SHORT TITLE:

MIGRATION OF ^{3}H-FUCOSE LABEL IN RAT CELLS.

ABSTRACT

Young rats were injected with ^{3}H-fucose and sacrificed after various times. In electron microscopic radioautographs of duodenal villus columnar cells and hepatocyes, at 2 minutes after injection the label was localized mostly over the Golgi apparatus. By 20 minutes, label appeared at all cell surfaces and in lysosomes. It is interpreted that the fucose label was incorporated by the Golgi apparatus into glycoproteins which migrated to lysosomes and to all cell surfaces to be added to the cell coat. A method developed to stain radioautographs of duodenal cells with phosphotungstic acid revealed glycoprotein in all labelled structures, including cytoplasmic vesicles which may transport the labelled glycoprotein to the cell surfaces. In light microscopic radioautographs of many other cell types, label appeared first over the Golgi region and later over cell secretions or over cell surfaces. Thus cell coat synthesis in all cells may be completed in the Golgi apparatus.

INCORPORATION OF ^{3}H-FUCOSE LABEL INTO GLYCOPROTEIN IN THE GOLGI APPARATUS OF RAT CELLS, AND ITS PASSAGE TO THE CELL COAT AND TO LYSOSOMES

by

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FOR JOY

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INTRODUCTION

In the past decade increasing attention has been given to the importance of the role played by carbohydrate substances in the structure and function of biological organisms, and enormous strides have been made towards an understanding of the structure and function of these substances. Only a few years ago, an assessment of the role played by carbohydrate macromolecules in organisms was limited to their presence in mucous secretion, in energy reserves such as starch or glycogen, and in structural materials such as connective tissue ground substances, cellulose or chitin (Harrow and Mazur, 1954). Yet, the refined techniques of modern biochemistry now indicate that carbohydrate residues are also present in an ever increasing number of proteins, including nearly all of the plasma proteins, many enzymes, hormones, antibodies, and the major structural proteins of animal tissues, the collagens. Especially interesting is the discovery of a layer of carbohydrate material at the surface of most or all animal cells.

The last few years have also seen a substantial beginning made in the understanding of the biosynthesis of carbohydrate substances, both in terms of the mechanisms involved and the sites in the cells where these mechanisms occur. Extremely helpful in these studies has been the utilization of isotopically labelled presursors of carbohydrates. In this thesis, the results obtained by radioautographically tracing the fate of a newly available carbohydrate precursor, ^{3}H-fucose, in rat tissues will be described.

Distribution of Carbohydrate Substances in Animal Tissues

Carbohydrate residues occur in four basic types of macromolecules in animal tissues:

1) Homopolysaccharides: which are polysaccharides containing only one type of monosaccharide residue, the most common example being glycogen.

2) Nucleic Acids: whose nucleoside components contain ribose or deoxyribose residues.

3) Glycolipids: The predominant glycolipids of mammalian systems are glycosylceramides. These have special structures which will not be discussed in this thesis (see reviews by Ledeen, 1966; Sweeley and Dawson, 1969; and McCluer, 1968), but contain the same sugar residues as found in many glycoproteins, ie. glucose, galactose, glucosamine, galactosamine, fucose, and sialic acid. Immunological evidence indicates a close chemical and metabolic relationship between these glycolipids and glycoproteins (McCluer, 1968).

4) Glycoproteins: These substances have been defined by Spiro (1970) as proteins which have carbohydrate covalently attached to their peptide portion, and their detailed structure is described below.

Structure and Distribution of Glycoproteins

The simple definition of glycoproteins given above although correct, masks the considerable confusion in defining the members of this class of substances in past years. Because of the great resistance with which these substances revealed their structures, it has been difficult to classify them, and many classifications have been proposed only to be later discarded or modified (Meyer, 1938; Meyer, 1945; Jeanloz, 1960; Gottschalk, 1962).

Indeed, the substances which are included in the above definition of glycoproteins constitute a very wide range of protein types as shown in Text-Table I (after Spiro, 1970). Nonetheless certain characteristic sugar residues are found in the carbohydrate portion of all of these

Group	Examples
Plasma	Fetuin (2), α_1-acid glycoprotein (3), transferrin (4), ceruloplasmin (5), haptoglobin (6), α_2-macroglobulin (7), Barium α_2-glycoprotein (8), fibrinogen (9), β-lipoprotein (10), thyroxine-binding globulin (11), corticosteroid-binding globulin (12)
Immunoglobulins	IgG (13), IgA (14), IgM (15)
Urine	Tam and Horsfall glycoprotein (16)
Hormones	Chorionic gonadotrophin (17), follicle-stimulating hormone (18), interstitial cell-stimulating hormone (19), thyroid-stimulating hormone (20), thyroglobulin (21)
Enzymes	Pancreatic ribonuclease (22, 23) and deoxyribonuclease (24), Taka-amylase (25), fungal glucose oxidase (26), stem bromelain (27), kidney γ-glutamyl transpeptidase (28), liver β-glucuronidase (29), N-acetylglucosaminidase (30), serum cholinesterase (31) and atropinesterase (32), horseradish peroxidase (33), fungal glucoamylase (34) and chloroperoxidase (35), pepsin and pepsinogen (36), yeast invertase (37)
Egg white	Ovalbumin (38), ovomucoid (39), avidin (40), ovotransferrin (41)
Mucins	Submaxillary glycoproteins (42, 43), ovarian cyst glycoproteins (44, 45), sulfated gastric (46), colonic (47), and submaxillary (48) glycoproteins
Connective tissue	Proteoglycans (49), collagens (50, 51), aorta glycoproteins (52, 53), acid glycoproteins of bone (54), keratan sulfate-proteins (55, 56)
Extracellular membranes	Glomerular basement membrane (57), lens capsule (58), Descemet's corneal membrane (59), Reichert's yolk sac membrane (60), annelid cuticle (61), plant cell wall (62), yeast cell wall (63)
Cellular membranes	Plasma membranes (64–69), intracellular membranes (70–73), platelet membranes (74), visual pigment (75), *E. coli* cell membrane (76)
Phytohemagglutinins	Soy bean (77), wax bean (78), potato (79), black locust (80), meadow mushroom (81)

Text-Table I

glycoproteins. These include D-galactose (Gal), D-Mannose (Man), D-glucose, (Glc), L-fucose (Fu), D-xylose (Xyl), L-arabinose (Arab), N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc),

and the N-acetyl and N-glycol derivatives of neuraminic acid (NAN, NGN) which constitute the sialic acids (SA). Until recently, acid mucopolysaccharides have usually been regarded as a separate class of carbohydrate macromolecules, but since most acid mucopolysaccharides are now known to be covalently bound to proteins, they may be considered as a special class of glycoproteins (proteoglycans). Their hexuronic acids, D-glucuronic acid (GlcUA) and L-iduronic acid (IdUA), should thus be added to the above list of sugar residues.

The carbohydrate portion of a glycoprotein molecule may constitute from less than 1% to more than 80% of the weight of the molecule (Spiro, 1970). Similarly the carbohydrate portion may be in the form of one prosthetic group, as in ovalbumin (Johansen, Marshall, and Neuberger, 1961), or as many as 800 groups, as in submaxillary glycoprotein (Graham and Gottschalk, 1960). The carbohydrate prosthetic groups range in size from monosaccharides or disaccharides, as in mucins (Graham and Gottschalk, 1960) or collagens (Spiro, 1969), to complex heteropolysaccharide units containing as many as seven of the above sugar types, as in plasma glycoproteins (Spiro, 1968).

Although the number of possible combinations and sequences of sugar types in these carbohydrate prosthetic groups is enormous, there is actually a very conservative utilization of structural patterns, with certain sequences recurring in many glycoproteins. In addition, there are only certain types of glycopeptide linkages used between the carbohydrate prosthetic groups and the polypeptide chain of the glycoproteins. On the basis of these structural features, a tentative chemical classification of carbohydrate prosthetic groups can be made, as shown in Text-Table II (condensed from Spiro, 1970).

	Glycopeptide bond			Carbohydrate unit		Glycoprotein[a]
	Amino acid	Sugar	Type	Composition[b]	Mol wt range	
1. a	Asn	GlcNAc	N-glycoside	NAN, NGN, Fu, Gal, GlcNAc, GalNAc, Man	2000–3500	Plasma proteins, fetuin hormones, immunoglobulins, thyroglobulin, porcine ribonuclease, basement membranes
b	Asn	GlcNAc	N-glycoside	Man, GlcNAc	1200–4000	Enzymes, egg white proteins, thyroglobulin, IgA immunoglobulin, soybean hemagglutinin
2. a	Ser, Thr	GalNAc[c]	O-glycoside	NGN, NAN, Fu, GalNAc, GlcNAc	203–1225[d]	Mucins, cell membranes, immunoglobulins, fetuin
b	Ser, Thr	GalNAc	O-glycoside	NAN, Fu, Gal, GlcNAc, GalNAc, sulfate	3500–10,000[d]	Cartilage keratan sulfate-protein[e]
3.	Ser	Xyl	O-glycoside	GlcUA, IdUA, GlcN (Ac, SO_4H) GalNAc, Gal, Xyl, O-sulfate	13,000–29,000[d]	Proteoglycans (protein complexes of chondroitin sulfates, dermatan sulfate, heparin, heparan sulfate)
4.	Hyl	Gal	O-glycoside	Glc, Gal	162–324	Basement membranes and collagens

Text-Table II

Thus some types of carbohydrate groups are linked to the polypeptide chain through an innermost N-acetylglucosamine residue which is attached by means of an N-glycosidic linkage to an asparagine residue of the polypeptide chain. Groups of this type include a large, extensively branched heteropolysaccharide group (1a in Text-Table II) occurring in plasma proteins, hormones, basement membranes, and other substances. The sequence of sugars in this group is usually as follows:

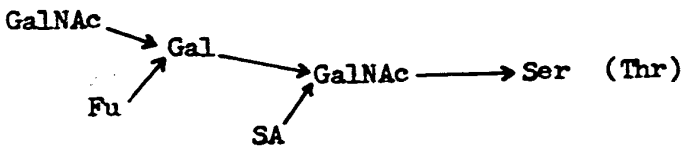
$$[\text{SA (or Fu)} \rightarrow \text{Gal} \rightarrow \text{GlcNAc}]_4 \longrightarrow (\text{Man, Man, Man, GlcNAc})\ \text{GlcNAc} \rightarrow \text{NHAsp}$$

The outer trisaccharides of this group, which always end in either sialic acid or fucose residues, are attached to mannose residues of the inner core (Wagh, Bornstein, Winzler, 1969).

A second type of asparagine linked prosthetic group occurring in many enzymes consists only of mannose and N-acetylglucosamine residues

(1b in Text-Table II).

In all mucins, and in some cell membrane glycoproteins, prosthetic groups occur which are attached through N-acetylgalactosamine to serine or threonine residues in the peptide chain by means of an O-glycosidic bond (2a Text-Table II). The sequence of sugars in this group is often:



In this case, sialic acid, fucose and sometimes N-acetylgalactosamine, are all terminal sugar residues.

A second type of serine (threonine) linked prosthetic group occurs in cartilage keratan sulfate-protein complexes (2b in Text-Table II). The basic structural pattern of this group is of mucopolysaccharide type and consists of a core of repeating N-acetylglucosamine and galactose residues. The sialic acid and fucose residues are believed to be attached in terminal positions in the carbohydrate chain. A group of sulfated glycoproteins have been recently described in gastric mucosa (Pamer, Glass, and Horowitz, 1968), colonic mucosa (Inoue and Yosizawa, 1966), and submaxillary gland (Bignardi et al., 1964), which contain fucose residues and may be closely related chemically to the above cartilage keratan sulfate.

A third type of carbohydrate-protein linkage has been found to attach most substances known as mucopolysaccharides to protein chains to form proteoglycans. Only hyaluronic acid has not as yet been clearly shown to be linked to protein (Spiro, 1970). The above linkage (3 in Text-Table II) consists of a sequence of two galactose residues and then

xylose which is O-glycosidically bound to serine residues in the protein chain, (Rodén, 1968), ie.

GlcUA→Gal→Gal→Xyl→Ser

The glucuronic acid residue at the left represents the first member of a chain of repeating disaccharides of hexuronic acid and hexosamine residues, which makes the carbohydrate group of these substances quite different from those of other glycoproteins; thus these substances constitute a specialized group.

Finally, a fourth type of glycopeptide bond, the O-glycosidic linkage of galactose to hydroxylysine (4 in Text-Table II) links the simple carbohydrate prosthetic groups of collagen to protein chains. In basement membrane collagens, all of these prosthetic groups are disaccharides containing an inner galactose and outer glucose residue, while in fibrillar collagens, up to 50% of the groups consist only of a single galactose residue (Spiro, 1969).

While most glycoproteins contain only one of the above types of carbohydrate groups, some contain more than one; thus thyroglobulin contains both of the above types of asparagine linked groups (1a and 1b) (Spiro, 1965), and basement membrane collagen contains asparagine linked heteropolysaccharide groups (1a) as well as hydroxylysine linked disaccharide groups (4) (Spiro, 1967).

Of special interest in the present thesis is the fact that L-fucose occurs as a terminal residue in several of the carbohydrate prosthetic groups listed in Text-Table II (1a, 2a, 2b), and therefore may be a residue in the carbohydrate moiety of plasma protein, fetuin, hormones,

thyroglobulin, immunoglobulins, mucins, cell membrane glycoproteins, basement membrane collagen, keratan sulfate-protein complexes, and sulfated glycoproteins of gastric and colonic mucosa and submaxillary glands.

The Presence of Glycoprotein at Cell Surface Membranes

Until quite recently, most animal cells were considered to be enclosed solely by a plasma membrane with no external coverings. Extraneous coats of carbohydrate nature were known to surround egg cells (Monné and Slauterback, 1950; Leblond, 1950; Soupart and Noyes, 1964) and protozoan cells (Bairatti and Lehmann, 1953; Pappas, 1954; Marshall and Nachmias, 1965; Szubinska, 1964), and there was indirect evidence that an intercellular cementing substance of carbohydrate nature (Rinaldini, 1958; Laws and Stickland, 1961) held together tissue culture and other cells (Moscona, 1952; Weiss, 1959; Rant Hebb and Wang-Chu, 1960). However, electron microscopy failed to reveal the presence of any cell coat along the outside of plasma membranes, except in the case of basement membrane type layers. (Fawcett, 1964) and the fuzzy coating on free surfaces of many epithelial cells (Burgos, 1960; Bonneville, 1961; Peachey and Rasmussen, 1961; Fawcett, 1962; Ito and Winchester, 1963; Ito, 1964).

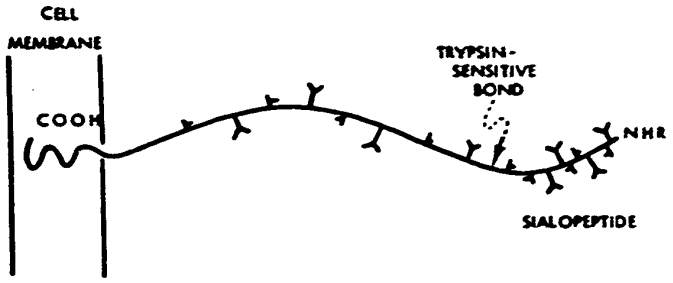
The existence of cell coat material on other cell surfaces was revealed only when histochemical stains specific for carbohydrate came into use and stained material was seen along all surfaces of tumor cells (Gasic and Berwick, 1962) and stratified epithelial cells (Wislocki, Fawcett and Dempsey, 1951), and along the lateral surfaces of intestinal epithelial cells (Puchtler and Leblond, 1958). In 1966 the ubiquitous presence of such staining was shown by Rambourg, Neutra and Leblond who demonstrated

carbohydrate material along the surfaces of over 50 cell types in the rat using the PA-Schiff and colloidal iron stains (Rambourg et al., 1966). With the successful adaption of these stains for ultrastructural use, Rambourg and Leblond demonstrated a similar staining of all surfaces in the electron microscope (Rambourg, and Leblond, 1967). The specificity of these stains indicated that the stained material was glycoprotein in nature since the periodic acid-Schiff stain, under the conditions used, stained only glycoproteins (Leblond at al., 1957). The staining of the coat by colloidal iron, which detects the acidic carboxyl and sulfuric acid groups of carbohydrates (Mowry, 1958, 1963), led some investigators to the conclusion that acid mucopolysaccharides were present in this coat (Ito, 1965). As pointed out by Rambourg et al. (1966), however, the staining could be equally well explained by the presence of acidic groups in sialic acid. The absence of acid mucopolysaccharides, at least in the apical surface coat of intestinal cells, has recently been shown by Forstner (1969) who showed that hexuronic acids were not present at this site.

Within the past few years, a number of electron microscopic histochemical techniques, with varying degrees of specificity for glycoprotein substances (ie.periodic acid silver-methenamine, periodic acid chromic acid silver methenamine, phosphotungstic acid at low pH, colloidal iron, colloidal thorium, ruthenium red, lanthanum, concanavalin A) have been developed, and the presence of a glycoprotein layer along cell surfaces has become well established. In addition, however, the existence of glycoprotein at cell surfaces has also been provided by several biochemical techniques such as cell electrophoresis, immunochemical, and microchemical techniques. While it is beyond the scope of this thesis to discuss these studies in detail (see reviews by Maddy, 1966; Benedetti and Emmelot, 1967;

Cook, 1968 ; and Winzler, 1970 a, b), some findings can be mentioned.

Quantative chemical determination of membrane composition indicates that most mammalian cell membranes contain between 2 and 8% carbohydrate (Winzler, 1970a). Some of this carbohydrate is in the form of glycolipids, but the remainder is glycoprotein. Although the nature of the carbohydrate components have not been extensively studied, neutral sugars such as galactose, mannose, fucose, and often some glucose are found, along with N-acetylglucosamine, N-acetylgalactosamine, and sialic acid. In erythrocyte membranes, on which most work has been done, a glycoprotein with a molecular weight of about 31,000 has been isolated (Kathan and Winzler, 1963). This glycoprotein has a high carbohydrate content of 60% and also has a very high content of serine and threonine, indicating that the carbohydrate prosthetic groups are probably of type 2a in Text-Table II. On the basis of information presently available, Winzler (1970[a]) has proposed a model for this glycoprotein and its association with the erythrocyte membrane as follows:



According to this model, the glycoprotein is associated with the lipoidal trilaminar plasma membrane by virtue of a region poor in carbohydrate and rich in lipophilic amino acids which are at the carboxyl end of the peptide chain.[1] The remainder of the chain has considerable carbohydrate attached

1. Recently evidence has been obtained that the polypeptide of this glycoprotein extends right through the lipoidal membrane and its inner part resides on the inner surface of the membrane (Bretscher, 1971).

as small oligosaccharides. The amino terminal end of the peptide chain is much enriched in carbohyrates, and this end forms the large sialopeptide released from intact erythrocytes by trypsin treatment, (Winzler, 1970).

The membrane fraction of intestinal brush borders has also been isolated (Overton et al., 1965; Eichholz, 1967, 1969; Forstner, et al., 1968), and shown to be rich in neutral sugars especially galactose (Eichholz and Winzler, unpublished). The biochemical composition of this fraction will be discussed in more detail in the Discussion section of this thesis.

The Presence of Glycoproteins in Lysosomes

The staining of lysosomes with the PA-Schiff technique in the light microscope (Davies, 1954; Novikoff, 1961; Koenig, 1962), and with periodic acid silver methenamine (Rambourg at al., 1969) and phosphotungstic acid (Bertolini, 1965; Rambourg, 1969; Rambourg, Bennett, Kopriwa, and Leblond, 1971) in the electron microscope, indicates that glycoproteins are present in these bodies. Recently it has been shown that many or all of the lysosomal hydrolytic enzymes are glycoproteins (Goldstone and Koenig, 1970) ie. β-glucuronidase (Plapp and Cole, 1967) DNA'se (Bernardi , 1968), N-acetyl-β-glucosaminidase (Robinson and Stirling, 1968) cathepsin C (Metrione, Neves and Fruton, 1966) acid phosphatase (Smith and Whitby, 1968) and aryl sulfatase (Allen and Roy, 1968). Thus the carbohydrate staining of these bodies is probab y due to the hydrolytic enzymes themselves, (Koenig, 1969).

Biosynthesis of Glycoproteins

Biosynthesis of the Protein Moiety of Glycoprotein

The protein moiety of glycoproteins is synthesized in the same fashion as other secretory proteins. The mechanism of this synthesis utilizing transfer, messenger and ribosomal RNA, has now been well established both in cell free systems (Siekevitz, 1952; Zamecnik and Keller, 1954; Nirenberg

and Matthaei, 1961), and, more recently, in cell fractions (Siekewitz and Palade, 1960; Redman, Siekewitz and Palade, 1966). The use of fractionation techniques has also established the ribosomes of the rough endoplasmic reticulum as the intracellular site of synthesis of secretory protein chains (Redman, 1968; 1969; Hicks, 1969) (although Glick and Warren (1969) report synthesis at the cell surface in HeLa cells). Additional evidence that the rough endoplasmic reticulum is the site of incorporation of amino acids into secretory protein molecules has been provided by the technique of radioautography. Thus when labelled amino acids were administerd to animals, radioautographic studies in protein secreting cells at both the light microscopic level (Warshawsky, Leblond and Droz, 1963) and the electron microscope level (Caro, 1961; Caro and Palade, 1964; Van Heynigen, 1964; Frank and Frank, 1969; Zagury et al., 1970; Bouteille, 1970) showed that the label first appeared in the rough endoplasmic reticulum.

Biosynthesis of the Carbohydrate Moiety of Glycoprotein

Mechanism

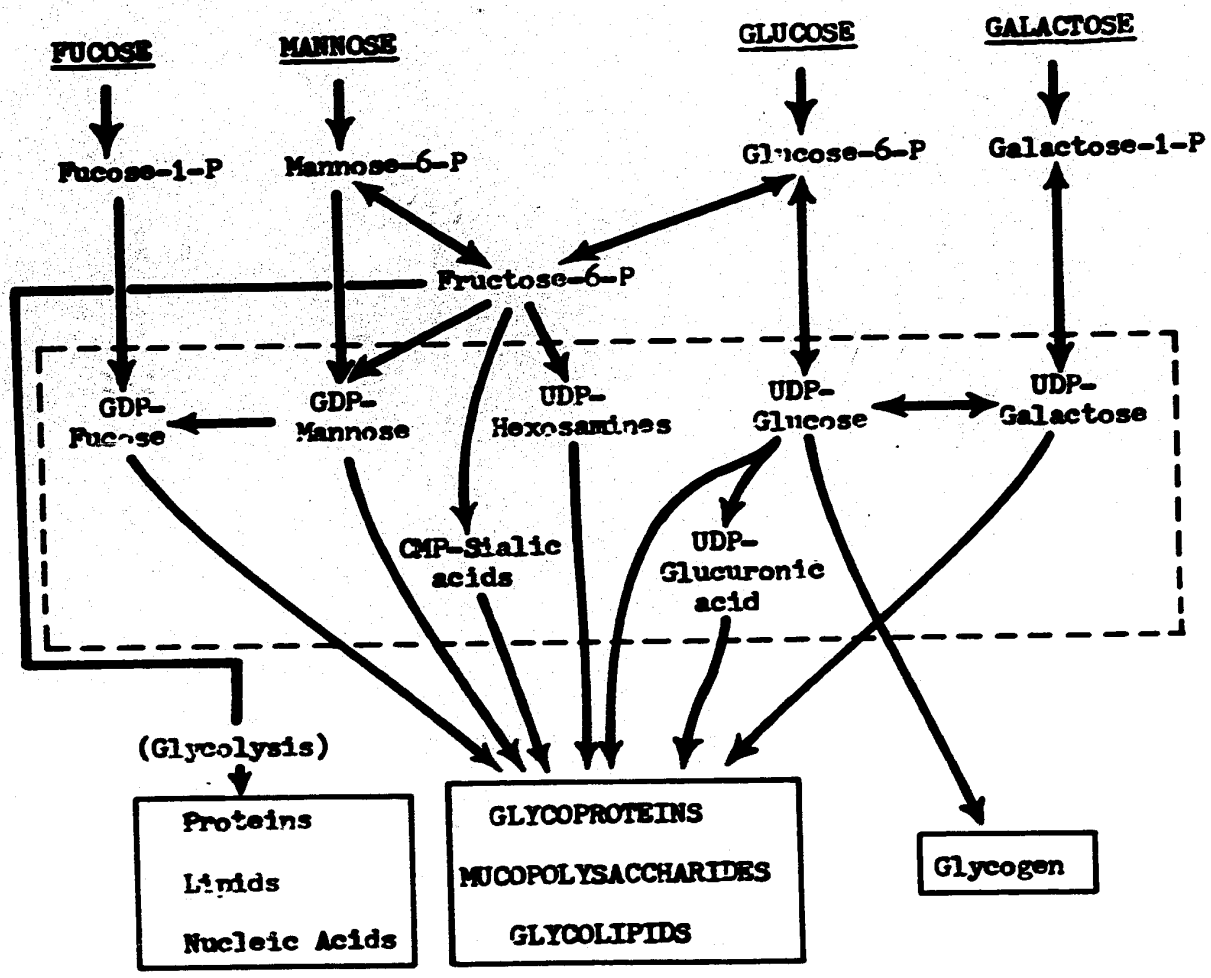
A large number of biochemical studies have now been carried out on the synthesis of the carbohydrate moiety of glycoproteins, and it is established that at least the major part of the carbohydrate moiety is added after synthesis of the polypeptide chains on the ribosomes is completed (Sarcione, 1964; Eyler and Cook, 1965; O'Brien at al., 1965; Molnar, Robinson and Winzler, 1965; Cook, Laico and Eyler, 1965; Lawford and Schachter, 1966; Spiro and Spiro, 1966; O'Brien, Canady, Hall and Neufeld, 1966; Simkin and Jameson, 1967; 1968; Maroz and Uhr, 1967; Hagopian, Bosmann and Eyler, 1968; Bosmann, Hagopian and Eyler, 1969; Molnar, Tetas, Chao, 1969; Herscovics, 1969, 1970; Schenkein and Uhr, 1970; Melchers, 1971; Choi, Knopf, and Lennox, 1971; Sherr and Uhr, 1971).

It is also fairly well established that the sugar residues are added onto the growing carbohydrate chain one at a time (Spiro and Spiro, 1966; Hagopian, Bosmann and Eylar, 1968; Molnar, Tetas and Chao, 1969; Herscovics, 1969, 1970). All of the sugars required for glycoprotein synthesis can be derived from glucose in the body (Dorfman, 1963; Spiro, 1963) (see Text-Figure I). To be added to a glycoprotein, however, sugars must first be linked to specific nucleotides (Leloir and Cardini, 1960) these sugar nucleotides then donate their respective sugars to growing carbohydrate chains in reactions catalyzed by highly specific glycosyl transferases which appear to be firmly bound to intracellular membranes (Novikoff and Goldfischer, 1961). A group of glycosyl transferases work in concert to assemble a carbohydrate unit, with the product of one enzymatic reaction becoming the substrate for the next reaction. The ability of this type of mechanism to produce carbohydrates of definite structure depends primarily on the specificity of these glycosyl transferases, which is directed both toward the sugar nucleotide as well as to the terminal sugar of the carbohydrate chain to which the new sugar is to be added (Hagopian, Bosmann and Eylar, 1968; Spiro, 1970).

Intracellular Sites of Synthesis of the Carbohydrate Moiety of Glycoprotein

Evidence from Biochemical Studies

Biochemical studies on the intracellular location of the addition of carbohydrate residues to growing glycoproteins have all made use of cell fractionation techniques, by means of which, fractions such as the ribosomal fraction, microsomal fraction (containing rough endoplasmic reticulum), smooth membrane fraction (containing smooth endoplasmic reticulum and probably Golgi apparatus), and a plasma membrane fraction could be obtained.



Text-Figure 1

These studies have shown that in the case of large heteropolysaccharide carbohydrate groups such as those in plasma proteins or in thyroglobulin (1a in Text-Table II), the inner core mannose and N-acetylglucosamine residues are added in the microsomal fraction (Sarcione, 1964; Molnar, Robinson and Winzler, 1965; Spiro and Spiro, 1966; Li, Li and Shetler, 1968). A controversy has existed over the site at which the innermost N-acetylglucosamine residue is added to the polypeptide chain. Considerable evidence has now been obtained, however, that N-acetylglucosamine residues may be added into the polypeptide chain while it is still growing on the ribosomes (Molnar et al., 1965; Lawford and Schachter, 1966; Molnar and Sy, 1967; Melchers and Knopf, 1967; Molnar, Tetas and Chao, 1969; Sherr and Uhr, 1971; Cowan and Robinson, 1970). In the carbohydrate prosthetic groups of membrane glycoproteins (2a in Text-Table II), the innermost N-acetylgalactosamine residue appears to become attached to serine or threonine residues of the polypeptide chain only in the smooth membrane fraction (Hagopian, Bosmann, and Eylar, 1968).

The more peripherally located sugar residues in carbohydrate side chains are added at later stages in the synthesis of the chains, and this addition appears to occur in the smooth membrane fraction. Thus galactose, fucose and sialic acid residues appear to be added in this fraction in a number of secretory cells (Molnar, Robinson and Winzler, 1965; Spiro and Spiro, 1965, 1968; Lawford and Schachter, 1966; Bosmann Hagopian and Eylar, 1969; Herscovics, 1969, 1970). In the case of collagen synthesis by HeLa cells, Hagopian, Bosmann and Eylar (1968) and Bos mann,(1969) have shown evidence that the glucose and galactose residues of collagen disaccharide carbohydrate groups (4 in Text-Table II)

are both added at the plasma membrane. In membrane glycoproteins, however (Hagopian, Bosmann and Eylar, 1968) and most other secretory proteins, the synthesis of the carbohydrate moiety appears to be completed within the cell. The addition of terminal sugars such as fucose appears to occur as a final step, as indicated by the very small amounts of this residue found in biochemical analyses of unsecreted immunoglobulin still within plasma tumor cells (Melchers, 1971; Choi, Knopf and Lennox, 1971).

Thus the weight of biochemical evidence favours the synthesis of the carbohydrate moiety of glycoproteins via the stepwise addition of sugars to growing carbohydrate side chains occurring at a number of sites in the cell. Some of the evidence supporting this concept derives from radiochemical studies after administration of radioactive sugars. Other evidence comes from the intracellular localization of the glycosyltransferases responsible for adding the sugars. The meaningful intracellular localization of these transferases has been a difficult task because of the inability of most cell fractionation techniques to differentiate intracellular organelles from one another. Thus while the smooth membrane fraction of cells was thought to contain the Golgi apparatus, identification could not be certain since the classical structure of the Golgi apparatus was not preserved (Hagopian, Bosmann and Eylar, 1968). Thus the results obtained from such studies could not be accurately correlated with cell morphology.

Recently, however, the methods of cell fractionation have been improved, and fractions thought to be rich in Golgi apparatus have been obtained (Morré and Mollenhauer, 1964; Hamilton, Morré, et al., 1967;

Morré et al., 1968; Wagner and Cynkin, 1969; Fleicher, Fleicher and Ozawa, 1969; Leelavathi et al., 1970; Morré, et al., 1970; Schachter et al., 1970). Analyses of these fractions for glycosyltransferases have indicated the presence of N-acetylglucosaminyltransferases (Wagner and Cynkin, 1969; Morré, Merlin and Keenan, 1969; Schachter et al., 1970), galactosyltranferases (Fleicher et al., 1969; Leelavathi et al., 1970; Morré, Merlin and Keenan, 1969; Schachter et al., 1970) and sialyltransferases (Schachter et al., 1970). This evidence implied a role of the Golgi apparatus in carbohydrate secretion. Such a role had also been indicated by radioautographic studies which traced the fate of protein precursors as described below.

Evidence From Radioautographic Studies On the Intracellular Migration of Newly Synthesized Proteins

When radioautographs of protein secreting cells are examined at various time intervals after administration of a labelled protein precursor, it is possible to trace the fate of proteins which have become labelled. When this type of study was carried out with pancreatic tissue, results obtained at the light microscopic level (Warshawsky, Leblond and Droz, 1963) and at the electron microscopic level (Caro, 1961; Caro and Palade, 1964; VanHeynigen, 1964) indicated that the amino acids were incorporated into protein macromolecules in the rough endoplasmic reticulum. When the fate of the labelled protein material in these cells was followed at later time intervals after injection, however, it was found that by 30 minutes after injection most of the labelled protein was no longer in the rough endoplasmic reticulum but was concentrated in the Golgi region of the cell. From here the labelled protein appeared to migrate to zymogen granules and thence to the acinar lumen. Similarly in thyroid follicular

cells, ^{3}H-leucine was radioautographically detected in the rough endoplasmic reticulum at 10 minutes after injection, but at later times was found to be concentrated in the Golgi region, and then at the apex of the cells (Nadler, Young, Leblond and Mitmaker, 1964). A similar pattern was observed in other glandular cells which synthesize and secrete protein, ie, in mammary gland cells, (Wellings and Philp, 1964), myelocytes (Fedorko and Hirsch, 1966), lymph node cells (Clark, 1966), ameloblasts (Warshawsky, 1966), osteoblasts (Frank and Frank, 1969), and plasma tumor cells (Clark, 1966). These studies indicated that the Golgi apparatus played a role in protein secretion, a concept not altogether new, since as long ago as 1914, Ramon Y Cajal had suggested, on the basis of the appearance of the Golgi apparatus in stimulated pancreatic acinar cells, that the Golgi apparatus might take part in the process of secretion by the formation of secretion granules (Ramon Y Cajal, 1914), and this interpretation was soon extended to many other secretory cells (Nassanow, 1923, 1924; Bowen, 1926). Similarly, the electron microscopic appearance of the Golgi apparatus in many cell types suggested the formation of secretion granules from Golgi saccules (Farquhar and Wellings, 1957; Dalton, 1961; Frei and Sheldon, 1961; Hirsch, 1961; Zeigel and Dalton, 1962; and many others).

Evidence from Histochemical Studies

Another approach to the problem of determining the intracellular site of the carbohydrate moiety of glycoproteins was to examine cells which had been specifically stained for glycoproteins using the PA-Schiff technique (Leblond et al., 1957). When such cells were examined, staining was observed in some cell secretions, and at the cell surfaces. In the cytoplasm, however, only one region took up stain, and this was the Golgi region, as observed in kidney cells (McManus, 1948), intestinal epithelial cells

(Gersh, 1949; Leblond, 1950; Moog and Wenger, 1952; Arzac and Flores, 1952; Casselman, 1955), thyroid follicular cells (Gersh, 1950), pituitary cells (Racadot, 1954) and spermatids (Leblond, 1950). When the periodic acid silver methenamine technique for use in the electron microscope was perfected by Rambourg, Hernandez and Leblond,(1969) and used to examine a variety of cell types in the rat, the Golgi apparatus was found to be stained in both secretory and non-secretory cells. In addition, a gradient was found to occur in the intensity of staining, with the last saccule on one side of each Golgi stack being heavily stained (mature face) while the last saccule on the other side showed little or no staining (immature face). A similar staining gradient of Golgi saccules was observed in many cells after staining with phosphotungstic acid at low pH (Rambourg, et. al., 1969) and in the columnar cells of small intestine after staining of acidic carbohydrate groups with colloidal iron (Wetzel, Wetzel and Spicer, 1966; Berlin, 1967).

These results suggested that glycoprotein material was being added onto secretory proteins as they passed through the Golgi apparatus.

Radioautographic Studies Utilizing Labelled Carbohydrate Precursors

The obvious tool for investigation of the intracellular sites of addition of carbohydrate residues to glycoproteins was the radioautographic observation of cells after administration of labelled carbohydrate precursors. Yet this method of investigation required that the precursors carry a label suitable for radioautographic use. Kumamoto (1956) had showed radioautographically that after an injection of ^{14}C-glucose into rats, the label could be traced to the secretion products in many cells, but with this isotope it was not possible to localize the intracellular sites of synthesis.

By 1964, however, ^{3}H-glucose had become available, and was injected by Peterson and Leblond into young rats. By radioautographically following the label in the light microscope, these authors showed that at early times after injection strong radioautographic reactions were localized over the supra nuclear Golgi region of many mucus secreting cells and chondrocytes (Peterson and Leblond, 1964a,b). In a subsequent electron microscopic radioautograhic study of colonic goblet cells, Neutra (née Peterson) and Leblond (1966) showed that at 5 minute intervals after an IP injection of ^{3}H-glucose, reaction was localized over the saccules of the Golgi apparatus itself. At later time intervals, the label migrated to nearby mucigen granules which then migrated to the cell apex to be disbursed into the lumen. These results were interpreted to indicate that the Golgi apparatus was the site of addition of the carbohydrate moiety of secretory glycoproteins in colonic goblet cells and probably other cells as well.

However, ^{3}H-glucose was not an ideal precursor for the study of glycoprotein synthesis. Although in colonic mucosa, most glucose was used for the synthesis of glycoprotein (Draper and Kent, 1963), in other tissues such as liver and striated muscle, much of the glucose label was incorporated into glycogen, while in many tissues such as liver and brain (Gaitonde et al., 1965), glucose was rapidly broken down to give rise to amino acids. Furthermore, when label was incorporated into the carbohydrate prosthetic groups of glycoproteins after ^{3}H-glucose injection, the label could reside in any number of different types of sugar residues since all residues occurring in the carbohydrate moiety could be derived from glucose (Dorfman, 1963; Spiro, 1963). Thus the label could occur

in residues near the attachment of the carbohydrate moiety to the polypeptide chain, as well as in residues in the middle portion or at the free ends of the carbohydrate moiety. Since sugar residues are added onto a growing carbohydrate prosthetic group one by one, this meant that label derived from ^{3}H-glucose could be incorporated into the carbohydrate moiety from the beginning of its synthesis until its completion.

Galactose, on the other hand, did not directly enter the glycolytic pathway (with the resultant formation of amino acids) as did glucose. Instead, exogenous galactose was first converted to UDP-galactose which may be directly utilized for synthesis of glycoproteins or glycolipids. Since galactose residues are located near the ends of many carbohydrate side chains, it could be expected that if the ^{3}H-galactose label was incorporated into such side chains as a ^{3}H-galactose residue, it would be added near the completion of synthesis of the side chain. When this sugar became commercially available in tritiated form, its fate was also traced by means of light microscopic radioautography in rat tissues by Neutra and Leblond, (1966) and by Bennett, (1967). The label was found to be incorporated in the Golgi region not only of most mucus secreting cells but also of many other cell types as well, some of which were generally regarded as non-secretory. These latter cells included intestinal columnar cells (Neutra and Leblond, 1966; Bennett, 1967) and kidney tubule cells, various duct cells, stratified epithelial cells, and the endothelial cells of capillaries (Bennett, 1967). In all such cases, the reaction was localized to the Golgi region at early time intervals, but at later times, was localized along cell surfaces

where it remained at the longest time interval studied (5 hours) after injection (Bennett, 1967). Since the surface of all cells studied in the rat had been shown to be the site of a surface coat composed of glycoproteins, (Rambourg, Neutra and Leblond, 1966), it was hypothesized that the label in these cells was migrating to the cell surface to be added to the cell coat. (Neutra and Leblond, 1966; Bennett, 1967).

At this time it was decided to investigate the above phenomenon in more detail, using electron microscopic radioautography. The cell type selected for this study was the duodenal villus columnar cell since the radioautographic reaction over the Golgi region of this cell type was particularly heavy after ^{3}H-galactose injection. This cell also lent itself to radioautographic studies at both the light and electron microscopic levels in that it was a fairly large polarized cell in which the Golgi apparatus occupied a well defined supranuclear position. This fact made interpretation of light microscopic radioautographs easier and afforded a greater degree of accuracy in assessing silver grains to specific organelles in electron microscopic radioautographs.

Experimental Plan and Resulting Contributions

1. Electron microscopic radioautographic studies were carried out on the duodenal villus columnar cells of the rat after injection of ^{3}H-galactose label. Radioautographs were examined from animals sacrificed as early as 2 minutes and as late as 5 hours after injection.

The results of these studies showed that the Golgi apparatus itself was the actual site of incorporation of ^{3}H-galactose label into glycoproteins. The results strongly indicated that this labelled glycoprotein migrated to the apical surface

> to be added to the cell coat, and also suggested some passage of label to the lateral cell coat. In addition, some evidence was provided concerning the mechanism of transport of this labelled material.[1]

One of the problems encountered with the use of ^{3}H-galactose as a precursor, however, was the presence of scattered label in the cytoplasm of the cells, especially at later time intervals after injection. This scattered labelling made it very difficult to determine whether or not reaction over the lateral cell membranes existed.

At this time a new precursor of the carbohydrate moiety of glycoproteins became commercially available, namely L-fucose-^{3}H. This sugar had the advantage over those previously used in that it was not converted to any other sugars and did not break down when injected into the body but instead was incorporated directly into glycoproteins as a fucose residue. Furthermore since fucose occurs only at the ends of the carbohydrate side chains of glycoproteins (Text-Table II) (Spiro, 1970), it would likely be one of the last sugars added. Thus the intracellular site at which ^{3}H-fucose label was incorporated into glycoproteins would probably represent the site of completion of synthesis of those glycoproteins. Because of these features, it was hoped that the use of this precursor would provide a clearer picture of glycoprotein synthesis and migration in the duodenal villus columnar cell. Therefore...

2. Radioautographic studies were carried out at both the light and electron microscopic levels on duodenal villus columnar cells from

1. Since the information obtained from the following radioautographic studies with ^{3}H-fucose largely superceded that obtained with ^{3}H-galactose, the results of this preliminary experiment are not directly reported in the Results section of this thesis, but are discussed in the Discussion section (see also Bennett, 1970).

animals sacrificed at time intervals varying from 2 minutes to 30 hours after ^{3}H-fucose injection.

> The results obtained from these studies confirmed the pathway of synthesis and migration of glycoproteins shown with ^{3}H-galactose. In addition, strong evidence was obtained for the passage of ^{3}H-fucose label from the Golgi apparatus to the lateral and basal surfaces of the cell and also for the passage of label from the Golgi apparatus to lysosomes. Grain counts performed on electron microscopic radioautographs and counts of labelled and unlabelled lysosomes provided a quantitative analysis of this migration of label.

At this time, a technique had been recently perfected by Rambourg (1969), whereby $\frac{1}{2}$ μ thick sections of glycol methacrylate embedded tissue could be stained specifically for glycoprotein using phosphotungstic acid at low pH, and then examined in the electron microscope. Therefore...

3. It was decided to attempt to combine the above technique with radioautography in order to see if the localization of ^{3}H-fucose label in the duodenal villus columnar cells correlated with positively stained structures.

> A technique was successfully devised in which $\frac{1}{2}$ μ thick sections of glycol methacrylate embedded duodenal tissue could be subjected to electron microscopic radioautography and then poststained specifically for glycoproteins using phosphotungstic acid. When radioautographs from animals sacrificed at different time intervals after ^{3}H-fucose injection were examined, the results showed that the label was almost completely localized to sites specifically stained for glycoproteins.

Another cell type which had exhibited a strong radioautographic reaction at its surfaces after ^{3}H-galactose injection was the hepatocyte. In view of the importance of this cell type, it was decided to investigate the distribution of ^{3}H-fucose label in this cell type at both the light and electron microscopic levels. Therefore...

4. Light and electron microscopic radioautographic studies were carried out on liver tissue from animals sacrificed at various time intervals after ^{3}H-fucose injection. The following observations were made:

> As in duodenal villus columnar cells, the ^{3}H-fucose label was at first localized in the Golgi complexes of these cells and later it appeared to migrate to the various cell surfaces and to lysosomes. Grain counts, and counts of labelled and unlabelled lysosomes provided a quantitative analysis of these phenomena.

Finally, it was of interest to know the general distribution of ^{3}H-fucose label in all rat tissues and to compare this to the distribution of previously used precursors. Therefore...

5. Radioautographs of paraffin sections of sample pieces of nearly all tissues and organs of the body were prepared and examined in the light microscope.

> ^{3}H-fucose was found to be incorporated into the Golgi region of a great variety of cell types and to undergo various fates. Among these were its passage into mucus and serous cell secretions, its passage into the ground substance of a number of tissues, and its passage to the cell surfaces of many cells.

MATERIALS AND METHODS

Radioautographic experiments were carried out *in vivo* using ^{3}H-fucose as a precursor, as summarized in Table I. The experimental animals were Sherman rats, usually of the male sex, and weighing approximately 30-40 grams. The animals were bred in the Department of Anatomy at McGill University.

Isotope Administration

The L-Fucose-^{3}H was purchased from New England Nuclear Corporation, Boston, Massachusetts, and had a specific activity of 4300 mc/mM. The isotope was dissolved in 90% ethanol. Immediately prior to use the isotope solution was evaporated to dryness in a Flash Evaporator (Buchler Instruments, Fort Lee, New Jersey); then the isotope was redissolved in 0.15 ml. of distilled water, and transferred to a 1 ml tuberculin syringe.

The isotope was administered to the animal in a single injection, intravenously via the external jugular vein. The external jugular vein was surgically exposed while the animal was under ether anaesthesia, and the syringe needle was carefully inserted into this vein at its junction with the cephalic vein.

Sacrifice of Animals and Initial Fixation of Tissues

Perfusion fixation

In all experiments, the animal was sacrificed by intracardiac perfusion of the animal with fixative. In order to ensure an adequate oxygen supply to the animal during the steps immediately prior to perfusion, a tracheotomy was performed on the animal as follows: at 2 minutes prior to the time of sacrifice, the animal was anaesthetized

TABLE I

RADIOAUTOGRAPHIC EXPERIMENTS

Experiment Number	Isotope	Specific Activity (mc/mM)	Dose (μc/g body wt)	Time of Sacrifice (after injection)	Number of Animals
1	L-Fucose-^{3}H	4300	120	2 min	2
2	L-Fucose-^{3}H	4300	120	5 min	1
3	L-Fucose-^{3}H	4300	120	10 min	1
4	L-Fucose-^{3}H	4300	120	20 min	2
5	L-Fucose-^{3}H	4300	120	35 min	2
6	L-Fucose-^{3}H	4300	120	1 hr	2
7	L-Fucose-^{3}H	4300	120	1½ hr	1
8	L-Fucose-^{3}H	4300	120	4 hr	3
9	L-Fucose-^{3}H	4300	120	22 hr	1
10	L-Fucose-^{3}H	4300	120	30 hr	1

with ether. The trachea was surgically exposed and a 2 mm vertical incision was made in it just above the manubrium. Through this incision, a polyethylene catheter (Venocath 16, No. 4816, Abbot Laboratories, North Chicago, Illinois) was inserted and secured in place with a cotton thread tied around the trachea. The catheter was then connected by means of a rubber tube to a stoppered glass vessel containing fresh cold ether. A mixture of 95% O_2 and 5% CO_2 was fed by another rubber tube into this glass vessel from a compressed source, and bubbled through the ether so that vapours were forced into the tube leading to the trachea. A by-pass shunt around the ether bottle was also provided so that the animal could receive a pure mixture of O_2/CO_2 without ether. Finally, a mechanism for artificial respiration of the animal after the thorax was opened, was provided by placing a glass tube containing a perforation (5mm diameter) between the catheter leading into the trachea and the rubber tube coming from the ether bottle. By placing a finger over the hole, ether vapours or O_2/CO_2 could be forced into the lungs, while removing the finger from the hole allowed the animal to expire air from the lungs.

Once the trachetomy was completed, a mid-sagittal line incision was made through the abdominal wall from the external genitalia to the sternum. Additional incisions were made along the inferior border of the rib cage on either side. The sternum was then sectioned mid-sagittally to open the thorax, at which time, an assistant commenced artificial respiration as described above. Then the diaphragm was cut along its insertion around the rib cage, and by grasping each half of the sternum with a pair of curved hemostats, the ribs were reflected laterally.

The right atrium of the heart was then perforated with fine scissors, and the heart was grasped between the thumb and forefinger of the left hand. A polyethylene catheter (Venocath-16 No. 4816, Abbot Laboratories, North Chicago, Illinois) with a bevelled tip at one end, and attached to a perfusion pump at the other, was then inserted directly into the left ventricle at the apex of the heart. The perfusion pump was started just before insertion of the catheter, in order to expel air bubbles from the catheter.

The perfusion pump (Ministaltic Pump, Cat. No. 72-894-21, Minostat Corp. 20 N. Moore St., New York 13, New York) was preset at dial reading 3. Fixative was stored in a 1 liter intravenous bottle (Abbo-Liter Container) suspended on an intravenous stand and fed into the pump by means of a disposable plastic tube (Venopak 78", Disposable Venoclysis Set, No. 4631 (13), Abbot Laboratories Limited, Montreal, Canada). Fixative was forced into the heart using the pump for five minutes, after which time the pump was turned off and the fixative allowed to flow by gravity alone. The flow was adjusted to 60 drops per minute, and was allowed to continue for an additional 15 minutes.

Perfusion Fixatives

The fixative used was 2.5% gluteraldehyde (diluted from TAAB 25% gluteraldehyde or Ladd 70% gluteraldehyde) in 0.05 M. Sorensen's phosphate buffer, with 0.1% sucrose and 0.25 or 1% non-radioactive L-fucose added to the buffered fixative. The final osmolality of the fixative was about 470 mOsM at a pH of 7.2.

LIGHT MICROSCOPY: Histological and Radioautographic Methods

Removal of Tissues

After perfusion was completed, the tissues were removed from the animal as follows: Segments of liver tissue were removed and processed

for eventual embedding in either paraffin or epon as described below. Then the remainder of the liver was dissected away to expose the stomach and intestines. The gastrointestinal tract was removed as a unit and immersed in 2.5% gluteraldehyde. Then segments of the stomach, duodenum, jejunum, ileum, and colon were taken and processed for embedding in paraffin or epon. Finally, pieces of most of the other organs and tissues of the animal were removed and processed for paraffin embedding.

Paraffin Embedding and Radioautography

Embedding

Tissues to be embedded in paraffin were placed in Bouin's fixative overnight, and were then further trimmed and transferred to 70% ethanol. Before transfer to 70% ethanol however, pieces of hard tissues, such as the knee joint, were decalcified by immersion in 4.13% disodium ethylenediaminetetra-acetic acid (EDTA) (Fisher Scientific Co.) with 0.44% sodium hydroxide added (isotonic at pH 7.4) at 4°C until they were soft enough for sectioning. After decalcification, these tissues were transferred to cold 0.15 M Sorensen's phosphate buffer (pH 7.2) overnight to remove the EDTA, and were then placed in 70% ethanol.

All tissues were dehydrated by transfer through a graded series of ethanol solutions, i.e. 1 hour in each of 70%, 95%, and 100% (2 changes) ethanol, and were then double embedded in celloidin/paraffin. In this procedure the tissues were successively immersed in the following solutions: 1 hour in 1:1 (V/V) methyl benzoate: ethanol, 1 hour in pure methyl benzoate, 24 hours in 2% celloidin (in methyl benzoate), 24 hours in 10% celloidin (in methyl benzoate), 24 hours in benzene (4 changes), ½ hour in 1:1 (V/V) benzene: paraffin, 1½ hours in pure paraffin at 52°C (3 changes). The soft tissues, about 30 in number, were then embedded in one paraffin block, while the hard tissues were

embedded in another block.

Staining of Paraffin Sections

Tissue sections 5 μ in thickness were mounted on glass slides; these were then deparaffinized and stained with one of the following staining procedures:

1) Routine hematoxylin and eosin staining.

2) The periodic acid Schiff (PA-Schiff) technique (McManus, 1948) for the detection of the vicinyl hydroxyl groups of glycogen and glycoproteins (Leblond et al, 1957; Pearse, 1960), followed by counter-staining with hematoxylin.

3) The colloidal iron stain for the detection of acidic carboxyl and sulfuric acid groups of carbohydrates (Mowry, 1958, 1963), followed by counterstaining with the Feulgen stain for nuclear DNA (Pearse, 1960).

Amylase Treatment

Deparaffinized sections on glass slides were immersed in fresh filtered saliva at 60 degrees C for 20 minutes. Control sections were subjected to a 60 degrees C water bath for the same time interval. The sections were subsequently stained with the PA-Schiff technique followed by hematoxylin.

Radioautography

Slides were radioautographed by the coating technique (Kopriwa and Leblond, 1962) using Kodak NTB 2 emulsion. After suitable exposure times, slides were exposed in groups of four, one slide stained with each of the three methods outlined above, and one slide treated with amylase.

Epon Embedding and Radioautography

Embedding

Pieces of liver and intestine were removed from the animal and

placed in a few drops of fixative on a sheet of dental wax. The liver tissue was cut into small pieces about 1 mm in diameter with a razor blade. Lengths of intestine were cut into short 'doughnut shaped' sections about 3 mm long, and then each 'doughnut shaped' section was cut in half so that two 'half-doughnuts' were formed. The tissues were then fixed for an additional 2-3 hours in the same fixative as used for perfusion, and rinsed overnight in cold 0.15 M Sorensen's phosphate buffer (pH 7.2).

The tissues were postfixed for 2 hours in 1% osmium tetroxide in 0.1 M Sorensen's phosphate buffer, and then dehydrated by transfer through a graded series of acetone solutions at room temperature as follows: 10 minutes in each of 30%, 50%, 70%, and 90% acetone, and then three 15 minute intervals in fresh 100% acetone.

Embedding was done in a mixture of 42.7 ml Epon 812, 43.2 ml. DDSA (Dodecenylsuccinic Anhydride) and 14.1 ml. NMA (Nadic Methyl Anhydride), with 2.0 ml. DMP-30 (2,4,6,-Tri(dimethylaminomethyl) phenol) added (All reagents from Fisher Scientific Co.). The tissues were transferred from the pure acetone to a mixture of 2 parts acetone: 1 part epon[1](1 hour), 1 part acetone: 2 parts epon (overnight), and then pure epon (at least 1 hour). The tissue pieces were then placed in inverted Beem capsules (Better Equipment Electron Microscopy, Bronx, New York), filled with epon mixture, and polymerized for 3-4 days in a 60 degrees C oven. The pieces of intestine were orientated in the capsules so that the lumen side of the 'half-doughnut' faced downwards.

Preparation of Sections for Radioautography

Epon blocks of liver or intestinal tissue were trimmed, and ½ micron thick sections were cut with glass knives using a Porter-Blum MT-1 Ultramicrotome. These were placed with a wire loop on glass slides.

1. "epon" refers to above mixture

Radioautography and Post-Staining of Sections

Slides were radioautographed by the coating technique (Kopriwa and Leblond, 1962) using Kodak NTB 2 emulsion. After suitable exposure times, the slides were developed and then stained by immersion in freshly filtered toluidine blue.

ELECTRON MICROSCOPY: Cytological and Radioautographic Methods

For electron microscopic studies, most of the tissue used was embedded in epon as described above, but in some studies, duodenal tissue was embedded in glycol methacrylate.

Epon Embedded Tissue

Preparation of Sections for Electron Microscopic Examination

Epon blocks of liver or intestinal tissue were trimmed, and ½ micron thick sections were cut with glass knives using a Porter-Blum MT-1 ultramicrotome. These were examined in the light microscope, and areas suitable for electron microscopic examination were selected. In the case of duodenal tissue, in all experiments except numbers 9 and 10 (Table I), the region of the lower third of the duodenal villi was chosen; in the latter experiments, the region of the apical third of the duodenal villi was chosen. The epon blocks were then further trimmed so as to include only the region chosen for examination. Ultrathin sections (gold-silver interference colors, 700-1000 A°) (Pease, 1964) were cut with either glass knives using a Porter-Blum MT 1 Ultramicrotome, or a diamond knife using a LKB Ultrotome ultramicrotome. Sections for morphological studies were placed directly on copper grids, stained with alcoholic uranyl acetate (Pease, 1964) for 10 minutes, followed by lead citrate (Reynolds, 1963) for 10 minutes, and then examined in a Siemens Elmskop I or Hitachi HS-7S electron microscope.

Preparation of Sections for Electron Microscopic Radioautography

Ultrathin sections were placed with a wire loop on glass slides coated

with celloidin [0.8% in iso-amyl acetate (Fisher Scientific Co.)], and dipped in liquid Ilford L4 emulsion (Ilford Limited, Ilford, England), using a semi-automatic device (Kopriwa, 1966). After suitable exposure times, the radioautographs were developed. Development was usually in D19b (Kodak formula) developer for 2 minutes at 20 degrees C (Kopriwa, 1967), but in some cases the radioautographs were developed in Elon/ascorbic acid developer for 6-8 minutes at 24 degrees C after gold latensification (modification of Salpeter and Bachmann, 1964). The radioautographs were fixed for 2 minutes in 24% sodium thiosulfate (Fisher Scientific Co.). The celloidin was then stripped from the slides by floating on water and the sections were picked up on copper grids.

For staining, the grids were floated, emulsion side down, over a drop of uranyl acetate for 10 minutes, and then over lead citrate for 30 minutes in a chamber free of carbon dioxide. These were then examined in a Siemens Elmskop I or Hitachi HS-7S electron microscope.

Radioautography of Glycol Methacrylate Embedded Tissue

Preparation of Tissues for Embedding

The initial treatment of tissues to be embedded in glycol methacrylate was the same as that described above for epon embedded tissues, i.e. pieces of duodenum were removed from a perfused animal, trimmed to the shape of 'half-doughnuts', fixed for an additional 2-3 hours in the same fixative as used for perfusion, and then rinsed overnight in cold 0.15 M Sorensen's phosphate buffer (pH 7.2).

The tissues were not post-fixed in osmium tetroxide, however, but were instead directly embedded in glycol methacrylate.

Embedding in Glycol Methacrylate (GMA)

The embedding technique used was that of Dr. A. Rambourg, after

E. Leduc and W. Bernhard (1967). All steps were carried out at 4 degrees C. The solutions used were the following: (All solutions were from Polysciences Inc., Rydal, Pennsylvania, 19046)

1) Butyl Methacrylate Solution

-100 ml. Butyl Methacrylate
-2 gm. LUPERCO CDB (Dichlorobenzoyl peroxide)
- filter the mixture

2) 80% GMA

-80 ml. GMA
-20 ml. distilled water

3) 97% GMA

-97 ml. GMA
-3 ml. distilled water

4) Final Solution

-7 parts 97% GMA
-3 parts Butyl Methacrylate Solution

5) Prepolymer

-Prepared in advance by heating a flask of the Final Solution over a bunsen burner very slowly and carefully until the exact moment when the Final Solution took on the consistency of honey. Then to stop further polymerization, the flask of Final Solution was quickly plunged into a bath of ice water. The timing of this step required considerable care since the Final Solution remained at the consistency of honey for only a few seconds before becoming further polymerized if the application of heat was continued. The prepolymer prepared by the above method could be kept indefinitely at -20 degrees C.

Since glycol methacrylate is water soluble, no dehydration of the tissues was necessary; from their overnight storage in 0.15 M Sorensen's phosphate buffer they were passed successively through two 10 minute rinses of 80% GMA, one 20 minute rinse of 97% GMA, one 20 minute rinse in a mixture of 1 part 97% GMA: 1 part Final Solution, and a final 20 minute rinse in pure Final Solution. The tissues were then kept overnight (12 hours) in Prepolymer, and then were embedded in Gelatin capsules. After all air

bubbles had been eliminated from the capsules, caps were placed on, and final polymerization was achieved by keeping the capsules under an ultraviolet light for 26 hours.

Preparation of Thin Sections for Electron Microscopic Examination

The blocks of glycol methacrylate embedded duodenum were trimmed, and 1 μ thick sections were cut with glass knives using a Porter-Blum MT - 1 Ultramicrotome. These were examined in the light microscope, and areas suitable for electron microscopic examination were selected. The blocks were then trimmed so as to include only the area chosen for examination. Ultrathin sections were cut with glass knives, and were stained with phosphotungstic acid by floating for 30 minutes on an aqueous solution of 1 % phosphotungstic acid (PTA) (Baker Chemical Co., Phillipsberg, N. J.) containing 10 ml of 36.5% (10 N) hydrochloric acid (Fisher Scientific Co.) per 100 ml solution. To transfer the sections from the microtome water bath to the staining solution, loops made of thin sheet plastic were used, and the sections remained in these loops while floating on the staining solution. The use of wire loops was avoided in order to prevent corrosion of the metal during staining. When staining was complete, the sections were removed from the staining solution by means of the loops, and washed for a few seconds by floating on distilled water. The washing period was restricted to only a few seconds to prevent unspecific staining by the remaining free phosphotungsic acid in the section at the elevated pH of the water bath. Then the sections were picked up on copper grids, allowed to dry, and examined in the electron microscope.

Preparation of ½ μ Sections for Electron Microscopic Radioautography

½ μ sections were cut and were placed with a wire loop on glass slides coated with celloidin (0.8% in iso-amyl acetate). These were dipped

in liquid Ilford L4 emulsion, using a semi-automatic device (Kopriwa, 1966). After suitable lengths of exposure time, the radioautographs were developed in Kodak Microdol developer for 4 minutes at 18 degrees C, followed by a water rinse, and then 10 minutes fixation in 30% sodium thiosulfate (Fisher Scientific Co.). The celloidin was then stripped off of the slides by floating on water, and the sections were picked up on platinum grids. Platinum grids were used since these were resistant to corrosion by the subsequently used PTA staining solution.

To remove the celloidin layer from the sections, the platinum grids were immersed in iso-amyl acetate for at least 5 minutes, after which they were rinsed by floating over night on distilled water. The sections were stained by floating the grids, section side down, for 20-60 minutes, on an aqueous solution of 1% phosphotungstic acid containing 3 ml of 36.5% (10 N) hydrochloric acid per 100 ml solution. The grids were then removed from the staining solution, washed by floating for a few seconds on distilled water, dried, and examined in a Siemens Elmskop I set at 60 or 80 K. V..

Analysis of Electron Microscopic Radioautographic Data

Counts of Silver Grains

In order to quantitatively analyse the distribution of ^{3}H-fucose label in cells at different times after injection of the label, counts of silver grains were made in radioautographs from animals sacrificed at different time intervals.

Since each silver grain was considered to represent a source of radioactivity in the underlying tissue, an attempt was made to classify each silver grain in terms of the type of organelle containing its radioactive source. In electron microscopic radioautographs this problem is complicated by the

fact that the source of the radioactivity that causes a grain to be formed is not necessarily located in organelles directly below the grain, but may be located some distance away. This is because a beta-ray emanating from a radioactive source in the tissue section may travel more than one micron, and could therefore strike silver bromide crystals in the emulsion other than the one or two crystals directly above it. Nadler (unpublished) has examined this problem and has concluded that in the case of a 0.1 μ thick tissue section covered by an emulsion consisting of a closely packed monolayer of spherical silver bromide crystals with 0.1 micron diameter, there would be a 95% probability that the source of a given grain would be located within a distance of 2250 A° from the center of the grain. This conclusion differs from that of Bachmann et al. (1968) that the probability of a silver grain being within 2250 A° of a point source is only 50%, but is in agreement with the data of Caro (1962) indicating that, with a 600 A° phage as radioactive source, 92% of the grains were located within a distance of 2250 A° from the edge of the source.

In a photographic print of an electron microscopic radioautograph magnified 30,000X, the 95% probability distance would be 6.7 mm. When electron micrographs in the present study were developed with D19 b developer for two minutes at 20 degrees C, as was usually the case, and viewed at 30,000X magnification, the average diameter of silver grains was found to be approximately 8mm. Thus it was reasoned that if a cellular organelle lay within a distance somewhat less than the width of a silver grain from the center of that silver grain, the organelle could be considered to be within the 95% probability distance from the center of the grain, and could therefore be considered as a possible source of radioactivity for that grain. To put it another way, any organelle which was separated

from a silver grain by less than half the latter's width could be considered a possible source for the grain.

For the purposes of grain counting, silver grains occurring over cells were classified as either being over specific organelles in the cell— such as the Golgi apparatus, lysosomes, or cell surfaces in duodenal columnar cells— or being over the remainder of the cytoplasm. It is obvious from the above discussion that any silver grain lying in the cytoplasm, separated by less than half its width from one of the above mentioned organelles, could have as its possible radioactive source not only the organelle in question but also the "remainder of cytoplasm" compartment. For reasons discussed more fully in the discussion section of this thesis, however, silver grains in the above situation were classified as being over the organelle near to which they were located. Only grains located over the cytoplasm further than one half their own width away from any of the organelles specifically mentioned above were listed as being over the remainder of the cytoplasm.

When the grain counts were completed, the percentage of the total grain count over each of the above cell components was calculated at each time interval studied.

Counts of Lysosomes

For analysis of the labelling of lysosomes in cells, a second approach was also used. Counts of labelled and unlabelled lysosomes were made from radioautographs. A lysosome was counted as labelled only if a silver grain lay over it or appeared to touch it. Lysosomes were classified as being either dense bodies, bodies with 1-3 vesicles, or multivesicular bodies, as described more fully in the results section. The percentage of labelled lysosomes of different types was calculated at each time interval after ^{3}H-fucose injection. By this method, a rough measure of the specific activity

of labelling of the lysosomal population at different time intervals was achieved.

Counts of Silver Grains Associated With Cytoplasmic Vesicles

In order to determine the importance of the association of some silver grains with cytoplasmic vesicles in duodenal villus columnar cells, counts were made of the number of silver grains associated with such vesicles. A method was then devised to determine the expected frequency of association of silver grains with vesicles if the silver grains were randomly distributed over the cell. The details of this method and the criteria used in making the above counts are described in more detail in the Results section of this thesis.

RESULTS

MORPHOLOGICAL AND RADIOAUTOGRAPHIC OBSERVATIONS ON DUODENAL TISSUE

Morphological Observations

Architecture of Duodenal Mucosa

Although the general structure of the small intestinal mucosa is well known and has been the subject of excellent reviews (see Toner, 1968), variations exist depending on the age and species of the animal under study, as well as on the particular region examined. Therefore a brief description of the duodenal tissue of young rats weighing approximately 40 grams is given.

In microscopic sections prepared from a cut surface of duodenal mucosa which is parallel to the length of the segment of duodenum from which it is taken, the mucosal epithelium is projected into the lumen as long, slender villi (Figure 1), each with a central core of lamina propria. The epithelium covering the villi consists mostly of tall columnar absorptive cells; interspersed with these are occasional goblet cells whose apical mucus filled cytoplasm (horizontal arrows) is unstained in this preparation. The cytoplasm of the villus columnar cells is quite heavily stained except for a region above the cell nucleus containing the Golgi apparatus; this area presents a negative Golgi image (oblique arrow).

Below the villi, the mucosal epithelium is invaginated to form crypts. The lumen of one crypt (vertical arrow) is seen to open into the space between the two central villi, and it can be seen that the epithelium lining the crypt is continuous with that covering the villi. The crypt epithelium contains undifferentiated crypt columnar cells, goblet cells, and some argentaffin cells and paneth cells.

Mucosal Dynamics

The classic studies of Leblond and his colleagues showed that cells of small intestinal crypts undergo constant renewal with a resulting migration of cells from the crypts to the tips of the villi where they are extruded. Leblond and Stevens (1948) estimated that the time taken by a crypt cell to migrate to the villus tip was around 1.57 days in the duodenum of adult rats. Walker and Leblond (1958) provided radioautographic evidence, after administration of labelled nucleic acid precursors, that duodenal crypt cells could migrate to the tip of the villi in mice in as little as 24 hours, while Messier and Leblond (1960) showed that the time taken in adult rats was between 24 and 48 hours.

It was desirable for the purposes of the present study to know the transit time taken for duodenal crypt cells to reach the tips of villi in 40 gram rats. The transit time was found to vary with the age of the animal in mice however (Lesher et al., 1961). Therefore in the present study, a series of radioautographic experiments was carried out in which rats were sacrificed at different time intervals after a single injection of ^{3}H-thymidine. The radioautographic results obtained from these experiments are shown in Table II.

TABLE II

No. of Animals	Time after ^{3}H-thymidine injection	Location in Duodenal Villus of Uppermost Labelled Columnar Cell Nuclei
1	12 hr	base of villus
1	24 hr	¼ way to apex of villus
1	36 hr	½ way to apex of villus
1	48 hr	apex of villus

Since, by the 48 hour time interval, labelled columnar cells extended right to the zone of cell extrusion at the tip of the villus, it is possible that some labelled cells had already been extruded by this time. Thus the time taken for cells in the duodenal crypt of 40 gram rats to incorporate ^{3}H-thymidine label and migrate to the villus tip was between 36 and 48 hours.

Cytology and Cytochemistry of Duodenal Villus Columnar Cells

As seen in figure 2, which shows a section of duodenal villus stained with PA-Schiff and hematoxylin, the duodenal villus columnar cells are long narrow cells whose nucleus occurs in the lower half of the cell. Interspersed with these cells are goblet cells whose mucus contents stain intensely with PA-Schiff. The apical striated border of the columnar cell stains intensely with PA-Schiff. The lateral cell surfaces are also definitely stained, but more lightly (vertical arrow). The basal cell surface and adjacent basement membrane bordering on the lamina propria core (LP) form a line of heavy stain. The columnar cell cytoplasm is mostly unstained, but patches of stain sometimes occur above the nucleus and represent elements of the Golgi apparatus (horizontal arrow). The PA-Schiff technique specifically stains glycoprotein and glycogen in paraffin sections. The staining of glycogen can be differentiated from that of glycoproteins by its abolishment by amylase treatment. In the present study amylase treatment had no effect on the PA-Schiff staining pattern observed in the duodenal villus columnar cells. This indicates that all of the structures stained by PA-Schiff in these cells are sites of glycoprotein material.

Fine Structure of Duodenal Villus Columnar Cells

In view of its functional importance, much investigation has been

carried out on the fine structure of the intestinal columnar absorptive cell, and a number of reviews have been written (Zetterqvist, 1956; Trier and Rubin, 1965; Toner, 1968). In 40 gram rats, however, the fine structure of duodenal villus columnar cells was found to differ in certain respects from that of adults. A short account of the structural details of these cells will therefore be given.

An electron micrograph at low magnification of the apical half of duodenal villus columnar cells is shown in figure 3. In the electron microscope, the apical striated border observed in light micrographs of columnar cells is seen to be made up of individual microvilli (mv). Just beneath this microvillus border is an area of cytoplasm occupied by horizontally running filaments of the terminal cell web (TW). The cytoplasm of the terminal web region does not contain mitochondria or cisternae of rough endoplasmic reticulum, but contains elements of smooth endoplasmic reticulum, some smooth surfaced cytoplasmic vesicles, and occasional lysosomes such as the multivesicular body (MVB) at the upper left of the figure. Lysosomes are also encountered in other regions of the cytoplasm, as shown by the presence of small dense bodies (D) above the nucleus (N). The Golgi apparatus in these cells consists of one or more stacks of flattened Golgi saccules (G) located in the cytoplasm above the nucleus. The lateral cell membranes (lmb) of these cells are often highly convoluted.

The microvillus or brush border of the cells is seen at higher magnification in figure 4. Attached to the outer leaflet of the plasma membrane of the microvilli is a distinct surface coat (SC) which is thicker over the tips of the microvilli than over their lateral surfaces. Within the microvilli are cores of central filaments (F). These pass

downwards to join the horizontally running filaments of the terminal web (TW) which in turn converge on the zonula adhaerens (ZA) of a junctional complex at the left of the figure. In the terminal web region of the cytoplasm, in these young animals, are a number of smooth surfaced cytoplasmic vesicles; these average about 140 mμ in diameter (vertical arrows). The membrane of these vesicles is similar in thickness to the apical cell membrane, and at some sites (horizontal arrows) there are indications that these vesicles may fuse with or derive from the apical surface membrane. The vesicles contain filamentous material similar in appearance to the surface coat. Also included in the terminal web cytoplasm are vesicles and tubules of smooth endoplasmic reticulum (SER). The membrane of these elements appears thinner and more irregular in outline than that of the above mentioned cytoplasmic vesicles, and the contents of their lumens are more electron dense.

The cisternae of rough endoplasmic reticulum occurring throughout the remainder of the cytoplasm are frequently found around the periphery of mitochondria (Figs. 3,6), and in this situation, ribosomes are often found attached to the cisternae only on the side facing the mitochondrion while the other side is bare (Fig.6). Another special feature of the cisternae of rough endoplasmic reticulum in these cells is their close association with stacks of Golgi saccules. Typical Golgi stacks are shown at high magnification in figures 5 and 7; here the stacks are cut in cross section through their central region, while in figure 6 and the lower left part of figure 7, Golgi stacks are shown cut in cross section through their peripheral region. Each Golgi stack is polarized, having an immature and a mature face, and the stack usually contains about five saccules. In columnar cells located near the base of duodenal villi, the saccules midway

between the two faces of the Golgi stacks are very flattened in their central portion (Figs. 5, 7: GS) and contain fairly dense material in their lumens. At their ends, however, these saccules are expanded, and their luminal contents are electron lucent. At the mature face of the Golgi stack, the saccules are still flattened in their most central portion, but their ends are even more enlarged to form Golgi vacuoles (GV). In cells further out on the villi, flattened portions of saccules are less often encountered, and most of the saccules are enlarged throughout (Fig. 3).

It is the immature face of the Golgi stack with which the rough endoplasmic reticulum comes into intimate association, for along this surface a cisterna of rough endoplasmic reticulum lies closely parallel to the most immature Golgi saccule, separated from it by a distance no greater than that separating the remaining Golgi saccules from one another (Figs. 5,6,lower left of 7). The fact that this cisterna is part of the rough endoplasmic reticulum is shown by the presence of ribosomes on the surface of the cisterna facing away from the Golgi stack (Fig. 5,6, lower left of 7), by the continuity of this cisterna with portions of rough endoplasmic reticulum outside of the Golgi apparatus (Fig. 6, lower left of 7), and by the fact that the luminal contents of this cisterna resemble those of other cisterna of rough endoplasmic reticulum rather than those of the Golgi saccules (Fig. 5,6, 7). Since the cisterna of endoplasmic reticulum adjacent to the Golgi stack has no ribosomes on its surface facing the Golgi stack, it could be considered a transitional element similar to that described in secretory cells (Zeigel and Dalton, 1962; Jamieson and Palade, 1967 a,b).

Small vesicles occur frequently near the ends of the Golgi saccules; some of these are smooth surfaced while others appear to have a fuzzy coat.

Somewhat larger coated vesicles are also sometimes seen in the Golgi region (Fig. 5: CV); these also occur in other regions of the cytoplasm such as the apical terminal web region (Fig. 4:CV). In some instances small vesicles are seen fusing with or budding off of the expanded ends of the Golgi saccules (Fig. 6: horizontal arrow). There is little evidence in these cells, however, of vesicles budding off of elements of transitional endoplasmic reticulum in the vicinity of the Golgi apparatus, such as described by Zeigel and Dalton (1962) and Jamieson and Palade (1967,a,b) in secretory cells.

Also occurring nearby the Golgi stacks are larger vesicles (Fig.7:V). These vesicles are very similar in size, appearance, and luminal contents, to the cytoplasmic vesicles described in the apical terminal web cytoplasm (Fig. 4: vertical arrows). Such vesicles also occur scattered in smaller numbers throughout the cytoplasm (see Fig. 3). In the Golgi region, it can be seen that these vesicles also resemble the nearby Golgi vacuoles (Figs. 6, 7: GV).

Other vesicles in the Golgi region,which are somewhat larger than those described above and contain a few internal vesicles, are designated as multivesicular bodies (Fig.7: MVB). The small internal vesicles contained in these bodies are similar in size and appearance to vesicles in the nearby cytoplasm (Fig. 7). Although these bodies contain a certain amount of amorphous matrix surrounding their internal vesicles, most of their content is electron lucent, and they are therefore designated as light multivesicular bodies. Another more frequently encountered type of multivesicular body in these cells contains more internal vesicles than the above type of multivesicular body, and these vesicles are embedded in a uniformly dense matrix (Figs. 3,6). The inset of figure 7 shows three

multivesicular bodies: one of the light type, a larger dark multivesicular body with fairly dense matrix, and a smaller one with very dense matrix. Some bodies are also observed which have as their predominant content a uniformly dense matrix, but which also contain a few internal vesicles (bottom of Figure 8).

Dense bodies of various sizes are often encountered in these cells. These bodies contain a dense matrix which is uniform except for the occasional inclusion of small granular elements (top of Figure 8), or one or two vesicles (Fig. 3, upper left of Fig. 7). Although dense bodies are usually seen as spherical bodies, sometimes narrow extensions lead away from the main body; in the lower left part of figure 7, such a narrow extension connects two dense bodies (D).

Radioautographic Observations on Duodenal Villus Columnar Cells

Light Microscopic Observations

Initial Uptake of ^{3}H-fucose Label

At 2 minutes after ^{3}H-fucose injection, a strong radioautographic reaction occurred over the duodenal villus columnar cells (Fig. 9) in which the great majority of silver grains were concentrated over the supranuclear Golgi region. The intensity of reaction was maximal in the cells of the lower third of the villi, and gradually decreased towards the apex. Only a comparatively light reaction was observed in the columnar cells of the duodenal crypts; yet here too, most of the reaction was supranuclear (vertical arrow). The radioautographic reaction at 5 minutes after ^{3}H-fucose injection is shown in figure 10. Again the reaction was mostly over the supranuclear Golgi region of the duodenal villus columnar cells, and only a few grains occurred elsewhere over the cells.

Distribution of Label Over Villus Columnar Cells at Later Time Intervals

At later time intervals after ^{3}H-fucose injection, the reaction over the duodenal villus columnar cells changed in intensity and especially in grain distribution. By 20 minutes and by 1 hour after injection (Fig. 11), the reaction was heavier than that seen at earlier time intervals, and silver grains appeared in substantial numbers not only over the supranuclear Golgi region, but, in addition, over the apical microvillus border of the cells, and over the lateral cell surfaces. The latter reaction was seen especially well in the region of the basal half of the columnar cells (horizontal arrow).

By 4 hours after ^{3}H-fucose injection, the labelling of the apical surface of the duodenal villus columnar cells had become even more intense

(Fig. 12). Heavy reaction remained over the lateral cell surfaces also (horizontal arrow).

By 30 hours after ^{3}H-fucose injection, the most heavily labelled villus columnar cells were no longer located in the lower third of the villi, but had migrated to the outer parts of the villi, while their place in the lower third was taken by poorly labelled crypt cells (Fig. 14). The fact that the poorly labelled cells in the lower third of the villi had been crypt cells at the time of ^{3}H-fucose injection was shown by the strong labelling of their nuclei (horizontal arrow) with ^{3}H-thymidine which had been injected into the animal at the same time as the ^{3}H-fucose. This finding was in accord with the results of the previously described ^{3}H-thymidine labelling study (Table II).in which the uppermost labelled crypt cells had migrated halfway up the villus by 36 hours after injection. In the heavily labelled columnar cells seen in the outer parts of the villi at this 30 hour time interval, reaction remained over the apical and lateral cell surfaces (Fig.13) although reaction over the Golgi region had diminished.

Electron Microscopic Observations of Epon Embedded Duodenal Tissue

Initial Uptake of ^{3}H-Fucose Label

When radioautographs of thin sections of duodenal villus columnar cells of animals sacrificed 2 minutes after ^{3}H-fucose injection were examined in the electron microscope, it was found that the great majority of silver grains were localized over the saccules of the supranuclear Golgi apparatus (Figure 15). At this early time interval, only a few grains occurred over other parts of the cell; thus in figure 15 three silver grains are seen over the cytoplasm of the apical half of the cells

(vertical arrows), one is seen over the lateral cell membrane (horizontal arrow), and one occurs just beneath the apical brush border in association with a cytoplasmic vesicle (oblique arrow).

When the distribution of silver grains over these cells was quantitatively analysed by means of grain counts, the results in Table III (and Graph I) were obtained. Within the limitations of the counting method used, as discussed in a later section of this thesis, the results indicated that, at 2 minutes after ^{3}H-fucose injection, over 80% of the silver grains were localized over the Golgi apparatus. Approximately 4% occurred over the lateral cell surface, and 1% over the apical cell surface; however almost no grains occurred over the basal cell surface. The nucleus accounted for about 1% of the silver grains, while approximately 4% lay over other cytoplasmic structures. Many of these latter grains appeared to be over mitochondria or over the cisternae of rough endoplasmic reticulum surrounding the mitochondria (Figure 15: vertical arrows).

Only occasional silver grains occurred over lysosomes at this 2 minute time interval. When counts of labelled, and unlabelled lysosomes were made, the results obtained (Table IV) showed that none of the multivesicular bodies were labelled and less than 2% of the dense bodies were labelled.

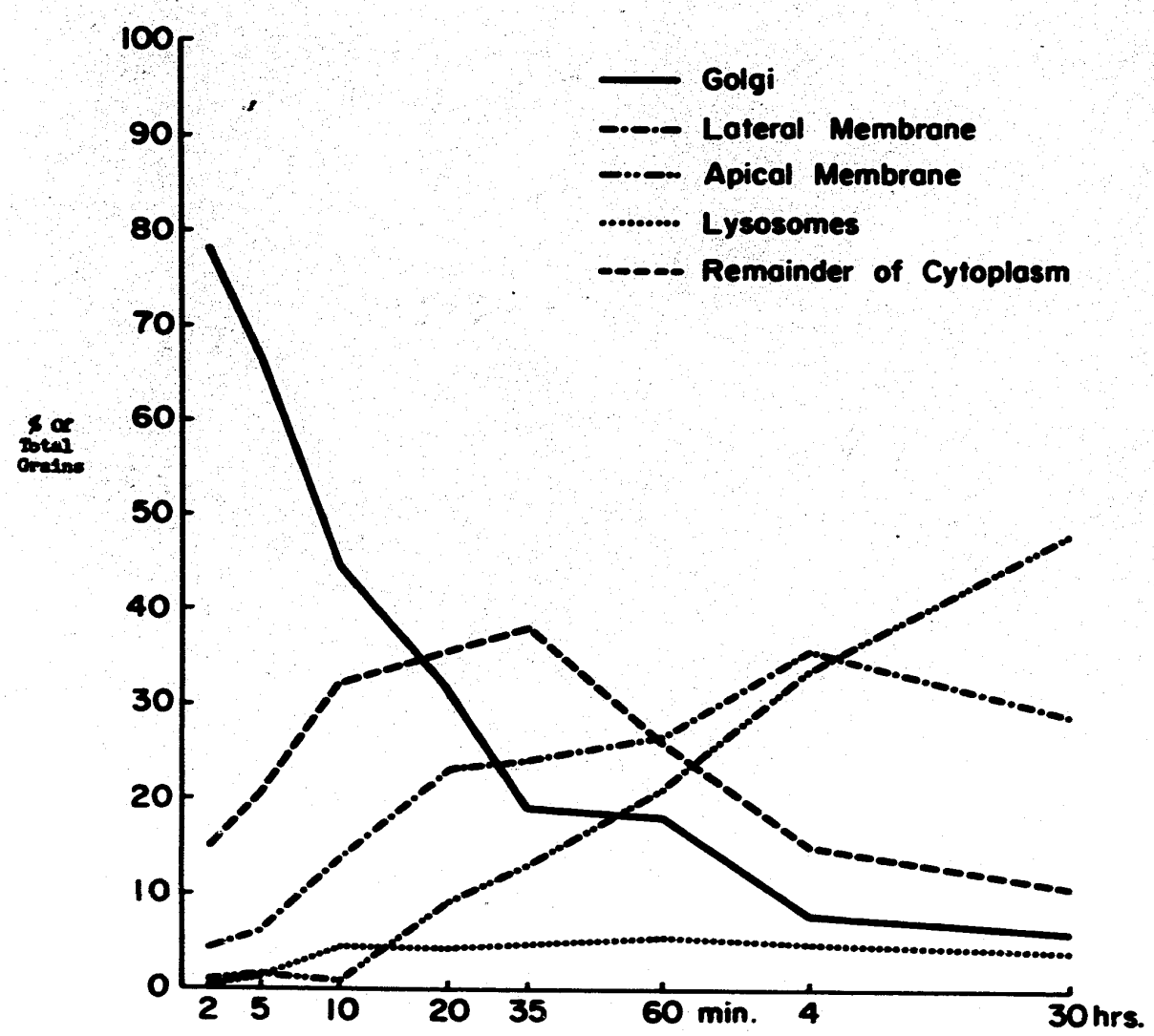
Figure 16 shows a radioautograph of the Golgi region of duodenal columnar cells from a second animal sacrificed 2 minutes after ^{3}H-fucose injection. Again, nearly all of the silver grains occur over the Golgi saccules except for one grain over a lateral membrane (vertical arrow), and one over the edge of a mitochondrion (horizontal arrow). None of the lysosomes in this figure are labelled.

At 5 minutes after ^{3}H-fucose injection, the distribution of silver

TABLE III

DISTRIBUTION OF SILVER GRAINS OVER COLUMNAR CELLS OF DUODENAL VILLI AT VARIOUS TIME INTERVALS AFTER AN INJECTION OF ^{3}H-FUCOSE

Time after ^{3}H-fucose injection	No. of grains counted	% over Golgi	% over lateral membrane	% over apical membrane	% over basal membrane	% over lysosomes	% over remainder of cytoplasm	% over nucleus
2 min	472	80.5	3.8	1.0	0.0	0.6	12.0	2.0
	595	76.0	4.5	0.7	0.2	0.3	18.3	0.0
5 min	2020	66.6	5.9	1.4	0.2	1.3	20.4	4.0
10 min	680	44.6	13.5	0.7	0.9	4.3	32.0	3.8
20 min	730	37.9	22.6	3.3	0.0	3.4	30.1	2.6
	963	22.9	23.6	14.8	0.5	5.0	31.8	1.2
35 min	1194	18.9	24.1	12.9	1.7	2.5	37.9	2.0
1 hr	2116	18.1	26.6	21.2	1.5	5.5	25.9	1.1
4 hr	2570	8.2	37.7	29.9	2.8	4.7	16.0	2.0
	1664	7.5	33.1	36.9	2.9	4.8	14.3	0.5
30 hr	667	6.1	28.9	47.8	1.8	4.0	10.8	0.4



Graph 1. Distribution of Silver Grains Over Columnar Cells Of Duodenal Villi At Various Time Intervals After An Injection Of ^{3}H-Fucose

TABLE IV

LABELLING OF LYSOSOMES IN COLUMNAR CELLS OF DUODENAL VILLI AT VARIOUS TIME INTERVALS AFTER AN INJECTION OF ^{3}H-FUCOSE

Time after ^{3}H-fucose injection	Uniform dense bodies		Bodies with 1-3 vesicles		Bodies with 4 vesicles (MVB)	
	No. counted	% labelled	No. counted	% labelled	No. counted	% labelled
2 min	99	2.2	4	0	16	0
	77	1.3	44	2.2	41	0
5 min	88	6.8	37	8.1	58	1.7
10 min	137	22.0	50	16.0	50	6.0
20 min	28	35.8	38	50.0	50	26.6
35 min	51	38.2	80	35.0	110	22.8
1 hr	168	25.0	94	69.1	86	62.8
4 hr	64	56.3	132	55.4	87	41.4
	214	57.0	205	64.0	98	61.2
30 hr	46	8.7	40	27.5	66	31.8

grains was similar to that at the 2 minute time interval. The percentage of grains over the cell surfaces, lysosomes, and remainder of the cytoplasm had risen slightly, however (Table III). Counts of labelled and unlabelled lysosomes (Table IV) showed that a significant number of lysosomes had now become labelled, ie. 7-8% of the dense bodies and bodies with 1-3 vesicles, and 1.7% of the multivesicular bodies.

Figures 17 and 18 show the radioautographic reactions over the Golgi region of columnar cells at the 5 minute time interval. Most of the grains shown are localized over the Golgi apparatus, but in figure 18, one dense body is seen to be labelled.

Distribution of Label Over Villus Columnar Cells at Later Time Intervals

At 10 minutes after ^{3}H-fucose injection (Figures 19 and 20) a heavy concentration of silver grains was still seen over the Golgi apparatus, but now several grains could be seen over other regions of the cells. Grain counts (Table III), showed that 44% of the silver grains were still localized over the Golgi apparatus.(G). Over 13% of the grains were now over the lateral cell membranes (lmb), although the apical and basal cell surfaces did not show significant reaction, being the site of 0.7% and 0.9% of the grains respectively.

Over 4% of the grains occurred over lysosomes at this time interval. Counts of labelled and unlabelled lysosomes in these cells (Table IV) showed that 22% of the dense bodies were now labelled, while 16% of bodies with 1-3 vesicles and 6% of multivesicular bodies were labelled. In figures 19 and 20, several dense bodies (D) and one multivesicular body (MVB) show labelling.

The remaining 20% of the silver grains at this 10 minute time interval lay over cytoplasmic organelles other than those mentioned above. Many

of these appeared to be associated with cisternae of rough endoplasmic reticulum surrounding mitochondria, but, others were associated with small cytoplasmic vesicles (oblique arrows).

By 20 minutes after ^{3}H-fucose injection (Figures 21 and 22), a heavy concentration of silver grains occurred not only over the Golgi apparatus (Fig. 22: G), but also over the apical microvillus border (Fig. 21: mv) and along the lateral surface membranes (Fig. 22: lmb). The localization of the silver grains along the lateral surface membranes was especially evident when the cells were cut in cross section as seen in figure 23. Finally, when the bases of the cells were examined (Fig. 24), a few silver grains were found to be localized over the basal surface membrane, although reaction over this surface was never as great in intensity as that over the lateral surface membranes.

Grain counts, carried out over the above cells (Table III) showed the presence of approximately 23% of the label over the Golgi apparatus, 24% over the lateral surface membranes, 15% over the apical surface membrane, and 1% over the basal surface membrane. Lysosomes also became quite heavily labelled by this time interval with over 35% of the dense bodies and over 25% of multivesicular bodies showing label (Table IV). The silver grains over lysosmes accounted for only a small portion (about 4%) of the total grain count, however (Table III), since the absolute number of grains localized over the surface membranes was much greater than that over lysosomes. In a second animal sacrificed 20 minutes after ^{3}H-fucose injection, a higher percentage of silver grains remained in the Golgi apparatus (37.9%) than in the animal described above. In this second animal, on the other hand, very few silver grains (3.3%) were found over the apical microvillus border (Fig. 25). In this respect

the radioautographic reaction seen in this second animal bore more resemblance to that seen in the animal sacrificed at 10 minutes than to that seen in the first animal sacrificed at 20 minutes after injection.

In both of the animals sacrificed at 20 minutes after injection as well as in an animal sacrificed at 35 minutes after injection, a concentration of silver grains occurred over the terminal web cytoplasm beneath the microvillus border (Figs. 21, 25). In all cases, several of the silver grains in this location were associated with small cytoplasmic vesicles which were also concentrated in the apical cytoplasm of these cells (Figs. 21,25: oblique arrows). Silver grains occurring over other parts of the cytoplasm of these cells were frequently associated with similar cytoplasmic vesicles as seen in figure 22 (oblique arrows).

At 1 hour after ^{3}H-fucose injection the grain distribution was quite similar to that seen at the 20 minute time interval, with many silver grains localized over the Golgi apparatus (G), the apical microvillus border (mv), and the lateral surface membranes (lmb). Many of the cell lysosomes were also labelled, such as the dense body (D) and multivesicular body (MVB) in figure 26. A fairly large percentage of the silver grains occurred over the remainder of the cytoplasm at this time interval, and many of these were associated with cytoplasmic vesicles (Fig. 26: arrows). No concentration of label was observed in the terminal web region of the cytoplasm, however.

By 4 hours after ^{3}H-fucose injection an intense reaction occurred

over the apical microvillus border of the cell and accounted for over 30% of the total grain count. Heavy reaction also occurred over the lateral surface membranes (Figs. 27-29: lmb), accounting for approximately 35% of the grain count. Lysosomal labelling reached its peak at this 4 hour time interval, with over 50% of the lysosomes in each of the classes being labelled (Table IV). Several labelled lysosomes are shown in figure 27 (horizontal arrows) and at higher magnification in figure 29.

Reaction over the Golgi apparatus (Fig. 27: G) had decreased by this time interval and now accounted for only 8% of the total grain count (Table III). Similarly, reaction over the remainder of the cytoplasm had decreased, and no concentration of silver grains occurred in the terminal web region.

Reaction over the apical microvillus border of columnar cells, at this and other time intervals, was not always of the same intensity over different columnar cells;thus in figure 30, a heavy radioautographic reaction is seen to occur over the microvillus border of the central cell, while only a light reaction occurs over the borders of the remaining cells. When several columnar cells are seen at low magnification (Fig. 31), some cells exhibit only a very light reaction over their apical surface (uncrossed arrow) while others exhibit a reaction of medium intensity (single-crossed arrows) and yet others exhibit an intense reaction (double-crossed arrows).

At 30 hours after ^{3}H-fucose injection, the most reactive duodenal columnar cells had migrated to the outer parts of the villi, as described in the previous section of this thesis dealing with light microscopic radioautographic observations on duodenal tissue. To examine the reactive duodenal columnar cells therefore, thin sections had to be made of the

apical third of the villi. When radioautographs of such sections were examined, a fairly heavy reaction was seen to remain over the microvillus border of the columnar cells (Figs. 32, 33: mv). Reaction also occurred over the lateral cell membranes (lmb) and over some lysosomes, notably multivesicular bodies (Fig.33: MVB). The percentage of the lysosome population which was labelled was greatly decreased from that seen at 4 hours after injection, however, particularly in the case of dense bodies which were now only 8.7% labelled.

The Association of Silver Grains With Cytoplasmic Vesicles

At all time intervals after ^{3}H-fucose injection, a certain percentage of the silver grains over duodenal columnar cells occurred over cytoplasmic structures other than the Golgi apparatus or lysosomes. The percentage of grains in this category at the different time intervals after injection is shown in Table III, and graphically illustrated in Graph I.

At the earliest time intervals studied (2 and 5 minutes), the percentage was fairly small (12-18%). This percentage increased rapidly with time, however, for by 10 minutes after ^{3}H-fucose injection, the percentage was over 30%, and by 35 minutes after injection, it was almost 40%. Then, at the later time intervals, the percentage of silver grains in this category decreased, and by 30 hours after injection, was only about 10% of the total grain count.

The silver grains in the above category were not uniformly distributed over the cytoplasm of the duodenal columnar cells, for more grains were usually found over the cytoplasm of the apical half of the cells than over the basal half. Furthermore, at 20 and 35 minutes after ^{3}H-fucose injection,

there was a pronounced concentration of these silver grains over the apical terminal web region of the cytoplasm (Figs. 21, 25). When the grains over the cytoplasm of cells from animals sacrificed at various time intervals after ^{3}H-fucose injection were examined closely, they frequently occurred over or close to the cytoplasmic vesicles described above. This was especially the case in the terminal web region of the cytoplasm where such vesicles were concentrated (Figs. 21 and 25: oblique arrows), but also occurred elsewhere in the cytoplasm (Figs. 22 and 26: oblique arrows, and Fig. 27: vertical arrows).

In order to quantitate the frequency of this association of silver grains with cytoplasmic vesicles, counts were made of all cytoplasmic silver grains which were unassociated with Golgi saccules, lysosomes, or cell surfaces (by the criteria outlined in the Methods section.) Then each grain was classified as to whether or not it was associated with a cytoplasmic vesicle (ie. whether or not it was within a distance equal to half its own diameter from such a vesicle). These counts were carried out in cells from animals sacrificed at all time intervals.

Within the limitations of the counting method used, as will be discussed in a later section of this thesis, the results obtained (Table V) showed that at 2 and 5 minutes after ^{3}H-fucose injection, less than 7% of the silver grains appeared to be associated with cytoplasmic vesicles. At 10 minutes after injection, however, over 40% of the grains appeared to be associated with vesicles, and in animals sacrificed at 20 and 35 minutes after injection, the percentages were 41% and 60%. At 1 hour after injection, about 38% of the grains were associated with vesicles, while by 4 and 30 hours, the percentage had fallen to only 18%. These figures indicated a rise and then fall in the percentage

TABLE V

DISTRIBUTION OF SILVER GRAINS OVER "REMAINDER OF CYTOPLASM" (TABLE III): FREQUENCY OF ASSOCIATION WITH CYTOPLASMIC VESICLES

Time after ^{3}H-fucose injection	No. of grains counted	Percentage of grains associated with cytoplasmic vesicles (I)	Expected percentage of grains associated with cytoplasmic vesicles if silver grains were randomly distributed (II)	I/II
2 minutes	14	7.1	19.8	0.4
	28	3.6	21.0	0.2
5 minutes	85	7.1	13.1	0.5
10 minutes	141	41.8	19.1	2.2
20 minutes	49	34.7	21.6	1.6
	89	41.6	12.1	3.4
35 minutes	116	60.3	13.5	4.5
1 hour	403	38.5	10.3	3.7
4 hours	280	18.2	3.9	4.6
	50	18.0	6.3	2.8
30 hours	44	18.2	12.5	1.5

of vesicle-associated grains with time after ^{3}H-fucose injection but they were not very meaningful in themselves because it was not known how frequently the silver grains would be associated with cytoplasmic vesicles if the silver grains were simply randomly distributed over the cytoplasm of the cell.

In order to determine this frequency, plastic overlay sheets were prepared which could be placed over radioautographic prints. On these transparent sheets, orderly rows of evenly spaced circles were drawn (49 circles per sheet). The diameter of each circle was twice the diameter of the average silver grain in the radioautographic print over which the plastic sheet was to be placed. When such a plastic overlay sheet was placed over a radioautographic print, each circle was taken to symbolically represent a silver grain plus a distance around that grain equal to half its own diameter. Since the pattern of the circles bore no relation to the underlying radioautographic print, the symbolic grains could be considered to be randomly distributed. The actual silver grains in the radioautographic print were ignored for the purposes of this procedure. For the symbolic silver grain of a circle in the sheet to be classified as a cytoplasmic grain unassociated with the Golgi apparatus, lysosomes, or cell surfaces, it was required that no part of any surface membrane, Golgi saccule, or lysosome (or nucleus) occur within the circle. Similarly, for the symbolic grain of a circle to be classified as being associated with a cytoplasmic vesicle, it was required that the circle contain such a vesicle. Thus the classification of the (randomly distributed) symbolic grains was carried out in exactly the same manner as was the classification of the actual silver grains in the counts above.

One further problem remained, however, for examination of radioautographs

showed that the columnar cells of different animals contained different numbers of vesicles; therefore it was necessary to determine the expected frequency of association of randomly distributed silver grains with cytoplasmic vesicles in each different animal. This was carried out, giving the results shown in Table V. Then for each animal, the ratio of the actual frequency of association of silver grains with cytoplasmic vesicles as compared to the expected frequency of randomly distributed grains with vesicles, was calculated.

If the actual silver grains over the cell cytoplasm in any animal were randomly distributed the above ratio could be expected to have a value of 1. If a ratio greater than 1 was obtained, an association of silver grains with vesicles greater than that expected due to chance was indicated. As shown in Table V, this ratio was not greater than 1 at the earliest time intervals, but from the 10 minute time interval onwards, it was considerably greater than 1, reaching a value of over 4 at 35 minutes after ^{3}H-fucose injection.

These results suggested that at the earliest time intervals after ^{3}H-fucose injection, most of the cytoplasmic silver grains outside of the Golgi apparatus were not associated with cytoplasmic vesicles, while at the later time intervals, many grains were associated with such vesicles.

Electron Microscopic Observations of Glycol Methacrylate Embedded Duodenal Tissue

When radioautographs of ½ micron thick sections of glycol methacrylate embedded duodenal tissue were examined in the electron microscope after staining with phosphotungstic acid at low pH, the results shown in figures 34-36 were obtained. These radioautographs were from an animal

sacrificed 4 hours after ^{3}H-fucose injection. Only certain cellular organelles were stained by the phosphotungstic acid stain. These included the apical microvillus border (Fig. 35, 36: mv), the lateral cell membranes (Fig. 34-36: lmb), the Golgi saccules (Fig. 34, 35: G), dense bodies (Figs. 34-36: D), multivesicular bodies (Fig. 36: MVB), and small vesicles concentrated in the terminal web region of the cytoplasm and scattered throughout the remainder of the cytoplasm (Figs. 34, 35: oblique arrows). The nucleus of the cell was not specifically stained but showed up in electron micrographs due to its own inherent electron density.

In the microvillus border (Fig. 35, 36: mv), the staining was localized to the surfaces of each individual microvillus, while the cytoplasmic core of the microvillus remained unstained. In the Golgi apparatus (Fig. 34: G), a transition in staining intensity occurred from one face of the Golgi stack to the other. As seen in the Golgi stack to the left of the nucleus in figure 34, the saccules along the immature (outer) face took up almost no stain (horizontal arrows), while the saccules and some vacuoles along the mature (inner) face were heavily stained (vertical arrows). In the Golgi stack to the right of the nucleus in figure 34, the plane of section was parallel to the flat surfaces of the stack of Golgi saccules, and in this plane it could be seen that the most mature saccule was fenestrated, or rather, consisted of a polygonal meshwork of tubules.

Dense bodies (Figs. 34-36: D) were sometimes found to be stained homogeneously throughout but often contained a more lightly stained center. In multivesicular bodies (Fig. 36: MVB), the matrix, and the walls of the internal vesicles were stained, but the contents of the internal

vesicles remained unstained. Similarly in the somewhat larger cytoplasmic vesicles scattered through the cytoplasm (Figs. 34, 35: oblique arrows), the vesicular wall stained but the contents remained unstained.

The radioautographic reaction occurring over these cells was very similar to that seen at the 4 hour time interval in thin epon sections (Figs. 27-29). A heavy reaction occurred over the apical microvillus border (Figs. 35, 36), and several grains appeared over the lateral cell membranes (Figs. 34-36). Many of the dense and multivesicular bodies in the cells were labelled (Figs. 34-36), and some grains occurred over saccules of the Golgi apparatus (Fig. 34).

RADIOAUTOGRAPHIC OBSERVATIONS ON LIVER TISSUE

Light Microscopic Observations

Initial Uptake of ^{3}H-fucose Label

Light microscopic radioautographs of liver tissue from animals sacrificed as early as 2 minutes after ^{3}H-fucose injection (Fig. 37) showed a fairly heavy uptake of label by hepatocytes. The reaction at low magnification took the form of localized clusters of silver grains (vertical arrow) over the liver cords midway between the sinusoids and between the cell nuclei. These areas are occupied by the bile canaliculi, near to which the Golgi complexes are located in these cells. Few grains occurred over the remaining cytoplasm of the hepatocytes, and no significant reaction could be seen along the sinusoidal surfaces of the liver cords (horizontal arrow).

Distribution of ^{3}H-fucose Label at Later Time Intervals

At 20 minutes after ^{3}H-fucose injection (Fig. 38), heavy grain clusters still overlay the bile canalicular regions of the cells (vertical arrow). In addition, however, a line of reaction could also be seen along the sinusoidal surface of the liver cords (horizontal arrow). Reaction over the remainder of the cytoplasm of the hepatocytes remained comparatively light.

By 4 hours after ^{3}H-fucose injection, the heavy grain clusters over the bile canalicular regions of the hepatocytes had almost completely disappeared (Fig. 39). Now only relatively light reaction remained over all of the cytoplasm of the hepatocytes. Over the sinusoidal surface of the liver cords, on the other hand, a heavy reaction persisted (horizontal arrow).

Electron Microscopic Observations

Initial Uptake of ^{3}H-fucose Label

The results obtained from examination of electron microscopic radioautographs of liver tissue at 2 minutes after ^{3}H-fucose injection confirmed the uptake of label by the Golgi apparatus of hepatocytes, for although the radioautographic reaction over these cells in thin sections was relatively light, even after 5 months of exposure, the large majority of silver grains present were localized over the Golgi saccules. Figure 40 shows the bile canalicular region of two adjacent hepatocytes. A lateral membrane separates the two cells, and is interrupted in the middle of the figure by a bile canaliculus (BC). In the cytoplasm of each of the hepatocytes, near the bile canaliculus, are seen stacks of Golgi saccules (G) which in these cells contain small lipoprotein granules. Scattered through the remaining cytoplasm are mitochondria, rough endoplasmic reticulum, and lysosomes--which in these cells are mainly of the dense body type--(D). All of the silver grains in this figure are localized over the Golgi saccules.

Grain counts carried out over these cells to quantitatively analyze the distribution of silver grains at different time intervals after ^{3}H-fucose injection yielded the results shown in Table VI. Within the limitations of the counting method used, the results showed that over 80% of the silver grains were localized over the Golgi apparatus at 2 minutes after injection. Less than 5% of the grains occurred over the cell surfaces, and no grains were found over lysosomes.

Distribution of ^{3}H-fucose Label at Later Time Intervals

The radioautographic reaction at 35 minutes after ^{3}H-fucose injection

TABLE VI

DISTRIBUTION OF SILVER GRAINS OVER HEPATOCYTES AT VARIOUS TIME INTERVALS AFTER AN INJECTION OF ^{3}H-FUCOSE

Time after ^{3}H-fucose injection	No. of grains counted	% over Golgi apparatus	% over Cell surfaces		% over lysosomes	% over Micro-Bodies	% over Rest of Cell
			sinusoidal	lateral			
2 min	21	81.0	0.0	4.8	0.0	0.0	14.0
35 min	910	59.1	7.0	3.4	10.0	1.0	19.5
4 hr	410	12.4	24.6	15.0	29.5	0.5	18.0

TABLE VII

LABELLING OF LYSOSOMES IN HEPATOCYTES AT VARIOUS TIME INTERVALS AFTER AN INJECTION OF ^{3}H-FUCOSE

Time after ^{3}H-fucose injection	Lysosomes	
	No. counted	% labelled
2 min	37	0.0
35 min	492	24.4
4 hr	330	42.1

is shown in figure 41. Some of the silver grains are still localized over Golgi saccules (G), while one grain is associated with a Golgi vacuole (vertical arrow). This figure also shows one of the few silver grains seen near a microbody (horizontal arrow). Another unlabelled microbody (MB) is shown in the lower left corner of the figure. Also shown are dense bodies (D) and a multivesicular body (MVB) which, in this figure, are unlabelled.

Counts of the distribution of silver grains over these cells at this 35 minute time interval showed that the percentage of grains over the Golgi apparatus had dropped to less than 60% while over 10% of the grains now appeared over the cell surfaces, and 10% appeared over the lysosomes (Table VI). When counts of labelled and unlabelled lysosomes in these cells were made, the results (Table VII) indicated that approximately 24% of the total population of lysosomes in these cells were now labelled.

By 4 hours after ^{3}H-fucose injection, the reaction over the Golgi apparatus of hepatocytes had further decreased, while labelling increased in other regions of the cells. In the radioautograph of the Golgi region of a hepatocyte shown in figure 42, no silver grains are seen directly over the Golgi saccules (G) but some are seen along the mature face of the Golgi stacks (short vertical arrows), and others are associated with Golgi vacuoles (horizontal arrows). The remainder of the silver grains in this figure are over dense bodies (D). Frequently the dense bodies in these cells occurred in clusters, as shown in figure 43; three silver grains are seen over this particular cluster, two lying over dense bodies, and one lying over both a dense body and a multivesicular body. A fourth silver grain in this figure lies adjacent to the cell surface bordering on a bile

canaliculus (BC). In the inset of figure 43, a heavily labelled multivesicular body (MVB) is shown.

Several grains also occurred over the sinusoidal surfaces of the hepatocytes at this 4 hour time interval as shown in figure 44 (arrows). Few grains occur over the cytoplasm of the hepatocytes in this figure, but some reaction is seen over the cytoplasm of a Kupffer cell (K).

Counts of the distribution of silver grains over the hepatocytes at this time interval showed that only about 12% of the silver grains remained over the Golgi apparatus, while nearly 40% occurred over the cell surfaces, and nearly 30% occurred over the lysosomes. Counts of labelled and unlabelled lysosomes showed that over 42% of the lysosomal population was now labelled.

RADIOAUTOGRAPHIC OBSERVATIONS ON OTHER TISSUES: A SURVEY OF THE DISTRIBUTION OF ^{3}H-FUCOSE LABEL IN THE RAT

^{3}H-fucose label was taken up into a great variety of cell types in the rat, in which it underwent various fates. Although nearly always localized to one part of the cytoplasm at early time intervals after injection, the reaction over different cell types changed in different ways at later time intervals, providing the means for some classification of the radioautographic reactions.[1]

Migration of ^{3}H-fucose Label to the Surfaces of Cells

In many cell types, the pattern of radioautographic reaction, as seen with the light microscope, was similar to that described in duodenal villus columnar cells and hepatocytes. These cell types include:

Villus Columnar Cells of Jejunum and Ileum

Near the base of the villi, villus columnar cells exhibited a radioautographic reaction similar in intensity and pattern of grain distribution to that described in the villus columnar cells of the duodenum. As one ascended the villus, however, the intensity of reaction rapidly diminished, and in the apical two thirds of the villi the columnar cells exhibited a negligible radioautographic reaction.

Columnar Cells of Small Intestinal Crypts

Columnar cells of the jejunal and ileal crypts exhibited a supranuclear reaction of medium intensity at early time intervals after ^{3}H-fucose injection. The reaction over duodenal crypt columnar cells was comparatively

1. In addition to the radioautographic reactions described in the following pages, reactions were also occasionally observed over a very few other cell types such as retinal pigment epithelial cells, osteoclasts, and certain cells of hemopoetic organs. Also, it should be mentioned that some tissues such as thyroid gland, and teeth were processed and analysed by co-workers in these experiments. Reactions over these tissues are not reported in this thesis, although the findings of these workers are mentioned in the discussion.

light (Fig. 9), but here too some grains were localized over the supranuclear cytoplasm (vertical arrow). With time the reaction over the supranuclear Golgi region decreased, and more and more silver grains became localized over the apical surface of the cells, so that by 4 hours after injection, a moderate apical surface reaction was present. By 30 hours after injection, many of the crypt cells had migrated to the lower parts of the villi, where they still exhibited a moderate apical surface reaction (Fig. 14).

Colonic Surface and Crypt Columnar Cells

Both of these cell types exhibited a fairly heavy radioautographic reaction similar to that seen over the villus columnar cells of the duodenum; thus at 10 minutes after ^{3}H-fucose injection (Fig. 45), reaction was localized over the supranuclear Golgi region of the surface columnar cells (vertical arrow) and crypt columnar cells (horizontal arrow). The intensity of the radioautographic reaction was somewhat less over crypt cells near the base of the crypts than over those nearer the surface. By 1 hour after ^{3}H-fucose injection the grain distribution over the cells had completely changed, and now the reaction was localized mainly over the apical surface of the cells, while the Golgi region and the base of the cells contained only little label (Fig. 46). In this feature, the reaction over these cells differed from that in duodenal villus columnar cells, for in the latter cells at 1 hour after injection, considerable reaction remained over the Golgi region, and many silver grains were seen over the lateral cell surfaces near the base of the cells (Fig. 11).

Duct Cells of the Submaxillary Gland

At early time intervals after ^{3}H-fucose injection (Fig. 47), the radioautographic reaction over these cells was localized to the supra-

nuclear region (horizontal arrows) while at later time intervals, such as at 4 hours after ^{3}H-fucose injection (Fig. 48), the supranuclear localization of silver grains were no longer present, and instead, a heavy line of reaction occurred over the lumenal surface of the cells (horizontal arrows). Various other duct cells in the body such as those lining the isthmus region of the oviduct in female rats and those lining the ductus epididymus in male rats, exhibited patterns of radioautographic reaction similar to that seen in the submaxillary gland duct cells.

Choroid Plexus Epithelial Cells

The radioautographic reaction over these cells at early time intervals after ^{3}H-fucose injection was localized in the form of paranuclear grain clusters (Fig. 49: oblique arrows), and the ventricular surface of these cells was only lightly labelled (vertical arrow). By 4 hours after injection, on the other hand (Fig. 50), the situation was completely reversed, with a heavy reaction occurring over the ventricular cell surface (vertical arrow) and only lighter labelling remaining over the cytoplasm. In some sites, lines of silver grains extended along the interface between adjacent cells (horizontal arrows), suggesting the localization of label over the lateral cell surfaces.

A radioautographic reaction very similar to the one described over the choroid plexus epithelial cells was seen over the epithelial cells of the ciliary body of the eye.

Tubule Cells of the Kidney Cortex

At the earliest time intervals after ^{3}H-fucose injection, substantial uptake of label was observed only in cells of certain tubules of the kidney cortex. The most prominent radioautographic reaction occurred over the cells of the long, straight ascending loops of Henle (Fig. 51: horizontal

arrow) in the medullary rays. When examined at higher magnification (Fig. 52) this reaction took the form of dense clusters of silver grains localized over the paranuclear cytoplasm of the cells (horizontal arrows). No significant reaction occurred over the nearby proximal convoluted tubules (PCT) or descending loops of Henle (vertical arrow). In the cortical labyrinth between the medullary rays, certain short segments of tubules exhibiting heavy reaction were seen (Fig. 51: vertical arrowheads); when examined closely, the cells of these tubules resembled those of distal convoluted tubules. However, profiles of unreactive distal convoluted tubules were frequently seen in the surrounding tissue. This indicated that either some parts of the distal convoluted tubules were reactive while others were not, or the reactive tubules represented parts of ascending loops of Henle which penetrated the cortical labyrinth to reach their respective glomeruli before becoming distal convoluted tubules.

At 20 minutes after ^{3}H-fucose injection (Fig. 53), the cells of the ascending loops of Henle still exhibited the most prominent radioautographic reaction in the kidney cortex; now the reaction was no longer localized, but covered the apical cytoplasm of the cells (horizontal arrow). At this time interval, a reaction of medium intensity also occurred over the cytoplasm of proximal convoluted tubule cells (PCT); at 10 minutes after ^{3}H-fucose injection some small localized grain clusters could be seen over the paranuclear cytoplasm of these cells, but by this time interval the labelling was diffuse in nature.

By 4 hours after ^{3}H-fucose injection (Fig. 54), the reaction over the proximal convoluted tubules (PCT) had become much heavier and was now the most prominent reaction over the kidney cortex. Most of the silver

grains were now localized over the apical brush border of the proximal convoluted tubule cells. The radioautographic reaction over the loops of Henle was still quite heavy (horizontal arrow), but was no longer as conspicuous as it had been in the earlier time intervals.

Endothelial Cells of Capillaries

Reactions over capillary endothelial cells constituted one of the most widespread radioautographic reactions in the body, occurring in practically all tissues. Such reactions were seen to special advantage in sections of cerebral cortex (Fig. 55) since nervous elements themselves did not incorporate much ^{3}H-fucose label. The reaction at 10 minutes after ^{3}H-fucose is shown in figure 55. At the center of the figure is an obliquely sectioned capillary whose endothelial cells exhibit a fairly strong radioautographic reaction (vertical arrows) in which the silver grains are localized over the cytoplasm at the end of the cell nucleus--a region occupied by the Golgi apparatus. Similar reactions could be seen over other smaller capillaries, often cut in cross section, such as those scattered throughout figure 55 (oblique arrows). Unless the lumen of such small capillaries could be clearly seen, it was sometimes difficult to differentiate a reactive endothelial cell of a capillary from other cell types in the tissue in which the capillary was located. Thus it is possible that the paranuclear grain clusters seen at early time intervals after ^{3}H-fucose injection in the connective tissue core of folds of choroid plexus (Fig. 49: horizontal arrow), in kidney glomeruli (Fig. 53: G), in pancreatic islets of Langerhans (Fig. 63: IL - horizontal arrows), and in various other sites, may represent reactions over capillary endothelial cells.

With time after injection, the initial localization of ^{3}H-fucose label in the endothelial cells gave way to an even distribution of the label along the length of the cells. Thus by 4 hours after ^{3}H-fucose injection, a continuous line of reaction was present along the length of the endothelial cells of cerebral cortex capillaries (Fig. 56: vertical arrows). Because of the thinness of the endothelial cells it was not possible at most sites to resolve with the light microscope whether or not the radioautographic reaction was localized along the lumenal surface of the cells. Inspection of sites where a nucleus was present (oblique arrows), however, showed that the radioautographic reaction occurred along the lumenal side of the nucleus.

Stratified Epithelial Membranes

All of the stratified epithelial membranes of the body exhibited uptake of ^{3}H-fucose label to a greater or lesser extent. Such stratified epithelial membranes included the epidermis of the skin, the bulb and root sheaths of hair follicles, the stratified epithelia of the cornea and conjunctiva, the stratified epithelia covering the tongue and lining the oral cavity, esophagus, and proximal stomach, and the stratified epithelia lining the urinary bladder and urethra.

In all of the above epithelial membranes, the same basic pattern of radioautographic reaction existed. At the early time intervals after ^{3}H-fucose injection, the reaction took the form of paranuclear grain clusters over the germinal and middle layers of the epithelial membrane. A reaction of this type can be seen over the conjunctival epithelium of an animal sacrificed 10 minutes after ^{3}H-fucose injection (Fig. 57). Although a certain amount of scatter is present, definite clusters of

silver grains occur beside the epithelial cell nuclei, especially over the middle layers of the membrane (vertical arrows).

At later time intervals after ^{3}H-fucose injection, the distribution of silver grains over stratified epithelial membranes was found to change completely. This change is seen when the reaction over the bulb of a hair follicle is examined at 4 hours after ^{3}H-fucose injection. (Fig. 58). The silver grains in many regions of the bulb are now localized almost exclusively over the cell surfaces, while only very light labelling occurs over the cytoplasm of the cells.

This strong localized reaction over the surface of the stratified epithelial cells was still present even at 30 hours after ^{3}H-fucose injection as seen in the obliquely cut section of conjunctival epithelium shown in figure 59. Now the most basal cells of the epithelial layer (those next to the connective tissue which is now heavily labelled) do not show a heavy reaction; however, the layers of cells further out in the membrane exhibit well localized surface reactions.

Migration of ^{3}H-fucose Label to Cell Secretions

Mucous Cells

^{3}H-fucose was taken up in varying amounts by all mucus secreting cells of the body after an injection of this isotope. In all mucous cell types, a similar pattern of reaction occurred: the ^{3}H-fucose label was at first localized to the supranuclear Golgi region of the cell, but later appeared in the cell secretion itself. Thus at early time intervals after ^{3}H-fucose injection a supranuclear radioautographic reaction was seen over duodenal villus goblet cells (Fig. 9: oblique arrow; Fig. 10 horizontal arrow), duodenal crypt goblet cells (Fig. 9: horizontal arrow),

colonic crypt and surface goblet cells (Fig. 45: oblique arrows); sub.-maxillary gland mucous cells (Fig. 47: vertical arrow), mucous acinar cells of tongue glands (Fig. 62: horizontal arrow), surface mucous cells of the stomach (Fig. 60), and mucous neck cells of the stomach glands (Fig. 60: horizontal arrows).

At later time intervals after ^{3}H-fucose injection, the radioautographic reaction over mucous cells appeared over the mucous secretion itself. Thus in the glandular portion of the stomach, by 4 hours after ^{3}H-fucose injection (Fig. 61), a very intense radioautographic reaction covered the mucous contents of the apical portions of the surface mucous cells which covered the mucosal surface and lined the gastric pits. The reaction over the gastric pits (vertical arrow)was of such intensity that the reactive areas often appeared solid black, and the underlying mucus was not visible. Heavy reaction also covered the layer of mucus already secreted into the stomach lumen. In the deeper portion of the stomach mucosa, long lines of reaction could be seen (horizontal arrows) which were continuous with the reaction over the gastric pits; these represented labelled mucus secreted into the lumens of the stomach glands by the mucous neck cells.

In some mucous cell types, such as the goblet cells of the colon, the rate at which the label migrated to the mucus secretion was much slower than in the above case; in these cells, for example, the reaction at 1 hour after ^{3}H-fucose injection (Fig. 46) was still at the very base of the mucous theca of the cells (oblique arrows), and had not appreciably migrated over the mucus even by 4 hours after injection.

Serous Cells

Serous cells in various sites of the body also exhibited uptake of label after ^{3}H-fucose injection, although the reaction was not usually as great in intensity as that over mucus secreting cells. At the earliest time intervals after injection, very little reaction usually occurred over cells of this type. By 20 minutes after injection, however, as seen in pancreatic acinar cells (Fig. 63), a reaction of medium intensity often occurred over the supranuclear cytoplasm (vertical arrows in Fig. 63). At later time intervals, the label migrated through the apical cytoplasm of the cells to the acinar lumen.

Cells Secreting Ground Substances of Tissues

Chondrocytes

Uptake of ^{3}H-fucose label by cartilage cells was not particularly heavy as compared to that exhibited by many other cell types. Nonetheless by 20 minutes after ^{3}H-fucose injection a definite reaction could be seen over the chondrocytes of epiphyseal plates (Fig. 64); the reaction took the form of clusters of silver grains localized over the paranuclear Golgi region of these cells (horizontal arrows). Reaction was heaviest over cells in the zone of elongation (left hand side of Fig. 64), and became lighter in the zone of hypertrophy (right hand side of Fig.64). In the latter cells the reaction was also more diffuse, although some grains were localized in paranuclear clusters (vertical arrows). At later time intervals after ^{3}H-fucose injection, label appeared diffusely throughout the cytoplasm of the chondrocytes, and reaction also appeared over the cartilage matrix surrounding these cells.

Osteoblasts

Osteoblasts took up substantial ^{3}H-fucose label at early time

intervals after ^{3}H-fucose injection as seen in figure 65, which shows the radioautographic reaction over the cancellous bone tissue of an epiphysis at 2 minutes after injection. The figure shows a number of bony spicules (B), some of which have central cores of calcified cartilage (C). Along the edges of the spicules are several osteoblast cells which exhibit a heavy radioautographic reaction. The silver grains over these cells are localized in dense clusters (vertical arrows) over the Golgi region adjacent to the nucleus (N). A considerable number of grains were also found scattered over the bone matrix at this time interval.

By 4 hours after ^{3}H-fucose injection (Fig. 66), the radioautographic reaction was localized mainly over the bony matrix itself (vertical arrows) near the interface between the bone and the osteoblasts (nucleus: N); only a small amount of label remained over the osteoblasts themselves. The scattered reaction seen over the bony matrix at the 2 minute time interval was not present at this time.

By 30 hours after ^{3}H-fucose injection, (Fig. 67), the radioautographic reaction over the bony matrix was seen as a heavy line of reaction (vertical arrows) located a considerable distance away from the osteoblasts (nucleus: N). Much less reaction occurred over the area of bony matrix between the line of heavy reaction and the osteoblasts. Within the bony spicules, some radioautographic reaction could also be seen over the bony matrix which surrounded lacunae containing osteocytes (oblique arrows). Many of these cells had exhibited localized reaction over their paranuclear cytoplasm at earlier time intervals.

Fibroblasts

Radioautographic reaction occurred over connective tissue in sites

all over the body after ^{3}H-fucose injection, and at late time intervals, this reaction became one of the most prominent reactions seen in the tissues.

At early time intervals after ^{3}H-fucose injection, the label was often localized in clusters adjacent to elongated nuclei. Such localizations were especially well seen in loose connective tissues such as the lamina propria of small intestinal villi (Fig. 10: vertical arrow), the lamina propria between colonic crypts (Fig. 45), and the loose connective tissue beneath conjunctival epithelium (Fig. 57: oblique arrow). It is possible that these localized grain clusters represent Golgi localized reactions over the cytoplasm of fibroblasts.

At later time intervals after ^{3}H-fucose injection the reaction over connective tissue became intense in many tissues. In the connective tissue underlying the conjunctival epithelium (Fig. 59), the connective tissue reaction not only filled the space between the conjunctival epithelium and the underlying striated muscle, but it also appeared to penetrate between the individual striated muscle fibers, making it difficult to determine whether the reaction at the surface of these fibers represented a cell surface reaction, or was reaction over strands of connective tissue between the muscle cells.

DISCUSSION

Metabolism of L-Fucose

L-fucose is not thought to occur as a free sugar in the blood of mammals (Cantarow and Trumper, 1962; Bell, 1962) although its presence has been shown in human milk (Heyns et al., 1956). In normal body metabolism, the fucose residues occurring in the carbohydrate moieties of glycoproteins are believed to be derived from GDP-fucose which in turn is derived by enzymatic conversion from GDP-mannose (Foster and Ginsburg, 1961; Segal and Topper, 1960) (see Text-Figure 1).

Free fucose can be utilized by animal tissues, however. After ^{14}C-fucose is injected intraperitonally into an animal, the label is rapidly cleared from the blood serum (Bekesi and Winzler, 1967) with only 1.5% of the administered dose remaining at 1 hour after injection. Similarly after an intravenous injection, the label is rapidly cleared from the blood (Bocci and Winzler, 1969).

Some label (30%) appears as free fucose in the urine (Coffey at al., 1964; Bekesi and Winzler, 1967), but other label is taken up by the cells of various tissues. To be used in metabolism, the fucose appears to be first phosphorylated and then converted to GDP-fucose (Bekesi and Winzler, 1967) (Text-figure, 1); the enzymes which catalyse these steps have been isolated from liver (Ishihara and Heath, 1968; Ishihara, Messaro and Heath, 1968). However other tissues can perform these conversions as well (Bekesi and Winzler, 1967; Kaufman and Ginsburg, 1968). In HeLa cells, exogenously administered fucose increases the concentration of intracellular GDP-fucose by 100 fold (Kaufman and Ginsburg, 1968), which probably occurs because GDP-fucose is thought to be directly formed from exogenous fucose, (Bekesi and Winzler, 1967);this pathway would thus bypass the two enzymatically catalized reactions by which GDP-fucose is

normally synthesized in these cells (mannose-1-P→ GDP-mannose→ GDP-fucose) and the rates of which are normally regulated by the intracellular level of GDP-fucose via feedback inhibition. This phenomenon may explain the observation of Bekesi and Winzler in rats that although labelled fucose was rapidly removed from the circulating blood with only 1.5% of the administered dose remaining at one hour, in most of the tissues the TCA soluble radioactivity, due in part to GDP-fucose, increased with time, reaching a peak at 40 and 50 minutes after injection in the small intestine and liver respectively (Bekesi and Winzler, 1967). This presence of labelled GDP-fucose in cells for as long as 50 minutes after injection of the label suggests that although the administration of labelled fucose by a single intravenous injection may approximate a pulse dose in terms of availability of labelled fucose to the cells from the circulating blood, the availability of labelled GDP-fucose within cells for glycoprotein synthesis would appear to last for a longer period. The possible importance of this fact will be discussed later in this thesis.

Fucose is not converted to glycogen (Shull and Miller, 1960; Coffey, Miller and Sellinger, 1964), and very little fucose is broken down into smaller metabolites, for after 6-10 hours, only 1.0-1.6% of the label appeared as CO_2 (Coffey, Miller and Sellinger, 1964; Bekesi and Winzler, 1967), and even this may not be due to metabolism of the ^3H-fucose by the rat tissues but rather by intestinal microflora (Bocci and Winzler, 1969). Indeed, when several tissues were hydrolyzed and then the hydrolysates were subjected to paper chromatographic analysis, fucose was the only radioactive component that could be demonstrated (Bekesi and Winzler, 1967). This is probably because of the irreversability of the

GDP-mannose→ GDP fucose conversion (Text-Figure 1) which prevents fucose from being converted to other monosaccharides, some of which could enter the glycolytic cycle. The main fate of the labelled GDP-fucose (Text-Figure 1) is to incorporate its fucose moiety into the carbohydrate side chains of various glycoproteins including those in intestine, liver, and brain, and in various surface membranes (Coffey et al., 1964; Kornfeld et al., 1965; Grollman and Marcus, 1966; Bekesi and Winzler, 1967; Kaufman and Ginsburg, 1968; Louisot et al., 1968; Bossman, Hagopian and Eylar, 1968b; Bocci and Winzler, 1969; Dutton and Barondes, 1970; Zatz and Barondes, 1970; Buck, Glick and Warren, 1970; Muramatsu and Nathenson, 1970; Herscovics, 1970). Labelling experiments indicate that here again there is no conversion to other sugar residues, for when ^{14}C-fucose is incorporated into glycoproteins, little if any label appears in sugar residues other than fucose (Bekesi and Winzler, 1966; Kaufman and Ginsburg, 1968; Herscovics, 1970). Bosmann, Hagopian and Eylar (1968) have shown the presence, in the smooth membrane fraction of HeLa cells, of two highly specific fucosyltransferases which transfer fucose residues from GDP-fucose into glycoprotein side chains. In preparations from human submaxillary gland and stomach, on the other hand, Chester and Watkins (1969) have shown the presence of three specific fucosyltransferases which incorporate fucose residues into glycoproteins with H, Le^a, Le^b blood group activity. The combination of these fucosyltransferases present appears to be genetically controlled by the blood group status of the individual. Thus injected free fucose is mostly used for glycoprotein synthesis in rats. The tissues whose glycoproteins are most heavily labelled after ^{14}C-fucose injection

are small intestine, liver, and blood serum , but all tissues contained some labelled protein-bound fucose (Bekesi and Winzler, 1967). It should be mentioned that fucose residues may also occur in some membrane glycolipids (Winzler, 70a) and the incorporation of some labelled exogenous fucose into membrane glycolipids has been demonstrated by Bosmann, Hagopian and Eylar (1969). This incorporation appears to occur in the smooth membrane fraction and might utilize the same fucosyltransferases as those which incorporate fucose into glycoproteins in this fraction (Bosmann, Hagopian and Eylar, 1968).

Interpretation of Results Obtained in Duodenal Villus Columnar Cells

Nature of Labelled Substances

Radioautographs of duodenal villus columnar cells after ^{3}H-fucose injection indicated that these cells incorporated considerable label. In attempting to interpret the nature of the substance or substances containing this label, certain conclusions can be drawn based on the preparative techniques used. Since small molecules would have been washed out of the tissue during the various steps of preparation for embedding, it may be assumed that the label resides in macromolecules. Furthermore, since many of the solutions through which the tissues were passed were lipid solvents, such as acetone, it is likely that most lipids were also lost (although some glycolipids may be retained, (Rambourg, et. al., 1966)). Since treatment with alpha amylase did not have any effect on the reaction over these cells, the label was not in glycogen. This leaves only three types of substances: nucleic acids, proteins and glycoproteins. The nucleic acids may be dismissed since their presursors (^{3}H-thymidine, ^{3}H-uridine) are taken up only in nuclei. So may proteins,

since their precursors (^{3}H-leucine) are taken up in basophilic regions of cells and nuclei. The ^{3}H-fucose label was not taken up in nuclei and rarely in basophilic regions, but usually in the Golgi apparatus. Thus it is likely that the fucose label was taken up in the last of the three substances mentioned: glycoprotein.

This contention is supported by strong biochemical evidence, as discussed in the previous section of this thesis, which shows that injected ^{14}C-fucose label is not converted to glycogen or appreciably broken down to smaller metabolites, but is instead incorporated into the fucose residues of the carbohydrate moieties of glycoproteins.

The heavy reaction over the duodenal villus columnar cells in the present study is in accord with the findings of both Coffey, Miller and Sellinger (1964), and Bekesi and Winzler (1967) that small intestine (and liver) were the most heavily labelled tissues after ^{14}C-fucose injection into rats. The authors of both of these works implied that the glycoprotein(s) in the small intestine might be mucin. However, in the present investigation, the mucin secreting goblet cells of duodenum, although reactive, took up less label than columnar cells did. Furthermore, goblet cells comprised only about one-twentieth of the epithelial cell population. Thus the glycoprotein analyzed by Coffey, Miller and Sellinger (1964) at 1 hour after injection of labelled fucose, that is, at a time when the present experiments showed much of the label at the cell surfaces, may have consisted mainly of cell coat material produced by columnar cells (Fig. 11). Since methods have recently developed for the isolation of a highly purified plasma membrane fraction from the brush border fraction of intestinal columnar cells, (Forstner et al., 1968b), these cells constitute an excellent model for the study of surface glycoproteins.

Forstner has demonstrated the incorporation of ^{14}C-glucosamine label into glucosamine and sialic acid residues of glycoprotein material associated with this plasma membrane fraction in rat small intestinal columnar cells (Forstner, 1968a, 1969). In a more recent paper, Forstner has shown the presence of three functionally distinct classes of glycoproteins in the rat small intestinal mucosa. One of these was associated with the brush border plasma membrane fraction of columnar cells as described above, and a second occurred in the lumen. These two classes were rapidly and highly labelled by ^{14}C-glucosamine. A third class remained poorly labelled and did not enter the brush border or luminal compartments; it was suggested that this glycoprotein might represent basement membrane. All of these glycoproteins were shown to contain fucose residues as well as sialic acid, hexosamine, and hexose residues (Forstner, 1970). The fact that they contained almost no hexuronic acid indicated that these substances consisted of glycoprotein rather than mucopolysaccharide.

Another type of evidence as to the nature of the substances into which the ^{3}H-fucose label may be incorporated in the present study, is provided by histochemical staining of the duodenal villus columnar cells. As shown in Figure 2, all of the surfaces of these cells are positively stained by the PA-Schiff stain, as is some of the cytoplasmic material in the Golgi region of the cells. Since this staining is not decreased by amylase treatment, it is not caused by glycogen, and may be considered to represent glycoprotein. In the electron microscope, the cell surfaces and Golgi apparatus were stained by the phosphotungstic acid stain (Figures 34-36). Although the specificity of this staining technique for glycoproteins has been questioned (Silverman and Glick, 1969; Glick and Scott, 1970; Scott and Glick, 1971), empirical evidence that aqueous

phosphotungstic acid when used at a low pH (less than pH 1) is reasonably specific for glycoprotein is very great, in that its staining pattern is idential to that of the PA-Schiff (Rambourg, Hernandez and Leblond, 1969; Rambourg, 1969a; Pease, 1970) technique or the PA silver-methenamine technique in the electron microscope--which are known to be specific for glycoproteins (in amylase treated sections). In addition Rambourg (1968) has performed a number of in vitro experiments with a variety of biological substances which support the conclusion that it is carbohydrates that combine with aqueousphosphotungstic acid at low pH. In the present study, labelling after ^{3}H-fucose injection was specifically localized at all time intervals to structures stained with phosphotungstic acid. These results are thus a further indication that the labelled material in these cells consists of glycoprotein. On the other hand, these findings provide added evidence for the specificity of the phosphotungstic acid technique for glycoproteins.

Site of Incorporation of ^{3}H-fucose Label

At two minutes after ^{3}H-fucose injection, electron microscopic radioautographs of duodenal villus columnar cells revealed that the great majority of silver grains occurred over the Golgi apparatus (Figs. 15 and 16). Grain counts at this time interval showed that almost 80% of the silver grains were localized over this organelle. Since, in the short time interval of two minutes, glycoprotein macromolecules which had incorporated ^{3}H-fucose label would probably not have migrated far, it can be assumed that the labelled glycoproteins occurring in the Golgi apparatus at this time incorporated their ^{3}H-fucose label within this same organelle. Since sugar residues are most likely added onto growing carbohydrate side chains of glycoproteins one by one, and since fucose occurs as a terminal residue

on the end of such chains, it would probably be one of the last sugars added. Hence the uptake of ^{3}H-fucose label in the Golgi apparatus within two minutes after injection indicated that the synthesis of at least some of the carbohydrate side chains of the glycoprotein molecules are completed in this organelle.

These results are in accord with those recently obtained in this department by Haddad, Smith, Herscovics, Nadler and Leblond (1971) in thyroid follicular cells and by Weinstock and Weinstock (1971) in odonto blasts. Thus evidence from all of these studies indicates that the completion of the carbohydrate moiety of glycoproteins (or at least some of their side chains) occurs in the Golgi apparatus, and corroborates the findings of the various biochemical studies which indicated that fucose label was incorporated into glycoproteins in the smooth membrane fraction (which would contain the Golgi apparatus).

But what of the sites of addition of those sugar residues located more internally on the carbohydrate side chains? Biochemical studies indicated that some of these were also added onto carbohydrate side chains in the smooth membrane fraction. Were they also added in the Golgi apparatus? A number of radioautographic studies have been now carried out which provide answers to these questions.

The results obtained from the electron microscopic radioautographic study of duodenal columnar cells after ^{3}H-galactose injection showed that this label was also taken up mostly in the Golgi apparatus at two minutes after injection (Fig. 68) (Bennett, 1970), indicating that galactose was also added on to carbohydrate side chains of glycoproteins within the Golgi apparatus. A similar localized uptake of ^{3}H-galactose label has recently

been reported in a number of cell types, including jejunal villus columnar cells of adult rats (Jersild, 1968), ileal villus columnar cells of cats (Ito, 1969), thyroid follicular cells (Whur, Herscovics, and Leblond, 1969; Haddad, 1971a), hepatocytes (Droz, 1966), ameloblasts (Weinstock, 1969), and plasma tumor cells (Zagury, Uhr, Jamieson and Palade, 1970). Thus galactose appears to be incorporated into carbohydrate side chains within the Golgi apparatus, showing that not only such terminal sugars as fucose, but also penultimate sugars such as galactose may be added in this organelle. This result is in accord with the recent localization of galactosyl transferases to Golgi rich cell fractions by a number of investigators (see Introduction).

In the case of sugars located more internally on the carbohydrate side chains, a different radioautographic pattern is obtained. Thus Whur, Herscovics and Leblond (1969) found that ^{3}H-mannose label was incorporated in the rough endoplasmic reticulum of thyroid follicular cells at early time intervals after administration. ^{3}H-glucosamine was found to be initially taken up in the rough endoplasmic reticulum of thyroid follicular cells (Haddad et al., 1971b), while in tumor plasma cells (Zagury et al., 1970), ^{3}H-glucosamine label was incorporated mainly in the rough endoplasmic reticulum, but also to a significant extent in the Golgi apparatus. These latter results were taken to indicate incorporation of some of the glucosamine into newly formed or forming polypeptides as innermost residues of carbohydrate side chains while the remainder were incorporated into more peripheral regions of the carbohydrate side chains and later in the intracellular life of the molecule.

In the duodenal villus columnar cells of the present investigation, light microscopic radioautographs showed that ^{3}H-mannose and ^{3}H-glucosamine

(and also ^{3}H-glucose and ^{3}H-ribose) were incorporated by the cells (Bennett, unpublished), but not to as great an extent as ^{3}H-galactose or ^{3}H-fucose. In addition, the label in each case was scattered over the cytoplasm at early time intervals in a pattern similar to that observed soon after an injection of ^{3}H-leucine (Bennett, unpublished). These results suggest that these labelled sugars (or labelled sugars metabolically derived from these in the case of ^{3}H-glucose and ^{3}H-ribose) were taken up into glycoproteins mostly in the rough endoplasmic reticulum. In colonic surface columnar cells, on the other hand, a fairly sharply localized reaction was seen over the Golgi region at 5 minutes after ^{3}H-glucosamine injection (Bennett, unpublished), suggesting that most of the labelled glucosamine in these cells is incorporated within the Golgi apparatus.

Thus radioautographic studies support the concept of sugars being added onto growing side chains of glycoproteins one by one, and in addition show that the sugars are added as the molecules pass from the site of polypeptide synthesis in the rough endoplasmic reticulum to the Golgi apparatus. Those sugars nearest to the ends of the carbohydrate chains are added last in the Golgi apparatus.

The Significance of Silver Grains Occurring Outside of the Golgi Apparatus at Early Time Intervals

In the present study, not all of the ^{3}H-fucose label was localized to the Golgi apparatus of duodenal villus columnar cells at 2 minutes after injection. Thus in figures 15 and 16 some grains occur over the cytoplasm outside the Golgi region, and some others occur over lateral membranes. Grain counts (Table III) at this time interval showed the presence of about 4% of the grains over the lateral cell membranes. This labelling could

suggest incorporation of ^{3}H-fucose label into glycoproteins at the cell membranes themselves. However since much fewer grains occurred over the apical or basal cell membranes, and those occurring over the lateral cell membranes were over regions of these membranes near to the Golgi apparatus, it is quite possible that these grains represented labelled glycoprotein which has already migrated from the Golgi apparatus to the cell surface.

Most of the silver grains over the cytoplasm outside of the Golgi region at this early time interval appeared to be over the edges of mitochondria or over cisternae of rough endoplasmic reticulum surrounding mitochondria. At all time intervals a certain proportion of silver grains occurred in these locations. It is possible that this label represents incorporation into material unrelated to the synthesis and migration of material through the Golgi apparatus. Conceivably some fucose breaks down into metabolites which give rise to amino acids and these are incorporated into proteins in the cisternae of rough endoplasmic reticulum. On the other hand, Bosmann has shown that ^{14}C-labelled fucose, as well as ^{14}C-labelled galactose, mannose, and glucose, are incorporated into glycoproteins by isolated rat liver mitochondria (Bosmann and Martin, 1969) suggesting that the scattered ^{3}H-fucose label in this study could be incorporated by the mitochondria themselves. Indeed the localization of such silver grains appears very similar to that of silver grains in electron microscopic radioautographs of kidney tubule cells obtained by Bergeron and Droz, (1969) at early time intervals after injection of ^{3}H-leucine to 40 gram rats. These authors interpreted that this label was incorporated into newly synthesized mitochondrial protein.

Migration of ^{3}H-fucose Label to Cell Surfaces

Although most of the ^{3}H-fucose label was confined to the Golgi apparatus at early time intervals after injection, label accumulated rapidly over the cell surface membranes, with time. The lateral cell membranes became labelled most rapidly (Table III, Graph 1), but by 20 minutes (Figs. 21-24) after injection, both apical and lateral cell surfaces were substantially labelled. These results strongly suggest that labelled material migrates from the Golgi apparatus to the cell surfaces. The fact that those surfaces nearer to the Golgi apparatus (lateral surfaces) became more rapidly labelled than those further away (apical surface), suggests that labelled material migrates away from the Golgi apparatus at a somewhat uniform rate, reaching those regions of surface membranes near to the Golgi apparatus sooner than regions further away.

Two problems must be considered, however, in accepting the interpretation that the above reactions over duodenal columnar cells indicate the migration of label from the Golgi apparatus to cell surfaces. The first problem relates to the localization of label to the cell surfaces. As outlined in the Materials and Methods section of this thesis, any silver grain occurring within a distance equal to half its own diameter from a cell surface was classified as being over that cell surface. It was explained that any grain within this distance of the surface membrane could have the surface membrane as its possible radioactive source, according to the calculations of N. J. Nadler in this department, (unpublished) (see also Nadler, 1971). It is equally possible, of course, that the radioactive source for the grain could lie in the region of the cytoplasm near to the surface membrane. It was reasoned,

however, that at least in the case of the lateral and basal cell surfaces this region of cytoplasm did not appear to differ in any way from the remaining cell cytoplasm, and since most of the remaining cytoplasm was not heavily labelled, it was unlikely that this region of cytoplasm adjacent to the cell surface membrane would be specifically labelled. Thus the grains in this region were assigned to the surface membrane. Along the apical cell surface, bordered by the terminal web cytoplasm, the situation was somewhat different as will be discussed later on in this thesis.

The second problem relates to the fact that the amount of label in the cells did not remain constant over the time period studied, but rather increased during the first four hours, and fell again by 30 hours. (Compare reaction at 1 hour (Fig 11 - exp. 3 wks) with reaction at 2 minutes (Fig. 9 - exposed 8 weeks). This would indicate that in terms of the availability of the immediate precursor for incorporation into glycoproteins, ie. GDP-fucose, a pulse dose of very short duration is not provided by a single IV injection of the isotope. According to biochemical observations (Coffey at al., 1964) the level of labelled fucose in the circulating blood rapidly falls after injection reaching only 1.5% of the original by 1 hour. However, the level of non-protein bound labelled intermediates, perhaps representing fucose-1-P and GDP-fucose reaches a peak by 1 hour (Bekesi and Winzler, 1967) indicating that the availability of GDP-fucose for incorporation into glycoproteins may be fairly long. This is suggested in the present study by the increase in total reaction over duodenal villus columnar cells over this one hour period. This is also suggested by the continued substantial reaction over the Golgi apparatus at the 20 minute and 1 hour time intervals (Figs. 22 and 26), for the continued presence of label in this organelle would suggest that new label is

continually being incorporated.

Under the above conditions, the change in percentage distribution of silver grains at different time intervals does not in itself prove a transfer of labelled material from one cell component to another. Nonetheless, the fact that the Golgi is heavily labelled at early time intervals while the cell surfaces contain almost no label, and yet by 20 minutes the cell surfaces become heavily labelled, strongly suggests that the label of these cell surfaces comes from the Golgi apparatus. Additional evidence of this pathway comes from the fact that the Golgi loses most of its labelling by 4 hours after injection (Figs. 27 and 35) while the cell surfaces are more heavily labelled than before. The present study therefore suggests that intestinal columnar cells manufacture their own cell coat and that the synthesis of this material is completed in the Golgi apparatus.

The first evidence that the apical surface coat material of intestinal columnar cells was produced independently by each cell rather than by neighbouring goblet cells was provided by Ito (1965) and Ito and Revel (1966) who showed that when pieces of cat intestine were incubated with ^{3}H-glucose and other labelled sugars and the labels were followed by electron microscopic radioautography, radioautographic reaction appeared first over the cytoplasm of the columnar cells, and after 1 hour, over the cell coat of the apical surface.

The possible intracytoplasmic site of the cell coat synthesis was suggested by light microscopic radioautographic studies showing the early localization of ^{3}H-galactose label to the Golgi region of duodenal villus columnar cells after ^{3}H-galactose injection into young rats, followed by appearance of the label at the striated border, (Neutra and Leblond, 1966; Bennett, 1967). This early localization of ^{3}H-galactose

label was confirmed in the electron microscope by Jersild (1968), but this author did not show the passage of label to the apical surface. In more recent in vitro radioautographic studies, Ito (1969) reported incorporation of tritium-labelled mannose and galactose into the Golgi apparatus of cat intestinal cells at early time intervals, followed by appearance of label at the apical surface coat at later times.

Recent evidence from biochemical fractionation studies has also indicated a migration of labelled material from the Golgi apparatus to the apical surface of intestinal columnar cells. Thus Forstner (1969) injected ^{14}C-glucosamine into adult rats, and after suitable time intervals fractionated the small intestinal mucosa. At 1 hour after injection the most radioactive glycoprotein occurred in the microsomal fraction (containing smooth endoplasmic reticulum and fragments of Golgi apparatus). By 3 hours however, microsomal radioactivity had diminished and now the heaviest labelling was of the glycoproteins of a purified brush border plasma membrane fraction, thus indicating a migration of labelled glycoprotein from the microsomal fraction to this fraction.

Migration of Label to the Lateral Cell Surface

In the radioautographic studies of Ito and Revel cited above (Ito, 1969), no evidence of migration of label from the Golgi apparatus to the lateral and basal surfaces of the cells was found, and it was suggested that the glycoprotein coatings of these surfaces were relatively stable components that turned over less actively than the coating on the apical cell surface (Ito, 1969). In the above mentioned light microscopic studies of the radioautographic reaction over rat duodenal columnar cells after ^{3}H-galactose injection, some reaction did appear over the lateral cell surfaces at later time intervals, suggesting that some ^{3}H-galactose

label did migrate from the Golgi apparatus to these surfaces (Bennett, 1967). When reaction over these cells after ^{3}H-galactose injection was examined in the electron microscope (Bennett, 1970), as part of the preliminary studies for the present investigation, it was found that the ^{3}H-galactose label was at first strongly localized to the Golgi apparatus of the cells (Fig. 68), but later also appeared at the apical cell surface (Fig. 69: mv). It can be seen from this figure, that many silver grains also occurred over the lateral cell membranes, However, numbers of silver grains appeared over the cytoplasm of the cells as well, perhaps due to the incorporation into protein in the rough endoplasmic reticulum of labelled amino acids derived from metabolites of the ^{3}H-galactose or to incoporation into mitochondrial glycoprotein (Bosmann and Martin, 1969). This scattered reaction made it difficult to establish with certainty that a reaction occurred over the lateral cell surfaces. In the electron microscopic radioautographs after ^{3}H-fucose injection, this localization to lateral cell membranes is more convincingly shown (Figs. 23, 23 27).

Migration of Label to the Basal Cell Surface or Basement Membrane

A few silver grains appeared over the basal surface of duodenal villus columnar cells at later time intervals after ^{3}H-fucose injection (Figs. 24 and 28). However, the percentage of grains over this surface was much smaller than that over the lateral cell surfaces. This is partly explained by the much smaller surface area of thebasal membrane, but even in photographs, the number of grains occurring over the basal surface membrane per unit length appeared to be smaller than that over the lateral membranes. Grains occurring over the basal surface membrane also, of necessity, lay over the nearby basement membrane. A number of

studies in recent years indicate that epithelial cells are at least partly responsible for the synthesis of their underlying basement membrane (Kurtz and Feldman, 1962; Hay and Revel, 1963; Revel, 1965; Young and Ocumpaugh, 1966; Pierce and Nakane, 1967; Oshima, et al., 1967; Kenyon, 1969; Nadol and Gibbons, 1970; Striker and Smuckler, 1970) and there is evidence that the Golgi apparatus is involved in this synthesis (Revel, 1965; Trelstad, 1970). Fucose is not a component of one type of the carbohydrate side chains of basement membrane collagens (type 4 in Text-Table II) (Spiro, 1969), but does occur in a second type of carbohydrate side chain (type 1a in Text-Table II) (Spiro, 1967). In the present study, however, the fact that the reaction over the basal cell surface is no heavier than the lateral surfaces, and perhaps is lighter suggests that ^{3}H-fucose label does not migrate, at least in large amounts, from the Golgi apparatus of these cells to their underlying basement membrane.

Mode of Transport of Labelled Glycoprotein from the Golgi Apparatus to The Cell Surfaces.

In their radioautographic studies on cat intestinal cells, Ito and Revel (1968) and Ito (1969) reported that no definite cytoplasmic structure, such as a vesicular or tubular system, could be identified in the cells, that might be responsible for the transport of labelled glycoprotein from the Golgi apparatus to the apical cell coat.

In the duodenal villus columnar cells of the young rats used in the present study, however, a number of smooth surfaced cytoplasmic vesicles were observed, which averaged about 140 mμ in diameter. These vesicles occurred in all regions of the cytoplasm. but were especially concentrated in two areas, the apical terminal web region of cytoplasm

beneath the microvillus border (Fig. 4), and the vicinity of the Golgi apparatus (Fig. 7: V). The contents of these vesicles were mostly electron lucent, but the vesicles contained some filamentous material which was similar in appearance to the cell coat covering the microvilli (Fig. 7). The membranes of the vesicles is similar in thickness to the apical cell membrane, and while vesicles were almost never seen in the process of fusing with (or being derived from) the apical surface membranes, occasional profiles (Fig. 4: horizontal arrow) indicated that such a process might occur. In the Golgi region (Fig. 7) it could be seen that the cytoplasmic vesicles resemble Golgi vacuoles (GV) suggesting that they might arise from these. (See also Fig. 68). Thus while it was possible that these cytoplasmic vesicles were derived by pinocytosis of the apical cell surface, it was also possible that these vesicles formed from Golgi vacuoles and were the carriers of cell coat material to the cell surface.

When electron microscopic radioautographic studies on duodenal villus columnar cells were carried out after ^{3}H-galactose injection it appeared that many of the silver grains which occurred over the cytoplasm between the Golgi apparatus and the apical microvillus border were close to or over these vesicles. This was especially the case in the terminal web region of the cytoplasm just beneath the microvillus border where such vesicles were somewhat concentrated (see Fig. 3 of Bennett, 1970), for in contrast to the findings of Ito (1969), silver grains were found to be concentrated in this region of the cytoplasm after ^{3}H-galactose injection. This fact in itself suggested a relationship between the silver grains and the vesicles.

In electron microscopic radioautographic studies after ^{3}H-fucose injection, a frequent association between cytoplasmic silver grains and vesicles was again observed at intermediate time intervals (10-60 minutes) after injection (Figs. 19, 20, 21, 22, 25, 26). If these vesicles were carriers of labelled material from Golgi apparatus to cell surfaces, it would be expected that the percentage of label in the cell compartment in which they were located (ie. "Remainder of Cytoplasm"), would be highest at the time at which the most active migration of label occurred and at which the association of silver grains with these vesicles was most apparent (ie. 10-60 minutes after ^{3}H-fucose injection). That this was indeed the case, is shown in Table III and Graph 1, which show that the percentage of grains in this category ("Remainder of Cytoplasm") rose from 12% at 2 minutes after injection to 40% at 35 minutes, and then fell to 25% by 4 hours and 10% by 30 hours. Since the labelling of this compartment is cut almost at its peak (Graph I) by the line representing the falling percentage of label over the Golgi apparatus, this suggests that the label in this compartment originates from the Golgi apparatus. Furthermore, the percentage of labelling over the cell surfaces was rapidly rising at this time interval. Thus it appeared feasible that the cytoplasmic labelling, or at least part of it, represented cell coat material in transit from the Golgi apparatus to the cell surfaces.

When actual grain counts were carried out to determine the association of silver grains in this category with cytoplasmic vesicles as described in the Results section, the results shown in Table V were obtained. It should be pointed out that the condition required for a silver grain to be considered as associated with a vesicle, ie. that the

grain be located not more than half its own diameter away from such a vesicle, does not mean that all such grains were associated with vesicles, because other compartments in the nearby cytoplasm could also have been the radioactive source for such grains. The resolution of the electron microscopic radioautographic method is not sufficient to establish with certainty the association of a silver grain with such a small structure as the cytoplasmic vesicle described here. What is important to remember here is that the same conditions were required for an actual silver grain to be considered as associated with a vesicle as were required for a symbolic randomly distributed silver grain to be considered as associated with a vesicle (as outlined in the method for calculating the expected percentage of silver grains associated with vesicles if the silver grains were randomly distributed - Results Section). Thus the association of the two types of silver grains (actual, and randomly distributed symbolic) with vesicles could be compared at different time intervals and it is the comparison that is significant. If the actual silver grains were randomly distributed over the cytoplasm, the ratio of $I/_{II}$ in Table V would be expected to be 1. If the silver grains were localized over some componant other than the vesicles, the ratio could be less than 1. If, on the other hand, the silver grains were associated with cytoplasmic vesicles to an extent greater than random chance, the ratio would be greater than 1.

As shown in Table V, at 2 and 5 minutes after injection, the ratio was less than 1, suggesting that the silver grains were associated with some component other than the vesicles. This finding is in accord with the observation that many of the grains appeared to be over rough endoplasmic reticulum or mitochandria at this time. By 10 minutes, on

the other hand, the ratio was greater than 1, and by 35 minutes, the ratio was 4.5. In other words, 4.5 times as many silver grains were associated with vesicles as would be expected if the association was by chance alone. By 30 hours after injection the ratio had again fallen to 1.5 suggesting that only a few grains were now associated with cytoplasmic vesicles.

Thus the above results, while unable to demonstrate with certainty that any one silver grain was associated with a cytoplasmic vesicle, strongly suggest that an association of silver grains with cytoplasmic vesicles does exist, and indicates therefore that these vesicles may be carriers of labelled cell coat glycoprotein from the Golgi apparatus to the cell surfaces. The fact that these vesicles stain positively for the presence of glycoprotein (Figs. 34 and 35) supports this concept.

Morphological studies relating to vesicles in intestinal columnar cells present two types of evidence. On the one hand, studies on the small intestine of neonatal rodents, have shown that intestinal columnar cells can incorporate maternal antibody from the lumen via pinocytotic vesicles and transport it to large supra-nuclear vacuoles (Clark, 1959; and others). However, there are indications that the vesicles observed in the present study are not of pinocytic origin. For example, no evidence of pinocytosis of the apical surface membrane comparable to that observed in the study of Clark (1959) was observed. Furthermore, Graney has shown (1968) that only cells in the ileal region of the small intestine have this pinocytotic capacity. Indeed in the present study, ileal cells were examined and found to exhibit evidence

of this pinocytotic capacity, but the appearance in these cells was quite different from that observed in the duodenum. Rodewald (1971) has recently presented evidence that columnar cells of the proximal intestine can pinocytose significant amounts of tracer from the lumen, but the animals studied were only 10 days old (as compared to approximately 4 weeks in the present study),and the cells were located at a distance considerably further away from the pyloris (ie. into the jejunum) than those in the present study. Thus the vesicles in the present study do not appear to be pinocytotic in origin.

Several other morphological studies,on theother hand, have indicated that vesicles similar to those in the present study, may be important in transferring material to the cell surface. Thus in an electron microscope study of mouse colonic columnar cells, Wetzel et al., (1966), showed smooth surfaced vesicles in the apical cytoplasm beneath the striated border (their Figure 3), which closely resemble the vesicles observed in the present study. When their tissue was stained with iron stains for acidic carbohydrate material, reaction occurred on the surface coat and in these apically located vesicles, as well as on similar vesicles adjacent to Golgi saccules, and on the Golgi saccules themselves. These authors suggested that the iron-stained material is synthesized in the Golgi apparatus and transported to the cell surface by means of these vesicles. Similar results were obtained in intestinal cells in the mouse colon by Weinstock (1968), in Xenopus by Bonneville and Weinstock (1970), and in small intestinal cells of fetal rat (Vollrath, 1971).

In Amoeba cells, the work of Stockem (1969) has demonstrated

the formation of vesicles from Golgi saccules which are very similar to the ones observed in the present study. These vesicles appear to travel to the cell surface where they fuse with the surface plasma membrane with the result that the membrane of the vesicle becomes part of the surface membrane. As in the present study, the vesicles contain filamentous material which resembles the filaments of the cell coat, and when the vesicular membrane becomes part of the cell membrane, the filaments become the cell coat. Staining for acid mucopolysaccharides with colloidal iron stained the filamentous surface coat, the filamentous material within the vesicles, and similar material in the cisternae of the saccules of the maturing half of the Golgi stack. Wise and Flickinger (1970), on the other hand, have shown that when Amoeba cells pinocytose tracers such as ferritin or horse radish peroxidase, the tracers end up in food vacuoles, but never appear in the Golgi apparatus. Thus, in contrast to the earlier suggestion of Daniels (1964), the Golgi apparatus does not appear to be formed from the plasma membrane in these cells. Staining of the cells for glycoproteins with the periodic acid silver-methenamine technique revealed their presence in the cell coat and in the cisternae of saccules on the maturing face of the Golgi apparatus and in vesicles. Therefore, like Stockem (1969) these authors suggest that the Golgi apparatus may contribute both membranes and cell coat to the surface of the cell via vesicles. Similar findings have been reported by Grove, Bracker and Morré in cells at the tips of fungal hyphae (1970).

In the mitotic telophase stage of plant cells, Whaley and Mollenhauer (1963) and also Hepler and Newcomb (1967) have observed that

numerous Golgi vesicles congregate on a plane equidistant from the daughter nuclei, and fuse to form the new plasma membranes which divide the cells. In a similar phase of mitosis in other plant cells (Whaley and Mollenhauer, 1963) the Golgi vesicles were filled with secretion product, and when the vesicles fused to form the plasma membrane, the secretory material ended up in the extracellular space between the cells. A similar phenomenon has been observed in many other instances where Golgi derived vesicles carry material to the cell surface. For example the studies of Brown (1969) and Brown et al., (1970) in Chrysophycean algae, have shown that Golgi saccules, in which scales have been formed, migrate to the cell surface and fuse with the plasma membrane whereby the saccule membrane is inserted into the plasma membrane while the scale is released to the surrounding wall. Palade (1959) showed that the vesicular wall of zymogen granules became part of the apical plasma membrane upon release of the granular contents into the acinar lumen. This finding has since been reported by others (Palade et al., 1961; Ito and Winchester, 1963; Stanley and Trier, 1965).

In rat transitional epithelium, Hicks (1966) has provided evidence that in transitional epithelium cells of rat bladder, Golgi derived fusiform vesicles may add membrane to the surface plasma membrane when the bladder expands, but when the bladder contracts, the plasma membrane invaginates and pinches off the fusiform vesicles into the cytoplasm. These vesicles can again be incorporated into the plasma membrane if needed, this serving as a store of excess membrane.

Two other types of evidence indicate that vesicles may transport labelled material from the Golgi apparatus to the cell surface. One

type comes from other reports of labelled vesicles in electron microscopic radioautographic studies. Thus in salamander epidermal cells and fibroblasts, after ^{3}H-proline administration Revel (1965) showed label over cytoplasmic vesicles similar to those of the present study. These were thus interpreted to be carriers of labelled collagen to the cell exterior. Similarly in HeLa cells after administration of ^{3}H-glucosamine, Reith, Oftebro, and Seljelid presented evidence (1970) that labelled material migrates from the Golgi apparatus to the cell surface coat. These authors also reported concentration of label over small vesicles in the apical cytoplasm although this was not convincingly demonstrated.

The other type of evidence comes from the fractionation studies of Forstner (1969), who showed that the cell sap fraction obtained from preparations of intestinal mucosa after ^{14}C-glucosamine administration always contained material of low radioactivity and concluded on this basis that labelled material migrated to the cell surface via a vesicular mechanism rather than travelling freely in the cell sap.

In summary, a great variety of morphological studies in various cell types have shown that Golgi derived vesicles can and do fuse with the surface membrane of cells and contribute membranes to this cell surface, [1] Thus the possibility of the cytoplasmic vesicles in the intestinal columnar cells of the present study playing a role similar to this is quite feasible.

Differences in Apical Surface Reaction from Cell to Cell

Although the intensity of apical surface reaction at 35 minutes to 4 hours after ^{3}H-fucose injection was fairly constant among most cells, as seen in the light microscope in figures 11 and 12, sometimes the reaction differed greatly over adjacent cells (Figs. 30 and 31). Although in these

1. For excellent review of this aspect of Golgi apparatus function see, Northcote, (1971).

radioautographs no difference in the amount of cell coat along the surface of the different cells was seen, Mukherjee and Williams (1967) have shown that in the mouse jejunum, the thickness of the cell coat changes abruptly from cell to cell, with some cells exhibiting a fairly thick (0.1 μ) coat and others having almost none (their Figure 4). The present radioautographic findings reflect this situation exactly (Fig. 12) and indicate that some cells are synthesizing cell coat material more rapidly than others. Both the above findings of Mukherjee and Williams and the present findings are further evidence that each columnar cell produces its own surface coat.

Nature of Labelled Material At Cell Surfaces of Duodenal Villus Columnar Cells

As mentioned previously, the staining of the cell surfaces of these cells by the periodic acid Schiff and phosphotungstic acid staining techniques indicates that the material present at these surfaces is glycoprotein in nature. Furthermore, the biochemical analysis of labelled material in a purified brush border plasma membrane fraction of rat intestinal cells by Forstner (1970) after injection of ^{14}C-glucosamine, showed that the labelled material was glycoprotein. But what was the nature of this glycoprotein material? The presence of alkaline phosphatase has been shown at the microvillar surface of rat duodenal villus columnar cells by Hugon and Borgers (1968), and this enzyme has been shown to be a glycoprotein (Portman et al., 1960). However recent studies have shown that the brush border is also the site of a number of disaccharidases, aminopeptidases and other digestive enzymes (see Dobbins, 1969; Greenberger, 1969; and Gardner, Brown and Laster, 1970; for reviews). Forstner, in a recent study (1971) has shown

that when ^{14}C-glucosamine is injected into a rat and a brush border plasma membrane fraction is obtained, a five minute digestion with papain releases surface particles containing 90% of the sucrase activity (as described previously by Oda and Seki, (1965), and Johnson (1967, 1969)), and also 45% of the radioactivity. Chromatographic studies have revealed that label is associated with each of four enzymes: maltase, sucrase, β-naphthylamidase, and alkaline phosphatase. Staining of the chromatographs with PA-Schiff stain indicated that all of these enzymes were glycoprotein in nature. Thus, at least at the apical surface of the duodenal villus columnar cells in the present study, it appears likely that the ^{3}H-fucose label resides in a number of glycoproteins some of which may be the above enzymes.

In the case of the lateral and basal cell membranes, much less is known about their chemical structure, although the presence of occasional alkaline phosphatase has been shown (Hugon and Borgers, 1968). The results of the present study indicate, however, that the glycoprotein components of these surfaces are perhaps not so different from those of the apical surface as previously thought (Ito, 1969), for both components at least appear to be derived from the Golgi apparatus, and both appear to become labelled at approximately the same rate.

Turnover of Cell Coat Material

When radioautographs of duodenal villus columnar cells were examined at 30 hours after injection, the percentage of silver grains over the cell surfaces (approx. 75%) was not very different from that observed at 4 hours (approx. 72%). However, the reaction had decreased in intensity as seen by comparing the apical surface reaction in figure

29 (4 hours) which was exposed for 7 weeks, with that in figure 33 (30 hours) which was exposed for 14 weeks. This decrease in reaction intensity suggests that cell coat material is continually lost with time. In radioautographic studies on adult cat intestinal cells by Ito and Revel (Ito, 1969), much of the reaction over the microvilli was lost by 6 hours after a pulse dose of labelled precursor, and these authors estimated the turnover time of the surface coat to be between 4 and 10 hours. In the present study, however, the turnover time may be much longer, for as indicated above, reaction remains over the microvilli at 30 hours after injection of ^{3}H-fucose. Longer time intervals after ^{3}H-fucose injection have not yet been studied in the present investigation, but would probably not yield further information for as shown in figure 14, the reactive villus columnar cells extend right to the zone of cell extrusion at the tips of the villi by 30 hours and it is possible that some have already been lost. The results of the thymidine labelling experiments indicate that all of the reactive cells would be lost between 36 and 48 hours after injection.

One problem related to the turnover of cell coat material is whether only the cell coat itself turns over, or whether the underlying plasma membrane to which it is attached also turns over. In other words, does the cell coat always remain attached to its underlying membrane, and must they be considered as one unit in terms of biogenesis and turn over? Ito showed that the cell coat is not removed from healthy cells by EDTA or by various mucolytic agents (Ito, 1969) although it does decrease in cells being extruded from the tips of villi. He suggested however, that the reason the coat

does not decrease after mucolytic agents may be due to its continual replacement by newly synthesized coat. In the present study, if the cell coat is carried to the cell surface attached to the inner surface of vesicles, which fuse with the plasma membrane, so that their inner surface becomes the outer cell surface, then it would be implied that the cell coat and underlying membrane are acting as one unit.

That segments of whole unit membrane can be provided to the surface plasma membrane is shown by a number of studies, as mentioned previously (p. 99). In addition, the Golgi apparatus has been shown to be the site where membrane changes from endoplasmic reticulum-like at the immature face to plasma membrane-like at the mature face (Grove, Bracker and Morré, 1968, 1970) (see also Keenan and Morré, 1970). thus making it an ideal source for new plasma membranes.

Since the vesicles in the present study appear to fuse with the cell membrane between the bases of adjacent microvilli, the newly added membrane would be inserted at the base of the microvilli, and since the label migrates to the tips of the microvilli, this would imply that the membrane also migrates to the tips of the microvilli, further implying a turnover of plasma membrane as well as of cell coat.

It may be a mistake, however, to think of the unit membrane and its cell coat as one static unit. A number of biochemical and histological studies have indicated that some cell types especially protozoan or tumor cells can form new plasma membrane quite rapidly (Gasic and Gasic, 1962; Nachmias, 1966; Kraemer, 1966; Warren and Glick, 1968; Marcus and Schwartz, 1968). However several of these studies are based on techniques such as observing the time taken to completely replace sialic acid residues

removed by neuraminidase treatment, and do not prove that the entire cell membrane is being reformed rather than just the glycoprotein surface coat. In addition, there are other studies which show a continuous loss of newly synthesized glycoprotein into the medium containing such cells (Molnar et al, 1965a; Kraemer, 1967) suggesting that only the surface coat may turnover. Finally, other recent studies indicate that the cell membrane may be more fluid than originally thought, and that components can diffuse quickly through it. Such evidence is provided by the fact that when cells containing different types of antigens are made to fuse, the different antigens rapidly become diffusely mixed throughout the plasma membrane covering the combined cells, as detected by florescence microscopy (Edidin, 1971). Similar findings have been found in the case of phospholipids (Peterson and Rubin, 1970) when fibroblasts in culture came in contact. Again, however, it is not possible to prove that all of the membrane components diffuse rather than just surface glycoprotein or phospholipids. In the case of the present study, however, these findings indicate it is not possible to assume turnover of apical cell membrane simply on the basis of migration of label from the base of the microvilli to their tips.

Furthermore, the villus intestinal epithelium may constitute a case where turnover of the labelled material is not occurring very rapidly for not only do the microvilli of the columnar cells increase in length rapidly at the base of the villi (where reaction in the present study was heaviest), but the levels of disaccharidases (Dahlqvist and Nordstrom, 1966) and alkaline phosphatase (Dahlqvist and

Nordstrom, 1966; Moog and Grey, 1967) increase from the base to the apex of the villi. Nonetheless, evidence has been shown for turnover of disaccharidases which is more rapid than could be accounted for by cell loss from the tips of villi (Das and Grey, 1970; James et al., 1971).

Possible Functions of Cell Surface Coat

Although the function of the surface coat is still not well understood, there is evidence that it may have a number of different roles with some of these having more importance in some cells than in others. Thus the cell coat may play a role in the processes of membrane permiability, binding of substances to cells and induction of pinocytosis, cell adhesion, cell-to-cell recognition, gamete recognition, cellular immunity, etc.(see review by Winzler, 1970a). In the small intestine, special functions may be assigned to the cell coat covering the microvillar surface, for as mentioned above, the cell coat at this site contains many digestive enzymes. Thus the brush border acts as a digestive surface where the final hydrolysis of carbohydrates and proteins occurs (see reviews by Greenberger, 1969; Dobbins, 1969; Crane, 1968, 1969; Rubin, 1970; Gardner et al., 1970). The surface coat at this site may also act as a barrier, for when dogs are exposed to prolongued shock and then volume repeated, the intestinal mucosa is abnormally permeable and the intestine is extremely susceptable to tryptic digestion. At this same time the PA-Schiff coat on the microvilli is lost (Bounous, et al., 1966). The fact that cells about to be extruded from villi tend to lose their surface coat (Ito, 1965), may indicate that it is the loss of the positive surface coat that leads to their extrusion (Forstner, 1969).

Migration of ^{3}H-fucose Label to Lysosomes

Morphology of Lysosomes in Duodenal Villus Columnar Cells

As described in the Results section, two main types of lysosomal bodies occurred in these cells: dense bodies which contained a uniformly dense matrix, and multivesicular bodies, which contained many internal vesicles. Most of the multivesicular bodies contained a matrix as dark as the dense bodies, but in some there was little or no matrix and these were referred to as light multivesicular bodies. A spectrum of morphological types was thus seen: at one end were light multivesicular bodies with little or no dense matrix but some internal vesicles; then came bodies with some dense matrix between their vesicles; then dark multivesicular bodies with considerable dense matrix and many internal vesicles; then bodies with mostly uniform dense matrix but containing a few vesicles; and finally at the other end were dense bodies with a uniformly dense matrix. A somewhat similar spectrum of lysosome types has been described by Hugon and Borgers (1968) in rat duodenal villus columnar cells. These authors showed that these bodies contained acid phosphatase and therefore could be considered lysosomes. In accord with the reported staining of lysosomes by the PA-Schiff technique (Davies, 1954; Novikoff, 1961; Koenig, 1962), the bodies in the present study were found to stain with phosphotungstic acid at low pH (Figs. 34-36). As mentioned in the Introduction, the glycoproteins present in these bodies probably represent the lysosomal hydrolases themselves (Koenig, 1969).

For the purpose of assessing the labelling of lysosomes in the present study, they were arbitrarily classified into three groups: dense bodies whose contents were uniformly dark, bodies which showed 1-3 vesicles

in section, and bodies showing four or more vesicles. Only the last ones were called multivesicular bodies.

Migration of Label to Lysosomes

Although at 2 minutes after ^{3}H-fucose injection, most of the label was localized to the Golgi apparatus and almost no grains occurred over lysosomes, by 5 minutes, a significant number of grains were found over dense bodies and bodies with 1-3 vesicles (Table IV). Over two-thirds of the labelled bodies were close to the Golgi apparatus at this early time. Between 10 and 35 minutes after injection labelling increased rapidly in all three types of bodies, although more slowly in multivesicular bodies than in the others. By 1 and 4 hours after injection labelling was heavy over all threetypes of bodies (Table IV, Figs. 26, 27, 29, 31, 35, 36). These results were interpreted to indicate that labelled glycoprotein, whose synthesis was completed in the Golgi apparatus, migrated to the lysosomes.

The results of this study are in accord with the observations of some other investigatiors. Thus Cohn, Fedorko and Hirsch (1966) administered ^{3}H-leucine to mononuclear phagocytes, and using electron microscopic radioautography, demonstrated the passage of labelled protein from the endoplasmic reticulum through the Golgi apparatus to lysosomes. Similar findings were obtained in myelocytes by Fedorko and Hirsch (1966), and label was found to be incorporated into hepatocyte lysosomes six hours after injection of ^{3}H-leucine into rats by Droz (1966). Incorporation of labelled sugars into lysosomal bodies has also been occasionally reported, as in HeLa cells after ^{3}H-glucosamine administration (Reith, Oftebro and Seljelid, 1970), in lymphocytes stimulated by phytohemagglutinin after ^{3}H-galactose administration (Anteunis et al., 1969), and in hepato-

cytes and spinal ganglion cells after ^{3}H-galactose administration (Rambourg and Droz, 1969).

In the present study, the fact that a number of lysosomes were labelled at 10 minutes after ^{3}H-fucose injection (Figs. 19 and 20; Table IV), ie. before the cell surfaces became heavily labelled, indicates that the lysosomes receive their newly-completed glycoprotein directly from the Golgi apparatus rather than indirectly from the cell surface. This concept is supported by the fact that over two-thirds of the lysosomes labelled at the 5 and 10 minute time intervals were located close to the Golgi apparatus.

It has been suggested that in many cell types multivesicular bodies evolve into dense bodies (Gordon, Miller and Bensch, 1965; Smith and Farquhar, 1966; Hugon and Borgers, 1968). In the present study, however, dense bodies were labelled more quickly than multivesicular bodies (Table IV), and they also lost their label sooner, being only about 8% labelled at 30 hours after injection while multivesicular bodies remained over 30% labelled. While these results do not indicate that the above transition does not exist in these cells, they suggest that the dense bodies did not receive their newly synthesized glycoprotein from multivesicular bodies. On the contrary, it is possible that the multivesicular bodies received some of their label from labelled dense bodies, or by a transformation of dense bodies into multivesicular bodies. The fact that the bodies with 1-3 vesicles in this study exhibit a labelling pattern intermediate between that of dense and multivesicular bodies, could suggest that these bodies are a transition stage in the evolution of one type of body to the other.

It is equally possible, however, that the three types of bodies each receive their label independently from the Golgi apparatus, but at varying rates. Similarly, differences in the rate of turnover between dense bodies and multivesicular bodies could explain the differences in labelling at the 30 hour time interval (Table IV).

The mechanism of transfer of labelled glycoprotein from the Golgi apparatus to lysosomes in these cells is not yet clarified. However, several possibilities may be considered. One possibility could be the direct transformation of Golgi saccules into lysosomes, as reported in thyroid follicular cells (Seljelid, 1967), kidney adenoma cells (Seljelid, 1966), intestinal crypt columnar cells (Moe, Rostgaard and Behnke, 1965) and promyelocytes (Bainton and Farquhar, 1966; Wetzel, Horn and Spicer; 1967). Dense bodies were often seen close to saccules of Golgi stacks in the present study (ie. Fig. 7), and dense bodies in this location were more quickly labelled (Fig. 18) than those bodies further away from the Golgi apparatus. Morphological evidence of transitional stages between Golgi saccules and dense bodies was not evident in routinely stained sections, however, although in sections stained with phosphotungstic acid, a transition was sometimes suggested (Fig. 34, left).

A second possibility is the transport of labelled glycoprotein from the Golgi apparatus to lysosomes via small Golgi vesicles (Novikoff, Essner and Quintana, 1964; Gordon, Miller and Bensch, 1965; Cohn, Fedorko and Hirsch, 1966; Friend and Farquhar, 1967), as indicated by observations suggesting fusion of Golgi vesicles with these bodies (Gordon, Miller and Bensch, 1965; Cohn, Fedorko, and Hirsch, 1966; Hirsch, Fedorko and Cohn, 1968) as well as by the presence of acidphosphatase in all these structures (Novikoff, Essner and Quintana, 1964; Smith and Farquhar, 1966). Small

vesicles were sometimes seen near dense and multivesicular bodies in the present study, although evidence of fusion was rarely observed.

A third possibility is derived from the finding of Golgi related tubules in negatively stained Golgi fractions (Ovtract, Morré and Merlin, 1969), or in thick sections of glycol embedded material stained for glycoproteins with phosphotungstic acid at low pH (Rambourg, 1969). The occasional presence of tails on lysosomes which was observed in this study (ie. Fig. 19, lower left), and by others (Novikoff, Essner and Quintana, 1964; Hugon and Borgers, 1968; Holtzman, 1969) suggests that, at least in the early stages of their formation, lysosomal bodies may be connected to Golgi tubules and that labelled material could be transported to them via these tubules from the Golgi apparatus.

The above possible pathways are not mutually exclusive and labelled glycoprotein could migrate from the Golgi apparatus to the lysosmes by more than one pathway.

In summary, the radioautographic results obtained in duodenal columnar cells indicated that the completion of synthesis of glycoproteins in these cells occurs in the Golgi apparatus. From here some label appears to migrate to the cell surfaces by means of small cytoplasmic vesicles where it is added to the cell coat. Other labelled glycoprotein migrates to lysosomes, although the mode of its transport has not yet been clarified. It is interesting to note here the hypothesis of Eylar (1965), that the presence of carbohydrate on a secretory protein acts as a chemical label which promotes its transport into the extracellular environment. Yet in the present study, glycoprotein was taken up into lysosomes and did not appear to leave the cell interior. In this context, the vacuome concept of DeDuve (1969) is of interest since according to this concept the lysosomes could be considered as part of the

external environment of the cells which has become internalized.

Interpretation of Results Obtained in Hepatocytes

Site of Incorporation of ^{3}H-fucose Label

At two minutes after ^{3}H-fucose injection, over 80% of the silver grains over hepatocytes were localized to the Golgi apparatus (Table VI) (Fig. 40). Thus, as in the case of duodenal villus columnar cells, it can be interpreted that the Golgi apparatus is the site of completion of synthesis of glycoproteins in these cells. ^{3}H-galactose label is also initially taken up in the Golgi apparatus of these cells as shown in earlier light microscopic radioautographic experiments (Bennett, 1967) and in the electron microscope by Droz (1966).

Distribution of ^{3}H-fucose Label at Later Time Intervals

At 35 minutes after ^{3}H-fucose injection, approximately 60% of the silver grains remained over the Golgi apparatus in hepatocytes (Table VI). However, as seen in figure 41, many of these grains now occurred over Golgi vacuoles which contained electron dense granules. These granules were interpreted to be albumen by Bruni and Porter (1965) who showed morphological evidence that this material migrated within Golgi vacuoles to the space of Disse where it was released. More recent evidence indicates that these granules are lipoprotein (Ashworth et al., 1966) and are probably the precursors of very low density lipoprotein (VLDL) in the blood (Mahley, Hamilton and Lequire, 1969; Claude, 1970). Recent studies however, in which labelled amino acids have been traced in hepatocytes using electron microscopic radioautography (Glaumann and Ericsson, 1970) or biochemical fractionation techniques (in which purified Golgi fractions were used) (Glaumann and Ericsson, 1970; Peters,

Fleicher and Fleicher, 1971), show that albumen is synthesized on the rough endoplasmic ribosomes of these cells and is then transported through the Golgi apparatus to the sinusoidal cell surface and the space of Disse. The role of the Golgi apparatus, in the case of albumen transport appears to be a rather passive one since no carbohydrate appears to be added to migrating albumen (Peters, Fleicher and Fleicher, 1971). Thus the labelling in the present study would not represent uptake into albumen but could represent addition of ^{3}H-fucose residues into other plasma proteins, since most of these are glycoproteins (Text-Table I). On the other hand, since considerable label remains at the sinusoidal cell surface (Fig. 39 and 44) and at lateral cell surfaces (Fig. 43) at 4 hours after infection (Table VI) and also at 30 hours, the material being labelled in the Golgi apparatus at early time intervals is likely glycoprotein destined to be added to the cell coat.

Considerable label also appears in lysosomes at later time intervals, so that 24 % of the bodies are labelled by 35 minutes, and 42% by 4 hours (Table VII). Most of the lysosomes in these cells are of the dense body type similar to those shown to contain acid phosphatase by Novikoff, (1961). However, some are multivesicular bodies and these are also labelled (Fig. 43). No support was found for the idea that microbodies are the precursors of multivesicular bodies (Bruni and Porter, 1965) since microbodies did not show significant uptake of label at any time interval. Rather, the results of the present study indicate that as in duodenal villus columnar cells, the lysosomes derive their newly completed glycoprotein directly from the Golgi apparatus.

Interpretation of Results Obtained From Light Microscopic Radioautographs Of Other Tissues

As described in the Results section, ^{3}H-fucose label was incorporated by a great variety of cell types in the rat. In practically every cell type in which a substantial radioautographic reaction occurred, the label was localized at early time intervals to one part of the cytoplasm. In many cell types this reactive region could be recognized as the Golgi region by virtue of its location, ie. the supra nuclear region in polarized columnar cells. In other cell types, the reactive region was in an area often occupied by the Golgi apparatus such as the paranuclear areas in stratified epithelial cells (Fig. 57). Thus in practically all reactive cells the evidence suggested that the ^{3}H-fucose label was incorporated in the Golgi apparatus indicating that glycoprotein synthesis was completed in this organelle. In different cell types the reaction changed in different ways at later time intervals. Two basic patterns could be discerned however.

1) Migration of ^{3}H-fucose to the Surfaces of Cells

In many cell types the pattern of migration resembled that observed in duodenal villus columnar cells in the light microscope, with the label localized to the Golgi region at early time intervals, but localized over the cell surfaces at later time intervals. The pattern of labelling at later time intervals was not identical in all of these cell types. Thus in duodenal villus columnar cells (Fig. 11 and 12), choroid plexus cells (Fig. 50), and stratified epithelial cells (Fig. 58 and 59). reaction appeared to occur over the whole surface of the cells at later time intervals while in other cells such as colonic columnar cells (Fig. 46), submaxillary gland duct cells (Fig. 48), and perhaps kidney proximal convoluted tubule cells (Fig. 54), reaction

appeared to occur over the apical cell surface only. Since the studies of Rambourg, Neutra and Leblond (1966) and Rambourg and Leblond (1967) have shown the presence of glycoprotein material along all surfaces of most cell types in the rat, the above result indicates that in some cell types surface glycoproteins at certain cell surfaces take up label to a greater extent than other cell surfaces.

While in certain cell types, such as colonic columnar cells, it is possible to be fairly certain that label along a particular surface membrane represents cell coat material, it should be mentioned that this was not always the case. In endothelial cells, for example, it is not possible, due to the thinness of the cell along most of its length, to tell if only one surface is labelled (Fig. 56); where nuclei occur, however (oblique arrows), the reaction occurs along the luminal side of the nucleus suggesting that the label resides along the luminal cell surface. This would agree with the findings of Rambourg and Droz (unpublished) that ^{3}H-galactose label was localized along the luminal surface of blood capillary endothelial cells (and pancreatic acinar cells).

A second problem of interpretation occurs in cells with some known secretory function. Thus the apical surface reaction seen over kidney proximal convoluted tubule cells (Fig, 54) could represent label in the apical surface coat, but could also represent secreted urinary glycoprotein (Keutel, 1965).

Finally, a third problem was the presence of heavy reaction over extracellular material such as collagen or connective tissue ground substance at late time intervals after ^{3}H-fucose injection (Fig. 59).

This heavy reaction permeated between cells such as the fibers of striated muscle at the bottom of figure 59, making it very difficult to discern whether the radioautographic reaction at the surface of these fibers represented a cell coat reaction, or a reaction over strands of connective tissue between the cells.

Within the limitations of interpretation imposed by the light microscopic technique, however, the present results suggest that many different cell types incorporate ^{3}H-fucose into glycoproteins in their Golgi complexes and this labelled glycoprotein then migrates to the cell surface to be added to the cell coat. Indeed, when very long exposure times are examined, almost all cells in the body give some indication of exhibiting a cell surface reaction. Thus the above pattern of reaction may represent a common mechanism whereby all cells renew their cell coats. The fact that many of these cell surface reactions remained heavy at 30 hours after injection suggests that the cell coat material may not turnover very rapidly in many cell types.

2) Migration of ^{3}H-fucose into Cell Secretions

As described in the results, ^{3}H-fucose label in the present study was also taken up into mucous secretions, serous secretions, and the secretions of connective tissue cells into ground substances. Little discussion of these reactions is necessary for the purposes of the present investigation except to mention that glycoproteins are known to occur in most of these secretions (see Text-Table II).

Comparison of Overall Distribution of ^{3}H-fucose Label in Tissues with That of ^{3}H-galactose.

In terms of its overall distribution in rat tissues, as detected

by light microscopic radioautography, ^{3}H-fucose label bore a fairly close resemblance to ^{3}H-galactose label. In certain features the uptake differed from that of ^{3}H-galactose, however. For example, ^{3}H-fucose label was never taken up into glycogen. On the other hand, the intense reaction seen over connective tissue at long time intervals after ^{3}H-fucose reaction, was not observed after ^{3}H-galactose. Certain individual cells such as pancreatic centroacinar cells heavily incorporated ^{3}H-galactose label but not ^{3}H-fucose label, and such differences in individual cellular uptake occurred in several other organs.

In addition, the localization of reactions to the Golgi region of cells tended to be more precise after ^{3}H-fucose injection than after ^{3}H-galactose reaction. Similarly in cells which exhibited cell surface reactions, the localization of label to the cell surfaces was more precise after ^{3}H-fucose than after ^{3}H-galactose.

Thus, as seen with each of the previous labelled sugars studied, ^{3}H-fucose has its own specific pattern of cellular uptake in tissues reflecting the ability of various cells to absorb this sugar, the distribution of fucosyltransferases, and the content of fucose residues in the various substances being secreted by the different cells.

SUMMARY AND CONCLUSIONS

The present investigation began as the result of some evidence obtained during the course of preliminary light microscopic radioautographic studies on the distribution of ^{3}H-galactose label in rat tissues. This evidence showed that label was taken up in the Golgi region of intestinal columnar cells at early time intervals after ^{3}H-galactose injection into rats, but at later times, the label appeared at the apical surface of the cells. Since the surface was the site of a prominent microvillus cell coat of glycoprotein nature, the results suggested that this surface coat might be synthesized in the Golgi apparatus of the cells.

It was decided to investigate this phenomenon using electron microscopic radioautography after ^{3}H-galactose injection into young rats. The results obtained from this study showed that in duodenal villus columnar cells the ^{3}H-galactose label was taken up in the Golgi apparatus itself at early times, and later migrated to the apical cell surface. Furthermore, a method of transport for this labelled material was indicated in the form of small cytoplasmic vesicles which appeared to arise from Golgi vacuoles and migrate to the apical surface membrane. Thus it was interpreted that ^{3}H-galactose was incorporated into the carbohydrate side chains of glycoproteins which migrated to the apical cell surface to be added to the cell coat.

At this time, a new isotope label ^{3}H-fucose became available. Since this sugar did not convert to other sugars or break down, it was considered to be a better precursor for the carbohydrate moiety of glycoproteins. Therefore radioautographic experiments were carried out at the light and electron microscopic levels with this labelled sugar in

rat tissues. These studies produced the following results:

In duodenal villus columnar cells the ^{3}H-fucose label was incorporated, at early time intervals after injection, into glycoproteins in the Golgi apparatus. Since fucose occurs as a terminal residue, on the carbohydrate side chains of glycoproteins it could be concluded that the Golgi apparatus is the site of completion of synthesis of glycoproteins or at least some of their side chains in this cell type.

At later time intervals after ^{3}H-fucose injection, label appeared over not only the apical surface of these cells but also over their lateral and basal surfaces. These results indicated that labelled glycoprotein migrated from the Golgi apparatus to all of the cell surfaces and suggested that the carbohydrate material along the lateral cell surfaces might not be as different from that on the apical surface as previously thought. As in the radioautographic studies after ^{3}H-galactose injection, some label was found to be associated with cytoplasmic vesicles. Quantitative analysis of this association of label with vesicles strongly suggested that these vesicles were the means of transport of label to the cell surfaces.

Label also appeared in lysosomal bodies at later time intervals. This finding suggested that lysosomes receive their newly labelled glycoproteins, perhaps representing the lysosomal hydrolases themselves, from the Golgi apparatus, although the mechanism of transport was not clarified.

A technique was successfully devised in which $\frac{1}{2}$ μ thick sections of glycol methacrylate embedded duodenal tissue could be subjected to electron microscopic radioautography and then poststained specifically

for glycoproteins using phosphotungstic acid. When radioautographs from animals sacrificed at different time intervals after ^{3}H-fucose injection were examined, the results showed that the label was almost completely localized to sites specifically stained for glycoproteins.

In liver hepatocytes, the distribution of ^{3}H-fucose label was also investigated by electron microscopic radioautography at various times after injection. The results obtained again indicated that the synthesis of glycoprotein material was completed in the Golgi apparatus, and that some of the labelled glycoprotein migrated to the cell surfaces perhaps to be added to the cell coat while other labelled glycoprotein migrated to the cell lysosomes.

Finally, light microscopic radioautographic investigations carried out to investigate the distribution of ^{3}H-fucose label in a variety of rat tissues, showed that the ^{3}H-fucose label after incorporation into the Golgi region of cells at early time intervals had two main fates: migration to cell secretions (mucous, serous or ground substances), or migration to the surface of cells. This latter phenomenon occurred in a great variety of cell types and indicates that the pathway of synthesis of cell coat material observed in the electron microscope in duodenal villus columnar cells and in hepatocytes may represent a common pathway by which all cells manufacture their cell coat.

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Fig. 1: Paraffin section of duodenal mucosa. Stained with hematoxylin and eosin. 210 X

In the upper two thirds of the figure the duodenal epithelium is projected into the intestinal lumen as four villi, each with a central core of lamina propria. Below the villi the epithelium is invaginated to form crypts. The lumen of one crypt (vertical arrow) is seen to open into the space between the two central villi, and it can be seen that the epithelium lining this crypt is continuous with that covering the villi. The villus epithelium consists mainly of columnar absorptive cells interspersed with occasional goblet cells. The mucous thecae of the goblet cells are unstained (horizontal arrows). The villus columnar cells have heavily stained cytoplasm except for an area above the cell nucleus which contains the Golgi apparatus; this area presents a negative Golgi image (oblique arrow).

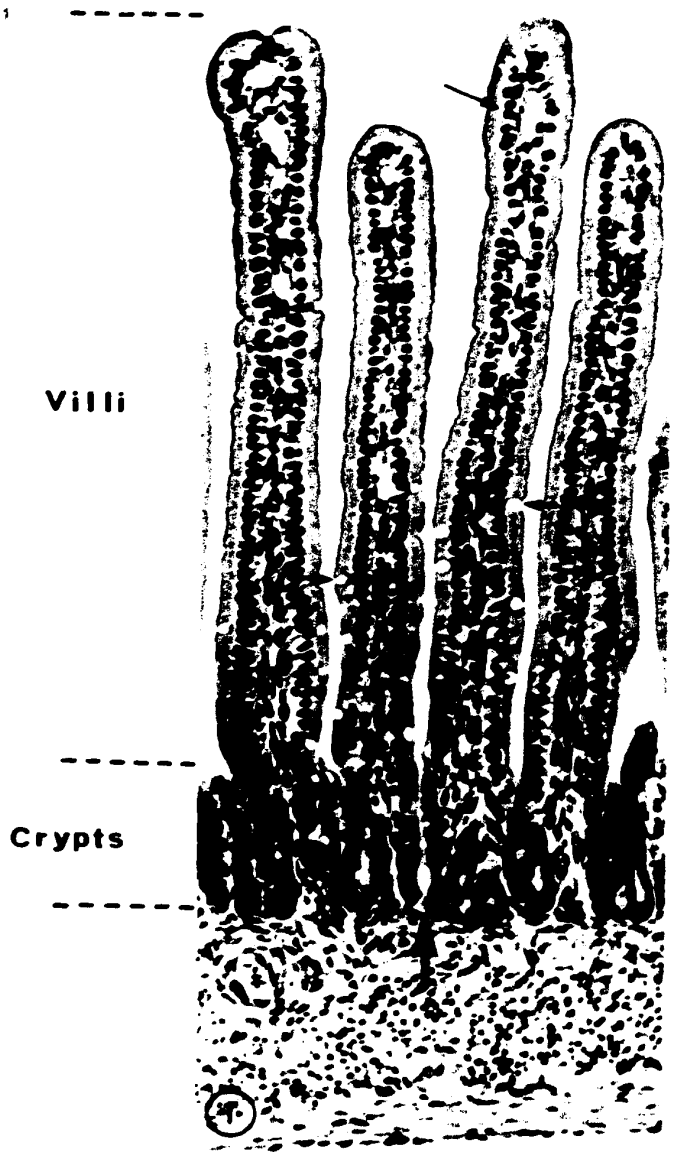

Villi
Crypts

Fig. 2: Paraffin section of part of a duodenal villus cut longitudinally. Stained with PA-Schiff and hematoxylin. 760 X

The layer of epithelial cells covering the inner lamina propria core (LP) of the villus consists mainly of columnar absorptive cells interspersed with occasional goblet cells. Between the bases of the columnar cells, near the lamina propria core, the nuclei of some lymphoid cells are evident. The mucus contents of the goblet cells stain intensely with the PA-Schiff stain as does the apical striated border of the columnar cells. The lateral surface membranes of the columnar cells are also definitely stained, but more lightly (vertical arrow). The columnar cell cytoplasm is mostly unstained, but patches of stain sometimes occur above the nucleus and represent elements of the Golgi apparatus (horizontal arrow).

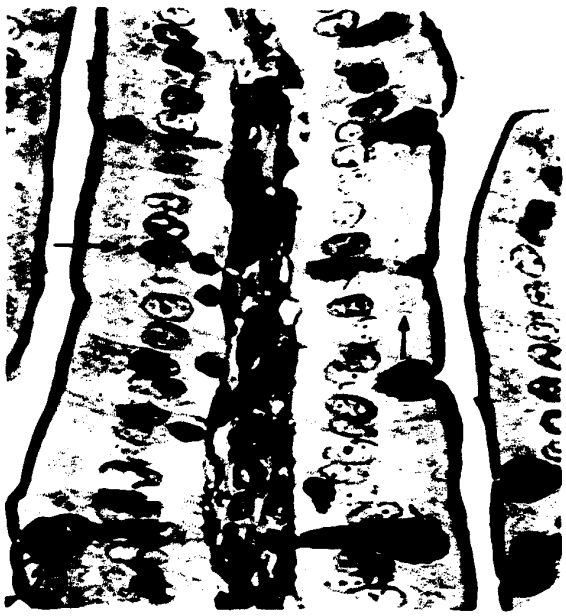


Fig. 3: Electron micrograph of the apical half of duodenal villus columnar cells cut in slightly oblique section. Stained with uranium and lead. 10,000 X.

In the electron microscope, the apical striated border observed in light micrographs of columnar cells is seen to be made up of individual microvilli (mv). Just beneath this microvillus border is an area of cytoplasm occupied by horizontally running filaments of the terminal cell web (TW). The cytoplasm of the terminal web region does not contain mitochondria or cisternae of rough endoplasmic reticulum, but contains elements of smooth endoplasmic reticulum, some smooth surfaced cytoplasmic vesicles, and occasional lysosomes such as the multivesicular body (MVB) at the upper left of the figure. Lysosomes are also encountered in other regions of the cytoplasm, as shown by the presence of small dense bodies (D) above the nucleus (N). The Golgi apparatus in these cells consists of one or more stacks of flattened Golgi saccules (G) located in the cytoplasm above the nucleus. The lateral cell membranes (lmb) of these cells are often highly convoluted.


mv
G
G

Figs. 4–8: Electron micrographs of duodenal villus columnar cells. Stained with uranium and lead.

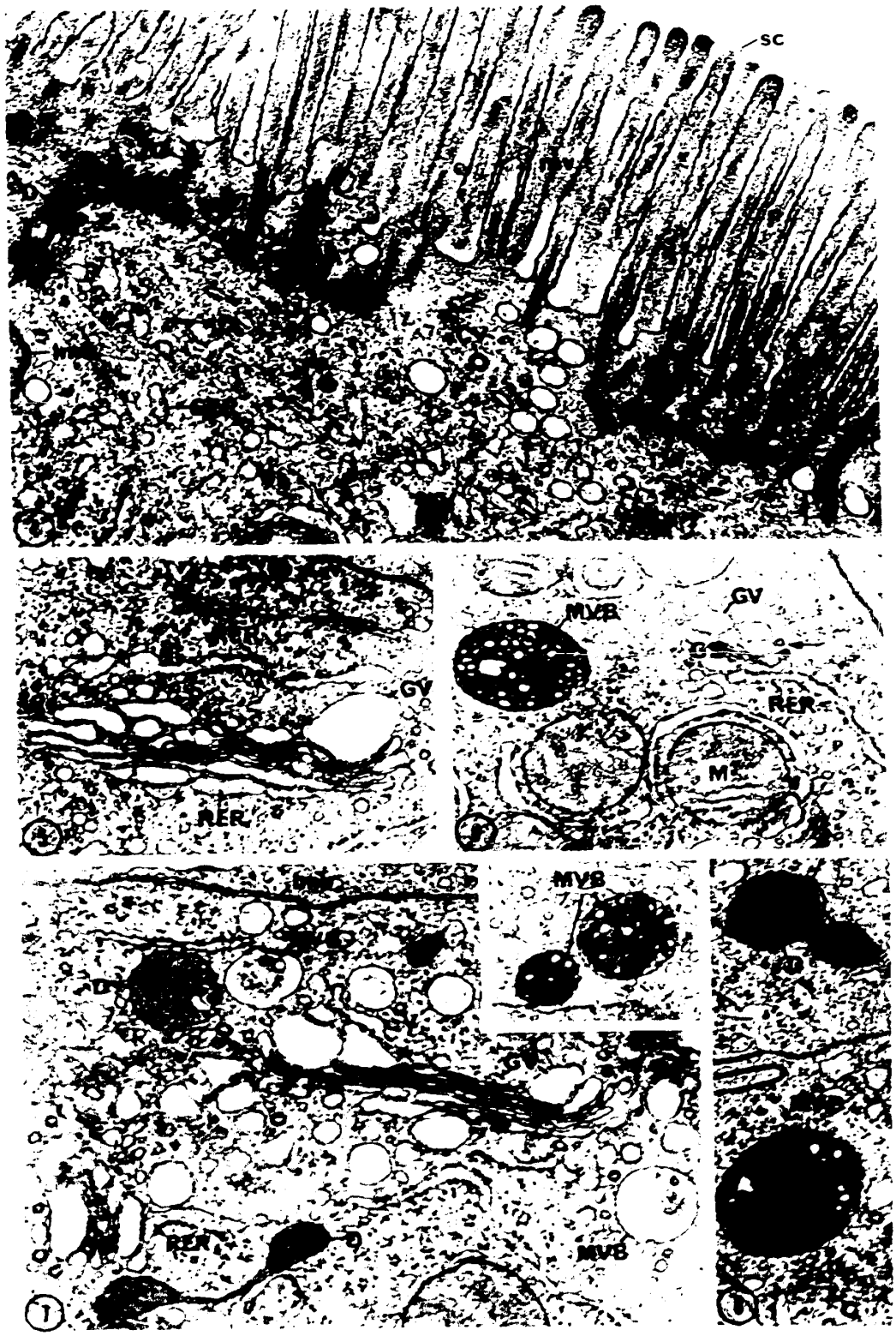
Fig. 4: The apical cytoplasm of columnar cells bordered by a row of microvilli (mv). Attached to the outer leaflet of the plasma membrane of the microvilli is a distinct surface coat (SC) which is thicker over the tips of the microvilli than over their lateral surfaces. Within the microvilli are cores of central filaments (F). These pass downwards to join the horizontally running filaments of the terminal web (TW) which in turn converge on the zonula adhaerens (ZA) of a junctional complex at the left of the figure. In the terminal web region of the cytoplasm, is a number of smooth surfaced cytoplasmic vesicles averaging about 140 mμ in diameter (vertical arrows). The membrane of these vesicles is similar in thickness to the apical cell membrane, and at some sites (horizontal arrow) there are indications that these vesicles may fuse with or derive from the apical surface membrane. The vesicles contain filamentous material similar in appearance to the surface coat. Also included in the terminal web cytoplasm are vesicles and tubules of smooth endoplasmic reticulum (SER). The membrane of these elements appears thinner and more irregular in outline than that of the above mentioned cytoplasmic vesicles, and the contents of their lumens are more electron dense. (ZO: zonula occludens; lmb: lateral cell membrane; CV: coated vesicle). 33,000 X

Fig. 5: Golgi region of cytoplasm. Running horizontally across the figure is a stack of Golgi saccules (GS); this stack is polarized with its immature face directed towards the bottom of the figure and its mature face towards the top. The saccules midway between the two faces of the stack are very flattened in their central portion, and contain dense material in their lumens. At their ends these saccules are expanded and their luminal contents are electron lucent. At the mature face of the Golgi stack the saccules are even more enlarged at their ends, forming Golgi vacuoles (GV). Along the immature face of the Golgi stack, a cisterna of rough endoplasmic reticulum (RER) is located closely parallel to the most immature Golgi saccule, separated from it by a distance equal to that between adjacent Golgi saccules. Some ribosomes can be seen along the surface of the cisterna away from the Golgi stack. The luminal contents of this cisterna are similar in electron density to those seen in other cisternae of rough endoplasmic reticulum (RER) in the surrounding cytoplasm. Near the top of the figure is a fairly large coated vesicle (CV), while other smaller vesicles occur near the Golgi saccules. 28,000 X

Fig. 6: An area of cytoplasm containing a large dark multivesicular body (MVB), two mitochondria (M), and a Golgi stack (GS) sectioned through its peripheral region. Along the immature face of the Golgi stack, a cisterna of rough endoplasmic reticulum is seen (RER), which leads away from the Golgi apparatus where it becomes covered by ribosomes on both of its surfaces (right side of figure). In the Golgi stack itself, small vesicles appear to arise from the ends of the central saccules (horizontal arrow). In the lower part of this figure, a cisterna of rough endoplasmic reticulum is wrapped around the two mitochondria. The surface of the cisterna near to the mitochondria is studded with ribosomes while the other surface has almost none. The dark multivesicular body shown here contains a uniformly dense matrix in which are several internal vesicles; most of these have light interiors, but some are filled with material as dense as the matrix itself. (GV: Golgi vacuoles). 28,000 X

Fig. 7: Golgi region of columnar cell. A horizontally orientated Golgi stack (GS), cut through its central region, is seen in the middle of the figure while a vertically orientated stack, cut through its peripheral region, is seen at the left. A cisterna of rough endoplasmic reticulum (RER) occurs along the immature face of the left hand Golgi stack. Near to the Golgi stack is a number of cytoplasmic vesicles (V). These are similar in size and appearance to the cytoplasmic vesicles seen in the apical cytoplasm in figure 4. Somewhat larger vesicular structures contain small internal vesicles and therefore are classified as multivesicular bodies (MVB); these are of the light type since they do not contain a uniformly dense matrix. In the inset of this figure, three multivesicular bodies are seen (MVB). the smallest body at the upper left is a light multivesicular body; containing only a small amount of moderately electron dense matrix between its few vesicles. The large dark multivesicular body at the right contains a fairly dense matrix throughout. The contents of some of its internal vesicles are light while others are dense. Finally, the multivesicular body at the lower left , has a very dense matrix throughout. In the cytoplasm of themain figure are two dense bodies (D), both with a uniform matrix of moderate electron density. In the right hand portion of the upper dense body one vesicle is seen having a very dense interior. Beside this vesicle, the limiting membrane of the dense body appears to be invaginated. The lower dense body consists of two enlarged vesicular portions connected by a long narrow stock. (lmb: lateral cell membrane). 33,000 X
inset 28,000 X

Fig. 8: Cytoplasm of two columnar cells. In the upper cell are two dense bodies (D). These contain a fairly uniform dense matrix; however some granular material is present near their periphery, and a vesicular profile is seen in the right hand portion of the larger dense body. In the lower cell is a larger dark multivesicular body (MVB). This body has as its predominant content a uniformly dense matrix, but it also contains a few internal vesicles. Some of these have light interiors, but others have contents as dense as the matrix itself and are difficult to see. 33,000 X


SC
MVB
GV
RER
MT
MVB
RER
MVB
MVB

Figs. 9-14: Light microscopic radioautographs of epon sections of duodenal tissue after ^{3}H-fucose injection. Stained with toluidine blue.

Fig. 9: Section of duodenal mucosa; 2 minutes after ^{3}H-fucose injection. Exposed 8 weeks.

The base of one duodenal villus and parts of the bases of adjacent villi are seen in this figure along with the underlying duodenal crypts. A strong radioautographic reaction occurs over the villus columnar cells, in which most of the silver grains are concentrated over the supranuclear Golgi region (nucleus: N). A fairly strong radioautographic reaction is also seen over the supranuclear region of villus goblet cells (oblique arrows), while a lighter reaction occurs over goblet cells in the crypts (horizontal arrow). Only a very light radioautographic reaction occurs over the crypt columnar cells, yet here too, a few silver grains can often be seen over the supranuclear region of the cells (vertical arrow). 400 X

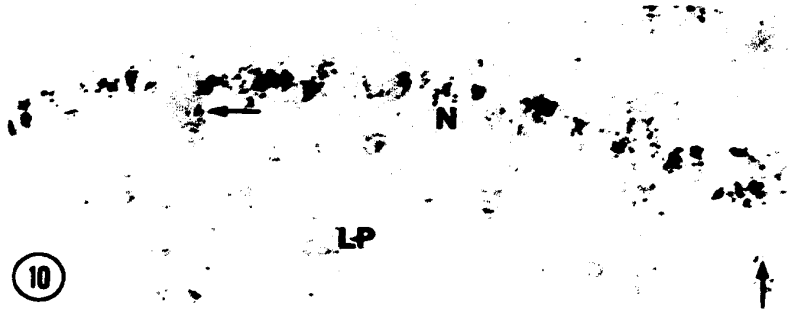
Fig. 10: Part of the basal third of a duodenal villus; 5 minutes after ^{3}H-fucose injection. Exposed 6 weeks.

A row of villus columnar and goblet cells extends horizontally across the figure, bordered above by part of the intestinal lumen, and below by the lamina propria core of the villus. A strong radioautographic reaction is seen over the supranuclear region of the villus columnar cells (nucleus: N) and also over goblet cells (horizontal arrow). In both cases very few silver grains occur elsewhere over the cells. Reaction over the underlying lamina propria (LP) is light at this time interval, but small clusters of silver grains occur over the paranuclear cytoplasm of occasional cells (vertical arrow). 900 X

Fig. 11: Part of the basal third of a duodenal villus; 1 hour after ^{3}H-fucose injection. Exposed 3 weeks.

The radioautographic reaction over the villus columnar cells is heavier than that seen at the earlier time intervals, and the distribution of silver grains has changed greatly. Now heavy reaction occurs not only over the supranuclear Golgi region of the cells (nucleus: N) but also over their apical striated border and along their lateral cell surfaces. This latter reaction is especially well seen in the region of the basal half of the columnar cells (horizontal arrow). 1000 X

9



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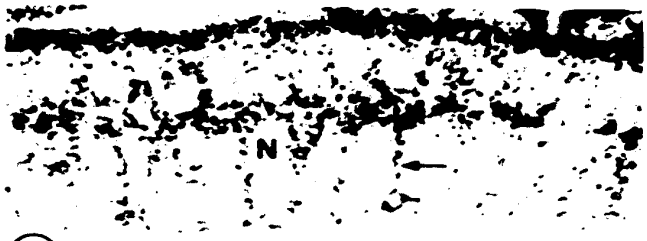

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