Differential and Analytical Subfractionation of Rat Liver Components Internalizing Insulin and Prolactin†

John J. M. Bergeron,* Norman Searle, Masood N. Khan, and Barry I. Posner
Departments of Anatomy and Medicine, McGill University, Montréal, Québec, Canada H3A 2B2
Received August 5, 1985

ABSTRACT: Receptor-mediated endocytosis of ^{125}I-insulin and ^{125}I-prolactin into liver parenchymal cells has been studied by quantitative subcellular fractionation. Differential centrifugation yielded three particulate fractions, N (nuclear), ML (large granule), and P (microsomes), and a final supernatant (S). Quantitative differences in the extent and rates of accumulation of ^{125}I-insulin and ^{125}I-prolactin into the fractions were observed. The acidotropic agent chloroquine and the microtubule disrupting agent colchicine were administered separately to rats. The agents increased significantly the $T_{1/2}$ of hormone clearance from the liver and augmented the accumulation of both ligands in the low-speed ML fraction. However, differences in the rates of accumulation of insulin and prolactin into all cell fractions were still maintained. Analytical centrifugation of each of the particulate fractions was carried out in order to determine if different endocytic components were specific to insulin or prolactin internalization. This was not the case. An "early" endosomal component of density 1.11 was identified in microsomes. A "late" endosome of density 1.10 was identified in the large granule (ML) fraction. Both endosomal components appeared to accumulate insulin and prolactin but at different rates. Marker enzyme analysis identified the presumed plasma membrane component in microsomes (density ~1.155). This component showed a significant difference in the rate of loss of ^{125}I-insulin ($T_{1/2} \sim 4.1$ min) as compared to that of ^{125}I-prolactin ($T_{1/2} \sim 12.7$ min). A further difference in the handling of the ligands was observed in early endosomes. Chloroquine treatment of rats led to an increased retention of insulin but not prolactin in the early endosomes while the uptake of both hormones into late endosomes was augmented. On the basis of these observations, we conclude that insulin and prolactin are handled differently by the hepatocyte. First, the rate of uptake of insulin into endosomes from plasma membrane is faster. Second, early endosomes are the probable site of initiation of insulin but not of prolactin degradation.

The in vivo uptake of ^{125}I-insulin and ^{125}I-prolactin into lipoprotein-containing structures of rat liver has been documented by the techniques of in vivo radioautography and subcellular fractionation (Bergeron et al., 1979, 1983; Josefsberg et al., 1979; Khan et al., 1982, 1985a,b; Posner et al., 1980, 1982). Thus, Golgi fractions especially enriched in lipoprotein-filled components concentrated intact ligand in structures similar to those that had been shown previously to be highly enriched in specific receptors for these ligands (Bergeron et al., 1973, 1978; Posner et al., 1978, 1979). At least two distinct receptor-enriched lipoprotein-containing components of low and high density, respectively, could be delineated by Percoll gradient centrifugation of Golgi fractions (Khan et al., 1982) and by metrizamide or Percoll gradient analyses of light mitochondrial fractions (Khan et al., 1981). These endosomal components were shown conclusively to be distinct from bona fide Golgi components by Percoll gradient centrifugation (high-density component; Khan et al., 1981, 1982) and by the diaminobenzidine-shift protocol (low-density component; Kay et al., 1984). In addition to low- and high-density lipoprotein-containing structures, we have observed a small, empty, smooth-surfaced vesicular component that accumulated ligand at very early times (Josefsberg et al., 1979; Posner et al., 1980, 1982a,b). From kinetic studies, we suggested that a sequential transport of ligand from the small empty vesicles to the larger low-density then higher density lipoprotein-filled structures occurred (Khan et al., 1982; Posner et al., 1982a).

However, the time courses of labeled insulin and labeled prolactin accumulation in these components were different (Posner et al., 1982a). Furthermore, the acidotropic agent chloroquine (de Duve, 1983) greatly augmented the accumulation of ^{125}I-insulin in endosomes in Golgi fractions (Posner et al., 1982b) with a far less pronounced effect noted for prolactin uptake into the same fractions (Khan et al., 1985b). As well, a differential effect of the microtubule disrupting agent colchicine was observed on the accumulation of ^{125}I-insulin compared to ^{125}I-prolactin in Golgi fraction endosomes (Posner et al., 1982c).

Past studies have focused on selected subcellular components. In the present study, liver has been fractionated after the injection of radiolabeled insulin or prolactin. Crude nuclear (N) fractions, large granule (ML) fractions, and microsomal (P) fractions were isolated by following a well-established protocol for differential centrifugation (Amar-Costesec et al., 1974; de Duve et al., 1955). In this way, we have examined quantitatively the total distribution of internalized insulin and prolactin in the hepatocyte. In addition, the effect of the agents chloroquine and colchicine on the total cellular distribution of the internalized hormones has been evaluated. Finally, we have studied the biochemical characteristics of the endosomal components. This has been carried out by a modified analytical centrifugation approach (de Duve, 1975) in order to evaluate the distribution of marker enzymes and internalized ligand in endosomal components resolved from the parent particulate fractions (N, ML, P) generated by differential centrifugation. With this approach, we have addressed the question as to why insulin and prolactin appear to behave

† Supported by grants from the MRC of Canada, the NCI of Canada, and the USPHS. J.J.M.B., M.N.K., and B.I.P. are Chercheurs Boursiers du Québec, and N.S. was supported by an MRC postdoctoral fellowship.
* Address correspondence to this author at the Department of Anatomy.

0006-2960/86/0425-1756$01.50/0

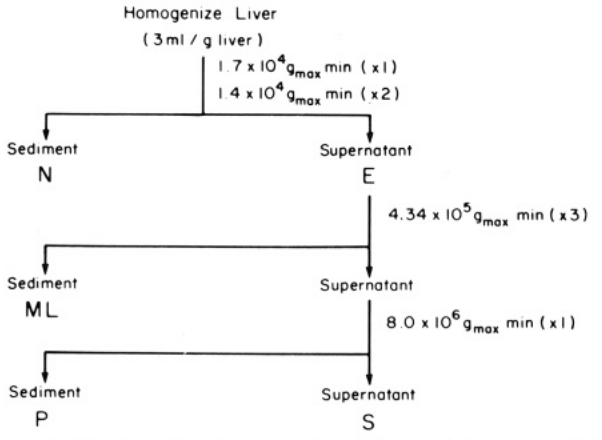

FIGURE 1: Fractionation seheme employed for preparing nuclear (N), large granule (ML), microsomal (P), and cell-soluble (S) fractions from liver homogenates following the protocol of Amar-Costesec et al. (1974). As described by de Duve et al. (1955) for the purposes of balance sheets of enzymic activities and the distribution of radioactivity, the homogenate is considered the sum of the cytoplasmic extract (E) and nuclear (N) fractions. The rotors and centrifuge tubes used are described under Experimental Procedures.

differently during receptor-mediated endocytosis into liver parenchymal cells.

Experimental Procedures

Hormones, Inhibitors, Enzyme Assays, and Protein Determinations. Insulin (24 units/mg) was generously provided by Connaught Laboratories (Willowdale, Ontario). Ovine prolactin (oPRL, NIH-P-S10, 26.4 IU/mg) was kindly provided by the Pituitary Hormone Distribution Program of NIAMKDD (Bethesda, MD). The hormones were iodinated and purified by Sephadex chromatography as described previously (Posner et al., 1982b,c) to specific activities of 150–180 μCi/μg (^{125}I-insulin) and 120–180 μCi/μg (^{125}I-prolactin). Radioiodinated hormones were prepared 1 day prior to experimentation. Chloroquine was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in 0.9% NaCl to a final concentration of 10 mg/mL, and adjusted to pH 7.4 with 1.0 N NaOH (Posner et al., 1982b). Colchicine was purchased from Sigma, dissolved in 0.9% NaCl to a final concentration of 10 mg/mL, and adjusted to pH 7.4 (Posner et al., 1982c).

Acid phosphatase (EC 3.1.32), galactosyltransferase (EC 2.4.1.38), glucose-6-phosphatase (EC 3.1.3.9), and 5′-nucleotidase (EC 3.1.3.5) were assayed as described previously (Bergeron et al., 1982; Khan et al., 1982). Protein content was determined with the Bio-Rad kit as recommended by the manufacturers (Bio-Rad, Mississauga, Ontario).

Animals and Injections. Female Sprague-Dawley rats 8–10 weeks of age and ca. 160–190 g of body weight were obtained from Canadian Breeding Laboratories (St. Constant, Québec) and fasted overnight prior to sacrifice. Control rats were injected with saline (ip) 1 h before use. Chloroquine-treated rats received 10 mg/200 g of body weight (ip) at 2 h and again at 1 h before sacrifice and received O_2 to assure a high survival rate. Colchicine-treated animals received 10 mg/100 g of body weight by a single ip injection 3 h before sacrifice. ^{125}I-Insulin or ^{125}I-prolactin was injected into the external jugular vein of each rat [0.15–0.2 mL; (30–33) × 10^6 dpm] under ether anaesthesia. Subcellular fractionation experiments were carried out on rats sacrificed usually at 2, 5, 10, and 20 min after ^{125}I-insulin injection and at 5, 15, 30, and 60 min after the injection of ^{125}I-prolactin.

Subcellular Fractionation. Differential centrifugation was carried out as described by Amar-Costesec et al. (1974) in buffered (4 mM imidazole, pH 7.4) 0.25 M sucrose. Briefly

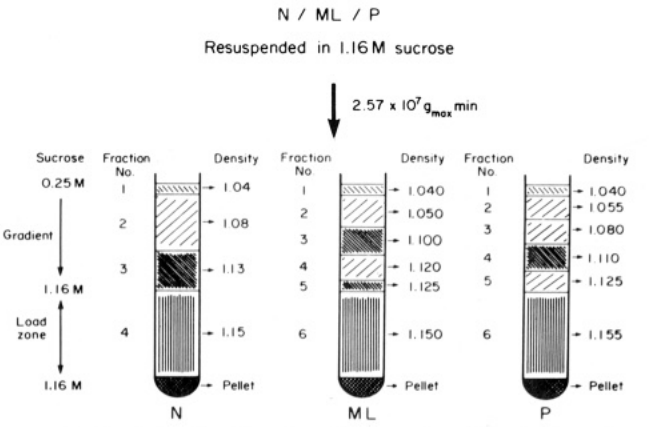

FIGURE 2: Analytical fractionation scheme employed for the floatation of endocytic components from the parent N, ML, and P particulate fractions as described under Experimental Procedures.

(Figure 1), after being minced with scissors, liver was homogenized with one up-and-down stroke of a motor-driven Teflon pestle (3 mL/g wet weight of liver). The homogenate was centrifuged at 3100 rpm for 10 min in a Beckman SW28 rotor and the supernatant saved. The pellet was returned to the homogenizer and rehomogenized with one up-and-down stroke in the original sucrose volume. The homogenate was then recentrifuged at 2800 rpm in the SW28 rotor for 10 min. The procedure was repeated once more. The pellet fraction was resuspended in buffered sucrose and referred to as the N fraction.

The resultant supernatants were combined and referred to as the cytoplasmic extract E. As described by Amar-Costesec et al. (1974) and de Duve (1971, 1975), the starting homogenate was considered the sum of the E + N fractions.

The cytoplasmic extract (E) was further centrifuged in an angle Ti-60 Beckman rotor at 25 000 rpm for 6 min 40 s. The pellet was washed twice in buffered sucrose and centrifuged at the same speed. After resuspension in buffered sucrose, the fraction was referred to as the ML (large granule) fraction.

The supernatants from the preparation of the ML fraction were combined and centrifuged for 40 min at 45 000 rpm in the angle Ti-60 rotor. After resuspension of the pellet in buffered sucrose, this fraction was referred to as the P fraction. The final supernatant was designated the S (cell soluble) fraction.

Relative specific activities were calculated as the percent of homogenate (E + N) enzyme activity or percent radioactivity per percent of homogenate (E + N) protein in that fraction (de Duve, 1971, 1975). The differential centrifugation studies on the distribution of ^{125}I-insulin in normal, colchicine-treated and chloroquine-treated rats were carried out on six occasions with one rat used for each time point (2, 5, 10, and 20 min after iv injection), i.e., a total of 72 rats. The differential centrifugation studies on ^{125}I-prolactin distribution were carried out on five occasions, i.e., a total of 60 rats.

A modified analytical subfractionation (Figure 2) of the N, ML, and P fractions was carried out by resuspending the individual fractions in 1.16 M sucrose over which a continuous sucrose gradient (0.25–1.15 M sucrose) was formed (volume gradient:volume load 1:1). Following centrifugation for 1.5 h in an SW40 Beckman rotor (285000g_{max}), four subfractions and the pellet were recovered from the N gradient, on the basis of the visibility of bands in the gradient. From the top to the bottom of the gradient, these were subfraction 1 consisting of a 0.75-mL volume at a density of 1.04, subfraction 2 (3.75 mL, ρ = 1.08), subfraction 3 (2.6 mL, ρ = 1.13), and subfraction 4 (5.4 mL, ρ = 1.15). The pellet was resuspended

in 0.25 M sucrose–imidazole, pH 7.4. For the gradient generated from the parent ML fractions, six fractions and the pellet were recovered again as based on the visibility of bands generated in the gradient. These were subfractions 1 (0.8 mL, ρ = 1.04), 2 (2.25 mL, ρ = 1.05), 3 (2 mL, ρ = 1.11), 4 (1.8 mL, ρ = 1.12), 5 (0.75 mL, ρ = 1.125), and 6 (5.2 mL, ρ = 1.15). The pellet was resuspended as above. The fractions recovered from the gradient of the parent P fraction consisted of subfractions 1 (0.7 mL, ρ = 1.04), 2 (1.73 mL, ρ = 1.055), 3 (1.6 mL, ρ = 1.08), 4 (1.9 mL, ρ = 1.11), 5 (1.4 mL, ρ = 1.125), and 6 (6 mL, ρ = 1.155). The pellet was resuspended as above. Sucrose densities were determined on a calibrated Abbe refractometer (Mark II, American Optical, Buffalo, NY). The results are based on a total of ten fractionations.

For some experiments, a light mitochondrial fraction (L) and the microsomal (P) fraction were generated by a method to be described in detail elsewhere,[1] and discontinuous gradient centrifugation was employed to float endocytic components from the parent particulate L and P fractions.[1]

Subcellular Cytochemistry and Electron Microscope (EM) Radioautography. Three female rats were each injected with 375 × 10^6 dpm of ^{125}I-prolactin (182 μCi/μg), and fractions were prepared that were highly enriched in internalized ligand. These were fraction 3 (ρ = 1.10) floated from the ML fraction by sucrose gradient centrifugation and fraction 4 (ρ = 1.11) floated from the P fraction (see Figure 2). After a 20-min fixation in 1% glutaraldehyde, pH 7.4 at 4 °C, samples were filtered and then processed for electron microscope radioautography as previously described (Khan et al., 1982). Additional samples were also fixed and filtered, but then the filters were washed in 100 mM sodium acetate (pH 5.0) followed by a 30-min incubation in a modified acid phosphatase cytochemical reaction medium (Barka & Andersen, 1962), which consisted of 8.7 mM β-glycerophosphate in 20 mM sodium acetate, pH 5, and 2.1 mM lead nitrate. After 30 min at 37 °C, filters were rinsed in buffer and processed for electron microscope radioautography as described by Khan et al. (1982).

Results

Rate of Loss of Radioactivity from Liver Homogenates. The majority of ^{125}I-insulin was lost from the circulation with a $T_{1/2}$ of ca. 1.5 min while that of ^{125}I-prolactin was measured as 2 min (data not shown). Both radiolabeled hormones were retained by the liver but then lost at different rates (Figure 3). Thus, after an initial lag of ca. 5 min, radiolabeled insulin was cleared from the liver with a $T_{1/2}$ of ~6 min. By contrast, prolactin showed a greater lag of 15 min before radioactivity was lost, with a $T_{1/2}$ then noted of ca. 17 min. Colchicine- and chloroquine-treated rats revealed retarded rates of clearance for both hormones from the liver with a significant retention of radiolabel noted at 20 min for ^{125}I-insulin and 60 min for ^{125}I prolactin.

Differential Centrifugation. Differential centrifugation of liver homogenates into the standard homogenate (fractions E + N), the N fraction (crude nuclear), the ML fraction (large granule), the P fraction (microsomes), and the S fraction (cell soluble) revealed the P fraction as the locus of the highest concentration and quantity of radioactivity at 2 min for ^{125}I-insulin and 5 min for ^{125}I-prolactin (Figure 4). For ^{125}I-insulin (Figure 4A, control), a temporal increase in the proportion of radioactivity in S was observed while the proportion of radiolabel in P decreased with time. Little radiolabel

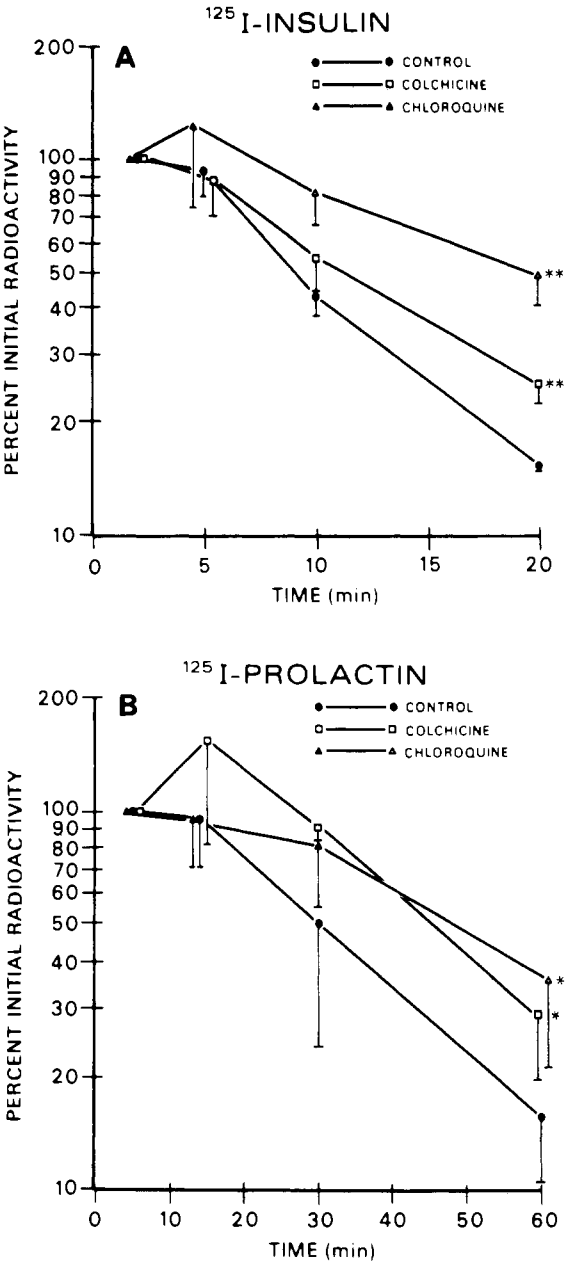

FIGURE 3: Rate of loss of radioactivity from liver homogenates (E + N) from control [(○) n = 6], colchicine-treated [(●) n = 6], or chloroquine-treated [(△) n = 6) rats ± SD after the injection of ^{125}I-insulin (A) or ^{125}I-prolactin (B) as described under Experimental Procedures. Statistical analysis indicated a significant inhibitory effect of colchicine and chloroquine on the loss of ^{125}I-insulin (**, $p < 0.01$) or ^{125}I-prolactin (*, $p < 0.05$) at the indicated times.

was observed in the ML fraction. For ^{125}I-prolactin, however (Figure 4B, control), an increase in the proportion of radioactivity in the ML fraction was observed coincident with the loss of radioactivity in P from 5 to 15 min and before an increase in the proportion of radioactivity in S (at 30 and 60 min). Previous studies have shown that radioactivity in particulate fractions represented largely undergraded ligand while radiolabeled insulin or prolactin in S (cell-soluble fraction) represented exclusively degraded material (Josefsberg et al. 1979; Khan et al., 1982; Posner et al., 1982a,b).

Chloroquine and colchicine effected significant retention of the proportion of ^{125}I-insulin in both P and ML fractions with a corresponding decrease in the proportion of radioactivity in S. Interestingly, colchicine caused a greater proportion of ^{125}I-insulin to accumulate in the ML fraction than did chloroquine (Figure 4A). Similar observations were made of the

[1] M. N. Khan, S. Savoie, J. J. M. Bergeron, and B. I. Posner, submitted for publication.

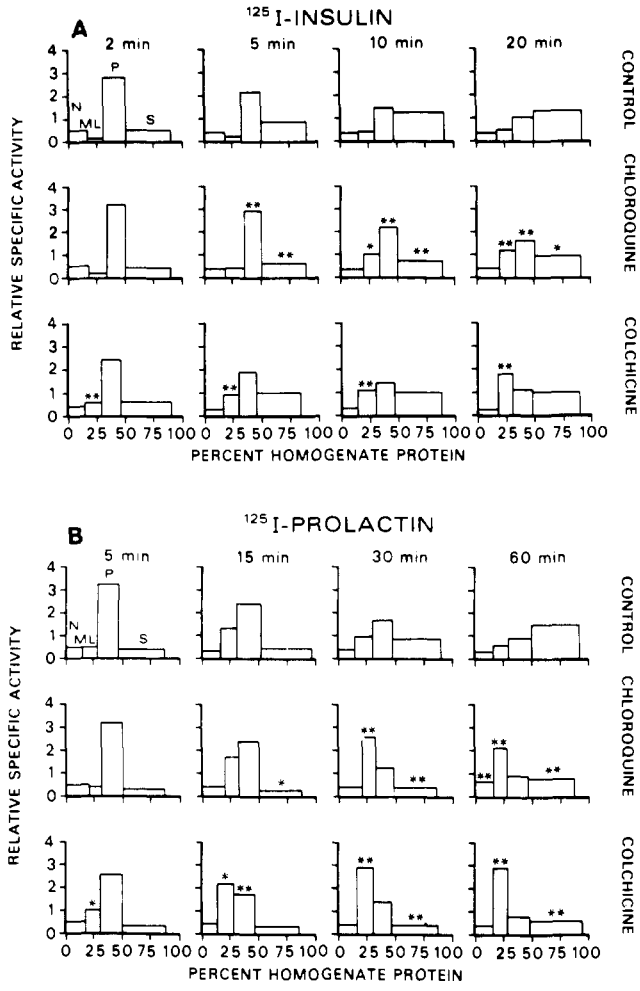

FIGURE 4: Modified de Duve plots of the distribution of radioactivity in N, ML, P, and S subcellular fractions (derived from the liver homogenate of Figure 1) at defined times after the injection of ^{125}I-insulin (A) or ^{125}I-prolactin (B) into female rats. Liver homogenates subfractionationed from chloroquine- or colchicine-pretreated rats are also indicated. Significant differences in the radioactive content of fractions in chloroquine- or colchicine-treated animals as compared to control rats are indicated (*, $p < 0.05$; **, $p < 0.01$).

effects of these inhibitors on the accumulation of ^{125}I-prolactin except for an even greater effect of colchicine on ^{125}I-prolactin accumulation in the ML fraction (Figure 4B).

Enzymic analyses of the standard fractions revealed the majority (74%) of the Golgi apparatus marker galactosyltransferase and of the ER marker glucose-6-phosphatase (63%) in microsomes with 47% of the plasmalemma marker (5′-nucleotidase) also observed in microsomes. The highest proportion of acid phosphatase, the marker for lysosomes, was in the ML fraction (44%) with only small amounts of the homogenate galactosyltransferase (1.2%), glucose-6-phosphatase (7.1%), and 5′-nucleotidase (7.1%) activities recovered in the fraction. The distributions of these enzyme activities were comparable to those described by De Duve et al. (1955) and more recently and extensively by Amar-Costesec et al. (1974).

Endosome Floatation. Appropriate sucrose gradients were reconstructed in order to fractionate the majority of the radioactivity away from the bulk of the protein. Trial and error resulted in the final choice of the sucrose gradient described in Figure 2. At early times after the injection of ^{125}I-insulin (Figure 5), radioactivity in the P fraction was distributed between two peaks of $\rho = 1.11$ ($P_{1.11}$) and $\rho = 1.155$ ($P_{1.155}$); the proportion of radioactivity in both peaks decreased temporally (Figure 5). Although two similar fractions ($\rho = 1.10$, $ML_{1.10}$; $\rho = 1.15$, $ML_{1.15}$) were floated from the ML fraction, the proportion of radioactivity in these fractions increased with time (Figure 5). Only one major peak of radioactivity was observed in the N fraction at ca. 1.15 in density.

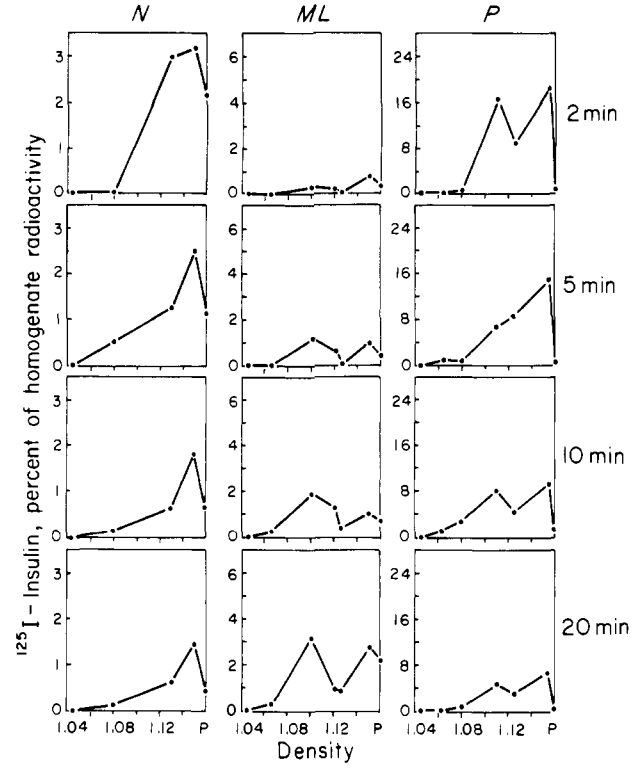

FIGURE 5: Distribution of radioactivity in fractions floated in a continuous sucrose gradient as described under Experimental Procedures. The results are taken from animals injected with ^{125}I-insulin. At noted times, the percent of homogenate (E + N) radioactivity was determined for each recovered fraction whose density is noted on the abscissa. P refers to the pellet, which was resuspended in 0.25 M sucrose–4 mM imidazole, pH 7.4.

The results were generally similar for ^{125}I-prolactin uptake (Figure 6) except that from 5 to 15 min the proportion of radioactivity in the $P_{1.155}$ fraction decreased while that in $P_{1.11}$ increased. Furthermore, the proportion of radiolabel in $ML_{1.10}$ increased markedly over the same time interval (Figure 6) but decreased thereafter concurrent with an increase in the proportion of radiolabel in the S fraction at 30 and 60 min (Figure 6). There was negligible radioactive content in the subfractions floated from the N fraction.

For both ligands, therefore, earliest labeling was found in the $P_{1.155}$ fraction followed closely by labeling of the $P_{1.11}$ fraction and finally the $ML_{1.10}$ fraction. Estimates of the rates of change in ^{125}I-insulin and ^{125}I-prolactin contents in the $P_{1.155}$, $P_{1.11}$, and $ML_{1.10}$ components at different times after injection were calculated as described in Figure 7. This was carried out by first calculating the radiolabel in each component as a percent of the maximum homogenate radioactivity and then normalizing to the maximum radioactive content for each component. These rates of change in ligand content ($T_{1/2}$) were first order and therefore stochastic. They were significantly ($P < 0.01$) faster for ^{125}I-insulin in $P_{1.155}$ ($T_{1/2} \sim 4.1$ min) and $P_{1.11}$ ($T_{1/2} \sim 4.2$ min) components as compared to that for the ^{125}I-prolactin content in the same fractions ($T_{1/2} \sim 12.7$ and 8.3 min for the $P_{1.155}$ and $P_{1.11}$ fractions, respectively). No significant difference was noted for the rates of change of ^{125}I-insulin ($T_{1/2} \sim 11.3$ min) or ^{125}I-prolactin content ($T_{1/2} \sim 10$ min) in the $ML_{1.10}$ fraction (Figure 7).

Enzymic analyses of the subfractions revealed (Figure 8) that the majority of protein, galactosyltransferase, acid

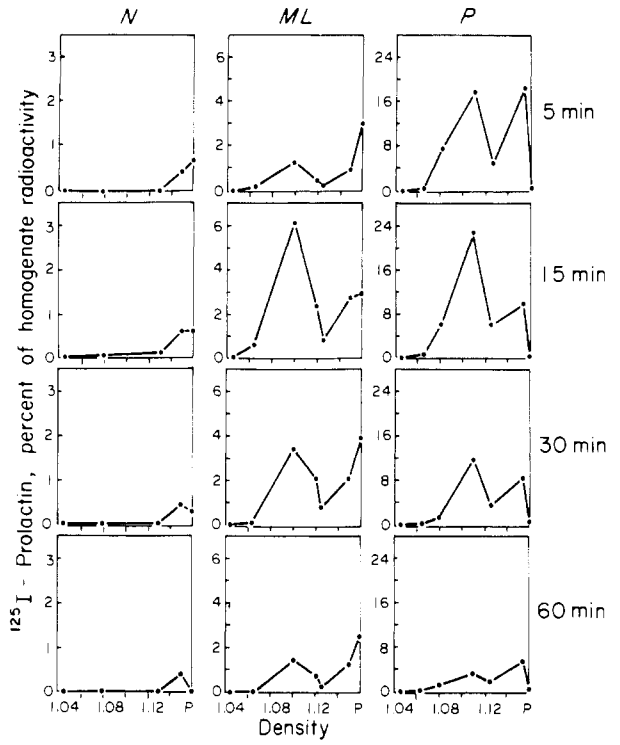

FIGURE 6: Distribution of radioactivity in fractions isolated after ^{125}I-prolactin injection. Conditions otherwise similar to those of Figure 5.

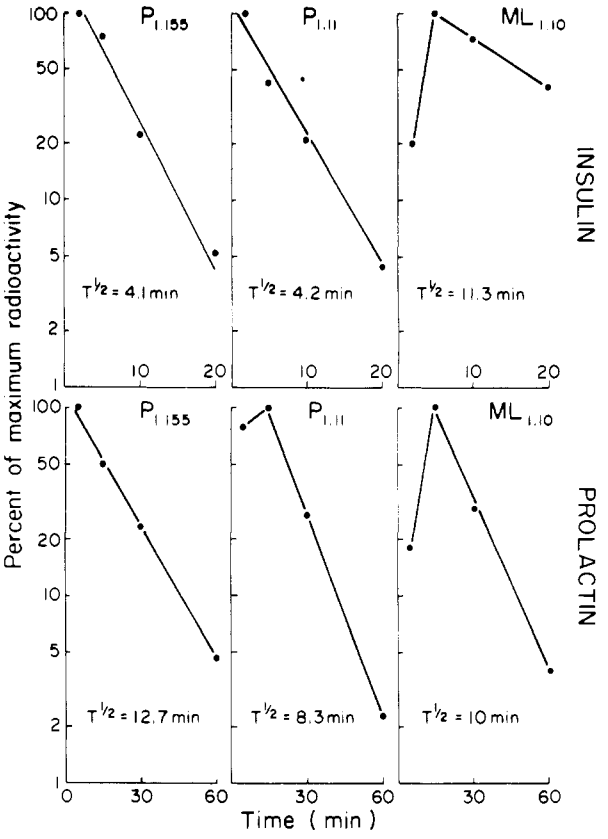

FIGURE 7: Rates of ^{125}I-insulin (upper) and ^{125}I-prolactin (lower) loss from subcellular components. The proportion of ^{125}I-insulin (Figure 5) or ^{125}I-prolactin (Figure 6) radioactivity in selected subcellular components ($P_{1.155}$, $P_{1.11}$, $ML_{1.10}$) for each time interval was multiplied by the proportion of maximal homogenate radioactivity for that time interval (Figure 3). The data were then normalized by expressing each point as a percent of the maximum radioactive content of each fraction. Regression analysis indicated a coefficient of correlation $r > 0.99$ for each compartment. The rates of ^{125}I-insulin loss from the $P_{1.155}$ and $P_{1.11}$ compartments were significantly ($P < 0.01$) more rapid than that of ^{125}I-prolactin from the same compartments as evaluated by Student's t test with no significant difference observed for hormone loss from the $ML_{1.10}$ compartment. These studies further show the later labeling of the $ML_{1.10}$ endosomal fraction as compared to the $P_{1.155}$ compartment (presumed plasma membrane).

phosphatase, glucose-6-phosphatase, and 5′-nucleotidase activities was clearly displaced from the major endocytic fractions containing radioactivity (i.e., $\rho = 1.10$ from ML and $\rho = 1.11$ from P). The $ML_{1.10}$ and $P_{1.11}$ components therefore did not fractionate with the bulk of any of the marker enzymes that were evaluated. Furthermore, the $T_{1/2}$ of ligand in these endocytic components (Figure 7) indicated that the $P_{1.11}$ fraction contained early endosomes while the $ML_{1.10}$ fraction was enriched in later endosomes.

Attempts at determining the locus of the ligands internalized in chloroquine- and colchicine-treated animals were carried out by a modification of the above method for endosome fractionation. First, a light mitochondrial (L) fraction was prepared rather than a large granule (ML) fraction. Control experiments revealed that the majority of ligand uptake into ML was recovered in the L fraction at considerably higher enrichment. Second, step gradients were constructed above the resuspended L (light mitochondrial) and P fractions. This served to distinguish endocytic components ($\rho < 1.13$) from heavier lysosomes and plasma membranes, i.e., $\rho > 1.13$ (unpublished results). The results (Figure 9) indicated that chloroquine treatment of rats led to a 1.5-fold increase of insulin uptake into light endocytic components floated from the P fraction (Figure 9b) and a 2-fold increase in the ^{125}I-insulin content of later endocytic components floated from the L fraction (Figure 9a). Colchicine treatment resulted in a lower radioactive content in light endocytic components derived from microsomes but in an increased proportion of homogenate ^{125}I-insulin in the later endocytic components floated from L (Figure 9a,b). Significant effects of chloroquine and colchicine were observed in components found in the L fraction at densities > 1.13 (Figure 9c), where the majority of lysosomal enzyme activity was found (not shown). Label was rapidly lost from the heavy components (Figure 9d) of the P fraction where the plasma membrane marker 5′-nucleotidase was found (not shown).

By contrast, similar experiments on ^{125}I-prolactin uptake showed only a minor effect of chloroquine on ligand accumulation in light endocytic components derived from the P fraction (compare panel b with panel f of Figure 9). The effect of chloroquine or prolactin accumulation in later endocytic components floated from L (1.6-fold, Figure 9e) was, however, comparable to that observed for the chloroquine enhancement of insulin retention in the same fraction (1.5-fold, Figure 9a). The effect of colchicine in diminishing prolactin uptake in early endocytic components (Figure 9f) while increasing ligand uptake in later endocytic components (Figure 9e) was similar to that shown for ^{125}I-insulin in the same fractions (Figure 9a,b). The effects of chloroquine on ^{125}I-prolactin uptake into heavier components ($\rho > 1.13$, Figure 9g,h), although slightly displaced in time, were generally similar to the observations for ^{125}I-insulin uptake into the same fractions (Figure 9c,d).

Characterization of Endosomes. Evaluation of the enzymic and radioactivity contents of fractions from the analytical gradients (Figures 5, 6, and 8) revealed considerable enrichment of internalized hormone relative to the marker enzyme activities that were evaluated (Table I). The $ML_{1.10}$ fraction was especially enriched in ligand with the major enzymic activity found to be acid phosphatase. For the $P_{1.11}$ endosomal fraction, the enrichment of internalized hormone was less (relative specific activity ca. 50–70-fold) with the most

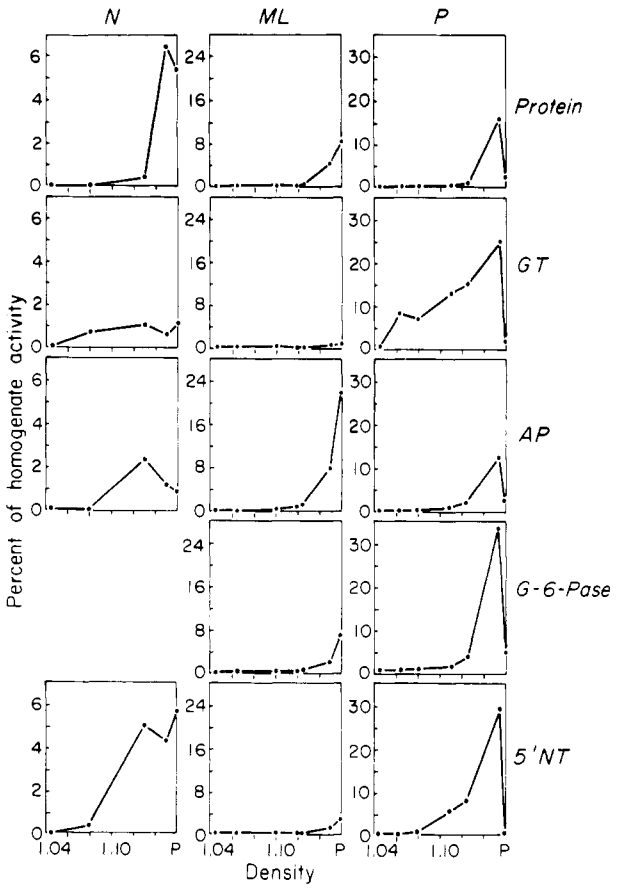

FIGURE 8: Distribution of marker enzymes in sucrose gradients processed identically with those of Figures 4 and 5. Assays for protein (n = 5), galactosyltransferase (GT, n = 3), acid phosphatase (AP, n = 3), glucose-6-phosphatase (G6Pase, n = 1), and 5′-nucleotidase (5′NT, n = 1) were carried out on each fraction recovered at the noted density as described under Experimental Procedures.

Table I: Proportion and Enrichment of Homogenate Marker Enzymes and Internalized Hormone in Endosome Fractions[a]

	endosome fraction			
	$ML_{1.10}$		$P_{1.11}$	
constituent	activity[b] (%)	RSA[c]	activity (%)	RSA
protein	0.03 ± 0.01		0.33 ± 0.1	
galactosyltransferase	0.26 ± 0.10	8.7	13.00 ± 2.0	39.4
acid phosphatase	0.51 ± 0.30	17.0	1.00 ± 0.1	3.0
5′-nucleotidase	0.14	4.7	5.60	17.0
glucose-6-phosphatase	0.11	3.7	0.8	2.4
^{125}I-insulin	3.10 (20 min)	103.0	16.4 (2 min)	49.6
^{125}I-prolactin	6.10 (15 min)	203.0	23.1 (15 min)	70.0

[a] The data are summarized for the major endosomal fractions described in Figures 5, 6, and 8. The proportions of radiolabeled hormone in each fraction at the times of peak uptake into these fractions are indicated. [b] Percent of homogenate activity. [c] RSA (relative specific activity) or enrichment of marker enzymes or internalized hormone calculated as described under Experimental Procedures.

prominent enzyme activity found to be galactosyltransferase (relative specific activity ca. 40-fold).

Morphological analysis of the endosome fractions (Figure 10) revealed ca. 300 nm diameter components, which were filled with lipoprotein in the $ML_{1.10}$ subfraction (Figure 10a). These components were overlaid by silver grains when assessed by radioautography. The significant enrichment of acid phosphatase in the $ML_{1.10}$ fraction prompted a combined acid phosphatase and radioautographic study (Figure 10b), which indicated that at least a portion of the radiolabeled components were reactive for acid phosphatase. Electron microscope radioautography of the $P_{1.11}$ endosomal fraction was also carried out. This fraction consisted of vesicular and small tubular components. As for the $ML_{1.10}$ fraction, however, label was found predominantly over the lipoprotein-filled structures (Figure 10c).

Discussion

Receptor-Mediated Endocytosis of Insulin and Prolactin. During the process of receptor-mediated endocytosis, we and others [reviewed in Bergeron et al. (1985)] have proposed that many ligands including insulin and prolactin are internalized through components of the endosomal apparatus with a final destination usually in a degradative compartment, probably the lysosome. In the present study, we have compared the kinetics of ^{125}I-insulin and ^{125}I-prolactin transport through endocytic components of liver parenchymal cells by carrying out differential and analytical fractionation for the internalized hormones in parallel. Since past studies have shown that the uptake of insulin and prolactin is into liver parenchymal cells by a receptor-mediated process with minimal contribution from endothelial and Kupffer cells (Bergeron et al., 1979), the conclusions we have derived here are specific to the endocytic components of hepatocytes.

We have observed differences in the handling of ^{125}I-insulin and ^{125}I-prolactin by the hepatocyte. These differences could be explained as follows: ^{125}I-insulin and ^{125}I-prolactin may be internalized through different endocytic components with endosomes specific to each ligand transforming into lysosomes at different rates. Alternatively, the two ligands could be transported at different rates through the same endosomes. Selective ligand degradation might occur at an endosomal locus. As discussed below, the data favor the latter view.

The differential centrifugation studies showed that particulate ^{125}I-insulin was enriched in the microsomal (P) fraction at all time intervals while ^{125}I-prolactin was evident in both ML and P fractions at 15- and 30-min postinjection. A differential sensitivity of the ligands to the acidotropic agent chloroquine (Bergeron et al., 1985; de Duve, 1983; Khan et al., 1985a,b; Posner et al., 1982b) was also observed. In chloroquine-treated rats, the rate of loss of ^{125}I-insulin was more markedly retarded than that of ^{125}I-prolactin. As well, both the microtubule inhibitor colchicine and the acidotropic agent chloroquine led to a greater proportional accumulation of either ligand in the ML (large granule) fraction. Thus, the possibility of alternate pathways for insulin and prolactin transport required consideration. Attempts were carried out to resolve this by determining if an analytical approach (de Duve, 1971, 1975) to monitor the distribution of internalized ligand would identify components unique to either insulin or prolactin uptake. Within the resolution of the approach, this did not appear to be the case. Thus, from parent microsomal (P) fractions, early endocytic components of ρ = 1.11 were resolved away from the plasmalemma marker enzyme 5′-nucleotidase (ρ = 1.155). From parent ML fractions, a later endocytic component of ρ = 1.10 was resolved from lysosomes (ρ > 1.15). Both ligands were enriched in the $P_{1.11}$ and $ML_{1.10}$ endosomes although the apparent time course of transit for each labeled hormone through these components was different. The analytical approach therefore not only defined the same endocytic components for both insulin and prolactin but also provided new information on the distribution of endocytic components during subcellular fractionation [see as well Debanne et al. (1984)].

A major difference in the handling of ^{125}I-insulin and ^{125}I-prolactin was localized surprisingly to the compartment enriched in 5′-nucleotidase activity, i.e., the presumed plasma membrane ($P_{1.155}$; Figure 7). Whereas insulin was internalized

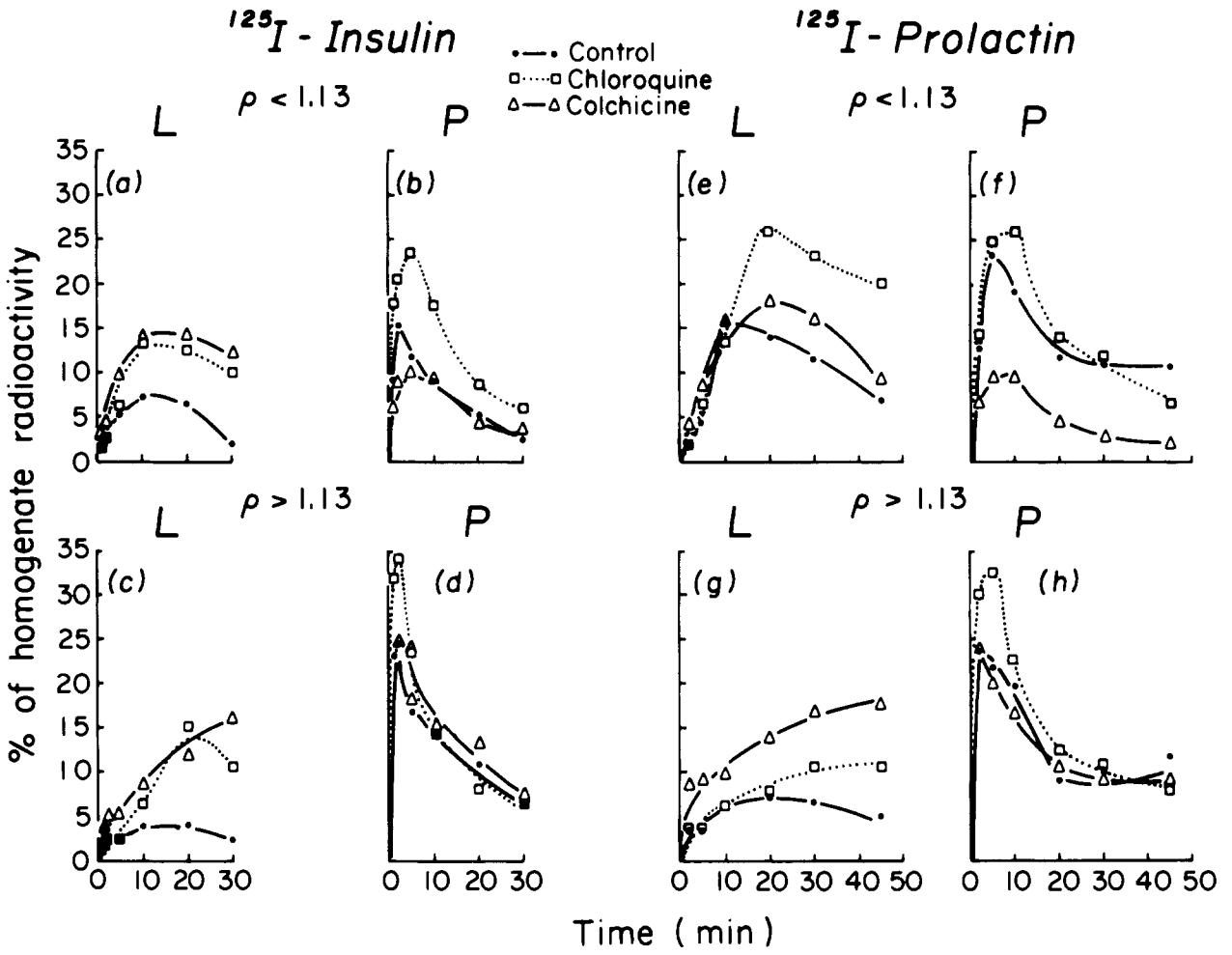

FIGURE 9: Effect of chloroquine or colchicine treatment of rats in accumulation of ^{125}I-insulin or ^{125}I-prolactin in endocytic components (ρ < 1.13) floated up in discontinuous sucrose gradients from parent L or P fractions. The radioactive content of endocytic fractions (ρ < 1.13, upper panels) is compared to that of heavier components (ρ > 1.13, including the bulk of lysosomes and plasma membranes). Enzymic analyses confirmed that the majority of the lysosomal and plasma membrane marker enzymes were at densities > 1.13 (data not shown).

from this fraction ($P_{1.15S}$) with a $T_{1/2}$ of ca. 4 min, prolactin internalization was approximately 3 times slower (cf. Figure 11). A difference in the rate of uptake at the presumed cell surface accounted for some but not all of the differences in the rates of processing of insulin and prolactin. Since only a 5-min lag was observed before the loss of ^{125}I-insulin was observed (Figure 3), then a significant portion of ^{125}I-insulin loss must have occurred within the same (early) endosome in which prolactin loss was minimal. The possibility that this was effected via degradation was supported by studies with chloroquine-treated rats. The more marked retention of insulin but not prolactin in crude microsomal endosomes (Figure 9) is consistent with the commencement of insulin but not prolactin degradation in the early $P_{1.11}$ endosome. Acidotropic agents such as chloroquine, monensin, etc. are known to accumulate in acidic components with the consequent preservation of ligand–receptor complexing (Bergeron et al., 1985; Posner et al., 1982b; Khan et al., 1985a; de Duve, 1983; Harford et al., 1983; Wolkoff et al., 1984) and the inhibition of the activity of acid proteases (de Duve et al., 1974). We have previously documented that insulin binding to its receptor was more markedly inhibited at low pH (i.e., pH 6) than that of prolactin binding to its receptor [cf. Khan et al. (1985b)]. A scenario in which insulin but not prolactin was dissociated and degraded in the $P_{1.11}$ endosome is consistent with our data and the known actions of acidotropic agents.

The effects of the microtubule inhibitor colchicine provided valuable information on the subcellular distribution of endosomes following colchicine treatment but did not shed light on the reasons for the different handling of insulin and prolactin by hepatic parenchymal cells. Morphologic studies have shown that colchicine treatment of rats leads to the accumulation of large lipoprotein-filled endocytic structures near the periphery of the hepatocyte (Bergeron et al., 1983; Redman et al., 1975). These very large structures (often exceeding 450 nm in diameter) would be expected to sediment in the ML fraction instead of normal sedimentation into the P fraction. The colchicine-induced redistribution of ligand as evaluated by differential and analytical centrifugation is therefore most probably due to a size change in the endosomes themselves.

Characterization of Endosomal Components. The analytical gradients were not designed to prepare endosomes but rather to identify the sedimentation properties of endocytic components as compared to established marker enzymes. Nevertheless, the enrichment of ligand within the endocytic components was considerable (Table I). The $P_{1.11}$ endosome fraction was enriched ca. 50–70-fold in ligand. The only marker enzyme of comparable enrichment was the Golgi apparatus marker, galactosyltransferase. In other studies we have clearly shown that galactosyltransferase is not a bona fide constituent of endosomes (Kay et al., 1984); therefore, the $P_{1.11}$ fraction is contaminated with Golgi elements. The later $ML_{1.10}$ component was considerably enriched (100–200-fold) in ligand with no marker enzyme activity comparably enriched in the fraction. However, by combined acid phosphatase cytochemistry and EM radioautography, it was concluded that a mi-

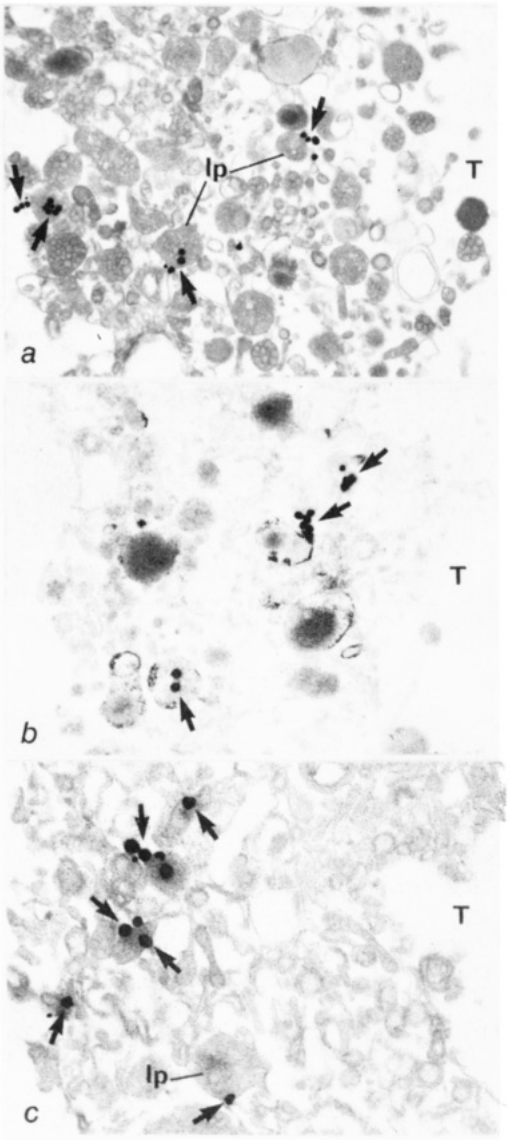

FIGURE 10: Electron microscope radioautography and acid phosphatase cytochemistry of major endosome fractions processed 15 min after the injection of ^{125}I-prolactin as described under Experimental Procedures. Radioautography of the $ML_{1.10}$ fraction (a) reveals silver grains (arrows) associated with lipoprotein-filled (lp) vesicular structures. Magnification 12400×; exposure 64 days. In b, silver grains (arrows) are associated with weakly acid phosphatase staining components. Magnification 12400×; exposure 64 days. The $P_{1.11}$ endosomal fraction reveals silver grains (arrows) associated with smaller lipoprotein-containing (lp) structures. Magnification 21000×; exposure 64 days. All micrographs have been oriented with the top of the filtered pellicle to the right (T).

nority of the acid phosphatase activity of the $ML_{1.10}$ endosome was indigenous to ligand-containing endosomes. These may represent a subpopulation of late endosomes in transition to lysosomes, and it may be within such structures tht dissociation of prolactin from its receptor and consequent degradation of prolactin begin. Such an interpretation is favored by the studies with chloroquine-treated rats showing a marked chloroquine effect on prolactin retention in the crude endosomes floated from the "L" (light mitochondrial) fractions (Figure 9).

By EM radioautography, both the early $P_{1.11}$ and the late $ML_{1.10}$ endosomes contained lipoproteins. Similar structures have been observed to accumulate in vivo insulin (Bergeron et al., 1979), prolactin (Bergeron et al., 1983), asialoglycoprotein (Courtoy et al., 1984; Deschuytenees et al., 1983; Stockert et al., 1980), and lipoprotein (Chao et al., 1981; Handley et al., 1981; Hornick et al., 1984). Indeed, the lipoprotein-filled content of liver endosomes probably represents internalized lipoprotein (Hornick et al., 1985).

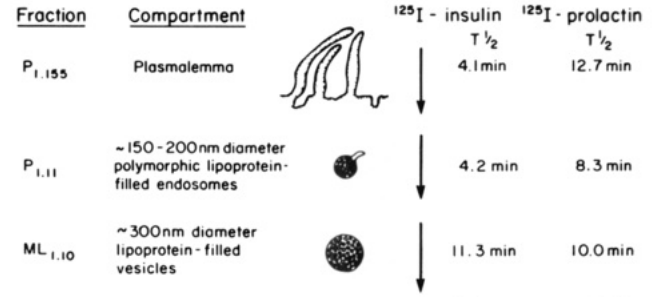

FIGURE 11: Receptor-mediated endocytosis of ^{125}I-insulin and ^{125}I-prolactin in vivo. Following binding to the plasmalemma ($\rho \sim 1.155$), ligand is internalized into early lipoprotein-filled endosomes of $\rho \sim 1.11$ found in the P fraction and then into similar looking but larger endosomes of $\rho \sim 1.10$ found in ML and L fractions. The major difference in the handling of insulin and prolactin is attributed to the plasmalemma. The rapid kinetics of insulin degradation suggest that insulin degradation must already begin in early ($P_{1.11}$) endosomes.

These studies therefore considerably extend our previous observations on the structures internalizing insulin and prolactin. The analytical approach has defined clearly the distinct handling of insulin and prolactin at the presumed cell membrane of the hepatocyte ($P_{1.155}$ fraction). This approach has also identified two distinct endosomal components found in microsomal ($P_{1.11}$) and ML ($ML_{1.10}$) fractions, respectively, both of which participate in the receptor-mediated endocytosis of insulin and prolactin (Figure 11). The $ML_{1.10}$ component is identified as the unique vesicle previously found in the light mitochondria (L) fraction (Khan et al., 1981) as well as the high-density structure containing low levels of acid phosphatase activity previously found in the Golgi intermediate fraction (Khan et al., 1982). The $P_{1.11}$ fraction probably corresponds to the early low-density endosome found in the Golgi light and intermediate fractions (Khan et al., 1982), which has been subsequently resolved from Golgi elements (Kay et al., 1984). Differential handling of insulin and prolactin by heptic parenchymal cells is probably due to selective degradation of insulin within early components of the endosomal apparatus.

ACKNOWLEDGMENTS

We thank Dr. C. E. Smith for his participation in the cytochemical studies.

Registry No. Insulin, 9004-10-8; prolactin, 9002-62-4.

REFERENCES

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., & Berthet, J. (1974) *J. Cell Biol. 61*, 201–212.

Barka, T., & Andersen, P. J. (1962) *J. Histochem. Cytochem. 10*, 741–753.

Bergeron, J. J. M., & Posner, B. I. (1981) in *Receptor-Mediated Binding and Internalization of Toxins and Hormones* (Middlebrook, J. L., & Kohn, L. D., Eds.) pp 197–218, Academic Press, New York.

Bergeron, J. J. M., Evans, W. H., & Geschwind, I. I. (1973) *J. Cell Biol. 59*, 771–776.

Bergeron, J. J. M., Posner, B. I., Josefsberg, Z., & Sikstrom, R. (1978) *J. Biol. Chem. 253*, 4058–4066.

Bergeron, J. J. M., Sikstrom, R. A., Hand, A. R., & Posner, B. I. (1979) *J. Cell Biol. 80*, 427–443.

Bergeron, J. J. M., Rachubinski, R. A., Sikstrom, R. A., Posner, B. I., & Paiement, J. (1982) *J. Cell Biol. 92*, 139–146.

Bergeron, J. J. M., Resch, L., Rachubinski, R., Patel, B. A., & Posner, B. I. (1983) *J. Cell Biol. 96*, 875–886.
Bergeron, J. J. M., Cruz, J., Khan, M. N., & Posner, B. I. (1985) *Annu. Rev. Physiol. 47*, 383–403.
Chao, Y. S., Jones, A. L., Hradek, G. T., Windler, E. E. T., & Hand, R. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 597–601.
Courtoy, P. J., Quintart, J., & Baudhuin, P. (1984) *J. Cell Biol. 98*, 870–876.
Debanne, M. T., Bolyos, M., Gauldie, J., & Regoeczi, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2995–2999.
de Duve, C. (1971) *J. Cell Biol. 50*, 20D–55D.
de Duve, C. (1975) *Science (Washington, D.C.) 189*, 186–194.
de Duve, C. (1983) *Eur. J. Biochem. 137*, 391–397.
de Duve, C., Pressmann, B. C., Gianetto, R., Wattiaux, R., & Appelmans, F. (1955) *Biochem. J. 60*, 604–617.
de Duve, C., de Bousey, T., Poole, B., Trouct, A., & Tulkens, P. (1974) *Biochem. Pharmacol. 23*, 2495–2531.
Deschuyteneer, M., Prieels, J. P., & Mosselmens, R. C. (1983) *Biol. Cell (1981) 50*, 17–29.
Handley, D. A., Arbeeny, C. N., Eder, H. A., & Chien, S. (1981) *J. Cell Biol. 90*, 778–787.
Harford, J., Wolkoff, A. W., Ashwell, G., & Klausner, R. D. (1983) *J. Cell Biol. 96*, 1824–1828.
Hornick, C. A., Jones, A. L., Renaud, G., Hradek, G., & Havel, R. J. (1984) *Am. J. Physiol. 246*, 6187–6194.
Hornick, C. A., Hamilton, R. L., Spaziani, E., Enders, G. H., & Havel, R. J. (1985) *J. Cell Biol. 100*, 1558–1569.
Josefsberg, Z., Posner, B. I., Patel, B., & Bergeron, J. J. M. (1979) *J. Biol. Chem. 254*, 209–214.
Kay, D. G., Khan, M. N., Posner, B. I., & Bergeron, J. J. M. (1984) *Biochem. Biophys. Res. Commun. 123*, 1144–1148.
Khan, M. N., Posner, B. I., Verma, A. K., Khan, R. J., & Bergeron, J. J. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4980–4984.
Khan, M. N., Posner, B. I., Khan, R. J., & Bergeron, J. J. M. (1982) *J. Biol. Chem. 257*, 5969–5976.
Khan, M. N., Savoie, S., Khan, R. J., Bergeron, J. J. M., & Posner, B. I. (1985a) *Diabetes 34*, 1025–1030.
Khan, R. J., Khan, M. N., Bergeron, J. J. M., & Posner, B. I. (1985b) *Biochim. Biophys. Acta 838*, 77–83.
Posner, B. I., Josefsberg, Z., & Bergeron, J. J. M. (1978) *J. Biol. Chem. 253*, 4067–4073.
Posner, B. I., Josefsberg, Z., & Bergeron, J. J. M. (1979) *J. Biol. Chem. 254*, 12494–12499.
Posner, B. I., Patel, B. A., Verma, A. K., & Bergeron, J. J. M. (1980) *J. Biol. Chem. 255*, 735–741.
Posner, B. I., Khan, M. N., & Bergeron, J. J. M. (1982a) *Endocr. Rev. 3*, 280–298.
Posner, B. I., Patel, B. A., Khan, M. N., & Bergeron, J. J. M. (1982b) *J. Biol. Chem. 257*, 5789–5799.
Posner, B. I., Verma, A. K., Patel, B. A., & Bergeron, J. J. M. (1982c) *J. Cell Biol. 93*, 560–567.
Redman, C. M., Banerjee, D., Howell, K., & Palade, G. E. (1975) *J. Cell Biol. 66*, 42–59.
Stockert, R. J., Haimes, H. B., Morell, A. G., Novikoff, P. M., Novikoff, A. B., Quintana, N., & Sternlieb, I. (1980) *Lab. Invest. 43*, 556–563.
Wolkoff, A. W., Klausner, R. D., Ashwell, G., & Harford, J. (1984) *J. Cell Biol. 98*, 375–381.

Phosphorylation Reduces the Affinity of Protein 4.1 for Spectrin†

Paul S. Eder,‡ Chu-Jing Soong, and Mariano Tao*
Department of Biological Chemistry, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60612
Received September 16, 1985

ABSTRACT: The phosphorylation of protein 4.1 by the membrane kinase and casein kinase A has been investigated. Each of these kinases catalyzed the incorporation of 2 mol of phosphate per mole of protein 4.1. The presence of both kinases in the reaction mixture did not lead to an increase in the incorporation of phosphates into the protein. An analysis of the acid hydrolysis products of the ^{32}P-labeled protein 4.1 indicated that the radioactivities were distributed between phosphothreonine and phosphoserine in a ratio of about 2 to 1. The effects of phosphorylation on the binding of protein 4.1 to spectrin were investigated by using sucrose density gradient centrifugation. The affinity of protein 4.1 for spectrin was reduced about 5-fold, from a K_D of 2×10^{-6} M to a K_D of 9.4×10^{-6} M, by phosphorylation. The phosphorylation of spectrin, on the other hand, appeared to increase slightly its affinity for protein 4.1. The results suggest that phosphorylation may lead to a relaxation of the cytoskeletal network and the formation of a more flexible membrane structure that is important to red cell function.

The human erythrocyte membrane contains an extensive cytoskeletal network that has been suggested to play an important role in the control of cell shape and deformability and the distribution of intramembrane particles and surface receptors (Marchesi, 1979, 1983; Branton et al., 1981; Tao & Conway, 1982). Detailed investigations of the composition, structure, and assembly of this membrane cytoskeletal network have shown that the network is formed by the interactions of three membrane proteins, spectrin, actin, and band 4.1 (Branton et al., 1981; Marchesi, 1983). Spectrin, the major protein of the erythrocyte membrane skeleton, is composed of two nonidentical subunits, α (M_r 240000) and β (M_r 220000), which associate to form double-stranded, fiberlike flexible heterodimers (Branton et al., 1981). These spectrin dimers can further assemble in a head to head arrangement into tetramers and higher oligomers (Morrow et al., 1981; Marchesi, 1983). The tetrameric form has been suggested to represent the major species of spectrin and the physiological functional unit in normal red cell ghosts (Liu & Palek, 1980;

† This work was supported by the National Institutes of Health (AM-23045) and by the Chicago Heart Association.
‡ Present address: Department of Biochemistry, The University of Iowa, Iowa City, IA 52242.

0006-2960/86/0425-1764$01.50/0