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Research Article

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Alveolar Cells: Incorporation of Carbohydrate into Protein and Evidence for Intracellular Protein Transport

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ABSTRACT Alveolar cells incubated with radioactive glucosamine, galactose, and mannose incorporate radioactivity into protein, that is, into material insoluble in cold and hot trichloroacetic acid and not extracted by lipid solvents. This incorporation is incompletely inhibited by puromycin hydrochloride. The kinetics of the subcellular distribution of radioactivity are consistent with a precursor-product relationship between microsomal protein and the protein of particles sedimenting at 15,000 *g*. It is thus suggested that alveolar cells incorporate these substrates intact into protein at the microsomal level with subsequent transfer of this newly formed material to particles sedimenting at 15,000 *g*.

INTRODUCTION

The procedure described by Myrvik, Leake, and Oshima (1) and later modified by others (2) allows large numbers of cells to be obtained by lung lavage. Moore and Schoenberg (3) have shown that most of the cells so obtained are derived from the cells that line normal alveoli and, hence, are referred to in this paper as alveolar cells as suggested by Bertalanffy (4). These cells have been studied in some detail with particular regard to their phagocytic, enzymatic, and certain other metabolic functions. Their biosynthetic properties

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have been studied in less detail, particularly their ability to incorporate radioactive substrates into newly formed protein.

This paper presents the results of an investigation of the *in vitro* incorporation of radioactivity into protein of cells obtained by lung lavage of *M. bovis* (BCG) treated rabbits (2). Radioactive sugars and glucosamine served as substrates. The experiments demonstrate that these cells do incorporate radioactivity into protein. The kinetics of the subcellular distribution of radioactivity are consistent with a precursor-product relationship (5) between microsomal protein and the protein of particles sedimenting at 15,000 *g*. It is thus suggested that alveolar cells incorporate the substrates into protein at the microsomal level with subsequent transfer of this newly formed material to particles sedimenting at 15,000 *g*.

METHODS

Preparation of alveolar cells. New Zealand, white male rabbits weighing about 2 kg were given 20 mg of heat-killed BCG intravenously, suspended in a 1 ml solution of 0.9% sodium chloride and 0.01% Tween 80 (2). 3-4 wk later the rabbits were sacrificed by air embolism, the trachea clamped, and the lungs, heart, and adherent structures removed from the chest en block and placed on ice. Structures adherent to the trachea and lungs were carefully removed and the external surface of the lungs rinsed with cold tap water.

To collect the alveolar cells, 50 ml of cold Hanks' solution (6) was injected into the clamped trachea. It was then unclamped and the lungs gently kneaded. This procedure was repeated several times and the washings kept on ice. The cells were harvested by centrifuging the washings at 5000 *g* for 10 min at 0°. They were resus-

pended in 5 volumes (v/wet wt) of Hanks' solution and this suspension was used to make the various reaction mixtures. Examination with the light microscope demonstrated that greater than 85% of these cells were large mononuclear cells, and remaining cells were lymphocytes, polymorphonuclear leukocytes, and red cells. Electron microscopy demonstrated that about one-half of the cells had abundant lamellar bodies characteristic of the so-called type II granular pneumocytes (7).

Incubation of alveolar cells. All incubations were performed the day the cells were collected, in glass vessels at 37° at pH 7.4 using Hanks' solution as the suspending medium with air as the gas phase. In the experiments on the subcellular site of incorporation of radioactive substrates, the reactions were stopped by adding equal volumes of ice cold Hanks' solution and by maintaining the mixture of 0–4°. Cells to be pulse labeled with glucosamine-1-¹⁴C hydrochloride were incubated with this substrate for 40 min in Hanks' medium. The reaction was stopped and the cells were washed three times with large volumes of this medium, resuspended, and then re-incubated for varying time intervals. In all other experiments the reactions were stopped by the addition of cold 20% trichloroacetic acid (TCA). The protein was isolated and assayed for radioactivity as described below. The radioactive materials used were DL-leucine-1-¹⁴C,¹ D-glucosamine-1-¹⁴C,¹ D-galactose-1-¹⁴C,¹ and D-mannose-1-¹⁴C.¹

Subcellular fractionation. All procedures were performed between 0 and 4°. After incubation, the cells were harvested by centrifugation at 1000 *g* for 10 min and resuspended in 10 volumes (v/wet wt of cells) of medium A, which contained 0.35 M sucrose, 0.035 M KHCO₃, 0.004 M MgCl₂, and 0.025 M KCl (8). Cell disruption was effected by homogenization in a Potter-Elvehjem homogenizer for 3 30-sec periods. The homogenate was centrifuged for 10 min at 1500 *g*; the supernatant fluid was removed and saved and the sediment suspended in 3 ml of medium A, homogenized, and centrifuged in an identical fashion. The sediment was then discarded, after which the two supernatant fluids were combined and centrifuged for 10 min at 15,000 *g*. The resulting supernatant material was diluted between two and three times (v/v) with medium B, which contained 0.9 M sucrose, 0.004 M MgCl₂, and 0.025 M KCl (8), and centrifuged for 60 min at 105,000 *g* in a Beckman model L ultracentrifuge² to sediment the microsomal fraction. The 105,000 *g* microsomal pellets were washed twice with medium A and suspended by gentle homogenization in this medium. The microsomal supernatant material was designated supernatant fraction.

Protein isolation. To precipitate the protein, equal volumes of cold 20% TCA were added to the sample to be assayed for radioactivity; these mixtures were allowed to stand in ice for 20 min, and were then centrifuged. The supernatant fluid was decanted, the sedi-

ment was washed three times with cold 5% TCA, and then extracted with 2.0 ml of ethyl ether-acetone-chloroform (2:2:1 v/v) at 50° for 30 min. Organic solvents were removed by decantation, the residues were air dried, and extracted with 5% TCA at 90° for 15 min. The supernatant fluid was removed and saved for determination of ribonucleic acid (RNA). The protein precipitates were dissolved in 0.2–1.0 ml of 0.2 M NaOH by heating at 80° for 30 min.

Assay for radioactivity. Radioactivity was measured in 0.1–0.2 ml samples of the NaOH solution of proteins. To these, 1–2 ml of hydroxide of hyamine (*p*-diisobutylcresoxyethoxyethyl) dimethylbenzylammonium hydroxide in methanol,³ and subsequently 10 ml of toluene containing 1 mg/ml of PPO (2,5-diphenyloxazole)¹ and 0.25 mg/ml of POPOP (*p*-bis[2-(5-phenyloxazolyl)]-benzene) were added.¹ Counting efficiency was determined by recounting each sample after adding toluene-¹⁴C as an internal standard. All measurements were corrected for efficiency and expressed as disintegrations per minute (dpm). Sufficient counts were obtained to have a counting accuracy of 97%.

Isolation of the sugar components. A quantity of cells (5 ml) was incubated at 37° for 2½ hr with 40 μc of glucosamine-1-¹⁴C. The reaction was stopped by chilling and the cells harvested by centrifugation at 1000 *g* for 10 min, whereupon they were homogenized in 4 ml of water. TCA (20%) was added in a volume equal to the total volume of the homogenate and the mixture allowed to stand on ice for 20 min. The insoluble material was collected by centrifugation and washed several times with water. The residue was hydrolyzed at 80° for one hr in 0.1 N HCl, and the remaining residue was hydrolyzed in 2.0 N HCl at 100° for 6 hr. HCl was removed from both hydrolysates by repeatedly bringing them to dryness in a rotary evaporator at 25°. Material remaining was suspended in water for paper chromatography. This procedure was repeated using galactose-1-¹⁴C as the substrate.

The material was subjected to descending chromatography on Whatman No. 1 paper with butanol-pyridine-water (6:4:3 by volume) and ethyl acetate-pyridine-water (10:4:3 by volume). Glucosamine, galactose, mannose, glucose, fucose, *N*-acetyl-glucosamine, and *N*-acetyl-galactosamine were run as standards.⁴ Sugars were detected with silver nitrate (9) and the hexosamines and *N*-acetylhexosamines by the method of Partridge (10). Radioactivity was located by an actigraph III paper-strip scanner.⁵

Chemical methods. Ribonucleic acid was measured using soluble yeast RNA⁶ as a standard (11). Crystallized bovine albumin⁴ was used as the standard for the protein determinations (12). Estimates of variances were carried out by conventional methods (13).

³ Packard Instrument Co., Inc., Downers Grove, Illinois.

⁴ Mann Research Laboratories, Inc., N. Y.

⁵ Nuclear Chicago Corp., Des Plaines, Ill.

⁶ General Biochemicals, Chagrin Falls, Ohio.

¹ New England Nuclear Corp., Boston, Mass.

² Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.

RESULTS

Time curve of substrate incorporation. Fig. 1 shows specific activities of protein of the cells from one rabbit, incubated in one instance with DL-leucine-1-¹⁴C and in the other with D-glucosamine-1-¹⁴C. Leucine incorporation is linear for 40 min after which it rises asymptotically. The curve of glucosamine incorporation rises less steeply initially, the ascending limb assuming a sigmoid shape. This delay in initial rise in specific activity when glucosamine is the labeled substrate rather than leucine is similar to that reported by Spiro and Spiro for incorporation of these substrates into thyroglobulin (14). They suggested this was due to the larger number of intermediate reactions and pools traversed by the sugar, compared to leucine, before incorporation into protein.

pH optimum for glucosamine incorporation. Maximum incorporation of glucosamine takes place at about pH 7.4. The buffers were acetate at pH 6.4 and 6.8, phosphate at 7.0 and 7.5, Tris-maleate at pH 7.6, 7.7, and 7.8. No change in pH took place during the 20 min incubation period as measured directly by a micro pH electrode.

Effect of puromycin on incorporation. Previous work in this laboratory demonstrated that in these

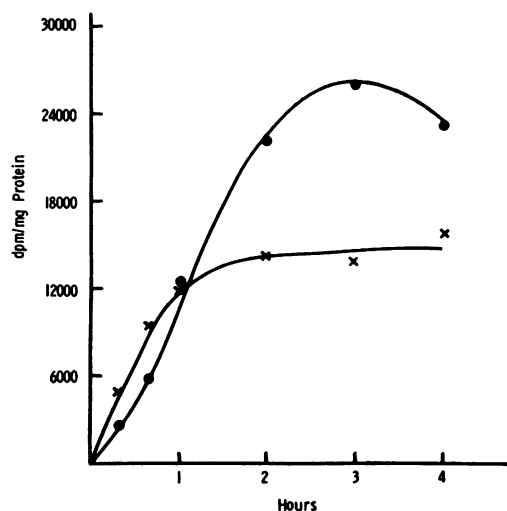


FIGURE 1 Time course of substrate incorporation into alveolar cell protein. Suspensions: 0.2 ml of alveolar cells in Hanks' solution, pH 7.4, was incubated for the times indicated at 37° with either D-glucosamine-1-¹⁴C (SA, 10.9 mc/mmmole; 0.11 μ mole, ●—●) or DL-leucine-1-¹⁴C (4.77 mc/mmmole; 0.11 μ mole, ×—×) in a final volume of 0.225 ml.

TABLE I

Effect of Puromycin on Incorporation of Radioactivity into Alveolar Cell Protein with Glucosamine-1-¹⁴C as Substrate

Time	Protein specific activity		
	Control	Puromycin treated	Inhibition
<i>min</i>	<i>dpm/mg</i>		<i>%</i>
20	116	92	21
40	434	258	41
60	718	267	63

cells, puromycin (2.5×10^{-8} M) inhibits leucine incorporation into protein by greater than 99% (15). Table I reveals the effect of puromycin dihydrochloride⁷ in the same concentration on glucosamine incorporation. Maximum inhibition was seen after 60 min of incubation. These data are consistent with that reported by others (14, 16–21), indicating incomplete inhibition of glucosamine incorporation into protein and suggesting that some of the incorporation occurs beyond the ribosomal level.

Subcellular fractionation. Because of the heterogeneity of the material sedimented at 15,000 *g*, we have named this fraction by the centrifugal force used to sediment the particles rather than assigning it a structural designation. In an attempt to delineate the degree to which the fractionation procedure varied from one incubation mixture to another during an experiment, the total RNA and total protein content of each subcellular fraction was measured for each incubation mixture. The RNA:protein ratio of each subcellular fraction was calculated for each incubation mixture. From this was derived the mean RNA:protein ratio \pm SD for each subcellular fraction in each experiment. In the experiment where glucosamine-1-¹⁴C was the substrate, the mean RNA:protein ratio \pm 1SD for the 15,000 *g*, microsomal, and supernatant fractions were 0.05 ± 0.01 , 0.15 ± 0.04 , and 0.01 ± 0.01 , respectively. With galactose-1-¹⁴C these values were 0.05 ± 0.01 , 0.14 ± 0.03 , and 0.03 ± 0.02 , whereas with mannose-1-¹⁴C as substrate the ratio of RNA:protein in these fractions was 0.05 ± 0.01 , 0.22 ± 0.07 , and 0.03 ± 0.02 , respectively. These values indicate rea-

⁷ Nutritional Biochemical Corp., Cleveland, Ohio.

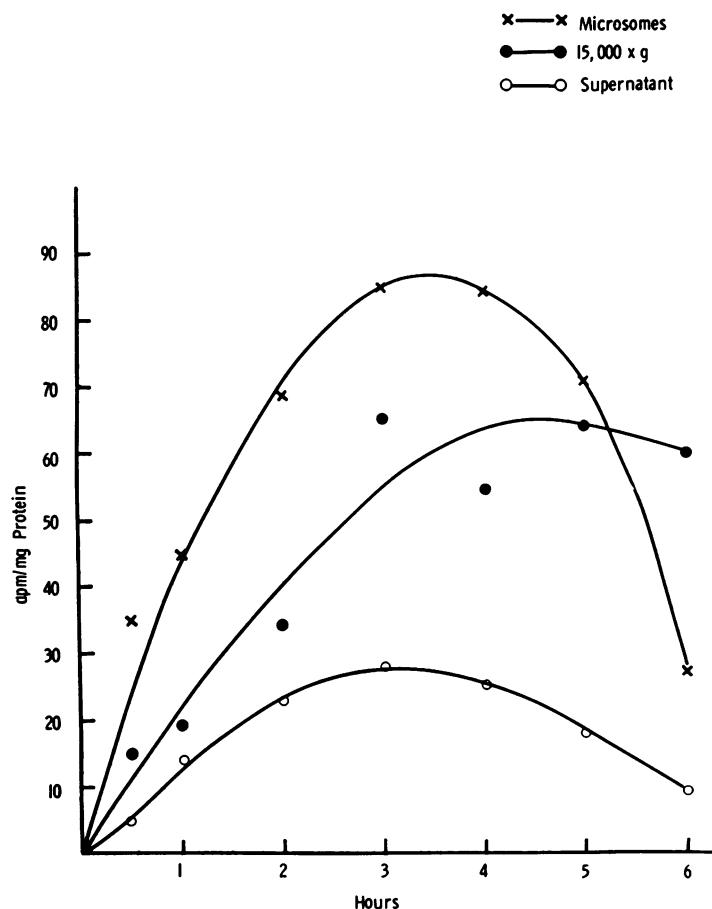


FIGURE 2 Time course of protein specific activity in subcellular fractions of alveolar cells during incubation with D-glucosamine-1-¹⁴C. 2-ml suspensions of alveolar cells were incubated at 37° and pH 7.4 with 0.025 ml of D-glucosamine-1-¹⁴C (SA, 10.36 mc/mmole; 0.12 μmole) for the times indicated.

sonable uniformity of subcellular fractionation among the various incubation mixtures of each experiment.

Subcellular site of incorporation of D-glucosamine-1-¹⁴C. Fig. 2 demonstrates the variation with time of protein specific activity in the various subcellular fractions when D-glucosamine-1-¹⁴C was the substrate. Initially, the microsomal fraction had the highest specific activity with a peak occurring between 3-4 hr. Thereafter, the specific activity of the protein of this fraction fell and the curve crossed that of the 15,000 g fraction. The shape of the curves satisfies the criteria for a precursor-product relationship as described by Zilvermit, Entenman, and Fishler (5). Experiments with galactose-1-¹⁴C and mannose-1-¹⁴C as substrates demonstrated similar curves.

Fig. 3 reveals the total protein-bound radioactivity in subcellular fractions of alveolar cells during incubation with D-glucosamine-1-¹⁴C. 2-ml suspensions of alveolar cells were incubated at 37° and pH 7.4 with 0.025 ml of D-glucosamine-1-¹⁴C (SA, 10.9 mc/mole; 0.11 μmole) for the times indicated.

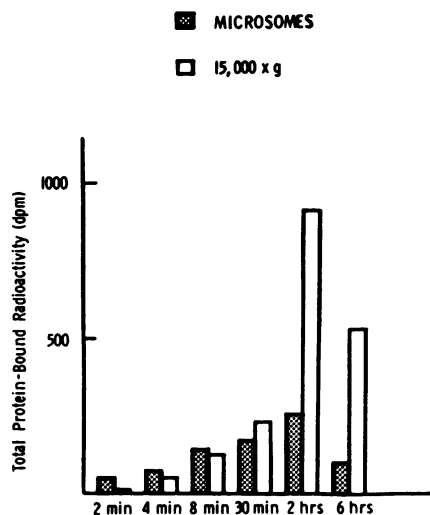


FIGURE 3 Distribution of total protein-bound radioactivity in subcellular fractions of alveolar cells during incubation with D-glucosamine-1-¹⁴C. 2-ml suspensions of alveolar cells were incubated at 37° and pH 7.4 with 0.025 ml of D-glucosamine-1-¹⁴C (SA, 10.9 mc/mole; 0.11 μmole) for the times indicated.

crossomal fraction. By 30 min, the 15,000 *g* fraction contained most of the protein-bound radioactivity. At 2 hr the total protein-bound radioactivity was threefold greater in the 15,000 *g* fraction compared to the microsomal fraction whereas at 6 hr, although both fractions had less protein-bound radioactivity, it was now about 5.5 times greater in the 15,000 *g* fraction than in the microsomal fraction.

Since these data are consistent with either incorporation of radioactivity into protein at the microsomal level with subsequent transfer to particles sedimenting at 15,000 *g*, or incorporation at more than one subcellular site but at different rates, the result of pulse labeling the cells was determined. Fig. 4 shows the time course of the protein specific activity after a 40 min pulsed incubation of D-glucosamine-1-¹⁴C with alveolar cells. The specific activity of the cell homogenate protein indicates that net incorporation ceased after the 1st hr after the pulse. This corresponds with the peak of microsomal protein specific activity, after which the specific activity of the microsomal protein fell below that of 15,000 *g* fraction. The specific activity of the protein of this

latter fraction continued to rise for 2 hr after the pulse and remained above that of the microsomal fraction, clearly suggesting a precursor-product relationship. The protein specific activity in the supernatant fluid remains relatively constant throughout most of the experiment.

Nature of isotopic compounds appearing in alveolar cell protein. In the previous paragraphs it has been implied that the sugars were incorporated intact into protein. It is necessary to determine this because the possibility exists that the radioactivity in the sugar has been converted into radioactive amino acids and hence perhaps protein turnover is also being measured. Thus, one could explain the data in Table I by suggesting that, at early times incorporation of sugar (not inhibited by puromycin) is being measured, whereas at latter times the incorporation of amino acids derived from the radioactive sugars is being measured and, hence the greater inhibition by puromycin. It will be noted in Table I that incorporation of radioactivity has virtually ceased between the 40- and 60-min points. If this inhibition of incorporation of radioactivity is due to inhibition of amino acid incorporation by puromycin

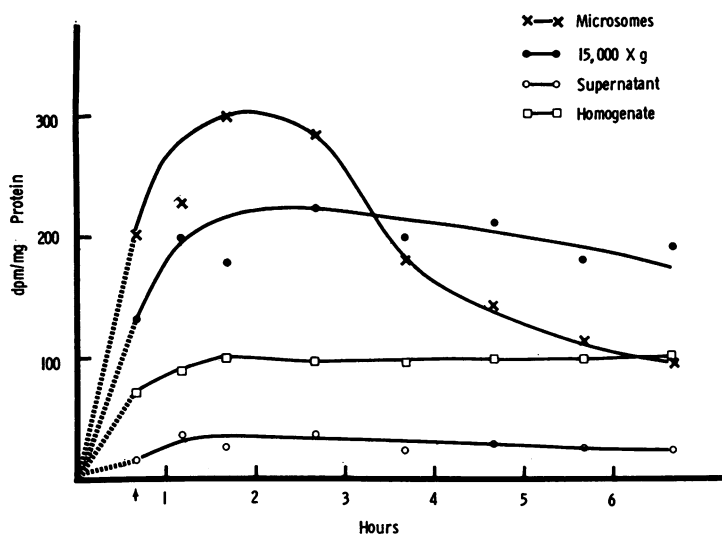
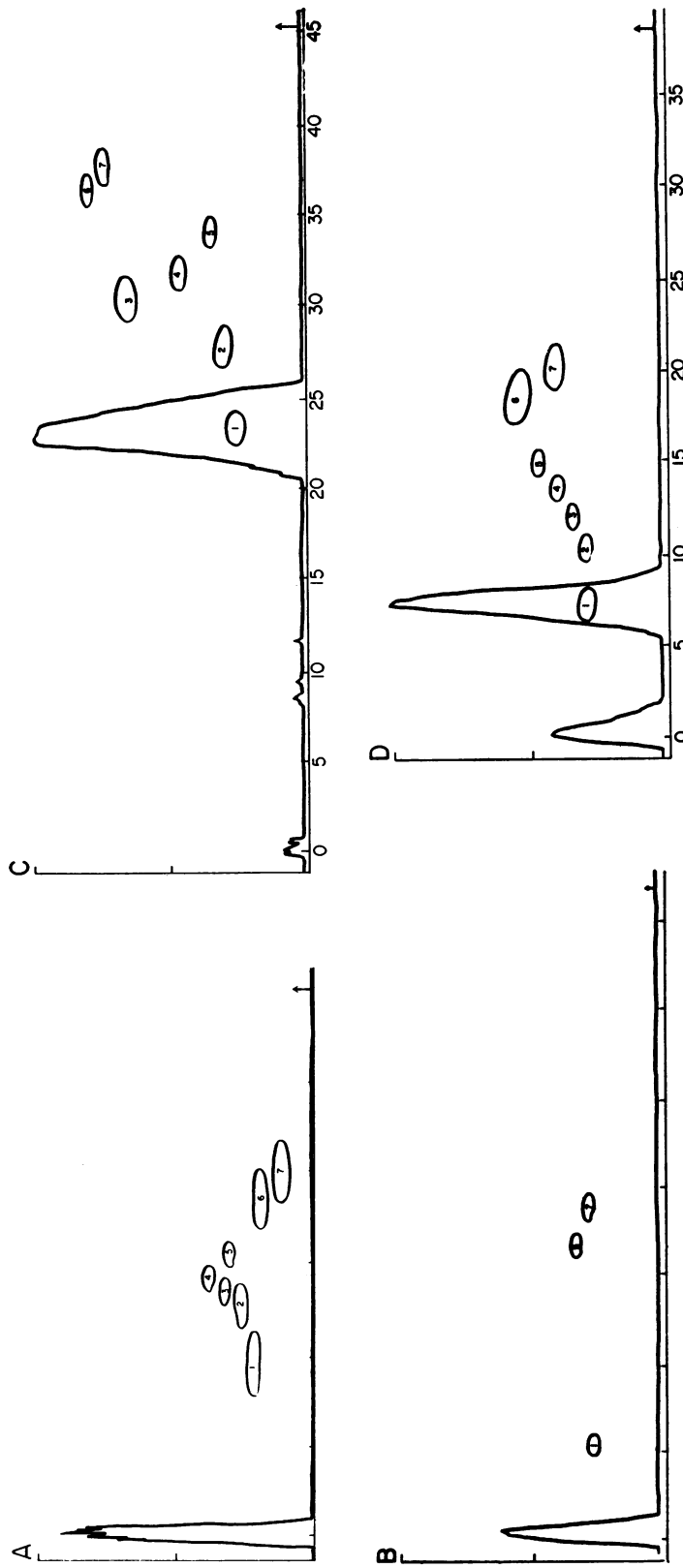


FIGURE 4. Time course of protein specific activity in subcellular fractions of alveolar cells after a pulsed incubation with D-glucosamine-1-¹⁴C. A 20 ml suspension of alveolar cells in Hanks' medium was incubated at 37° and pH 7.4 with 0.5 ml of D-glucosamine-1-¹⁴C (SA, 10.9 mc/mmole; 2.2 μ moles) for 40 min. The reaction was stopped by chilling the cells which were three times washed with cold Hanks' medium and resuspended to a final volume of 20 ml. 2-ml samples were then reincubated for the times indicated. Arrow indicates end of pulse; ---- indicates presumed specific activity during pulsed incubation with D-glucosamine.



The solvent for graphs A and C were ethyl acetate-pyridine-water (10:4:3) and for B and D, butanol-pyridine-water (6:4:3). Full scale for graphs A-D are: 50, 300, 50, and 300 cpm, respectively. The numerals on the abscissa represent centimeters from the origin and the arrows indicate the solvent front. Circles represent location of standards. Numbers within circles in ascending numerical order represent glucosamine galactose, glucose, mannose, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine.

FIGURE 5 Paper chromatography of material released by acid hydrolysis of trichloroacetic acid insoluble material. 5 ml of alveolar cells were incubated at 37° in Hanks' solution for 2½ hr with 40 µc of glucosamine-1-¹⁴C and the reactions then stopped with 20% TCA. Graphs A and B represent descending chromatography of the hydrolysate obtained by hydrolysis of the TCA insoluble material in 0.1 N HCl at 80° for 1 hr. Graphs C and D represent chromatography of the hydrolysate from the 6 hr hydrolysis in 2 N HCl at 100°.

after this 40–60-minute period, and if conversion to amino acids with subsequent incorporation into protein plays a significant role in our data, the amino acids should be seen in the material to be assayed for radioactivity after 40–60 min of incubation. To evaluate this possibility, we examined the nature of the isotopic material appearing in alveolar cell protein after a 2½ hr incubation with glucosamine-1-¹⁴C and also galactose-1-¹⁴C. In experiments where the TCA insoluble material was hydrolyzed in 0.1 N HCl for 1 hr and chromatographed after removing the HCl, all the radioactivity remained at the origin (Fig. 5). However, when the material hydrolyzed for 6 hr in 2.0 N HCl, and chromatographed, two radioactive peaks were always detected. One small peak remained at the origin and probably represents unhydrolyzed protein. The other radioactive substance moved at the same *R_f* as its nonradioactive standard (Fig. 5). The same results were obtained when galactose-1-¹⁴C was the substrate. This indicates that these radioactive substrates were incorporated into protein largely unchanged and that conversion to amino acids did not take place to any detectable extent. Based on previous work by others (20–23), it is probable that the incorporation was through a nucleotide intermediate, but that these compounds were not detected by the isolation procedures used in this study.

DISCUSSION

Studies on the biosynthesis of proteins in pancreas and liver have produced strong evidence suggesting intracellular transport of secretory proteins (24, 25). This has been particularly well studied in the pancreas by Palade and Siekevitz and their associates who have postulated that digestive enzymes are synthesized on ribosomes attached to the rough endoplasmic reticulum. These enzymes are subsequently transported across this membrane to enter the cisternae of the rough endoplasmic reticulum. They later appear at the periphery of the Golgi region, then aggregate in its central condensing vacuoles, after which by progressive filling and condensation of their content, finally become zymogen granules (26–29).

In this regard, it is important to emphasize that the type II granular pneumocytes, which are known to be present in lung washings (3), are thought to perform a secretory function, in particular, to form at least part of the acellular al-

veolar lining layer (30). Recent electron microscopic studies have demonstrated the bulk release of material thought to be protein by these cells (31). Sorokin has interpreted the ultrastructural organization of the type II granular pneumocyte to be consistent with the thesis that intracellular transport and extracellular release of proteinaceous material takes place by these cells (32). The kinetics of the subcellular distribution of radioactivity into alveolar cell protein (Figs. 3–5) may be interpreted in several ways. The first of which, consistent in a general way with Sorokin's thesis of transport, indicates that the sugars are incorporated into protein at the microsomal level with subsequent transfer of this newly formed radioactive protein to particles sedimenting at 15,000 *g*. However, the kinetic data are also consistent with simultaneous incorporation at two or more subcellular sites, but at different rates. Finally, the observed kinetics of incorporation may be due to different degrees of stability of the labeled protein in the different subcellular fractions.

Most previous studies of carbohydrate incorporation into protein have used liver, thyroid, and Ehrlich's tumor ascites cells. Of these studies, those concerned with the subcellular site of incorporation of sugars into protein have indicated that the carbohydrate components of newly formed protein are added to the protein core at the microsomal level (16–18, 33–36). This thesis is based primarily on the kinetics of the subcellular distribution of radioactivity. These findings in other tissues indirectly favor the single-site hypothesis for alveolar cells. Furthermore, in light of the kinetics of the pulse-labeling experiments (Fig. 4), the multiple-site alternative would require the unlikely possibility that amino sugar incorporation into protein by particles sedimenting at 15,000 *g* increases at the same rate that incorporation into microsomal protein falls, in order to maintain net synthesis in the cell constant as indicated by the constancy of the protein specific activity in the cell homogenate. Because of these considerations, the alternative, which indicates intracellular protein transport in alveolar cells, is considered most probable. However, the data do not eliminate the possibility that the observed kinetics might be due to different degrees of stability of the labeled fractions.

Although the cells studied are a mixed population, rather than only type II granular pneumocytes (3), it is still intriguing to relate this evidence for intracellular protein transport to the production of the acellular substance lining the alveoli. This lining layer is thought to contain surface active materials (lung surfactants) which are responsible for much of the stability of the small pulmonary air spaces (37). However, the precise contribution of the lung surfactants to the entire acellular lining layer is not clear. Pattle and Thomas (38) and Klaus, Clements, and Havel (39) suggested that this surface-active material is a lipid-protein complex. Biochemical analyses of lung homogenates or washings obtained by lung lavage have shown that most of the surface tension-lowering ability of this presumed complex resides in its phospholipid components, particularly dipalmitoyl lecithin (38-41). Little is known about the biochemical makeup of the protein component of these lung surfactants beyond the demonstration by Abrams and Taylor (42) that it has the electrophoretic mobility of an alpha globulin. The histochemical studies of Macklin (43), Chase (44), and Groniowski and Biczyskova (45) suggest that carbohydrate-protein moieties are present in the alveolar lining layer, but it is not known if these substances are part of the protein moiety of lung surfactants. However, these findings are particularly interesting because granular pneumocytes thought to synthesize and secrete surfactant contain cytoplasmic inclusions (lamellar bodies), which are thought to be rich in carbohydrate-protein material because they are diastase-resistant, periodic acid-Schiff positive (46). These lamellar bodies, whose presence is associated with the ability of lung extracts to lower surface tension (47-49), are present in the same subcellular fraction of lung homogenates which contain most of the surface activity (50). One could thus speculate that part of the lung surfactant and, hence the alveolar lining layer, is a carbohydrate-protein moiety synthesized at the microsomal level and transported to the lamellar bodies, known to be in the 15,000 g fraction. This material would then be released from the cell to form the alveolar lining layer. Furthermore, since, in the liver at least, the enzymes required in the final stages of lecithin biosynthesis are microsomal enzymes (51, 52), some type of intracellular transport seems to be required for the localization of the surface ac-

tivity in the fraction sedimenting at a lower centrifugal force than the microsomes. However, the cells commonly referred to as alveolar macrophages may be responsible for the present findings. Studies with radioautography may answer this question.

ACKNOWLEDGMENTS

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