The Effect of Triton WR-1339 on the Subcellular Distribution of Trypan Blue and ^{125}I -Labelled Albumin in Rat Liver

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1. The density-gradient distribution patterns of acid phosphatase, Trypan Blue and denatured ^{125}I -labelled albumin were studied by discontinuous sucrose- and isopycnic sucrose-density-gradient centrifugation on combined heavy and light mitochondrial (M+L) fractions of liver isolated from normal rats and from rats injected with Triton WR-1339. 2. The results obtained from the subfractionation of the M+L pellet of normal animals indicate that the equilibrium density of Trypan Blue and acid-insoluble radioactivity is the same as that for acid phosphatase, which suggests they are bound by a common membrane to form a distinct subcellular population of lysosomal nature. 3. In contrast, the analysis of the isopycnic gradients obtained on subfractionation of M+L pellets of liver isolated from rats treated with Triton WR-1339 show that the acid-insoluble radioactivity has an equilibrium density around 1.21, whereas the acid hydrolases, including cathepsin D, show the characteristic shift to an equilibrium density of around 1.12. Trypan Blue is distributed along the gradient with distinct peaks at densities 1.22 and 1.12. 4. Similar equilibrium-density distribution patterns were obtained with M+L pellets isolated from rats pretreated with Triton WR-1339 but not injected with Trypan Blue. 5. Treatment of the rats with Triton WR-1339 does not affect albumin digestion of isolated intact lysosomes despite the fact that most of the cathepsin D and the albumin ingested by phagocytosis are located in different vacuoles. 6. It is concluded from these experiments that in the liver of animals treated with Triton WR-1339 ^{125}I -labelled albumin is located within heterophagosomes which do not fuse with heterolysosomes containing the non-ionic detergent Triton WR-1339. The inability of these two lysosomal populations to fuse is not due to Trypan Blue.

The experiments of Griffiths *et al.* (1966) gave rise to the idea of a new form of lysosome pathology caused by the uptake of enzyme inhibitors into phagolysosomes. Trypan Blue (Lloyd *et al.*, 1968), suramin (Smeesters & Jacques, 1968; Wills, 1952) and sodium aurothiomalate (Persellin & Ziff, 1966; Norton *et al.*, 1968; Ennis *et al.*, 1968) are such inhibitors, and experimental evidence to support a role of intralysosomal inhibitors for these compounds was reported by Davies *et al.* (1971). In their work it was demonstrated that lysosomes isolated from animals pretreated with these compounds and incubated in Tris-acetate buffer at pH 7.4 containing 0.25 M-sucrose had a decreased digestive capacity compared with lysosomes isolated from normal animals. It was argued by these authors that the decreased digestive capacity was the result of intralysosomal inhibition of the enzymes responsible for protein catabolism. An alternative possibility is that in the treated rats the

lower digestive capacity reflects an interference with the fusion between the lysosomes (containing the enzyme) and phagosomes (containing the substrate).

The possibility that Trypan Blue interferes with membrane fusion is examined for Trypan Blue in the present paper by studying the sedimentation behaviour in sucrose gradients of lysosomes isolated from normal rats and rats treated with Triton WR-1339. The work is based on the observation of Wattiaux *et al.* (1963) that the non-ionic detergent Triton WR-1339 is selectively taken up and accumulates within lysosomes with the result that the equilibrium density of the lysosomes is lowered and they float on a sucrose gradient. In this way lysosomes can be separated from mitochondria and peroxisomes. The uptake of ^{125}I -labelled albumin into liver lysosomes of rats treated with Triton WR-1339 also has been investigated by using a technique described by Mego *et al.* (1967). This technique measures the proteolysis of ^{125}I -labelled albumin ingested by phagocytosis, within isolated intact liver lysosomes. Some of the work described in this paper has been reported briefly (Davies, 1973).

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Materials and Methods

Chemicals

[125 I]Iodide was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. and bovine serum albumin (type II), haemoglobin (bovine, type II), and Triton X-100 were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. A commercial sample of Trypan Blue (Williams Ltd., Hounslow, Middx., U.K.) was freed from salt and converted into the free acid as described by Lloyd & Beck (1966). An aqueous 1% (w/v) solution of the dye was prepared for injections. Triton WR-1339 was a gift from Hilton-Davis Chemicals, Fawdon, Newcastle-upon-Tyne, U.K. All other chemicals were of analytical grade from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Preparation of denatured 125 I-labelled bovine serum albumin. The method described by Davies *et al.* (1971) was used.

Enzyme assays. Acid phosphatase (EC 3.1.3.2) was assayed at pH 5.0 by using *p*-nitrophenyl phosphate (10 mM) as substrate (Torriani, 1960) and preincubating the fractions for 10 min to inactivate microsomal phosphatases as described by Neil & Horner (1964). Acid proteinase (cathepsin D; EC 3.4.4.23) was assayed at pH 3.8 by using haemoglobin (2%, w/v) as substrate, the released tyrosine residues being determined by the method of Anson (1937).

Determination of Trypan Blue. The method described by Lloyd *et al.* (1968) was used.

Determination of protein. This was carried out by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Animal experiments. Male (250–300 g) and female (180–200 g) Wistar rats were used. In the experiments with Triton WR-1339 the animals were injected with the detergent in a dose of 850 mg/kg body wt. 96 h before they were killed. Trypan Blue (75 mg/kg body wt.) was injected 24 h before the animals were killed. All rats were given an intravenous injection, into the femoral vein, of denatured 125 I-labelled bovine serum albumin (2.5 mg of protein/kg body wt.). The rats were killed 0.5 h after the albumin injection, except in one experiment, when the animals were killed 1.0 h or 2.5 h after injection.

Preparation of the combined light and heavy mitochondrial fraction of rat liver. Rats were killed by a blow on the head, decapitated and bled. The liver was quickly removed and placed in ice-cold 0.25 M-sucrose (adjusted to pH 7.4 with NaHCO_3) and homogenized with a Potter-Elvehjem-type Teflon-on-glass homogenizer. The homogenate was diluted with 0.25 M-sucrose (10 ml/g of liver) and centrifuged for

10 min at 4°C and 1000g (r_{av} , 7.4 cm) in a MSE High-Speed 18 refrigerated centrifuge with rotor 69181. The supernatant was carefully removed and centrifuged at 16500g for 20 min. The supernatant was removed and the pellet (the M+L pellet) subfractionated as described below.

Subfractionation of the M+L pellet. (a) The M+L pellet was subfractionated on a discontinuous sucrose gradient by a method described by Leighton *et al.* (1968). The M+L pellet obtained above by differential centrifugation was gently suspended in 45% (w/w) sucrose ($\rho = 1.21$) (1 ml of sucrose solution/g of liver processed). The suspension (25 ml) was transferred into a centrifuge tube for the SW 25.2 rotor of the Beckman L2 preparative ultracentrifuge. On top of the M+L suspension two additional sucrose solutions, one with a density of 1.155 (20 ml) and the other of 1.06 (10 ml) were layered carefully. The SW 25.2 rotor was used in the Beckman L2 ultracentrifuge and the tubes were centrifuged for 2.5 h at 4°C and 75500g (r_{av} , 10.8 cm). The resultant bands were removed separately, the volume of each recorded and labelled as subfractions A, B, C, D, E and F (Fig. 1). The pellet present at the bottom of the centrifuge tube was suspended in distilled water to a final volume of 25 ml.

(b) The combined M+L fraction obtained by differential centrifugation was also subfractionated on a continuous sucrose gradient. The combined pellet was resuspended in 0.25 M-sucrose (1 ml of sucrose/g of liver processed) and layered on the top of a continuous sucrose gradient prepared in tubes no. 303305 (Beckman Instrument Co.) from two sucrose solutions in water respectively 23.15% (w/w) and 53.92%

Fig. 1. Purification of lysosomes from a combined M+L fraction of rat liver by discontinuous gradient centrifugation

Preparation of the gradient and conditions of centrifugation are described in the Materials and Methods section. The letters on the right-hand side of the figure correspond to the number of fractions collected.

(w/w) sucrose. The total volume in each centrifuge tube was 5.4 ml, made up of 0.8 ml of M+L suspension and 4.6 ml of sucrose gradient. The tubes were centrifuged for 2.5 h at 4°C and 105 000g (r_{av} , 6.5 cm) in the SW 50L rotor of the Beckman L2 preparative ultracentrifuge. At the end of the experiment each tube was divided into fractions, usually ten, by gravity flow. Each fraction was analysed for acid phosphatase, protein and total trichloroacetic acid-insoluble radioactivity. In some experiments cathepsin D activity was also determined. In experiments with M+L fractions obtained from animals pretreated with Trypan Blue the concentration of the bis-azo dye was determined. In all density-gradient isopycnic-centrifugation experiments the results were first expressed as a percentage of the sum of the amount recovered in all the fractions. In most of the experiments this value did not differ significantly from the value obtained from the M+L fraction placed on the gradient, except for Trypan Blue where the presence of protein interferes with the determination. The calculated results were represented in the form of density-gradient histograms [as described by Beaufay *et al.* (1964)]. In all diagrams the surface area of each block is equal to the percentage of the component present in the appropriate fraction and the total surface area of the diagrams is equal to 100.

To compare the results of several different gradient experiments in this investigation the base of the observed density-distribution histogram covering the range of the gradient between 1.25 and 1.09 was divided into eight equal sections of density increments ($\Delta\rho$) 0.02. The areas of the histogram included in each density increase of 0.02 were then represented by rectangular blocks of appropriate height to form a new histogram. The ordinates refer to % of the total recovered activity present in the subfractions and the

shaded areas represent material outside the upper limit of the gradient.

Incubation of lysosomes containing ^{125}I -labelled albumin. The method described by Davies *et al.* (1971) was used to determine the extent of breakdown of labelled albumin in isolated intact lysosomes. An M+L fraction prepared by differential centrifugation was resuspended carefully in 0.01 M-Tris-acetate buffer, pH 7.4, containing 0.25 M-sucrose and immediately incubated at 22°C. Samples were removed at intervals up to 2 h and assayed for trichloroacetic acid-soluble radioactivity (a measure of the digestion of labelled albumin) and for non-sedimentable trichloroacetic acid-insoluble radioactivity (a measure of the extent of lysosome rupture during the incubation).

Results

Subfractionation of combined M+L pellet by using a discontinuous sucrose gradient

Table 1 shows the results of an experiment carried out to subfractionate the combined (M+L) pellet obtained from animals pretreated with Triton WR-1339 and injected with ^{125}I -labelled albumin. As expected from the work of Leighton *et al.* (1968) subfraction B was rich in acid phosphatase and contained over 60% of the enzyme recovered from the gradient. On the basis of increased specific activity, fraction B represents nearly a 10-fold purification of acid phosphatase over the starting material (M+L pellet) (specific activity: M+L fraction, 0.086 unit/mg of protein; fraction B, 0.844 unit/mg of protein). In contrast the acid-insoluble radioactivity did not follow the pattern described above for acid phosphatase and little or no acid-insoluble radioactivity

Table 1. Results of subfractionation of an M+L fraction of rat liver by discontinuous-sucrose-gradient centrifugation

Fresh liver from four animals previously injected with Triton WR-1339 (850 mg/kg) and denatured ^{125}I -labelled albumin were used to prepare an M+L fraction by differential centrifugation. The subfractionation was carried out as shown in Fig. 1 and as described in the Materials and Methods section. The recovery for acid phosphatase (*p*-nitrophenyl phosphatase) was 95%, and for the acid-insoluble radioactivity 91%, of the amount applied to the gradient. Units of activity for acid phosphatase are expressed as μmol of *p*-nitrophenol released/min.

Fraction	Acid phosphatase		Radioactivity (% of total acid-insoluble radioactivity recovered)
	(units/fraction)	(% of total activity recovered)	
A	—	—	1.0
B	42.74	63.75	1.15
C	—	—	0.33
D	6.36	9.48	2.0
E	5.34	7.96	24.3
F	12.60	18.79	71.26

Table 2. Separation of lysosomes from an M+L fraction of liver isolated from rats treated with Triton WR-1339 and Trypan Blue

Fresh liver from animals previously injected with Triton WR-1339 (850mg/kg), Trypan Blue (75mg/kg) and ^{125}I -labelled albumin was used to prepare an M+L fraction by differential centrifugation. The subfractionation was carried out as shown in Fig. 1, and as described in the Materials and Methods section. The recovery for acid phosphatase (*p*-nitrophenyl phosphatase) was 99.5%, for the acid-insoluble radioactivity 97.0%, and for the Trypan Blue 91.2% of the amount applied to the gradient. Units of activity for acid phosphatase are μmol of *p*-nitrophenol released/min.

Fraction	Acid phosphatase		Radioactivity (% of total acid-insoluble radioactivity recovered)	Trypan Blue	
	(units/fraction)	(% of total activity recovered)		(μg /fraction)	(% of total recovered)
A	—	—	0.9	—	—
B	38.29	57.3	1.9	54.2	33.5
C	—	—	0.79	—	—
D	5.57	8.3	4.0	17.4	10.75
E	4.94	7.4	9.7	68.7	42.45
F	18.0	26.9	81.2	21.5	13.3

was present in subfraction B, the majority of which was recovered in subfractions E and F.

Table 2 shows results of a similar experiment carried out to subfractionate an M+L pellet isolated from a rat pretreated with Triton WR-1339 as described above but in addition injected with Trypan Blue. Treatment of the animal with Trypan Blue did not alter essentially the distribution of acid phosphatase or acid-insoluble radioactivity. The total yield of acid-insoluble radioactivity in subfraction B was 2% of the total recovered from the gradient compared with 57% for acid phosphatase. On the other hand, 34% of the Trypan Blue recovered in the experiment was found in fraction B.

Subfractionation by using density-gradient isopycnic centrifugation

A series of density-gradient isopycnic-centrifugation experiments were carried out with combined M+L fractions of rat liver. Three main series of experiments were undertaken. They differ in the nature of the pretreatment of the animal before injection with labelled protein.

In the first experiment the animals were pretreated with Trypan Blue (75 mg/kg) 24 h before receiving an intravenous injection of denatured ^{125}I -labelled albumin. Fig. 2 shows the results of an experiment in which an M+L fraction was subfractionated by isopycnic density-gradient centrifugation. This represents the subfractionation of granules which have a lower capacity to digest albumin ingested by phagocytosis (Davies *et al.*, 1971). Examination of the

gradient (Fig. 2) indicates that the equilibrium density of acid phosphatase and hence of lysosomes is between 1.21 and 1.23, which is in good agreement with the results published for rat liver by Beaufay *et al.* (1964). In addition acid-insoluble radioactivity and Trypan Blue each show peaks of distribution similar to that of acid phosphatase. In the M+L fraction most of the protein content is associated with mitochondria and therefore the distribution of protein on the gradient can be used in place of cytochrome oxidase to follow the distribution of mitochondria. The protein forms a band at a density between 1.19 and 1.21 and is distinct from acid-insoluble radioactivity, acid phosphatase and Trypan Blue.

The second experiment was undertaken with animals pretreated with Triton WR-1339 4 days before receiving an injection of labelled albumin. The results of the subfraction of the M+L granules isolated from these animals show the expected shift of acid hydrolases (represented by acid phosphatase in Fig. 3) to an equilibrium density below 1.14. In the experiment most of the acid hydrolase activity was separated from the mitochondria, as indicated by the equilibrium density for protein on the gradient. In contrast the acid-insoluble radioactivity behaves differently and participates only to a very minor extent in the shift observed with the acid hydrolases. Comparison of the distribution of the labelled protein from Triton WR-1339-treated rats with that from the untreated animals (see Fig. 2) shows a broader distribution with a slight displacement of the peak to a density of around 1.21. In view of this shift and the complete separation from the majority of the acid hydrolases of the M+L fraction the possibility that

Fig. 2. Distribution patterns of acid-insoluble radioactivity, acid phosphatase, Trypan Blue and protein for combined M+L fraction of rat liver as established by isopycnic density-gradient centrifugation

Rats were injected with ^{125}I -labelled albumin (2.5 mg of protein/kg) 24 h after a single injection of Trypan Blue (75 mg/kg) and killed 0.5 h later. A sample (0.8 ml) of the combined M+L fraction was subfractionated in a linear sucrose gradient in a SW 50L rotor for 150 min at 4°C and 105000g by using the Beckman L2 ultracentrifuge (see the Materials and Methods section for full details). Fractions were collected by gravity flow and assayed for (a) acid-insoluble radioactivity, (b) acid phosphatase, (c) protein and (d) Trypan Blue. The method for representation of the results is described in the Materials and Methods section. When more than one experiment was performed diagrams show the average results and the number of experiments in parentheses.

the labelled protein was associated with the mitochondria and not enclosed within membranes was examined. To explore this possibility ^{125}I -labelled albumin was included in an M+L fraction isolated

Fig. 3. Influence of a single injection of Triton WR-1339 on the density-distribution patterns of a combined M+L pellet obtained from an animal injected with ^{125}I -labelled albumin

Rats were injected with ^{125}I -labelled albumin (2.5 mg/kg) 4 days after a single injection of Triton WR-1339 (850 mg/kg) and killed after 0.5 h. A sample of the combined M+L fraction of the liver was subfractionated on a linear sucrose gradient. The conditions of the centrifugation and the presentation of the results are the same as in Fig. 2. Fractions were assayed for (a) acid-insoluble radioactivity, (b) acid phosphatase and (c) protein. Numbers of experiments are given in parentheses.

from a rat pretreated with Triton WR-1339 but injected with 0.9% NaCl in place of ^{125}I -labelled albumin. Analysis of the gradient (Fig. 4) demonstrates that the acid-insoluble radioactivity did not sediment with the mitochondria. In this experiment the distribution patterns of protein and acid phosphatase were similar to those obtained when the animals received intravenous ^{125}I -labelled albumin (see Fig. 3).

Fig. 5 shows the results obtained from the M+L fraction isolated from rats pretreated with Triton WR-1339 and Trypan Blue and represents the third series of isopycnic gradient experiments. Treatment

Fig. 4. Density distribution of ^{125}I -labelled albumin incorporated into a prepared combined M+L fraction of rat liver

Rats were injected with 0.9% NaCl instead of labelled protein 4 days after receiving an injection of Triton WF-1339 (850mg/kg) and killed after 0.5h. ^{125}I -labelled albumin ($20\mu\text{l}$, containing $25\mu\text{g}$ of protein) was mixed with the combined M+L fraction (0.8ml), kept at 4°C for 30min and then subfractionated as described in Fig. 2. The fractions were assayed for (a) acid-insoluble radioactivity, (b) acid phosphatase and (c) protein. Results are for a typical experiment.

of the rats with Trypan Blue did not affect the distribution of protein or modify the sedimentation behaviour of the acid hydrolases or acid-insoluble radioactivity. The results illustrated in Fig. 5 indicate that the majority of the acid phosphatase and acid cathepsin activities exhibit a distinct shift towards a lower equilibrium density. Acid-insoluble radioactivity failed to show this displacement. The findings that Trypan Blue was distributed along the gradient with distinct peaks at between 1.21 and 1.23 and around 1.12 agree with the results of the discontinuous gradient recorded in Table 2.

Additional experiments were carried out to explore the effect of time on the distribution of acid-insoluble radioactivity within lysosomes of Triton WR-1339-treated animals. The results of these experiments are

Fig. 5. Density-distribution patterns of combined M+L fraction of the liver of rats injected with Triton WR-1339, Trypan Blue and ^{125}I -labelled albumin

Rats were injected with Triton WR-1339 (850mg/kg) and 3 days later with Trypan Blue (75mg/kg). Finally (24h after the Trypan Blue), they received an intravenous injection of labelled protein and were killed 0.5h later. A combined M+L fraction of liver was prepared and subfractionated as described in Fig. 2. The fractions were assayed for (a) acid-insoluble radioactivity, (b) acid phosphatase, (c) protein, (d) cathepsin D and (e) Trypan Blue. Numbers of experiments are given in parentheses.

Fig. 6. Density-distribution patterns of the combined M+L fraction of the liver of rats injected with Triton WR-1339 and ^{125}I -labelled albumin

Rats were injected with ^{125}I -labelled albumin (2.5 mg/kg) 4 days after injection of Triton WR-1339 (850 mg/kg) and killed after (a) 1.0 h and (b) 2.5 h and the fractions assayed for acid-insoluble radioactivity. The conditions of the centrifugation of the combined M+L fraction of liver and the method for the representation of the results is the same as described in Fig. 2.

shown in Fig. 6 and demonstrate that ^{125}I -labelled albumin does not accumulate within Triton WR-1339-filled lysosomes for a period of at least 2.5 h after intravenous injection of the labelled protein.

Effect of Triton WR-1339 on the intralysosomal digestion of denatured ^{125}I -labelled albumin

In view of the above results, which indicated that the labelled albumin and the majority of acid hydrolases (including cathepsin D) were located in different vacuoles, the effect of Triton WR-1339 on the digestive capacity of isolated rat liver lysosomes was studied by the method of Davies *et al.* (1971).

Suspensions of liver lysosomes isolated from control rats and Triton WR-1339-treated rats were incubated at 22°C in 'osmotically protected' (i.e., containing 0.25 M-sucrose) buffer, pH 7.4. In seven separate control incubations the percentage of the total radioactivity that was soluble in trichloroacetic acid increased by 16.45 (s.d. ± 3.51) over a 2 h incubation period. This is a measure of the digestive capacity of the isolated lysosomes.

During the same seven experiments the non-sedi-

mentable trichloroacetic acid-insoluble acid increased by 11.37% (s.d. ± 5.9). This represents the degree of lysosome rupture, since it is a measure of albumin originally found within lysosomes and that has become non-sedimentable.

With Triton WR-1339-treated animals in seven experiments the trichloroacetic acid-soluble radioactivity increased by 17.37% (s.d. ± 5.9) and the non-sedimentable radioactivity by 11.28% (s.d. ± 2.27).

These results show that pretreatment of the animals with Triton WR-1339 had little or no effect on either albumin digestion or lysosome breakage.

Discussion

The results in the present paper on the isopycnic centrifugation of a combined M+L fraction of liver isolated from normal rats show that Trypan Blue and ^{125}I -labelled albumin both have an equilibrium density of 1.22. In the same experiment the equilibrium density of mitochondria, as represented by protein, is 1.20, whereas the distribution of acid phosphatase shows that the equilibrium density of the lysosome population is 1.22. Thus since acid phosphatase, Trypan Blue and ^{125}I -labelled albumin can be differentiated from the mitochondria on the basis of equilibrium density, it is likely that the Trypan Blue and ^{125}I -labelled albumin are associated with lysosomes rather than mitochondria.

It has been reported by Wattiaux *et al.* (1963) that a single injection of Triton WR-1339 alters the equilibrium density of rat liver lysosomes in a sucrose gradient, which permits the separation of lysosomes from mitochondria and peroxisomes. To investigate further whether Trypan Blue and ^{125}I -labelled albumin are associated with lysosomes advantage was taken of this unique property of Triton WR-1339. In the isopycnic gradient experiments described in the present paper a single injection of Triton WR-1339 caused a distinct shift of acid phosphatase towards the lower densities of the sucrose gradient. Trypan Blue showed a bimodal distribution with one peak in the 1.12 region and a second at 1.21. However, what was particularly noteworthy was that the ^{125}I -labelled albumin, newly ingested by phagocytosis, did not accumulate in the Triton WR-1339-filled lysosomes to any significant amount, but was distributed on the gradient with a peak at a density of 1.21. The results of the isopycnic centrifugation of combined M+L fractions isolated from rats injected with Triton WR-1339 therefore indicate that two distinct populations of lysosomes are present. The first population, designated the light-lysosome fraction, contains Triton WR-1339 and most of the acid hydrolases present in the M+L fraction and is characterized by an equilibrium density of about 1.12. The second population contains the ^{125}I -labelled albumin, newly ingested by phagocytosis, and acid

hydrolases but no Triton WR-1339 and is characterized by an equilibrium density of 1.21. The second population has been designated the heavy lysosome fraction and presumably corresponds to the lysosome fraction described in the experiments carried out with rats injected with Trypan Blue but not Triton WR-1339. ^{125}I -labelled albumin is found in the heavy lysosome fraction irrespective of whether Trypan Blue has been given. Thus the possibility that Trypan Blue in some way prevents the uptake of labelled protein into light lysosomes can be ruled out.

The conclusion that ^{125}I -labelled albumin located in the heavy lysosome fraction is associated with lysosomes and does not represent endocytic vacuoles devoid of hydrolytic enzymes is borne out by the results of the incubation experiments carried out with M+L fractions isolated from rats treated with Triton WR-1339 and injected with labelled albumin. During the incubation proteolytic digestion of the ^{125}I -labelled albumin, ingested by phagocytosis, took place and its progress was monitored by measuring the release of acid-soluble radioactivity (see the Results section). This finding can be explained only if the labelled albumin was concentrated within secondary lysosomes (see Mego & McQueen, 1965; Mego *et al.*, 1967; Davies *et al.*, 1971).

The present investigation has provided evidence that after intravenous injection into Triton WR-1339-treated rats, ^{125}I -labelled albumin is found in secondary lysosomes (digestive vacuoles), probably as a result of fusion of endocytic vacuoles containing the labelled albumin with vesicles containing acid hydrolases. The evidence from the sucrose-gradient experiments indicates that the light lysosomes do not contribute to the enzyme content of the digestive vacuoles. If they do it is to a minor extent and their contribution is such that the equilibrium density of the digestion vacuoles are unaltered on a sucrose gradient. Thus it appears that the ^{125}I -labelled albumin newly ingested by phagocytosis does not fuse with Triton WR-1339-filled lysosomes but only with primary lysosomes. It has been suggested that membranes of primary lysosomes fuse with newly formed vacuoles but only rarely with older vacuoles (Dingle, 1968; de Duve, 1969). The present findings are consistent with this idea.

Gradient experiments similar to those described in the present paper but with different markers have been carried out by several workers. Extensive investigations have been made by Jacques (1968) on liver M+L fractions of rats injected with horseradish peroxidase. This enzyme, which can be shown by biochemical and histochemical techniques to be present in liver lysosomes, does not accumulate in Triton WR-1339-filled lysosomes. On the other hand, sucrose and dextran (Wattiaux, 1966), desialylated caeruloplasmin (Gregoriadis *et al.*, 1970), and invertase-containing liposomes (Gregoriadis & Ryman,

1972) all accumulate in the light lysosomes of Triton WR-1339-treated rats. Further, it must be recalled that Trypan Blue accumulates in light lysosomes (see also Lloyd *et al.*, 1968).

Finally, it remains to discuss the mechanism that governs the uptake of exogenous materials into Triton WR-1339-filled lysosomes. It is noteworthy that sucrose, dextran, and Trypan Blue, all of which accumulate in light lysosomes, are resistant to attack by lysosomal enzymes. On the other hand, ^{125}I -labelled albumin and horseradish peroxidase are both susceptible to lysosomal cathepsins. It is therefore possible that only exogenous material slowly digested or resistant to enzymic attack by lysosome acid hydrolases concentrates in Triton WR-1339-filled lysosomes. Although there are obvious objections in drawing conclusions from limited experimental results, it is a reasonable supposition that Triton WR-1339-filled lysosomes represent inert residual bodies and do not participate in one of the main functions of lysosomes; namely the digestion of protein taken into the cell by phagocytosis.

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References

- Anson, M. L. (1937) *J. Gen. Physiol.* **20**, 565–574
- Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964) *Biochem. J.* **92**, 184–205
- Davies, M. (1973) *Biochem. Soc. Trans.* **1**, 202–203
- Davies, M., Lloyd, J. B. & Beck, F. (1971) *Biochem. J.* **121**, 21–26
- de Duve, C. (1969) in *Lysosomes in Biology and Pathology*, (Dingle, J. T. & Fell, H. B., eds.), part 1, pp. 3–40, North-Holland Publishing Co., Amsterdam
- Dingle, J. T. (1968) *Brit. Med. Bull.* **24**, 141–145
- Ennis, R. S., Granda, J. L. & Posner, A. S. (1968) *Arthritis Rheum.* **11**, 756–764
- Gregoriadis, G. & Ryman, B. E. (1972) *Biochem. J.* **129**, 123–133
- Gregoriadis, G., Morrell, A. G., Sternlieb, I. & Scheinberg, I. H. (1970) *J. Biol. Chem.* **245**, 5833–5837
- Griffiths, A., Lloyd, J. B. & Beck, F. (1966) *Biochem. J.* **100**, 45P–46P
- Jacques, P. (1968) Thesis, University of Louvain
- Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S. & de Duve, C. (1968) *J. Cell Biol.* **37**, 482–513
- Lloyd, J. B. & Beck, F. (1966) *J. Embryol. Exp. Morphol.* **16**, 29–39
- Lloyd, J. B., Beck, F., Griffiths, A. & Parry, L. M. (1968) in *Interaction of Drugs and Subcellular Components in Animal Cells* (Campbell, P. N., ed.), pp. 171–202, J. and A. Churchill Ltd., London
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mego, J. L. & McQueen, J. D. (1965) *Biochim. Biophys. Acta* **100**, 136–143

- Mego, J. L., Bertini, F. & McQueen, J. D. (1967) *J. Cell Biol.* **32**, 699–707
- Neil, M. W. & Horner, M. W. (1964) *Biochem. J.* **92**, 217–224
- Norton, W. L., Lewis, D. C. & Ziff, M. (1968) *Arthritis Rheum.* **11**, 436–443
- Persellin, R. H. & Ziff, M. (1966) *Arthritis Rheum.* **9**, 57–65
- Smeesters, C. & Jacques, P. J. (1968) *Excerpta Med. Found. Int. Congr. Ser.* **166**, 82
- Torriani, A. (1960) *Biachim. Biophys. Acta* **38**, 460–469
- Wattiaux, R. (1966) Thesis, University of Louvain
- Wattiaux, R., Wibo, M. & Baudhuin, P. (1963) *Lysosomes, Ciba Found. Symp.* 176–195
- Wills, E. D. (1952) *Biochem. J.* **50**, 421–425