IN VITRO INCORPORATION OF [^{3}H]THREONINE AND [^{3}H]GLUCOSE BY THE MUCOUS AND SEROUS CELLS OF THE HUMAN BRONCHIAL SUBMUCOSAL GLAND

A Quantitative Electron Microscope Study

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ABSTRACT

Incorporation of [^{3}H]threonine and [^{3}H]glucose by the mucous and serous cells of the human bronchial submucosal gland has been studied over 8 h using, for the first time, in vitro pulse labeling and electron microscope autoradiography. In assessing the autoradiographs, two methods were compared, the circle analysis and the recently described hypothetical grain analysis.

Preliminary studies showed formaldehyde to be the most suitable fixative. Chemical analysis of tissue revealed that [^{3}H]threonine was incorporated into the polypeptide moiety of the bronchial gland product and that metabolites of [^{3}H]-glucose were incorporated into the carbohydrate.

Tritiated threonine was first localized in the endoplasmic reticulum of both mucous and serous cells and later migrated to the Golgi apparatus, while metabolites of [^{3}H]glucose localized first mainly in the Golgi apparatus. From here, both radioactive precursors were next identified in vacuoles and, finally, in secretory granules. The mucous cell incorporated strikingly more of both radioactive precursors than the serous cell. Thus, it seems that oligosaccharides of mucous and serous cell glycoproteins are synthesized mainly in the Golgi apparatus and added there to the polypeptide core which is synthesized in the endoplasmic reticulum.

The relationship of the mucous cell to the serous cell is discussed. It seems that under "normal" conditions each cell represents a different line but that injury may transform a serous cell into a mucous cell.

The human bronchial submucosal gland includes two important types of secretory cell, the mucous and serous. These cells have different ultrastructural features (33) and have been shown by serial reconstruction to comprise their own individual and discrete regions of the gland (34). Histochemical studies have shown that the mucous cell contains four different types of acidic glycoprotein (25) that may occur either alone or in certain combinations but are not found together in a single cell. In the serous cell, however, both sialo- and sulfomucin always occur together (26). In chronic bronchitis, the pattern of hypertrophy of mucous acini is rather different from that of serous acini

(11), in that the number of mucous acini per unit area of gland is known to increase while that of the serous acini does not.

This paper deals with the in vitro incorporation of [^{3}H]threonine and [^{3}H]glucose metabolites by both the mucous and serous cells of the human bronchial submucosal gland. The use of this tissue allows both cells to be studied under the same environmental conditions. Pulse labeling and electron microscope autoradiography have been used. Two methods for the quantification of the autoradiographs, the circle analysis (53) and the hypothetical grain analysis (6), were applied and their results compared. Particular attention has been given to the organelles generally associated with the production of the secretory products, endoplasmic reticulum, Golgi apparatus, vacuoles, and secretory granules.

In this study the results show the pathways for the incorporation of the two radioactive precursors to be similar for both the mucous cell and the serous cell, although the timing and degree of labeling in the two cells are different. The relationship is discussed between the mucous cell and the serous cell which are compared with secretory cells at other sites.

Preliminary studies were carried out to establish that the radioactive precursors were being incorporated by the explant into protein and glycoprotein and to decide on the optimal fixative for study of the human bronchial submucosal gland.

MATERIAL AND METHODS

Organ Culture and Radioactive Precursors

Specimens were available from 12 patients undergoing pneumonectomy or lobectomy for bronchiectasis or carcinoma of the lung. A ring of bronchus was taken from the lobe immediately after resection: the most proximal half centimeter was used for routine histology and estimation of gland wall ratio (41); the remainder was prepared for organ culture. Organ culture was carried out using the modification of Trowell's technique (49) described by Sturgess and Reid (47). Two radioactive precursors were used, DL-[G-^{3}H]threonine and D-[6-^{3}H]glucose (Radiochemical Centre, Amersham, England). The dilution of these precursors in TC 199 depended on the experimental procedure.

Preliminary Studies

Before the high resolution autoradiographic study, preliminary studies were carried out (*a*) to establish that the radioactive precursors were being incorporated into the acid glycoprotein molecule, and (*b*) to decide the optimal fixative for the human bronchus.

Incorporation of Radioactive Precursors

Explants (10–12 mg) were prepared and incubated with 250 μCi/ml of either [^{3}H]threonine or [^{3}H]glucose. For half the explants, 1 mg/ml puromycin dihydrochloride was added to the incubation medium. After 60 min the tissue was removed and washed three times in either 1% glucose or threonine, then sonicated and digested with Pronase in 0.1 M Tris buffer at 37°C for 18 h (32) and centrifuged. Trichloroacetic acid (TCA) was added to the supernatant to give a final concentration of 5% (35, 44) and the supernatant was centrifuged. The supernatant was neutralized with sodium hydroxide, ethanol was added to give a final concentration of 75% (3, 12), and the mixture was left at 4°C for 2 h and centrifuged. Both the Pronase and TCA precipitates were given two further washes with either distilled water or TCA, respectively.

Precipitates, dissolved in 1 ml of 0.5 N sodium hydroxide, and 1 ml aliquots of the supernates were dialyzed against water for 4 days at 4°C. After dialysis, 0.25-ml aliquots were taken and diluted with Insta-gel (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. Thus, the percentage of total radioactivity retained in all the precipitates and supernates could be estimated.

Trichloroacetic acid precipitation showed that 24% of [^{3}H]threonine was incorporated into proteins (Table I) and that puromycin prevented 75% of this incorporation. Ethanolic precipitation of glycoproteins showed that 58% of [^{3}H]glucose metabolites were in this precipitate: puromycin reduced this incorporation by approximately 80%.

Selection of Optimal Fixative

The amount of radioactive precursor held in the tissue after fixation and incubation at 4°C (52) was established using scintillation counting and light microscope examination of sections.

Scintillation Counting

Explants were incubated with 250 μCi/ml of either [^{3}H]threonine or [^{3}H]glucose for 60 min. For some explants 1 mg/ml of puromycin dihydrochloride was also added to the medium, while others were treated with a "cold" rinse immediately after incubation and before fixation. Explants were fixed for 2 h in one of the following: 2% glutaraldehyde, 10% formaldehyde, or Karnovsky fixative (24), all in cacodylate buffer (pH 7.2). After fixation and postfixation in osmium tetroxide, the explants were processed for electron microscopy through graded alcohols and epoxy propane. All fixatives and processing fluids were saved and aliquots of these were diluted with Insta-gel for scintillation counting. The dehydrated tissue was combusted in a Packard Tritium

Oxidizer and diluted with Insta-gel. Thus, the percentage of the total radioactivity retained could be estimated for each fluid and each specimen. At least two explants were treated with each fixative.

Because of the widely different amounts of gland included in each specimen, the amount of uptake showed a wide variance even when assessed by one technique. These results show that Formalin binds less [^{3}H]threonine to the bronchial gland than either glutaraldehyde or Karnovsky's fixative (Table II). It seems from Table III that puromycin before Formalin fixation reduces glucose incorporation, but this probably only reflects a smaller amount of gland present in the explant. A cold rinse did not aid the removal of radioactive precursors from the tissue except in the case of [^{3}H]threonine before fixation with glutaraldehyde (Table III).

Incubation at 4°C

After equilibration, explants were incubated with 250 μCi/ml of either [^{3}H]threonine or [^{3}H]glucose at 4°C for 10 min. The fixatives used in this experiment included glutaraldehyde for 90 min, Karnovsky's fixative for 90 min, osmium tetroxide for 60 min, formaldehyde fixation for 4 and 18 h, and formaldehyde for 60 min followed by secondary fixation in glutaraldehyde for 90 min all in cacodylate buffer (pH 7.2). This last technique was used in an attempt to improve morphological quality. Two explants were used for each fixative. The fixed explants were processed for light microscopy, embedded in wax, and sections were cut at 4 μm and stained with the periodic acid-Schiff technique (25). Using Kodak AR 10 (Eastman Kodak Co., Rochester, N.Y.), stripping film autographs were prepared.

CONTROLS: Control explants were incubated in either [^{3}H]threonine or [^{3}H]glucose at 37°C for 10 min before fixation in either glutaraldehyde or Formalin, while the others were incubated at 4°C without the addition of radioactive precursor.

QUANTIFICATION: The nonspecific binding of the radioactive precursors to the tissue was assessed by counting the number of silver grains over mucous and

TABLE I

[^{3}H]Threonine and [^{3}H]Glucose in the Precipitate and Supernatant after Dialysis, with and without Added Puromycin (% of Total Radioactivity)

Puromycin	[^{3}H]threonine		[^{3}H]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) TCA wash, supernatant 2	3	11	2	5
(D) TCA wash, supernatant 3	2	1	—	1
(E) TCA wash, supernanant 1 then precipitated with ethanol	4	14	58	12
(F) Alcohol wash	27	14	24	44

TABLE II

Radioactivity Retained by Tissue andFluids Used during Processing for Electron Microscopy after Incubation with Either [^{3}H]Threonine and [^{3}H]Glucose (Expressed as a Percentage of Total Recovered)

	[^{3}H]Threonine			[^{3}H]Glucose		
	Formalin	Glutaraldehyde	Karnovsky	Formalin	Glutaraldehyde	Karnovsky
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 (×3)		0.2	0.1	0.2	0.1	0.3
Epoxy propane		0.1				0.2

TABLE III

[³H]Threonine and [³H]Glucose Recovered from the Tissue and Fixative with (+) and without (−) Added Puromycin and with (+) or without (−) a Cold Rinse Before Fixation

	[³H]Threonine				[³H]Glucose			
	Formalin		Glutaraldehyde		Formalin		Glutaraldehyde	
Fixative used	−	+	−	+	−	+	−	+
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
Cold rinse								
Tissue	8.1	12.0	35.9	22.2	31.4	30.1	33.2	37.4
Cold rinse		66.6 } 85.0		36.5 } 75.8		38.4 } 66.0		32.5 } 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

The greatest loss during the rest of the tissue processing procedure was 3.1% but most samples showed a smaller loss than this, approximately 1.5%.

serous acini using a squared graticule unit (0.1-mm squares) and an oil immersion lens. For each fixative 10 fields from each of the two slides were assessed. Background counts were made in areas containing no gland. Formalin-fixed tissue was taken as a reference, and each of the other fixatives was compared with this, using Student's *t* test. At 4°C, over both mucous and serous cells, the three fixation techniques that included Formalin showed less activity for [³H]threonine than the others. In the case of [³H]glucose, only glutaraldehyde bound more to both the mucous and serous cell than Formalin.

Morphological Preservation

The ultrastructural appearance after each fixative was compared with that obtained after glutaraldehyde. The main difference was found in the serous secretory granule. The granules appeared as discrete, electron-dense structures (Fig. 1) after glutaraldehyde or Karnovsky's fixative, and as electron-lucent granules after formaldehyde fixation for 4 h or osmium tetroxide, although both formaldehyde fixation for 18 h and formaldehyde fixation for 60 min followed by fixation with glutaraldehyde showed the granules to be a mixture of lucent and dense (Fig. 2). The mucous cells showed a similar appearance after all fixatives with electron-lucent, usually confluent granules.

Thus, after incubation of tissue with radioactive precursors, both scintillation counting of tissue and examination of 4-μm sections after incubation at 4°C showed that formaldehyde caused the least nonspecific binding. On the basis of these findings and the morphological preservation, it was decided to fix in formaldehyde for 18 h.

High Resolution Autoradiography: Autoradiography of 1-μm Sections

Explants were incubated for periods of 20 min–8 h in either 50 μCi/ml [³H]threonine or 25 μCi/ml [³H]-glucose. Incubations of 60 min and less were "pulse" labeled and their results are recorded in minutes. Where explants were incubated for 2–8 h, this included a 60-min pulse label and a "chase" incubation in TC 199 alone for the remaining time: these are described in hours. After incubation, the explants were fixed in 10% formaldehyde and processed for electron microscopy (32).

Sections, 1 μm thick, were taken from the Araldite-embedded blocks and autoradiographs prepared using the dipping technique with Ilford L4 emulsion diluted 1:1 with distilled water at 50°C. After 10 days of exposure at room temperature, the autoradiographs were developed in Microdol-X, fixed in Kodafix, stained with toluidine blue, and examined with an oil immersion lens.

Quantification

The regional distribution of silver grains over each of 10 serous and 10 mucous acini in each of two slides was recorded. For mucous acini, four regions were assessed, the base, midregion, apex, and lumen. For serous acini, three regions were assessed within each acinus, the base, midregion, and apex (the lumen of serous acini cannot be satisfactorily identified by the light microscope). The degree of labeling was assessed by using a method similar to that described by Sturgess (46). Heavy labeling noted by a (+) indicated that there were 40–70 grains/100 μm^2, (±) indicated 10–30 grains and (−) less than 10 grains/100 μm^2. The background was assessed in a similar manner over regions of section that contained no

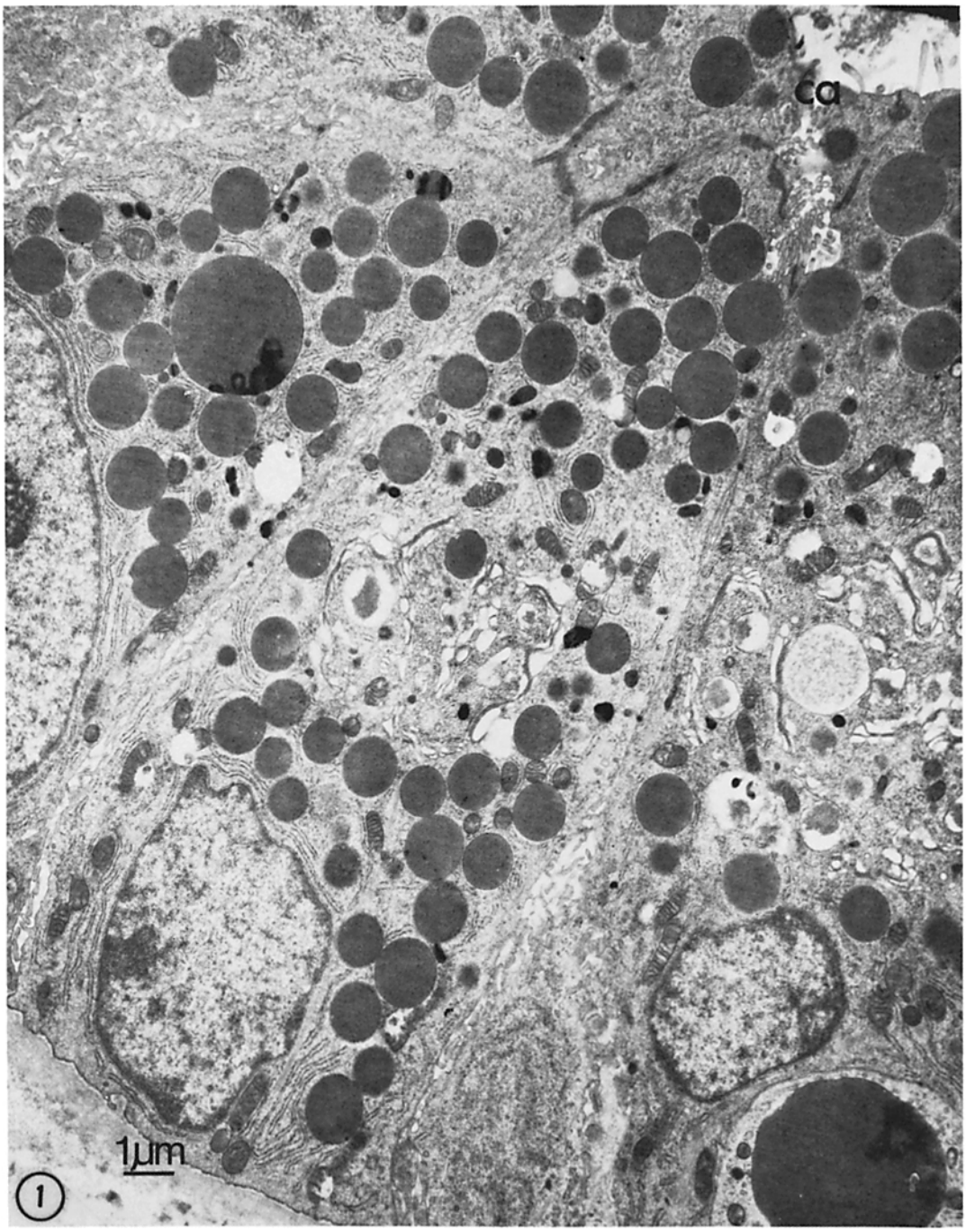

FIGURE 1 Electron micrograph of part of a serous acinus around a canaliculus (*ca*). After primary fixation in glutaraldehyde, the serous granules characteristically are seen as electron-dense, discrete structures. × 7,500.

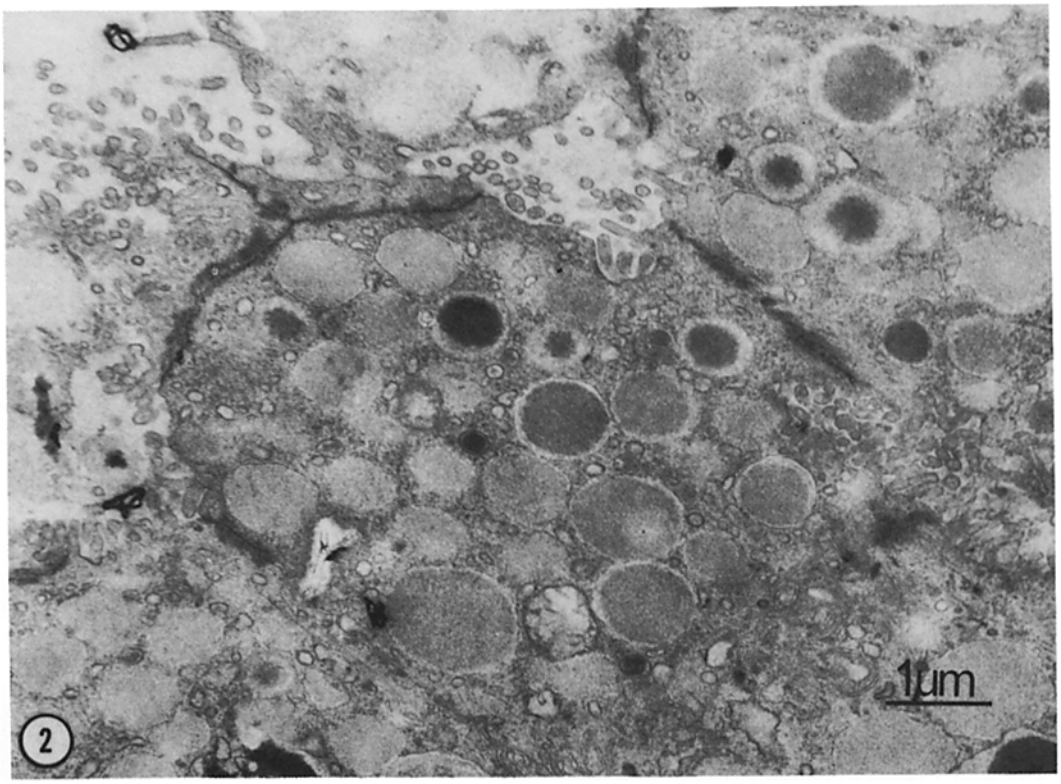

FIGURE 2 Electron micrograph of part of a serous acinus after primary fixation in formaldehyde for 18 h. The granules are discrete with varying electron density. × 14,000.

gland, and the number was always less than 10 grains/1,000 μm^2. The results are expressed as a mean percentage of 20 acini. The acinus was considered to be a unit since most of the cells in one acinus show a similar pattern of labeling.

Electron Microscopic Autoradiographs

Explants were incubated with either 250 μCi/ml [^{3}H]glucose or 500 μCi/ml [^{3}H]threonine and then fixed and processed as described for the 1-μm sections. Serial, gold-colored sections (~1000 Å) were cut on an LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.) with glass knives. The sections were mounted on a glass slide previously coated with a layer of Formvar and a thin layer of carbon (~50 Å). A monolayer of Ilford L4 emulsion was applied to the slide using a dipping apparatus and exposed in a light-tight box at room temperature. The exposure time for [^{3}H]glucose was 4 wk, and that for [^{3}H]threonine, 10 wk. The autoradiographs were developed in Microdol-X for 3 min and fixed in Kodafix. Sections were floated from the slide using hydrofluoric acid (39), mounted on copper grids, stained with 1% uranyl acetate in methanol and lead citrate (42), and examined in an AEI EM6B (AEI Scientific Apparatus, Inc., Elmsford, N.Y.). Micrographs were printed to a magnification of × 25,000.

CONTROLS: No positive or negative chemography was encountered. Background counts were assessed over the Formvar film in areas surrounding the section, and were found to be less than 1 silver grain per 1,000 μm^2 or less than 0.001 silver grains per unit area, i.e. 1 μm^2.

Quantification of Autoradiographs

Two methods of analysis were applied to the electron microscope autoradiographs, the circle analysis (53) and the recently described hypothetical grain analysis which allows for "cross fire" of disintegrations between neighboring organelles (6), thus enabling a more accurate assessment of the source of the radioactive disintegration. For these methods, pictures were taken of the first region of serous and mucous cells encountered in the section. At least six micrographs were analyzed to include two from the cell base, two from midregion, and two from the apex (32). More micrographs were analyzed if 100 silver grains were not included in the six pictures.

Circle Analysis

The circle, point, and grain analysis were carried out as described by Williams (53). The half radius (HR) for

the study was 2,720 Å, and this was equivalent to circles measuring approximately 14.00 mm in diameter (43).

For the circle and grain analysis the organelles were grouped as shown in Table IV although some items were later combined since few circles fell over them. It should be noted that "endoplasmic reticulum" included ribosomes and cellular ground substance; the junctional region ER/Golgi apparatus contained the smooth surfaced vesicles that bud from the endoplasmic reticulum; the "vacuoles" corresponded to "condensing vacuoles" (as described by Jamieson and Palade, 21); and canaliculi were assessed only for the serous cell.

The analysis allows calculation of the specific activity per organelle. This is the same as the relative specific activity described by Williams (53) and is 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 absolute uptake per organelle was also calculated for each section by dividing the total number of silver grains over a given type of organelle by the total number of circles falling over that type of organelle. By comparing explants incubated for different periods, the time of maximum absolute uptake for each organelle could be assessed. Two specimens were studied for each time and the mean value was obtained.

Absolute Uptake by Whole Cell Including Comparison between Base and Apex

The time of maximum absolute uptake for the whole of the mucous or serous cell was assessed by dividing the total number of silver grains by the total number of circles for each cell at each time. To compare the cell base with the apex, a card with a hole 12.5 × 8.5 cm was superimposed over each of six micrographs of the cell base and six of the apex, for both mucous and serous cells, at each incubation time. A shift of silver grains from cell base to apex could thus be detected.

Relative Area of Organelles in Each Cell

Using sequential analysis of the data obtained in the point analysis, it was possible to calculate the relative areas of various organelles within each of the cell types.

Hypothetical Grain Analysis

For the hypothetical grain analysis and actual grain analysis, the method of Blackett and Parry (6) was followed. Only four regions of the cell were studied: (*a*) the endoplasmic reticulum, with which were included all regions save (*b*) Golgi apparatus, including vacuoles and vesicles, (*c*) granules, and (*d*) nucleus. Three times, 60 min, 4 h, and 8 h, were analyzed. A computer program devised by these workers was used to process the data. from which the χ^2 test for significance was calculated, and an assessment of activity per unit area for each organelle and the grains per grid point falling over organelles were derived: in each case, standard errors were determined.

TABLE IV

Grouping of Organelles for Circle Analysis

Orgenelle	Combined organelles
Endoplasmic reticulum (ER)	
ER/Golgi apparatus	ER/Golgi apparatus
Golgi apparatus	Golgi apparatus
Vacuole, Granule	Granule
ER/vacuole, ER/granule	ER/granule
Nucleus	Nucleus
Canaliculus	Canaliculus
ER/mitochondrion, ER/membrane, ER/canaliculus, ER/other, ER/lysosome	ER/other

RESULTS

6 of the 12 specimens were obtained from patients with chronic bronchitis as judged by a raised gland-wall ratio, i.e. >0.38 (41), while the other six had gland-wall ratios within normal limits.

High Resolution Autoradiography: Autoradiography of 1-μm Sections

The results of the 1-μm sections are described below by reference to acini and to the pattern of incorporation, but only by reference to heavy labeling, i.e. where there is a concentration of 40–70 silver grains/100 μm^2 of acinar tssue and where 50% or more of acini in the regions base, midregion, apex, and lumen are labeled to this concentration. An example of these results is shown in Table V.

[^3H]threonine: Mucous Acini

The mucous acini showed little labeling before 40 min, when 90% of acini were labeled at the base

TABLE V

Density of Silver Grains in Regions of Mucous Acinus after Incubation with [^3H]Threonine for Various Times—Expressed as a Percentage of the Acinar Region Showing the Given Grain Density

	Acinar region											
	Basal			Mid			Apical			Lumen		
	Grain density											
Time	+*	±‡	−§	+*	±‡	−§	+*	±‡	−§	+*	±‡	−§
20 min		80	20		20	80			100			100
40 min	90	10		30	70			20	80			100
60 min	70	10	20	100			50	40	10		30	70
2 h	90	10		100			80	20			40	60
3 h	20	70	10	100			60	40			60	40
4 h		100		100			100				30	70
5 h		100		100			80	20			50	50
6 h		100		100			70	30		10	50	40
7 h		100		70	30		100			60	40	
8 h		100		50	50		100			90	10	

* + = 40–70 silver grains/100 μm^2.

‡ ± = 10–30.

§ − = 10.

Underline denotes more than 50% of the acinar region heavily labeled.

(Table V). From 60 min, heavy labeling of acini was found in the midregion and apex. Heavy labeling of the lumen was found by 7 h.

Serous Acini

From 60 min to 6 h, the acini showed heavy apical labeling. At 2, 3, 7, and 8 h, heavy labeling was seen also at the base, and from 2 h, in the midregion.

[^3H]glucose: Mucous Acini

From 60 min, heavy labeling occurred in the midregion, from 5 h in the apical region, and by 7 h in the lumen. Only at 2 h was heavy labeling seen at the base of mucous acini.

Serous Acini

Similarly in the serous acini, little labeling occurred before 60 min, when it was virtually confined to the midregion. In the base, from 2 h, heavy labeling was found, and in the apex, from 3 h.

Thus, with the light microscope, different labeling patterns are apparent for [^{3}H]threonine and [^{3}H]glucose. For [^{3}H]threonine, localization in the mucous acini was first encountered at 40 min in the basal region, while for the serous acini the initial localization at 60 min was at the apex. Metabolites of [^{3}H]glucose, on the other hand, for both mucous and serous acini, localized initially at 60 min in the midregion.

Electron Microscope Autoradiography: Circle Analysis

The detailed results for absolute uptake and specific activity of organelles appear in Meyrick's thesis (32). In this paper, only the smooth curves for the organelles generally associated with glycoprotein synthesis are given.[1] Comparison of the actual grain distribution over organelles with a random distribution (circle analysis) using the χ^2 test to assess significance established whether or not the distribution was random.

[^3H]threonine

For the *mucous* cell, at all times the results of the χ^2 test were all highly significant ($P = <0.001$). For the *serous* cell, the results were statistically significant for most explants (Table VI). The five explants in which the results were

[1] After consultation with Professor B. Benhamin, Professor of Actuarial Science, City University, London, it was not considered necessary to fit mathematical curves to data, but, instead, smooth curves were drawn freehand through the data to interpret the trend.

TABLE VI

Probability Values for the Nonrandom Distribution of Silver Grains within the Serous Cell after Incubation in [³H]Threonine

Incubation time	1 (P = <)	2 (P = <)
20 min	0.2	0.001
40 min	0.9	0.1
60 min	0.05	0.05
2 h	0.02	0.1
3 h	0.1	0.1
4 h	0.5	0.01
5 h	0.01	0.05
6 h	0.05	0.05
7 h	0.3	0.2
8 h	0.001	0.001

<0.05 = statistically significant = nonrandom distribution. <0.2 = trend to nonrandom distribution. >0.2 = not significant = random distribution.

not significant showed uptake but demonstrated no particular concentration in any organelle. The random distribution indicated by these five results does not suggest a specific pattern of departure from the general trend.

ABSOLUTE UPTAKE: Although all explants were removed from the [³H]threonine by 60 min, for the *mucous* cell, maximum absolute uptake was not found for the endoplasmic reticulum until 4 h, for the Golgi apparatus until between 4 and 5 h, and for the vacuoles and granules until 7 h (Fig. 3). For the *serous* cell, maximum absolute uptake was also achieved for the endoplasmic reticulum and Golgi apparatus at 4 h, and rather earlier, at 5 h, for the vacuoles and granules (Fig. 4).

Specific Activity per Organelle:

Mucous cell

Maximal specific activity was achieved at 60 min over the endoplasmic reticulum and junctional region ER/Golgi apparatus (Fig. 5) and at 4 h over the Golgi apparatus (Fig. 6). The vacuoles showed activity at 60 min and 2 h, and the granules alone showed maximal specific activity at 7 and 8 h (Fig. 7). Because of the small volume occupied by vacuoles, these are taken together with the granules for statistical analysis. For the nucleus, absolute maximum uptake and maximum specific activity were each at 60 min.

Serous Cell

The analysis rather suggests two pathways for the incorporation: (*a*) [³H]threonine is initially incorporated into the endoplasmic reticulum as shown for the mucous cell or (*b*) at an early stage it is incorporated into the granules directly; the results of the 1-μm sections also suggest this. At 40 min, maximum specific activity was found over the endoplasmic reticulum and junctional region ER/Golgi apparatus (Figs. 8 and 9 *a*). Between 60 min and 2 h, the maximum occurred over the Golgi apparatus (Fig. 9 *b*). An initial peak was found at 60 min for the vacuoles and granules (Fig. 8), and a second peak of activity for the granules alone at 8 h. The initial peak of activity may be apparent rather than real, representing cross fire from the closely situated endoplasmic reticulum (vide infra).

Also of interest are the canaliculi, situated external to the serous cell, that include the lumen of the serous acinus and invaginating processes both into and between the serous cells; absolute uptake and specific activity over these rose steadily to 8 h. Maximum absolute uptake for the nucleus was detected between 4 and 8 h, and the specific activity was maximal by 40 min.

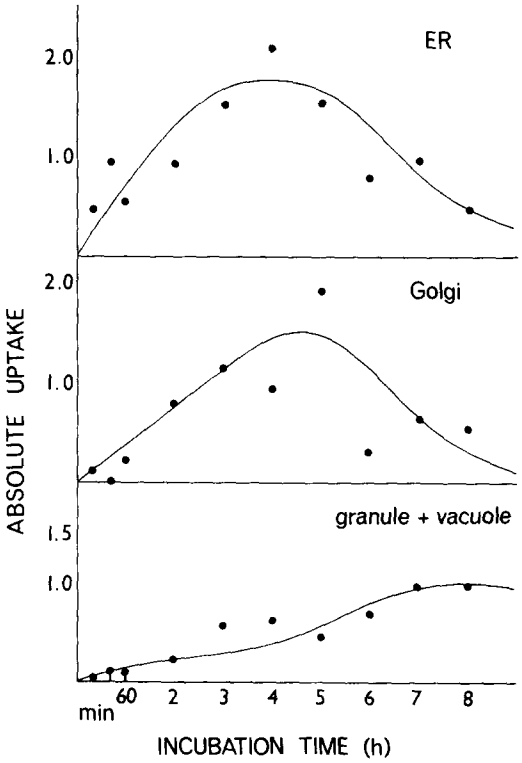

FIGURE 3 Smooth curves representing the trend of points over 8 h for absolute uptake of [³H]threonine by organelles of the mucous cell (a point represents the mean for the given time).

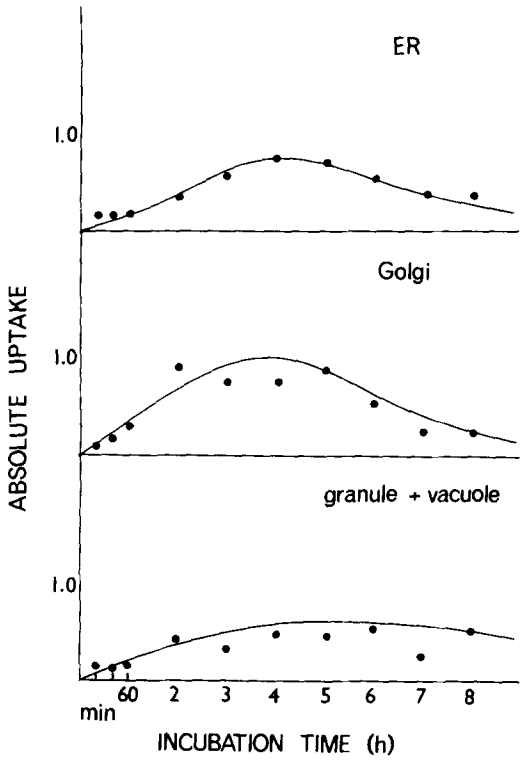

FIGURE 4 Smooth curves representing the trend of points over 8 h for absolute uptake of [^{3}H]threonine by organelles of the serous cell (a point represents the mean for the given time).

[^3H]*glucose*

For the *mucous* and *serous* cell at all times the results of the χ^2 test were all highly significant ($P = <0.001$).

ABSOLUTE UPTAKE: For the *mucous* cell, absolute uptake by the Golgi apparatus was maximal at 60 min. Uptake was still increasing at 8 h in the vacuoles, granules, and endoplasmic reticulum (Fig. 10). For the *serous* cell, maximum uptake was similarly found in the Golgi apparatus at 60 min, while for all other organelles earlier. For the endoplasmic reticulum, maximum uptake was found between 3 and 4 h. and for the vacuoles and granules at 6 h (Fig. 11).

Specific Activity per Organelle: Mucous Cell

In the mucous cell, maximum specific activity was encountered at 60 min in the Golgi apparatus (Figs. 12, 13) and the vacuoles. Activity was still increasing in the granules at 8 h (Fig. 12). A small peak of activity was found between 60 min and 2 h in the endoplasmic reticulum. Little activity was encountered in the junctional region ER/Golgi apparatus at any time. Little absolute uptake or specific activity for the nucleus was found at any time.

Serous Cell

At 60 min the maximum specific activity was found over the Golgi apparatus, endoplasmic reticulum, and junctional region ER/Golgi apparatus (Figs. 14 and 15 *a*). For the vacuoles an initial peak was seen between 60 min and 2 h, and for the

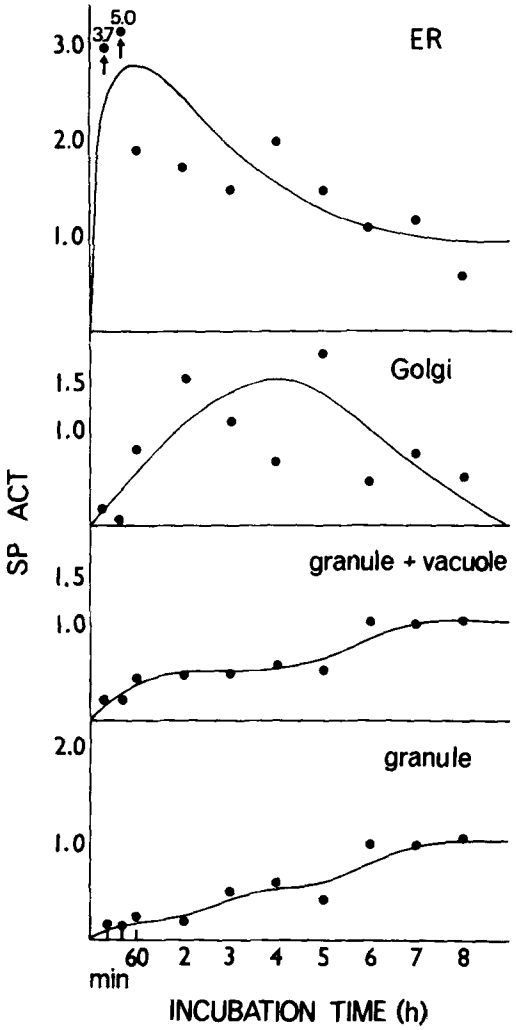

FIGURE 5 Smooth curves representing the trend of points over 8 h for the sp act of organelles of the mucous cell after incubation with [^{3}H]threonine (a point represents the mean for the given time).

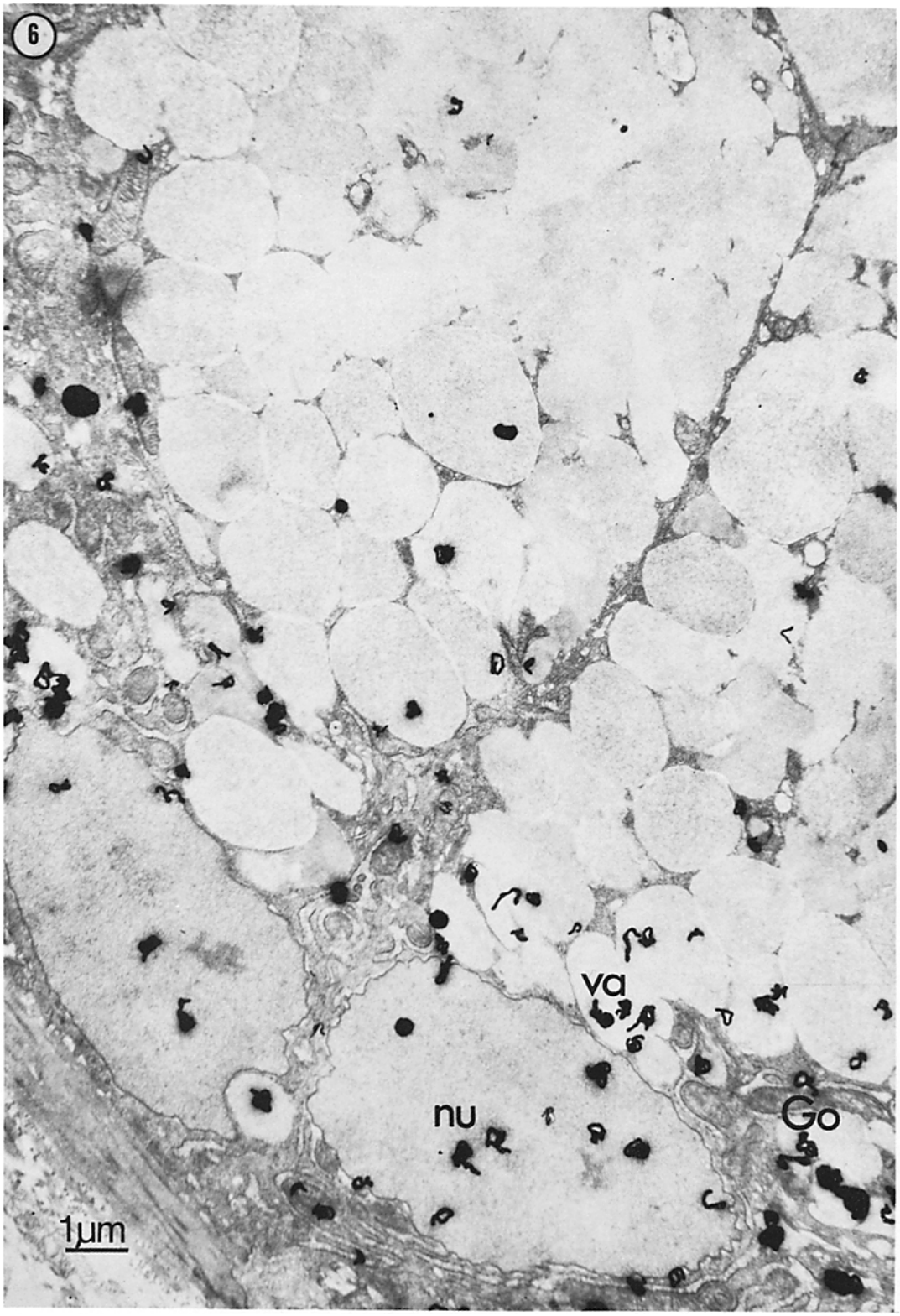

FIGURE 6 Electron micrograph of part of a mucous acinus after incubation with [^{3}H]threonine for 4 h. Silver grains are localized over the base of the cell only, over the Golgi apparatus (*Go*), vacuoles (*va*), and nucleus (*nu*). × 10,500.

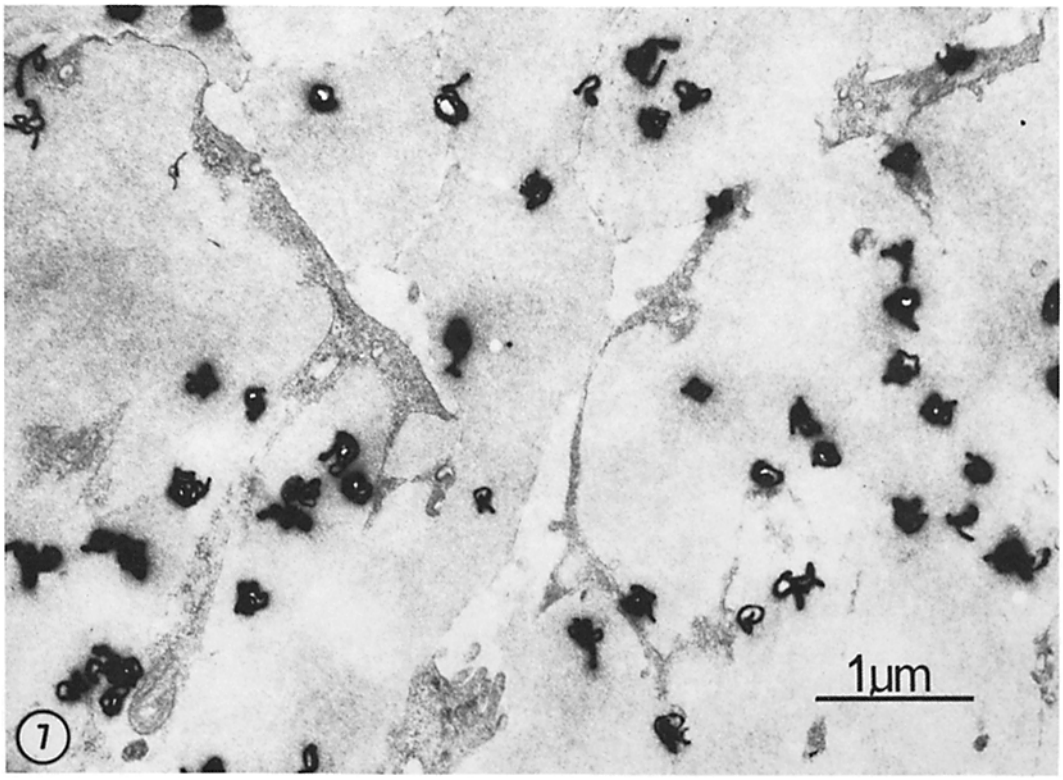

FIGURE 7 Electron micrograph of part of the apical region of a mucous acinus after incubation with [^{3}H]threonine for 8 h. Silver grains are numerous over the cell apex which is seen to be discharging. × 19,000.

granules alone at 6 h (Fig. 15 *b*). The specific activity and absolute uptake for the canaliculi were still increasing at 8 h. Little incorporation of [^{3}H]glucose was found for the nucleus.

Absolute Uptake by Mucous and Serous Cells

[^{3}H]THREONINE: Maximum absolute uptake by the *mucous* cell and *serous* cell was achieved at 3 h. By 6 h a decrease in grain density had occurred, indicating discharge from the cell (Table VII). For the *mucous* cell, during the first 5 h the grain density was heaviest at the cell base, but from 6 to 8 h it was similar at the base and apex. Grain density at the base and apex of the *serous* cell was similar at all times and only at 7 h was it decreasing.

[^{3}H]GLUCOSE: Maximum absolute uptake for the *mucous* cell was found at 3 h and remained at this level. For the first 6 h, grain density was greatest at the cell base; at 7 and 8 h, at the apex (Fig. 16).

Maximum uptake for the *serous* cell was seen at 5 h, and from this time it decreased. At all times, as for [^{3}H]threonine, grain density at the base and apex was similar throughout the serous cell (Fig. 17).

Volume of a Given Organelle within the Mucous and Serous Cell

Sequential analysis revealed that in the *mucous* cell the secretory granules occupy the major part of the cell, with the endoplasmic reticulum in second place. 75% of the mucous cell apex is occupied by the granules (Table VIII). In the *serous* cell the endoplasmic reticulum occupies the major part of the cell, with the granules in second place (Table VIII), and even in the apex of the cell the endoplasmic reticulum predominates.

The circle analysis has shown that for both mucous and serous cells the pattern of incorporation of [^{3}H]threonine and [^{3}H]glucose metabolites is different. For the mucous cell, as for the serous cell, [^{3}H]threonine is initially localized in the

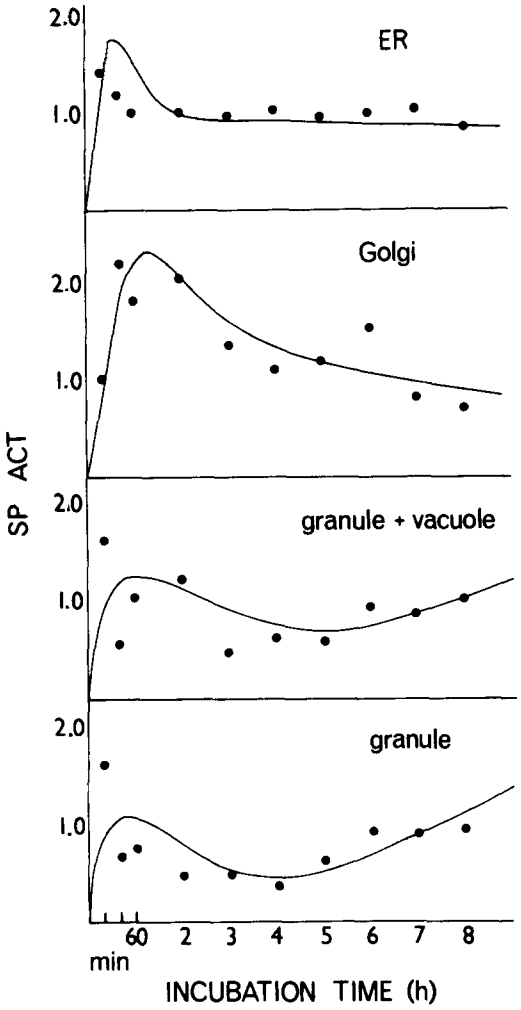

FIGURE 8 Smooth curves representing the trend of points over 8 h for the sp act or organelles of the serous cell after incubation with [^{3}H]threonine (a point represents the mean for the given time).

endoplasmic reticulum; the radioactive precursor then migrates to the Golgi apparatus, vacuoles, and granules. The results for the serous cell also suggest the possibility of an alternative pathway whereby incorporation occurs directly into the granules.

In contrast, metabolites of [^{3}H]glucose are found to localize initially in the Golgi apparatus; from here, the metabolites travel to the vacuoles and finally are seen in the mature secretory granules. Evidence of discharge of newly formed mucous granules is found at 7 h, and for the serous cell at 6 h. At all times, absolute uptake of [^{3}H]-threonine and [^{3}H]glucose by the mucous cell was greater than for the serous cell.

Hypothetical Grain Analysis

Using this method, three specimens were analyzed for both mucous and serous cell uptakes after incubation with either [^{3}H]threonine or [^{3}H]glucose at 60 min, 4 and 8 h. The grains per grid point (absolute uptake) and activity per unit area (specific activity) were assessed (39). In general, the results showed patterns of incorporation that were similar to that described using the circle analysis.

The important difference between the two methods of analysis (the figures for these results only are shown) is shown by the serous cell after 60 min of incubation with [^{3}H]threonine, when, with the hypothetical grain analysis, the granules show a small activity both per unit area (Fig. 18) and as grains per grid point (Fig. 19). This suggests that the peak for the granules found at this time, with the circle analysis, and also with the 1-μm sections, could have arisen from cross fire between organelles.

Chi-Squared Test

The results for the χ^2 test show (Table IX) better levels of confidence for the serous cell after incubation with either [^{3}H]threonine or [^{3}H]glucose using the hypothetical grain analysis, whereas for the mucous cell higher levels of confidence are gained using the circle analysis.

Incorporation of Radioactive Precursors by Other Cells of the Human Bronchial Submucosal Gland

Several other cell types are encountered in the human bronchial gland, but only in small numbers (33). Over the small, clear cell, few if any silver grains were found to localize after incubation with either [^{3}H]threonine of [^{3}H]glucose. The myoepithelial cells, situated at the base of the acinar cells, on the other hand, did incorporate both radioactive precursors but to a lesser concentration than the acinar cells, but, because of the small relative area occupied by these cells, quantitative analysis of the silver grains was not attempted.

DISCUSSION

Incorporation of [^{3}H]threonine and [^{3}H]glucose by the mucous and serous cells of the human bron-

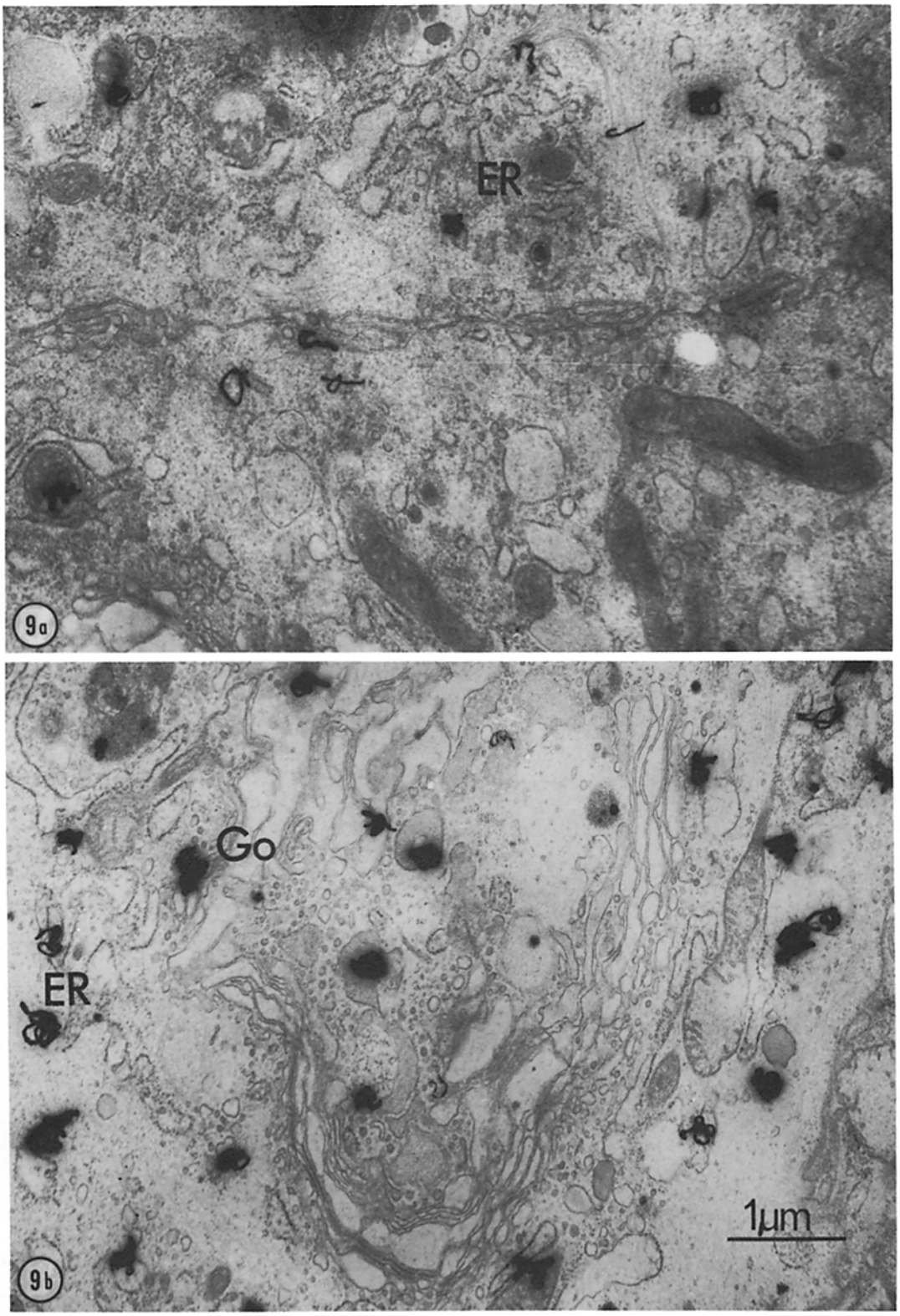

FIGURE 9 *a* and *b* Electron micrographs of part of a serous cell after incubation with [^{3}H]threonine (*a*) for 40 min: silver grains are localized over the endoplasmic reticulum. × 18,750. (*b*) for 2 h: silver grains are now localized over both the endoplasmic reticulum (ER) and the Golgi apparatus (*Go*) × 18,750.

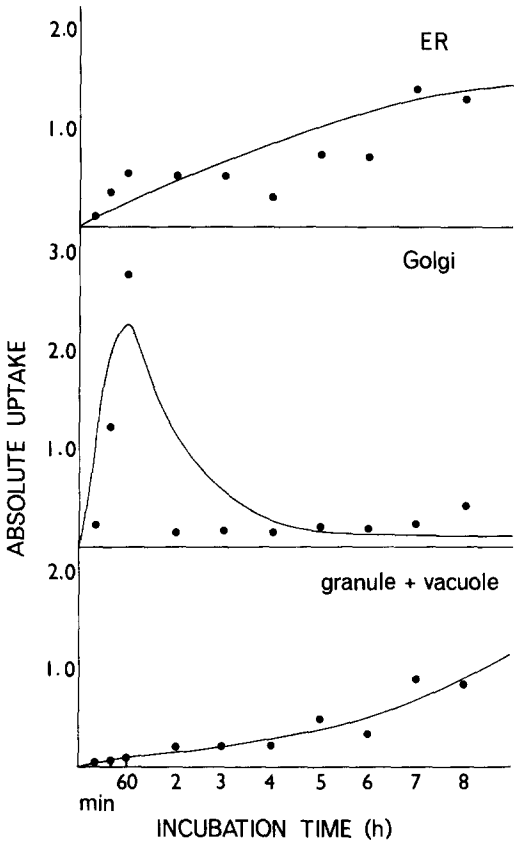

FIGURE 10 Smooth curves representing the trend of points over 8 h for absolute uptake of [^{3}H]glucose by organelles of the mucous cell (a point represents the mean for a given time).

chial submucosal gland has, for the first time, been studied using an organ culture technique, pulse labeling, and electron microscope autoradiography. The incorporation has been followed for periods up to 8 h. It seems that for each cell, [^{3}H]threonine is initially incorporated into the endoplasmic reticulum and that metabolites of [^{3}H]glucose are added to the protein core mainly in the Golgi apparatus. The concentration of both radioactive precursors was found to be higher for the mucous cell than the serous.

Although similarities are seen between the mucous cell and serous cell there are obvious differences which make it difficult to regard them as a single cell type. Since they are mixed throughout the bulk of the gland, its inhomogeneity makes fractionation techniques inapplicable. Precipitation studies, particularly when combined, as here, with electron microscope autoradiography and quantitative analysis, offer the best means of tracing the incorporation of radioactive precursors. Only a gland such as the pancreas, which contains large clumps of one type of secretory cell, allows the use of cell fractionation techniques (20, 21).

Mucous and Serous Cell Metabolism

Both mucous and serous cells have been shown to produce an acid glycoprotein (25, 26), and in the tissue analysis, a preliminary analysis in this study, both glucose and threonine have been found in the macromolecules. After 60 min of incubation, [^{3}H]threonine was found in the protein (TCA) fraction of the bronchial gland. This doubtless represents an early stage of glycoprotein since it has been shown, in sheep colonic mucosa, that threonine is incorporated exclusively into the peptide chain of the glycoprotein molecule (1) and represents 20% of all amino acids present in mucus

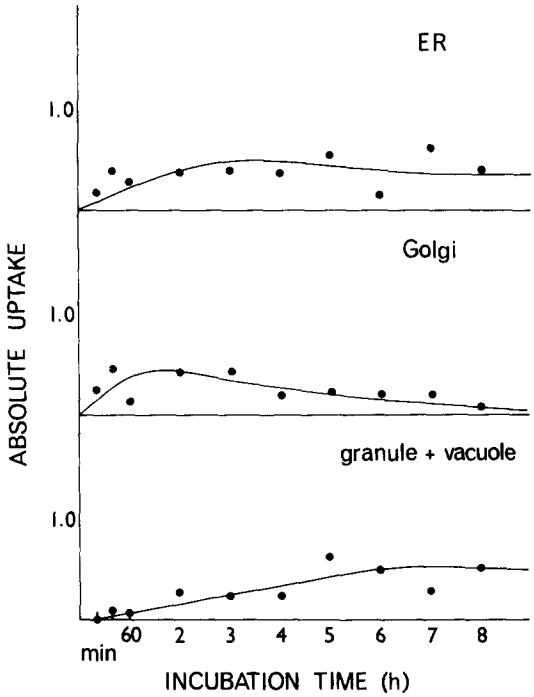

FIGURE 11 Smooth curves representing the trend of points over 8 h for absolute uptake of [^{3}H]glucose by organelles of the serous cell (a point represents the mean for the given time).

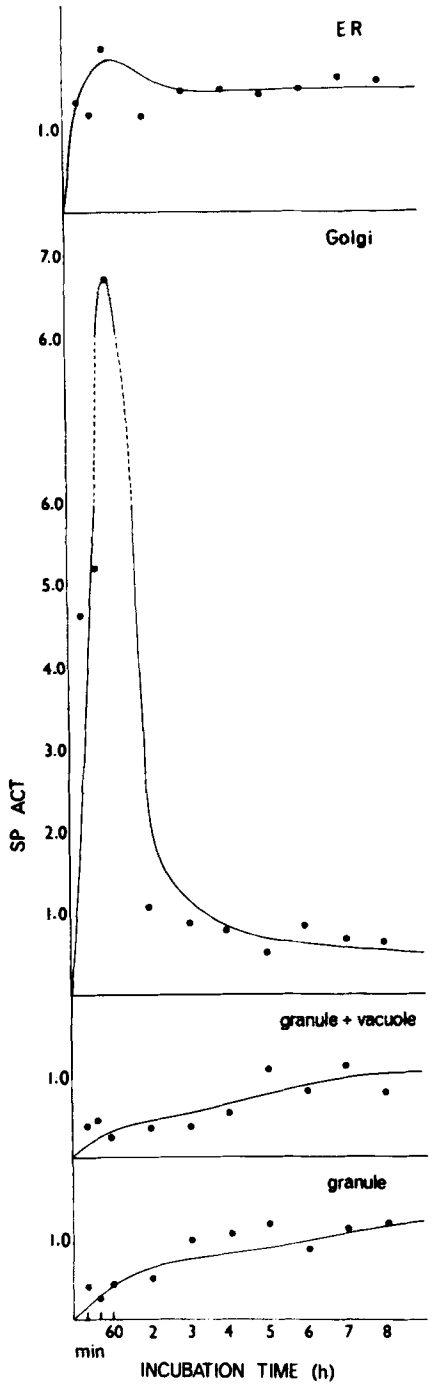

FIGURE 12 Smooth curves representing the trend of points over 8 h for the sp act of organelles of the mucous cell after incubation with [^{3}H]glucose (a point represents the mean for the given time).

(17). Glucose, on the other hand, is at this time shown by ethanolic precipitation to be in the glycoprotein.

Inhibition of glucose incorporation by puromycin is caused presumably by interference with formation of the polypeptide chain of the glycoprotein. Similar results were found for the calf thyroid (45) where almost complete inhibition of peptide synthesis was obtained with puromycin, but after further incubation with [^{14}C]glucose variable degrees of inhibition of sugar incorporation were also found; it seemed that the most peripherally located sugar, sialic acid, was least affected.

Bronchial mucus contains little glucose or mannose but substantial amounts of fucose, galactose, glucosamine, galactosamine, and sialic acid (8, 10). It is likely that [^{3}H]glucose is converted into these sugars and that the partial inhibition of [^{3}H]glucose incorporation by puromycin suggests the step-by-step addition of the sugars. Probably the addition of the terminal sugars, sialic acid and fucose, to the preformed peptide core continues while attachment of the more centrally located sugars of the glycoprotein molecule, galactose, galactosamine, and glucosamine, which occurs at an earlier phase, depends on new synthesis of polypeptides. In sheep colonic mucosa, incorporation of [^{14}C]glucose into acid glycoprotein was found to be principally in the sialic acid group while the protein moiety was unlabeled (12).

Selection of Optimal Fixative for Bronchial Gland

The results of the search for the optimal fixative for the bronchial gland after its incubation with a radioactive amino acid agree with those found for the liver (40) and retina (18). A previous study with [^{3}H]mannose (51), however, reported that nonspecific binding did not occur with sugars, but our results after incubation with [^{3}H]glucose at 4°C indicate that it may.

Of the three methods that included formaldehyde, formaldehyde for 18 h gave the best tissue preservation when compared to that obtained with glutaraldehyde, suggesting that longer fixation periods allow more cross-linking of protein to occur and thus aid protein stability. Baker and McCrae (4), however, concluded from their studies with mouse exocrine pancreas that little was to be

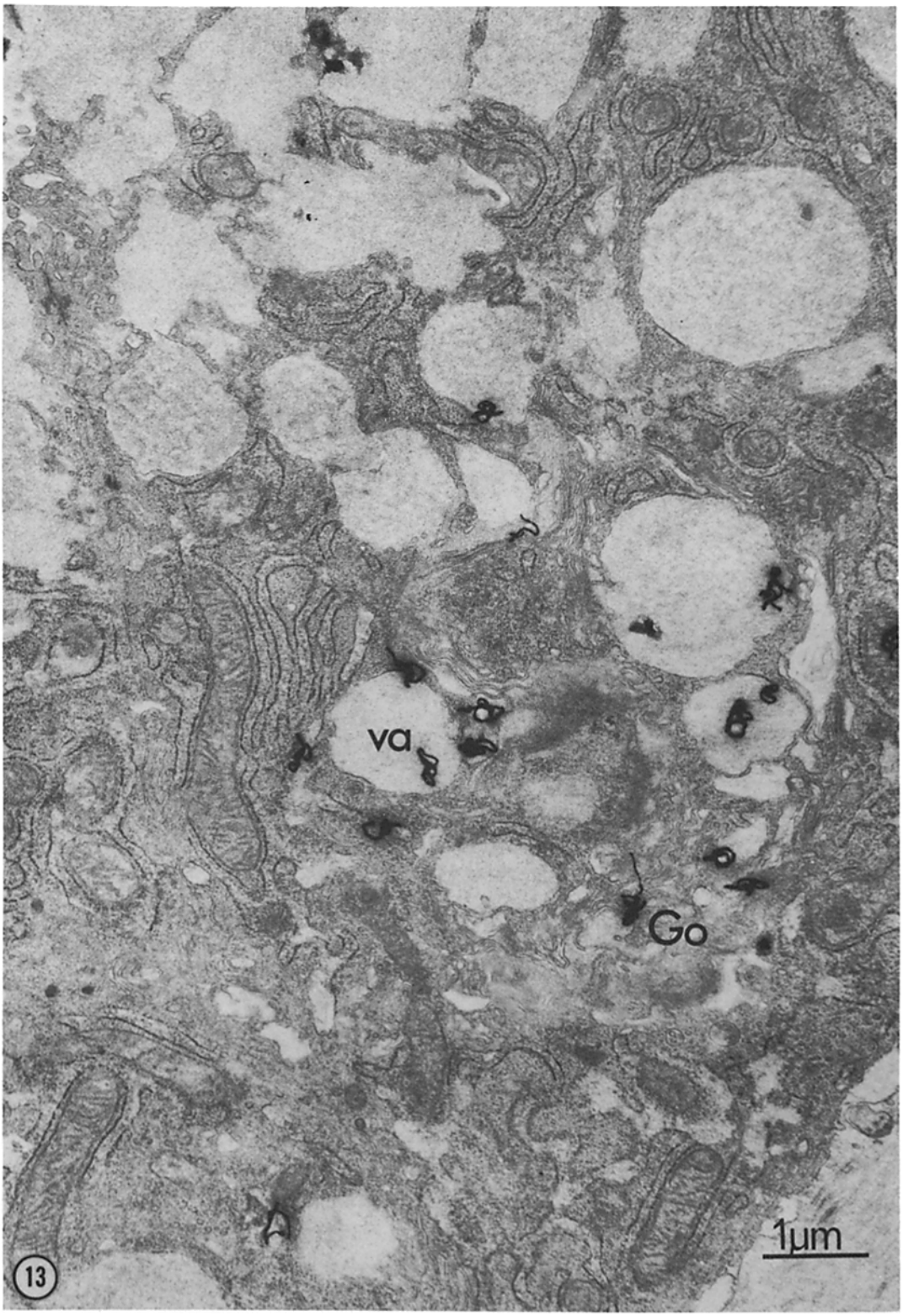

FIGURE 13 Part of a mucous cell after incubation with [^{3}H]glucose for 40 min. Silver grains are localized over the Golgi apparatus (*Go*) and vacuoles (*va*). × 17,000.

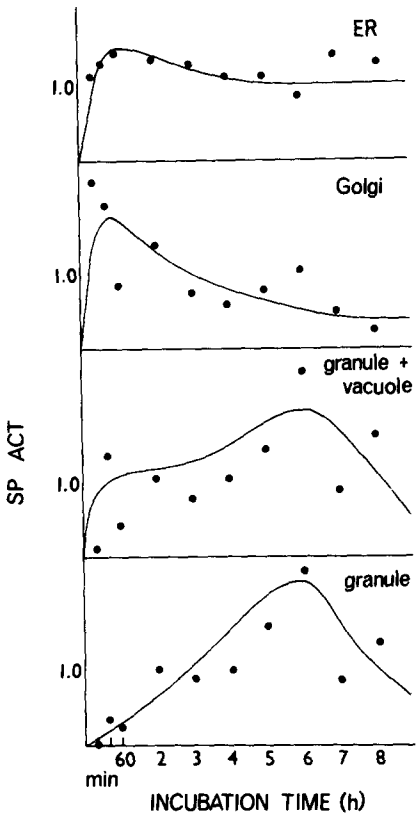

FIGURE 14 Smooth curves representing the trend of points over 8 h for the sp act of organelles of the serous cell after incubation with [^{3}H]glucose (a point represents the mean for the given time).

gained by fixing in formaldehyde for periods longer than 45 min.

Light Microscopic Autoradiography

After incubation with [^{3}H]threonine, examination of 1-μm sections as used in the present study added little to the findings of Sturgess and Reid (47) for either mucous or serous acini. In the present experiments, [^{3}H]glucose was found to localize initially in the midregion of the mucous and serous cells, where the Golgi apparatus is situated. This differs from the report of Sturgess and Reid (47) in which initial localization was reported at the base, a discrepancy perhaps explained by the better resolution and localization possible with a 1-μm section and by a slight difference in the way the acinus was arbitrarily divided into regions in order to assess grain concentration.

Quantification of High Resolution Autoradiographs

Two methods of quantification of electron microscope autoradiographs have been used, the circle analysis (53) and the hypothetical grain analysis (6). The circle analysis has been used in many studies (14, 15, 38, 54), but this is the first time that the hypothetical grain method has been used to compare uptakes a radioactive precursor after various times of incubation. This method has certain advantages over the circle analysis, calculation of standard errors is possible, and the method takes into account the effect of cross fire from one organelle to another by disintegrations, and allows detection of radioactive disintegrations from a line source such as a membrane as well. Against the advantages, one must set the considerable disasvantage that, at present, the method is more time consuming.

The results obtained by the two methods were similar for each experiment, save for the serous cell after incubation with [^{3}H]threonine for 60 min The circle analysis only showed a high activity associated with granules. It thus seems that labeling of the granules is only apparent and is doubtless due to cross fire from other organelles, which, in this case, as shown by sequential analysis, would seem to be the endoplasmic reticulum. When organelles are distributed throughout the cell as in the case of the serous cell, the hypothetical grain analysis seems preferable, but for cells with a regional distribution of organelles, such as the mucous cell, there is no such advantage.

Relationship of Mucous to Serous Cell

While mucous and serous cells are similar in certain respects, it is perhaps more useful to concentrate on their differences. They have previously been shown to be similar in that both produce an acid glycoprotein (25, 26), have a similar response to drugs (48), drain into the same duct system (34), and are present within the gland as well as within the airway epithelium (23). Gland reconstruction has shown that each cell type is segregated in a different region of the tubule (34), and histochemical studies have demonstrated different populations of granule for each cell type (25, 26). With the electron microscope, the serous cell has been shown to have discrete, electron-dense granules, the mucous cell, electron-lucent, mainly confluent ones. The serous cell discharges

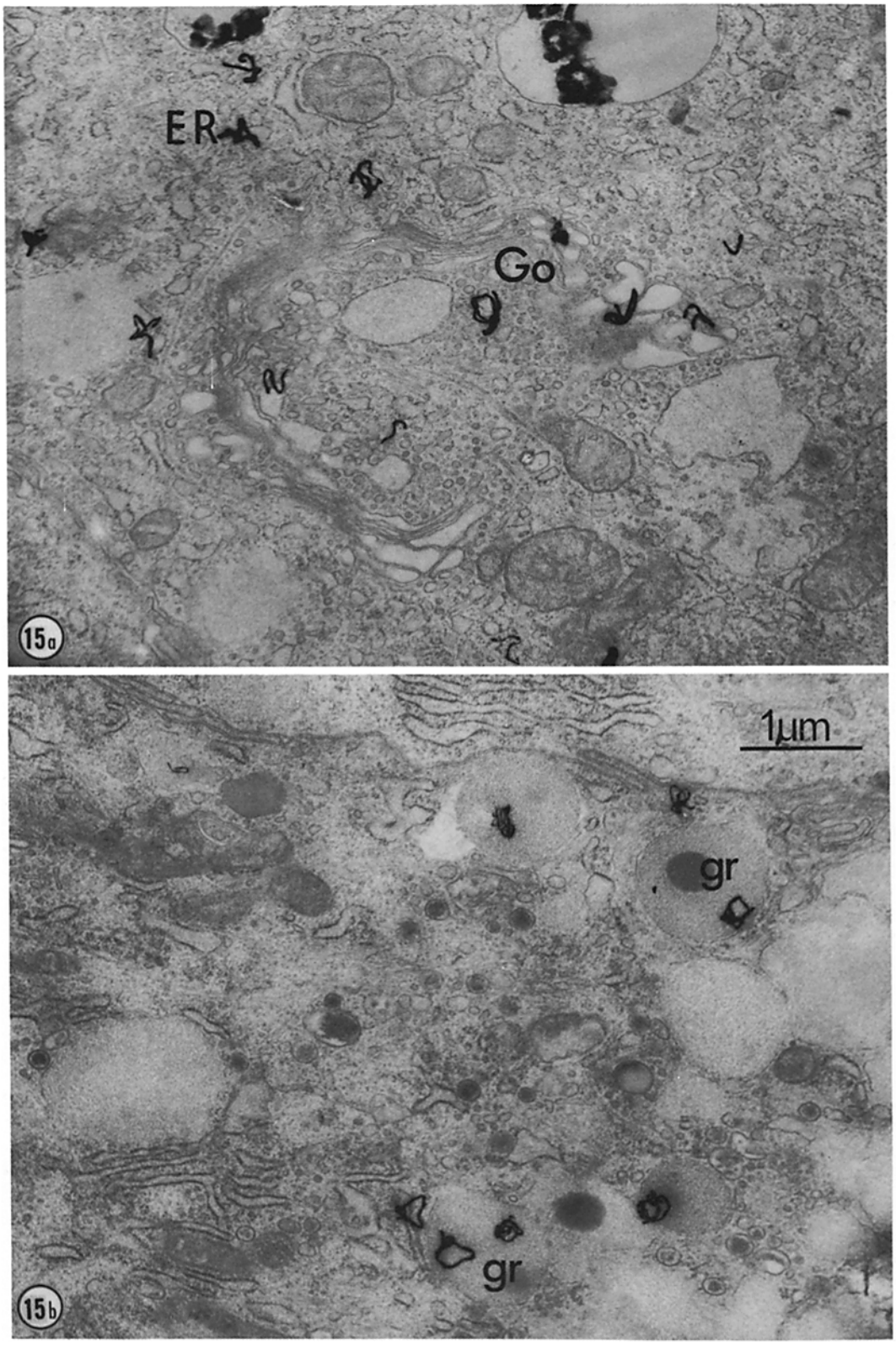

FIGURE 15 *a* and *b* Electron micrograph of part of a serous cell after incubation with [^{3}H]glucose (*a*) for 2 h: silver grains are localized over the Golgi apparatus (*Go*) and the endoplasmic reticulum (*ER*). × 18,750. (*b*) for 6 h: grains are localized over the secretory granules (*gr*). × 18,750.

TABLE VII

Mean Number of Grains Per Unit Area (4 μm²) over Mucous and Serous Cells after Incubation with [³H]Threonine

Incubation time	Mucous			Serous		
	No. Grains	No. Circles	Grains/ Circles	No. Grains	No. Circles	Grains/ Circles
20 min	178	768	0.2	164	926	0.2
40 min	168	804	0.2	164	797	0.2
60 min	389	1,164	0.3	289	1,159	0.3
2 h	844	1,046	0.8	480	880	0.6
3 h	904	860	1.1	820	1,176	0.7
4 h	862	825	1.0	755	1,061	0.7
5 h	841	806	1.0	628	845	0.7
6 h	754	678	1.1	513	1,067	0.5
7 h	781	882	0.9	401	1,181	0.3
8 h	802	1,054	0.8	393	923	0.4

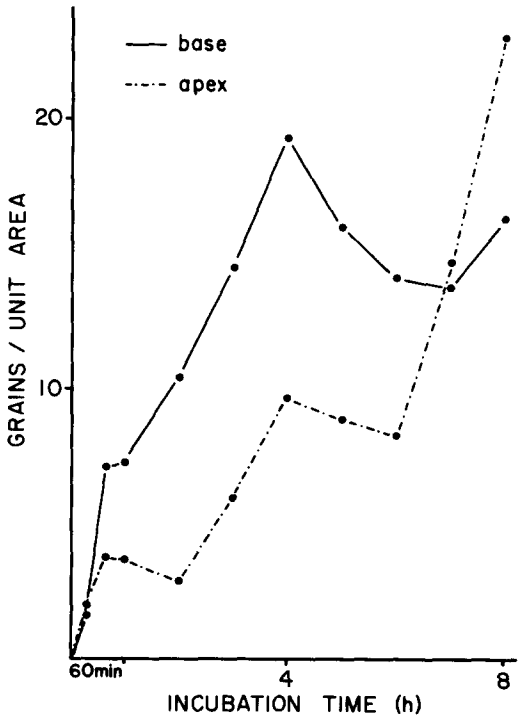

FIGURE 16 Silver grains per unit area (12.5 × 8.5 cm) over base and apex of the mucous cell after incubation with [³H]glucose (each point represents the mean).

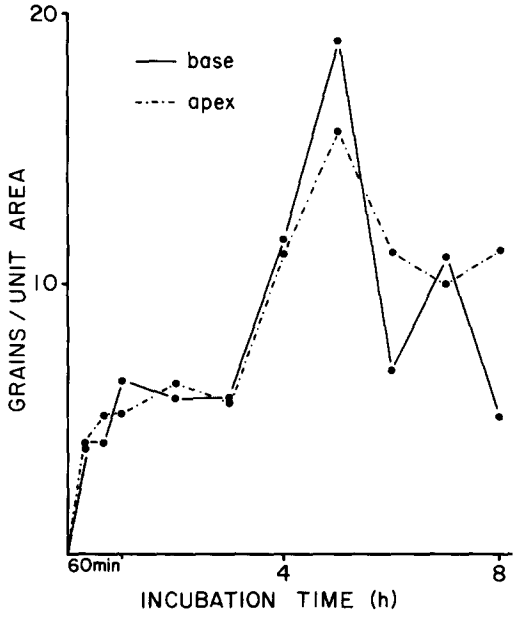

FIGURE 17 Silver grains per unit area (12.5 × 8.5 cm) over base and apex of the serous cell after incubation with [³H]glucose (each point represents the mean).

its granules singly, by a merocrine process (2, 16), whereas the mucous cell would seem to discharge batches of confluent granules. Canaliculi have been found to be associated only with the serous cell (33). In cases of hypertrophy of the bronchial gland (11), both cell types are always present although it is the mucous acini that mainly increase in number and their tubules that dilate while the serous cell appears to be present in normal numbers.

Transformation of serous to mucous cells has been suggested for the surface epithelium of the rat airway, in which an epithelial serous cell has recently been described (23). Transitional forms were rarely found in control rats, but were frequent in cases of airway "irritation" induced by

TABLE VIII

Progressive mean (12 Blocks) Showing Total Volume Occupied by Organelles of Mucous and Serous Cells and Their Total Volume at Base and Apex

Organelles	Mucous			Serous		
	Whole cell	Base	Apex	Whole cell	Base	Apex
	%	%	%	%	%	%
Endoplasmic reticulum	29.3	35.9	20.5	62.7	60.2	65.4
Granule + vacuole	52.2	32.7	73.1	17.7	14.1	26.6
Golgi apparatus	8.0	7.7	3.4	6.1	8.1	3.3
Mitochondria	1.6	2.5	0.8	3.9	3.0	1.9
Membrane	0.8	1.4	0.1	1.7	1.5	2.3
Nucleus	7.1	19.5	2.0	6.9	11.9	0.0
Lysosome + others	0.9	0.2	0.0	1.0	1.0	0.4

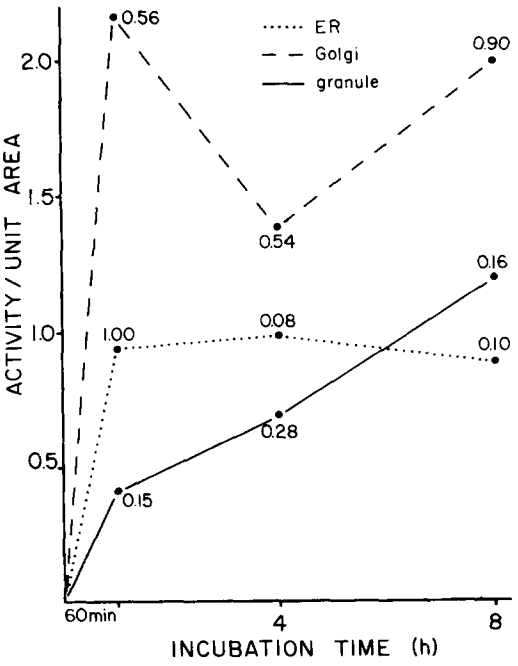

FIGURE 18 Activity per unit area for the organelles of the serous cell after incubation with [^{3}H]threonine, obtained by the hypothetical grain analysis (each point represents the mean, numbers, the standard error). Note the low activity for the granules at 60 min.

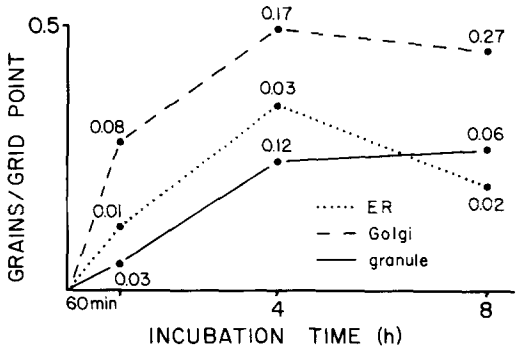

FIGURE 19 Grains per grid point for organelles of the serous cell after incubation with [^{3}H]threonine, obtained by the hypothetical grain analysis (each point represents the mean, numbers the standard error). These points show low values similar to those of the absolute uptake, obtained by the circle analysis.

either cigarette smoke or isoprenaline injection (22). Previous studies of the bronchial gland have also implied that in cases of gland hypertrophy associated with irritation the serous cell may undergo transformation to a mucous cell (46, 47).

Mitosis has been found in both mucous and serous cells of bronchial submucosal gland as shown in the human by in vitro incorporation of [^{3}H]thymidine[2] and in the rat[3] after injection of colchicine. Whereas the surface epithelium includes basal and undifferentiated or intermediate cells that can be regarded as "stem" cells (7, 23), no such cell type is seen in the gland.

The present studies have revealed further similarities and differences. Again, similarities are found in that both mucous and serous cells show the same patterns of incorporation for [^{3}H]threonine and [^{3}H]glucose. [^{3}H]Threonine was initially incorporated into the endoplasmic reticulum; the labeled metabolite migrated to the Golgi apparatus, then to the vacuoles, and was finally seen in the mature secretory granules. Since labeling of the Golgi lamellae was found, it seems that they

[2] Meyrick, B. Unpublished observations.

[3] Lamb, D. Unpublished observations.

TABLE IX

Hypothetical Grain Analysis—X^2 Test

Time	[^{3}H]Threonine		[^{3}H]Glucose	
	Mucous	Serous	Mucous	Serous
60 min	0.55*	0.81*	0.003	0.95*
4 h	0.24	0.88*	0.68*	0.75*
8 h	0.41	0.60*	0.10	0.27

* Represents a high level of confidence.

are involved in the synthesis of the secretory proteins, glycoproteins, and that they later form the vacuoles. On the other hand, the metabolites of [^{3}H]glucose were initially localized in the Golgi apparatus, in its lamellae, vesicles, and associated vacuoles of less than 1 μm. Such labeling of the different regions of the Golgi apparatus perhaps reflects the addition of different metabolites of [^{3}H]glucose in each of the zones. From here the radioactivity migrated to the larger vacuoles and finally was found in the secretory granules.

Differences were also encountered. First, the volumes of the various organelles within mucous and serous cells were different, as were their distributions; for example, there was relatively more endoplasmic reticulum in the serous cell than in the mucous cell, and it was distributed throughout the entire cell, whereas in the mucous cell it was mainly in the base. Second, after incubation with [^{3}H]threonine, the serous cell showed maximum activity over the Golgi apparatus more rapidly than did the mucous cell, which may suggest that the mucous cell molecule is more complex. Third, at all times, for both radioactive precursors, the concentration per unit area was less in the serous cell than in the mucous cell, although the ratio of [^{3}H]threonine to [^{3}H]glucose in the granules of both cells was approximately 1:1. This may mean that the secretory molecules of the serous cell are more dilute or again less complex than those of the mucous cell, which may occur because there is relatively less polymerization. In a recent study (28), sputum samples with a high dry weight were found to be more viscous than those with a low dry weight. This suggests that the mucous cell may secrete a more viscous glycoprotein than the serous cell. Finally, the concentration of both [^{3}H]-threonine and [^{3}H]glucose metabolites in the mucous cell granules was still increasing after 8 h of incubation, while for the serous cell a decrease in the overall amount of radioactivity was found at 7 h after incubation with [^{3}H]threonine and at 6 h with [^{3}H]glucose, suggesting a shorter cycle for the serous granules than for the mucous granules.

Thus, it would seem that under "normal" conditions mucous and serous cells are separate cell lines, although experimental studies suggest that, at least in the surface epithelium of rat airway, irritation causes a transformation of a serous to a mucous cell (22). Such a transformation effect would seem to be less important for the serous and mucous cells of the bronchial gland.

In the present study [^{3}H]glucose is found at all times in moderate amounts over the endoplasmic reticulum, and perhaps indicates the synthesis of cytoplasmic glycoproteins. However, the possibility that some [^{3}H]glucose is added in the endoplasmic reticulum cannot be completely excluded since Zagury et al (55), studying a plasma cell tumor, found glucosamine to be incorporated in both the endoplasmic reticulum and the Golgi apparatus. Mannose has also been found to be incorporated in the cisternal space of the thyroid (52), but, since little of this sugar is found in bronchial mucus, this would not seem important. Most sugars have been reported to be added in the Golgi apparatus: these include glucose (37), galactose (13, 36, 52), fucose (5), and glucosamine (29).

The metabolic function of the nuclear incorporation of [^{3}H]threonine seen at 60 min is not clear. As amino acid labeling is abolished when cycloheximide is added to the incubation medium (27), it almost certainly arises from protein metabolism. Hopkins and Farquhar (19) reported similar findings in cells isolated from rat anterior pituitary.

Comparison with Secretory Cells at Other Sites

Although the mucous and serous cells of the bronchial gland show similar patterns of incorporation of radioactive precursors, they are not alike ultrastructurally. For this reason, it is interesting to compare the pattern of amino acid incorporation in the serous cell with that in other secretory cells that are ultrastructurally similar. The incorporation of [^{3}H]threonine by the serous cell of the bronchial gland shows the same pattern as the incorporation by the zymogen cell of the guinea pig exocrine pancreas (20, 21) or as the incorporation of [^{3}H]leucine by the acinar cell of the rabbit parotid (9).

On the other hand, comparison of the timing of these events shows several differences. For the

serous cell of the bronchial gland, maximum labeling of the vacuoles with [^{3}H]threonine is seen at 2 h, but for the zymogen cell and parotid cell it is apparent earlier, at 40 min. The serous cell and parotid cell show maximum labeling of granules at 6 h, while the zymogen cell shows this at 2 h. Thus, the serous cell of the bronchial gland would appear to synthesize peptides at a slower rate than either the pancreatic zymogen cell or the parotid cell and to have a slower secretory cycle than the zymogen cell but not the parotid cell, in which granule storage has been shown to be prolonged (9).

Function of Serous Cell

Because of the similarities found between the zymogen cell of the exocrine pancreas, the acinar cell of the parotid, the Paneth cell (29, 50), and the serous cell of the bronchial gland, it is likely that the bronchial serous cell also produces a zymogenic granule. Lysozyme has been demonstrated in the Paneth cell, in the salivary acinar cell (30), and in tracheobronchial secretions (31). In the airways, such enzymes might help in the release and breakdown of mucous granules and facilitate their removal from the secretory tubule. It may be that the serous cell also contributes to the periciliary liquid layer of the bronchus, a fluid that is thought to be less viscid than the sheets of mucus that rest on the ciliary tips.

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