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Autor: Ryser, H.J.-P.

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The Uptake of Foreign Proteins by Mammalian Cells and the Functions of Pinocytosis

H. J.-P. Ryser

Most of us have been brought up with the concept that living cells, like semipermeable bags, exchange small molecular solutes with the environment but exclude all foreign macromolecules from entering. It is, of course, still true that the plasma membrane represents an effective barrier to macromolecular intrusions, but it has become evident in the last ten to fifteen years that this barrier is not all exclusive and that foreign macromolecules of many types and sizes can be taken up by mammalian cells. This major reassessment is the result of two independent developments which occurred in the fields of cytology and biology.

In cytology the notion of a strict exclusion of macromolecules was first put into question by the observations of Lewis on membrane movements in macrophages [1]. This author noticed by microscopy that the plasma membrane seemed to give rise to small vesicles, which in time-lapse cinematography were seen to migrate towards the cell inside in a fashion that could be described as "cell drinking". Using the greek word for drinking, Lewis called this process pinocytosis, in contrast to phagocytosis which means cell eating. Mast and Doyle, Holter and Marshall, Chapman-Andresen and others [2–4] found that pinocytosis was even more spectacular in amoebae, where the vesicles seemed to arise at the lower end of long funnels or invaginations of the cell membrane. Chapman-Andresen and Holter were the first to point out that pinocytosis might serve nutritional functions [4]. They showed that glucose which does not cross the cell membrane of amoebae could be detected by autoradiography in pinocytotic vesicles, and later on in their surrounding and in the cytoplasm [4]. It was easy to generalize from there and propose that what happened to glucose in amoebae, could well happen to macromolecules in other cells. The notion that pinocytosis might be a way whereby mammalian cells take up macromolecules, first suggested by Danielli [5], was promoted forcefully by Bennett [6] and was later substantiated by numerous electron microscopic studies using tracer molecules such as haemoglobin, ferritin and peroxydase [7–11]. All such studies, regardless of the tissue under consideration showed the localization of macromolecules in pinocytotic vesicles and related vacuoles. It became obvious from studies of Straus [12] and DeDuve [13] that pino-

cytotic vesicles not only fused with each other but fused with primary lysosomes to receive lysosomal enzymes and become secondary lysosomes or digestive vacuoles. In an early electron microscope study on the uptake of ferritin, we convinced ourselves that these processes did occur in tumor cells [14]. We were surprised at that time to find that ferritin was never detected in the unstructured part of the cytoplasm and always found within vesicles or vacuoles [14]. This observation can be conveniently assumed to mean that pinocytosis is the only way by which macromolecules are taken up by mammalian cells. But it can also be taken to suggest that, after all, pinocytosis might be a meaningless process, since ingested macromolecules are segregated in membrane-bound structures where, it is believed, they undergo enzymatic degradation. Why call it a cellular uptake if macromolecules do not reach the cytoplasm, but remain in enclaves of extracellular space? How can pinocytosis serve a transport function if transported material is destroyed before reaching the cytoplasm? These questions have been part of the criticism voiced against the relevance of measurements of protein uptake. And these questions would have been very difficult to answer without the help of a second major development that took place in another area of biology.

This concurring event was the demonstration of genetic transformation in bacteria and of nucleic acid infections in mammalian cells [15–17]. It established that foreign nucleic acids presented to live bacterial or mammalian cells could not only get into cells, but also express their biological potential inside host cells. Thus, even though infrastructural studies did not establish the presence of foreign macromolecules elsewhere than in vesicles and vacuoles, biological experiments demonstrated that some of them must reach intracellular sites of action. In all probability those that reach these sites are just too few to be traced in their path. This leaves some uncertainty as to the mechanisms of transport of the "elected few". On the one hand it cannot be entirely ruled out that they penetrate without the help of membrane vesiculation. On the other hand, the weight of the evidence and the trend in cell biological thinking strongly suggest that the macromolecules destined to function are indeed taken up by pinocytosis and escape from cytoplasmic vesicles and vacuoles in a second step of transport. Some of this evidence will be discussed later on, but it can be pointed out already that uptake vesicles and vacuoles are labile and rather transient structures, influenced by pharmacological agents, and subject to constant reorganization. Major changes may, therefore, be expected to occur at the level of vacuolar membranes leading either to an escape of enclosed material or to its specific transfer to other structures more closely related to cell functions. In summary, it is quite safe to postulate that pinocytosis is involved in the transport of those macromolecules that express themselves in host cells. Measuring the penetration of proteins into cytoplasmic vacuoles, therefore, has more meaning than simply measuring an irrelevant capture and destruction process. Moreover, regardless of the proportion of breakdown, the first

step of this process, namely, the capture itself, is likely to serve a number of functions. Finally, no matter how macromolecules are transported, the very fact that some of them perform specific functions in host cells is of such fundamental importance that it calls for concentrated studies. These were enough reasons for us to focus upon the process of macromolecular transport. Our first concern was to define a model system in which the penetration of a labelled macromolecule could easily be measured. We chose to work with a tissue culture system using an established line of mouse tumor, Sarcoma S 180 II and to use a commercially available radioactive protein, iodinated human serum albumin as a marker [18].

Measurement and stimulation of albumin uptake

The procedure we have been following is a very simple one: we grow sarcoma cells as monolayers in small tissue culture flasks, and at time zero, replace the growth medium with a very similar medium containing labelled albumin. At intervals, this radioactive medium is washed off, the cells are rinsed, detached with trypsine, washed several times and treated with trichloroacetic acid. The cellular protein is counted for specific activity.

Reproducible measurements of net uptake can be obtained when the following three pitfalls are avoided: namely, when the experiments are done with healthy and intact cells; when a reincorporation of labelled amino acid is ruled out; and third, when a distinction is made between adsorption to the cell surface and intracellular uptake. The use of monolayer cultures and of procedures such as dye exclusion tests, growth tests, and control exposures gave us adequate assurance with regard to the first point. Special *in vitro* studies indicating that monoiodotyrosin cannot be utilized for *de novo* protein biosynthesis showed us that we did not need to worry about reincorporation of I^{131}-labelled aminoacids [19]. Experiments carried out at low temperature taught us how to distinguish between adsorption and uptake. They showed that a 2-minute exposure to labelled medium at 2° C and at 37° C led to identical levels of albumin binding, that could be lowered by successive washings. A 2-minute or 2-hour exposure at 2° C gave essentially identical values, suggesting that adsorption could be measured in at least two ways: short exposures at higher temperature or prolonged exposures at 2° C. At temperatures higher than 4° C, exposures led to an albumin-binding increasing both with time and temperature. These increases resisted repeated cell washings. They correlated to the amount of ferritin detected as a function of time in the electron microscopic study. They represented, therefore, a net uptake of albumin into cytoplasmic vesicles. This net uptake increases linearly with the albumin concentration in the medium and is not susceptible to conventional metabolic inhibitors. Its order of magnitude is 10^4–10^5 molecules per cell per hour. Since the amino acid content of this albumin load represents only a small percentage of the amount of amino acids that the cell can transport actively and incorporate into new proteins, it appears unlikely that under physiological conditions pinocytosis of proteins would

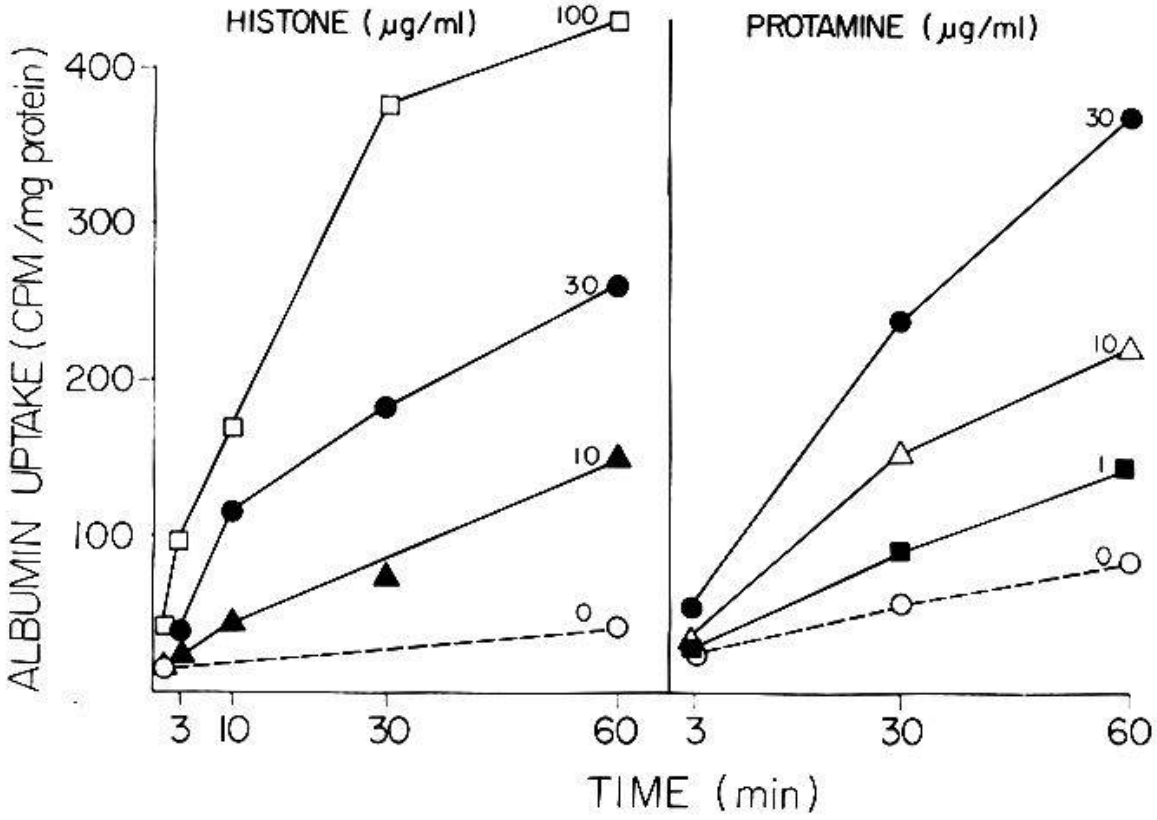

Fig. 1. Effect of increasing concentrations of calf thymus histones (10, 30, 100 μg/ml) and salmon sperm protamine (1, 10, 30 μg/ml) on the time curve of uptake of I^{131}-albumin. The first time points give an estimate of the albumin adsorption to the cell surface. Net uptake is indicated by increases in activity above these initial values. In the controls (interrupted lines), the magnitude of the net uptake is about 1.5–2 times that of the adsorption. The presence of 30 μg/ml of the basic proteins increases the net uptake to 6.4 times (protamine) and 9 times (histone) that of the control. The ordinate gives the number of I^{131} disintegrations per min per mg of cell protein.

represent a meaningful source of nitrogen [18]. We believe that pinocytosis performs a number of more subtle functions, that will be outlined later on.

The low magnitude of protein uptake provided a good baseline for studying conditions that might enhance the process. Amos and his group had already suggested that protamin enhanced the penetration of bacterial RNA into mammalian cells [20]. Both protamins and histones, when used in our system, enhanced albumin uptake to a very marked extent (Fig. 1). Different histone fractions, however, showed different effects [21], the one rich in arginine being considerably more active than the one rich in lysine (Fig. 2). This finding is puzzling in view of the fact that both fractions have very comparable ratios of basic to acidic amino acids, and comparable molecular weights. It suggests that the basicity of the protein cannot alone account for the effect and that perhaps specific sequences of amino acids are determining. The use of synthetic homopolymers demonstrated that poly-L-arginine was indeed more effective than poly-L-lysine in promoting albumin uptake. But the picture was further complicated by the finding that poly-arginine was no more active than polyhistidine, polyornithine and poly-

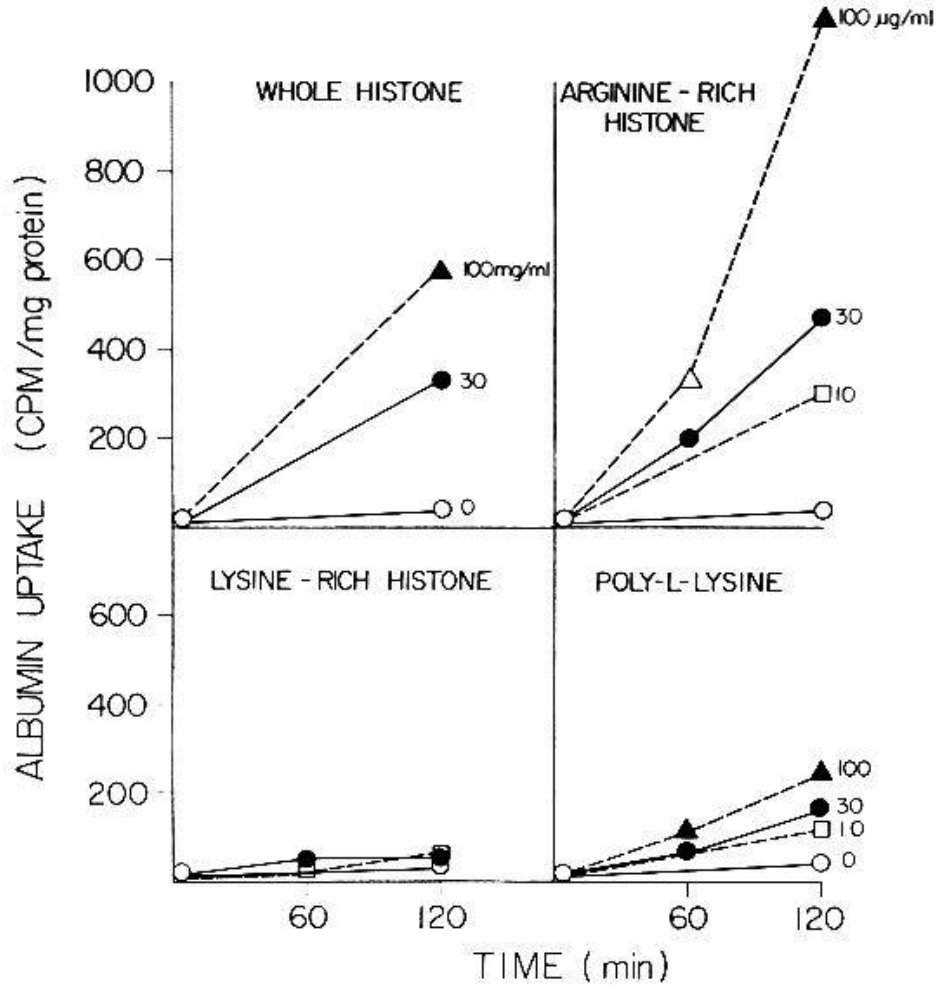

Fig. 2. Effect of three different histone preparations and of poly-L-lysine (MW 50,000) upon the time course of uptake of labelled albumin. The different compounds have been tested at the following concentrations in µg/ml: 10 (open squares), 30 (closed circles), and 100 (triangles). Controls are indicated with open circles. Ordinate as in Fig. 1.

D-lysine [21, 22]. Thus the two isomers of lysine turned out to differ markedly in their effect, particularly when used at higher concentrations (Fig. 3). It is interesting to note that these two optical isomers differ profoundly in yet another biological function, namely, their ability to elicit an immune response [23, 24]. The way in which immunogenicity may relate to uptake in this instance is not yet clear. The similarity of effects of D-lysine, and the three other basic polyamino acids is shown in Fig. 4 where the curve to the left goes through the mean values obtained with polyornithine and where the different symbols about the curve are measurements obtained with one of the other three compounds. Clearly this dose-effect curve is higher and steeper than those of histones, protamines and DEAE-Dextran. The stimulations are up to 50 times and the threshold concentrations are surprisingly low. The four most active basic polyamino acids produced significant effects at concentrations of 0.01 µg per ml, *i.e.* in the case of polyornithine (MW 200,000) at 5×10^{-11} moles per liter, or in terms of population, in amounts

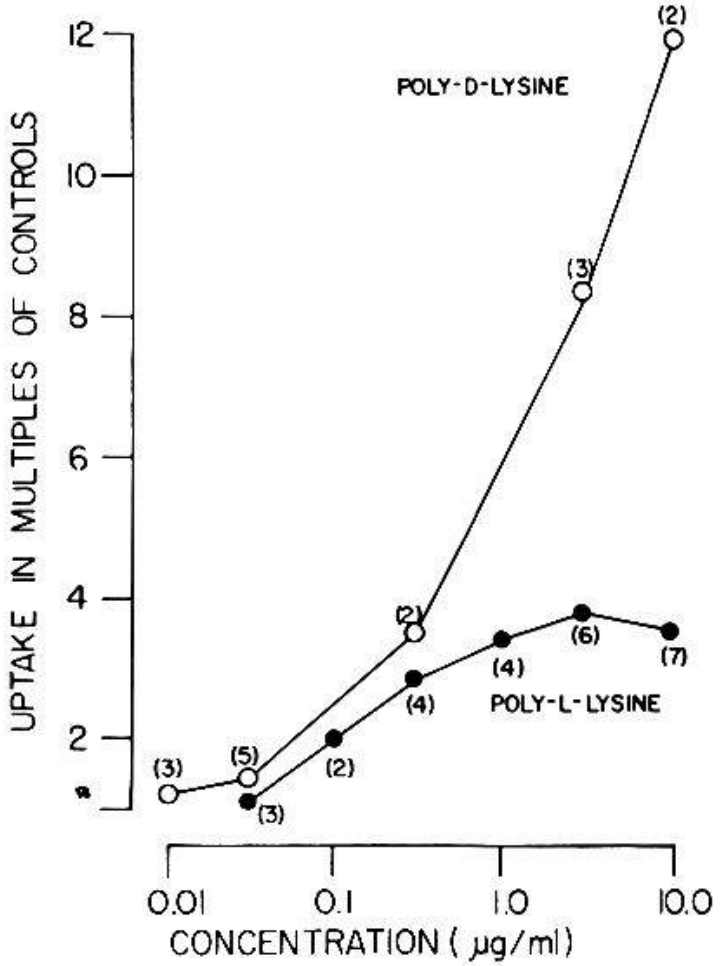

Fig. 3. Semilogarithmic plotting of the dose–effect relationship for the two optical isomers of polylysine. Ordinate: uptake of labelled albumin expressed as multiple of the control values obtained for each experiment. The points are averages of the number of experiments indicated in parenthesis. Incubation for 2 hours at 37° C. in Eagle's medium without serum. The molecular weights were 70,000 and 113,000 for the L- and D-form of polylysine respectively.

of 2000 molecules per cell. This high potency warranted further studies of the mode of action of these basic polymers.

The first point to determine was whether these basic compounds remained on the cell membrane or entered into the cell. Using fluorescein-labelled histones and polyaminoacids, HANCOCK could demonstrate fluorescent droplets distributed in all areas of the cytoplasm including the paranuclear regions [25]. The fluorescence extracted from exposed cells was strong enough to be measured spectrofluorometrically. The measurements showed a much greater penetration of basic macromolecules than of albumin [21]. Experiments in which cells were exposed to both forms of label and in which half of the cells were used to measure the penetration of one of the labels showed furthermore that the penetration of different basic compounds correlated quite closely with their enhancing effect on albumin uptake [21]. This suggests not only an association but a causal relation between polymer and albumin uptake. What is the essence of this relation? Gel chromato-

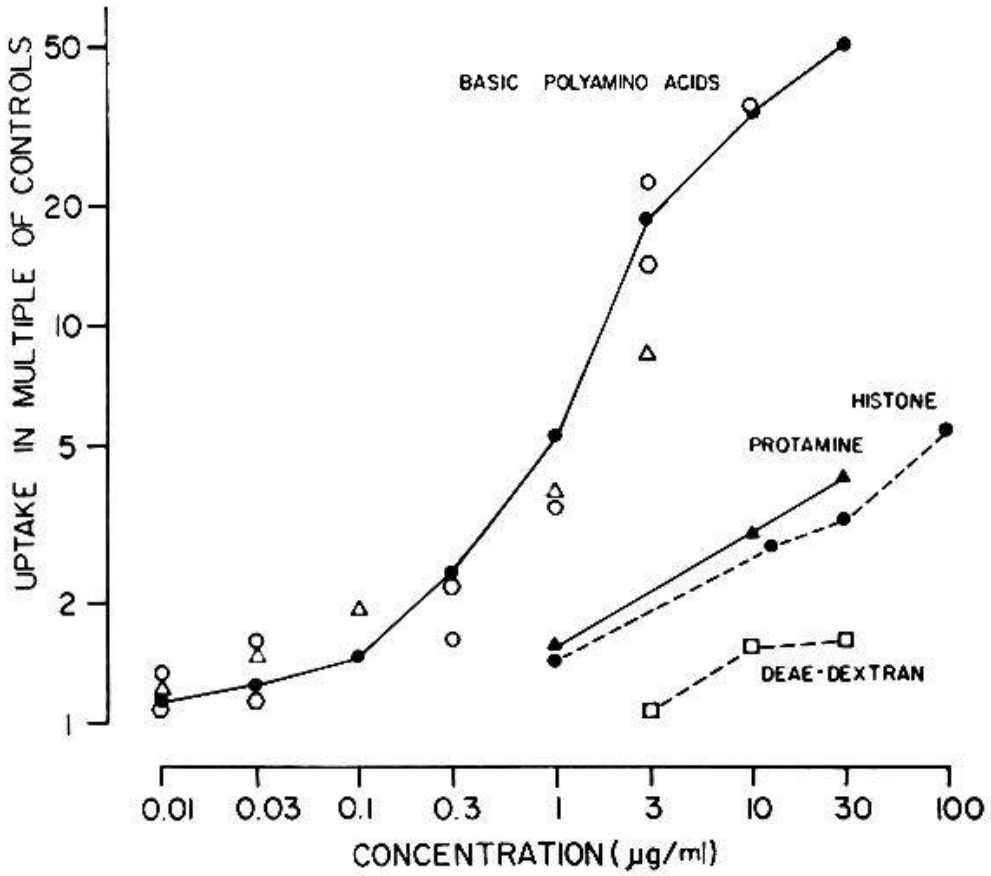

Fig. 4. Semilogarithmic plotting of the dose-effect relationships for four different basic polyamino acids, two basic proteins and a non-peptidic basic polymer (DEAE-Dextran MW 2×10^6). Ordinate as in Fig. 3. The points are means of 2–15 experiments. The curve to the left goes through the mean values obtained with poly-L-ornithine MW 200,000. The other basic polyamino acids tested are poly-D-lysine MW 113,000 (open triangles), poly-L-arginine MW 28,000 (open hexagons), and poly-L-histidine MW 8,000 (open circles). Incubation for 2 hours, 37° C, in Eagle's medium without serum.

graphy of a mixture of poly-L-ornithine MW 200,000 and labelled albumin, passing through a column of Sephadex G 200, shows that the two do not engage in any obvious *in vitro* complex formation, since the elution pattern of albumin is unchanged by the presence of poly-L-ornithine. Moreover, a complex formed *in vitro* would probably be taken up in a fixed stoichiometric ratio, and this was not found to be the case, since the ratio changed markedly with the concentration of albumin in the medium. Rather than to assume a complexing of the two macromolecules prior to uptake, we suggest that albumin is carried into the cell because of its presence on or around the membrane when cationic polymers penetrate into the cell. The question of the mechanism of enhancement must, therefore, be rephrased: What is it about the basic compounds that makes them penetrate so readily into cells and carry albumin in their wake? One factor besides the charge has now been identified; namely, the size of the basic molecule. The role of the size was suggested already by the early observation that diamines such as spermine and spermidine of molecular weights around 200 have no effect on albumin uptake, as if they were too small for the job. Series of

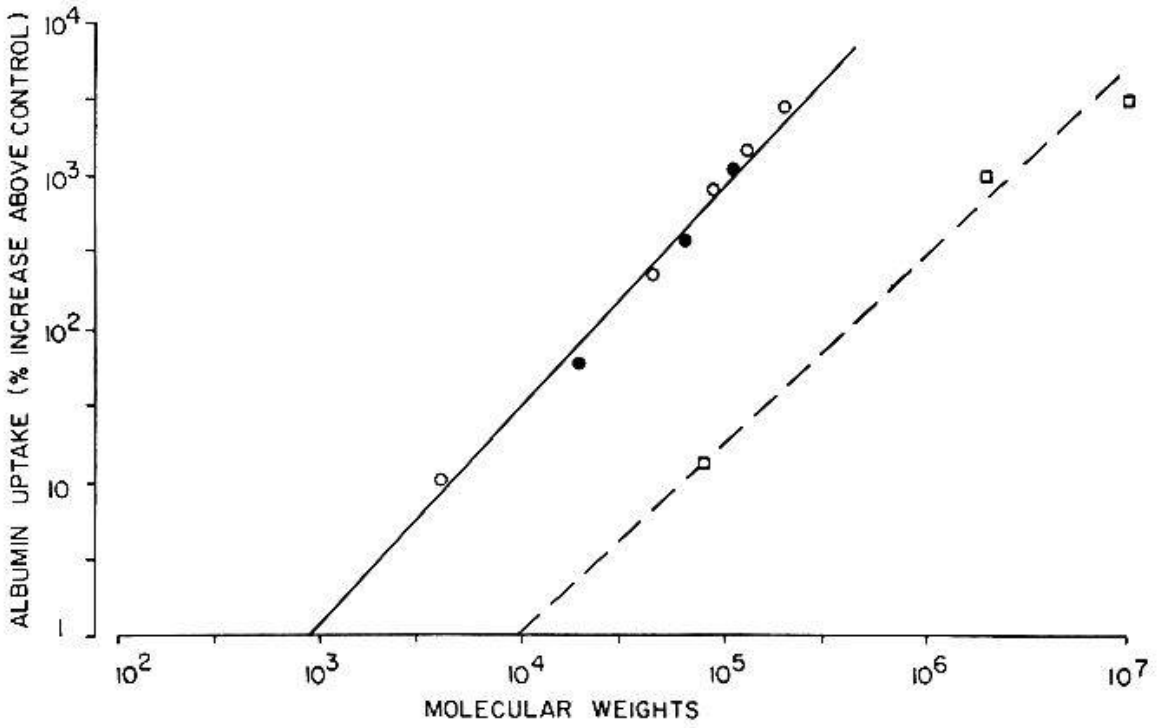

Fig. 5. Correlation between activity and molecular weight of polymers in homologous series of poly-L-ornithine (closed circles), poly-D-lysine (open circles) and DEAE-Dextrans (squares). Ordinate: Stimulation of albumin uptake expressed in percent increases above the uptake of control cells. The experimental data, obtained with 3 μg/ml of the various compounds, were corrected for molarity to express the effect of a uniform concentration of 1.5^{-8} moles/l.

identical polymers differing only by their size were tested at the standard concentration of 3 μg/ml. Homologous polymers of decreasing sizes had decreasing effects even though the smaller molecular compounds were tested at higher molar concentrations [26]. This meant that on a molar basis the drop of effect with size was bound to be even more pronounced. This prediction was confirmed by comparing equimolar concentrations of the various agents or by converting the measured effects of 3 μg/ml into molar effects. Both procedures gave identical results and revealed a strictly linear relation between effects and molecular weights [26] (Fig. 5). The line of Fig. 5 is sufficiently straight to be extended to intersect the abscissa. The intersection corresponds to a molecular weight of about 600–1000 depending upon experimental conditions. These molecular weights are well above the size of spermine and spermidine, which explains quite adequately why these amines are unable to enhance albumin uptake. The figure of 600–1000 is also reminiscent of the minimal size required in experimental immunology to elicit an immune response with synthetic oligopeptides [23, 27]. The linear correlation between enhancement and molecular size has an unexpected meaning when viewed in the context of the other correlation mentioned above, between enhancement of albumin uptake and penetration of polymers. Since the enhancement is in fact due to a faster penetration of the polymer into the cell, it follows that the penetration of the polymers

themselves should increase linearly with their size. This inescapable deduction says, in other words, that large molecules penetrate more easily into cells than smaller ones, a view that at first seems to shake established concepts of membrane transport. It underlines the fact that the transport of small solutes has little in common with the transport of macromolecules. There is evidence from different sectors of biology, that this finding about the effect of molecular size has general validity. It had been shown that labelled DNA was taken up more effectively by HeLa cells when presented as supramolecular aggregates [28, 29]. Immunologists, on the other hand, have known for some time that particulate antigens elicit different responses than soluble ones [30] and that the immunogenicity of synthetic polypeptides increases with their size [27, 31]. One will have to get used to the view that in the case of macromolecules, the bigger ones apparently have the better chance to get in. What does this tell about the mechanisms of penetration? The simplest explanation of this effect of size is that, in order to get in, macromolecules must make multiple attachments to the cell membrane. An increasing size raises the likelihood of multiple and simultaneous attachment. One would, therefore, expect that a rigid polymer or one in which the binding groups are further apart, would also be less active. Our data on the effect of three different sizes of DEAE-Dextran are consistent with this view. They show the same linear relation between effect and size. But they indicate that larger sizes are required to produce effects comparable to those of the basic polyamino acids (Fig. 5). Thus, the potency of the Dextrans, expressed on a weight basis is lower than that of the polypeptides. Our theory proposes that simultaneous attachments must occur, but does not at this moment specify the nature of these attachments. Future investigations will clarify this problem. Although there is some uniformity in the action of four basic polymers, one remembers the differences seen between the two optical isomers of polylysines and between the two types of histones. These unexplained findings indicate that rather subtle characteristics of secondary and tertiary structures may influence the effectiveness of simultaneous attachments. In summary, it appears that other factors as well as charge and size are involved in this striking membrane effect, and that a further study of these effects will bear on several aspects of membrane structure. The molecular mechanisms that follow a multiple attachment of polymers to membrane constituents are not known. However, newer models proposed for the plasma membrane are very helpful in this regard and may at least suggest plausible mechanisms involving the structural proteins of the cell membrane [26, 32].

Uptake of other proteins and other macromolecules

Leaving these questions for the future, let us turn to those points that can be answered now by simple experiments. Will basic polymers enhance the penetration of other proteins and other macromolecules in a variety of

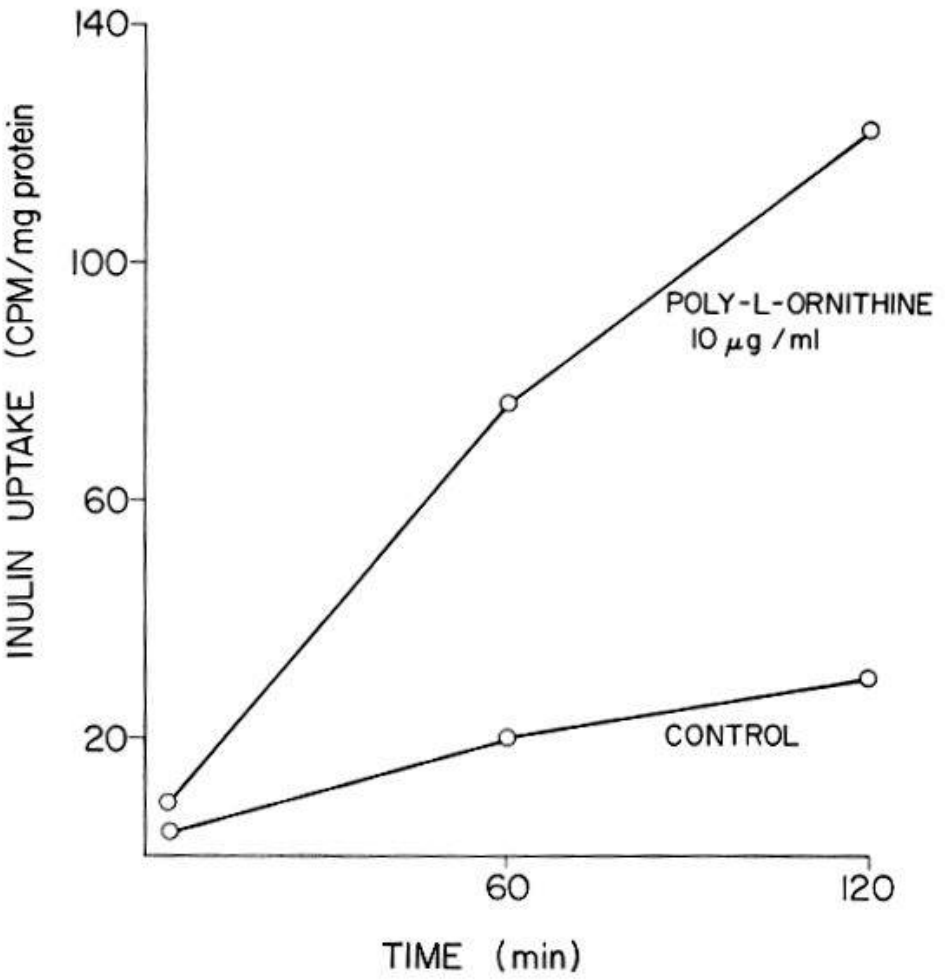

Fig. 6. Binding and uptake of inuline-C^{14} as a function of time, in the presence of 10 μg/ml poly-L-ornithine, MW 200,000 (upper curve). The first time points give an estimate of the adsorption of inuline to the cell surface. Increases above these initial values indicate a net uptake of inuline. The ratio of uptake: adsorption is greater for inuline (6:1) than for albumin (2:1), but the enhancement of uptake produced by 10 μg/ml poly-L-ornithine is considerably smaller (5 times the control uptake versus 34 times for albumin, see Fig. 1 and Fig. 4). Incubation for 2 hours, 37° C, in Eagle's medium without serum containing inuline-C^{14} 50 μc/ml.

cell types? Can they be considered to be tools given to biologists to introduce into any cells the macromolecule of their choice? The compound which in our view was the least likely to penetrate into cells was inuline. This polysaccharide is used in the clinic and in physiological research to determine the volume of the extracellular space. This determination is based upon the premise that inuline does not penetrate into cells. Our experiments showed that this assumption is not correct, that inuline is taken up by sarcoma cells and that this penetration is enhanced although to a lesser degree by poly-L-ornithine [33] (Fig. 6).

Among the macromolecules of greater biological interest, we tested desoxyribonucleic acids and prepared P^{32}-labelled DNA from Bacillus subtilis. Experiments showed that the uptake of this foreign DNA compared in many respects to the uptake of albumin. It had a comparable temperature de-

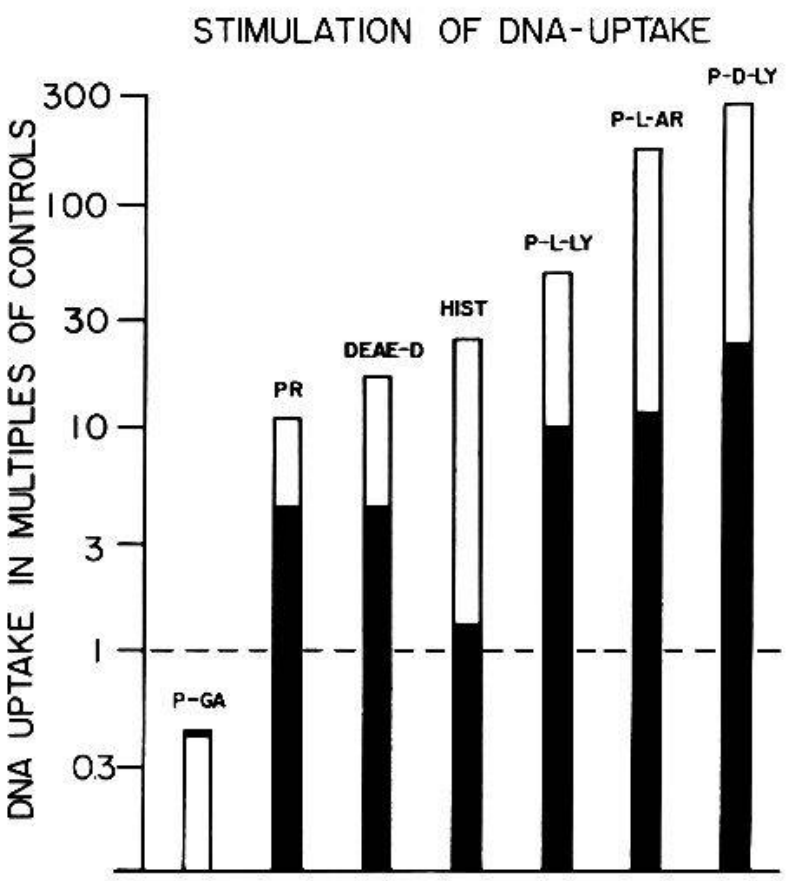

Fig. 7. Uptake of P^{32}-DNA by tumor cells in the presence of basic proteins and polyamino acids. The horizontal interrupted line gives the uptake of the controls. Ordinate: multiples of the control uptake. The dark and light columns correspond to the effect of two concentrations of the tested compounds, namely 1.0 μg/ml (dark) and 10.0 μg/ml (light). The first column shows the inhibitory effect of an acidic polymer, poly-L-glutamic acid MW 79,000. The other columns, from left to right show the effects of protamin (salmon sperm), DEAE-Dextran MW 2×10^6, crude histone (calf thymus), poly-L-lysine MW 70,000, poly-L-arginine MW 28,000, and poly-D-lysine MW 113,000. Although the pattern of stimulation is similar for DNA and albumin, the degree of overall enhancement is considerably greater for DNA (see Fig. 4). This difference may be related to the larger size of the molecule and possibly to the formation of DNA-aggregates in vitro. Incubation: 60 min at 37° C in Eagle's medium (1% horse serum) containing 20 μg DNA per ml. The activity of the medium was approximately 10^{-2} μc/ml (8.8×10^3 counts/min/ml). The DNA was isolated from Bacillus subtilis grown overnight in the presence of labelled phosphate.

pendance, a comparable time-curve, and more importantly it was enhanced qualitatively in similar fashion by different basic compounds (Fig. 7). It is difficult in experiments with P^{32}-DNA to exclude some reincorporation of labelled phosphate into newly synthetized DNA. The data of Fig. 7, however, indicate that this incorporation is quantitatively negligible, since the pattern of enhancement is identical for albumin and DNA. In both cases the D-form of polylysine is more active than the L-form, which in turn is more active than lysine-rich histones. Moreover, poly-L-glutamic acid inhibits both DNA and albumin uptake. The analogy suggests that both macromolecules may be carried into the cell passively under the influence

of the basic compounds, and that cationic polymers may indeed be tools of general value to biologists. It is of interest at this point to mention a few instances in which poly-L-ornithine and other polymers have been used with success, since their membrane effect was described. Virologists studying the cytopathic action of infectious ribonucleic acids have found it possible to enhance these biological effects with polyamino acids in concentrations of 1–20 μg/ml [34]. Studies on the penetration of whole viruses have also revealed positive effects of poly-L-ornithine and poly-L-lysine [35, 36]. Virologists engaged in the study of the mode of action of interferon have now been able to enhance the protection afforded by this protein by giving it to cells with poly-L-ornithine [37]. In this instance poly-L-ornithine has contributed to clarifying the mode of action of interferon, since it was still debated whether interferon acted from outside or inside the cell. Similarly it was unclear whether a large toxic protein, like diphtheria toxin, needed to be taken up by cells in order to act. Recent experiments by MOERING and MOERING indicate that cells insensitive to diphtheria toxin are rendered sensitive by the presence of poly-L-ornithine in the medium [38]. As in the case of interferon, these data imply that the two biologically active proteins are ingested before they exert their function. The last two examples are of particular interest to us because they relate to foreign proteins performing specific biological functions in mammalian host cells. Encouraged by these examples, one may predict that in the coming years, other cases will be identified in which a foreign protein penetrates into a host cell to exert biological functions. A number of candidates are already on the scene. For instance, the nerve growth factor isolated from mammalian tissues by LEVI-MONTALCINI and her group [39], and the cell growth protein isolated from fetal calf serum by PUCK and his group [40] are likely to belong in this category. Certain viral antigens can influence the metabolism of mammalian cells, independently of their immunogenic effects. It is likely that they do so by first getting into cells [41]. Several phases of the immune response itself may, of course, be connected with the capture of macromolecules either by macrophages or lymphocytes [30]. Furthermore, the question whether protein hormones might have to penetrate into their target cells is still unresolved [42]. The use of basic compounds might be helpful to clarify this question.

The turnover of albumin in host cells

The pessimism that could have derived from the finding that, in thin sections, no trace of ferritin is detected outside of vesicles and vacuoles can now be cast away. The fact remains, however, that a large percentage of the proteins transported into mammalian cells is rapidly destroyed. It was of obvious importance, therefore, to study the kinetics of this breakdown and the factors that might influence the process.

This problem was approached in the following way. Cells of Sarcoma S 180 grown in suspension were labelled with I^{131}-albumin under optimal conditions. They were

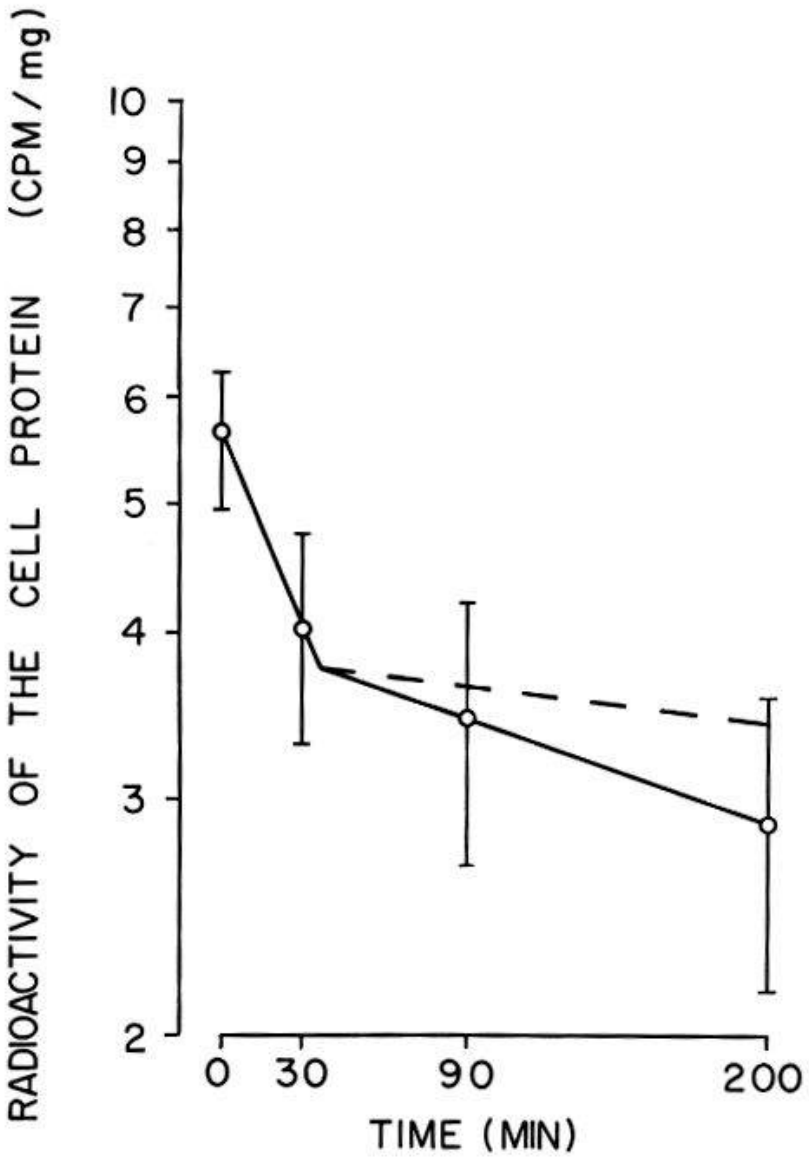

Fig. 8. Loss of acid insoluble radioactivity from I^{131}-labelled tumor cells in the course of a reincubation in non-radioactive medium. Cell labelling: incubation of 60 minutes at 37° C in Eagle's medium (1% horse serum) containing 30 μg/ml of I^{131} albumin. After washing, the cells were incubated in their regular growth medium. The interrupted line represents the isotope dilution that would be due to cell growth. The brackets give the standard deviations of results obtained in different experiments.

washed free of radioactive medium and reincubated in a label-free medium. At various times, both the remaining activity of the cell protein and the newly appeared activity in the medium were analysed. The specific activity of the protein, plotted semilogarithmically against time decreased linearly in a biphasic fashion [43, 44] (Fig. 8).

A first phase of rapid degradation accounted for a loss of about $^2/_3$ of the initial label within less than 60 minutes. The second phase of degradation was too fast to be due to an isotope dilution related to growth, but too slow to eliminate the remaining activity within 8 hours. In no experiments did this elimination go further than 90%. It can be concluded that cells have a very effective mechanism to destroy ingested foreign proteins, but that this breakdown does not discard the totality of the ingested albumin. The per-

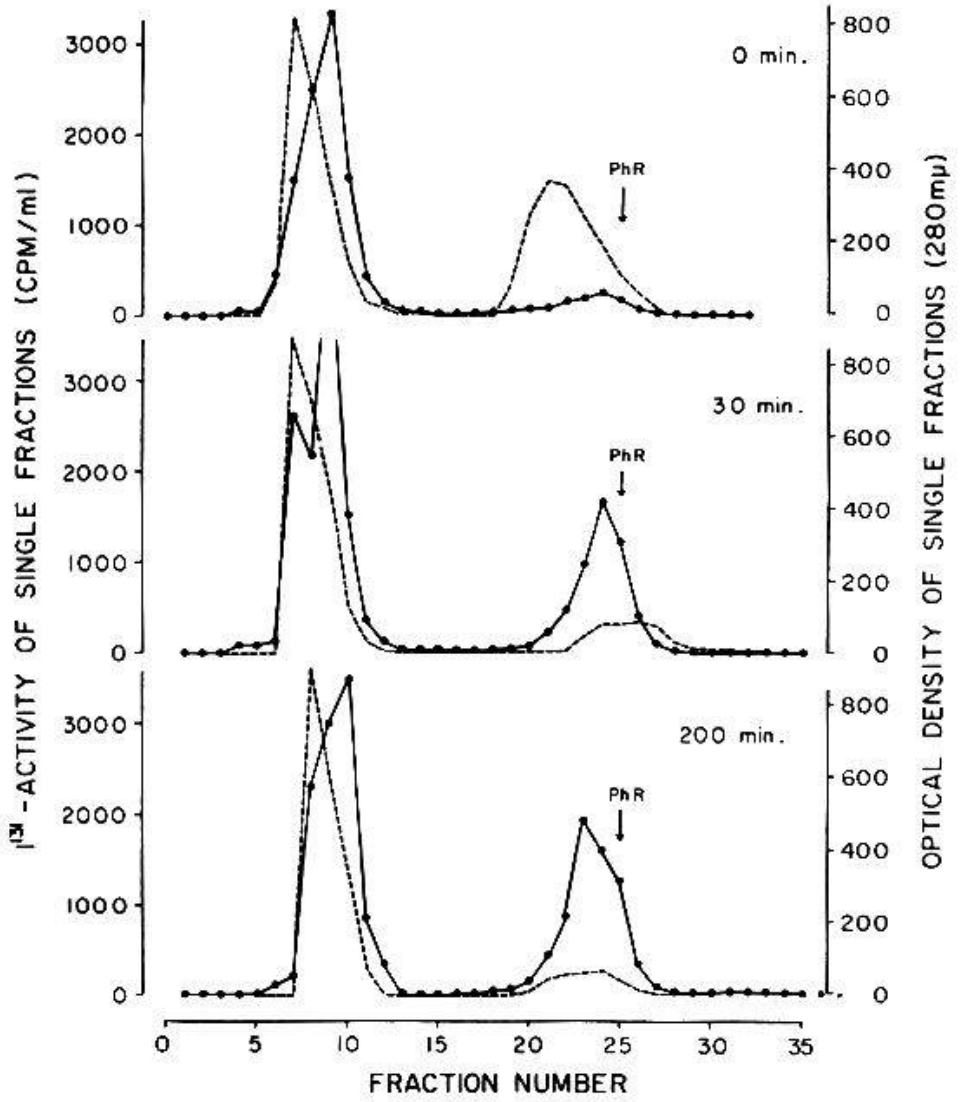

Fig. 9. Analysis of the radioactivity found at three different times in the reincubation medium. Gel filtration on Sephadex G-75 (K-tris buffer, pH 7.4). The cells were labelled as indicated in Fig. 8. Reincubation was carried out in Eagle's medium containing 2% horse serum. The interrupted profiles represent the optical density of single fractions measured at 280 mμ. The I^{131} radioactivity of the fractions (closed circles) was measured in a liquid scintillation counter. The arrow (PhR) indicates the maximum in the elution of the marker phenol red MW 346. There is an increase with time of the amount of small molecular labels detectable in the medium.

sistence of even a minimal fraction is enough to allow for the performance of possible functions related to foreign proteins. Because of the biphasic nature of the process, it is tempting to suggest that albumin finds access to different compartments in the cell. The fast degradation measured in the first phase could occur in the secondary lysosomes, whereas the second slower phase may suggest an extra-lysosomal breakdown. Electron microscopy, however, does not yet provide the desired support for this hypothesis [14].

At various times during albumin degradation, the incubation medium was analysed for its content of I^{131}-degradation products. The amount of small molecular label, negligible at first, increased with time and reached a sizable proportion of the total radioactivity present in the medium. Chromato-

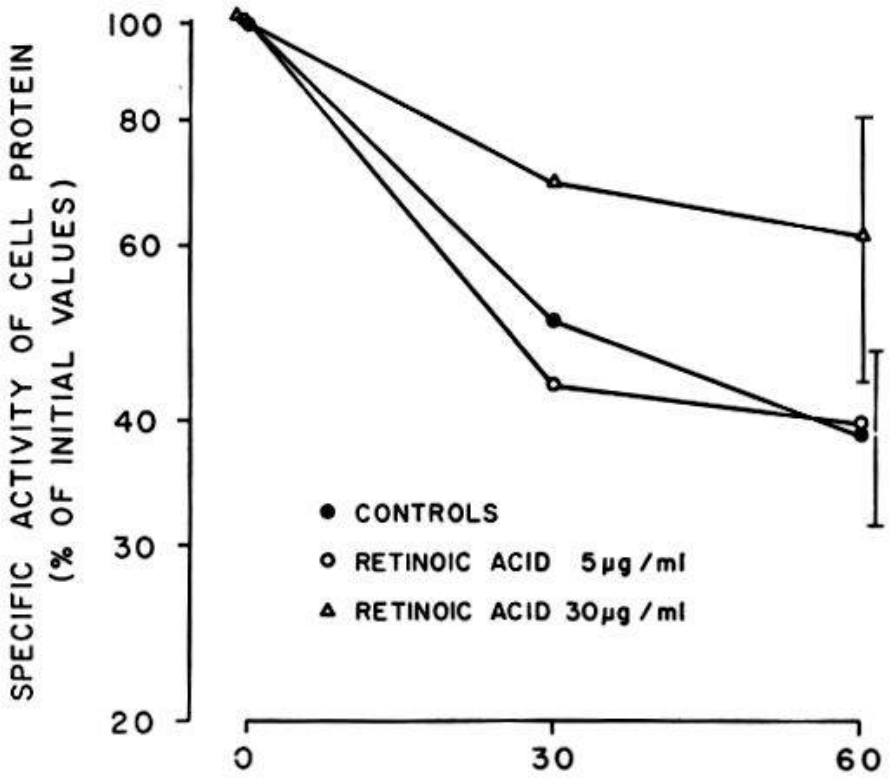

Fig. 10. Effect of retinoic acid on the degradation of ingested I^{131}-albumin. The control curve (closed circle) represent the loss of acid insoluble activity from tumor cells labelled, washed and reincubated as indicated in Fig. 8 (closed circles). The other two curves correspond to cells which were preincubated for 2 hours in the presence of retinoic acid prior to, as well as during the labelling. The radioactivity of all cell samples was first expressed in counts/min/mg of cell protein and thereafter expressed as % of the radioactivities measured at time zero.

graphy on Sephadex G 75 revealed a peak of activity corresponding to a molecular size of about 300–400 [44] (Fig. 9). Further resolution of this fraction on Sephadex G 15 indicated the presence of three metabolites, the smallest of which was identified by paper chromatography as monoiodotyrosine [45]. Similar gel and paper chromatographies were carried out with cells homogenized at various times of reincubation. They gave comparable results, but revealed in addition the presence of several intermediate fractions. According to their pattern of elution, these labelled intermediates correspond probably to oligopeptides of a size that is just too large to readily diffuse from cytoplasmic vacuoles into the medium [45]. These results prove that the degradation of I^{131}-albumin is not only a rapid but also an extensive digestive process yielding small peptides and aminoacids. There is no doubt that this digestion may limit the function of biologically active foreign macromolecules in host cells.

It would be of obvious interest to know how to inhibit this degradation, and our investigations have led us to believe that such inhibitions can indeed be achieved. For instance, a lesser degradation is observed when cells are incubated at temperatures lower than 25° C, or when they are subjected to pharmacological agents known to act upon lysosomes. It has been

established by the studies of FELL and DINGLE [46] that one such agent, vitamin A, enhances the fusion of lysosomes with the plasma membrane and as a consequence, depletes the cell of lysosomal enzymes. One might expect, therefore, that treating the cells with vitamin A would interfere with the process of intracellular digestion. In our experiments, a water-soluble derivative of vitamin A, retinoic acid, did inhibit the intracellular breakdown of albumin, as shown in Fig. 10. Similar data were obtained with another drug, chloroquine, which has the property of stabilizing lysosomal membranes [45]. In this case, the drug apparently inhibits protein breakdown by preventing the fusion of lysosomes and pinocytotic vesicles. These data and interpretations strengthen the view that lysosomes play a major role in the intracellular breakdown of foreign macromolecules. There is no experimental evidence as yet correlating rate of breakdown and function of a biologically active macromolecule. We have postulated, however, that the procedures used by virologists to enhance infections with viral RNA do not act only on the uptake, but also on the intracellular fate of the nucleic acids [47]. Clearly, enhancement of uptake and inhibition of breakdown are complementary facets of the same critical question: How does one enhance the biological effects of foreign macromolecules in host cells? Our work on both aspects of the problem should contribute to answering this question.

Other functions of pinocytosis

The essence of our presentation so far has been to indicate that foreign macromolecules are taken up into cellular vesicles and vacuoles, that most of them are destroyed and that some can escape and exert specific biological functions in the cell.

Since the majority of macromolecules may not escape unharmed from membrane-bound structures, it is legitimate to wonder whether this trapping does not itself serve a purpose. It may be viewed essentially as a defense device protecting the cells against the intrusion of unwanted macromolecules. But beyond this view there now are strong indications that the captured macromolecules might have functions of their own, depending upon their nature and upon the type of cells involved. Whereas in our experimental system, albumin appears to be broken down rather rapidly, it is known that other cells such as oocytes or intestinal epithelia handle other foreign proteins in a more conservative fashion. Egg cells, for instance, can take up proteins by pinocytosis and accumulate them in the form of yolk material [48]. Intestinal cells of newborn mammals take up γ-globulines by pinocytosis and rather than destroying them, transport them from the luminal to the serosal side of the cell where they enter the blood stream [49, 50]. In these two cases pinocytotic vacuoles appear to serve respectively as storage containers and transport bags. At the other end of the spectrum, in cells where foreign proteins are rapidly digested, the enzymatic hydrolysis might give rise to active breakdown products, which would act either in the

cell or be released into the blood. Many of the peptides that possess biological activity are activated by the proteolytic splitting of a masking group. Such activations may well in some cases take place within pinocytotic vacuoles.

In addition to the transcellular transport of immunoglobulines by the intestinal epithelium, there may also be a transport mechanism in which proteins would bind small molecules, penetrate with them into the cell and release them in the course of their own proteolytic breakdown. We have now obtained conclusive evidence that such a transport can occur and be associated with dramatic biological effects [51]. Although our experimental evidence was obtained with a heavy metal that is toxic to the cell, there is no doubt that the model may apply to other molecules like drugs and that under circumstances, such a transport might be of therapeutic advantage. A well-known candidate for such a protein-mediated uptake is, of course, vitamin B_{12}, which is not absorbed without the help of B_{12}-binding protein. The hypothesis that vitamin B_{12} is carried into cells by pinocytosis is appealing, but still awaiting confirmation [52]. These examples suffice to suggest that the functions associated with the penetration of protein into mammalian cells may be numerous, diverse and fundamental. It is too early to assess the relative importance of the different roles we have assigned to pinocytosis but it is useful to keep in mind that they do not all require the conservation of an intact macromolecule. The most intriguing aspect is, of course, that specific foreign proteins taken up by pinocytosis might participate in the control of cell growth, and the regulation of cell function and metabolism.

Summary

It is established that pure viral nucleic acids can be infectious to mammalian tissues and can transform normal into tumor cells. There is no doubt, therefore, that foreign macromolecules can get into cells and express their biological potential in a host. Little is known, however, about the transport mechanisms responsible for these effects. It is not clear whether other macromolecules, transported in similar fashion, might mediate other important biological functions. Electron microscopic studies have demonstrated in a large variety of tissues that tracer proteins (ferritin, hemoglobin, peroxydase) are taken up by pinocytosis. Pinocytotic vesicles fuse with each other and with primary lysosomes to become digestive vacuoles. Because tracers are not seen outside of these structures, one may ask whether this process represents a meaningful transport mechanism. This question has been tested experimentally by measuring the uptake and the fate of a labelled protein in order to define the characteristics of both transport and intracellular turnover of a foreign macromolecule. The experiments were carried out in cultures of established tumor cell lines. The marker most widely used was radioiodinated human serum albumin. The major results to-date can be summarized as follows: The uptake of albumin requires little metabolic energy. It can be inhibited by polyglutamic acid and strongly enhanced by

basic polyamino acids. Different proteins are taken up at different rates. Uptake is influenced by factors such as charge, molecular size, optical isomerism, and amino acid sequence. The breakdown of ingested protein can be influenced experimentally. Most of our data on the transport of proteins apply also to other macromolecules. Special attention has been focused upon correlations between uptake and ultimate biological effects of foreign proteins and upon the possible functions of pinocytosis.

Zusammenfassung

Es ist eine Tatsache, daß aus Viren gewonnene Nukleinsäuren normale tierische Zellen infizieren und in Tumorzellen umwandeln können. Es besteht daher kein Zweifel, daß fremde Makromoleküle in das Innere einer Wirtszelle einzudringen und dort ihr biologisches Potential zur Entfaltung zu bringen vermögen. Die für diese Wirkungen verantwortlichen Transportmechanismen sind jedoch nur wenig bekannt. Man weiß nicht, ob sie auch andere Makromoleküle transportieren und ob z. B. fremde Proteine in ihrem Wirt bedeutende biologische Funktionen ausüben können. Durch die Verwendung des Elektronenmikroskopes konnte bewiesen werden, daß gewisse markierte Proteine wie Ferritin, Hämoglobin und Peroxydase durch Pinozytose in zahlreiche Gewebe eindringen. Die Pinozytosebläschen vereinigen sich untereinander und mit primären Lysosomen um Verdauungsvakuolen zu bilden. Da die Spuren niemals außerhalb der Bläschen und Vakuolen beobachtet werden, kann man sich fragen, ob dieser Vorgang einen eigentlichen Transport darstellt. Diese Frage wurde durch Messung der zellulären Penetration und des Stoffwechselgeschehens eines radioaktiven Proteins geprüft, um gleichzeitig sowohl den Transport- als auch den Degradationsparameter zu bestimmen. Die Versuche wurden an Kulturen tumoraler Zellen ausgeführt; als markierte Substanz diente in den meisten Fällen das radiojodierte Albumin. Die wesentlichsten Ergebnisse können wie folgt zusammengefaßt werden. Die Penetration des Albumins benötigt nur wenig Energie. Sie wird durch die Polyglutaminsäure gehemmt und durch Polymere basischer Aminosäuren stark erhöht. Verschiedene Proteine unterscheiden sich im Penetrationsgrad. Der Vorgang ist von Faktoren wie Belastung und molekulärer Größe, optischem Isomerismus und Sequenzen der Aminosäuren abhängig. Die Degradation der aufgenommenen Proteine kann experimentell beeinflußt werden. Die meisten unserer Ergebnisse über den Transport der Proteine sind auch für andere Moleküle gültig. Besondere Aufmerksamkeit wurde der Korrelation zwischen Aufnahme und biologischen Auswirkungen fremder Proteine und den möglichen Funktionen der Pinozytose geschenkt.

Résumé

Il est établi que des acides nucléiques extraits de virus peuvent être infectieux et peuvent transformer des cellules animales normales en cellules

tumorales. Il n'y a donc pas de doute que des macromolécules étrangères peuvent pénétrer et exprimer leur potentiel biologique au sein d'une cellule-hôte. Les mécanismes de transport responsables de ces effets sont toutefois mal connus. L'on ne sait pas s'ils transportent aussi d'autres macromolécules et si, par exemple, des protéines étrangères peuvent aussi exercer des fonctions biologiques importantes dans leurs hôtes. L'emploi du microscope électronique a permis de démontrer que certaines protéines traceuses telles que la ferritine, l'hémoglobine et la peroxydase pénètrent par pinocytose dans de nombreux tissus. Les vésicules de pinocytose fusionnent entre elles et avec des lysosomes primaires pour former des vacuoles digestives. Comme les traces ne sont jamais observées en dehors des vésicules et vacuoles, on peut se demander si ce processus représente un véritable transport. Cette question a été examinée en mesurant la pénétration cellulaire et le sort métabolique d'une protéine radio-active, de manière à définir à la fois les paramètres du transport et de la dégradation. Les expériences ont été faites sur des cultures établies de cellules tumorales avec, comme marqueur principal, de l'albumine radio-iodée. Les résultats essentiels peuvent se résumer de la façon suivante. La pénétration d'albumine nécessite peu d'énergie. Elle est inhibée par l'acide polyglutamique et fortement augmentée par des polymères d'acides aminés basiques. Différentes protéines diffèrent par leur taux de pénétration. Le processus dépend de facteurs tels que charge et taille moléculaires, isomérisme optique et séquences d'acides aminés. La dégradation de protéines ingérées peut être influencée expérimentalement. La plupart de nos résultats sur le transport de protéines sont valables aussi pour d'autres macromolécules. La corrélation entre pénétration et différents effets biologiques est à l'étude, de même que les fonctions diverses qui semblent être remplies par la pinocytose de protéines.

Riassunto

E' stato dimostrato che gli acidi nucleici estratti dai virus possono essere infettivi e che possono trasformare le cellule animali in cellule tumorali. Non c'è dubbio quindi, che degli acidi nucleici estranei possano penetrare ed esprimere il loro potenziale biologico all'interno di una cellula ospite. I meccanismi di trasporto responsabili di questi fatti sono tuttavia mal conosciuti. Non si sa se trasportano anche altre macromolecole e se per esempio delle proteine estranee possano pure esercitare funzioni biologiche importanti nei loro ospiti. L'uso del microscopio elettronico ha permesso di dimostrare che certe proteine quali la ferritina, l'emoglobina e la perossidasi, penetrano per pinocitosi in numerosi tessuti. Le vescicole dovute a pinocitosi si uniscono tra di loro e con dei lisosomi primari per formare delle vescicole digestive. Dato che le molecule trasportate non sono mai state trovate nemmeno in tracce fuori delle vescicole e dei vacuoli, ci si può domandare se questo processo rappresenti un vero trasporto. Questa questione fu esaminata misurando la penetrazione cellulare e la funzione metabolica di una proteina radio-

attiva, in maniera da definire contemporaneamente i parametri del trasporto e della degradazione. Le esperienze furono fatte su delle culture costituite da cellule tumorali e con l'albumina radioiodata quale sostanza marcante principale. I risultati essenziali possono essere riassunti nel seguente modo. La penetrazione dell'albumina richiede poca energia. Essa è inibita dall'acido poliglutamico e fortemente aumentata dai polimeri degli aminoacidi basici. Proteine diverse si differenziano grazie alla loro forza di penetrazione. Il processo dipende da fattori quali la carica e la grandezza molecolare, l'isomerismo ottico e le sequenze degli aminoacidi. La degradazione delle proteine ingerite può essere influenzata sperimentalmente. La maggior parte dei nostri risultati sul trasporto delle proteine sono validi anche per altre macromolecole. La correlazione tra penetrazione ed i diversi effetti biologici è attualmente allo studio, come pure le diverse funzioni che sembrano essere esercitate dalla pinocitosi delle proteine.

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Address of the author: Until August 1st 1969: Department of Pharmacology, Harvard Medical School, Boston Mass. 02115, USA. – After August 1st 1969: Department of Cell Biology and Pharmacology, University of Maryland Medical School, Baltimore, Md. 21201, USA.

Discussion

A. de Weck, Berne:

Dr Ryser has shown us very effectively that basic proteins and especially basic polypeptides such as polylysines are very effective in fostering the uptake of macromolecules by cells. This seems to be due to the peculiar effect of clusters of basic charges on the cell membrane. Basic proteins and peptides permit to substances not only to enter the cells but also to leave them. We have shown for example that polyamines such as polylysines are very effective histamine liberators and cause as well wheal-and-erythema reactions in man as anaphylactic reactions in guinea pigs and degranulation of mast cells. These effects on the permeability of the membrane appear to be due to the positive charges present on these molecules. Substitution of the free amino groups (e.g. by succinylation) completely abolishes the activity.

H. J.-P. RYSER:

Answer to the comments of Dr. DE WECK:

It is well known that histones and basic polycations in higher concentrations are toxic to cells. Toxicity increases with time and temperature of exposure and varies with other experimental conditions. Cells in suspension are more susceptible than monolayers. Serum added to the medium has a protective effect. For monolayers kept in medium with 1% horse serum for 60 min at 37° C, the thresholds of toxicity are of the order of 30–100 μg/ml for basic polyamino acids and histones respectively. Subcultures of monolayers exposed to these conditions still show perfectly normal growth. As is pointed out in Dr. HANCOCK's paper (see R. HANCOCK, this bulletin, p. 386, Fig. 3), a number of investigators, unaware of these facts have carried out biological experiments with histones and polyamino acids at concentrations that are clearly toxic. The interpretation of such results is open to questions. The phenomena described in our presentation have all been obtained with concentrations that are well below toxic levels.