

## MACROPHAGE PLASMA MEMBRANE

### II. STUDIES ON SYNTHESIS AND TURNOVER OF PROTEIN CONSTITUENTS\*

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Alveolar macrophages have many features which make them particularly suitable subjects for studies on plasma membrane biogenesis and turnover: they are readily obtained in large numbers as an essentially pure population from the pulmonary tract of Calmette-Guerin bacillus (BCG)-stimulated rabbits (1); plasma membranes of excellent purity can be separated from them (2); they can be maintained *in vitro* with little or no cell division or cell death (3); and they actively engage in pinocytosis and phagocytosis (4), activities of particular interest in relation to plasma membrane life history.

The previous paper presented our findings on the isolation of a highly purified plasma membrane preparation and the partial biochemical characterization of the protein components of the membrane. This report presents radioactive labeling studies on the biosynthesis and metabolic turnover rate of these macrophage plasma membrane proteins.

#### *Materials and Methods*

*Macrophages.*—Alveolar macrophages were obtained from BCG-stimulated rabbits as previously described (1, 5).

*Leucine Incorporation into Protein.*—The incorporation of  $^3\text{H}$ -leucine into trichloroacetic acid (TCA)-precipitable protein was studied essentially as described by Manchester and Young (6). The standard incubation mixture consisted of a suspension of twice-washed alveolar macrophages ( $1 \times 10^9$ ) at a concentration of  $1 \times 10^7$  cells/ml in minimal essential medium (MEM)<sup>1</sup> without leucine containing 10% dialyzed normal rabbit serum.  $^3\text{H}$ -leucine (5  $\mu\text{Ci/ml}$ , specific activity 19.7 Ci/mole) was added and the mixture incubated for an indicated time period with shaking at 37°C in an atmosphere of 95% O<sub>2</sub>:5% CO<sub>2</sub>. At the end of the incubation period, the mixture was placed in an ice bath and cold nonradioactive leucine was added to a final concentration of 10 mM. Incorporation of  $^3\text{H}$ -leucine into whole-cell

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<sup>1</sup> Abbreviation used in this paper: MEM, minimal essential medium.

protein was determined on a fraction of 0.5 ml of the incubation mixture containing  $5 \times 10^6$  cells. The cells were washed twice with MEM containing "cold" leucine. The cell button was precipitated by the addition of 3 ml of 10% TCA containing 10 mM nonradioactive leucine and the mixture heated to 90°C for 15 min. The resulting precipitate was separated by centrifugation, washed twice in 5% TCA containing 5 mM leucine, and once each with ethanol ether (1:1) and ether. The protein precipitate was dissolved in 1 N NaOH, and the radioactivity assayed using Bray's solution (7) in a Nuclear Chicago liquid scintillation spectrometer (Nuclear Chicago Corporation, Des Plaines, Ill.). Incorporation of  $^3\text{H}$ -leucine into the macrophage plasma membrane was determined by isolation of the plasma membrane of the remaining cells in the incubation mixture using the glutaraldehyde-fixation method (2). Samples of the lyophilized membrane preparation were dissolved in 1 N NaOH and the total radioactivity assayed as described above.

*Choline Incorporation Studies.*—The incorporation of  $^3\text{H}$ -choline into whole cell and membrane lipid was determined by adding to the incubation mixture 5  $\mu\text{Ci}^3\text{H}$  choline/ml (specific activity 72.7 mCi/mM). The MEM used in this system contained leucine. The reaction was terminated by placing the incubation mixture in an ice bath and adding nonradioactive choline to a final concentration of 10 mM. Incorporation of the isotope into the whole cells was determined on a fraction of  $5 \times 10^6$  cells of the final incubation mixture. The cells were washed twice in MEM containing 10 mM of unlabeled choline, precipitated with 10% TCA-choline solution, heated to 90°C for 15 min, and washed twice with the TCA solution. The final precipitate was extracted by the Folch method (8) and a sample of the lipid extract assayed for radioactivity using a toluene phosphor. Incorporation of the  $^3\text{H}$ -choline into the plasma membrane fraction was determined essentially as described above. The isolated plasma membrane fraction was subjected to Folch extraction and the lipid extract assayed for radioactivity.

*Glucosamine Incorporation Studies.*—The incorporation of  $^3\text{H}$ -glucosamine into whole cells and membrane protein was determined by adding to the incubation mixture 5  $\mu\text{Ci}^3\text{H}$ -glucosamine/ml (specific activity 1300 mCi/mM). The incubation mixture contained MEM with leucine. Processing and analysis were carried out essentially as described for the leucine studies except that the wash solutions contained 10 mM nonradioactive glucosamine.

*"Pulse-Chase" Experiments with  $^3\text{H}$ -Leucine.*—In these studies two incubation flasks were used, each containing an identical leucine incubation mixture. After 90 min of incubation, the reaction was terminated in flask A and the incorporation of  $^3\text{H}$ -leucine into whole-cell protein and plasma membrane protein determined as described above. The incubation mixture in flask B was placed in an ice bath and unlabeled leucine was added to a final concentration of 10 mM. The cells were spun down and washed twice with MEM containing 10 mM nonradioactive leucine and 2% rabbit serum. The cells were then incubated in MEM containing 10 mM unlabeled leucine and 10% rabbit serum. At the end of the chase period, the radioactivity of the whole cell and plasma membrane was determined. Cell viability at the termination of 3 hr and 6 hr chase periods was >95% as determined by trypan blue exclusion.

*Phagolysosomal Membrane  $^3\text{H}$ -Leucine Incorporation.*—In these studies, the cells in the incubation mixture were labeled by exposure for 90 min to  $^3\text{H}$ -leucine. The reaction was terminated and the cells washed as described above. The labeled cells ( $1 \times 10^6$ ) were then incubated in MEM containing 10 mM unlabeled leucine, 10% rabbit serum, and  $1 \times 10^{11}$  latex beads for 30 min. The phagolysosomal particles were separated (2) and the labeling pattern of the isolated interiorized membrane studied.

*Thin Layer Chromatography.*—A sample of Folch extract of membrane labeled with  $^3\text{H}$ -choline was concentrated under nitrogen and placed on a silica gel thin layer plate (silica gel 1-B, Baker-Flex, J. T. Baker Chemical Co., Phillipsburg, N. J.). Ascending chromatography was performed using chloroform:methanol:water (65:25:4). The chromatogram was cut into 1

cm strips and radioactivity assayed. Nonradioactive choline, lysolecithin, and phosphatidyl choline standards were also chromatographed on the same plate to serve as marker  $R_f$  values.

*Polyacrylamide Gel Electrophoresis.*—This was done as described in the previous paper (2). Scans of the stained polyacrylamide gels were performed using the Gilford linear transport gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Radioactivity labeling patterns of the membrane proteins were determined by slicing the gels and determining the radioactivity of the individual fractions. The individual fractions were dried for 18 hr at 37°C, solubilized in 0.1 ml  $H_2O_2$  and 2 ml Soluene (Packard Instrument Co., Zurich, Switzerland), and radioactivity determined using a toluene phosphor (9).

#### RESULTS

*$^3H$ -Leucine Incorporation into Plasma Membrane.*—The kinetics of incorporation of  $^3H$ -leucine into whole cell and plasma membrane protein are shown in

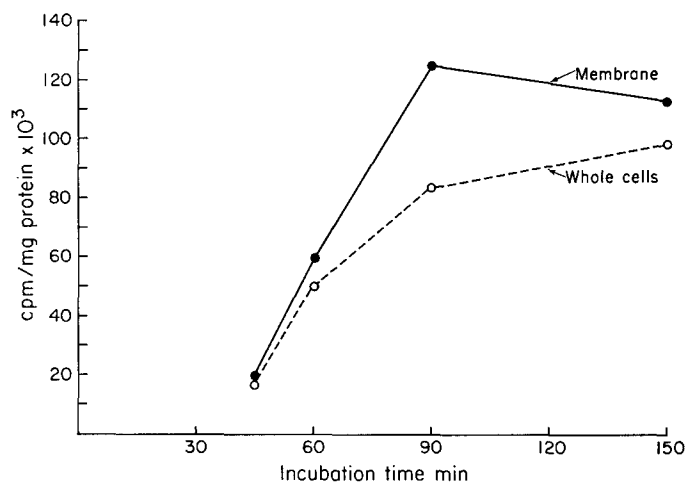


FIG. 1. Incorporation of  $^3H$ -leucine into whole cell and plasma membrane protein.

Fig. 1. Very little radioactivity was incorporated during the first 45 min of incubation. This lag period, which presumably was due to time required for transport into the cell and equilibration with the various intracellular pools, was followed by a relatively linear increase in labeling at up to 90 min of incubation. Thereafter the incorporation rate decreased. The amount of radioactivity incorporated into the plasma membrane at each time point represented 1–3% of the total label incorporated into whole cells.

The radioactive labeling pattern of individual plasma membrane proteins was studied by solubilizing the labeled membrane proteins in phenol-urea-acetic acid, separating them on acrylamide gel, slicing the gel into strips, dissolving the gel strips in hydrogen peroxide, and measuring radioactivity. Fig. 2 demonstrates the labeled-membrane protein patterns obtained from the cells

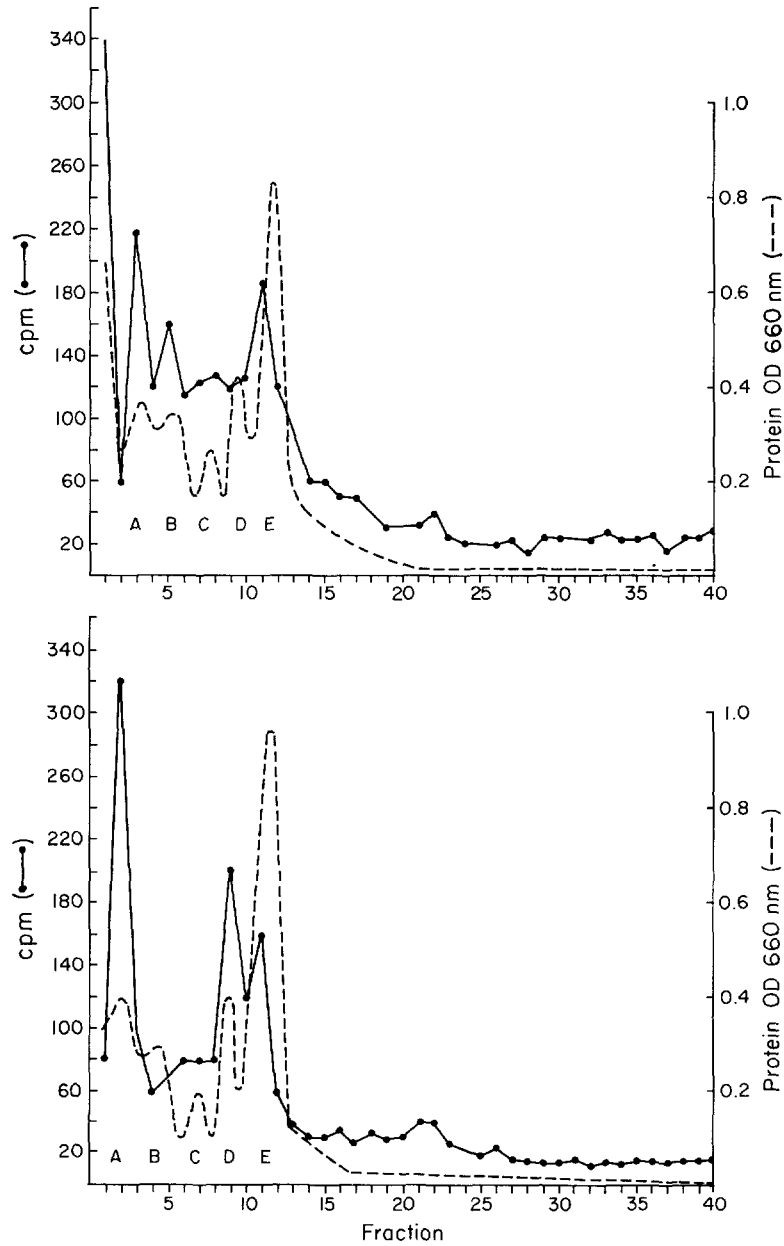


FIG. 2. Gel electrophoresis of labeled plasma membrane proteins demonstrating incorporation of  $^3\text{H}$ -leucine into specific membrane proteins. The upper section is an analysis on cells incubated in the medium containing radioactive leucine for 90 min. The lower section presents similar analysis on membranes from cells labeled for 150 min. Cathode toward the right.

of two separate experiments, one incubated for 90 min and the other for 150 min. The membrane proteins obtained from cells exposed to the isotope for 90 min revealed significant incorporation into bands A, B, and E. Membrane proteins from cells incubated with  $^3\text{H}$ -leucine for 150 min showed a different pattern in terms of the relative intensity of labeling among the various membrane components. The difference was particularly marked with respect to membrane protein bands B and D; B was less labeled and D more labeled after 150 min than was the case after 90 min of incubation. Since these analyses were performed at different times with different animals, some of the differences might have been due to experimental variability. Repeated studies, however, showed the patterns after a given time of incubation to be quite reproducible,

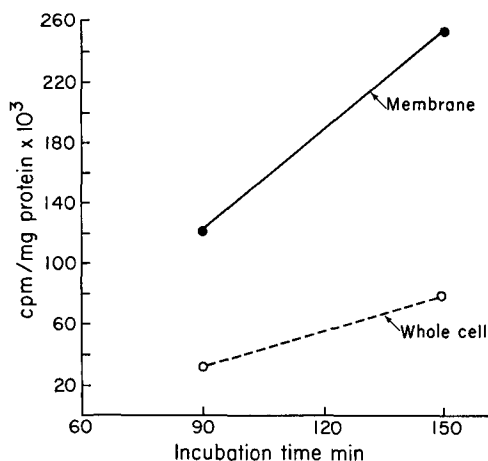


FIG. 3. Incorporation of  $^3\text{H}$ -choline into whole cell and plasma membrane lipid.

and confirmed the differences in band labeling between the 90 and 150 min specimens. The protein staining of these various plasma membrane preparations was reasonably constant. Gel electrophoresis of a mixture of nonradioactive membrane protein with  $^3\text{H}$ -leucine revealed no evidence of nonspecific adsorption of the label to the individual membrane components.

*$^3\text{H}$ -Choline Incorporation into Plasma Membrane.*—The incorporation of  $^3\text{H}$ -choline into whole cell and plasma membrane lipid is shown in Fig. 3. Of interest was the fact that the plasma membrane lipid displayed a higher specific radioactivity than did the whole cell lipid. Thin layer chromatography of the radioactive lipid fraction derived from the isolated plasma membrane preparation revealed that the label was primarily incorporated into lecithin (Fig. 4). A smaller amount of labeled material was detected in an area migrating with an  $R_f$  typical of lysolecithin.

The  $^3\text{H}$ -choline labeling pattern of the individual plasma membrane proteins was investigated by gel electrophoretic analysis of the membrane proteins obtained from cells incubated with the isotope for 150 min (Fig. 5). Some radioactive label remained at the origin of the gel. Of the proteins migrating into the gel only the two most rapidly migrating bands D and E showed evidence of incorporation of the radioactive label. Gel electrophoresis of a mixture of nonradioactive membrane protein with  $^3\text{H}$ -choline revealed no evidence of nonspecific adsorption of the label to the individual membrane components.

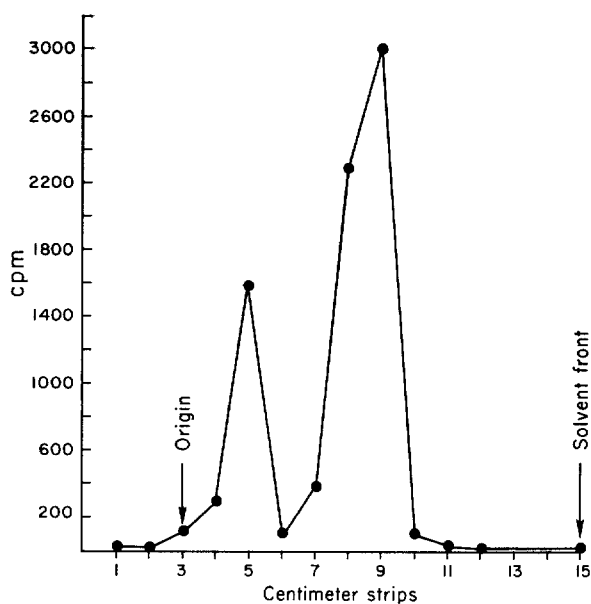


FIG. 4. Thin layer chromatogram of the radioactive lipid fraction obtained from plasma membranes isolated from cells incubated with tritiated choline. The major faster moving fraction migrated with an  $R_f$  value identical to lecithin. The slower moving fraction moved with an  $R_f$  identical to a lysolecithin marker. Choline remained at the origin.

*$^3\text{H}$ -Glucosamine Incorporation into Plasma Membrane*—The  $^3\text{H}$ -glucosamine labeling pattern of the individual plasma membrane proteins was demonstrated by gel electrophoretic analysis of the membrane proteins obtained from cells incubated with the isotope for 90 min (Fig. 6). Two protein bands, A and E, showed incorporation of the label. In addition a significant portion of the radioactivity remained at the origin of the gel. Gel electrophoresis of a mixture of nonradioactive membrane protein with  $^3\text{H}$ -glucosamine revealed no evidence of nonspecific adsorption of the label to the individual membrane protein migrating into the gel.

*Pulse-Chase Experiments with  $^3\text{H}$ -Leucine*.—These experiments were done to

obtain information on the turnover rate of the plasma membrane proteins. The alveolar macrophages were exposed to radioactive label for 90 min, then washed thoroughly to remove extracellular label and chased by incubation for various time periods in medium containing an excess of nonradioactive leucine. The results of separate experiments utilizing chase periods of 3 and 6 hr are shown in Fig. 7. The calculated half-life of the plasma membrane protein in both experiments was approximately the same, 7-8 hr. The calculated half-life for the whole-cell protein was slightly longer, 9-11 hr.

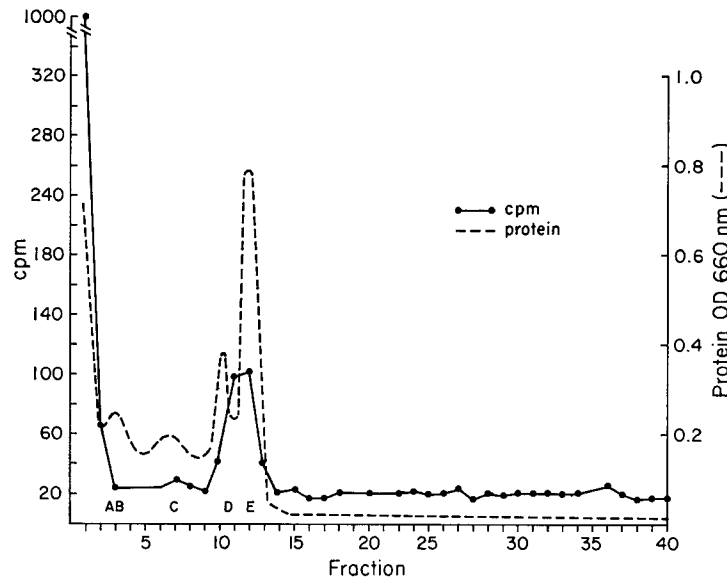


FIG. 5. Gel electrophoresis of plasma membrane proteins after incubation of whole cells in the medium containing  $^3\text{H}$ -choline for 150 min. Radiolabel is present in two bands migrating rapidly toward the cathode at the right.

In order to study the turnover of individual membrane proteins, the radioactivity labeling pattern as determined by gel electrophoresis was compared from membranes derived from cells incubated with the radioactive label for 90 min and from cells subsequently incubated in a nonradioactive medium. The results of a 6 hr chase experiment are shown in Fig. 8. The radioactive labeling pattern following the 90 min exposure was similar to the previous 90 min  $^3\text{H}$ -leucine incorporation study. Protein bands A, B, and E showed the highest relative degree of incorporation. As in the previous study, a significant amount of radioactive label remained at the origin of the gel. The labeling pattern of the plasma membrane preparation obtained from the cells after the 6 hr chase period revealed that the radioactivity of each of the major protein peaks had

decreased to approximately the same extent. Of some interest was the appearance of a small rapidly moving band of increased radioactivity in the gels of the 6 hr chase preparation. This band (fraction 22) was not present in the gel of the 90 min pulse preparation, and did not stain for protein.

*<sup>3</sup>H-Leucine Incorporation into Phagolysosomal Membrane.*—The radioactivity labeling pattern of the interiorized plasma membrane obtained from phagolysos-

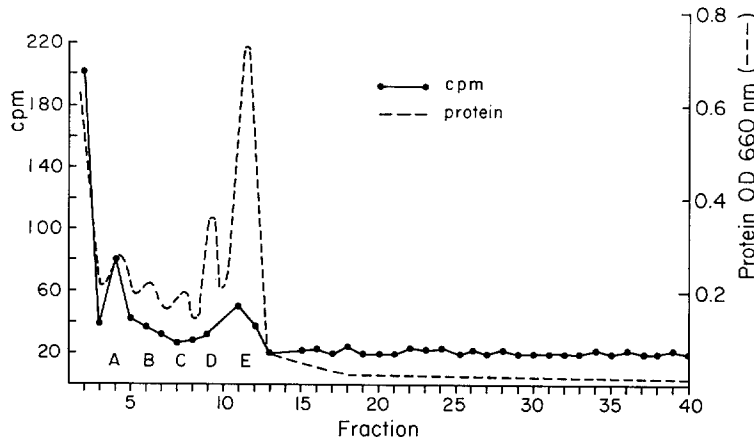


FIG. 6. Gel electrophoresis of plasma membrane proteins demonstrating incorporation of radiolabel into specific membrane proteins after incubation of whole cells in the medium containing  $^3\text{H}$ -glucosamine for 90 min. The cathode is toward the right.

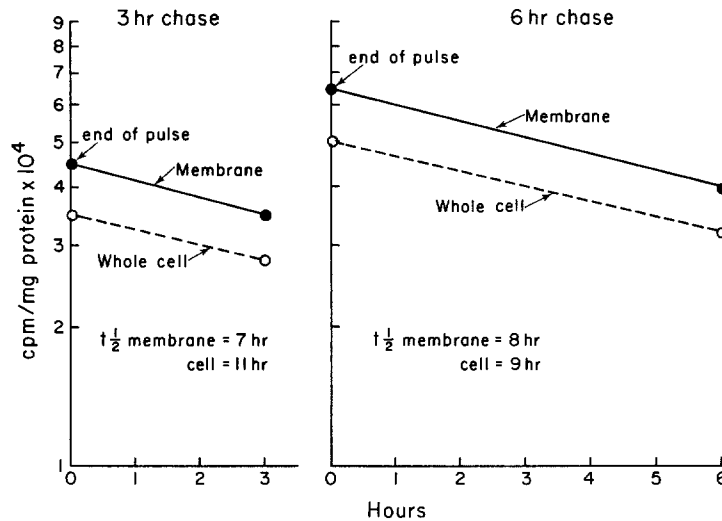


FIG. 7. Pulse-Chase experiments demonstrating the rapid plasma membrane turnover.



somal particles isolated from cells incubated with  $^3\text{H}$ -leucine and then allowed to ingest polystyrene particles is shown in Fig. 9. The phagolysosomal membrane protein pattern generally resembled the plasma membrane protein pattern. Protein bands A, B, C, and E displayed similar or identical electro-

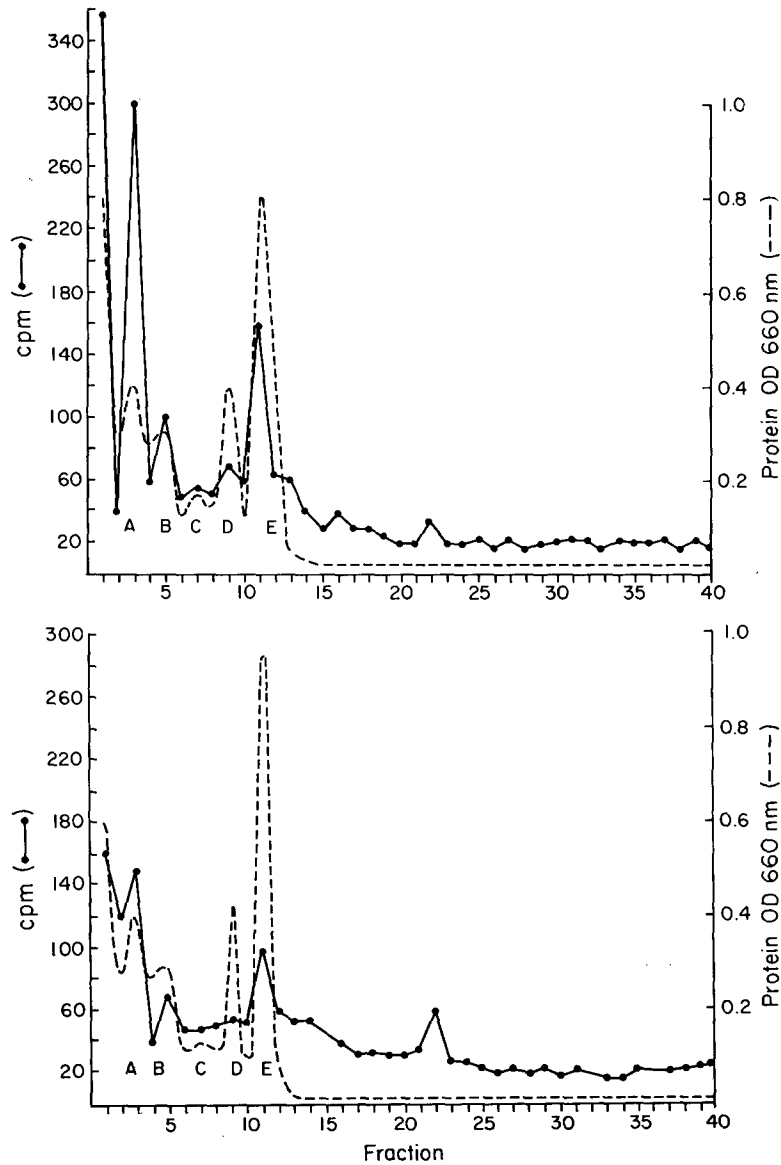


FIG. 8. Gel electrophoresis of labeled plasma membrane proteins obtained from the cells exposed to  $^3\text{H}$ -leucine for 90 min (upper section) and subsequently incubated in a nonradioactive medium for 6 hr (lower section). The cathode is toward the right.

phoretic mobility in the phagolysosomal membrane preparation and in the plasma membrane preparation. However protein D of the plasma membrane preparation was not seen in the phagolysosomal membrane protein preparation. The radioactive labeling pattern of the phagolysosomal membrane proteins showed evidence of incorporation of  $^3\text{H}$ -leucine into the several individual protein components. Band C showed the heaviest protein staining and also showed the greatest amount of radioactive labeling. Phagolysosomal membranes con-

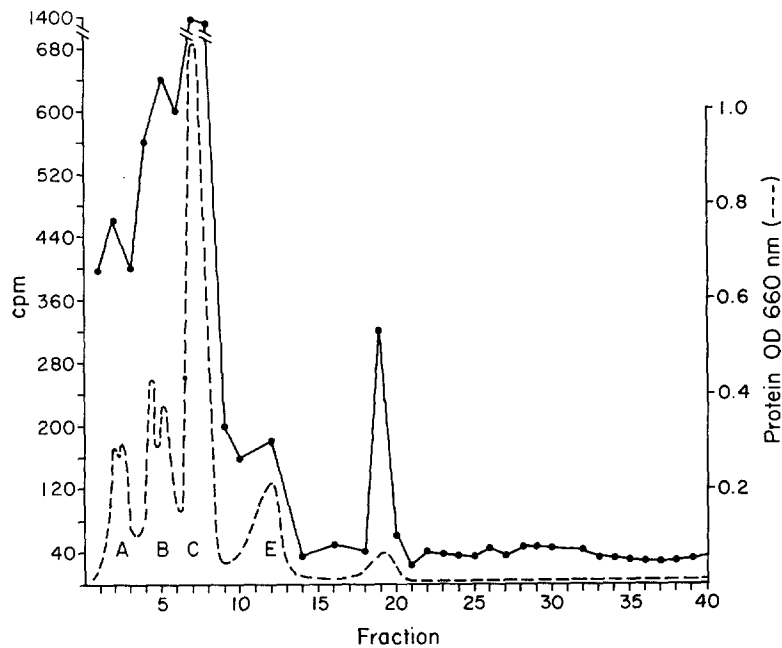


FIG. 9. Phagolysosomal membranes. Gel electrophoresis of interiorized plasma membrane proteins obtained from phagolysosomal particles isolated from cells incubated with  $^3\text{H}$ -leucine and then allowed to ingest polystyrene particles.

tained a rapidly moving protein band (fraction 19) barely detectable on the protein scan but showing high degree of radioactivity; no comparable area was found in the gel electrophoretic analyses of the plasma membrane proteins.

#### DISCUSSION

Some of the limitations of the present studies should be pointed out. They deal with only one class of major components in the plasma membrane, namely protein, and it is entirely possible that the lipid membrane constituents might exhibit somewhat different behavior. Furthermore, technical problems make it impossible to study biogenesis and turnover of all of the membrane proteins.

The isolated membranes are only about two-thirds soluble in phenol-urea-acetic acid, and a small fraction of the solubilized material is aggregated or otherwise altered so that it does not migrate into the acrylamide gel. Thus the final analyses are made on approximately 50% of the membrane proteins. However a number of components are resolved in the gels, and the findings are quite reproducible so that we feel certain that the results do shed light on plasma membrane synthesis and degradation in macrophages.

The incorporation of radioactive leucine into membrane protein by macrophages is rapid and easily demonstrated. The techniques employed do not allow precise quantitations of this synthetic step, i.e. per cent of membrane synthesized per hour, but it is possible to determine whether the individual protein components become labeled at the same or at different rates. These components display a heterogeneous labeling rate pattern with time as may be seen in a comparison of bands B and D at 90 and 150 min as shown in Fig. 2. The findings thus suggest that the macrophage plasma membrane proteins are synthesized and assembled independently of one another. The biogenesis of this structure is apparently a multistep process, probably involving the formation first of a membrane basic structure or matrix which is subsequently further molded or modified by the synthesis and insertion of additional protein components.

Our studies demonstrating the preferential incorporation of labeled carbohydrate and lipid precursors into separate plasma membrane components are compatible with the chemical heterogeneity of these proteins as demonstrated in the previous paper with special staining of the gel electrophoresis bands (2). The two protein bands staining for lipid were the same rapidly migrating bands which showed evidence of  $^3\text{H}$ -choline incorporation into lecithin and probably lysolecithin. Two bands incorporated glucosamine, whereas only one band near the origin stained for carbohydrate with the PAS technique. Isolation of these individual membrane constituents which differ significantly in chemical composition should be of great interest in dissecting further the structure-function relationships of the membrane proteins.

In contrast to the variations in labeling rates of the membrane protein bands during synthesis, the disappearance of label after removal and chase of the radioactive precursor proceeded at approximately the same rate for each of the protein bands in the gels. This result suggests that the major pathway for membrane catabolism in this cell involves removal in bulk of membrane segments large enough to include all of the protein components rather than the degradation of individual proteins by specific mechanisms. Since the macrophages exhibit pinocytosis under the conditions employed for the pulse-chase studies, removal of membrane in relatively large pieces by this process seems the most likely basis for the disappearance observed. Interiorized plasma membrane, even if unaltered in structure and function, would not be recovered in the plasma membrane fraction obtained by the glutaraldehyde procedure, since

this procedure involves removal of the cytoplasm and membranous elements embedded in it, and recovery only of the large surface membrane sheets.

The loss of label during the chase period is rapid and progressive. If this loss is due mainly to removal by endocytosis, it follows then that the interiorized membrane is in large part degraded rather than returned to the surface membrane in a shuttle system.

The half-life of the membrane protein as determined in these experiments is a maximum. The true turnover rate may be even more rapid if certain artifacts (e.g. reutilization of label) have significantly influenced our results. The half-life found for the macrophage plasma membrane protein (7–8 hr) is much shorter than that found for rat hepatocyte endoplasmic reticulum (75–113 hr) by Omura, Siekivitz, and Palade (10) or that found for rat hepatocyte microsomal membranes (50 hr) by Arrias, Doyle, and Schimke (11). The markedly shorter half-life observed by us may simply be due to the fact that we studied a different membrane and a different cell. Certainly the mechanisms for synthesis and for degradation of membrane proteins might be quite different in a thin cytomembrane such as endoplasmic reticulum than in the thicker plasma membrane.

As mentioned above, it seems likely that endocytosis which is characteristic of macrophages may be the major factor accounting for the rapid turnover of the plasma membrane proteins in this cell. The cells were maintained during the chase period in a medium containing 10% serum, conditions known to favor a moderate rate of pinocytosis (3). It is not possible to quantitate the amount of surface membrane brought into the cells, but it is not unreasonable to suppose that half of the plasma membrane might be interiorized over the course of a 7 hr period. Studies on turnover rate of membrane proteins in macrophages maintained in high and in low serum media, thus leading to high and low pinocytosis rates during the chase period, should serve to confirm the role of pinocytosis in membrane breakdown.

The studies reported here on analysis of protein bands of phagolysosomal membranes confirm the concept of bulk removal of membrane proteins by endocytic processes, and also provide some information on rapid degradation of interiorized plasma membrane, as evidenced by the disappearance of band D from the patterns, and the appearance of a rapidly migrating band of radioactivity perhaps due to a low molecular weight membrane-protein digestion product. Much further work needs to be done on the fate of interiorized membrane constituents. It is of course possible that various constituents may behave quite differently; some may be completely degraded whereas others may be retained or only partially altered and perhaps reutilized. In any event the picture that emerges of macrophage plasma membrane life history is a dynamic one with high levels of membrane synthesis and degradation in the steady or resting state. The site and mechanism of plasma membrane biogenesis in this cell is unknown.

It seems likely that plasma membrane breakdown in macrophages is due in large part to interiorization in the form of pinocytic or phagocytic vacuoles, and subsequent partial or complete degradation within lysosomes.

#### SUMMARY

Rabbit alveolar macrophages were incubated *in vitro* with radioactive protein precursors. Plasma membranes were isolated from these cells, dissolved in phenol-urea-acetic acid, and separated by acrylamide gel electrophoresis.  $^3\text{H}$ -leucine was rapidly incorporated into membrane protein. The rate of labeling with  $^3\text{H}$ -leucine was markedly different from one protein band to another, indicating heterogeneous or multistep synthesis and assembly of proteins in the alveolar macrophage plasma membrane.

Cells incubated with  $^3\text{H}$ -choline incorporated this compound into membrane lecithin. On gel electrophoresis the label derived from choline was located in the two bands migrating most rapidly towards the cathode. Studies on cells incubated with  $^3\text{H}$ -glucosamine revealed incorporation of label into two protein bands, one located near the origin and the other migrating rapidly towards the cathode.

The *in vitro* techniques were also employed for pulse-chase studies to gain information on rate of turnover of macrophage plasma membrane proteins. This turnover rate was rapid, with a half-life of approximately 8 hr. The radioactivity disappeared from the several protein bands at the same rate, suggesting bulk removal of membrane rather than catabolism of the individual proteins *in situ*. Endocytosis seems a likely mechanism to account for a major part of the plasma membrane removal.

Studies on the protein components of phagolysosomal membranes from cells which had been labeled with  $^3\text{H}$ -leucine revealed the presence of all of the major labeled protein bands characteristic of the plasma membrane except one, thus confirming the bulk interiorization of large segments or units of plasma membrane by endocytic processes.

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