# ORGAN CULTURE OF THE HUMAN BRONCHIAL SUBMUCOSAL GLAND: AN ELECTRON MICROSCOPIC STUDY OF THE UPTAKE OF RADIOACTIVE GLUCOSE AND THREONINE

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## ABSTRACT

Disturbance of the secretion of the bronchial submucosal gland is an important feature of many lung diseases. Organ culture studies combined with light microscopic autoradiography have recently added to our understanding of the glands.

In the present study, using organ culture and two recently described quantitative techniques for electron microscopic autoradiography, it has been possible to show, for the first time, the progression of $H^3$-threonine and $H^3$-glucose through organelles of both the mucous and serous cell into the macromolecular glycoprotein component of the secretion.

Scintillation counting and examination of light microscopic autoradiographs after incubation at $4^{\circ}C$, showed that optimal fixation of the gland, after incubation in either $H^3$-threonine or $H^3$-glucose, was achieved by fixation for 18 hours in formalin.

Chemical identification, after precipitation of protein and glycoprotein demonstrated that puromycin inhibited the incorporation of $H^3$-threonine

into the protein and as a result of this $H^3$-glucose uptake was also inhibited.

While the pattern of incorporation for the mucous and serous cells was similar - $H^3$-threonine was incorporated into the protein core in the endoplasmic reticulum and metabolites of $H^3$-glucose were added to a side chain of the protein core in the Golgi apparatus. Major differences in the degree of uptake of both radioactive precursors by the mucous and serous cell underlines a fundamental difference between them.

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# CHAPTER I

## HISTORICAL INTRODUCTION

In man, the mucus secreting cells of the tracheobronchial tree are located at two sites; the goblet cells in the surface epithelium and the mucous and serous cells in the submucosal glands. The latter site is probably the more important of the two as it is estimated to represent 40 times the volume of the surface epithelial goblet cells (Tappan and Zalan, 1963; Reid, 1964).

In chronic bronchitis, there is a hypersecretion of mucus from both the submucosal glands and the goblet cells of the airways, while the increased susceptibility to infection in cystic fibrosis has been attributed to alterations in the mucous secretions of the tracheobronchial tree. Thus, a study of the production of mucous secretion by the mucous and serous cells of the submucosal glands is relevant to bronchial hypersecretory states.

This thesis attempts to trace, using electron microscopic autoradiography, the incorporation of an amino acid (tritiated threonine) and a sugar (tritiated glucose) into the secretion formed in the mucous and serous cells of the human bronchial glands. In tracing the incorporation of $H^3$-threonine and $H^3$-glucose into these cells, particular emphasis has been placed on the organelles generally associated with the production of secretory products; the endoplasmic reticulum, the Golgi apparatus and the secretory granules.

Autoradiography is a technique employed to demonstrate the position of radioactive metabolites in an organ or cell by reducing the silver halides in a photographic emulsion which has been superimposed over the tissue section. The passage of the charged ion through the silver halide causes the formation of a latent image, which is later made permanent by the process of development.

The historical introduction is concerned with 5 main topics:

Anatomy of the bronchial submucosal glands
Previous studies of the uptake of radioactive precursors by the human bronchial submucosal glands
Radioactive uptake by mucous and serous cells at other sites
High resolution autoradiography
Quantification of electron microscopic autoradiographs.

## ANATOMY OF THE BRONCHIAL SUBMUCOSAL GLANDS

### Position and Distribution

As long ago as 1602, Laurentius described the glands in the respiratory tract of Man. In 1838, Rokitansky found mucous glands in the posterior wall of the trachea and main

bronchus, and later Kolliker (1853) described them in the larynx and trachea.

Bonne (1901), stated that the glands were found only in the walls of the airways containing cartilage. Policard and Galy (1945) found that the amount of gland present in the airways was proportional to the airway calibre and that in Man, airways less than 1 mm in diameter, do not contain gland.

The distribution of the glands has also been studied by bronchography (Duprez and Mampuys, 1953) and the majority of gland ducts were found in the proximal wall.

More recent work has supported these findings (de Haller, 1969; Meyrick, Sturgess and Reid, 1969), and it is now accepted that the bronchial submucosal glands are found in the airways that contain cartilage in their wall and they may extend as far as the last plate of cartilage. The glands are situated mainly internal to the epithelium and between the cartilage plates but sometimes they do extend external to the cartilage. Many glands are also situated in the muscular posterior wall of the trachea and main bronchus.

In the normal trachea, Thurlbeck, Benjamin and Reid (1961) estimated that there is one gland per

square millimetre of mucosal surface, that is, 6,000 glands are present, which is one sixth of the number in the whole bronchial tree.

Structure

One of the first descriptions of the human bronchial glands was by Rokitansky (1838); he was able to comment that in 'catarrhe des bronches' gross dilatations of these glands occurred.

The glands were reported to be epithelial in origin by Bowman (1847) being formed by involutions of the surface epithelium. He described the gland as a 'complicated structure, arranged in a membranous form, consisting of a closely packed mass of secretory tubules'. The secretory portion of the gland - the terminal vesicles - was surrounded by a basement membrane, which was continuous through the duct region with the mucous membrane.

Kolliker (1853) described the glands as racemose, 'in which, round and elongated glandular vesicles are situated on the ultimate ends of the secretory ducts'.

One of the first descriptions of the detailed microscopic structure of the bronchial glands was in

1898 by Fuchs-Wolfring. In her studies of the dog and rabbit, she found the glands to be comprised of two cell types - mucous, and albuminous or serous cells. The serous cells were found as clusters or demilunes of Giannuzzi and, in the dog, thought to secrete into the mucous tubule lumen by capillaries between the mucous cells, while in the rabbit, direct into a lumen.

The presence of both mucous and serous cells in the tracheal glands of the cat as well as the dog was reported by Watjen (1921). Schaeffer (1920) was the first to describe these two cell types in the human respiratory tract. Later, de Haller and Reid (1965) described the acini according to their morphological appearance, 'distended' (mucous) and 'non-distended' (serous) acini.

In 1945, Policard and Galy had stated that the gland tubules could be divided into three groups according to their cell type:

1 mucous

2 serous

3 mainly mucous with serous demilunes.

They described the mucous and serous cells as distinct cell types, the gland being predominantly mucous. Lamb (1969) however, reported the gland to be

composed of equal proportions, as judged by point-counting of area, of mucous and serous cells.

Meyrick, Sturgess and Reid (1969), using serial sections from a block of normal bronchus which had been cut parallel to the surface epithelium, reconstructed a human bronchial submucosal gland by superimposing tracings obtained with a modified Edinger apparatus. They found that mucous and serous cells comprised their own separate and discrete regions. Secretory tubules were mucous at their origin from the duct system and serous cells were found mainly as clusters at the distal ends of the tubules, although occasionally serous buds were seen to arise from the sides of the mucous tubules. A duct system to the gland was also described in detail.

## Histochemical Features

Different staining characteristics have been described for mucous and serous cells (Burkl, 1952; McCarthy and Reid, 1964; Lamb and Reid, 1969, 1970). In general the majority of serous cells contain neutral glycoprotein and the mucous cells acid glycoprotein.

## Ultrastructure

The electron microscopic appearance of the mucous and serous cells was also found to be different; the mucous cell is characterised by electron lucent, confluent granules and the serous cell by dense, discrete granules. The fine structure of the human bronchial submucosal glands have been little studies, although the secretory cells do resemble exocrine cells described in other regions of the respiratory tract (Rhodin, 1959).

The ultrastructure of the mucous and serous cells of the gland were outlined in 1965 by Bensch, Gordon and Miller; these workers also described basket cells (myoepithelial cells) and the innervation of the gland and reported the occurrence of Kultschitsky cells within the acinus.

Geieking (1968) reported on the structure of the human bronchial submucosal glands and he suggested that the serous cell granules were lysosomal in structure.

In 1970, Meyrick and Reid gave a more detailed description of the bronchial glands after fixation in glutaraldehyde and post-fixation in osmium tetroxide. They found six cell types as well as motor nerves

within the acinus of the gland:

mucous cells

serous cells

myoepithelial cells

small clear cells (probably lymphocytes)

Kultschitsky cells

mast cells.

Only the mucous and serous cells will be described here as this thesis is only concerned with these two cell types.

Mucous cell

The mucous cells are columnar in shape, measuring 25-30 µm in height, they have an irregular, basally situated nucleus and are arranged around a definite lumen. The apical regions of the cells are packed with moderately electron lucent secretory granules, 300-1800 nm in diameter. The membrane of the larger granules is not evident and these granules appear to fuse together. An extensive Golgi apparatus is found above the nucleus. Mitochondria and rough endoplasmic reticulum are frequent, most being found in the basal portion of the cell. Free ribosomes, multivesicular bodies and occasional lipochondria are also identified.

Serous cell

The serous cells are arranged around a small lumen, their shape resembles a truncated cone and they measure approximately 25 μm in height. The nucleus is spherical and basal. Granules, mostly electron dense and 300-1000 nm in diameter, are found mainly in the apical region of the cells, close to the luminal cell membrane and the intercellular canaliculi that are found between serous cells. Occasionally a dense core gives a halo appearance to the serous granule. After primary fixation with osmium tetroxide the granules have a similar density to the mucous cell granule that has been fixed in glutaraldehyde.

Long and ovoid mitochondria and rough endoplasmic reticulum in parallel arrays are situated mainly in the lower portion of the cell. Throughout the cell free ribosomes are abundant and lysosomes and lipid bodies are found. The Golgi apparatus is supranuclear and multivesicular bodies are occasionally seen. Numerous vesicles and tubules are seen in the cell cytoplasm adjacent to canaliculi and close to the apical membrane.

## PREVIOUS STUDIES OF THE UPTAKE OF RADIOACTIVE PRECURSORS BY THE HUMAN BRONCHIAL SUBMUCOSAL GLANDS

All previous studies concerned with the uptake of radioactive precursors by these glands have been with the light microscope.

### Uptake of $H^3$-glucose

In 1969, using organ culture techniques, Lamb reported that it was possible to trace $H^3$-glucose through the bronchial glands. After short periods of incubation, $H^3$-glucose was localized in the base of the mucous cell and at four hours, the cells were labelled throughout with some grains over the adjacent lumen.

Reports on the uptake of $H^3$-glucose and $S^{35}$-sulphate appeared later, for the mucous cell (Lamb, 1969; Lamb and Reid, 1969) and for the serous cell (Lamb, 1969; Lamb and Reid, 1970). Only the results of the work with $H^3$-glucose are referred to here. For the mucous cell, a similar pattern of $H^3$-glucose incorporation to that described by Sturgess and Reid (1972a) was found (vide infra).

The localization of $H^3$-glucose, in the serous cell, depended on the amount of intracellular secretory product and the duration of the experiment. With short incubation periods, if the cell had only apical secretion granules, uptake was in the upper two thirds of the cell, with the greatest concentration over the granules. If the cells were nearly filled with secretion, uptake was initially found at the basal fringe of the granules: with longer incubation periods, in both situations, uptake was found throughout the whole cell.

Sturgess (1970) and Sturgess and Reid (1972a) also reported on the secretory activity of the human bronchial submucosal glands in vitro. These workers used both a carbohydrate precursor ($H^3$-glucose) and an amino acid precursor ($H^3$-threonine). Using $H^3$-glucose, for the mucous cell, localization was initially (30 minutes) at the cell base. After two hours of incubation with the radioactive precursor, although the label was still seen in the base, some was also present in the basal portion of the secretory granules and between two - four hours the cell apex gradually filled with the label. Labelling of the apical edge of the cell was seen at four hours. A few silver grains were seen in the lumen at two hours and by four hours, 50% of the cells were discharging

labelled mucus. After six hours the concentration of grains within the gland lumen gradually increased.

In the serous cell, at 30 minutes, the $H^3$-glucose was also localized at the cell base and at one-two hours, just basal to the secretory granules. Between two-four hours, the label spread through the granule region and at four hours the label was concentrated at the cell apex and the apico-lateral borders. Secretion was seen in the dilated serous acinar lumens at four-six hours.

## Uptake of $H^3$-threonine

Tritiated threonine was first seen in the basal region of the mucous cell at 30 minutes (Sturgess and Reid, 1972a), it was then quickly distributed either throughout the whole cell or to the basal and apical regions of the cell, suggesting in the latter case, an association with the endoplasmic reticulum of the cell, not the secretory granules. Longer periods of incubation increased the concentration of the silver grains, but did not alter their position. After three hours incubation, 50% of the cells were discharging.

Similarly with the serous cell, the grains first appeared in the basal region at 30 minutes and

by one hour, the grains were distributed throughout the whole cell. The precise time at which the serous cell discharged could not be detected.

## Studies using similar radioactive precursors in other species

A moderate blackening (of silver grains) over mucous cells and an intense blackening over serous cells of the rat tracheal glands was noted four hours after subcutaneous injection of $S^{35}$-methionine by Leblond, Everett and Simmons in 1957, indicating protein synthesis within these cells.

Neutra and Leblond (1966a) gave $H^3$-glucose subcutaneously to 100g rats and found, using light microscopic autoradiography, localization, 15 minutes later, in the Golgi region of the mucous cells of the tracheal glands. In 1966(b), these workers studied the uptake of $H^3$-glucose and $H^3$-galactose by the Golgi region of various cells in the rat. Among these results, it was reported that neither the serous or mucous cells of the tracheal glands localized $H^3$-galactose, but with $H^3$-glucose a large number of silver grains (+++) were found over the mucous cells but very few ($\pm$) over the serous cells.

Chakrin, Baker, Spicer, Wardell, De Sanctis and Dries (1972) studied the synthesis and secretion of the intact, innervated canine trachea. Light microscopic autoradiography revealed incorporation of $H^3$-glucosamine by the tracheal epithelium and the glands, the amount of incorporation depending on the duration of incubation. The labelling was dense in the apical portion of the cell at six hours and over the luminal surface of the cells at ten hours.

## RADIOACTIVE UPTAKE OF MUCOUS AND SEROUS CELLS AT OTHER SITES

It is generally considered that the serous cells of the human bronchial submucosal glands closely resemble the acinar cells of the exocrine pancreas (Palade, 1966), while the mucous cells resemble the goblet cells of the small intestine (Bierring, 1962; Freeman, 1966) and the sublingual gland (Scott and Pease, 1959; Kim, Nasjleti and Han, 1972).

### Incorporation of proteins by the pancreatic acinar (serous) cell

Since most of the work on the synthesis of proteins has been carried out in the pancreatic acinar cell, this

will be taken as a model for the 'serous' cell type. Tritiated leucine is the radioactive amino acid that mainly has been studied. No reports have been found concerned with the incorporation of $H^3$-threonine - the one used in the present study. The results of Sturgess (1970), in bronchi, point to the incorporation of $H^3$-leucine and $H^3$-threonine being similar.

Three types of protein may be recognized by intracellular behaviour (Leblond, 1965).

1. Secretory proteins - synthesized in the cell for discharge
2. Sedentary proteins - mainly attributed to local intracellular turnover
3. Proteins for cell renewal - active protein synthesis is found to be directly proportional to the number of cells being produced (Leblond et al, 1956).

In 1956, using light microscopic autoradiography, Leblond et al were able to show, four hours after subcutaneous injection of $S^{35}$-methionine, intense blackening over the acinar cells of the rat exocrine pancreas, in the region of the zymogen granules.

In 1961(a), Caro was able to demonstrate using

electron microscopic autoradiography of guinea pig pancreas, that 20 minutes after intravenous injection of $H^3$-leucine, 70% of the developed silver grains lay over the Golgi apparatus and the nearby zymogen granules. The Golgi apparatus was therefore thought to be the site of protein concentration.

In 1963, Warshawsky, Lebond and Droz showed, using light microscopic autoradiography, that after intraperitoneal injection into the rat of $H^3$-glycine, leucine or methionine, a peak for incorporation of label occurred, between 10 and 30 minutes, in the basal region of the cell, at 30 minutes in the proximal zymogen region and at four hours, over the distal zymogen granules.

In 1964, Caro and Palade, and Van Heynigen studied the uptake of $H^3$-leucine by the pancreas but only the former study is described here as it is in greater detail. Caro and Palade (1964) gave $H^3$-leucine intravenously to guinea pigs. Using 0.4 $\mu$m methacrylate sections for light microscopic autoradiographs, at 4-10 minutes, the grains were found to lie in the basal region, between 10-30 minutes, in the 'centrosphere' region (near to the Golgi apparatus), at 60 minutes, the Golgi apparatus and granules and between 1-15 hours, over the granules and the lumen. Grains were not seen in the lumen before 2-3 hours. Using electron

microscopic autoradiography, silver grains were seen over the rough endoplasmic reticulum and some over smooth surfaced vesicles associated with the Golgi apparatus at five minutes. More grains were found over the smooth surfaced vesicles at six minutes and at 20 minutes, grains were found over the condensing vacuoles of the Golgi apparatus. At 45 minutes, the condensing vacuoles and the nearby granules were labelled and at four hours, the label was mainly over the granules. This work supported the idea of a link between the rough endoplasmic reticulum and the Golgi complex, provided by the smooth surfaced vesicles.

In 1967a, Jamieson and Palade using fractionation procedures and electron microscopic autoradiography, applied to 'in vitro' pancreatic slices of guinea pig pancreas, found that at three minutes, the $H^3$-leucine was in the rough microsome region of the pellet (i.e. rough endoplasmic reticulum and transitional elements) and at ten minutes, in the smooth microsome region (i.e. smooth surfaced vesicles), this provided more evidence for this link. These authors also (1967b) examined the zymogen pellet in a similar manner and found some labelling in this fraction at 20 minutes, with the peak labelling at 40 and 60 minutes. In a parallel electron microscope autoradiographic study of this fraction, no label was found over the zymogen granules until 60 minutes. The labelling at earlier

times was due to disintegrations from the condensing vacuoles.

In 1968(a), with cycloheximide, Jamieson and Palade reduced protein synthesis by 95%. Using electron microscope autoradiography and cell fractionation they were able to show that after 40 minutes of inhibition, three minute pulse label and a 37 minute chase in culture medium + cycloheximide, intracellular transport of the proteins formed during the pulse label continued at approximately 80% efficiency of the control level. With increasing periods of time the efficiency decreased. This work demonstrated that protein synthesis is independent of the rate of intracellular transport.

Jamieson and Palade (1967c; 1968b) further reported on the effect of inhibitors of glycolysis (fluoride, iodoacetate), respiration ($N_2$, cyanide and Actinomycin D) and oxidative phosphorylation (dinitrophenol, oligomycin) on intracellular transport, with simultaneous inhibition of protein synthesis (see 1968a). The inhibitors of oxidative phosphorylation and respiration were found to block intracellular transport while glycolysis inhibitors did not. Again using electron microscopic autoradiography and cell fractionation, the energy dependent step for intracellular transport was thought to be located between the transitional elements of the endoplasmic reticulum and the smooth surfaced vesicles of the Golgi apparatus.

Recently Castle, Jamieson and Palade (1972) studied the secretory process of the parotid acinar cell of rabbit. Using electron microscopic autoradiography they were able to show that the synthesis, transportation and storage of the granules was similar to that described for the guinea-pig pancreas. The time period for maturation of the granule was however greatly increased; mature granules began to localize grains at three hours (one hour in the pancreas), labelled granules at the cell apex were rarely seen and the acinar lumina were not significantly labelled even at six hours (significant at two hours in the pancreas).

## Incorporation of carbohydrates into the 'serous' type cell

No studies dealing specifically with the incorporation of radioactive sugars into serous cells have been carried out. Neutra and Leblond (1966b) mention that after intraperitoneal injection, the pancreatic acinar cells of the rat incorporate a little $H^3$-glucose (±) and a little more $H^3$-glucosamine (+).

In 1967, Bennett injected rats with $H^3$-glucose, mannose or galactose and fixed specimens from various organs at 15 minutes, one and 15 hours. In the serous type cells, e.g. Paneth cell and acinar cell of the

pancreas, the label always appeared first in the Golgi region and then later in the granules. The greatest intensity of silver grains was obtained with glucose, less with galactose and least with mannose.

## Incorporation of proteins into mucous cells

Few studies concerning the synthesis of proteins by mucous cells have been made. Using the light microscope, Jennings and Florey (1956) gave DL-($S^{35}$)methionine to two mice and found that the silver grains obtained had no relation to the mucus in the cell, but were found all over the epithelial cells of the stomach and intestine, particularly the gastric faveolae.

In 1957,Leblond et al reported localization of $S^{35}$-methionine, at four hours, over the perinuclear region of mucous cells in the sublingual gland and small intestine, and over the granules of the mucous cells at 24 and 36 hours.

## Incorporation of carbohydrates into mucous cells

In 1964(a) Peterson and Leblond gave D-glucose-6-$H^3$ subcutaneously to four day old rats and studied various organs from the animals. Using the light microscope, the Golgi zone was recognized by stains demonstrating carbohydrates i.e.

periodic acid Schiff, coloidal iron and Maillet's technique for demonstrating the Golgi apparatus. After administration of $H^3$-glucose, autoradiography revealed labelling in this region by 5 - 15 minutes that remained here for one hour, after one hour, the labelling was seen in the secretory material. Differences in amount of label over cells in various regions were noted, e.g. at 15 minutes, the mucous cells of the colon surface epithelium of the trachea and sublingual gland exhibited large numbers of silver grains (+++) and the mucous cells of the tracheal glands and the submaxillary gland less (+).

Peterson and Leblond (1964b) also did parallel experiments with glucose labelled, in the C-1 and C-6 position, with tritium: no difference was found using either labelling position. It is therefore likely that the entire 6-carbon chain of glucose is used as a building block for synthesis.

Neutra and Leblond (1966a) studied the uptake of $H^3$-glucose into colonic goblet cells of rat after intraperitoneal injection. Using light microscopic autoradiography, at five minutes, a U-shaped band of grains was found above the nucleus, the U open toward the cell apex, more grains were found in the same region at 20 minutes; at one hour, some grains were localized in the central mucigen (mucous granule) region and at

1.5 hours, grains were appearing in the apical mucigen and at four hours, the majority of grains were in the apical supranuclear region. Using electron microscopic autoradiography, at five minutes, grains were localized over the Golgi lamellae, none were found over the rough endoplasmic reticulum: at 20 minutes the grains were over both the Golgi lamellae and the mucigen granules. By 40 minutes nearly all the grains had left the Golgi apparatus, at one and 1.5 hours, more granules were labelled away from the Golgi apparatus and by four hours, although the label varied from cell to cell, the grains were found mainly in the apical region.

Neutra and Leblond (1966b) also compared the uptake of $H^3$-galactose to $H^3$-glucose in the Golgi regions of various cells which synthesize carbohydrates. The mucous cells of the sublingual and tracheal gland were found to have a high uptake of $H^3$-galactose (+++), the sublingual gland, a moderate uptake of $H^3$-galactose (++), but the mucous cells of the tracheal glands showed no localization of $H^3$-galactose; the submaxillary gland had some uptake of $H^3$-glucose (+) but none for $H^3$-galactose. Although not all mucous cells incorporated $H^3$-galactose, in those that did, localization was better than for $H^3$-glucose. This may be due to some of the glucose being broken down during glycolysis, and its catabolism into amino acids, nucleotides and lipids etc. For this reason $H^3$-leucine was also injected into rats and localization, at six and

30 minutes, suggesting that the widespread scatter of $H^3$-glucose may be caused by its synthesis into proteins.

Bennett (1967) also studies the mucous cells of various tissues of rat after administration of $H^3$-glucose, galactose and mannose; he reported similar findings for mucous cells to those already stated (see page 31 ) for serous cells.

General pattern and pathway of protein synthesis

A pattern for the synthesis and transport of proteins has been established from the forementioned work. It seems similar for both mucous and serous cells.

Newly synthesized secretory proteins are transported into the intracisternal space of the rough endoplasmic reticulum, for example, grains are located in the intracisternal space of the mouse thyroid follicular cells after injection with $H^3$-leucine (Nadler, 1965). From there it travels to the Golgi apparatus, via smooth surfaced vesicles, to join the 'immature' or distal face of the Golgi apparatus. The condensing vacuole is thought to arise from the 'mature' or proximal face of the Golgi apparatus. The mature granule arises directly from the condensing vacuole after further maturation, thought

by Hokin and Huebner (1967), to represent extrusion of water from the granule and possibly transport of additional protein across the zymogen granule membrane.

## Suggested alternate routes for intracellular transport of proteins

Although the above pattern is now generally accepted as the pathway for protein synthesis, other routes had been suggested. In 1953, Weiss suggested that zymogen granules were derived directly from the ergastoplasm (endoplasmic reticulum). Small buds were pinched from the tubules of ergastoplasm sacs and as these migrated to the cell apex, secretion accumulated within the lumen and the granules enlarged. Palade in 1956 also thought that the zymogen granule could be stored in the endoplasmic reticulum. Similar conclusions were reached for the albumen-secreting cells of the hen oviduct by Hendler, Dalton and Glenner (1957); they stated that protein synthesis occurred in the ergastoplasm and found no evidence to indicate involvement of the Golgi apparatus. In 1965, Ichikawa reported zymogen granules in the rough endoplasmic reticulum of the canine pancreas, in both controls and animals stimulated with pancreozymin. All these reports found the intracisternal granules to be infrequent and pondered that they may be caused by a block in the

the intracellular transport system.

Jamieson and Palade (1971) drew attention to the absence of silver grains over the Golgi saccules (lamellae) (1967b); they suggested that the smooth vesicles may go directly to the condensing vacuole, bypassing the Golgi saccule, or alternatively the level of radioactivity in the Golgi saccules may be too low to detect. They therefore stimulated the pancreatic acinar cells to secrete with carbamycholine. The condensing vacuoles and granules virtually disappeared by three hours and granules were seen in the Golgi saccules which seemed to take over the role of the condensing vacuole.

It has also been suggested that the secretory proteins leave the microsomes in a soluble form and pass through the cell sap to form zymogen granules in the apical region or go directly into the gland lumen (Redman and Hokin, 1959; Morris and Dickman, 1960).

## General pattern and pathway of carbohydrate synthesis

As a general rule sugars are added tc the secretory product in the Golgi apparatus. Little work has been done to show the exact site of the addition. From the Golgi

apparatus the sugar migrates to the cell apex in the newly formed granules.

In the columnar cells of the cat intestine (Ito, 1965) and the rat duodenal epithelium (Bennett, 1970), $H^3$-glucose and $H^3$-galactose were reported to localize initially in the Golgi region and at one hour the label was found over the apical cell coat. In 1970, Zagury, Uhr, Jamieson and Palade studied the synthesis and secretion of IgG by in vitro incorporation of $H^3$-galactose and glucosamine and $H^3$-leucine into plasma cells from mouse myeloma tumour. They found that $H^3$-galactose was added to the molecule in the Golgi region but $H^3$-glucosamine, a sugar centrally placed in the carbohydrate side-chain, was incorporated either in the Golgi region or in the rough endoplasmic reticulum. The formed immunoglobulins were thought to be transported to the plasma membrane for discharge by way of Golgi derived smooth surfaced vesicles.

## Discharge of secretion granules

### Mucous Cell

In the non-secreting phase, the mucous cell has cytoplasmic projections from the free apical edge; these disappear during the cycle due to build up of mucus within the cell causing the cell to bulge into the lumen. Fusion and confluence of the mucous granules is also observed at this stage (Bierring, 1962; Freeman, 1966). When the cell becomes overdistended the apical cell membrane ruptures and some of the granules and membranes are released (Palay, 1958; Freeman, 1966).

Neutra and Leblond (1966a) however, thought that in the rat colonic goblet cell, the mucigen granules were released singly and the goblet cell granules rarely fused together. Martinez-Hernandez, Nakane and Pierce (1972) reported fusion of granules with the apical cell membrane leading to release of single granules in the mouse submaxillary gland. Both groups suggested that rupture or loss of granule membranes within the cell may be due to poor fixation.

Kim et al (1972) stimulated the mucous cells of the rat sublingual gland with isoprenaline and pilocarpine; discharge of mucous secretion was complete by two hours. Large vacuoles were found in the cytoplasm at one hour,

these were limited by smooth vesicles, similar to those associated with the Golgi apparatus. At two hours, a distinct membrane had formed around the vacuole and the secretory cycle started again. This work strongly suggests that many mucous granules may be released at one time.

Serous cell

The secretory phase of the serous type granules differs from that of the mucous cell. The granule migrates to the cell apex, where the granule membrane fuses with the cell membrane thus exposing the granule to the lumen, without a break occurring in the cell membrane: this is merocrine secretion or exocytosis. This pattern has been found in many tissues e.g. guinea pig pancreas (Palade, 1959), rat and mouse parotid gland (Parks, 1962) and Von Ebner's gland of the rat (Hand, 1972).

Since the serous granules are rarely seen to discharge, secretion has been studied mainly in the stimulated gland. In this type of experiment an increase in lumen diameter is seen to accompany a massive discharge of granules into the lumen (Simson, 1969; Amsterdam, Ohad and Schramm, 1969 and Jamieson and Palade, 1971). Fusion of zymogen granules has been reported, but only after prior

fusion with the cell membrane (Ichikawa, 1965; Amsterdam et al, 1969).

## HIGH RESOLUTION AUTORADIOGRAPHY

The basic principles for electron microscopic autoradiography are the same as for the light microscope (for light microscope, see Belanger and Leblond, 1946; Pelc, 1947 and Pelc and Howard, 1952). The factors determining the resolution of the autoradiograph are the photographic process as well as the thickness of the section, the emulsion and the intervening layer (see page 49 ). Thus the resolution of electron microscope autoradiographis is ten times better than that of the light microscope, but 100 times poorer than the resolution of the electron microscope (Jacob, 1971).

## Evolution of electron microscopic autoradiography

The combination of electron microscopy and autoradiography was first attempted by Comer and Skipper (1954), who examined tracks emitted from radioactive mineral grains. The sample was mixed with warm C2 photographic emulsion and dropped or spread on to a formvar coated slide. After development the specimen was floated off the slide and mounted on an electron microscope grid. In 1956, Liquier-Milward studied the first biological specimen; nuclei were labelled with Cobalt$^{60}$ ($\beta$ emmission), dilute Ilford G5 emulsion was poured over the specimen that was then mounted on a formvar coated slide. Exposure was carried out in a thick walled lead box placed in a coal-mine at a depth of 600 metres. The results of these two experiments were not encouraging since the resolution obtained did not exceed that of the light microscope.

Three years later an improvement in resolution was achieved by O'Brien and George (1959). They used methacrylate embedded yeast cells labelled with Polonium$^{210}$. Sections were mounted on a carbon coated grid and then coated with Eastman Kodak NTA emulsion diluted 1:1 with distilled water.

In 1959, George and Vogt devised a loop method for applying emulsion. A wire loop was dipped into melted 45°C) Eastman Kodak NTA emulsion, withdrawn and applied to the grid before the emulsion dried. Later, George (1961) modified this by adding a detergent (0.05% sodium lauryl

sulphate) to the emulsion hoping to give a more even distribution of silver halide crystals.

## Techniques for producing a monolayer

A monolayer of closely packed silver halide crystals is an essential for electron microscopic autoradiography; this combines the maximum sensitivity with the maximum resolution of the emulsion.

Tritium ($H^3$) was first used by Van Tubergen in 1961. He labelled Escherichia coli with $H^3$-thymidine and applied Kodak V-1055 emulsion (smaller grains than previously used) to sections mounted on formvar and carbon coated grids. He modified the George and Vogt (1959) technique by allowing the emulsion to gel in the loop, in order to achieve a uniform layer of silver halide crystals, before blowing it on to the sections. In 1962, Caro and Van Tubergen published a full description of the 'loop' technique for obtaining a monolayer that is still used by many workers.

Harford and Hamlin (1961a and b) tried a different approach for consistently increasing the number of grains found in autoradiographs. They labelled HeLa cells with $H^3$-thymidine, coated them with Kodak NTB-3 and placed the sections, during exposure, between two poles of a magnet (10,000 gauss). They reported an increase in the number of

grains due, they suggested, to the path of the particles being bent by the strength of the magnetic field and thus increasing the number of hits. Caro (1961b), using theoretical calculations, stated that magnetic fields of such low intensity could have no such effect; the same conclusion was also reached by Fromme and Zessack (1966).

Pelc, Coombs and Budd (1961) found that an even distribution of grains could be obtained if grids were not used until after the completion of the photographic process (it had been noticed that clumping of grains was prone to occur around the grid bars). The sections were placed on a formvar film stretched over a hole, 7 mm diameter, drilled in a Perspex slide. This method was described in full in 1964 by Budd and Pelc.

In 1961, Caro and Palade described a method whereby the grid, attached to a slide by a piece of sticky tape, was dipped directly into the emulsion.

A dipping technique for light microscopic autoradiography was described by Kopriwa and Leblond (1962) and in 1966 Kopriwa developed a semiautomatic machine for dipping electron microscope sections mounted on formvar coated slides into emulsion. The use of a machine allows a slow, constant speed of withdrawal thus ensuring a uniform reproducible

monolayer of silver grains.

Silk, Hawtrey, Spence and Gear (1961) attempted to make their own emulsion in an effort to bring the sections and emulsion into close apposition. Sections were mounted on formvar/carbon coated grids and a thin layer of silver was evaporated on to the tissue. This was then exposed to bromine vapour and covered with a film of aqueous gelatin. Unfortunately the results obtained were suspect since the silver grains arising from the $H^3$-thymidine were found over the cytoplasm, not the nucleus.

Centrifugation techniques for precipitating the emulsion in a uniform monolayer were also tried (Koehler, Muhlether and Frey-Wyssling, 1963 and Rohr, Kleitmann and Ochlert, 1965) but these were found to be time consuming and with no advantage over the loop technique.

Today the most commonly used techniques for electron microscopic autoradiography are the loop technique of Caro and Van Tubergen (1962) and the dipping technique (Kopriwa, 1966). Ilford L4 emulsion with a silver halide crystal diameter of 1200-1400Å, first used by Caro and Van Tubergen (1962) is mostly used. Other emulsions have been tried e.g. Gavaert

NUC-307 with silver halide crystals 700Å in diameter (Granboulan, 1965) and Eastman Kodak NTE with a crystal 500-700Å in diameter (Bachmann and Salpeter, 1964).

## Contrast of electron microscopic autoradiographs

Various techniques to enhance the contrast of the electron microscopic autoradiographs have been tried. Comer and Skipper (1954) used diastase to remove the gelatin from the emulsion and Hampton and Quastler (1961) a 0.2% solution of pepsin. Przybylski (1961) enhanced section contrast with an 0.2% solution of trypsin at 38°C for one hour and Silk et al (1961) removed the gelatin in warm water overnight after fixing the emulsion in a non-hardening fixer (15% aqueous sodium thiosulphate). Revel and Hay (1961) showed the benefits of using Karnovsky's (1961) lead stain; in addition to staining the section they found the emulsion to be partially removed by alkaline hydrolysis. Other methods include removal by acetic acid (Granboulan, Granboulan and Bernhard, 1962) and removal of the gelatin by sublimation in an electron microscope beam (Meek and Moses, 1963).

It was suggested that the removal of the gelatin may result in loss or movement of the silver grains (Przybylski, 1961; Hampton and Quastler, 1961 and

Revel and Hay, 1963). This question is still unanswered and should be checked for in each investigation where staining is carried out after the photographic process.

A detailed account of the practice and application of electron microscopic autoradiography was given by Jacob in 1971.

## Resolution

As previously stated the resolution of electron microscopic autoradiography is not as good as the resolving power of the electron microscope. Two factors are responsible for this loss, the photographic error and the geometric error. The resolution of electron microscopic autoradiography is defined as 'the radius of a circle about a point source which contains half the silver grains produced by that source' (Bachmann and Salpeter, 1965).

### Photographic error

When an ionising particle hits a silver bromide crystal, a latent image is formed. This may be anywhere in the crystal and not at the point of impact, thus the maximum distance between the path of the

particle and the latent image is equal to the diameter of the silver bromide crystal (Bachmann and Salpeter, 1965). A smaller silver halide might be thought to improve the photographic error, but with a monolayer of silver grains this can lead to a loss in sensitivity in the emulsion. If the crystal size is too small there is an increased chance that the particle might pass through the emulsion without forming a latent image. The optimal size for the silver halide crystal is one that combines the highest sensitivity with the best resolution for that emulsion. For practical purposes the latent image is assumed to be the mid-point of the coiled silver grain and the grain is assumed to grow randomly.

According to Caro and Van Tubergen (1962) the maximal error due to the photographic process may be expressed as the sum of the diameter of the undeveloped silver bromide crystal (a) and one half of the size of the developed grain (b). However, Bachmann and Salpeter (1965) found that on average, the photographic error (Ep) was less than this and they calculated it to be

$$Ep = \sqrt{\frac{a^2}{5} \quad \frac{b^2}{12}}$$

Geometric error

Radiation leaves a decaying atom in a specimen i.e. the source, in a random manner but only estimates of its position are possible.

Caro (1962), calculated that the greatest geometric error was approximately equal to the thickness of either the emulsion or section, whichever is the greater. Bachmann and Salpeter (1965) and Salpeter and Bachmann (1965), calculated that one half of all developed grains lie within a circle of radius 1.7HD (see page 84 ) around a point source.

$$HD = \frac{te}{3} + \frac{ti}{1} + \frac{ts}{2}$$

where te = thickness of emulsion

ti = thickness of intervening layer (e.g. carbon layer)

ts = section thickness (Figure I-1).

Therefore the Final error as calculated by Bachmann and Salpeter is the square root of the sum of the geometric error and the photographic error squared

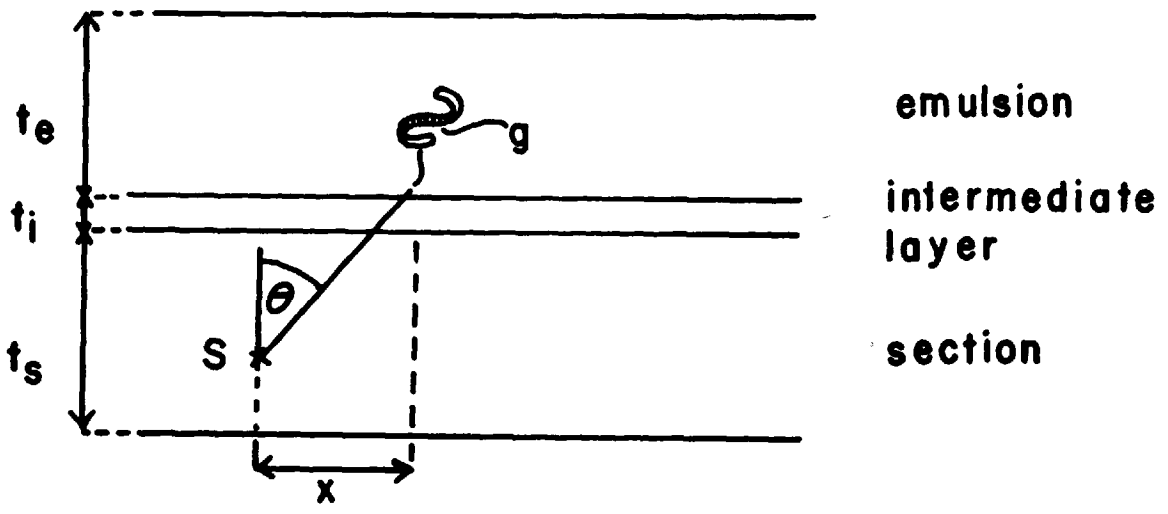
$$\text{final error} = \sqrt{Ep^2 + Eg^2}$$

Since the source of radiation is a biological section, this should be as thin as possible to give the best resolution. In practice when very thin sections are used, the grains are fewer because of the low content of

FIGURE I-1

Schematic representation of an autoradiograph preparation. The radioactive sources (S) are located in the section; the disintegration follows a straight line in the section and the intermediate layer, but is scattered in the emulsion layer: X is the projected distance from the source (S) to the developed grain (g), $t_s$, thickness of specimen; $t_e$, thickness of emulsion; $t_i$, thickness of intervening layer (after Bachmann and Salpeter, 1965, Figure 5).

FIGURE I-1



radioactivity within the section, therefore sections ( ~~ 1000Å) thicker than those generally used for electron microscopy are obtained with a resultant loss of resolution.

## QUANTIFICATION OF ELECTRON MICROSCOPIC AUTORADIOGRAPHS

### Origin of Technique

The techniques used for 'quantifying' components of biological material were originally derived from the methods used by geologists and metallurgists. In 1847, a geologist named Delesse postulated that the sum of the areas of components of rocks, obtained by weighing paper tracings, was proportional to their volume. Rosiwal (1898) stated that, provided the distribution of particles was homogeneous, volumes of the particles could be computed from linear measurements with calipers.

### Application to Biological Material

Chalkley (1843), was the first person to apply these methods to biological material. His random sampling technique consisted of a five point system on the diaphragm of the ocular (the middle point was used for focusing on the specimen); this method was originally used for estimating nuclear/cytoplasmic ratios. Later, Chalkley,

Cornfield and Parks (1949) used a finite line and this aided calculations of cell complexity.

In 1954, Elias and Cohen used geometric analysis on electron micrographs of rat liver. In the 1960's, these arduous, quantification techniques were increasingly applied to electron micrographs (e.g. Laud, 1962; Weibel and Knight, 1964; Weibel, Kistler and Scherle, 1966; Staubli, Hess and Weibel, 1969).

## Application to Electron Microscopic Autoradiography

Whereas the first methods for 'analysing' electron microscopic autoradiographs were purely subjective, nowadays the interpretation of autoradiographs takes into account the resolution and the 'expected' grain distribution around a point source. These points have been discussed in detail by Caro, 1962; Pelc, 1963; Bachmann and Salpeter, 1965.

In 1962, Caro derived some hypothetical curves for the distribution of silver grains around a point source. Maunsbach (1966) and Coimbra and Leblond (1966) plotted histograms showing the frequency of silver grains at varying distances from an organelle and compared them to Caro's 'curves': these curves were found to apply only to highly organized tissue, e.g. striated muscle.

The quantitative method of Salpeter, Bachmann and Salpeter (1969) is based on the assumption that a given organelle is labelled. The structure being studied is fitted to one of a series of shapes, e.g. an ellipsoid or oval shape would fit a Golgi apparatus, this area is then called a 'bin'. The perpendicular distance from the mid-point of the silver grain to the closest parameter of the 'bin' is measured. This is converted to HD (see page 84 ) for a line source or HR for a point source. The HD is the distance from a hot line within which half of the developed grains fall. The HR is used for a point source and this is equal to 1.7HD. A histogram is then constructed and compared with the 'Universal curves' given for the various shapes in the above paper.

Williams (1969) described a rather simpler method of quantification. Using this method it is not necessary to assume that any organelle is labelled. Circles of diameter 1.7HD (Salpeter et al, 1969) are superimposed in a random grid fashion over a micrograph, and the structures within these circles are noted; this gives the 'effective area' or an estimate of the expected number of silver grains that would occur if the grains were random. The silver grains are also surrounded by a circle of the same size and the structures over which the grains lie is noted, this gives the 'actual' grain count. Using these two findings Chi-squared test for significance is applied to find if

the grains are distributed in a random manner over the section.

Recently, Blackett and Parry (1973) have described a method that combines both of the above methods. A theoretical or hypothetical distribution of grains is obtained as in both of the above, but with the aid of computer derived directions and distances. The circle analysis of Williams is then applied to these random grains and compared to the 'actual' grain distribution. The numbers in the columns obtained for the various organelles and junctional regions are manipulated by varying their activity, until a similar pattern to the 'actual' grain distribution is acquired. This is done by repeated application of the Chi-squared test. It is possible using this method to pin-point activity in junctional or 'cross-fire' regions.

# CHAPTER II

## MATERIAL AND METHODS

## MATERIAL

Adult human bronchi were obtained from patients undergoing pneumonectomy or lobectomy for carcinoma of the lung, at the Brompton, London Chest and Charing Cross Hospitals (Table II-1).

Bronchi were collected immediately on removal from the body. The proximal end of the bronchus was taken and placed in a nutrient medium, TC 199 (Glaxo Laboratories Ltd). The length of bronchial cuff obtained depended on the size of the stump attached to the specimen and on the location of the tumour. The specimen was discarded if infiltration of the tumour into the bronchus was found. A ring of bronchus 0.3 - 0.5 cm deep was taken from the most proximal end of the bronchial specimen for routine histological examination and examination of the gland/wall ratio (Reid, 1960).

The specimens obtained from the London Chest and Charing Cross Hospitals were found to be suitable for organ culture and electron microscopic examination if the bronchial ring was transported immediately in TC 199 in a refrigerated container and cultured within one hour of removal from the body. Tissue that had been removed from the body for more than one hour and post-mortem material was found to be unsuitable.

TABLE II-1

DETAILS OF MATERIAL STUDIED

| Study | Patient's name | Hospital No | Age | Gland/wall ratio | Smoking habit/ day | Operation | Diagnosis | EM No. |
|---|---|---|---|---|---|---|---|---|
| Scintillation counting | GH | CX325208 | 63 | 0.24 | 20 | R.Pn/ectomy | Adeno-Ca. | |
| | PB | B29323 | 49 | 0.35 | Not stated | Completion Pn/ectomy | Broncho-pulmonary fistula and Bronchiectasis | |
| | HC | LC77028 | 59 | 0.48 | 50-60 | R.Pn/ectomy | Squamous Ca. | |
| Incubation at 4°C | CB | LC78841 | 67 | 0.55 | 30 | R.M.L. L/ectomy | Adeno-Ca. | |
| | SS | B32485 | 63 | 0.43 | 15 | R.U.L/ectomy | Adeno-Ca. | |
| Light microscope | AV | B40401 | 58 | 0.38 | 50 | L.Pn/ectomy | Squamous Ca. | |
| Electron microscope | HH | B39177 | 61 | 0.26 | 40-60 | L.Pn/ectomy | Squamcus Ca. | 457 |
| | GB | B30218 | 61 | 0.40 | 5-10 | R.Pn/ectomy | Adeno-Ca. | 402 |
| | WK | LC77949 | 58 | 0.39 | 1 oz | L.Pn/ectomy | Oat cell Ca. | 411 |
| | TS | B34713 | 48 | 0.27 | 30-40 | L.Pn/ectomy | Oat cell Ca. | 421 |
| | JR | B37334 | 53 | 0.29 | 30 | L.Pn/ectomy | Squamous Ca. | 448 |
| | SB | B38219 | 48 | 0.46 | 20-30 | R.Pn/ectomy | Squamous Ca. | 455 |

Ca = Carcinoma
All patients were male
All received Omnopon and Scopolamine as premedication, as well as Thiopentane and Pancurare during anaesthesia

# METHODS

## Organ culture technique

A modification (Sturgess, 1970) of Trowell's technique (1959) for the culture of mature organs was used for the culturing of human bronchial submucosal glands. In this method the cultures are maintained on a nutrient medium, TC 199, in an atmosphere of 5% carbon dioxide in oxygen at 37°C.

### Preparation

Preparation of tissue was identical to that described by Sturgess (1970). Strips of tissue, approximately 1-2 by 10-15 mm, were dissected from the bronchial rings. These are referred to as explants. Two or three explants were laid on to sterile lens tissue, and placed on a raised aluminium supporting grid in a sterile Petri dish (Figure II-1). The space underneath the grid was filled with culture medium, until the explants just rested on the meniscus of the fluid. The explants were then incubated or equilibrated at 37°C for one hour.

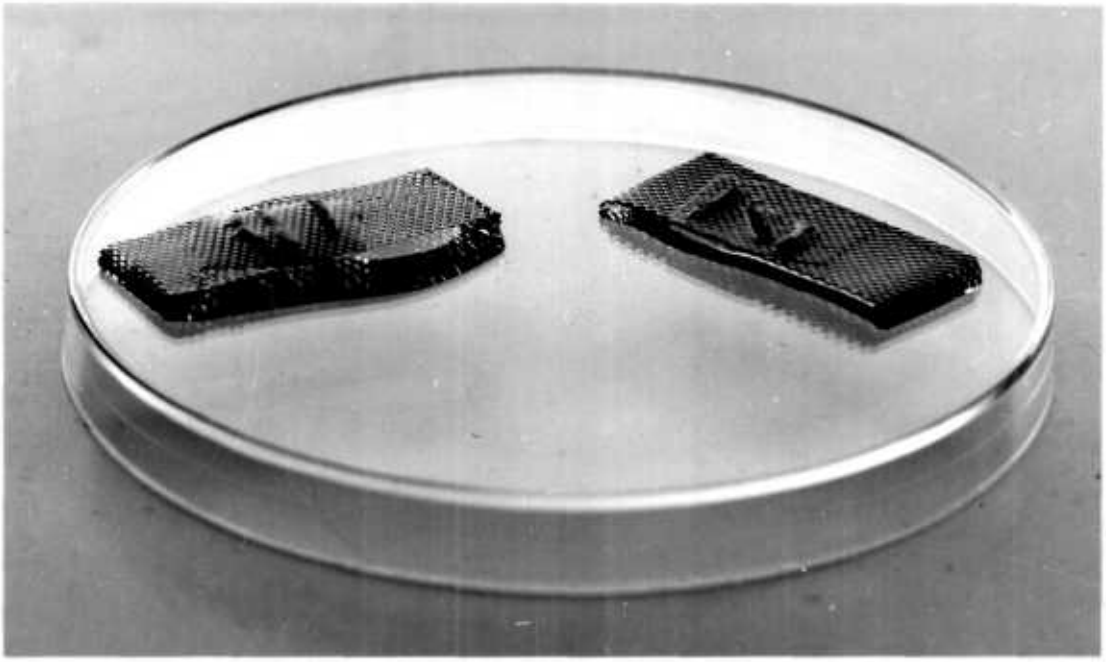
## Addition of radioactive precursor

After equilibration, the culture fluid was replaced with TC 199 containing radioactive precursor at a selected concentration (vide infra). The Petri dish and contents were again incubated at 37°C and explants were removed at

FIGURE II-1

View of grid supporting explants, showing the culture medium below the grid. The correct amount of culture fluid is present under the right hand grid, just moistening the lens tissue. In the case of the right hand grid, excess medium is present.

FIGURE II-1



20, 40 and 60 minutes. Incubation periods up to and including 60 minutes are referred to as 'pulse' labels. The remaining explants, after incubation for 60 minutes in radioactive precursor + TC 199, were removed and washed three times with approximately 5 ml of TC 199. They were then placed on clean lens tissue and grids in a clean Petri dish and culture fluid alone was placed under the grid. The Petri dish was returned to the incubator for the 'chase' incubation. During the 'chase' incubation, explants were removed at hourly intervals - 1-7 hours - thus making a total incubation time of eight hours, i.e. 60 minute 'pulse' label plus a seven hour 'chase' incubation. Thus specimens were obtained at 20, 40 and 60 minutes, 2, 3, 4, 5, 6, 7 and 8 hours.

Radioactive Precursors

Two radioactive precursors were used in these studies:

D-Glucose-6-$H^3$

DL-Threonine-$H^3$(G).

These were obtained in aqueous solution from the Radiochemical Centre, Amersham (Table II-2). The half-life of tritiated compounds was 12½ years and therefore variation in activity in these experiments was negligible.

TABLE II-2

RADIOACTIVE PRECURSORS

| Radioactive tracer ($H^3$) | Reference No | Specific activity ($\mu Ci/mM$) | % decomposition /annum |
|---|---|---|---|
| D-Glucose-6-$H^3$ | TRK 85 | 1000 | 10 |
| DL-Threonine-$H^3$(G) | TRA 91 | 80 | 5 |

Decomposition of these chemicals at +2°C was 10% per annum for tritiated ($H^3$) glucose and 5% for tritiated ($H^3$) threonine; tritiated compounds were discarded when their activity had decomposed by 5%.

Dose of radioactive precursor given

In previous light microscope experiments in this Department (Lamb, 1969; Sturgess and Reid, 1972a), a dose of 10μCi/ml was used when culturing bronchial gland explants. A larger dose of radioactive precursor was necessary for electron microscopic autoradiography due to the thinness of the sections used (1/40 of a light microscope section). Three doses were tried in order to find an appropriate amount. Explants were exposed to 100, 250 and 500μCi/ml of $H^3$-glucose or $H^3$-threonine at 37°C for one hour.

Tissues were fixed in glutaraldehyde or formalin and processed for electron microscopy. Electron microscope and 1 μm light microscope sections were prepared for autoradiography and exposed for varying lengths of time.

1. One μm light microscope sections were exposed for two, four and eight days. This gave an estimate of the exposure time needed for electron microscopic autoradiographs.

2. Electron microscope sections (1000Å) were exposed for four, eight and ten weeks.

The procedure for fixation and autoradiography will be described later.

The dose of radioactive precursor giving an adequate number of silver grains for quantification in a reasonable exposure time, was found to be 250μCi/ml for $H^3$-glucose with an exposure time of four weeks, and 500μCi/ml for $H^3$-threonine with an exposure time of ten weeks. A concentrated solution (X10) of TC 199 was used as a diluent for the aqueous radioactive precursor, as the addition of such large amount of radioactive precursor to single strength TC 199 made the culture medium too dilute to maintain adequately the growth of the explant.

## Fixation and Processing

### Selection of a fixative

Although glutaraldehyde is the fixative most commonly used for the fixation of electron microscopic specimens, it has recently been reported (Peters and Ashley, 1969) that glutaraldehyde is capable of binding free radioactive amino acids to tissue. Since $H^3$-threonine was used in the present experiments, preliminary studies using various fixation techniques were carried out. These are listed in the order

in which they were done,

i) scintillation counts

ii) light microscopic examination after incubation at $4^{\circ}C$.

i) Scintillation counts

These were carried out to show the amount of radioactive precursor lost during the process of fixation and processing.

Cold rinses Some explants were washed well in either 1% cold aqueous threonine or glucose (three changes in five minutes), this was thought to assist the removal of unbound radioactive precursor from the tissue.

Puromycin Some explants were incubated with the addition of 1 mg/ml of puromycin dihydrochloride (see Appendix) to both the equilabration and radioactive incubation medium. It was thought that this would inhibit incorporation of radioactive amino acids to the tissue.

All the explants were prepared and cultured as above for one hour at $37^{\circ}C$ in either $H^3$-threonine or $H^3$-glucose. At least two explants from each group were then fixed in each of the following:

1. 4% formalin in cacodylate buffer
2. 2% glutaraldehyde in cacodylate buffer
3. a modified Karnovsky (1965) fluid (see Appendix).

The explants were then processed as outlined in Table II-3. All the processing fluids were saved in separate containers for analysis of the radioactive content.

Dilution for scintillation counts The tritium activity in the samples was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer (model No 3375). Samples (0.25 ml) were diluted in Insta-Gel (10 ml), a scintillator developed by Packard Instruments Company, Inc. The counting efficiency of each sample was estimated by the automatic external standard ratio (A.E.S.) method. The cold rinse and fixation fluids were diluted 1 in 100 with distilled water prior to final dilution as the counts for these samples were very high. Each sample was counted for a 20 minute period.

Oxidation of tissue After the processing procedure, the remaining tissues were combusted in a Packard Tritium Oxidizer, diluted in 10 ml of Insta-Gel and counted in the Scintillation Spectrometer for 20 minute periods. The results of the scintillation

TABLE II-3

PROCESSING SCHEDULE OF TISSUE FOR ELECTRON MICROSCOPY

1. Fix tissue
2. Wash tissue in cacodylate buffer (X3) and leave in final wash at $4^{\circ}C$, overnight
3. Post-fix in 1% osmium tetroxide at room temperature for one hour
4. Wash in distilled water (X3)
5. Dehydrate in 70% methanol - 20 minutes
6. Dehydrate in 90% methanol - 20 minutes
7. Dehydrate in 100% methanol - 20 minutes (X3)
8. Epoxy propane - 30 minutes
9. Embed in Araldite.

counting are given in Chapter III.

ii) Light microscopic examination after incubation at 4°C

Explants were prepared as above and incubated with either $H^3$-glucose or $H^3$-threonine (250μCi/ml). Experimental explants were incubated at 4°C for ten minutes and controls were incubated with and without added radioactive precursor at 37°C for ten minutes. The explants were fixed in each of a range of fixatives and left for varying times (Table II-4).

The explants were then processed for light microscopy in a Histokinette Automatic Processing Machine and embedded in wax at 60°C (Sturgess, 1970).

Four μm sections were cut and stained with the periodic acid Schiff technique (Culling, 1963). Using the stripping technique (Pelc, 1947; Sturgess, 1970), autoradiographs were prepared using Kodak AR 10 stripping film. The slides were exposed in a light-tight box at 4°C for four days before developing in Kodak D 19 at 20°C for five minutes and fixing in Fix-Sol (Johnson's of Hendon) diluted 1 in 10 with distilled water for 15 minutes.

Quantification was carried out using an oil immersion lens (X100) and X8 eyepieces. One of the

TABLE II-4

INCUBATION TEMPERATURE, FIXATIVE AND FIXATION TIME USED FOR EXPLANTS AFTER INCUBATION IN EITHER $H^3$-THREONINE OR $H^3$-GLUCOSE FOR TEN MINUTES

| FIXATIVE | FIXATION TIME (HRS) | INCUBATION TEMPERATURE($^{\circ}$C) |
|---|---|---|
| Formalin | 4.0 | +4 |
| Formalin | 18.0 | +4 |
| Glutaraldehyde | 1.5 | +4 |
| Osmium tetroxide | 1.0 | +4 |
| Karnovsky | 1.5 | +4 |
| Formalin + 1.0)<br>Glutaraldehyde 1.5) | 2.5 | +4 |

18 explants were used in all

All incubations were for 10 minutes

eyepieces contained a squared graticle unit, the side of each square measuring 0.1 mm, the number of silver grains in each of ten squares was counted for both mucous and serous cells. A total of 20 squares was counted for each slide and two slides for each fixative were examined. The results are given in Chapter III.

For fixation, the explants were taken from the incubator and put immediately into the fixative. After ten minutes, they were cut into 1-2 mm cubes and replaced in fixative for the remainder of the fixation period. The tissue blocks were processed and embedded in Araldite as outlined in Table II-3.

Cutting One μm sections were cut from the blocks using an LKB Ultratome III and a glass knife and then stained with 1% Toluidine blue in 1% sodium tetraborate. These sections were examined to see if any gland was present within the block and whether preservation of tissue was adequate. Further 1 μm sections were also cut for light microscopic autoradiography. If submucosal glands were present, serial sections of a regular light gold interference colour ( ~~1000Å) were cut for high resolution autoradiography. Care was taken to obtain the light gold colour in an attempt to standardize section thickness throughout the experiment. Salpeter (1967) has shown that judgement

of section thickness in the water bath around the knife is accurate to 10-15%. Slightly thicker sections are used for electron microscopic autoradiography, than for routine electron microscopy, as this helps to increase the contrast of the section and also increases the amount of radioactivity present.

Sections were also obtained for routine electron microscopy.

## Autoradiography of 1 μm sections

One μm sections were cut to check the effectiveness of the radioactive label. Glass slides were cleaned in chromic acid, washed in running water for one hour, rinsed in distilled water and dried. Two or three sections, 1 μm thick, were placed on to the slide. The sections were coated, using a dipping technique (see page 76 ), under a red light, and then dried and left to expose in a light-tight box at room temperature for four-eight days. The emulsion was then developed in Kodak D-19 developer for five minutes at 20°C, fixed in Kodafix, diluted 1 in 3 with distilled water, for two minutes and washed thoroughly in distilled water. The sections were stained with 1% Toluidine blue in borax and examined with the light microscope using an oil immersion lens. A well labelled 1 μm section at four days indicated a four week exposure for electron microscopic autoradiographs.

## High resolution autoradiography

The method used in the present studies is essentially similar to that described in 1964 by Salpeter and Bachmann. Microscope slides were cleaned in chromic acid, washed in running tap water for one hour, rinsed in distilled water and oven dried. These were then dipped into a Koplin jar containing 0.5% formvar in chloroform and again oven dried. The slide was then coated with a thin layer of carbon using an A.E.I. evaporation unit. Ribbons of sections were then placed on the slide. The sections were left in the lower third quarter of the slide, as this was the region where the monolayer of silver halide grain was found (vide infra).

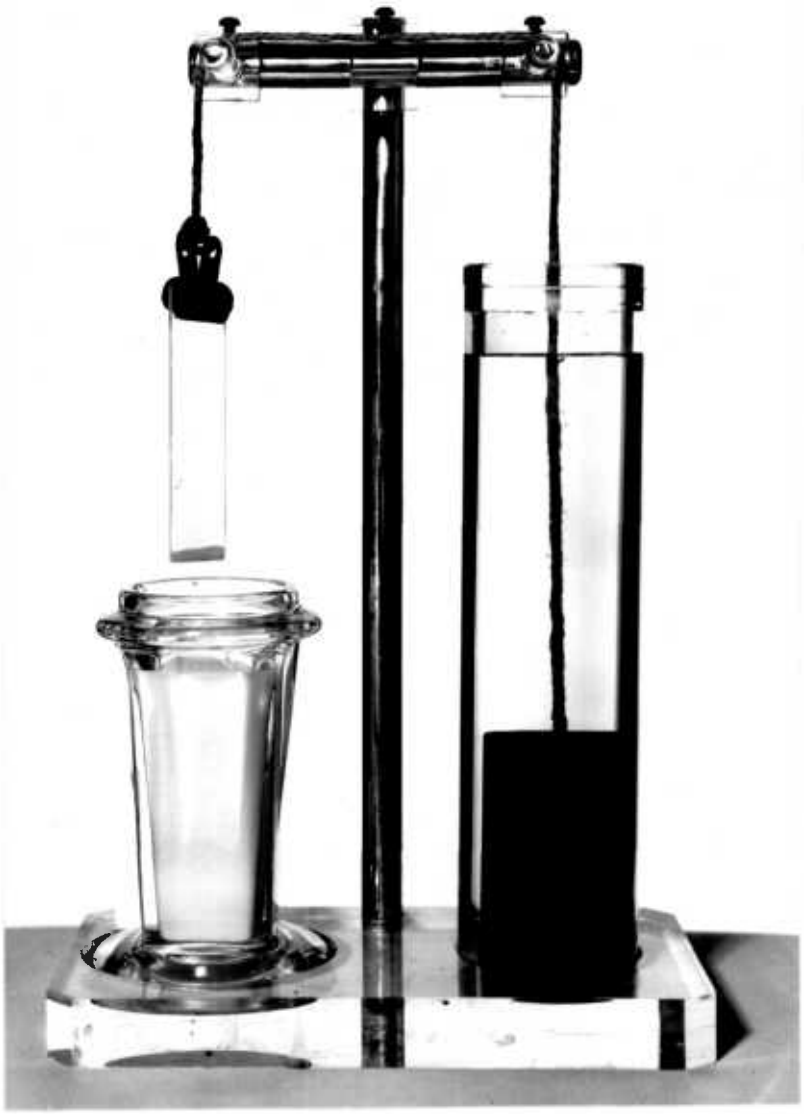
## Application of emulsion

A monolayer of silver halide grains was achieved using Ilford L4 emulsion and a simplified dipping apparatus (Kopriwa, 1966), under a red safety light. The apparatus comprised a Koplin jar, in which the emulsion was placed, a clamp stand, over which was placed a piece of string, one end of which was attached to a clip which held a slide and the other end to a lead weight, in an oil dashpot; this regulated the withdrawal rate of the slide from the emulsion (Figure II-2). The slide for coating was attached to the apparatus by the clip and put into the emulsion, when the slide was released it was withdrawn at a regular, even and reproducible rate. The rate at which

FIGURE II-2

Apparatus used for coating the electron microscope sections, mounted on slides, with a monolayer of silver halide crystals.

FIGURE II-2



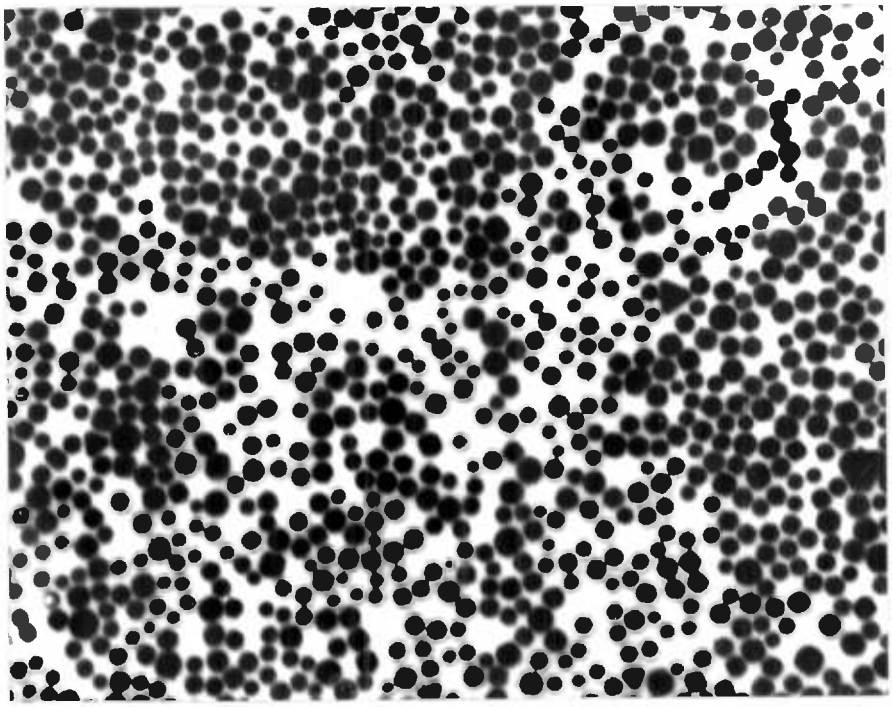
the slide is withdrawn has a bearing on the emulsion thickness. Several dilutions of emulsion were tried until a monolayer of silver halide grains was produced. It was found that if 40 ml of distilled water at 50°C was added to 10g of Ilford L4 emulsion, stirred thoroughly and left to cool for ten minutes before the slide was dipped, a tightly packed monolayer was formed in the third quarter of the slide. The presence of the monolayer was checked by examining the layer in the electron microscope (Figure II-3).

For 1 μm sections, Ilford L4 emulsion was diluted 1:1 with distilled water at 50°C and left for ten minutes before the slide was dipped.

Storage of emulsion

It was found during the course of these experiments, that storage of emulsion in the same refrigerator as osmium tetroxide solution caused fixation of the undeveloped silver halide crystals, resulting in loss of silver grains in the final autoradiograph. For this reason emulsion and osmium tetroxide were stored in separate refrigerators.

FIGURE II-3



Exposure

After dipping, the slides were left to dry for 30-60 minutes and then stored in a light-tight box containing silica gel at room temperature for the appropriate time.

Development and fixation

After exposure the slides were developed in Kodak Microdol-X developer at 20°C for three minutes. Caro and Van Tubergen (1962) found that at three minutes a plateau was reached for silver grains and further development produced an increase in background. Development of the latent image produces long filamentous grains, growing out of the surface of the crystal. Present within the silver halide crystal are a number of atoms of metallic silver, called the 'latent-image', in the presence of a weak reducing agent, i.e. developer; these will catalyse the conversion of the whole crystal into silver, the undeveloped crystals are dissolved out in the fixer. Fixation was carried out using Kodafix, diluted 1 in 3 with distilled water for two minutes. The slides were then washed thoroughly in distilled water.

Mounting of sections on grids

The slides were drained until nearly dry, then with a sharp point, a square was scored into the emulsion and carbon film around the section. A drop of 10% hydrofluoric acid was carefully placed to one side of this square, this was then drawn into the score mark and allowed to seep under the section and lift it. The section was then transferred to a Petri dish containing distilled water and mounted on to the shiny side of a copper grid (LKB Instruments Limited, 200 mesh, 85 μm spaces).

## Controls

Two controls were tested with each group of autoradiographs.

1. A block of unlabelled bronchial submucosal gland was sectioned, processed for autoradiography and examined with the electron microscope. This was a check for positive chemography.

2. A 'prepared' slide was dipped into the emulsion, dried and fogged by exposing to the light. The emulsion was developed, fixed and examined in the electron microscope. This was a check for negative chemography and for emulsion efficiency.

## Chemography

This is the chemical interaction of the source with the emulsion layer. Two types of chemography are recognized, positive and negative. Positive chemography is anatomical in distribution and may be related to specific cells, this is demonstrated by the presence of grains in the negative control. Negative chemography is generalised fading caused by uneven drying of the emulsion or may be caused by the tissue. It is recognized by a regional lowering in number of grains in the positive control. In the present studies neither negative nor positive chemography was found.

## Staining

The sections were stained with alcoholic uranyl acetate followed by lead citrate (Reynolds, 1963) after mounting on to copper grids. The lead citrate dissolved away the gelatin in the emulsion layer; some workers have suggested that this may cause movement of the developed silver grain, therefore an unstained section was also examined. No movement of grains was seen.

## Light Microscopy

Explants were fixed in buffered formalin (Lamb, 1969) and processed for light microscopy. Sections were cut at 4 μm and one stained with haematoxylin and eosin and another with the Alcian blue/periodic acid Schiff technique (Jones and Reid, 1973). The sections were examined for tumour invasion and the gland/wall ratio was obtained. Light microscopic autoradiography was carried out, but because of the high dose of radioactive precursor used for the experiments, the material was found to be unsuitable.

## Light microscopic autoradiographs of 1 μm sections

A separate light microscope experiment was carried out because of the unsuitability of the 4 μm sections. Explants were incubated in either 50μCi/ml $H^3$-threonine or 25μCi/ml $H^3$-glucose for 20 minutes to eight hours at 37°C (see page 105 ). Explants were then fixed overnight at 4°C, Processed and embedded in Araldite (Table II-3).

One μm sections were cut and autoradiographs obtained after ten days exposure. The sections were examined with an oil immersion lens (X100) and X8 eyepieces.

The method of assessment of uptake by acini is given with the results for $H^3$-threonine, in Chapter IV and for

$H^3$-glucose, in Chapter V.

Examination of electron microscopic autoradiographs

The sections were examined using an A.E.I. EM6B. Plates were taken at a magnification of 5,000. These were taken in a random manner, orientation of the section was not attempted. At least eight different micrographs of serous cells were taken, these did not overlap and were taken in the first region of serous cells encountered and included basal, apical and mid-regions of cells; mucous cells were examined in a similar manner. The photographic plates (Ilford EM5 plates) were developed in Universal PQ Developer (Johnson's of Hendon) diluted 1 in 5 with water and fixed in Kodafix, diluted 1 in 4 with water, for five minutes at 20°C.

Enlarging of the plates was carried out using a Leitz Focomat IIC at a magnification of 5, making the final print magnification X25,000; this was printed on to 15 x 12 inch Kodak single weight, glossy, bromide paper. The prints or micrographs were developed in Ilford Contrast FF diluted 1 in 5 with water and fixed in Kodafix diluted 1 in 8 with water at 20°C.

## Quantification of electron microscopic autoradiographs

Two methods were used for quantifying the electron microscopic autoradiographs - 1) the circle analysis of Williams (1969) and 2) the hypothetical grain analysis of Blackett and Parry (1973). This thesis deals mainly with the circle analysis of Williams, the latter, recently described method is included for the sake of completeness and the results are therefore not as comprehensive.

When collecting data from electron microscopic autoradiographs several requirements should be considered.

1. Resolution The resolution of the technique is defined as the radius of a circle within which 50% of the developed grains will fall. Salpeter et al (1969), have calculated that for a 1,000Å section, using Ilford L4 emulsion (1,400Å in thickness) and developing in Microdol-X, the resolution or half-distance (HD) is 1,600Å. The half distance (HD) is equal to the distance from a hot line source of tritium within which half of the developed grains fall. For a point source, half radius (HR) is used and this is equal to 1.7 HD.

The method used for this present experiment is identical to that quoted above, therefore the HD equals

1,600Å, and the HR, 2,720Å. Thus circles of 14.0 mm are approximately equal to the HR of this experiment using prints at a magnification of X25,000.

2. Section thickness In order to allow comparison between experiments, care must be taken in obtaining uniform and even sections. In these present experiments, serial sections of a pale gold colour were used.

3. Emulsion thickness A tightly packed monolayer is essential for this technique to give maximum resolution. If the monolayer is not tightly packed, the radioactive decays may miss the silver halide crystals completely and if too tightly packed, i.e. more than one layer - more than one latent image may be formed, accompanied by a loss of resolution.

With these features established, a method of analysis, similar to that of Williams (1969), can be described. This will now be referred to as the circle analysis.

## Circle analysis

Random sampling methods were applied because of the small amount of tissue used for electron microscopy. Date was collected in three ways, from each of two specimens at each incubation time.

Circle analysis

A list of organelles (single items) found in the mucous and serous cells was drawn up, to this was added a list of combination or junctional regions over which circles (14.0 mm) diameter could possibly fall. Some groups, since few circles fell over them, were later put into the 'others' group (Table II-5). It should be noted that the ribosomes and cellular ground substance were included with the endoplasmic reticulum (ER), that the term vacuole was applied to the pale electron lucent granules situated near to the Golgi apparatus and that the latter included both lamellae and some small associated vesicles; the majority of the smooth-surfaced vesicles were included in the junctional region, ER/Golgi apparatus. Canalculi were only found in association with serous cells.

The data was collected with the aid of a screen or a grid; this consisted of a series of circles, 14.0 mm diameter, drawn on to a 14 x 18" X-ray film at one inch intervals (Figure II-4), at the centre of each circle was a dot. The circles were placed one inch apart ( ~~ 1.1 μm), so that in general no organelle had more than one point fall on it (Weibel, 1972). A regular grid was used as no problems with periodicity of cell structure arose. The grid was allowed to settle in a random manner over the

TABLE II-5

SPECIMEN ANALYSIS FOR THE MUCOUS CELL AFTER INCUBATION FOR 40 MINUTES IN $H^3$-THREONINE, SHOWING THE RESULTS OF THE CIRCLE, POINT AND GRAIN ANALYSIS AND EXPECTED GRAINS FOR THE $x^2$ TEST

| ORGANELLES | No circle | % | No point | % | Actual grains | % | Expected grains |
|---|---|---|---|---|---|---|---|
| Single items | | | | | | | |
| Endoplasmic reticulum (ER) | 17 | 4.4 | 91 | 23.7 | 11 | 8.7 | 5.6 |
| Golgi apparatus - lamellae | 4 | 1.0 | 10 | 2.6 | 10 | 7.9 | 1.3 |
| vacuoles and vesicles | 29 | 7.6 | 22 | 5.7 | 29 | 23.0 | 9.5 |
| granules | 113 | 29.5 | 237 | 61.9 | 13 | 10.3 | 37.2 |
| vacuole | 0 | 0.0 | 5 | 1.3 | 0 | 0.0 | 0.0 |
| nucleus | 7 | 1.8 | 12 | 3.1 | 0 | 0.0 | 2.3 |
| canaliculus | not used for the mucous cell | | | | | | |
| Junctional regions | | | | | | | |
| ER/Golgi apparatus | 6 | 1.6 | 0 | 0.0 | 3 | 2.4 | 2.0 |
| ER/granule | 171 | 44.6 | 0 | 0.0 | 38 | 30.2 | 56.2 |
| ER/vacuole | 2 | 0.5 | 0 | 0.0 | 0 | 0.0 | 0.7 |
| ER/nucleus | 9 | 2.3 | 0 | 0.0 | 3 | 2.4 | 2.9 |
| ER/mitochondria | 5 | 1.3 | 2 | 0.5 | 4 | 3.2 | 1.6 |
| ER/membrane | 11 | 2.9 | 1 | 0.3 | 5 | 4.0 | 3.6 |
| ER/lysosome | 3 | 0.8 | 3 | 0.8 | 0 | 0.0 | 1.0 |
| ER/canaliculus | not used for the mucous cell | | | | | | |
| others | 6 | 1.6 | 0 | 0.0 | 10 | 7.9 | 2.0 |
| Total | 383 | | 383 | | 126 | | |

The figures in the point column for the junctional regions represent points found over the organelle other than endoplasmic reticulum.

FIGURE II-4

Grid used for the circle analysis. The organelle or the junctional region surrounded by circles (circle analysis) and organelle over which the central point fell (point analysis), were noted. X 16,500.

FIGURE II-5

Grid used for the hypothetical grain analysis. The organelle under each cross-over point was considered as the source of a radioactive disintegration (e.g. as shown here Golgi apparatus). A computer derived direction and distance was measured from the source and the region surrounded by the circle was considered as the position of a hypothetical grain (e.g. as shown here N.W. 32.0 mm = Golgi apparatus to nucleus). X 16,500.

FIGURE II-4

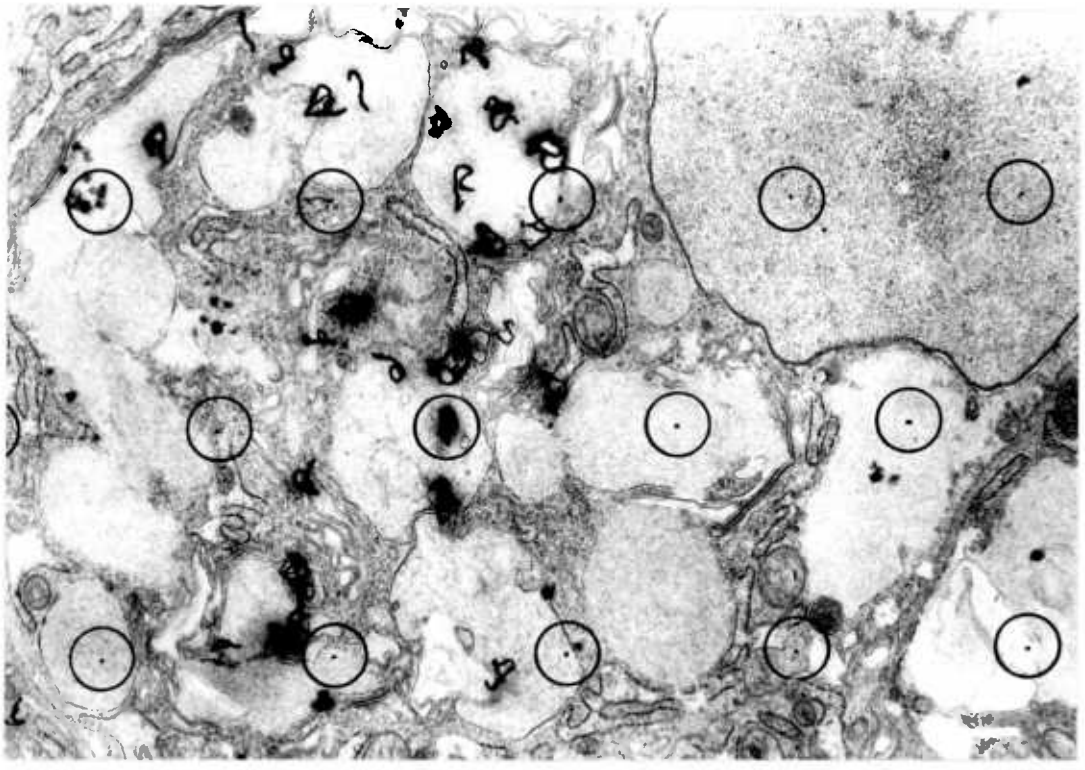
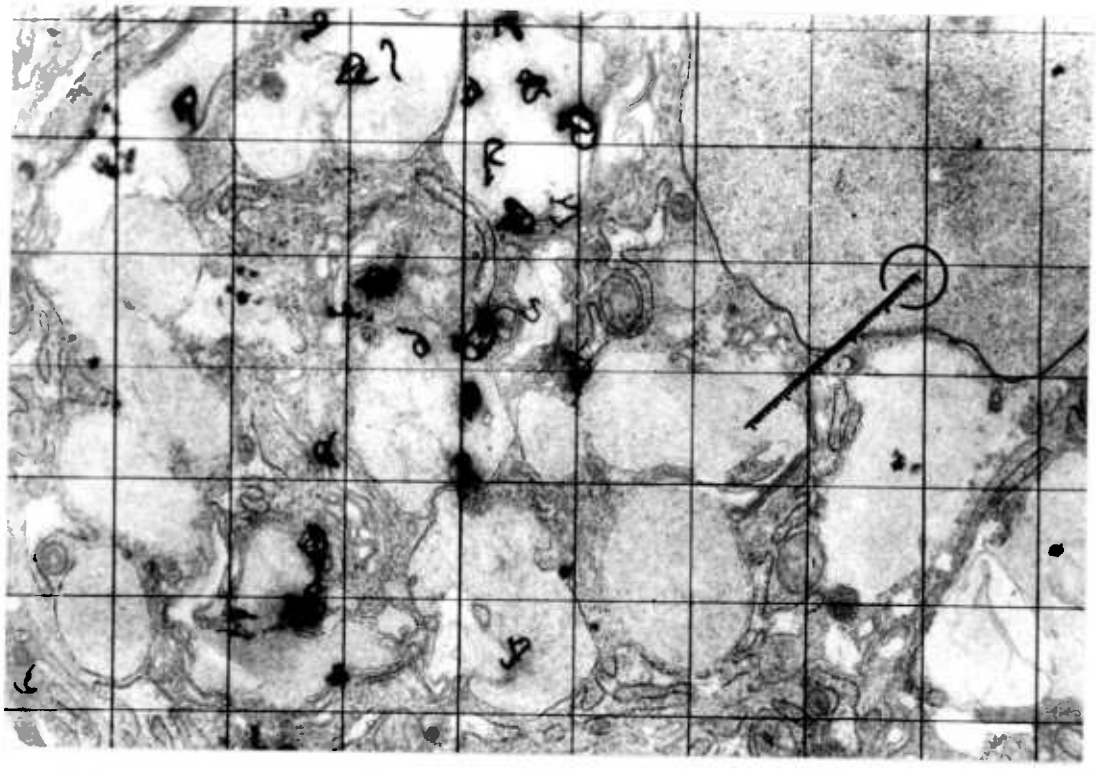


FIGURE II-5



print and data of two types was collected simultaneously.

Point analysis

Additional information was obtained by noting the organelle over which the centre point of each circle fell. This data was collected according to the organelles shown in Table II-6.

Grain count

The position of the silver grains within the cell was noted. Circles, 14.0 mm diameter, were placed over each silver grain and the organelle or junctional region enclosed by the circle was noted in an identical manner to that of the circle analysis.

Background count

Background counts for all specimens showed less than one silver grain per 1,000 sq μm.

Using the circle analysis and the actual grain count, the expected grain frequency (Table II-5) was calculated and the Chi-squared test for significance applied. In this way it can be determined if the

TABLE II-6

LIST OF ORGANELLES USED IN THE POINT ANALYSIS OF BOTH MUCOUS AND SEROUS CELLS

SINGLE ITEMS

Endoplasmic reticulum (ER) - includes ribosomes and cellular ground substance

Granule

Golgi apparatus

Vacuole

Nucleus

Mitochondrion

Membrane

Lysosome

Others

Canaliculus - serous cell only

silver grains are associated with a particular organelle or if they are randomly distributed.

## The hypothetical grain method

The Blackett and Parry method (1973), combines the circle analysis of Williams (1969) and the mathematical considerations of resolution described by Salpeter et al (1969). Thus, the method assesses the activity within regions of a cell and takes into account the geometric shapes and patterns of these regions. Using this method, data was obtained for four groups - (I) endoplasmic reticulum (ER), this group included all organelles save (II) granules (III) Golgi apparatus and (IV) nucleus. Data was collected in two ways.

### Actual grain analysis

This was carried out in an identical manner to the grain count described for the circle analysis.

### Hypothetical grain analysis

A grid of one inch squares was superimposed over a print, magnification X25,000. Each cross-over point

of the grid was considered to be the site of a radioactive disintegration (similar to the point analysis of Williams, 1969). Random distances and directions obtained from a computer programme, were then measured from these sites of radioactive disintegration (Figure II-5) and the new position was considered to be the hypothetical silver grain produced by this disintegration. A circle, 14.0 mm diameter, was placed over this hypothetical grain and its location and the source of the disintegration noted. This data was best noted in the form of a table (Table II-7), the horizontal column shows the source of the hypothetical grain and the vertical, the hypothetical grain. The 'cross-fire' between each region is shown as the scattering along the row. The Table is easily modified to exhibit different distributions of hypothetical grains by varying the activity within a region, e.g. if it is thought that there is twice as much activity in the granules as in other regions, one would multiply the granule row by two. The final column (Table II-7) shows the grains per unit area for each of the four organelles and shows the Golgi apparatus to be the most active for this time. The site of the hypothetical grains are then altered according to these figures, the expected grains calculated and the Chi-squared test for significance applied to show the goodness of fit, i.e. the similarity between the actual and expected grains. Blackett and Parry have recently designed a computer programme for this calculation.

TABLE II-7

SPECIMEN ANALYSIS FOR THE SEROUS CELL AFTER 60 MINUTE INCUBATION IN $H^3$-THREONINE, USING THE HYPOTHETICAL GRAIN ANALYSIS

| Source of hypothetical grain | SITE OF HYPOTHETICAL GRAIN | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ER | ER/ granule | ER/Golgi apparatus | ER/ nucleus | granule | granule/ Golgi apparatus | Golgi apparatus | nucleus | Total grains | Grains/* unit area |
| Endoplasmic reticulum(ER) | 380 | 87 | 28 | 4 | 10 | 3 | 12 | 0 | 524 | 0.12 |
| granule | 16 | 60 | 2 | 1 | 50 | 1 | 1 | 0 | 131 | 0.05 |
| Golgi apparatus | 9 | 6 | 36 | 0 | 1 | 2 | 52 | 0 | 106 | 0.28 |
| nucleus | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 19 | 23 | 0.05 |
| Total | 405 | 153 | 66 | 9 | 61 | 6 | 65 | 19 | 784 | |
| Actual grain distribution | 49 | 16 | 17 | 0 | 4 | 3 | 13 | 1 | 103 | |
| Expected grains | 50 | 16 | 14 | 1 | 4 | 1 | 16 | 1 | 103 | |
| $x^2$ | 0 | 0 | 0.8 | 0 | 0 | 0 | 0.7 | 0 | $x^2_4 = 1.5$ | |
| | | | | | | | | | $p = 85\%$ | |

* = Grains per unit area = activity ascribed to each organelle to give the best fit for actual and hypothetical grains, i.e. the lowest $x^2$ value

## Chemical Analysis

In order to establish whether the radioactive precursors were being incorporated into the carbohydrate and protein moieties of the human bronchial submucosal gland, a series of precipitation studies were carried out.

Explants were incubated for one hour at 37°C with either 250μCi/ml $H^3$-threonine or $H^3$-glucose. The explants were washed three times in cold rinses, blotted and weighed, then chopped into small pieces and placed in a test tube with 1 ml of distilled water. The tissue pieces were then sonicated at 0°C for two minutes (periods of 30 seconds) at 20 kilocycles/second (M.S.E. Ultrasonicator). One ml of pronase in 0.1M TRIS buffer was added to the test tube to give a final dilution of 1 mg/ml. This was incubated at 37°C overnight and then spun at 2,500g for 30 minutes. The supernatant B (pronase) was removed from the precipitate A, which was then washed twice with 5 ml of distilled water and spun as above; all three supernatants were pooled (supernatant B).

## Trichloracetic acid precipitation of proteins

Trichloracetic acid (50%) was added to supernatant B to give a final concentration of 5%, and then mixed by inversion and spun at 2,500g for 30 minutes. The supernatant E, was removed from precipitate B, and

washed twice with 5 ml of 5% trichloracetic acid (supernatants C and D), these were all saved.

Ethanolic precipitation of glycoproteins

Supernatant E was neutralized with 50% sodium hydroxide and then three times the volume of ethanol was added. This was left at 4°C for two hours and then spun at 2,500g for 30 minutes. The supernatant F was removed from precipitate E and then the precipitate washed twice with 75% ethanol. All three supernatants were pooled (supernatant F).

From each of the supernatants, 0.25 ml was taken and diluted with 10 ml of Insta-Gel Emulsifier for scintillation counting.

All the above precipitates (A, B and E) were dissolved in 1 ml of 0.5N sodium hydroxide and 1 ml aliquots from each of the supernatants (C, D and F) were dialysed against distilled water for four days at 4°C. After dialysis the fluids were weighed and 0.25 ml was taken from each and diluted with Insta-Gel for scintillation counting.

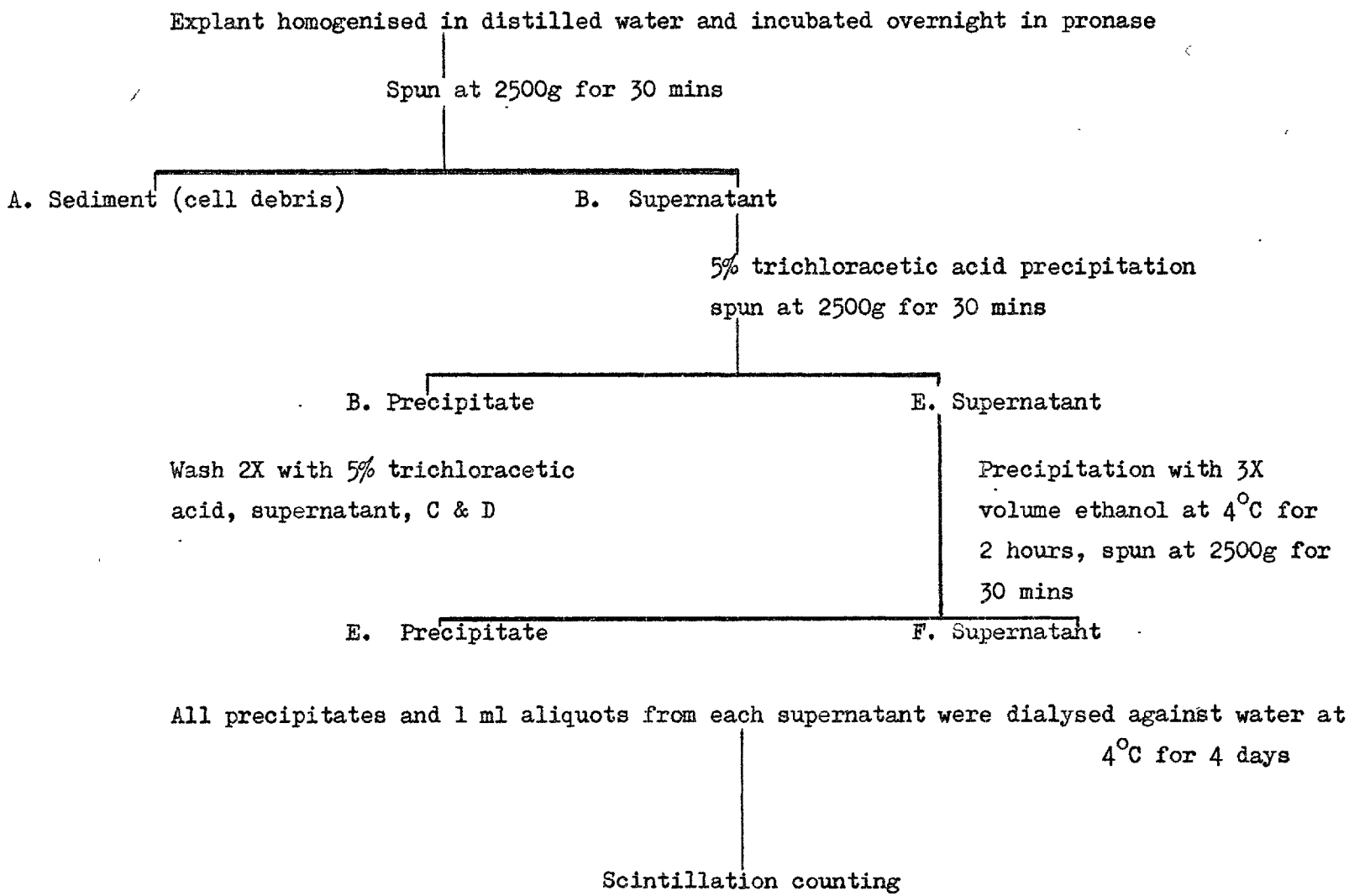
Puromycin

The above chemical analysis was also carried out after the addition of1mg/ml of puromycin dihydrochloride to the incubation medium.

For summary of the methods see Scheme II-1. Results are given in Chapter III.

Scheme II-1

CHEMICAL ANALYSIS



# CHAPTER III

## SELECTION OF OPTIMUM FIXATIVE FOR EXPLANTS AFTER INCUBATION WITH EITHER $H^3$-THREONINE OR $H^3$-GLUCOSE - INCLUDING CHEMICAL ANALYSIS

Several experiments were carried out to determine the best fixative for use with explants, after incubation with either $H^3$-threonine or $H^3$-glucose; that is the one giving the best morphological preservation yet not binding the radioactive precursor to the tissue in a non-specific manner. Peters and Ashley (1967) have shown that radioactive amino-acids ($H^3$-leucine) are bound to tissue non-specifically by glutaraldehyde and to a lesser amount by osmium tetroxide, but not by formalin.

## SCINTILLATION COUNTING

For three different fixatives - formalin, glutaraldehyde and a modified Karnovsky's fixative - the ability to bind the radioactive precursor to the tissue in a non-specific way has been investigated. The processing fluids and combusted tissue were examined in order to estimate the amount of radioactivity lost during each stage of processing (Table III-1).

Because of the inhomogeneity of the human bronchial wall, the amount of radioactivity retained in the tissue and the degree of non-specific binding is difficult to assess, but a trend can be detected. In an effort to counteract this inhomogeneity, the posterior wall of the bronchus was used, as this region contains most gland and no cartilage.

The results show a marked increase in the amount of

TABLE III-1

$H^3$-THREONINE AND $H^3$-GLUCOSE RETAINED BY THE TISSUE AND BY EACH FLUID USED DURING PROCESSING FOR ELECTRON MICROSCOPY (EXPRESSED AS A PERCENTAGE OF TOTAL RECOVERED)

| | $H^3$-Threonine | | | $H^3$-Glucose | | |
|---|---|---|---|---|---|---|
| | Form. | Glut. | Karn. | Form. | Glut. | Karn. |
| Tissue | 5.4 | 55.3 | 21.0 | 34.1 | 28.2 | 41.0 |
| Fixative | 92.0 | 37.2 | 72.3 | 56.4 | 63.1 | 49.8 |
| Buffer | 2.0 | 4.0 | 3.8 | 7.2 | 6.5 | 7.5 |
| Osmium tetroxide | 0.3 | 2.0 | 2.0 | 0.7 | 0.8 | 0.5 |
| Distilled water | 0.1 | 0.4 | 0.2 | 0.5 | 0.4 | 0.1 |
| 70% methanol | 0.1 | 0.4 | 0.3 | 0.5 | 0.4 | 0.2 |
| 90% methanol | 0.1 | 0.4 | 0.3 | 0.4 | 0.5 | 0.4 |
| 100% methanol(X 3) | | 0.2 | 0.1 | 0.2 | 0.1 | 0.3 |
| Epoxy propane | | 0.1 | | | | 0.2 |

Form. = Formalin
Glut. = Glutaraldehyde
Karn. = Karnovsky

non-specific $H^3$-threonine retained by the tissue after fixation in Karnovsky's fixative and even more after glutaraldehyde, indicating that formalin is the fixative of choice for $H^3$-threonine.

Little difference was found in the amount of $H^3$-glucose retained in the tissue after any of the three fixatives (the small difference shown is probably due to the varying amount of gland in each explant). These results indicate that any of the three fixatives may be used to preserve the tissue after incorporation of $H^3$-glucose as none appear to cause non-specific binding.

Not all subsequent experiments included Karnovsky's fixative as it was felt that this fixative had no advantage over formalin alone.

## Cold rinse

The effect of using a cold rinse prior to fixation with either glutaraldehyde or formalin was investigated. It was thought that this might help to remove the unbound radioactive precursor from the tissue and thus minimize non-specific binding.

There was little difference in the percentage of total radioactivity (Table III-2) seen in the tissue

TABLE III-2

THE EFFECT OF A COLD RINSE PRIOR TO FIXATION AND PROCESSING AFTER INCUBATION WITH $H^3$-THREONINE OR $H^3$-GLUCOSE

(Percentages are given as in Table III-1)

| | $H^3$-threonine | | | | $H^3$-glucose | | | |
|---|---|---|---|---|---|---|---|---|
| | Formalin | | Glutaraldehyde | | Formalin | | Glutaraldehyde | |
| Tissue | 8.1 | 12.0 | 39.5 | 22.2 | 31.4 | 30.1 | 33.2 | 37.4 |
| Cold rinse | | 66.6 | | 36.5 | | 38.4 | | 32.5 |
| | | 85.0 | | 75.8 | | 66.0 | | 55.3 |
| Fixative | 89.8 | 18.4 | 56.5 | 39.3 | 66.0 | 27.6 | 59.4 | 22.8 |
| Buffer | 1.5 | 2.0 | 2.0 | 2.1 | 2.5 | 3.0 | 4.1 | 4.0 |
| Osmium tetroxide | 0.2 | 0.3 | 1.2 | 0.5 | 0.4 | 0.3 | 2.0 | 2.3 |
| Distilled water | 0.1 | 0.1 | 0.3 | 0.2 | 0.1 | 0.1 | 0.5 | 0.3 |
| 70% methanol | 0.1 | 0.2 | 0.3 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 |
| 90% methanol | 0.1 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.3 |
| 100% methanol(X 3) | 0.1 | 0.1 | 0.1 | 0.0 | 0.2 | 0.2 | 0.3 | 0.2 |
| Epoxy propane | | 0.2 | | | | | 0.1 | |

when a cold rinse was used. The fixative alone appeared to remove as much radioactive precursor as the cold rinse and fixative together, except in the case of $H^3$-threonine fixed in glutaraldehyde, where the cold rinse did remove rather more than the glutaraldehyde alone.

The scintillation counts show that for $H^3$-threonine, formalin is the most suitable fixative since glutaraldehyde and Karnovsky's fixative both are associated with non-specific attachment of the radioactive precursor to the tissue (Tables III-1 and 2).

A cold rinse is not necessary when $H^3$-glucose is used. On the other hand it would seem desirable before fixing tissue, labelled with $H^3$-threonine, in glutaraldehyde and also before chemical analysis, since in this case, the tissue is not fixed.

## Puromycin

Similar experiments to those reported on page 100 were carried out after the explants had been incubated for 60 minutes at 37°C in $H^3$-threonine or $H^3$-glucose to which puromycin had been added. Puromycin is an

inhibitor of protein synthesis (Yarmolinsky and De La Haba, 1959) and therefore was thought to reduce incorporation of $H^3$-threonine into the cellular secretion of the gland, thus reducing the amount of radioactivity left in the tissue.

The results (Table III-3) suggest that in the case of $H^3$-threonine, puromycin had little effect on the amount of $H^3$-threonine taken up by the tissue. It evidently therefore, effects synthesis. (see page also )

In the case of $H^3$-glucose, puromycin did appear to reduce the uptake, especially in the case of tissue fixed in formalin.

## LIGHT MICROSCOPIC EXAMINATION AFTER INCUBATION AT 4°C

The effects of six different fixation techniques were compared after incubation for ten minutes at 4°C with either $H^3$-threonine or $H^3$-glucose; the tissues were then studied using light microscopic autoradiography. The fixatives used were: glutaraldehyde, osmium tetroxide, Karnovsky's fixative, formalin for four hours, formalin for 18 hours and formalin with post-fixation in glutaraldehyde (Table III-4). The last two methods were used in an attempt to improve the morphological preservation obtained with

TABLE III-3

$H^3$-THREONINE AND $H^3$-GLUCOSE RECOVERED WITH (+) AND WITHOUT (-) ADDED PUROMYCIN (EXPRESSED AS A PERCENTAGE OF TOTAL RECOVERED)

| | $H^3$-threonine | | | | $H^3$-glucose | | | |
|---|---|---|---|---|---|---|---|---|
| Fixative used | Formalin | | Glutaraldehyde | | Formalin | | Glutaraldehyde | |
| Puromycin | - | + | - | + | - | + | - | + |
| Tissue | 11.3 | 17.5 | 37.5 | 43.6 | 36.4 | 18.1 | 37.5 | 31.9 |
| Fixative | 86.0 | 79.5 | 51.5 | 49.2 | 54.8 | 68.2 | 53.9 | 58.2 |
| Buffer | 2.3 | 2.0 | 5.5 | 4.4 | 6.2 | 9.6 | 6.4 | 7.4 |
| Osmium tetroxide | 0.2 | 0.5 | 3.5 | 1.9 | 0.9 | 1.0 | 0.8 | 1.0 |
| Distilled water | 0.1 | 0.1 | 0.5 | 0.3 | 0.5 | 0.7 | 0.4 | 0.4 |
| 70% methanol | 0.1 | 0.1 | 0.6 | 0.5 | 0.6 | 0.8 | 0.4 | 0.4 |
| 90% methanol | | 0.1 | 0.6 | 0.1 | 0.4 | 0.9 | 0.5 | 0.4 |
| 100% methanol (X3) | | 0.1 | 0.2 | | 0.2 | 0.5 | 0.1 | 0.2 |
| Epoxy propane | | 0.1 | 0.1 | | | 0.2 | | 0.1 |

TABLE III-4

THE DURATION, FIXATION AND TEMPERATURES USED FOR INCUBATION OF EXPERIMENTAL AND CONTROL EXPLANTS IN EITHER $H^3$-THREONINE OR $H^3$-GLUCOSE

| FIXATIVE | INCUBATION temp (°C) | FIXATION time(hrs) | FIXATION temp(°C) |
|---|---|---|---|
| Experimental | | | |
| 1. Glutaraldehyde | 4 | 1.5 | 18 |
| 2. Osmium tetroxide | 4 | 1.0 | 18 |
| 3. Karnovsky | 4 | 1.5 | 18 |
| 4. Formalin | 4 | 4.0 | 18 |
| 5. Formalin | 4 | 18.0 | 4 |
| 6. Formalin + Glutaraldehyde | 4 | 1 + 1.5 | 18 |
| Controls | | | |
| 7. Formalin | 37 | 4.0 | 18 |
| 8. Glutaraldehyde | 37 | 1.5 | 18 |
| 9. Formalin + glutaraldehyde (no added radioactive precursor) | 37 | 1 + 1.5 | 18 |

formalin for four hours (vide infra).

Using the light microscope and a squared graticule unit, counts were made in 20 fields of the number of grains that lay over mucous and serous acini. Background counts were carried out in the same manner over tissue free from gland. The mean count per field was obtained. These are shown in Table III-5, for $H^3$-threonine and Table III-6 for $H^3$-glucose, together with the background counts for each specimen.

Assuming that little penetration and incorporation of the label into macromolecules occurs during incubation at $4^{\circ}C$, any label that is detected can be ascribed to non-specific binding of free substrate to the tissue.

The Student's t-test was applied between formalin and each of the other fixatives, it being assumed from the previous work that formalin was the optimum fixative (Table III-7).

## $H^3$-threonine

After incubation in $H^3$-threonine (Table III-7), a significantly greater amount of label is bound to the tissue after fixation in glutaraldehyde and Karnovsky than formalin ($p = <0.001$); osmium tetroxide also binds more than formalin ($p = < 0.01$), but not as much as the other

TABLE III-5

MEAN GRAIN COUNTS OVER MUCOUS, SEROUS ACINI AND BACKGROUND AFTER INCUBATION WITH $H^3$-THREONINE AFTER VARIOUS FIXATION TECHNIQUES

| FIXATIVE | Mucous grains/field | | Serous grains/field | | Background grains/field | |
|---|---|---|---|---|---|---|
| Experimental at 4°C | | | | | | |
| 1. Glutaraldehyde | 8.5 | 0.233* | 13.0 | 0.330* | 40.1 | 0.553* |
| 2. Osmium tetroxide | 1.1 | 1.202 | 3.4 | 1.401 | 1.0 | 0.394 |
| 3. Karnovsky | 14.7 | 0.375 | 34.1 | 0.733 | 40.1 | 1.059 |
| 4. Formalin (4 hrs) | 0.5 | 1.290 | 1.0 | 1.670 | 0.8 | 0.416 |
| 5. Formalin (18 hrs) | 0.7 | 0.259 | 1.4 | 0.305 | 0.8 | 0.290 |
| 6. Formalin + glutaraldehyde | 0.6 | 0.220 | 1.3 | 0.330 | 1.9 | 0.458 |
| Controls at 37°C | | | | | | |
| 7. Formalin (4 hrs) | 2.7 | 0.334 | 1.6 | 0.399 | 0.9 | 0.326 |
| 8. Glutaraldehyde | 16.2 | 1.474 | 34.0 | 1.632 | 40.1 | 1.059 |
| 9. Formalin + glutaraldehyde (no added radioactive precursor) | 0.6 | 0.220 | 1.4 | 0.498 | 0.7 | 0.260 |

* = standard error of the mean

TABLE III-6

MEAN GRAIN COUNTS OVER MUCOUS, SEROUS ACINI AND BACKGROUND AFTER INCUBATION WITH $H^3$-GLUCOSE AFTER VARIOUS FIXATION TECHNIQUES

| FIXATIVE | Mucous grains/field | | Serous grains/field | | Background grains/field | |
|---|---|---|---|---|---|---|
| Experimental at 4°C | | | | | | |
| 1. Glutaraldehyde | 2.2 | 0.489* | 2.6 | 0.258* | 1.5 | 0.428* |
| 2. Osmium tetroxide | 0.9 | 0.233 | 1.5 | 0.370 | 0.9 | 0.314 |
| 3. Karnovsky | 1.2 | 0.103 | 1.6 | 0.401 | 0.9 | 0.348 |
| 4. Formalin (4 hrs) | 0.7 | 0.213 | 1.0 | 0.451 | 0.4 | 0.163 |
| 5. Formalin (18 hrs) | 0.8 | 0.233 | 1.1 | 0.432 | 0.5 | 0.213 |
| 6. Formalin + glutaraldehyde | 0.7 | 0.266 | 1.0 | 0.397 | 0.5 | 0.242 |
| Controls at 37°C | | | | | | |
| 7. Formalin (4 hrs) | 8.4 | 1.739 | 7.3 | 1.160 | 1.6 | 0.339 |
| 8. Glutaraldehyde | 7.3 | 2.680 | 5.9 | 0.900 | 2.3 | 0.558 |
| 9. Formalin + glutaraldehyde (no added radioactive precursor) | 0.7 | 0.212 | 1.1 | 0.410 | 0.4 | 0.194 |

* = standard error of the mean

TABLE III-7

COMPARISON OF FIXATIVES ASSUMING

THAT FORMALIN IS THE OPTIMUM

| | $H^3$-threonine | | $H^3$-glucose | |
|---|---|---|---|---|
| | Mucous | Serous | Mucous | Serous |
| Formalin at 4°C/Formalin + Glutaraldehyde at 37°C (no added radioactive precursor) | | | | |
| Formalin/Glutaraldehyde at 37°C | $p = < 0.001$ | $p = < 0.001$ | $p = < 0.01$ | $p = < 0.01$ |
| Formalin/Osmium tetroxide at 4°C | | $p = < 0.01$ | | |
| Formalin/Karnovsky at 4°C | $p = < 0.001$ | $p = < 0.001$ | | |
| Formalin (4 hrs)/ Formalin (18 hrs) at 4°C | | | | |
| Formalin at 4°C/Formalin + Glutaraldehyde at 37°C | | | | |
| Formalin (4 hrs) at 4°C/ Formalin at 37°C | $p = < 0.001$ | | $p = < 0.001$ | $p = < 0.001$ |
| Glutaraldehyde at 4°C/ Glutaraldehyde at 37°C | $p = < 0.001$ | $p = < 0.001$ | $p = < 0.001$ | $p = < 0.001$ |
| Formalin (4 hrs)/ Glutaraldehyde at 37°C | $p = < 0.001$ | $p = < 0.001$ | $p = < 0.001$ | $p = < 0.001$ |
| Formalin (4 hrs)/Formalin + Glutaraldehyde at 37°C (no added radioactive precursor) | | | | |

Only the figures that are statistically significant are given

two and this only over serous acini. Results were similar for formalin at four hours, formalin at 18 hours and formalin followed by secondary fixation in glutaraldehyde.

After incubation of explants in $H^3$-threonine at 37°C, there was a significant increase of label over both mucous and serous acini in comparison with glutaraldehyde at 4°C and formalin at 37°C ($p = <0.001$). Explants fixed in formalin showed significantly more label over the mucous acini only after incubation at 37°C than at 4°C.

Background counts

For $H^3$-threonine, significant differences between the background counts and those over mucous and serous acini were found for glutaraldehyde and Karnovsky's fixative ($p = <0.001$) and for the serous acini alone, after fixation in osmium tetroxide ($p = <0.01$). All three formalin fixation techniques showed no significant difference.

$H^3$-glucose

For $H^3$-glucose, of all fixatives tested, only glutaraldehyde at both 4°C and 37°C bound significantly more radioactive precursor to both mucous and serous acini ($p = <0.001$, Table III-7), than formalin either at 4°C or 37°C.

After incubation with $H^3$-glucose at 37°C and fixation in either formalin or glutaraldehyde there was significantly more radioactive label over both mucous and serous acini in comparison to incubation at 4°C ($p = <0.001$, Table III-7).

Background counts

For all fixatives, no significant difference between the background counts and those over mucous and serous acini was found, when explants were incubated at 4°C.

The number of grains lying over mucous and serous acini was also compared. For $H^3$-threonine, all fixatives with the exception of formalin showed a significantly greater number of grains to lie over the serous acini (Table III-8).

TABLE III-8

COMPARISON OF GRAIN COUNTS FOR MUCOUS AND SEROUS ACINI AFTER INCUBATION WITH $H^3$-THREONINE AND $H^3$GLUCOSE

| FIXATIVE | $H^3$-THREONINE Mucous/Serous |
|---|---|
| Experimental | |
| 1. Glutaraldehyde at 4°C | $p = < 0.001$ |
| 2. Osmium tetroxide at 4°C | $p = < 0.02$ |
| 3. Karnovsky at 4°C | $p = < 0.001$ |
| 4. Formalin at 4°C (4 hrs) | |
| 5. Formalin at 4°C (18 hrs) | |
| 6. Formalin + glutaraldehyde at 4°C | |
| Controls | |
| 7. Formalin at 37°C | |
| 8. Glutaraldehyde at 37°C | $p = < 0.001$ |
| 9. Formalin + glutaraldehyde at 37°C (no added radioactive precursor) | |

Only the figures that are statistically significant are given. There was no statistical difference of grain counts over mucous and serous cells with $H^3$glucose.

The results after incubation at 4°C are similar to those of the scintillation counts (see page 104). When using $H^3$-threonine, formalin would seem to be the fixative of choice, since glutaraldehyde and Karnovsky's fixative each cause non-specific binding of the $H^3$-threonine to the tissue. Osmium tetroxide seems to be better than glutaraldehyde and Karnovsky's fixative, but it is rarely used for electron microscopic autoradiography since it is thought to give rise to chemography (see page 81 ), especially when, as in our case, there is no carbon layer to protect the section from the emulsion.

When using $H^3$-glucose either formalin or Karnovsky's fixative seem to be adequate.

From all the above results it was decided to fix the explants incubated in either $H^3$-threonine or $H^3$-glucose in formalin: a cold rinse was not necessary (for duration of fixation, vide infra).

## MORPHOLOGICAL PRESERVATION

The morphological preservation obtained after fixation in formalin for either four or 18 hours, followed by post-fixation in osmium tetroxide, or formalin followed by post-fixation in glutaraldehyde and then osmium tetroxide, was

examined in order to establish which of the three gave the most similar ultrastructural picture to that of glutaraldehyde. Glutaraldehyde is the fixative most commonly used for tissue to be examined with the electron microscope.

The mucous cell showed little ultrastructural difference after fixation in formalin to that of glutaraldehyde. All granules appeared electron lucent and some were confluent.

The serous cell did show some differences. In general all organelles were similar, but marked differences were found for the granules. After glutaraldehyde fixation, the granules appear as discrete, dense homogeneous structures (Figure III-1). Fixation in formalin for fours, caused the granules to appear electron lucent, with disruption of their limiting membrane although the granules were still discrete; a similar picture was obtained for formalin followed by post-fixation in glutaraldehyde. Fixation in formalin for 18 hours produced better results. All secretory granules were discrete and homogeneous and many, although not all, were dense (Figure III-2). This enabled easy identification of serous and mucous cells.

Thus it was decided to use formalin fixation for 18 hours for the explants.

FIGURE III-1

Part of a serous acinus around a canaliculus (ca). After primary fixation in glutaraldehyde and post-fixation in osmium tetroxide, the serous granules characteristically are seen as electron dense, discrete structures. X 10,500.

FIGURE III-1

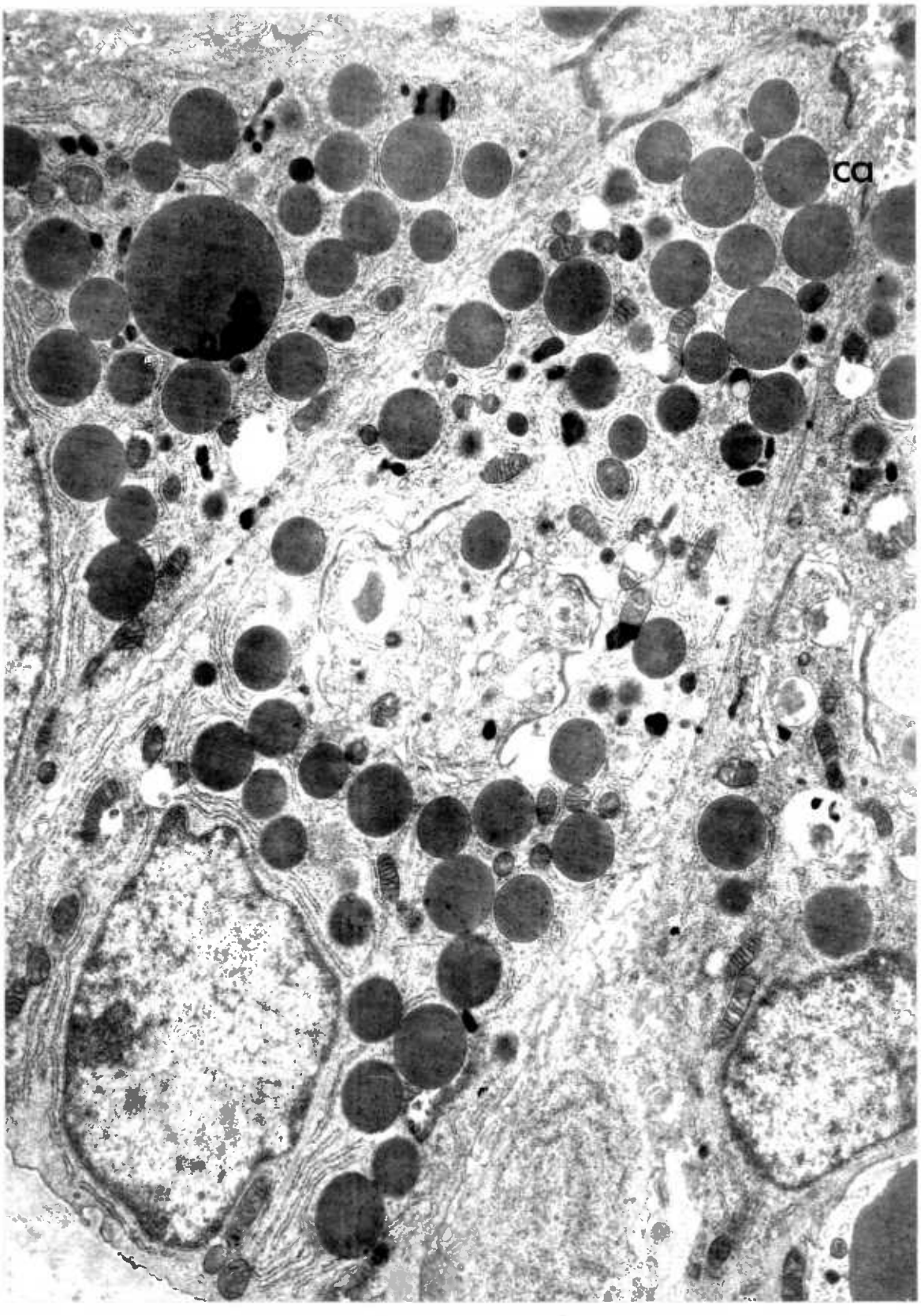
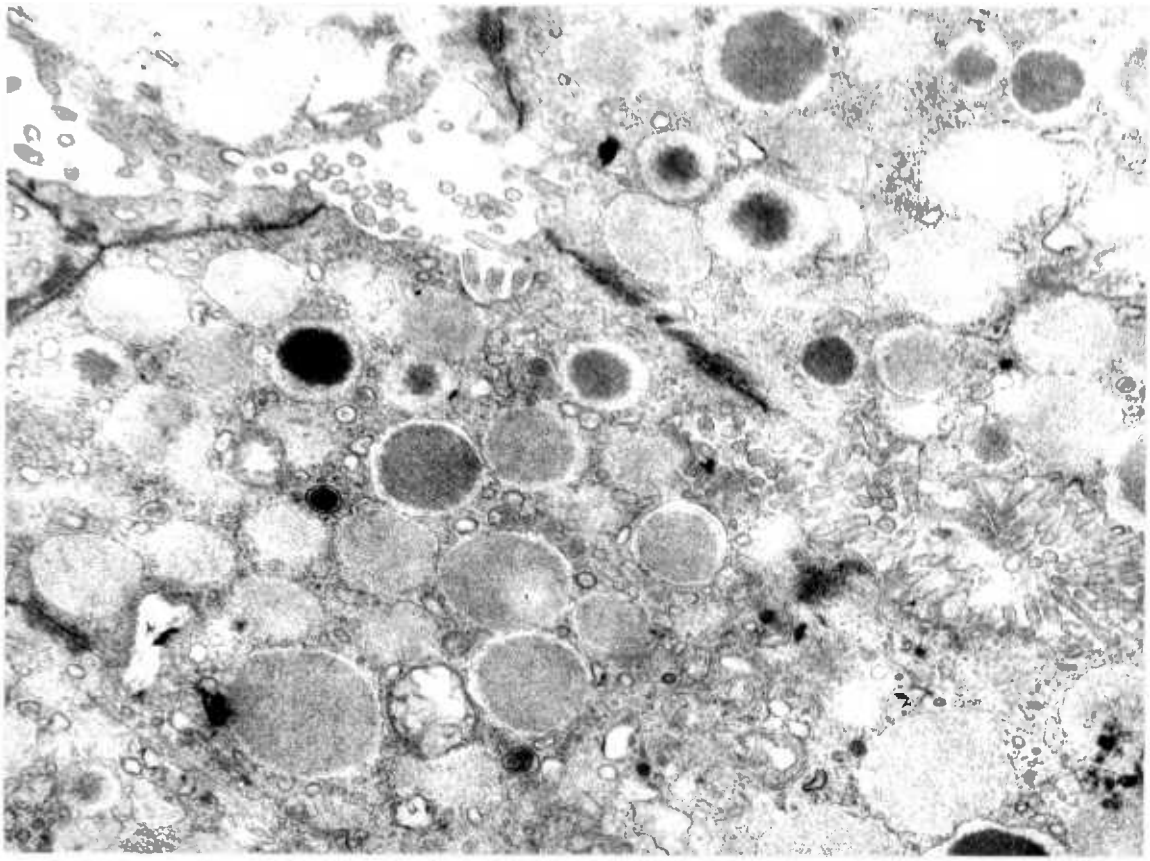


FIGURE III-2

Part of a serous cell after primary fixation for 18 hours in formalin and post-fixation in osmium tetroxide. The granules appear as discrete structures with varying electron densities. X 10,500.

FIGURE III-2



## CHEMICAL ANALYSIS

Precipitation techniques for protein and glycoproteins were applied to homogenates of bronchial gland explants that had been incubated for 60 minutes with either $H^3$-threonine or $H^3$-glucose; half the explants were incubated with added puromycin. All the explants used were from the posterior wall and weighed 10 - 12 mg (wet weight). The radioactivity within the precipitates and in measured aliquots of supernatants were measured by scintillation counting after dialysis.

The results of these counts are summarised in Table III-9. The percentage of the total for all the supernatants and precipitates are shown. The most important feature for $H^3$-threonine was that 24% of the label was precipitated by trichloracetic acid (indicating incorporation of the $H^3$-threonine into proteins). After treatment with puromycin, only 6% of the label was found in this precipitate, showing that puromycin had inhibited by 75%, the incorporation of $H^3$-threonine into the proteins.

Little $H^3$-glucose was precipitated by trichloracetic acid (2%), but 58% of the label was precipitated by ethanol which is reported to precipitate glycoprotein and perhaps a small amount of carbohydrate. Puromycin inhibited this incorporation into proteins by approximately 80%.

TABLE III-9

$H^3$-THREONINE AND $H^3$-GLUCOSE FOUND IN THE PRECIPITATES AND SUPERNATANTS AFTER DIALYSIS WITH AND WITHOUT ADDED PUROMYCIN

| | $H^3$-threonine | | $H^3$-glucose | |
|---|---|---|---|---|
| | Without % | With % | Without % | With % |
| A. CELL DEBRIS | 40 | 54 | 14 | 34 |
| B. WATER/PRONASE, SUPERNATANT then precipitated with trichloracetic acid | 24 | 6 | 2 | 4 |
| C. TRICHLORACETIC ACID WASH, SUPERNATANT 2 | 3 | 11 | 2 | 5 |
| D. TRICHLORACETIC ACID WASH, SUPERNATANT 3 | 2 | 1 | - | 1 |
| E. TRICHLORACETIC ACID WASH, SUPERNATANT 1 then precipitated with ethanol | 4 | 14 | 58 | 12 |
| F. ALCOHOL WASH | 27 | 14 | 24 | 44 |

The results would suggest that in the case of $H^3$-threonine, puromycin does not inhibit uptake into the cell, but does hinder incorporation. The inhibition of uptake of $H^3$-glucose, suggests that a block occurs in the synthesis of the polypeptide chain, to which the glucose is linked.

## SUMMARY

The optimum fixative for electron microscopic autoradiography is the one that gives the best morphological preservation without causing non-specific binding of radioactive precursor to the tissue.

Several techniques have been used in order to decide on the optimum fixative for the mucous and serous cells of the human bronchial submucosal gland after incubation, in either $H^3$-threonine or $H^3$-glucose at $37^{\circ}C$, for 60 minutes.

Using scintillation counts, the non-specific binding effects of formalin, glutaraldehyde and Karnovsky's fixative were assessed.

With $H^3$-threonine, both glutaraldehyde and Karnovsky's fixative bound more radioactive precursor to

the tissue than formalin.

With $H^3$-glucose, all three fixatives gave similar and satisfactory results..

The effect of a cold rinse prior to fixation in either formalin or glutaraldehyde was assessed.

After incubation with $H^3$-threonine, a cold rinse removed no more excess label than fixation in formalin alone. A cold rinse prior to fixation in glutaraldehyde did aid the removal of excess $H^3$-threonine.

After $H^3$-glucose, the cold rinse removed no more excess than either glutaraldehyde or formalin alone.

The effect of puromycin on the uptake of $H^3$-threonine and $H^3$-glucose was examined.

After incubation with $H^3$-threonine, puromycin had little effect on the amount of radioactivity found in the tissue.

After $H^3$-glucose, puromycin did seem to inhibit incorporation of $H^3$-glucose by the tissue.

Light microscopic autoradiographs were examined after six fixation techniques: explants had been incubated in either $H^3$-threonine or $H^3$-glucose for ten minutes at $4^oC$. It was assumed that any labelling found could be ascribed to non-specific binding of the radioactive precursor by the fixative.

For $H^3$-threonine, primary fixation in formalin gave the least non-specific binding - formalin at four hours and 18 hours and formalin for one hour followed by post-fixation in glutaraldehyde. Glutaraldehyde and Karnovsky's fixative both bound significantly more $H^3$-threonine to the tissue than formalin and osmium tetroxide, significantly more to the serous acini only.

For $H^3$-glucose, only glutaraldehyde bound significantly more label to the tissue than formalin.

When grain counts over mucous acini were compared to those over serous acini, glutaraldehyde, Karnovsky's fixative and to a lesser extent osmium tetroxide all bound significantly more $H^3$-threonine to the serous acini. Significant differences, for grain counts over mucous and serous acini were not found after incubation in $H^3$-glucose.

Primary fixation in formalin for 18 hours followed by post-fixation in osmium tetroxide gave the best morphological preservation and allowed easy identification of mucous and serous cells.

Using precipitation techniques, after incubation at 37°C for 60 minutes in either $H^3$-threonine and $H^3$-glucose, with and without the addition of puromycin to the incubation medium, 24% of the $H^3$-threonine was found in the protein precipitate (TCA). If puromycin was added to the incubation medium much less label was recovered in the protein precipitate, indicating that approximately 20% of $H^3$-threonine is incorporated into the protein core.

After incubation with $H^3$-glucose for one hour, precipitation techniques showed 58% of the label to be in the glycoprotein precipitate (ethanol). Incubation with puromycin inhibited 80% of this, indicating that approximately 50% of the $H^3$-glucose is incorporated into the glycoprotein of the tissue, and that puromycin caused a block in the synthesis of the glycoprotein, evidently of the polypeptide chain.

# CHAPTER IV

## INCORPORATION OF $H^3$-THREONINE BY MUCOUS AND SEROUS CELLS OF THE HUMAN BRONCHIAL SUBMUCOSAL GLAND

The pattern of incorporation of $H^3$-threonine into the mucous and serous cells has been studied in the human bronchial submucosal gland, using both light and electron microscopy.

Explants were incubated in radioactive precursor for 20, 40 or 60 minutes, these exposures are referred to as 'pulse' labels. The passage of the 60 minute 'pulse' label through the cell was then studied for periods up to eight hours. This was achieved by removing the explants from the radioactive precursor after the 60 minute 'pulse' label and then incubating in the nutrient medium, TC199, alone for periods of one - seven hours -the 'chase' incubation-thus making the overall incubation times up to eight hours. In describing the results, incubation times reported in minutes are 'pulse' labels and those referred to in hours include both the 'pulse' and 'chase' label, e.g. a three hour incubation represents the 60 minute 'pulse' label followed by a two hour 'chase' incubation. Thus, it is the total incubation time that is used to qualify the 'chase' label.

The light microscopic autoradiographs were quantitatively analysed: this showed the regional distribution of the silver grains within acini over an eight hour period of time.

Quantitative analysis of the electron microscopic autoradiographs allowed assessment both of 1. the total uptake or absolute amount of radioactivity within an organelle and 2. the relative concentration of silver grains associated with each organelle - called the specific activity. Study of both these features over a time span allowed the time of maximum total uptake and relative activity for each organelle to be determined.

From data obtained during this analysis two other features were also assessed, (i) the total content of radioactivity in the mucous and serous cell at each time examined and (ii) the volume occupied by each organelle within the mucous and serous cell.

## A. LIGHT MICROSCOPIC AUTORADIOGRAPHY

Ten acini of each cell type were examined. The regional distribution of silver grains within each acinus was noted. For mucous acini, four regions, based on cell levels were assessed - the base, mid-region, apex and lumen: for the serous acini, only three regions were identifiable, the base, mid-region and apex, since the lumen was difficult to identify with the light microscope.

The degree of labelling was assessed using a simplified system similar to that described by

Sturgess (1970), in which a chart was used, showing various grain densities. In the present study, only three grain densities were recognized, (i) heavy labelling, noted by a +, indicating that there were 40 - 70 silver grains per 100 sq μm (ii) little labelling, ±, indicating 10 - 30 grains per 100 sq μm and (iii) -, indicating less than ten grains per 100 sq μm. Background labelling was found to be less than ten grains per 100 sq μm. The results were expressed as a percentage of the ten acini. The acinus could be considered a unit since most of the cells in any one acinus showed a similar pattern of labelling (Figure IV-1).

The description of the results deals with the regional distribution of the silver grains over a period of eight hours, comments are made only when 50% or more of the acini are heavily labelled (+) in any one region. Table IV-1 shows the results obtained using light microscopic autoradiography for the mucous acini and Table IV-2 for the serous acini.

## Mucous acini

After incubation in $H^3$-threonine for 20 minutes, little labelling (±) was found in the cell base (region 1) (Table IV-1); between 40 minutes and two hours, this

FIGURE IV-1

Light microscopic autoradiograph (1 $\mu$m) after two hours incubation with $H^3$-threonine. Localization of silver grains is seen in the base, mid-region and apex of the mucous acinar cells (regions 1-3 respectively), but not in the lumen. All cells show a similar pattern of labelling. Toluidine blue X 1,500

FIGURE IV-1

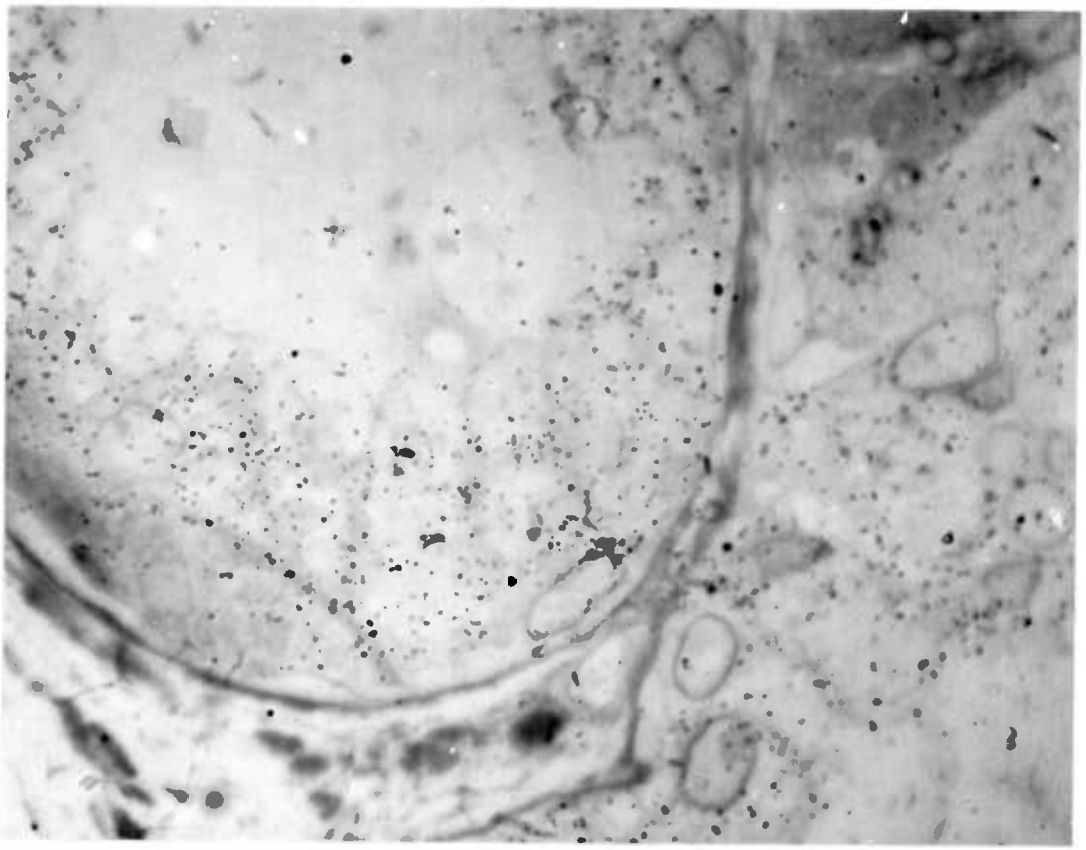


TABLE IV-1

REGIONAL DISTRIBUTION OF SILVER GRAINS SHOWING THE GRAIN DENSITY AS THE PERCENTAGE FOR EACH REGION OF MUCOUS ACINUS AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Regions | 1 | | | 2 | | | 3 | | | 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grain density | + | ± | − | + | ± | − | + | ± | − | + | ± | − |
| 20 mins | | 80 | 20 | | 20 | 80 | | | 100 | | | 100 |
| 40 mins | 90 | 10 | | 30 | 70 | | | 20 | 80 | | | 100 |
| 60 mins | 70 | 10 | 20 | 100 | | | 50 | 40 | 10 | | 30 | 70 |
| 2 hrs | 90 | 10 | | 100 | | | 80 | 20 | | | 40 | 60 |
| 3 hrs | 20 | 70 | 10 | 100 | | | 60 | 40 | | | 60 | 40 |
| 4 hrs | | 100 | | 100 | | | 100 | | | | 30 | 70 |
| 5 hrs | | 100 | | 100 | | | 80 | 20 | | | 50 | 50 |
| 6 hrs | | 100 | | 700 | | | 70 | 30 | | 10 | 50 | 40 |
| 7 hrs | | 100 | | 70 | 30 | | 100 | | | 60 | 40 | |
| 8 hrs | | 100 | | 50 | 50 | | 100 | | | 590 | 10 | |

Region 1 = base of acinus
Region 2 = mid-region
Region 3 = apex
Region 4 = lumen
\+ = 40-70 silver grains per 100 sq μm
± = 10-30
− = 10

Line denotes heavy labelling, 50% or more, of acini in this region.

region was definitely labelled, and from three - eight hours, little labelling persisted.

At 20 and 40 minutes, few grains were found in the mid-region (region 2) of the acinus, but from 60 minutes - seven hours, definite localization in all acini were found. By eight hours, only 50% of acini showed heavy labelling in this region.

In the apical region (region 3), at 20 minutes, no grains were found and very few at 40 minutes, by 60 minutes, heavy localization was found in 50% of acini. At two hours, 80% of acinar apices were heavily labelled and this remained at eight hours, although the percentage of labelled acini varied between 60 and 100% during this time. Few grains, from 60 minutes - six hours, were found in the acinar lumen, and heavy localization here only occurred at seven and eight hours.

## Serous acini

Little labelling of the serous acini occurred until 60 minutes (Table IV-2) when 50% of acini showed heavy apical labelling. This localization persisted until the seventh hour when, as at eight hours, little labelling was found.

TABLE IV-2

REGIONAL DISTRIBUTION OF SILVER GRAINS SHOWING THE GRAIN DENSITY AS THE PERCENTAGE FOR EACH REGION OF SEROUS ACINUS AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Regions | 1 | | | 2 | | | 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Grain density | + | ± | − | + | ± | − | + | ± | − |
| 20 mins | | | 100 | | 20 | 80 | | 40 | 60 |
| 40 mins | 10 | 90 | | | 20 | 80 | | 90 | 10 |
| 60 mins | 10 | 90 | | 20 | 80 | | 50 | 50 | |
| 2 hrs | 60 | 40 | | 70 | 20 | 10 | 40 | 60 | |
| 3 hrs | 80 | 20 | | 80 | 20 | | 90 | 10 | |
| 4 hrs | | 100 | | 60 | 40 | | 80 | 20 | |
| 5 hrs | 10 | 90 | | 50 | 50 | | 80 | 20 | |
| 6 hrs | 30 | 70 | | 40 | 60 | | 70 | 30 | |
| 7 hrs | 90 | 10 | | 90 | 10 | | 10 | 90 | |
| 8 hrs | 70 | 30 | | 80 | 20 | | 30 | 70 | |

Region 1 = base of acinus
Region 2 = mid-region
Region 3 = apical
\+ = 40-70 silver grains per 100 sq μm
± = 10-30
− = 10

Line denotes labelling, 50% or more, of acini in this region.

During the first 60 minutes little labelling was found in the mid-region, but at two hours, 70% of acini were labelled. This persisted throughout the remainder of the experiment with localization ranging between 50 - 90%, only at six hours did the percentage of acini labelled in this region fall to 40%.

By 60 minutes, 50% of the acinar apices were heavily labelled, although only 40% were labelled at two hours. From three - six hours, heavy labelling of the acinar apices was again seen. At seven and eight hours, little labelling was found in this region.

In the mucous acini, with $H^3$-threonine, little labelling is found at 20 minutes and this is in the basal region. The heavier localization at 40 minutes is also in the basal region (probably the rough endoplasmic reticulum), but by three hours the labelling in this region has diminished. At 60 minutes, all acini are definitely labelled in the mid-region (probably the Golgi apparatus). Here labelling persists until six hours, suggesting that the $H^3$-threonine that is initially found in the basal region has now travelled to the mid-region. From 60 minutes, heavy labelling is seen in the mid-region. At 60 minutes, localization of grains is also seen in the apical region, but at first

the localization is due to granules that are not adjacent to the lumen. Only from four hours are granules in this luminal region labelled. At seven and eight hours, heavy localization of silver grains in the lumen is found.

For the serous acini, the progression of precursor that can be identified through the mucous acinus is not apparent. Certainly heavy localization of silver grains is seen at 60 minutes but it is at the apex, unlike the mucous acini where localization at this time is still basal. From as early as two hours, the labelling appears to be general, throughout the serous acini. These results show that the mucous and serous acini of the human bronchial submucosal glands exhibit a different pattern for localization of $H^3$-threonine.

This was analysed further by electron microscopic autoradiography.

## B. HIGH RESOLUTION AUTORADIOGRAPHY

Table IV-3 summarises the specimens used for the analysis of the electron microscopic autoradiographs, for both mucous and serous cells and the incubation times of the tissue. All figures in the following results are from these specimens.

The results for the light microscopic autoradiographs were described with reference to acini but those following, from the electron microscope, are described by reference to cells.

Two methods were used to analyse quantitatively the electron microscope autoradiographs.

1. The circle analysis of Williams (1969). Details of this method are given in Materials and Methods (page 85).

2. The hypothetical grain method of Blackett and Parry (1973). Details for this method are given in Materials and Methods (page 92).

This latter method of analysis has only recently been developed and has not previously been used to show uptake of a radioactive precursor over a period of time.

TABLE IV-3

SPECIMENS USED FOR THE ELECTRON MICROSCOPIC STUDIES WITH $H^3$-THREONINE

| INCUBATION TIME | STUDY 1 | STUDY 2 |
|---|---|---|
| 20 minutes | 448/1 | 457/1 |
| 40 | 448/3 | 457/3 |
| 60 | 418/7 | 457/4 |
| 2 hours | 418/9 | 457/7 |
| 3 | 418/11 | 455/2 |
| 4 | 421/2 | 455/4 |
| 5 | 421/3 | 455/6 |
| 6 | 421/4 | 455/8 |
| 7 | 421/5 | 455/10 |
| 8 | 421/6 | 455/12 |

The incorporation of $H^3$-threonine by mucous and serous cells will be described in this chapter by reference to both these methods - the mucous cell first. The following chapter deals with the incorporation of $H^3$-glucose by these two cell types.

## METHOD

### Selection of Micrograph

Before analysing the uptake of $H^3$-threonine it is important to describe how the micrographs for detailed examination were selected. For the circle analysis, two specimens for each incubation time were analysed, but for the hypothetical grain method, only one.

The regions of the autoradiographs to be photographed were selected in a random manner, in that the fields used for quantitative analysis represented the first region of mucous and serous cell encountered in the section; up to 12 fields were photographed of each cell type. The pictures were taken to include at least two from the basal region of the cell, two from the apex and two from the mid-region. The first two pictures from each region were then analysed. More micrographs were analysed however, if the total grain count was less than 100.

Because of the high magnification used (total

magnification X 25,000), micrographs of a whole cell in a coronal plane were rarely obtained. There was for this reason perhaps a bias towards the mid-region of the cell, since the mid-region might be included in both the apical and basal picture as well as the one taken specifically for this region. Figure IV-2 shows a montage of a mucous cell taken at a magnification of 5,000, the magnification used for this study, and shows the regions of a cell usually included in one picture. The serous cell was similarly divided.

## Circle Analysis

The circle, point and grain analysis were carried out as described on page 86. Using these results the Chi-squared test and the specific activity and absolute uptake by organelles, from each specimen at each incubation time were calculated.

### Chi-squared test

The $x^2$ test was applied, using all the organelles and also the junctional regions as shown in Table IV-4 (column 1), but some of these items gave a low expected value, that is of less than five, the minimum figure for a satisfactory $x^2$ test. The expected value was here

FIGURE IV-2

Montage of mucous cells showing the amount of cell contained in a micrograph taken at a magnification of 5,000. In general it can be seen that micrographs taken at three levels (base, mid-region and apex) would include the majority of the cell.

Fixation: Glut + $OsO_4$. X 13,000 approx.

FIGURE IV-2

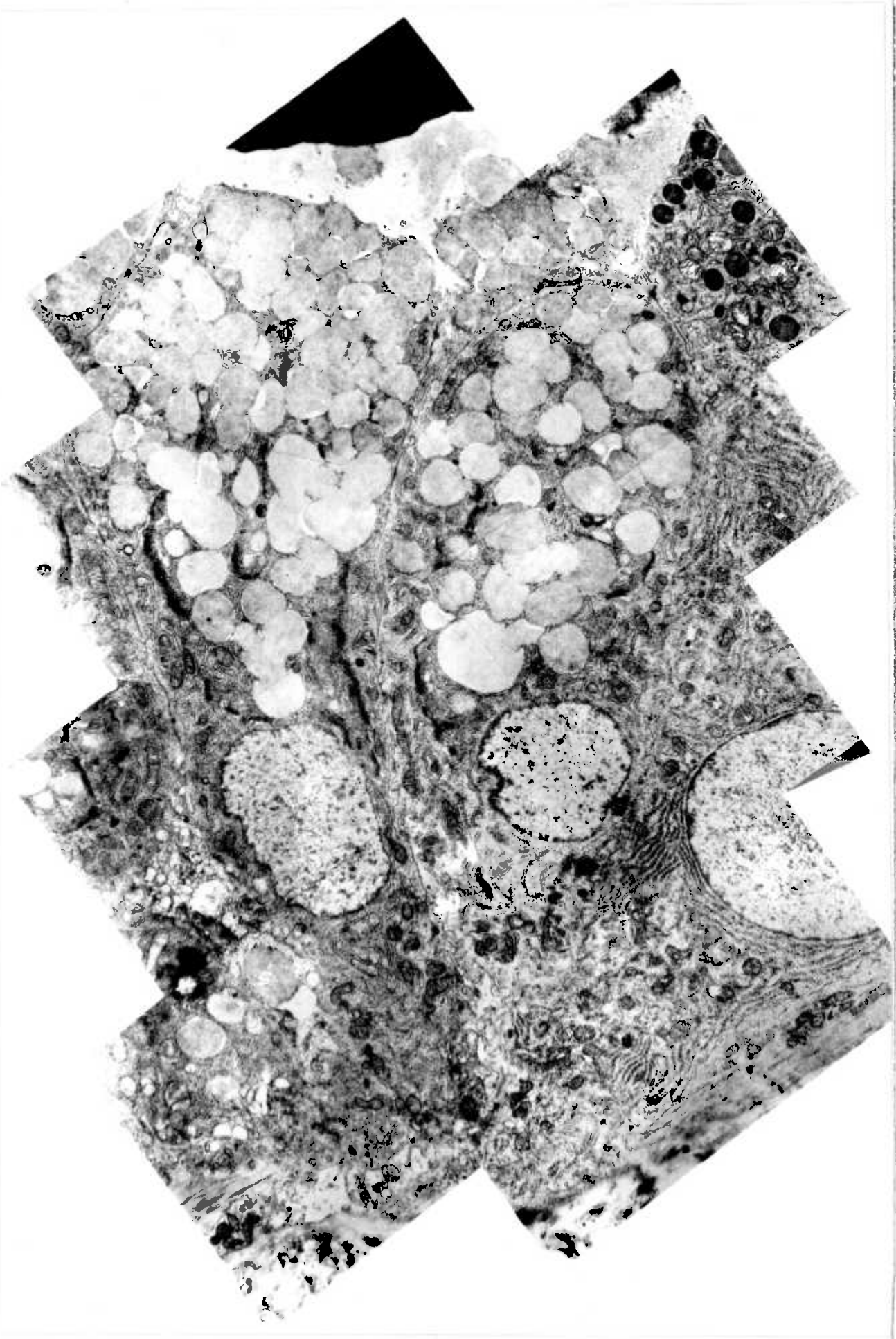


TABLE IV-4

SHOWING HOW ORGANELLES WERE COMBINED

TO GIVE A MORE ACCURATE $x^2$ TEST

| ORGANELLE | COMBINED ORGANELLES |
|---|---|
| Endoplasmic reticulum (ER) | |
| ER/Golgi apparatus | |
| Golgi apparatus | |
| vacuole ) | granule |
| granule ) | |
| ER/vacuole ) | ER/granule |
| ER/granule ) | |
| nucleus | |
| canaliculus | |
| ER/mitochondrion ) | ER/other |
| ER/membrane ) | |
| ER/canaliculus ) | |
| ER/other ) | |
| ER/lysosome ) | |

taken as the number of circles falling over a particular organelle, divided by the total number of circles multiplied by the total number of actual silver grains. It therefore was decided to combine some of these regions that gave a rather low expected value. Thus the groups vacuole and granule were combined as were ER/vacuole and ER/granule. The groups ER/granule and ER/Golgi apparatus were kept as separate items but all other junctional regions were added together and called ER/other (Table IV-4). The $x^2$ test was then applied to these new groups (Table IV-5).

Specific activity

The specific activity is a measure of the concentration of radioactivity within an organelle. Specific activity was calculated by dividing the percentage of total number of silver grains over a particular organelle by the percentage of total number of circles over the same organelle.

The specific activity can be used in two ways:

(i) The relative activity of each organelle in a particular section can be compared.

(ii) The time of maximum specific activity for each organelle can be determined, by comparing the specific activity of the organelle after incubation in

TABLE IV-5

SPECIMEN ANALYSIS ($x^2$ TEST) OF THE MUCOUS CELL AFTER INCUBATION WITH $H^3$-THREONINE FOR 40 MINUTES: ORGANELLES COMBINED IN AN ATTEMPT TO REACH EXPECTED VALUES OF MORE THAN FIVE

| ORGANELLES | No. Circles | % | No. Points | % | Actual Grains (A) | % | Expected Grains (E) |
|---|---|---|---|---|---|---|---|
| Single items | | | | | | | |
| Endoplasmic reticulum(ER) | 17 | 4.4 | 91 | 23.8 | 11 | 8.7 | 5.6 |
| Golgi apparatus | 22 | 8.6 | 32 | 8.3 | 39 | 30.9 | 10.8 |
| Granules + Vacuoles | 113 | 29.5 | 242 | 63.2 | 13 | 10.3 | 37.2 |
| Nucleus | 7 | 1.8 | 12 | 3.1 | | | 2.3 |
| Canaliculus | not used for mucous cell | | | | | | |
| Junctional regions | | | | | | | |
| ER/Golgi apparatus | 6 | 1.6 | | | 3 | 2.4 | 2.0 |
| ER/granule + ER/vacuole | 173 | 45.2 | | | 38 | 30.2 | 56.9 |
| ER/other | 34 | 8.9 | 6 | 1.6 | 22 | 17.5 | 11.2 |
| TOTAL | 383 | | 383 | | 126 | | |

$x^2$ test of comparison against E, $\frac{\text{No circles/organelle}}{\text{Total No circles}} \times$ Total actual grains

on the assumption given $Px^2$ d.f.6 $< 0.001$

the radioactive precursor for various times. This is independent of total uptake by the cell (vide infra) and of the explant. When comparing the specific activities at different times it is difficult to obtain a smooth curve due to inevitable, if minor variation in both section and emulsion thickness and in the varying specific activity of different batches of radioactive precursor.

In the present study (ii) is used and the results will be referred to as the relative concentration.

Uptake of radioactive precursor

In the present study, an extension of the circle analysis has been developed allowing the uptake of radioactive precursor in absolute terms to be determined and will be referred to as 'absolute uptake'. This is determined by dividing the actual number of silver grains by the actual number of circles for each incubation time. Comparison of these figures gives the actual uptake of radioactive precursor for each organelle so that the time at which it is at maximum can be detected.

## Hypothetical Grain Analysis

This method gives an estimate of the activity in various regions of sections and takes into account the geometric shape and position of the silver grains within the section. The measured distribution of these points around a thin line source is used to construct a hypothetical grain distribution which is then compared to the distribution of actual grains (Table II-7). Different activities are then ascribed to various regions until satisfactory agreement with the actual grains is obtained using the $x^2$ test for significance.

The method for analysing the actual grains is identical to the grain count of the circle analysis (page 90 ). A more detailed description of the hypothetical grain analysis is described in Material and Methods (page 92).

One specimen only from each of three incubation times - 60 minutes, four and eight hours, were examined after incubation in $H^3$-threonine. Both mucous and serous cells were analysed. Four items were studied:

(i) rough endoplasmic reticulum (ER), this group included all organelles except for the following

(ii) granule including vacuole

(iii) Golgi apparatus - including vesicles and lamellae and

(iv) nucleus.

Dr N. Blackett and Miss D. Parry assisted in the

analysis and interpretation of the data obtained. A computer programme has recently been devised by these workers and was used to process the data. The percentage area and the percentage activity for each organelle at each time was calculated. By dividing the latter by the former an estimate of the activity per unit area was obtained. The activity per unit area corresponds to the relative concentration of silver grains obtained using the circle analysis. The number of silver grains per grid point was also determined by dividing the actual number of silver grains for each specimen by the total number of grid points superimposed over the micrographs and multiplying this figure by the activity per unit area. This corresponds to the absolute activity of the circle analysis.

The computer also calculated standard errors for these results, by random selection of another nine sets of hypothetical grains based on the Poisson distribution of those already counted, and then comparing these new figures to the actual silver grains using the $x^2$ test for significance.

Only (i) grains per grid point and (ii) activity per unit area will be described here, and these separately.

## MUCOUS CELL

### Circle Analysis

The results of the chi-squared tests for the mucous cell after incubation in $H^3$-threonine, at all times were highly significant with p values of $< 0.001$.

### Absolute uptake and relative concentration of $H^3$-threonine

The results for the relative concentration of silver grains (specific activity) and the absolute uptake of organelles are described together. Tables IV-6 and IV-7 give the values for the absolute and relative uptake respectively for each of the two specimens at each incubation time and the mean for the pair. The following Figures represent the smoothed curves, done in consultation with a statistician, showing the trend of the points given in Tables IV-6 and IV-7. Only these curves are described in the results.

Initial incorporation of $H^3$-threonine by the mucous cell was in the endoplasmic reticulum. Absolute uptake was found to be maximal at four hours, and from four - eight hours, decreased (Table IV-6 and Figure IV-3). These results could indicate one of two patterns of behaviour either that by four hours, total uptake was

TABLE IV-6

THE ABSOLUTE UPTAKE OF TWO STUDIES AND THEIR MEAN FOR ORGANELLES IN THE MUCOUS CELL AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 0.44 | 1.00 | 0.81 | 1.00 | 1.00 | 1.73 | 1.71 | 1.16 | 1.15 | 0.60 |
| | 2 | 0.48 | 0.89 | 0.27 | 0.83 | 2.02 | 2.41 | 1.33 | 0.40 | 0.73 | 0.30 |
| | m | 0.46 | 0.94 | 0.54 | 0.91 | 1.53 | 2.07 | 1.52 | 0.78 | 0.94 | 0.45 |
| ER/Golgi | 1 | 0.40 | 1.00 | 3.00 | 1.38 | 1.62 | 0.80 | 2.66 | 1.05 | 0.90 | 1.00 |
| | 2 | 0.38 | 0.55 | 0.08 | 0.10 | 2.00 | 1.88 | 1.00 | 0.25 | 0.80 | 0.50 |
| | m | 0.39 | 0.77 | 1.54 | 0.74 | 1.81 | 1.34 | 1.83 | 0.65 | 0.85 | 0.75 |
| Golgi | 1 | 0.00 | 0.00 | 0.33 | 0.85 | 0.79 | 0.79 | 2.39 | 0.35 | 0.89 | 0.75 |
| | 2 | 0.26 | 0.03 | 0.11 | 0.73 | 1.50 | 1.13 | 1.42 | 0.26 | 0.42 | 0.37 |
| | m | 0.13 | 0.01 | 0.22 | 0.79 | 1.14 | 0.96 | 1.90 | 0.30 | 0.65 | 0.56 |
| Granule | 1 | 0.01 | 0.22 | 0.19 | 0.32 | 1.00 | 0.56 | 0.37 | 0.87 | 1.55 | 1.31 |
| | 2 | 0.06 | 0.03 | 0.06 | 0.15 | 0.10 | 0.70 | 0.59 | 0.54 | 0.39 | 0.65 |
| | m | 0.03 | 0.12 | 0.12 | 0.26 | 0.55 | 0.63 | 0.48 | 0.70 | 0.92 | 0.98 |

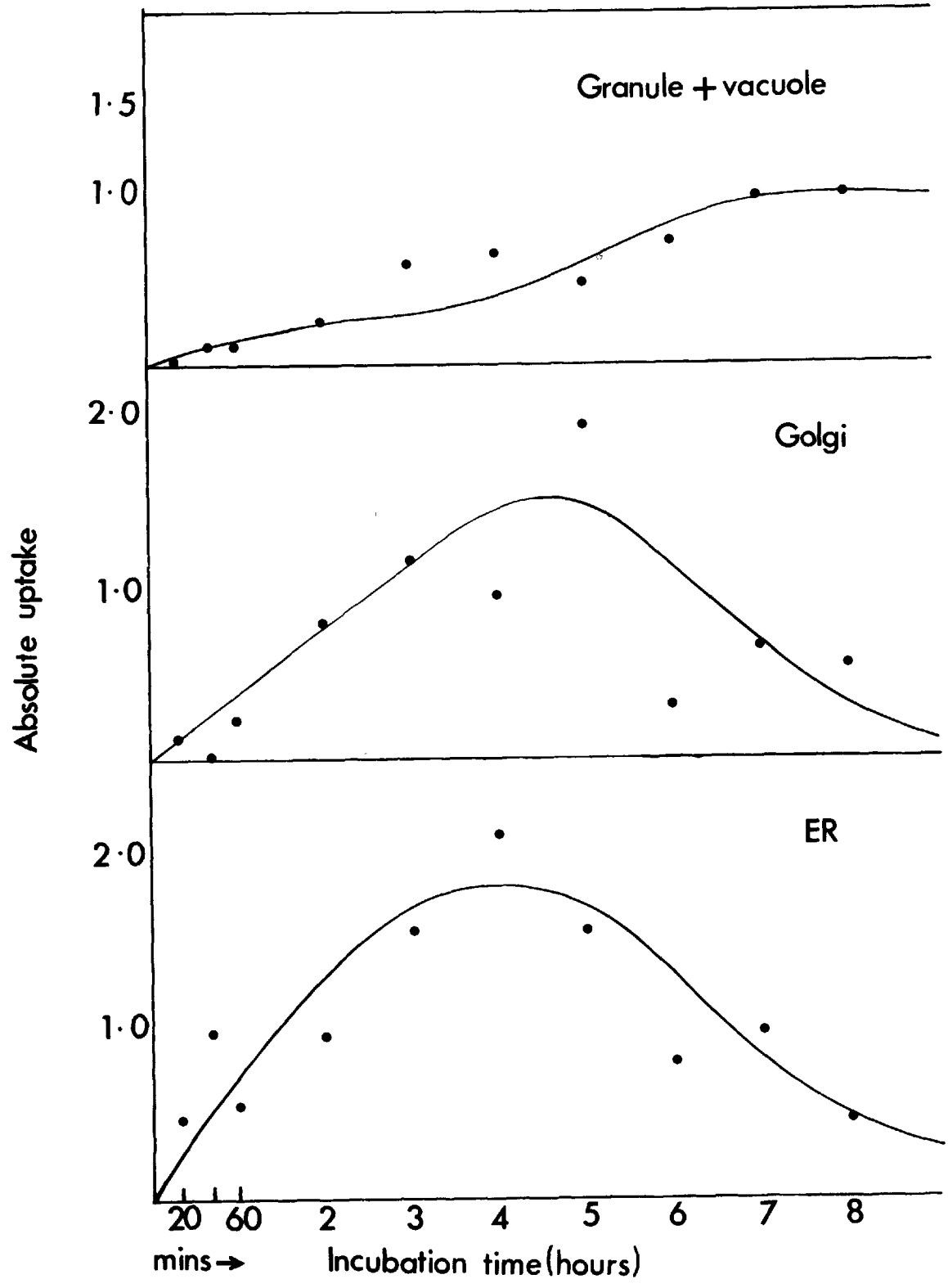
| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ER/granule | 1 | 0.07 | 0.13 | 0.31 | 0.35 | 0.19 | 1.18 | 1.23 | 0.84 | 1.47 | 1.70 |
| | 2 | 0.22 | 0.23 | 0.10 | 0.17 | 0.65 | 1.02 | 1.28 | 1.13 | 0.54 | 0.85 |
| | m | 0.14 | 0.18 | 0.20 | 0.26 | <u>1.29</u> | 1.10 | 1.25 | 0.98 | 1.00 | 1.27 |
| ER/others | 1 | 0.14 | 0.09 | 0.29 | 0.62 | 0.84 | 1.01 | 0.75 | 0.52 | 0.98 | 0.97 |
| | 2 | 0.29 | 0.43 | 0.21 | 0.33 | 1.03 | 1.56 | 1.09 | 0.48 | 0.60 | 0.48 |
| | m | 0.21 | 0.26 | 0.25 | 0.47 | 0.93 | <u>1.28</u> | 0.92 | 0.50 | 0.79 | 0.72 |
| Nucleus | 1 | 0.00 | 1.45 | 0.77 | 0.70 | 0.77 | 0.75 | 1.33 | 0.89 | 0.71 | 1.36 |
| | 2 | 0.17 | 0.19 | 0.33 | 0.45 | 1.17 | 0.89 | 0.84 | 0.48 | 0.32 | 0.68 |
| | m | 0.08 | 0.82 | 0.55 | 0.57 | 0.97 | 0.82 | <u>1.08</u> | 0.68 | 0.51 | 1.02 |

Maximum absolute uptake for each organelle is underlined.

FIGURE IV-3

Smoothed curves representing the trend of points for absolute uptake of $H^3$-threonine by organelles of the mucous cell after incubation for various times (the dots represent the mean for each time).

FIGURE IV-3


Granule + vacuole
1·5
1·0
Golgi
2·0
1·0
ER
2·0
1·0
Absolute uptake
20
60
2
3
4
5
6
7
8
mins →
Incubation time (hours)

complete and decrease in activity was as a direct result of activity being transferred to other organelles or, it may be that uptake was still occurring after four hours, although more $H^3$-threonine was being transferred to other organelles thus causing this decrease. Observation of other organelles did not elucidate this matter. At 60 minutes, the relative concentration of silver grains was maximum for the endoplasmic reticulum (Table IV-7 and Figure IV-4). These results indicate that the initial incorporation of $H^3$-threonine is into the endoplasmic reticulum, although absolute uptake by this organelle continues beyond this time.

From the endoplasmic reticulum, $H^3$-threonine passed to the junctional region, ER/Golgi apparatus. Between four and five hours, absolute uptake for this region was maximal and then diminished (Table IV-6). At 60 minutes, the maximum relative concentration of silver grains was found to be associated with the ER/Golgi apparatus (Table IV-7).

Radioactive precursor was next seen in the Golgi apparatus. At five hours, maximum absolute activity was found here and from this time it decreased (Table IV-6 and Figure IV-3). At four hours, the relative concentration of silver grains was maximal over the Golgi apparatus (Table IV-7 and Figures IV-4 and IV-5). For the Golgi apparatus, both results

TABLE IV-7

THE SPECIFIC ACTIVITY (RELATIVE CONCENTRATION OF SILVER GRAINS) OF TWO STUDIES AND THEIR MEAN FOR ORGANELLES IN THE MUCOUS CELL AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 5.28 | 6.68 | 2.05 | 1.41 | 0.92 | 1.70 | 1.55 | 1.44 | 0.92 | 0.70 |
| | 2 | 2.07 | 3.33 | 1.70 | 2.05 | 2.02 | 2.25 | 1.34 | 0.72 | 1.40 | 0.42 |
| | m | 3.67 | 5.01 | 1.88 | 1.73 | 1.47 | 1.97 | 1.45 | 1.08 | 1.16 | 0.58 |
| ER/Golgi | 1 | 4.74 | 6.74 | 7.52 | 1.95 | 1.49 | 0.79 | 2.41 | 1.31 | 0.73 | 1.02 |
| | 2 | 1.66 | 2.17 | 0.53 | 0.25 | 1.95 | 1.76 | 1.00 | 0.33 | 1.64 | 0.83 |
| | m | 3.20 | 4.46 | 4.03 | 1.10 | 1.72 | 1.28 | 1.71 | 0.82 | 1.19 | 0.93 |
| Golgi | 1 | 0.00 | 0.00 | 0.85 | 1.19 | 0.72 | 0.77 | 2.16 | 0.46 | 0.71 | 0.42 |
| | 2 | 0.04 | 0.14 | 0.73 | 1.80 | 1.46 | 1.05 | 1.41 | 0.46 | 0.78 | 0.58 |
| | m | 0.20 | 0.07 | 0.79 | 1.50 | 1.09 | 0.91 | 1.79 | 0.46 | 0.75 | 0.50 |
| Granule | 1 | 0.12 | 0.29 | 0.38 | 0.16 | 0.92 | 0.55 | 0.34 | 1.08 | 1.24 | 1.07 |
| | 2 | 0.27 | 0.07 | 0.10 | 0.28 | 0.10 | 0.62 | 0.55 | 0.97 | 0.75 | 1.02 |
| | m | 0.20 | 0.18 | 0.24 | 0.22 | 0.51 | 0.58 | 0.45 | 1.02 | 1.00 | 1.05 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ER/granule | 1 | 0.85 | 0.91 | 0.81 | 0.55 | 1.79 | 1.16 | 1.12 | 1.06 | 1.18 | 1.11 |
| | 2 | 0.98 | 0.90 | 1.24 | 0.49 | 0.64 | 0.95 | 1.30 | 1.97 | 1.05 | 1.33 |
| | m | 0.92 | 0.91 | <u>1.48</u> | 0.52 | 1.22 | 1.06 | 1.21 | 1.52 | 1.12 | 1.22 |
| ER/others | 1 | 1.75 | 0.67 | 0.61 | 0.89 | 1.97 | 1.00 | 0.69 | 0.65 | 0.78 | 0.44 |
| | 2 | 0.93 | 1.69 | 1.33 | 0.82 | 1.01 | 1.46 | 1.12 | 0.85 | 1.17 | 0.76 |
| | m | <u>1.34</u> | 1.18 | 0.97 | 0.86 | 0.99 | 1.23 | 0.91 | 0.75 | 0.96 | 0.60 |
| Nucleus | 1 | 0.00 | 3.01 | 1.95 | 0.99 | 0.71 | 0.74 | 1.20 | 1.10 | 0.51 | 1.02 |
| | 2 | 0.72 | 0.76 | 2.18 | 1.12 | 1.14 | 0.83 | 0.85 | 0.85 | 0.61 | 1.05 |
| | m | 0.36 | 1.89 | <u>2.07</u> | 1.06 | 0.93 | 0.70 | 1.03 | 0.98 | 0.56 | 1.04 |

Maximum specific activity for each organelle is underlined.

FIGURE IV-4

Smoothed curves representing the trend of points for the specific activity (relative concentration of silver grains) of organelles of the mucous cell after incubation with $H^3$-threonine for various times (the dots represent the mean for each time.

FIGURE IV-4

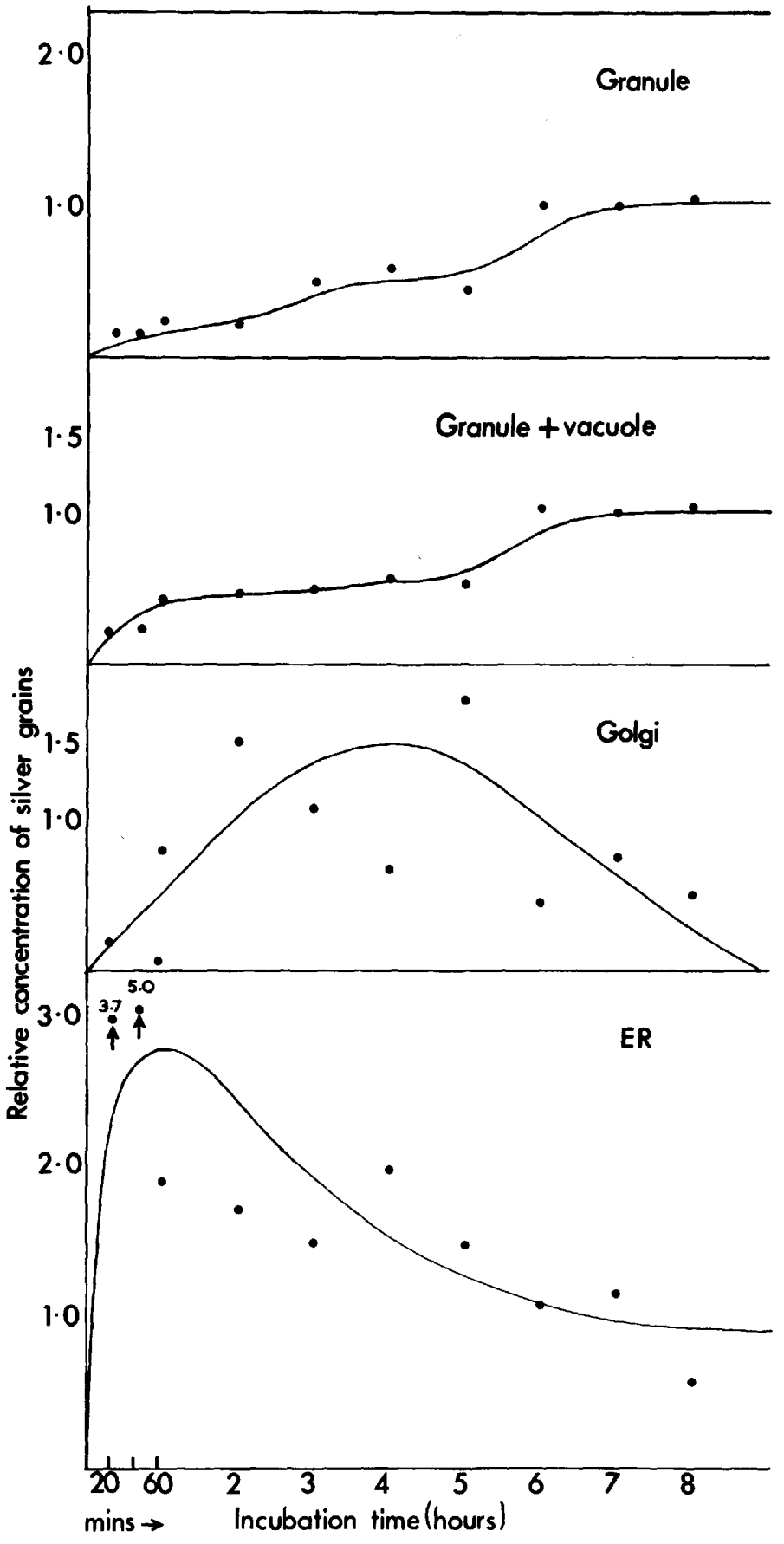
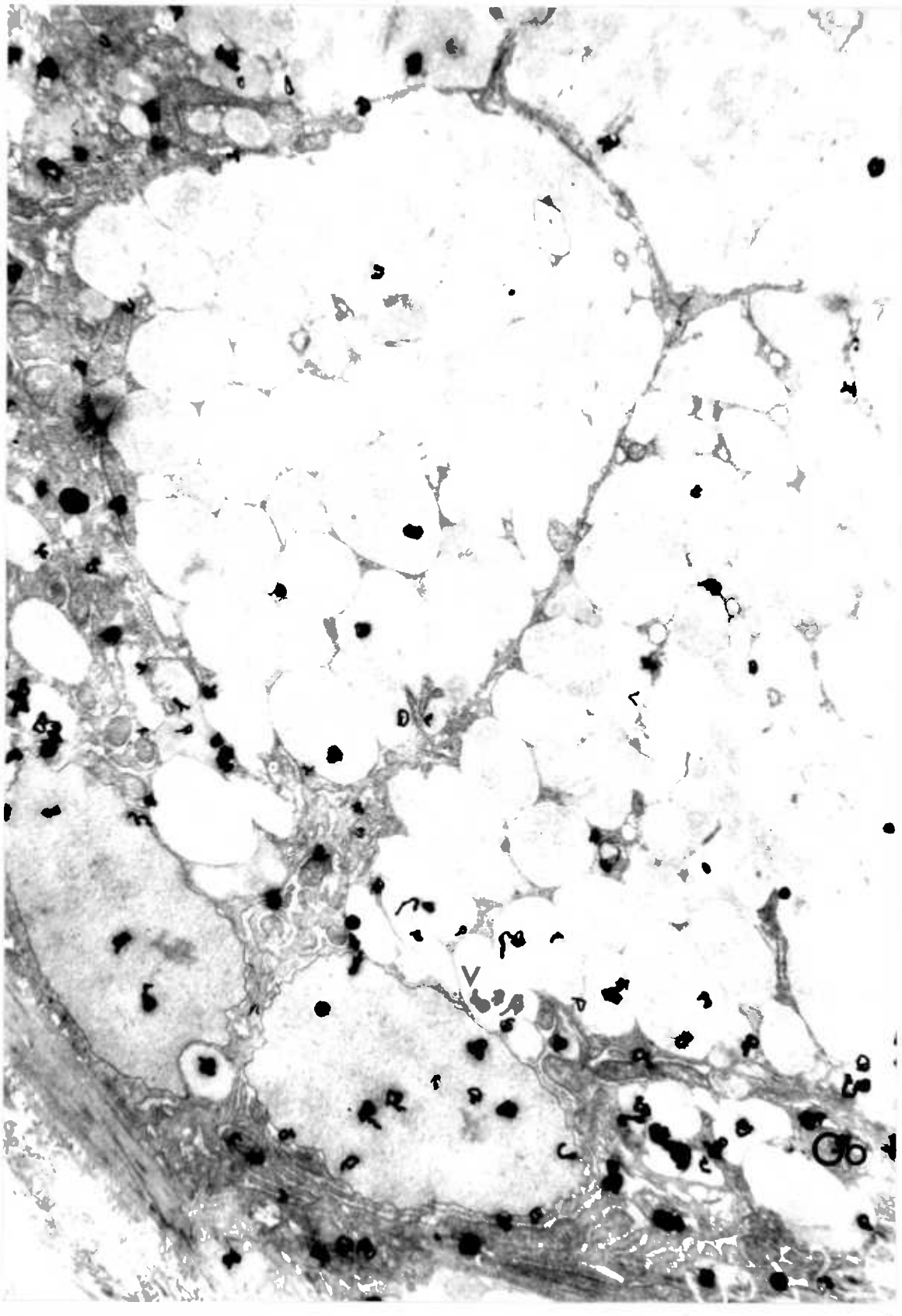

Granule
Granule + vacuole
Golgi
ER
2·0
1·0
1·5
1·0
1·5
1·0
3·0
2·0
1·0
3.7
5.0
Relative concentration of silver grains
20
60
2
3
4
5
6
7
8
mins →
Incubation time (hours)

FIGURE IV-5

Mucous cell after incubation for four hours with $H^3$-threonine. Localization of silver grains is evident over the Golgi apparatus (Go) and the vacuoles (v). X 9,000.

FIGURE IV-5



show a later time of incorporation of $H^3$-threonine than the endoplasmic reticulum and thus suggest that radioactivity has travelled from the latter to the Golgi apparatus.

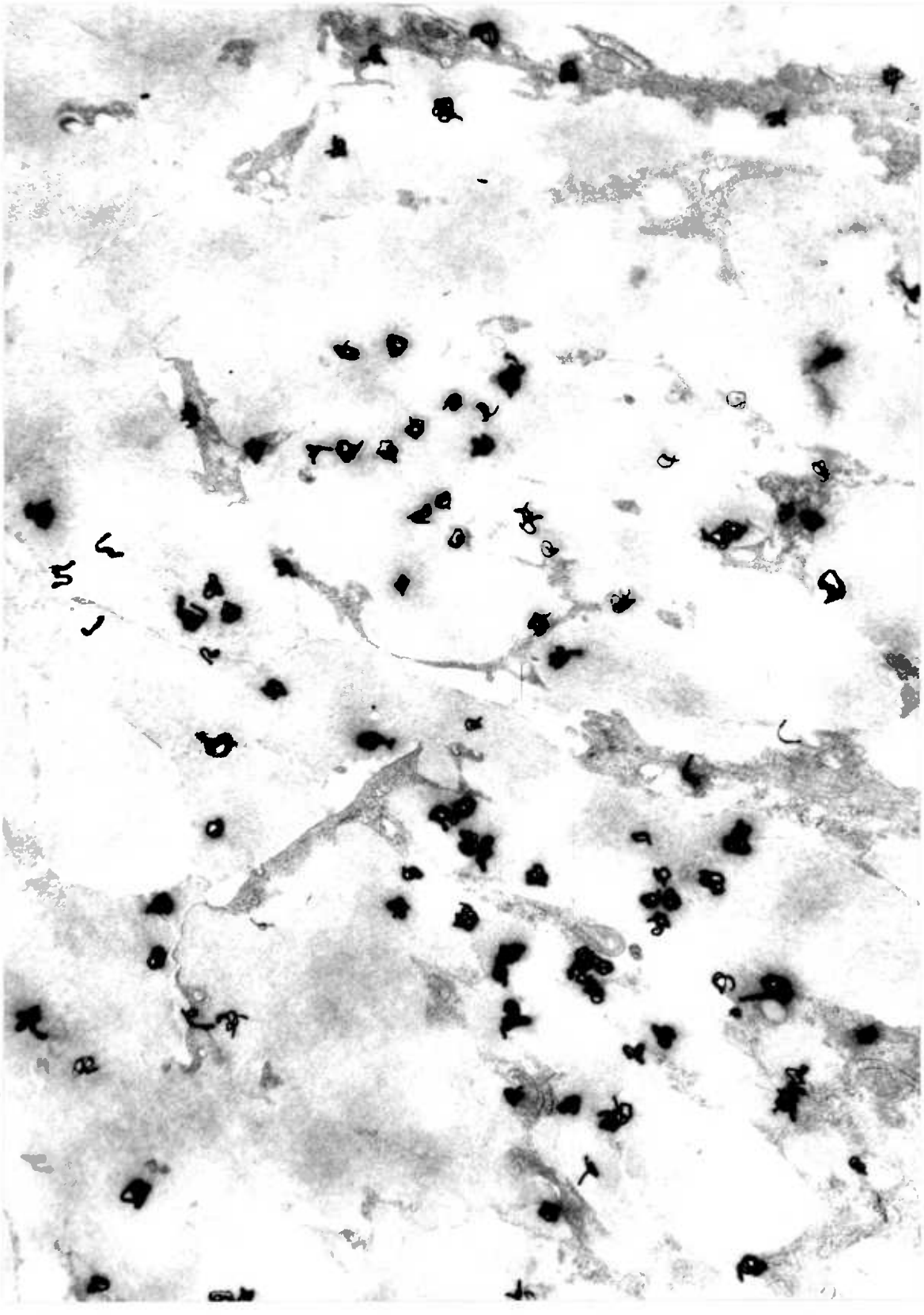
The junctional region, ER/Golgi apparatus (see above) showed features for uptake that reflect its two component organelles, and support the suggestion that radioactivity moves from the endoplasmic reticulum to the Golgi apparatus.

Activity was next encountered in the vacuoles (this is an immature secretory granule, situated close to the Golgi apparatus). Because of the small volume of cell occupied by the vacuole, the results for this organelle are considered with those of the granule. At seven hours, the absolute uptake by the granules alone was maximum as was that for the granules and vacuoles combined (Table IV-6 and Figures IV-3). The relative concentration of silver grains over the granule was also maximal at seven hours (Table IV-7 and Figures IV-4 and IV-6) and was again similar if the vacuoles were included, but at 60 minutes and two hours, a higher relative activity was found for the granules if the vacuoles were added (Table IV-7 and Figures IV-4 and IV-5). This result indicates that the vacuole is indeed an immature secretory granule and the pathway of radioactive precursor is from the Golgi apparatus to the vacuole and then to the granule.

FIGURE IV-6

After incubation with $H^3$-threonine for eight hours, silver grains are numerous over the granules at the apex of the mucous cell, which is seen to be discharging. X 15,000.

FIGURE IV-6



It should also be noted that if no further uptake occurs by the tissue from four hours onwards, all activity found in the granule must be derived from other organelles.

By six hours, the absolute uptake in the junctional region, ER/granule was maximal (Table IV-6) and remained at this level at eight hours. The relative concentration of silver grains was at maximum for this region at five hours and decreased slightly by eight hours (Table IV-7), the longest incubation time examined in this study. A smaller peak was also seen at 60 minutes, this is doubtless due to the activity of the endoplasmic reticulum. The incorporation of $H^3$-threonine by this junctional region would thus seem to reflect both its component organelles.

By four hours, maximum absolute activity was found in the junctional region ER/others and from four - eight hours, this activity decreased (Table IV-6). At 60 minutes, the relative concentration of silver grains was at maximum for this region (Table IV-7). The level of incorporation for this junctional region, ER/others is less than that of the endoplasmic reticulum alone, this would reflect insignificant amounts of activity within the other organelles (mitochondria, membranes and lysosomes) of this region.

By four hours, maximum absolute activity was found in the nucleus (Table IV-6), from this time until eight hours, a decrease in activity appeared. At 60 minutes, relative concentration of silver grains in the nucleus was maximal (Table IV-7). Protein synthesis has been reported to occur in the nucleus and these results would support this.

Thus, $H^3$-threonine is initially incorporated into the endoplasmic reticulum of the mucous cell. From here, the radioactive precursor is transported to the Golgi apparatus and then to the secretory granules via the vacuoles.

From the above results for the absolute uptake of $H^3$-threonine by the organelles of the mucous cell, it would seem that uptake continues beyond the initial 60 minute 'pulse' label. The additional 'uptake' by the organelles must therefore be derived from the tissue of the explant itself, and may be due to an excess of $H^3$-threonine being trapped in the tissue, despite the washing procedure after the 'pulse' labelling (see page 205 for absolute uptake by the cell).

Maximum total uptake for all various organelles save the granules and the junctional region, ER/granule, occurs between four and five hours, then decreases, but

for these two regions uptake reaches maximum at eight hours, for the granules, and at five hours for the ER/granule and then remains at this level. This would suggest that activity in the granules is derived from other organelles in the cell (see page 205 for absolute uptake by whole cell).

## Hypothetical Grain Analysis

(i) Grains per grid point (Absolute uptake)

At 60 minutes (Table IV-8 and Figure IV-7), apart from the nucleus, the endoplasmic reticulum showed the greatest uptake of $H^3$-threonine. At four hours, absolute uptake by this organelle had increased and at eight hours decreased.

At 60 minutes, the Golgi apparatus showed little uptake, but at four hours, the maximum absolute uptake was in this organelle; at eight hours activity had decreased.

From 60 minutes to eight hours, absolute uptake by the granules increased.

At 60 minutes, maximum absolute activity was found in the nucleus, at four hours uptake had decreased and by eight hours increased again.

TABLE IV-8

THE PERCENTAGE AREA, PERCENTAGE ACTIVITY, GRAIN PER GRID POINT AND ACTIVITY PER UNIT AREA FOR THE MUCOUS CELL AFTER INCUBATION IN $H^3$-THREONINE FOR 60 MINUTES, 4 AND 8 HOURS

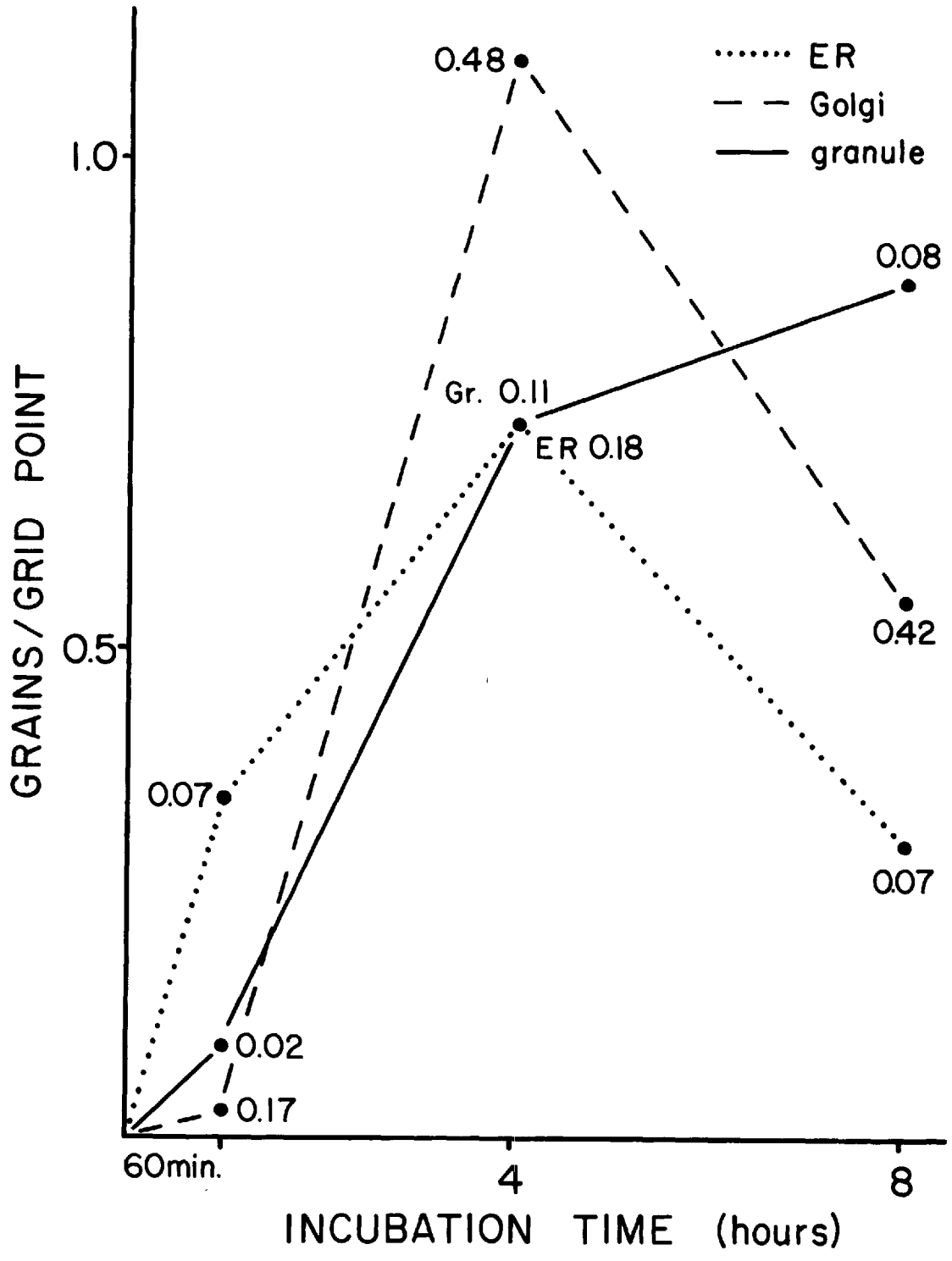
| Incubation time | Organelle | Area % | Activity % | | Grains per grid point | | Activity per unit area | |
|---|---|---|---|---|---|---|---|---|
| 60 minutes | ER | 28.2 | 51.3 | 6.6* | 0.36 | 0.07* | 1.82 | 0.28* |
| | granule | 58.7 | 28.8 | 6.5 | 0.10 | 0.02 | 0.49 | 0.12 |
| | Golgi | 4.6 | 1.3 | 3.0 | 0.10 | 0.17 | 0.28 | 0.42 |
| | nucleus | 8.5 | 18.6 | 2.5 | 0.30 | 0.20 | 2.21 | 0.26 |
| 4 hours | ER | 44.6 | 53.1 | 10.0 | 0.79 | 0.18 | 1.19 | 0.20 |
| | granule | 42.8 | 34.9 | 9.6 | 0.69 | 0.11 | 0.82 | 0.24 |
| | Golgi | 6.6 | 10.3 | 4.4 | 1.25 | 0.48 | 1.55 | 0.75 |
| | nucleus | 6.0 | 1.6 | 1.1 | 0.16 | 0.14 | 0.28 | 0.21 |
| 8 hours | ER | 32.8 | 16.4 | 2.3 | 0.30 | 0.07 | 0.50 | 0.07 |
| | granule | 54.2 | 71.7 | 4.3 | 0.90 | 0.08 | 1.32 | 0.10 |
| | Golgi | 2.4 | 0.0 | 1.5 | 0.48 | 0.42 | 0.01 | 0.75 |
| | nucleus | 10.6 | 11.9 | 5.3 | 0.72 | 0.15 | 1.13 | 0.04 |

* = standard error of the mean

FIGURE IV-7

Grains per grid point for organelles of the mucous cell after incubation with $H^3$-threonine for various times (figures represent the standard error of the mean for each point).

FIGURE IV-7


0.48
ER
Golgi
granule
1.0
0.08
Gr. 0.11
ER 0.18
0.5
0.42
GRAINS/GRID POINT
0.07
0.07
0.02
0.17
60min.
4
8
INCUBATION TIME (hours)

These results confirm those of the circle analysis. Absolute uptake in the endoplasmic reticulum and the Golgi apparatus continues until at least the fourth hour. From this time it is difficult to assess whether uptake ceases or whether more activity is passed to other regions of the cell than incorporated by the endoplasmic reticulum and Golgi apparatus.

Absolute uptake by the granules reaches maximum after the other organelles, so it is presumed that the activity found in the granules has travelled to this region from other organelles. Only for the nucleus does the absolute uptake differ from that obtained by the circle analysis. The erratic behaviour is probably explained by the small percentage area of the nucleus and that it is a distinct, isolated organelle within the cell.

(ii) Activity per unit area (relative concentration of silver grains)

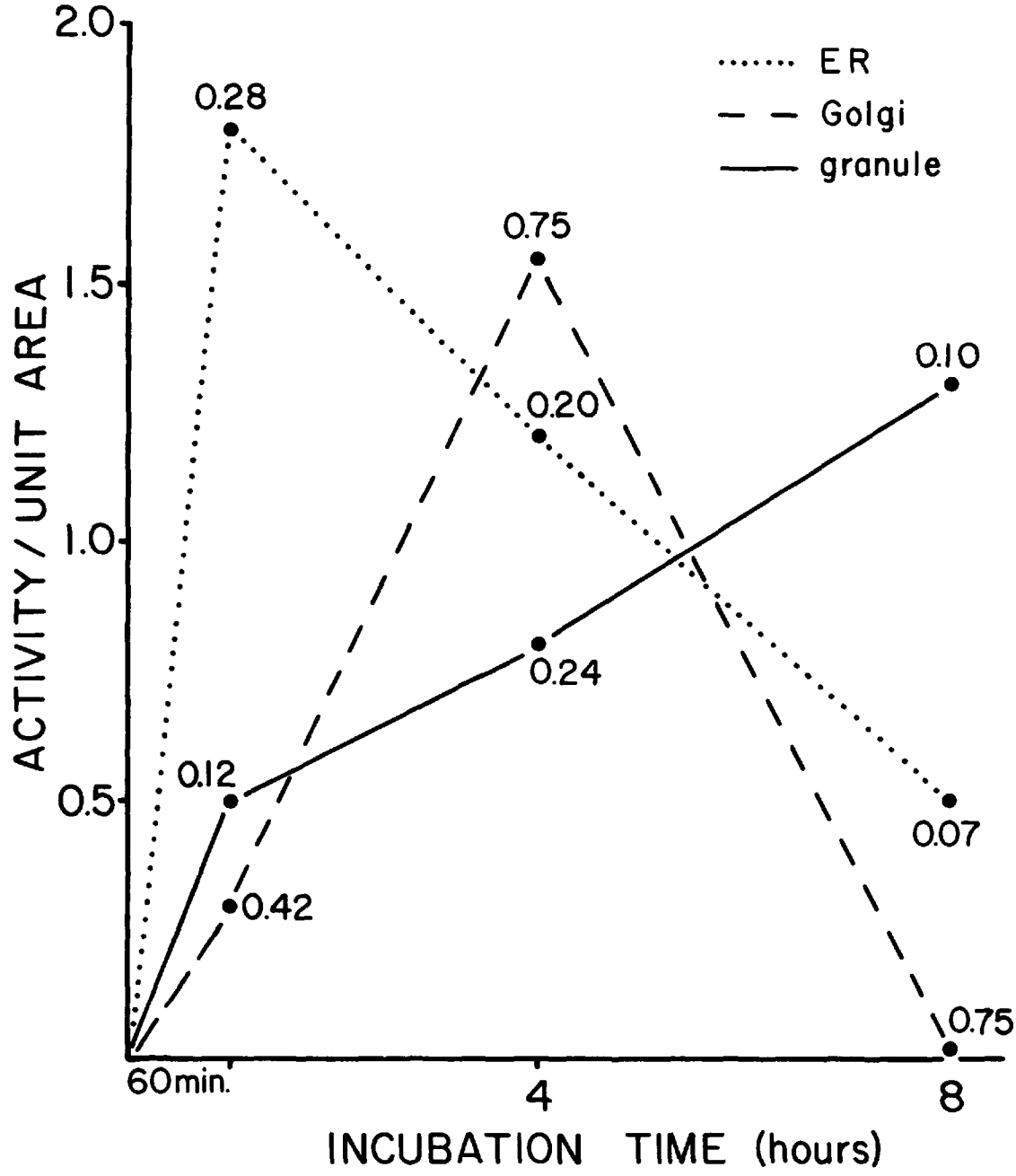
For the mucous cell, after incubation in $H^3$-threonine (Table IV-8 and Figure IV-8), at 60 minutes, much of the activity per unit area was in the endoplasmic reticulum, this decreased at four and eight hours.

In the Golgi apparatus at 60 minutes, little activity was seen, this had increased at four hours and decreased again at eight hours.

FIGURE IV-8

Activity per unit area for organelles of the mucous cell after incubation with $H^3$-threonine for various times (figures represent the standard error of the mean for each point).

FIGURE IV-8


2.0
1.5
1.0
0.5
ACTIVITY / UNIT AREA
ER
Golgi
granule
0.28
0.75
0.10
0.20
0.24
0.12
0.07
0.42
0.75
60min.
4
8
INCUBATION TIME (hours)

From 60 minutes to eight hours, activity per unit area in the granules progressively increased.

Activity for the nucleus was maximum at 60 minutes, but fell at four and eight hours.

These results again agree with those of the circle analysis. Tritiated threonine is initially incorporated into the endoplasmic reticulum, from here it travels to the Golgi apparatus and finally is seen in the secretory granules. Incorporation into the nucleus is also seen at 60 minutes.

Chi-squared test

For the mucous cell, after incubation for 60 minutes in $H^3$-threonine, the $x^2$ analysis of the pattern of distribution for the silver grains gave a value of $p = 0.55$. This represents a fairly high level of significance. At four hours, the level of confidence achieved was only $p = 0.24$ and at eight hours, a higher level of confidence was again reached, $p = 0.41$.

In conclusion, for the mucous cell, quantitative analysis of both light microscopic autoradiography and high resolution autoradiography show the same pattern for

the incorporation of $H^3$-threonine.

Initial incorporation, i.e. at 60 minutes, as studied with either the light or electron microscope, is found to be basal, the region containing most of the rough endoplasmic reticulum (page 220). At 2 hours, heavy labelling with the light microscope is found in the mid-region of acini. The more precise analysis of the electron microscope shows that at four hours, the maximum concentration of silver grains is found in the Golgi apparatus. With the light microscope, heavy localization of silver grains over the apical region of acini is seen from four hours, but not until seven and eight hours is the luminal region heavily labelled. This corresponds to the findings of the electron microscope, where at seven and eight hours, the maximum concentration of silver grains is in the secretory granules.

Using the light microscope, heavy labelling of the acinar lumen was found at seven and eight hours, indicating discharge from the mucous cell.

## SEROUS CELL

The serous cell was analysed in the same way as the mucous cell.

### Circle Analysis

The results of the chi-squared tests for the serous cell after incubation in $H^3$-threonine for various times are shown in Table IV-9. In most cases the results are statistically significant. Four exceptions should perhaps be noted, these are the results at 40 minutes and four hours in study 1 and at seven hours in both studies. The randomness of these results does not suggest a specific pattern of departure from the general trend.

### Absolute uptake and relative concentration of $H^3$-threonine

The figures for the absolute uptake of $H^3$-threonine by the organelles of the serous cell, after incubation in $H^3$-threonine, are shown in Table IV-10 and Figure IV-9. The specific activity values or relative concentration of silver grains over organelles within the serous cell are shown in Table IV-11 and Figure IV-10. Only the smoothed

TABLE IV-9

THE PROBABILITY VALUES FOR THE SEROUS CELL AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Incubation time | 1. ($p =<$) | 2. ($p =<$) |
|---|---|---|
| 20 minutes | 0.2 | 0.001 |
| 40 minutes | 0.9 | 0.1 |
| 60 minutes | 0.05 | 0.05 |
| 2 hours | 0.02 | 0.1 |
| 3 hours | 0.1 | 0.1 |
| 4 hours | 0.5 | 0.01 |
| 5 hours | 0.01 | 0.05 |
| 6 hours | 0.05 | 0.05 |
| 7 hours | 0.3 | 0.2 |
| 8 hours | 0.001 | 0.001 |

$<0.05$ = statistically significant = non random distribution
$<0.2$ = trend to non-random distribution
$<0.2$ = not significant = random distribution

curves, showing the trend of the points for both the absolute uptake and the relative concentration of silver grains for each organelle will be described.

Initial incorporation of $H^3$-threonine by the serous cell was in the endoplasmic reticulum. Absolute uptake for the endoplasmic reticulum was maximum at four hours (Table IV-10 and Figure IV-9), and from this time decreased. At 20 minutes, the relative concentration of silver grains was maximal for the endoplasmic reticulum (Table IV-11 and Figures IV-10 and IV-11). These results, like those of the mucous cell, indicate that initial incorporation of $H^3$-threonine is in the endoplasmic reticulum, although further uptake by this organelle continues beyond this time.

From the endoplasmic reticulum, the activity passed to the junctional region, ER/Golgi apparatus. At four hours, the uptake for this region was at maximum and then diminished (Table IV-10). At 40 minutes, the maximum relative concentration of silver grains was found for the ER/Golgi apparatus (Table IV-11 and Figure IV-11).

Radioactivity is next seen in the Golgi apparatus. At four hours, maximal absolute uptake was found in the Golgi apparatus, this then decreased (Table IV-10 and Figure IV-9). At 60 minutes, the relative concentration

TABLE IV-10

THE ABSOLUTE UPTAKE OF TWO STUDIES AND THEIR MEAN FOR ORGANELLES IN THE SEROUS CELL AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 0.15 | 0.10 | 0.26 | 0.42 | 0.40 | 0.66 | 0.64 | 0.77 | 0.59 | 0.61 |
| | 2 | 0.15 | 0.27 | 0.11 | 0.30 | 0.79 | 0.81 | 0.75 | 0.33 | 0.22 | 0.20 |
| | m | 0.15 | 0.18 | 0.18 | 0.36 | 0.59 | 0.73 | 0.69 | 0.55 | 0.40 | 0.40 |
| ER/Golgi | 1 | 0.18 | 0.36 | 0.46 | 0.71 | 0.60 | 0.82 | 0.93 | 0.53 | 0.72 | 0.76 |
| | 2 | 0.00 | 0.44 | 0.00 | 0.18 | 1.36 | 0.46 | 1.00 | 0.00 | 0.17 | 0.00 |
| | m | 0.09 | 0.40 | 0.23 | 0.44 | 0.98 | 0.64 | 0.96 | 0.26 | 0.44 | 0.38 |
| Golgi | 1 | 0.09 | 0.10 | 0.37 | 1.40 | 0.75 | 0.70 | 1.00 | 0.54 | 0.34 | 0.24 |
| | 2 | 0.11 | 0.29 | 0.27 | 0.47 | 0.78 | 0.83 | 0.80 | 0.55 | 0.21 | 0.25 |
| | m | 0.10 | 0.19 | 0.32 | 0.93 | 0.76 | 0.76 | 0.90 | 0.54 | 0.27 | 0.24 |
| Granule | 1 | 0.16 | 0.00 | 0.18 | 0.42 | 0.11 | 0.45 | 0.33 | 0.76 | 0.33 | 0.78 |
| | 2 | 0.16 | 0.25 | 0.16 | 0.42 | 0.61 | 0.50 | 0.55 | 0.28 | 0.19 | 0.22 |
| | m | 0.16 | 0.12 | 0.17 | 0.42 | 0.36 | 0.47 | 0.44 | 0.52 | 0.26 | 0.50 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ER/granule | 1 | 0.12 | 0.08 | 0.24 | 0.31 | 0.52 | 0.65 | 1.35 | 1.09 | 0.95 | 0.87 |
| | 2 | 0.08 | 0.09 | 0.08 | 0.28 | 1.14 | 1.00 | 0.75 | 0.31 | 0.17 | 0.27 |
| | m | 0.10 | 0.08 | 0.16 | 0.29 | 0.83 | 0.82 | <u>1.05</u> | 0.70 | 0.56 | 0.57 |
| ER/others | 1 | 0.11 | 0.07 | 0.14 | 0.17 | 0.30 | 0.53 | 0.93 | 0.59 | 0.53 | 0.60 |
| | 2 | 0.03 | 0.21 | 0.10 | 0.27 | 0.68 | 0.48 | 0.69 | 0.35 | 0.16 | 0.24 |
| | m | 0.07 | 0.14 | 0.12 | 0.22 | 0.49 | 0.60 | <u>0.81</u> | 0.47 | 0.34 | 0.37 |
| Nucleus | 1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.47 | 0.78 | 0.57 | 0.80 | 0.18 | 1.11 |
| | 2 | 0.05 | 0.77 | 0.25 | 0.24 | 1.25 | 0.12 | 0.72 | 0.36 | 0.27 | 0.42 |
| | m | 0.02 | 0.38 | 0.12 | 0.12 | <u>0.86</u> | 0.45 | 0.64 | 0.58 | 0.22 | 0.76 |
| Canaliculi | 1 | 0.00 | 0.09 | 0.08 | 0.28 | 0.30 | 0.12 | 0.75 | 1.05 | 0.53 | 2.50 |
| | 2 | 0.07 | 0.00 | 0.00 | 0.00 | 0.42 | 1.33 | 0.37 | 0.00 | 0.42 | 0.00 |
| | m | 0.03 | 0.04 | 0.04 | 0.14 | 0.36 | 0.72 | 0.56 | 0.52 | 0.47 | <u>1.25</u> |

Maximum absolute uptake for each organelle is underlined.

FIGURE IV-9

Smoothed curves representing the trend of points for absolute uptake of $H^3$-threonine by organelles of the serous cell after incubation for various times (the dots represent the mean for each time).

FIGURE ~~IV-9~~ V. 7. 5 p. 266

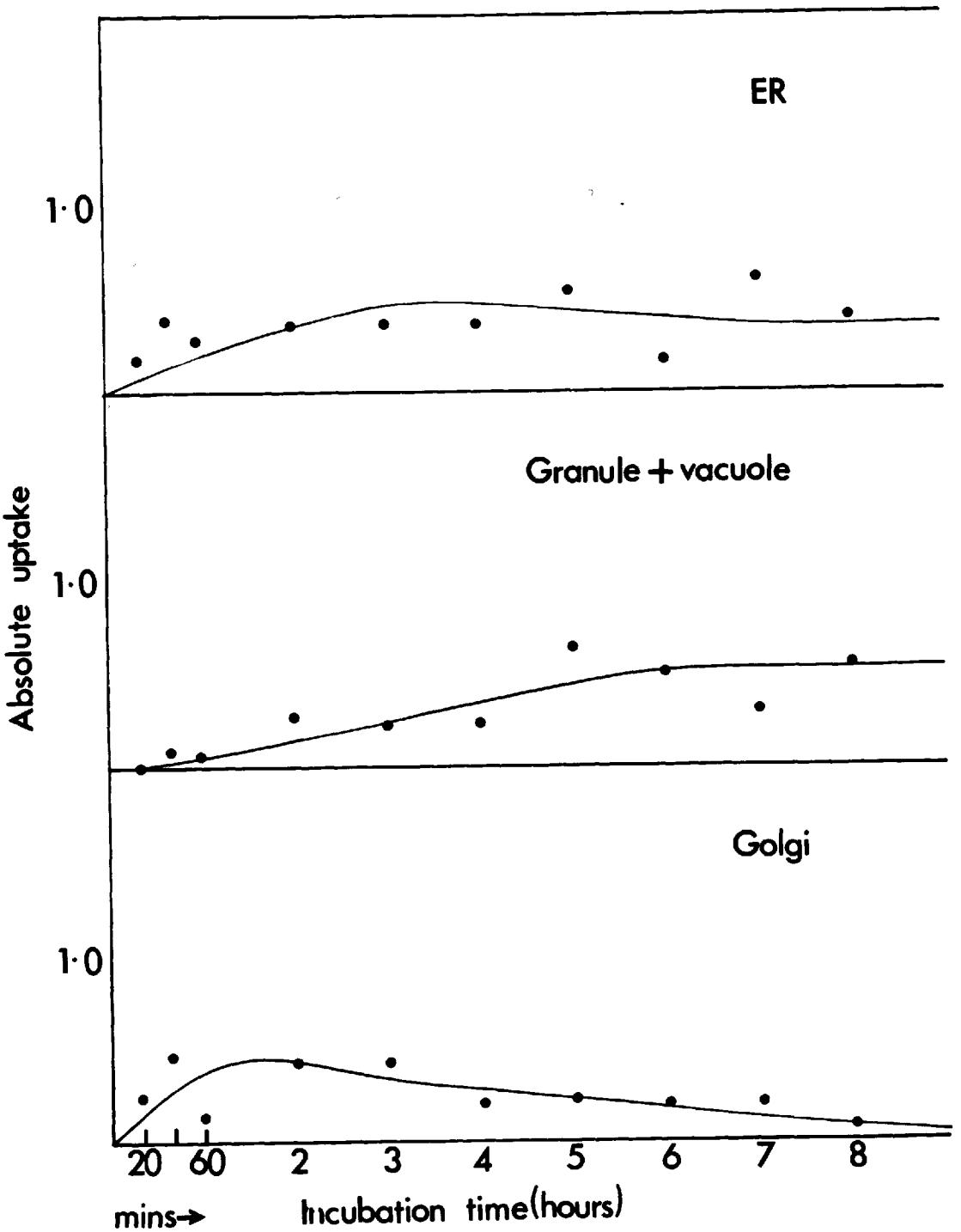


TABLE IV-11

THE SPECIFIC ACTIVITY (RELATIVE CONCENTRATION OF SILVER GRAINS) OF TWO STUDIES AND THEIR MEAN FOR ORGANELLES IN THE SEROUS CELL AFTER INCUBATION IN $H^3$-THREONINE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 1.13 | 1.15 | 1.11 | 1.07 | 0.99 | 1.04 | 0.81 | 0.99 | 1.01 | 0.83 |
| | 2 | 1.74 | 1.21 | 0.88 | 0.97 | 0.92 | 1.08 | 1.10 | 1.01 | 1.11 | 0.88 |
| | m | 1.44 | 1.18 | 1.00 | 1.02 | 0.96 | 1.06 | 0.96 | 1.00 | 1.06 | 0.86 |
| ER/Golgi | 1 | 1.52 | 3.89 | 1.89 | 1.82 | 1.47 | 1.29 | 1.17 | 0.67 | 1.23 | 0.00 |
| | 2 | 0.00 | 2.00 | 0.00 | 0.58 | 1.57 | 0.61 | 1.42 | 0.00 | 0.84 | 1.03 |
| | m | 0.76 | 2.95 | 0.95 | 1.20 | 1.52 | 0.95 | 1.30 | 0.34 | 1.04 | 0.52 |
| Golgi | 1 | 0.74 | 3.20 | 1.51 | 3.56 | 1.83 | 1.10 | 1.25 | 0.69 | 0.58 | 0.32 |
| | 2 | 1.30 | 1.31 | 2.10 | 1.52 | 0.89 | 1.11 | 1.17 | 2.40 | 1.07 | 1.08 |
| | m | 1.02 | 2.25 | 1.81 | 2.04 | 1.36 | 1.10 | 1.21 | 1.55 | 0.83 | 0.70 |
| Granule | 1 | 1.35 | 0.00 | 0.74 | 1.07 | 0.27 | 0.71 | 0.42 | 0.92 | 0.73 | 1.06 |
| | 2 | 1.88 | 1.12 | 1.30 | 1.38 | 0.71 | 0.67 | 0.79 | 0.91 | 0.96 | 0.98 |
| | m | 1.61 | 0.56 | 1.02 | 1.22 | 0.48 | 0.68 | 0.61 | 0.94 | 0.85 | 1.02 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ER/granule | 1 | 1.10 | 0.95 | 0.81 | 0.76 | 1.28 | 1.00 | 1.71 | 1.36 | 1.46 | 1.04 |
| | 2 | 0.94 | 0.39 | 0.37 | 0.25 | 1.28 | 1.33 | 1.10 | 0.97 | 0.78 | 1.22 |
| | m | 1.02 | 0.67 | 0.59 | 0.51 | 1.28 | 1.17 | <u>1.41</u> | 1.17 | 1.12 | 1.13 |
| ER/others | 1 | 0.95 | 0.79 | 0.69 | 0.45 | 0.73 | 0.84 | 1.02 | 0.75 | 1.01 | 1.03 |
| | 2 | 0.51 | 0.97 | 0.87 | 1.40 | 0.78 | 0.65 | 1.01 | 1.09 | 0.81 | 0.67 |
| | m | 0.73 | 0.88 | 0.78 | 0.93 | 0.76 | 0.75 | <u>1.02</u> | 0.92 | 0.91 | 0.85 |
| Nucleus | 1 | 0.00 | 0.00 | 0.00 | 0.00 | 1.15 | 1.23 | 0.72 | 1.01 | 0.32 | 1.80 |
| | 2 | 0.77 | 3.49 | 1.97 | 1.45 | 1.44 | 0.17 | 1.08 | 1.09 | 1.36 | 1.51 |
| | m | 0.39 | <u>1.75</u> | 0.99 | 0.73 | 1.30 | 0.70 | 0.90 | 1.05 | 0.84 | 1.66 |
| Canaliculi | 1 | 0.00 | 1.02 | 0.36 | 0.73 | 0.73 | 0.20 | 0.94 | 1.34 | 0.91 | 0.00 |
| | 2 | 0.87 | 0.00 | 0.00 | 0.00 | 0.49 | 1.44 | 0.55 | 0.00 | 2.11 | 3.37 |
| | m | 0.44 | 0.51 | 0.18 | 0.37 | 0.61 | 0.82 | 0.75 | 0.67 | 1.51 | <u>1.69</u> |

Maximum specific activity for each organelle is underlined.

FIGURE IV-10

Smoothed curves representing the trend of points for the specific activity (relative concentration of silver grains) of organelles of the serous cell after incubation with $H^3$-threonine for various times (the dots represent the mean for each time).

FIGURE IV-10

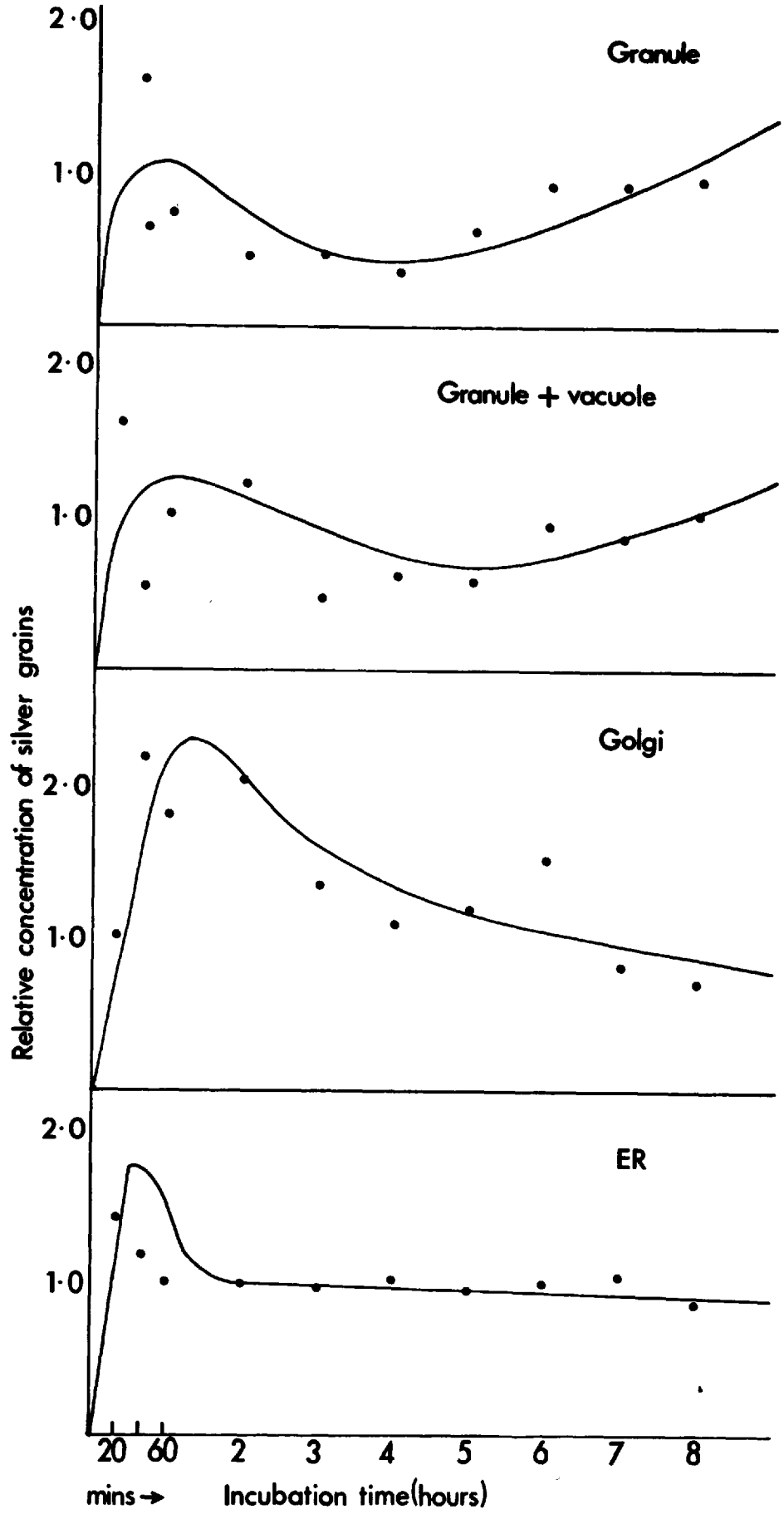
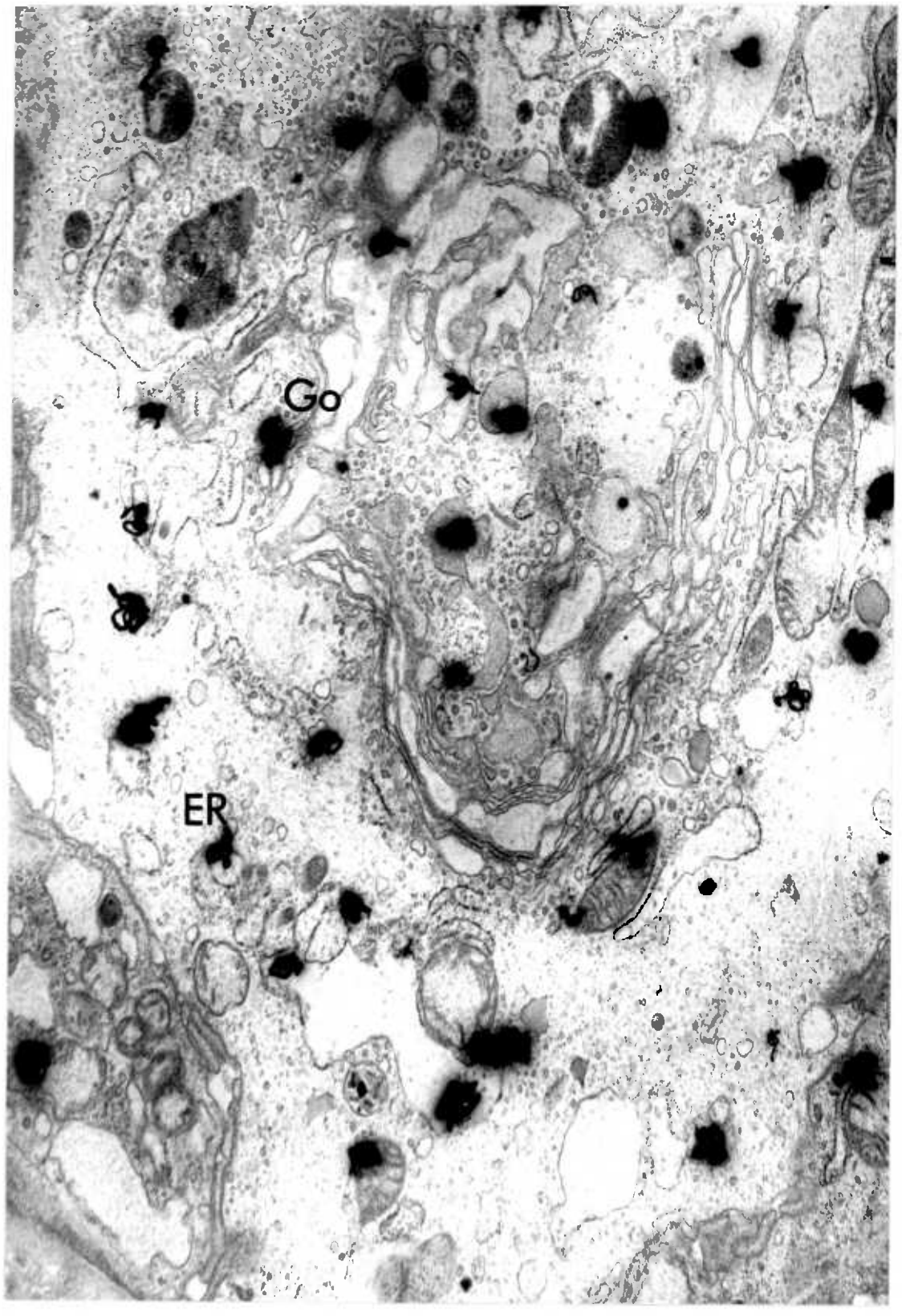

Granule
Granule + vacuole
Golgi
ER
2·0
1·0
Relative concentration of silver grains
20
60
2
3
4
5
6
7
8
mins →
Incubation time(hours)

FIGURE IV-11

Part of a serous cell, after two hours incubation with $H^3$-threonine. Localization of silver grains is found over the endoplasmic reticulum (ER) and the Golgi apparatus (Go). X 20,000.

FIGURE IV-11



of silver grains was at maximum over this region (Table IV-11 and Figures IV-10 and IV-11). The results for the maximum relative concentration of silver grains over the Golgi apparatus occurs at a later time than that for the endoplasmic reticulum and the junctional region, ER/Golgi apparatus. This would support the suggestion that the $H^3$-threonine moves from the rough endoplasmic reticulum to the Golgi apparatus. This timing contrasts markedly with that for the mucous cell where the maximum relative concentration of silver grains for the Golgi apparatus was not seen until later at five hours.

The results for the absolute uptake of all three of these organelles is maximum at a similar time (four hours), but differences in time of uptake would be difficult to detect since radioactivity passes through these three organelles in a short space of time (60 minutes). Specimens from 60 minutes onwards were only examined at hourly intervals.

Radioactivity was found in the vacuoles at a similar time to the Golgi apparatus. The results for the vacuole are considered together with those for the granules, since the volume of the cell occupied by the vacuoles is small. At four hours, absolute uptake by the granules alone and with the

vacuoles combined was at maximum (Table IV-10 and Figure (IV-9), this level of uptake then fell slightly during the remainder of the experiment. At 40 minutes, considering the granules alone (without vacuoles), the relative concentration of silver grains was at a peak, this activity then decreased until four hours and from this time again increased to reach a second peak at eight hours (Table IV-11 and Figure IV-10). A similar pattern was found when the vacuoles were added to the granules; the first peak occurred between 40 and 60 minutes, but this was more pronounced than that of the granules alone. The relative concentration of silver grains remained at a higher level in this combined group until seven and eight hours, when the second peak was reached.

The first peak in relative concentration of silver grains for the granules was unexpected and could be caused by three factors:

1. serous granules may be synthesized very rapidly (approximately 40 minutes)
2. $H^3$-threonine may be more readily available to the serous cell from the canaliculi than from the surrounding tissue and granules therefore may incorporate $H^3$-threonine directly
3. labelling of the granules may be only apparent.

This latter factor is preferred. For discussion of these factors see page 311.

The higher relative concentration of silver grains found when the granules and vacuoles are combined would seem to lend support to the suggestion that the vacuoles are the precursors of the secretory granule. The first peak of maximum relative concentration of silver grains for the combined group occurs at a similar time to that of the Golgi apparatus and thus lends further support for a link between the Golgi apparatus and the vacuoles. The second peak for the serous granules, at eight hours, represents a longer secretory cycle and is similar to that described for the mucous cell. From these results it would seem that some at least of the radioactive precursor passes from the Golgi apparatus to the vacuoles and finally localizes within the secretory granules. The explanation for the first peak is however, at this point uncertain.

Between four and five hours, absolute uptake in the junctional region ER/granule was at maximum, and then decreased (Table IV-10). The relative concentration of silver grains showed a small peak between 20 and 40 minutes (Table IV-11), doubtless due to the endoplasmic reticulum of this region; the maximum concentration of silver grains was found at four hours, this level then fell slightly until eight hours. The incorporation of $H^3$-threonine by the region would therefore seem to reflect both of the component organelles.

At five hours, maximum absolute uptake was found for the junctional region ER/others, from five - eight hours this decreased (Table IV-10). Between 40 and 60 minutes, the relative concentration of silver grains in this region was maximum (Table IV-11). The incorporation of $H^3$-threonine by this junctional region, ER/others would seem to be mainly into the endoplasmic reticulum, little would appear to be in the 'other' organelles.

Between four and five hours, absolute uptake by the nucleus was at maximum, from this time a decrease was seen (Table IV-10). At 40 minutes, maximum relative concentration of silver grains was found for the nucleus (Table IV-11). A similar maximum was found for the nucleus of the mucous cell and would indicate that protein synthesis might occur within the nucleus.

For the canaliculi, situated external to the serous cell, absolute uptake was still increasing at eight hours (Table IV-10). The relative concentration of silver grains over this region also continued to rise at eight hours (Table IV-11). This would suggest that the number of serous cell granules discharging had not reached maximum by eight hours.

Thus, a similar pattern of incorporation of $H^3$-threonine can be described for the serous cell as found for the mucous cell. Tritiated threonine is initially incorporated into the endoplasmic reticulum, from here it is transported to the Golgi apparatus and finally localizes in the granules having passed through the vacuoles.

Because of the peak in relative concentration of silver grains in the granules at 40 minutes, a supplementary or alternative pathway must also be considered. Incorporation of $H^3$-threonine would seem initially to localize in the granules at the apex, the radioactivity perhaps being transported from the canaliculi by vesicles. These granules may be immature at this stage and could then pass to the Golgi apparatus for the addition of other components of the secretory product. When the granules are fully mature they migrate again to the lateral and apical surfaces around the canaliculi for discharge.

If the mucous and serous cells are considered to incorporate $H^3$-threonine by similar pathways, the only difference found is in the timing of the various sequences. For the mucous cell, silver grains are initially localized in the endoplasmic reticulum at 60 minutes and for the serous cell, at 20 minutes. Maximum relative concentration of silver grains in

the Golgi apparatus is at four hours in the mucous cell, and at 60 minutes for the serous cell. For the granules of both cell types, at approximately seven hours, the maximum relative concentration of silver grains is found.

This would indicate that the initial synthesis of the secretory product of the serous cell is faster than that of the mucous cell, although the final product, the secretory granule, is fully formed for both cell types, at a similar time. Maturation of the serous granule would therefore seem to take longer to complete than that of the mucous cell.

The results of the absolute uptake of $H^3$-threonine by the organelles of the serous cell, in a similar manner to the mucous cell (see page 166 ), show that uptake continues after removal of the explant from the 'pulse' label. Maximum absolute uptake is reached for most organelles at four hours, slightly longer times are obtained for the junctional regions, ER/granule and ER/others, and the canaliculi showed increasing uptake even at eight hours. This latter region is situated outside the serous cell. Uptake of $H^3$-threonine by all the organelles of the serous cell is less than by those of the mucous cell (see page 205 for absolute uptake by the whole cell).

## Hypothetical Grain Analysis

Using this method, three specimens were analysed for the serous cell, after incubation in $H^3$-threonine for 60 minutes, four and eight hours. The (i) grains per grid point and (ii) activity per unit area will be described.

### (i) Grains per grid point (absolute uptake)

At 60 minutes (Table IV-12 and Figure IV-12), the Golgi apparatus showed the maximum grains per grid point for this time, at four hours uptake had increased and remained at this level at eight hours.

At 60 minutes, little absolute uptake was found for the endoplasmic reticulum, uptake increased at four hours and fell again at eight hours.

Few grains per grid point, at 60 minutes, were seen for the granules, by four hours an increase was apparent and at eight hours, a further slight increase.

At 60 minutes, the nucleus exhibited little absolute uptake, but at four and eight hours this increased.

In general these results confirm those of the circle analysis. Absolute uptake of $H^3$-threonine by

TABLE IV-12

THE PERCENTAGE AREA, PERCENTAGE ACTIVITY, ACTIVITY PER GRID POINT AND ACTIVITY PER UNIT AREA FOR THE SEROUS CELL AFTER INCUBATION IN $H^3$ THREONINE, FOR 60 MINUTES, 4 AND 8 HOURS

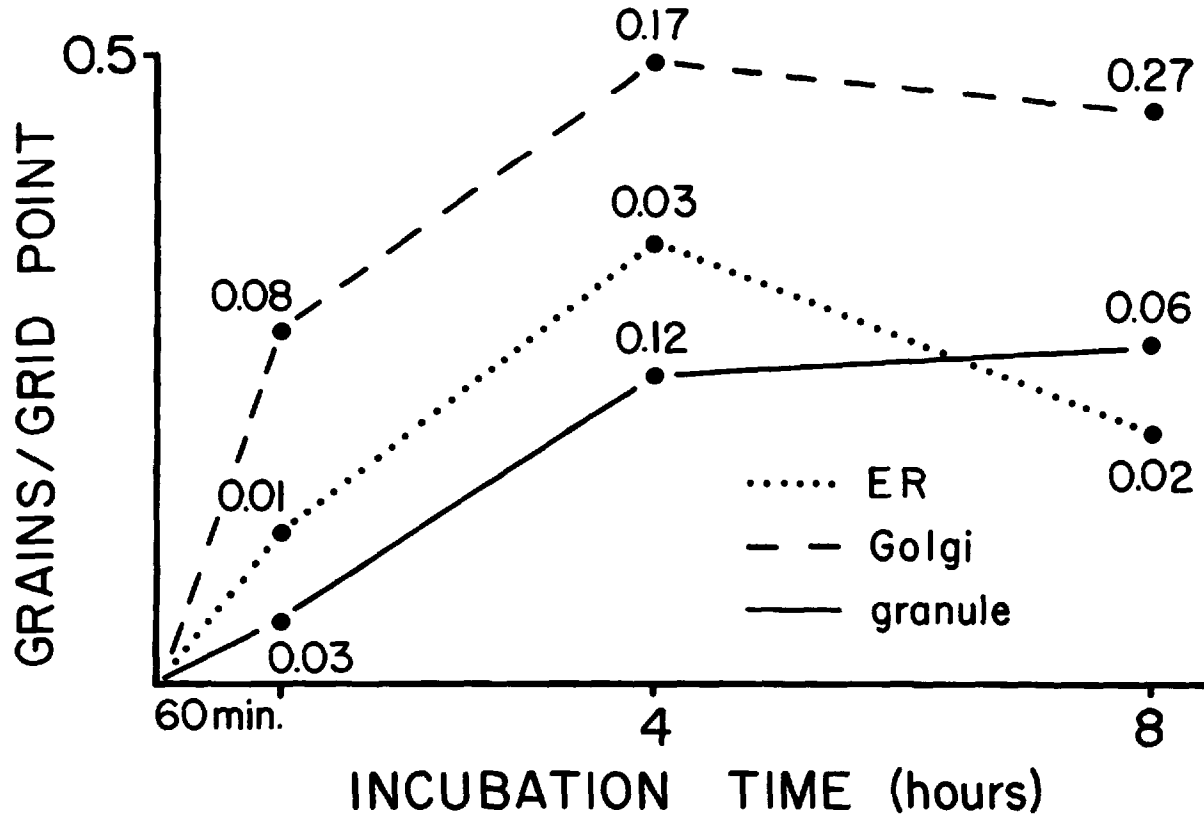
| Incubation time | Organelle | Area % | Activity % | | Grains per grid point | | Activity per unit area | |
|---|---|---|---|---|---|---|---|---|
| 60 minutes | ER | 66.8 | 62.8 | 6.8* | 0.12 | 0.01* | 0.93 | 1.00* |
| | granule | 16.7 | 6.8 | 3.4 | 0.05 | 0.03 | 0.40 | 0.15 |
| | Golgi | 13.5 | 29.3 | 7.7 | 0.28 | 0.08 | 2.16 | 0.56 |
| | nucleus | 2.9 | 1.1 | 0.5 | 0.05 | 0.02 | 0.48 | 0.37 |
| 4 hours | ER | 70.2 | 69.4 | 4.4 | 0.35 | 0.03 | 0.98 | 0.08 |
| | granule | 10.2 | 7.0 | 2.6 | 0.24 | 0.12 | 0.68 | 0.28 |
| | Golgi | 10.3 | 14.3 | 4.6 | 0.49 | 0.17 | 1.39 | 0.54 |
| | nucleus | 9.3 | 9.2 | 3.2 | 0.35 | 0.13 | 0.98 | 0.49 |
| 8 hours | ER | 58.1 | 46.5 | 5.8 | 0.20 | 0.02 | 0.89 | 0.10 |
| | granule | 31.9 | 34.7 | 5.4 | 0.27 | 0.06 | 1.19 | 0.16 |
| | Golgi | 4.6 | 8.4 | 3.8 | 0.45 | 0.27 | 1.99 | 0.90 |
| | nucleus | 5.4 | 10.4 | 3.2 | 0.48 | 0.14 | 0.12 | 1.16 |

* = standard error of the mean

FIGURE IV-12

Grains per grid point for organelles of the serous cell after incubation with $H^3$-threonine for various times (figures represent the standard error of the mean for each point).

FIGURE IV-12


0.5
GRAINS/GRID POINT
0.17
0.27
0.03
0.08
0.06
0.12
0.01
0.02
0.03
ER
Golgi
granule
60min.
4
8
INCUBATION TIME (hours)

the serous cell continues in the Golgi apparatus and the endoplasmic reticulum until at least four hours, when uptake may stop or alternatively transport of activity from this organelle to other regions may exceed uptake.

Absolute uptake for both the granules and the nucleus reach maximum at eight hours. This would suggest, especially in the case of the granules, that uptake by this organelle could be as a result of transport of the radioactive precursor from other organelles. In the case of the nucleus, this would seem improbable, since protein synthesis is thought to occur in the nucleus itself and therefore the need for cytoplasmic protein is unlikely. Alternatively, this unexpected finding for the nucleus may be due to the distinct, compact region occupied by this organelle in the cell or even to impending mitotic activity.

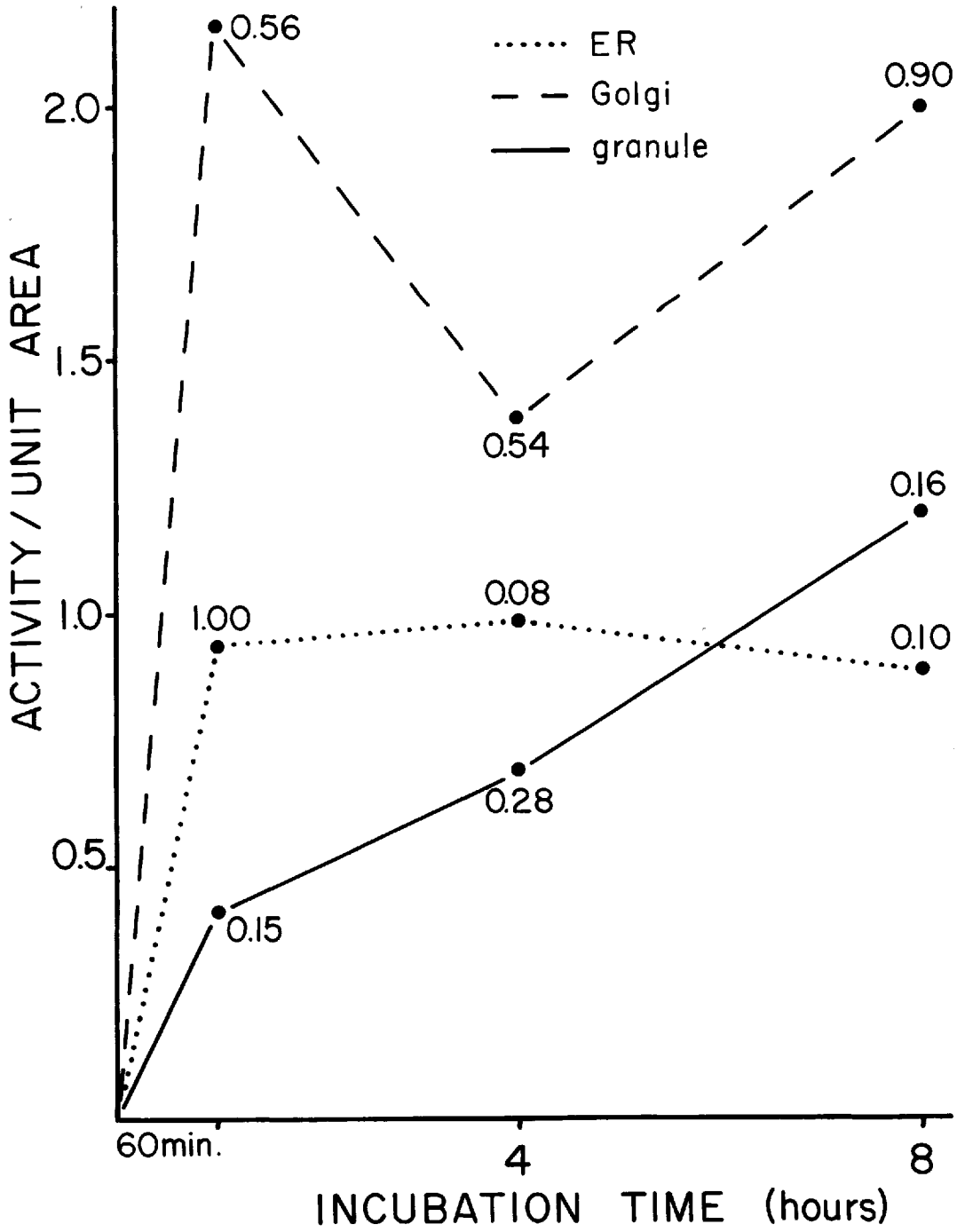
(ii) Activity per unit area (relative concentration of silver grains)

For the serous cell, after incubation in $H^3$-threonine, at 60 minutes (Table IV-12 and Figure IV-13), maximum activity per unit area was found in the Golgi apparatus, at four hours this decreased, and eight hours increased. The standard error for this organelle is rather large

FIGURE IV-13

Activity per unit area for organelles of the serous cell after incubation with $H^3$-threonine for various times (figures represent the standard error of the mean for each point).

FIGURE IV-13


ER
Golgi
granule
ACTIVITY / UNIT AREA
2.0
1.5
1.0
0.5
0.56
0.54
0.90
0.16
0.08
1.00
0.10
0.28
0.15
60min.
4
8
INCUBATION TIME (hours)

and it is therefore felt, in view of the results of the circle analysis, that these results may represent an overall downward trend (decrease) of activity.

For the endoplasmic reticulum, a constant level of activity per unit area was found at all times.

At 60 minutes, little activity was found in the granules, at four and eight hours this activity increased.

Little activity, at 60 minutes, was found in the nucleus, at four hours this increased, and at eight hours fell.

For the serous cell, it would seem from the results of the hypothetical grain analysis, that initial incorporation of $H^3$-threonine is into the Golgi apparatus and that the endoplasmic reticulum plays no part in the synthesis of protein - clearly an unlikely event. In view of the observations made with the circle analysis however, this would not seem to be the case, since at 20 minutes (an earlier time than studied for the hypothetical grain analysis), localization was found in the endoplasmic reticulum, which by 60 minutes, had passed to the Golgi apparatus.

With the hypothetical grain analysis, no evidence of the double peak, found for the granules in the circle analysis, is apparent. These results are not conflicting if it is recognized that the hypothetical grain analysis makes allowance for 'cross-fire'. Thus, the early peak, seen with the circle analysis is likely to represent 'cross-fire' from adjacent organelles, probably the endoplasmic reticulum, rather than a localization within the granules.

With these points in mind it becomes apparent that the serous cell incorporates $H^3$-threonine in a similar manner to that described for the mucous cell (see page 174 ). Initial incorporation is into the endoplasmic reticulum, activity then passes to the Golgi apparatus and finally into the granules, by way of the vacuoles. Incorporation of $H^3$-threonine into the nucleus is seen at four hours.

Chi-squared test

At 60 minutes, for the serous cell after incubation in $H^3$-threonine, the $x^2$ analysis of the pattern of distribution for the silver grains gave a value of $p = 0.81$, at four hours $p = 0.88$ and at eight hours $p = 0.60$. For all times these represent a high level of confidence.

In conclusion, for the serous cell, light and electron microscopic quantitative analysis of autoradiographs show conflicting patterns for incorporation of $H^3$-threonine.

With the light microscope, little incorporation of $H^3$-threonine is found before 60 minutes into serous acini, whereas with the electron microscope, at 20 minutes, maximum incorporation is seen in the endoplasmic reticulum and into the granules at 40 minutes. With the light microscope, from 60 minutes, the serous acini show intermittent localization to all three regions of the acini. With the electron microscope, at 60 minutes, $H^3$-threonine localizes in the Golgi apparatus and the vacuoles, the radioactive precursor then passes to the granules.

Localization of $H^3$-threonine in the canaliculi, with the electron microscope, increases over the eight hours of the experiment, indicating that the peak of serous cell discharge has not been achieved even at eight hours.

The localization of $H^3$-threonine, at 40 minutes, in the granules is thought to be due to 'cross-fire', the source of the silver grain being situated in a nearby organelle, probably the endoplasmic reticulum.

In the course of the present study, total uptake of $H^3$-threonine by the mucous and serous cell was assessed as well as the volume of a given organelle within both cell types.

## TOTAL UPTAKE OF $H^3$-THREONINE BY MUCOUS AND SEROUS CELLS: GRAINS PER UNIT AREA

By dividing the total number of silver grains by the total number of circles counted for each incubation time, an estimate of the number of grains per unit area was obtained, i.e. a measure of grain density. This estimate does not take into account the distribution of grains associated with particular organelles. One circle was found to represent approximately 4 μm of cell. Table IV-13 shows the values obtained and Figure IV-14 the number of grains per unit area.

The mean number of silver grains per unit area (grain density) was also counted over the base and apex of both cell types. A card, with a hole 12.5 x 8.5 cm was superimposed over at least six micrographs for each cell type, at each incubation time, for each of two specimens. A shift of silver grains from the base to the apex of the cell could thus be detected. Table IV-14 shows the mean number of grains counted for the mucous (Figure IV-15) and the serous cell (Figure IV-16).

TABLE IV-13

MEAN NUMBER OF GRAINS PER UNIT AREA (4 sq μm) OVER THE MUCOUS AND SEROUS CELL AFTER INCUBATION WITH $H^3$-THREONINE

| INCUBATION TIME | No. Grains | No. Circles | Grains / Circles |
|---|---|---|---|
| | | MUCOUS CELL | |
| 20 minutes | 178 | 768 | 0.23 |
| 40 minutes | 168 | 804 | 0.21 |
| 60 minutes | 389 | 1164 | 0.33 |
| 2 hours | 844 | 1046 | 0.81 |
| 3 hours | 904 | 860 | 1.05 |
| 4 hours | 862 | 825 | 1.04 |
| 5 hours | 841 | 806 | 1.04 |
| 6 hours | 754 | 678 | 1.11 |
| 7 hours | 781 | 882 | 0.88 |
| 8 hours | 802 | 1054 | 0.76 |
| | | SEROUS CELL | |
| 20 minutes | 164 | 926 | 0.18 |
| 40 minutes | 164 | 797 | 0.21 |
| 60 minutes | 289 | 1159 | 0.25 |
| 2 hours | 480 | 880 | 0.55 |
| 3 hours | 820 | 1176 | 0.70 |
| 4 hours | 755 | 1061 | 0.71 |
| 5 hours | 628 | 845 | 0.74 |
| 6 hours | 513 | 1067 | 0.48 |
| 7 hours | 401 | 1181 | 0.34 |
| 8 hours | 393 | 923 | 0.43 |

FIGURE IV-14

Mean number of grains per unit area (4 sq $\mu$m) over both the mucous and serous cell after incubation with $H^3$-threonine for various times.

FIGURE IV-14

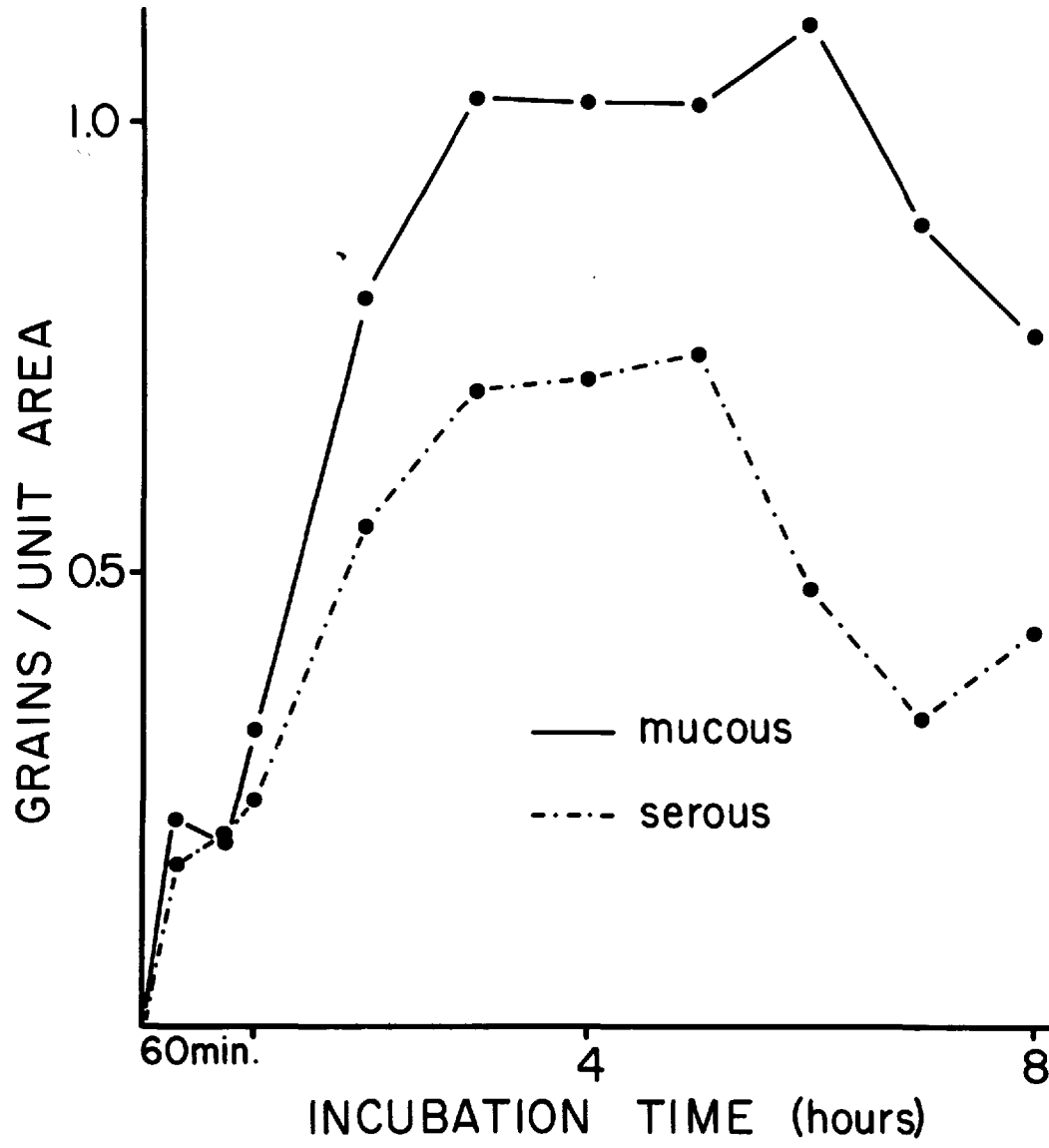

1.0
0.5
GRAINS / UNIT AREA
mucous
serous
60min.
4
8
INCUBATION TIME (hours)

TABLE IV-14

MEAN NUMBER OF GRAINS PER UNIT AREA (12.5 x 8.5 cm) OVER THE BASE AND APEX OF BOTH THE MUCOUS AND SEROUS CELL AFTER INCUBATION WITH $H^3$-THREONINE

| | MUCOUS | | SEROUS | |
|---|---|---|---|---|
| Incubation time | Base | Apex | Base | Apex |
| 20 minutes | 4.3 | 1.6 | 1.3 | 1.7 |
| 40 minutes | 4.0 | 1.5 | 4.5 | 3.1 |
| 60 minutes | 10.5 | 3.1 | 5.4 | 1.1 |
| 2 hours | 9.8 | 5.1 | 8.6 | 7.4 |
| 3 hours | 25.6 | 7.7 | 12.0 | 10.4 |
| 4 hours | 26.2 | 5.8 | 15.3 | 15.8 |
| 5 hours | 33.6 | 8.5 | 15.2 | 18.0 |
| 6 hours | 20.5 | 24.3 | 16.0 | 16.9 |
| 7 hours | 17.8 | 17.2 | 9.6 | 10.6 |
| 8 hours | 20.6 | 17.0 | 11.1 | 16.2 |

FIGURE IV-15

Mean number of grains per unit area (12.5 x 8.5 cm) over the base and apex of the mucous cell after incubation with $H^3$-threonine for various times.

FIGURE IV-15

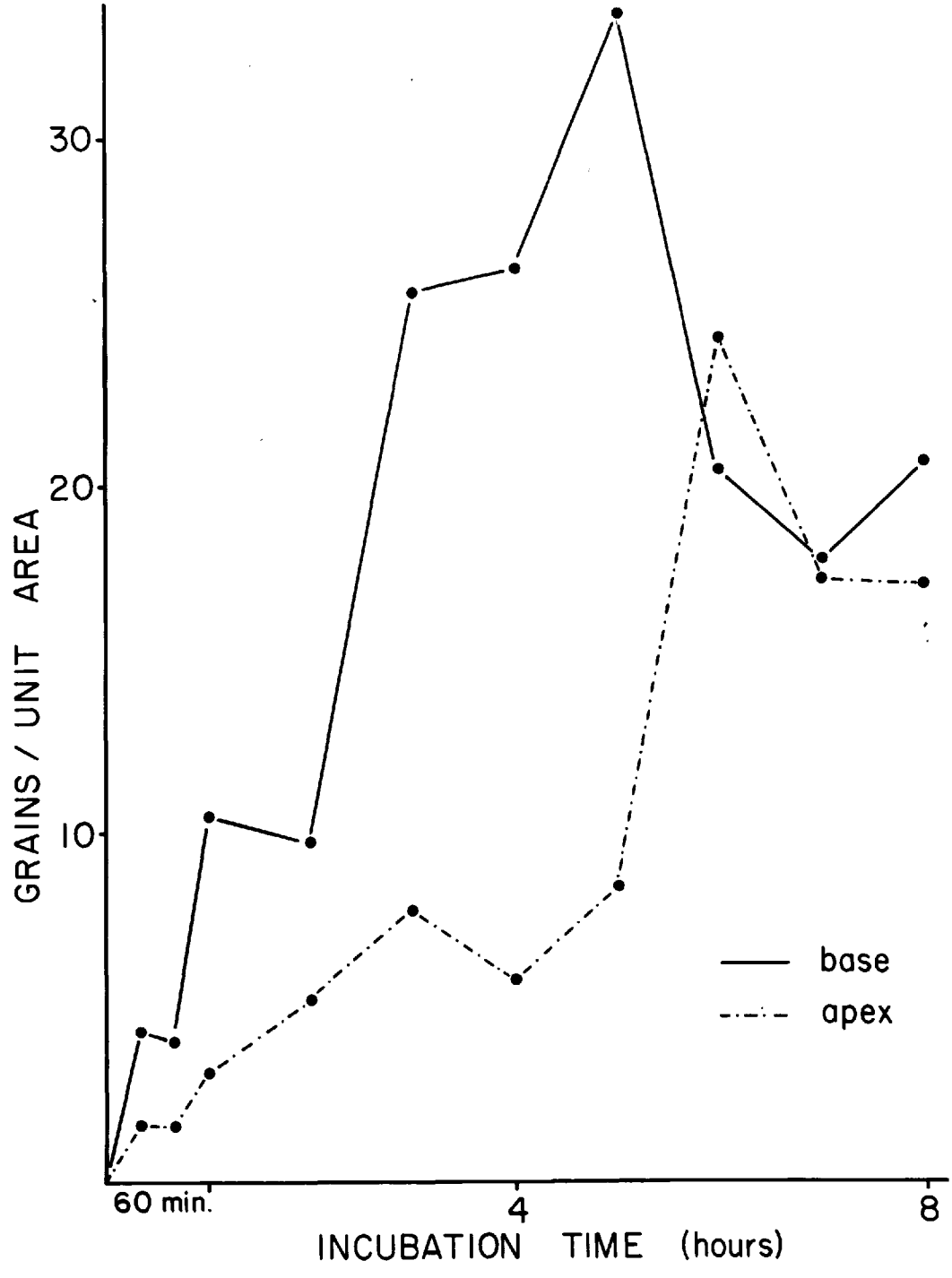
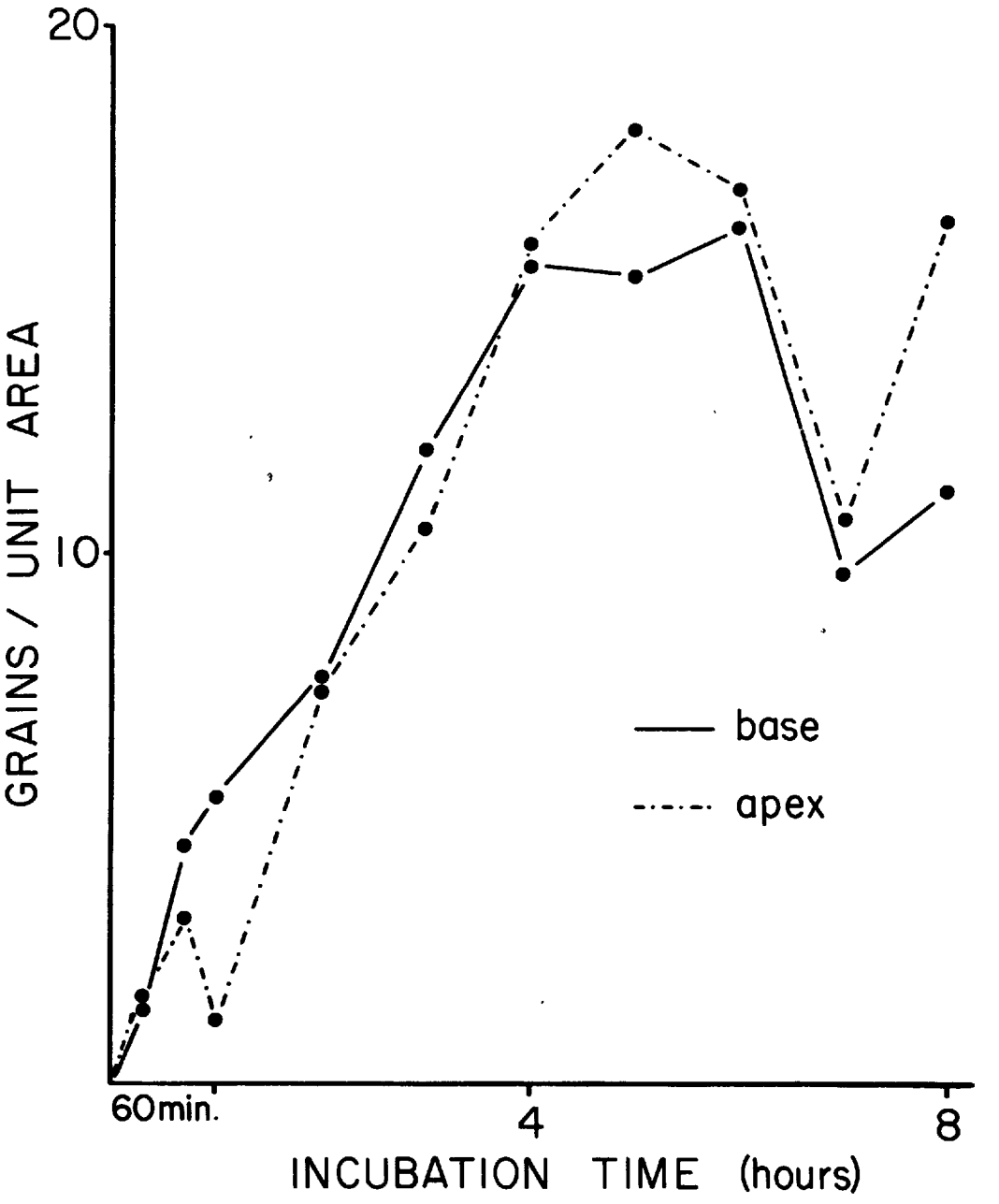

30
20
10
GRAINS / UNIT AREA
base
apex
60 min.
4
8
INCUBATION TIME (hours)

FIGURE IV-16

Mean number of grains per unit area (12.5 x 8.5 cm) over the base and apex of the serous cell after incubation with $H^3$-threonine for various times.

FIGURE IV-16


20
10
GRAINS / UNIT AREA
base
apex
60min.
4
8
INCUBATION TIME (hours)

## Mucous Cell

For the mucous cell, the maximum uptake of $H^3$-threonine was reached at three hours, this was maintained until six hours after which grain density fell (Table IV-13 and Figure IV-14). The high grain density at six hours may be just a chance finding. Localization of grains was first seen in the cell base (Table IV-14 and Figure IV-15). At six hours, most of the silver grains were found in the cell apex, indicating that the $H^3$-threonine had travelled to the cell apex, evidently from the endoplasmic reticulum to the granules. At seven and eight hours, the majority of silver grains are again in the cell base, although grain density has decreased, probably because those in the cell apex have been secreted.

## Serous Cell

Uptake of $H^3$-threonine by the serous cell reached maximum between three - four hours, this was slightly less in amount than that by the mucous cell. A decrease in grain density was apparent by five hours (Table IV-13 and Figure IV-14). Throughout the serous cell, the grain density at base and apex were similar (Table IV-14 and Figure IV-16), the silver grain over the cell base just predominating until five hours and from this time, those in the apex. Grain density in the base and apex decreased during six and seven hours suggesting discharge, while at eight hours, an increase was seen in both regions,

rather more prominent in the apex.

The overall uptake of $H^3$-threonine for both the mucous and serous cell is shown by determining the number of silver grains per unit area. For both cell types, uptake increases up to three hours, suggesting that by this time uptake is complete. Since the mucous cell is not seen to discharge granules before seven hours, the suggestion that uptake continues beyond four hours but the level remains the same because of discharge can probably be discounted. For the serous cell, discharge is seen a little earlier, at six hours. Thus maximum absolute uptake, which is seen to continue after the initial 'pulse' incubation, is probably due to utilization of extracellular $H^3$-threonine within the explant.

Comparison of grain densities over the base and apex of the mucous and serous cell suggests that in the mucous cell, $H^3$-threonine passes from the base to the apex, this being a reflection of the regional distribution of the organelles. For the serous cell, on the other hand, at all times studied, a similar grain density is seen at the base and the apex, this is no doubt due to the widespread scatter of organelles. Grain densities suggest discharge from the mucous cell at seven and eight hours, this closely agrees with the results of the light microscopic

autoradiographs. For the serous cell, discharge is suggested from six hours.

## VOLUME OF A GIVEN ORGANELLE WITHIN A CELL

Using sequential analysis, it was possible to calculate, from the data obtained by point counting, the percentage of total volume occupied by a given organelle in either the mucous or serous cell. The results shown (Tables IV-15 and IV-16) are those for the $H^3$-threonine incubated explants.

Sequential analysis revealed that most of the mucous cell was comprised of granules and next came the endoplasmic reticulum. (Table IV-15 shows the progression expressed as a mean for each organelle, the final mean being in column 12). For the serous cell (Table IV-16), the endoplasmic reticulum occupied most of the cell with the granules in second place.

Using the data obtained in the point analysis for individual micrographs it was possible to compare the concentration of organelles in the base of the cell with those in the apex (Table IV-17).

It is apparent from these results that the proportion of the various organelles in the mucous cell is different from the serous. The endoplasmic reticulum comprises 29% of the mucous cell and 67% of the serous cell, while the

TABLE IV-15

PROGRESSIVE MEAN FOR THE DISTRIBUTION OF ORGANELLES WITHIN THE MUCOUS CELL

| No. of blocks | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Endoplasmic reticulum | 22.0 | 22.6 | 23.2 | 27.1 | 26.5 | 28.3 | 28.6 | 28.3 | 28.8 | 29.5 | 29.3 |
| Granule | 60.2 | 60.8 | 60.4 | 51.5 | 54.0 | 51.9 | 52.0 | 51.5 | 50.1 | 49.1 | 49.5 |
| Golgi | 9.1 | 8.8 | 7.9 | 8.3 | 7.9 | 8.3 | 8.0 | 8.6 | 8.4 | 8.3 | 8.0 |
| Vacuole | 0.7 | 0.9 | 1.0 | 1.7 | 1.5 | 1.3 | 1.2 | 1.1 | 1.6 | 1.8 | 1.7 |
| Mitochondria | 2.1 | 1.6 | 1.5 | 1.6 | 1.4 | 1.5 | 1.5 | 1.7 | 1.6 | 1.5 | 1.6 |
| Membrane | 0.4 | 0.4 | 0.3 | 0.6 | 0.6 | 0.7 | 0.7 | 0.7 | 0.8 | 0.8 | 0.8 |
| Nucleus | 5.0 | 4.3 | 4.6 | 8.3 | 7.2 | 6.9 | 6.7 | 7.2 | 6.4 | 6.9 | 7.1 |
| Lysosome | | 0.2 | 0.2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.3 | 1.1 | 1.0 | 0.9 |
| Others | | | | | | | | | | | |

TABLE IV-16

PROGRESSIVE MEAN FOR THE DISTRIBUTION OF ORGANELLES WITHIN THE SEROUS CELL

| No. of blocks | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Endoplasmic reticulum | 56.9 | 58.2 | 58.8 | 58.9 | 60.0 | 60.8 | 60.9 | 60.6 | 60.3 | 60.1 | 60.7 |
| Granule | 14.6 | 16.9 | 19.3 | 19.2 | 16.4 | 14.9 | 14.7 | 14.8 | 14.7 | 15.2 | 14.8 |
| Golgi | 7.8 | 6.5 | 5.8 | 6.3 | 7.0 | 6.6 | 6.4 | 6.0 | 6.2 | 6.1 | 6.1 |
| Vacuole | 1.6 | 1.9 | 2.2 | 2.0 | 2.2 | 3.0 | 2.7 | 2.9 | 2.7 | 2.6 | 2.4 |
| Mitochondria | 3.2 | 3.2 | 2.5 | 2.5 | 2.7 | 3.0 | 3.4 | 3.6 | 3.8 | 3.8 | 3.9 |
| Membrane | 2.4 | 2.2 | 1.9 | 1.7 | 1.6 | 1.5 | 1.5 | 1.5 | 1.8 | 1.8 | 1.7 |
| Nucleus | 3.5 | 4.2 | 4.0 | 4.2 | 5.5 | 6.0 | 6.4 | 6.8 | 6.8 | 6.8 | 6.9 |
| Lysosome | 1.0 | 1.1 | 0.9 | 0.9 | 0.9 | 0.9 | 1.0 | 0.9 | 1.0 | 0.9 | 0.9 |
| Others | | | | | | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 |
| Canaliculi | 8.9 | 5.9 | 4.4 | 3.6 | 3.1 | 2.6 | 2.6 | 2.3 | 2.3 | 2.3 | 2.1 |

TABLE IV-17

MEAN PERCENTAGE OF TOTAL VOLUME OCCUPIED BY A SINGLE ORGANELLE IN THE BASE AND APEX OF THE MUCOUS AND SEROUS CELL (POINT ANALYSIS)

| | MUCOUS | | SEROUS | |
|---|---|---|---|---|
| | Base | Apex | Base | Apex |
| Endoplasmic reticulum | 33.4 | 20.5 | 60.2 | 65.4 |
| Granules | 32.7 | 73.1 | 14.1 | 26.2 |
| Golgi | 7.7 | 3.4 | 8.1 | 3.3 |
| Mitochondria | 2.5 | 0.79 | 3.0 | 1.9 |
| Membrane | 1.4 | 0.1 | 1.5 | 2.3 |
| Nucleus | 19.3 | 2.0 | 11.9 | 0.0 |
| Lysosome | 0.2 | 0.0 | 1.0 | 0.4 |

granules occupy 50% of the total volume of the mucous cell and only 11% of the serous cell.

In the base of the mucous cell, the endoplasmic reticulum, granules and nucleus predominate while at the apex, the granules alone; a greater proportion of all other organelles is found in the cell base than in the apex.

For the serous cell, the endoplasmic reticulum and the nucleus again predominate in the cell base but although the granules are found mainly in the cell apex, they do not predominate even here. As judged by the area it occupies, the major constituent of the whole cell is the endoplasmic reticulum, the percentage being similar for both the base and the apex. The Golgi apparatus, mitochondria and lysosomes are also more frequent in the cell base than the apex. The cell membrane is more abundant at the apex, probably because of the canaliculi present.

These findings could mean either that the mucous and serous cell are different cell types or that they represent different stages of a secretory cycle, but since each cell type is in a different region of the secretory tubule, the former would seem more likely.

## SUMMARY

The incorporation of $H^3$-threonine by the mucous and serous cells of the human bronchial submucosal gland has been studied using both light and electron microscopic autoradiography.

For the mucous cell, both studies gave similar results, but the electron microscope considerably amplified our understanding of the intracellular pathways. Tritiated threonine initially localized at 60 minutes, in the endoplasmic reticulum of the cell as well as the junctional region, ER/Golgi apparatus. Maximum localization of the radioactive precursor was seen at five hours in the Golgi apparatus, although before this time localization was increasing in this region, indicating passage from the endoplasmic reticulum to the Golgi apparatus. From 60 minutes, activity was found in the vacuoles (immature secretory granules), and then passed gradually to the granules. From six hours, localization in the granules reached maximum. Using light microscopic autoradiography, at seven and eight hours, the lumens of the mucous acini were heavily labelled.

Maximum uptake by the granules and the junctional region, ER/granule occurred at a later

time than all other organelles and would therefore suggest that activity in the granules was derived from other intracellular organelles.

For the serous cell, light and electron microscopic autoradiography seemed to show somewhat conflicting results for the incorporation of $H^3$-threonine. Using the light microscope, from two hours, localization of silver grains appeared to be generally through the cell. However, the improved resolution obtained with the electron microscope did show a pattern of incorporation similar to that of the mucous cell. At 20 minutes, maximum concentration of $H^3$-threonine was found in the endoplasmic reticulum, at 40 minutes, in the junctional region ER/Golgi apparatus and at 60 minutes in the Golgi apparatus. At 60 minutes the radioactive precursor was also in the vacuoles. At seven and eight hours, maximum localization was in the granules, this would suggest a similar pattern of incorporation to that of the mucous cell. Localization was also found in the granules at 40 minutes, this is difficult to explain and may be real or due to 'cross-fire' between organelles. At eight hours, localization of silver grains in canaliculi was still increasing.

Maximum uptake for all organelles was reached at four hours, save for the junctional regions ER/Granule and ER/others, where this was slightly extended. Uptake in the canaliculi was still increasing at eight hours. For all organelles, maximum uptake of $H^3$-threonine for the serous cell was less in amount than for the mucous cell.

By estimating grain density (grains per unit area) for the whole cell, the maximum activity of $H^3$-threonine was obtained at three hours for both mucous and serous cells. Grain density decreased at seven hours, indicating discharge from the mucous cell and at six hours, for the serous cell.

If the grain density over the base was compared to that of the apex, for the mucous cell, initially grain density was found to predominate in the cell base, at six hours, in the cell apex and at seven and eight hours again in the cell base. This indicated the passage of radioactive precursor from the base to the apex and discharge from the cell between six and seven hours. For the serous cell, grain density was similar at all times for the base and the apex, at seven hours, there was a decrease in grain density indicating discharge.

The volume occupied by individual organelles within mucous and serous cells and the distribution of these organelles in the base and apex of the cell was found to differ.

# CHAPTER V

## INCORPORATION OF $H^3$-GLUCOSE BY MUCOUS AND SEROUS CELLS OF THE HUMAN BRONCHIAL SUBMUCOSAL GLAND

Using both light and electron microscopic autoradiography, the pattern of incorporation of $H^3$-glucose by the mucous and serous cells of the human bronchial submucosal gland has been studied.

Explants were incubated in $H^3$-glucose in the same way as described for $H^3$-threonine for periods up to eight hours, i.e. 20, 40 or 60 minute 'pulse' label plus a one - seven hour 'chase' incubation.

Quantitative analysis of light microscopic autoradiography revealed the regional distribution of silver grains within acini over a period of eight hours.

Using quantitative analysis for the electron microscopic autoradiographs 1. the total uptake or absolute amount of radioactivity within an organelle and 2. the relative concentration of silver grains associated with each organelle (specific activity) was calculated. The time of maximum total uptake and maximum relative concentration of silver grains for each organelle was assessed with reference to the analysis for each incubation time.

Using this data, the total uptake of $H^3$-glucose by the mucous and serous cell at each time examined, was also calculated.

## A. LIGHT MICROSCOPIC AUTORADIOGRAPHY

The same method has been applied to explants incubated in $H^3$-glucose as described in Chapter IV (page 129). Table V-1 shows the results of the light microscopic autoradiography for the mucous acini after incubation in $H^3$-glucose and Table V-2, for the serous acini. The degree of labelling (+, $\pm$ or -) at each level (region one - four for mucous or one - three for serous) within the acinus is recorded as a percentage.

Tritiated glucose, like $H^3$-threonine, showed similar labelling for the majority of mucous and serous cells in an acinus (Figure V-1).

### Mucous Acini

After incubation in $H^3$-glucose, little labelling was found anywhere in the mucous acini until 60 minutes (Table V-1) when all showed heavy labelling in the mid-region (region 2). Before 60 minutes, the few ($\pm$) silver grains found were virtually all in the mid-region. Localization to this region persisted up to six hours, when a reduction in number of acini labelled in this region was found.

At 40 minutes, a few grains were found over the apical region of only some acini. There was a slight

FIGURE V-1

One $\mu$m light microscopic autoradiograph, showing part of a mucous acinus after incubation for 60 minutes with $H^3$-glucose. Localization of silver grains is seen in the mid-region of the acinar cells. All cells show a similar pattern of labelling.
Toluidine blue X 1,500

FIGURE V-1

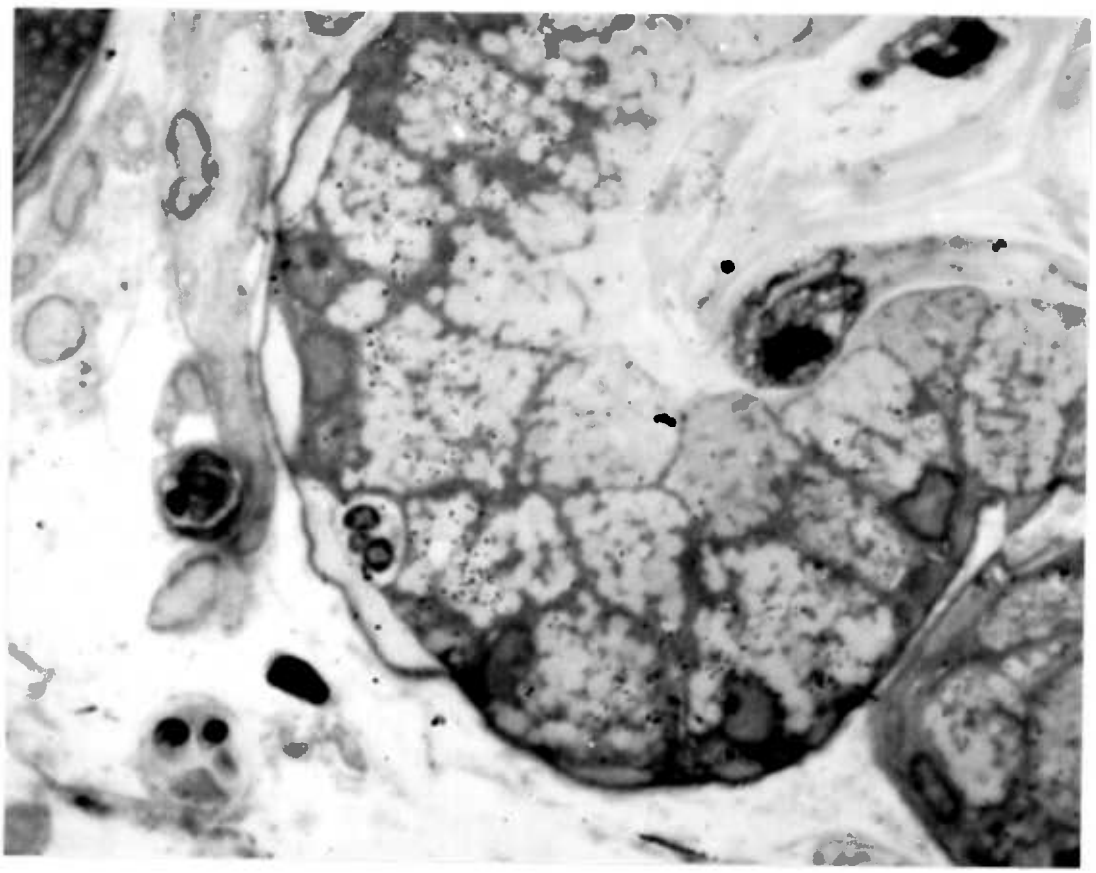


TABLE V-1

REGIONAL DISTRIBUTION OF SILVER GRAINS SHOWING THE GRAIN DENSITY AS THE PERCENTAGE FOR EACH REGION OF MUCOUS ACINUS AFTER INCUBATION IN $H^3$-GLUCOSE FOR VARIOUS TIMES

| Regions | 1 | | | 2 | | | 3 | | | 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grain density | + | ± | − | + | ± | − | + | ± | − | + | ± | − |
| 20 mins | | 40 | 60 | 10 | 80 | 10 | | | 100 | | 10 | 100 |
| 40 mins | | 10 | 90 | | 100 | | | 40 | 60 | | | 100 |
| 60 mins | 20 | 30 | 50 | 100 | | | | 30 | 70 | | | 100 |
| 2 hrs | 70 | 30 | | 100 | | | | 50 | 50 | | 10 | 90 |
| 3 hrs | | 60 | 40 | 100 | | | 20 | 50 | 30 | | 10 | 90 |
| 4 hrs | | 100 | | 100 | | | 40 | 60 | | | 20 | 80 |
| 5 hrs | | 100 | | 100 | | | 80 | 20 | | | 70 | 30 |
| 6 hrs | | 100 | | 60 | 40 | | 100 | | | 20 | 70 | 10 |
| 7 hrs | | 100 | | 80 | 20 | | 70 | 30 | | 50 | 40 | 10 |
| 8 hrs | | 100 | | 80 | 20 | | 100 | | | 90 | 10 | |

Region 1 = base of acinus
Region 2 = mid-region
Region 3 = apical
\+ = 40-70 silver grains per 100 sq μm
± = 10-30
− = 10

Line denotes heavy labelling, 50% or more, of acini in this region.

increase in the number of acini showing labelling here until four hours but between five - eight hours, most acini (70 - 90%) were definitely labelled in the apical region (region 4). Few grains were found in the lumen until seven and eight hours when they were heavily labelled, 50% and 90% respectively.

At any one time few silver grains appeared over the basal region (region 1) of the mucous acini, save at two hours when 70% of acini were heavily labelled in this region, but by three hours, again, few grains were found.

## Serous Acini

Little labelling of the serous acini occurred until 60 minutes (Table V-2), when 70% of the acini were heavily labelled again in the mid-region: this labelling persisted throughout the remainder of the experiment, since at all times between 50 - 100% of acini were labelled here.

Few grains were found in the acinar apical region between 40 minutes and two hours, but from three - eight hours, 50 - 100% of acini were heavily labelled in this region.

TABLE V-2

REGIONAL DISTRIBUTION OF SILVER GRAINS SHOWING THE GRAIN DENSITY AS THE PERCENTAGE FOR EACH REGION OF SEROUS ACINUS AFTER INCUBATION IN $H^3$-GLUCOSE FOR VARIOUS TIMES

| | SEROUS ACINI | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Regions | 1 | | | 2 | | | 3 | | |
| Grain density | + | ± | − | + | ± | − | + | ± | − |
| 20 mins | | | 100 | | | 100 | | | 100 |
| 40 mins | | 20 | 80 | | 70 | 30 | | 50 | 50 |
| 60 mins | 10 | 40 | 50 | 70 | 10 | 20 | | 40 | 60 |
| 2 hrs | 60 | 40 | | 80 | 20 | | | 50 | 50 |
| 3 hrs | 40 | 40 | 20 | 100 | | | 100 | | |
| 4 hrs | 50 | 50 | | 70 | 20 | 10 | 60 | 40 | |
| 5 hrs | 40 | 60 | | 50 | 50 | | 50 | 50 | |
| 6 hrs | 60 | 40 | | 100 | | | 100 | | |
| 7 hrs | 80 | 20 | | 70 | 30 | | 100 | | |
| 8 hrs | 90 | 10 | | 90 | 10 | | 90 | 10 | |

Region 1 = base of acinus
Region 2 = mid-region
Region 3 = apical
\+ = 40-70 silver grains per 100 sq μm
± = 10-30
− = 10

Line denotes heavy labelling, 50% or more, of acini in this region.

At two hours, 60% of acini were heavily labelled in the basal region, this persisted throughout the experiment with perhaps a slight increase in number of labelled acini, although at no time were all acini heavily labelled at their base.

For the mucous acini, after incubation in $H^3$-glucose, initial heavy localization is found at 60 minutes and this in the mid-region (probably in the Golgi apparatus). Only at two hours is the basal region heavily labelled in many acini, otherwise little localization occurred in this region. This initial incorporation of $H^3$-glucose contrasts with that of $H^3$-threonine, which at 40 minutes, is found mainly in the basal region (probably in endoplasmic reticulum). Between five - eight hours, heavy localization of $H^3$-glucose is found in the apical region and by seven and eight hours, most of the acinar lumens are labelled. For $H^3$-threonine, apical localization occurred earlier - at 60 minutes, although luminal localization was similar for both cell types at seven and eight hours.

For the serous acini, initial heavy localization of $H^3$-glucose occurs at 60 minutes and this, as for the mucous acinus, is also in the mid-region. At two

hours, localization is to the base and the mid-region, while from three - eight hours, the silver grains are distributed through all regions of the cell, but with fewer grains at the base. From two hours, the pattern of $H^3$-glucose incorporation into the mid-region of the serous acini is in general, similar to that for $H^3$-threonine. From three - eight hours, localization of $H^3$-glucose is maintained in the apical region while for $H^3$-threonine at seven and eight hours, localization in this region decreases. Basal localization of $H^3$-glucose from two - eight hours slightly increases, but for $H^3$-threonine, heavy localization was found only at two and three hours and at seven and eight hours. These results largely indicate a similar pattern of incorporation of $H^3$-glucose by both mucous and serous cells. However, with reference to the results of the previous chapter, the mucous and serous cells of the human bronchial submucosal glands exhibit different patterns for incorporation of $H^3$-glucose and $H^3$-threonine.

## B. HIGH RESOLUTION AUTORADIOGRAPHY

Table V-3 lists the specimens of mucous and serous cell used in the analysis of the electron microscopic autoradiographs. The incubation times for each specimen is also shown. All the following results are from these specimens.

In an identical manner to that for $H^3$-threonine, both 1. the circle analysis of Williams (1969) and 2. the hypothetical grain analysis (Blackett and Parry, 1973), were used to analyse quantitatively the electron microscopic autoradiographs.

### MUCOUS CELL

#### Circle Analysis

The circle, point and grain analysis were carried out as described on page 86. From these results, the chi-squared test for significance, the specific activity and the total uptake by organelles from each specimen at each incubation time were calculated.

The results of the chi-squared test for the mucous cell, after incubation in $H^3$-glucose, were all highly significant with p values of $< 0.001$.

TABLE V-3

SPECIMENS USED FOR THE ELECTRON MICROSCOPIC STUDIES WITH $H^3$-GLUCOSE

| INCUBATION TIME | STUDY 1 | STUDY 2 |
|---|---|---|
| 20 minutes | 411/5 | 457/2 |
| 40 " | 411/7 | 457/4 |
| 60 " | 402/1 | 457/6 |
| 2 hours | 402/3 | 457/8 |
| 3 " | 402/5 | 455/1 |
| 4 " | 402/7 | 455/3 |
| 5 " | 421/9 | 455/5 |
| 6 " | 421/10 | 455/7 |
| 7 " | 421/11 | 455/19 |
| 8 " | 421/12 | 455/11 |

Absolute uptake and relative concentration of $H^3$-glucose

Initial incorporation of $H^3$-glucose by the mucous cell was in the Golgi apparatus. Absolute uptake was found to be maximal at 60 minutes and from this time was seen to decrease (Table V-4 and Figure V-2). This indicates that the Golgi apparatus was saturated with $H^3$-glucose at this time ('chase' incubation in radioactive precursor was 60 minutes). At 60 minutes the relative concentration of silver grains over the Golgi apparatus was at its maximum (Table V-5 and Figures V-3a and 4).

From the Golgi apparatus the radioactivity was found to pass into the vacuoles and then the secretory granules. Absolute uptake by the granules alone and the granules and vacuoles combined was found still to be increasing at eight hours (Table V-4 and Figure V-2). The relative concentration of silver grains over the granules alone increased rapidly during the first two hours (Table V-5 and Figure V-3b), from two - eight hours, a more gradual increase occurred. When the vacuoles and granules were combined, the initial rapid increase of silver grains occurred by 60 minutes. These results would indicate that radioactivity reaches the secretory granules by localising first in the vacuoles. The vacuoles and Golgi apparatus appear to incorporate $H^3$-glucose at similar times (60 minutes), this is no doubt because the vacuoles are closely associated to the

TABLE V-4

THE ABSOLUTE UPTAKE OF TWO STUDIES AND THEIR MEAN FOR ORGANELLES IN THE MUCOUS CELL AFTER INCUBATION IN $H^3$-GLUCOSE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 0.07 | 0.08 | 0.85 | 0.73 | 0.60 | 0.52 | 0.37 | 0.71 | 1.25 | 1.08 |
| | 2 | 0.10 | 0.64 | 0.24 | 0.29 | 0.44 | 0.15 | 1.09 | 0.69 | 1.53 | 1.48 |
| | m | 0.08 | 0.36 | 0.54 | 0.51 | 0.52 | 0.33 | 0.73 | 0.70 | 1.39 | 1.28 |
| ER/Golgi | 1 | 0.23 | 0.38 | 0.50 | 0.22 | 0.10 | 0.16 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | 0.20 | 0.50 | 0.20 | 0.00 | 0.05 | 0.00 | 0.88 | 0.26 | 0.81 | 0.91 |
| | m | 0.21 | 0.44 | 0.35 | 0.11 | 0.07 | 0.08 | 0.44 | 0.13 | 0.40 | 0.45 |
| Golgi | 1 | 0.21 | 0.15 | 5.50 | 0.50 | 0.18 | 0.24 | 0.08 | 0.20 | 0.29 | 0.19 |
| | 2 | 0.24 | 1.32 | 0.10 | 0.32 | 0.16 | 0.08 | 0.37 | 0.37 | 0.20 | 0.67 |
| | m | 0.22 | 1.23 | 2.80 | 0.41 | 0.17 | 0.16 | 0.22 | 0.28 | 0.24 | 0.43 |
| Gran. & Vac. | 1 | 0.03 | 0.13 | 0.13 | 0.33 | 0.10 | 0.29 | 0.10 | 0.29 | 0.11 | 0.82 |
| | 2 | 0.00 | 0.10 | 0.11 | 0.21 | 0.36 | 0.10 | 0.89 | 0.48 | 1.69 | 0.88 |
| | m | 0.01 | 0.11 | 0.12 | 0.27 | 0.23 | 0.19 | 0.49 | 0.38 | 0.90 | 0.85 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Granule | 1 | 0.02 | 0.04 | 0.13 | 0.32 | 0.11 | 0.35 | 0.10 | 0.30 | 0.11 | 0.82 |
| | 2 | 0.03 | 0.10 | 0.07 | 0.11 | 0.32 | 0.11 | 0.89 | 0.39 | 1.69 | 0.91 |
| | m | 0.02 | 0.07 | 0.10 | 0.21 | 0.21 | 0.23 | 0.49 | 0.34 | <u>0.90</u> | 0.86 |
| ER/granule | 1 | 0.01 | 0.10 | 0.38 | 1.02 | 0.12 | 0.53 | 0.17 | 0.56 | 0.33 | 0.40 |
| | 2 | 0.00 | 0.22 | 0.16 | 0.33 | 0.61 | 0.13 | 0.79 | 0.61 | 0.85 | 1.00 |
| | m | 0.00 | 0.16 | 0.27 | 0.67 | 0.36 | 0.33 | 0.48 | 0.58 | 0.59 | <u>0.70</u> |
| ER/others | 1 | 0.06 | 0.06 | 0.19 | 0.47 | 0.27 | 0.27 | 0.16 | 0.96 | 0.11 | 0.40 |
| | 2 | 0.07 | 0.60 | 0.11 | 0.06 | 0.17 | 0.07 | 0.57 | 0.26 | 0.34 | 0.09 |
| | m | 0.06 | 0.33 | 0.15 | 0.26 | 0.22 | 0.17 | 0.33 | <u>0.61</u> | 0.22 | 0.24 |
| Nucleus | 1 | 0.00 | 0.10 | 0.03 | 0.05 | 0.04 | 0.04 | 0.00 | 0.06 | 0.14 | 0.08 |
| | 2 | 0.00 | 0.00 | 0.00 | 0.07 | 0.05 | 0.00 | 0.22 | 0.18 | 0.26 | 0.29 |
| | m | 0.00 | 0.05 | 0.01 | 0.06 | 0.04 | 0.02 | 0.11 | 0.12 | <u>0.20</u> | 0.18 |

Maximum absolute uptake for each organelle is underlined.

FIGURE V-2

Smoothed curves representing the trend of points for the absolute uptake of $H^3$-glucose by organelles of the mucous cell after incubation for various times (the dots represent the mean for each time).

FIGURE V-2

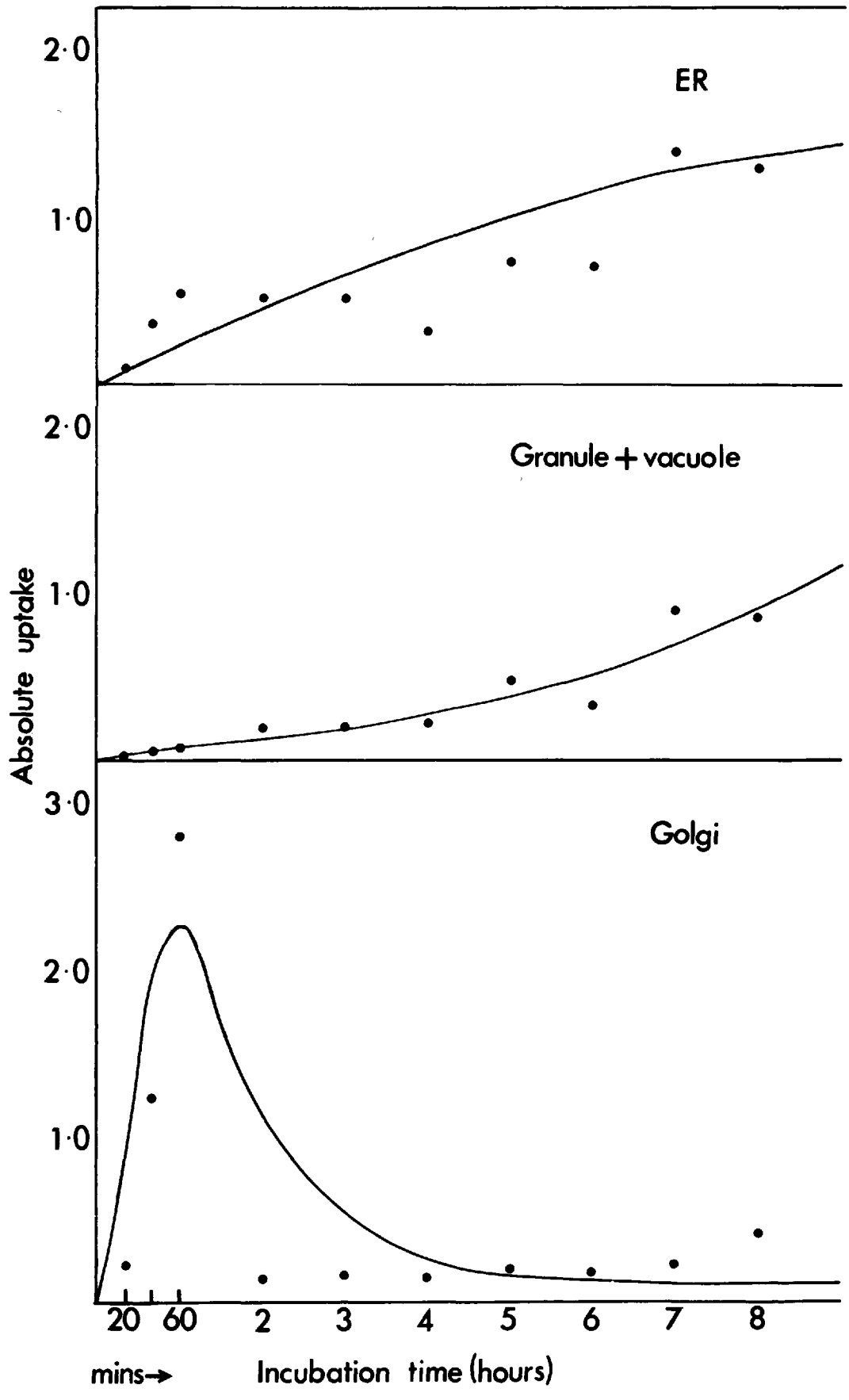

2·0
1·0
ER
2·0
1·0
Granule + vacuole
Absolute uptake
3·0
2·0
1·0
Golgi
20
60
2
3
4
5
6
7
8
mins→
Incubation time (hours)

TABLE V-5

THE SPECIFIC ACTIVITY (RELATIVE CONCENTRATION OF SILVER GRAINS) OF TWO CASES AND THEIR MEAN FOR ORGANELLES IN THE MUCOUS CELL AFTER INCUBATION IN $H^3$-GLUCOSE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 1.81 | 0.37 | 2.47 | 1.28 | 1.39 | 1.66 | 1.51 | 1.69 | 1.66 | 1.69 |
| | 2 | 0.90 | 2.00 | 1.50 | 1.07 | 1.57 | 1.38 | 1.37 | 1.33 | 1.63 | 1.53 |
| | m | 1.35 | 1.19 | 1.99 | 1.18 | 1.48 | 1.52 | 1.44 | 1.51 | 1.65 | 1.61 |
| ER/Golgi | 1 | 5.44 | 1.66 | 1.47 | 0.38 | 0.09 | 0.50 | 1.17 | 0.00 | 0.00 | 0.00 |
| | 2 | 4.21 | 1.50 | 0.25 | 0.00 | 0.15 | 0.00 | 0.00 | 0.51 | 0.87 | 0.94 |
| | m | 4.82 | 1.58 | 0.86 | 0.19 | 0.12 | 0.25 | 0.58 | 0.25 | 0.43 | 0.47 |
| Golgi | 1 | 4.94 | 6.40 | 32.46 | 0.86 | 1.70 | 0.76 | 0.46 | 0.47 | 1.09 | 0.51 |
| | 2 | 4.30 | 4.01 | 0.75 | 1.25 | 0.56 | 0.75 | 0.52 | 1.16 | 0.22 | 0.70 |
| | m | 4.62 | 5.21 | 16.71 | 1.06 | 0.85 | 0.76 | 0.49 | 0.82 | 0.66 | 0.61 |
| Gran. & Vac. | 1 | 0.71 | 0.58 | 0.39 | 0.57 | 0.60 | 0.00 | 1.18 | 0.94 | 0.44 | 0.42 |
| | 2 | 0.05 | 0.32 | 0.09 | 0.16 | 0.17 | 1.00 | 0.98 | 0.71 | 1.80 | 1.16 |
| | m | 0.38 | 0.45 | 0.24 | 0.36 | 0.38 | 0.54 | 1.08 | 0.82 | 1.12 | 0.79 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Granule | 1 | 0.71 | 0.20 | 0.39 | 0.55 | 0.82 | 1.11 | 1.06 | 1.00 | 0.44 | 1.23 |
| | 2 | 0.10 | 0.32 | 0.50 | 0.44 | 1.13 | 1.00 | 1.30 | 0.76 | 1.80 | 1.17 |
| | m | 0.41 | 0.26 | 0.44 | 0.50 | 0.98 | 1.06 | 1.18 | 0.88 | 1.12 | <u>1.20</u> |
| ER/granule | 1 | 0.36 | 0.43 | 1.13 | 1.77 | 1.22 | 1.69 | 1.05 | .0.22 | 1.26 | 0.90 |
| | 2 | 0.30 | 0.67 | 1.10 | 1.20 | 2.22 | 1.20 | 1.00 | 1.21 | 0.91 | 1.02 |
| | m | 0.33 | 0.55 | 1.11 | 1.48 | <u>1.72</u> | 1.44 | 1.02 | 0.71 | 1.08 | 0.96 |
| ER/others | 1 | 1.56 | 0.31 | 0.42 | 0.81 | 0.70 | 0.86 | 0.52 | 2.28 | 0.42 | 0.43 |
| | 2 | 0.28 | 1.81 | 0.36 | 0.27 | 0.61 | 0.81 | 0.75 | 0.51 | 0.41 | 0.60 |
| | m | 0.92 | 1.06 | 0.39 | 0.54 | 0.65 | 0.83 | 0.63 | <u>1.39</u> | 0.41 | 0.51 |
| Nucleus | 1 | 0.00 | 0.46 | 0.04 | 0.09 | 0.31 | 0.15 | 0.00 | 0.16 | 0.53 | 0.18 |
| | 2 | 0.06 | 0.00 | 0.00 | 0.33 | 0.02 | 0.00 | 0.29 | 0.28 | 0.28 | 0.30 |
| | m | 0.03 | 0.23 | 0.02 | 0.21 | 0.16 | 0.07 | 0.14 | 0.22 | <u>0.40</u> | 0.24 |

Maximum absolute uptake for each organelle is underlined.

FIGURE V-3a

Smoothed curve representing the trend of points for the specific activity (relative concentration of silver grains) of the Golgi apparatus of the mucous cell after incubation with $H^3$-glucose for various times (the dots represent the mean for each time).

FIGURE V-3a

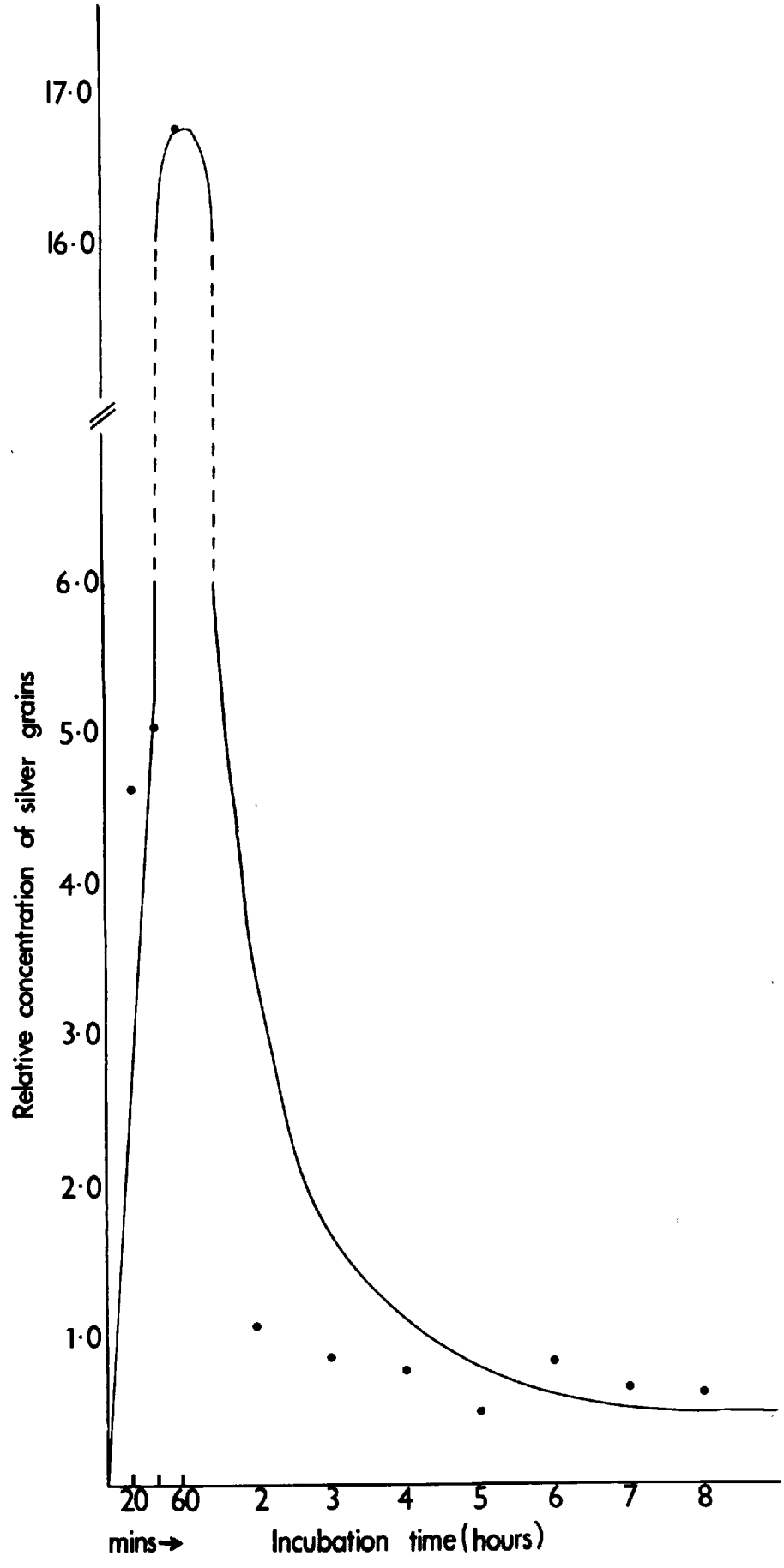

17·0
16·0
6·0
5·0
4·0
3·0
2·0
1·0
Relative concentration of silver grains
20
60
2
3
4
5
6
7
8
mins→
Incubation time (hours)

FIGURE V-3b

Smoothed curves representing the trend of points for the specific activity (relative concentration of silver grains) of organelles of the mucous cell after incubation with $H^3$-glucose for various times (the dots represent the mean for each time).

FIGURE V-3b

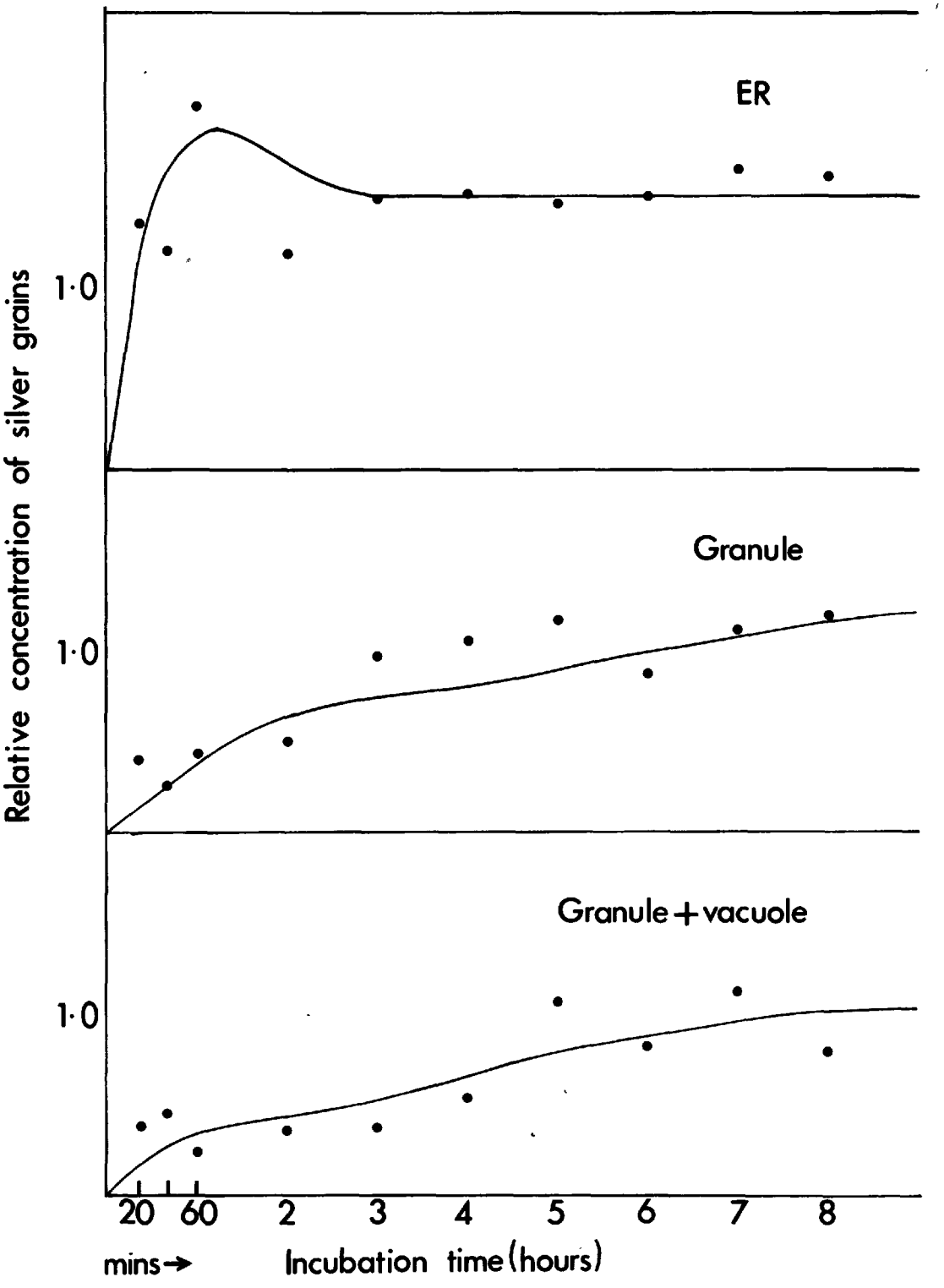
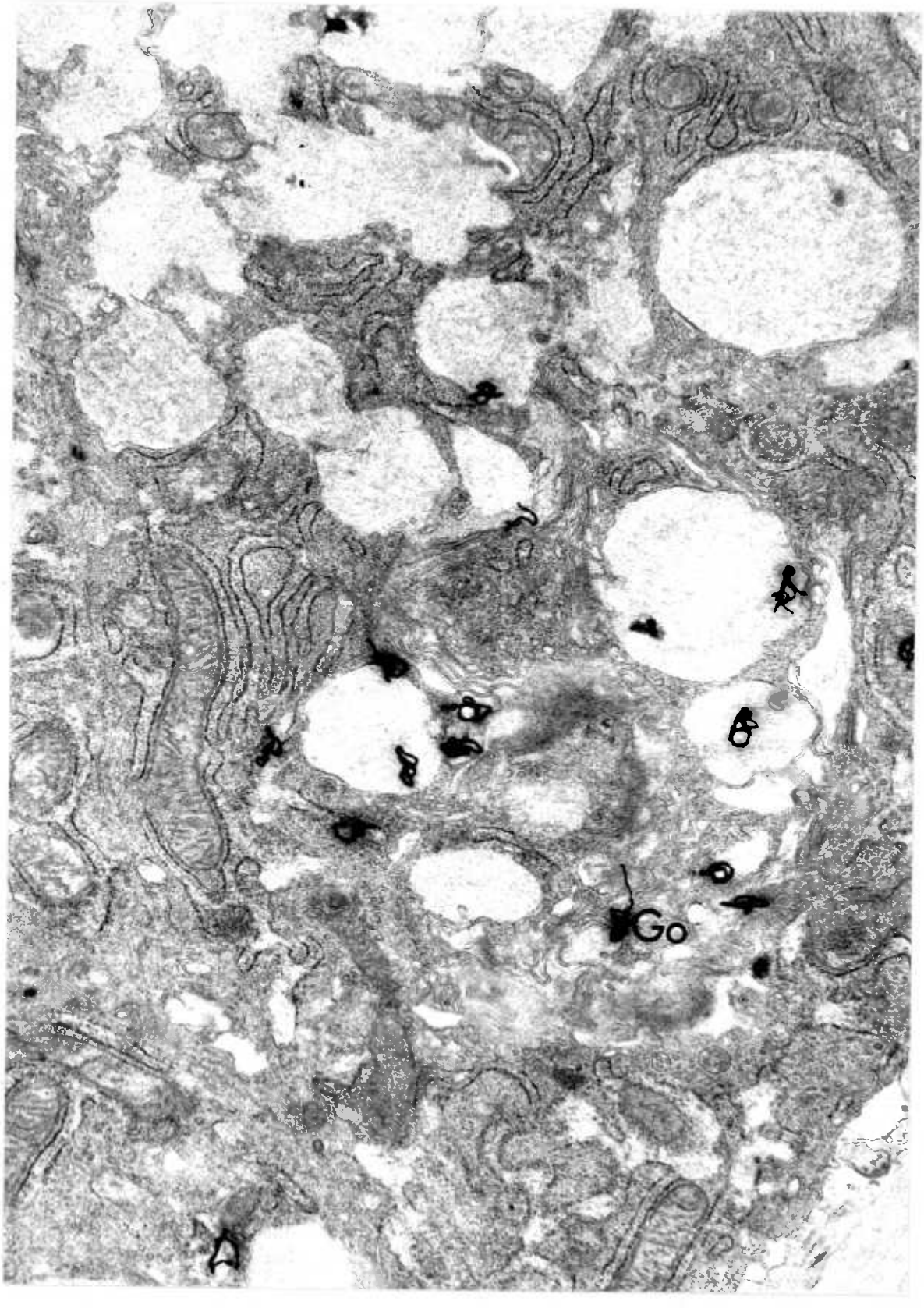

ER
Granule
Granule + vacuole
Relative concentration of silver grains
1·0
1·0
1·0
20
60
2
3
4
5
6
7
8
mins →
Incubation time (hours)

FIGURE V-4

After incubation with $H^3$-glucose for 40 minutes, localization of silver grains in the mucous cell is evident over the Golgi apparatus (Go).
X 20,000.

FIGURE V--4



Golgi apparatus. The granules, at eight hours, still appear to be localizing radioactivity.

At eight hours, absolute uptake by the endoplasmic reticulum was still increasing (Table V-4 and Figure V-2). Between 60 minutes and two hours, the maximum relative concentration of silver grains was found for this region (Table V-5 and Figure V-3b). From this time to three hours, the activity fell slightly and remained at this fairly high level for the remainder of the experiment.

Absolute uptake for the junctional region, ER/Golgi apparatus, was at maximum at 60 minutes, then from 60 minutes - three hours fell, from three - eight hours a steady, gradual increase was seen (Table V-4). At 20 minutes, the maximum relative concentration of silver grains was found for this region (Table V-5). This pattern of absolute uptake by this junctional region reflects its component organelles, the Golgi apparatus and the endoplasmic reticulum.

Absolute uptake by the junctional region ER/granule was increasing at eight hours (Table V-4). From nought to four hours the relative concentration (Table V-5) of silver grains in this region increased rapidly and from four - eight hours, the increase was slower. This pattern of incorporation is similar to

that described for the granules, but the more rapid increase in the level of activity is perhaps due to the endoplasmic reticulum of this junctional region.

At six hours, absolute uptake by the junctional region ER/others (Table V-4), was at maximum, from this time to eight hours a decrease was seen. Between 60 minutes and two hours, a peak in the relative concentration of silver grains in this region occurred (Table V-5), a second peak was also apparent between six and seven hours. The first peak is doubtless due to activity in the endoplasmic reticulum and the latter peak, to localization of radioactivity in 'others', which includes mitochondria, membranes and lysosomes.

Absolute uptake by the nucleus (Table V-4), continued until eight hours, although uptake was very small. The relative concentration (Table V-5) of silver grains over this region was also increasing at eight hours.

Tritiated glucose is initially incorporated into the Golgi apparatus of the mucous cell, from here radioactivity passes to the vacuoles and finally to the secretory granules. The endoplasmic reticulum does not seem to play a large part if any, in the synthesis of the carbohydrate component of the secretory product.

Absolute uptake by the Golgi apparatus of the mucous cell after incubation in $H^3$-glucose, is complete at 60 minutes, whereas absolute uptake for all other organelles is still increasing at eight hours. This would suggest either that activity in other regions is derived from the Golgi apparatus or that all organelles save the Golgi apparatus, which would appear to be saturated at 60 minutes, continue to utilize any excess $H^3$-glucose that remained in the tissue even after the washing procedure prior to the 'chase' incubation (see page 288 for absolute or total uptake by whole cell).

## Hypothetical Grain Analysis

The method for analysing the hypothetical grain method is described on page 92. Details for the analysis are identical to those given for the mucous and serous cells after incubation in $H^3$-threonine (page 148).

The results of the (i) grains per grid point and (ii) the activity per unit area are described in this chapter for the mucous and serous cell after incubation in $H^3$-glucose.

(i) Grains per grid point (Absolute uptake)

At 60 minutes (Table V-6 and Figure V-5) the Golgi apparatus showed the maximum uptake of $H^3$-glucose, this had fallen at four hours and remained at about this level at eight hours.

At 60 minutes, a lesser uptake was seen in the endoplasmic reticulum, similar levels of uptake were found at four and eight hours.

Little uptake at 60 minutes was seen in the granules but this rapidly increased at four, to reach maximum uptake at eight hours.

Little uptake, for the nucleus, was found at any of the three times studied.

These results would agree with those for the absolute uptake, using the circle analysis. Maximum uptake of $H^3$-glucose at 60 minutes, is found in the Golgi apparatus while uptake in the granules increases from nought - eight hours, which would suggest that the radioactive precursor travels from the Golgi apparatus to the granules. The endoplasmic reticulum shows moderate uptake of $H^3$-glucose at a similar level for each of the three times examined, suggesting that

TABLE V-6

THE PERCENTAGE AREA, PERCENTAGE ACTIVITY, GRAINS PER GRID POINT AND ACTIVITY PER UNIT AREA FOR THE MUCOUS CELL AFTER INCUBATION IN $H^3$GLUCOSE FOR 60 MINUTES, 4 AND 8 HOURS

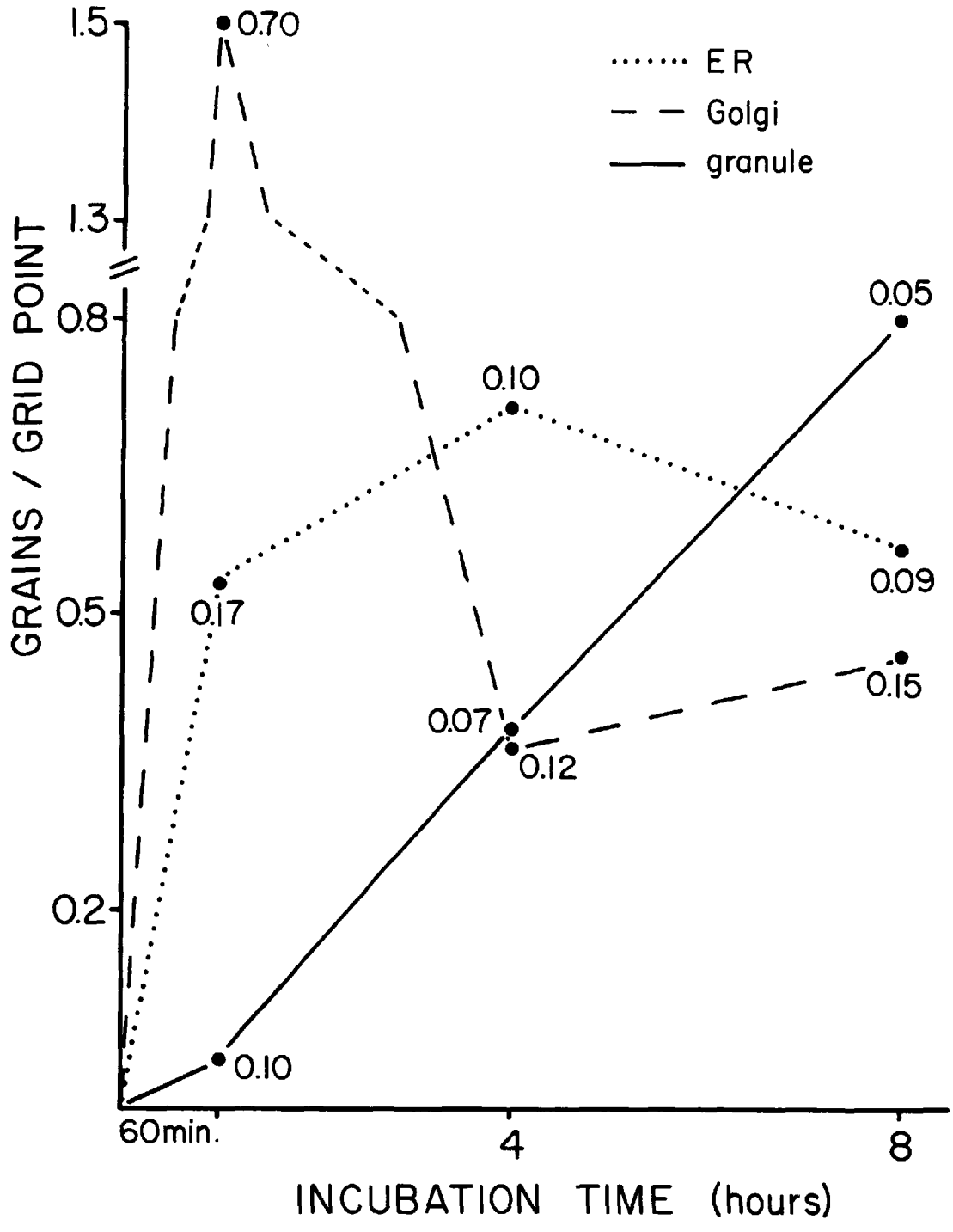
| Incubation time | Organelle | Area % | Activity % | Grains per grid point | | Activity per unit area | |
|---|---|---|---|---|---|---|---|
| 60 minutes | ER | 38.1 | 72.6 | 0.52 | 0.17* | 1.81 | 0.17* |
| | granule | 54.7 | 9.4 | 0.04 | 0.10 | 0.23 | 0.11 |
| | Golgi | 3.1 | 16.9 | 1.50 | 2.49 | 1.50 | 0.70 |
| | nucleus | 4.1 | 1.1 | 0.07 | 0.05 | 0.30 | 0.34 |
| 4 hours | ER | 36.4 | 59.9 | 0.71 | 0.10 | 1.64 | 0.19 |
| | granule | 33.1 | 29.5 | 0.38 | 0.07 | 0.89 | 0.15 |
| | Golgi | 10.8 | 9.2 | 0.36 | 0.12 | 0.85 | 0.28 |
| | nucleus | 19.6 | 1.4 | 0.03 | 0.03 | 0.07 | 0.08 |
| 8 hours | ER | 33.7 | 29.0 | 0.55 | 0.09 | 0.73 | 0.18 |
| | granule | 49.7 | 62.3 | 0.80 | 0.05 | 1.36 | 0.10 |
| | Golgi | 9.2 | 6.6 | 0.46 | 0.15 | 0.80 | 0.20 |
| | nucleus | 7.4 | 2.1 | 0.18 | 0.09 | 0.01 | 0.03 |

* = standard error of the mean

FIGURE V-5

Grains per grid point for organelles of the mucous cell after incubation with $H^3$-glucose for various times (figures represent the standard error of the mean for each point).

FIGURE V-5


1.5
1.3
0.8
0.5
0.2
GRAINS / GRID POINT
0.70
0.10
0.17
0.10
0.07
0.12
0.05
0.09
0.15
ER
Golgi
granule
60min.
4
8
INCUBATION TIME (hours)

little, if any, radioactivity passes from this organelle to other regions. Little uptake is found for the nucleus at any time.

(ii) Activity per unit area (relative concentration of silver grains)

At 60 minutes, maximum activity was found in the Golgi apparatus, this activity had fallen sharply by four hours and was similar to this level at eight hours (Table V-6 and Figure V-6).

The maximum activity for the endoplasmic reticulum was also seen at 60 minutes, and fell at four and eight hours.

For the granules, at 60 minutes, little activity was seen, but this increased at four and eight hours.

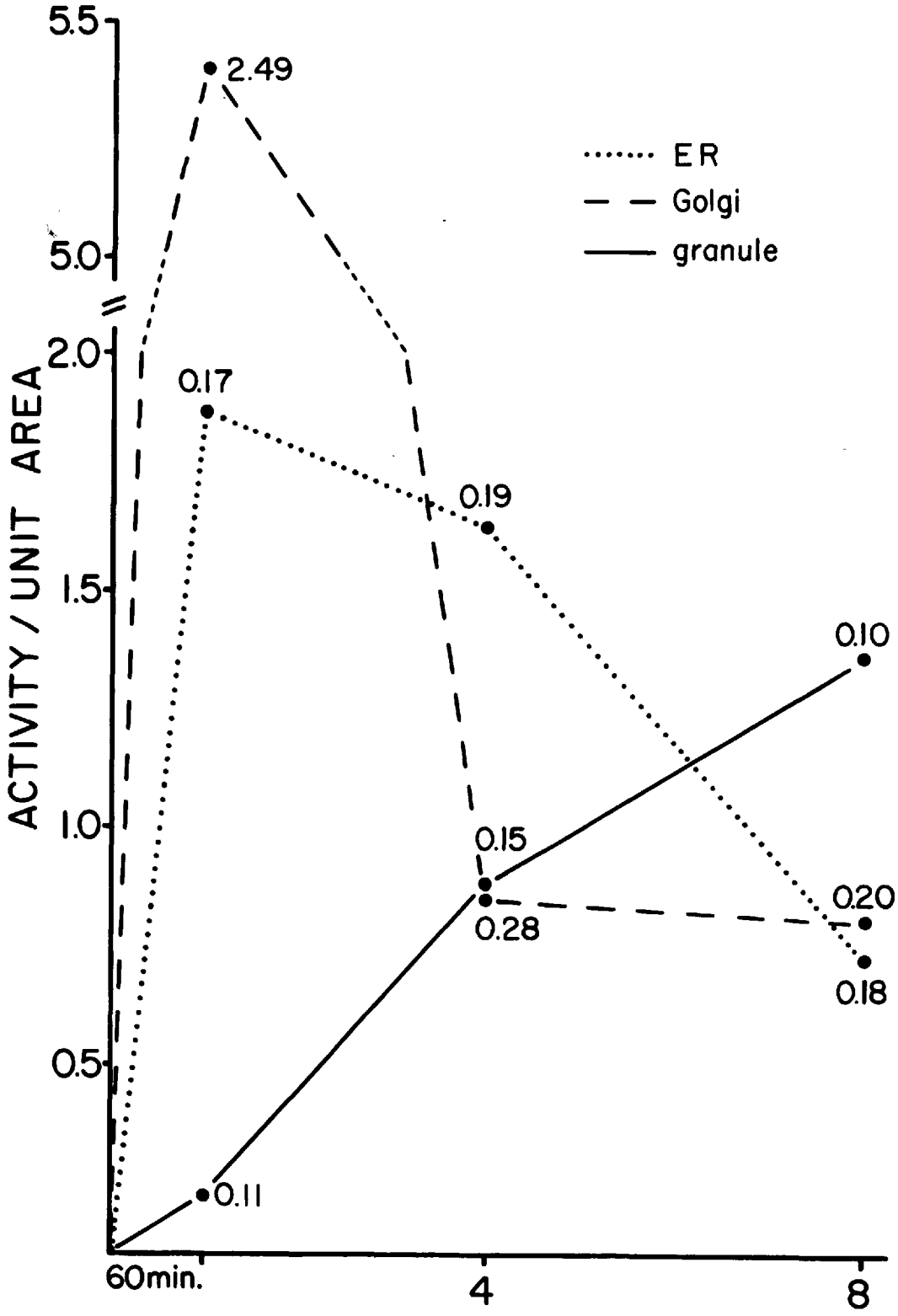
Little activity was found for the nucleus at any time.

The results of this present analysis again agree with those of the circle analysis. For the mucous cell after incubation for 60 minutes in $H^3$-glucose, initial incorporation is seen in the Golgi apparatus and from here it travels to the secretory granules.

FIGURE V-6

Activity per unit area for organelles of the mucous cell after incubation with $H^3$-glucose for various times (figures represent the standard error of the mean for each point).

FIGURE V-6


5.5
5.0
2.0
1.5
1.0
0.5
ACTIVITY / UNIT AREA
2.49
0.17
0.19
0.10
0.15
0.20
0.28
0.18
0.11
60min.
4
8
ER
Golgi
granule

Moderate incorporation is seen into the endoplasmic reticulum but since the level of activity does not decrease it is thought to be unlikely that much, if any, activity passes from this region to the Golgi apparatus or the granules. Little incorporation of $H^3$-glucose is found in the nucleus.

Chi-squared test

For the mucous cell, after incubation in $H^3$-glucose for 60 minutes, the $x^2$ analysis of the pattern of distribution for the silver grains gave a value of only $p = 0.003$. This represents a low level of confidence. At four hours, $p = 0.68$, a high level of confidence and at eight hours, $p = 0.10$.

In conclusion, for the mucous cell, quantitative analysis shows the same pattern of incorporation for $H^3$-glucose, using both light and electron microscopic autoradiography.

At 60 minutes, initial heavy localization, with the light microscope is seen in the mid-region of the acinar cells, with the electron microscope, this initial incorporation is found to be in the Golgi apparatus (situated in the mid-region of the cell).

From the Golgi apparatus, labelling passes to the vacuoles. At five hours, with the light microscope, heavy labelling of the acinar apices also occurs, although from as early as 40 minutes, the labelling in this region is steadily increasing. Using the electron microscope, from nought - eight hours, a rise in the relative concentration of silver grains, as well as in the absolute uptake of radioactive precursor, is seen over the granules.

Heavy labelling of the acinar lumen is found at seven and eight hours, using light microscopy, indicating the secretion of granules from the mucous cell, this is a similar discharge time to that seen after incubation in $H^3$-threonine.

In contrast to the pattern of incorporation of $H^3$-threonine by the mucous cell, the endoplasmic reticulum does not appear to play a part in carbohydrate synthesis. It would seem to be in the Golgi apparatus that this moiety of the secretory product is added to the protein core which, as shown in Chapter IV, is formed in the endoplasmic reticulum.

## SEROUS CELL

The serous cell was analysed in an identical manner to the mucous cell after incubation in $H^3$-glucose.

### Circle Analysis

For the serous cell, after incubation in $H^3$-glucose, the chi-squared tests were all highly significant ( $p = < 0.001$).

### Absolute uptake and relative concentration of $H^3$-glucose

The absolute uptake values and those of the relative concentration of silver grains (specific activity) for each organelle are shown in Tables V-7 and V-8, and depicted in Figures V-7 and V-8. Only the smoothed curves showing the trend of the points for both of these will be described.

Initial incorporation of $H^3$-glucose by the serous cell was seen in the Golgi apparatus. Absolute uptake was maximal between 60 minutes and two hours and from this time a decrease was found (Table V-7 and Figure V-7). Between 20 and 40 minutes, the relative concentration of silver grains over this region was at its maximum (Table V-8 and Figures V-8 and V-9). These results like those for the mucous cell indicate that initial

TABLE V-7

THE MEAN ABSOLUTE UPTAKE FOR EACH ORGANELLE IN THE SEROUS CELL OF TWO SPECIMENS AND THEIR MEAN, AFTER INCUBATION IN $H^3$-GLUCOSE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 0.15 | 0.65 | 0.33 | 0.40 | 0.45 | 0.32 | 0.97 | 0.14 | 1.01 | 0.31 |
| | 2 | 0.20 | 0.10 | 0.21 | 0.29 | 0.27 | 0.39 | 0.15 | 0.19 | 0.21 | 0.50 |
| | m | 0.17 | 0.37 | 0.27 | 0.34 | 0.36 | 0.35 | 0.56 | 0.16 | 0.61 | 0.40 |
| Granule | 1 | 0.00 | 0.00 | 0.07 | 0.33 | 0.38 | 0.31 | 1.16 | 0.38 | 0.45 | 0.86 |
| | 2 | 0.00 | 0.07 | 0.02 | 0.14 | 0.16 | 0.15 | 0.16 | 0.66 | 0.18 | 0.26 |
| | m | 0.00 | 0.03 | 0.04 | 0.23 | 0.27 | 0.23 | 0.66 | 0.52 | 0.31 | 0.56 |
| Golgi | 1 | 0.21 | 0.71 | 0.21 | 0.76 | 0.75 | 0.16 | 0.36 | 0.12 | 0.33 | 0.17 |
| | 2 | 0.32 | 0.22 | 0.10 | 0.09 | 0.10 | 0.21 | 0.10 | 0.31 | 0.08 | 0.00 |
| | m | 0.26 | 0.46 | 0.15 | 0.42 | 0.42 | 0.18 | 0.23 | 0.21 | 0.20 | 0.08 |
| Gran. & Vac. | 1 | 0.00 | 0.07 | 0.07 | 0.37 | 0.34 | 0.33 | 1.16 | 0.42 | 0.45 | 0.86 |
| | 2 | 0.00 | 0.11 | 0.07 | 0.18 | 0.12 | 0.13 | 0.10 | 0.56 | 0.17 | 0.23 |
| | m | 0.00 | 0.09 | 0.07 | 0.27 | 0.23 | 0.23 | 0.63 | 0.49 | 0.31 | 0.54 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ER/Golgi | 1 | 0.04 | 0.37 | 0.88 | 0.45 | 0.40 | 0.30 | 0.68 | 0.22 | 0.22 | 0.21 |
| | 2 | 0.14 | 0.14 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.20 | 0.00 |
| | m | 0.09 | 0.25 | <u>0.44</u> | 0.22 | 0.20 | 0.15 | 0.34 | 0.11 | 0.21 | 0.10 |
| ER/granule | 1 | 0.09 | 0.36 | 0.10 | 0.25 | 0.28 | 0.26 | 1.10 | 0.24 | 0.62 | 0.67 |
| | 2 | 0.31 | 0.06 | 0.28 | 0.28 | 0.17 | 0.26 | 0.20 | 0.35 | 0.14 | 0.23 |
| | m | 0.20 | 0.19 | 0.19 | 0.26 | 0.22 | 0.26 | <u>0.65</u> | 0.29 | 0.38 | 0.45 |
| ER/others | 1 | 0.10 | 0.43 | 0.14 | 0.25 | 0.30 | 0.33 | 0.62 | 0.09 | 0.41 | 0.28 |
| | 2 | 0.14 | 0.07 | 0.15 | 0.11 | 0.19 | 0.35 | 0.09 | 0.17 | 0.15 | 0.33 |
| | m | 0.12 | 0.25 | 0.14 | 0.18 | 0.24 | 0.34 | <u>0.35</u> | 0.13 | 0.28 | 0.30 |
| Canaliculi | 1 | 0.00 | 0.20 | 0.00 | 0.35 | 0.40 | 0.10 | 1.40 | 0.10 | 1.06 | 0.62 |
| | 2 | 0.02 | 0.16 | 0.00 | 0.00 | 0.00 | 0.11 | 0.00 | 0.00 | 0.00 | 0.57 |
| | m | 0.01 | 0.16 | 0.00 | 0.17 | 0.20 | 0.10 | 0.70 | 0.05 | 0.53 | <u>0.59</u> |
| Nucleus | 1 | 0.00 | 0.06 | 0.09 | 0.03 | 0.00 | 0.00 | 0.66 | 0.08 | 0.00 | 0.07 |
| | 2 | 0.07 | 0.00 | 0.00 | 0.05 | 0.16 | <u>0.17</u> | 0.00 | 0.08 | 0.08 | 0.03 |
| | m | 0.03 | 0.03 | 0.04 | 0.04 | 0.08 | 0.08 | 0.33 | 0.08 | 0.04 | 0.05 |

Maximum absolute uptake for each organelle is underlined.

FIGURE V-7

Smoothed curves representing the trend of points for the absolute uptake of $H^3$-glucose by organelles of the serous cell after incubation for various times (the dots represent the mean for each time).

FIGURE ~~V-7~~ IV 9 to page 182

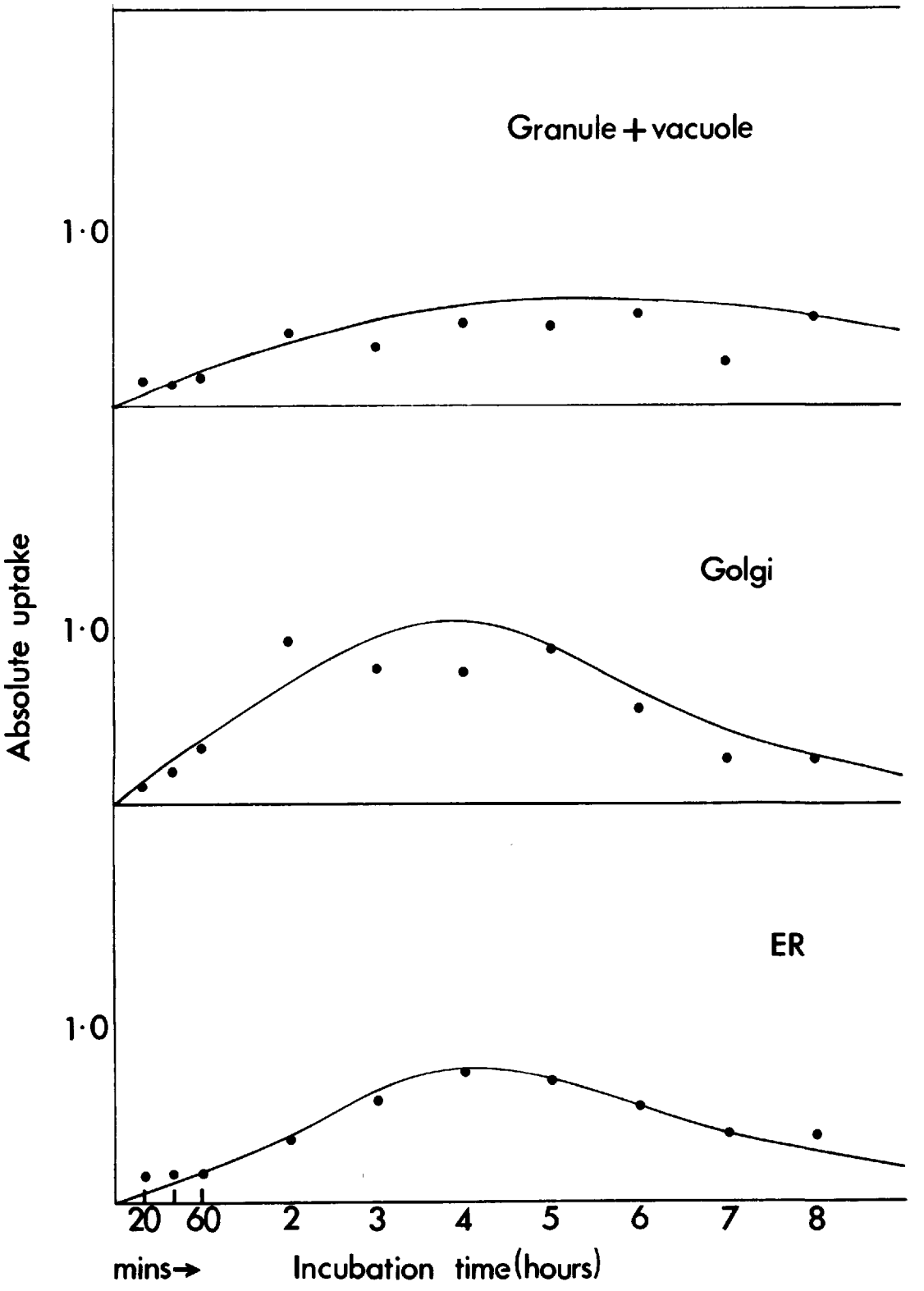


TABLE V-8

THE MEAN SPECIFIC ACTIVITY (RELATIVE CONCENTRATION OF SILVER GRAINS) OF ORGANELLES IN THE SEROUS CELL OF TWO SPECIMENS AND THEIR MEAN AFTER INCUBATION IN $H^3$-GLUCOSE FOR VARIOUS TIMES

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E.R. | 1 | 1.04 | 1.46 | 1.62 | 1.22 | 1.35 | 1.09 | 1.09 | 0.92 | 1.55 | 0.87 |
| | 2 | 1.20 | 1.07 | 1.28 | 1.44 | 1.15 | 1.17 | 1.15 | 0.79 | 1.24 | 1.56 |
| | M | 1.12 | 1.26 | 1.45 | 1.33 | 1.25 | 1.13 | 1.12 | 0.86 | 1.40 | 1.22 |
| Granule | 1 | 0.00 | 0.00 | 0.37 | 1.01 | 1.13 | 1.05 | 1.31 | 2.48 | 0.69 | 2.39 |
| | 2 | 0.00 | 0.75 | 1.17 | 1.00 | 0.67 | 0.92 | 1.81 | 2.69 | 1.06 | 0.83 |
| | M | 0.00 | 0.38 | 0.27 | 1.01 | 0.90 | 0.99 | 1.56 | 2.58 | 0.88 | 1.61 |
| Golgi | 1 | 2.66 | 1.61 | 1.04 | 2.33 | 1.25 | 0.54 | 0.41 | 0.76 | 0.51 | 0.49 |
| | 2 | 1.84 | 2.24 | 0.63 | 0.50 | 0.25 | 0.60 | 1.15 | 1.30 | 0.51 | |
| | M | 2.25 | 1.93 | 0.84 | 1.42 | 0.75 | 0.57 | 1.03 | 0.78 | 0.51 | 0.25 |
| Gran. & Vac. | 1 | 0.08 | 1.55 | 0.37 | 1.13 | 0.95 | 1.12 | 1.31 | 2.62 | 0.69 | 2.39 |
| | 2 | 0.00 | 1.14 | 0.44 | 1.00 | 0.64 | 0.95 | 1.50 | 2.29 | 1.06 | 0.83 |
| | M | 0.04 | 1.34 | 0.40 | 1.06 | 0.79 | 1.03 | 1.40 | 2.45 | 0.87 | 1.61 |

| Incubation time | | 20 mins | 40 mins | 60 mins | 2 hrs | 3 hrs | 4 hrs | 5 hrs | 6 hrs | 7 hrs | 8 hrs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ER/Golgi | 1 | 0.00 | 0.81 | 4.26 | 1.36 | 0.04 | 0.00 | 0.77 | 1.41 | 0.34 | 0.58 |
| | 2 | 0.82 | 1.40 | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 1.17 | 0.00 |
| | M | 0.41 | 1.10 | 2.13 | 0.68 | 0.02 | 0.02 | 0.38 | 0.70 | 0.75 | 0.29 |
| ER/granule | 1 | 0.42 | 0.80 | 0.51 | 0.87 | 0.90 | 0.98 | 1.22 | 1.53 | 0.95 | 1.87 |
| | 2 | 1.78 | 0.80 | 1.52 | 1.50 | 0.91 | 0.90 | 1.17 | 1.39 | 0.84 | 0.73 |
| | M | 1.10 | 0.80 | 1.01 | 1.18 | 0.90 | 0.94 | 1.19 | 1.46 | 0.89 | 1.30 |
| ER/others | 1 | 0.49 | 0.97 | 0.68 | 0.78 | 0.61 | 1.11 | 0.69 | 0.55 | 0.62 | 0.78 |
| | 2 | 0.66 | 0.69 | 0.51 | 0.55 | 0.82 | 1.07 | 0.75 | 0.71 | 0.90 | 0.95 |
| | M | 0.57 | 0.83 | 0.59 | 0.66 | 0.71 | 1.09 | 0.72 | 0.63 | 0.76 | 0.86 |
| Canaliculi | 1 | 0.00 | 0.44 | 0.96 | 1.06 | 0.62 | 0.32 | 0.57 | 0.64 | 1.63 | 1.73 |
| | 2 | 0.14 | 1.63 | 0.00 | 0.00 | 0.00 | 0.31 | 0.00 | 0.00 | 0.00 | 1.78 |
| | M | 0.07 | 1.03 | 0.48 | 0.53 | 0.31 | 0.31 | 0.78 | 0.32 | 0.81 | 1.75 |
| Nucleus | 1 | 0.00 | 0.14 | 0.46 | 0.10 | 0.15 | 0.00 | 0.75 | 0.52 | 0.00 | 0.21 |
| | 2 | 0.41 | 0.00 | 0.00 | 0.67 | 0.69 | 0.54 | 0.00 | 0.33 | 0,50 | 0.10 |
| | M | 0.20 | 0.07 | 0.23 | 0.38 | 0.42 | 0.27 | 0.37 | 0.42 | 0.25 | 0.15 |

Maximum specific activity for each organelle is underlined.

FIGURE V-8

Smoothed curves representing the trend of points for the specific activity (relative concentration of silver grains) of organelles of the serous cell after incubation with $H^3$-glucose for various times (the dots represent the mean for each time).

FIGURE V-8

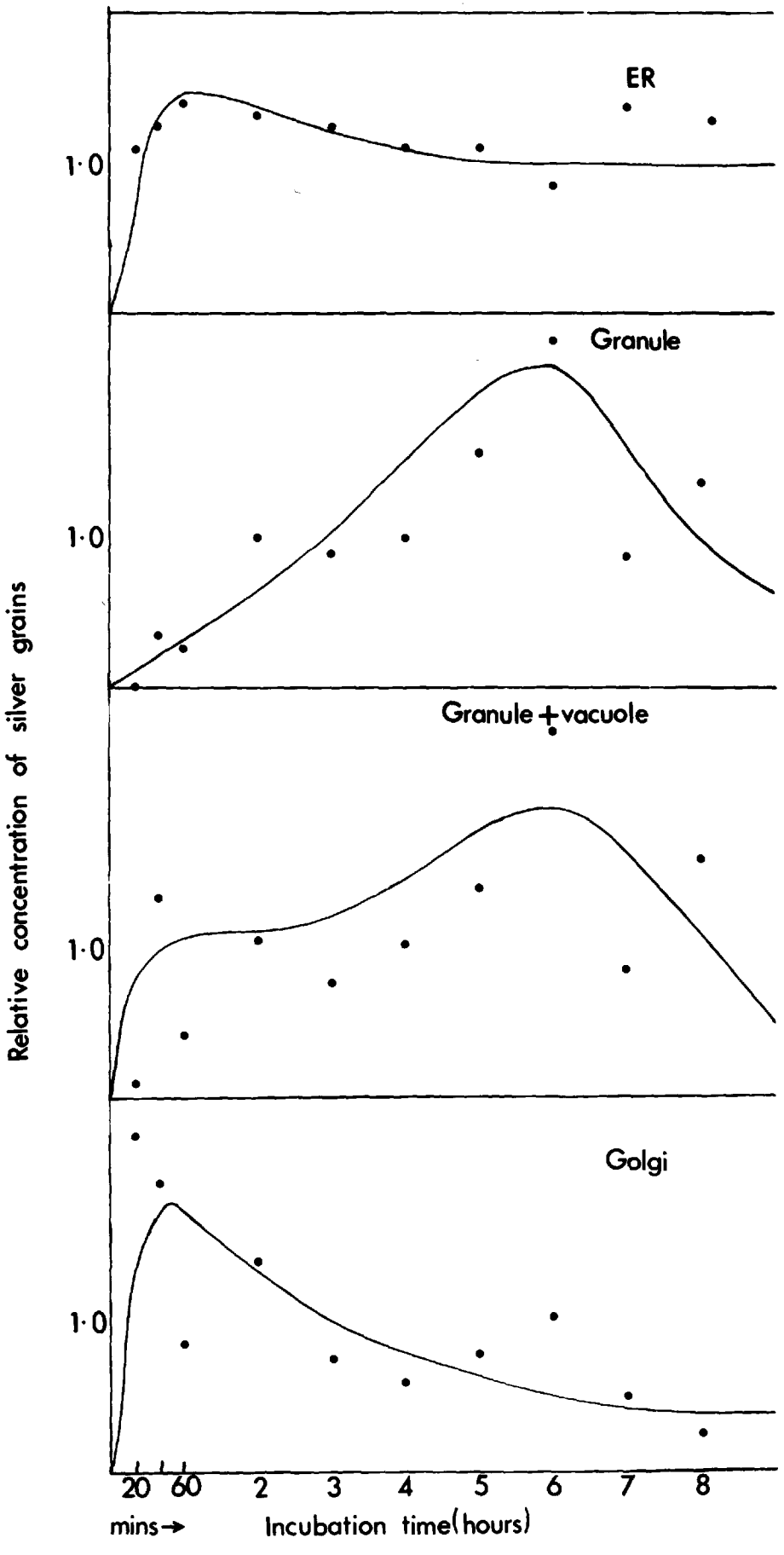
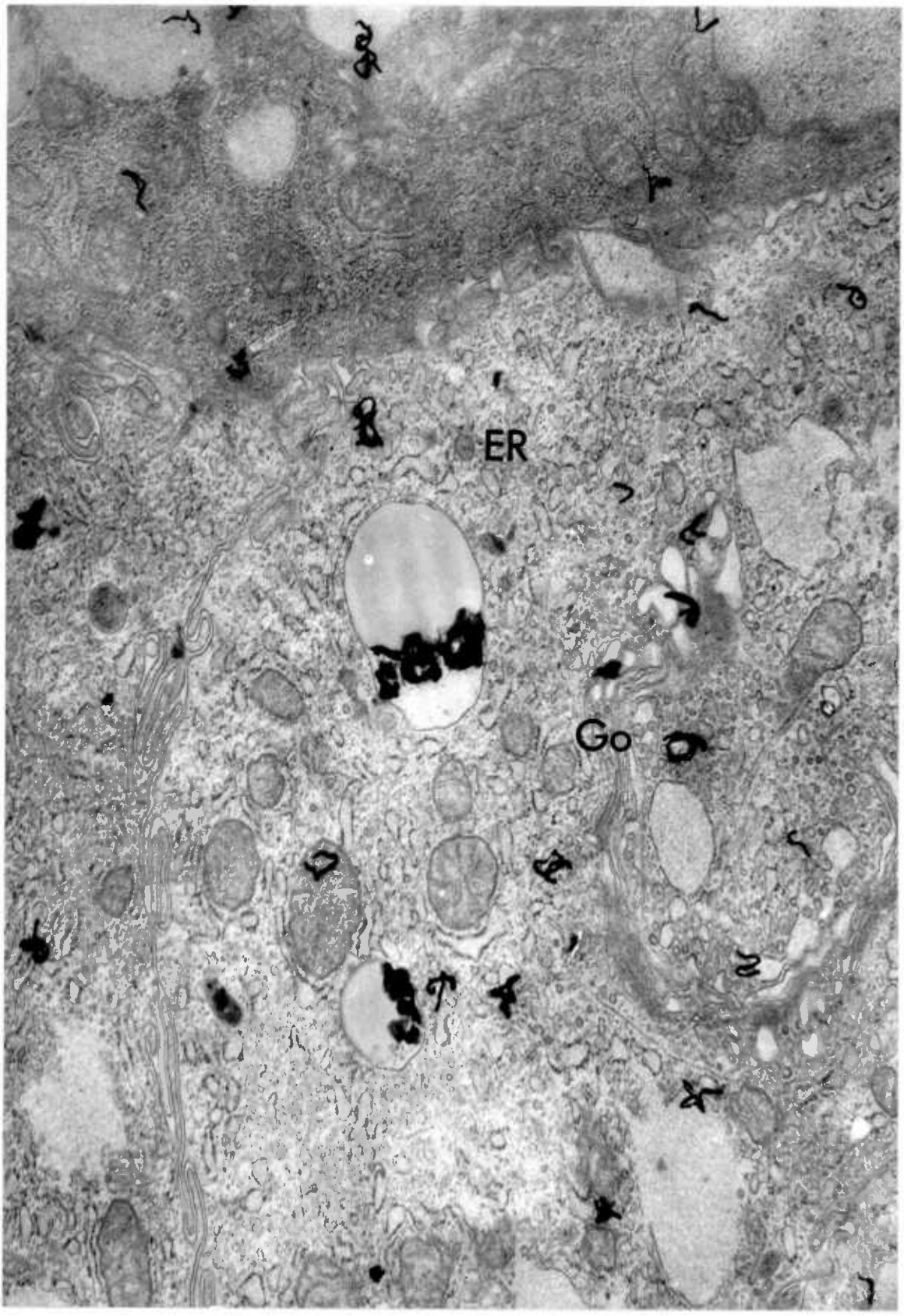

ER
Granule
Granule + vacuole
Golgi
1·0
1·0
1·0
1·0
Relative concentration of silver grains
20
60
2
3
4
5
6
7
8
mins →
Incubation time (hours)

FIGURE V-9

Part of a serous cell after two hours incubation with $H^3$-glucose. Localization of silver grains is seen over the endoplasmic reticulum (ER) and the Golgi apparatus (Go). X 20,000.

FIGURE V-9



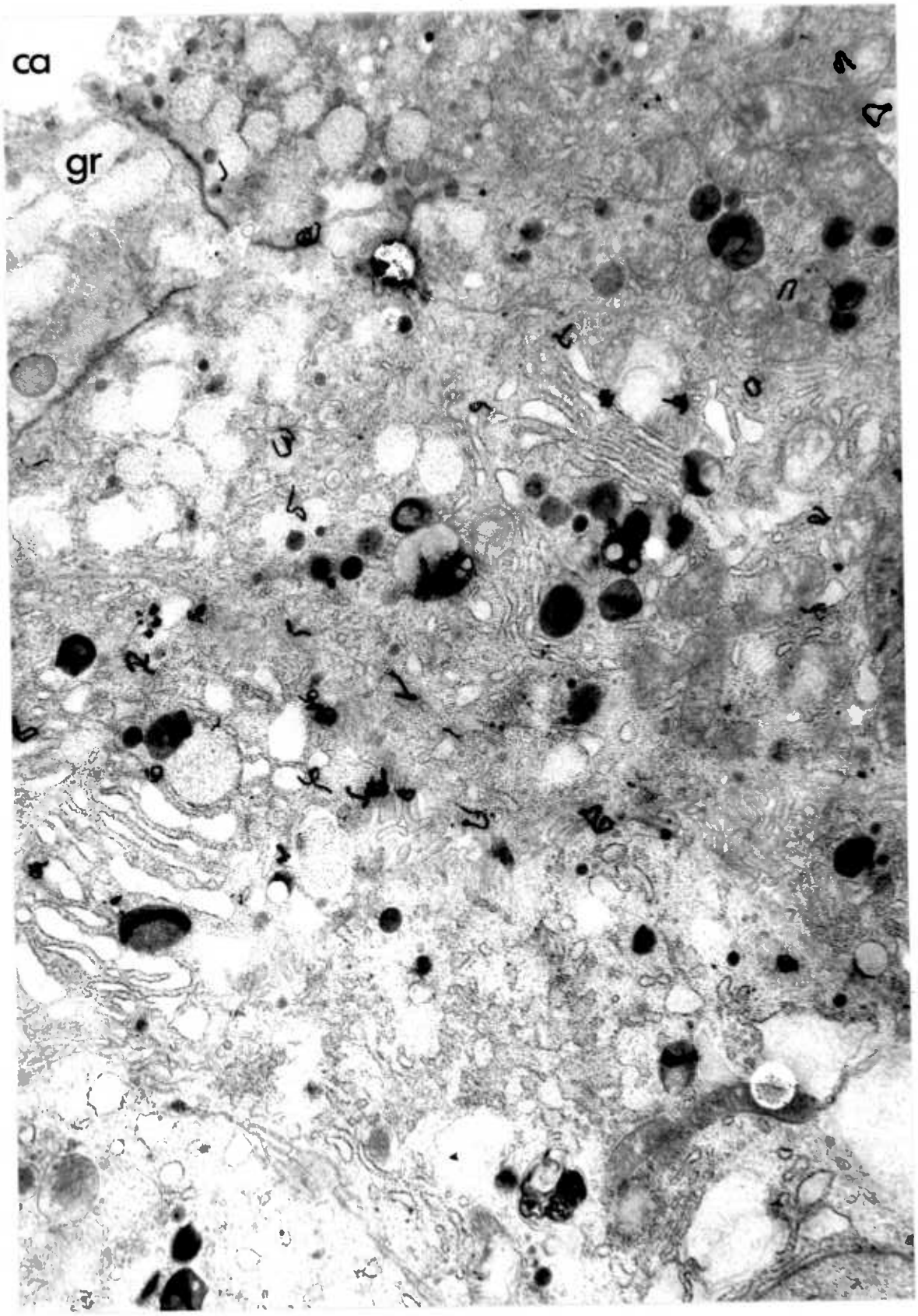
incorporation of $H^3$-glucose is in the Golgi apparatus. Absolute uptake of the radioactive precursor, as for the mucous cell, would again seem to have reached saturation for this organelle at approximately 60 minutes, the time representing the end of the 'pulse' label.

From the Golgi apparatus the radioactive precursor travels to the vacuoles and then to the secretory granules. The smoothed curve for the vacuoles is again described in combination with that of the granules. Absolute uptake for the granules alone as well as the granules and vacuoles combined, was maximal at five hours and remained at this level until eight hours (Table V-7 and Figure V-7). The relative concentration for the granules alone was at maximum between five and six hours, for the granules and vacuoles combined a similar maximum was found between five and six hours (Table V-8 and Figures V-8 and V-10), but up to 60 minutes, a rapid increase in the concentration of grains occurred that was not seen for the granules alone. These results would therefore support the suggested passage of the radioactive precursor from the Golgi apparatus to the vacuoles and then to the secretory granules. This is a similar pathway to that described for the mucous cell after incubation in $H^3$-glucose.

FIGURE V-10

After incubation with $H^3$-glucose for eight hours, localization of silver grains appears to be throughout the serous cell (perhaps associated with the endoplasmic reticulum), but circle analysis of all serous cells incubated for this time reveals that both the canaliculi (ca) and the granules (gr) show a high relative concentration of silver grains. X 15,000.

FIGURE V-10



Absolute uptake for the endoplasmic reticulum was at maximum between four and five hours, and then decreased (Table V-7 and Figure V-7). At 60 minutes, the relative concentration of silver grains (Table V-8 and Figures V-8 and V-9) in this region was maximal, but only decreased slightly over the remaining seven hours.

Absolute uptake for the junctional region, ER/Golgi apparatus was found to be at maximum at two hours (Table V-7), and then decreased. At approximately 60 minutes, the relative concentration of silver grains for this junctional region was seen to be maximal (Table V-8). The behaviour of this junctional region therefore closely resembles that of the Golgi apparatus.

For the junctional region, ER/granule, absolute activity was still increasing at eight hours (Table V-7). Two peaks were found for the relative concentration of silver grains (Table V-8), the first between 60 minutes and two hours and the second between five and six hours. The first peak is doubtless a reflection of the radioactivity within the endoplasmic reticulum and the second the granules.

At three hours, absolute uptake was maximum for the junctional region, ER/others and remained at a similar level for the remainder of the experiment (Table V-7).

Between two and three hours, the relative concentration of silver grains was maximal in this region (Table V-8). These results indicate that much of the activity found in this region is from the endoplasmic reticulum portion of the junctional region, however some, although little radioactivity is also suggested in the 'others' portion (mitochondria, membranes and lysosomes).

At five hours, absolute uptake for the nucleus was at maximum and from this time decreased (Table V-7). At three hours, the relative concentration of silver grains in this region was maximal (Table V-8), although, at all times, the specific activity for this region was low.

Absolute activity for the canaliculi (situated external to the serous cell), was still increasing at eight hours (Table V-7). Up to 60 minutes, the relative concentration of silver grains for this region rose rapidly, between 60 minutes and three hours, the silver grains remained steady and from four - eight hours, again increased rapidly (Table V-8 and Figure V-10).

Initial incorporation of $H^3$-glucose by the serous cell is seen in the Golgi apparatus. This activity then passes to the vacuoles, then to the granules and is finally secreted into the canaliculi. In a similar

manner to the mucous cell, the endoplasmic reticulum does not appear to be involved in the synthesis of the carbohydrate moiety of the secretory product.

Between 60 minutes and two hours, absolute uptake by the Golgi apparatus is at maximum. Uptake by all other organelles reaches a maximum value only after longer incubation times. This would again suggest, as for the mucous cell after incubation in $H^3$-glucose, that the radioactivity incorporated by these other organelles may be derived from the Golgi apparatus, or that the Golgi apparatus is 'saturated' with $H^3$-glucose at about 60 minutes and the other organelles alone utilize the excess radioactive precursor retained in the tissue after the washing procedure prior to the 'chase' incubation. Uptake of $H^3$-glucose by all the organelles of the serous cell is less in amount than by those of the mucous cell (see later for absolute or total uptake by the whole cell).

## Hypothetical Grain Analysis

The results for the (i) grains per grid point and (ii) activity per unit area for the serous cell after incubation in $H^3$-glucose are shown in Table V-9 and Figures V-11 and V-12 .

(i) Grains per grid point (absolute uptake)

At 60 minutes, both the Golgi apparatus and endoplasmic reticulum showed similar uptake of $H^3$-glucose (Table V-9 and Figure V-11). At four hours, for the Golgi apparatus, uptake fell, and at eight hours, remained at this level. For the endoplasmic reticulum at four hours, the uptake increased and at eight hours, fell.

At 60 minutes, very little uptake was found for the granules, at four hours, uptake for this organelle was maximum for this time, and by eight hours, uptake decreased.

For the nucleus, very little uptake was found at 60 minutes, four and eight hours.

These results, in general, agree with those of the circle analysis. At 60 minutes, uptake by the Golgi apparatus and the endoplasmic reticulum is similar, uptake at four hours, for the Golgi apparatus decreases, while that for the granules increases. The uptake for the endoplasmic reticulum remains at similar level for all three times examined. This suggests that $H^3$-glucose, is initially incorporated into both the Golgi apparatus and the endoplasmic reticulum; the activity in the Golgi apparatus then passes to the granules while that of the endoplasmic reticulum seems to remain in this region and would thus seem to play little, if any,

TABLE V-9

THE PERCENTAGE AREA, PERCENTAGE ACTIVITY, GRAINS PER GRID POINT AND ACTIVITY PER UNIT AREA FOR THE SEROUS CELL AFTER INCUBATION IN $H^3$-GLUCOSE FOR 60 MINUTES, 4 AND 8 HOURS

| Incubation time | Organelle | Area % | Activity % | Grains per grid point | | Activity per unit area | |
|---|---|---|---|---|---|---|---|
| 60 minutes | ER | 65.4 | 94.1 | 0.18 | *** | 1.40 | 0.11* |
| | granule | 18.2 | 0.6 | 0.00 | | 0.00 | 0.13 |
| | Golgi | 3.4 | 4.7 | 0.18 | | 2.11 | 1.73 |
| | nucleus | 13.1 | 0.6 | 0.00 | | 0.08 | 0.17 |
| 4 hours | ER | 68.3 | 76.9 | 0.36 | 0.02* | 1.10 | 0.06 |
| | granule | 16.5 | 23.1 | 0.45 | 0.12 | 1.38 | 0.37 |
| | Golgi | 4.9 | 0.0 | 0.00 | 0.05 | 0.25 | 0.29 |
| | nucleus | 10.3 | 0.0 | 0.00 | 0.06 | 0.01 | 0.13 |
| 8 hours | ER | 73.6 | 53.8 | 0.22 | *** | 1.26 | *** |
| | granule | 14.4 | 6.2 | 0.07 | | 0.42 | |
| | Golgi | 3.7 | 0.0 | 0.00 | | 0.00 | |
| | nucleus | 8.3 | 0.0 | 0.00 | | 0.05 | |

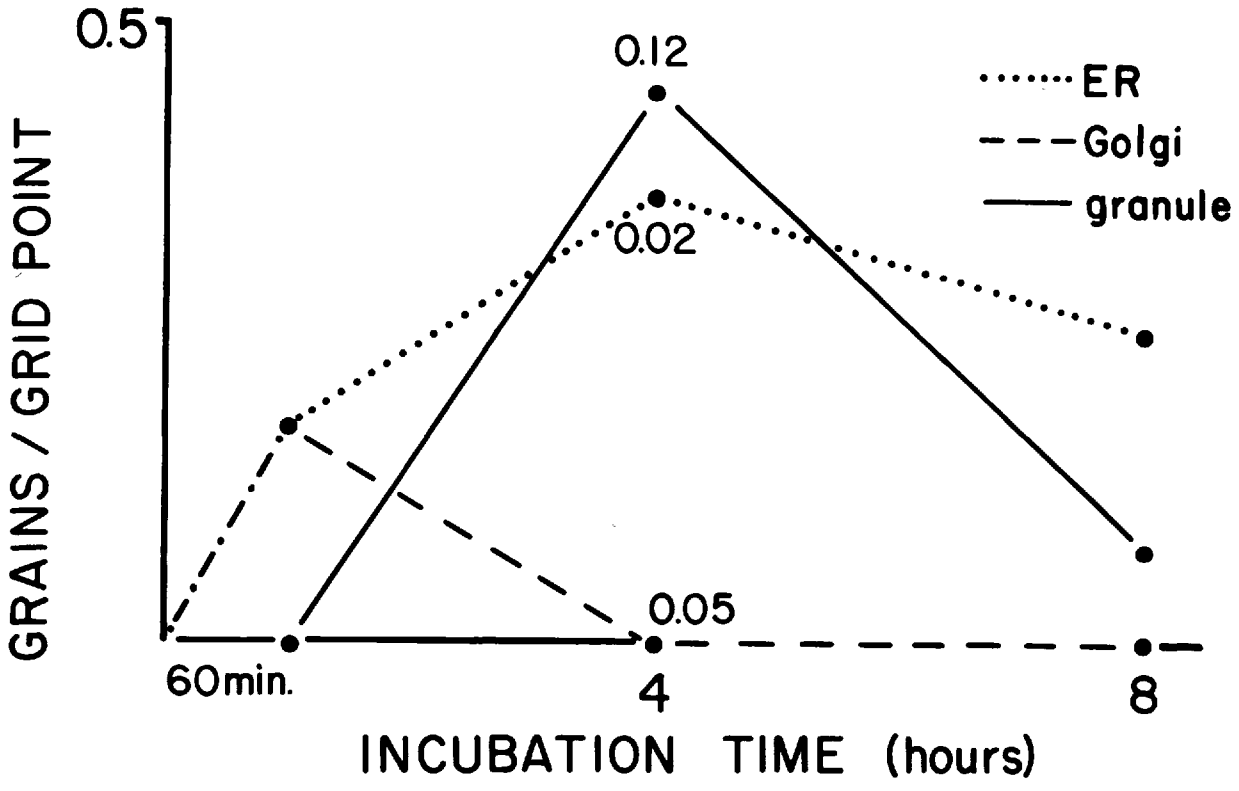
* = Standard error of the mean

*** = Computer unable to calculate standard error of the mean

FIGURE V-11

Grains per grid point for organelles of the serous cell after incubation with $H^3$-glucose for various times (figures represent the standard error of the mean for each point).

FIGURE V-11


0.5
0.12
ER
Golgi
granule
0.02
0.05
GRAINS / GRID POINT
60min.
4
8
INCUBATION TIME (hours)

part in the synthesis of carbohydrates. Little uptake is seen into the nucleus.

(ii) Activity per unit area (relative concentration of silver grains).

At 60 minutes, maximum activity was seen in the Golgi apparatus (Table V-9 and Figure V-12), this fell sharply by four hours, and at eight hours, continued to decrease.

At 60 minutes, little activity was seen in the granules, at four hours, maximum activity was seen in this region and by eight hours, had decreased.

For the endoplasmic reticulum, at 60 minutes, moderate activity occurred and at four and eight hours similar activity remained.

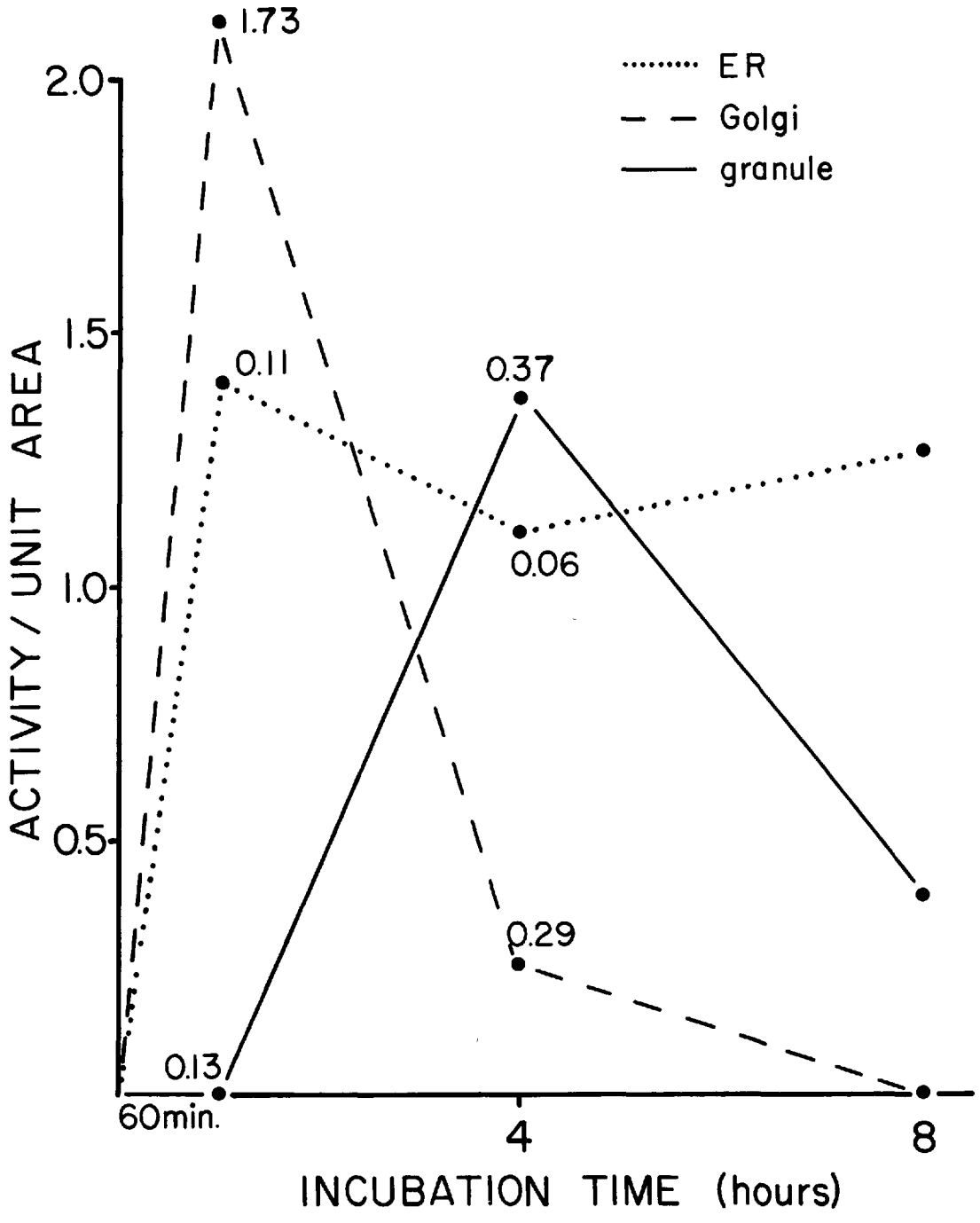
Little activity was found for the nucleus at any of the three times examined.

These results again agree with those of the circle analysis. After incubation in $H^3$-glucose, initial incorporation is seen in the Golgi apparatus of the serous cell, this activity then travels to

FIGURE V-12

Activity per unit area for organelles of the serous cell after incubation with $H^3$-glucose for various times (figures represent the standard error of the mean for each point).

FIGURE V-12


1.73
2.0
1.5
1.0
0.5
ER
Golgi
granule
0.11
0.37
0.06
0.29
0.13
ACTIVITY / UNIT AREA
60min.
4
8
INCUBATION TIME (hours)

the granules. Since the moderate activity found in the endoplasmic reticulum does not decrease it is thought that this organelle passes little, if any, radioactivity to other regions of the cell. The nucleus incorporates little $H^3$-glucose.

Chi-squared test

For the serous cell, after incubation for 60 minutes in $H^3$-glucose, the $x^2$ test for the pattern of distribution of the silver grains gave a value of $p = 0.95$, at four hours, $p = 0.75$ (these both represent high levels of confidence), and at eight hours, a value of only $p = 0.27$.

In conclusion, for the serous cell, using both light and electron microscopic autoradiography, quantitative analysis has revealed a similar pattern of incorporation of $H^3$-glucose.

At approximately 60 minutes, using the light microscope, initial heavy localization of silver grains is in the mid-region of the acinus. Using the electron microscope, initial incorporation is found at this time in the Golgi apparatus.

At three hours, using light microscopy, heavy localization of silver grains is at the apices of acini. Using the electron microscope, at five and six hours, maximum activity is found in the granules although activity in this region can be seen to be increasing before this time.

Up to eight hours, localization of $H^3$-glucose continues to increase in the canaliculi, suggesting that the peak of serous cell discharge may not yet have been reached, even at eight hours.

This pattern of incorporation is similar to that described for the mucous cell after incubation in $H^3$-glucose. When these results are compared to those after incubation in $H^3$-threonine for both cell types, it is apparent that different patterns occur for the incorporation of amino acids ($H^3$-threonine) and carbohydrates ($H^3$-glucose). Tritiated threonine is initially incorporated into the endoplasmic reticulum, thus indicating that this orgenelle synthesises amino acids (protein core of the secretory product), whereas $H^3$-glucose is initially incorporated into the Golgi apparatus, the region of the cell where the sugars are formed and also where they are added as a side chain to the preformed protein core.

## INCORPORATION OF RADIOACTIVE PRECURSORS BY OTHER CELLS OF THE HUMAN BRONCHIAL SUBMUCOSAL GLAND

Several other cell types were encountered in this study, but only in small numbers. Over both the small, clear cell and the collecting duct cell, few if any silver grains were found to localize after incubation in either $H^3$-threonine or $H^3$-glucose. The myoepithelial cells, situated at the base of the acinar cells on the other hand, did incorporate both radioactive precursors, but because of the small relative area of these cells, quantitative analysis of the silver grains over them was not attempted.

The total uptake of $H^3$-glucose by the mucous and serous cell was assessed in a similar manner to that described for $H^3$-threonine.

## TOTAL UPTAKE OF $H^3$-GLUCOSE BY MUCOUS AND SEROUS CELLS: GRAINS PER UNIT AREA

These methods are described on page 205 and give an estimate of the number of silver grains per unit area, i.e. grain density. Table V-10 and Figure V-13 show the grain density for the whole mucous and serous cell. Table V-11 and Figures V-14 and V-15 show the grain density over the base and apex of both cell types.

TABLE V-10

MEAN NUMBER OF GRAINS PER UNIT AREA (4 sq μm) OVER THE MUCOUS AND SEROUS CELL AFTER INCUBATION WITH $H^3$-GLUCOSE

| INCUBATION TIME | No. Grains | No. Circles | Grains / Circles |
|---|---|---|---|
| | | MUCOUS CELL | |
| 20 minutes | 60 | 1400 | 0.04 |
| 40 minutes | 221 | 831 | 0.27 |
| 60 minutes | 242 | 712 | 0.34 |
| 2 hours | 510 | 903 | 0.56 |
| 3 hours | 576 | 761 | 0.76 |
| 4 hours | 496 | 880 | 0.56 |
| 5 hours | 594 | 784 | 0.76 |
| 6 hours | 389 | 827 | 0.47 |
| 7 hours | 538 | 820 | 0.66 |
| 8 hours | 1135 | 1164 | 0.98 |
| | | SEROUS CELL | |
| 20 minutes | 89 | 515 | 0.17 |
| 40 minutes | 218 | 1171 | 0.19 |
| 60 minutes | 360 | 1196 | 0.30 |
| 2 hours | 349 | 1005 | 0.35 |
| 3 hours | 442 | 920 | 0.48 |
| 4 hours | 448 | 742 | 0.60 |
| 5 hours | 662 | 742 | 0.89 |
| 6 hours | 377 | 1055 | 0.36 |
| 7 hours | 340 | 1121 | 0.30 |
| 8 hours | 282 | 830 | 0.34 |

FIGURE V-13

Mean number of grains per unit area (4 sq μm) over both the mucous and serous cell after incubation with $H^3$-glucose for various times.

FIGURE V-13

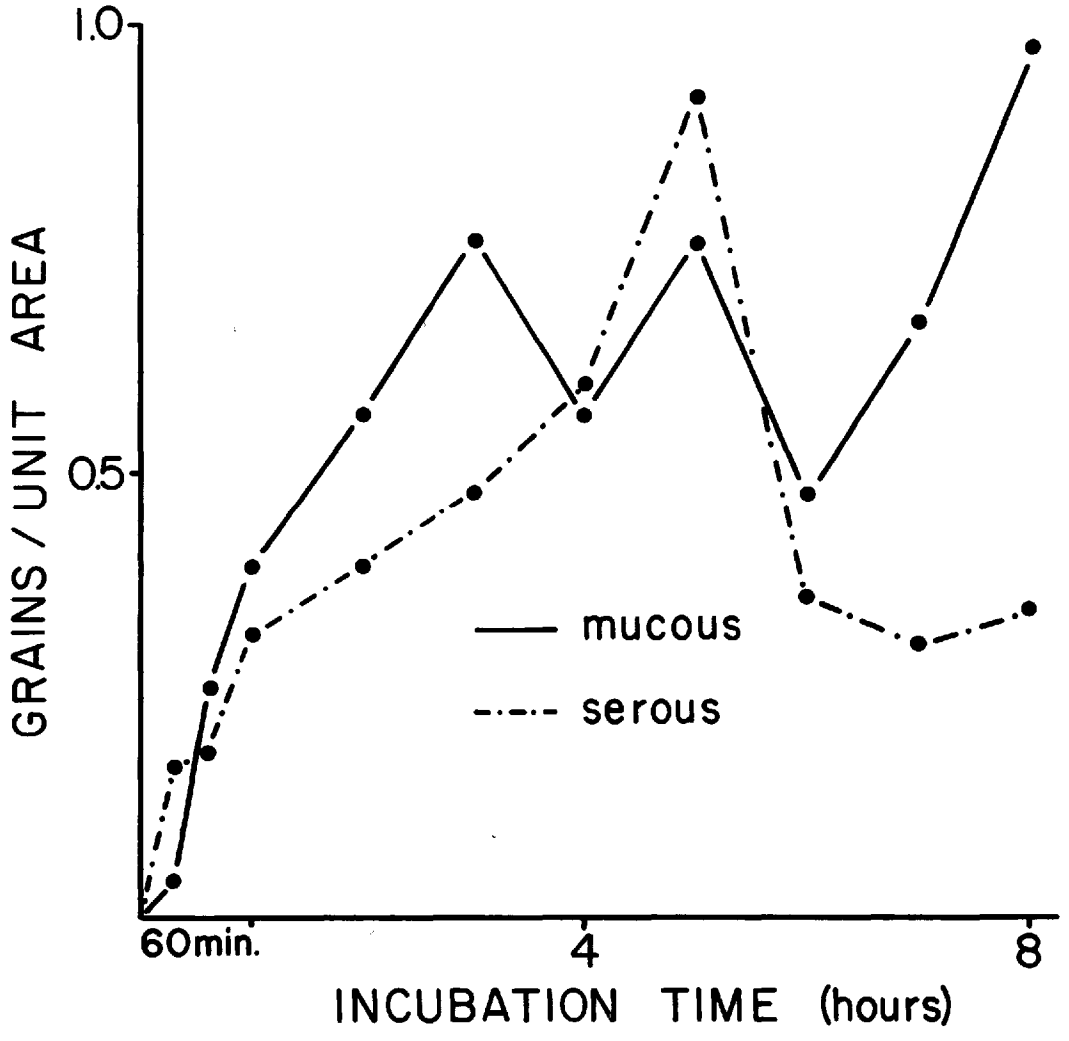

1.0
0.5
GRAINS / UNIT AREA
mucous
serous
60min.
4
8
INCUBATION TIME (hours)

TABLE V-11

THE MEAN NUMBER OF GRAINS PER UNIT AREA (12.5 x 8.5 cm) OVER THE BASE AND APEX OF BOTH THE MUCOUS AND SEROUS CELL AFTER INCUBATION WITH $H^3$-GLUCOSE

| | MUCOUS | | SEROUS | |
|---|---|---|---|---|
| Incubation time | Base | Apex | Base | Apex |
| 20 minutes | 1.5 | 2.0 | 4.1 | 3.9 |
| 40 minutes | 7.0 | 3.8 | 4.1 | 5.1 |
| 60 minutes | 7.3 | 3.7 | 6.4 | 5.2 |
| 2 hours | 11.3 | 2.9 | 5.7 | 6.3 |
| 3 hours | 14.5 | 6.0 | 5.8 | 5.6 |
| 4 hours | 19.3 | 9.7 | 11.7 | 11.1 |
| 5 hours | 16.0 | 8.9 | 19.0 | 15.7 |
| 6 hours | 14.1 | 8.3 | 6.8 | 11.2 |
| 7 hours | 13.8 | 14.6 | 11.0 | 10.0 |
| 8 hours | 16.3 | 23.0 | 5.2 | 11.2 |

FIGURE V-14

Mean number of grains per unit area (12.5 x 8.5 cm) over the base and apex of the mucous cell after incubation with $H^3$-glucose for various times.

FIGURE V-14

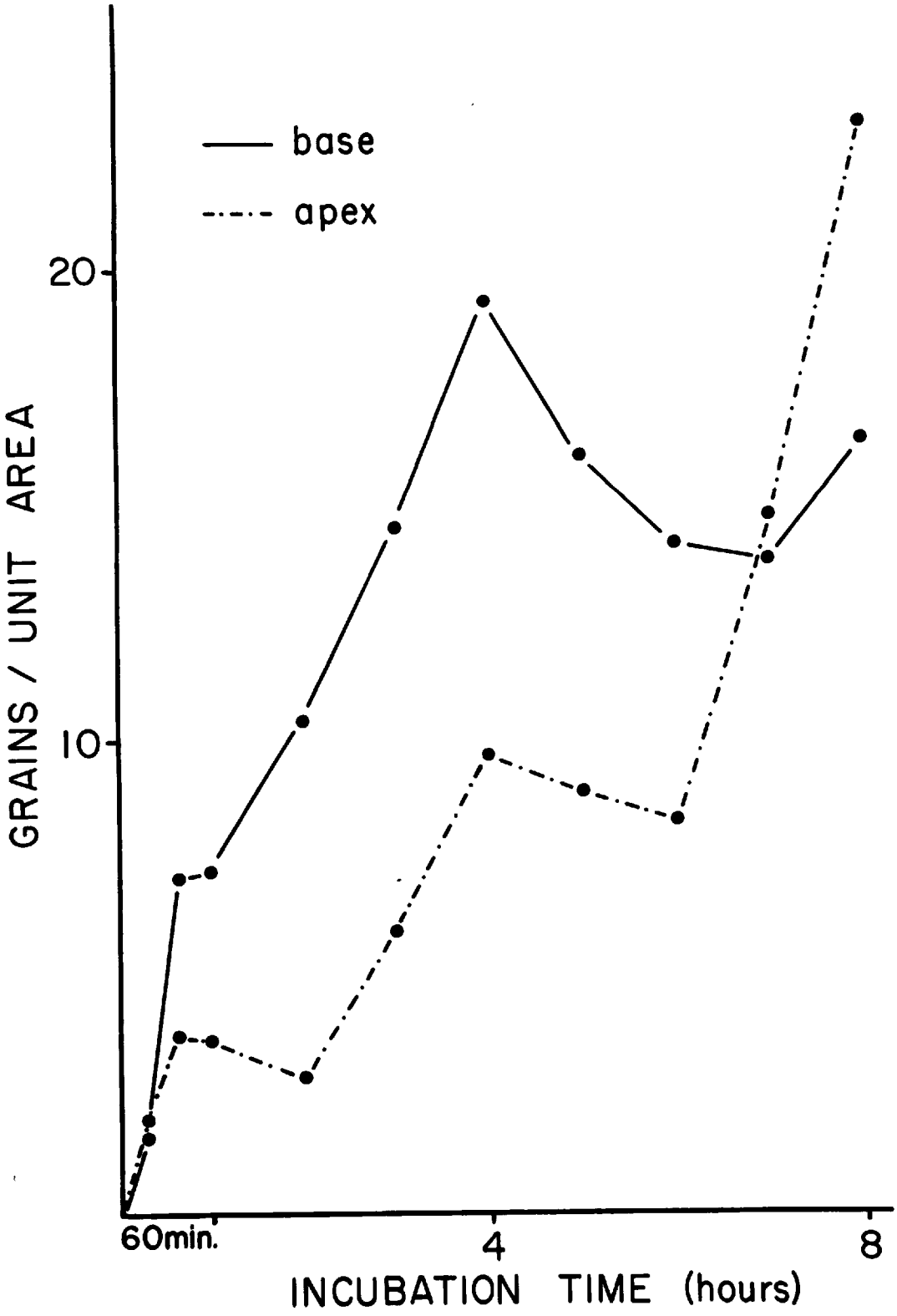
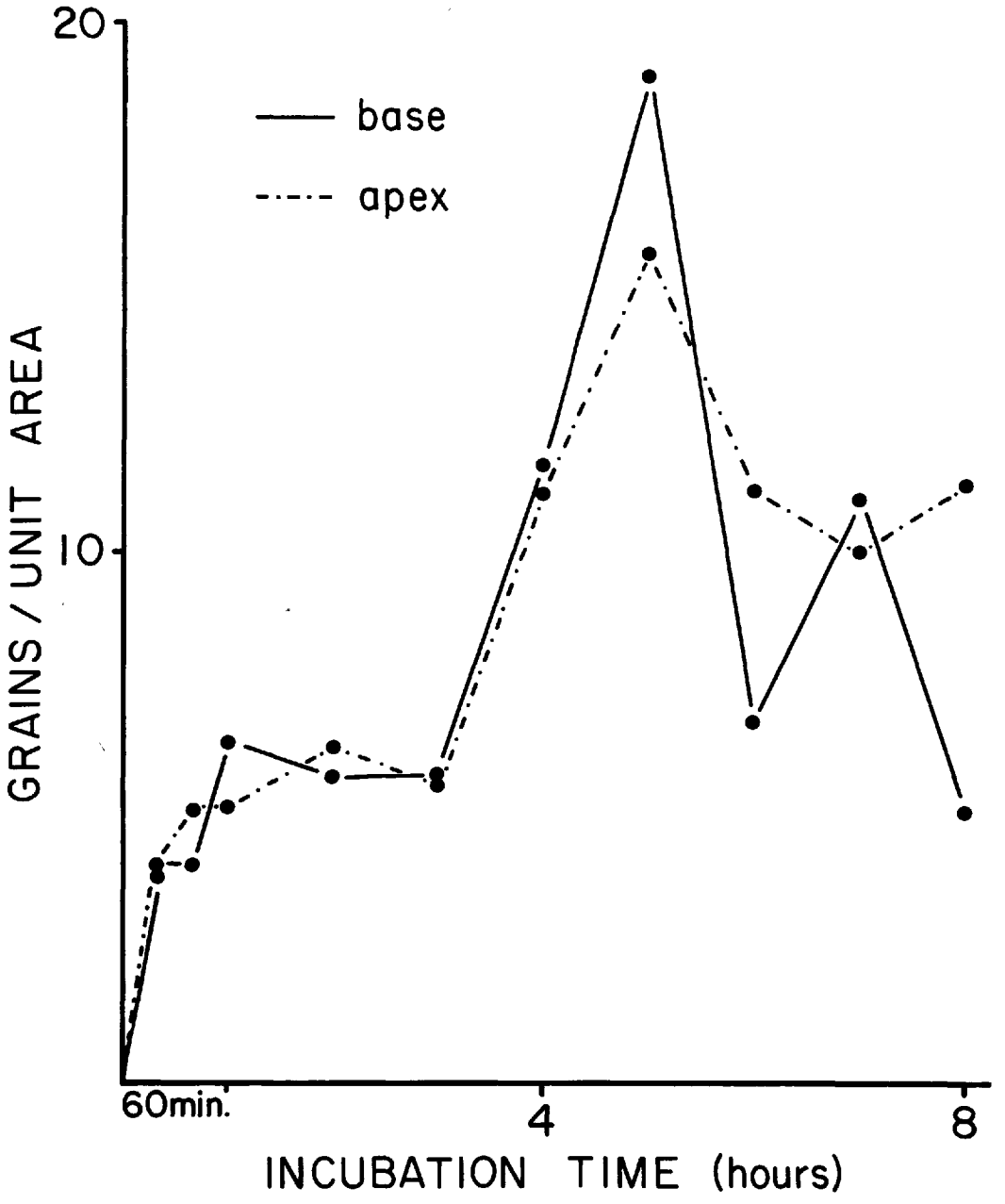

base
apex
20
10
GRAINS / UNIT AREA
60min.
4
8
INCUBATION TIME (hours)

FIGURE V-15

Mean number of grains per unit area (12.5 x 8.5 cm) over the base and apex of the serous cell after incubation in $H^3$-glucose for various times.

FIGURE V-15


20
10
GRAINS / UNIT AREA
base
apex
60min.
4
8
INCUBATION TIME (hours)

## Mucous Cell

Little incorporation of $H^3$-glucose was obtained before 40 minutes. Maximum grain density was obtained between three and four hours and remained at a similar level for the remainder of the eight hours. The higher grain density at eight hours was probably a chance finding. Grain density in the base of the mucous cell predominated until seven hours, when the grain density in the cell apex was higher. No evidence of secretion was found.

## Serous Cell

Maximum uptake of $H^3$-glucose by the serous cell was obtained at five hours and from this time decreased, indicating discharge. Throughout the serous cell, at all times, grain density was similar at the base and apex.

The overall uptake of $H^3$-glucose for both mucous and serous cells has been studied by determining the number of silver grains per unit area. For the mucous cell, maximum uptake was found between three and four hours, a similar time to that for maximum incorporation of $H^3$-threonine by both mucous and serous cells. Since no evidence of mucous cell secretion is found, this

would suggest that the uptake of $H^3$-glucose is complete by this time (three - four hours). For the serous cell, maximum uptake is obtained at five hours and by six hours, evidence of discharge is apparent. It is therefore probable that the maximum absolute uptake of $H^3$-glucose by the cell, seen with the electron microscope at four hours for the mucous and five hours for the serous cell is due to utilization of unbound radioactive precursor within the tissue.

Comparison of grain densities over the base and apex of both mucous and serous cells, as for the results of $H^3$-threonine, show that in the mucous cell, $H^3$-glucose passes from the cell base to the apex of the cell, this being a reflection of the regional distribution of organelles. For the serous cell, at all times studied, grain density due to $H^3$-glucose is similar in the base and apex of the cell; similar findings were also reported for $H^3$-threonine.

Grain densities due to $H^3$-glucose show no evidence of discharge from the mucous cell. Using the light microscope, heavy labelling of the mucous acinar lumens was found at seven and eight hours. This discrepancy is difficult to explain. For the serous cell, discharge is suggested from six hours.

## SUMMARY

Using both light and electron microscopic autoradiography, the incorporation of $H^3$-glucose by the mucous and serous cells of the human bronchial submucosal gland has been studied.

For both mucous and serous cells, both studies gave similar results. Tritiated glucose was initially localized, at 60 minutes, in the Golgi apparatus. Activity then travelled to the vacuoles (this was particularly noticeable in the mucous cell) and at six hours, maximum localization of silver grains was found for the serous cell granules, while at eight hours, for the mucous granules, activity was still increasing. Using light microscopic autoradiography, at seven and eight hours, the lumens of the mucous acini were heavily labelled. Labelling of serous canaliculi, at eight hours, was shown by electron microscopic autoradiography, still to be increasing.

Maximum uptake of $H^3$-glucose by the Golgi apparatus, for both cells occurred at approximately 60 minutes. All other unassociated organelles (i.e. not including ER/Golgi apparatus) reached maximum uptake only after longer incubation times, indicating either that activity for all other organelles was derived from the Golgi apparatus or that at 60 minutes the Golgi apparatus was saturated with $H^3$-glucose and thus the remaining

organelles utilized the remaining unbound radioactive precursor.

For all organelles of the serous cell, maximum uptake of $H^3$-glucose was less in amount than for the mucous cell.

Grain density (grains per unit area) for the whole cell was estimated. Maximum uptake of $H^3$-glucose, for the mucous cell, was found to occur between three and four hours and for the serous cell, at five hours. Grain density decreased at six hours for the serous cell, suggesting discharge of $H^3$-glucose, while for the mucous cell, no such decrease was seen.

Comparison of the grain density at the base of the cell to that at the apex showed that for the mucous cell, grain density at the base predominated until seven hours, when at seven and eight hours, the majority of grains were in the apex. This indicated the passage of $H^3$-glucose from the base to the apex of the cell. For the serous cell, grain density was similar at all times for the base and apex, at six hours, a decrease in grain density occurred indicative of discharge.

# CHAPTER VI

## DISCUSSION

Using chemical analysis, $H^3$-threonine has been shown to be incorporated into the proteins, and $H^3$-glucose into the glycoprotein side-chains, of the mucous and serous cells of the human bronchial submucosal gland.

Formalin was found to be the optimum fixative for explants after incubation with either $H^3$-threonine or $H^3$-glucose; glutaraldehyde bound both radioactive precursors to the tissue in a random manner.

After incubation with $H^3$-threonine, the results of the light and electron microscopic autoradiographs were comparable for the mucous cell, while those for the serous cell were not. With the light microscope, random incorporation of $H^3$-threonine was suggested for the serous cell, while the electron microscope suggested two patterns of incorporation, (i) a similar pattern of uptake as shown for the mucous cell and (ii) incorporation directly into the granules. The more definite patterns obtained with the electron microscope are doubtless due to the improved resolution of this technique.

After incubation with $H^3$-glucose, the results for the light and electron microscopic autoradiographs were comparable for both mucous and serous cells and both cell types showed similar patterns of incorporation.

Before analysing the results for the uptake of $H^3$-threonine and $H^3$-glucose by the bronchial gland, the reasons for using these two radioactive precursors may be considered as well as their specificity.

## $H^3$-THREONINE

Threonine has been shown by Havez and Biserte (1969) to represent approximately 20% of all amino acids present in mucus. In sheep colonic mucosa, Allen and Kent (1968) have shown that threonine goes exclusively into the polypeptide chain of the glycoprotein molecule. In a pilot study, Sturgess (1970), examined three tritiated amino acid precursors - leucine, methionine and threonine - and found threonine to be the most actively incorporated into explants of human bronchial submucosal gland.

In the present study, using trichloracetic acid precipitation, it has been shown that 24% of available $H^3$-threonine is incorporated into the protein of the submucosal gland. Incubation with added puromycin inhibited this incorporation by approximately 75%. Trichloracetic acid has been shown to precipitate proteins by Baer, Bringaze and McNamee (1954) and Morris and Dickman (1960), while most glycoproteins are not precipitated (Schultze and Heremans, 1966).

## $H^3$-GLUCOSE

In 1966(b), Neutra and Leblond outlined a scheme for the utilization of $H^3$-glucose by secretory cells. They showed the pathways to be complex and the end products variable, e.g. proteins, nucleic acids and mucopolysaccharides. However, other workers have found, at least for the mucous cell, that glucose is incorporated mainly on to the terminal sugar groups. Incorporation of glucose-I-$C^{14}$ into sheep colonic mucus has been found by Draper and Kent (1963), to be principally into the sialic acid group, whereas the protein moiety was unlabelled.

Using histochemical and autoradiographic studies of the human bronchial gland, Lamb (1969) demonstrated that the removal of sialic acid by sialidase or acid hydrolysis also removed radioactive labelling from the intracellular secretion of the mucous cell. From this it was concluded that the majority of $H^3$-glucose is included in the terminal sialic acid group.

In the present study, precipitation with ethanol (Aminoff, Morgan and Watkins, 1950; Baer et al, 1954 and Draper and Kent, 1963) indicated that 58% of $H^3$-glucose was incorporated into the glycoprotein of the human bronchial submucosal gland. Puromycin inhibited this incorporation by 80%. As puromycin inhibits protein synthesis only, it is surmised that the block occurred in the synthesis of the polypeptide chain to which the

glucose is linked, thus causing a decrease in the uptake of $H^3$-glucose into the carbohydrate moiety of the gland. Similar results were found for the thyroid (Spiro and Spiro, 1966), where almost complete inhibition of peptide synthesis was obtained with puromycin. Variable degrees of inhibition for sugar incorporation were obtained and the most peripherally located sugar (sialic acid) was most affected.

Using light microscopic autoradiography, Sturgess (1970) and Sturgess and Reid (1972a) studied the localization of $H^3$-threonine and $H^3$-glucose into both mucous and serous cells of the human bronchial gland and were able to assess the pathway for their incorporation as well as the secretory activity of the gland and the turnover of various components of the glycoprotein.

Since the present work combines both electron microscopy and autoradiography it enables more precise localization of $H^3$-threonine and $H^3$-glucose in both mucous and serous cells. In some cases activity arising from radioactive precursors can be ascribed to individual organelles.

# FIXATION

## Non-specific binding effect

It has been shown recently that glutaraldehyde and to a lesser extent osmium tetroxide both bind radioactive amino acids to tissue in a non-specific manner (Peters and Ashley, 1967 and Hodson and Marshall, 1967). This indicates that chemically bifunctional fixative reagents, that can cause cross-linking, may cause a label of low molecular weight to be irreversibly bound to the tissue (Williams, 1969). This effect seems only to be of consequence when the tissue is fixed in the presence of a high activity radiochemical (Bergeron and Droz, 1968), as used in the present study.

## $H^3$-threonine

Using scintillation counts, both glutaraldehyde and Karnovsky's fixative were found in the present study, to bind more $H^3$-threonine to the tissue than formalin. It had also been suggested that washing tissue in a 'cold' rinse after incubation in a radioactive amino acid, aids the removal of the unbound radioactive precursor (Williams, 1972). This was found to be the case for explants incubated with $H^3$-threonine and fixed in glutaraldehyde, but no improvement was found for formalin. Both these studies indicated that glutaraldehyde binds $H^3$-threonine to the

tissue in a non-specific manner and that formalin is the more suitable fixative.

In a further study, non-specific binding of $H^3$-threonine was prevented by incubating the explants at $4^oC$. Formalin was shown again to cause least non-specific binding of $H^3$-threonine. It was noted that glutaraldehyde, Karnovsky's fixative and osmium tetroxide all bound more $H^3$-threonine to the serous cells than to the mucous cells. This effect may be explained by the more active cross-linking of proteins by these fixatives when compared to formalin. Alternatively, this finding could suggest that formalin does not preserve adequately the proteins of the serous cell, but since no loss of glucose was apparent (see page 113 ), this would argue that the glycoprotein moiety of the serous cell was not lost, rather that glutaraldehyde, Karnovsky's fixative and osmium tetroxide all bound more non-specific $H^3$-threonine to serous cells than to mucous cells. The reason for this is obscure.

A greater amount of protein is thought to be present in the serous cell than the mucous cell (see page 324 ). Background counts and those over mucous and serous cells were similar after formalin fixation, indicating that non-specific binding did not occur with this fixative and agreed with earlier findings (Peters and Ashley, 1967 and Hodson and Marshall, 1967).

It was therefore concluded that either formalin alone of glutaraldehyde with a 'cold' rinse could be used to fix explants of human bronchial submucosal gland after incubation with $H^3$-threonine. In the particular situation reported here however, non-specific binding of radioactive aminoacids was not easily detected because of the inhomogeneity of the tissue, so it was felt that the results obtained with formalin were more reliable.

$H^3$-glucose

Carbohydrate precursors have not been shown to produce this non-specific binding artefact (Vanha-Perttula and Grimley, 1970). In the present study, scintillation counts revealed that all fixatives were suitable and that 'cold' rinses did not aid the removal of excess $H^3$-glucose.

The light microscope experiment at $4^oC$ demonstrated however, that glutaraldehyde bound significantly more $H^3$-glucose to both mucous and serous cells than formalin.

Thus any of the fixatives save for glutaraldehyde could be used to fix tissue after incubation with $H^3$-glucose.

## Morphological preservation

Since the now classic report by Sabatini, Bensch and Barrnett (1963) on the preservation of tissue by aldehydes, glutaraldehyde with secondary fixation in osmium tetroxide is the fixation technique most commonly used for electron microscopy. In the light of recent results, formalin would seem to be preferable for use with electron microscopic autoradiography.

Unfortunately, formalin fixation either with post-fixation in osmium tetroxide, or with post-fixation in glutaraldehyde and then osmium tetroxide, although adequately preserving the mucous cell, showed marked differences for the preservation of the serous cell granules when compared to the fixation of glutaraldehyde. Differentiation between mucous and serous cells became difficult, but fixation in formalin for 18 hours did allow easy identification of mucous and serous cells. The better preservation obtained with formalin for 18 hours suggests that the longer fixation period allows more cross-linking of protein to occur and aids the stability of the proteins. This conflicts with the results of Baker and McCrae (1966), who concluded that little was to be gained by fixing in formalin for longer than 45 minutes.

## LIGHT MICROSCOPIC AUTORADIOGRAPHY

Examination of 1 μm, toluidine blue stained, Araldite sections, as well as the use of a 'pulse' labelling system for all explants incubated with either $H^3$-threonine or $H^3$-glucose, has added to the observations of Sturgess and Reid (1972a), using 5 μm paraffin sections. More accurate localization of silver grains over the acinus and hence the cell has been possible.

### Pattern of incorporation as visualized with the light microscope

In the present study, the pattern of incorporation of $H^3$-threonine by the mucous and serous acini of the human bronchial submucosal gland was found to be different for each cell.

For the mucous acini, initial localization was found at the base and the labelling then passed through the cell and into the lumen. For the serous acini, discrete localization of labelling was not found, at all times it was diffuse. Similar findings were reported by Sturgess and Reid (1972a).

In contrast incorporation of $H^3$-glucose by mucous and serous acini in the present study was similar. The use of 1 μm sections showed initial localization of

$H^3$-glucose in the mid-region of acini. This contrasts with the results of Sturgess (1970) and Sturgess and Reid (1972a) who reported initial localization in the cell base.

## HIGH RESOLUTION AUTORADIOGRAPHY

### $H^3$-threonine

Using electron microscopic autoradiography, the pattern of incorporation of $H^3$-threonine by the mucous cell of the human bronchial submucosal gland followed a similar pattern to that described by Jamieson and Palade (1964) for the uptake of $H^3$-leucine by the acinar cell of the guinea pig pancreas. The present electron microscopic findings were also comparable to those of the light microscope. The results for the serous cell, however, suggested two pathways for the incorporation of $H^3$-threonine, (i) similar to that of the mucous cell although slight time differences occurred for each stage and (ii) rapid incorporation of $H^3$-threonine directly into the serous granules. This last observation may be real or apparent and caused by one of three factors.

1. Synthesis of the serous granules may occur rapidly, but this would seem unlikely since the second peak of activity was not seen until much later (seven hours) and would therefore represent a longer secretory cycle.

2. Tritiated threonine may be more readily available to the serous granules from the canaliculi than to the base of the cell from the surrounding tissue. In this case the granules may incorporate $H^3$-threonine directly from the canaliculi, perhaps by pinocytic activity. These pinocytic vesicles then may fuse directly with the secretory granules. In 1969, Simson suggested that restitution of granules in the rat parotid gland, after stimulation with isoprenaline (isoproteranol), occurred initially around the intracellular canaliculi and the luminal edge with the appearance of small dense granules. These were thought to migrate and fuse with other secretory products formed within the extensive Golgi apparatus. In the present experiments, no such secretogogue was applied to the explants. Since the serous granules were therefore secreting in an essentially normal manner it is unlikely that the granules themselves were labelled at this early stage.

3. The labelling of the serous granules may be only apparent. The source of the silver grains could be situated outside the granule. The endoplasmic reticulum is the organelle that is most commonly found in close association with the granules and may therefore be the source of the radioactive disintegration. Thus, the apparent labelling of the granules could be caused by

'cross-fire' from adjacent organelles (Blackett and Parry, 1973 and Parry and Blackett, 1973).

This last, factor 3, would seem to be the most plausible explanation for the labelling of the granules at the earlier time. So it is considered that the mucous and serous cells of the human bronchial submucosal gland have similar patterns for incorporation of $H^3$-threonine. A similar pattern of incorporation is also reported for other sites, e.g. the endocrine pancreas (Howell, Kostianovsky and Lacy, 1969), the parotid gland (Amsterdam, Ohad and Schramm, 1968) and neutrophilic leucocytes (Fedorko and Hirsch, 1966).

The following is the predicted pathway for the incorporation of $H^3$-threonine by the mucous and serous cells and is compared to the incorporation of $H^3$-leucine in the acinar cells of the exocrine pancreas.

Initial concentration of $H^3$-threonine is seen to be in the endoplasmic reticulum. Synthesis of exportable protein has been established to occur in association with the polysomes of the rough endoplasmic reticulum of the pigeon pancreas (Redman, Siekevitz and Palade, 1966) and the hepatic parenchymal cell (Redman, 1969). In the mucous cell of the human bronchial submucosal gland, this stage was found to be maximal at 60 minutes, and in the serous cell at 20 minutes. In the acinar cell of the exocrine pancreas, uptake was seen at ten minutes.

After the proteins are synthesized by the polysomes, they are transferred to the cisternal space. Blobel and Sabatini (1970) and Sabatini and Blobel (1970), have recently demonstrated that the peptides are vectorially transferred across the membrane into the cisternal space during the course of growth of the molecule. The nascent peptide is seen to grow in a channel or groove in the large subunit of the ribosome and penetrates the membrane of the endoplasmic reticulum through a patent orifice which may be continuously or intermittently open.

From the endoplasmic reticulum, the nascent peptide travels to either the lamellae of the Golgi apparatus or to the 'condensing' vacuoles. This passage has been shown to occur in the acinar cell of the exocrine pancreas, via smooth surfaced vesicles which are seen to bud from the endoplasmic reticulum and are situated mainly in the region of the Golgi apparatus (Caro and Palade, 1964 and Jamieson and Palade, 1967a). Similar vesicles were seen, in both mucous and serous cells of the human bronchial submucosal gland.

In the present study, the smooth surfaced vesicles were contained mainly in the junctional region, ER/Golgi apparatus. The maximum activity was found in this region, at 60 minutes for the mucous cell and at 50 minutes for the serous cell. The

exocrine pancreas showed activity in this region at ten minutes. The resolution obtained using electron microscopic autoradiography did not show conclusively whether the smooth surfaced vesicles travelled to either the Golgi lamellae or to the 'condensing' vacuoles. However, in the resting acinar cell of the exocrine pancreas, the results of Jamieson and Palade (1967a) using electron microscopic autoradiography and cell fractionation techniques, suggested that these vesicles travel directly to the 'condensing' vacuole, but if the cell is stimulated to discharge with carbamycholine and secretion is allowed to reform, the smooth surfaced vesicles appear to deliver their radioactive content ($H^3$-leucine) to the lamellae of the Golgi apparatus (Jamieson and Palade, 1971b). Direct tubular connections have been shown between the rough endoplasmic reticulum and the Golgi apparatus by Claude (1970), in the hepatic parenchymal cell.

In the present study, activity for the vacuoles ('condensing' vacuoles) of the mucous cell was seen from 60 minutes with maximum at three hours and for the serous cell, from 60 minutes to two hours.

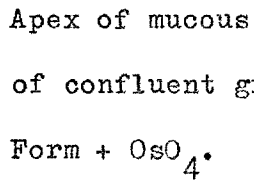
Finally the vacuoles enlarge and migrate to the apical surface of the cell. Maximum activity was seen for the mucous granules at six hours and for the serous granules, at seven hours. This contrasts with the zymogen granules of the exocrine pancreas where maximum activity was found at 60 minutes.

Discharge from both mucous and serous cells was occurring at eight hours, while the cells of the exocrine pancreas discharge at two hours. In the case of the mucous cell of the human bronchial submucosal gland, at one time, several granules are seen to discharge (Figure VI-1) as well as fragments of other organelles, e.g. ribosomes and mitochondria. For the serous cell, typical exocrine secretion occurs where the granule fuses with either the apical or lateral membrane around a canaliculus and the contents are extruded without a break occurring in the cell membrane (Figure VI-2).

The present study has shown that the pattern of incorporation for $H^3$-threonine by the mucous and serous cells of the human bronchial submucosal gland is similar and is comparable to the incorporation of amino acids by other secretory cells.

Nuclear incorporation of $H^3$-threonine, seen in the present study at 60 minutes, has also been reported by Hopkins and Farquhar (1973) for cells dissociated from rat anterior pituitaries. The metabolic function of this uptake is not clear, but it is almost certainly due to protein metabolism as the nuclear labelling is abolished when cyclohexamide (protein synthesis inhibitor) is added to the incubation medium (Lockhart Ewart and Taylor, 1971). As yet it is unclear if the nuclear protein is (i) formed within the nucleus (ii) formed within the cytoplasm and subsequently migrates to the

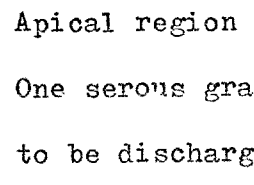
FIGURE VI-1



Apex of mucous cell showing discharge of confluent granules (↓).

Form + $OsO_4$. X26,000.

FIGURE VI-2



Apical region of a serous cell. One serous granule (gr) appears to be discharging into a canaliculus (ca).

Form + $OsO_4$. X26,000

FIGURE VI-1

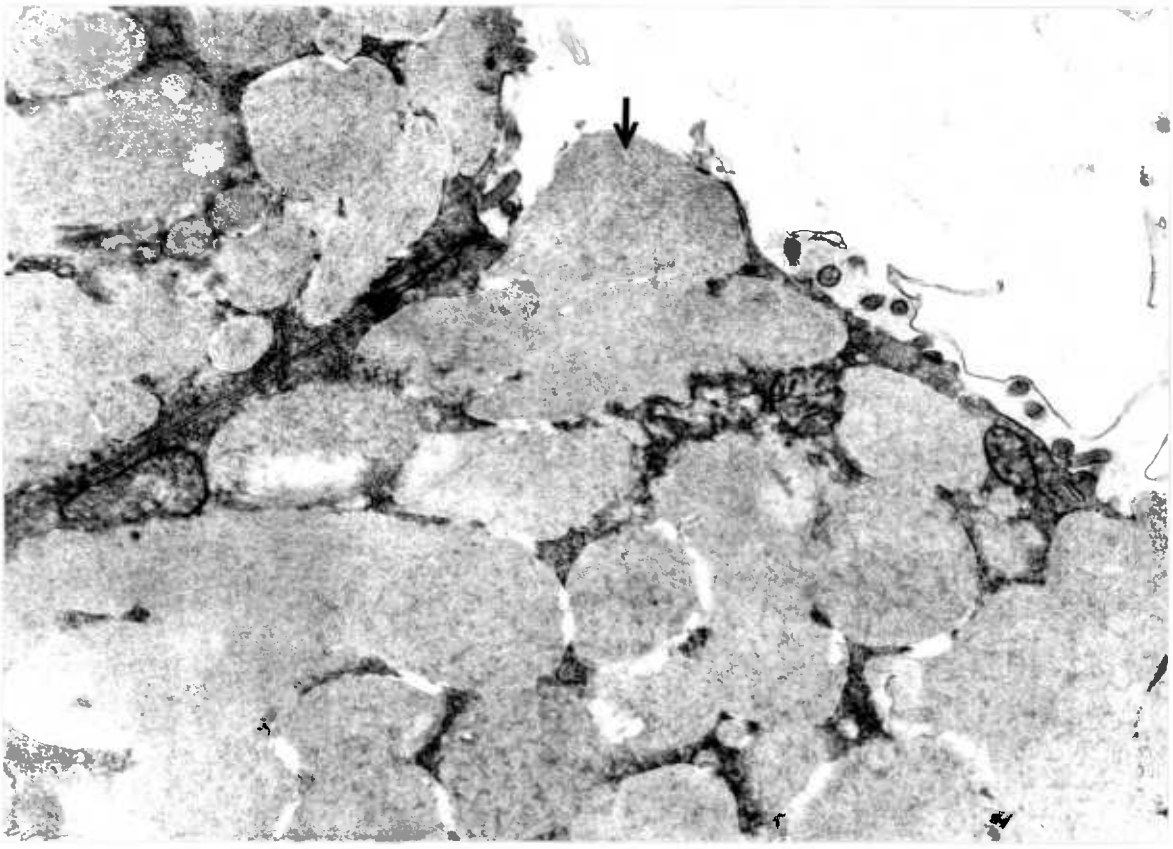
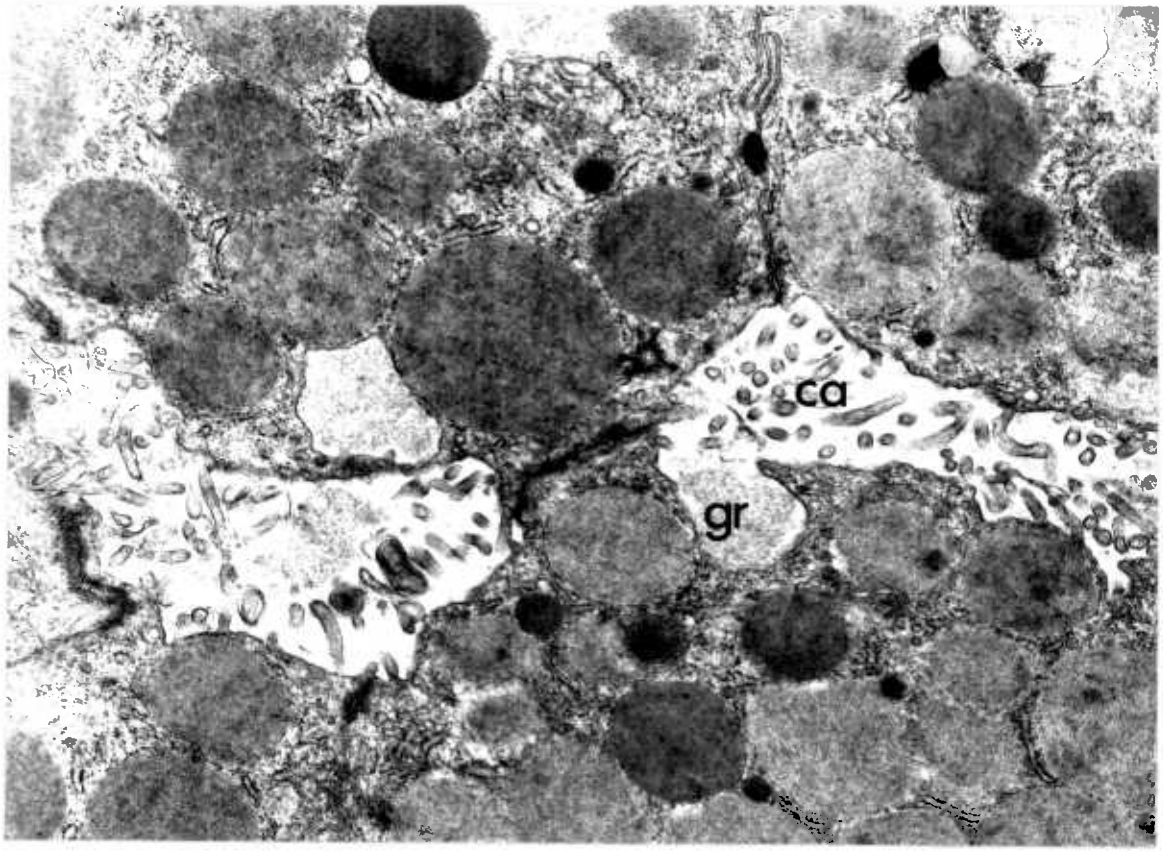


FIGURE VI-2



nucleus (iii) certain proteins are formed in the nucleus while others are formed in the cytoplasm (Wu and Warner, 1971).

## $H^3$-glucose

The electron and light microscopic findings were similar for both mucous and serous cells, and were comparable to $H^3$-glucose incorporation into other secretory cells (Neutra and Leblond, 1966a and b).

The following is the predicted pathway for the incorporation of $H^3$-glucose into the mucous and serous cells of the human bronchial submucosal gland. Initial concentration of $H^3$-glucose is seen in the Golgi apparatus, the radioactive precursor then passes to the vacuoles and finally localizes in the secretory granules.

Many other sugars have been reported to be added to the polypeptide chain in the Golgi apparatus, e.g. galactose, to the Golgi apparatus of rat thyroid (Whur, Hersovics and Leblond, 1969) and the columnar cells of the rat duodenum (Bennett, 1970 and Bennett and Leblond, 1971), fucose, to the columnar cells of the rat small intestine (Bennett and Leblond, 1970) and glucosamine, to rat hepatocytes (Droz, 1966). Incorporation of $S^{35}$-sulphate has also been shown to occur in the Golgi apparatus (Berg and Young, 1971).

In the present study, $H^3$-glucose appeared over the endoplasmic reticulum in moderate amounts at all times studied. Since this labelling persisted it does not seem likely to be related to the formation of secretory products, it is more likely to be associated with sedentary proteins. However, the possibility that some $H^3$-glucose is added in the endoplasmic reticulum cannot be completely excluded. Certain of the carbohydrate moieties have been reported to be added to the polypeptide chain after this has passed into the cisternal space. Whur, Hersovics and Leblond (1969), reported the addition of $H^3$-mannose in the cisternal space of the endoplasmic reticulum and Zagury, Uhr, Jamieson and Palade (1970) that $H^3$-glucosamine incorporation into the IgG molecule of a plasma cell tumour, appeared to take place in the endoplasmic reticulum as well as the Golgi apparatus. Earlier fractionation techniques also suggested this double site of incorporation for glucosamine, in the liver (Molnar, Robinson and Winzler, 1965) and in the intestine (Louisot, Frot-courtaz, Bertagnolio, Got and Colobert, 1967).

From these results it is apparent that only terminal sugars, i.e. sialic acid and fucose, are added in the Golgi apparatus, while sugars that are situated close to the protein core, i.e. N-acetylhexosamine and mannose, are added in the endoplasmic reticulum; other sugars, for example glucosamine, that are situated mid-way between these ('bridging' sugars) may be added either in the

endoplasmic reticulum or the Golgi apparatus or in both.

In the present study it is apparent that $H^3$-threonine is incorporated into the protein core at the endoplasmic reticulum and that $H^3$-glucose is added to this protein core at the Golgi apparatus as a terminal sugar, probably sialic acid (Lamb, 1969).

## RATE OF SYNTHESIS

The present study reveals a slower rate of synthesis and discharge of secretory material for the bronchial glands than for other organs, e.g. the acinar cells of the exocrine pancreas, where Nadler (1963) calculated that the mean life span of 'exportable' protein was 52.4 minutes, labelling of the granules was found at 60 minutes (Caro and Palade, 1964) and significant labelling of the lumina was apparent at two hours (Castle et al, 1972). Recently Cutler and Chaudhury (1973), described the secretory cells of the convoluted tubules of rat submandibular gland to have a cycle of eight hours and Simson (1969) described a cycle of six hours for the rat parotid gland.

The length of the cycle may be dependant on external stimuli. The cells of the exocrine pancreas discharge in response to the stimulus of eating and therefore behave in an irregular manner which is dependant on the feeding habits of the individual. Formation of new granules

therefore would need to be rapid since stimuli may be frequent. Discharge from the mucous and serous cells of the bronchial gland as well as the parotid and submandibular gland occurs continuously but may be increased by external stimuli. The continuous secretion doubtless has some effect on the rate of the cell cycle.

## DIFFERENCES BETWEEN MUCOUS AND SEROUS CELLS

Previous reports have suggested that the serous cells undergo transformation to mucous cells in cases of gland hypertrophy associated with irritation (Sturgess, 1970). Sturgess and Reid (1972b) have also shown that mucous and serous cells have a similar response to drugs.

This concept of transformation of 'serous' to mucous cells has also been suggested for the surface airway epithelium of the rat lung. Transition forms were found rarely in the airways of control rats, but in cases of irritation of the airway epithelium by isoprenaline and cigarette smoke, were frequent (Jeffery, 1973).

The relationship between mucous and serous cells is not clearly understood. Histochemical and ultrastructural studies show obvious differences so it may be that these cells are two distinct types. The present results lend support to this hypothesis and the following summarizes the evidence.

1. Although mucous and serous cells both follow the predicted pathways for the incorporation of radioactive amino acid and carbohydrate precursors, the absolute uptake per organelle for both $H^3$-threonine and $H^3$-glucose was less for the serous cell than for the mucous. This would suggest less active synthesis in the serous cell.

2. Mucous and serous cells have been shown to comprise their own separate and discrete regions within the secretory tubules (Meyrick et al, 1969).

3. Mitotic activity has been seen (Lamb, 1973) in the mucous and serous cells of the rat bronchial submucosal gland and have also been found in the course of the present study. This would suggest that each cell line undergoes multiplication.

4. Although the ultrastructure of the mucous cell granules was similar for all fixatives, that of the serous cell altered greatly depending on the fixative used. Primary glutaraldehyde fixation gave the majority of serous granules an electron dense appearance, although occasional two-toned granules were seen.

Glutaraldehyde has been shown to be a more efficient fixative for proteins than formalin (Hopwood, 1969), formalin and osmium tetroxide have been found to form poor cross-linkages (Hopwood, 1970). This last

observation suggests that the serous granules are comprised mainly of proteins while the mucous cells have little. Granules in other serous type cells, e.g. zymogen granules have also been shown to contain much protein (Amsterdam and Schramm, 1966).

5. The histochemical studies of Lamb (1969) demonstrated a wider range of glycoproteins in the mucous cell than in the serous cell. In the serous cell, both sialo and sulpho-mucin always occurred together while in the mucous cell at least four different glycoproteins were found (i) sialic acid sensitive to sialidase (ii) sialic acid resistant to sialidase (iii) sulphate staining for sulphate after hydrolysis and (iv) sulphate not staining for sulphate after acid hydrolysis. The four histochemical types did not all occur in the same mucous cell at the same time, but either alone or in certain combinations.

6. Using the Alcian blue (pH 2.6)/periodic acid Schiff technique, the majority of mucous granules have been found to stain with Alcian blue, indicating the presence of an acid glycoprotein, while many of the serous granules stained magenta and only a few blue, indicating that the serous cell secretion is mainly a neutral glycoprotein. A correlation between the electron density and the periodic acid Schiff staining of granules has been suggested (Selzman and Liebelt, 1962; Staley and Trier, 1965 and Moe, 1968). In the present

study, the serous granules mainly stained magenta and were electron dense after glutaraldehyde fixation, while the mucous cell had a low electron density and stained for acid glycoprotein.

7. The mucous and serous granules discharge in different manners. The serous granules are secreted individually, while the mucous granules are secreted in clusters (confluent granules).

8. The proportion of the cell volume occupied by a given organelle was found to be different in the mucous and serous cell. The volume of endoplasmic reticulum is of particular interest. In the case of the serous cell 60.7% of the cell volume is occupied by the endoplasmic reticulum, while of the mucous cell only 29.4%. Jamieson (1972) states that '..... typical of cells that are highly specialized to synthesize large amount of exportable protein, 60% of the volume of the cell is occupied by the elements of rough endoplasmic reticulum'.

The different localization of silver grains in the mucous and serous cell is doubtless a reflection of the localization of certain of the organelles within a cell, especially the endoplasmic reticulum and the secretory granules. The mucous cell shows distinct zoning of these two organelles with the endoplasmic reticulum mainly in the base and the granules in the apex. The

serous cell has endoplasmic throughout the cell and the granules, although fewer than for the mucous cell, are mainly situated in the apex. Therefore, radioactive precursor in the endoplasmic reticulum of the serous cell would show as a widespread scatter of grains and activity could thus be assigned to other organelles as a result of 'cross-fire'.

The high proportion of endoplasmic reticulum suggests that the serous cell secretes a more proteinaceous product than the mucous cell. Alternatively, if the mode of discharge is considered, the secretion of the serous cell might appear to be continuous while that of the mucous cell, cyclic. If this is the case it may be that the rough endoplasmic reticulum of the mucous cell increases in volume during synthesis.

These facts indicate that under 'normal' conditions, mucous and serous cells behave as two distinct cell types. However, the studies of Sturgess (1970) and Jeffery (1973) suggest that after irritation, 'serous' type cells may transform to mucous cells.

## SIMILARITY BETWEEN SEROUS AND ZYMOGEN GRANULES

Obvious ultrastructural similarities are seen between the granules of the serous cell and those of the acinar cell

of the exocrine pancreas. The granules of the mucous cell however, differ in their ultrastructural appearance.

Recently, Lamb and Reid (1970) have shown the presence of $S^{35}$-sulphate in the serous cells of the human bronchial submucosal gland, using light microscopic autoradiography. With the electron microscope, $S^{35}$-sulphate has also been found in the zymogen granules of the exocrine pancreas (Berg and Young, 1971). This fact together with the similar ultrastructural features and synthetic pathways for both these cells would lead one to suspect that they have a similar function. It may be that the serous granules of the bronchial gland, as the zymogen granules of the exocrine pancreas, contain enzymes that on release break down the mucous granules and thus facilitate the removal of mucus from the secretory tubule.

## COMPARISON OF THE TWO METHODS USED FOR QUANTIFICATION OF ELECTRON MICROSCOPIC AUTORADIOGRAPHS

In recent years the need for statistical analysis of electron microscopic autoradiography has become apparent. As yet these methods are still in their infancy. In the present study, two such methods have been applied and compared. For the first time, the hypothetical grain method has been applied to a study of the uptake of a radioactive precursor over a time span. As shown here, these methods gave consistent results. However, the hypothetical grain method

has certain advantages over the circle analysis; calculation of the standard error is possible and it takes into account 'cross-fire' of silver grain from one organelle to another as well as allowing radioactive disintegrations from line sources such as membranes, to be detected. Against these obvious advantages, one must set the major disadvantage, that collection of data for the hypothetical grain method is, at present, more arduous than that for the circle analysis.

It is of interest that in the present study, only one major discrepancy was found; this was for the serous cell after incubation in $H^3$-threonine. After 60 minutes incubation, with the circle analysis, a high relative concentration of silver grains was associated with the granules. With the hypothetical grain method a low concentration was found. The high concentration found with the circle analysis is doubtless due to 'cross-fire' from other organelles, probably the endoplasmic reticulum. Thus, it would seem that for cells with definite regional distribution of organelles such as the mucous cell, both methods give similar results but for cells with a widespread scatter of organelles such as the serous cell, the hypothetical grain analysis is preferable. As these quantification methods stand at present, it is to be hoped that modifications will be made to these rather tedious techniques while maintaining their reliability.

## GENERAL SUMMARY

The incorporation of $H^3$-threonine and $H^3$-glucose into the mucous and serous cells of the human bronchial submucosal gland has, for the first time, been studied by electron microscopic autoradiography. Uptake and discharge has been followed over a period of eight hours. The following summarizes the pattern of incorporation. The detailed results are given at the end of each chapter of results.

After preliminary studies, formalin was found to be the most suitable fixative. Chemical analysis revealed that $H^3$-threonine was incorporated into the protein moiety of the bronchial gland and $H^3$-glucose into the carbohydrate moiety.

The passage of $H^3$-threonine was different to that of $H^3$-glucose. For the mucous cell, $H^3$-threonine localized first in the endoplasmic reticulum, passed to the Golgi apparatus, then the vacuoles and finally to the granules. Evidence of discharge was found at seven hours. On the other hand, $H^3$-glucose localized initially in the Golgi apparatus, passed to the vacuoles and finally to the granules. Again it seemed that discharge occurred at seven hours.

A similar pattern of incorporation for both

radioactive precursors was found for the serous cell, although slight time differences occurred at some stages. An alternative pathway for the incorporation of $H^3$-threonine, where localization is directly into the granules, was a possibility.

Maximum absolute uptake by the organelles of both the mucous and serous cells, after incubation with either radioactive precursor was not reached until four hours, except in the case of the Golgi apparatus of the mucous cell after incubation with $H^3$-glucose when maximum absolute uptake was found at 60 minutes. This, as well as the total radioactive uptake by the whole cell, indicated that utilization of additional radioactive precursor occurred after removal from the 'pulse' label. Less uptake was found in the serous cell than in the mucous cell of either radioactive precursor and for each organelle. A decrease in grain density for the serous cell, indicating discharge, was seen at seven hours after incubation with $H^3$-threonine and at six hours, after incubation with $H^3$-glucose.

For the mucous cell, maximum grain density due to either $H^3$-threonine or $H^3$-glucose was found to pass from the base to the apex of the cell, but for the serous cell, grain density was similar at all times studied, in the base and apex.

The volume within a cell occupied by various organelles was found to vary in the mucous and serous cell.

These findings suggest that the protein core of the glycoprotein is formed in the endoplasmic reticulum of both the mucous and serous cell of the human bronchial submucosal gland. The attachment of the oligosaccharide to the protein core occurs in the Golgi apparatus.

The general pattern of incorporation of $H^3$-threonine and $H^3$-glucose by the mucous and serous cell seems to be similar, although evidence suggests that these cells are different cell types while both producing a glycoprotein with acidic side-chains.

# APPENDIX

# FIXATIVES

All fixatives were diluted with Cacodylate buffer.

## Cacodylate Buffer

0.1M Sodium cacodylate - 100.0 ml

0.1N Hydrochloric acid - 8.3 ml

Diluted to 200 ml with distilled water (pH 7.2)

## Glutaraldehyde

This was obtained from Taab Laboratories as a 25% aqueous solution. A 2.5% solution was usually used.

Glutaraldehyde - 5.0 ml

Diluted to 50 ml with cacodylate buffer + 5 drops of 1% aqueous calcium chloride.

## Formalin

4 gm Paraformaldehyde was placed in

0.1M Sodium cacodylate - 50.00 ml

0.1N Hydrochloric acid - 4.15 ml

1% aqueous calcium chloride - 10 drops

Diluted to 80 ml with distilled water.

This was heated to 70°C with occasional stirring, cooled, filtered and made up to 100 ml with distilled water.

Modified Karnovsky's Fixative

This was made up as for formalin, but after cooling and filtering 10 ml glutaraldehyde was added before the solution was made up to 100 ml.

Osmium Tetroxide

1g of osmium tetroxide was dissolved in 100 ml cacodylate buffer. The osmium tetroxide was obtained from Johnson Matthey Chemicals Ltd in glass ampoules containing 0.5 gm.

## EMBEDDING MEDIUM

Araldite

All specimens for electron microscopy were embedded in Araldite epoxy resin (CIBA).

| | | |
|---|---|---|
| Epoxy resin | (CY212) | - 1 lb |
| Hardener | (HY960) | - 1 lb |
| Accelerator | (DY064) | - 13.5 ml |
| Di-n-butyl phthalate | | - 25.0 ml |

Araldite was made up in bulk and stored in bottles in deep-freeze; the bottles were thawed and used as required.

## CHEMICAL ANALYSIS

### Pronase

10 mg pronase was dissolved in 10 ml 0.1 TRIS buffer, pH 8.0, containing 0.0015M calcium acetate. One drop of toluene was added to stop the growth of moulds.

### Puromycin

10 mg puromycin dihydrochloride + 40 µl N sodium bicarbonate was dissolved in 5 ml of TC 199. This was diluted 1:1 with either more TC 199 or with radioactive precursor, giving a final concentration of approximately 1 mg/ml M.W. puromycin = 544.5.

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