ON THE SITE OF SULFATION IN COLONIC GOBLET CELLS

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ABSTRACT

The location of bound S^{35} in the goblet cell of the rat colon at time points from 2 to 60 minutes after administration of S^{35} as sodium sulfate has been observed *in vivo* and *in vitro* by radioautographic techniques. Grains were first observed by electron microscopy over the stacked lamellae of the paranuclear part of the Golgi apparatus. The label was subsequently found associated with the supranuclear Golgi lamellae and was then seen associated with the smooth membranes limiting the mucin granules in the goblet. Finally, between ½ and 1 hour, the secreted mucus product in the crypts became radioactive. Neither mitochondria nor the endoplasmic reticulum was labeled. It is concluded that the Golgi apparatus is the organelle in which sulfation occurs.

INTRODUCTION

The intestinal goblet cell, within which we have attempted to localize the sites of sulfate binding, consists of a basal "foot" portion containing the nucleus, most of the mitochondria, and the granular endoplasmic reticulum. The apical half, the goblet proper, is limited by a thin rim of cytoplasm enclosing the mass of smooth membrane-limited mucin droplets filling the goblet. Between the base of the goblet and the nucleus, and in gradual transition with the mucin droplets, there are bilamellar stacks of flattened membranes and approximated vesicles, the latter varying from fifty to several hundred millimicrons in diameter. Both smooth membranes and vesicles constitute the Golgi apparatus of this cell (10, 11, 16, 36). In addition to the supranuclear Golgi structures (Fig. 6), one may observe these stacked membranes and vesicles in a paranuclear location extending in a basal direction. The content of these vesicles is similar in texture and density to that of the mucin droplets in the goblet proper.

About a decade ago, the uptake of radioactive sulfate from $S^{35}O_4$ in the mucous membranes of the digestive tract was demonstrated in radioautograms (1, 2, 35), and a correlation with mucopolysaccharide synthesis was suggested (23). With refinements of radioautographic techniques the localization of S^{35} in goblet cells was shown to be supranuclear (24). This showed that the Golgi apparatus was the area in which S^{35} was concentrated, and it was inferred that the Golgi apparatus might be the site of sulfation (17, 24).

Theoretical considerations on the applications of radioautography to electron microscopic specimens have been presented recently (8, 40). These studies have shown that radioautographic techniques using isotopes emitting beta particles of relatively high energy (0.154 to 0.167 Mev) allow sufficiently high resolution so that they can be

profitably applied in electron microscopy. In the present study these methods were used in an attempt to demonstrate at higher resolution the intracellular pathway of bound radiosulfate in relation to the production of mucus by the colonic goblet cells of the rat.

In all previous studies of the goblet cells the earliest time studied after S^{35} administration was 60 minutes (2, 3, 13, 14, 24). Since at this time the label is already evident in the secretion in the glandular lumen, it was deemed desirable to study the uptake at much earlier times in order to demonstrate the intracellular site at which sulfate is first bound, as well as the subsequent pathway of sulfate through the cell, so that more precise evidence could be gained as to the site at which sulfation of the secretory product occurs.

MATERIALS AND METHODS

Experimental

IN VIVO EXPERIMENTS: $S^{35}O_4$, as sodium sulfate of high specific activity, diluted with balanced salt solution, was administered to white Sherman rats, of both sexes and ranging in weight from 60 to 140 grams, in doses corresponding to 20 to 60 μc per gram body weight. Specimens of the descending colon were removed under ether anesthesia at 5, 10, 15, 20, 30, and 60 minutes after injection of sulfate, which was intravenous for the 5, 10, and 15 minute experiments and either intravenous or intraperitoneal for the longer time points. In some experiments a diluting dose of sodium sulfate was administered intraperitoneally 8 minutes after injection of radiosulfate.

IN VITRO EXPERIMENTS: The capacity of surviving pieces of isolated colonic tissue to bind radiosulfate has been previously reported (39). For "pulse labeling," tissue from the descending colon was excised and subdivided into blocks about 2 × 2 mm. These fragments were incubated for 2, 4, 6 and 10 minutes in a tissue culture medium consisting of balanced salt solution, 0.2 per cent lactalbumin hydrolysate, and 5 per cent serum containing 600 μc of radioactive sulfate per milliliter. This was followed by immersion in 0.050 per cent cold Na_2SO_4 in medium for 30, 45, 60, and 120 seconds, respectively.

FIXATION AND EMBEDDING: All the above-described tissues were fixed in phosphate-buffered 1 per cent OsO_4 for 60 to 90 minutes at 0 to 4°C, dehydrated through alcohols, and embedded in a prepolymerized mixture of butyl and methyl methacrylates. Sections in the "silver-gold" range (about 900 A) were cut for electron microscopy, and "thick" sections, ½ to 2μ, were mounted on "subbed" glass slides.

Radioautography

The methods used were those described by Caro and van Tubergen (9).

PHASE CONTRAST MICROSCOPY: The methacrylate was removed from the thick sections with amyl acetate and the slides were coated with liquified Kodak NTB_2 emulsion by dipping. Exposure times ranged from 2 to 7 weeks. Slides were developed in Kodak D-19 at 20° for 2 minutes.

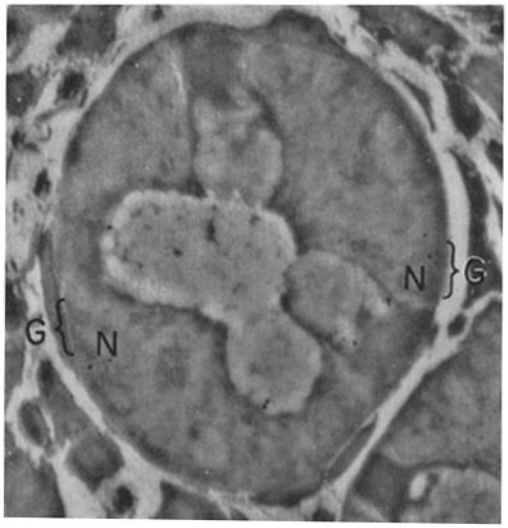
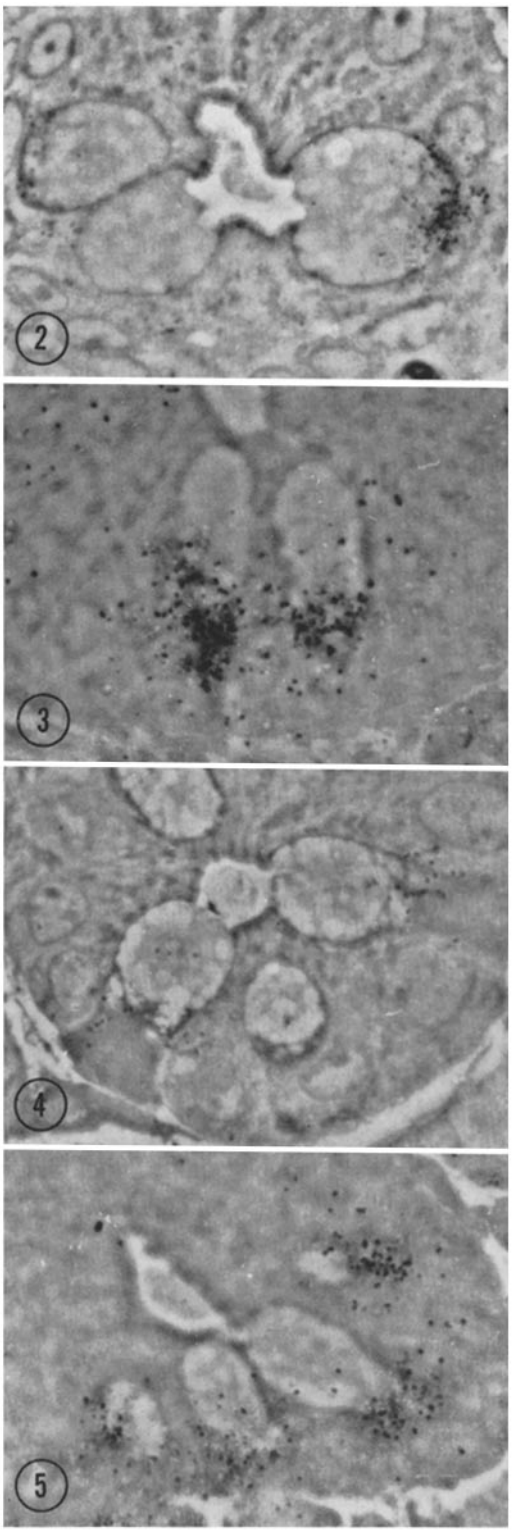


FIGURE 1 Radioautograph of a colonic crypt in cross-section after a 2 minute pulse label *in vitro* with $Na_2S^{35}O_4$. Grains overlie the para- and supranuclear area of three goblet cells. Grains also overlie tangential sections through the basal parts of two goblet cells (*G*) which are recognizable by the "dark" cytoplasm. *N*, nucleus. Phase contrast. × 1870.

ELECTRON MICROSCOPY: Sections on grids were covered with a film of Ilford L-4 emulsion retained on a copper wire loop. Exposure times in the various experiments ranged from 6 to 8 weeks. Grids were developed in Kodak D19 for 2 minutes at 20°C or Microdol for 4 minutes at 20°C. Some grids were stained with Karnovsky lead stain (25) for the dual purpose of enhancing contrast and removing gelatin. The grids were examined in an RCA EMU 2 E and a Siemens Elmiskop.

RESULTS

In all experiments uptake of S^{35} by the colonic goblet cell was observed in both the light and electron microscopic radioautographs.



The Early Time Points

Two minutes after immersion in $S^{35}O_4$-containing medium, sulfate was already bound in the para- and supranuclear regions in a few of the goblet cells (Fig. 1). In both the *in vivo* and *in vitro* preparations, at 4, 5, 6, and 10 minutes grains were observable over moderate numbers of goblet cells in the thick sections (Figs. 2 to 5). The intensity of the labeling varied among the goblet cells, but the grains were preponderantly in the para- and supranuclear regions of the cell. In the electron micrographs the grains were predominantly associated with the supra- and paranuclear stacked lamellae of the Golgi apparatus (Figs. 6 to 10). The electron microscopic radioautographs permitted clear distinction between the smooth membranes of the Golgi apparatus and the granular membranes of the endoplasmic reticulum. Grains were never found overlying the granular endoplasmic reticulum, nor were any found associated with mitochondria. At these times grains were not associated with the mucin droplets or membranes constituting the contents of the goblet proper.

The Intermediate Time Points

At 15, 20, and 30 minutes heavy labeling was observed in the majority of goblet cells. Again, the localization was principally supranuclear, with, however, a moderate number of grains over the basal half of the goblet proper (Figs. 11 and 12). In the electron microscope the grains were associated mostly with the lamellae of the Golgi apparatus, including those which border the sides of the basal half of the goblet proper. In addition, some grains were observed over the basal contents of the goblet proper, apparently associated with the smooth membranes that limit the mucin droplets within this structure.

The Late Time Point

In agreement with previous workers (2, 13, 24), extensive labeling of goblet cells was observed at

FIGURES 2 through 5 Radioautographs of colonic crypts 5 and 10 minutes after intravenous injection of $Na_2S^{35}O_4$ are shown in Figs. 2 and 3. Figs 4 and 5 are comparable radioautographs 6 and 10 minutes after pulse labeling *in vitro* with $Na_2S^{35}O_4$. The intensity of labeling varies among the goblet cells, but the preponderance of grains overlies the para- and supranuclear areas. Phase contrast. × 2000.

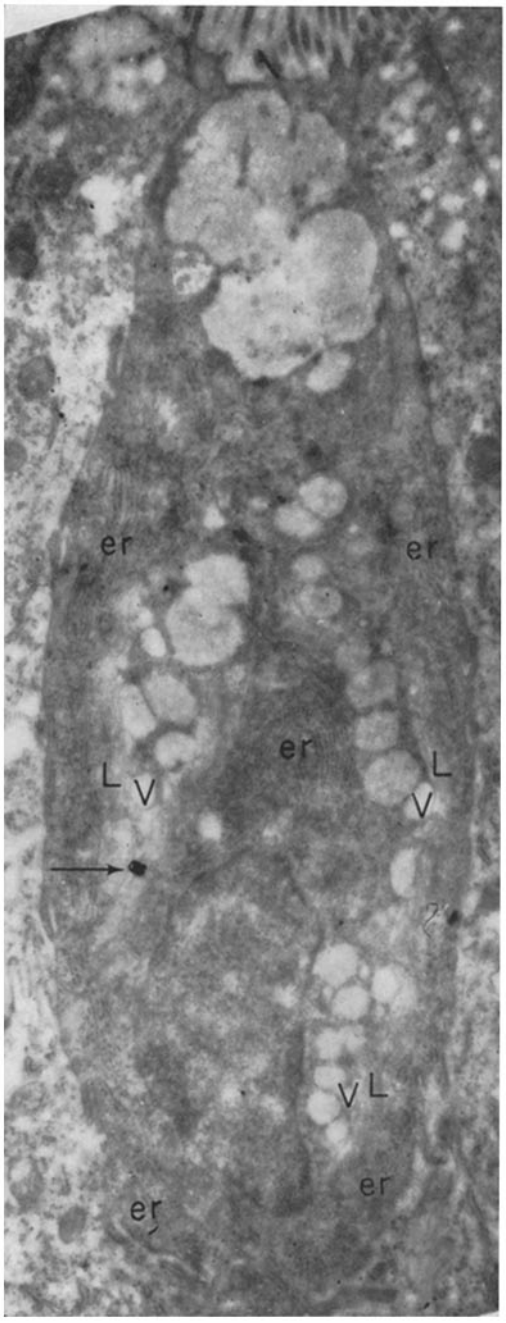


FIGURE 6 Longitudinal section of rat colonic goblet cell 5 minutes after intravenous injection of $Na_2S^{35}O_4$. Lamellae (*L*) and vesicles (*V*) of the Golgi apparatus are noted in para- as well as supranuclear position. They are clearly distinguishable from the granular endoplasmic reticulum (*er*). A single grain (arrow) overlies such lamellae to the left of the nucleus. × 9000.

60 minutes, at which time secreted mucus in the crypt lumen was also radioactive. Sixty minutes after intraperitoneal injection a very high proportion of goblet cells at all levels of the crypts was overlaid with a large number of grains (Fig. 13). As reported by Jennings and Florey (24), in the goblet cells of the upper ⅔ (neck) of the crypts the label tended to be concentrated in the supranuclear zone, whereas the label was distributed throughout the whole goblet in the cells of the deep (fundic) third of the crypts (Fig. 14). This probably reflects a more rapid rate of secretion in the latter cells. In the electron microscope, grains were found in the supranuclear Golgi zone, throughout the goblet, and in the secretory product in the glandular lumens as well (Figs. 15 to 18). The grains seen over the goblet itself were associated with the smooth membranes which enclose the individual mucin droplets rather than being randomly dispersed over these droplets.

At 60 minutes, as in all earlier time points studied, grains were not found in association with the granular endoplasmic reticulum or mitochondria, nor was there any association of grains with the absorptive cells or stromal elements.

DISCUSSION

Most of the recent morphologic studies (5, 16, 18–22, 36) and the radioautographic study of Jennings and Florey (24) extend the much earlier suggested association of the Golgi apparatus and secretion (6, 15, 33). In the present study, at all the early time points there was a consistent association of grains with the para- and supranuclear lamellae of the Golgi apparatus, and the membranes of these lamellae are the earliest site of sulfate binding demonstrable by the methods used. The accuracy of this localization is supported by the calculations of Pelc (40) which indicate that, with S^{35}, silver grains are formed within 0.2 μ of the S^{35} source in intracellular structures.

The preferential association of the grains with the smooth membranes limiting the mucin droplets may mean that sulfate becomes associated with a polysaccharide in the Golgi area, the sulfated product then remaining at the periphery of the mucin granule close to its limiting membrane as it progresses to the apex of the cell. More likely, however, sulfate, having become bound to membranes which become part of the Golgi

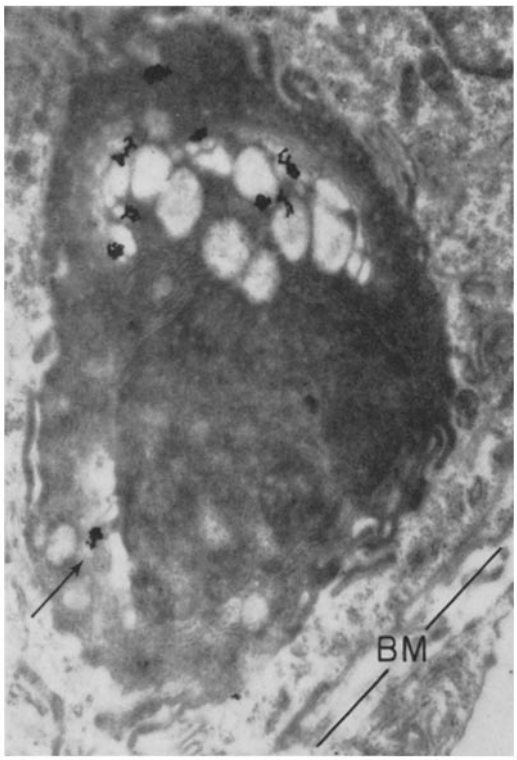


FIGURE 7 Radioautograph of the basal portion of a goblet cell 5 minutes after intravenous injection of $Na_2S^{35}O_4$. Note the proximity to the basement membrane (*BM*) of the paranuclear grain (arrow) which overlies smooth membranes. The remaining grains overlie the supranuclear portion of the Golgi apparatus. × 9,600.

apparatus, may progress toward the cell apex if these membranes "flow" (4, 34) towards this pole as the mucin droplets are moved to the glandular lumen.[1]

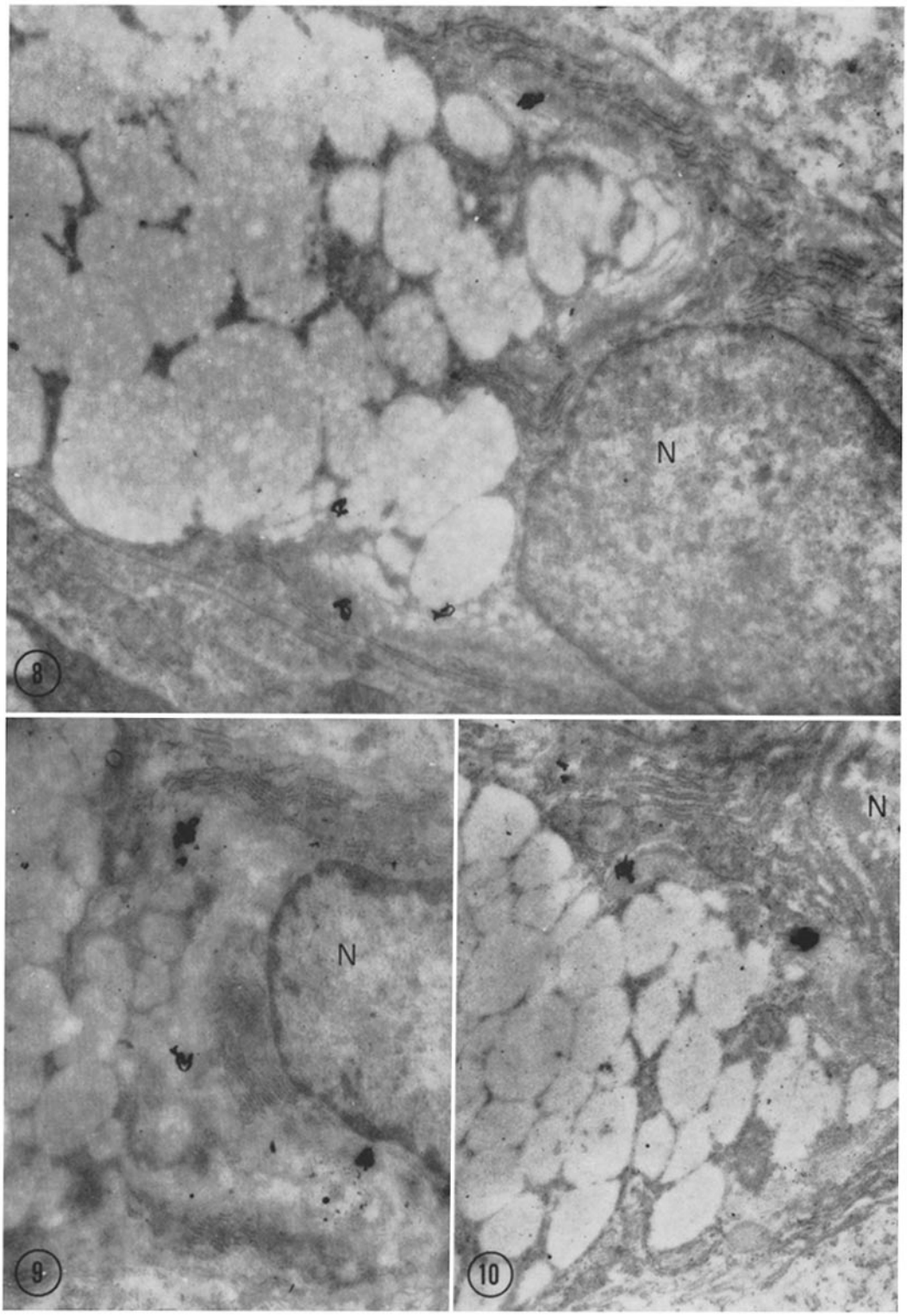
Knowledge of the detailed chemistry of colonic mucins is relatively incomplete (27, 45). It is known that one of the minor components is a sulfated mucosubstance, but the way in which a mucosubstance becomes sulfated is poorly understood. It has been assumed that this esterification is an exchange requiring a high energy bond and takes place by transfer of sulfate from phosphoadenosine phosphosulfate (30, 31). It has been shown that combination of sulfate as phosphoadenosine phosphosulfate and its subsequent transfer to mucopolysaccharide (30, 43) and certain other acceptors can occur in the presence of cell-free preparations of colonic mucosa (28, 37, 44). Kent *et al.* (29), in studying S^{35} incorporation in duodenal mucosubstances, describe a diffusible sulfated fraction, presumed to be sulfate ester, in addition to the non-dialysable sulfated mucopolysaccharide. While it is possible that a precursor substance of lower molecular weight becomes sulfated rather than the fully polymerised polysaccharide, radioautograms can only demonstrate the sulfate combined with polymerised or formed components.

Radioactive sulfate evidently does not simply exchange with the sulfate of an already sulfated mucopolysaccharide, but rather uptake by mucus-secreting cells reflects *de novo* sulfation of a polysaccharide or precursor substance (39). Biochemical studies (38) have shown that exposure of sulfated mucus to radioactive sulfate does not result in exchange, and in the present *in vitro* studies, in which an opportunity was provided for sulfate exchange at all levels of the goblet cell as well as in the secretory product, at the early time points grains were found only in association with the Golgi structures and not over the apical part of the goblet or over the secretion. These observations add reassurance that specific sites of sulfate trapping and esterification were being observed in the radioautograms.

Previous observations (24, 38) indicate that S^{35} administered as sulfate is not used in the synthesis of sulfur-containing amino acids. Since $S^{35}O_4$ uptake was confined exclusively to the goblet cells, none being observed in other epithelial cells or stromal cells of the colon, it is added evidence that $S^{35}O_4$ is used almost exclusively for the synthesis of ester-sulfated mucopolysaccharide.

Although the study has yielded information concerning the transit of bound sulfate from structures associated with the Golgi apparatus of the goblet cell to the secreted product, it tells nothing about the mechanism(s) whereby sulfate moves from the incubation medium or the blood stream to the Golgi apparatus of the goblet cell. This may take place within 2 minutes, an observation which is perhaps not surprising since it has been shown biochemically that, in the case of cartilage,

[1] The peripheral location of grains closely associated with the medium-sized or larger vesicles of the Golgi apparatus might also be explained by the statistical fact that the more probable location of grains over a uniformly labeled disc would be peripheral (L. Caro, personal communication).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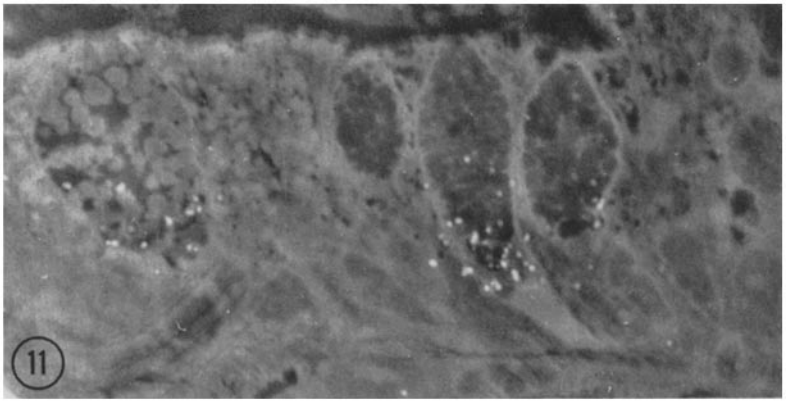

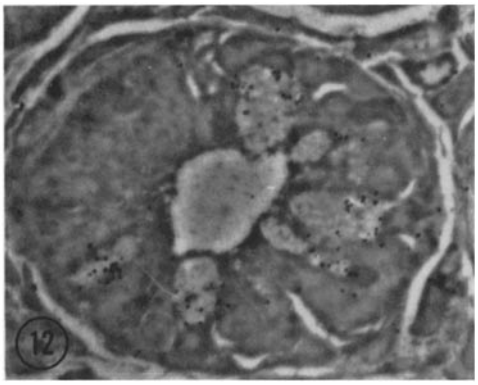

FIGURES 11 and 12 At 15 and 30 minutes after intraperitoneal injection of $Na_2S^{35}O_4$, grains were observed over the basal half of the goblet proper as well as over the Golgi area. Fig. 11 is negative phase contrast. Fig 11, × 3000; Fig. 12, × 1700.

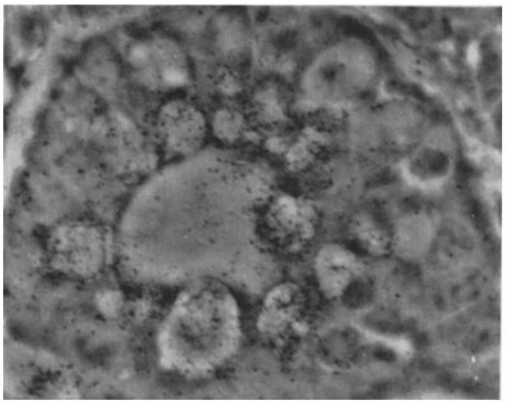

FIGURE 13 At 60 minutes after intraperitoneal injection of $Na_2S^{35}O_4$ the label has progressed through the apical half of the goblet proper and is evident in the secreted mucus in the crypt lumen. Phase contrast. × 1700.

FIGURES 8 to 10 In each figure the basal pole of the cell is toward the right margin. A portion of the nucleus (*N*) is visible. Fig. 8 shows 4 grains overlying stacked Golgi lamellae at the base of the goblet proper 5 minutes after intravenous injection of $Na_2S^{35}O_4$. In Fig. 9, 4 minutes after *in vitro* "pulse" labeling, a paranuclear grain as well as supranuclear grains are seen. Fig. 10 shows grains in supranuclear position 10 minutes after "pulse" labeling. All grains are associated with smooth lamellae. ×10,400.

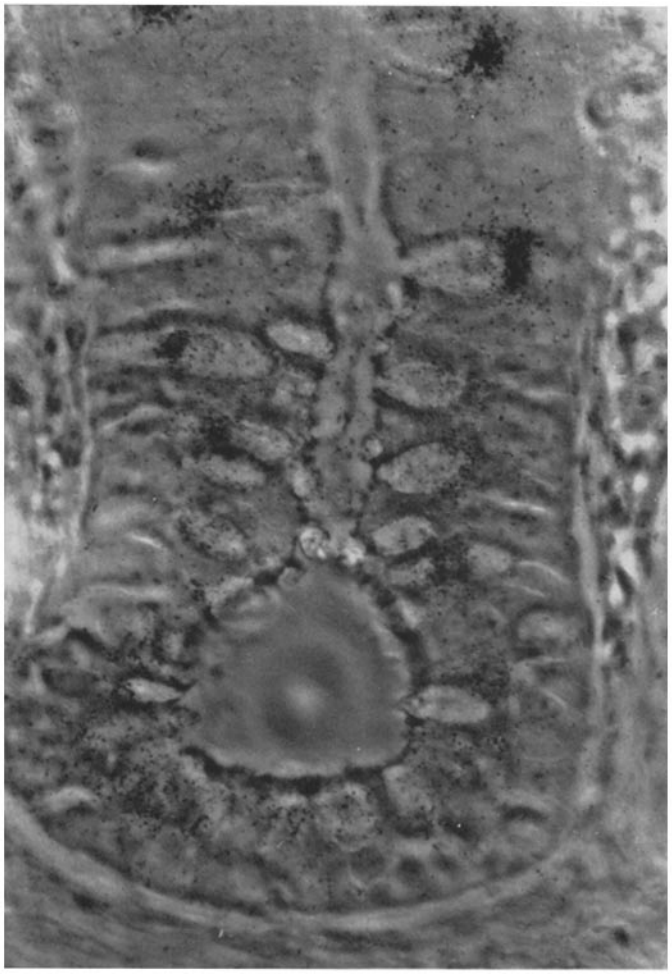

FIGURE 14 A longitudinal section of a crypt 60 minutes after intraperitoneal injection of $Na_2S^{35}O_4$. There is a dispersion of grains over the goblet proper in the cells of the deep (fundic) portion of the crypt, indicating greater rapidity of secretion in these cells. Above this level, in the neck of the crypt, a supranuclear concentration of grains is noted. Phase contrast. ×1400.

sulfation of polysaccharide may take place in as brief a time as five minutes (46).

In transit from blood to cell membrane, sulfate must pass across (or possibly in between) the capillary endothelial cells, the capillary basement membrane, and the ground substance of the lamina propria. In cartilage, the rapidity of movement of inorganic sulfate through the ground substance is remarkable (7).

The sulfate must then traverse the basement membrane of the crypt of Lieberkühn and be presented at the plasma membrane of the epithelial cells. It is known that some cell types, *e.g.* muscle and nerve, do not permit entry of sulfate ion (12). However, in the case of cells such as goblet cells, chondrocytes, and fibroblasts, which accumulate sulfate, some mechanism must facilitate entry and retain this ion in the cell. In cartilage, Whitehouse and Boström (46) suggest a transport mechanism (pump) at the cell surface, energized by ATP, which transfers this anion to an intracellular pool of inorganic sulfate. The nature of the mechanisms which facilitate entry of sulfate into these cells and which bind it there is not known. It is probable that receptor sites are necessary (47), whether at the surface or on endo-

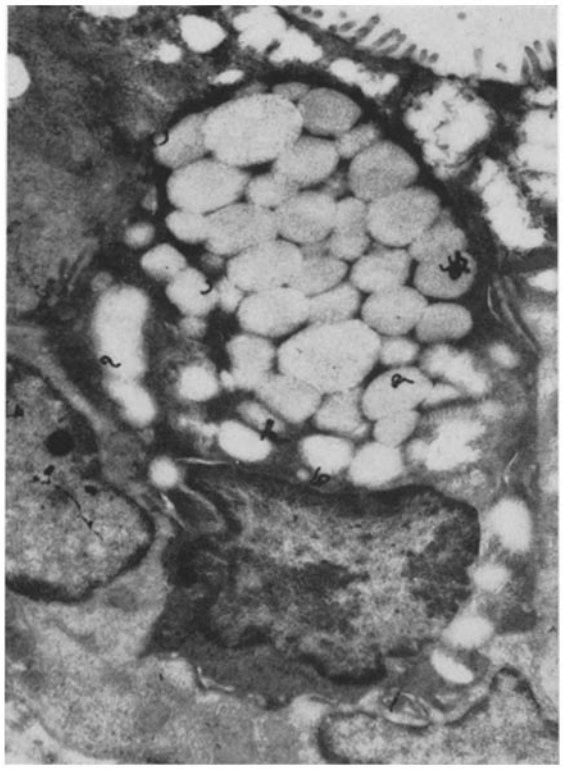

FIGURE 15 Radioautograph of a goblet cell 60 minutes after intraperitoneal injection of $Na_2S^{35}O_4$. Grains are present throughout the goblet proper at this time point. Approximately × 9000.

cellular membranes, but the existence of an ion pump in the plasma membrane comparable to that known for cations can only be surmised.

Failure to find an association of grains with the endoplasmic reticulum or with the mitochondria contrasts with the observations of Winters *et al.* (47) concerning the high degree of inorganic sulfate uptake by isolated mitochondria of kidney epithelium. Absence of grains over the endoplasmic reticulum and the mitochondria of the goblet cells does not necessarily mean that these structures do not participate in sulfate transport or trapping. The possibilities remain that in the goblet cells these organelles have a transient role which does not lead to an accumulation of bound sulfate, or that sulfate in these organelles is in a form extractable by the preparatory techniques for electron microscopy.

The final secretory product is a mucopolysaccharide, probably complexed with protein, and it is presumed that the protein moiety is synthesized in the granular endoplasmic reticulum. While the organelles associated with the production of the polysaccharide component are not known, some authors have suggested that this type of synthesis (at least in the case of glycogen) is associated with smooth membranes (26, 41, 42, but see also 32). Our observations concern that fraction of the product which is sulfated. With the method of high resolution radioautography, our studies have demonstrated the intracellular pathway of bound radiosulfate. The Golgi lamellae were shown to be the first site of sulfate binding and it seems reasonable to suggest that these membranes are the principal site of sulfation.

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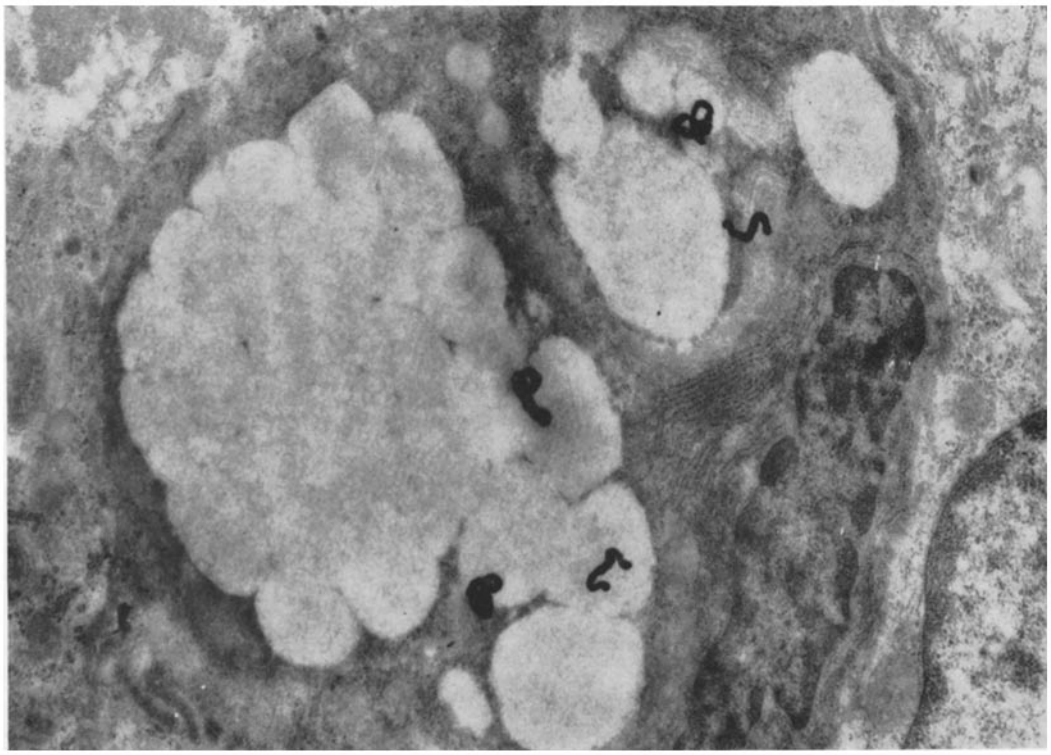
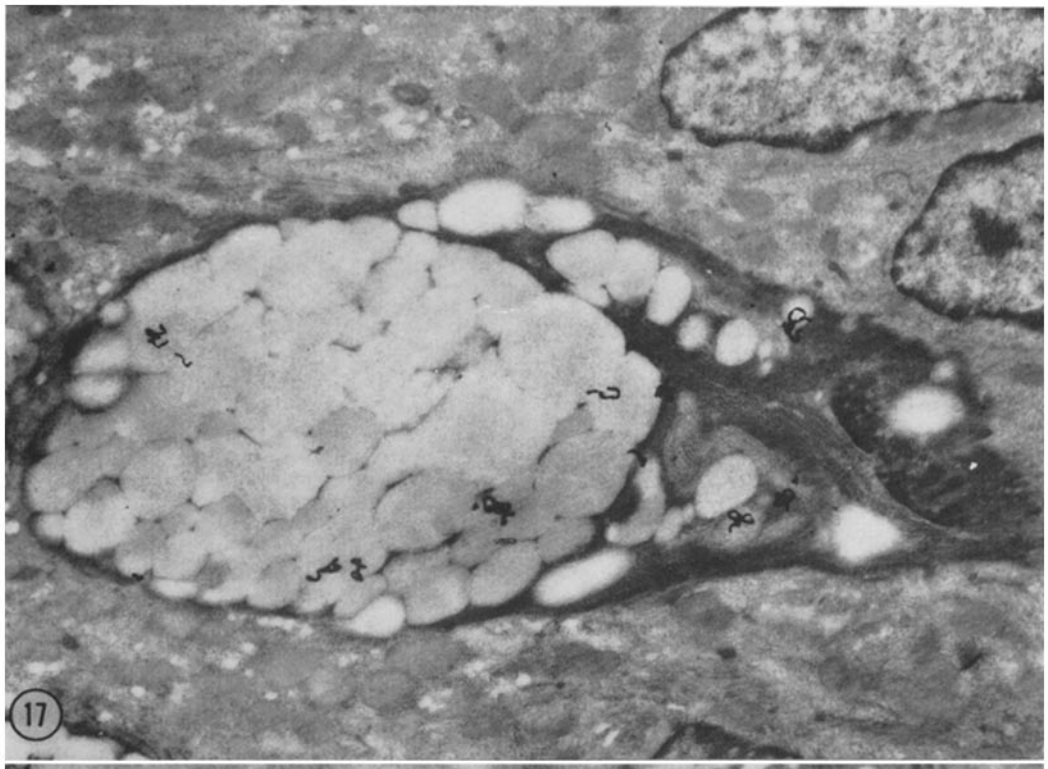


FIGURE 16 A tangential section through the basal portion of a goblet cell 60 minutes after intraperitoneal injection of $Na_2S^{35}O_4$. Some of the grains in the supranuclear region overlie the Golgi lamellae which are clearly distinguishable from the granular endoplasmic reticulum. Other grains are associated with the base of the goblet proper. Approximately ×21,000.

FIGURE 17 In this longitudinal section of a goblet cell 60 minutes after intraperitoneal injection of $Na_2S^{35}O_4$, grains associated with the Golgi area as well as grains over the goblet proper are evident. Approximately ×13,000.

FIGURE 18 Numerous grains are evident in the apical half of this goblet cell 60 minutes after intraperitoneal injection of $Na_2S^{35}O_4$. In this figure, as well as in Fig. 17, the grains appear to be associated with the membranes limiting the mucin droplets. Label is also present in the mucus of the crypt lumen. Approximately ×16,000.



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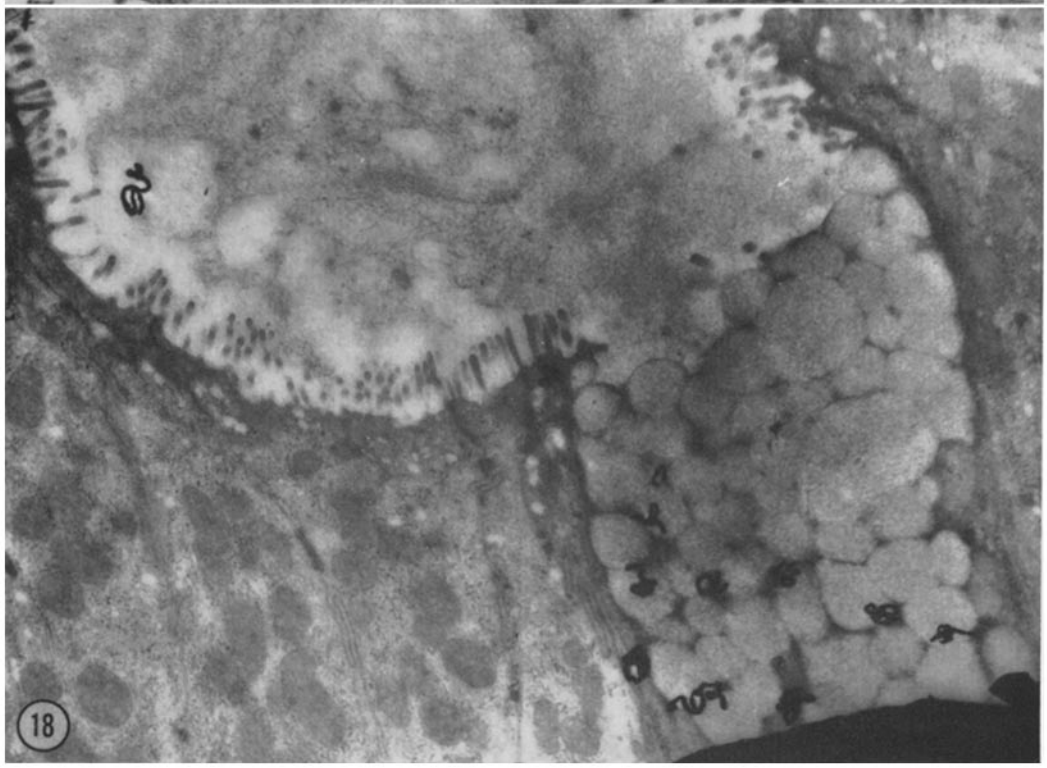

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