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MORPHOLOGICAL OBSERVATIONS ON THE CELLULAR AND SUBCELLULAR DESTINATION OF INTRAVENOUSLY ADMINISTERED LIPOSOMES

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Summary.—The cellular and subcellular fate of intravenously administered liposomes was traced by light and electron microscopy, using nitroblue tetrazolium (NBT) as a marker. Liposomes were found almost exclusively within phagocytic vacuoles of cells of the reticuloendothelial system. Fusion of lysosomes with phagocytic vacuoles and liposomal degradation were clearly visible. Liposomes may find wide application in the study and treatment of metabolic, infectious and neoplastic disease of phagocytic cells.

CONVENTIONAL systemic drug therapy is generally nonspecific in application. Although the action of the agent is usually dependent upon a qualitative or quantitative metabolic peculiarity of the target cell or organism, normal cells are also frequently adversely affected. In addition, the drug is itself vulnerable to metabolic modification and immunological reactions before reaching its site of action. A carrier system capable of isolating therapeutic agents and of directing them to specific target cells would therefore be of advantage.

Liposomes, lipid spherules consisting of a closed system of concentric lipid bilayers alternating with aqueous compartments (Bangham, 1968) have been proposed as a vehicle for the transport of enzymes (Gregoriadis, Leathwood and Ryman, 1971) and drugs (Gregoriadis, 1973) in the treatment of inherited enzyme deficiencies and other disorders in man. However, the use of such a form of treatment must depend upon the specific localization of the liposome entrapped material after intravenous administration. Although there is some evidence that substances within liposomes become associated with the lysosomes of the liver and spleen of experimental animals (Gregoriadis and Ryman, 1972*a*,*b*) their precise cellular destination and the fate of the carrier liposomes have not been ascertained.

To answer these questions, the fate of intravenously administered liposomes was investigated by light and electron microscopy. Nitroblue tetrazolium (NBT) was selected as the liposomal marker because reduction, either spontaneously within cells or artificially *in vitro*, converts it from a pale yellow water soluble compound to a dark blue insoluble diformazan, visible by light microscopy and under certain circumstances by electron microscopy, which does not diffuse from the site of reduction (Nachlas *et al.*, 1957).

MATERIALS AND METHODS

Entrapment of dye within liposomes.—The sources and grades of egg lecithin, cholesterol and phosphatidic acid used in the preparation of liposomes have been described elsewhere (Gregoriadis and Ryman, 1972*b*). NBT was purchased from Sigma Chemical Co., reduced nicotinamide adenine dinucleotide (NADH) from Boehringer, and phenazine methosulphate from British Drug Houses.

For the preparation of NBT containing liposomes, egg lecithin, cholesterol and phosphatidic acid (molar ratio 7 : 2 : 1) (Gregoriadis and Ryman, 1972*b*) were dissolved in $CHCl_3$ and after rotary evaporation under vacuum the thin lipid layer obtained was dispersed with 2 ml of an aqueous NBT solution (2 and 10 mg/ml). The liposomal suspension was allowed to stand at room temperature for 3–5 hours, sonicated (Gregoriadis *et al.*, 1971) and then centrifuged at 100,000 *g* for 30 min. The liposomal pellet was washed with and suspended in 1% NaCl. Entrapped NBT was measured spectrophotometrically after reduction to diformazan and extraction with pyridine. To 0·1 ml of the suspension was added 0·9 ml of a 5 mmol/l aqueous solution of NADH and 0·1 ml of 75 mmol/l aqueous solution of phenazine methosulphate. The mixture was sonicated as above for 15 sec, mixed with 9·0 ml of pyridine and measured at 515 nm against a mixture of water and pyridine (1 : 9 v/v). The concentration of diformazan was computed from a standard curve constructed with known amounts of NBT reduced to diformazan by an identical procedure.

Liposomes containing diformazan were prepared by first entrapping an aqueous solution of NBT (2 mg/ml) and NADH (10 mmol/l) as described above. Three hours after sonication, reduction of the entrapped and non-entrapped NBT was promoted by the addition of 0·1 ml of a 10 mmol/l aqueous solution of phenazine methosulphate. Liposome entrapped diformazan was separated from free diformazan by molecular sieve chromatography on a Sepharose 6B (Pharmacia) column (1 × 25 cm). The concentration of entrapped diformazan was measured spectrophotometrically after extraction as described above. To ensure that the diformazan was associated with liposomes and not with "free" lipids that did not participate in the formation of liposomes, $Na^{131}I$ was coentrapped with diformazan and a comparison made between the radioactivity and diformazan concentration in the fractions eluted from the column.

Diformazan containing liposomes and liposomes alone were applied to carbon films (Valentine, Shapiro and Stadtman, 1968) and examined in the electron microscope unstained and negatively stained with a 2% aqueous solution of sodium silicotungstate.

Animal experiments.—Male albino rats (Wistar) weighing 100–120 *g* were injected *via* the tail vein with 1·0 ml of the following solutions: (a) liposome entrapped NBT (1·0 or 5·0 mg NBT and 40·0 mg lipids) suspended in 1% NaCl; (b) 1·0 or 5·0 mg NBT in 0·36 mol/l dextrose. A single rat was injected with a suspension of liposomes (40·0 mg lipid) and non-entrapped NBT (5·0 mg/ml) in 0·36 mol/l dextrose; (c) liposome entrapped diformazan (1·0 mg diformazan and 40·0 mg lipids); (d) a mixture of 0·9% NaCl and rat serum containing 1·25 mg diformazan prepared as follows: 1·0 ml of an aqueous solution of NBT (2·5 mg/ml) was mixed with 0·9 ml serum containing NADH (10 mmol/l final concentration). NBT was subsequently reduced by the addition of 0·1 ml of 0·15 mmol/l phenazine methosulphate. The preparation was then dialysed overnight against 2 l of 0·9% NaCl.

The animals were decapitated at time intervals up to 20 hours and specimens of brain, myocardium, lung, liver, kidney, pancreas, iliac lymph node, small and large intestine and spleen were fixed in 10% formaldehyde for 24 hours. Paraffin embedded sections were examined by light microscopy unstained and after staining with a saturated solution of tartrazine (Raymond Lamb) in 2-ethoxyethanol (Hopkins and Williams). Kidney sections were stained with the PAS technique (Ham and Leeson, 1961). Smears of blood and bone marrow were also examined unstained and after staining with Leishman's stain (British Drug Houses).

Samples of spleen, liver and kidney from a rat injected with NBT containing liposomes (1·0 mg NBT) and killed 2 hours later, and from an untreated rat were fixed in 3% glutaraldehyde (Sabatini, Bensch and Barrnett, 1963) in 0·2 mol/l sodium cacodylate buffer, pH 7·4. Trimmed blocks of approximately 1 mm^3 were washed overnight in 0·1 mol/l sodium cacodylate, pH 7·4, containing 0·25 mol/l sucrose, post-fixed for 2 hours in phosphate buffered 1% osmium tetroxide (Millonig, 1961), dehydrated in a graded series of ethanol solutions and embedded in epoxy resin (Luft, 1961). Sections 1 μm thick were cut on a Sorvall MT-2

TABLE.—*The Light Microscopic Localization of Diformazan at Various Times after the Intravenous Injection of NBT in the Oxidized and Reduced Forms, Associated and Unassociated with Liposomes, into Rats*

Group	Preparation administered	Dose: 5 mg Number of animals	Dose: 1 mg Number of animals	Time (h)	Spleen	Site	Liver	Site	Kidney	Site	Bone marrow	Site
(a)	NBT in liposomes	1	1	0·5	0		0		+		0	
		1	1	1	0		0		+ +	Distal	0	
		1	1	3	+	Macrophages	0	Kupffer cells	+ +	convoluted	+	Macrophages
			1	20	+ + +	(Neutrophils)	+	(Neutrophils)	+	tubules	+	(Neutrophils)
(b)	Free NBT	6		0				Immediate death				
			1	0·25	0		0		+ +	Distal convoluted	0	
			1	20	+	Macrophages	+	Hepatocytes	+	tubules	0	
(c)	Diformazan in liposomes		2	1	+ + +	Macrophages (Neutrophils)	+ +	Kupffer cells (Neutrophils)	0		+	Macrophages (Neutrophils)
(d)	Free diformazan		1	1	0		0		0		0	

ultramicrotome, mounted on glass slides and examined unstained by phase contrast microscopy. Ultrathin sections were examined unstained, and after contrast staining with alcoholic uranyl acetate (Watson, 1958) followed by lead citrate (Reynolds, 1963), in an AEI EM 6B electron microscope.

RESULTS

All 6 rats injected with 5·0 mg of NBT either alone or together with, but unentrapped in, liposomes (Table, Group b) convulsed and died almost immediately. No other rats showed any side-effects after injection.

Liposome entrapped NBT

Light microscopy.—Typical punctate, dark blue deposits of diformazan were found in the spleen, liver, kidney and bone marrow. Very occasionally diformazan was seen in circulating neutrophils and monocytes (Table, Group a). The intensity of staining was independent of the amount of NBT administered.

Splenic deposits, which were always confined to the red pulp, first became visible 3 hours after injection and were very dense by 20 hours (Fig. 1) 1 μm thick epoxy resin embedded sections examined unstained by phase contrast microscopy (Fig. 2) demonstrated that the dye crystals were present within phagocytic vacuoles of the sinusoidal lining cells and occasional neutrophils.

Deposits were visible only in the liver after 20 hours where they showed the same distribution in Küpffer cells as diformazan containing liposomes (Fig. 3). No staining of parenchymal cells was observed.

Diformazan reaction products were present in renal tubular epithelium, predominantly the distal tubules, as early as 30 minutes after injection (Fig. 4). Initially the deposits were localized to the basal region but after 20 hours they were scattered throughout the cell: phase contrast microscopy of 1 μm unstained sections showed their localization within small vacuoles (Fig. 5).

Scanty diformazan was seen in macrophages and, less commonly, in neutrophils of the bone marrow from 3 hours onwards.

Electron microscopy.—Specimens examined by electron microscopy 2 hours after injection showed abundant liposomes in phagocytic vacuoles of varying size within splenic sinusoidal lining cells (Fig. 6), liver Küpffer cells and occasionally in renal tubular and glomerular mesangial cells. In most instances the liposomes showed considerable condensation and shrinkage (Fig. 6), but in favourable circumstances typical concentric lamellae could be seen (Fig. 7) similar to those of the negatively stained liposomes (Fig. 8). Dense body lysosomes were frequently seen in close proximity to, or actually fused with, the liposome containing vacuoles (Fig. 6, 7). Unstained sections from the same blocks revealed diformazan crystals within the phagocytic vacuoles (Fig. 9), but these were largely obscured after contrast staining with uranyl acetate and alkaline lead, either separately or in combination, due to the intense staining of liposomes within the same vacuoles (Fig. 6). Lipsomes were also observed within vacuoles in neutrophils in the spleen and liver (Fig. 10). There was no evidence of liposomes or diformazan crystals within the parenchymal cells of the liver in stained or unstained sections.

Nothing resembling either the diformazan or liposomes was seen in the tissues from the control animal.

Free NBT

As early as 15 minutes after the administration of free NBT, abundant deposits of diformazan were observed in the basal region of cells of the distal convoluted tubules of the kidney (Table, Group b). Such deposits appeared identical to those observed following the injection of liposome entrapped NBT, and showed similar dispersion throughout the cells by 20 hours. In addition, very occasional intravacuolar needle-shaped crystals were found within hepatocytes (Fig. 11) and splenic macrophages.

Liposome entrapped diformazan

The close association between the radioactivity and the absorbance at 515 nm after column chromatography of the mixture of liposomes, diformazan and $Na^{131}I$ indicated co-entrapment of ^{131}I and diformazan within the liposomes (Fig. 12).

Uptake of lipsome entrapped diformazan was observed in splenic and bone marrow macrophages and in Küpffer cells of liver 1 hour after injection (Table, Group c). The distribution and density of the deposits resembled that seen 20 hours after the injection of liposome entrapped NBT, except that the kidney was not stained. Occasional diformazan deposits were present within neutrophils in the bone marrow, spleen and liver and, very rarely, in peripheral blood neutrophils and monocytes.

No dye crystals could be seen when free liposomes with entrapped diformazan were examined unstained by electron microscopy. Control liposomes and those containing diformazan both showed the typical lamellated structure described by

EXPLANATION OF PLATES

FIG. 1.—Spleen 20 hours after the injection of liposome entrapped NBT showing diformazan deposits in red pulp (rp) but not white pulp (wp). Tartrazine × 280.

FIG. 2.—Spleen 2 hours after injection of liposome entrapped NBT showing intravacuolar diformazan within sinusoidal lining cells (arrows). Unstained 1 μm epoxy section, phase contrast × 2,400.

FIG. 3.—Liver 20 hours after injection of liposome entrapped diformazan. Intense punctate diformazan deposits are present within Küpffer cells. Tartrazine × 800. Insert × 1,950.

FIG. 4.—Kidney 1 hour after the injection of liposome entrapped NBT showing diformazan deposits in the basal region of the distal tubules. Tartrazine × 380.

FIG. 5.—Kidney 2 hours after the injection of liposome entrapped NBT. Intravacuolar diformazan crystals are visible in tubular epithelium. Unstained 1 μm epoxy section, phase contrast × 2,000.

FIG. 6.—Spleen 2 hours after injection of liposome entrapped NBT. Phagocytic vacuoles within a macrophage contain ingested liposomes. Numerous lysosomes can be seen in close proximity to or fused with (arrows) the vacuole membranes. Uranyl acetate and lead citrate × 18,000.

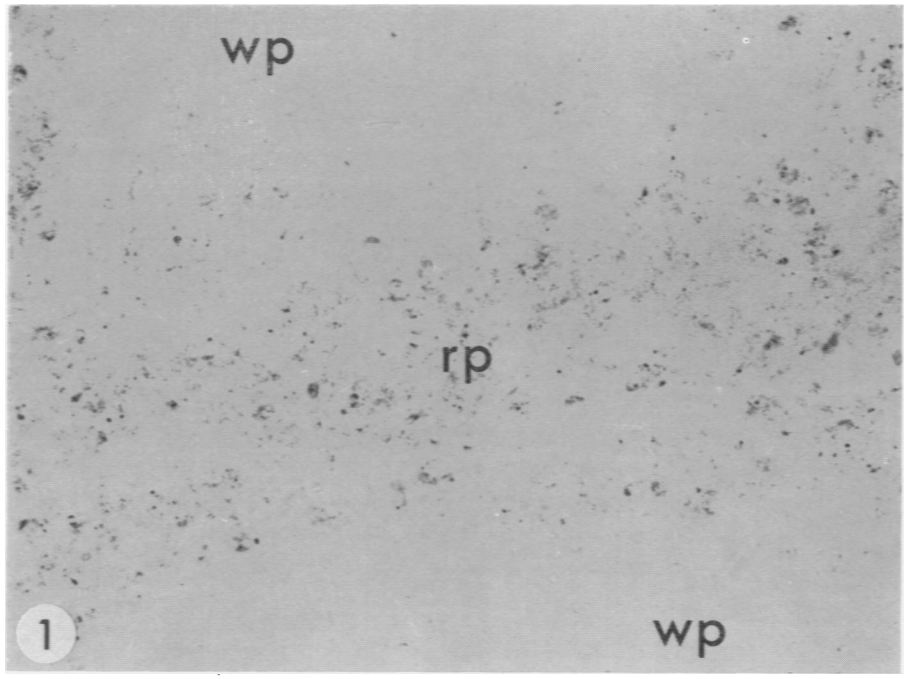
FIG. 7.—Spleen 2 hours after injection of liposome entrapped NBT. Detail of phagocytic vacuole showing the concentric lamellar appearance (arrows) of ingested liposomes. A lysosome (l) can be seen adjacent to the vacuolar membrane. Uranyl acetate and lead citrate × 136,000.

FIG. 8.—Liposome containing diformazan. Sodium silicotungstate × 136,000.

FIG. 9.—Spleen 2 hours after injection of liposome entrapped NBT. Electron opaque diformazan deposits (arrows) are present within phagocytic vacuoles of a macrophage. Unstained ultrathin section × 17,000.

FIG. 10.—Liver 2 hours after injection of liposome entrapped NBT. Liposomes can be seen within phagocytic vacuoles of a neutrophil within the lumen of a sinusoid. Uranyl acetate and lead citrate × 12,000.

FIG. 11.—Liver 20 hours after the injection of free NBT showing an intravacuolar diformazan crystal within a parenchymal cell. Tartrazine × 2,850.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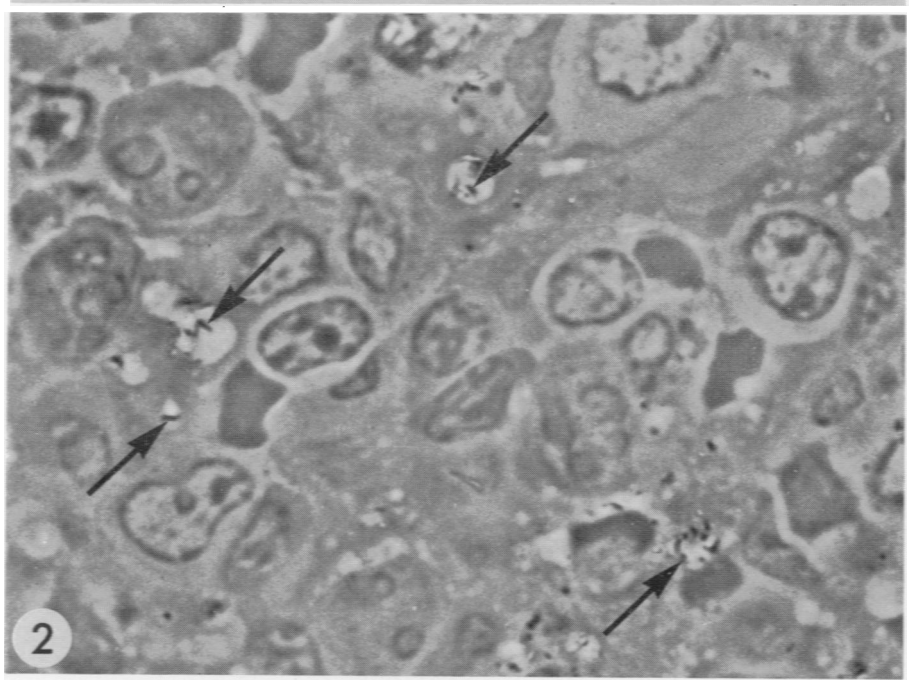
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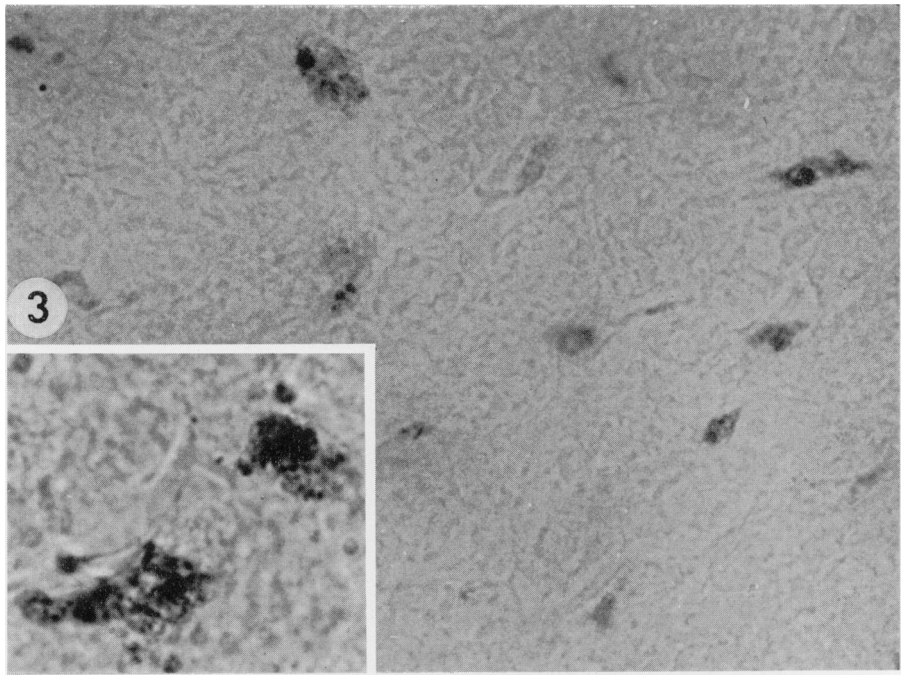
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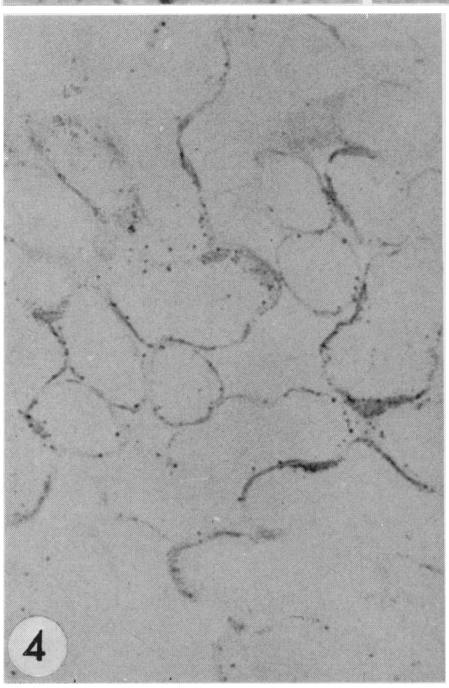
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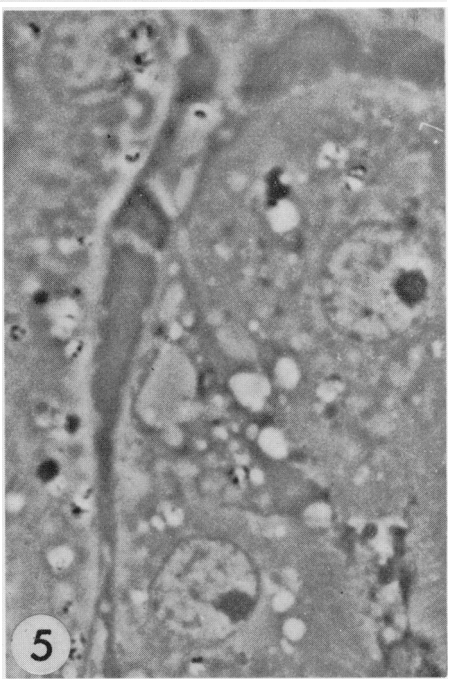
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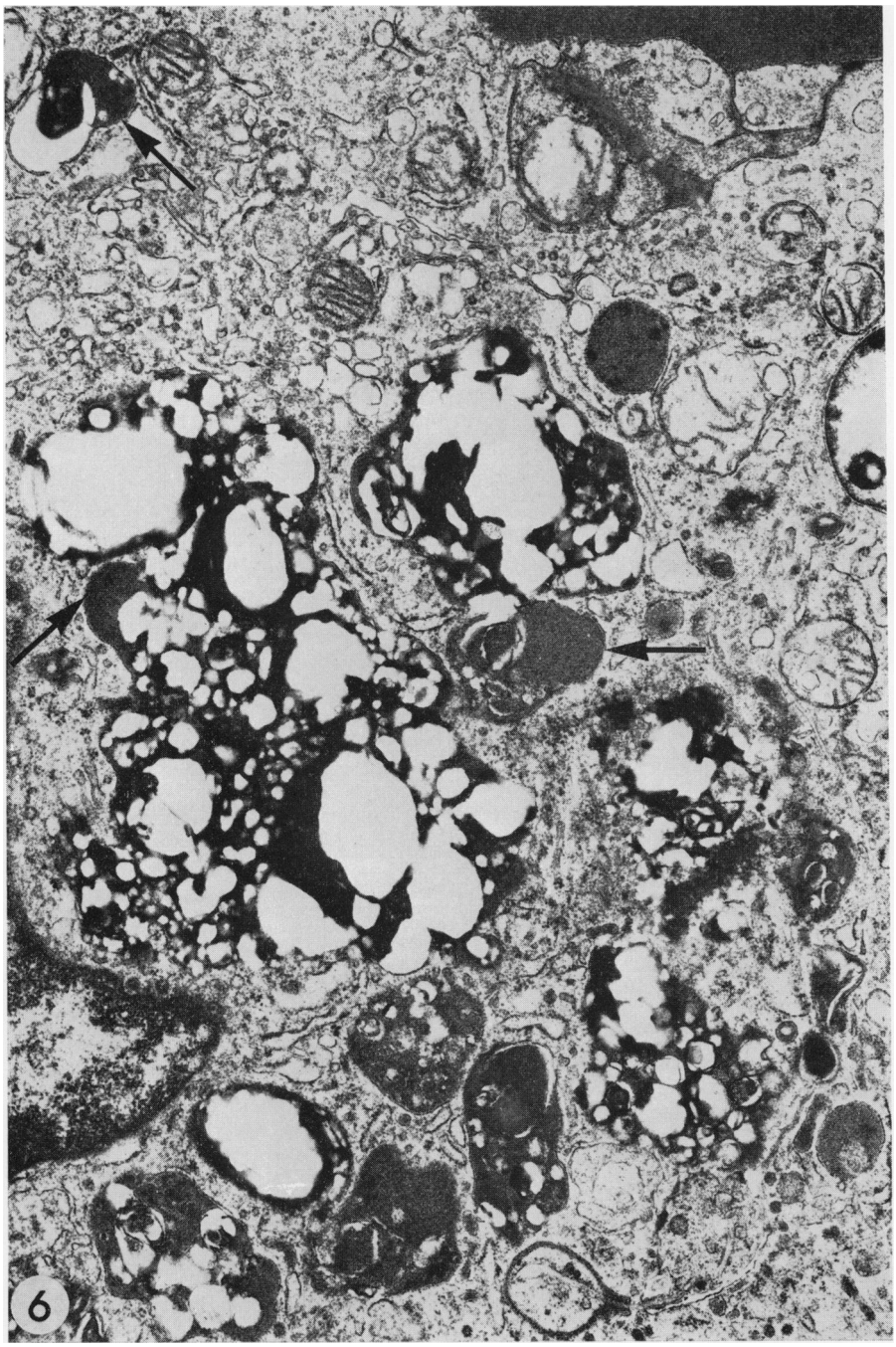
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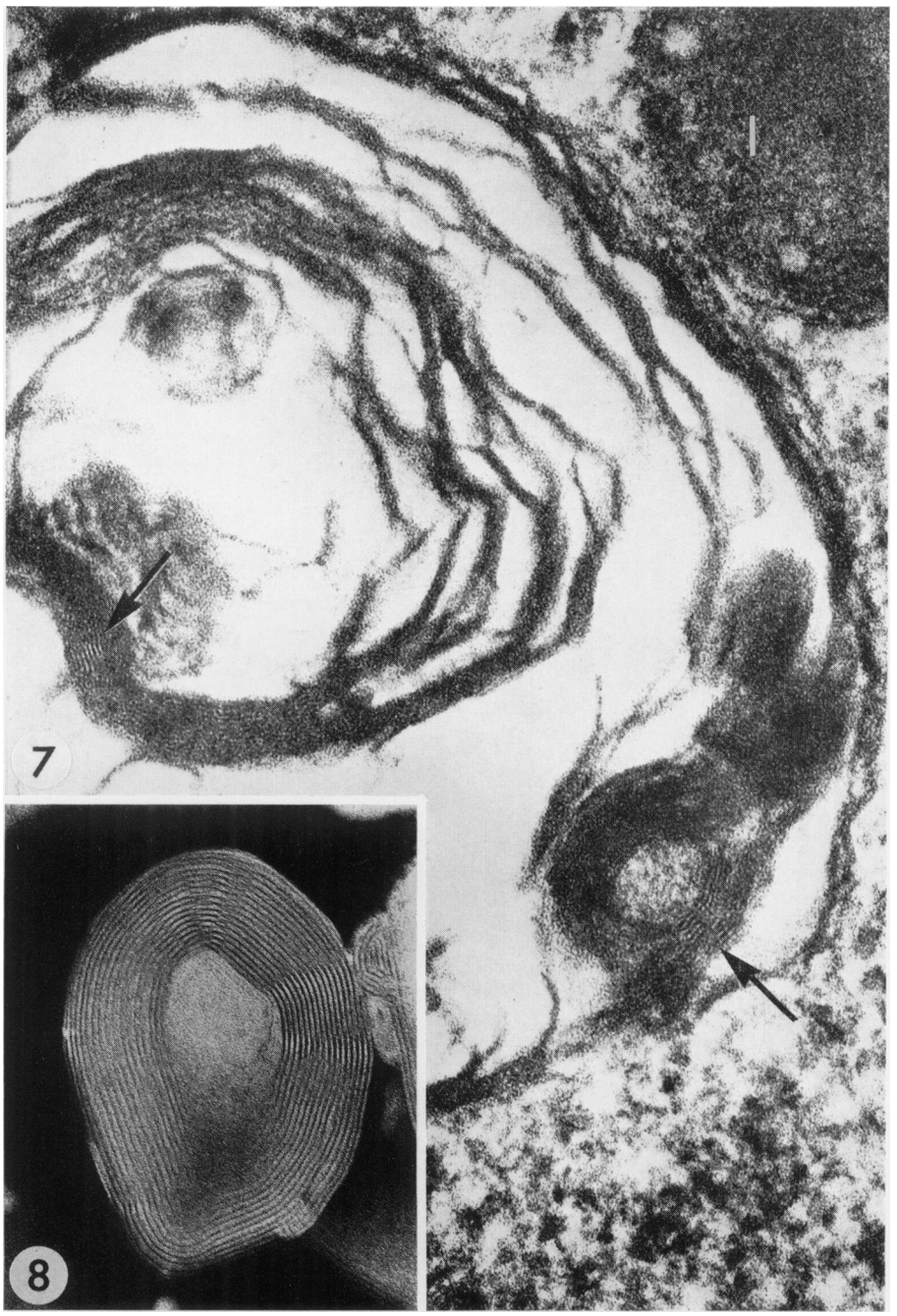
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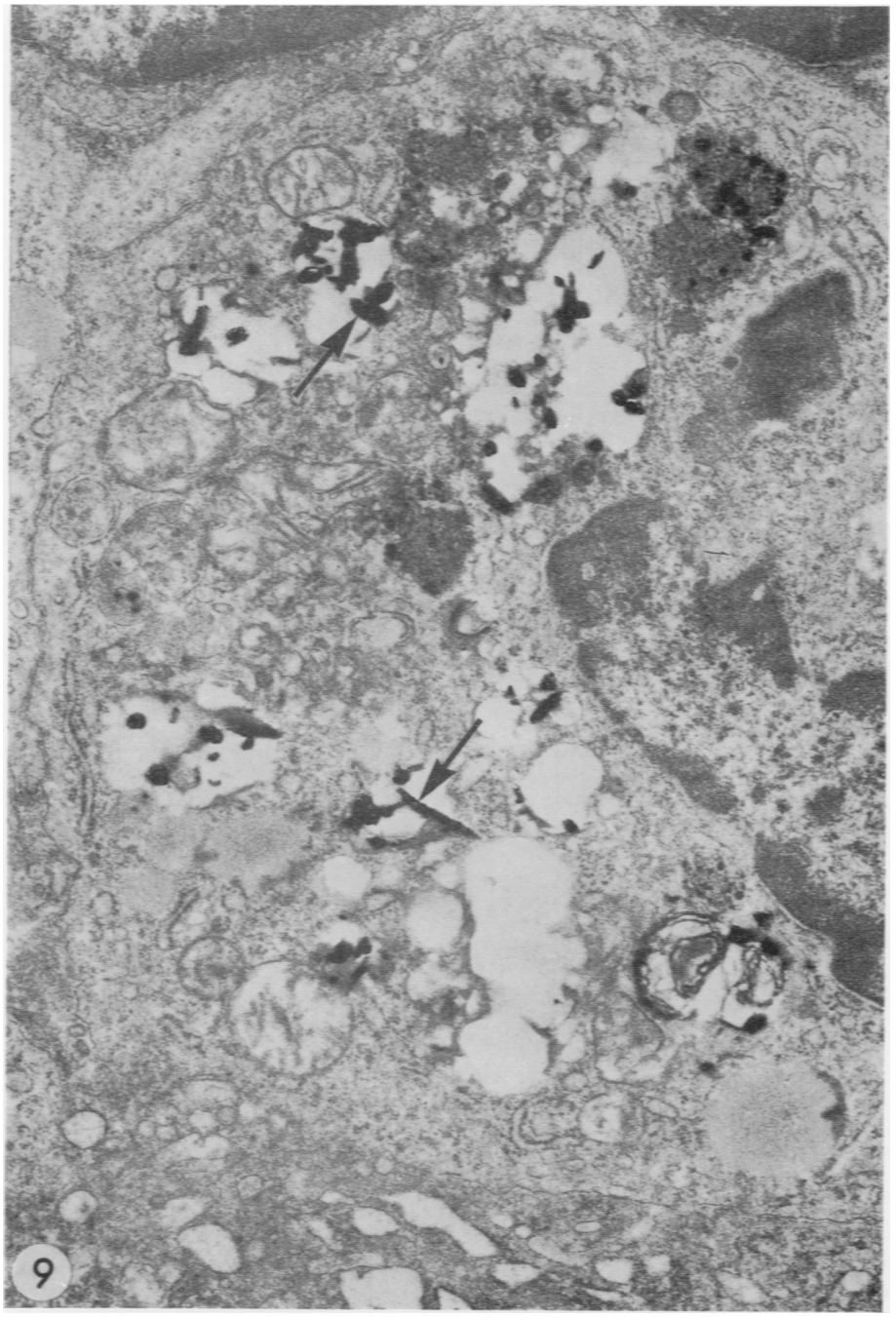
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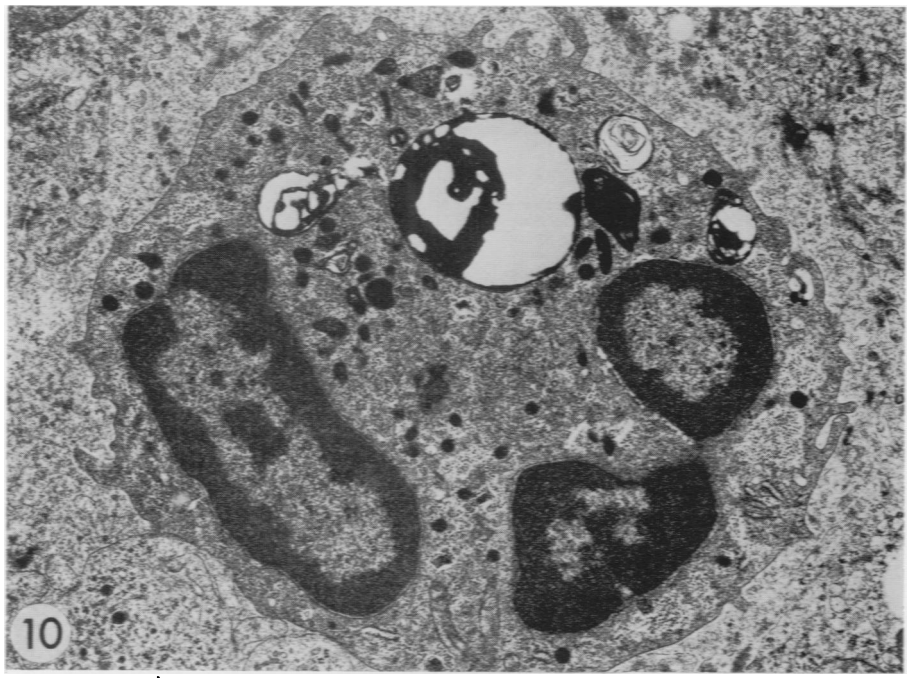

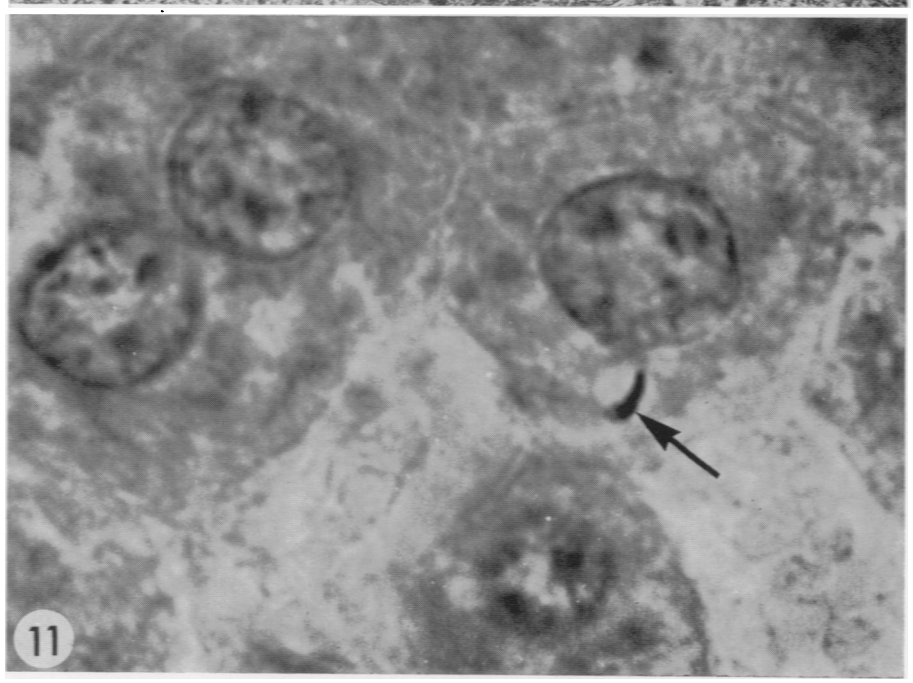

Segal, Wills, Richmond, Slavin, Black and Gregoriadis

others (Bangham, 1968) after negative staining with sodium silicotungstate (Fig. 8).

Non-entrapped diformazan

No staining was observed in any of the tissues of the rat injected with non-entrapped diformazan (Table, Group d).

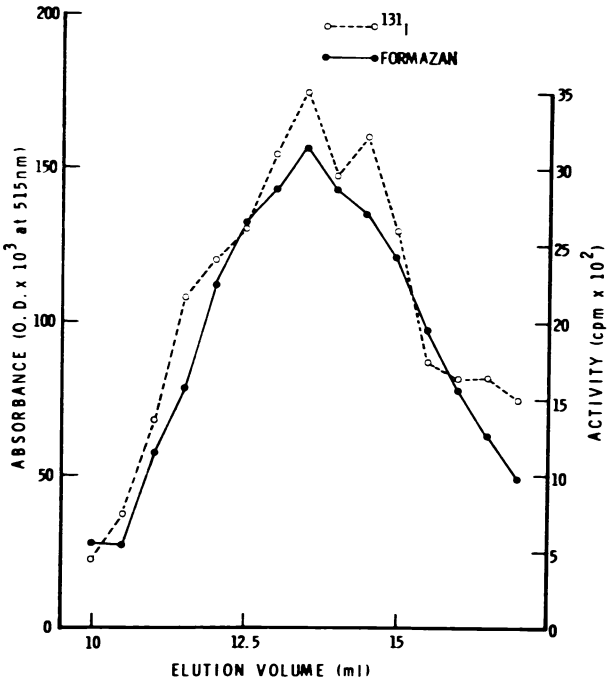

FIG. 12.—Molecular sieve chromatography of liposomes containing diformazan and $Na^{131}I$.

DISCUSSION

NBT has proved to be a useful liposomal marker and when administered entrapped in liposomes, diformazan deposits were observed within cells of the reticuloendothelial system and in neutrophils (Fig. 1–7, 9, 10). The localization of diformazan could be correlated with the presence of liposomes within phagocytic vacuoles by the combined use of light, phase contrast and electron microscopy.

A delay was observed before the reduction of liposome entrapped NBT. Since macrophages and neutrophils reduce dye very rapidly after endocytosis (Nathan, Baehner and Weaver, 1969), the lag period is probably a measure of the rate of liposomal degradation within the cell. When diformazan is present within free liposomes it is electron lucent and thus must be released from the liposome, probably in the oxidized form, to allow crystal formation. Structural disorganization of ingested liposomes was apparent by electron microscopy (Fig. 6). Although partially attributable to processing artefacts, much of the degradation probably results from the action of lipases released from lysosomes fused with liposome containing vacuoles (Fig. 6).

The distribution of diformazan deposits following the injection of liposomes containing NBT was somewhat of a hybrid between the uptake of liposome entrapped diformazan by phagocytic cells and of free NBT by renal tubular cells, possibly by pinocytosis. This probably resulted from the administration of a mixture of NBT within liposomes, and free NBT, sequestered between the liposomes during centrifugation and released on resuspension and intravenous injection. Minimal uptake of liposomes by the kidney has also been observed in rats injected with ^{131}I-albumin containing liposomes (Gregoriadis and Ryman, 1972*a*).

Diformazan is insoluble in water but it remains in solution in serum bound to lipoproteins (Segal, Putman and Minchin-Clarke, 1973). Its continued circulation in this form after administration without liposomes could account for the absence of focal tissue deposition.

The present study showed that hepatic uptake of liposomes was confined to the Küpffer cells. This is in conflict with a previous report in which autoradiographic evidence from rat liver preparations 3 minutes after the injection of liposomes containing cholesterol-^{3}H in their structure suggested that they were present in both Küpffer cells and parenchymal cells (Gregoriadis and Ryman, 1972*a*). Although no exchange of cholesterol-^{3}H was observed with blood or cell surfaces (Gregoriadis and Ryman, 1972*a*), it is possible that in this experiment an interchange of cholesterol-^{3}H occurred between liposomes and hepatocytes.

Liposomes have been proposed as carriers of enzymes or drugs in the treatment of inherited enzyme deficiencies and other disorders in man (Gregoriadis and Buckland, 1973). The findings of this study suggest that liposomes might be of use in the investigation and treatment of diseases involving phagocytic cells. Moreover, the immediate and striking protective action afforded by liposomes was evident from the survival of animals injected with an otherwise lethal dose of NBT.

The potential of so highly selective an approach to therapy is great.

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