

# Fusion of Sequentially Internalized Vesicles in Alveolar Macrophages

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**Abstract.** Previously we reported that internalized ligand-receptor complexes are transported within the alveolar macrophage at a rate that is independent of the ligand and/or receptor but is dependent on the endocytic apparatus (Ward, D. M., R. S. Ajioka, and J. Kaplan. 1989. *J. Biol. Chem.* 264:8164-8170). To probe the mechanism of intracellular vesicle transport, we examined the ability of vesicles internalized at different times to fuse. The mixing of ligands internalized at different times was studied using the 3,3'-diaminobenzidine/horseradish peroxidase density shift technique. The ability of internalized vesicles to fuse was dependent upon their location in the endocytic pathway. When ligands were administered as tandem pulses a significant amount of mixing (20-40%) of vesicular contents was observed. The pattern of mixing was independent of the ligands employed (transferrin, mannosylated BSA, or alpha macroglobulin), the order of ligand addition, and temperature (37°C or 28°C). Fusion was restricted to a brief period immediately after internalization. The amount of fusion in early endosomes did not increase when cells, given

tandem pulses, were chased such that the ligands further traversed the early endocytic pathway. Little fusion, also, was seen when a chase was interposed between the two ligand pulses. The temporal segregation of vesicle contents seen in early endosomes was lost within late endosomes. Extensive mixing of vesicle contents was observed in the later portion of the endocytic pathway. This portion of the pathway is defined by the absence of internalized transferrin and is composed of ligands en route to lysosomes. Incubation of cells in iso-osmotic medium in which Na<sup>+</sup> was replaced by K<sup>+</sup> inhibited movement of internalized ligands to the lysosome, resulting in ligand accumulation within the late endocytic pathway. The accumulation of ligand was correlated with extensive mixing of sequentially internalized ligands. Although significant amounts of ligand degradation were observed, this compartment was devoid of conventional lysosomal markers such as acid glycosidases. These results indicate changing patterns of vesicle fusion within the endocytic pathway, with a complete loss of temporal ligand segregation in a prelysosomal compartment.

**L**IGANDS internalized as a result of receptor-mediated endocytosis may have different fates. Some ligands, such as Tf, are recycled to the cell surface complexed to their receptor (van Renswoude et al., 1982). Others dissociate from their receptor, and while the receptor is recycled, the ligand is sent to the lysosome where it is either stored or degraded (Anderson and Kaplan, 1983). In other cases, the ligand-receptor complex may be degraded as a unit in the lysosome (Stoscheck and Carpenter, 1984). The fate of internalized ligands and receptors appears to result from the interplay of three factors: the changing environment of the endosomal lumen (i.e., decreased pH) (Tycko and Maxfield 1982), inherent structural features of the receptor (Davis et al., 1987), and the time necessary for receptor-ligand dissociation relative to the rate of vesicle movement (Linderman and Lauffenburger, 1988).

The endocytic apparatus can be morphologically and biochemically differentiated based on the types of receptors

and/or ligands present within vesicles. Vesicles in the early portion of the endocytic apparatus contain occupied and unoccupied receptors. Vesicles in the later portion of the endocytic apparatus can be divided into two categories, those that are recycled back to the cell surface and contain unoccupied receptors and/or apo Tf-receptor complexes (referred to herein as recycling endosomes), or late endosomes that are being targeted to the lysosome and contain either free ligands or ligand-receptor complexes. Late endosomes exhibit a different polypeptide composition (Schmid et al., 1988) and a much lower pH than either early endosomes or recycling endosomes (Tycko and Maxfield, 1982; Murphy et al., 1984).

From a study of the movement of receptors and ligands within the endocytic apparatus, we concluded that internalized receptors and ligands exhibit cohort behavior; that is, they move at a rate which is defined by the internalized vesicles, and which is content independent (Ward et al., 1989).

One explanation for cohort behavior is that movement is both vesicular and ordered. That is, once internalized, vesicles do not intermix but progress through the endocytic apparatus in order of internalization. In HeLa cells, the contents of vesicles containing ligands or receptors internalized at different times do not mix and remain temporally segregated (Ajioka and Kaplan, 1986). A recent study by Salzman and Maxfield (1988) has suggested, however, that fusion of sequentially internalized ligands may occur in early and late stages in the recycling process. Their studies suggest that in CHO cells, Tf-containing and fluid-containing vesicles fuse in early endosomes, while in other cell types fusion may occur later in the endocytic pathway.

These contrasting observations raise questions about the general applicability of results obtained in any one cell type; i.e., what features are common to all cells and which are cell type dependent? We have in the past contrasted results obtained in HeLa cells, a cultured nondifferentiated cell line, with those obtained in alveolar macrophages, a highly differentiated cell type specialized for endocytic activities. Common features between the two widely disparate cell types may reflect general properties of the endocytic system. To this end, we have examined the question of temporal segregation of internalized ligands in alveolar macrophages using the DAB/HRP density shift technique as described initially by Courtoy et al. (1984). Our results indicate that immediately after internalization there is a narrow "window" in which sequentially formed vesicles can fuse. Within 2 min of internalization, fusion of these sequentially formed vesicles radically decreased and no further fusion of vesicles was observed in the early part of the endocytic apparatus. Ligands internalized at different times, which did not mix in the early portion of the endocytic cycle, mixed in a prelysosomal compartment at later stages of the endocytic cycle. Ligand degradation appeared to be initiated within this vesicle, although this compartment does not contain lysosomal glycosidases.

## Materials and Methods

### Cells

Rabbit alveolar macrophages were obtained by bronchial lavage (Myrvik et al., 1961), and the cells cultured as described previously (Kaplan, 1980). All cells were incubated for 45 min at 37°C before use (Kaplan and Keogh, 1982).

### Materials

Alpha-macroglobulin (aM-T)<sup>1</sup> was isolated from rabbit plasma and aM-<sup>125</sup>I-T was prepared as described (Kaplan, 1982). Mannosylated-BSA (man-BSA) was obtained from E. Y. Laboratories, Inc. (San Mateo, CA). Human Tf was isolated by the method of Sawatzki (1981). Man-BSA and Tf were iodinated using iodogen (Pierce Chemical Co., Rockford, IL) as described by Wiley and Cunningham (1982). HRP was obtained from Sigma Chemical Co. (St. Louis, MO). Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and diluted according to the manufacturer's instructions.

### Subcellular Fractionation

All operations were performed at 0°C. Monolayers were washed with Hank's balanced salts solution (HBSS), and cells removed using a cell

scraper. Cell pellets were resuspended in 0.25 M sucrose in 10 mM Tris-HCl and 1 mM EDTA, pH 7.2. Cells were homogenized in a precooled, tight-fitting Dounce homogenizer, centrifuged at 800 g for 10 min, and the supernatant was applied to 27% Percoll at 59,000 g (average) for specified times. Gradients were fractionated bottom to top and refractive indices measured using a refractometer (Bausch & Lomb Inc., Rochester, NY) (22°C). Typically 10–30% vesicle breakage was observed as measured by release of internalized ligand or the lysosomal enzyme B-*n*-acetylhexosaminidase. Endosomal fractions were pooled and incubated with DAB and H<sub>2</sub>O<sub>2</sub> as described previously (Ward et al., 1989). The percentage of radioactivity "shifted" by DAB was calculated by measuring the amount of radioactivity in fractions one and two in samples exposed to DAB, and then subtracting the amount of radioactivity in similar fractions from samples that were not exposed to DAB. This value was then divided by the maximum amount of DAB shift observed in fractions one and two from the positive control (ligands administered simultaneously).

## Methods

The binding of all ligands was measured as described for the binding of aM-<sup>125</sup>I-T (Kaplan, 1980) using  $1 \times 10^{-8}$  M for each radiolabeled ligand and 1 mg/ml HRP unless otherwise noted. The method of Kaplan and Nielson (1979) was used to distinguish between surface bound and internalized aM-T. Surface and internalized man-BSA and Tf, as well as ligand degradation products, were measured as previously described (Ward et al., 1989). K<sup>+</sup> containing buffers were used as described by Baenziger and Fiete (1982) except that the buffer contained amino acids, glucose, and vitamins in concentrations similar to that of Hank's minimal essential medium. No difference was observed, however, in ligand uptake, degradation, and movement in iso-K<sup>+</sup> buffers with or without these modifications. All experiments were performed at least three times. The radioactivity in each fraction of the Percoll gradients was determined as the percentage of total radioactivity applied to the gradient.

## Additional Procedures

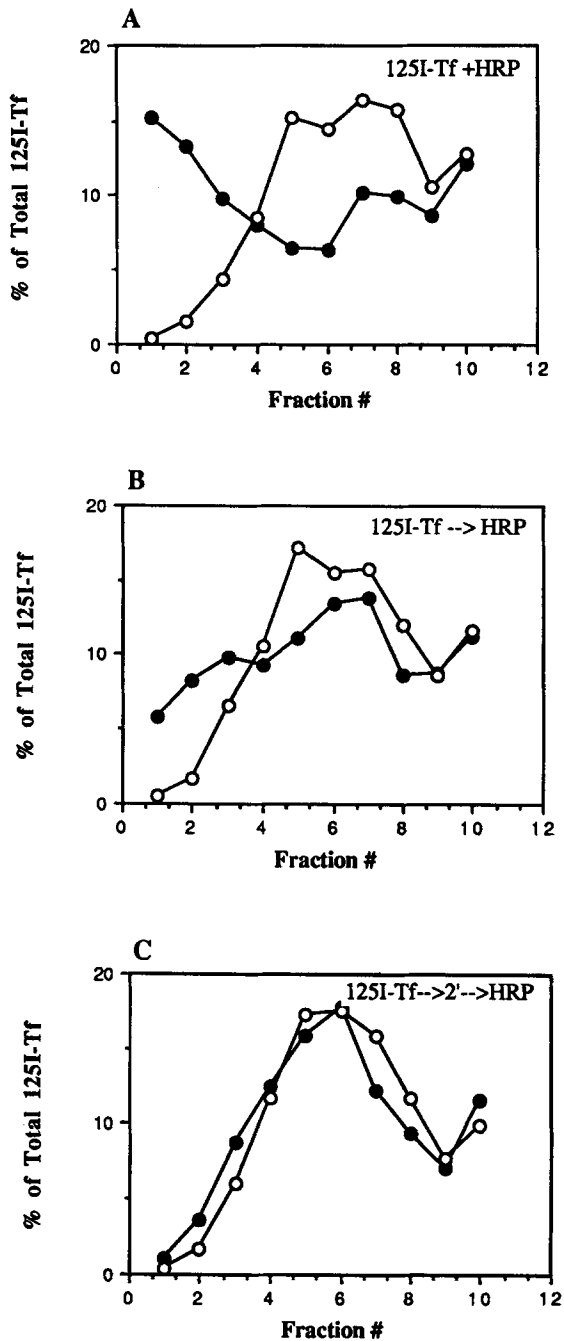
Protein determinations were performed as described by Lowry et al. (1951) or when Percoll was present by the procedure of Vincent and Nadeau (1983), using BSA (fraction V; Sigma Chemical Co.) as a standard. The activities of alkaline phosphodiesterase and glycosidases were measured as described by Edelson and Erbs (1978) and Kaplan (1978), respectively.

## Results

The approach used to study temporal segregation of internalized ligands in alveolar macrophages was similar to that used in a previous study in HeLa cells (Ajioka and Kaplan, 1986). Cells are allowed to internalize a radiolabeled ligand, washed and then incubated with HRP as a second ligand. The cells are homogenized, an endosomal fraction obtained and that fraction incubated with H<sub>2</sub>O<sub>2</sub> and DAB. If the contents of the vesicles mix then the distribution of the radioiodinated marker will exhibit an increase in buoyant density because of the peroxidase catalyzed polymerization of DAB. Studies in our laboratory and elsewhere have demonstrated the validity of this approach to study vesicular movement; the critical features being that only peroxidase-containing vesicles exhibit a density shift, and that all of the manipulations involved (homogenization, gradients, DAB exposure) do not induce mixing of vesicle contents (Courtoy et al., 1984; Ajioka and Kaplan, 1986 and 1987; Ward et al., 1989). An advantage of using alveolar macrophages is that they internalize HRP as a result of either fluid phase pinocytosis or receptor-mediated endocytosis, the latter as a consequence of binding of HRP to the mannose terminal glycoprotein receptor (Stahl et al., 1980; Kaplan and Nielson, 1979a,b).

When endosomal fractions were obtained from cells that had been incubated simultaneously with HRP and <sup>125</sup>I-Tf

1. *Abbreviations used in this paper:* aM-T, alpha-macroglobulin-trypsin complex; man-BSA, mannosylated BSA.



**Figure 1.** Examination of early endosomes for fusion of vesicles containing sequentially pulsed HRP and  $^{125}\text{I}$ -Tf. Cells were incubated for 2 min at  $37^\circ\text{C}$  with either  $^{125}\text{I}$ -Tf and HRP (A) or with  $^{125}\text{I}$ -Tf alone. All samples were then placed at  $0^\circ\text{C}$ , washed, and surface ligand was removed. An aliquot of cells incubated with  $^{125}\text{I}$ -Tf alone was then placed at  $37^\circ\text{C}$  and incubated for 2 min with HRP, either immediately (B) or after a 2-min lag at  $37^\circ\text{C}$  (C). The cells were placed at  $0^\circ\text{C}$ , homogenized, endosomes isolated, and then incubated with  $\text{H}_2\text{O}_2$  and DAB as described in Materials and Methods. (●) Samples incubated with DAB and (○) the gradient profile of samples incubated without DAB.

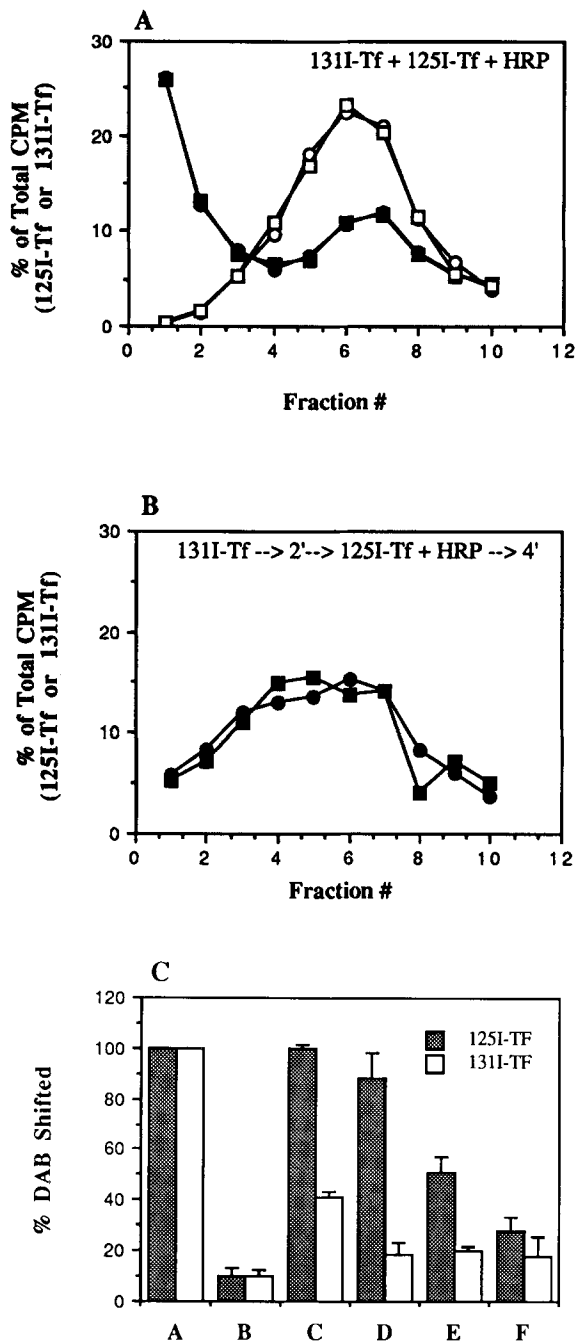
for 2 min at  $37^\circ\text{C}$ ,  $\sim 30\text{--}40\%$  of  $^{125}\text{I}$ -Tf exhibited a density shift (Fig. 1 A). In the absence of HRP, there was no change in the buoyant density of the internalized ligand (data not shown). The amount of material shifted when both ligands

are given simultaneously reflects the limitation of the technique and is independent of the ligand. Similar results were obtained using  $^{125}\text{I}$ -man-BSA or  $\text{aM}\cdot^{125}\text{I}$ -T (data not shown). The presence of radioactivity at the top of the gradient represents vesicles that have been disrupted and whose contents are not subject to the shift (Ajioka and Kaplan, 1987). There may also be vesicles which, while they appear intact, are probably damaged and do not shift upon DAB exposure (Ward et al., 1989; Stoorvogel et al., 1988). The percentage of shifting observed varied between experiments by  $\sim 10\text{--}20\%$ . To normalize for this variation, we performed positive (two ligands given simultaneously) and negative (minus DAB or HRP) controls for each experiment.

#### *Movement of Ligands in the Early Endocytic Pathway*

To determine whether vesicle fusion occurred within early endosomes, cells were incubated with  $^{125}\text{I}$ -Tf for 2 min washed, stripped of surface bound ligand, chased with media at  $37^\circ\text{C}$  for specified times and then incubated with HRP for 2 min. In cells that were given the two ligands as tandem pulses, there appeared to be a reproducible amount of  $^{125}\text{I}$ -Tf shifted to a higher density (Fig. 1 B). As the time between the pulses increased, the amount of material shifted decreased to background levels (Fig. 1 C).

To insure that the decrease in the amount of material subject to the density shift was not the result of ligands moving into a compartment that might be more susceptible to damage than the initial vesicle, cells were incubated with  $^{131}\text{I}$ -Tf followed by an incubation with  $^{125}\text{I}$ -Tf and HRP. In this instance, the presence of  $^{125}\text{I}$ -Tf serves two purposes. (a) It acts as an internal control for vesicle damage, and (b) it also allows us to define the demarcation between early and late endosomes. The results of this experiment are shown in Fig. 2, in which A and B are examples of the gradients with A being a short and B an extended incubation with the radiolabeled ligands. Data from three different experiments are summarized in C. When the ligands are given as a tandem pulse  $\sim 40\%$  of the  $^{131}\text{I}$ -Tf was subject to a density shift (Fig. 2 C, bar C). When a chase period was interposed between the pulses, the amount of  $^{131}\text{I}$ -Tf subject to the shift decreased, while the amount of  $^{125}\text{I}$ -Tf subject to the shift was relatively unchanged (Fig. 2 C, bar D). 3–4 min after being internalized, HRP's ability to effect a density shift of  $^{125}\text{I}$ -Tf was diminished (Fig. 2 C, bars E and F), indicating that separation of the two ligands had occurred. The time frame of separation is similar to that demonstrated for hepatocytes (Stoorvogel et al., 1987). The decrease in the ability of  $^{131}\text{I}$ -Tf to be shifted (2 min) occurred before the decrease in the ability to shift  $^{125}\text{I}$ -Tf (3–4 min); demonstrating that the lack of fusion did not reflect an increase in vesicle damage. We consistently observed a broadening of the gradient profile when analyzing vesicle preparations obtained from cells incubated with iodinated Tf (Fig. 2 A versus B). This change in gradient profile was not the result of variability in gradients or centrifugation conditions. Samples run on second gradients exhibited similar profiles to the first gradient. Changes in gradient profile were not seen with ligands other than Tf, and the change in gradient profile was only seen with extended incubations. Short incubations (2 min) with iodinated Tf showed a "typical" profile (Fig. 2 A), whereas, with further incubation (4–6 min), the gradient profile demonstrated broadening (Fig. 2 B). One possible explanation is



**Figure 2.** Use of  $^{131}\text{I}$ -Tf and  $^{125}\text{I}$ -Tf to define the degree of mixing in the early endocytic pathway. Cells were incubated at  $37^\circ\text{C}$  for 2 min with  $^{125}\text{I}$ -Tf,  $^{131}\text{I}$ -Tf, and HRP (A and C, column A),  $^{125}\text{I}$ -Tf and  $^{131}\text{I}$ -Tf (C, column B), or  $^{131}\text{I}$ -Tf alone. All samples were then placed at  $0^\circ\text{C}$ , washed, and surface ligand was removed. The cells were incubated with  $^{131}\text{I}$ -Tf alone were then incubated for 2 min at  $37^\circ\text{C}$  with  $^{125}\text{I}$ -Tf and HRP, either immediately (C, column C) or after a 2-min lag (C, columns D–F). The samples were washed and harvested immediately after the end of the incubation with ligands (C, columns C and D) or were allowed to incubate for a further 2 min (column E) or 4 min (B and C, column F) at  $37^\circ\text{C}$  without additional ligand. The purpose of this incubation was to allow the ligand to move further through the recycling pathway and measure the time of separation between  $^{125}\text{I}$ -Tf and HRP. In C, the data represent the average of the three separate experiments. The values obtained for the amount of  $^{131}\text{I}$ -Tf subject to the density shift between cells exposed to a tandem pulse (column C) and those that had

that the broadening reflects movement of Tf into recycling endosomes that may exhibit a higher density.

This experiment was also performed at  $28^\circ\text{C}$  to slow the rate of vesicle movement and thus increase our resolution. We again observed a consistent amount (20–40%) of mixing of vesicle contents when the ligands were given as tandem pulses (data not shown). The degree of mixing was reduced when chase periods as short as 2 min were inserted between sequential exposure of cells to ligand. At  $37^\circ\text{C}$ , the demarcation between early and late endosomes occurred 3–4 min after ligand internalization, whereas, at  $28^\circ\text{C}$ , it occurred at 6–8 min after ligand exposure (data not shown). In cells given tandem pulses of ligand, mixing only occurred in the earliest portion of the endocytic pathway. No additional mixing was observed when tandemly internalized ligands were allowed to further traverse the early portion of the endocytic pathway. Additionally, the order of ligand administration did not affect the extent of mixing observed (data not shown).

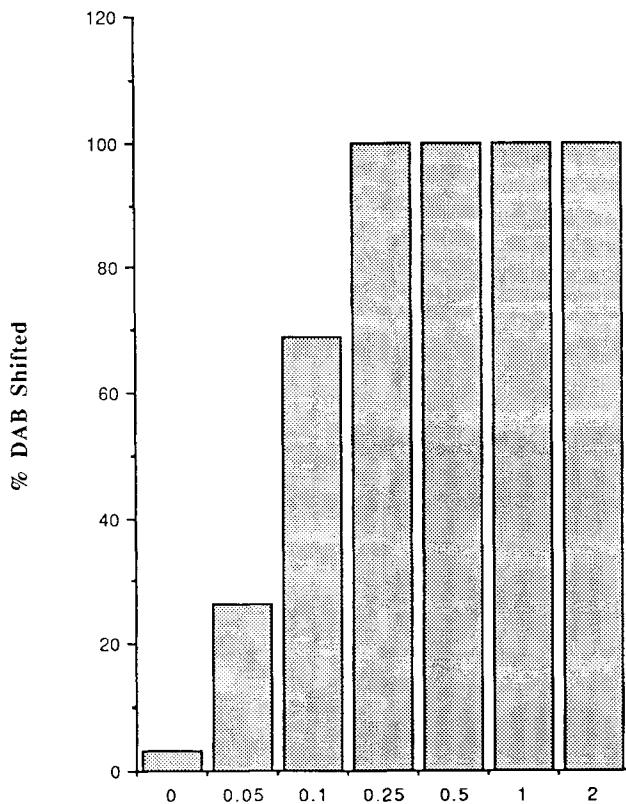
One possible explanation for the lack of mixing seen in the earliest part of the endocytic pathway is that an increase in vesicle volume diluted the concentration of HRP to the point where it could not effect a complete density shift, leading to an underestimation of the amount of vesicle fusion. To examine this possibility we titrated the amount of HRP required to give a density shift. The concentration of HRP usually employed was 1.0 mg/ml, and as demonstrated in Fig. 3, changes in density were seen with concentrations as low as 0.05 mg/ml. Thus, we should be able to detect fusion events even under conditions that result in a 20-fold dilution of HRP.

Previous studies in alveolar macrophages demonstrated that different ligands and receptors move through the intracellular endocytic apparatus at the same rate, suggesting that there are no receptor or ligand specific rates (Ward et al., 1989). Based on this result, we predicted that in the early portion of the endocytic pathway, other ligands would behave similarly to Tf. To test this hypothesis we employed  $\text{aM}\cdot^{125}\text{I}$ -T as a ligand. This ligand is routed to the lysosome and provides information on the early and late parts of the endocytic pathway but not on the behavior of recycling vesicles. Within the early part of the pathway the results were similar to that obtained with Tf. The amount of mixing seen as a result of tandem pulses was 20–40% of control, and dropped to near background levels when a 2-min chase was interposed between the different ligand pulses (Fig. 4 A). Identical results were observed using  $^{125}\text{I}$ -man-BSA as a ligand (data not shown). These experiments demonstrate that regardless of the ligand, administration of ligands as tandem pulses resulted in a significant amount of mixing in early endosomes. The degree of mixing was again reduced when a chase period was interposed between the two pulses.

#### **Fusion of Sequentially Internalized Ligands in Late Endosomes**

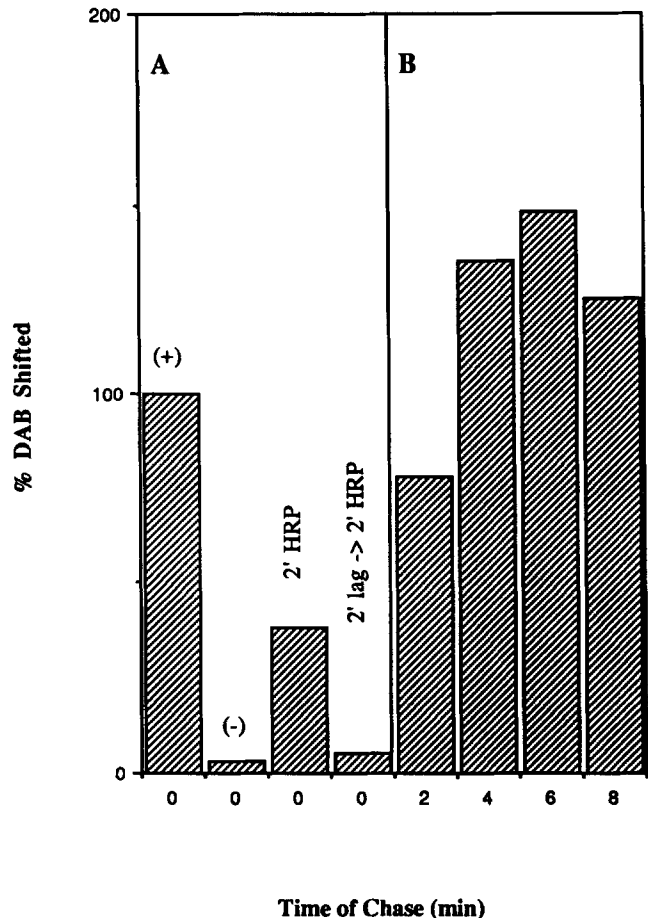
Except for a short period of time immediately after internalization, ligands within the early endocytic pathway remained

a 2-min chase interposed between the pulses (column D) were analyzed statistically using a *t* test and exhibited a *p* value of 0.001. ( $\square$ )  $^{131}\text{I}$ -Tf, and ( $\circ$ )  $^{125}\text{I}$ -Tf with closed symbols represent samples exposed to DAB.



**Figure 3.** Determination of the amount of HRP required to obtain a density shift. Cells were incubated at 37°C for 2 min with aM-<sup>125</sup>I-T and varying concentrations of HRP (0.01–2.00 mg/ml). Samples were then placed at 0°C, surface ligand was removed, and subcellular fractionation was performed as described. Endosomal peaks were pooled and incubated with DAB and H<sub>2</sub>O<sub>2</sub> before application to the second Percoll gradient. The data is expressed as the amount of density shift at each HRP concentration relative to that seen in cells exposed to 2.0 mg/ml HRP.

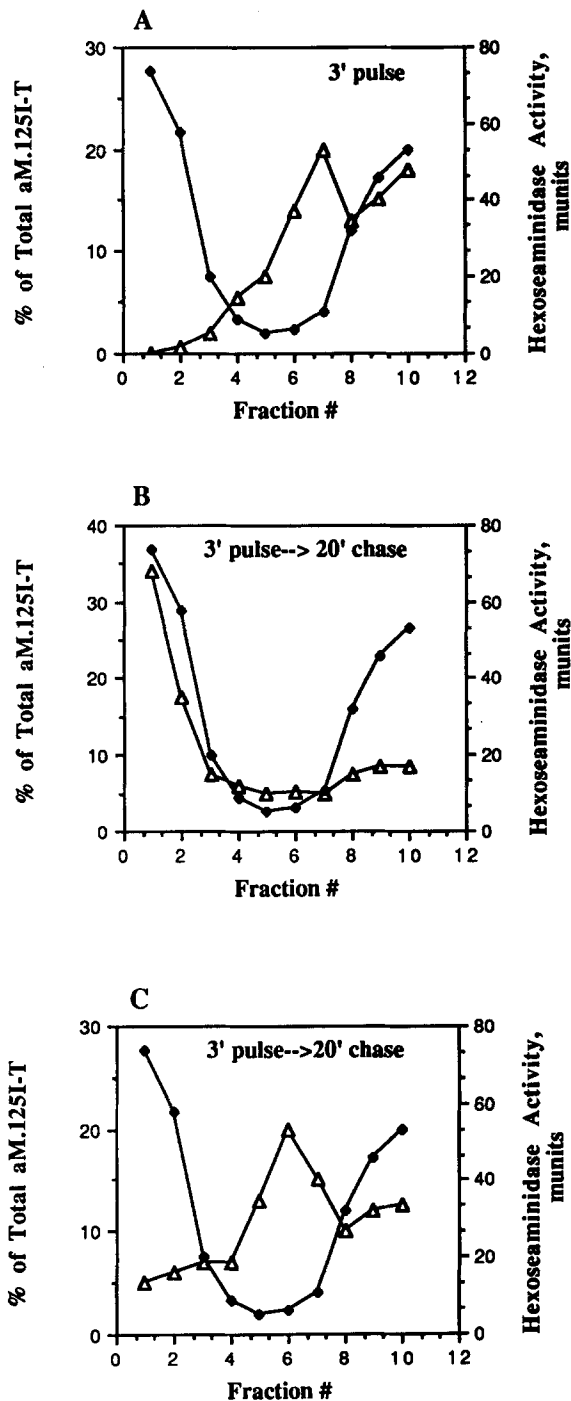
in separate compartments. Extensive mixing of sequentially internalized ligands occurred, however, as the ligands traversed the endocytic apparatus moving from early to late endosomes. Fig. 4 B is a continuation of the experiment presented in Fig. 4 A except that cells were further incubated at 28°C after the initial administration of HRP. This protocol allowed us to assay sequential endosome fusion in different parts of the late endocytic pathway prior to the lysosome. As the ligands move through the endocytic cycle, the amount of vesicle fusion increased. For example, the amount of mixing after administration of the two ligands with a 2-min chase interposed was <10%. When these cells were incubated for an additional 2 min in the absence of HRP, the amount of mixing increased to 80%. We consistently ( $n = 5$ ) observed greater density shifts of sequentially added ligands in late endosomes than observed when ligands were given simultaneously (see Discussion). Similar results were obtained using <sup>125</sup>I-man-BSA (data not shown). The fact that we can see fusion in the late pathway demonstrates that the lack of fusion in the early part of the endocytic pathway cannot be



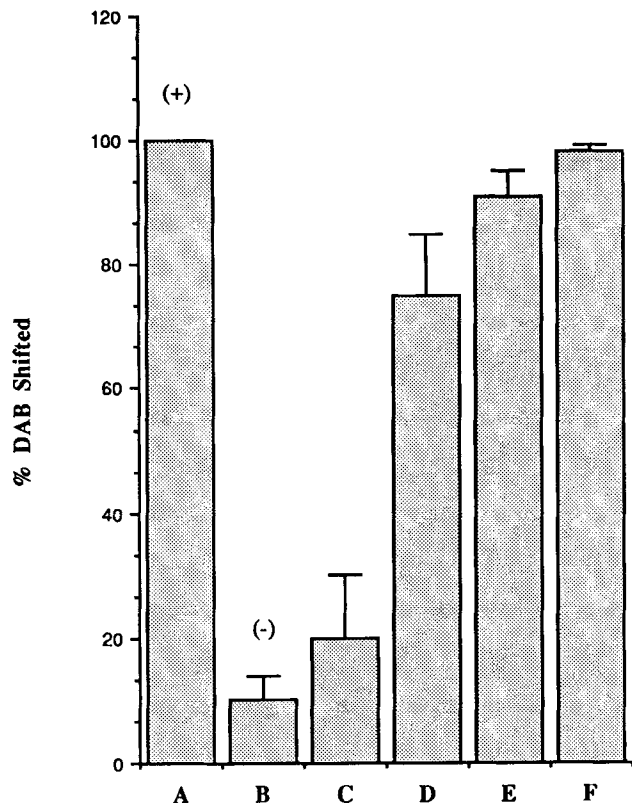
**Figure 4.** Mixing of tandem and sequentially added aM-<sup>125</sup>I-T and HRP in cells incubated at 28°C. Cells were incubated with either aM-<sup>125</sup>I-T and HRP (+), or aM-<sup>125</sup>I-T alone (-) at 28°C for 4 min, placed back at 0°C, and surface ligand was removed. Cells incubated with aM-<sup>125</sup>I-T only were placed at 28°C and incubated with HRP for 2 min either immediately or after a 2 min-lag (A). Those cells given a 2-min lag before the HRP pulse were placed at 0°C, surface HRP was removed, and then incubated at 28°C for an additional 2–8 min, to allow movement of ligand further through the endocytic apparatus. All samples were homogenized, endosome isolated and incubated with DAB/H<sub>2</sub>O<sub>2</sub>. The times given in the figure represent the total time at 28°C after HRP administration. (+) Represents ligands given simultaneously and (-) those that never received HRP or DAB. The percentage of radioactivity shifted by DAB is calculated as described in Materials and Methods.

ascribed to dilution of HRP concentration as a result of an increase in vesicle volume.

The above experiment was performed at 28°C, because movement of ligand to the lysosome was too rapid at 37°C to obtain quantifiable results. To determine if mixing between late endocytic compartments was simply an artifact of reduced temperature, we attempted to find methods by which we could block movement of ligands to the lysosome at 37°C. Baenziger and Fiete (1982) demonstrated that incubation of hepatocytes, in media in which Na<sup>+</sup> was replaced by K<sup>+</sup> (iso-K<sup>+</sup>), resulted in a decrease in ligand degradation but not receptor–ligand internalization, or receptor recycling. Continued receptor recycling resulted in the accumu-



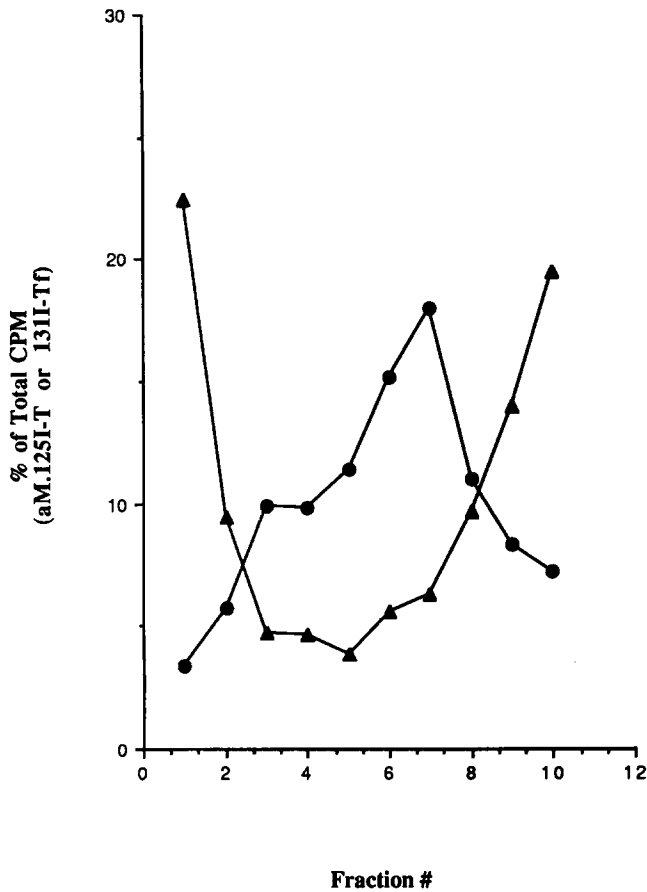
**Figure 5.** Iso- $K^+$  buffers block movement of ligand to the lysosome. Cells were incubated in  $Na^+$  or  $K^+$  buffers at  $37^\circ C$  for 60 min before ligand exposure. The cells were incubated with  $aM.125I-T$  at  $37^\circ C$  for 3 min, placed at  $0^\circ C$ , and surface ligand was removed. Cells were then incubated at  $37^\circ C$  for an additional 20 min in the specified buffer. The cells were harvested, homogenized, and the homogenate was applied to a Percoll gradient. The distribution of radioactivity ( $\Delta$ ) and B-N-acetylhexoseaminidase activity ( $\blacklozenge$ ) were determined. *A* is a representative radioactive profile as both  $Na^+$  and  $K^+$  samples were identical after a 3-min incubation. *B* represents cells incubated further in  $Na^+$  and *C* represents cells after an additional 20 min in  $K^+$ .



**Figure 6.** Cells in iso- $K^+$  buffers accumulate sequentially given ligands in late endosomes. Cells were incubated in iso- $K^+$  buffers at  $37^\circ C$  for 60 min and then incubated either in the presence of  $aM.125I-T$  and HRP (*A*) or with  $aM.125I-T$  alone (*B*) for 5 min at  $37^\circ C$ . The cells were placed at  $0^\circ C$ , washed, surface bound ligand was removed, and then incubated at  $37^\circ C$  for an additional 10 min. Additional cells, which had only been exposed to  $aM.125I-T$ , were then given a 2-min pulse of HRP, placed at  $0^\circ C$ , and excess HRP was removed. The cells were harvested after a further incubation in iso- $K^+$  buffers for 2 min (*C*), 4 min (*D*), 6 min (*E*), and 8 min (*F*). The data are expressed as the percentage shifted upon DAB exposure and are calculated as described in Materials and Methods.

lation of ligand in a nonlysosomal compartment. Incubation of macrophages in iso- $K^+$  buffers at  $37^\circ C$  prevented movement of internalized  $aM.125I-T$  to lysosomes (Fig. 5). Based on the distribution of radioactivity across the gradient the rate of movement of ligand to the lysosome was decreased by  $>85\%$ .

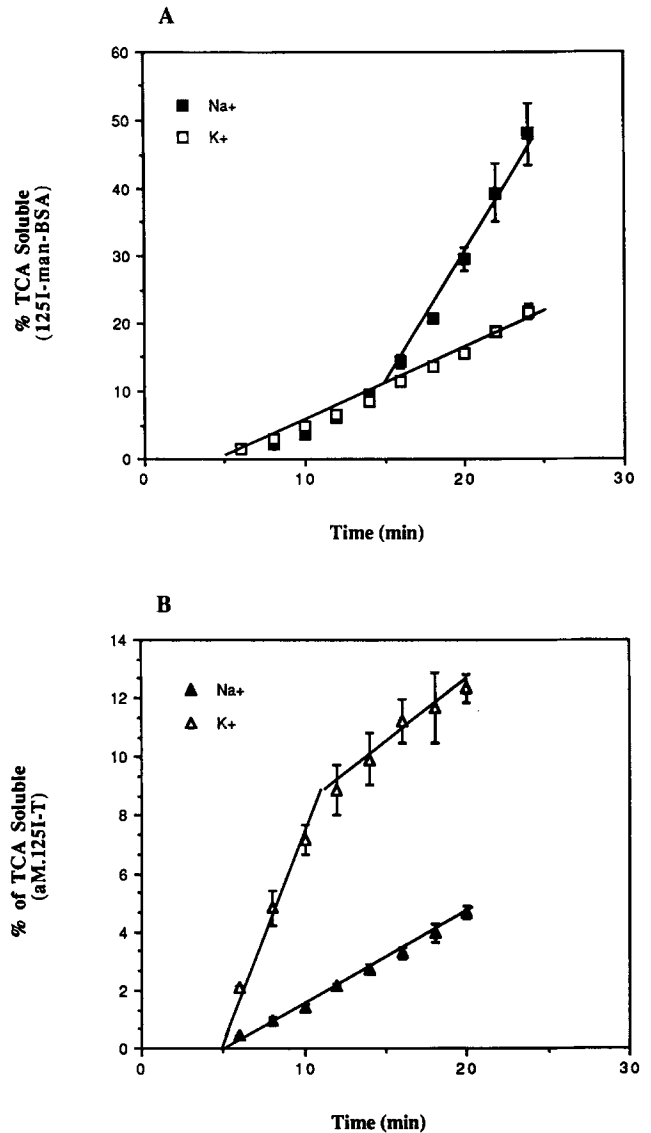
Accumulation of ligand implies fusion of vesicles containing ligands internalized at different times, or the presence of large numbers of prelysosomal vesicles. To distinguish between these two hypotheses, we determined whether sequentially internalized ligands entered the same compartment. Cells incubated in iso- $K^+$  buffer were given a pulse of  $aM.125I-T$  for 5 min, chased in the absence of ligand for 10 min, and then were given a 2-min pulse of HRP (Fig. 6). Control experiments (ligands administered simultaneously) demonstrated that incubated cells in iso- $K^+$  buffers did not affect the density shift (*A* and *B*). There was only a limited amount of mixing (20%) immediately after internalization of the second ligand (Fig. 6, column *C*), but, upon further incubation, the degree of mixing increased dramatically (columns *D-F*).



**Figure 7.** <sup>131</sup>I-Tf does not mix with aM.<sup>125</sup>I-T and HRP in iso-K<sup>+</sup> buffers in late endosomes. Cells were incubated in iso-K<sup>+</sup> buffers for 60 min at 37°C and then incubated with aM.<sup>125</sup>I-T and HRP at 37°C for 5 min. The cells were placed at 0°C, washed, and surface ligand was removed. After an additional 10 min at 37°C, cells were incubated with <sup>131</sup>I-Tf for an additional 2–10 min. Cells were then placed at 0°C, surface ligand was removed, homogenized, and the density shift or radiolabel was measured. (●) <sup>131</sup>I-Tf; (▲) aM.<sup>125</sup>I-T. This figure represents a 6-min incubation with <sup>131</sup>I-Tf.

To prove that the loss of temporal segregation occurred only in late endosomes, we took advantage of the fact that this vesicle population is defined by the absence of Tf. Cells incubated in iso-K<sup>+</sup> were pulsed for 5 min with aM.<sup>125</sup>I-T and HRP, chased for 10 min, and then incubated with <sup>131</sup>I-Tf for periods up to 10 min. At specified times, the cells were homogenized and the endosomal fraction analyzed. Regardless of the length of exposure of cells to Tf, there was no measurable density shift of <sup>131</sup>I-Tf, whereas aM.<sup>125</sup>I-T invariably shifted density. A representative experiment is demonstrated in Fig. 7 in which cells were incubated with <sup>131</sup>I-Tf for 6 min. By measuring <sup>131</sup>I-Tf radioactivity in cell homogenates, we determined that incubation of cells in iso-K<sup>+</sup> buffer did not affect the recycling rate of Tf (6–8 min; data not shown). Since we saw mixing of aM.<sup>125</sup>I-T with HRP within 4 min of addition (Fig. 4), we conclude that Tf does not mix with the previously internalized HRP. The lack of mixing with Tf defines the HRP containing compartment as being a late endosome.

In repeating these experiments with <sup>125</sup>I-man-BSA, we



**Figure 8.** Degradation of <sup>125</sup>I-man-BSA and aM.<sup>125</sup>I-T in iso-K<sup>+</sup> buffers. Cells incubated in either iso Na<sup>+</sup> or iso K<sup>+</sup> buffers were exposed to either <sup>125</sup>I-man-BSA (A), or aM.<sup>125</sup>I-T (B), for 3 min at 37°C. Cells were then placed at 0°C, washed, and surface ligand was removed and then placed back at 37°C in Na<sup>+</sup> or K<sup>+</sup> buffers. At selected times, samples were removed and the amount of TCA soluble radioactivity in the media determined. The data are expressed as the percentage of TCA soluble radioactivity in the medium relative to the total radioactivity in the cell at the start of the experiment.

observed that the ligand was rapidly degraded in iso-K<sup>+</sup> incubated cells (data not shown). Measurement of the accumulation of aM.<sup>125</sup>I-T also indicated a decrease in cell associated ligand. These results are discrepant with previous reports on the effect of iso-K<sup>+</sup> buffers on ligand degradation (Baenziger and Fiete, 1982; Wolkoff, 1984), prompting us to further examine ligand catabolism in cells incubated in iso-K<sup>+</sup> buffers. Measurement of the rate of degradation of <sup>125</sup>I-man-BSA in Na<sup>+</sup>-containing buffers suggests a multiphasic curve (Diment and Stahl, 1985; Fig. 8 A). The greatest rate of degradation was obtained at 12–13 min after

internalization, at which point the curve appeared monophasic. Ligand degradation was also seen in cells exposed to iso-K<sup>+</sup> buffers, although the curve appeared less complex with the maximum rate being initiated at 5 min. The rate of degradation in iso-K<sup>+</sup>-incubated cells was ~30% that seen in Na<sup>+</sup>-incubated cells. Surprisingly, we observed a higher rate of aM·<sup>125</sup>I-T degradation in iso-K<sup>+</sup>-incubated cells than in Na<sup>+</sup>-incubated cells (Fig. 8 B). It should be pointed out, however, that the rate of degradation of aM·<sup>125</sup>I-T is much slower than <sup>125</sup>I-man BSA, and over the time course studied, only a small percent of the internalized aM·<sup>125</sup>I-T was degraded. During the time period in which these measurements were made, ligand was found in compartments that do not contain B-n-acetylhexosaminidase, suggesting that the compartment is not a lysosome, at least by conventional definition.

## Discussion

Within the intracellular endocytic pathway, the properties of internalized receptor-ligand complexes are affected by a changing intravesicular milieu. As a result, ligands and receptors may have different eventual fates, either degradation in lysosomes, or movement back to the cell surface. Changes in the intravesicular milieu appear to be sequential, and follow a defined pattern. For example, early endosomes are typified by a higher pH than late endosomes. These changes suggest the existence of a temporal order in the endocytic pathway. Another example of order in the endocytic pathway is the fact that the contents of vesicles internalized at different times do not intermix; thus they are temporally segregated. This was first observed in a study of Tf receptor behavior in HeLa cells (Ajioka and Kaplan, 1986). Recently, Salzman and Maxfield (1988) reported that they were able to confirm these observations on the lack of mixing in the early part of the endocytic pathway in HeLa cells, although they observed mixing in the later part of the recycling pathway of HeLa cells. Furthermore, they observed mixing of sequentially added ligands and fluid in the early, as well as later, portions of the endocytic apparatus in CHO cells. There are a number of possible explanations for the difference between results in the two studies, including the choice of ligands employed, the methodology used to assay mixing, the conditions at which the cells were maintained (i.e., reduced temperatures and different procedures used to remove surface bound ligand), as well as different cell types.

In this study, we examined temporal segregation of internalized ligands in alveolar macrophages. Use of this cell type allowed us to examine the behavior of a number of different ligands in a single cell type. We observed a changing pattern in the ability of vesicles, internalized at different times, to fuse.

### *Fusion of Sequentially Internalized Vesicles*

We observed a limited amount of ligands in cells given tandem pulses of ligand. The mixing was seen only when ligands were administered as tandem pulses. If a chase period of 2-min was interposed between the two pulses, the amount of mixing was radically decreased. The degree of mixing was independent of the ligand employed or the order of ligand addition. This result is different from that obtained

in our previous study in HeLa cells (Ajioka and Kaplan, 1986) in which we observed no mixing between ligands in sequentially internalized compartments. In that study, we did not use tandemly administered pulses, but had always employed a chase between the pulses. We have repeated those experiments using tandem pulses and still did not observe any mixing (data not shown). Since the techniques were the same, we conclude that this reflects a cell type specific difference in the early portion of the endocytic pathway.

There are two hypotheses that may explain the mixing seen as a result of tandem pulses: (a) intracellular mixing of vesicle contents is restricted to the earliest portion of the endocytic pathway, or (b) the observed mixing of vesicle contents occurs at the cell surface. It is possible that a certain percentage of early endosomes fuse with the cell surface; the contents of these endosomes are mixed with newly added ligand bound to cell surface receptors; and both ligands are then internalized together. There is evidence to support the immediate fusion of early endosomes with the cell surface, a process referred to as diacytosis or retroendocytosis (Green-span and St. Clair, 1984; Chang and Kullberg, 1984; Marshall, 1985; McKinley and Wiley, 1988). We can demonstrate that ~15% of internalized ligand reappears at the cell surface (McVey Ward, D., S. Davis-Kaplan, and J. Kaplan, manuscript in preparation), a level that is lower than the amount of fusion.

Fusion of early endosomes does not increase when cells given tandem pulses are chased such that the ligands further traverse the early endocytic pathway, or is not seen when a lag time is interposed between the two ligand pulses. These observations suggest that mixing is restricted to the earliest portion of the endocytic pathway, and temporal ligand segregation is maintained throughout the remainder of the early endocytic pathway. We have little information on mixing in the recycling pathway in alveolar macrophages. To study ligand mixing in recycling endosomes in HeLa cells, we used the conjugate Tf-HRP and <sup>125</sup>I-Tf as markers of recycling vesicles (Ajioka and Kaplan, 1986). In alveolar macrophages, we were unable to demonstrate DAB shifts using Tf-HRP even in those samples given both ligands simultaneously. These cells contain only 10<sup>4</sup>Tf receptors as compared to 10<sup>6</sup> in HeLa cells, and we were unable to obtain enough intracellular Tf-HRP to effect a density shift. We did, however, consistently observe a broadening of our gradient profiles when iodinated-Tf was allowed to further traverse the endocytic pathway. One possible explanation for the profile broadening may be the changing endosome population; that is, from early to recycling endosomes.

### *Ligand Intermixing in the Late Endocytic Pathway*

There is a breakdown in temporal segregation of sequentially internalized ligands in late portions of the endocytic pathway. This part of the pathway is defined by the absence of internalized Tf, and contains ligands which are en route to the lysosome. Ligands that showed no mixing in the early part of the endocytic pathway showed levels of mixing in late endosomes that were equivalent to or greater than cells in which the ligands were given simultaneously. One explanation for the observed level of DAB shiftable material (>100% of control levels; cf., Fig. 4 B) is that there are ligand specific early endosomes that later mix their constituents. There are



studies that are for (Gorman and Poretz, 1987; Goldberg et al., 1987) and against this possibility (Stoorvogel et al., 1987) thus leaving this possibility still plausible. Another interpretation may be that early endosomes are more susceptible to breakage or damage than late endosomes. If this is true, then, as ligand progresses into late endosomes, the amount of DAB shifting observed would increase. Regardless of which interpretation is accurate, our data indicate the presence of a pool of late endosomes in which ligands mix extensively before being delivered to the lysosome. The level of mixing was independent of the ligand employed, the order of administration of the ligand, whether the ligand was internalized by fluid phase or receptor mediated endocytosis, and the temperature at which the cells were maintained during the course of the experiment (18, 28, or 37°C; data not shown). Mixing of late endocytic contents could be observed in cells incubated at 37°C in iso-Na<sup>+</sup> buffers, although it was difficult to quantify because of the rapidity at which molecules were moved to the lysosome.

Baenziger and Fiete (1982) reported that hepatocytes incubated in iso-K<sup>+</sup> buffer internalized asialoglycoproteins, but did not degrade them or send them to the lysosome allowing ligand to accumulate in late endosomes. It has recently been suggested that the effect of iso-K<sup>+</sup> buffers is mediated by an acidification of the cytosol (Samuelson et al., 1988). This suggestion is consistent with the demonstration that cytosol acidification can alter endosomal movement (Davoust et al., 1987). Since ligand continues to accumulate within the cell, we reasoned that the compartment in which these ligands accumulated might be the compartment in which temporal ligand segregation was abolished. We demonstrated that movement of ligand to lysosomes was inhibited in macrophages incubated in iso-K<sup>+</sup> media (Fig. 5), and that ligands internalized at different times were capable of mixing (Fig. 6). Even in iso-K<sup>+</sup> buffers Tf-containing vesicles do not exhibit mixing with previously internalized ligands, demonstrating that when mixing did occur the compartments involved were part of the late endocytic pathway, and that incubation in iso-K<sup>+</sup> per se did not induce mixing.

One of the striking features of the studies of Baenziger and Fiete (1982, 1986) was the observation that incubation of hepatocytes in iso-K<sup>+</sup> buffer inhibited ligand degradation. A small amount of ligand degradation was observed in the studies of Wolkoff et al. (1984). They ascribed this low rate of degradation to an incomplete inhibition of ligand movement to lysosomes. We were therefore surprised to see a significant rate of degradation of man-BSA in macrophages incubated in iso-K<sup>+</sup> media. The rate of degradation was 30–50% of that seen in iso-Na<sup>+</sup>-incubated cells. We could not detect significant amounts of ligand in lysosomes (Fig. 5); therefore, degradation did not result from "escape" of internalized molecules to the lysosome. Rather, we think that it may represent the action of cathepsin-like proteases present in late endocytic compartments. This was first described in macrophages by Diment and Stahl (1985) and later demonstrated in other cell types ranging from Swiss 3T3 (Roederer et al., 1987) to *Xenopus* oocytes (Opresko and Karpf, 1987). It is unclear why previous studies, which used hepatocytes and asialoglycoproteins, did not see more ligand degradation. One plausible explanation is that the ability to observe degradation is not only cell type dependent but ligand specific. man-BSA was degraded at a lower rate in late

endosomes than in lysosomes; however, aM-T appeared to be degraded at a higher rate in the late endosome than it was in the lysosome, although it should be recognized that the absolute degradation rate of this ligand is much lower than for man-BSA, implying differences in protease activity in the two compartments.

What does temporal segregation or fusion suggest about the endocytic apparatus? Studies using in vitro endosome fusion systems have demonstrated substantial levels of fusion among early endosomes (Davey et al., 1985; Braell, 1987; Diaz et al., 1988; Gruenberg et al., 1989). Indeed, the majority of early endosomes are capable of in vitro fusion. This level of fusion is much higher than is seen using in vitro assays, as demonstrated in this and previous studies (Ajioka and Kaplan, 1986; Salzman and Maxfield, 1988). Furthermore, as demonstrated directly by the studies here and implied by others, inhibitors that prevent movement of ligand to lysosomes but not ligand accumulation (Baenziger and Fiete, 1982, 1986; Mueller and Hubbard, 1986; Schmid et al., 1988; Samuelson et al., 1988) demonstrate that there must be a late endosomal component capable of fusion with incoming vesicles, from which membrane can be recycled. Yet, to date, no in vitro fusion assays have demonstrated fusion of early internalization compartments with late compartments, or late compartments with each other, although Mullock et al. (1989) recently developed an in vitro endosome-lysosome fusion system. The observation that there is extensive fusion of late endosomal compartments in vivo but not in vitro suggests that the present in vitro assays are lacking some critical component. The fact that early endosomes are capable of fusion with each other in vitro, but do not do so in vivo, or only to a limited degree, implies that in vivo they must be under some physical restraint that prevents fusion. For example, if they were tethered to cytoskeletal elements, these elements could specify a rate of movement and assure that movement occurs in a vectorial manner. Studies using agents which disrupt microtubules have not demonstrated any discernible effect on either internalization or movement in the early endocytic pathway, although small effects were observed in late endosomes (Gruenberg et al., 1989). Studies are currently underway to address these issues.

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