis (Ledeen et al., 1968a), the fatty acid distribution of nine brain gangliosides was described. All had at least one NANA unit and all had similar fatty acid compositions (very high C18:0) with two notable exceptions: "G_{3A}" (NANA-NANA-gal-glc-Cer) and "G_6" (NANA-gal-glc-Cer). These two

gangliosides had fatty acid compositions in which C16:0, 18:0 and 18:1 concentrations were prominent. With the exception of these latter two gangliosides, all the rest of the gangliosides were of the galNAc-gal-glc-Cer stem. It would seem then, that glycolipids with 16:0 or 18:1 would be more likely to have a NANA unit added early in biosynthesis and end up as "short chain" ganglioside. The glycolipids lacking these particular fatty acids could have a NANA unit added at a later stage, after a longer oligosaccharide chain containing galNAc was synthesized. The presence of 16:0 or 18:1 fatty acids would not exclude either metabolic pathway, but simply make one pathway more likely because of cellular environmental placement or enzyme specificity conferred by a particular fatty acid.

Again, if the fatty acid composition of dog small intestines is examined (Vance et al., 1966; McKibbin, 1969), a similar situation exists. The fatty acid composition of the ceramide pentaglycoside (galNAc-galNAc-gal-gal-glc-Cer) differed from that of the gangliosides (NANA-gal-gal-glc-Cer) in that stearic acid was significantly higher. This would suggest that a cerebroside (glc-Cer), which could be a precursor of either molecule, with stearate as its fatty acid, would be more likely to be directed away from ganglioside formation. That is, the enzyme(s) responsible for the addition of NANA does not function as efficiently when this fatty acid is present, as when some other fatty acid is present.

Finally, the hydroxy fatty acid patterns of four glycolipids were examined in human kidney (Makita, 1964; Martensson, 1966) and human blood serum, spleen and liver (Svennerholm and Svennerholm, 1963b). These four glycolipids were ceramide mono-(glc-Cer), di-(gal-glc-Cer),

trihexoside (gal-gal-glc-Cer), and aminoglycolipid (galNAc-gal-gal-glc-Cer). It is seen that the aminoglycolipids (and sometimes the ceramide trihexoside) had either no hydroxy fatty acids or a remarkably lower content than the ceramide mono- and dihexosides. This finding may indicate that the enzyme which adds either terminal glycose unit to the trihexoside or aminoglycolipid may have lower activity when a hydroxy fatty acid is present in its substrate. On the other hand, this does not appear to be true in mouse ascites sarcoma (Gray, 1965).

More information is needed concerning fatty acid composition of individual glycolipids so that these patterns in asialoglycolipids and gangliosides, in particular, can be compared. Understanding why fatty acid compositions vary among glycolipids of the same tissue may provide a key to understanding a part of the mechanism of glycolipid biosynthesis.

3. The Long Chain Amine Portion.

A detailed analysis of the long chain bases is omitted in most of the literature concerning glycolipid structure. This portion of the glycolipid molecule is by far the most neglected when compared with the abundant information available on the carbohydrate and fatty acid portions.

There is a number of long chain amines which are known to occur in mammalian glycolipids:

(a) 4-sphingenine (sphingosine) - an 18-carbon chain, having hydroxyl groups on carbons 1 and 3, an amino group on carbon 2, and a trans double bond between carbon 4 and 5.

(b) Sphinganine (dihydrosphingosine) - similar to sphingosine, but saturated at carbons 4 and 5.

(c) 4-eicosasphingenine (eicosisphingosine) - a 20-carbon chain similar to sphingosine but having two additional carbon atoms at the methyl end of the chain. This base is interesting in that in ox and calf brain it occurs only in association with gangliosides (Stanacev and Chargaff, 1965; Sambasivarao and McCluer, 1965). In this ganglioside fraction, sphingosine and eicosisphingosine each accounted for roughly 40-50% of the long chain amines, and dihydrosphingosine was about 3-5%. Eicosisphingosine has been reported in the gangliosides of human, calf, rabbit, dog, rat, and ox brain and is absent in the asialoglycolipids of these same tissues (Sambasivarao and McCluer, 1965). It has also been detected in the neutral glycolipids of human kidney in small amounts (Martensson, 1966).

(d) Eicosasphinganine (dihydroeicosisphingosine) - similar to eicosisphingosine, but saturated at carbons 4 and 5. It may be present in small amounts in ox brain gangliosides (1-2%) (Stanacev and Chargaff, 1965; Sambasivarao and McCluer, 1964).

(e) 4D-hydroxysphinganine (phytosphingosine) - similar to dihydrosphingosine except there is a hydroxyl group on carbon 4. This base has been reported to occur in human kidney (Martensson, 1966).

(f) Shorter chain bases with 16 and 17 carbon atoms have been reported to occur in minor amounts in human kidney (Martensson, 1966).

Sphingosine appears to be the predominant base in most tissues studied, but further analyses are needed on all tissues as regards long chain base composition.

Table I. Chemical Linkages of Glycose Units in Glycolipids.

Glycose units	References
glc-Cer (1→1)	Hakomori, 1967; Makita and Yamakawa, 1964; Stoffyn et al., 1968; Eto et al., 1968; Ledeen and Salsman, 1965; Ledeen et al., 1968a; Handa and Yamakawa, 1964; Kuhn and Wiegandt, 1963; Handa and Handa, 1965; Klenk and Padberg, 1962.
gal-Cer (1→1)	Kishimoto et al., 1968; Makita, 1964; Yamakawa et al., 1962a.
gal-glc (1→4)	Hakomori, 1967; Makita and Yamakawa, 1964; Stoffyn et al., 1968; Eto et al., 1968; Ledeen and Salsman, 1965; Ledeen et al., 1968a; Klenk and Padberg, 1960; Handa and Yamakawa, 1964; Kuhn and Wiegandt, 1963; Miyatake et al., 1968; Makita et al., 1964; Makita and Yamakawa, 1964; Kuhn and Wiegandt, 1964; Handa and Handa, 1965; Yamakawa et al., 1962b.
glcNAc-gal (1→3)	Hakomori, 1967; Eto et al., 1968; Yamakawa et al., 1962; 1965; Kuhn and Wiegandt, 1964.
galNAc-gal (1→3)	Miyatake et al., 1968; Makita et al., 1964.
galNAc-gal (1→4)	Ledeen and Salsman, 1965; Kuhn and Wiegandt, 1963.
gal-glcNAc (1→3)	Hakomori, 1967; Eto et al., 1968.
gal-glcNAc (1→4)	Yamakawa et al., 1960; Kuhn and Wiegandt, 1964.
gal-galNAc (1→3)	Kuhn and Wiegandt, 1963.

Table I (Continued)

NANA- or NGNA-gal (2→3)	Handa and Handa, 1965; Klenk and Padberg, 1962; Ledeen and Salsman, 1965; Ledeen et al., 1968a; Handa and Yamakawa, 1964; Kuhn and Wiegandt, 1963.
NANA- or NGNA-NANA (2→8)	Ledeen et al., 1968a; Handa and Handa, 1965.
fucose-glcNAc (1→4)	Hakomori, 1967.
gal-gal (1→3)	Eto et al., 1968.
gal-gal (1→4)	Yamakawa et al., 1962b; 1965; Miyatake et al., 1968; Makita et al., 1964; Makita and Yamakawa, 1964.
SO_4^- -gal (→3)	Makita and Yamakawa, 1964; Makita, 1964; Yamakawa et al., 1962b; Stoffyn et al., 1968.

Table II. Articles Dealing with the Fatty Acid Composition of Glycolipids.

Tissue (species)	References
Blood Plasma or Serum (human)	Svennerholm and Svennerholm, 1963a,b.
Brain (human)	Sambasivarao and McCluer, 1964; Ledeen et al., 1968a; Eeg-Olofsson et al., 1966; Klenk et al., 1962; Ledeen and Salsman, 1965.
(bovine)	Sambasivarao and McCluer, 1964.
(dog)	Sambasivarao and McCluer, 1964.
(ox)	Stanacev and Chargaff, 1965.
(pig)	Kishimoto et al., 1968.
(rabbit)	Sambasivarao and McCluer, 1964.
Erythrocytes (human)	Hakomori and Strycharz, 1968; Yamakawa et al., 1963.
(cat)	Handa and Handa, 1965.
(dog)	Handa and Yamakawa, 1964.
(hog)	Miyatake et al., 1968.
(rabbit)	Eto et al., 1968.
Intestine (dog)	Vance et al., 1968; McKibbin, 1969.
Kidney (human)	Rapport et al., 1964; Martensson, 1966; Martensson, 1963.
Lens of Eye (human)	Feldman et al., 1966.
Leukocytes (human)	Miras et al., 1966.
Liver (human)	Eeg-Olofsson et al., 1966; Svennerholm, 1963b.

Table II (Continued).

Lung (pig)	Gallai-Hatchard and Gray, 1966.
Spleen (human)	Wagner, 1964; Makita and Yamakawa, 1962; Suomi and Agranoff, 1965; Svennerholm and Svennerholm, 1963b.
(bovine)	Makita and Yamakawa, 1962.
(equine)	Makita and Yamakawa, 1962.
Tumor Cells Ascites Sarcoma (mouse)	Gray, 1965.

CHAPTER III. DISTRIBUTION OF GLYCOLIPIDS IN MAMMALIAN TISSUES

Glycolipids are probably constituents of the cells of every mammalian tissue. Tissues of various mammalian species in which glycolipids have been isolated or detected are listed with the pertinent references in Table III. For convenience, references pretaining to asialoglycolipids and gangliosides are separated.

Tissues may show characteristic distributions of various glycolipid types (McKibbin, in press). A comparison of several tissues which contain neutral glycolipids of the galNAc-gal-gal-glc-Cer stem is shown in Table IV. From this table it can be seen that no tissue is exactly like any other thus far studied. Human kidney (Martensson, 1963; Makita, 1964) and human placenta (Svennerholm, 1965) have similar distributions, but differ markedly in the ratio of neutral to acidic glycolipids: 1:1 for placenta and 5:1 in kidney (Svennerholm, 1965). The significance of these unique distributions is, at the present time, unknown, but it has been speculated that this phenonmenon could be related to cell recognition (McKibbin, 1969).

It is important to note that values shown in Table IV must be regarded as only tentative because recoveries of various glycolipids during isolation appear to be a function of carbohydrate chain length, and recoveries of the aminoglycolipid in human serum or plasma was estimated to be 71% (Vance and Sweeley, 1967) and less than 50% (Svennerholm and Svennerholm, 1963a,b). In mouse ascites sarcoma cells, recovery of total lipid hexose was estimated as 76% (Gray, 1965) after purification procedures.

Complete evaluation of glycolipid patterns between species and tissues within species, must await further investigation. At the

present time, the accumulating data concerning glycolipid distribution suffer from: (1) incomplete and differential recoveries of glycolipid types during isolation procedures, (2) inattention by investigators to minor glycolipids having five or more glycose residues which may show important organ and tissue specificity and (3) the lack of confirmation of evidence by more than one laboratory.

Table III. Occurrence of Asialoglycolipids and Gangliosides in the Tissues of Various Mammalian Species.

Tissue (species)	References	
	asialoglycolipid	ganglioside
Adrenal Medulla (bovine)	------	Ledeen et al., 1968b
Blood Plasma or Serum (human)	Svennerholm and Svennerholm, 1963a, b; Vance and Sweeley, 1967.	------
Brain (human)	Klenk and Doss, 1962; Eeg-Olofsson et al., 1966.	Dain et al., 1962; Klenk et al., 1962; Klenk and Gielen, 1961, 1963; Korey and Gonatas, 1963; Johnson and McCluer, 1963; Eeg-Olofsson, 1966; Kuhn et al., 1961; Ledeen and Salsman, 1965; Ledeen et al., 1968a; Penick et al., 1966; Sambasivarao and McCluer, 1964; Svennerholm, 1963a; Suzuki, 1964.
(bovine)	Yamakawa et al., 1962a.	Dain et al., 1962; Kuhn et al., 1961; Sambasivarao and McCluer, 1964; Suzuki, 1964.

Table III (Continued).

(dog)	------	Sambasivarao and McCluer, 1962; Dain et al., 1962.
(guinea pig)	Eichberg et al., 1964.	Eichberg et al., 1964; Wherrett and McIlwain, 1962.
(rabbit)	------	Sambasivarao and McCluer, 1964.
(rat)	Seminario et al., 1964.	Sambasivarao and McCluer, 1964; Seminario et al., 1964.
(ox)	------	Stanacev and Chargaff, 1965.
(pig)	Kishimoto et al., 1968.	------
Erythrocytes (human)	Hakomori and Strycharz, 1968; Yamakawa et al., 1962a,b; 1960; 1965; Vance and Sweeley, 1967; Klenk and Lauenstein, 1952.	Booth, 1963; Hakomori and Strycharz, 1968; Yamakawa et al., 1965.
(bovine)	Yamakawa et al., 1960.	Yamakawa et al., 1960.
(cat)	------	Yamakawa et al., 1960; Handa and Handa, 1965.
(dog)	------	Handa and Yamakawa, 1964.

Table III (Continued).

(equine)	Yamakawa et al.,1960; Klenk and Lauenstein, 1952; 1953; Klenk and Wolter, 1952.	Yamakawa et al., 1960; Klenk and Padberg, 1962; Handa and Yamakawa, 1964; Klenk and Lauenstein, 1952; 1953; Klenk and Wolter, 1952.
(hog)	Miyatake et al., 1968.	------
(guinea pig)	Yamakawa et al., 1960.	------
(sheep)	Yamakawa et al., 1960.	------
(rabbit)	Eto et al., 1968; Yamakawa et al., 1960.	------
Intestine (dog)	Vance et al., 1966; McKibbin, 1969.	Vance et al., 1966; McKibbin, 1969.
Kidney (human)	Martensson, 1966; Makita, 1964; Makita and Yamakawa, 1964; Makita et al., 1964; Martensson, 1963; Rapport et al., 1964; Stoffyn et al., 1968.	Makita, 1964.
(rat)	Green and Robinson, 1960.	------

Table III (Continued).

Lens of Eye (human)	Feldman et al., 1966.	Feldman et al., 1966.
Leukocytes (human)	Miras et al., 1966.	------
Liver (human)	Svennerholm and Svennerholm, 1963b.	Eeg-Olofsson et al., 1966.
(rat)	Green and Robinson, 1960.	------
Lung (pig)	Gallai-Hatchard and Gray, 1966.	Gallai-Hatchard and Gray, 1966.
Placenta (human)	Svennerholm, 1965.	Svennerholm, 1965.
Salivary Gland Submaxillary (rat)	Pritchard, 1967.	------
(dog)	Smith, unpublished	?
Spleen (human)	Wagner, 1964; Makita and Yamakawa, 1962; Suomi and Agranoff, 1965; Svennerholm and Svennerholm, 1963b.	Svennerholm, 1963b
(bovine)	Makita and Yamakawa, 1962.	------
(equine)	Makita and Yamakawa, 1962.	------
(ox)	Rapport et al., 1960; Klenk and Rennkamp, 1942.	Klenk and Rennkamp, 1942.

Table III (Continued).

(rat)	Green and Robinson, 1960.	------
Tumor Cells (human)		
Carcinoma		
1. epidermold	Rapport et al., 1960.	------
2. unspecified	Rapport et al., 1961.	------
Adenocarcinoma		
1. bronchiogenic	Hakomori and Jeanloz, 1964.	Hakomori and Jeanloz, 1964.
2. gastric	Hakomori et al., 1967; Hakomori and Jeanloz, 1964.	Hakomori and Jeanloz, 1964.
3. cecal	Hakomori et al., 1967.	------
4. lung	Hakomori et al., 1967.	------
(mouse) ascites-sarcoma	Gray, 1965.	------
mastocytoma	Green and Robinson, 1960.	------

Table IV. Distribution of Various Neutral Glycolipids Expressed as Dry Weight Percentages of the Four Glycolipids Isolated.

Tissue	glc-Cer	gal-glc-Cer	gal-gal-glc-Cer	galNAc-gal-gal-glc-Cer	galNAc-galNAc-gal-gal-glc-Cer
*Human Erythrocytes[1]	2.3	11.4	10.7	75.6	---
Human Kidney[2]	5-7	10-12	25-30	45-50	---
Human Kidney[3]	12	7.8	28	52	---
Human Liver[4]	15-20	65-70	7-10	8-12	---
Pig Lung[5]	15	7.0	54	24	---
Human Serum and Plasma[4,6]	40-50	45-55	5-10	2-5	---
*Human Plasma[1]	39.4	30.7	14.2	15.7	---
Human Placenta[7]	9.5	19	24	47	---
*Dog Whole Intestine[8]	33.0	24.8	12.4	---	29.6
Mouse Ascites Sarcoma Cells[9]	55	21	9	15	---

*Values reported in μmoles in original articles and converted into μgm. using molecular weights of glycolipids assuming a sphingosine base and C20:0 fatty acid: 667; 829; 991; 1190; 1389.

1. Vance and Sweeley, 1967.

2. Martensson, 1963.

Table IV (Continued).

3. Makita, 1964.

4. Svennerholm and Svennerholm, 1963b.

5. Gallai-Hatchard and Gray, 1966.

6. Svennerholm and Svennerholm, 1963a.

7. Svennerholm, 1965.

8. McKibbin, 1969.

9. Gray, 1965.

CHAPTER IV. SUBCELLULAR DISTRIBUTION OF GLYCOLIPIDS IN MAMMALIAN TISSUES

The subcellular distribution of asialoglycolipids with more than one glycose residue has not been studied in any tissue. However, the distribution of cerebrosides, sulfatides or lipid sialic acid was examined in brain tissues of guinea pigs (Wolfe, 1961; Wolfe and McIlwain, 1961; Wherrett and McIlwain, 1962; Eichberg et al., 1964) and rats (Green and Robinson, 1960; Seminario et al., 1964; Lapetina et al., 1967) and in liver and kidney of rats (Green and Robinson, 1960).

1. Ganglioside Subcellular Distribution.

Ganglioside subcellular distribution has been studied only in brain tissues of rodents (Wolfe, 1961; Wolfe and McIlwain, 1961; Wherrett and McIlwain, 1962; Eichberg et al., 1964; Seminario et al., 1964; Lapetina et al., 1967). In these reports, individual gangliosides were not isolated, and the gangliosides were assayed as a class by sialic acid determinations. These brain gangliosides were extracted quantitatively from chloroform-methanol whole lipid extracts by a solvent fractionation technique (Folch et al., 1957).

Comparison of ganglioside distributions with respect to protein in rodent brains indicated that gangliosides were relatively concentrated in the microsomal fraction (Eichberg et al., 1964; Wherrett and McIlwain, 1962; Seminario et al., 1964) and in the synaptosomes or cell fractions related to synaptic endings (Seminario et al., 1964; Eichberg et al., 1964). Lapetina and coworkers reported synaptic membranes had the highest concentration of gangliosides, while other fractions were low (Lapetina et al., 1967). Of interest was the finding

that all subcellular fractions, as well as myelin particles and synaptosomes, had a remarkably similar ganglioside pattern when examined by thin layer chromatography (Eichberg et al., 1964).

2. Asialoglycolipid Subcellular Distribution.

The subcellular distribution of the cerebrosides and sulfatides was studied in several species and tissues. The distribution of the sulfatides was examined in the brain, kidney and liver of the rat (Green and Robinson, 1960). These authors reported that sulfatides were distributed very differently in the subcellular fractions of the three organs studied. Although the chemical identity of the sulfatides was well established, these results are subject to criticism since the solvent extraction method of Lees and coworkers was used (Lees et al., 1959). This method does not give quantitative recovery of sulfatides from the total lipid extract. Additionally, the method was originally worked out on bovine brain tissue and its use on other tissues might also be questioned. Finally, values for the subcellular fractions were not compared with the whole homogenates, and differences in subcellular distributions in tissues mentioned above may be due to differential recoveries of the sulfatides.

Cerebrosides plus sulfatides distributions were reported in guinea pig brain (Eichberg et al., 1964). These lipids were isolated using the alumina column technique of Long and Staples (1961) after solvent extraction of the gangliosides. According to these latter authors, this technique gave quantitative recovery of the cerebrosides and sulfatides although the values of only one recovery experiment were quoted. It is interesting to note that lipid values for only two experiments (two pooled samples of four animals) and in some cases values for only one experiment were reported.

Cerebroside subcellular distributions were determined in rat brain by direct analysis of the total lipid extract after removal of gangliosides (Seminario et al., 1964). The cerebroside values were based on galactose determinations multiplied by a mass factor of 4.6. If rat brain contains asialogalactolipids other than cerebrosides and sulfatides, the cerebroside values reported by Seminario and coworkers would be high.

3. Glycolipids of Individual Subcellular Fractions and Organelles.

a. Nuclear fraction.

In human brain, the relative amounts of cerebrosides were found to be greater in nuclei than in whole brain (Tryell and Richter, 1951); it was later suggested that these results should be confirmed by more modern methods (Fleisher and Rouser, 1965). Most preliminary nuclear pellets were crude preparations and usually contained cellular debris and whole unbroken cells. Nuclei which were purified past this initial crude stage by density gradient centrifugation, were assayed for cerebroside plus sulfatide and ganglioside in guinea pig brain (Eichberg et al., 1964). When related to protein, nuclear cerebrosides plus sulfatides concentration was less and ganglioside concentration unchanged when compared to the homogenate.

b. Mitochondrial fraction.

Several authors have reported on the glycolipid composition of mitochondria with quite variable results. Rat brain mitochondria were reported to have 18% of the lipid accounted for by cerebrosides (Lovtrup and Svennerholm, 1963), while in guinea pig brain mitochondria, cerebrosides accounted for less than 1% (Eichberg et al., 1964). The higher values of Lovtrup and Svennerholm were criticized by Eichberg

and coworkers who suggested that sucrose contamination might account for the discrepancy. Gangliosides, cerebrosides and sulfatides were reported to be essentially absent from mitochondrial fractions of guinea pig brain (Eichberg et al., 1964) and gangliosides from mitochondria of rat brain (Lovtrup and Svennerholm, 1963). Cerebrosides were also reported to be absent from highly purified mitochondria of bovine heart, liver, and kidney (Fleisher et al., 1967). The method of specific identification and quantitation of cerebrosides in this latter paper was not described.

c. Microsomal fraction.

In rodent brain, the microsomal fraction was reported to be enriched in gangliosides (Eichberg et al., 1964; Wolfe, 1961; Wolfe and McIlwain, 1961; Lapetina et al., 1967).

4. Summary.

Because some tissues are characterized by the presence of unique structures and organelles, such as the myelin particles and synaptosomes of brain, comparison of glycolipid subcellular distributions between tissues may be meaningless. Even within brain, correlation of glycolipid values is difficult because of differences in species, methods of assay, and cell fractionation procedures employed (Lapetina et al., 1967).

The foregoing discussion indicates that the subcellular distribution of glycolipids has been studied extensively only in rodent brains, and in no case have individual glycolipids been identified and quantitated, except for the sulfatides, and distributions reported for this compound are questionable.

CHAPTER V. METABOLISM OF GLYCOLIPIDS IN MAMMALIAN AND AVIAN TISSUES

Tissues which have been studied in detail as regards their glycolipid composition (Penick, Meisler, and McCluer, 1966) have been shown to contain a number of structurally similar glycolipids which closely resemble each other in their lipid component and show homology in their carbohydrate portion. For this reason, experimental evidence has been sought by a number of investigators to establish a metabolic relationship between glycolipids such as the cerebrosides, which contain only one glycose unit, and the asialoglycolipids and gangliosides containing from two to seven glycose units.

1. Biosynthesis.

Incorporation of radioactive glucose, galactose, and hexosamines into brain glycolipids has been reported in a number of studies *in vivo*. In 7-22 day old rats, radioactive glucose and/or galactose was rapidly incorporated into various glycolipids (Radin *et al.*, 1957; Burton *et al.*, 1963; Suzuki and Korey, 1963; Hauser, 1964). Glucosamine was shown to be incorporated into asialoglycolipid and gangliosides of 8-14 day old rats (Burton *et al.*, 1963). Radioactive galactose (Radin *et al.*, 1957) and glucose (Hauser, 1964) have been reported to be incorporated into sulfatides. These papers indicated that incorporation of glucose, galactose, or glucosamine occurred most actively between 7-22 days, post partum.

In 10-day-old rats, an attempt to incorporate radioactive cerebroside, labeled in the acyl or glucose moiety, into the more highly glycosylated glycolipids after intracerebral and intraperitoneal injection yielded negative results (Kanfer, 1965). Since no

radioactivity was found in the more complex glycolipids of brain, kidney, spleen, and liver, Kanfer concluded that cerebrosides were not precursors for the more complex glycolipids.

In vitro studies on intact organs include the incorporation of radioactive glucose into cerebrosides of liver and blood in isolated, perfused rat liver (Kean, 1966) and incorporation of S^{35}-sulfate into rat submandibular salivary gland tissue sulfatides (Pritchard, 1967). It was not possible, however, to identify sulfatides from the chromatographic evidence presented in the latter report.

In vitro studies involving homogenized tissue preparations have supplied most of the information now known concerning:

(a) cell fractions responsible for glycolipid biosynthesis,

(b) biosynthetic pathways by which the more highly glycosylated glycolipids are made from cerebrosides,

(c) glycose carrier molecules involved in glycolipid biosynthesis, and

(d) cell fractions in which catabolic enzymes are localized and the sequence of degradation of glycolipids (catabolism of glycolipids will be discussed in a following section).

a. Biosynthesis of cerebroside.

The following pathway for galactocerebroside was reported in rat brain homogenates:

1. sphingosine + UDP-galactose $\longrightarrow$ psychosine (galactosyl sphingosine) + UDP. The enzyme which catalyzed this reaction was found in the 105,000g pellet or microsomal fraction (Cleland and Kennedy, 1960).

2. psychosine + stearyl CoA $\xrightarrow{ATP}$ galactocerebroside. The enzyme which catalyzed this reaction was also found in the microsomal fraction or the 100,000 pellet (Brady, 1962).

Another study showed that 8% of the radioactive galactose used in rat brain subcellular fractions was incorporated into galactocerebrosides. 50% of this incorporated galactose was accounted for by the microsomal fraction (Burton et al., 1958a).

b. Biosynthesis of sulfatide.

Radin et al. (1957) concluded from their turnover studies following injections of 1-C^{14}-galactose into rats that cerebroside sulfate was made from cerebroside. In in vivo experiments on rat brain, incorporation of glucose carbon into galactocerebrosides was always greater than incorporation into galactosulfatides both in 20-day-old and adult animals. From these data, it was suggested by Hauser (1964) that sulfatides were synthesized from cerebrosides. Subsequently, it was reported that S-35 sulfate was incorporated in vitro and in vivo into developing rat brain, with a peak of incorporation reached when the animal was between 18-22 days old (McKhann et al., 1965). In vitro addition of cerebroside to the incubation mixture was reported to "markedly" increase sulfate incorporation into sulfatides. "A variety of other lipids" (not identified) had no effect. The highest incorporation of S^{32}-sulfate was in the microsomal fraction. It would seem that sulfatides are synthesized from cerebrosides in rat brain, but the evidence is far from conclusive.

c. Biosynthesis of ceramide diglycoside.

Glucocerebroside has been shown to react with UDP-galactose to yield a ceramide diglycoside, gal-glc-Cer. This reaction is catalyzed by rat spleen preparations (Hauser, 1967).

d. Biosynthesis of oligoglycosides and gangliosides.

Ceramide dihexosides may be pivotal in glycolipid biosynthesis, for several alternate pathways for the synthesis of larger glycolipids have been reported, all of which have gal-glc-Cer as a precursor.

(i) Ceramide trihexoside precursor.

The same rat spleen preparation which catalyzed the synthesis of ceramide dihexoside from cerebroside (Hauser, 1967) also catalyzed the biosynthesis of gal-gal-glc-Cer from gal-glc-Cer and UDP-galactose (Hildebrand and Hauser, 1968).

(ii) Ceramide dihexoside as a ceramide trihexoside (containing N-acetyl hexosamine) precursor.

gal-glc-Cer + UDP-N-acetylgalactosamine ⟶ galNAc-gal-glc-Cer + UDP. This reaction has been reported to be catalyzed by a rat brain preparation (Handa and Burton, 1966) and by embryonic chicken brain preparations (Steigerwald et al., 1966). According to the latter investigators, the ceramide dihexoside accepted both N-acetyl galactosamine and sialic acid, but N-acetylgalactosamine incorporation was only 10% of sialic acid incorporation.

(iii) Ceramide dihexoside as a ganglioside precursor.

Steigerwald et al. (1966) have reported that embryonic chicken brain preparation also catalyzed the following reactions.

1. gal-glc-Cer + CMP-NANA ⟶ galglcCer (hematoside)
   NANA

2. gal-glc-Cer + UDP-galNAc $\xrightarrow[Mn^{++}]{\text{detergent}}$ galNAc-gal-glc-Cer
   NANA NANA (Tay-Sachs' ganglioside)

Their ganglioside migrated as authentic Tay-Sachs' ganglioside on TLC in four solvent systems and the N-acetyl hexosamine:glucose: galactose:sialic acid:sphingosine ratio was 1.00:0.93:0.92:1.05:0.66.

Although no data were given in support of this, these investigators stated that their embryonic chicken brain preparation also catalyzed these additional reactions.

3. Tay-Sachs' ganglioside + UDP-gal ⟶ monosialoganglioside

4. monosialoganglioside + CMP-NANA ⟶ disialoganglioside

The evidence accumulated thus far by a number of different investigators, mentioned in the foregoing discussion, indicates that in rat brain, embryonic chicken brain, and rat spleen, the glycolipids were synthesized by the addition of one glycose unit at a time to a precursor molecule. There was also evidence that gangliosides were indeed synthesized from asialoglycolipids. Finally, the carrier molecule for glucose, galactose, and N-acetyl galactosamine was UDP and the carrier molecule for N-acetylneuraminic acid was CMP.

In dog whole small intestinal tissue, ceramide mono-, di, tri-, and pentaglycosides, but not tetraglycosides, are present (Vance et al., 1966; McKibbin, 1969). This opens the possibility that two glycose molecules (i.e., two galactosaminyl molecules) may be added as a unit. An alternate possibility, which seems more likely, is that the activity of the enzyme which catalyzes the conversion of the tetra- to the pentaglycoside is relatively high.

2. Catabolism of Glycolipids.

Just as the biosynthesis of glycolipids appears to occur in a stepwise fashion, i.e., one glycose unit added at a time, so too does the degradation of glycolipids, with each terminal glycose unit being cleaved sequentially.

a. Neuraminidases or sialidases.

Neuraminidase which cleaves sialic acid from glycolipids containing one, two or three sialic acid residues have been isolated from calf brain (Leibovitz and Gatt, 1968). This enzyme (or enzymes) had little activity toward sialyllactose or "a glycoprotein" preparation rich in sialic acid. These findings suggested that this enzyme showed substrate specificity for glycolipids. Additionally, the enzyme had very little activity without detergents, had a pH optimum of 4.4, and removed the sialic acid of Tay-Sachs' ganglioside only after removal of the terminal galactosamine residue with hexosaminidase from calf brain (Frohwein and Gatt, 1967a).

Listed below are some purified gangliosides which are substrates for crude calf brain neuraminidase and the products of the enzyme's action according to Leibovitz and Gatt (1968).

SUBSTRATE	PRODUCT
gal-galNAc-gal-glc-Cer[1] NANA NANA NANA	gal-galNAc-gal-glc-Cer[2] (MAJOR PRODUCT) NANA NANA
gal-galNAc-gal-glc-Cer[2] NANA NANA	Not determined, but sialic acid released.

1. NANA-2,3-Galβ-1,3-GalNAcβ-1,4-(NANA-2,8-NANA-2,3)-Galβ-1,4-Glcβ 1,1-(2-N-acyl)-sphingosine.

2. Galβ-1,3-GalNAc-β-1,4-(NANA-2,8-NANA-2,3)-Gal-β-1,4-Glc-β 1,1-(2-N-acyl)-sphingosine.

gal-galNAc-gal-glc-Cer[3] NANA NANA	Not determined, but sialic acid released.
gal-galNAc-gal-glc-Cer[4] NANA	No sialic acid released.
galNAc-gal-glc-Cer[5] NANA	No sialic acid released.
gal-gal-glc-Cer[6] NGNA	Not determined, but sialic acid released.

Although it is not known whether one or more neuraminidases are involved, the conclusion from these results is that all sialic acid residues can ultimately be hydrolyzed by the crude calf brain enzyme preparation if the sialic acid residue is linked to either a galactose moiety which has a free hydroxyl group on carbon 4 or to a sialic acid moiety. Possibly, for activity, neuraminidase(s) requires that the sialic acid moiety be glycosidically linked to a carbon atom which itself is bonded to two adjacent hydroxylated carbon atoms.

b. N-Acetyl hexosaminidases.

There have been two ceramide tetraglycosides containing a terminal N-acetylgalactosamine isolated: a "globoside" from human erythrocyte stroma (Makita et al., 1964; Yamakawa et al., 1965) and the "Tay-Sachs' ganglioside" from human brain tissue (Ledeen and Salsman, 1965). A calf brain enzyme, β-N-acetylhexosaminidase, cleaved the terminal N-acetylgalactosamine moiety of both these glycosphingolipids (Frohwein

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3. NANA-2,3-Gal-β 1,3-GalNAc-β 1,4-(NANA-2,3)-Gal-β 1,4-Glc-β 1,1-(2-N-acyl)-sphingosine.

4. Galβ 1,3 + GalNAc-β 1,4-(NANA-2,3)-Gal-β 1,4-Glcβ 1,1-(2-N-acyl)-sphingosine.

5. GalNAc-β 1,4-(NANA-2,3)-Gal-β 1,4-Glcβ 1,1-(2-N-acyl)-sphingosine.

6. NGNA-2,3-Gal-β 1,4-Glc-β 1,1-(2-N-acyl)-sphingosine.

and Gatt, 1967b). This enzyme also cleaved the terminal N-acetyl-galactosamine moiety from a ceramide triglycoside (galNAc-gal-glc-Cer). The triglycoside was prepared by Gatt and Rapport (1966) from an ox brain ceramide tetraglycoside. The "Tay-Sachs' ganglioside" inhibited the activity of this enzyme when the ceramide triglycoside was the substrate.

The fact that the enzyme was particulate, harvested from the subcellular fraction between 1000g and 20,000g, and had a pH optimum of 3.8 (Frohwein and Gatt, 1967a,b) suggested that the enzyme was lysosomal in origin. Two other enzymes, β-N-glucosaminidase and β-N-galactosaminidase, obtained from the 100,000g supernatant of calf brain, failed to cleave the terminal N-acetylgalactosamine from the "Tay-Sachs' ganglioside", the "globoside" or the ceramide triglycoside. The substrate specificities of the three enzymes mentioned above are compared (Frohwein and Gatt, 1967a).

SUBSTRATE	ENZYME		
	1*	2*	3*
p-Nitrophenyl O-β-(2-acetamido-2-deoxy)-D-glucopyranoside	100%	100%	100%
p-Nitrophenyl O-β-(2-acetamide-2-deoxy)-D-galactopyranoside	22%	4%	1100%
β-phenyl N-acetylglucosaminide	54%	100%	85%

c. β-Galactosidases.

There were several crude enzyme preparations isolated from brain tissue which hydrolyzed terminal galactose units from glycolipids. Since the enzyme preparations were only partially purified and often

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*1. β-N-acetylhexosaminidase
2. β-N-acetylglucosaminidase
3. β-N-acetylgalactosaminidase

showed activity toward several substrates, it is possible that the preparations contained more than one enzyme, as suggested by Bowen and Radin (1968b).

Gatt and Rapport (1966b) have described the isolation of a β-galactosidase which has subsequently been shown to hydrolyze the terminal galactose unit from: gal-galNAc-gal-glc-glc-Cer, gal-galNAc-(NANA) gal-glc-Cer, gal-gal-glc-Cer, gal-glc-Cer, gal-gal-Cer, but showed no activity toward gal-Cer or psychosine (galactosylsphingosine) (Gatt and Rapport, 1966a; Gatt, 1967). This enzyme required the detergents, cholate or taurocholate, for activity. Additional detergent, in the form of Triton X-100, further enhanced activity (Gatt and Rapport, 1966a). This enzyme also hydrolyzed the glycosidic linkage of lactose. A β-galactosidase was also isolated from calf brain, but has not been studied as extensively as that isolated from rat brain. The procedures used in the isolation of these enzymes were suggestive of a lysosomal location.

The enzyme had the highest specific activity in the subcellular fraction that sedimented between 1,000 and 13,000g, a pH optimum of 4.5, and was stimulated by taurocholate. Its subcellular distribution also suggested lysosomal origin.

d. <u>β-Glucosidase</u>.

An ox brain β-glucosidase has been reported which catalyzed the conversion of glucosyl ceramide to ceramide and glucose (Gatt and Rapport, 1966b; Gatt, 1966a). This enzyme had a pH optimum of 4.5, and was stimulated by a mixture of detergents such as Triton X-100 and sodium cholate or taurocholate. This enzyme hydrolyzed nitrophenyl

β-D-glucopyranoside but not the β-glucosidic linkages of cellobiose, gentiobiose, methyl β-glucoside or salicin.

e. Ceramide monoglycosidases.

An enzyme which catalyzed the breakdown of both glucosyl- and galactosyl ceramide was described in rat whole small intestinal tissue (Brady et al., 1965a). It was localized between the 600g and 8400g pellet and was membrane bound. Both gluco- and galactosphingosine inhibited the enzyme (68% and 58% respectively).

An enzyme which is specific for the breakdown of glucocerebroside to glucose and ceramide was described in the 100,000g supernatant of rat and human splenic tissue (Brady et al., 1965b). In the most highly purified preparations, this enzyme was inactive toward galactocerebroside and o-nitrophenyl-β-D-galactopyranose. P-nitrophenyl-β-D-glucopyranose was cleaved at about 40% the rate of glucocerebroside, but the ratio of enzymatic activity with p-nitrophenyl-β-D-glucopyranose as substrate compared to that with glucocerebroside decreased during purification. This suggested that the two compounds were substrates for different enzymes.

An enzyme from the 100,000g supernatant of pig brain homogenate which catalyzed the hydrolysis of galactocerebroside to galactose and ceramide was reported (Hajra and Radin, 1965; Hajra et al., 1966). It had a pH optimum of 4.5 and sodium cholate or taurocholate were essential for activity of the enzyme. The enzyme preparation liberated ceramide from gal-glc-Cer, glc-Cer, and cerebronoyl psychosine (galactosyl ceramide with hydroxy fatty acid).

f. Ceramide triglycosidase.

An enzyme which catalyzed the hydrolysis of the terminal galactose moiety of human kidney gal-gal-glc-Cer was demonstrated in brain, liver,

kidney, spleen and small intestine of rat (Brady et al., 1967b). This enzyme was specific for the ceramide trihexoside, having no activity on gal-glc-Cer, gal-Cer, or glc-Cer. In rat small intestine, 25% of the total activity found in the homogenate was present in the 700g pellet while 57% was found in the 12,000g pellet, a subcellular fraction associated with lysosomes in liver (Bjorntorp et al., 1965). The low pH optimum (5.0) and the subcellular location were suggestive of a lysosomal association, although this was not investigated. The intestinal enzyme is membrane bound and non-competitively inhibited by glucosylsphingosine. The ceramide mono- and dihexoside failed to inhibit the enzyme. A deficiency of a similar enzyme in kidney has been implicated as the defect in Fabry's disease, which is characterized in part by an accumulation of ceramide trihexoside (Brady et al., 1967a).

g. Ceramidase.

A ceramidase was reported in rat brain which catalyzed not only the breakdown of ceramide to fatty acid and sphingosine, but the synthesis of ceramide from these compounds (Gatt, 1963; 1966b). This enzyme was found in a subcellular fraction which was isolated between 1500-15000g and appeared to be membrane bound. Whether or not this enzyme was contained within lysosomes was not determined. Ceramidase activity was also found in rat liver and kidney homogenates.

3. Summary.

The subcellular distribution of the enzymes involved in the biosynthesis of glycolipids appeared to be associated with the microsomal fraction. Catabolic enzymes were associated with several different subcellular fractions. These were the supernatant and particulate

fractions which sedimented at lower gravity forces (1000-20,000g) than those associated with the microsomal fractions. The catabolic enzymes associated with the particulate fractions required treatment with a detergent or sonication for release and were probably lysosomal in origin.

PART TWO: EXPERIMENTAL

CHAPTER VI. CELL FRACTIONATION MUCOSA FROM DOG SMALL INTESTINE

1. Introduction.

Cell fractionation offers a method of subdividing the cell into less complex fractions which nevertheless retain some structural and functional integrity. Reference to cell fractionation of dog intestine was lacking in the literature, although there were several articles dealing with the general principles of cell fractionation (Schneider, 1959; Allfrey, 1959; Schneider and Kuff, 1964; Fleischer and Rouser, 1965; Mathias, 1966).

Articles dealing with cell fractionation of intestinal tissue of other animals, i.e., man (Dawson and Isselbacher, 1960), guinea pig (Glover and Green, 1957; Hubscher and Sherratt, 1962), rat (Dawson and Isselbacher, 1960; Robinson, 1962; Hubscher and Sherratt, 1962; Brady et al., 1965a; Allard et al., 1957), cat (Hubscher et al., 1962; Hubscher and Sherratt, 1962) failed to show the degree of purity of the subcellular fractions.

There are several important principles which must be considered when tissue cell fractionation is undertaken (Schneider, 1959):

(1) selection of tissue

(2) method of cell homogenization

(3) selection of centrifugal forces for cell fractions

(4) determination of purity and cytological identification of fractions.

These principles will be discussed briefly in the following paragraphs.

2. Selection of Tissue.

Tissues which are made up of one cell type present a less complicated problem for cell fractionation than those containing a variety of cell

types. Because of the many cell types associated with the various tissues of the intestine, it was natural to seek a way to eliminate some of this variety.

The method chosen was a mechanical scraping of the mucosa lying between the ampulla of Vater and the appendix. Our initial observations, and those of others (Glover and Green, 1957), indicated that only the outermost portions of the villi of the intestinal mucosa were obtained by this method. The fact that a recent investigation (Vance et al., 1966) indicated that the major portion of the intestinal glycolipid was derived from the mucosa made this method even more attractive.

3. Method of Cell Homogenization.

The object of cell fractionation is the rupture of the plasma membrane without disrupting the intracellular structures. There are several instruments now available which are routinely used to accomplish this end:

(a) Waring blendor

(b) Teflon pestle tissue grinder* (Potter-Elvehjem)

(c) Dounce ball-type homogenizer**

The method using the Waring blendor had an advantage over the other methods mentioned above in that large amounts of tissue can be used with a minimum of effort. This method, however, can be damaging to subcellular particles (Mathias, 1966) and was one of the procedures used to activate lysosomes (Gianetto and de Duve, 1955).

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* A. H. Thomas Co., No. 4288-B, Philadelphia 5, Pennsylvania.

** Blaessig Glass Specialties, No. 518, 45 Atlantic Ave., Rochester 9, New York.

The other two methods were supposedly less damaging to subcellular particles (Mathias, 1966) but the three methods were not compared on the same tissues with all subcellular fractions. There is, however, an investigation (Witter et al., 1953) which compared ATP-ase activity of rat liver mitochondria prepared by the Waring blendor and a "pestle-type" homogenizer. Yields of mitochondria were not discussed, but the Waring blendor method produced mitochondria with slightly more ATP-ase activity than those produced by the "pestle-type" homogenizer. ATP-ase activity is related to mechanical damage of the mitochondria (Robert Glaze, private communication).

4. Selection of Centrifugal Forces for the Cell Fractions.

While the general procedures for cell fractionation by the differential centrifugation method are similar for most tissues, each tissue is unique and a method developed for one tissue may have to be modified if applied to another (Schneider, 1959).

Our initial approach to this problem was influenced by a procedure used on human liver (Bjorntorp et al., 1965) in which the homogenate in 0.25M sucrose was spun at a number of increasing centrifugal forces to give a total of seven fractions plus the supernatant. In the present study, the final choice of centrifugal force used to obtain the nuclear fraction, however, was arrived at somewhat arbitrarily and was retained primarily because the nuclear pellet produced was quite manageable.

5. Determination of Purity and Cytological Identification of Fractions.

Since one of the purposes of this investigation was to establish whether or not certain glycolipids isolated from dog intestine are related to a particular cell fraction, it was necessary to demonstrate the degree to which one cell fraction was contaminated by another. This

is best accomplished if a "balance sheet" is used in which the concentration of various enzymes, DNA and protein from all the fractions are tabulated and compared with total concentration in the homogenate (Schneider, 1959). Once some relative purification has been obtained by this method, light and electron microscopy can be used as additional checks for purity, as well as to determine the morphology of the various components of the fractions. This important optical monitoring of the subcellular fractions, however, will have to be done in connection with future work.

6. Materials and Methods.

Three dogs were sacrificed with an overdose of sodium pentobarbital, the entire small intestine was removed and immediately immersed in ice. The intestine was cut into small segments approximately six inches long and each segment opened longitudinally, washed with tap water and placed in iced 0.25M sucrose. Each segment was immersed in iced 0.9% NaCl and scrubbed with gauze to remove adhering mucous, then placed back into iced 0.25M sucrose. The mucosa was stripped off with a glass slide and placed immediately into ice cold 0.25M sucrose in a 600 ml. beaker (approximately 30 grams of mucosa in 300 ml. of sucrose per beaker). Approximately 120-160 grams of intestinal mucosa, which consisted of the outermost portion of the intestinal villi, were usually obtained from a single small intestine. The mucosal tissue was homogenized with a Potter-Elvehjem homogenizer (Teflon pestle) with a 300 ml. capacity and squeezed through two thicknesses of cheese cloth. 1000 ml. of homogenate were used for obtaining the subcellular fractions. The remainder of the homogenate (except for 35 ml. which were used for enzyme and chemical assays to determine purity of fractions) was lyophilized. The

lyophilized residue was extracted by refluxing for 1-1/2 hours with 95% ethyl alcohol - ethyl ether in 3:1 ratio (Bloor's reagent). This extract was used for lipid determinations.

The nuclear pellet was obtained by centrifuging 1000 ml. of homogenate at 1.1 x 10^3g for 30 minutes. The pellet obtained was made to 900 ml. with 0.25M sucrose, resuspended and centrifuged again at 1.1 x 10^3g for 30 minutes. The pellet obtained from the second centrifugation was resuspended to 1000 ml. in 0.25M sucrose.

The supernatants from the two nuclear pellets were combined and centrifuged at 7.7 x 10^3g for 30 minutes. The pellet obtained was resuspended to 300 ml. in 0.25M sucrose and centrifuged again at 7.7 x 10^3g for 30 minutes. This second mitochondrial pellet was resuspended to 250 ml. in 0.25M sucrose.

The two mitochondrial supernatants were combined and centrifuged at 105.6 x 10^3g for 30 minutes to obtain a microsomal pellet and the supernatant. The microsomal pellet was resuspended to 250 ml. in 0.25M sucrose and the volume of the supernatant recorded. 35 ml. were saved from the resuspended nuclear, mitochondrial, microsomal pellets and the supernatant for assays to determine purity of fractions. The remainder was lyophilized and extracted for glycolipid determinations as described for the homogenate.

The procedure followed for obtaining the subcellular fractions of dog intestinal mucosa is outlined in Figure 1.

a. Chemical and enzyme assays used to determine purity of fractions.

DNA was determined by the diphenylamine method of Schneider (Schneider, 1945). Cytochrome oxidase was assayed polarographically by measuring oxygen consumption with a Clark electrode at 29-30°C in

an assay method described by Schnaitman and coworkers (1967). The Clark electrode was calibrated using distilled water as a standard. No attempt was made to determine the true oxygen saturation in the assay medium used. Acid phosphatase was assayed as described by Appelmans and coworkers (1955); glucose-6-phosphatase was determined by the method of de Duve and coworkers (1955). Inorganic phosphate released in both the acid phosphatase and glucose-6-phosphatase assay methods was determined by the method of Fisk and Subbarow (1925). Total nitrogen was determined by the method of Koch and McMeekin (1924) modified for lipid material (McKibbin and Taylor, 1949).

Enzyme assays were done within 24 hours after fractionation was completed on the homogenate and subcellular fractions which had been maintained at 0°C. DNA and nitrogen determinations were done on subcellular fractions which had been frozen at -27°C for periods of three days to several weeks.

7. Results.

The results of cell fractionations on the intestinal mucosa of three dogs are presented in tabular form in Tables V through XII.

From Table V it can be seen that the small intestine, on the average, yielded 143 gm. of mucosa (wet weight). This did not represent the entire weight of mucosa, but only that part which could be scraped off with a glass slide, i.e., the outermost portions of the intestinal villi. It was found that there was an average of 11.7 mg. of nitrogen per gram of wet mucosa. The values for the three individual dogs fell within 9% of the mean value. Rat intestinal mucosa (one experiment) was reported to contain 22.7 mg. of nitrogen per gram of tissue (Allard et al., 1957).

A report on rat intestinal mucosa (which was collected and homogenized in essentially the same manner as reported in this paper) gave the following percentage yields for nitrogen in the subcellular fractions (one experiment): nuclear, 10.5; mitochondrial, 21.6; microsomal, 22.4; supernatant, 44.4; recovery, 98.7 (Allard et al., 1957). In Table VI-B, the percentages of nitrogen in the mitochondrial fraction (3.7%) and microsomal fraction (9.3%) were lower than values reported for rat intestinal mucosa. This difference possibly was due to incomplete cell breakage and to the higher gravity forces used in dog intestinal mucosa for the nuclear and mitochondrial fractions than those used in rat mucosa. In general, the reproducibility from dog to dog was good in terms of percent nitrogen yielded per fraction, with the greatest variation occurring in the nuclear and supernatant fractions.

In Table VII it can be seen that most of the DNA is confined to the nuclear fraction. Although some of this was accounted for by unbroken cells, the specific concentration of DNA in terms of mg. of deoxyadenosine per mg. nitrogen (Table VII-C) was increased twofold for the nuclear fraction when compared to the homogenate. Further purification of the nuclear fraction will be necessary before it can be considered to be representative of nuclei, however.

The values for cytochrome oxidase activity are presented in Table VIII. The average recovery of total cytochrome oxidase activity in the mitochondrial fraction was 29%. This low percentage was a result of incomplete cell breakage and the relatively high gravitational force used for the nuclear pellet (1.1×10^3). The specific activity of the mitochondrial fraction was 7.9 times that of the

homogenate, an almost eightfold purification of the mitochondrial fraction when compared to the homogenate (Table VIII-C). The nuclear fraction showed 1.4 times the specific activity of the homogenate. The supernatant, while recorded as having no activity (Table VIII-A), actually had inhibitory activity as the values obtained for the supernatant were routinely lower than the blank used for that assay.

The acid phosphatase activity recorded in Table IX (Part 1) was unbound activity. One experiment (Dog 3) was performed in which the homogenate and subcellular fractions were preincubated for 3 hours at 37°C before substrate was added. This procedure gave total activity of acid phosphatase (Appelmans et al., 1955). When compared with unbound activity, the total acid phosphatase activity was increased in the homogenate and all the subcellular fractions with the exception of the supernatant, which remained the same (see Table IX (Part 2)-A). On subsequent animals, only unbound activity was determined. 52% of the acid phosphatase activity occurred in the supernatant (Table IX (Part 1)-A). The specific activity, however, was greatest in the microsomal fraction, being greater than the homogenate by a factor of 1.7 (Table IX (Part 1)-C). There was considerable variation in specific activity in the microsomal fraction, and the average value for the specific activity was not representative of individual dogs. The percentage distributions were perhaps a better index than specific activities for determining the reproducibility of the cell fractions from dog to dog (Table IX (Part 1)-B). The average values were more representative of individual dogs. The specific activity of the supernatant fraction was greater by a factor of 1.3 when compared to that of the homogenate (Table IX (Part 1)-C).

The glucose-6-phosphatase activity seemed to present two distinct patterns. Dogs 4 and 6 had roughly three times the activity that Dog 3 had as judged from the homogenates (Table X-A). Some individual variation was seen in the microsomal fraction, where the activity of Dog 4 was 4.7 fold greater and that of Dog 6 was 6.8 fold greater than Dog 3. The supernatants for all three dogs had similar total activities. The nuclear fractions of Dogs 4 and 6 also showed greater activity than Dog 3, but this fraction represented whole cells as well as nuclei. These two distinct patterns were also seen in the percentage distributions of enzyme (Table X-B). In Dog 3, most of the activity appeared in the nuclear and supernatant fractions; in Dogs 4 and 6, most appeared in the nuclear and the microsomal fractions. The specific activity of glucose-6-phosphatase was less in the nuclear and supernatant fractions and greater in the microsomal fractions than in the homogenate (Table X-C). The activity in the microsomal fraction was 1.8, 3.5 and 4.9 fold greater in Dogs 3, 4, and 6 respectively when compared to the homogenate. In the mitochondrial fraction, the specific activity was lower in Dog 3 and higher in Dogs 4 and 6 when compared to the homogenate.

Table XI is a summary of the percentage distributions and recoveries of the various parameters measured. The nuclear fraction had almost all the DNA and approximately 2/3 of the cytochrome oxidase activity. The nuclear pellet was contaminated by all other fractions. It represented a partially purified fraction, however, because roughly 1/3 of the cytochrome oxidase, 3/4 of the acid phosphatase, and 2/3 of the glucose-6-phosphatase had been removed from the homogenate in producing the nuclear pellet. The mitochondrial

fraction was small, representing only 3.7% of the nitrogen, and so percentages of all enzyme activities and DNA might be expected to be small. Cytochrome oxidase percentage, however, was quite high when compared to DNA and other enzyme activity percentages. The microsomal fraction had minor amounts of DNA and cytochrome oxidase, less than 1/7 of the acid phosphatase activity and more glucose-6-phosphatase activity than any other marker, although only 1/3 of the activity was represented in this fraction. The supernatant had minor amounts of DNA, no cytochrome oxidase activity, about 1/2 of the acid phosphatase activity and 1/5 of the glucose-6-phosphatase activity. The average recoveries for three experiments of DNA, nitrogen, and enzyme activities were very good, the lowest being 93.5% for glucose-6-phosphatase.

Table XII is a summary of the average ratios of percent of DNA concentration or enzyme activity per percent nitrogen. It was from these data that some idea of relative purification of fractions and cell breakage was best obtained. The homogenate had ratios of 1:1:1:1. The nuclear fraction was best represented by DNA although cytochrome oxidase specific activity was high. The nuclear fraction was the least pure of all the fractions. Some whole villi with intact cells were seen in this fraction on microscopic examination. The mitochondrial fraction, from this table, appeared to be the purest fraction as cytochrome oxidase showed the greatest increase in specific activity of any of the parameters measured. The microsomal fraction was best represented by glucose-6-phosphatase and would be the best place to study structures associated with this enzyme. Unbound acid phosphatase specific activity was highest in the microsomal fraction, although this activity was high in the supernatant fraction also. The

lysosomes, which are often monitored by acid phosphatase activity, were reported to be distributed in the mitochondrial and to a lesser extent in the microsomal fraction in rat and human liver (Bjorntorp et al., 1965; Berthet and de Duve, 1951). However, this activity may not be a completely reliable indicator for all lysosomes (Beaufay et al., 1964). In dog intestinal mucosa, unbound acid phosphatase activity appeared to be most abundant in the supernatant fraction. Purification of lysosomes should begin in this fraction only if the acid phosphatase activity could be shown to be associated with intact lysosomes.

8. Discussion.

Cell fractionation of intestinal mucosa of any mammalian species with the monitoring of the classical subcellular markers has not been reported. This paper provides such information for dog small intestinal mucosa and establishes a base line for cell fractionation of dog intestinal mucosa. With this information, individual subcellular fractions can be further purified in order to study the glycolipid composition without the necessity of complete cell fractionation each time. This cell fractionation procedure represents an important first step in the purification of nuclei, mitochondria, membranes associated with glucose-6-phosphatase activity, and lysosomes.

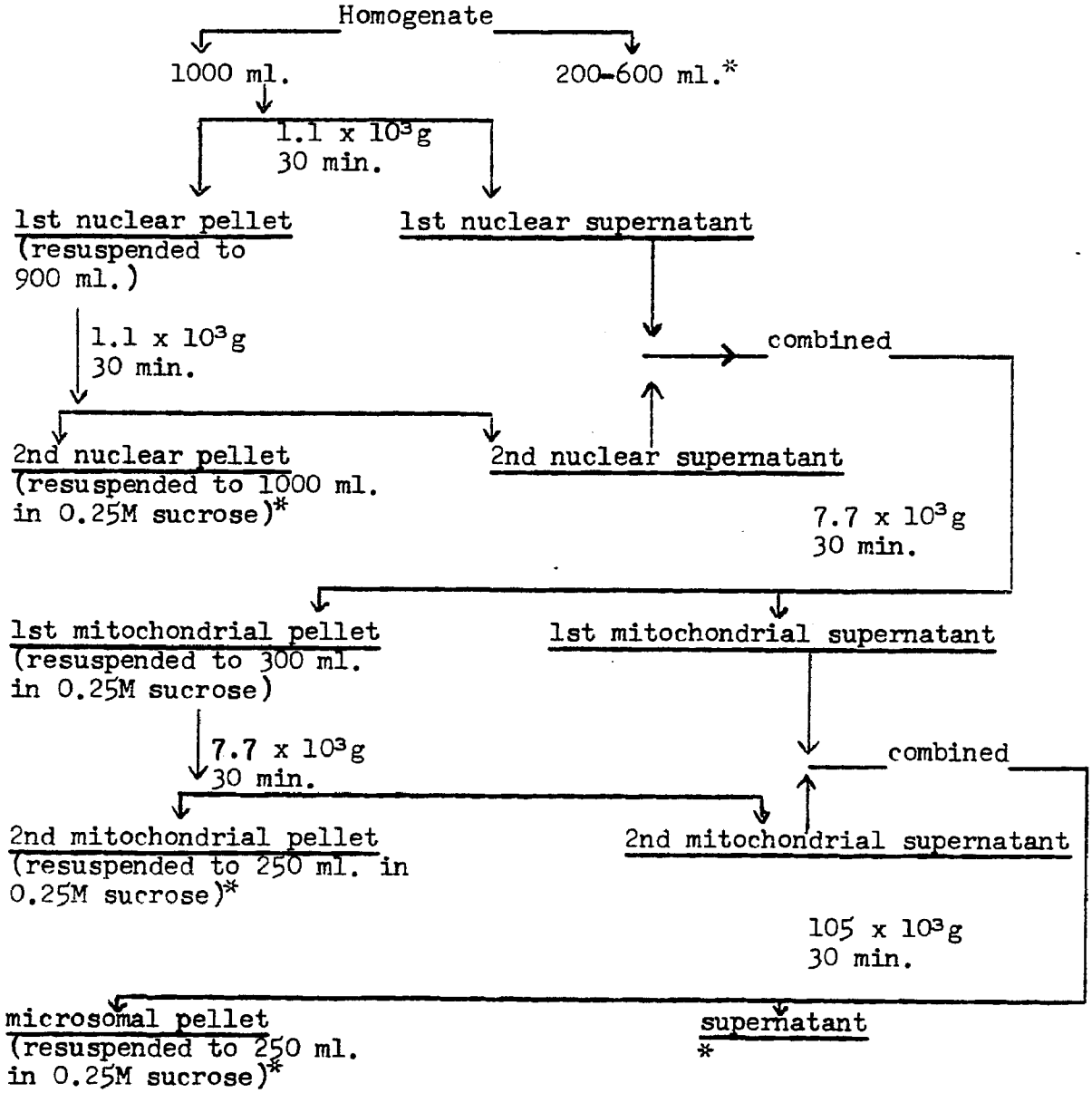
In the nuclear fraction, the high cytochrome oxidase activity could be accounted for in part by incomplete cell breakage and in part by the relatively high gravities used to obtain the nuclear pellet. 600-800g are more frequently used to obtain the nuclear pellet. 1.1×10^3g, however, yielded a nuclear pellet which was much more manageable than that obtained by using 800g. The nuclear pellet might be reduced in cytochrome oxidase by using 800g rather than 1.1×10^3g.

If bound acid phosphatase activity can be taken as an index of intact lysosomes, the fact that total and unbound activities were the same in the supernatant (one experiment, Dog 3) might suggest that lysosomes, as discrete organelles, were not present in the supernatant. This observation must be resolved by further experimentation and electron microscopy if the glycolipid constituents of lysosomal membranes are to be determined. If lysosomal membranes are not intact in the supernatant, then the microsomal fraction would provide a better source for the purification of intact lysosomes.

Cell breakage is estimated to be roughly 66% on the basis that approximately 1/3 of the acid phosphatase and glucose-6-phosphatase activity remained in the nuclear fraction. Additional evidence for this estimate could be obtained if centrifuging the homogenate at 600-800g would produce a nuclear pellet with 1/3 less cytochrome oxidase activity than the nuclear pellet produced at 1.1×10^3g. This, however, must await future experimentation.

Figure 1. Scheme for Cell Fractionation of Dog Intestinal Mucosa.

120-160 gm. mucosa, wet weight, (1 gm. mucosa/10 ml. 0.25M sucrose) were homogenized with a Potter-Elvehjem homogenizer (Teflon pestle) with ten strokes and squeezed through two thicknesses of cheese cloth. This yielded 1200-1600 ml. of homogenate.



All centrifugal gravities are for the maximum tube radii.

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* 35 ml. saved for assays; remainder lyophilized and extracted for glycolipid determinations.

Table V. Mg. Nitrogen per Gm. of Intestinal Mucosa (wet weight) as Calculated from Homogenate.

Dog #	3	4	6	Average	Average Protein (gm/gm tissue)
Nitrogen (mg/gm)	11.7	10.7	12.8	11.7	0.073
Total gms. mucosa (wet weight)	126	161	143	143	-----

Table VI. Subcellular Distribution of Nitrogen.

A. Mg. nitrogen per ml. of homogenate or subcellular fraction. Calculations were made with the homogenate and subcellular fractions adjusted to a total of 1000 ml. each.

Dog #	3	4	6	Average
Fraction				
Homogenate	1.48	1.72	2.00	1.73
Nuclear	0.649	0.920	0.859	0.809
Mitochondrial	0.069	0.055	0.065	0.063
Microsomal	0.157	0.154	0.169	0.160
Supernatant	0.705	0.587	0.752	0.681

B. Percentage distribution and recoveries of nitrogen in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	
Nuclear	43.9	53.4	42.5	46.6
Mitochondrial	4.7	3.2	3.3	3.7
Microsomal	10.6	8.9	8.5	9.3
Supernatant	47.7	34.1	37.6	39.8
Total Recovery	107	99.6	91.9	99.5

Table VII. Subcellular Distribution of DNA.

A. Mg. desoxyadenosine per ml. of homogenate or subcellular fraction. Calculations were made with the homogenate and subcellular fractions adjusted to a total of 1000 ml. each.

Dog #	3	4	6	Average
Fraction				
Homogenate	0.850	0.735	1.120	0.902
Nuclear	0.735	0.750	1.020	0.835
Mitochondrial	0.016	0.014	0.007	0.012
Microsomal	0.019	0.019	0.017	0.018
Supernatant	0.053	0.030	0.042	0.042

B. Percentage distribution and recoveries of DNA in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	
Nuclear	86.5	102	91.1	93.2
Mitochondrial	1.9	1.9	0.6	1.5
Microsomal	2.2	2.6	1.5	2.1
Supernatant	6.2	4.1	3.8	4.7
Total Recovery	96.8	111	97.0	102

Table VII (Continued).

C. Specific concentration in mg. desoxyadenosine per mg. nitrogen.

Dog #	3	4	6	Average
Fraction				
Homogenate	0.574	0.427	0.560	0.520
Nuclear	1.13	0.815	1.19	1.04
Mitochondrial	0.23	0.26	0.11	0.20
Microsomal	0.12	0.12	0.10	0.12
Supernatant	0.075	0.051	0.056	0.061

Table VIII. Subcellular Distribution of Cytochrome Oxidase Activity.

A. µGm. atoms O/min/ml of homogenate or subcellular fraction. Calculations were made with the homogenate and subcellular fractions adjusted to a total of 1000 ml. each.

Dog #	3	4	6	Average
Fraction				
Homogenate	10	9.2	7.2	8.8
Nuclear	6.2	7.4	4.4	6.0
Mitochondrial	3.4	2.7	1.7	2.6
Microsomal	0.65	0.26	0.29	0.40
Supernatant	-	-	-	-

B. Percentage distribution and recoveries of cytochrome oxidase activity in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	
Nuclear	62	80	61	68
Mitochondrial	34	29	24	29
Microsomal	6.5	2.8	2.4	3.9
Supernatant	-	-	-	-
Total Recovery	103	112	88	100

Table VIII (Continued).

C. Specific activity in μgm. atoms O per mg. nitrogen for 1 minute incubation at 29-30°C.

Dog #	3	4	6	Average
Fraction				
Homogenate	6.7	5.4	3.7	5.3
Nuclear	9.5	8.1	5.2	7.6
Mitochondrial	49	49	27	42
Microsomal	4.1	1.7	1.7	2.5
Supernatant	-	-	-	-

Table IX (Part 1). Subcellular Distribution of Unbound Acid Phosphatase Activity.

A. μGm. phosphorus released per ml. of homogenate or subcellular fraction for 15 minutes incubation at 37°C. Calculations were made with the homogenate and subcellular fractions adjusted to a total of 1000 ml. each.

Dog #	3	4	6	Average
Fraction				
Homogenate	77.1	98.6	121	99.0
Nuclear	22.3	28.8	28.3	26.5
Mitochondrial	2.7	2.8	5.0	3.5
Microsomal	7.5	13.4	26.0	15.6
Supernatant	41.8	55.2	56.9	51.3

B. Percentage distribution and recoveries of acid phosphatase activity in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	
Nuclear	29.0	29.2	23.4	27.2
Mitochondrial	3.5	2.8	4.1	3.5
Microsomal	9.7	13.5	21.4	14.9
Supernatant	54.4	56.0	47.0	52.4
Total Recovery	96.5	102	95.9	98.0

Table IX (Continued).

C. Specific activity in mg. phosphorus released per mg. of nitrogen for 15 minutes incubation at 37°C.

Dog #	3	4	6	Average
Fraction				
Homogenate	0.052	0.057	0.061	0.057
Nuclear	0.034	0.031	0.033	0.033
Mitochondrial	0.041	0.051	0.077	0.056
Microsomal	0.048	0.087	0.154	0.096
Supernatant	0.059	0.094	0.076	0.076

Table IX (Part 2). Subcellular Distribution of Acid Phosphatase for Dog #3 Only. Total, Unbound and Bound Acid Phosphatase Activity.

A. Acid phosphatase activity in μgm. phosphorus released per ml. of homogenate or subcellular fraction for 15 minutes incubation at 37°C. Calculations were made with the homogenate and subcellular fractions adjusted to a total of 1000 ml. each.

(Total activity - unbound activity - bound activity)

Dog #3 Fraction	Total	Unbound	Bound
Homogenate	112	77.1	34.7
Nuclear	30.9	22.3	8.6
Mitochondrial	4.0	2.7	1.3
Microsomal	10.8	7.5	3.3
Supernatant	42.0	41.8	0.2

B. Specific activity in mg. phosphorus released per mg. of nitrogen for 15 minutes incubation at 37°C. Percentage distributions and recoveries of acid phosphatase activity in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #3	Specific Activity		Percentage Distribution	
Fraction	Unbound	Bound	Unbound	Bound
Homogenate	0.052	0.023	100	100
Nuclear	0.034	0.013	29.0	27.6
Mitochondrial	0.041	0.019	3.5	3.5
Microsomal	0.048	0.021	9.7	9.6
Supernatant	0.059	0.000	54.4	37.6
Recovery			96.5	78.3

Table X. Subcellular Distribution of Glucose-6-Phosphatase Activity.

A. µGm. phosphorus released per ml. of homogenate or subcellular fraction for 15 minutes incubation at 37°C. Calculations were made with the homogenate and subcellular fractions adjusted to a total of 1000 ml.

Dog #	3	4	6	Average
Fraction				
Homogenate	117	332	368	272
Nuclear	44.8	129	101	150
Mitochondrial	2.4	20.7	20.0	14.4
Microsomal	22.2	103	151	92.2
Supernatant	45.4	53.9	58.3	52.5

B. Percentage distribution and recoveries of glucose-6-phosphatase activity in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	
Nuclear	38.2	39.0	27.4	34.9
Mitochondrial	2.0	6.2	5.4	4.5
Microsomal	19.0	31.2	41.1	30.4
Supernatant	38.7	16.3	15.9	23.6
Total Recovery	97.9	92.7	89.8	93.5

Table X (Continued).

C. Specific activity in mg. phosphorus released per mg. of nitrogen for 15 minutes incubation at 37°C.

Dog #	3	4	6	Average
Fraction				
Homogenate	0.079	0.192	0.184	0.152
Nuclear	0.069	0.140	0.119	0.109
Mitochondrial	0.037	0.375	0.307	0.240
Microsomal	0.142	0.670	0.895	0.569
Supernatant	0.064	0.091	0.086	0.080

Table XI. Summary of Percentage Distributions and Recoveries of the Subcellular Markers and Nitrogen for Three Dogs.

Percentage distribution and recoveries of nitrogen, DNA, acid phosphatase and glucose-6-phosphatase in centrifugal fractions of dog intestinal mucosa homogenate. Means and ranges of three experiments.

Centrifugal Fraction	xg	min.	DNA	Cytochrome Oxidase	Acid Phosphatase	Glucose-6-Phosphatase	Nitrogen
Nuclear	1.1×10^3	30	93.2 (86.5-102)	68 (61-80)	27.2 (23.4-29.2)	34.9 (27.4-39.0)	46.6 (42.5-43.9)
Mitochondrial	7.7×10^3	30	1.5 (0.6-1.9)	29 (24-34)	3.5 (2.8- 4.1)	4.5 (2.0- 6.2)	3.7 (3.2- 4.7)
Microsomal	105×10^3	30	2.1 (1.5-2.6)	3.9 (2.4-6.5)	14.9 (9.7-21.4)	30.4 (19.0-41.1)	9.3 (8.5-10.6)
Supernatant	-	-	4.7 (3.8-6.2)	0 (0)	52.4 (47.0-56.0)	23.6 (15.9-38.7)	39.8 (34.1-47.7)
Recovery			102 (96.8-111)	101 (88-112)	98.0 (95.9-102)	93.5 (89.8-97.9)	99.5 (91.9-107)

Table XII. Average Ratios for Three Experiments for the Subcellular Markers**

Centrifugal Fraction	DNA	Cytochrome Oxidase	Acid Phosphatase	Glucose-6-Phosphatase
Homogenate	1.00	1.0	1.00	1.00
Nuclear	2.00	1.5	0.58	0.75
Mitochondrial	0.41	7.9	0.95	1.22
Microsomal	0.23	0.42	1.60	3.27
Supernatant	0.12	-	1.32	0.59

** Average ratios in percent of DNA, acid phosphatase, or glucose-6-phosphatase per percent nitrogen (Calculated from Table XI).

CHAPTER VII. DETERMINATION OF THE OPTIMUM HYDROLYSIS TIMES FOR THE CARBOHYDRATES OF CERTAIN GLYCOLIPIDS OF DOG INTESTINE USING A SIMPLE HETEROGENEOUS HYDROLYSIS TECHNIQUE

1. Introduction.

Glycolipids, as the name implies, are compounds which are made up of a carbohydrate and a lipid component. In the total lipid extraction of any tissue, the glycolipids coexist with the other classes of lipids, notably the neutral lipids and the phospholipids. In the separation of glycolipids from the other classes of lipids, and in their purification and chemical determination, it is the carbohydrate moieties of these compounds which are routinely measured.

Since the lipid portion of the glycolipids, as well as the other classes of lipids present, may interfere with the carbohydrate assays used (Radin, 1958), these lipids must be removed before reliable carbohydrate determinations can be performed. This is accomplished by hydrolysis in acid, which not only breaks the chemical bonds which link the lipid and carbohydrate portions of the glycolipids, but the bonds which link the carbohydrate moieties to each other as well. The lipids are then extracted from this aqueous acid hydrolysate with a nonpolar organic solvent, such as chloroform.

There are two general types of hydrolyses which can be used to separate the lipid and carbohydrate portions of glycolipids: (1) in-phase or homogeneous and (2) out-of-phase or heterogeneous hydrolysis. In the homogeneous hydrolysis, the lipids are in solution, while in the heterogeneous hydrolysis, they are not.

Glycolipid analysis would be greatly simplified if all the glycolipid carbohydrates could be hydrolyzed from the lipid portion of the molecule and from each other by using a single normality of acid at a single temperature for a certain length of time. There are some indications in the literature that optimum hydrolysis conditions vary with the particular carbohydrate to be assayed. For instance, in one report (Vance, Shook and McKibbin, 1966), hexosamines were assayed after being hydrolyzed for 3.5 hours in 2.5N or 3.0N HCl, while glucose and galactose were assayed after being hydrolyzed for 3.0 hours in 2.0N HCl, and sialic acid was determined without prior hydrolysis. Blix hydrolyzed "glucoproteins" which evidently contained hexosamine for 14 hours at 100°C in 2.0N HCl (Blix, 1948). Suzuki and Korey (1963) used 0.1N HCl at 100°C for 25 minutes to release sialic acid resistant to neuraminidase and 1.0N HCl at 100°C for 16 hours to hydrolyze carbohydrates other than sialic acid. While these are only a few examples of hydrolysis conditions which are used to break the glycosidic bonds in which various carbohydrates are involved, they serve to illustrate that a multiplicity of hydrolysis conditions have been used.

The hydrolysis methods which were in use in this laboratory at the initiation of this investigation were all of the homogeneous type. In general, for this type of hydrolysis, several steps are necessary: (1) the lipid sample to be hydrolyzed, in an organic solvent, is placed in a round bottom flask and taken to dryness; (2) the acid is added along with ethyl alcohol, or some similar solvent (which solubilizes the nonpolar lipids in the polar acid), and is heated for a certain length of time; (3) the ethyl alcohol is removed under vacuum and

chloroform added for the extraction of the lipids; (4) the aqueous and chloroform phases are transferred quantitatively to a centrifuge tube and centrifuged; (5) the aqueous phase is then transferred quantitatively to a reaction flask for colorimetric development.

In preliminary attempts to determine the concentrations of the major glycolipids in canine intestinal mucosa subcellular fractions, it became apparent that a hydrolysis method was needed which required smaller amounts and less manipulation of the glycolipid samples than the procedure described above. Additionally, this method must lend itself well to the hydrolysis of large numbers of samples simultaneously.

The method which was chosen was a simplification of a heterogeneous hydrolysis described by Radin (Radin, 1958) and is described in detail in the following section. Since the hydrolysis method was changed from a homogeneous to a heterogeneous type, it became necessary to determine the optimum hydrolysis times for the various carbohydrates known to be associated with canine intestinal glycolipids. The data which ultimately led to a choice of hydrolysis times are presented in "3. Results."

2. Materials and Methods.

The lipid sample, in organic solvent, was placed in culture tubes (13 x 100 mm, capacity 8 ml.) with teflon lined plastic caps (Kimble Products, Owens-Illinois, Toledo, Ohio 43601, Catalog No. 45066-A). The organic solvent was removed with a stream of nitrogen gas at 40°C.

a. Hydrolysis of lipid for glucose, galactose, and hexosamine assays.

2.5N hydrochloric acid was delivered with a 1 ml. ground glass syringe with a Chaney-stop attachment and a glass tip with a ground glass joint (Misco Scientific, 1825 Eastshore Highway, Berkeley, California 94710, Catalog No. S-4464-2-B). Carbohydrate standards were

made by making 1 ml. of aqueous standard to 10 ml. with 2.78N HCl, in a volumetric flask. This procedure yielded standard in 2.5N HCl. A similar procedure was used to make 2.5N HCl for hydrolysis, except 1 ml. of water was used instead of aqueous standard.

Acid blanks, lipid samples and standards in acid were placed in an air oven at 100°C for 8 hours. These were taken from the oven, cooled in ice water and neutralized with approximately 0.4 ml. of 6.24N NaOH and approximately 0.6 ml. of 0.1M tris. 1 ml. of chloroform was added to the culture tubes and the plastic caps screwed tight. The NaOH, tris and chloroform were delivered to the culture tubes with glass syringes with Chaney-stops and glass tips. The tubes were shaken and centrifuged. The upper aqueous phase contained the hydrolyzed carbohydrates and aliquots were taken from this upper layer for carbohydrate determinations. The lower chloroform layer contained lipid and was not used.

b. Hydrolysis of lipid for sialic acid assays.

The hydrolytic procedure for sialic acid (N-acetyl neuraminic acid) determinations was exactly as described for that used for glucose, galactose, and hexosamine except the 2.78N HCl, 6.24N NaOH, and 0.1M tris were diluted 1 to 25 in water to give respectively 0.111N HCl, 0.248N NaOH, and 0.004M tris. 1 ml. of sialic acid standard or water was diluted 1 to 10 with 0.111N HCl to give a final acid normality of 0.1N. Hydrolyses were carried out in an air oven at 100°C for 1 hour.

In this work, five methods were used to determine carbohydrate concentrations: (1) an anthrone method (Bailey, 1958) which gives a color with both glucose and galactose; (2) glucose oxidase method (Washko and Rice, 1961) by which glucose can be specifically determined;

(3) galactose oxidase which can utilize both galactose and galactosamine as substrates (Roth et al., 1965; Avigad et al., 1962); (4) a hexosamine method (Elson and Morgan, 1933) as modified by Blix (1948) with which galactosamine is determined and (5) a sialic acid method (Svennerholm, 1957) as modified by Miettiner and Takki-Luukkainen (1959).

For convenience, volumes and amounts of carbohydrate used in the various assays are given in Table XIII.

3. Results.

It became necessary to solve several problems before this hydrolysis and neutralization method could be used, however, and these are listed below:

(a) The glass syringes had to be adjusted to deliver a known final volume.

(b) The accuracy of the method had to be ascertained. Since a slight variation in pH occurred from tube to tube on neutralization of the acid with NaOH and tris, it also became necessary to determine if this variation affected the accuracy of the method.

(c) The optimum hydrolysis time at 100°C for the various carbohydrate moieties assayed had to be determined.

a. Adjusting the glass syringes.

This was done empirically, by delivering water to the mark of a 1 ml. volumetric flask. When this was accomplished, the Chaney-stop was tightened and not adjusted further. This syringe was used to deliver the HCl. It was found that the syringe with a Chaney-stop could consistently deliver to the mark from day to day. Then 1 ml. of acid was delivered to a 2 ml. volumetric flask and enough NaOH and tris added with two additional syringes with Chaney-stops to make to

the mark. The final pH was between 7 and 8.

This procedure was quite tedious to perform and could take several hours to accomplish. Once the syringes were set, however, they never needed to be adjusted again (provided the stop was not changed). Any desired pH could be obtained by varying the normality of the NaOH.

b. Determining the accuracy of the method and the effect of pH variation on the accuracy of the method.

For this step, four carbohydrate standards (glucose, galactose, N-acetyl galactosamine, and N-acetyl neuraminic acid) were made up in HCl, neutralized, extracted with chloroform, and centrifuged. Aliquots were taken from the aqueous phase and carbohydrate concentrations and pH determined. For glucose, two experiments of 40 samples each were run, while for the other three carbohydrate standards, two experiments of 20 each were run.

The optical densities of all the samples of each experiment were averaged and this average optical density was equated with the theoretical concentration of the aqueous phase. Percent error was correlated with the pH statistically by running a correlation coefficient. The results are tabulated in Table XIV along with the average error for single samples and duplicate samples.

c. Determination of optimum hydrolysis times for glycolipid carbohydrates.

Using the hydrolysis method described in the Materials and Methods section, experiments were run to ascertain the optimum hydrolysis conditions for the release of glucose, galactose, hexosamine and sialic acid.

Figure 2 shows the results of hydrolysis of a pure glycolipid containing only glucose in 2.5N HCl at 100°C over a 10-hour period. One experiment in duplicate was run. This experiment showed that the greatest yield of glucose occurred between 6 and 10 hours. It did not give any information regarding the percent of the total glucose this yield represented. This information was obtained by measuring the glucose contained in the glucocerebroside without hydrolysis by a direct anthrone method. The value obtained by the direct anthrone method was taken as the "true value", and the value obtained by the glucose oxidase after hydrolysis in 2.5N HCl at 100°C from 0 to 10 hours was compared to the "true value". Two experiments in duplicate were run and averaged. Figure 3 shows that 100 percent of the glucose was released by 6 hours, and this value remained constant from 6 to 10 hours.

The optimum hydrolysis time for the removal of sialic acid was determined on both purified ganglioside (Pierce Chemical Company, Box 117, Rockford, Illinois, Catalog No. 337 501) and a "glycolipid mixture", free of other lipids, from dog intestine. This mixture included two asialoglycolipids and at least one ganglioside. Two experiments in duplicate were done on each of these preparations. The hydrolysis was carried out in 0.1N HCl at 100°C from 0 to 2.5 hours for both glycolipid preparations (Figure 4). For the purified ganglioside, the experimental value was within 4 percent of the theoretical value calculated for the dry weight of the ganglioside. The value of sialic acid obtained for the "glycolipid mixture" after heterogeneous hydrolysis was between 5 and 7 percent higher than that obtained by a homogeneous hydrolysis. The homogeneous hydrolysis used had a final HCl concentration of 0.46N

(Sweeley and Klionsky, 1963; modified by J. M. McKibbin, personal communication).

Figure 5 shows the results of the hydrolysis of a glycolipid mixture which had one hexosamine containing glycolipid and two glycolipids which did not contain hexosamine. The hydrolysis was carried out in 2.5N HCl at 100°C for 1 to 24 hours. One and sometimes two experiments in duplicate were run at various time intervals. There was no significant difference in the values of hexosamine over this time period. Hexosamine values, obtained after heterogeneous hydrolysis, were from 2 to 10 percent higher than those obtained by a homogeneous hydrolysis (Vance et al., 1966).

The hydrolysis of a "purified" glycolipid which contains glucose, galactose and galactosamine is shown in Figure 6. Two experiments in duplicate were done. The analysis of this compound is given in detail elsewhere (Vance et al., 1966). The molar ratio of glucose to galactosamine for the formula proposed is 1:2. Actually, the experimental molar ratio is 1:1.69, so the compound may consist of a family of pentaglycosides with a variable carbohydrate sequence. This experiment supported the evidence presented in Figures 2, 3, and 5 in that optimum yields of glucose occurred between 6 and 10 hours, while the hexosamine, under the same conditions, was hydrolyzed more rapidly (in this particular case, by three hours).

Figure 7 simply points out the need for hydrolyzing standards along with unknown glycolipid samples, particularly in the case of glucose and sialic acid. 20 percent of the glucose and 5 percent of the galactose was degraded by 8 hours in 2.5N HCl at 100°C, and 6 percent of the sialic acid was degraded by 1 hour in 0.1N HCl at 100°C. The

amount of degradation of standards was calculated in the following manner. Standards which were heated in acid at 100°C for various lengths of time were neutralized and assayed. These values were compared to the optical density of standards made up in acid and neutralized immediately. Because N-acetylgalactosamine was used for a hexosamine standard, there was very little color development with the standards which had not been subjected to acid and heat. This phenomenon occurred because the amino group of hexosamine must be free before a pyrrole ring can be formed for color development with P-dimethylaminobenzaldehyde (Elson and Morgan, 1933). Therefore, standards subjected to acid plus heat for 3 to 10 hours were compared with the 1-hour standards. There was no significant change over this period of time.

Calculations of glycolipid values based on unhydrolyzed sugar standards, then, will result in low experimental values. On the other hand, if the stability in heated acid of glycosylated sugars is greater than free sugar standards, then experimental values for glycolipids based on sugar standards heated in acid will be high. The use of hydrolyzed sugar standards seems justified since 80 percent of glycolipid glucose (Figure 3), the most difficult of the glycolipid sugars to hydrolyze, was released within the first 2 hours of an 8-hour hydrolysis.

4. Discussion.

The heterogeneous method of hydrolysis of glycolipids described in this paper was shown to compare favorably with results obtained by homogeneous hydrolysis both on purified glycolipids and glycolipid mixtures. The techniques utilized for preparing the samples for hydrolysis, and subsequent neutralization, involves very little time because syringes with Chaney-stops were used rather than pipettes. This method had the

added advantages that the procedures of hydrolysis, neutralization and lipid extraction are all done in the same culture tube. Additionally, there was enough aqueous hydrolysate (2 ml.) to take 0.5 ml. aliquots for three different assays. For instance, aliquots for glucose, hexosamine, and galactose assays were all taken from the same tube after a single hydrolysis.

When stored at 4°C, carbohydrates in the hydrolysate were stable for at least one day, and hexosamine was stable for 2 days. Hydrolysis, neutralization and extraction could therefore be done on one day and carbohydrate assays done one or two days later.

Large numbers of hydrolyses can be done at one time by a single individual, and as many as 76 hydrolyses were carried out at a single time.

Blanks, standards and samples were routinely done in duplicate and hydrolyzed simultaneously. Standards were hydrolyzed along with glycolipid samples to avoid introducing error due to degradation of carbohydrates. If optical densities of duplicates varied by more than 10 percent, they were rejected and repeated. This was done because it was found, in a series of determinations of carbohydrate standards, that whenever the average of two duplicates was over 5 percent in error when compared with the known value, the variation in the optical density was in excess of 10 percent.

The hydrolysis conditions used for glucose, galactose, and hexosamine were 2.5N HCl for 8 hours at 100°C; for sialic acid, 0.1N HCl for 1 hour at 100°C. While the glucose which is linked directly to the lipid in dog intestinal glycolipids was more slowly released than galactosamine, both were released by 8 hours under the conditions cited.

a. Anthrone with chloride.

It is known that chloride ion enhances the anthrone color. Lunt and Sufcliffe (1953) reported that 1 percent NaCl increased the sensitivity of anthrone by 17 percent. Scott and Melvin (1953) reported that 0.36 percent HCl in a 3 milligrams percent solution of dextran introduced a +13.3 percent error and that 0.1 percent solution of sodium chloride in the same dextran solution resulted in a +9.5 percent error. Fales and coworkers (1961) reported a 25 percent augmentation in anthrone color with glucose by 0.1M chloride solution and that fluoride, bromide and iodide also enhance anthrone color.

The chloride concentration in the hydrolysates of the method described in this paper was 1.25M. It was found that the anthrone optical density per micromole of glucose with 1.25M chloride ion was 1.6 times greater than glucose without chloride ion.

This enhancement of anthrone color by chloride ion can be used advantageously if lipid extracts contain insufficient chloride to introduce any significant error. Glucose standards and purified glucocerebrosides were heated separately in 2.5N HCl for 8 hours. The concentrations of glucose in these hydrolysates were determined both by glucose oxidase and anthrone with 1.25M chloride ion. When the glucose values obtained by these two methods were compared, the following results were obtained: (1) for 40 duplicate glucose standards, the average error between the two methods was ± 3.20 percent; (2) for purified dog glucocerebroside, the average error was ± 4.31 percent for six duplicate determinations.

Table XIII. Quantities and Volumes Used for Various Carbohydrate Assays and Approximate Optical Densities Produced.

Assay	μMoles needed for hydrolysis	μMoles needed for assay	ml. of hydrolysate needed for assay	total reaction volume (ml.)	approximate O.D. produced*
glucose by anthrone with 1.25M Cl^-	0.575	0.144	0.5	5.5	0.250
galactose oxidase	0.224	0.056	0.5	1.0	0.325
glucose oxidase	0.575	0.144	0.5	2.5	0.250
galactosamine	0.605	0.151	0.5	6.5	0.300
sialic acid	0.200	0.100	1.0	4.0	0.270

*Because of the relatively high optical densities produced by the concentrations cited, the amounts needed for hydrolysis could be reduced by as much as two or three times. A light path of 10 mm. was used to obtain the optical density values.

Table XIV. Accuracy of Methods, Correlation Between pH Variation and Error, and Other Data.

Method	Exp. No.	Correlation Coefficient	No. of Samples	"t" Value	Aver. percent error of method (single detrs.)	Aver. percent error of method (duplicate detrs.)
glucose oxidase	1	0.02	40	0.09	± 2.93	± 2.57
	2	-0.08	40	0.34	± 2.45	± 2.44
galactose oxidase	1	-0.59	20	3.11	± 4.42	± 3.70
	2	-0.43	20	2.02	± 2.41	± 1.95
galactosamine	1	-0.02	20	0.09	± 1.46	± 1.17
	2	-0.12	20	0.51	± 1.66	± 1.19
sialic acid	1	-0.15	20	0.65	± 1.50	± 1.36
	2	-0.16	20	0.69	± 2.92	± 2.24

There was no significant error introduced by pH variation after neutralization for all methods listed except for Experiment No. 1 of galactose oxidase. In this experiment, 35 percent of ± 4.42 percent was accounted for by variation in pH. But since the overall average error was ± 4.42 percent, the method was acceptable. These two galactose oxidase experiments were repeated with essentially the same results.

Table XIV (Continued).

Method	Exp. No.	Mean pH	Aver. Deviation from mean pH	pH range from mean pH
glucose	1	8.32	±0.10	+ 0.28 to - 0.22
oxidase	2	8.33	±0.11	+ 0.27 to - 0.54
galactose	1	8.72	±0.15	+ 0.38 to - 0.37
oxidase	2	8.83	±0.07	+ 0.17 to - 0.18
galactosamine	1	7.02	±0.17	+ 0.48 to - 0.97
	2	7.04	±0.12	+ 0.26 to - 0.44
sialic	1	5.81	±0.03	+ 0.69 to - 0.71
acid	2	5.92	±0.41	+ 1.03 to - 1.92

FIGURE 2

DETERMINATION OF OPTIMUM HYDROLYSIS TIME FOR GLUCOCEREBROSIDE

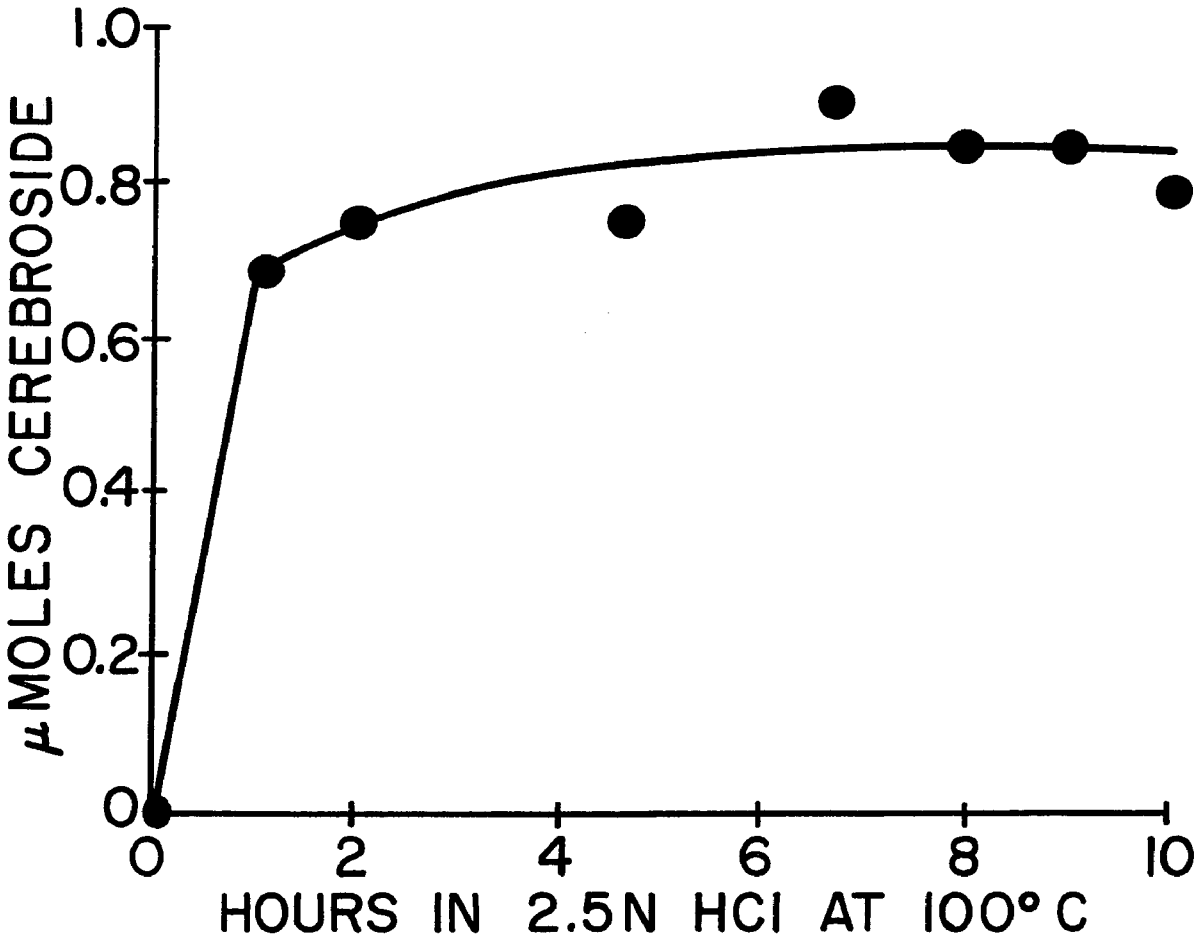


FIGURE 3

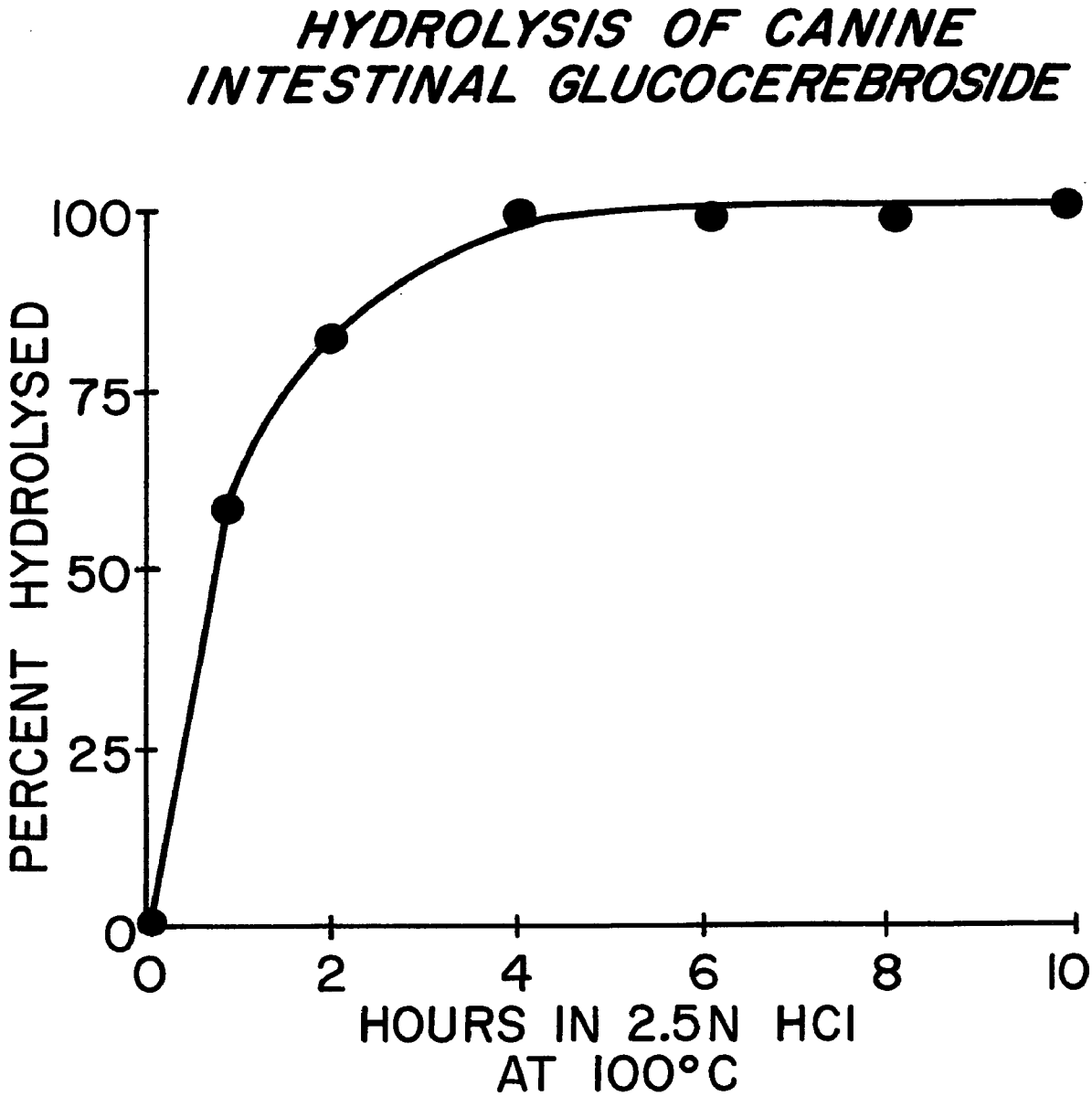

HYDROLYSIS OF CANINE
INTESTINAL GLUCOCEREBROSIDE
PERCENT HYDROLYSED
100
75
50
25
0
0
2
4
6
8
10
HOURS IN 2.5N HCl
AT 100°C

FIGURE 4

DETERMINATION OF OPTIMUM HYDROLYSIS TIME FOR GLYCOLIPID SIALIC ACID

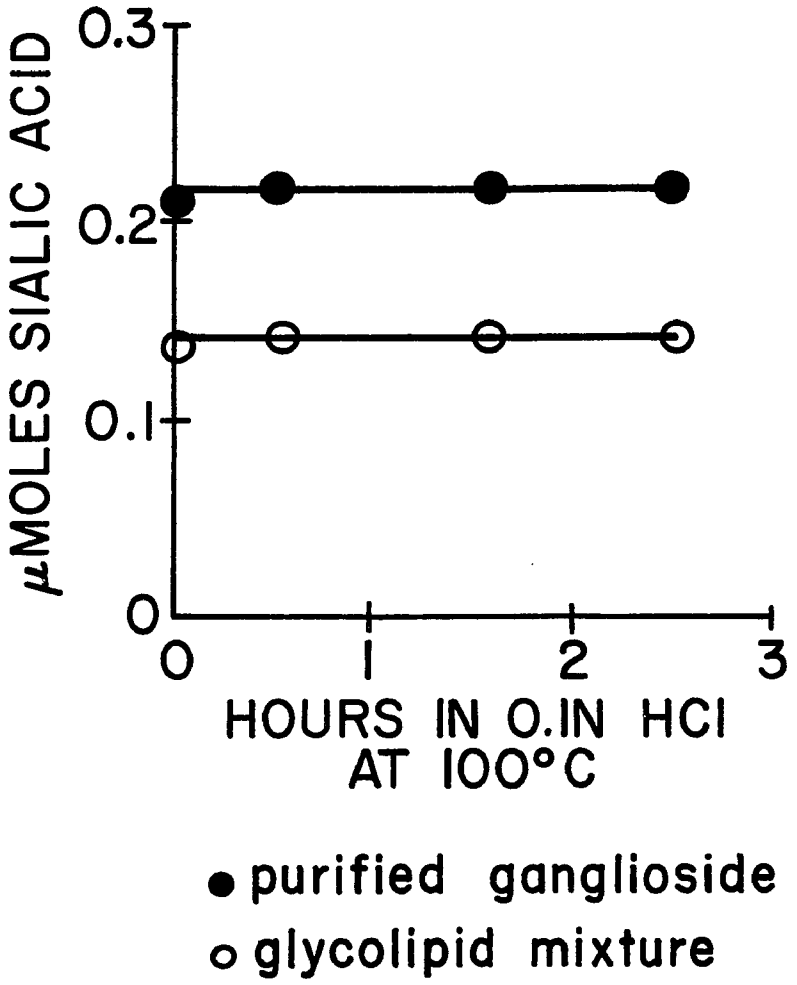


FIGURE 5

DETERMINATION OF OPTIMUM HYDROLYSIS TIME FOR GLYCOLIPID HEXOSAMINE

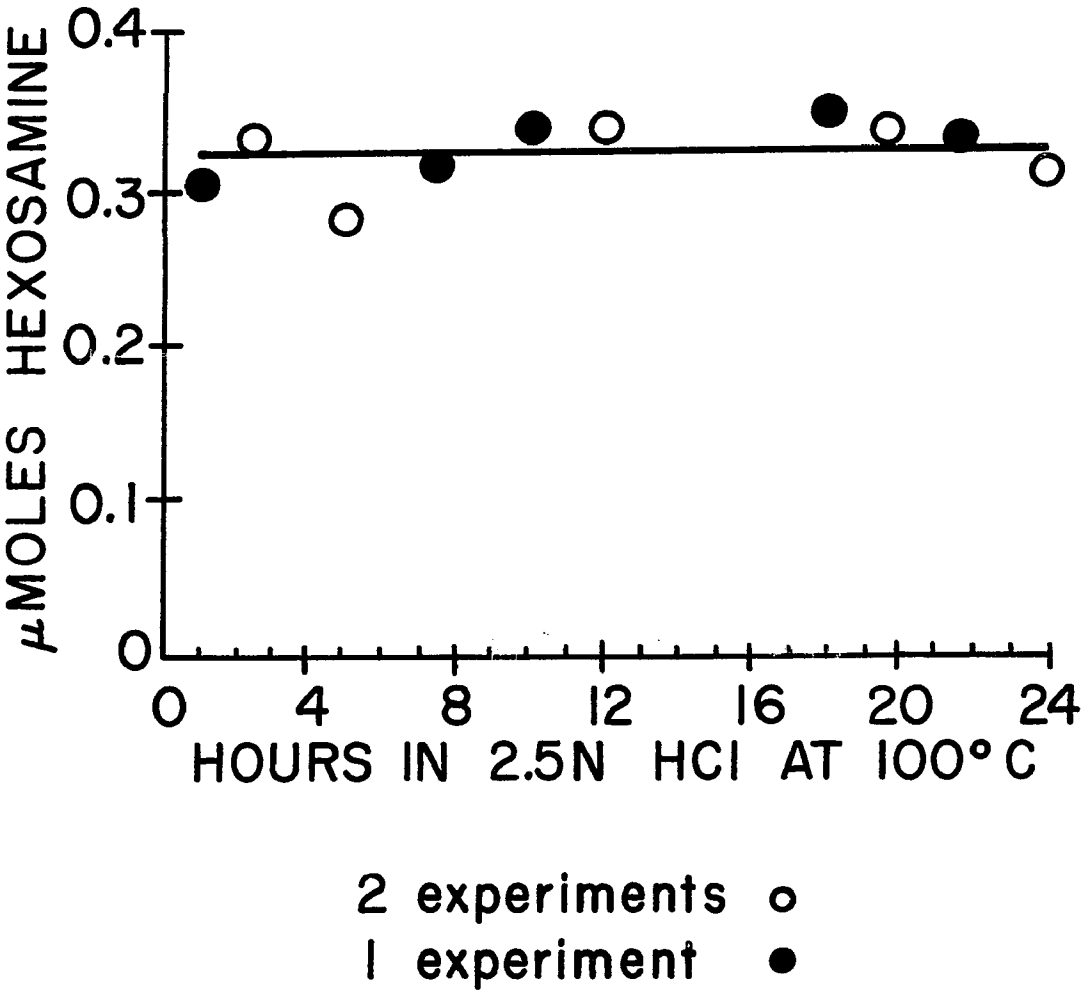


FIGURE 6

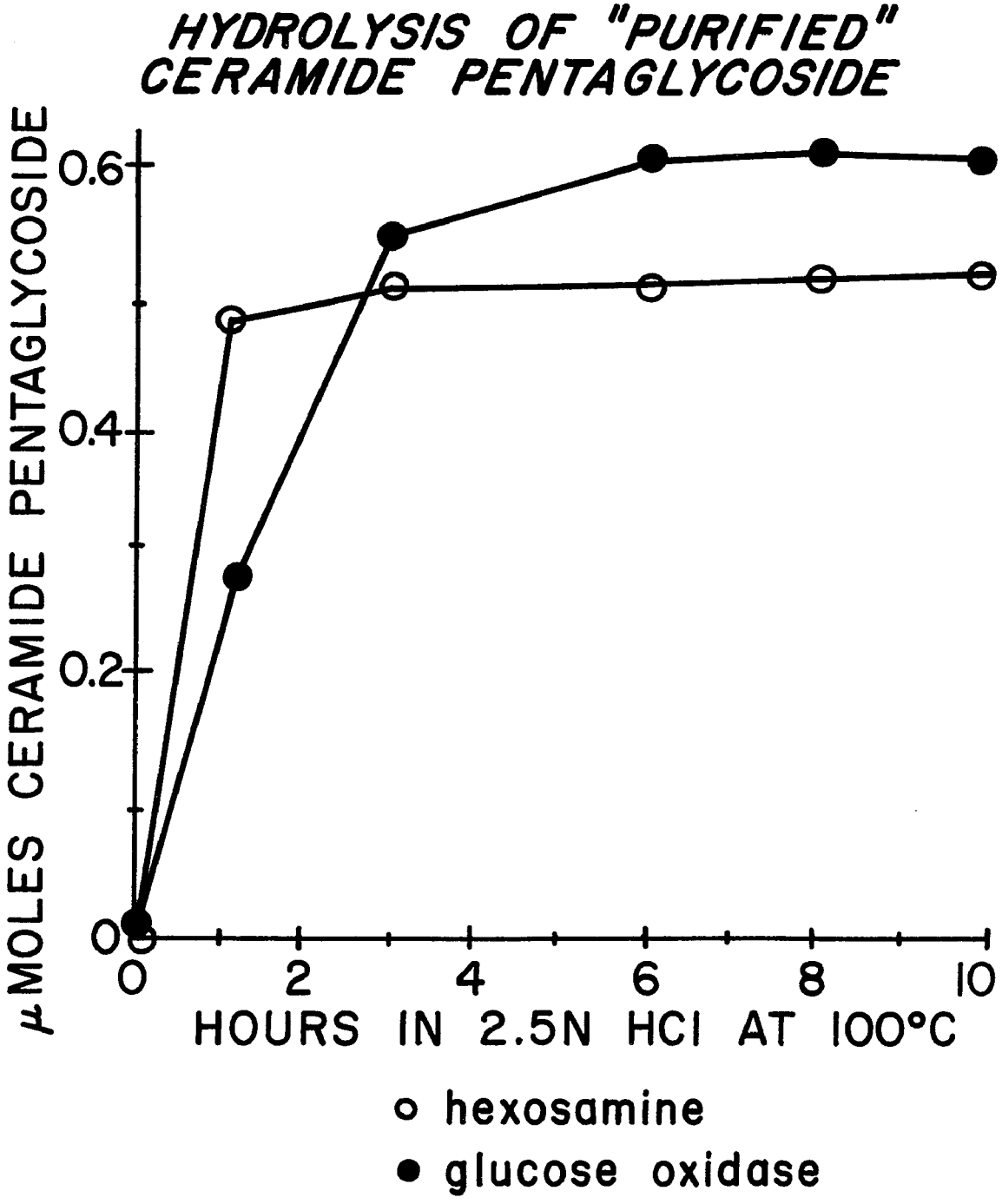
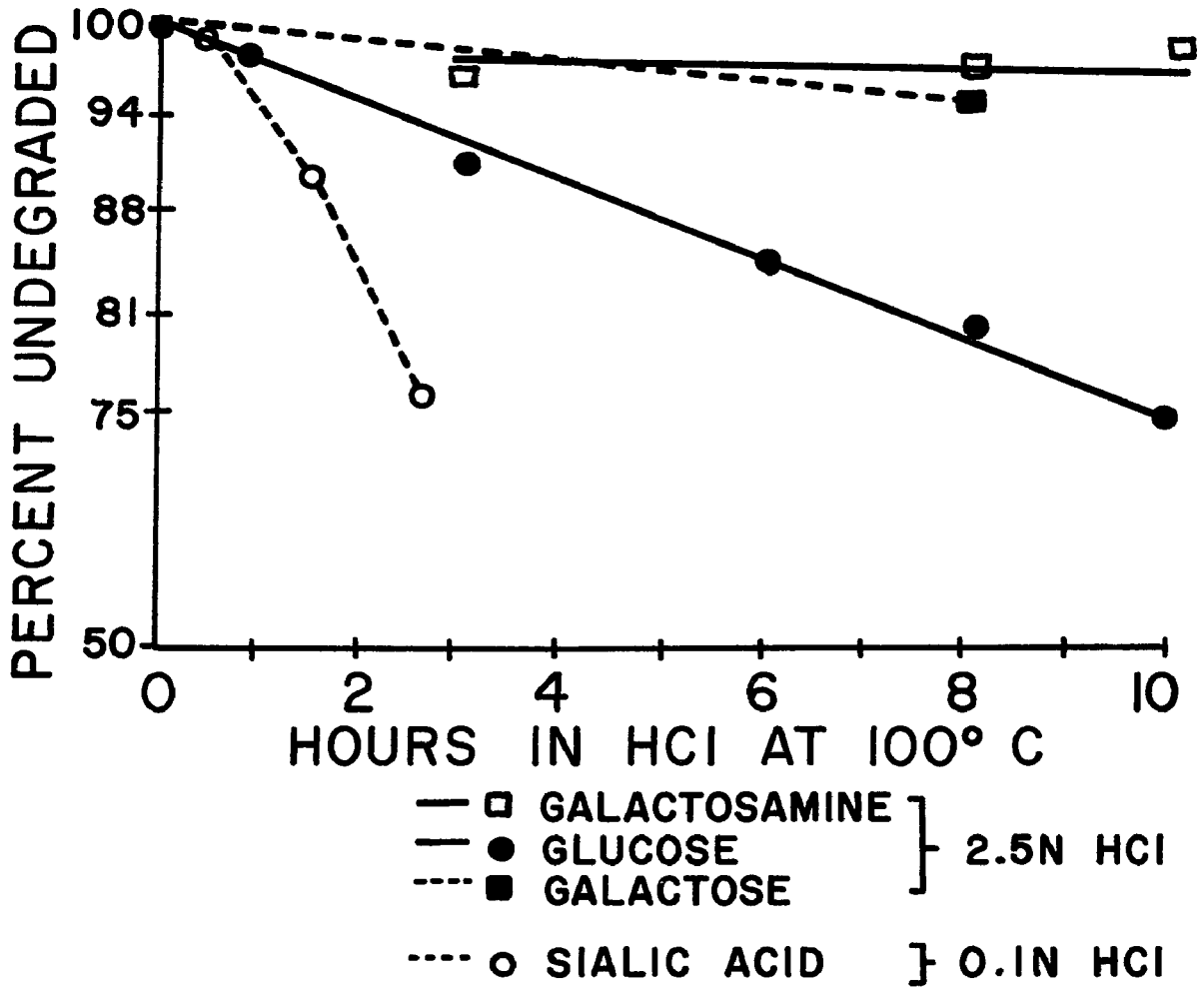

HYDROLYSIS OF "PURIFIED" CERAMIDE PENTAGLYCOSIDE
μMOLES CERAMIDE PENTAGLYCOSIDE
0.6
0.4
0.2
0
0
2
4
6
8
10
HOURS IN 2.5N HCl AT 100°C
o hexosamine
● glucose oxidase

FIGURE 7

DEGRADATION OF VARIOUS CARBOHYDRATE STANDARDS



CHAPTER VIII. LIPID DETERMINATIONS AND PRELIMINARY PURIFICATION OF THE GLYCOLIPIDS OF DOG INTESTINAL MUCOSA SUBCELLULAR FRACTIONS.

1. Materials and Methods.

a. Lipid extraction and preliminary purification of glycolipids.

The homogenate and subcellular fractions of dog intestinal mucosa were lyophilized and weighed. Twenty ml. of Bloor's reagent (95% ethyl alcohol-ethyl ether in 3:1 ratio) were used per gram of lyophilized residue for extraction of the lipids. Since some of the lyophilized material adhered to the lyophilizing flasks, 1 ml. of distilled H_2O per gram of residue was used to wash the flasks. This volume of H_2O was divided into two equal portions and the flasks washed twice. This water wash was added to the lyophilized tissue in Bloor's reagent and the total mixture refluxed for one and a half hours.

The lipid extracts for the intestinal tissue were subsequently filtered through medium grade sintered glass and the residue washed twice with one sixth of the volume of Bloor's reagent used for extraction of the lyophilized tissue. The filtered lipid extract was concentrated to dryness under nitrogen and the residue redissolved in 40-120 ml. $CHCl_3$ and an equal volume of 0.25M $MgCl_2$. The chloroform solutions of the total lipids of the intestinal homogenate and subcellular fractions were washed with 0.25M $MgCl_2$ (McKibbin et al., 1961). These washings were repeated until aliquots of the $MgCl_2$ washings gave no color with anthrone reagent (Bailey, 1958) and then washed two additional times. In some cases, particularly with the homogenate and nuclear fractions, twenty-five washings failed to produce aliquots which were negative to anthrone.

To remove any remaining sucrose, the total lipid extract was taken to dryness, and the residue emulsified with H_2O and $CHCl_3$. These emulsions were dialyzed in 3/8 in. dialysis tubing (Union Carbide Corporation, Food Products Division, 6733 65th St., Chicago, Illinois 60638) against running tap water for 3 days at room temperature. The contents of the dialysis tubing were concentrated under nitrogen and brought to volume in $CHCl_3$. Despite extensive washings and dialysis of the total lipid extract, there was thin layer chromatographic evidence that the sucrose was never successfully removed (for evidence, see Chapter IX). These chloroform solutions were analyzed for lipid content.

The total lipids in $CHCl_3$ of the homogenates and subcellular fractions were put on silicic acid columns. The amounts of silicic acid, celite, the internal diameters of the columns, and the amount of solvent used for eluting the columns were determined by relating the lipid phosphorus of the homogenates and subcellular fractions to the lipid phosphorus and cross sectional area of the silicic acid columns previously reported by Vance et al., (1966). The silicic acid columns were eluted with 2, 10, 17, 50, and 80% methanol in chloroform. These eluents were designated S-2, S-10, S-17, S-50 and S-80, respectively. Fraction S-2 was shown to contain no glycolipid (Vance et al., 1966). Fractions S-10 and S-17 and fractions S-50 and S-80 were combined and designated S-10-17 and S-50-80, respectively. These fractions were taken to dryness under nitrogen and the residue redissolved in $CHCl_3$.

The S-10-17 eluents of the homogenate and subcellular fractions were put on Florisil columns and eluted the 5, 35, 50, 60% methanol in chloroform and 100% methanol. These eluents were designated F-5, F-35,

F-50, F-60 and F-100, respectively. F-5 contained no glycolipid (J. M. McKibbin, personal communication). Eluents F-35 and F-50 and eluents F-60 and F-100 were combined and designated F-35-50 and F-60-100, respectively. The S-50-80 eluents of the homogenate and subcellular fractions were put on Florisil columns and eluted with 35 and 75% methanol in chloroform and 100% methanol. These eluents were designated F-35, F-75, and F-100, respectively. The F-50 eluent has been reported to contain 90% of the glycolipid (Vance et al., 1966). The F-75 and F-100 eluents were combined and designated F-75-100. The amounts of Florisil, the internal diameters of the columns, and the amount of solvent used for eluting the Florisil columns were determined by relating the lipid phosphorus to previously reported values. In the case of the S-10-17 eluents, the lipid phosphorus was related to 848 μmoles P loaded on a 13 mm I.D. column (Vance et al., 1966). With the S-50-80 eluents, the lipid phosphorus was related to 2700 μmoles P loaded on a 17 mm I.D. column (Vance et al., 1966; J. M. McKibbin, personal communication). The F-35-50 and F-75-100 eluents were further resolved on thin layer chromatography (see Chapter IX).

b. Chemical determinations.

Lipid phosphorus was determined by the perchloric acid method of Harris and Popat (1954). Chemical determination of total "galactose" by anthrone, hexosamine, and sialic acid were described in detail in Chapter VII. Nitrogen determinations on the homogenates and subcellular fractions of dog intestinal mucosa were described in Chapter VI.

2. Results.

The lipid values for the crude nuclear fraction of Dog 3 were all low due to incomplete lipid solubilization after extraction. This was

the first subcellular fraction extracted and the H_2O wash was not used. The procedure described above was used subsequently on all other fractions.

The total lipid phosphorus per 1000 ml. of homogenate or subcellular fraction is shown in Table XV-A. The values for the homogenate of three different dogs were very similar, with an average of 13.5×10^2 μmoles phosphorus per 1000 ml. Expressed as μmoles lipid phosphorus per gram of fresh mucosa, an average value of 13.4 was found (Table XIX). These values were lower than that of 16.9 μmoles per gram fresh mucosa (J. M. McKibbin, personal communication). This latter value was reported for whole mucosa, while the former was for the outermost portions of the intestinal villi. 63% of the lipid phosphorus was accounted for by the crude nuclear fraction, while approximately 8, 21, and 7% was accounted for by the mitochondrial, microsomal, and supernatant fractions respectively (Table XV-B). When lipid phosphorus total nitrogen ratios for the subcellular fractions were compared to the homogenate, the mitochondrial and microsomal fractions were enriched and the supernatant was very low (Table XV-C, D). The crude nuclear fraction was also enriched, but not as much as the mitochondrial and microsomal fractions.

The average value for lipid hexosamine per 1000 ml. of homogenate was 28.5 μmoles (Table XVI-A) or 0.283 μmoles per gram of fresh tissue (Table XIX). These values were lower than that of 0.369 μmoles per gram fresh whole mucosa (J. M. McKibbin, personal communication). Of interest was the finding that the μmolar lipid hexosamine/lipid phosphorus ratio was 2.1×10^{-2} for both whole intestinal mucosa and the outermost portion of the intestinal mucosa villi. Table XVI-B shows

that 69% of the lipid hexosamine was found in the crude nuclear pellet, while the microsomal fraction accounted for 28%. When compared to nitrogen, it was found that the microsomal fraction was three times as enriched in lipid hexosamine as was the homogenate (Table XVI-D). Hexosamine determinations on the supernatant yielded negative results. Only compounds which migrated on thin layer chromatograms with glycolipid standards having five or more sugar residues contained hexosamine (see Chapter IX). However, there are gangliosides in intestinal mucosa whose composition is unknown, and these may also contain hexosamine.

The percentage distribution of nitrogen, lipid phosphorus and lipid hexosamine in the subcellular fractions of three dogs is given in Table XVII. Lipid phosphorus and hexosamine distributions were similar, but differed from nitrogen distribution. Lipid phosphorus and lipid hexosamine values, however, were unrelated in whole tissue and the subcellular fractions. This was illustrated by the considerable variation in the phospholipid/lipid hexosamine molar ratio among individual dogs (Table XVIII-A, B). For instance, in the homogenates this ratio in Dogs 3 and 4 was 1.5 times greater than in Dog 6. In the mitochondrial fraction, this ratio in Dog 3 was 1.7 times greater than in Dog 6 and 1.4 times greater in Dog 4. Similar variations were found in the microsomal fraction where this ratio in Dog 3 was 1.2 times that in Dog 4 and 1.8 times that in Dog 6. With respect to nitrogen, both lipid phosphorus and lipid hexosamine were enriched in the microsomal fraction when compared to the homogenate (Tables XV-D and XVI-D). The ratios of percent lipid phosphorus to percent lipid hexosamine for three different dogs were compared in Table XVIII-B. A larger percentage of lipid hexosamine than lipid phosphorus was found in the microsomal fraction as

indicated by an average ratio of 0.75. In the mitochondrial fraction, a ratio of 1.33 was found. This value indicated that a larger percentage of lipid phosphorus than lipid hexosamine was present in the mitochondrial fraction.

Recoveries of lipid anthrone "galactose" and lipid phosphorus from silicic acid columns for subcellular fractions from two different dogs is given in Table XX. The average recoveries for ten columns was 91% for lipid "galactose" and 100% for lipid phosphorus.

Recoveries of lipid "galactose" from Florisil columns for two different dogs is given in Table XXI. The F-35-50 eluents from ten Florisil columns contained an average of 50% of the lipid "galactose". The F-60-100 eluents from nine columns was 31%. Together the average recovery of the S-10-17 lipid "galactose" from Florisil columns was 81%. Recoveries of the S-50-80 lipid "galactose" from ten Florisil columns was 62%. The total recovery of all lipid "galactose" put on Florisil columns was 72%. Since 92% of the "galactose" in the whole lipid extracts was recovered from silicic acid columns, an average of 66% of the original whole lipid extract "galactose" was accounted for after Florisil column chromatography. Although the lipid "galactose" values were taken as a measure of a glycolipid, a compound which migrated with sucrose was shown by thin layer chromatography to be present in the F-75-100 eluent from Florisil columns. This would mean that values for lipid "galactose" from the S-50-80 eluents from silicic acid columns, as well as the F-75-100 eluents, were partly accounted for by sucrose.

3. Discussion.

Lipid determinations on subcellular fractions which have been reported have either used pooled tissues (Eichberg et al., 1964;

Seminario et al., 1964; Lapetina et al., 1967) or tissues from individual animals (Wolfe, 1961; Wherrett and McIlwain, 1962; Green and Robinson, 1960). In the case where individual animals were used, the results of only one experiment were reported. In this thesis, lipid analyses on three different animals were reported.

When percent lipid phosphorus/percent nitrogen ratios of the microsomal fractions were compared with those of the homogenates, the microsomal fractions were enriched in phospholipid in dog intestinal mucosa and rodent brain (Seminario et al., 1964; Eichberg et al., 1964). A comparison of percent lipid hexosamine/percent nitrogen ratios of the homogenate and microsomal fraction of dog intestinal mucosa indicated that the microsomal fractions were enriched three-fold. The only compounds which contained hexosamine which have been isolated from the homogenate have been compounds migrating on thin layer chromatography with a ceramide pentaglycoside and a ceramide hexaglycoside. Only the hexaglycoside has been isolated from the microsomal fraction. Since large losses were sustained in isolating these compounds, it is possible that pentaglycoside was present in the other subcellular fractions. Another compound which has been identified in both the homogenate and microsomal fraction, and which could be an additional source of hexosamine, was a compound which migrated on thin layer chromatography with a ganglioside standard, G-2 (see Chapter IX). These compounds may be concentrated in the microsomal fraction, but since this fraction contains structures which are membranous in nature, a more logical conclusion is that membranes in general contain a spectrum of glycolipids, and are enriched in phospholipid and glycolipid.

The possibility that certain subcellular organelles contain unique glycolipids which other organelles in the same cell lack is an intriguing idea, and one which originally prompted the present investigation. The isolation, quantitation, and comparison on a micromolar basis of individual glycolipids was impossible because of large losses encountered during their purification. Only 66% of the anthrone lipid material in the original lipid extract could be accounted for after silicic acid and Florisil column chromatography. Additional losses were sustained as individual glycolipids were isolated by preparative thin layer chromatography. Using pooled whole small intestine, McKibbin reported 82% recovery of the original whole lipid hexose (McKibbin, 1969). The procedures for isolating glycolipids from pooled dog whole intestine (Vance et al., 1966; McKibbin, 1969) were modified in the isolation of glycolipids from the subcellular fractions of individual dogs. Smaller amounts of tissue (and of glycolipids) necessitated combining silicic acid eluents for Florisil column chromatography. These silicic acid eluents were treated separately in pooled dog whole intestine. This procedure may have resulted in poor recovery. Purification procedures of glycolipids by column chromatography are, of course, empirical. The recoveries of glycolipid using procedures described in this chapter could perhaps be improved by using larger eluting solvent volumes, but this must await future work.

Recovery of glycolipids after column chromatography by other investigators on other tissues has been variable. In mouse ascites-sarcoma cells, Gray reported a recovery of 76% of the hexose of the original lipid extract (Gray, 1965). Makita and Yamakawa (1962)

reported recoveries of lipid hexose of 60-80% from silicic acid columns of human, equine, and bovine spleen lipid. In human kidney 89-92% of the hexose in the original lipid extract was accounted for (Martensson, 1963; 1966). Gallai-Hatchard and Gray (1966) accounted for 97% of the lipid glucose in whole lipid extract of pig lung. The column chromatography methods described in this thesis for the purification of glycolipids did not yield adequate recoveries for a micromolar comparison of different glycolipids in various subcellular fractions. The column procedures are, however, adequate for purification of glycolipids from individual dog small intestinal mucosa for structural characterization. This is of some importance as evidence for differences of mucosal glycolipids in individual dogs is presented in Chapter IX.

Table XV. Subcellular Distribution of Total Lipid Phosphorus

A. In μmoles phosphorus per 1000 ml. homogenate or subcellular fraction.

Dog #	3	4	6	Average
Fraction				
Homogenate	14.0×10^2	13.2×10^2	13.3×10^2	13.5×10^2
Nuclear	362(918*)	876	743	846*
Mitochondrial	104	95.4	119	106
Microsomal	313	233	313	286
Supernatant	62.9	75.3	138	92.1

B. Percentage distribution and recoveries of lipid phosphorus in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	
Nuclear	25.9(65.9*)	66.5	55.7	(62.7*)
Mitochondrial	7.4	7.2	8.9	7.8
Microsomal	22.4	17.7	23.4	21.2
Supernatant	4.5	5.7	10.3	6.8
Total Recovery	60.2	97.1	98.3	(98.5*)

Table XV (Continued).

C. Lipid phosphorus/total fraction nitrogen ratios in μmoles lipid phosphorus per mg. nitrogen.

Dog #	3	4	6	Average
Fraction				
Homogenate	0.945	0.764	0.667	0.792
Nuclear	(1.41*)	0.952	0.865	(1.08*)
Mitochondrial	1.51	1.73	1.83	1.69
Microsomal	1.99	1.51	1.85	1.78
Supernatant	0.089	0.128	0.183	0.133

D. Average "percentage ratios" calculated as a ratio of percent lipid phosphorus/percent nitrogen.

Dog #	3	4	6	Average
Fraction				
Homogenate	1.00	1.00	1.00	1.00
Nuclear	(1.50*)	1.25	1.31	(1.35*)
Mitochondrial	1.58	2.26	2.69	2.18
Microsomal	2.11	1.98	2.76	2.28
Supernatant	0.09	0.17	0.28	0.18

* Figures in parentheses are projected values obtained by subtracting the total value of all subcellular fractions (other than the nuclear fraction) from the homogenate of Dog #3, or using such values to obtain an average value.

Table XVI. Subcellular Distribution of Total Lipid Hexosamine

A. In μmoles hexosamine per 1000 ml. homogenate or subcellular fraction.

Dog #	3	4	6	Average
Fraction				
Homogenate	24.2	24.2	37.2	28.5
Nuclear	(16.5*)	19.4	21.8	(19.2*)
Mitochondrial	1.28	1.60	2.59	1.82
Microsomal	6.66	5.97	12.1	8.23
Supernatant	0	0	0	0

B. Percentage distribution and recoveries of lipid hexosamine in centrifugal fractions of dog intestinal mucosa homogenate.

Dog #	3	4	6	Average
Fraction				
Homogenate	100	100	100	100
Nuclear	26.7(68.1*)	79.3	58.5	(68.6*)
Mitochondrial	5.3	6.6	6.0	6.0
Microsomal	27.6	24.7	32.4	28.2
Supernatant	0	0	0	0
Total Recovery	59.6(100*)	111	96.9	103

Table XVI (Continued).

C. Lipid hexosamine/nitrogen ratios in μmoles lipid hexosamine per gram nitrogen.

Dog #	3	4	6	Average
Fraction				
Homogenate	16.3	14.0	18.6	16.3
Nuclear	(25.4*)	21.1	25.3	(23.9*)
Mitochondrial	18.6	29.1	39.9	29.2
Microsomal	42.4	38.8	71.4	50.9
Supernatant	0	0	0	0

D. Average "percentage ratios" calculated as a ratio of percent lipid hexosamine/percent nitrogen.

Dog #	3	4	6	Average
Fraction				
Homogenate	1.00	1.00	1.00	1.00
Nuclear	(1.55*)	1.49	1.38	(1.47*)
Mitochondrial	1.13	2.06	1.82	1.67
Microsomal	2.60	2.78	3.81	3.06
Supernatant	0	0	0	0

* Figures in parentheses are projected values obtained by subtracting the total value of all subcellular fractions (other than the nuclear fraction) from the homogenate of Dog #3, or using such values to obtain an average value.

Table XVII. Average Percentage Subcellular Distributions of Nitrogen, Lipid Phosphorus, and Lipid Hexosamine for Three Dogs

	Nitrogen	Lipid Phosphorus	Lipid Hexosamine
Fraction			
Homogenate	100	100	100
Nuclear	46.6	(62.7*)	(68.6*)
Mitochondrial	3.7	7.8	6.0
Microsomal	9.3	21.2	28.2
Supernatant	39.8	6.8	0
Total Recovery	99.5	(98.5*)	(103*)

* Figures in parentheses are projected values obtained by subtracting the total value of all subcellular fractions (other than the nuclear fraction) from the homogenate of Dog #3, or using such values to obtain an average value.

Table XVIII. Relations of Lipid Phosphorus and Glycolipid Hexosamine in Subcellular Fractions

A. Molar ratio of lipid phosphorus to lipid hexosamine.

Dog #	3	4	6	Average
Fraction				
Homogenate	57.8	54.5	35.9	49.4
Nuclear	(55.8*)	45.2	34.1	(45.0*)
Mitochondrial	81.2	59.2	45.8	62.2
Microsomal	47.0	39.0	25.9	37.3
Supernatant	0	0	0	0

B. Ratio of percent lipid phosphorus to percent lipid hexosamine.

Dog #	3	4	6	Average
Fraction				
Homogenate	1.00	1.00	1.00	1.00
Nuclear	(0.96*)	0.84	0.95	(0.92*)
Mitochondrial	1.40	1.09	1.48	1.33
Microsomal	0.81	0.72	0.72	0.75
Supernatant	0	0	0	0

* Figures in parentheses are projected values obtained by subtracting the total value of all subcellular fractions (other than the nuclear fraction) from the homogenate of Dog #3, or using such values to obtain an average value.

Table XIX. μMoles Lipid Phosphorus and Lipid Hexosamine per Gm. of Mucosa (wet weight).

Dog #	3	4	6	Average
Phosphorus	13.7	13.2	13.1	13.4
Hexosamine	0.240	0.242	0.366	0.283

Table XX. Recoveries of Lipid Anthrone "Galactose" and Lipid Phosphorus from Silicic Acid Columns for Dogs 4 and 6. All Determinations Done in Duplicate.

Dog Fraction	On Column	μM Galactose Recovered S-10-17	S-50-80	Total Recovered	Percent Recovered
4 Homogenate	112	50.5	56.5	107	95.5
4 Nuclear	149	54.8	88.5	143	95.8
4 Mitochondrial	12.3	2.95	7.83	10.8	87.8
4 Microsomal	54.3	19.7	32.3	52.0	95.8
4 Supernatant	14.2	3.48	9.55	13.0	91.5
6 Homogenate	113	40.3	45.5	85.8	75.9
6 Nuclear	155	49.8	91.5	141	91.0
6 Mitochondrial	29.7	8.87	17.9	26.8	90.2
6 Microsomal	65.8	32.3	33.7	66	100
6 Supernatant	34.5	5.79	24.3	30.1	87.2

Average Recovery (all columns) 91.1%

Table XX (Continued).

Dog Fraction	On Column	μM Phosphorus Recovered S-10-17	S-50-80	Total Recovered	Percent Recovered
4 Homogenate	534	247	303	550	103
4 Nuclear	713	472	413	785	110
4 Mitochondrial	52.8	25.1	25.1	50.2	95.1
4 Microsomal	151	77.5	76.4	153.9	102
4 Supernatant	60.6	22.8	35.5	58.3	96.2
6 Homogenate	418	181	205	386	92.3
6 Nuclear	595	396	260	656	110
6 Mitochondrial	84.0	45.6	40.7	86.3	103
6 Microsomal	203	101	98.1	199	98.0
6 Supernatant	115	21.2	78.3	99.5	86.5

Average Recovery (all columns) 99.6%

Table XXI. Recoveries of Lipid Anthrone "Galactose" from Florisil Columns.

A total of 631 μmoles "galactose" put on all columns, with 451 μmoles "galactose" recovered. All determinations done in duplicate.

	S-10-17					S-50-80		
	μM "galactose" F-35-50			μM "galactose" F-60-100		μM "galactose" F-75-100		
Dog Fraction	On Column	Recovered	% Recovered	Recovered	% Recovered	On Column	Recovered	% Recovered
4 Homogenate	48.5	31.2	64.3	7.80	16.1	54.8	37.5	68.4
4 Nuclear	53.0	31.1	59.0	14.8	28.9	77.8	61.4	78.9
4 Mitochondrial	1.95	0.788	40.4	0.704+	36.1	5.64	2.18	38.6
4 Microsomal	17.6	7.95	45.2	5.96	33.9	28.8	15.3	53.1
4 Supernatant	2.51	1.03	41.0	0.834	33.2	7.45	4.77	64.0
6 Homogenate	35.8	21.0	58.7	9.00	25.1	52.1	30.5	58.5
6 Nuclear	48.1	22.4	46.6	11.6	24.1	89.4	54.5	61.0
6 Mitochondrial	7.46	2.68	35.9	2.04	27.3	15.0	11.2	74.7
6 Microsomal	29.7	16.6	55.9	9.15*	-	31.7	22.1	69.7
6 Supernatant	2.71	1.52	56.1	1.43+	52.8	21.0	11.5	54.8
Average % Recovered			50.3		30.8			62.2

Average total % recovered from S-10-17: 81.1

+ Single determination. * Eluent lost; value estimated from average recovery of other Florisil columns.

CHAPTER IX. THE SEPARATION, ISOLATION, AND IDENTIFICATION OF GLYCOLIPIDS FROM DOG INTESTINAL MUCOSA SUBCELLULAR FRACTIONS BY THIN LAYER CHROMATOGRAPHY

1. Introduction.

Prior to this investigation, separation of glycolipids of whole dog intestine and dog intestinal mucosa were accomplished by silicic acid, Florisil, and DEAE-cellulose column chromatography (Vance et al., 1966; McKibbin,1969). Preparative thin layer chromatography, using Silica Gel G developed in chloroform-methanol-water systems (65:35:8) (McKibbin, 1969) and (75:25:4) (J. M. McKibbin, personal communication) were also used. Only the shorter chain glycolipids which contain one, two, and three glycose units per molecule were separated using column techniques exclusively (McKibbin, 1969). The pentaglycoside was isolated using column techniques and countercurrent solvent fractionation (Vance et al., 1966). After silicic acid and Florisil column chromatography, ceramide tri- and pentaglycoside, and a ganglioside were isolated using preparative thin layer chromatography (McKibbin, 1969). In the articles mentioned above, tissues from several animals were pooled and used as starting material for lipid extraction. In the separation and isolation of glycolipids from subcellular fractions of intestinal mucosa of individual dogs, the small amounts of material available precluded all but preliminary column techniques and required preparative thin layer chromatography.

In the course of their purification, glycolipids were divided into two groups by silicic acid columns. One group contained glycolipids possessing one, two and three sugars, but no gangliosides. The second

group contained glycolipids with three or more sugars and the gangliosides. These two groups were passed separately through Florisil columns to remove phospholipid. The two groups of glycolipids were then subjected to thin layer chromatography and so members of one group did not interfere with members of the other.

Three new solvent systems (C, D, and E) for analytical and preparative thin layer chromatography (TLC) were developed by this author for the isolation of intestinal mucosa glycolipids and are presented below. Systems C and D were used analytically and preparatively. System E and Systems F and G (which were modifications of System E) were used analytically.

2. Material and Methods.

a. Thin layer chromatography.

(i) Solvent systems.

For convenience, the solvent systems used will be referred to by the letter system given below.

System	Solvent System (all volume ratios)	Sorbant
A	chloroform-methanol-water (65:35:8)	Silica Gel G
B	chloroform-methanol-water (75:25:4)	Silica Gel G
C	chloroform-methanol-conc. NH_4OH (40:80:25)	Silica Gel G
D	pyridine-acetone-diethylamine-water(10:90:21:19)	Silica Gel G
E	chloroform-methanol-water-diethylamine (110:90:40:105)	Neutral Aluminum Oxide
F	chloroform-methanol-water-diethylamine (110:90:36:72)	Neutral Aluminum Oxide
G	chloroform-methanol-water-diethylamine (110:90:38:88)	Neutral Aluminum Oxide

(ii) Glycolipid standards.

Glycolipids, including Hexaglycosides D-1 and D-2, were used as

thin layer chromatography standards. They were isolated from whole dog intestine by Dr. J. M. McKibbin and their composition has been described (Vance et al., 1966; McKibbin, 1969). Hexaglycosides D-1 and D-2 were pooled and separated into two components by System C. The faster migrating component was designated Hexaglycoside I and the slower, Hexaglycoside II. Their chemical analysis has not been described.

(iii) Sorbants and detection.

Silica Gel G (American Optical Corp.) in a ratio of 5 gm. per 10 ml. distilled H_2O or Neutral Aluminum Oxide (Brinkman Instruments, Inc.) in a ratio of 3 gm. per 4 ml. distilled H_2O were mixed in a rubber stoppered Erlenmeyer flask and spread on clean glass plates (20 x 20 cm.). 0.25 mm. thicknesses of these sorbants were used for analytical TLC and 0.50 mm. thicknesses were used for preparative TLC. When the layers became dull in appearance, they were put in a 95°C oven over night (14-16 hours). Glycolipids were detected on analytical thin layer by spraying with 0.2% naphthoresorcinol in 95% ethyl alcohol:20% H_2SO_4 (1:1, v/v) and heating at 110°C for 10-15 minutes. Glycolipids gave a blue or purple color with naphthoresorcinol-H_2SO_4 spray. Glycolipids were detected on preparative thin layer by spraying with 0.0005% Rhodamine 6G and examining under ultraviolet light. Sucrose could be detected by the naphthoresorcinol-H_2SO_4 reagent, but not by Rhodamine 6G.

b. Starting material.

Mixtures of glycolipids from dog intestinal mucosa homogenates and subcellular fractions were obtained by passing whole lipid extracts through silicic acid and Florisil columns. These procedures were described in Chapter VIII.

c. Chemical analysis of glycolipids.

The methods used were described in Chapter VI.

d. Hydrolysis of glycolipids for paper chromatography.

Glycolipids were hydrolyzed as described in Chapter VI except that hydrolyzed samples were not neutralized. Instead, the HCl hydrolysates in culture tubes were cooled and shaken with 2 ml. $CHCl_3$. An emulsion usually formed which was broken by freezing and centrifuging. The upper HCl layer was transferred to another culture tube and taken to dryness under vacuum, rinsed with 0.2 ml. distilled H_2O, and taken to dryness again. 0.05-0.07 ml. distilled H_2O was added to the tube and this was spotted on Whatman chromatography paper.

e. Descending paper chromatography.

Sugars were separated on Whatman chromatography paper No. 1 using n-butanol-pyridine-water (5:3:2) (System II) or n-butylacetate-acetic acid-water (3:2:1) (System III) developed for 40 hours as described by Masamune and Yosizawa (1953). System II separated glucosamine HCl, galactosamine HCl, glucose, galactose, fucose, and mannose (mannose and fructose migrated together in this system). System III was used to separate fructose and mannose. Detection of sugars was by $AgNO_3$, in acetone sprayed with NaOH as described by Trevelyan and coworkers (1950).

3. Results.

a. The separation of glycolipid standards of dog whole intestine.

(i) Separation of mono-, di, and trihexosides.

In dog whole intestine, there are four glycolipids which fall into this group, a glucocerebroside, sulfatide, dihexoside, and trihexoside (Vance et al., 1966; McKibbin, 1969). The sulfatide is exclusively galactosulfatide (McKibbin, personal communication). All but the sulfatide were detected in and isolated from intestinal mucosa. These

glycolipids, exclusive of the sulfatide, were separated preparatively using System B. In this system, the cerebroside had the greatest Rf and the trihexoside the least, with the dihexoside migrating between (Figures 15,16). On analytical TLC, the sulfatide migrated slightly in front of the trihexoside (Figures 15,16). An even better separation of the mono-, di-, and trihexoside was obtained using Silica Gel G impregnated with sodium borate (Young and Kanfer, 1965) and developed in System A.

(ii) Separation of oligohexosides.

There is no single solvent system which will adequately separate this group of glycolipids. In glycolipid preparations of subcellular fractions originally prepared in 0.25M sucrose, sucrose may also be present and together with the gangliosides and other glycolipids, make up a complex mixture of compounds. The ceramide trihexoside and sucrose were separated from all other glycolipids in this group using System A (Figure 8). Once the sucrose and the trihexoside were separated from this group of glycolipids, the gangliosides, pentaglycosides and hexaglycosides were separated preparatively from each other using System C (Figures 9, 27, 29). The gangliosides migrated closest to the solvent front followed by the pentaglycoside and finally the hexaglycoside. Trihexosides and pentaglycoside were not separated by this system (Figure 9). The hexaglycosides D-1 and D-2, which were isolated from pooled tissues, were probably mixtures of two or more glycolipids as indicated by chemical analysis (McKibbin, 1969) and their behavior in System C. This system separated them into two spots (Figures 9, 19, 27). Hexaglycosides isolated from subcellular fractions of intestinal mucosa of individual dogs migrated in this system as one spot (Figure 29). Hexaglycosides can be further separated using System E (Figures 10, 30, 31),

System F (Figure 11) or System G (Figure 12). Preparative TLC using these systems was not pursued. In subcellular fractions of intestinal mucosa of individual dogs, hexaglycosides of Dog 4 migrated as one spot while hexaglycosides of Dog 6, in some instances, migrated as two spots (Figures 30, 31). This may indicate a difference in the structure of glycolipids in individual dogs. These hexaglycosides might be further resolved by redeveloping the thin layer plate in the same solvent system.

(iii) Separation of gangliosides from oligohexosides.

There were at least two gangliosides which were isolated from whole intestine. These two, and possibly a third, may exist in intestinal mucosa, but have not yet been isolated and characterized. The gangliosides were designated G-1, G-2, and G-3. Although G-3 has not been shown to contain sialic acid, its behavior on thin layer was similar to known gangliosides. G-1 and G-2 were isolated from a complex mixture of whole intestine glycolipids which also contained trihexoside and pentaglycoside. G-1 and G-2 were used for standards for TLC and both contained sialic acid. G-1 may be a ganglioside which was previously described (Vance et al., 1966). The structure of G-2 is unknown. In System A, both these compounds migrated behind the pentaglycoside and were separated from the pentaglycoside except in the presence of hexaglycosides (Figure 8). The hexaglycoside migrated with G-1 and pentaglycoside as seen in Figures 8, 26, and 27, Channel 2. Gangliosides and oligohexosides were separated using System C(Figures 9, 27, 29) or System D (Figures 13, 14, 21, 22, 33). Systems E, F and G also separated the pentaglycosides and hexaglycosides from the gangliosides (Figures 11, 12). In these systems, the latter migrated nearest the solvent front,

and the former occupied an intermediate position between the gangliosides and hexaglycosides.

It is interesting to note that in the neutral System A, G-1 and G-2 migrated behind the pentaglycoside, and the trihexoside and the pentaglycoside were separated (Figure 8). However, in the basic Systems C, D, E, F, and G, the trihexoside and pentaglycoside were not separated, and the gangliosides migrated in front of the pentaglycoside (Figures 9, 11, 12, 13, 14, 29). G-3 migrated ahead of the pentaglycoside in System D and this was the only evidence that it was a ganglioside (Figures 21, 22, 33, 34). G-3 has not been detected in any other system and may even be an artefact.

b. Isolation and identification by TLC of glycolipids from subcellular fractions of intestinal mucosa of individual dogs.

(i) Glucocerebrosides.

Compounds which migrated with cerebroside standards in System B were detected in the homogenate, crude nuclear, mitochondrial, and microsomal fractions of Dogs 4 and 6 and in the supernatant of Dog 6 (Figures 15, 16). The supernatant fraction of Dog 4 was used up in preliminary analysis. Cerebrosides were isolated by preparative thin layer chromatography from the homogenate and microsomal fractions of Dogs 4 and 6 and the crude nuclear fraction of Dog 6 (Figure 23). Paper chromatography of hydrolysates of these fractions revealed only glucose, and the presence of glucose was verified with glucose oxidase. The cerebrosides of intestinal mucosa appeared as two or three spots which were probably due to differences in fatty acid chain length and/or the presence of hydroxy fatty acids (Figures 15, 16, 23). Recoveries of cerebrosides and dihexosides by preparative TLC are given in Table XXII.

(ii) Sulfatide.

This compound has been isolated from whole intestine and found to be exclusively galactocerebroside (J. M. McKibbin, personal communication). It was not detected in intestinal mucosa subcellular fractions. F-35-50 eluents were used for analytical and preparative thin layer chromatography. Since these eluents represented only 50% of the hexose of the S-10-17 lipids, the possibility remains that sulfatide was present in intestinal mucosa.

(iii) Dihexoside.

Compounds which migrated with ceramide dihexoside standards were detected in the homogenate, crude nuclear, mitochondrial, and microsomal fractions of Dogs 4 and 6 and the supernatant of Dog 6 (Figures 15, 16). The supernatant of Dog 4 was used up in preliminary analyses. Dihexosides were isolated by preparative thin layer chromatography from the homogenate and microsomal fractions of Dog 4 and 6 and the crude nuclear fraction of Dog 6 (Figure 24). Paper chromatography of hydrolysates of these fractions revealed only glucose and galactose. Chemical analyses by glucose oxidase and galactose oxidase indicated that glucose and galactose were in a 1:1 molar ratio (Table XXIII). Recoveries of cerebrosides and dihexosides by preparative TLC are given in Table XXII. The dihexosides migrates as two or three spots which were probably due to differences in fatty acid chain length and/or hydroxy fatty acids (Figures 15, 16, 24).

(iv) Trihexoside.

Compounds which migrated in System A with ceramide trihexoside standards were detected in the homogenate, mitochondrial, microsomal, and supernatant fractions of Dogs 4 and 6 and in the crude nuclear

fraction of Dog 6 (Figures 17, 18). These were isolated by preparative thin layer chromatography from the homogenate and the crude nuclear and microsomal fractions of Dogs 4 and 6 (Figure 25). This chromatographic identification is valid in that no other glycolipids thus far isolated from or detected in whole intestine or intestinal mucosa migrated with the trihexoside in this solvent system.

(v) Pentaglycoside.

The pentaglycoside could not be identified by a single solvent system on thin layer chromatography in the presence of trihexoside, hexaglycoside and gangliosides. Two systems were necessary to identify the pentaglycoside: System A to differentiate the trihexoside and pentaglycoside, and System C to differentiate the pentaglycoside from gangliosides and hexaglycoside. The trihexoside and pentaglycoside migrated together in this latter system (Figure 9). The value of System C is illustrated by comparison of Figures 26 and 27, Channel 2. Four glycolipids which migrated as one spot in System B were separated in System C. Compounds which migrated with the pentaglycoside have been isolated from the homogenate of Dog 4 and the crude nuclear fraction of Dog 6 and detected in the crude nuclear fraction of Dog 4 (Figures 26, 27). Chemical analysis of the isolated pentaglycosides indicated the presence of hexosamines by the Elson-Morgan reaction. Glucose could not be quantitated because of the small amounts of pentaglycoside isolated. Large losses were encountered in purifying the pentaglycosides. An average of 62% of the total anthrone of fraction S-50-80 which was put on Florisil columns was recovered. For this reason, the possibility that pentaglycoside was present in other subcellular fractions remains.

(vi) Hexaglycoside.

The hexaglycoside was separated from all the other known glycolipids of intestine using System C, if sucrose was absent (Figure 9). This system was used preparatively to isolate the hexaglycosides. Sucrose migrated with the hexaglycoside in this system, and these two compounds were separated with System A (Figure 8). Compounds which migrated with hexaglycoside standards in two systems, A and C, have been isolated from the homogenate and microsomal fractions of Dogs 4 and 6 and the crude nuclear fraction of Dog 6 (Figures 28, 29). The compound was also detected in the crude nuclear fraction of Dog 4 (Figure 27, Channel 2). They were further resolved analytically using System E. The "hexaglycosides" of Dogs 4 and 6 were different, Dog 6 having a faster moving component which Dog 4 lacked (Figures 30, 31). Paper chromatography of the hydrolysates of these compounds showed that Dog 4 had only glucosamine and no (or very little) galactosamine and Dog 6 had both glucosamine and galactosamine (Figures 35, 36). These findings suggested that glycolipids with six or more sugars may differ in structure in individual dogs.

(vii) Gangliosides.

Compounds which migrated with and behind ganglioside standards G-1 and G-2, and in front of the pentaglycoside in System D were detected in the homogenate, mitochondrial, microsomal, and supernatant fractions of Dogs 4 and 6 and in the crude nuclear fraction of Dog 6 (Figures 21, 22). A compound (G-3) which migrated behind G-2 is prominent in all these fractions and may be a ganglioside which has not been previously described. G-3 is better seen in Figure 33 where a fraction containing compounds which migrated with G-1, G-2, and hexaglycoside had an

additional compound (G-3). The absence of trihexoside and pentaglycoside in this fraction is evidenced in Figure 34.

(viii) Sucrose.

In homogenization of tissue in 0.25M sucrose for subcellular fractionation, sucrose might have been carried along with glycolipids as they were purified. There was some evidence that this occurred in the present work. Compounds which migrated with sucrose standards in System A were detected in the homogenate, mitochondrial, microsomal, and supernatant fractions of Dogs 4 and 6 and the crude nuclear fraction of Dog 6 (Figures 17, 18). These compounds were isolated from the homogenate, crude nuclear, and microsomal fractions of Dogs 4 and 6 (Figure 32). However, paper chromatography of the hydrolysates of these compounds in two solvent systems (Systems I and II) indicated the presence of glucose but not fructose. Instead of fructose, there was a double spot which migrated behind the amino sugar standards (glucosamine HCl and galactosamine HCl). However, other evidence suggested that the compound isolated (and shown in Figure 32) was sucrose. This thin layer chromatogram (Figure 32) was sprayed with naphthoresorcinol-H_2SO_4 reagent and heated in a 110°C oven. The compounds and the sucrose standard developed a magenta color within 5 minutes which turned blue on further heating. Sialic determinations on the whole lipid extracts of the homogenate and all subcellular fractions of Dogs 4 and 6 yielded only mahogany or light amber colors. Sialic acid determinations on the nuclear fractions of Dog 4 gave the characteristic blue color indicative of the presence of sialic acid only after the compound which migrated with sucrose on TLC was removed. Finally, when authentic sucrose was added to sialic acid standards and sialic acid determinations done, clear mahogany or amber colors were obtained.

4. Discussion.

In the isolation of pure glycolipids, methods which permit unambiguous identification and subsequent isolation of these compounds are of paramount importance. In this thesis, three new solvent systems for thin layer chromatography were described. Their usefulness has been in the separation of gangliosides, pentaglycosides and hexaglycosides. The hexaglycosides, which may actually be a mixture of complex glycolipids, were also further resolved. The separation of the penta- and hexaglycosides, and the resolution of the hexaglycosides from dog intestine had been a serious problem. These new solvent systems will be important tools in the isolation of hexaglycosides and gangliosides of dog intestine. These solvent systems will also be useful in the elucidation of precursor-product relationships in biosynthetic and catabolic pathways. Using these systems, preliminary evidence for differences in the structure of hexaglycosides from different dogs has been obtained. This finding is of considerable interest and suggests that arbitrary pooling of dog tissue for the study of the hexaglycosides should be avoided. Biosynthetic precursors of the hexaglycosides should also be studied in individual dogs. The relationship of the hexaglycosides to dog blood group specificity will be pursued in future work.

An interesting finding was that compounds which migrated with sucrose standards on thin layer chromatography were still present in the homogenate and all subcellular fractions, even after the extensive purification procedures. The failure to eliminate sucrose by washing procedures alone was confirmed in dog mucosa (J. M. McKibbin, personal communication) and also encountered in subcellular fractionation of guinea pig brain (Eichberg *et al.*, 1964). The identification of sucrose in relation to

glycolipids was also of importance in interpretating analytical data for total hexose.

Qualitatively, there was no striking difference in the glycolipid pattern of the homogenates and subcellular fractions of dog intestinal mucosa. A similar finding has been reported in guinea pig brain gangliosides (Eichberg et al., 1964). In this latter report, density gradient centrifugation was used, a procedure which results in rather pure subcellular fractions. Even myelin and synaptosomes had a ganglioside spectrum like all other subcellular fractions. These results might indicate that all glycolipids are ubiquitous in the membranes of all cell organelles.

In general, available evidence suggests that recoveries of glycolipids from preparative thin layer chromatography can be expected to decrease as the length of the carbohydrate chain increases. Recoveries of mono- and dihexosides from thin layer chromatography have been reported to be from 80-100% (McKibbin, personal communication; Suomi and Agranoff, 1965; Svennerholm and Svennerholm, 1963a; Vance and Sweeley, 1967). Recoveries for tetraglycoside containing an amino sugar have been reported to be 71% (Vance and Sweeley, 1967) and 50% (Svennerholm and Svennerholm, 1963a). Recovery of glycolipids with six or more sugar residues was reported to be "extremely poor" (Yamakawa et al., 1965) and of ganglioside 90-100% (Svennerholm, 1963b; Suzuki, 1964). Recoveries from preparative TLC of the mono- and dihexosides from dog intestine subcellular fractions were unsatisfactory for a molar comparison of these glycolipids. A possible source of poor recovery may have been the method used to concentrate the Silica Gel G eluent. Large volumes of solvent were used for this purpose, and reduction of this volume was

first accomplished using a large round bottom flask. This flask was rinsed three times with solvent which was transferred to a 35 ml. pear-shaped flask. The contents of this small flask were taken to dryness, and rinsed three times with solvent, which was taken to volume. An insoluble residue which could have been glycolipid was a constant finding on the walls of the small flask. An additional problem was that a single separation on preparative TLC was frequently inadequate. There seemed to be an affinity between glycolipids having different carbohydrate chain lengths and between glycolipids and sucrose. Glycolipids which were visualized with Rhodamine 6G under U.V. light as single bands, would contain other components when re-chromatogrammed in the same solvent system. These glycolipids were separated again on preparative TLC with resultant further losses. Recoveries of glycolipids from subcellular fractions were adequate, however, to allow preliminary chemical analysis on glycolipids from individual dog intestinal mucosa subcellular fractions. This was of some interest, for with the possible exception of sulfatide (Green and Robinson, 1960), no individual glycolipids have been isolated from the subcellular fractions of any other tissues. In this thesis, mono-, di-, tri-, penta- and hexaglycosides have been isolated and identified with TLC. Future work on individual dog intestinal tissue (which has not been subdivided by cell fractionation) will be easier utilizing the hydrolysis and TLC methods developed for use in this problem.

Table XXII. Recoveries of Ceramide Mono- and Dihexoside from Preparative Thin Layer Chromatography.

Dog Fraction	μmoles galactose on plates	No. TLC Plates Used	μmoles galactose recovered	% Recovered
4 Homogenate	28.2	3	16.8	59.6
4 Microsomal	6.44	1	4.83	75.6
6 Homogenate	19.1	2	11.4	59.7
6 Nuclear	20.5	2	18.1	88.3
6 Microsomal	14.9	2	9.70	65.1

"Galactose" was determined by the anthrone method of Bailey, and calculated from galactose standards.

Table XXIII. Molar Ratios of Glucose and Galactose in Ceramide Dihexosides Isolated from Dog Intestinal Mucosa Subcellular Fractions.

Dog Fraction	Molar Ratio of Glucose:Galactose
4 Homogenate	1.01
4 Microsomal	1.15
6 Homogenate	1.00
6 Nuclear	0.990
6 Microsomal	1.01

Glucose was determined by glucose oxidase method and galactose by galactose oxidase method.

Figure 8. The migration of glycolipid standards and sucrose in System A. This system was used to separate the trihexoside and sucrose from other glycolipids. Channels: 1. trihexoside, 2. pentaglycoside, 3. (a) pentaglycoside (b) ganglioside G-1 (c) ganglioside G-2, 4. hexaglycoside D-1, 5. hexaglycoside D-2, 6. sucrose. (System A; naphthoresorcinol-H_2SO_4 detection).

Figure 9. The migration of glycolipid standards in System C. This system separated the gangliosides, pentaglycoside and hexaglycosides. The trihexoside and pentaglycoside were not separated. Channels: 1. trihexoside. 2. pentaglycoside. 3. hexaglycoside I. 4. hexaglycoside I. 5. hexaglycoside II. 6. ganglioside G-1. 7. ganglioside G-2. (System C; naphthoresorcinol-H_2SO_4 detection).

Figure 8

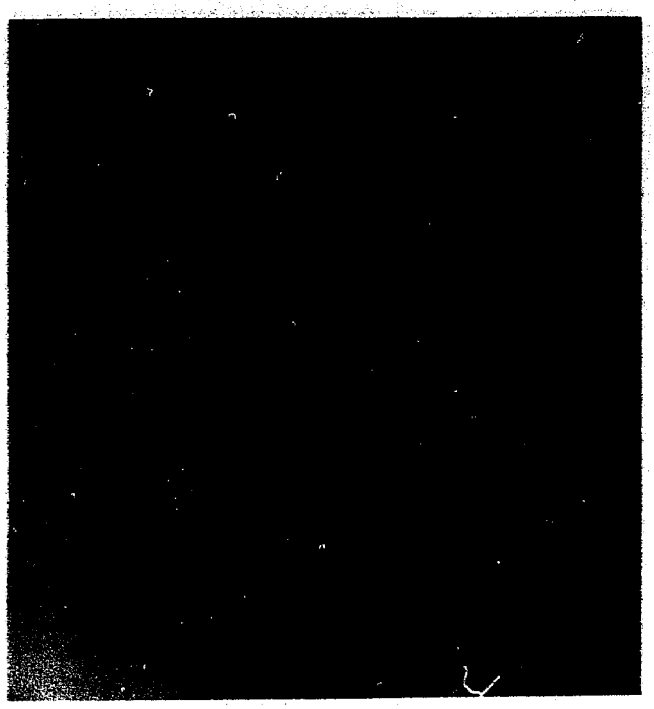


Figure 9

Figure 10. The migration of glycolipid standards in System E. Pentaglycoside was separated from hexaglycoside and hexaglycosides D-1 and D-2 were separated into three components. Channels: 1. hexaglycoside D-1. 2. hexaglycoside D-2. 3. pentaglycoside. (System E; naptho-resorcinol-H_2SO_4 detection).

Figure 11. The migration of glycolipid standards in System F. Hexaglycosides D-1 and D-2 were separated into three components. Ganglioside G-1, pentaglycoside, and hexaglycosides were well separated. Channels: 1. hexaglycoside D-1. 2. hexaglycoside D-2. 3. pentaglycoside. 4. (a) ganglioside G-1. (System F; naphthoresorcinol-H_2SO_4 detection).

Figure 12. The migration of glycolipid standards in System G. Trihexoside and pentaglycoside were not separated. Hexaglycosides were separated into two components. Gangliosides G-1 and G-2 were separated. Channels: 1. trihexoside. 2. pentaglycoside. 3. hexaglycoside I. 4. hexaglycoside I. 5. hexaglycoside II. 6. ganglioside G-1. 7. ganglioside G-2. (System G; naphthoresorcinol-H_2SO_4 detection).

Figure 10

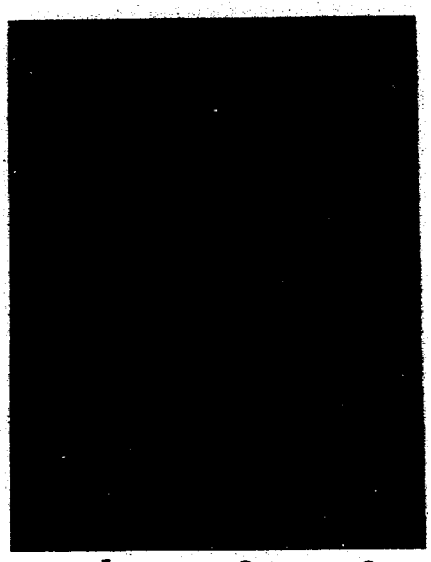


Figure 11

Figure 12

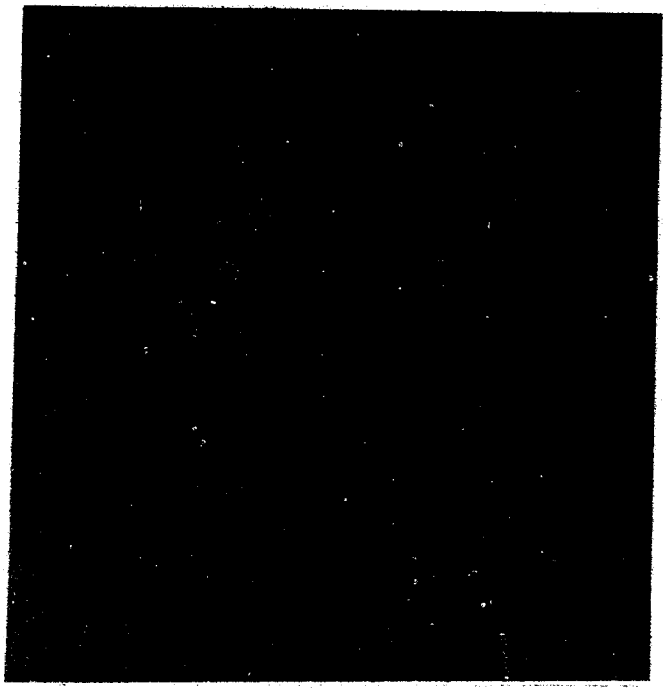


Figure 13. The migration of glycolipid standards in System D. Trihexoside and pentaglycoside were not separated. Gangliosides G-1, G-2, pentaglycoside and hexaglycosides were well separated. Channels: 1. trihexoside. 2. pentaglycoside. 3. mixture of glycolipids containing (a) ganglioside G-1 (b) ganglioside G-2 (c) pentaglycoside. 4. hexaglycoside D-1. 5. hexaglycoside D-2. (System D; naphthoresorcinol-H_2SO_4 detection).

Figure 14. The migration of glycolipid standards in System D. Gangliosides, pentaglycoside and hexaglycosides were well separated. Hexaglycosides D-1 and D-2 migrated differently. Channels: 1. pentaglycoside. 2. hexaglycoside D-1. 3. hexaglycoside D-2. 4. ganglioside G-1. 5. ganglioside G-2. (System D; naphthoresorcinol-H_2SO_4 detection).

Figure 15. Ceramide mono- and dihexosides from subcellular fractions of Dog 4. These compounds were detected in the homogenate, microsomal and supernatant fractions of Dog 4. Channels: 1. homogenate. 2. microsomal fraction. 3 and 4. glycolipid standards (a) monohexoside (b) sulfatide (c) dihexoside (d) trihexoside. 5. supernatant fraction. (System B; naphthoresorcinol-H_2SO_4 detection).

Figure 16. Ceramide mono- and dihexosides from subcellular fractions of Dog 6. These compounds were detected in the homogenate, crude nuclear, mitochondrial, microsomal and supernatant fractions. Channels: 1. homogenate. 2. crude nuclear fraction. 3 and 4. glycolipid standards (a) monohexoside (b) sulfatide (c) dihexoside (d) trihexoside. 5. mitochondrial fraction. 6. microsomal fraction. 7. supernatant fraction. (System B; naphthoresorcinol-H_2SO_4 detection).

Figure 13

Figure 14

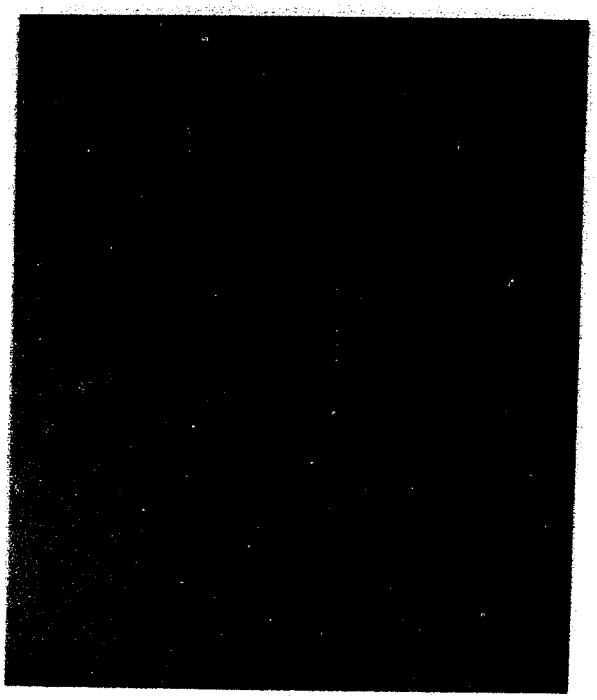
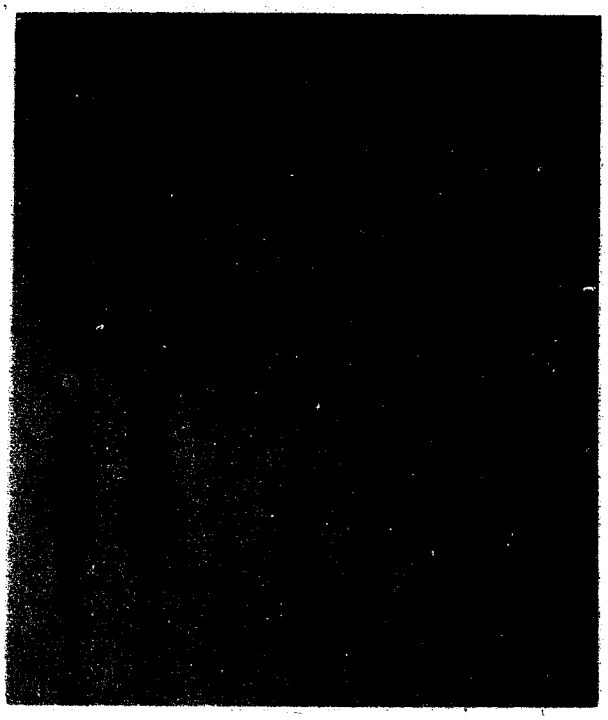


Figure 15

Figure 16



Figures 17 and 18. Glycolipid patterns in System A of oligohexosides and gangliosides of the subcellular fractions of Dogs 4 and 6. Compounds which migrated with trihexoside and sucrose standards were detected in all fractions. (System A; naphthoresorcinol-H_2SO_4 detection).

Figure 17. Dog 4. Channels: 1. homogenate. 2. mitochondrial fraction. 3. glycolipid standards (a) trihexoside (b) pentaglycoside (c) ganglioside G-2 (d) sucrose. 4. microsomal fraction. 5. supernatant fraction.

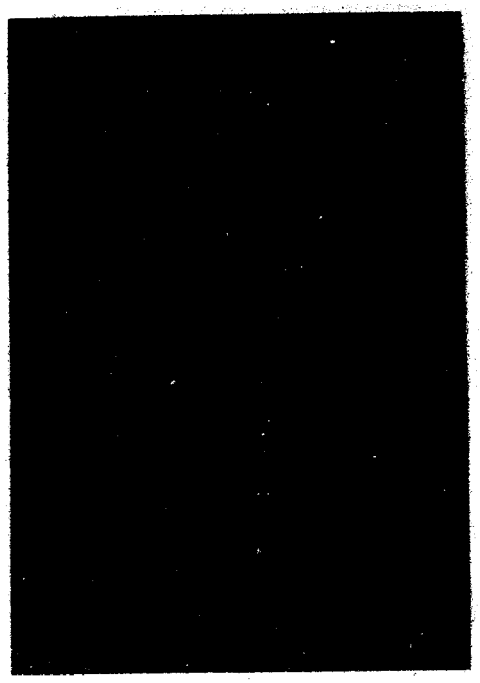
Figure 18. Dog 6. Channels: 1. homogenate. 2. crude nuclear fraction. 3. glycolipid standards (a) trihexoside (b) pentaglycoside (c) ganglioside G-2 (d) sucrose. 4. mitochondrial fraction. 5. microsomal fraction. 6. supernatant fraction.

Figures 19 and 20. Glycolipid patterns in System C of oligohexosides and gangliosides of the subcellular fractions of Dogs 4 and 6. No striking differences were seen in the subcellular fractions. Sucrose and hexaglycoside I were not separated. (System C; napthoresorcinol-H_2SO_4 detection).

Figure 19. Dog 4. Channels: 1. homogenate. 2. mitochondrial fraction. 3 and 4. glycolipid standards (a) pentaglycoside (b) sucrose (c) hexaglycoside I (d) hexaglycoside II. 5. microsomal fraction. 6. supernatant fraction.

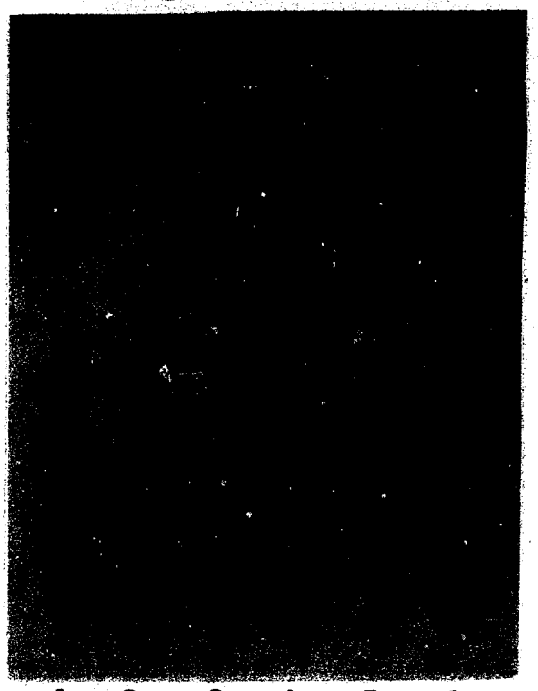
Figure 20. Dog 6. Channels: 1. homogenate. 2. crude nuclear fraction. 3 and 4. glycolipid standards (a) pentaglycoside (b) sucrose (c) hexaglycoside I (d) hexaglycoside II. 5. mitochondrial fraction. 6. microsomal fraction. 7. supernatant fraction.

Figure 17



1 2 3 4 5

Figure 18



1 2 3 4 5 6

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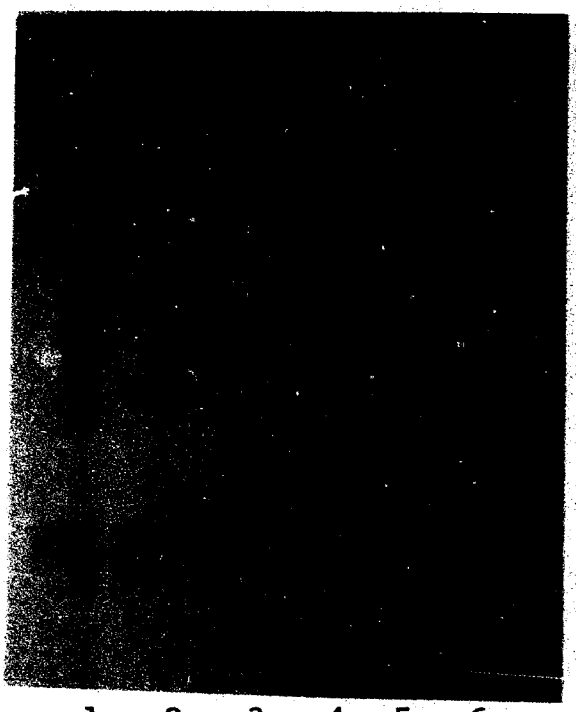
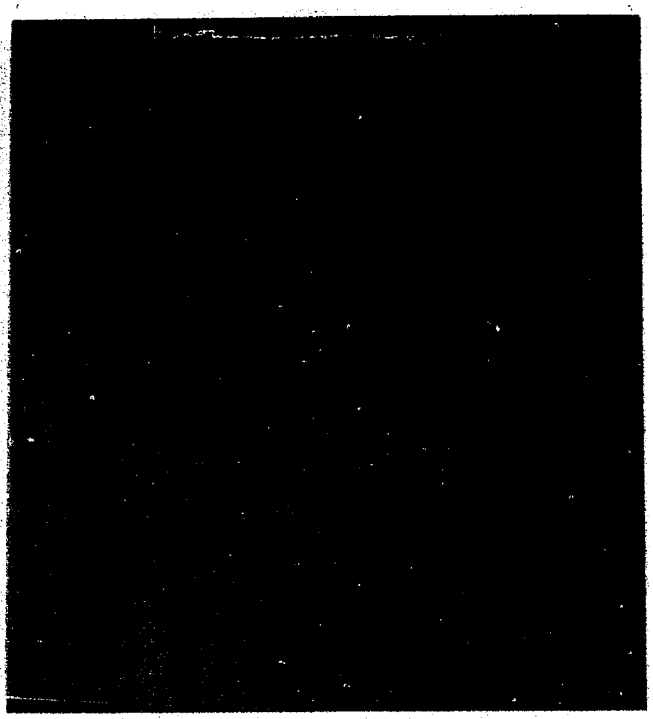


1 2 3 4 5 6

Figure 20



1 2 3 4 5 6 7

Figures 21 and 22. Glycolipid patterns in System D of oligohexosides and gangliosides of Dogs 4 and 6. No striking differences were seen in the subcellular fractions. Note the presence of a compound (G-3) which migrated between the ganglioside G-2 (f) and the pentaglycoside (g). (System D; naphthoresorcinol-H_2SO_4 detection).

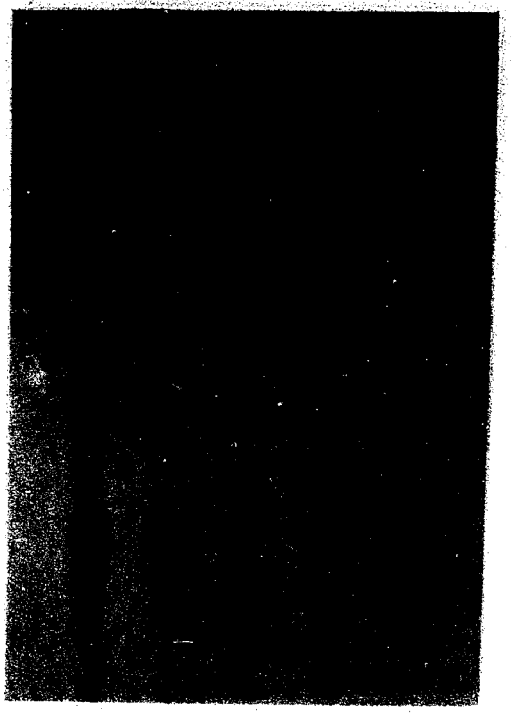
Figure 21. Dog 4. Channels: 1. homogenate. 2. mitochondrial fraction. 3 and 4. glycolipid standards (e) ganglioside G-1 (f) ganglioside G-2 (g) pentaglycoside (h) sucrose. 5. microsomal fraction. 6. supernatant fraction.

Figure 22. Dog 6. Channels: 1. homogenate. 2. crude nuclear fraction. 3 and 4. glycolipid standards (e) ganglioside G-1 (f) ganglioside G-2 (g) pentaglycoside (h) sucrose. 5. mitochondrial fraction. 6. microsomal fraction. 7. supernatant fraction.

Figure 23. Isolation of ceramide monohexoside from subcellular fractions of Dogs 4 and 6. Channels: 1. Dog 4, homogenate. 2. Dog 4, microsomal fraction. 3. glycolipid standards (i) monohexoside (k) dihexoside. 4. Dog 6, homogenate. 5. Dog 6, crude nuclear fraction. 6. microsomal fraction. (System B; naphthoresorcinol-H_2SO_4 detection).

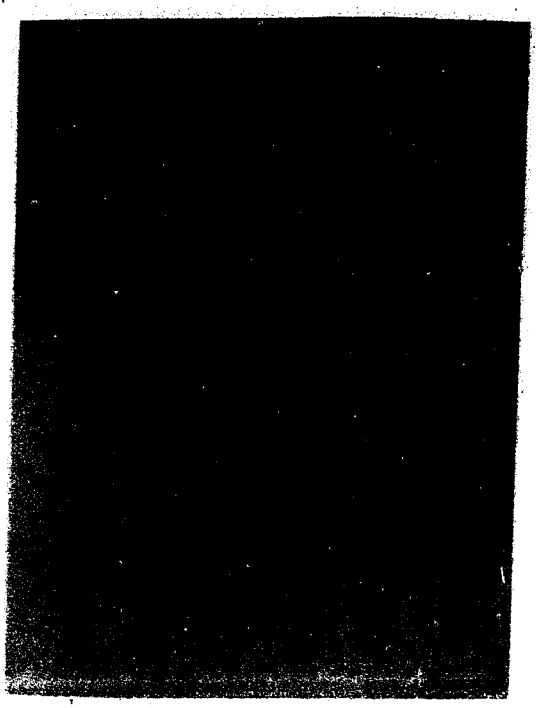
Figure 24. Isolation of ceramide dihexoside from subcellular fractions of Dogs 4 and 6. Channels: 1. Dog 4, homogenate. 2. Dog 4, microsomal fraction. 3. glycolipid standards (i) monohexoside (k) dihexoside. 4. Dog 6, homogenate. 5. Dog 6, crude nuclear fraction. 6. Dog 6, microsomal fraction. (System B; naphthoresorcinol-H_2SO_4 detection).

Figure 21



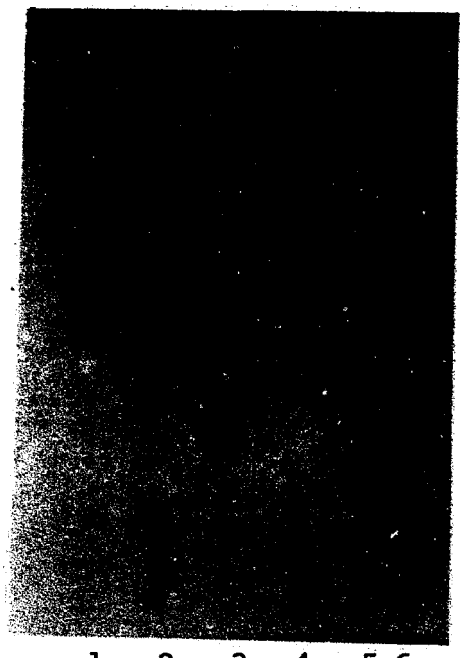
1 2 3 4 5

Figure 22



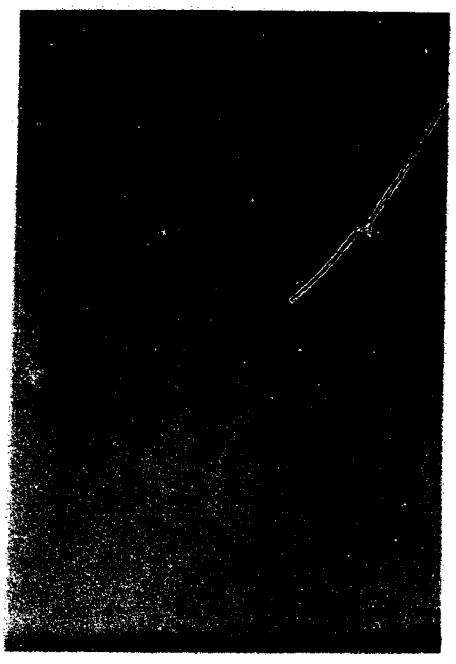
1 2 3 4 5 6

Figure 23



1 2 3 4 5 6

Figure 24



1 2 3 4 5 6

Figure 25. Isolation of ceramide trihexoside from subcellular fractions of Dogs 4 and 6. Only the standards and trihexosides gave a blue color with naphthoresorcinol-H_2SO_4 spray. Channels: 1. Dog 4, homogenate. 2. Dog 4, crude nuclear fraction. 3. Dog 4, microsomal fraction. 4. glycolipid standards (e) dihexoside (f) trihexoside (g) pentaglycoside. 5. Dog 6, homogenate. 6. Dog 6, crude nuclear fraction. 7. Dog 6, microsomal fraction. (System A; naphthoresorcinol-H_2SO_4 detection).

Figures 26 and 27. Isolation of compounds migrating with ceramide pentaglycoside standards in Systems A and C. The value of the new solvent system, System C, was apparent when Channel 2 of Figures 26 and 27 were compared. This comparison showed that a mixture of glycolipids migrated as one spot in System A, and four spots in System C.

Figure 26. Channels: 1. Dog 4, homogenate. 2. Dog 4, crude nuclear fraction. 3. glycolipid standards (e) trihexoside (f) pentaglycoside (g) ganglioside G-2. 4. Dog 6, crude nuclear fraction. (System A; naphthoresorcinol-H_2SO_4 detection).

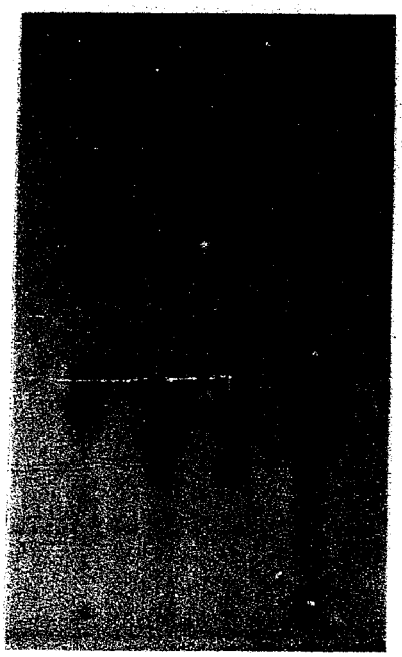
Figure 27. Channels: 1. Dog 4, homogenate. 2. Dog 4, crude nuclear fraction. 3. glycolipid standards (e) ganglioside G-1 (f) pentaglycoside (g) hexaglycoside I (h) hexaglycoside II. (System C; naphthoresorcinol-H_2SO_4 detection).

Figure 28. Isolation of ceramide hexaglycosides from subcellular fractions of Dogs 4 and 6. These compounds were neither trihexoside or sucrose. Only the standards and the hexaglycosides gave a blue color with naphthoresorcinol-H_2SO_4 spray. Compare with Figures 29 and 30. Channels: 1. Dog 4, homogenate. 2. Dog 4, microsomal fraction. 3. glycolipid standards (e) trihexoside (f) hexaglycoside I (g) sucrose. 4. Dog 6, homogenate. 5. Dog 6, crude nuclear fraction. 6. Dog 6, microsomal fraction. (System A; naphthoresorcinol-H_2SO_4 detection).

Figure 25

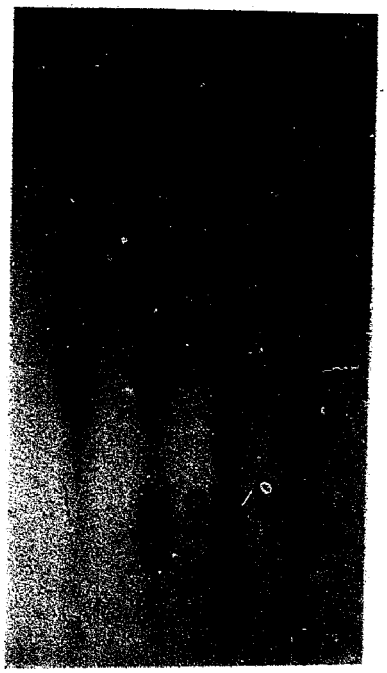
1 2 3 4 5 6 7

Figure 26



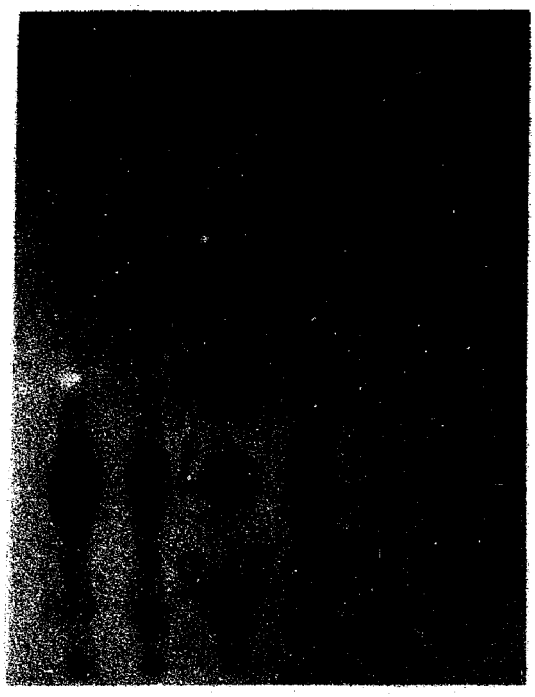
1 2 3 4

Figure 27



1 2 3 4

Figure 28



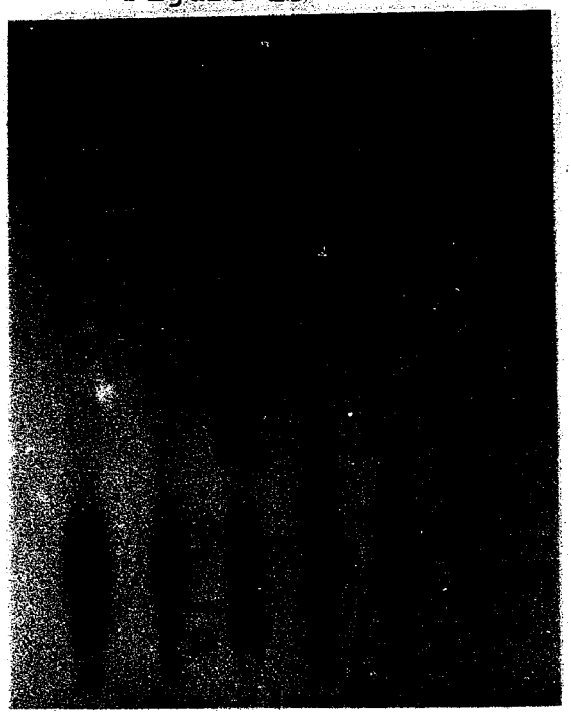
1 2 3 4 5 6

Figure 29. Isolation of hexaglycosides from subcellular fractions of Dogs 4 and 6. These compounds were neither gangliosides G-1, G-2, G-3 nor pentaglycoside. Only the standards and hexaglycosides gave a blue color with naphthoresorcinol-H_2SO_4 spray. Compare with Figures 28 and 30. Channels: 1. Dog 4, homogenate. 2. Dog 4, microsomal fraction. 3. glycolipid standards: (m) ganglioside G-1 (n) pentaglycoside (o) hexaglycoside I (p) hexaglycoside II. 4. Dog 6, homogenate. 5. Dog 6, crude nuclear fraction. 6. Dog 6, microsomal fraction. (System C; naphthoresorcinol-H_2SO_4 detection).

Figures 30 and 31. Differences in migration of hexaglycosides from individual dogs in System E. Hexaglycosides of Dog 4 had a single component, while those of Dog 6, in some cases, had two components. Only the components shown in Figure 31 gave a blue color with naphtho-resorcinol-H_2SO_4 spray. Compare with Figures 28 and 29. Channels: 1. Dog 4, homogenate. 2. Dog 4, microsomal fraction. 3. glycolipid standard, hexaglycoside I. 4. glycolipid standard, hexaglycoside II. 5. Dog 6, homogenate. 6. Dog, crude nuclear fraction. 7. Dog 6, microsomal fraction. (System E; naphthoresorcinol-H_2SO_4 detection).

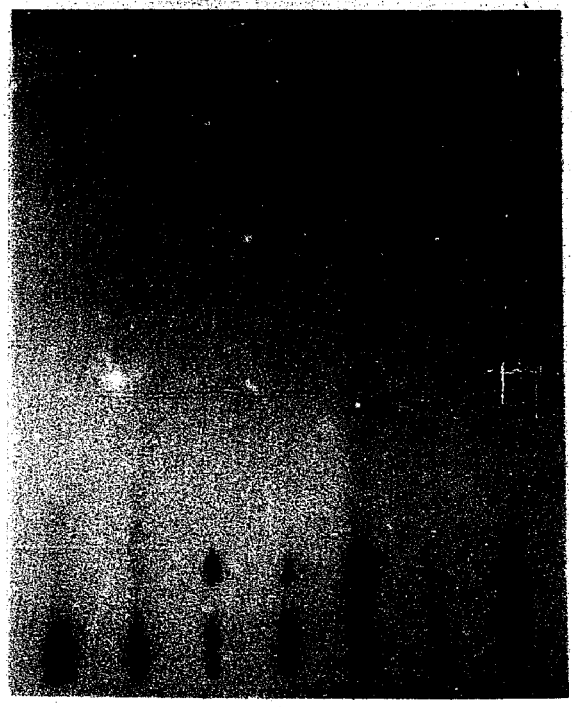
Figure 31. An enlargement of Figure 30.

Figure 29



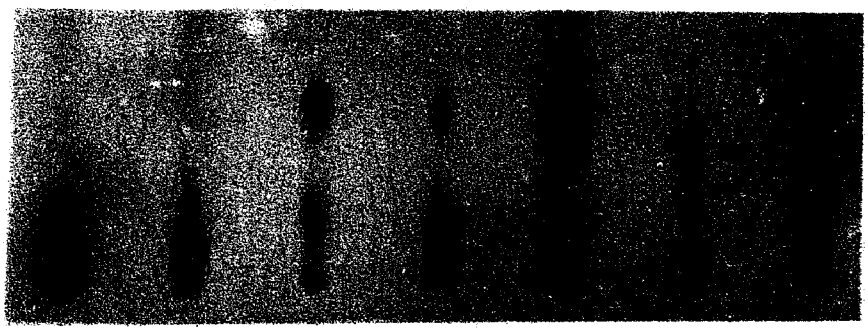
1 2 3 4 5 6

Figure 30



1 2 3 4 5 6 7

Figure 31



1 2 3 4 5 6 7

Figure 32. Isolation of sucrose from subcellular fractions. In System A, sucrose had the lowest, and ganglioside G-2 the next lowest, Rf when compared to all other glycolipids in dog whole intestine. Channels: 1. Dog 4, homogenate. 2. Dog 4, crude nuclear fraction. 3. Dog 4, microsomal fraction. 4. standards (o) ganglioside G-2 (p) sucrose. 5. Dog 6, homogenate. 6. Dog 6, crude nuclear fraction. 7. Dog 6, microsomal fraction. (System A; naphthoresorcinol-H_2SO_4 detection).

Figures 33 and 34. Detection of ganglioside G-3, a previously undescribed compound. G-3 (t) was neither ganglioside G-1 (r), G-2 (s), hexaglycoside (u), trihexoside (w), nor pentaglycoside (x). Other glycolipids of small intestine migrated in front of, or behind, the standards shown. G-3 was demonstrated only with System D. A mixture of glycolipids which migrated as one spot in System A (Figure 34; Channel 2), migrated as four spots in System D (Figure 33, Channel 1).

Figure 33. Channels: 1. Dog 4, homogenate glycolipid mixture. (t) ganglioside G-3 (u) hexaglycoside. 2. standards (r) ganglioside G-1 (s) ganglioside G-2. (System D; naphthoresorcinol-H_2SO_4 detection).

Figure 34. Channels: 1. glycolipid standards (w) trihexoside (x) pentaglycoside. 2. Dog 4, homogenate glycolipid mixture. (System A; naphthoresorcinol-H_2SO_4 detection).

Figure 32

1 2 3 4 5 6 7

Figure 33

1 2

Figure 34

1 2

Figures 35 and 36. Paper chromatography of acid hydrolysates of hexaglycosides from Dogs 4 and 6. These were isolated from small intestinal mucosa subcellular fractions of individual dogs. These paper chromatograms showed that hexaglycosides isolated from Dog 4 had glucosamine, while those isolated from Dog 6 had both glucosamine and galactosamine. The origin is toward the bottom of the Figures. Channel: 1. Dog 4. homogenate. 2. Dog 4, microsomal fraction. 3. sugar standards. 4. Dog 6, homogenate. 5. Dog 6, crude nuclear fraction. 6. Dog 6, microsomal fraction. (System II; detection by $AgNO_3$ in acetone, followed by NaOH).

Figure 36. Enlargement of Figure 35 which shows the area of the amino sugars.

Figure 35

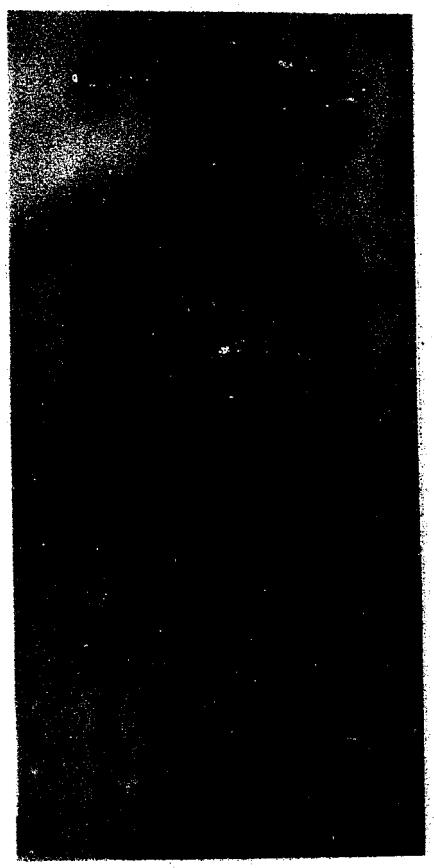
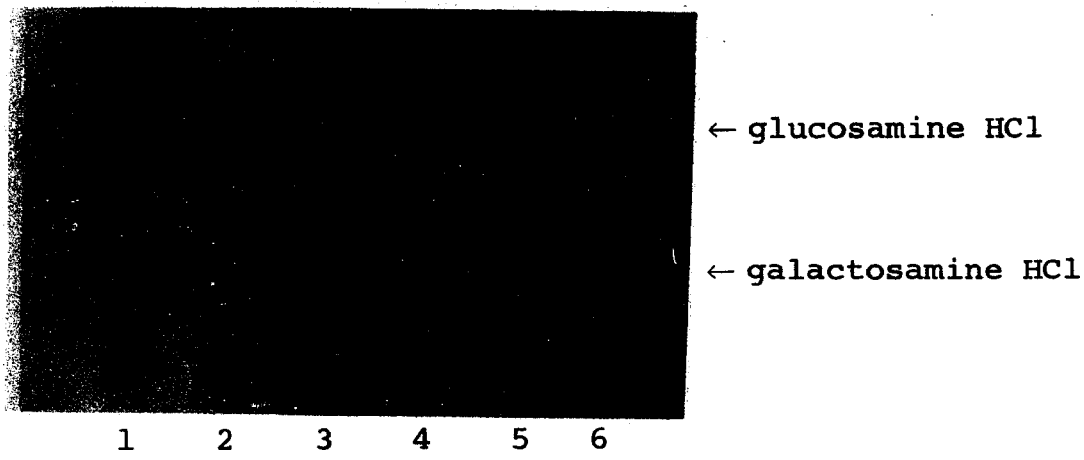


Figure 36



PART THREE: SUMMARY

The survey of the literature includes chapters on structure, distribution, subcellular distribution, and metabolism of glycolipids in various mammalian tissues. Tables include fifteen different glycosidic linkages found in glycolipids with references for each type of linkage and for fatty acid composition of glycolipids. References are given according to organ or tissue and mammalian species for gangliosides and asialoglycolipids.

The experimental section includes the subcellular distribution of DNA, cytochrome oxidase, unbound acid phosphatase, glucose-6-phosphatase, nitrogen, lipid phosphorus, and lipid hexosamine for three dogs (Table XXIV). When the percentages for these parameters were compared to percentages of nitrogen in the subcellular fractions the average ratios shown in Table XXV were found.

Of particular interest was a threefold increase of lipid hexosamine in the microsomal fraction when compared to the homogenate.

Evidence was presented for the optimum hydrolysis conditions for dog intestinal glycolipids using a simple heterogenous hydrolysis technique.

The whole lipid extracts of the homogenates and subcellular fractions were separately passed first through silicic acid and then Florisil columns. 66% of the anthrone reacting material in the original lipid extract was accounted for after these procedures.

The following individual glycolipids were identified by thin layer chromatography (TLC) in dog subcellular fractions: glucocerebroside, ceramide di- and trihexoside in the homogenate and all subcellular

fractions; ceramide pentaglycoside in the homogenate and nuclear fraction; compounds which migrated with ceramide hexaglycoside on TLC in the total homogenate, crude nuclear, and microsomal fractions. Because of incomplete recoveries from column techniques, it is possible that sulfatide, penta- and hexaglycoside were present in all subcellular fractions. Thin layer and paper chromatographic evidence was presented for structural heterogeneity of the ceramide hexaglycosides in individual dogs. Hexaglycosides from one dog contained both glucosamine and galactosamine, while hexaglycosides from another dog contained only glucosamine. Despite extensive washing of the original lipid extract with 0.25M $MgCl_2$ and subsequent dialysis, sucrose was identified by TLC in the homogenate and all subcellular fractions.

Three new TLC solvent systems were presented: chloroform-methanol-concentrated NH_4OH, 40:80:25 (System C) and pyridine-acetone-diethylamine-H_2O 10:90:21:19 (System D) on Silica Gel G; chloroform-methanol-H_2O-diethylamine, 110:90:36:72 (System E) on Neutral Aluminum Oxide. Systems C and D were used for preparative and analytical TLC, and System E was used for analytical TLC. Systems C, D, and E all separated the gangliosides, ceramide penta- and hexaglycosides from each other. Pentaglycosides and trihexosides were not separated by these Systems. These Systems also separated two gangliosides. System D allowed the detection of a compound which was tentatively interpreted to be a third ganglioside. System E further resolved the ceramide hexaglycosides into two components.

Table XXIV. Summary of Percentage Distributions and Recoveries for Three Dogs for the Subcellular Enzyme Markers, Nitrogen, Lipid Phosphorus and Lipid Hexosamine. The values found for the homogenate were taken as 100%.

Subcellular Fraction	Crude Nuclear (1.1×10^3g) 30 min.	Mitochondrial (7.7×10^3g) 30 min.	Microsomal (105×10^3g) 30 min.	Supernatant	Recovery
DNA	93.2 (86.5-102)	1.5 (0.6-1.9)	2.1 (1.5-2.6)	4.7 (3.8-6.2)	102 (96.8-111)
Cytochrome Oxidase	68 (61-80)	29 (24-34)	3.9 (2.4-6.5)	0 (0)	101 (88-112)
Acid Phosphatase	27.2 (23.4-29.2)	3.5 (2.8-4.1)	14.9 (9.7-21.4)	52.4 (47.0-56.0)	98.0 (95.9-102)
Glucose-6-Phosphatase	34.9 (27.4-39.0)	4.5 (2.0-6.2)	30.4 (19.0-41.1)	23.6 (15.9-38.7)	93.5 (89.8-97.9)
Lipid Phosphorus	62.7 (55.7-66.5)	7.8 (7.2-8.9)	21.2 (17.7-22.4)	6.8 (4.5-10.3)	98.5 (97.1-98.3)
Lipid Hexosamine	68.6 (58.5-79.3)	6.0 (5.3-6.6)	28.2 (24.7-32.4)	0 (0)	103 (96.9-111)
Nitrogen	46.6 (42.5-53.4)	3.7 (3.2-4.7)	9.3 (8.5-10.6)	39.8 (34.1-47.7)	99.5 (91.9-107)

Table XXV. Ratios Found by Dividing the Percent Subcellular Markers, Lipid Phosphorus, and Lipid Hexosamine by the Percent Nitrogen.

	Subcellular Fraction				
	Homogenate	Crude Nuclear	Mitochondrial	Microsomal	Supernatant
DNA	1.00	2.00	0.41	0.23	0.12
Cytochrome Oxidase	1.0	1.5	7.9	0.42	-
Acid Phosphatase	1.00	0.58	0.95	1.60	1.32
Glucose-6-Phosphatase	1.00	0.75	1.22	3.27	0.59
Lipid Phosphorus	1.00	1.35	2.18	2.28	0.18
Lipid Hexosamine	1.00	1.47	1.67	3.06	-

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