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COMMENTARY*

LYSOSOMOTROPIC AGENTS

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THE TERM "lysosomotropic" is used in this paper to designate all substances that are taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake. This property characterizes many biologically active agents, and generally bears an intimate relationship to their pharmacological, therapeutic, toxic or pathogenic effects. Only recently has this relationship come to be recognized, and it is as yet taken into account very infrequently in pharmacological interpretations.

This subject is of more than academic interest. Lysosomes are involved in many pathological situations, and our growing understanding of this role has brought to light the need in therapeutics for lysosomotropic agents endowed with certain well-defined properties. Such considerations may provide useful guidelines for drug development, testing or screening.

Perhaps the most interesting aspect of lysosomotropism is that it can be conferred artificially on almost any substance, by suitable coupling with an appropriate carrier. As will be shown, this device can serve as a means not only of acting selectively in or on lysosomes, but also of directing drugs preferentially into a chosen cell type. Enough such examples are already known to indicate that this field of research offers rich possibilities for a novel kind of drug design, with applications to many different diseases, including among others microbial infections, parasitoses, leukemia and cancer.

Properly reinterpreted, the literature on lysosomotropism is immense and goes back more than a century, to the early studies on "vital staining", "cellular storage", "vacuolar transformation" and related phenomena, which we now know to be almost invariably manifestations of lysosomotropism. No attempt has been made to cover this literature. Even contemporary works have been cited sparingly, being selected primarily for their illustrative value and direct relevance to practical applications.

* The Commentary Editors are aware that this contribution is much longer than any other Commentary so far published or accepted. The policy remains that Commentaries should be brief but this is not an inflexible rule. When a new and important concept requires additional space to be fully and adequately described then an exception, as in this case, will be made.

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As to the lysosomes themselves, we have assumed that the reader is familiar with the main properties and functions of these particles and with the cellular mechanisms whereby they exert their physiological roles and participate in the production of disease. Background information on these topics can be found in a number of reviews and treatises.[1–7]

GENERAL PHARMACOLOGICAL MECHANISMS

Entry into lysosomes

As illustrated in Fig. 1, there are three distinct modes of entry into the lysosomes. The main route is by endocytosis, which depends on enclosure within vacuoles derived from invaginations of the plasma membrane. In most cases, these vacuoles subsequently fuse with lysosomes, either primary or secondary, and thus become part of the lysosome system. The materials that can be taken up in this manner include a variety of small molecules, all major groups of macromolecules, insoluble particles, as well as such complex objects as viruses, bacteria, parts of other cells, and even whole cells. There is thus practically no limit to what can be introduced into lysosomes and this unique accessibility is one of the features that make these particles an ideal target for drugs. Another useful property of endocytosis is its specificity. Different materials are taken up at different rates, and these rates vary considerably, both in absolute magnitude and relative to each other, from one cell type to another.

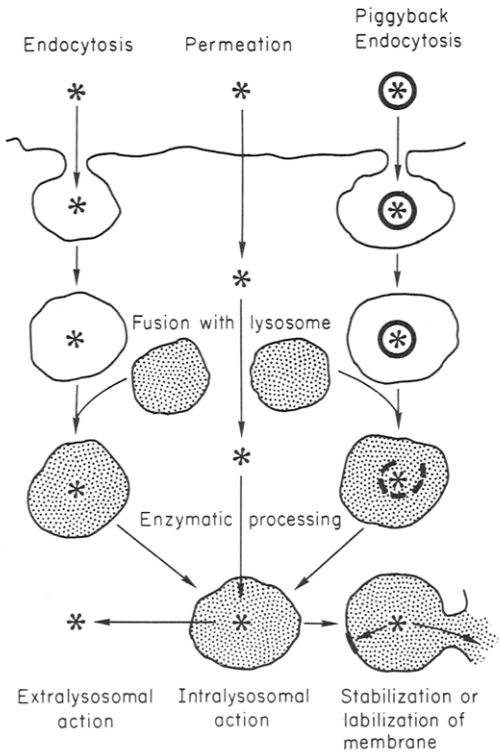

FIG. 1. Mechanisms of entry and modes of action of lysosomotropic agents.

As a result of this, preferential uptake by certain cells is the rule rather than the exception for materials entering by the endocytic route.

A special and particularly interesting form of endocytosis is that described in Fig. 1 as piggyback endocytosis. This term was first used by Sbarra *et al.*[8] to describe the nonselective uptake of solutes accompanying the induction of phagocytosis in leukocytes. We use it here in a slightly different sense, which actually fits better the image it evokes, to designate the uptake of substances that enter lysosomes as part of an endocytizable complex, and are then released in free form as a result of processing of this complex within the lysosomes. This mechanism operates in a number of physiological as well as pathological situations. It has considerable potential in pharmacology and therapeutics, as will be shown in this paper.

A number of substances are known that accumulate selectively within lysosomes, sometimes at a very rapid rate and in enormous amounts, in spite of a freely permeant character. The kinetics of uptake of these substances preclude the endocytic route and indicate that they must enter by permeation. A trapping mechanism must account for their selective intralysosomal accumulation.

In addition to the three routes depicted in Fig. 1, materials also enter lysosomes through the autophagic segregation of cytoplasmic constituents, and, in the particular case of secretory products, through crinophagy or fusion of a secretion granule with a lysosome. Through these mechanisms and also through phagocytosis of fragments or debris of other cells, substances that have initially been concentrated in cell structures other than lysosomes may eventually end up in lysosomes and even become concentrated there. They will, however, do so much more slowly than typical lysosomotropic substances.

Intralysosomal processing

Materials entering lysosomes immediately become exposed to an acid milieu, pH most probably between 4 and 5, and to a collection of some 40 or more digestive enzymes capable by their concerted or sequential action of extensively dismantling a large variety of complex materials, including most naturally occurring macromolecules as well as the cell structures that are made of them. This processing has two important aspects, one negative and one positive. It restricts the number of biologically active lysosomotropic agents to those that are sufficiently resistant to lysosomal inactivation to produce an effect. On the other hand, it allows the use of a variety of precursors capable of giving rise to an active agent through lysosomal digestion.

Modes of action

Lysosomotropic agents can act in three different ways, shown schematically in Fig. 1. The agent can remain in the lysosomes, and act by altering the intralysosomal milieu. It can do this by its sheer bulk, causing the lysosomes to swell to accommodate the incoming material together with the water it attracts. The result is a storage syndrome, a condition more or less well tolerated depending on the type of cell involved, on the size of the deposits and on the degree of reversibility of the situation. This consequence of lysosomotropism is responsible for a number of toxic manifestations and iatrogenic diseases. In addition to storage, many specific effects can be exerted inside the lysosomes, for instance enzyme activation or inhibition, changes in local pH and ionic composition, clearing effects of various sorts by physical or

chemical means, antibiotic and other chemotherapeutic effects, neutralization of toxins, etc.... Several such examples will be described.

Agents entering lysosomes can act also by modifying the properties of the lysosomal membrane, increasing or decreasing its ability to contain lysosomal digestion and to protect the surrounding cytoplasm against damage by the lysosomal enzymes. Such stabilizing or labilizing effects can be accomplished by a variety of steroids and other lipid-soluble substances. Some of these, like cortisone and hydrocortisone, have been widely publicized as lysosomal modifiers, and for this reason would probably most readily be called lysosomotropic by pharmacologists. They are, however, not lysosomotropic in the sense in which this word is used here since they probably associate nonselectively with all cytomembranes and their action is not restricted to the membrane of lysosomes. As will be pointed out, true lysosomotropic agents offer possibilities for more specific effects exerted on the lysosomal membrane from within.

In addition to these effects directed toward the lysosomes themselves, a variety of extralysosomal effects can be obtained also with the help of lysosomotropic agents, if these are of the piggyback complex or precursor type. The effect in this case is not produced by the agent itself, but by a permeant substance that arises from the agent through lysosomal processing and then diffuses out of the lysosomes to act elsewhere in the cell. In this kind of application the lysosome is a tool rather than a target. It simply serves to set free drugs that have been selectively directed into certain cells by piggyback endocytosis.

THERAPEUTIC INDICATIONS

The number of diseases in which some form of lysosomotropic therapy may be expected to be beneficial is very large. Among them, many implicate the lysosomes themselves as either sites or agents of the pathological process. Others do not involve lysosomes, but provide interesting opportunities of more selective chemotherapeutic or other interventions based on lysosomotropism.

Intralysosomal effects

There are many conditions in which we may wish to change the intralysosomal milieu, to either correct intrinsic deficiencies or counteract exogenous alterations. Only the most representative indications of this sort will be listed here.

Conditions characterized by lysosomal overloading. This term covers all conditions in which lysosomes become loaded with excessive amounts of certain materials. The condition can be induced, for instance by the administration of undigestible lysosomotropic substances; or it can be inborn and result from a severe congenital deficiency of one of the lysosomal enzymes, the recognized pathogeny of most of the known genetic storage diseases[7] (Table 1). Intermediates between these two types also occur, for instance in such cases where the necessary enzyme or enzymes exist in limited amounts adequate under normal conditions, but insufficient to handle an excessive influx of substrate. Such a mechanism probably accounts for the deposition of protein in proximal tubule cell lysosomes in the nephrotic kidney,[9] and for that of cholesteryl ester in the lysosomes of arterial smooth muscle cells in experimental atheroma.[10]

Overloading of lysosomes may also result from the presence in the lysosomal system of inhibitors of lysosomal hydrolases. The inhibitor may be an administered

TABLE 1. CONDITIONS CHARACTERIZED BY LYSOSOMAL OVERLOADING

Induced by
Inorganic compounds
Iron (hemochromatosis), thorium (thorotrastosis), silica (silicosis), asbestos (asbestosis), carbon particles, etc. ...
Organic micromolecules
Sucrose, mannitol, basic dyes, drugs, etc. ...
Organic macromolecules
Proteins, polysaccharides (dextran, etc. ...), synthetic polymers (Triton WR-1339, polyvinylpyrrolidone, etc. . . .)
Inborn
Lipidoses
Sulfatidosis, lactosyl-ceramidosis; Gaucher, Krabbe, Tay-Sachs, Sandhoff, Fabry, Farber, Niemann-Pick and Wolman diseases
Mucolipidoses
Types I, II (I-Cell disease) and III, fucosidosis, mannosidosis, G_{M1}-gangliosidosis, mucosulfatidosis
Mucopolysaccharidoses
Hurler, Hunter, Sanfilippo, Morquio, Scheie and Maroteaux-Lamy diseases; chondroitin 4- and 6-sulfaturia, β-glucuronidase deficiency
Others
Type II glycogenosis, aspartylglycosaminuria, cystinosis, acid phosphatase deficiency, ceroid-lipofuscinosis

lysosomotropic agent, or a substance accumulating in the lysosomes as a secondary consequence of a primary enzyme defect.[11] Finally, lysosomal storage may occur as the end result of complex chains of events in which the lysosomes themselves are entirely innocent. For instance, the increased lysosomal storage of lipofuscin that occurs with ageing is believed to be a secondary consequence, mediated through autophagy, of the formation of this undegradable material elsewhere in the cell.[12]

Whatever its cause or causes, lysosome overloading is harmful; it may lead to widespread degenerative alterations of cells and tissues, to severe functional deficiencies, and even to death of the patient. Although not necessarily the only possible approach, introduction into the lysosomes of an appropriate clearing agent obviously represents a logical way of combating such conditions. Depending on circumstances, the agent may be an enzyme, or an activator, or an anti-inhibitor, or a substance that may assist the stored material out of the lysosomes. In all cases, lysosomotropism is necessary to convey the agent to the right intracellular site.

There are situations where clearing of the lysosomes is chemically and physically impossible. The only possible curative measure left is then exocytic discharge of the lysosomal contents into the extracellular spaces. Lysosomotropism, in the sense adopted in this paper, may conceivably be, but does not have to be, a property of drugs capable of inducing exocytosis. Whether such clearing of the lysosomes can be achieved without serious consequences to the cells or to their environment remains to be established.

Diseases due to lysosomal rupture. Cases are known or suspected in which cells are damaged or killed as a result of an injury from within to the membrane of their lysosomes. Examples are silicosis[13] and other respiratory diseases due to particulate pollutants,[14] gout,[15] certain cases of photosensitization,[16] and possibly also infections

in which membranolytic toxins are released from phagocytized microorganisms. The streptococcal streptolysins could act in this manner.[17]

All these conditions represent possible indications for lysosomotropic agents capable of neutralizing or destroying the substance attacking the lysosomal membrane, or of preventing its formation.

Bacterial infections. In many infectious diseases, for instance, those caused by mycobacteriae, brucellae, listeriae, salmonellae, the causal agent survives and proliferates intracellularly, most often within the confines of the vacuolar system into which it has been introduced through phagocytosis. It is obvious that if we wish to reach the intracellular bacteria with an appropriate agent, we must use one with the right tropism, and capable in addition of acting under the conditions prevailing within the vacuoles occupied by the bacteria. We may wish to do so either with a bacteriostatic or bactericidal agent directed against the bacteria, or with substances altering the intravacuolar microenvironment so as to make it incapable of supporting bacterial growth.

In the choice or design of such drugs, it is very important to take into consideration which part of the vacuolar system is occupied by the bacteria. If these dwell mostly within lysosomes, as seems to be the case in leprosy for instance,[18] we have the advantage of having at our disposal the whole battery of lysosomal enzymes for processing of a carrier, with the restriction that the agent itself must be stable and active in the intralysosomal milieu. A number of cases have, however, been described recently in which phagocytized infective agents fail to reach lysosomes because phagosome-lysosome fusion is inhibited.[19–21] What is needed here is a "phagosomotropic" drug, possibly a much more difficult thing to achieve than a lysosomotropic drug. Alternatively and perhaps preferably, attempts could be made to counteract the inhibition of phagosome-lysosome fusion.

Lysosomotropic antibiotics can be very useful also in extracellular infections, in which the causal agent has been found to escape the killing action of drugs or seric factors by taking refuge within the vacuolar system of nonbactericidal cells.[22–24]

Parasitoses. Many parasitic organisms, for instance toxoplasmae, leishmaniae, trypanosomes and plasmodia, occupy the vacuolar system of the host at some or all stages of the infectious process. The therapeutic problems created by these parasitoses thus resemble those encountered in intracellular bacterial infections, even including the problems raised by the actual location of the microorganism within the vacuolar system of the host. Toxoplasma, for instance, has been shown to inhibit phagosome-lysosome fusion and to reside only in phagosomes.[25] This property characterizes the live microorganism; phagosomes containing dead toxoplasmae fuse regularly with lysosomes. The location of other intracellular parasites has not yet been elucidated.

In many cases a second approach is rendered possible by the fact that the parasite itself feeds largely or exclusively by phagocytosis, making it potentially vulnerable to lysosomotropic drugs directed against its own lysosome system.

Viral infections. As recently reviewed by Dales,[26] most viruses penetrate into cells by a mechanism of viropexis which is closely related to phagocytosis. However, they usually escape from phagosomes or release their genetic information before reaching the lysosomes. Reovirus is exceptional in that it not only invades the lysosomes but actually takes advantage of the lysosomal proteases for uncoating of the genetic

material. Lysosomes may also be involved at later stages of viral infections, as targets or mediators of cytotoxic effects.[27] These mechanisms lend themselves to a variety of possible interferences by lysosomotropic drugs.

Effects on lysosomal membrane

Stabilizing effects. We have mentioned above some of the conditions in which rupture of the lysosomal membrane is well documented as playing a direct pathogenic role. Many more situations exist in which such a role is suspected on the basis of circumstantial evidence. A related pathogenic mechanism is the extracellular release of lysosomal enzymes, as a result for instance of "regurgitation during feeding".[28] Together these two mechanisms are believed to be causally involved in the damage to cells and extracellular structures characteristic of numerous chronic degenerative and inflammatory affections. Many workers believe that anti-inflammatory agents such as cortisone and hydrocortisone exert their effects at least partly by modifying the lysosomal membrane, rendering it less leaky or fragile, and perhaps less capable of fusing with plasma membranes to allow exocytic discharge of the lysosome contents.[29]

At present this whole area is still somewhat controversial, due to the lack of definitive evidence in most instances. It provides an interesting field of exploration with lysosomotropic derivatives of the alleged stabilizers capable of releasing the drugs directly within the lysosomes.

Labilizing effects. There are no known indications where labilization of the lysosomal membrane may be expected to be beneficial to cells. As a mechanism of cell destruction, however, the device holds considerable promise, were it not for its general lack of specificity. This drawback could be obviated by the use of carriers taken up preferentially by the target cells. By suitable coupling to such a carrier, membranolytic agents could be made to be selectively released within the lysosomes of undesirable cells to cause their demise by lysosomal rupture.[30,31] Cancer cells, infectious protozoa, fungi and other eukaryotic parasites, as well as certain lines of lymphocytes involved for instance in graft rejection, could be eradicated in this manner.

Extralysosomal effects

As pointed out above, lysosomotropism can serve as a device for directing a drug towards certain cells. In principle, almost any disease can be an indication for this kind of therapeutic approach, to the extent that an effect restricted to the target cells, or particularly intense on them, is desirable and feasible. Cancer chemotherapy is, of course, a prime example.

AGENTS ENTERING BY PERMEATION

Kinetics

Early students of vital staining were quick to recognize the existence of profound differences in staining characteristics between soluble dyes of the neutral red type and acidic dyes such as trypan blue that either form micelles or bind strongly to plasma proteins. The basic dyes are taken up by almost any cell type in a matter of minutes, in contrast with the acidic dyes which become detectable in cytoplasmic particles

only in phagocytic cells and after prolonged exposure. Entry by permeation vs endocytosis is of course responsible for this difference.

Obviously, if a freely permeant substance accumulates in lysosomes, some trapping mechanism must be operative within these particles. Intralysosomal trapping by protonation most likely accounts for the majority of such substances. Exceptionally, other mechanisms may be involved.

Trapping by protonation. It has long been known that many weak acids and bases can diffuse readily across biological membranes in unionized form, but not or very slowly in ionized form. If the membrane separates two regions of different pH, acids accumulate on the basic side, bases on the acid side, as a result of this permeability difference. This phenomenon has led to many interesting applications. In pharmacology, for instance, it has served to alter the rate of excretion of drugs through manipulation of the urinary pH.[32] In cell biology, it has provided a method for measuring the internal pH of cells and subcellular organelles.[33] It also furnishes the most satisfactory explanation for the intralysosomal accumulation of weakly basic substances, as exemplified by neutral red.

Let us consider the penetration of a weak base:

$$B + H^+ \rightleftharpoons BH^+$$

through the plasma membrane into the cytoplasm and then through the lysosomal membrane into the lysosomes. We will use subscripts to identify the parameters connected with each compartment as indicated in Fig. 2. We will assume that the permeability characteristics of the plasma membrane and of the lysosomal membrane are the same, and that the unprotonated form of the base has a permeability coefficient P, and the protonated form p. A and V denote area and volume respectively.

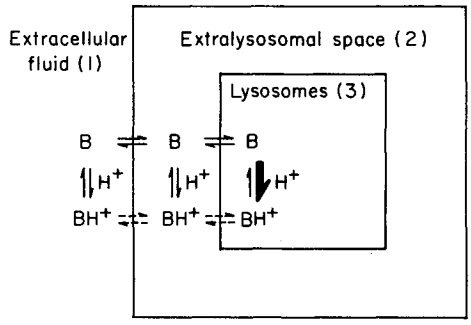

FIG. 2. Model of intralysosomal trapping of weak bases. Numbering of compartments refers to subscripts in equations.

We will define C_i as the total concentration of base in the i-th compartment.

$$C_i = [B]_i + [BH^+]_i \tag{1}$$

We will assume that the diffusion rates in the cytoplasm and in the lysosomes are sufficiently fast so that membrane permeability is rate-limiting. Then in the lysosomes:

$$\frac{dC_3}{dt} = \frac{A_3}{V_3}\{P([B]_2 - [B]_3) + p([BH^+]_2 - [BH^+]_3)\} \tag{2}$$

Substituting:

$$\alpha = \frac{p}{P} \tag{3}$$

$$K = \frac{[B]\,[H^+]}{[BH^+]} \tag{4}$$

we can write:

$$\frac{dC_3}{dt} = \frac{A_3 P}{V_3}\left\{\frac{\alpha\,[H^+]_2 + K}{[H^+]_2 + K}C_2 - \frac{\alpha\,[H^+]_3 + K}{[H^+]_3 + K}C_3\right\} \tag{5}$$

Replacing the complicated coefficients of the concentration:

$$\gamma_i = \frac{\alpha\,[H^+]_i + K}{[H^+]_i + K} \tag{6}$$

we get:

$$\frac{dC_3}{dt} = \frac{A_3 P}{V_3}(\gamma_2 C_2 - \gamma_3 C_3) \tag{7}$$

For the concentration change in the extralysosomal space:

$$\frac{dC_2}{dt} = \frac{P}{V_2}\{A_2(\gamma_1 C_1 - \gamma_2 C_2) - A_3(\gamma_2 C_2 - \gamma_3 C_3)\} \tag{8}$$

It is possible to solve equations (7) and (8) explicitly, but the solution would be very cumbersome. Consequently we will discuss the general properties of the solution and present some particular examples of solutions obtained by the finite difference method with the help of a computer. If F is the ratio of the total concentration of the substance in the lysosomes to that in the extracellular medium, it is clear from equations (7) and (8) that in the steady state:

$$F = \frac{\gamma_1}{\gamma_3} \tag{9}$$

and from equation (6) we can see that when α and K are small relative to the hydrogen ion concentration:

$$F \simeq \frac{[H^+]_3}{[H^+]_1} \tag{10}$$

That is, the concentration ratio for the lysosomotropic substance is equal to the ratio of the hydrogen ion concentration in the lysosomes to that in the fluid outside the cell. This is in fact the maximum concentration ratio that will be reached by any substance.

Figure 3 shows the effect of the value of α on steady state concentration ratios of bases of various pK's assuming the pH in the lysosomes to be 4 and that in the medium to be 7. Higher values of α decrease the concentration ratio for all substances, but bases with higher pK's are more sensitive to this effect. On the other hand, bases with pK's below 8 can never achieve the maximum concentration ratio

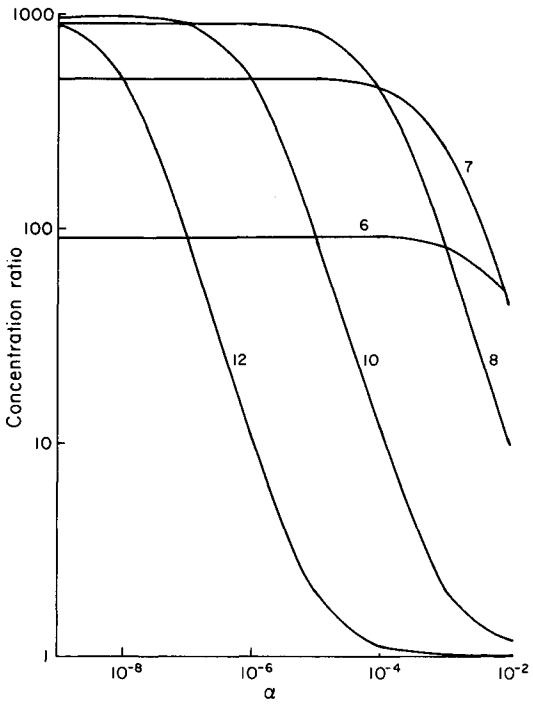

FIG. 3. Influence of α and of pK on steady state concentration of base in lysosomal compartment (pH = 4·0) relative to extralysosomal compartment (pH = 7·0). Numbers on curves identify pK of base.

even when α equals 0. Consequently substances with a pK around 8 are the most likely to show the kind of lysosomotropism we are discussing. As we will see below, bases with high pK's would be expected to penetrate the lysosomes very slowly.

Obviously the kinetics of penetration will differ from cell type to cell type depending on the cell parameters V_2, V_3, A_2 and A_3. For two cell types we can make reasonable estimates of the values of these parameters. The volume of lysosomes in rat liver is given by Weibel *et al.*[34] as 0·0068 cm^3/cm^3 tissue, or about 0·0065 $cm^3\ g^{-1}$, and by Baudhuin[35] as 0·004 $cm^3\ g^{-1}$. We will adopt a value of 0·005 $cm^3\ g^{-1}$, which, multiplied by the surface to volume ratio of $1{\cdot}1 \times 10^5\ cm^{-1}$ given by Baudhuin[35] for hepatic lysosomes, corresponds to a surface area of $5{\cdot}5 \times 10^2\ cm^2\ g^{-1}$. To estimate the area of the plasma membrane, we take as a basis the fact that marker enzymes such as 5'-nucleotidase or alkaline phosphodiesterase I are purified to a maximum of about 35-fold with respect to proteins in the best preparations of purified plasma membranes.[36]* From this we deduce that plasma membranes contain approximately 3 per cent of the total liver proteins, occupying a volume of about 0·15 $cm^3\ g^{-1}$ (assuming that 40 per cent of their wet weight is protein, compared with 20 per cent for whole liver). With a thickness of 10 nm, this leads to a surface area

* The best values given in the literature are of the order of 25- to 30-fold. Recent work by Dr. J. Remacle in our Louvain Laboratory indicates that such preparations are still significantly contaminated by microsomal elements. Eliminating this contamination increases the purification ratio of plasma membrane markers to a maximum of 35-fold.

of $1{\cdot}5 \times 10^4$ cm^2 g^{-1}, which amounts to a little over 5 times the value of $2{\cdot}8 \times 10^3$ cm^2 per cm^3 given by Weibel *et al.*[34] for the surface area of hepatocytes considered as smooth bodies. A 5-fold increase in surface area due to infoldings of the membrane and to irregularities in cell shape seems quite plausible. We will assume that some 80 per cent of the surface area estimated in this manner, or $1{\cdot}2 \times 10^4$ cm^2 g^{-1}, belongs to spaces of Disse and is available for permeation from the blood.

Cellular parameters of fibroblasts are somewhat uncertain and undoubtedly vary greatly from one culture to another. In the cultures studied by Van Hoof,[11] lysosomes in rat fibroblasts occupied a volume of 0·038 cm^3, and had a total membrane surface area of $3{\cdot}8 \times 10^3$ cm^2, per cm^3 of cells. According to measurements made on normal sections through such monolayers, the average thickness of the cells is 2 μm (3·5 μm if the section includes the nucleus). Estimates based on protein content lead to the same value. Thus the upper surface area of the sheet is of the order of 5×10^3 cm^2/cm^3 of cells. Due to cell overlapping, membrane folding and probable accessibility of the lower surface of the sheet to diffusible molecules, the area of plasma membrane involved in permeation must be at least 2–3 times this value. We will assume $1{\cdot}2 \times 10^4$ cm^2/cm^3 of cells.

With these values, we can estimate the kinetics of penetration of lysosomotropic substances. In Fig. 4 is shown the influence of pK on the time needed for rat liver lysosomes to become filled to half the steady state concentration of a weak base having a permeability coefficient of 10^{-3} cm min^{-1} for the free base and of 0 for the protonated form. We assume the extracellular concentration of the substance to remain

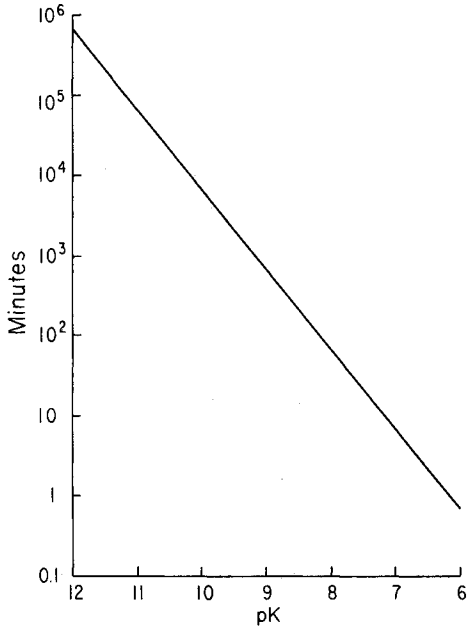

FIG. 4. Influence of pK on rate of penetration of base into lysosomes, assuming $P = 10^{-3}$ cm min^{-1}, p = 0, intralysosomal pH = 4·0, and extralysosomal pH = extracellular pH = 7·0. Ordinate refers to the time needed for the intralysosomal concentration in hepatocytes to reach half its steady state value.

constant, and the pH to be 7 in the cytoplasm and external fluid and 4 in the lysosomes. For pK values sufficiently higher than the lysosomal pH, the permeation rate decreases by a factor of 10 for every unit increase of pK. This indicates again the suitability of 8 as the pK of a lysosomotropic drug that enters by permeation.

The kinetics of permeation into the cytoplasm and lysosomes depends on the cellular parameters. Figure 5 shows the way in which concentration in these two intracellular compartments would change in liver cells exposed to a weak base with a pK of 8, the other assumptions being those adopted for Fig. 4. The comparable situation for fibroblasts is shown in Fig. 6.

In both cell types, uptake follows a biphasic course. There is an initial rapid phase, during which the cytoplasm fills up to the concentration required for the rate of permeation into the lysosomes to match the intracellular penetration. This concentration stands to the outside concentration in a ratio approximately equal to the ratio of plasma membrane surface area to surface area of plasma membrane plus lysosomal membrane (0·96 for liver and 0·75 for fibroblasts). After that, permeation into the lysosomes becomes rate-limiting and penetration into the cytoplasm proceeds more slowly.

The final distribution of substance between the two compartments after the steady state has been reached depends, of course, on their relative volume. The fraction L of intracellular substance occupying the lysosomes is given by:

$$L = \frac{C_3 V_3}{C_2 V_2 + C_3 V_3} \tag{11}$$

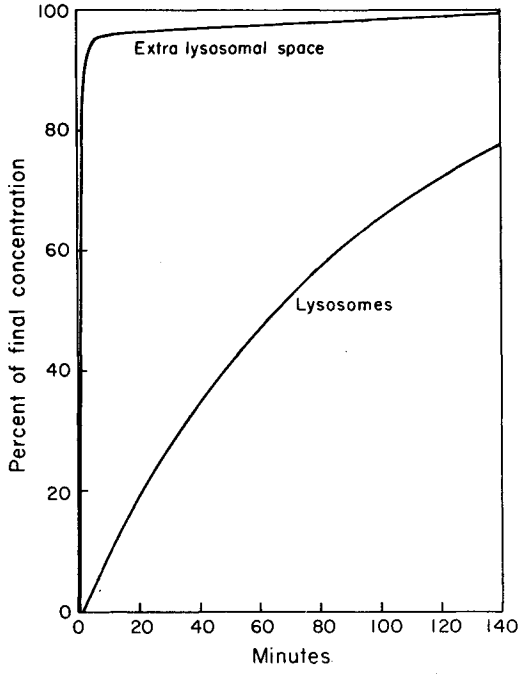

FIG. 5. Kinetics of penetration of weak base into cytoplasm and lysosomes of rat hepatocytes: pK = 8·0, other conditions as in Fig. 4. Note that in final steady state, concentration is 1000-fold, and total amount 5-fold, higher inside than outside lysosomes.

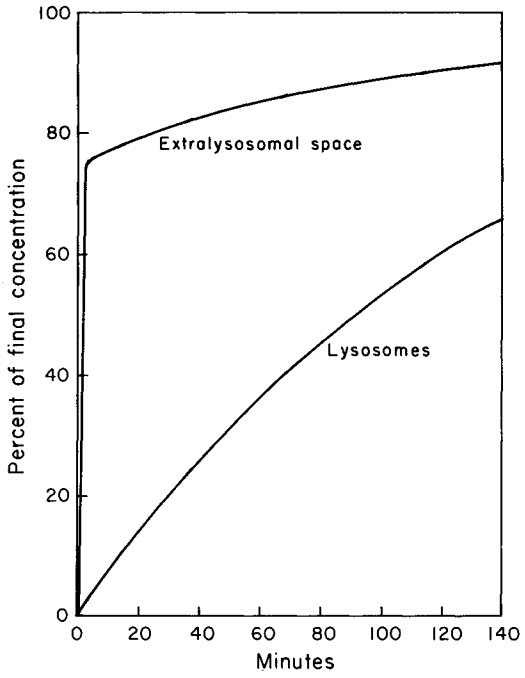

FIG. 6. Kinetics of penetration of weak base into cytoplasm and lysosomes of rat fibroblasts: same conditions as in Fig. 5. Note that in final steady state concentration is 1000-fold, and total amount almost 40-fold, higher inside than outside lysosomes.

For the conditions of Figs. 5 and 6, L amounts to about 0·82 in hepatocytes and 0·97 in fibroblasts.

In the foregoing calculations, several simplifying assumptions have been made. First, the pH inside the lysosomes has been assumed to remain constant, which can be true only if the amount of base taken up is small with respect to the buffering capacity of the lysosomes or if protons are supplied to the lysosomes to replace those lost by protonation. We don't know the buffering capacity of the lysosomes, but it cannot possibly be high enough to support the massive uptakes that have been observed, for instance with chloroquine. As will be mentioned below, this substance easily reaches absolute concentrations in excess of 20 mM inside lysosomes, with little apparent rise in intralysosomal pH, as evidenced by concentration ratios of several hundred-fold. No proton reserve can account for this and the participation of a proton pump appears to us for this reason to be mandatory. Most likely, this pump provides the driving force for the continuing uptake of base. Some circumstantial evidence supporting the existence of an ATP-dependent proton pump in the lysosomal membrane has been reported.[37] Conceivably, proton secretion may become rate-limiting for the uptake of bases of low pK or of high permeability coefficient.

Another assumption we have made is that the lysosomal volume remains constant. This is likely to be invalid under conditions where continuing uptake is possible. Since the lysosomal contents will remain in osmotic equilibrium with the cytoplasm as the weak base is taken up into the lysosomes, water will be taken up as well. Indeed, extensive dilatation of the lysosomes generally accompanies the uptake of weak

bases. This phenomenon makes it even more imperative to postulate the existence of a proton pump.

In our theoretical analysis we have assumed simple mass action, using concentrations in all equations. If, as seems likely, the activity coefficient of the substance in question is depressed in concentrated solution, then the concentration factor achieved in lysosomes could be even higher than that calculated. For example, an isotonic solution of chloroquine sulfate is twice as concentrated as would be calculated from the ions present.[38]

In addition, we have discounted all losses of lysosomotropic substance due to metabolism or digestion, and we have neglected the influence of cellular compartmentation on the distribution of the substance in the extralysosomal space. The latter assumption is clearly an oversimplification. Basic dyes may be expected to bind to nucleic acids, in the nucleus and in the ribosomes. On the other hand, they will tend to be excluded from mitochondria.[39]

It should be noted that our equations are applicable also to the movement of a weak acid, in which case of course α will be greater than unity and the directions of all movements are reversed. Weak acids are kept out or driven out of lysosomes, and may therefore be termed negatively lysosomotropic. This point is of great importance since it may explain the inefficiency of acidic drugs in cases where an intralysosomal action is required.

Other trapping mechanisms. Dingle and Barrett[40] have extracted from kidney lysosomes an acidic substance of apparent glycolipid nature with a strong affinity for acridine orange and other cationic compounds, and also for a nonionic hydrocarbon, dimethylbenzathracene. It is not known whether this substance occurs in the lysosomes of other cells nor how important its role in the trapping of lysosomotropic agents may be. In our opinion, it cannot, for simple reasons of stoichiometry, be considered a substitute for an energy-dependent proton pump when it comes to the uptake of large quantities of weak bases such as neutral red or chloroquine. But it could account for the lysosomal binding of metals, and for that of the hydrocarbons studied by Allison and Young.[41,42]

Examples

Basic dyes. The granules that concentrate basic dyes have been clearly identified as lysosomes,[40–45] but the concentration mechanism remains a matter for conjecture. One reason for this uncertainty lies in the apparently contradictory nature of the available evidence. On one hand, many observations, going back to the early investigations of neutral red uptake by Nassonov[46] in 1930, indicate that the uptake of basic dyes is an energy-dependent process. On the other hand, there is clear proof that cells can be stained at low temperature,[40,44,47] and even that isolated lysosomes take up weak bases *in vitro*.[40,43,45,48] The work of Robbins *et al.*[44,47] on the uptake of acridine orange by HeLa cells provides a clue to the solution of this paradox, indicating that the requirement for energy may depend on the amount of dye taken up. In the absence of an energy supply, small amounts of dye can be efficiently concentrated in lysosomes due simply to the natural proton reserve of these particles and their content in binding acidic compounds of the type described by Dingle and Barrett.[40] However, to sustain continuing uptake of dye and the ensuing dilatation of

the lysosomal compartment and accumulation of large quantities of dye, energy has to be supplied.

All available evidence, including the effect of pH on dye uptake,[45,47] is consistent with our proposal that the dyes permeate in unprotonated form and are trapped by protonation, with a proton pump located in the lysosomal membrane providing the driving force for continuing uptake. It is interesting that, except for the participation of a proton pump, this mechanism was convincingly demonstrated to be operative in the uptake of brilliant cresyl blue into the central vacuole of *Nitella*, by the extensive kinetic investigations carried out almost 50 years ago by Marian Irwin.[49] But the relevance of her work to vital staining seems not to have been noted.

From the pathological point of view, vacuolation as a result of basic dye uptake is associated with increased evidence of autophagy and membrane breakdown.[44,50] Exposure to the dyes is toxic and can even be lethal, possibly as a result of photochemical damage to the lysosomal membrane.[16,51,52]

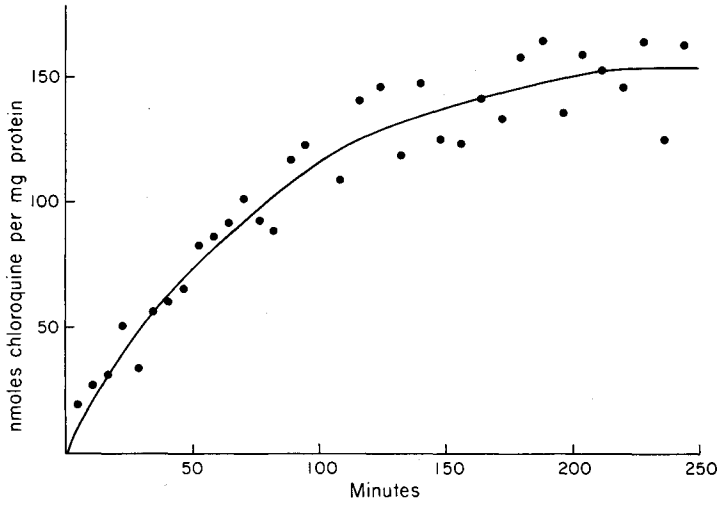

FIG. 7. Penetration of chloroquine into rat fibroblasts exposed *in vitro* to 100 μM chloroquine. Experimental conditions as described by Wibo and Poole.[54]

Chloroquine. Cells exposed to chloroquine take up this compound avidly, concentrating it many-fold over the surrounding medium in a matter of minutes (Fig. 7). Intense cytoplasmic vacuolation accompanies this process (Fig. 8). There is clear evidence, both morphological and biochemical, that the vacuoles are the site of accumulation of the drug and that they are of lysosomal nature.[14,41,42,53,54]

The amounts of drug that can be taken up by cells are enormous. For instance, rat fibroblasts exposed to chloroquine for 200 min at pH 7·4 contained 20 μmoles of drug per cm^3 of cell water if the outside concentration was 100 μM, and 8 μmoles per cm^3 if the outside concentration was 10 μM.[54] The concentration in the lysosomes must be even higher. These findings are consistent with our model of proton pump driven uptake provided the lysosomal pH is assumed to be not far from 4·0. Chloroquine is a weak base with an appropriate pK of 8·1.[55] Its uptake is accelerated

by a rise in extracellular pH,[54,55] as required by the model. Reports on energy dependence are conflicting,[53,56] but can be reconciled, as for the basic dyes, by the observation that energy is not required for the initial phase of uptake, but is needed for continuing accumulation.[56]

The rapid rate of chloroquine uptake is somewhat surprising, since the drug has a second basic group, of pK 10·1,[55] and therefore occurs in only trace amounts of unprotonated form at physiological pH. From the kinetic data of Wibo and Poole[54] and the cellular parameters we have adopted above for rat fibroblasts, we estimate that the permeability coefficient of the membranes to free chloroquine base must be at least 2·5 cm min^{-1}. This is much higher than has been reported for any hydrophilic compound. On the other hand, for a lipid-soluble substance crossing a 5 nm thick lipid layer, it corresponds to a diffusion coefficient of 2×10^{-8} cm^2 sec^{-1}, not an unacceptable value. Unfortunately, we have been unable to find quantitative data on membrane permeability to lipophilic substances in the literature.*

Permeation in monoprotonated form is conceivable, but would require the participation of a special permease. Otherwise retention of the monoprotonated dyes becomes incomprehensible. On the other hand, endocytosis cannot possibly account for the observed rates of uptake, especially since chloroquine has actually been found to inhibit pinocytosis.[53]

It is likely that many of the known biological effects of chloroquine, including perhaps its antimalarial action, are directly related to its lysosomotropism. On one hand, the high degree of vacuolation caused by the drug is probably harmful to many cells, especially neurones. It is associated with evidence of autophagy,[53,57] and could explain the retinal damage and other neurological side effects that complicate the use of chloroquine. On the other hand, at the concentration reached inside lysosomes, chloroquine could seriously affect the activity of some of the lysosomal enzymes and thereby impair lysosomal digestion. Indeed, chloroquine has been found to inhibit the breakdown of endogenous proteins[54] and mucopolysaccharides[57a] in fibroblasts and that of exogenous proteins in macrophages (Fig. 9). *In vitro* experiments have shown it to be an inhibitor of an acid chondromucoprotease present in cartilage,[58] and of cathepsin B_1,[54] one of the lysosomal proteases believed to be particularly important in the initiation of proteolysis.[59]

Chloroquine is taken up by erythrocytes infected with sensitive plasmodia,[60–62] but not by normal erythrocytes; its uptake is distinctly slower in erythrocytes containing chloroquine-resistant plasmodia.[60,62] The drug produces typical vacuolation and autophagy in the parasite, indicative of a lysosomal localization.[63,64] It is tempting to assume that it exerts its cytotoxic activity by way of the lysosomes.[42] In line with this hypothesis, Homewood *et al.*[65] have proposed that chloroquine may act by inhibiting hemoglobin digestion in the plasmodia. The theory given for this inhibition is the raising of the intralysosomal pH by consumption of hydrogen ions during the protonation of the chloroquine. However this cannot be the explanation if concentration of the drug is a function of lysosomal acidity, as we assume. An alter-

* As this paper was being sent to press, an interesting paper on the passage of acetylsalicylic acid across a lipid bilayer has appeared.[56a] It is shown in this paper that diffusion of the uncharged free acid across the bilayer occurs at a very rapid rate, corresponding to a permeability coefficient of the order of 40 cm min^{-1}. Diffusion across the adjacent unstirred water layers is much slower and is normally rate-limiting unless it is facilitated by a proton exchange reaction.

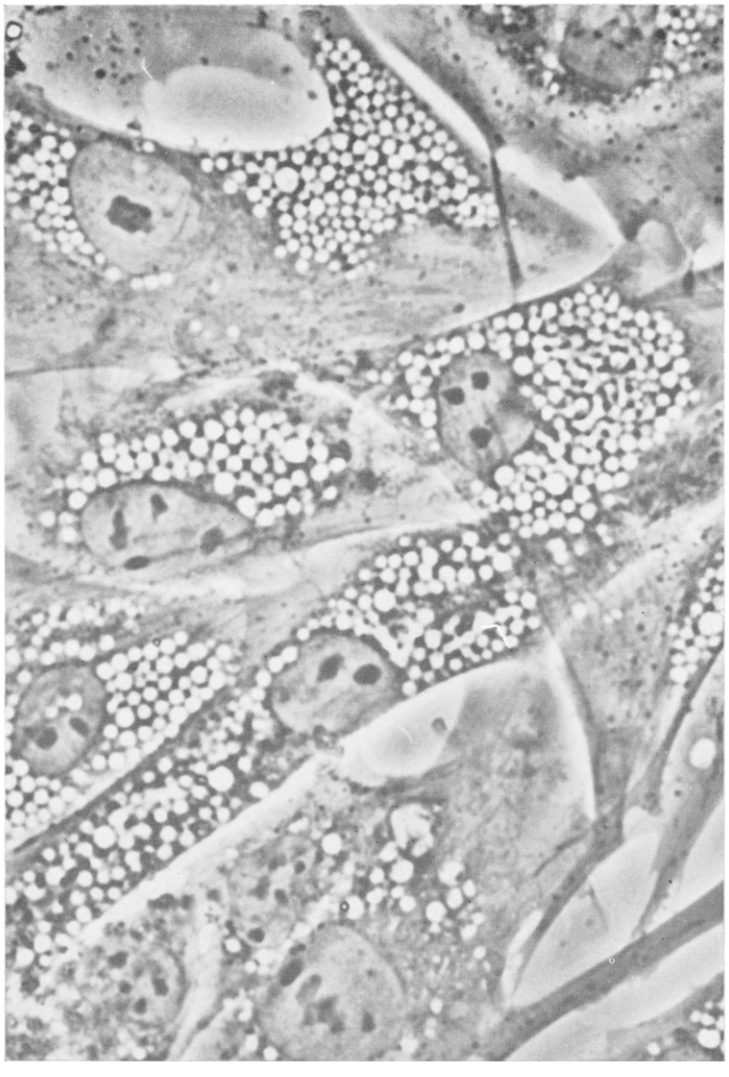

FIG. 8. Vacuolation of rat fibroblasts exposed for 200 min to 100 μM chloroquine (see Fig. 7). Phase contrast: $\times 1500$.

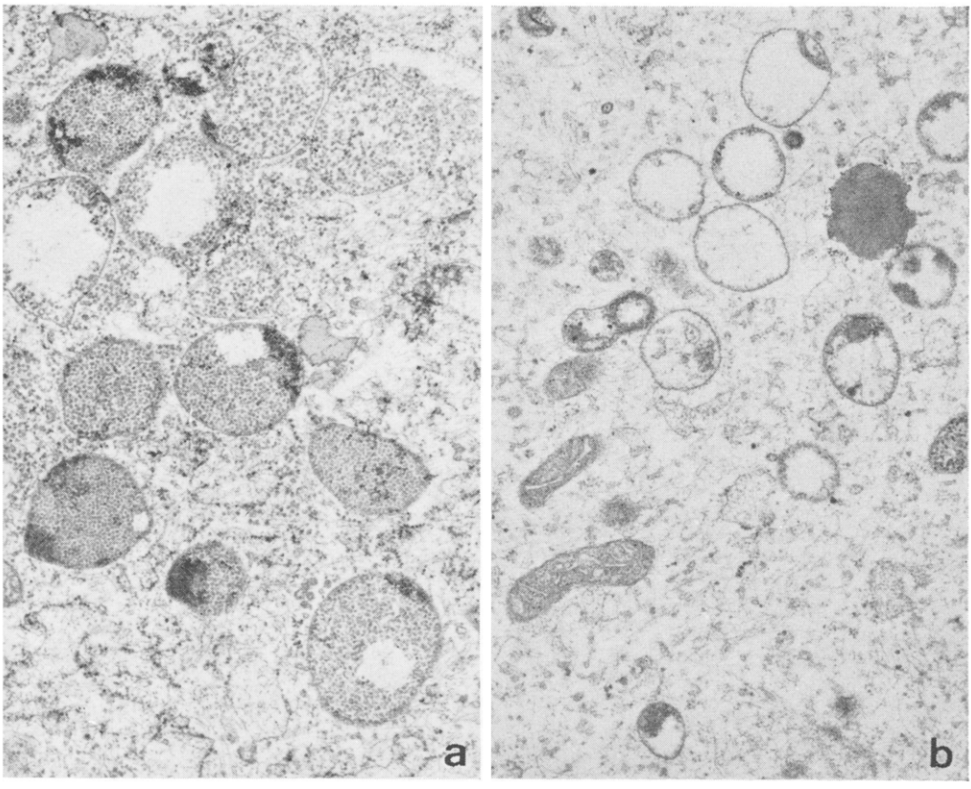

FIG. 12. Effect of the addition of acid α–glucosidase, purified from human placenta, on cultured fibroblasts from patients with type II glycogenosis (Pompe). (a) Untreated cell in which all lysosomes are loaded with glycogen. ×17,300. (b) Same culture after addition of enzyme. Biochemical analysis demonstrated that enzyme was present within the cells and that glycogen content had dropped to a normal value. Most lysosomes were cleared from their glycogen. ×22,500. Unpublished results of Th. de Barsy and J. G. Leroy.

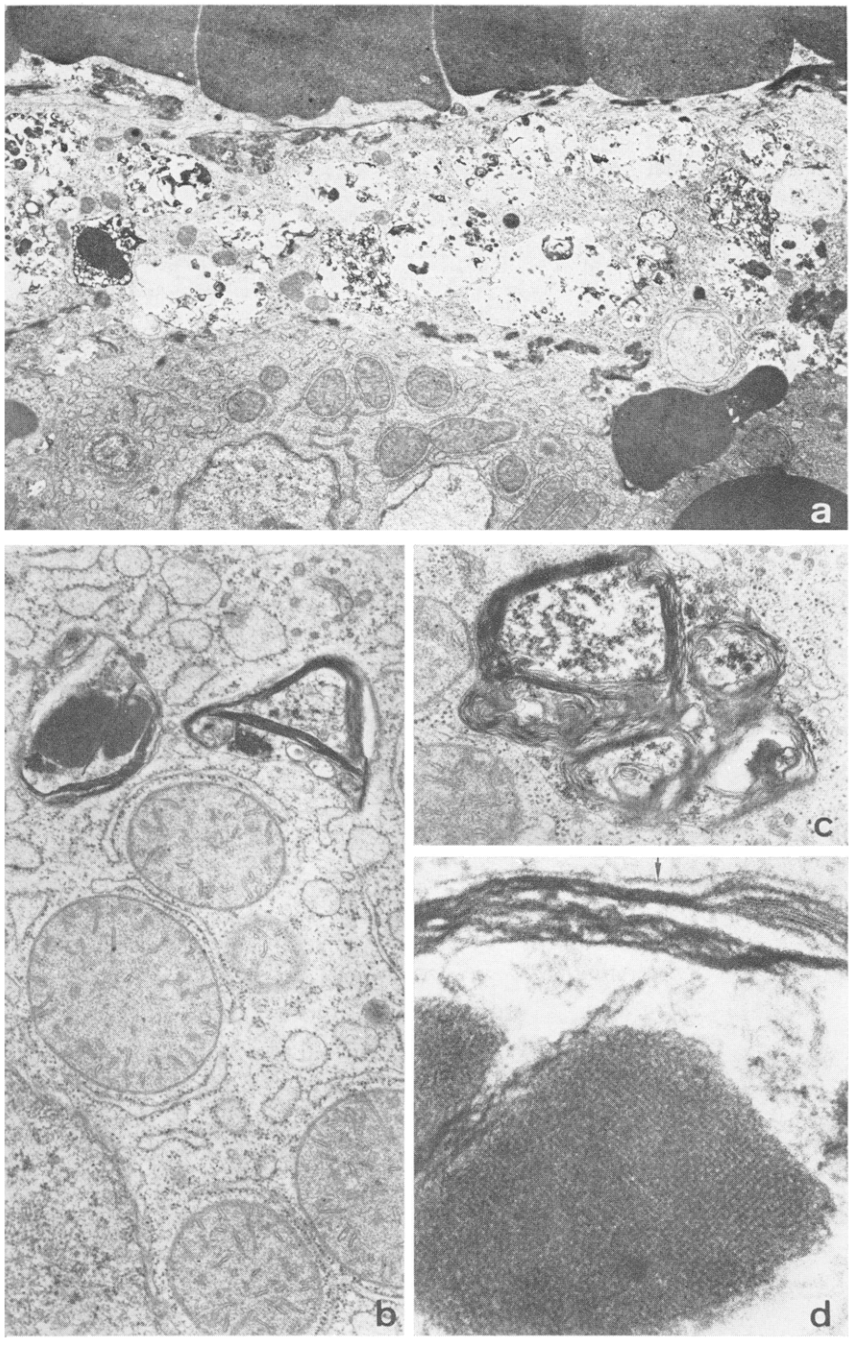

FIG. 15. Ultrastructural aspect of new-born rat liver, 7.5 hr after i.v. injection of liposome-enclosed antibodies directed against purified acid α-glucosidase. The liposomes were made of phosphatidylethanolamine, cholesterol and stearylamine in a molar ratio of 7:2:1. (a) Low power magnification showing a Kupffer cell dilated by numerous vacuoles which contain osmiophilic lamellar or amorphous debris, probably residues of largely digested liposomes. Part of the sinusoid and hepatocytes without particularities, are also visible. ×5600. (b) Hepatocyte with two lysosomes, each one containing a liposome. ×22,000. (c) Aspect at higher magnification of a lysosome in a hepatocyte. Multilamellar osmiophilic structures are easily recognizable. ×30,600. (d) Enlargement of a fragment of Fig. 15b, showing the various organization patterns of the lipid components constituting the liposomes. The arrow points to the lysosomal membrane. ×96,200. Unpublished observations of Th. de Barsy, P. Devos and F. Van Hoof.

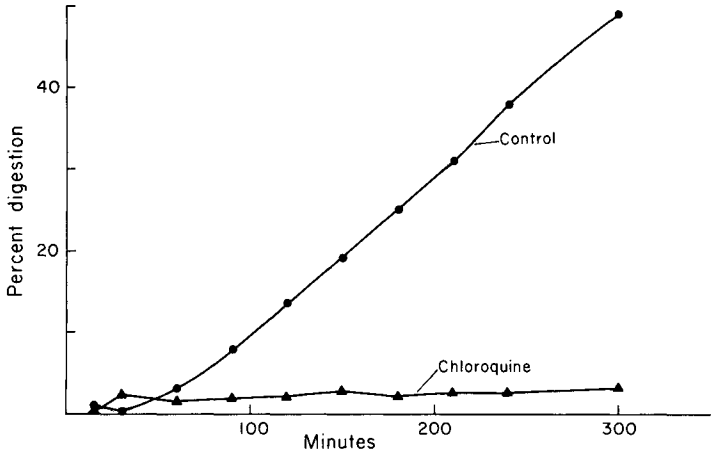

FIG. 9. Inhibition by chloroquine of intracellular digestion of leucine-labelled bacteria in mouse macrophages. Cells were exposed for 1 hr to heat-killed leucine-labelled *Streptococcus faecalis*, washed free of unphagocytized bacteria, and incubated further with and without 100 μM chloroquine. Digestion is measured by percentage of total radioactivity soluble in trichloroacetic acid. Unpublished results of J. Chudzik and B. Poole.

native explanation is a direct inhibition of proteolytic enzymes. In this event, however, the hemoglobin digesting enzyme of the plasmodia must be different from the cathepsin D that accomplishes this role in mammals, since cathepsin D is not inhibited by chloroquine.

There is evidence that other anti-malarials also are concentrated in lysosomes.[41,42] They could therefore act like chloroquine by virtue of their lysosomotropism.

Chloroquine has been shown by Weissmann[5] to stabilize the lysosomal membrane *in vitro*. Evidence of *in vitro* labilization has been presented,[66] but almost certainly reflects increased mechanical fragility of the lysosomes as a result of their increase in volume. To what extent the anti-inflammatory effect of chloroquine is due to stabilization of the lysosomal membrane[5] and to inhibition of proteolysis[58] is not known.

Streptomycin. This antibiotic is known to have little effect on intracellular bacteria, even those that are highly sensitive to it *in vitro*, and this phenomenon was originally ascribed to the inability of the drug to enter cells.[67,68] More recent experiments have invalidated this explanation, showing that streptomycin does in fact penetrate into cells, slowly but steadily, to the point of reaching an intracellular concentration 3–4 times that in the medium.[69] The possibility remained that the drug may fail to reach the sites occupied by the bacteria in the vacuolar system, suggesting that its efficiency might be enhanced significantly if it were administered in lysosomotropic form. Exploratory experiments initiated as a first step toward the development of such a form did however disclose that streptomycin is naturally lysosomotropic.[70,71] In rat fibroblasts exposed to the antibiotic, uptake goes on for some 3–4 days, at a rate almost directly proportional to the concentration of the drug in the medium (Fig. 10). In cells fractionated after the plateau was reached, the bulk of the drug taken up was shown to be associated with lysosomes, where it could be estimated to be concentrated almost 100-fold over the outside medium.

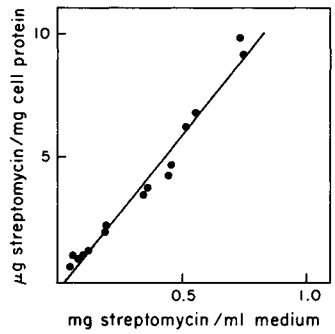

FIG. 10. Kinetics of uptake of streptomycin by rat fibroblasts. Ordinate gives cell content of drug after 4 days exposure to concentration indicated in abscissa. Unpublished results of P. Tulkens.

On a semi-quantitative level, the kinetics of lysosomal concentration of streptomycin are compatible with the protonation theory. Streptomycin is a basic compound, theoretically retainable by protonation. But it has three basic groups, including two strongly basic guanidino groups, and should therefore be expected to permeate very slowly (Fig. 4) and perhaps, if α is not low enough, to reach lower steady state levels (Fig. 3) inside lysosomes than do weak bases. This is exactly what is found. Here again, however, a very high permeability coefficient to the unprotonated form must be assumed, since the concentration of this form in the outside medium must be almost vanishingly low.

It should be noted that in the case of streptomycin, penetration by the alternative route of endocytosis cannot be entirely ruled out, especially if the antibiotic is taken up in association with serum proteins that are endocytized selectively. Nonselective endocytosis (see below) would require a pinocytic rate of the order of 0·65 ml per g of wet cells per day, or 4 times the value estimated for rat liver by Wattiaux.[72]

Whatever the mode of entry of streptomycin into the lysosomes, its low antibiotic activity on bacteria growing intracellularly deserves an explanation. Several factors have to be taken into account. Entry of the drug is very slow and it may be that insufficient time was allowed for its penetration in the experiments where negative results were recorded. Indeed, it has recently been found by Chang[73] that streptomycin will kill intracellular bacteria in cells exposed for a sufficiently long time to large amounts of the antibiotic. It is also possible that the bacteria were located mostly in phagosomes rather than in lysosomes, as mentioned above. Finally, the intralysosomal acidity is very unfavorable to the action of streptomycin: the LD_{50} of streptomycin for *Bacillus subtilis* was found to be 24 times larger at pH 5 than at pH 7.[71] Thus the concentration mechanism is to some extent self-defeating in that it relies on physical conditions that depress biological activity.

Other agents. Allison and Young[41,42] have listed the numerous compounds that have been seen to accumulate within lysosomes. In addition to those already mentioned, their list includes a variety of substances such as polybenzenoid hydrocarbons, uroporphyrin, vitamin A and others, that lack a suitable basic group and must be retained by another mechanism.[40] The kinetics of penetration of these substances have not been studied and their mode of uptake is not known. The possibility that some of their biological properties may depend on lysosomotropism must be kept

in mind. There is good evidence that lysosomes laden with light-absorbing compounds are involved in a number of photochemical injuries.[16,51,52] Lysosomal accumulation of carcinogenic hydrocarbons has been implicated in the development of cancer.[51,74]

AGENTS ENTERING BY ENDOCYTOSIS

Kinetics

The kinetics of endocytosis have been examined by Jacques[75] who distinguishes fluid endocytosis and adsorptive endocytosis. These two categories will be referred to here as nonselective and selective.

Nonselective endocytosis. Also termed passive, this kind of endocytosis is due to simple entrainment of solutes within fluid droplets taken in by pinocytosis. It obeys the following equation:

$$\frac{dQ}{dt} = vC_1 \tag{12}$$

in which: Q is the amount of endocytized substance; C_1 is the extracellular concentration of the substance; and v is the endocytic activity of the cells expressed in terms of the volume of fluid taken in per unit of time.

Selective endocytosis. Preferential uptake takes place for all substances capable of combining with certain sites on the plasma membrane.

Let R be the maximum amount of substance the membrane can bind per unit surface area (surface density of receptor sites); K_s be the concentration of substance at which the membrane is half-saturated (dissociation constant of the membrane–substance complex); and θ be the average surface to volume ratio of the endocytic vacuoles (in first approximation, three times their reciprocal average radius).

Then:

$$\frac{dQ}{dt} = v\left(C_1 + \theta R \frac{C_1}{K_s + C_1}\right). \tag{13}$$

Cellular specificity

So far very few comparative studies have been made of the pinocytic activities of different cell types, especially with respect to their chemical specificity. Such studies are essential for the development of lysosomotropic drugs relying on selective endocytic uptake by the target cells for the achievement of cell-specific effects. There are indications in the literature that they may yield very rewarding results.

For instance, Ryser[76] has found the uptake of iodinated albumin by an established tumor-cell line, Sarcoma S 180 II, to be stimulated by basic polypeptides and inhibited by polyglutamate. In contrast, polyglutamate and other polyanions were found by Cohn and Parks[77] to be among the most effective inducers of pinocytosis in mouse peritoneal macrophages; polycations were very toxic to these cells.

Of considerable interest also is the work of Morell and co-workers[78–81] who have shown that the uptake of ceruloplasmin and other glycoproteins into the lysosomes of hepatocytes is increased considerably by removal of sialic acid and uncovering of a terminal galactosyl residue in the oligosaccharide side-chains. Here again, the

mouse macrophage behaves entirely differently: fetuin fails to induce pinocytosis in this type of cell after desialation.[77]

Examples

Enzymes. After the discovery by Hers of the first inborn lysosomal disease (for a review see Ref. 7), it was pointed out that such enzyme deficiencies are uniquely open to replacement therapy since the normal endocytic route could be used to convey the missing enzyme to the lysosomes.[82] This idea was first put to a test by Jacques and Bruns[83] who found that hepatic sucrose injury could be prevented in rats pretreated with yeast invertase. Detailed studies[84] indicated that after intravenous injections, the invertase is taken up largely by the liver, where it decays slowly, with a half-life of 5·4 days, residing within particles resembling lysosomes in many respects. The enzyme is definitely active intracellularly and is strategically placed to hydrolyze sucrose molecules entering by pinocytosis, thereby preventing vacuolation caused by the intralysosomal accumulation of the disaccharide (Fig. 11). Cohn and Ehrenreich[85] found subsequently that invertase can also be used in a curative way, on macrophages vacuolated by exposure to sucrose.

A natural model system was provided by the finding that fibroblasts from patients with storage diseases will, when cultured under appropriate conditions, display signs of the disease,[86] and that these anomalies can be corrected by normal human serum[87] and by factors secreted by fibroblasts of different genotype.[88] Especially in the hands of Neufeld and her co-workers,[89] these observations have yielded rich dividends, including the discovery of new enzymes and that of a selective mechanism of uptake of some lysosomal enzymes. At present, correction of a lysosomal defect in fibroblasts from patients by added factors has been accomplished successfully in a dozen different genetic storage diseases[89,90] (Fig. 12). In most cases, the corrective factor has been clearly identified as the deficient enzyme, uptake of the enzyme by

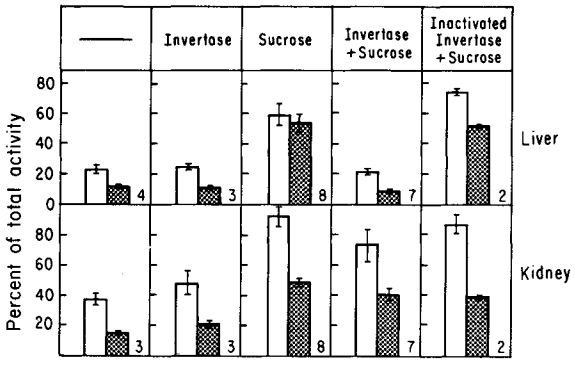

FIG. 11. Prevention of sucrose injury by pretreatment with invertase. Open bars show free activity, shaded bars unsedimentable activity of acid phosphatase in 0·25 M sucrose homogenates of liver and kidney of control rats, rats pretreated with yeast invertase (0·1 mg/g body weight i.v., about 2 days before sacrifice), sucrose (7·65 ml of 50% (w/w) solution, i.p., for 200 g rat, about 1 day before sacrifice), invertase + sucrose, and heat-inactivated invertase + sucrose. Increase in free and unsedimentable enzyme activity denotes increased fragility of lysosomes due to sucrose-induced swelling. This increase is prevented completely by active invertase in liver, only slightly in kidney. Shown are means ± S.E.M. and number of animals. Results of Jacques and Bruns,[83,84] with the kind permission of the authors.

the cells has been demonstrated, and correction has been assessed biochemically by the breakdown of endogenous storage material or of endocytized exogenous material or of both. Absolute proof that the corrective enzyme is taken up by endocytosis and is stored in the lysosomes where it exerts its clearing effect has not been provided, but the circumstantial evidence supporting this interpretation is overwhelming.

In the course of such studies, Bach *et al.*[91] made the remarkable finding that α-L-iduronidase, the enzyme deficient in Hurler and Scheie diseases, was taken up in a highly selective manner by the fibroblasts of a Hurler patient. After 48 hr, the cells had removed up to 40 per cent of the enzyme present in the medium, as against less than 1% of ^{125}I-labeled albumin. It was subsequently found by Hickman and Neufeld[92] that human fibroblasts selectively take up also bovine liver β-glucuronidase and *N*-acetyl-β-glucosaminidase isolated from the medium in which normal human fibroblasts have been grown, but that they fail to do so for any of the three glycosidases if these originate from medium in which I-cells had been grown (I-cell disease is a condition in which cultured fibroblasts display enlarged lysosomes associated with the deficiency of many lysosomal enzymes). The authors conclude from their findings that these lysosomal enzymes, and perhaps others, carry a recognition site for a receptor on the fibroblast membrane, and they suggest that I-cell disease may be a genetic deficiency affecting the synthesis of this site. Selective uptake of *N*-acetyl-β-glucosaminidase could be abolished by mild treatment of the enzyme with periodate under conditions that do not impair enzyme activity, suggesting that the recognition site is of carbohydrate nature.[93]

How many other cell types besides fibroblasts possess the appropriate receptor is not known. Another interesting question, unanswered at the present time, concerns the number of lysosomal enzymes that possess the recognition site. Selective uptake of purified *N*-acetyl-β-glucosaminidase could not so far be demonstrated in Tay-Sachs[94] or Sandhoff disease.[95]

The success of *in vitro* enzyme replacement therapy has encouraged numerous attempts on patients. Measures tried so far include injections of enzyme preparations, among them purified human enzymes, administration of human plasma or leukocytes, and in a number of cases kidney transplantation as a means of ensuring a continuous supply of the missing enzyme. According to the proceedings of a recent symposium at which most clinicians engaged in this kind of work have confronted their experiences,[90] replacement therapy has not proved very successful so far in congenital storage diseases, with the possible exception of Fabry disease. Major difficulties, in addition to the obvious technological and immunological problems associated with any enzyme therapy, seem to be, on one hand the avidity of the liver for the injected enzymes, and on the other hand the endothelial and other barriers that limit the accessibility of many cells to injected proteins, in particular of neurones, which are severely affected in so many of these diseases. Insufficient pinocytic activity or lack of suitable receptors in certain cell types may create additional complications. It is, however, not excluded that with better knowledge these problems may yield to appropriate molecular engineering.

Antibodies. Immunoglobulins are known to be taken up within lysosomes, but to be rather resistant to catheptic proteolysis.[96–99] Thanks to these properties, lysosomal digestion can be interfered with in living cells by exposure to inhibitory antibodies raised against lysosomal enzymes. This was accomplished by Tulkens *et al.*[97]

in fibroblasts exposed to antisera prepared against a complete lysosomal extract and containing antibodies inhibitory to several lysosomal hydrolases, and by Dingle *et al.*[100] in macrophages exposed to a strongly inhibitory anti-cathepsin D antibody. In both experiments, the evidence suggests that the inhibitory effects were exerted inside the lysosomes by pinocytized antibody molecules. Fibroblasts treated with anti-lysosome antiserum developed typical morphological signs of a storage syndrome, their lysosomal compartment increasing from 4 to 25 per cent of the total cell volume in less than 4 days.[11,97]

Polysaccharides. Dextran accumulates in lysosomes, as was first demonstrated morphologically by Daems,[101] and later confirmed biochemically thanks to the observation that lysosomes become denser, both in liver[36,72] and in spleen,[102] after injection of dextran. The mechanism and kinetics of dextran uptake have not been investigated. Penetration of the polymer by endocytosis seems most likely. Other uncharged polysaccharides such as levan, inulin and ficoll probably behave in the same way.

Dextran which is used medically as a plasma substitute and as a vehicle for iron, is not innocuous.[103] Its toxicity could very well be due to its accumulation in, and very slow clearance from, lysosomes.

Acidic polysaccharides are much more efficient inducers of pinocytosis in macrophages than their uncharged congeners.[77] They are also considerably more toxic. For instance, dextran sulfate, which accumulates in lysosomes,[104] renders animals highly susceptible to bacterial infection, probably by impairment of their macrophage function.[105] This could be the case also of carrageenan, a sulfated polygalactose extracted from seaweeds, widely used in the food industry as an emulsifier. A potentially dangerous derivative of native carrageenan, obtained by partial acid hydrolysis, is used in the therapy of peptic ulcers. This substance, which accumulates in the lysosomal system,[106] resists the action of purified rat liver lysosomes, and interferes with many acid glycosidases (Van Hoof, unpublished data).

Triton WR-1339. This nonhemolytic nonionic detergent is perhaps the best known of lysosomotropic agents, owing to its practical value in allowing the isolation of hepatic lysosomes.[107] It came to be used for this purpose in a very roundabout fashion.

Triton WR-1339 was first synthesized as part of a series of polyoctylphenolpolyethyleneglycols for possible use, in aerosol form, as a fluidifier of bronchial secretions. It was soon found to produce considerable hyperlipemia and hypercholesterolemia.[108] This observation stimulated a large number of investigations, including one on liver lysosomes which was prompted by an earlier finding showing that cholesterol is a potent stabilizer of lysosomes *in vitro*.[109] It seemed of interest to find out whether this effect also takes place *in vivo*. In the course of these experiments, it was found that injection of Triton WR-1339 causes a very marked decrease in the density of hepatic lysosomes, and the subsequent analysis of this phenomenon showed it to be the consequence of massive intralysosomal storage of the detergent.[72,110] In plasma, Triton WR-1339 is bound to lipoproteins;[111] it is cleared very slowly, apparently by nonselective endocytosis.[72] Numerous other tissues also have been found to store the detergent in their lysosomes.

In a completely different area, Triton WR-1339 was used by Cornforth *et al.*[112] as a means of studying the effect of experimental hyperlipemia on the course of tuber-

culosis infection. Thus the remarkable protection afforded by the detergent against *Mycobacterium tuberculosis* was discovered. This effect was subsequently shown to be indirect and mediated by monocytes,[113] and to extend also to other intracellular parasites, including leishmaniae and trypanosomes.[114,115] There seems to be little doubt that Triton WR-1339, as well as the related "Macrocyclon", owes its anti-infectious properties to its lysosomotropism, rendering the vacuolar apparatus inhospitable to certain invading microorganisms. But how it does this is not clear. Its effect has been correlated both with an inhibitory action on lysosomal lipolysis[116] and with the accumulation of a heat-stable inhibitor of mycobacterial growth, apparently of lipid nature and neutralized by bovine serum albumin.[117] The situation has been rendered even more puzzling by the recent finding that tuberculous bacilli seem to proliferate mostly in phagosomes rather than in lysosomes, and to have the property of inhibiting phagosome-lysosome fusion.[19,20] So far, no satisfactory hypothesis bringing together these apparently mutually contradictory facts has been put forward.

Early reports that Triton WR-1339 has anti-tumor properties[118–120] have been followed by conflicting data concerning its effect on the metastatic spread of tumor cells. Both inhibition of this process[121,122] and its acceleration[123,124] have been described. It is not known whether these effects bear any relationship to the lysosomotropic properties of the detergent.

Polyvinylpyrrolidone and polyvinylpyridine N-*oxide.* Polyvinylpyrrolidone, originally employed as a plasma substitute, is still used as a slow-release agent mostly in posterior pituitary hormone therapy. Its administration in diabetes insipidus is responsible for an iatrogenic disease usually displaying benign dermatological symptoms,[125] but sometimes progressing to death.[126] The polymer is stored in lysosomes,[13,127] as is the related substance polyvinylpyridine-*N*-oxide, which has been used successfully by Allison *et al.*[13] to protect macrophages against silica injury. Apparently the polymer, thanks to its great hydrogen-bonding capacity, coats injected silica crystals and prevents them from interacting with the lysosomal membrane and causing its rupture.[14]

Trypan blue and suramin. Trypan blue is the prototype of those nonpermeant dyes that accumulate in lysosomes following pinocytic uptake.[128] It probably enters cells in combination with plasma proteins,[129] and should perhaps be better described as entering by piggyback endocytosis. Which of the two, protein or dye, is the carrier in this case has not been determined. It could very well be the acidic dye, acting similarly to fluorescein which has been shown to accelerate considerably the uptake of the proteins to which it is bound.[130,131] Suramin, which has replaced trypan blue as a trypanocidal agent, binds very strongly to plasma proteins.[129] It is also taken up within lysosomes.[132,133]

Lloyd and co-workers[128,133–135] have made the interesting suggestion that trypan blue and related compounds may owe their main pharmacological and toxic properties, in particular their teratogenic activity, to their ability to inhibit lysosomal enzymes. They have adduced considerable evidence in support of this hypothesis, including the direct demonstration of impaired proteolysis within isolated lysosomes.[133] The mechanism of action of the dyes could, however, involve more than simple intralysosomal enzyme inhibition, since trypan blue was found to inhibit also the pinocytic uptake of proteins by isolated yolk sacs.[135] It is interesting that

simultaneous inhibition of protein uptake and of intralysosomal proteolysis was similarly observed in fibroblasts exposed to inhibitory anti-lysosome antibodies.[97]

We are not aware of any lysosome-oriented studies dealing with the trypanocidal effect of the drugs. That this effect may be directly related to the drugs being lysosomotropic for the parasites represents an intriguing possibility.

Micrococcin. Of interest from a historical point of view are the attempts that were made more than 20 years ago by Markham, Florey and their co-workers[136–138] to use a phagocytizable insoluble antibiotic, micrococcin, as antituberculous agent. Their results were disappointing, probably because of the low activity of the drug used. The basic concept, of using an antibiotic with the right tropism, was obviously correct.*

Small molecules. It has long been known that injection of hypertonic sucrose causes extensive cell vacuolation in the kidneys and liver. Similar injuries have been observed in a variety of cell types exposed to sucrose *in vitro*. There is undisputable evidence, both morphological and biochemical, that the vacuoles are swollen lysosomes filled with pinocytized sucrose.[72,85,139–142]

The phenomenon of sucrose vacuolation is a particular case of a general phenomenon, which is the osmotic swelling of lysosomes that accumulate large quantities of an osmotically active solute. We have encountered it above as a consequence of the intralysosomal trapping of permeant bases. When the mode of entry is endocytosis, vacuolation depends on the inability of the solute both to permeate at a significant rate across the lysosomal membrane and to be converted by hydrolysis to permeant products. Cohn and Ehrenreich[85,143] have made an ingenious application of these principles in their studies on the induction of macrophage vacuolation by various oligosaccharides and peptides.

The term "osmotic swelling" must be accepted cautiously and should not be taken to imply that the lysosomes are hypertonic with respect to their surrounding. Fusion of small lysosomes with each other and with incoming pinocytic vacuoles, to form vacuoles of increasing size, probably accounts largely for the increase in total lysosomal volume, which may not require much increase in membrane surface area.

AGENTS ENTERING BY PIGGYBACK ENDOCYTOSIS

Kinetics

For drugs entering by piggyback endocytosis, at least three kinetic factors must be considered: (1) the rate of endocytic uptake of the complex; (2) the rate of intracellular processing leading to release of the drug inside the lysosomes; (3) the rate of diffusion of the drug out of the lysosomes. For drugs that are inactivated at a measurable rate inside the lysosomes, this process must be taken into account as well.

Entry. In most cases, the kinetics of penetration of a drug entering cells by piggyback endocytosis will be governed by those of the carrier. It may, however, happen that combination with the drug may modify favorably or unfavorably the rate of uptake of the carrier. In any case, we would expect uptake of the complex to follow

* We are indebted to Dr. G. Mackaness for drawing our attention to this interesting attempt.

the kinetics of selective endocytosis (equation 13, p. 2513), which in first approximation may be represented by the simpler formula:

$$\frac{dQ}{dt} = v0R \frac{C_1}{K_s + C_1} \tag{14}$$

In this equation, there are four cellular parameters that can be exploited to achieve selective uptake by the chosen target cells:

v, the overall pinocytic activity of the cells; θ, a measure of the amount of membrane internalized for a given pinocytic activity; R, the surface density of binding sites on the cell membrane; and K_s, the dissociation constant of the substrate–membrane complex.

The choice is obviously very wide. Another interesting feature of piggyback endocytosis comes to light when it is compared to permeation, which obeys the following equation:

$$\frac{dQ}{dt} = AP(C_1 - C_2) \tag{15}$$

which includes two cellular parameters:

A, the surface area of the plasma membrane, and P, its permeability coefficient to the drug.

At zero time ($C_2 = 0$), the ratio E of the rate of penetration of the drug in combination with an endocytizable carrier to its rate of permeation in free form is given by:

$$E = \frac{D}{K_s + C_1} \tag{16}$$

in which:

$$D = \frac{v0R}{AP} \tag{17}$$

We see that:

$$E < 1 \text{ if } C_1 > D - K_s$$
$$E = 1 \text{ if } C_1 = D - K_s$$
$$E > 1 \text{ if } C_1 < D - K_s$$

In other words, provided K_s is smaller than D, penetration by piggyback endocytosis always becomes more rapid than permeation when the outside concentration falls below a certain threshold. This means that with a favorable combination of parameters pharmacological effects may be obtained at distinctly lower drug concentration when intracellular penetration of the drug occurs by piggyback endocytosis than when it does by permeation.

Processing. After endocytic uptake, a finite time, often of the order of 10–15 min, is required for the endocytic vacuole to come into contact and fuse with a lysosome. After this delay, the rate of release of the drug will depend on the sensitivity of the drug-carrier bond to enzymatic rupture and on the richness of the lysosomes in the required enzymatic activity. If intralysosomal acidity plays a role, the lysosomal pH will also be important.

Exit. The rate of permeation of the free drug out of the lysosomes obviously represents a crucial factor whether an intralysosomal or an extralysosomal action is aimed at. It must be as slow as possible in the former case, and sufficiently fast in the latter. These conditions are all the more stringent, the more sensitive the drug to lysosomal inactivation.

Examples

With antibodies as carrier. The role of cytophilic antibodies in stimulating phagocytosis by leukocytes and macrophages has been known for a long time and could be used to direct drugs into cells that have Fc receptors on their membrane. In addition, there is now increasing evidence that binding to immunological determinants of the membrane may initiate endocytosis.[144] A well documented example occurs in connection with the "capping" phenomenon.[145–149]

A number of attempts have been made to use antibodies[150–157] or lectin-like substances[158–159] able to combine specifically with certain surface antigens of cells, as carriers of cytotoxic agents. Although the complexes were not specifically designed to be lysosomotropic and their mechanism of action has not been studied, entry by endocytosis followed by intralysosomal processing could have mediated some of the observed effects, especially when the target of the cytotoxic agent (alkylating agent or diphtheria toxin) was itself intracellular. Other mechanisms must however be considered, including a simple synergistic effect unrelated to any carrier role of the antibody.[157]

With proteins other than antibodies as carrier. A number of workers have attached antitumoral agents to certain proteins believed to be preferentially taken up by tumor tissues,[160–167] though here again a lysosome-mediated effect was not considered nor examined experimentally.

Rogers and Kornfeld[168] have made an ingenious application of the discovery by Morell and co-workers[78–81] that hepatocytes have an endocytic receptor for desialated glycopeptides with terminal galactosyl residues on their side-chains. Using asialofetuin glycopeptide as carrier, they have been able to increase the rate of hepatic uptake of albumin or lysozyme as much as 10-fold by chemically coupling these proteins to the vector peptide. They explicitly mention the possible use of this technique "to specifically induce the hepatic uptake of other substances such as drugs".

Liver necrosis in intact animals and selective killing of macrophages in culture have been accomplished by Fiume and co-workers[169–171] by means of amanitin, an inhibitor of RNA polymerase II, conjugated with bovine serum albumin. The hepatic toxicity of this complex could be further enhanced by coupling with fluorescein[172] which we have seen increases the pinocytic uptake of proteins.[130,131] Although the authors offer no direct evidence of a lysosomotropic mode of action, this seems very likely. This interpretation implies that amanitin, which is a cyclic octapeptide, can diffuse out of the lysosomes, since its target enzyme is located in the nucleus.

The microbial diphtheria toxin and two highly toxic seed proteins, ricin and abrin, could well turn out to be natural lysosomotropic agents of the piggyback type. Made up of a simple polypeptide chain of 60,000–65,000 MW, they are easily split into two fragments neither of which is toxic to intact cells. This splitting is however necessary for the toxins to manifest their activity, which depends on inhibition of protein synthesis by one of the fragments (A). The other fragment (B) has a strong affinity for

the cell membrane and is required for penetration of the toxins.[173] It is not known as yet where the splitting takes place nor how the toxin enters cells. The possibility of endocytic uptake followed by lysosomal separation of the active A fragment from the B carrier is an attractive one, but requires a special mechanism to allow the A fragment, a polypeptide of 24,000 MW, to escape out of the lysosomes.

With DNA as carrier. Trouet *et al.*[174] have used DNA as vector for daunorubicin, a potent cytotoxic agent which is used in the treatment of leukemia. Daunorubicin owes its toxicity to its ability to form tightly bound intercalating complexes with DNA, thereby inhibiting gene duplication and transcription. Its clinical use is complicated by fast excretion, by high toxicity on rapidly dividing normal cells, and also by a poorly understood deleterious effect on heart muscle. It was reasoned that these drawbacks might be alleviated to some extent by administration of the drug in the form of a suitable lysosomotropic complex with a macromolecular carrier. Renal excretion, as well as uptake by heart cells, which probably do not pinocytize very much, should be curtailed, while the high endocytic activity reported for several types of tumor cells might ensure a preferential uptake of the drug by the target cells. DNA, a potent inducer of pinocytosis[77] easily degradable by lysosomes[175] promised to be a particularly convenient carrier, since its high affinity for the drug might make the need for a covalent linkage unnecessary.

Experimental testing confirmed these predictions. The DNA-daunorubicin complex showed the necessary physico-chemical properties, in being both stable under normal conditions, and broken with release of active drug by lysosomal digestion. The drug itself proved resistant to lysosomal inactivation, as required. When tested on a variety of isolated cell systems including several types of human leukemia and cancer cells, the complex was found to be as effective as the free drug in inhibiting cell growth and survival, at least in terms of the final degree of inhibition achieved. Kinetically the effects were equally rapid for the two forms of the drug, or sometimes even faster for the complex, when low doses were used, whereas at high dosage the complex acted more slowly than the free drug (Fig. 13). This is in conformity with the predicted behavior (equation 16). Morphological examination provided further confirmation that when carried by DNA, the drug is indeed supplied to the cells by way of the lysosomes, in agreement with the model of Fig. 1.[71] Finally, tests on normal mice and on mice inoculated either intraperitoneally or intravenously with

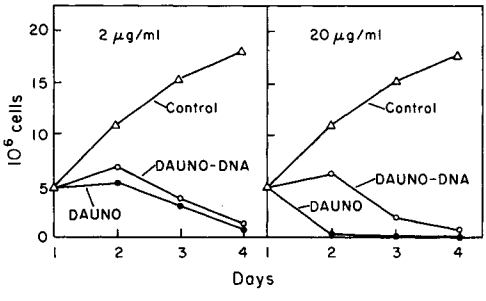

FIG. 13. Influence of free daunorubicin and of DNA-daunorubicin complex on growth and viability of Burkitt lymphoma cells, with two different concentrations of drug. Unpublished results of A. Trouet, D. Deprez-De Campeneere and M. Lamy.

L1210 leukemia cells showed the complex to be distinctly less toxic and therapeutically more effective than the free drug. As expected, the chemotherapeutic activity of the complex depends greatly on the administration route. To be effective, the complex has to be injected into the same compartment, peritoneal cavity or blood, as the inoculated cells.

More recently, a similar series of experiments has been performed with adriamycin, an antibiotic closely related to daunorubicin from which it differs only by the presence of an additional hydroxyl group. The DNA-adriamycin complex was found to have all the properties of the DNA-daunorubicin complex, and to have an even

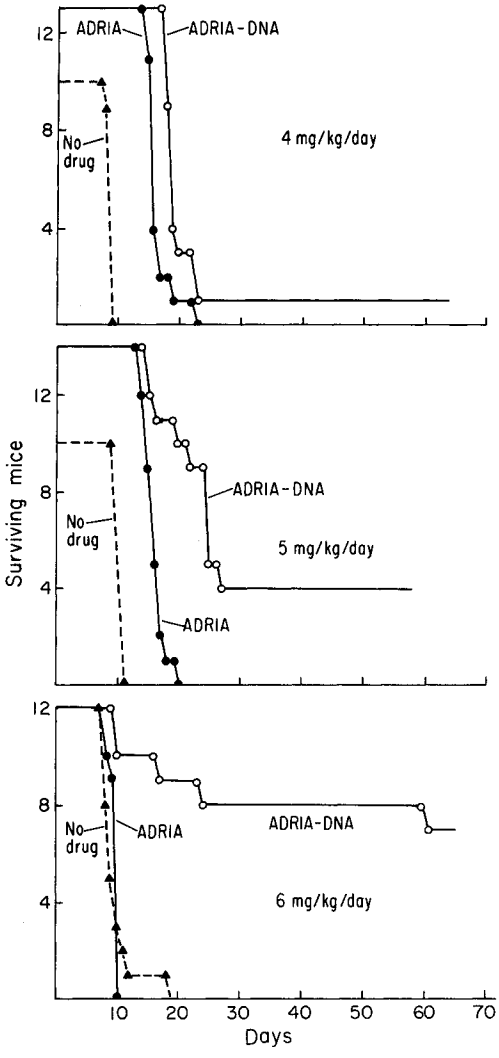

FIG. 14. Chemotherapeutic effect of free adriamycin and of DNA-adriamycin on L1210 leukemia. DBA/2 mice were inoculated on day 0 with 10^4 L1210 leukemic cells given i.v. Treatment consists of 5 daily i.v. injections, on days 1–5, of drug at dosage indicated. Unpublished results of A. Trouet, D. Deprez-De Campeneere and M. De Smedt-Malengreaux.

better therapeutic index than the latter. Its effectiveness on L1210 leukemia is illustrated in Fig. 14.

Prompted by the outcome of these experiments, cautious clinical trials were undertaken, first on terminal leukemia patients who had become resistant to all forms of therapy. The DNA complexes were tolerated remarkably well by these patients and evoked marked hematological responses, even in cases resistant to the free drug.[174,176] These trials were extended progressively. At present, the DNA complexes of daunorubicin and adriamycin are being tested in a number of clinical departments, both in Belgium and abroad. Very encouraging results have so far been obtained in the treatment of leukemia, lymphosarcoma and various solid tumors of children. For example, complete remission was achieved with daunorubicin-DNA in 12 out of 20 cases of non-lymphoblastic leukemia. This result, which compares favorably with the best statistics of others, becomes even more striking since it was attained at very low cost of toxicity; only one patient (age 77) died during the induction period.

With liposomes as carrier. The idea of using liposomes as a means of conveying active agents to the lysosomes was first proposed by Gregoriadis and Ryman[177,178] who showed that suitably prepared liposomes, when injected intravenously, are taken up very actively by liver and spleen cells, ending up within lysosomes. Several proteins incorporated in the liposomes could be delivered to the lysosomes by this device. That the proteins are subsequently released, presumably through the action of the lysosomal lipases, is indicated by a recent report by Gregoriadis and Buckland,[179] who have shown that sucrose vacuolation can be corrected in macrophages or fibroblasts by exposure to liposome-entrapped invertase.

Similar experiments were made in our laboratory with an antibody raised against purified rat acid α-glucosidase, a lysosomal enzyme. Intravenous injection of this antibody enclosed within liposomes to new-born rats caused a 40% inhibition of the hepatic α-glucosidase. Morphological examination of the liver showed clear evidence that the liposomes are taken up into the lysosomes of both Kupffer cells and hepatocytes, and digested there, especially in the Kupffer cells (Fig. 15).

A most interesting application of lysosomotropism by means of liposomes has been made by Rahman and co-workers,[180] who have successfully removed plutonium stored within liver lysosomes by administering a chelating agent, diethylaminetriamine pentaacetate, entrapped within liposomes. Here is an example of a substance, that would tend to be excluded from the lysosomes, and therefore incapable of exerting its clearing effect, by virtue of its anionic character (see p. 2508), being forced into the lysosomes by means of an appropriate carrier.

Liposomes loaded with antiviral antibody have been shown by Magee and Miller[181] to be 3000 to 10,000 times more effective than the free antibodies in protecting cells *in vitro* against a viral infection. The authors attribute this effect to surface protection by liposome-bound antibody attached to the cell membrane. They do not consider the alternative possibility of an intraphagosomal or intralysosomal action of the antibody.

With dextran as carrier. Dextran has been used as vehicle in the administration of iron salts.[182] The development of this preparation was in no way prompted by lysosomotropic considerations. However, it is not impossible that iron-dextran may owe its pharmacological property of slow releasing agent, and conversely its toxicity,

to its lysosomotropic character. Its administration to animals was found to induce marked changes in the hepatic lysosomes.[183]

With other carriers. Baehner *et al.*[184] have used latex particles as carriers for glucose oxidase and succeeded in restoring by these complexes the metabolic deficiency of leukocytes from chronic granulomatous disease patients. The metabolic defect due to a lack of H_2O_2 production during phagocytosis was alleviated in this manner. In this case, of course, there is no lysosomal processing of the carrier, nor is it needed since the bound enzyme is perfectly active.

Also relevant to this topic are the efforts that are being made to prolong the life of injected enzymes and decrease their immunogenicity by microencapsulation within artificial polymers.[185–187] In these cases, lack of uptake by the cells would be an advantage, unless an intralysosomal action is aimed at.

Numerous other possibilities obviously come to mind, especially if the cells of the reticulo-endothelial system are the main target, as for instance in certain infectious and parasitic diseases and in certain thesaurismoses (Gaucher, Niemann-Pick). Even the patient's own red blood cells or thrombocytes could provide a suitable vehicle, well tolerated and fully digestible. Erythrocytes are easily rendered phagocytizable, for instance by 24 hr incubation at 37° in physiological saline.[188]

GUIDELINES FOR THE DESIGN OF LYSOSOMOTROPIC AGENTS

Lysosomotropic therapy offers considerable scope for the design of a new class of drugs to be conveyed by piggyback endocytosis towards the lysosomes of their target cells. We will discuss briefly here the principles that should guide this kind of research.

Choice of drug

The first requirement of any drug destined to act within or by way of lysosomes is a sufficient degree of resistance to inactivation by the local pH and enzyme activities, to allow the desired effects to be exerted. Clearly, the drug must be more stable in the intralysosomal milieu if it is to remain and act within the lysosomes than if it only passes through them. This property is conveniently assessed by incubation at acid pH (around 4·5) in the presence of an extract of hepatic lysosomes, which are easily isolated.[107,189] Not all cell types, however, have the same complement of enzymes in their lysosomes, and further checks with the actual target cell lysosomes may be necessary.

Other requirements depend on the particular site and mode of action aimed at. If the drug is to act within the lysosomes, it should be retained, and preferably concentrated, in these particles, and it should be adapted to act under intralysosomal conditions. Molecular size, lipid solubility, the nature and pK of ionizable groups, all are physical characteristics that may be important in these respects. In practice, it is very important that the effectiveness of such drugs be tested under the right conditions. We have seen the importance of this in the case of streptomycin.

If the drug is to leave the lysosomes and act elsewhere in the cell, the same physical characteristics have to be considered, but in a different context. For instance, we have seen that weak acids will tend to be driven out of the lysosomes. However, escape out of the lysosomes is only a necessary condition of effectiveness, not a sufficient

one. The drug must be retained sufficiently inside the cell, it must be able to gain access to its target and it must be active under the local conditions.

There may be a need for a considerable amount of molecular engineering before the optimal physico-chemical characteristics are obtained. While this may be accomplished by the time-hallowed method of trial and error, the rational approach may well prove less laborious and more economical. But for such custom-making to be possible, the measurements of the target cell must be known. We have seen the important influence exerted on permeation kinetics by the volume and surface area of the lysosomal compartment, relative to other cellular parameters. Simple morphometric methods are available for the gathering of such data.[34,35] Once the data are known, kinetic equations valid for the target cell can be written, the influence of molecular properties can be adequately assessed, and the drug can be tailored to the needs.

A point of considerable importance relates to the toxicity of the drug. In view of the fact that binding to a carrier will often abolish or greatly reduce the pharmacological activity of a drug, as well as restrict its access to certain cell types or tissues, lysosomotropic forms of drugs may be considerably less toxic than the free drugs. We have seen examples of this fact with the DNA complexes of daunorubicin and adriamycin. Consequently, high toxicity should not be considered sufficient ground for rejecting a drug as a possible object for making a lysosomotropic derivative. In fact, such derivatives could possibly allow the use of a number of very active drugs that were never introduced into therapeutic practice owing to their excessive toxicity.

Choice of carrier

By definition, the carrier must be capable of binding the drug in a manner that is at the same time unaffected by the body fluids along the route of administration and reversible under intralysosomal conditions (see below), and it must be endocytizable. Preferably, it should be degradable by the lysosomes, although this may not be an absolute requirement, since storage of the carrier may be a small price to pay for the desired effect. Finally, the carrier must not interfere with the action of the drug and it must be of low toxicity, a requirement which includes lack of immunogenicity whenever prolonged or repeated treatment is foreseen. Carriers of human origin may be particularly advantageous in this respect.

To these general requirements must be added all the characteristics likely to favor preferential or selective uptake by the target cells and to stimulate their endocytic activity. From the limited amount of information presently available on this topic it can already be concluded that such characteristics exist. Charge, molecular size and particular carbohydrate groups have all been shown to affect the pinocytic uptake of molecules in a cell-specific manner.[76–81,91–93,190,191] Also exploitable are immunological determinants and possibly hormone receptors. Needed here is a wide and systematic survey of the endocytic idiosyncrasies of individual cell types.

A crucially important property of the carrier is represented by its ability to actually reach the target cells, when loaded with the drug. In the organism, different cells occupy different environments, which are separated from each other by special cell layers and by structural elements. Unless the drug–carrier complex can be introduced directly into the particular space occupied by the target cells, it must be capable of crossing the interposed barriers. Three processes are important in this respect: (1)

vesicular transport or diacytosis, dependent on endocytic uptake followed by exocytic discharge, a process carried out by endothelial and mesothelial cells; (2) passage through intercellular gaps, a phenomenon which can be aided by certain drugs; (3) filtration through macromolecular meshes such as exist, for instance, in basement membranes and related structures. The molecular size of the carrier may be very important in the latter respect, and studies on selective pinocytosis should focus on this aspect as well. For instance, it has been shown that a simple basic tetrapeptide, Thr-Lys-Pro-Arg or "tuftsin", is responsible for the stimulation of phagocytosis by the cytophilic γ-globulin leukokinin.[192] It is possible that small oligosaccharides or glycopeptides could mimic the specific effects of certain glycoproteins. It would be very useful to know the minimum size required for this to occur. Unfortunately, any property or intervention designed to favor passage across barriers will also tend to favor renal excretion, decreasing the duration of action of the drug. Maximum advantage has to be taken here of the mechanisms whereby the organism itself selectively transports molecules from one space to another.

Choice of linkage

As a rule, the linkage chosen should render the drug pharmacologically inactive, be stable in the environments to which the complex is to be exposed before reaching the lysosomes, and be broken within the lysosomes. There may, however, be exceptions to this rule. For instance, if a carrier is used for the sole purpose of directing a therapeutic agent into the lysosomes of certain cells, the desired effect may conceivably be achieved satisfactorily by using a linkage that does not abolish the activity of the agent, or even that is not split within the lysosomes. An example of this kind is provided by the work of Baehner *et al.*,[184] who attached glucose oxidase to latex particles in order to cause the enzyme to be phagocytized by the leukocytes of patients suffering of chronic granulomatous disease.

Within the above limits, the linkage can be covalent or not. Noncovalent linkages have the great advantage that they can be formed without the help of chemical technology. But they must involve very tight binding. Otherwise sufficient dissociation will occur in the body fluids to defeat the therapeutic purpose. Intralysosomal release of the drug may be favored by the local pH, if the linkage is acid-sensitive. Most often it will depend on digestion of the carrier. Consequently, the influence of drug binding on digestibility of the carrier needs to be investigated. This problem was encountered in the work on DNA daunorubicin.[174] It turned out that drug binding inhibits DNA breakdown to an extent that helps to slow down the undesirable attack of the complex by plasma deoxyribonuclease, but does not prevent its intralysosomal dissociation. Another important variable was recognized in this work, namely that of species differences. It was found that the toxicity of the DNA–daunorubicin complex is to some extent a function of plasma deoxyribonuclease activity, which varies greatly from one species to another and even between strains of the same species. Fortunately, it is particularly low in man.[193] The possibility that chronic administration of the carrier–drug complex may lead to an increased level of lytic enzymes in the plasma must also be kept in mind.[194] So far, no such untoward reaction has been observed in mice receiving DNA-complexes.

If a covalent linkage is used to bind the drug to the carrier, this linkage must either be split within the lysosomes, or leave the drug free to reach its target and act on

it after breakdown of the carrier. It is, for instance, conceivable that a fragment of carrier may remain bound to the drug after digestion is completed. This is no drawback if the resulting drug derivative has the required properties for therapeutic effectiveness. Requirements for sufficient stability and innocuousness before the complex reaches the lysosomes must of course also be met.

Choice of route of administration

This problem is closely related to that, already discussed, of accessibility of the target cells. Clearly, the route of administration must be such as to allow the carrier–drug complex to reach the target cells intact and in sufficient amounts. It should be as close as possible to the actual environment occupied by the target cells, so as to allow the use of slowly excreted long-acting drug complexes. As already mentioned, ancillary drugs may be necessary to favor penetration of the complex to the desired site.

CONCLUSION

Some substances are naturally lysosomotropic; others can be made so by suitable combination with a lysosomotropic carrier. When therapeutic exploitation of this property was first considered, it was simply a theoretical possibility based on our knowledge of lysosome physiology.[30,195] At present, it is an established reality, supported by a number of experimental examples, including at least one of proven clinical worth. A rich new field lies open for therapeutic research.

This research will have to be very different from conventional pharmacology and pharmaceutical chemistry. Its foundation will have to be cell biology; its main objects, the molecular determinants of permeation and endocytosis, the enzymatic activities of lysosomes, the characteristics of cytomembranes, especially plasma membranes and lysosomal membranes, the morphometric measurements of cells, the modalities of macromolecular transport across biological barriers. There is considerable fun in store, as well as the prospect of important therapeutic victories, for those who will engage in this kind of work.

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