Proc. Natl. Acad. Sci. USA
Vol. 76, No. 8, pp. 3824–3828, August 1979
Biochemistry

Insertion and turnover of macrophage plasma membrane proteins

(trinitrobenzene sulfonic acid/immunoprecipitation/sodium dodecyl sulfate/polyacrylamide gel electrophoresis)

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Contributed by Zanvil A. Cohn, June 4, 1979

ABSTRACT **The composition, insertion, and turnover of externally disposed proteins on the macrophage plasma membrane were analyzed. Cells labeled with [^{35}S]methionine were incubated with the nonpermeant reagent trinitrobenzene sulfonic acid to introduce the trinitrophenyl moiety on free amino groups of externally oriented membrane proteins. The cells were then incubated with rabbit anti-dinitrophenyl IgG and the immune complexes formed with the trinitrophenyl-proteins were isolated from detergent lysates of the cells by using fixed *Staphylococcus aureus* as the immunoadsorbent. Proteins isolated by this method were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The interval between the release of newly synthesized proteins from ribosomes and their appearance at the cell surface, where they became accessible to trinitrobenzene sulfonic acid, was studied in pulse-chase experiments. The "transit" time of four major membrane glycoproteins (48,000–310,000 M_r) ranged from 36 to 55 min and their appearance on the cell surface occurred in a relatively synchronous fashion. The turnover of most proteins of molecular weight above 50,000 was very slow ($t_{1/2} > 80$ hr) and was rather synchronous. Two exceptions were the 310,000 M_r protein, which was lost with a $t_{1/2} = 21$ hr, and a major glycoprotein (M_r 48,000), which exhibited more complex kinetics. Although the overall turnover of surface proteins was biphasic in nature, the rapid phase of protein loss was largely due to low molecular weight species.**

Trinitrobenzene sulfonic acid (TNBS), by virtue of its charge and size, does not penetrate rapidly into cells and has been used to label and to purify externally disposed plasma membrane proteins and lipids that have free amino groups (1–7). We have modified these methods to isolate biosynthetically labeled plasma membrane proteins after introduction of the trinitrophenyl (TNP) hapten by incubation with TNBS (unpublished data). Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoretic profiles of such proteins labeled with [1-^{14}C]glucosamine, [1-^{14}C]mannose, and [^{35}S]methionine were similar, suggesting that most of the surface polypeptides were glycosylated. There was also good agreement between electrophoretic profiles of labeled proteins isolated after TNBS incubation and surface polypeptides labeled with ^{125}I by lactoperoxidase and glucose oxidase (8). In this paper we applied the method of immunoprecipitation after TNBS derivatization of biosynthetically labeled mouse peritoneal macrophages to analyze the biogenesis and turnover of plasma membrane proteins.

MATERIALS AND METHODS

Cells. Peritoneal macrophages were collected from CD_2F_1 (BALB/c × DBA/2) mice (Flow Laboratories, McLean, VA) 4 days after the intraperitoneal injection of 1 ml of thioglycollate broth (Difco) (9). Cells were seeded at 3–6 × 10^6 cells per 60-mm tissue culture dish in α-modified Eagle's minimal medium containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Flow Laboratories) and were washed extensively 3–4 hr later to remove nonadherent cells.

Antiserum. Rabbit anti-dinitrophenyl (DNP) IgG was prepared as described (10), and aggregates were removed by centrifugation at 40,000 rpm (105,000 × *g*) for 30 min in a Beckman 40 rotor.

Labeling of Cells. Monolayers in 60-mm dishes were incubated for 15 min, or for 3 hr at 37°C, with 2.5 ml of methionine-free, α-modified Eagle's medium containing 10% dialyzed heat-inactivated fetal bovine serum and 60–100 μCi of [^{35}S]-methionine per dish (500 Ci/mmol; Amersham/Searle; 1 Ci = 3.7 × 10^7 becquerels).

Isolation of TNP-Substituted Surface Proteins. Externally disposed cell surface proteins from cells incubated with TNBS were isolated as follows: Briefly, [^{35}S]methionine-labeled macrophage monolayers were incubated with 10 mM TNBS (Pierce) dissolved in phosphate-buffered saline without divalent cations (P_i/NaCl) at pH 7.8 for 30 min at 4°C and washed with cold P_i/NaCl. The viable TNBS-derivatized cells were then incubated on ice for 20 min in P_i/NaCl containing 0.5 mg of rabbit anti-DNP IgG and 1 mg of bovine serum albumin per ml. The cells were washed again with cold P_i/NaCl, scraped off the dish, collected by centrifugation (1500 × *g*, 5 min), and lysed in 200 μl of lysis buffer [P_i/NaCl containing 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.5% Nonidet P-40 (Bethesda Research Laboratories, Rockville, MD), and 0.15 trypsin inhibitor units of aprotinin per ml (Sigma)]. Nuclei were removed by centrifugation (1500 × *g*, 5 min) and the supernatant, further clarified by centrifugation (23,000 × *g*, 20 min), was incubated for 15 min on ice with 100 μl of a 10% (wt/vol) suspension of formaldehyde-fixed *Staphylococcus aureus* (11, 12) suspended in salt/azide buffer (0.2 M NaCl/12.5 mM potassium phosphate/0.02% NaN_3, pH 7.4) containing 1 mg of bovine serum albumin per ml. The fixed bacteria were washed twice with 750 μl of salt/azide buffer by centrifugation, washed once with 750 μl of a detergent solution (0.1% $NaDodSO_4$/0.05% Nonidet P-40/0.3 M NaCl/10 mM Tris·HCl, pH 8.6), and finally washed with 1 ml of salt/azide buffer. After the last wash the pellet was resuspended in 30 μl of sample buffer (50 mM dithiothreitol/50 mM Na_2CO_3/12% sucrose/2% $NaDodSO_4$) to elute adsorbed proteins for gel electrophoresis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Samples were loaded onto 1-mm-thick 4–11% polyacrylamide slab gels (13) and electrophoresed at 50 mA per gel for 5–6 hr. Gels were processed for fluorography as described by Bonner and Laskey (14), and exposed on prefogged Eastman Kodak X-Omat R-1 film for autoradiography (15). Molecular weight standards used

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Abbreviations: P_i/NaCl, phosphate-buffered saline; DNP, dinitrophenyl; $NaDodSO_4$, sodium dodecyl sulfate; TNBS, trinitrobenzene sulfonic acid; TNP, trinitrophenyl.

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were myosin (220,000) (gift of Ann Hubbard, Yale University School of Medicine), β-galactosidase (135,000), bovine serum albumin (68,000), ovalbumin (43,000), and soybean trypsin inhibitor (21,500).

RESULTS

Proteins Immunoprecipitated from TNBS-Treated Cells. The method for the isolation of surface proteins depends on the selective introduction of the TNP hapten into externally oriented proteins and their subsequent immunoprecipitation with purified rabbit anti-DNP IgG (1–6). The labeled proteins immunoprecipitated from macrophages after 3 hr of incubation with [^{35}S]methionine are shown in Fig. 1. Approximately 1% of the total radioactivity incorporated into cell protein during a 3-hr period was isolated by this method. We have established that no radioactive proteins were immunoprecipitated when the incubations with TNBS or rabbit anti-DNP IgG were omitted (unpublished data). Five major polypeptides and seven to eight minor bands were resolved by NaDodSO$_4$ gel electrophoresis. Proteins of apparent M_r 310,000, 170,000, 100,000, and 45,000–50,000 were prominent in all experiments.

Insertion of Newly Synthesized Proteins into Plasma Membrane. Preliminary labeling experiments established that the incorporation of [^{35}S]methionine into protein ceased within minutes of cell transfer to nonradioactive medium. Thus, the rate at which newly synthesized proteins were transported to the plasma membrane, where labeled proteins became accessible to TNBS derivatization, could be measured in a series of pulse–chase experiments (Fig. 2). At various intervals after a 15-min pulse with [^{35}S]methionine, cells were incubated with TNBS and the TNP-proteins were isolated by immunoprecipitation. The amount of radioactivity in TNP-proteins isolated from the cell surface (Fig. 2*A*) increased by 3- to 4-fold after the pulse and reached a peak at 2 hr. This indicated a pronounced lag in the export of newly synthesized proteins to the cell surface. In contrast, the incorporated radioactivity in the total cell lysate decreased about 20% during the 5-hr period.

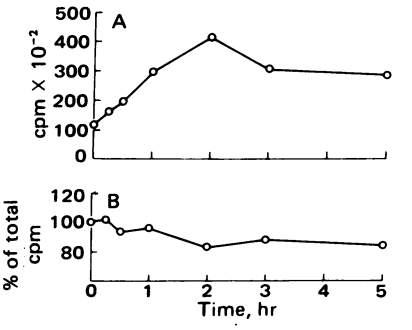

FIG. 2. Kinetics of appearance of labeled protein at the cell surface after a pulse of [^{35}S]methionine. Cells were pulsed with [^{35}S]-methionine for 15 min, washed, and chased in α-modified Eagle's medium containing 10% heat-inactivated fetal bovine serum. The end of the pulse was designated time 0. At pndicated intervals, cells were incubated on ice with TNBS and the TNP-proteins were isolated by immunoprecipitation and assayed for radioactivity (*A*). Radioactivity in the total cell lysate at indicated intervals, expressed as percent of initial incorporation, is shown in *B*.

Analysis of Newly Synthesized Plasma Membrane Proteins. The distribution of newly synthesized peptides that became accessible to derivatization by TNBS during the chase period is shown in Fig. 3. Immunoprecipitation of TNP-proteins immediately after the 15-min pulse resulted in little radioac-

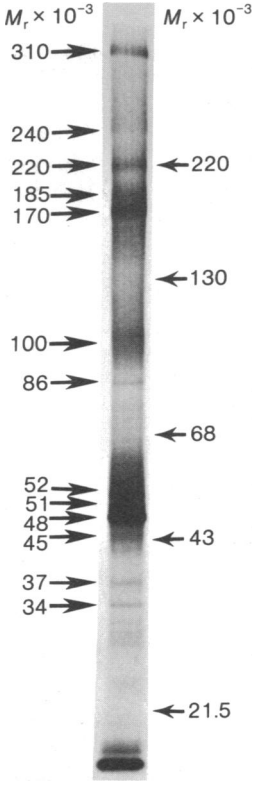

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{35}S]-methionine-labeled macrophage proteins immunoprecipitated from cells treated with TNBS and rabbit anti-DNP IgG. Arrows and numbers on the left denote the apparent M_r of the indicated bands. Arrows on the right indicate the position of the M_r standards.

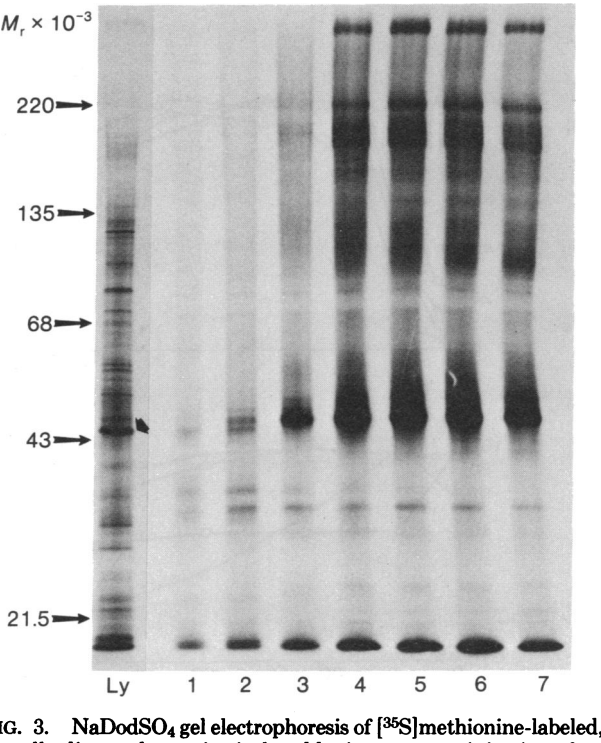

FIG. 3. NaDodSO$_4$ gel electrophoresis of [^{35}S]methionine-labeled, externally disposed proteins isolated by immunoprecipitation after TNBS treatment at various times after a 15-min pulse with [^{35}S]-methionine. Lane 1, immunoprecipitate immediately after the pulse. Lanes 2–7, precipitates from cultures chased in methionine-containing medium for 15 min, 30 min, 1 hr, 2 hr, 3 hr, and 5 hr, respectively. Ly, total cell lysate.

tivity and a restricted number of proteins (Fig. 3, lane 1). By 60 min, after the end of the pulse (Fig. 3, lane 4), a complete complement of membrane polypeptides was present. Major intracellular peptides in the lysate (Fig. 3, lane Ly) did not appear in the cell surface immunoprecipitates. When cells were first incubated with TNBS and then labeled with [^{35}S]methionine, there was no decrease in the amount of radioactive amino acid incorporated into protein and insignificant immunoprecipitation of labeled membrane polypeptides (see Fig. 6, lane 1). These results indicate that incubation with TNBS did not adversely affect cell viability and did not result in isolation of underivatized but labeled protein species.

A densitometric analysis of some of the major glycopeptides of Fig. 3 is presented in Fig. 4*A*. The $t_{1/2}$ for the appearance of the different peptides at the surface of the cell after the end of the 15-min [^{35}S]methionine pulse were as follows: 310,000 M_r peptide, 51 min; 170,000 M_r peptide, 42 min; 100,000 M_r peptide, 40 min; and 48,000 M_r peptide, 29 min. Insertion of these polypeptides is virtually complete by 60 min and occurs in a relatively synchronous fashion. The NaDodSO$_4$ gel electrophoresis patterns remain unaltered for 5 hr into the chase.

Turnover of TNP-Surface Proteins and Total Cellular Protein. Cells were labeled for 3 hr with [^{35}S]methionine and then chased in nonradioactive medium before derivatization with TNBS. The results (Fig. 5*A*) showed that surface polypeptides were lost with biphasic kinetics. The rapid phase had a $t_{1/2}$ of about 13 hr (correlation coefficient, 0.99) and accounted for about 60% of the initial surface-associated radioactivity. The slow component had a $t_{1/2}$ of 80 hr (correlation coefficient, 0.91). In similar experiments, total cellular protein labeled with [^{35}S]methionine decayed with a slow component of $t_{1/2}$ of 110 hr (Fig. 5*B*). The total protein on the dishes remained constant, indicating that the decrease in radioactivity was not due to cell loss (Fig. 5*B*).

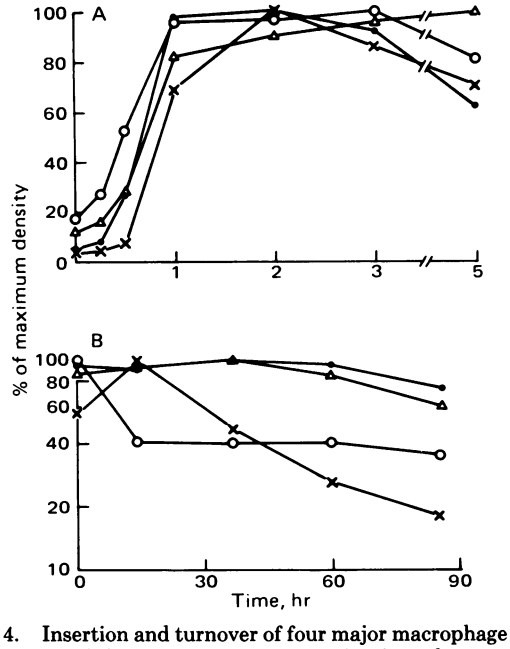

FIG. 4. Insertion and turnover of four major macrophage membrane proteins. (*A*) Rates of protein insertion into the membrane calculated from densitometric analysis of fluorogram shown in Fig. 3. (*B*) Kinetics of ^{35}S label loss from the same proteins calculated from densitometric analysis of fluorogram shown in Fig. 6. Results are expressed as percent of maximum density. Protein M_r: △, 100,000; ○, 48,000; ×, 310,000; ●, 170,000.

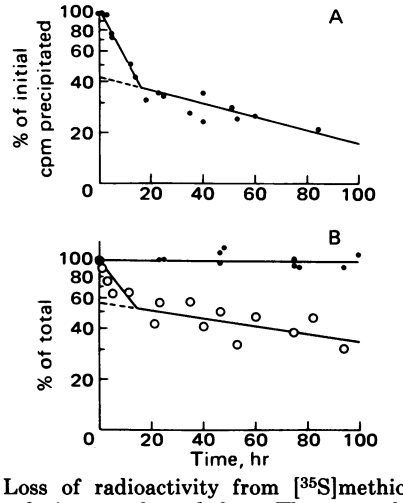

FIG. 5. Loss of radioactivity from [^{35}S]methionine-labeled macrophages during a prolonged chase. The macrophages were labeled with [^{35}S]methionine for 3 hr (time 0). (*A*) Loss of [^{35}S]methionine-labeled surface protein. The results of three experiments were pooled. (*B*) Loss of [^{35}S]methionine-labeled total protein (○). Total protein on the dishes (●). All results are expressed as percent of values at time 0.

Analysis of Turnover of Plasma Membrane Proteins. The analysis of plasma membrane proteins either by NaDodSO$_4$ gel electrophoresis (Fig. 6) or densitometry (Fig. 4*B*) indicated that turnover was somewhat asynchronous. Certain groups of polypeptides decayed slowly (100,000 and 170,000 M_r) with

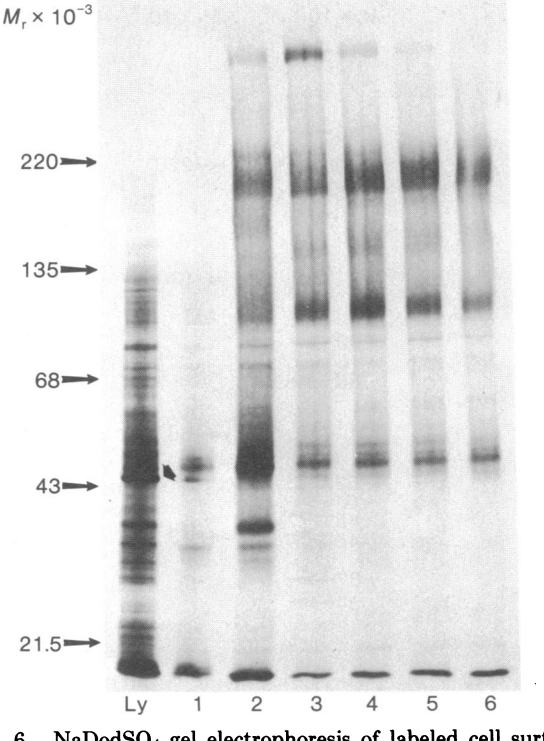

FIG. 6. NaDodSO$_4$ gel electrophoresis of labeled cell surface polypeptides at intervals after a 3-hr pulse with [^{35}S]methionine. Ly, cell lysate obtained at the end of the 3-hr labeling period. Lane 1, control in which the cells were incubated with TNBS *before* being labeled for 3 hr with [^{35}S]methionine. Lanes 2–6, immunoprecipitated peptides obtained from cells labeled for 3 hr and chased for 0 hr, 14 hr, 36 hr, 60 hr, and 84 hr, respectively, before TNBS treatment. Arrow marks the position of actin.

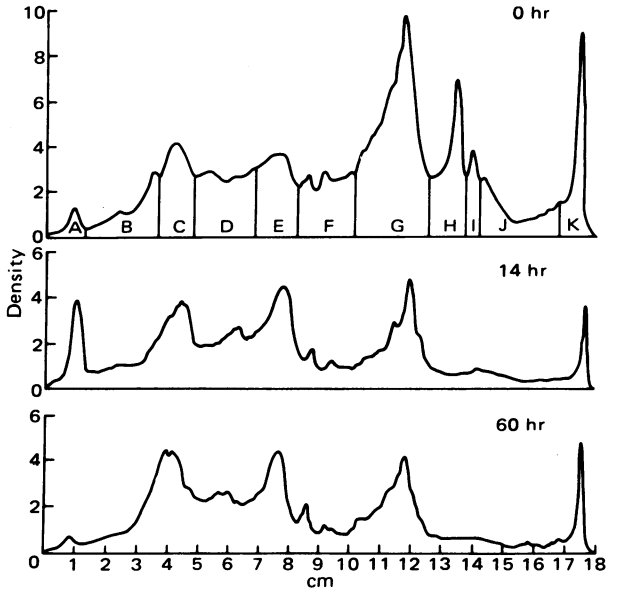

FIG. 7. Densitometric scans of macrophage membrane polypeptides at 0, 14, and 60 hr (Fig. 6, lanes 2, 3, and 5) after a 3-hr pulse with [^{35}S]methionine. Graphs represent distribution of density in each of the fluorograms scanned. See Table 1.

Table 1. Sum of density units in each of the regions indicated (top gel) in Fig. 7 of gels from 0, 14, and 60 hr after pulse

$M_r \times 10^{-3}$	Region	Density units 0 hr	14 hr	60 hr
310	A	7	19	3
	B	30	31	25
170	C	42	35	44
	D	54	43	46
100	E	46	44	43
	F	48	20	21
48	G	132	51	52
37	H	46	8	8
	I	15	4	3
	J	31	10	9
Gel front	K	33	10	14
Total density		484	275	268

half-lives of >80 hr, whereas others (48,000 and 310,000 M_r) turned over more rapidly. A more quantitative densitometric assessment of the turnover of individual molecular weight classes is shown in Fig. 7. Analyses were carried out at 0, 14, and 60 hr after the pulse of [^{35}S]methionine and correlated well with the overall kinetics seen in Fig. 5*A*. It appears that after the rapid phase of label loss in the first 14 hr that the majority of the polypeptides turn over rather synchronously. In fact, during the rapid phase the 48,000 M_r protein accounts for 40% of the total label loss.

DISCUSSION

Our studies have shown that immunoprecipitation of proteins from TNBS-treated biosynthetically labeled cells can be used to analyze the insertion and turnover of externally oriented plasma membrane proteins. Because the incubation with TNBS is performed at the end of the chase interval, the method introduces minimal perturbations to the cells. The TNBS derivatization method also offered the opportunity to study the biosynthesis of all the major proteins "tagged" by TNP in the same cell population.

After a 15-min pulse of [^{35}S]methionine, very few labeled proteins were present on the cell surface, but after 2 hr of chase in nonradioactive medium the full complement of radioactive glycoproteins was observed. Using these data, one can estimate the transit time of the externally disposed peptides inside the cell. The interval calculated for release of half of the newly labeled nascent chains from the ribosomes [assuming a biosynthetic rate of about 20 sec for hemoglobin chains (16, 17)] was subtracted from the experimentally determined $t_{1/2}$ for the appearance of the labeled polypeptide at the cell surface timed from the beginning of the pulse. The transit times thus calculated range from 55 min for the largest protein (310,000 M_r) to 36 min for the smallest protein (48,000 M_r). However, these differences would be minimized if the biosynthetic rate for glycosylated proteins synthesized on membrane-bound ribosomes was slower than that for hemoglobin.

The glycosylation of proteins and subsequent processing of the oligosaccharides are complicated events involving the initial transfer of oligosaccharide from a lipid–oligosaccharide precursor and subsequent "trimming" of some residues and addition of others to yield the "high mannose" and "complex" oligosaccharides found in the mature glycoproteins (18–20). The processing of the oligosaccharide chains after transfer to the nascent peptide chain is estimated to take from 20 to 60 min.

A distinction has been made by Atkinson (21, 22) between the rate of insertion of nonglycosylated membrane proteins, which are inserted within 1–2 min of completion of synthesis, and the glycosylated proteins of vesicular stomatitis virus and HeLa cells, which show a 12- to 30-min lag before insertion into plasma membrane. In other systems, transit times ranging from 30 min for splenocyte H-2 alloantigens (23) to 3 hr for lymphocyte surface IgM (24) and chicken muscle acetylcholine receptor (25) have been reported. The similarity between the rates of insertion of different macrophage membrane glycoproteins shown in Fig. 4*A* suggests that the proteins are inserted synchronously shortly after completion of processing of oligosaccharide side chains and is consistent with the insertion of proteins in membrane units as suggested previously (26).

The macrophage is an ideal cell type for turnover experiments because it does not divide and can be maintained in culture for long periods. The method for isolation of externally oriented plasma membrane proteins described here avoids possible artifacts due to perturbation of the cell during labeling with ^{125}I or NaB^3H_4. The turnover of TNP-labeled proteins showed biphasic kinetics, with approximately 60% of the radiolabeled surface peptides being lost with a $t_{1/2}$ of 13 hr and the remainder, with a $t_{1/2}$ of 80 hr. These results agree well with the turnover data for glycoproteins and ^{125}I-labeled proteins reported by Baumann and Doyle (27) for hepatoma tissue culture cells and are somewhat longer than the $t_{1/2}$ values of 2 and 25–33 hr reported for ^{125}I-labeled proteins of L cells by Hubbard and Cohn (28).

Rapid and slow phases of turnover have been observed in the turnover rates of individual proteins labeled with ^{125}I (28, 29). With one notable exception, we find a single rate of turnover for all proteins isolated after incubation with TNBS. As shown in Figs. 4*B* and 6, the majority of proteins (notably the 170,000 and 100,000 M_r species) are extremely stable, with $t_{1/2} > 80$ hr. The peptide of M_r 310,000 exhibits a single turnover rate of 21 hr. However, the major glycoprotein of apparent M_r 48,000 exhibited complex turnover kinetics, decreasing by 60% in the first 14 hr and remaining stable thereafter. We have not, however, ruled out the possibility that two proteins comigrate at this position.

Possible mechanisms for surface protein turnover include digestion in the lysosomal compartment after endocytosis, shedding from the cell surface, and extracellular digestion by proteases secreted from the cell or present in the serum. Emerson and Cone (29) suggest that the rapid phase of ^{125}I-labeled Ia antigen loss is due to shedding and is observed only when the cell is viable. This implies there must be heterogeneity of Ia antigen on the surface such that some is shed rapidly and other molecules remain anchored in the membrane. Baumann and Doyle (27) suggest that the trypsin-sensitive glycoproteins on the surface of hepatoma cells are degraded more rapidly than other surface proteins. It will be interesting to determine which of these routes is responsible for the decay of the macrophage surface glycoproteins described here.

These studies demonstrate the general applicability of the method of isolation of surface proteins after incubation of cells with TNBS to the questions of turnover and biogenesis of these proteins. The method is also applicable to study of the flow of exteriorly "tagged" proteins into intracellular compartments and to the analysis of proteins that span the lipid bilayer in phagosomes and pinosomes.

We thank Ira Mellman for stimulating discussions and Helen Plutner for her excellent technical assistance. This work has been supported by grants from the Jane Coffin Childs Memorial Fund and the Andrew W. Mellon Foundation, Biomedical Research Support Grant 5-S07-RR 07065, and Research Grant AI-14603 from the U.S. Public Health Service. G.K. is an International Fogarty Fellow of the U.S. Public Health Service.

1. Bonsall, R. W. & Hunt, S. (1971) *Biochim. Biophys. Acta* **249,** 281–284.
2. Arroti, J. J. & Garvin, J. E. (1972) *Biochim. Biophys. Acta* **255,** 79–90.
3. Tarone, G., Pratt, M. & Comoglio, P. (1973) *Biochim. Biophys. Acta* **311,** 214–221.
4. Vidal, R., Tarone, G., Peroni, F. & Comoglio, P. M. (1974) *FEBS Lett.* **47,** 107–112.
5. Gordesky, S. E., Marinetti, G. V. & Love, R. (1975) *J. Membr. Biol.* **20,** 111–132.
6. Tarone, G. & Comoglio, P. M. (1977) *Exp. Cell Res.* **110,** 143–152.
7. Roseman, M., Litman, B. J. & Thompson, T. E. (1975) *Biochemistry* **14,** 4826–4830.
8. Hubbard, A. L. & Cohn, Z. A. (1975) *J. Cell Biol.* **64,** 438–460.
9. Cohn, Z. A. & Benson, B. (1965) *J. Exp. Med.* **121,** 153–170.
10. Unkeless, J. C. (1977) *J. Exp. Med.* **145,** 931–947.
11. Kessler, S. W. (1975) *J. Immunol.* **115,** 1617–1624.
12. Kessler, S. W. (1976) *J. Immunol.* **117,** 1482–1490.
13. Neville, D. M., Jr. & Glassmann, H. (1979) *Methods Enzymol.* **32,** 92–102.
14. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
15. Laskey, R. A. & Mills, A D. (1975) *Eur. J. Biochem.* **56,** 335–341.
16. Knopf, P. M. & Lamfron, H. (1965) *Biochim. Biophys. Acta* **95,** 398–407.
17. Hunt, T., Hunter, T. & Munro, A. (1969) *J. Mol. Biol.* **43,** 123–133.
18. Robbins, P. W., Hubbard, C. S., Turco, S. J. & Wirth, D. F. (1977) *Cell* **12,** 893–900.
19. Tabas, I., Schlesinger, S. & Kornfeld, S. (1978) *J. Biol. Chem.* **253,** 716–722.
20. Hunt, L. A., Etchison, J. R. & Summers, D. F. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 754–758.
21. Atkinson, P. H. (1975) *J. Biol. Chem.* **250,** 2123–2134.
22. Atkinson, P. H. (1978) *J. Supramol. Struct.* **8,** 89–109.
23. Vitetta, E. S. & Uhr, J. W. (1975) *J. Immunol.* **115,** 374–381.
24. Vitetta, E. S. & Uhr, J. W. (1974) *J. Exp. Med.* **139,** 1599–1620.
25. Devreotes, P. N., Gardner, J. M. & Fambrough, D. M. (1977) *Cell* **10,** 365–373.
26. Doyle, D. Baumann, H., England, B., Friedman, E., Hou, E. & Tweto, J. (1978) *J. Biol. Chem.* **253,** 965–973.
27. Baumann, H. & Doyle, D. (1978) *J. Biol. Chem.* **253,** 4408–4418.
28. Hubbard, A. L. & Cohn, Z. A. (1975) *J. Cell Biol.* **64,** 461–479.
29. Emerson, S. G. & Cone, R. E. (1979) *J. Immunol.* **122,** 892–899.