AN ELECTRON MICROSCOPE AUTORADIOGRAPHIC STUDY OF
THE CARBOHYDRATE RECOGNITION SYSTEMS IN RAT LIVER

II. Intracellular Fates of the $^{125}\text{I}$-Ligands

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ABSTRACT

Electron microscope autoradiographic and biochemical methods were used to study the intracellular fates of several $^{125}\text{I}$-glycoproteins, known to be specifically bound and internalized by the different cell types in the liver. At the earliest times examined (1–2 min), $^{125}\text{I}$-asialo-glycoproteins (ASGP) were localized predominately along the sinusoidal front of hepatocytes. Analysis of the distribution of autoradiographic grains indicated that: (a) $\sim40$–$60\%$ of the $^{125}\text{I}$-ligand could be ascribed to the plasmalemma; (b) a significant fraction had already been internalized; yet (c) very little $^{125}\text{I}$-ligand was present in the lysosome-Golgi region. Between 4 and 15 min after administration of $^{125}\text{I}$-ASGPs, there was a dramatic redistribution of autoradiographic grains from regions of the plasmalemma and peripheral cytoplasm (30% decrease) to the lysosome-Golgi region (30% increase). At longer times (30 min), there was continued drainage of $^{125}\text{I}$-ASGP into this region. The grain density over secondary lysosomes was 60–90 times higher than that over recognizable Golgi elements, clearly indicating that lysosomes were the ultimate destination of the $^{125}\text{I}$-ASGP. However, no more than 60% of the total $^{125}\text{I}$-ligand could be localized to lysosome-rich regions of the hepatocyte, with the remaining 40% primarily in the intermediate cytoplasm. Biochemical evidence for proteolysis of the internalized $^{125}\text{I}$-ASGP (presumably within lysosomes) was obtained when $[^{125}\text{I}]-\text{mono-iodotyrosine}$ was found in the liver (i.e., hepatocytes) at times later than 15 min.

The temporal redistribution observed for mannose and $N$-acetylgalcosamine-terminated glycoproteins (ahexosamino-orosomucoid and agalacto-orosomucoid, respectively) in endothelial cells indicated that the $^{125}\text{I}$-ligands resided in macro-pinoscytic vesicles (1–15 min) before their ultimate residence in dense bodies (15 min). The same $^{125}\text{I}$-ligands were also localized to structures resembling secondary lysosomes in Kupffer cells. The lysosomal nature of these organelles was implied from the appearance of $[^{125}\text{I}]-\text{mono-iodotyrosine}$ in the liver at later times. $^{125}\text{I}$-$\beta$-glucuronidase followed the same intracellular pathway in both cell types but was not degraded.
In the preceding paper (17), we presented results of an electron microscope autoradiographic (EM-ARG) study on the cellular distribution of seven iodinated glycoproteins rapidly cleared from the circulating plasma by the liver. There appeared to be two distinct carbohydrate recognition systems present on different cell types in this tissue. That is, we found that hepatocytes, but not cells lining the sinusoids, specifically bound and internalized mannose on their oligosaccharide chains. Conversely, endothelial cells and Kupffer cells, but not hepatocytes, specifically bound and internalized mannose and/or N-acetylglucosamine-terminating glycoproteins. Other laboratories have reported similar results using different approaches (see introduction to preceding paper (17) for appropriate citations of earlier work.) In this paper, we present data on the changes in the intracellular distribution and on the metabolism of the various ligands that occur as a function of time after internalization.

MATERIALS AND METHODS

General

The preparation and iodination of the glycoproteins, as well as the microscopy and ARG of the liver, have been described (17).

In one EM-ARG experiment, 2.5 d before injection of 125I-asialo-fetuin (ASF), Triton WR-1339 (150-190 mg/100 g body wt) was injected intraperitoneally in sterile saline. This nonionic, nonhydrolyzable detergent has been shown to accumulate within the secondary lysosomes of both hepatocytes and Kupffer cells and thus to "mark" and increase the visibility of this intracellular compartment (29).

Analysis of Autoradiograms

INTRACELLULAR DISTRIBUTION OF 125I-ASIALOGLYCOPEPTIDES (ASGP): Micrographs of hepatocytes present in ARG preparations were taken at random at a standard magnification of 3,400. Quantitative analysis was performed on prints at × 9,200. A zoom microscope was placed over the micrograph, further enlarging selected regions 10 times (to × 92,000), and grain or point assignments were made. The intracellular distribution of ARG grains associated with hepatocytes at various times after ASGP administration was determined by counting grains overlying the following regions: (a) sinusoidal front microvilli; (b) four bands, each 2,000 Å in width, extending from the base of the sinusoidal plasmalemma 8,000 Å into the hepatocyte cytoplasm; (c) regions (usually near bile canaliculi) having recognizable Golgi structures (piles of cisternal elements, smooth-membranned vesicles, and vacuoles containing lipoprotein particles) and/or recognizable lysosomal structures (dense or residual bodies and vacuoles containing amorphous material); (d) the remaining cytoplasm (termed intermediate); and (e) the nucleus. These regions are outlined in Fig. 1. The area of each region was determined by using a calibrated grid (d = 2.4 cm, 80 points) and recording the type of region located at each intersection point.

It is apparent from Fig. 1a and b that the plasmalemma on the sinusoidal front covers numerous, irregular microvilli from 4,000 to 10,000 Å in length. This complicated picture necessitated our equating the microvillar region to the sinusoidal front plasmalemma, although the region also contains the space of Disse (external to the actual bilayer) and cytoplasmic matrix (internal to the bilayer). The decision to collect grains within four successive 2,000-Å bands of the hepatocyte peripheral cytoplasm was based on several considerations. First, it is apparent that a band 8,000 Å in thickness constitutes a coherent intracellular region in which there are few mitochondria and little endoplasmic reticulum, but many smooth vesicles, possibly originating from plasmalemma at the sinusoidal front (Fig. 1b). Secondly, the distance in which half the grains emanating from a line or point source will fall has been defined as the half-distance (HD) by Salpeter et al. and established as ~1,000 Å (850 ± 120 Å) for 125I under the conditions used in this study (26). Thus, the distribution of grains in successive 2-HD (2,000 Å) bands away from the base of the sinusoidal plasmalemma should reveal whether those grains originated from that structure (and were detected in surrounding regions due to "radiation spread") or from structures within the peripheral cytoplasm. In practice, ARG grains found within 2 HD of a structure can be ascribed to it, based on statistical considerations (27).

Both grain distribution and relative grain density (from grain and area measurements) were calculated for each intracellular region listed above.

ARG GRAIN DISTRIBUTION WITHIN THE LYSOSOME-GOLGI AREA OF HEPATOCYTES: The lysosome-Golgi region of hepatocytes was further subdivided into the following regions: (a) the Golgi complex, consisting of stacks of Golgi cisternae and neighboring vacuoles containing lipoprotein particles (e.g., Fig. 3c); (b) lysosomes (i.e., dense bodies, residual bodies, and

KEY WORDS carbohydrate recognition - liver - internalization - intracellular transport - lysosomes - degradation
FIGURE 1  EM-autoradiographs illustrating the intracellular compartments in the hepatocyte chosen for 125I-ASGP quantitative analysis. (a) The two solid lines along the sinusoidal lumen (SL) mark the outer and inner boundaries of the microvillar band (Mv); the hatched line is 8,000 Å from the base of the microvillar band; the arrow-heads mark successive 2,000-Å bands within the peripheral cytoplasm; lysosome-Golgi regions (L-G) are outlined. Bar, 1 μm; × 7,200. The micrographs used for grain and point counting were printed at × 9,200 and magnified to × 92,000 as described in Materials and Methods. (b) Higher magnification of the microvillar region (Mv) and peripheral cytoplasm of the hepatocyte. The microvilli vary in shape and length and extend into the space of Disse (DS). Coated vesicles (Cv) as well as vesicles with smooth membranes can be seen in the peripheral cytoplasm. Bar, 0.2 μm; × 23,000.
activity in the sample was measured in a gamma counter, suggesting rapid internalization of 125I-ASF. At 15 min, ARG grains were observed at significant distances within the cytoplasm, suggesting rapid internalization of 125I-ASF. At 15 and 30 min, grains were found in the lysosome-Golgi region of cells and were associated with bodies having the structural characteristics of secondary lysosomes (Figs. 3 b and c, and 4 a).

We have quantitated the ARG grain distribution and grain density over a number of intracellular compartments at four times after the injection of 125I-ASF and 125I-asialo-orosomucoid (ASOR). The data from the earliest time point (1 min, 125I-ASOR) are presented in Table I to illustrate the size of the various cellular compartments analyzed and the actual number of ARG grains counted in each compartment. The microvillar regions (see Fig. 1 for demarcation of compartments) represented 9.4% of the total cell area (volume) in the sections examined and yet 27.1% of the hepatocyte ARG grains were localized in this region. This means that there was a 2.9-fold concentration of grains over the microvilli. However, the highest grain density was not found at the sinusoidal front but within the cytoplasm 0-2,000 A from the base of the microvillar band. These results indicated that, as early as 1 min, 125I-ASOR was associated with structures other than (or in addition to) the sinusoidal plasmalemma. Another interesting finding was the absence of grains ~2,000-0 Â external to the microvillar band. If 125I-molecules were uniformly distributed over the microvilli, a percentage of the developed grains should have been found in this band, due to radiation spread (27). Their absence suggested a preferential association of 125I-ASOR with the base rather than the tips of microvilli, and/or with the plasmalemma in between the microvilli. We are currently investigating this possibility at times earlier than 1 min. Finally, the grain density over the Golgi and lysosome region (Table I) was quite low indicating negligible transport of 125I-ASOR to this region by 1 min.

Table II summarizes grain distributions 1-30 min after the injection of two 125I-ASGPs. As mentioned above (Materials and Methods), grains overlying the microvilli and as far as 2,000 Â (2 HD) into the hepatocyte peripheral cytoplasm could be statistically ascribed to the plasmalemma. Therefore, to simplify the presentation of data, these two regions were grouped together and classified as "plasmalemma." Because no more than one ARG grain was found 2 HD outside the microvillar region at any time examined, this region was omitted from all calculations. Finally, data from the three cytoplasmic bands of 2,000 Â each (columns 3-5 of Table I) were pooled under
FIGURE 2  EM-autoradiographs of livers fixed by perfusion 2 min (Fig. 2a) and 30 min (Fig. 2b) after i.v. injection of $^{125}$I-ASF. (a) At 2 min, ARG grains are localized primarily to the hepatocyte (Hep) cytoplasm adjacent to the sinusoidal lumen (SL). Bar, 1 μm; × 7,900. (b) At 30 min many ARG grains can be seen near bile canaliculi (BC), overlying structures morphologically identifiable as secondary lysosomes (Ly), but not Golgi (Go) elements. The fenestrations (Fen) of an endothelial cell (E) are evident in this micrograph. Bar, 1 μm; × 7,900.
FIGURE 3 EM-autoradiographs of hepatocytes present in livers fixed by perfusion 1 min (Fig. 3a) and 15 min (Fig. 3b and c) after i.v. injection of $^{125}$I-ASGP. (a) At 1 min, ARG grains representing $^{125}$I-ASOR (95 µg, 0.6 mCi injected) are localized predominantly to the base of the microvilli, where coated pits (Cp) can be seen. 15 d exposure; bar, 0.2 µm; × 30,500. (b) At 15 min, ARG grains representing $^{125}$I-ASF (800 µg, 2.4 mCi injected) are still localized near the sinusoidal lumen. Both coated pits (Cp) and coated vesicles (Cv) are present as well as smooth-membraned vesicles of varying diameters. 7 d exposure; bar, 0.2 µm; × 30,000. (c) At 15 min, $^{125}$I-ASF is also found overlying lysosomes (Ly) and unidentified structures (double arrowheads) near bile canaliculi. A Golgi complex (Go) filled with lipoprotein particles is nearby, but no ARG grains are associated with it. 7 d exposure; bar, 0.2 µm; × 30,000.
FIGURE 4 EM-autoradiographs of hepatocytes present in livers fixed by perfusion 30 min after i.v. injection of $^{125}$I-ASF. (a) ARG grains are localized to several lysosomes ($Ly$) near a bile canaliculus ($BC$). The structure associated with one ARG grain (double arrowhead) cannot be identified (termed "other" as described in Materials and Methods). 30 d exposure; bar, 1 μm; $\times$ 32,000. (b) 2.5 d after injection of Triton-WR-1339 and 30 min after $^{125}$I-ASF (220 μg, 2.6 mCi), ARG grains are localized to lysosomes of normal appearance ($Ly$) and those filled with the detergent ($T$). Again, some grains are associated with unidentified structures (double arrowhead), but none over recognizable Golgi elements ($Go$). 28 d exposure; bar, 1 μm; $\times$ 17,500.
TABLE I

Distribution of ARG Grains in Hepatocytes 1 min following Injection of 125I-ASOR

| Parameter                      | Cytoplasm                                                                 |
|--------------------------------|---------------------------------------------------------------------------|
|                                | Intracellular region                                                       |
|                                |                            | Microvillar band | 0-2,000 Å                   | 2,000-4,000 Å               | 4,000-6,000 Å              | 6,000-8,000 Å              | Intermediate cytoplasm | Lysosome-Golgi | Nucleus |
| Grain number (n = 372)         |                            | 1               | 101                          | 119                         | 48                          | 33                          | 13                         | 46             | 11      | 0       |
| % total grains                 |                            | 0.3             | 21.7                         | 32                          | 12.9                        | 8.9                         | 3.5                        | 12.3           | 2.9     | 0       |
| Area, μm² (7,369 μm² total)    |                            | 138             | 690                          | 255                         | 152                         | 131                         | 165                        | 4,733           | 386     | 718     |
| % total area                   |                            | 1.9             | 9.4                          | 3.5                         | 2.1                         | 1.8                         | 2.2                        | 64.2            | 5.2     | 9.8     |
| Grain density (Grains/100 μm²)|                            | 0.72            | 14.6                         | 46.7                        | 31.6                        | 25.2                        | 7.9                        | 0.9             | 2.8     | 0       |
| Relative grain density (1)*    |                            | 0.1x            | 2.9x                         | 9.1x                        | 6.1x                        | 4.9x                        | 1.6x                       | 0.2x            | 0.6x    | 0x      |

* Relative grain density was calculated by dividing the percent of ARG grains found in a compartment by the percent area of the compartment.

TABLE II

Distribution of ARG-Grains in Hepatocytes following Administration of 125I-ASGP

| Molecule | Time (min) | Plasma membrane (microvilli + 0-2,000 Å) | Peripheral cytoplasm (2,000-8,000 Å) | Intermediate cytoplasm | Lysosome-Golgi | Total grains counted |
|----------|------------|----------------------------------------|-------------------------------------|------------------------|---------------|---------------------|
| ASF♂     | 2          | 37                                     | 35                                  | 24                     | 1             | 327                 |
| ASF♀     | 4          | 29                                     | 26                                  | 32                     | 12            | 798                 |
| ASF♂♀    | 15         | 10                                     | 15                                  | 33                     | 41            | 684                 |
| ASF♀♀    | 30         | 14                                     | 14                                  | 19                     | 52            | 255                 |
| (ASF)♀♀  | 30         | 14                                     | 14                                  | 19                     | 52            | 255                 |
| ASF♂♀♀   | 30         | 7                                      | 8                                   | 22                     | 61            | 315                 |
| (Triton WR-1339)♀♀ | 30         | 7                                      | 8                                   | 22                     | 61            | 315                 |

* Animals were injected i.v. with the indicated 125I-protein, and the livers perfused, fixed, and processed as described.

♂ One 125I-ASF preparation, 1.2 mCi/mg, was used for the 2- (0.6 mg) and 30-min (0.9 mg) time points.
♀ One 125I-ASF preparation, 3 mCi/mg, was used for the 4- (0.8 mg) and 15-min (0.8 mg) time points.
♀♀ The 30-min grain distribution has been corrected for hydrolysis of 125I-ASF by normalizing 30-min grain density to that found at 15 min and then by adding grains to only the intermediate cytoplasm and lysosome-Golgi regions in proportion to their ratio at 15 min.
♀♀♀ 2.5 d prior to i.v. administration of 125I-ASF and sacrifice, the rat was injected intraperitoneally with Triton-WR-1339 as described in Materials and Methods.

The term "peripheral cytoplasm." The grain distributions and densities of 125I-ASOR 1 min after injection have already been discussed. However, it is relevant here to recall the kinetics of 125I-ASGP accumulation in the liver, as presented in Fig. 2 of the preceding paper (17). At 1 min, <50% of the maximal radioactivity was present in the liver and, yet, a significant fraction of the grains...
(40%) was already within the hepatocyte cytoplasm, indicating very rapid internalization of the bound $^{125}$I-ligand. At 2 min, >60% of the $^{125}$I-ASGP in the liver had been internalized (Table II). Between 2 and 4 min, $^{125}$I-ASGP continued to accumulate in the liver (Fig. 2, preceding paper [17], and our analysis indicated that transport beyond the peripheral and intermediate cytoplasm had commenced. That is, there was a significant concentration of ARG grains in the lysosome-Golgi region by 4 min (Table III, 4.3X vs. 0.4X at 2 min). As shown in the preceding paper, further accumulation of $^{125}$I-ASGP in the liver was negligible after ~4 min (see Fig. 2 of reference 17). In addition, the levels of acid-insoluble radioactivity remained constant to at least 15 min. Therefore, any changes in intracellular grain distribution between 4 and 15 min reflected redistribution under steady state conditions (most probably by intracellular transport), rather than net accumulation or net depletion (hydrolysis). It can be seen (Table II) that there was a significant decrease in ARG grains over the plasmalemmal region with a concomitant increase within the lysosome-Golgi region over this 10-min interval. There was significantly less (or no) change in the percentage or density of grains found in the two intervening cytoplasmic regions (Tables II and III).

Finally, the intracellular grain distribution at 30 min must be considered in light of the observed loss of radioactivity from the liver between 15 and 30 min (Fig. 2, preceding paper 117). From biochemical measurements made in this (see below) and other studies, it was clear that hydrolysis of the $^{125}$I-ligand was occurring, presumably within structures located in the lysosome-Golgi region, if not other regions as well. Despite the bias introduced by preferential loss from lysosomes, the grain distribution at 30 min reflected a continued drainage into the lysosome-Golgi region from both the peripheral and intermediate cytoplasmic compartments. If appropriate correction was made for the loss of grains between 15 and 30 min (assuming that the loss occurred only from the intermediate and lysosome-Golgi regions), the distribution was altered slightly (Table II). However, a maximum of only 60% of the ARG grains was found under any circumstances in the lysosome-Golgi region. If the ultimate destination of all $^{125}$I-ASF molecules was lysosomes, then a large percentage of these structures must lie outside the regions so designated in this study (see Discussion).

The temporal changes in ARG grain density paralleled those observed for the grain distribution. The relative densities found in each of the seven intracellular regions are presented in Table III to illustrate the shift in concentration of $^{125}$I-ligand through the peripheral cytoplasm. At the earliest times examined (1 and 2 min), the highest density of ARG grains was found in the peripheral cytoplasm. Even when the volume occupied by the space of Disse in the microvillar band was subtracted (~50%, data not shown), the concentration of $^{125}$I-ASGP did not equal that found in the

| Molecule | Time | Hepatocyte grain density | Microvillar band | Intermediate cytoplasm | Lysosome Golgi |
|----------|------|-------------------------|-----------------|------------------------|---------------|
| A. ASF    | 2    | 8.5                     | 2.2x            | 5.7x                   | 10.7x         |
| A. ASF    | 4    | 18.2                    | 1.6x            | 4.5x                   | 6.6x          |
| A. ASF    | 15   | 21.9                    | 0.9x            | 1.6x                   | 6.5x          |
| A. ASF    | 30   | 10.6                    | 0.5x            | 2.0x                   | 2.8x          |
| E. ASOR   | 1    | 35.1                    | 2.9x            | 9.2x                   | 6.3x          |
| F. ASOR   | 15   | 24.1                    | 0.2x            | 0.8x                   | 1.2x          |

*The micrographs used for the grain distributions (presented in Table II) were also used to measure the fractional area of each intracellular region. The relative grain density (percent grains/percent area) was then calculated.

§ The absolute grain density over hepatocytes was calculated as follows: (total number of grains over hepatocytes + total area containing those grains) / fractional decay of $^{125}$I (based on the number of days sections were exposed) + millicuries of $^{125}$I-protein injected.
peripheral cytoplasm, reinforcing the conclusion that ligand internalization was very rapid. At later times, the relative density in the periphery declined, with a concomitant increase only in the lysosome-Golgi region, until a 10- to 20-fold concentration was observed (Table III, D and F). Clearly, this region was the major destination for the internalized $^{125}$I-ASGP.

Localization of ARG Grains Within the Lysosome-Golgi Region

Qualitative examination of many peribiliary regions (e.g., Figs. 2b, 3c, and 4a) revealed that ARG grains were localized predominantly in bodies with a morphology typical of lysosomes. The lysosomal nature of many of these structures was confirmed by acid phosphatase cytochemistry (not shown). Another approach used to localize the internalized ligands in the lysosome-Golgi region was to mark the secondary lysosome population by injecting Triton WR-1339 into animals 2.5 d before injection of $^{125}$I-ASF. This detergent did not substantially alter the overall grain distribution in the hepatocyte (Table II), but it grossly affected the morphology of the peribiliary region (Fig. 4b).

Large lucent vacuoles containing some dense material were present throughout the lysosome-Golgi region. ARG grains representing $^{125}$I-ASF were associated with these vacuoles, as well as with dense bodies in the region. Again, no grains were observed overlying recognizable Golgi elements.

The results of a quantitative analysis of the grain distributions and densities over the different structures in the lysosome-Golgi region are presented in Table IV. We found significant amounts of $^{125}$I-ASOR present in lysosomes as early as 15 min after injection, and <2% of the grains overlying recognizable Golgi elements. The grain density in lysosomes was 57-90 times higher than that in the Golgi elements, clearly indicating that lysosomes, not Golgi elements, were the ultimate destination of the $^{125}$I-ASGP. However, 30-40% of the ARG grains in this region were localized to structures not identifiable as Golgi or lysosomal. Their nature is not yet known.

Metabolism of $^{125}$I-ASGP

The morphologic evidence presented above indicated that a majority of the $^{125}$I-ASGP internalized by hepatocytes was transported to lysosomes by 30 min. Therefore, we investigated the metabolic fate of the liver-associated $^{125}$I-ligand as a function of time. Our results can be summarized as follows (see Table V): (a) There was a decline in the total radioactivity present in the liver between 15 and 30 min after injection of $^{125}$I-ASOR (see also Fig. 2 of the preceding paper [17]); (b) the acid-soluble fraction of the remaining liver-associated radioactivity increased at later times, but never exceeded 20% of the total; (c) significant amounts of both $[^{125}]$MIT, a product of proteolysis, and $[^{125}]$iodide, a product of MIT deiodination (see reference 20), were detected in the acid-soluble fraction of the liver at both 15 and 30 min; (d) no $^{125}$I-products of intermediate size were found; and (e) neither plasma nor serum degraded $^{125}$I-ASGP (data not shown). These data suggested that hydrolysis of $^{125}$I-ASGP occurred in the liver (i.e., hepatocytes) followed by rapid removal of the soluble $^{125}$I-products ($[^{125}]$iodide). However, the modest increase in acid-soluble radioactivity in the blood did not correspond to the loss from

| Time after injection | Secondary lysosomes and dense bodies | Other | Secondary lysosomes and dense bodies | Other |
|----------------------|------------------------------------|-------|------------------------------------|-------|
| 15 min               | 1 (1.6%)                           | 42 (79%) | 17 (28%)                           | 0.103 (5.86) |
|                      | (10%)                              | (28%)  | (10%)                              | (5.86) |
| 30 min               | 2 (0.95%)                          | 123 (58.6%) | 85 (40.4%)                       | 0.056 (5.56) |
|                      | (10%)                              | (40.4%)| (10%)                              | (5.56) |

*$^{*}$ Identification of compartments was based on criteria outlined in Methods and Materials. Examples of the three categories are given in Figs. 3c and 4a and b.

$^{†}$ Percent of total grains counted.

$^{‡}$ Relative grain density, normalizing to the Golgi.
TABLE V
Characterization of Acid-Soluble Radioactivity Found in Different Compartments following in vivo Administration of 125I-Ligands

| Ligand       | Fraction          | Time (min) | Radioactivity recovered (cpm x 10⁴) | Acid-soluble % total recovered | V₀ | [125I]iodide | [125I]MIT |
|--------------|-------------------|------------|------------------------------------|-------------------------------|----|--------------|-----------|
| 125I-ASOR    | Liver             | 4          | 19.5                                | 2.0                           | 9.9 | 86.3        | 3         |
|              |                   | 15         | 20.4                                | 11.9                          | 6.9 | 66.2        | 16.2      |
|              |                   | 30         | 13.9                                | 11.5                          | 3.9 | 74.1        | 15.8      |
|              | Blood             | 4          | 3.0**                               | 4.9                           | ND |             |           |
|              |                   | 15         | 2.0                                 | 10.7                          | 2.5 | 89.3        | 6.3       |
|              |                   | 30         | 3.0                                 | 26.7                          | 1.9 | 95          | 3         |
| 125I-AGOR    | Intestinal contents | 30       | 0.03                                | 83                            | 8.9 | 87.8        | 3.3       |
|              | Stomach contents  | 30         | 0.3                                 | 76                            | 2.2 | 97.0        | 0         |

* Acid-soluble radioactivity was analyzed by gel filtration as described in Materials and Methods. Identification was based on identity with the elution profiles of standards.
‡ V₀, Void volume, as detected by blue Dextran.
§ Recovery of radioactivity applied to the Sephadex G-25 column ranged from 90 to 110%. Radioactivity not accounted for in the three regions listed was recovered between V₀ and 1- and comprised ~1-10% of the recovered 125I.
|| 125I-material at the V₀ was found to be intact 125I-glycoprotein by SDS-PAGE and present in the acid-soluble fraction as a result of imperfect separation of the two fractions.
¶ The presence of [125I]iodide in the acid-soluble fraction at 4 min reflects incomplete removal of unincorporated isotope during iodination of the glycoprotein and is a small fraction of the total radioactivity in the liver (0.863 x 2 = 1.73%).
** The rather high levels of radioactivity present in the blood between 5 and 15 min reflect the age (2 mo) and method of storage of the 125I-protein (in dilute solution, 0.20 mg/ml, at -20°C).
$$ ND, not determined.

the liver (see Table V in this study and Fig. 2 in the preceding paper [17]). Rather, the bulk of the released [125I]iodide at 30 min was found in the contents of the stomach and intestine (data for 125I-ASOR not shown but are comparable to that shown for 125I-agalacto-orosomucoid [AGOR] in Table V).

Intracellular Localization and Temporal Redistribution of 125I-AGOR 125I-Ahexosaminio-Orosomucoid AHor), 125I-ß-glucuronidase, and 125I-Mannobiosaminated-RNase A Dimer (M-RNase) in Cells Lining the Sinusoids

MORPHOLOGY: The four modified glycoproteins found associated with Kupffer and endothelial cells demonstrated qualitatively similar intracellular localizations 15 min after injection. Fig. 5 illustrates the distribution of 125I-AGOR in an endothelial cell and Kupffer cell. In endothelial cells, ARG grains were predominantly lying over dense bodies and vacuoles having light content (called macropinocytic vesicles [30]). The ARG grains in Kupffer cells were associated more often with structures having heterogeneous contents. Occasionally, grains were found in the vicinity of a Golgi complex but never over the stacks. 125I-AHor, 125I-ß-glucuronidase and 125I-M-RNase exhibited similar localizations.

Qualitative observations of endothelial cells indicated a temporal redistribution of the same four glycoproteins between 1 and 15 min after injection. At 1 min, grains representing 125I-AHor were found overlying areas of cytoplasm rich in coated vesicles (Fig. 6a). However, the diameter of the vesicles was small (1,200 Å) relative to the resolution of 125I ARG at the EM level (~1,000 Å), and therefore, analysis of the association of grains with these structures was not attempted. (To test the hypothesis that molecules were contained within these vesicles, statistical analysis of the grain distribution and correction for radiation spread would be required.) Significant numbers of
FIGURE 5  EM-autoradiographs of cells lining the blood sinusoid. Livers were fixed by perfusion 15 min after i.v. injection of $^{125}$I-AGOR (160 μg, 1 mCi). (a) A Kupffer cell (K) contains many ARG grains overlying lysosomes (Ly), few grains over the tubular invaginations (TI), and none over stacked elements of the Golgi (Go). 18 d exposure; bar, 1 μm; × 11,000. (b) Higher magnification of Kupffer cell lysosomes with associated ARG grains. Note the heterogeneous content. × 24,300. (c) An endothelial cell (E) contains ARG grains overlying macropinocytic vesicles (Mpv) and dense bodies (Db). Bar 1 μm; × 9,500. (d) Higher magnification of macropinocytic vesicles with associated ARG grains. × 22,000.
FIGURE 6  EM-autoradiographs of endothelial cells (E) present in livers fixed by perfusion 1 min (Fig. 6a) or 15 min (Fig. 6b) after i.v. injection of $^{125}$I-AHOR (90 μg, 0.6 mCi). (a) At 1 min, ARG grains are overlying regions rich in coated vesicles (Cv) or are associated with macropinocytic vesicles (Mpv). A sieve plate (Sp) is evident in this section. 15 d exposure; bar, 1 μm; × 12,000. (b) At 15 min, ARG grains are associated with either macropinocytic vesicles or dense bodies (Db). 15 d exposure; bar, 1 μm; × 9,500.

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grains were also found associated with macropinoctyes at 1 min but not with dense bodies. At 15 min, the grains were heavily concentrated in both macropinoctyes and dense bodies (Fig. 6 b). At 60 min, $^{125}$I-$\beta$-glucuronidase was located almost exclusively in dense bodies (micrograph not shown). Other $^{125}$I-ligands were not examined at times later than 15 min due to their hydrolysis and loss from the liver (see below).

**DISCUSSION**

The use of EM-ARG has allowed us to follow the $^{125}$I-ligands that are bound and internalized by the different cell types in the liver. Within the limits of the technique, we have established the kinetics of intracellular transport for ASGPs in hepatocytes. In addition, we have identified the intracellular compartments in which mannose/N-acetylglucosamine-terminating glycoproteins are found after internalization by Kupffer and endothelial cells.

**Intracellular Fate of $^{125}$I-ASGPs in Hepatocytes**

**EARLY EVENTS:** The present data would suggest that the binding sites (receptors?) for ASGP are nonrandomly distributed in the hepatocyte plasmalemma. That is, ARG grains at the earliest times examined were concentrated in the region of the sinusoidal domain more than in the lateral domain. However, preliminary data on isolated hepatocyte plasmalemma preparations suggest that binding sites are also present in the lateral domain. It is possible that the number of these sites in the sinusoidal domain of a single hepatocyte, or their efficiency in the internalization of ligand, exceeds the number of $^{125}$I-ASGP bound and internalized by one cell under the experimental conditions used in the present study.

Our data also suggest that the sites binding $^{125}$I-
ASGP 2 min after administration of the ligand are not uniformly distributed throughout the sinusoidal domain but are localized to membrane regions between the microvillar projections that extend into the space of Disse. This selective distribution may be explained by the presence of receptors in "coated pits" structures often seen in the intermicrovillar regions (Fig. 1b). Binding sites for several proteins destined for internalization have been localized to similar "coated" regions (or "coated pits") of the plasmalemma in fibroblasts (2, 12, 22), chicken oocytes (24), and human syncytial trophoblasts (19). Thus, these structures (and the corresponding coated vesicles that form from invaginations of coated pits [10, 25]) are favored candidates to contain ASGP receptors. Of course, binding sites might be randomly distributed in the absence of ligand and cluster only after interaction, as has been suggested for the epidermal growth factor receptor in human carcinoma cells (15, but see reference 21 for insulin receptor). Due to resolution limitations of the probe used in this study (125I resolution = 1,000 Å) and to rapid internalization of the ligand, we were unable to distinguish among the several alternatives.

Later events: Our quantitative EM-ARG results establish that the 125I-ASGPs are internalized very rapidly, because at 1 min (2 min maximum, 125I-ASOR) we found significant amounts of the ligand already within the cell. This distribution, in addition to the observation that 90% of the radioactivity at 2 min is sedimentable (100,000 g for 2 h) and thus particulate, suggests very strongly that the ligand is being internalized within an endocytic vesicle. The vesicles must be relatively small, because ARG grains found in the peripheral cytoplasm at early times are not associated with any distinctive structure. Again, ARG is not of sufficiently high resolution to allow identification of the presumed membranous structure.

Others have used subcellular fractionation to follow the early events of ASGP binding and uptake (14, 20). At 4 min, Labadie et al. (20) found 60% of the liver 125I-ASF in a microsomal fraction that, upon further subfractionation, yielded a preparation enriched in 125I-ASF and a plasma membrane enzyme activity (alkaline phosphodiesterase I). These investigators interpreted the parallel enrichment as evidence for the binding of 125I-ASF to sinusoidal front plasmalemma. The kinetics of 125I-ASF redistribution observed in our EM-ARG study would suggest that a large fraction of the 125I-ASF found in a microsomal fraction may be sequestered within endocytic vesicles, because we find a maximum of 29% of the 125I-ASF at the sinusoidal front at 4 min and 26% in the peripheral cytoplasm. Experiments designed to resolve this question are in progress.

Our EM-ARG study confirms the involvement of lysosomes in the metabolism of 125I-ASGP's and provides direct information on the kinetics of intracellular transport to this organelle. As early as 4 min, we found a significant amount (12%) of 125I-ligand in the lysosome-Golgi regions. At 15 min the value reached 42% and by 30 min was 52%. At each time point, more than half of the grains in this region could be localized to bona fide secondary lysosomes and <2% of Golgi elements. Bergeron et al. (4), using EM-ARG and a fine grain development, have reported that a substantial fraction (~15%) of 125I-insulin 10 min after internalization by hepatocytes (in vivo) could be localized to vacuoles containing lipoprotein particles and presumed to be Golgi (but see references 7 and 13). We were unable to localize 125I-ASF or 125I-ASOR to such structures, perhaps reflecting the different fates of the ASGPs vs. a polypeptide hormone or the difficulty in identifying structures underlying the filamentous ARG grains resulting from the emulsion and development used in our study. However, we could find at most 60% of the 125I-ASF in lysosome-rich regions of the hepatocyte, with the remaining 40% primarily in the intermediate cytoplasm. Because all of the internalized 125I-ASF is eventually degraded, the meaning of this rather high and persistent fraction in unidentified structures is uncertain. Several possible explanations are: (a) inefficient transport of endocytosed ligand to peribiliary lysosomes; (b) a random distribution of lysosomes throughout the cytoplasm; or (c) recycling of ASGP receptor to which the ligand remains bound, with eventual deposit in lysosomes. Answers must come from future work.

Several lines of evidence before results obtained in this study indicated that lysosomes were the ultimate destination of internalized ASGPs. First, there was evidence of hydrolysis of radiolabeled...
lysosomes, and the receptor is retrieved and recycling of these ^251-ligands was not performed.

Intracellular Fates of ^125^I-AGOR, ^125^I-AHOR, and ^125^I-ß-glucuronidase

A quantitative EM-ARG study of the kinetics of internalization and intracellular compartmentalization of these ^125^I-ligands was not performed. However, our tentative picture for endothelial cells based on the limited evidence so far available is: uptake into coated vesicles, followed rapidly by fusion with existing macropinosomal vesicles and/or dense bodies, and ultimate residence in dense bodies. Particles the size of ferritin vesicles have been traced through the same pathway (30). Others have reported that the macropinosomal vesicles and dense bodies contain lysosomal enzymes, but we were unable to demonstrate acid phosphatase cytochemically within either of these organelles. Nevertheless, the modified ^125^I-orosomucoids, AGOR and AHOR, were extensively degraded within 60 min of entry, as evidenced by the progressive loss of acid-precipitable radioactivity from the liver, the appearance of both ^125^I[MIT and ^125^I]iodide within the liver, their release into the circulation, and their ultimate accumulation in the stomach. Others have reported a similar metabolic pathway for RNase B, a mannose-terminating glycoprotein (5). Although ^125^I-ß-glucuronidase exhibited the same temporal redistribution observed for AGOR and AHOR in both endothelial cells and Kupffer cells, the molecule was not metabolized, a finding also reported by others (1).

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