emptied and washed, and the 24h efflux of inulin was determined; it is plotted against
the final swelling ratio from the experiment in which the sac was stretched. An unstretched
control is also included. In a similar unstretched sac, the half-time of raffinose escape was
140 min. Clearly inulin escapes faster than the polyethylene glycol of comparable mol-
ecular weight at all porosities, but at least 10 times slower than raffinose.
In further experiments inulin and polyethylene glycol were dialysed together. The
results confirmed that inulin efflux significantly exceeds polyethylene glycol efflux, and
that the latter is concentration dependent. However, they showed much less effect of sac
stretching on inulin efflux, indicating an interaction between inulin and polyethylene
glycol efflux. In the shortest of these experiments (24h) the mean ratio of inulin to poly-
ethylene glycol efflux was 6.4; the ratio of initial escape rates might therefore be much
greater.
In conclusion, the escape rate of inulin is at least six times that of polyethylene glycol
6000 at all the porosities studied. For comparison with this value, it can be estimated
from Figs. 1 and 2 that, under comparable conditions, the efflux of polyethylene glycol
4000 is about three times that of polyethylene glycol 6000, and inulin efflux is about
twice that of polyethylene glycol 4000. The elution of 95% of the inulin in 1.3 times the
void volume of Sephadex G-25 indicates a mean molecular weight of at least 5500 (cf.
Phelps, 1965). It is therefore apparent from the results that polyethylene glycols do not
pass in an appreciably extended configuration through membranes of porosity compar-
able with the renal glomerular membrane. They also show that inulin passes through a
cellophane membrane much more rapidly than would be expected from its molecular
volume of rotation, but at 0.1 or less of the rate for raffinose, which can be considered as a
sphere of diameter equal to the thickness of inulin.

Most of the work was done at the Biophysical Laboratory, Harvard Medical School, Boston,
Mass., U.S.A., under the tenure of a special Post-doctoral Fellowship (GM 34261) from the
U.S. Public Health Service.

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Phelps, C. F. (1965) Biochem. J. 95, 41-47

Centrifugal and Activation Methods for rapid Identification of
Lysosomotropic Drugs with the Use of Rat Liver Homogenates

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The successful development of lysosomology as applied to the therapy of cellular dis-
orders (Jacques, 1968, 1972) requires the design of simple quantitative methods allowing
systematic screening of drugs on the basis of their ability to rejoin specific intracellular
target compartments, e.g. exoplasmic vacuoles (Jacques, 1973). Two such approaches
belonging to biochemical cytology can presently be applied directly to the whole liver
homogenate, in order accurately to localize exogenous material (e.g. drugs) within cells:
analytical centrifugations and differential activation (Huybrechts-Godin & Jacques,
1974) of the various organelles.

When applied to the intracellular localization of exogenous material, the standard
analytical centrifugation techniques are not only exceedingly laborious, even in the most
favourable instances, but they also only provide part of the useful information (Jacques,
1968, 1974). In the peculiar case of lysosomotropic drugs selection, the most efficient
centrifugal method requires additional treatment of the animal by means of another
exogenous compound whose lysosomotropism had been previously established. Ancil-
lary compounds (e.g. Dextran, Triton WR-1339 or its macrocyclic analogues) must be
able to concentrate specifically within lysosomes and to reach therein sufficient concentrations to change their equilibrium density and thus allow their separation in appreciable amounts from all the other organelles, in a density gradient.

Table 1 reports the relative concentration of two primary drugs (suramin and amiodarone) and of a lysosomal marker enzyme (acid β-glycerophosphatase) in purified lysosomal fractions from discontinuous sucrose–water gradients which contained none of these substances when parallel treatment with the ancillary lysosomotropic compound had been omitted. Since these fractions were devoid of mitochondrial, microsomal, peroxisomal and nuclear organelles, it is tempting to conclude that nearly one-tenth of the intracellular suramin or amiodarone is located within vacuoles containing the ancillary compound and that some of these vacuoles, at least, are secondary lysosomes; however, no indication is provided as to the localization of the other nine-tenths of the primary drug.

### Table 1. Relative concentration of drugs and acid phosphatase in highly purified lysosomal fractions obtained in discontinuous sucrose–water gradients after parallel treatment of rats with 'lysosomotropic' ancillary compounds

Whole homogenate or combined mitochondrial fractions were submitted to gradient fractionation respectively in suramin and amiodarone experiments. Density limits of fractions: \( L_1 < 1.10; 1.10 < L_2 < 1.15; D > 1.34 \), at 0°C.

<table>
<thead>
<tr>
<th>Primary drug and reference enzyme</th>
<th>Ancillary lysosomotropic compound</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suramin and Acid phosphatase</td>
<td>Triton WR-1339</td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td>10.2</td>
<td>11.4</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>8.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Suramin</td>
<td>12.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>6.9</td>
<td>16.7</td>
</tr>
<tr>
<td>Amiodarone and Acid phosphatase</td>
<td>Triton WR-1339</td>
<td></td>
</tr>
<tr>
<td>Amiodarone</td>
<td>5.3</td>
<td>21.3</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>7.5</td>
<td>20.9</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Dextran</td>
<td>5.9</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td></td>
<td>33.5</td>
</tr>
</tbody>
</table>

### Table 2. Differential activation or release by digitonin

Tabulated values indicate digitonin concentrations (mg per g of tissue) leading to reported percentage of release.

<table>
<thead>
<tr>
<th>Organelle membrane injured</th>
<th>Marker or drug</th>
<th>Percentage of release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosomal</td>
<td>Four enzymes*</td>
<td>1.15 2.05 3.25</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>5'-Nucleotidase</td>
<td>2.00 5.00 7.40</td>
</tr>
<tr>
<td>Peroxisomal</td>
<td>Catalase</td>
<td>19.1 21.0 23.0</td>
</tr>
<tr>
<td>Golgian</td>
<td>Galactosyltransferase</td>
<td>29.5 43.0 75.00</td>
</tr>
<tr>
<td>Endoplasmic reticulum</td>
<td>Nucleoside diphosphatase</td>
<td>26.6 44.0 69.10</td>
</tr>
<tr>
<td>Mitochondrial external</td>
<td>Sulphite–cytochrome e reductase</td>
<td>35.0 50.0 66.7</td>
</tr>
<tr>
<td>Mitochondrial internal</td>
<td>Glutamate dehydrogenase</td>
<td>93.3 119.0 143.0</td>
</tr>
<tr>
<td>Unknown</td>
<td>Suramin</td>
<td>2.5 5.5 8.0</td>
</tr>
<tr>
<td></td>
<td>Horse-radish peroxidase</td>
<td>2.9 3.5 5.0</td>
</tr>
</tbody>
</table>

* Acid β-glycerophosphatase, β-galactosidase, β-N-acetylamino deoxyglucosidase and deoxyribonuclease.
In differential activation, advantage is taken of the different sensitivity of the various organelles towards digitonin which is added \textit{in vitro} to liver homogenates (Huybrechts-Godin & Jacques, 1974). The method can be applied to the localization of exogenous material without parallel treatment by ancillary lysosomotropic compounds. Table 2 illustrates the digitonin-induced release of latent marker enzymes characterizing several cytoplasmic organelles or substructures, and of two drugs utilized in applied lysosomology: horse-radish peroxidase and suramin. The totality of the intracellular drug could be found within cytoplasmic vacuoles representing either lysosomes, or vesicles deriving from the plasma membrane (heterophagosomes?) or both types of structures, in a tissue whose molecular and cellular dynamics cannot possibly have been altered by the ancillary lysosomotropic compounds required for the centrifugation technique. In addition, these results allow us to discard the hypotheses that, in our experimental conditions, a measurable proportion of any of the two drugs might have concentrated within Golgi structures, the lumen of endoplasmic reticulum or the interstitial compartments of mitochondria.


Jacques, P. J. (1968) \textit{Epuration plasmaticque de protéines étrangères, leur capture et leur destinée dans l'appareil vacuolaire du foie}, Librairie Universitaire, Louvain


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**Liposomes as Carriers \textit{in vivo} for Methotrexate**

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In addition to its uses in choriocarcinoma and leukaemia, methotrexate (aminomethylpteroyl glutamic acid) has been used by close arterial infusion in the treatment of liver cancer (Geddes & Falkson, 1970). Liposomes (phospholipid vesicles) injected intravenously into rats have been shown to be localized mainly in the liver; the use of liposomes as carriers for methotrexate was therefore investigated.

Methotrexate was entrapped in liposomes composed of egg phosphatidylcholine, cholesterol and dicetyl phosphate (molar proportions 5:5:1) and therefore having net negative charge; a pharmaceutical preparation of methotrexate sodium (Lederle Laboratories, Pearl River, N.Y., U.S.A.) was used. Entrapped and non-entrapped drug were separated by methods similar to those previously described for the entrapment of proteins (Gregoriadis & Ryman, 1972). At a final lipid concentration of 100mg/ml an entrapment of 18% of the methotrexate was obtained. There was no significant leakage of the drug out of the liposomes over a period of several days.

Male Wistar rats (100–125g) were injected in the tail vein with 1ml of a solution containing 1mg of methotrexate/ml (including 0.25μCi of $[^3H]$methotrexate) either in free solution or entrapped in liposomes (4mg of lipids/ml). Animals were killed at intervals after injection and the tissues assayed for radioactivity. The entrapment of methotrexate in liposomes markedly decreased the rate of clearance of the drug from the blood (Table 1), but the concentrations in both liver and spleen were increased, that in spleen by two orders of magnitude. The concentration of drug in the kidney after 30min was much lower when the drug was entrapped in liposomes than when it was free. This is the result of the rapid excretion of free drug into the tubules of the kidney, and the appearance of a large amount of the drug in the urine. After longer time-intervals the effect of entrapment

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