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FATE OF ASIALOFETUIN ENDOCYTOSED BY RAT LIVER

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We have investigated the endocytosis by rat liver of asialofetuin coupled to [^{125}I] tyramine cellobiose: [^{125}I] TCASF.Subcellular distribution of radioactive compounds was established after differential and isopycnic centrifugation and by analysing the fractions by SDS electrophoresis. Labelling secondary lysosomes was performed by injecting rats with Triton WR 1339 four days before injecting the protein. Results show that after being associated with endosomes [^{125}I] TCASF is recovered in organelles where they are subjected to a first degradation, the density of these organelles is practically not affected by Triton WR 1339 injection . Later the degradation products are associated with lysosomes whose density is markedly lowered by Triton WR 1339 treatment. These observations suggest that the first intracellular organelles where [^{125}I] TCASF is subjected to digestion are distinct from the secondary lysosome population. This could be in agreement with the hypothesis that supposes that endosomes acquire enzymes from primary lysosomes before fusion with secondary lysosomes.

After traveling through endosomes, most endocytosed substances reach lysosomes. The hydrolysis of these molecules takes place in these organelles, although according to recent results, it could begin in an endosomal compartment (Diment et al. 1988). Until now, it is not clear whether an endocytosed compound is delivered at random throughout the whole lysosome population or if different kinds of lysosomes are involved during the molecules stay in the lysosomal compartment, as suggested by the work of Berg et al. (1985). As first proposed by de Duve and Wattiaux (1966), two main classes of lysosomes could be considered: primary lysosomes and secondary lysosomes depending on whether they have not or they have previously been engaged in heterophagic or autophagic events. Therefore, an endocytosed compound can meet lysosomal hydrolases as a result of a fusion of an endosome with a primary or with a secondary lysosome. In the present work, we investigated the problem by using a method that allows the identification of lysosomes that have been already involved in a heterophagic process. We made use of rats injected with Triton WR 1339; the detergent is endocytosed by the liver, accumulates in lysosomes and strikingly decreases their density (Wattiaux et al. 1963). Therefore these secondary lysosomes can be easily detected after gradient centrifugation owing to their abnormally low equilibrium density. We followed the endocytosis of asialofetuin (ASF) which is mostly taken up by hepatocytes (Harford and Ashwell,1982) Labeling of this molecule was performed with [^{125}I] tyramine

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cellobiose in order to prevent the release from the lysosomes of the labelled degradation compounds after digestion of the endocytosed protein in these organelles (Berg et al.1985). Our results indicate that the endocytosed protein has first to pass through lysosomes that have not been involved in Triton WR 1339 endocytosis before reaching lysosomes containing the detergent.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing about 200 g. Asialofetuin (ASF) radiolabelled with iodinated tyramine-cellobiose (^{125}I-TC) was injected intraveinously (0.1 mg in 1 ml saline). Animals were killed at various times after injection; the liver was perfused with cold 0.15 M NaCl, removed and homogenised in ice cold 0.25M sucrose. Differential centrifugation was performed according to de Duve et al.(1955), except that the two mitochondrial fractions were isolated together. Density gradient experiments were done according to Beaufay et al. (1964). Iodination of tyramine-cellobiose and its subsequent binding to protein was carried out by the method of Pittman et al. (1983) as modified by Hysing et al. (1986). Fractions and sections of gradients were analysed by SDS-polyacrylamide gel electrophoresis under reductive conditions. Cathepsin C was assayed according to the method of Jadot et al. (1984), alkaline phosphodiesterase according to Beaufay et al. (1974).

RESULTS

Fig.1 illustrates the distribution of radioactivity amongst fractions isolated by differential centrifugation of liver homogenates prepared at increasing times after [^{125}I] TCASF injection. Early after injection radioactivity is mainly associated with the microsomal fraction; later it becomes more and more located in the total mitochondrial fraction.

Taking into account these results, isopycnic centrifugation in a sucrose gradient was performed on a microsomal fraction isolated 5 min after [^{125}I] TCASF injection and on mitochondrial fractions prepared 10 and 20 min. after injection. Fig.2 shows the results obtained with the microsomal fraction. The labelled molecules are present in low

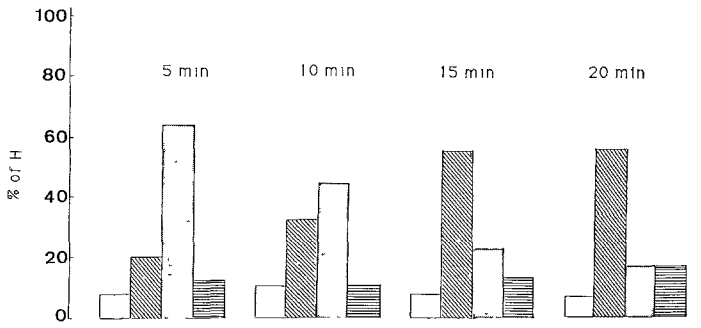

Fig.1

Distribution of radioactivity at increasing times after [125I] TCASF injection. The liver homogenates (H) were fractionated by differential centrifugation in a nuclear fraction ( ),a total mitochondrial fraction ( ) a microsomal fraction ( ) and an unsedimentable fraction( ).

density organelles. It is to be noted that at that time, as indicated by the measurement of acid precipitable radioactivity, [^{125}I] TCASF is mostly undegraded. On the same figure, is presented the distribution of alkaline phosphodiesterase, a marker enzyme for plasma membrane. It is distinctly different from that of radioactivity, indicating that the glycoprotein is no longer associated with plasma membrane. The distribution curve of radioactivity is similar to that of hepatocyte endosomes as described by Limet et al. (1985) and England et al.(1986). Fig.3 depicts the distribution of radioactivity after isopycnic centrifugation of a total mitochondrial fraction isolated 10 min and 20 min after [^{125}I] TCASF injection. After 10 min, the distribution of radioactivity is bimodal; a small peak is observed in low density zones, where as we have shown in Fig.2, endosomes are probably located. An important peak is seen in higher density regions. For sake of comparison, the distribution of a lysosomal enzyme, cathepsin C is given. It is obvious that the largest proportion of radioactivity is recovered where lysosomes equilibrate. After 20

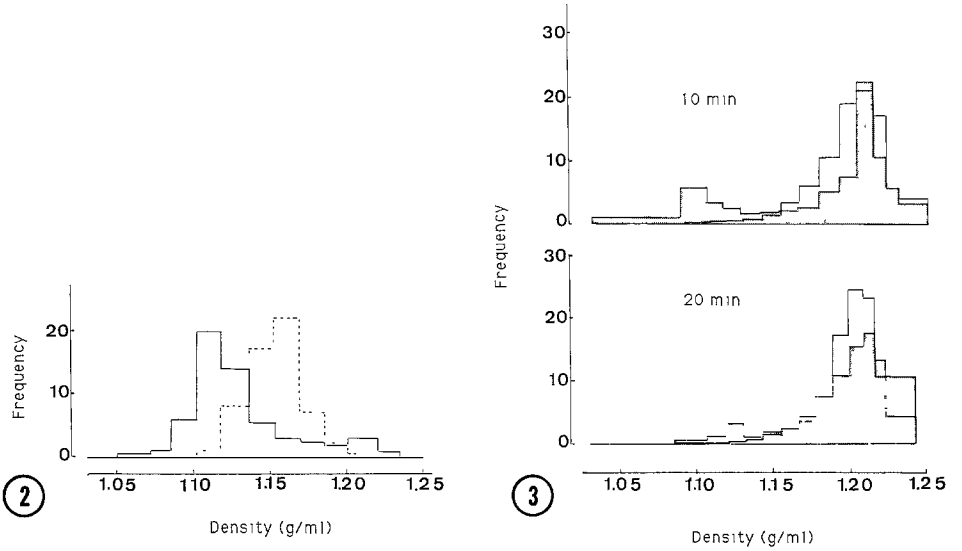

Fig.2

Density distribution histograms of radioactivity and alkaline phosphodiesterase after isopycnic centrifugation of a microsomal fraction. The particle preparation was isolated 5 min after [^{125}I] TCASF injection. Centrifugation was performed at 39 000 rev/min in the SW65 Spinco rotor. The time integral of the square angular velocity was 860 rad^2/ns. The sucrose gradient extended from 1.05 g/ml to 1.26 g/ml density. Ordinate: frequency $Q/\Sigma Q.\Delta\rho$ where Q represents the activity found in the fraction, ΣQ, the total activity recovered in the sum of the fraction and $\Delta\rho$ the increment of density from top to bottom of the fraction. (▭) radioactivity; (▭) alkaline phosphodiesterase.

Fig.3.

Density distribution histograms of radioactivity and cathepsin C after isopycnic centrifugation of a total mitochondrial fraction. The particle preparations were isolated 10 min and 20 min after [^{125}I]TCASF injection. Centrifugation was performed at 39 000 rev/min in the SW 65 Spinco rotor. The time integral of the square angular velocity was 144 rad^2/ns. The sucrose gradient extended from 1.05 g/ml to 1.26 g/ml. For explanation of the graph see legend of Fig.2. (▭) cathepsin C; (▭) radioactivity.

min, the radioactivity distribution is similar except that practically no labelled compounds are recovered in the endosomal region. In the next figure. (Fig.4), results of similar experiments are presented but the mitochondrial fractions were isolated from rats preinjected with Triton WR 1339 four days before [^{125}I] TCASF injection. The effect of the detergent on lysosomes is clearly illustrated by the distribution of cathepsin C, which is now situated in low density zones. After 10 min, the distribution of radioactivity is almost unaffected by Triton WR 1339 treatment. A small peak is still found in low density zones, an important one in high density fractions. Particularly striking is the fact that contrarily to what is seen in the normal animal, most of the radioactivity exhibits a distribution distinct from that of lysosomal enzyme cathepsin C. The situation is quite different after 20 min; at that time, the radioactivity distribution curve has been subjected to an important shift toward lower densities so that labelled molecules are now recovered to a large extent where the lysosomal cathepsin C is present.

Obviously the question is what is the nature of the radioactivity recovered in the gradient fractions since degraded labelled products remain in the organelles where they are generated owing to their tyramine cellobiose content. We analysed by SDS electrophoresis and autoradiography fractions obtained after isopycnic centrifugation of a total mitochondrial fraction isolated 10 min and 20 min after [^{125}I] TCASF injection, the animals having been injected with Triton WR 1339, 4 days before. Results are illustrated in Fig.5. After 10 min, degradation products are already well apparent in the mitochondrial fraction. They are mostly located in the high density regions of the gradient together with undegraded molecules. It is to be noted that a small amount of intact [^{125}I] TCASF is recovered in the low density fractions (fraction 5 and 6), corresponding to the small peak of radioactivity present where endosomes equilibrate. As indicated by the electrophoresis pattern, no intact [^{125}I] TCASF is present in the mitochondrial fraction isolated 20 min after injection. The fast migrating radioactive compounds are recovered in fractions of lower density than that of organelles with which labelled components where associated after 10 min. These fractions contain the secondary lysosomes loaded with Triton WR 1339 as illustrated in Fig.4.

DISCUSSION

According to our results, after being endocytosed, [^{125}I] TCASF is first recovered in organelles present in the microsomal fraction and exhibiting a low equilibrium density in a sucrose gradient. This is in agreement with previous observations of Limet et al. (1985) and England et al. (1986) in their investigations on endocytosis of galactosylated proteins by rat liver. These organelles are probably endosomes. Subsequently, the radioactive molecules are transferred to structures sedimenting in the mitochondrial fractions, that are endowed with a markedly higher density and equilibrate in sucrose gradient like lysosomes. Triton WR 1339 pretreatment of the animals allows us to distinguish two kinds of structures amongst these organelles depending on whether their density is affected or not by the detergent. The labelled substances are first associated with particles

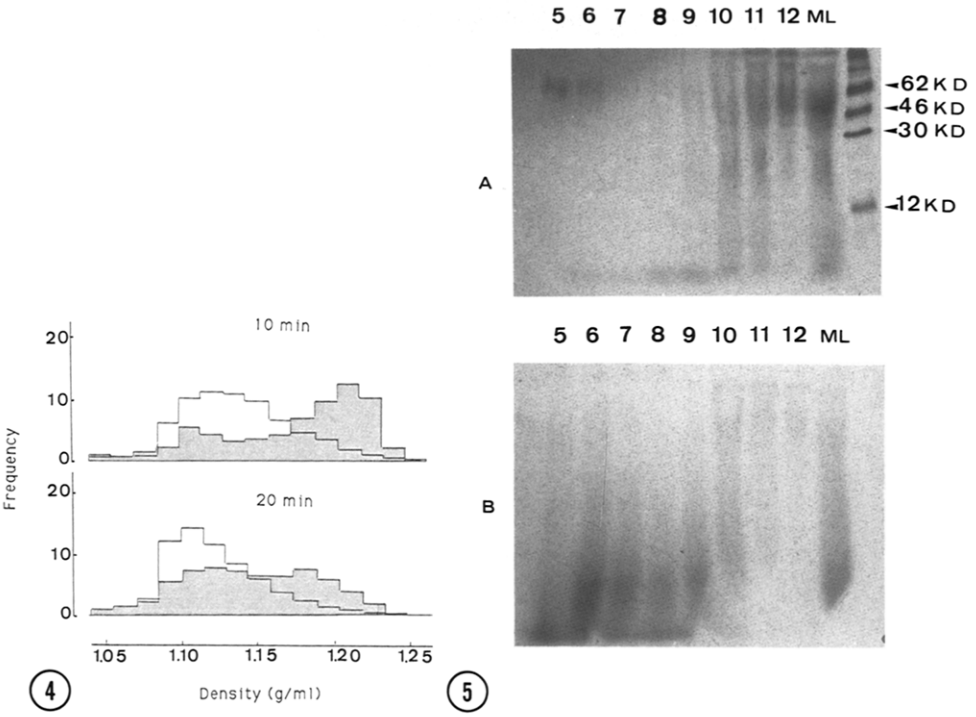

Fig.4

Density distribution histograms of radioactivity and cathepsin C after isopycnic centrifugation of a total mitochondrial fraction. Effect of Triton WR 1339. The particle preparations were isolated 10 min and 20 min after [^{125}I] TCASF injection. The rats were injected with Triton WR 1339 (170 mg in 1 ml saline) four days before receiving the [^{125}I] TCASF injection. For centrifugation conditions see legend of Fig.3. (▭) cathepsin C; (▨) radioactivity.

Fig.5

Proteolysis in organelles with which radioactivity is associated. Mitochondrial fractions were isolated 10 min (A) or 20 min (B) after [^{125}I] TCASF injection, the rats having been injected with Triton WR 1339 (170 mg/ml saline) four days before. The granule preparations were fractionated by isopycnic centrifugation in a sucrose gradient and the fractions analysed by SDS electrophoresis and autoradiography. The density of the fractions increases from left to right. ML: mitochondrial fraction before fractionation.

(particle A) whose density does not change after Triton WR 1339 treatment; later, they are found in structures (particle B) that like lysosomes become lighter after injection of the detergent. Degradation products are present in particles A, that therefore must contain proteolytic enzymes; however, low molecular weight degradation molecules appearing later, are mainly located in particles B. Thus apparently, [^{125}I]TCASF has to pass through two hydrolytic compartments after leaving endosomes. It is subjected to a partial degradation in the first one, being more extensively hydrolysed in the second one. The centrifugation behaviour of particles A and their propteolytic capacity strongly suggest that they are lysosomes; owing to the fact that they are not affected by Triton WR 1339, they might be considered as lysosomes that have not been involved in previous het-

erophagic events and originate from a fusion between endosomes or vesicles derived from endosomes and primary lysosomes.

Previous results of England et al (1986) can be paralleled with our observations. In their study of [^{14}C] sucrose asialofetuin endocytosis by rat liver, they found that the compound has to travel successively through two kinds of lysosomes that can be distinguished by their density in a Nycodenz gradient. However, the functionnal difference between these two populations of lysosomes is not apparent. Our work goes further; it shows that lysosomes that are first involved in the intracellular degradation of the endocytosed glycoprotein are probably "neoformed" secondary lysosomes that later transfer their content into "old" organelles.

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