STUDIES ON PROTEIN UPTAKE BY ISOLATED TUMOR CELLS

III. Apparent Stimulations due to pH, Hypertonicity, Polycations, or Dehydration and Their Relation to the Enhanced Penetration of Infectious Nucleic Acids

HUGUES J.-P. RYSER

From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Fixation of ^{131}I-serum albumin by Ehrlich ascites tumor cells in suspensions and sarcoma S-180 monolayers was measured under experimental conditions. Anaerobic incubation and inhibitors of the oxidative metabolism critically restricted the range of glucose concentrations capable of supporting cell life; in glucose concentrations higher than 10^{-2} M, Ehrlich cells suffered from their own acid production; in concentrations 10^{-2} M, lower than they underwent damage by starvation. Both types of damage were accompanied by increased albumin fixation unrelated to pinocytosis. Different procedures recommended to enhance the uptake of infectious viral RNA by animal cells in culture were tested for their ability to increase albumin uptake. They enhanced the penetration of both albumin and vital dyes and decreased the viability of cell populations. Their effect, therefore, is related to cell damage. It was postulated that reversible damage to cells favors RNA infection by leading to abnormal uptake processes and by decreasing intracellular digestion. This abnormal uptake is different from pinocytosis and also from the massive fixation of albumin to dead cells. The latter phenomenon is due to adsorption by intracellular sites exposed by disruption of the cell membrane. Polycations are able to induce all three forms of fixation depending on the experimental conditions.

A number of cytochemical and electron microscopic studies from this and other laboratories have demonstrated beyond doubt that protein molecules can be taken up by intact tumor cells in suspension (15, 42) and by different normal tissues in vivo (21, 23, 29, 49, 50). This uptake occurs by pinocytosis and micropinocytosis and is considered a normal function of living cells. Studies on the kinetics of this process have indicated that Ehrlich ascites carcinoma cells in suspension (41), as well as sarcoma 180 cells in monolayer (40), take up a small but statistically significant amount of albumin-^{131}I within 1 hr of exposure. The low rate of albumin uptake, measured in normal growth media, has spurred interest in devising ways to stimulate this transport. Work along this line has led to one remarkable instance of physiological stimulation and to several examples of pathological responses. On one hand, it has been shown that histones and basic polyamino acids such as poly-L-ornithine and poly-D-lysine can stimulate albumin

uptake severalfold at concentrations that are too low to interfere with normal cell growth (43). On the other hand, attempts to increase albumin uptake by other means have led only to apparent stimulation, due in reality to cell damage. Although the occurrence of such pseudostimulation in the measurement of macromolecular penetration has been pointed out (40, 41), recent literature indicates that the subject deserves more emphasis. This paper, therefore, illustrates and discusses some of the deceiving phenomena encountered in measuring the uptake of macromolecules. The presentation will be restricted to those experimental conditions most likely to be used in attempts to stimulate pinocytosis. Because, during the past 8 yr, there has been great interest in the possibility of stimulating the cellular uptake of nucleic acids, special consideration will be given to procedures recommended for this purpose. These include the use of hypertonic solutions (1, 18, 25, 32), polybasic macromolecules (2, 31, 47, 48), unphysiological pH (32), and cell dehydration (46). The influence of such procedures on the cellular uptake of albumin will be examined. This will lead to a reassessment of the mechanisms underlying the enhanced penetration of infectious nucleic acids, and will point to the need for distinguishing among three separate processes in order to account for the various forms of fixation of foreign macromolecules by mammalian cells.

METHODS

Cells, Incubation, and Counting

Procedures described elsewhere have been followed to prepare Ehrlich ascites carcinoma cells (41), and rat thymus lymphocytes (39), to incubate tumor cell suspensions in the presence of ^{131}I-labeled albumin, and to measure albumin uptake (41). Anaerobic conditions were achieved by maintaining an atmosphere of 95% N_2 and 5% CO_2 in a Dubnoff shaking incubator. Unless otherwise noted, the final glucose concentration of the incubation medium was 8×10^{-3} M.

Starvation conditions were realized by incubating cells anaerobically in the absence of glucose, or at very low glucose concentration. In a few experiments the conditions were modified to match those of Meizel and Kay (36) or Koch et al. (31). In experiments on the effect of hypertonic solutions, suspensions with twice the number of cells were diluted twofold, at time zero, with hypertonic solutions of the appropriate concentration. These solutions were rendered hypertonic by adding NaCl or sucrose to Tyrode-bicarbonate in amounts appropriate to achieve simple multiples of the osmotic pressure of a physiological saline. Experiments on the effect of nonphysiological pH were started by resuspending cells in a Tyrode-maleate buffer of appropriate pH. At the end of incubation the suspended cells were washed and treated with trichloroacetic acid. The radioactivity of the extracted precipitate was counted on filters in a low background β-counter (41).

Sarcoma 180 monolayers were handled as previously indicated (40). In dehydration experiments the growth medium was poured off and drained thoroughly by placing the T-flasks in a test tube rack with their unstoppered ends down; the monolayers were kept in this position at room temperature for 1 min–4 hr. Before adding albumin-^{131}I, whatever fluid had collected in the neck of the flask was drained. The label was added in a total volume of 0.2 ml to match the conditions described by Smull et al. (46). This volume was spread out by gravity as evenly as possible over the monolayer and left in contact with the cells for 5 min. The monolayers then

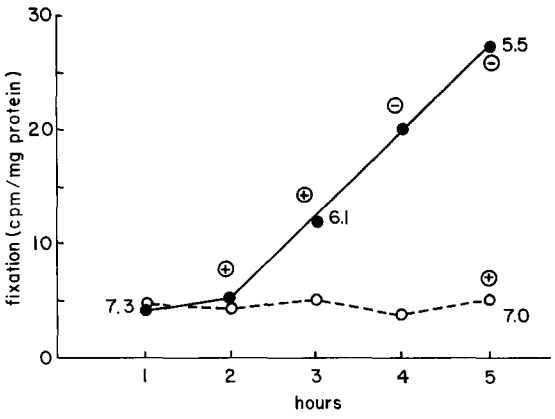

FIGURE 1 Time curve of albumin-^{131}I fixation by Ehrlich ascites tumor cells incubated at 37°C under anaerobic conditions (upper curve) compared with controls incubated aerobically (lower curve). Glucose concentration in the medium: 8×10^{-2} M. The numbers next to single points indicate the pH of samples taken at the given times. The signs + and − refer to the viability of the cell population as tested by reinjection of the sample intraperitoneally into mice. Uptake of albumin in control cells (aerobic incubation) is not detectable in all experiments with Ehrlich cell suspensions (41), but is easy to measure in a reproducible way with tumor cells grown in monolayers (40, 43).

were rinsed twice with a balanced salt solution, trypsinized, washed and treated with trichloroacetic acid, and processed as described (40). In these experiments the dye exclusion test (see below) was carried out directly on a monolayer treated in the same fashion up to the stage of the double rinsing. In some experiments the processing of either sarcoma 180 cells or Ehrlich cells was simplified by filtering the trichloroacetic acid-insoluble cell residue directly onto Millipore filters (Millipore Filter Corp., Bedford, Mass.) and washing it on the filter with 5% trichloroacetic acid. All results are expressed in radioactivity per mg of cell protein. The protein content was determined either gravimetrically, after protein purification, or chemically (34), on aliquots taken before trichloroacetic acid treatment.

Substances

^{131}I-human serum albumin, purchased from Abbott Laboratories, North Chicago, Ill. was checked chromatographically (40) for homogeneity and contamination with free ^{131}I. Experiments indicated that a low level of free ^{131}I of the order of 1–4% did not interfere with our measurements of albumin uptake. Within those limits, albumin-^{131}I was used without prior purification; it could be stored at 4°C for up to 3 wk after shipment without undergoing chromatographically detectable modification. The activity in the medium used in different experiments varied between 5×10^5 and 8×10^6 cpm/ml.

The polyamino acids and histones used to test the effect of polycations were described in an earlier study (43).

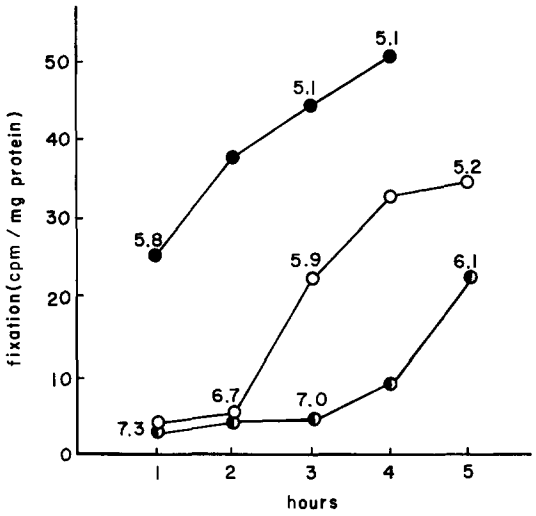

FIGURE 2 Influence of the buffer capacity of the medium on the albumin-^{131}I fixation by Ehrlich ascites tumor cells. Anaerobic incubation at 37°C in saline containing 8×10^{-2} M glucose and different Na-bicarbonate concentrations, namely 0.001 M (upper curve), 0.01 M (middle curve), and 0.07 M (lower curve). Characteristic pH values measured in the course of incubation are given next to the corresponding time points.

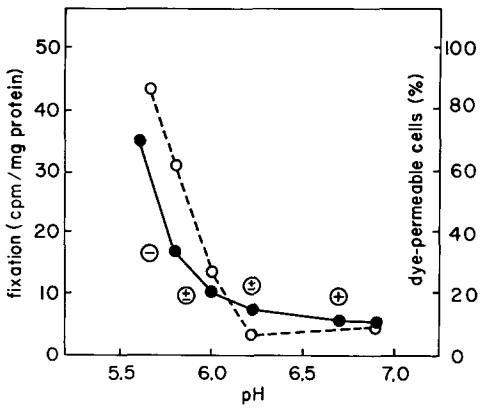

FIGURE 3 Fixation of albumin-^{131}I by Ehrlich ascites tumor cells incubated for 2 hr at different pH (closed circles). The increase in fixation in the acid region correlates with an increase in the percentage of cells permeable to nigrosine (open circles, interrupted line). The symbols + and − refer to the viability of the cell population (see Methods). Incubation at 37°C, under O_2/CO_2, in Tyrode solution buffered with 0.03 M maleate of different pH.

Dye Exclusion and Cell Viability Tests

The permeability to vital dyes was tested with either eosin Y (45) or nigrosine (28). The reliability and reproducibility of the procedure have been established and discussed elsewhere (20). The ability of Ehrlich cells to survive various exposures was tested by intraperitoneally reinjecting 5×10^7 cells into mice and by following the formation of an ascitic tumor in these mice. The test was called negative when no growth had developed 3 wk after inoculation. The outcome was designated ± when only minimal ascites was detected after 3 wk.

RESULTS

Pseudostimulation due to Low pH

The importance of pathological uptake resulting from exposure to pH 5–7 was first recognized in the course of experiments in anaerobic conditions. These experiments tested whether an increased rate of glycolysis was associated with increased protein uptake. This was of interest because a comparable association had been

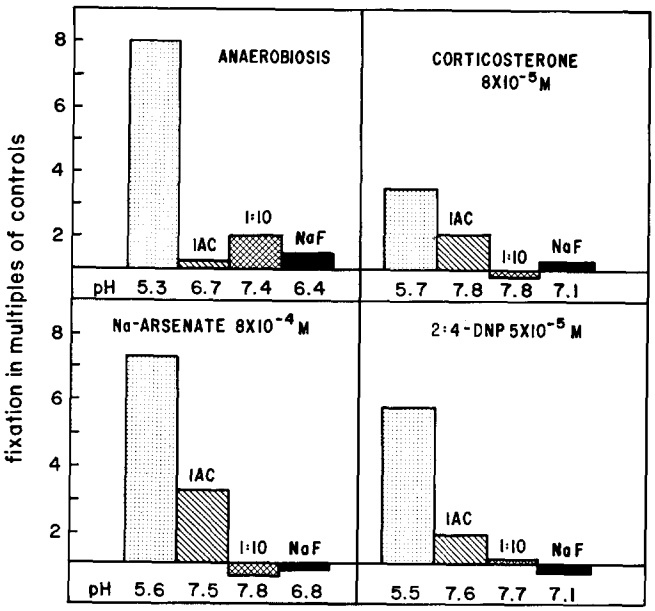

FIGURE 4 Fixation of albumin-^{131}I by Ehrlich ascites tumor cells in four different conditions which stimulate their rate of glycolysis. The increased glycolytic activity is indicated indirectly by the drop of pH in the incubation media (see figures below first column in each quadrangle), and by the corrective effect of adding Na-iodoacetate (IAC, 8×10^{-5} M), Na-fluoride (NaF, 5×10^{-3} M), or of lowering (1:10) the glucose concentration in the medium. Incubation for 5 hr (4 hr in the case of Na-arsenate) in 8×10^{-2} glucose and in aerobic conditions (except upper left). Results expressed in multiples of the albumin fixation by controls incubated under aerobic conditions without metabolic agents.

observed in white blood cells engaged in phagocytosis (37, 44). In the course of anaerobic incubation, a marked increase in albumin fixation could be measured in our system (Fig. 1). It became apparent, however, that this increase was associated, not with the rate of glycolysis, but with the acidification of the medium resulting from anaerobic acid production (see pH values in Fig. 1). When, for instance, the experiments of Fig. 1 were carried out in incubation media of increasing buffer capacity, glycolysis was not influenced by these modifications, but the protein fixation was delayed or suppressed (Fig. 2). Furthermore, fixation curves of increasing steepness and comparable in shape to that of Fig. 1 could be obtained even under aerobic conditions, when the cells were incubated in media of decreasing pH. In all three types of experiments, exposure to a low pH was accompanied by cell damage. In Fig. 1 severe cell damage is indicated by the failure of cells to divide when reinjected intraperitoneally into living mice. In order to demonstrate more clearly the association between cell damage and albumin fixation, cell samples were tested for their ability to exclude vital dyes, and the percentage of dye-permeable cells was plotted together with the albumin-^{131}I fixation measured at different pH (Fig. 3). That the two curves are parallel is striking.

Such artificial albumin fixations also could be observed when cells incubated under aerobic conditions were exposed to inhibitors of the oxidative metabolism, such as DNP (2,4-dinitrophenol), arsenate, and cyanide. By inhibiting oxidative substrate utilization, such compounds cause a relative increase of glycolysis (6) and acid production which, in our experiments (Fig. 4), led to the same type of pH effect. A more unexpected finding was the pronounced increase in albumin fixation caused by steroid hormones (Fig. 4). All four types of increases could be prevented by adding iodoacetate or NaF to the incubation mixture or by reducing the glucose concentration in the medium (Fig. 4). The pH values recorded in Fig. 4 indicate that in these conditions steroid hormones act like DNP or arsenate by stimulating glycolysis and, thereby, acidifying the incubation medium.

Most tumor cells differ from their normal counterparts in that they display a greater rate of anaerobic glycolysis. It could be anticipated, therefore, that normal cells would be less likely to undergo cell damage of the nature just described. In a comparison of thymus lymphocytes and lymphoma cells, it was found that anaerobiosis, DNP, arsenates, and steroid hormones did not elicit a glycolytic response in the lymphocytes which was strong enough to influence the pH of a well-buffered incubation medium. Consequently, only negligible increases in albumin fixation

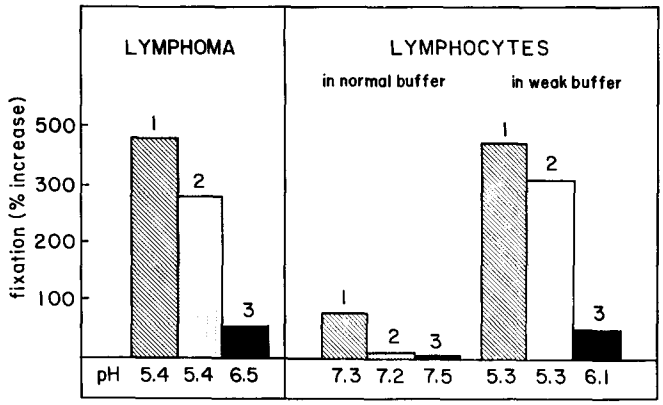

FIGURE 5 Comparison of the response of lymphocytes and lymphoma cells to three conditions which increase glycolysis. *1*, anaerobic incubation; *2*, aerobic incubation in the presence of 8×10^{-4} M Na-arsenate; *3*, aerobic incubation in the presence of 8×10^{-5} M corticosterone. Incubation for 5 hr in Tyrode solution containing 8×10^{-2} M glucose, and either 30 mM Na-bicarbonate (left and middle sections) or 0.3 mM Na-bicarbonate (section to the right). Ordinate: albumin fixation expressed as % increases above control values. The lymphoma, an ascitic tumor of thymic origin in the mouse, was kindly supplied by Dr. Emma Shelton.

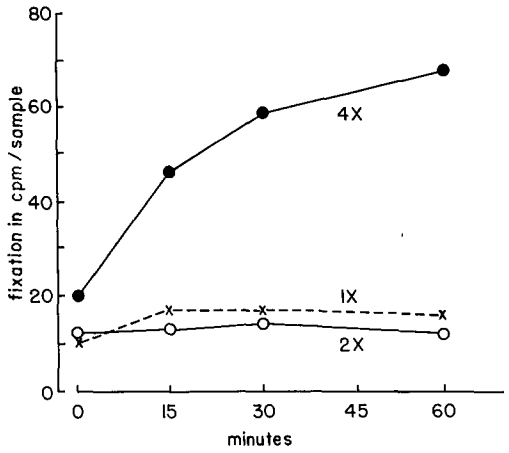

FIGURE 6. Fixation of albumin-^{131}I by Ehrlich ascites tumor cells incubated in isotonic (1 ×) and hypertonic saline (2 ×, 4 ×). Hypertonicity achieved by adding NaCl to Tyrode solution and expressed as multiple of the osmotic pressure of isotonic saline. The pH was 7.1–7.3 throughout.

were observed (Fig. 5, middle section). When, however, the buffer capacity of the medium was reduced strongly, these same conditions led to a glycolytic response that lowered the pH and increased the albumin fixation to values almost indistinguishable from those obtained with tumor cells (Fig. 5, right section). This effect could be prevented again by inhibiting glycolysis with iodoacetate or lowering the glucose concentration. Hence, both normal and tumor cells respond in a qualitatively similar way to factors that stimulate glycolysis and both are affected by low pH.

Pseudostimulation Produced by Hypertonic Media

Short exposure of cell cultures to hypertonic media is a procedure currently used by virologists to enhance the biological effects of nucleic acids on animal cells (1, 18, 25, 32). It was of interest, therefore, to explore whether this procedure also would enhance albumin uptake. The conditions of exposure were made to match as closely as possible those used by Colter et al. (14) in the Mengo RNA-L-cell system.

Doubling the osmotic pressure of the physiological saline by adding NaCl did not produce any significant change in protein fixation within a 60 min incubation (Fig. 6). Quadrupling the osmotic pressure, however, led to an immediate effect on albumin fixation, amounting to a 3- to 5-fold increase over the control values after exposures of 15 and 60 min, respectively (Fig. 6). Raising the NaCl concentration further produced greater effects (Fig. 7). They were of the same magnitude as those observed after exposure to low pH, and likewise were associated with cell damage. This association is indicated in Fig. 7 by the percentage of dye-permeable cells, and by the inability of severely exposed cells to grow

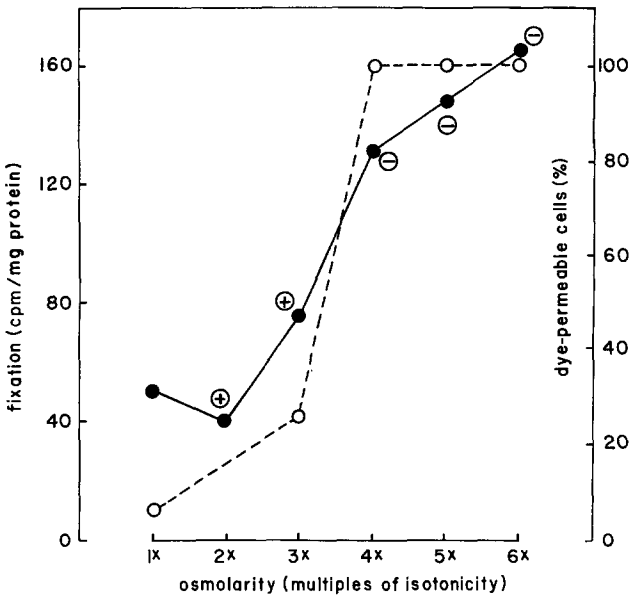

FIGURE 7 Effect of a 30 min exposure of Ehrlich ascites tumor cells to Tyrode solution of increasing osmotic pressures. Hypertonicity (abscissa) was produced by NaCl addition to medium and expressed as multiples of the osmotic pressure of isotonic Tyrode (control medium, 1 ×). Hypertonicity produces an increase in albumin fixation (closed circles) and a concomitant increase in the percentage of cells permeable to dye (open circles, interrupted line). Signs + and − refer to the viability of the cell population (see Methods).

in mice after intraperitoneal injection. The transition from a viable to a nonviable cell population occurred after 15–30 min exposure to a medium having four times the osmotic pressure of physiological saline. Other techniques of short-term exposure using tissue culture monolayers yielded similar results. Qualitatively similar but less pronounced effects were obtained with media made hypertonic by the addition of sucrose. Ellem and Colter (19) reported that the intensity of response to hypertonic sucrose varies with the cell type.

Pseudostimulation Initiated by Exposure to Polycations and Dehydration

The marked increases in albumin fixation observed after osmotic shock suggested that other manipulations used to enhance the infectivity of RNA preparations might have similar effects. Two other procedures known to serve the same purpose were tested in our system: (*a*) progressive dehydration (46), and (*b*) exposure to protamine (2), histones (47, 48), and basic polyamino acids (31), in concentrations up to 300 μg/ml. The experiments on polycations, performed on Ehrlich ascites tumor cells in suspension, are summarized in Table I. All three basic compounds tested produced a very marked, concentration-dependent increase of albumin fixation, which correlated very well with a concomitant increase in the percentage of injured cells. The data demonstrate also, as has been pointed out in a different context (43), that poly-L-ornithine is much more potent on a weight basis than either protamine or histones, and that, in this system, the first signs of abnormal permeability are observed with 3 μg of poly-L-ornithine per ml. In contrast, when administered to monolayers of Sarcoma S-180 incubated in Eagle's medium, poly-L-ornithine alters the permeability to dyes only at concentrations of 30 μg/ml (43) and produces physiological stimulation of albumin uptake at concentrations as low as 0.01 μg/ml.[1]

The experiments on the effect of dehydration, carried out on cell monolayers to match the conditions described by Smull and Ludwig (46), produced similar results: a simultaneous increase in the fixation of both albumin and vital dyes (Table II). A third procedure known to enhance RNA infectivity is the exposure of cells to an unphysiological pH (32). In the light of our data on pH (see Figs. 1–5) it can be assumed that the conditions found effective in the RNA system also would produce an abnormal permeability to albumin and dyes.

Pseudostimulation due to the Lack of Glucose

In the course of experiments on glucose requirement, it was found that Ehrlich ascites

[1] Ryser, H. J.-P. Data in preparation.

TABLE I

Correlation between Albumin Fixation and Cell Damage in Ehrlich Ascites Cell Suspensions Exposed to a Medium Containing Increasing Concentrations of Basic Proteins.

		30 min exposure		60 min exposure	
		Fixation (multiples of control)	Damage (% stained cells)	Fixation (multiples of control)	Damage (% stained cells)
	μg/ml				
Protamine	0	1.0	7.0	1.0	6.5
	100	4.9	14.5	5.0	22.0
	300	9.9	27.0	11.4	40.0
Histone	0	1.0	0.5	1.0	2.0
	100	4.8	9.5	4.0	13.0
	300	10.8	52.0	7.2	85.0
Poly-L-ornithine	0	1.0	8.0	1.0	9.1
	3	20.7	10.2	17.9	38.0
	10	—	35.5	11.0	45.2
	20	29.6	99.0	22.0	100.0
	100	69.5	99.4	54.7	100.0

The fixation, originally measured as cpm of albumin-^{131}I per mg of cell protein, is expressed here as multiples of the control values (fixation of controls = 1). The damage is indicated by the percentage of cells permeable to nigrosine. Incubation at 37°C, room atmosphere, in Eagle's medium containing 1% horse serum. The values are averages of two to four samples.

TABLE II

Correlation between Albumin Fixation and Cell Damage Seen in Cell Monolayers of Sarcoma-180 Exposed to Dehydration of Increasing Duration.

Duration of exposure to dehydration	Fixation (multiples of controls)	Damage (% of stained cells)
min		
0	1.0	9.3
30	3.6	12.5
60	—	15.4
90	6.0	32.0
120	4.5	39.2
180	7.6	100.0

Fixation and damage expressed as in Table I.

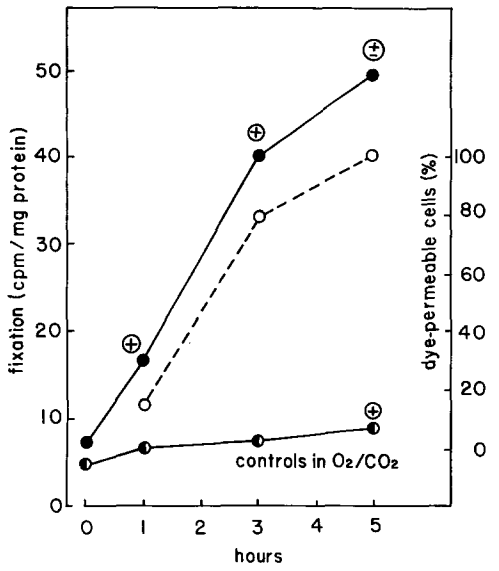

FIGURE 8. Effect of omitting glucose from the medium of Ehrlich ascites tumor cells incubated in anaerobic (upper curve) as opposed to aerobic conditions (lower curve). The increase in albumin fixation seen in anaerobiosis (closed circles, solid line) is accompanied by an increase in the percentage of dye-permeable cells (open circle, interrupted line). The pH of the medium after a 5 hr incubation was 7.5 in both conditions. The signs + and − refer to the viability of the cell population (see Methods).

tumor cells tolerated very well the absence of glucose in the medium, when incubated under aerobic conditions. Presumably they were capable of deriving enough metabolic energy from the oxidative utilization of endogenous substrates. However, in the absence of both glucose and oxygen, the cells developed a pronounced and time-dependent ability to bind albumin-^{131}I (Fig. 8). This effect was different from that obtained in anaerobiosis with high glucose concentrations (see Fig. 1), since it was not accompanied by a drop in the pH of the incubation medium. Yet, like the other increases in ^{131}I activity so far described, it also was associated with a change in cell permeability to vital dyes. Fig. 8 shows this correlation as it develops with time, and Fig. 9 depicts the albumin fixation as a function of the glucose concentration. In this experiment, the consequences of glucose deprivation appeared at an initial concentration of about 10^{-2} M, and became more pronounced with decreasing glucose concentrations. To make the curve of Fig. 9 complete and more intelligible, the samples exposed to the two highest glucose concentrations were treated with 8×10^{-5} M iodoacetate in order

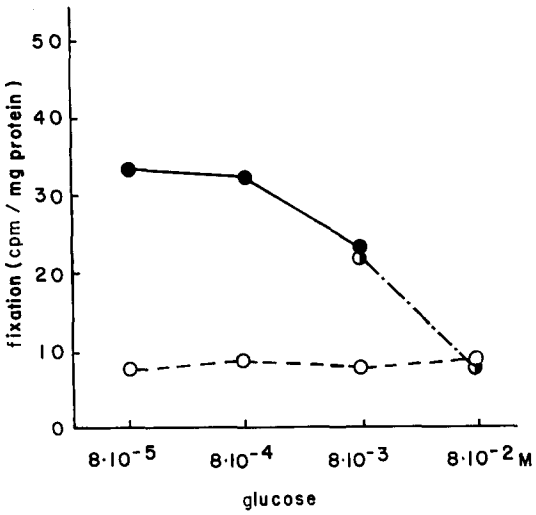

FIGURE 9. Effect of varying the glucose concentrations in the medium of Ehrlich ascites tumor cells incubated in anaerobic (upper curve) as opposed to aerobic conditions (lower curve). At the highest concentration (8×10^{-2} M), acidification of the medium was avoided by adding 8×10^{-5} M iodoacetate (half-closed circle). This addition did not affect the albumin fixation measured in 8×10^{-3} M glucose (compare closed and half-closed circles). The pH remained at 7.3–7.5 in all samples during a 5 hr incubation.

to inhibit excessive glycolysis and thus avoid the acidification of the medium described in Fig. 1. In this way a 7.3–7.5 pH was maintained at all glucose concentrations. The overlapping of the two values obtained with and without iodoacetate in 8×10^{-3} M glucose indicates that the inhibitor by itself did not damage the cells in these conditions. There can be little doubt that the increases in albumin fixation measured in these experiments result from cell injury by starvation.

The same association of albumin fixation and cell injury was observed in a number of other unphysiological conditions, such as exposure to detergents, heavy metal ions, or excessive concentrations of trypsin or metabolic inhibitors. Inappropriate handling of cells during preparation and incubation under suboptimal conditions produced noticeable damage. It was found, for instance, that washing Ehrlich ascites cells four times in cold saline after withdrawal from the animal, a procedure used in certain studies, produced a measurable impairment of dye exclusion. When incubated as concentrated suspensions in weakly buffered saline with inadequate glucose concentrations (36), such cells developed additional injury. This is shown in Table III, which uses as controls the data obtained with the same cells prepared and incubated under more favorable conditions.

TABLE III

Correlation between Albumin Fixation and Cell Damage to Ehrlich Ascites Tumor Cells Prepared and Incubated as Described by Meizel and Kay (36).

Incubation	Fixation		Damage (% stained cells)	
	Test	Control	Test	Control
min	*cpm/mg*	*cpm/mg*		
1	404	255	22.5	2.5
	616	420	25.5	16.5
15	—	—	31.8	2.5
			30.5	16.5
30	997	645	45.0	2.2
	1140	440	42.5	11.5

The test cells were washed four times in cold isotonic saline and incubated in isotonic saline containing 0.02 M glucose, 0.0125 M phosphate buffer at pH 7.4, and 0.1 M sucrose. Control cells were washed once in Tyrode solution at room temperature and incubated in Eagle's medium with 1% horse serum. Incubation temperature: 37°C. In both cases there was 0.5 ml of packed cells in 4 ml of medium (36).

DISCUSSION

Effect of pH

The pH artefact observed, when cells were incubated in high glucose concentrations, is particularly insidious because it lends itself to obvious misinterpretations. Finding, for instance, that albumin fixation is decreased by NaF appeared to bear out the view that albumin uptake, like phagocytosis (37, 44), depends on glycolysis. Following this line of thought, the increased fixation recorded under the effect of DNP or anaerobiosis could have been considered further evidence for a preferential use of glycotically supplied energy by this uptake process. Meizel and Kay (36) have followed this reasoning in the interpretation of similar data. In fact, the increased albumin fixation is not the expression of any physiological uptake process, but rather the result of a pathological response to low pH. Other reports on the uptake of macromolecules by Ehr-

lich ascite cells have failed to consider this potential artefact and, therefore are open to question (12, 13). It has been shown elsewhere that the use of Ehrlich ascites cells grown in mice is not suited ideally for the demonstration of small, quantitative differences in albumin uptake (40); the question whether, under optimal testing conditions, a correlation between uptake and glycolytic activity is at all detectable, will be examined in a later publication.[1]

Another pitfall must be avoided when interpreting increases in albumin fixation at low pH. It is known that in amebae albumin stimulates pinocytosis at acid, but not at neutral, pH (11). An increased albumin fixation by mammalian cells exposed to an acid medium, therefore, could be attributed, by analogy, to a stimulation of pinocytosis. Our data do not present any evidence for such a stimulation. They demonstrate, on the contrary, that Ehrlich ascites tumor cells, in contrast to amebae, suffer severe damage when exposed to an acid pH, and that gross changes in the permeability to macromolecules can account for the marked increase in albumin fixation. It is conceivable that some intensification of membrane movements might occur under the effect of low pH before the occurrence of cell damage. However, when measured in a cell population undergoing injury, a stimulation of this kind necessarily is obscured by the gross changes in permeability which prevail under these conditions.

The results of the comparison of normal and tumor lymphoid cells also call for a reappraisal of interpretation. It had been postulated that tumor tissues have a greater rate of pinocytotic protein uptake than normal tissues (8, 9). Fig. 5 illustrates the kind of marked difference in albumin fixation by normal and tumor cells that could have been interpreted erroneously as supporting this postulate. Upon closer consideration of the buffer capacity of media, however, the described difference only indicates that tumor cells are more prone than normal cells to be harmed by their own glycolytic acid production.

Besides this specific vulnerability, large tumors in situ are known to be vascularized poorly and to undergo central necrosis. Since there is undoubtedly a strong albumin fixation by dead cells, it can be assumed that the initial observations on the high rate of protein uptake by tumor tissues in vivo (9) were due, in large part, to a pathological uptake by injured cells. Since it first was expressed (40, 41), this critical view has received support from studies of Walker sarcoma in vivo, showing that necrotic areas of the tumor bind on the average 10 times more gamma macroglobulin than do nonnecrotic regions of the same tumor (27). Moreover, histological studies have indicated that necrotic centers are capable of retaining more vital dyes than the nonnecrotic periphery of the tumor (22), and that undamaged tumor cells are not characterized by any higher ability to take up fluorescein-labeled proteins (16).

Lack of Glucose

It is worth noting that the absence of glucose and oxygen does not change the environment in any way that could have a direct or specific effect on the cell membrane. In this case the impairment of the membrane function is secondary to a shutdown of the energy metabolism. Evidently the concentration of glucose necessary to maintain tumor cells in a viable state varies with the degree of oxygen saturation of the medium. Thus, for instance, a level of 8×10^{-5} M was tolerated well in aerobic, but proved *insufficient* in anaerobic conditions; on the other hand 8×10^{-2} M glucose was satisfactory in aerobic but *too high* in anaerobic conditions. It follows that, in anaerobiosis, the glucose concentration is a critical parameter that must be kept within a narrow range if severe cell damage from either cause is to be avoided.

The optimal glucose requirement also is likely to depend on the cell concentration in the medium. This is particularly true for tumors which, like the Ehrlich ascites carcinoma, are noted for a very high degree of glucose utilization. An excessive cell concentration, for instance, may create conditions of relative glucose starvation. Poor oxygenation of the medium also is more likely to happen in the presence of high cell concentrations and thus may narrow the range of glucose concentrations in which cells can remain healthy. Such unfavorable conditions of incubation seem to have prevailed in several published studies (12, 13, 36).

In a recent paper comparing the effect of 2×10^{-4} M and 2×10^{-2} M glucose on the incorporation of DNA by nuclei of Ehrlich ascites carcinoma cells (36), the unexplained high values obtained with the lower glucose concentration are strongly reminiscent of the starvation artefact just described, whereas the

marked response elicited by DNP reported in the same publication is superimposable on our data on DNP (Fig. 4) which, as we have seen, must be interpreted as a pH artefact due to an increased glycolysis in a medium of high glucose content. The nonphysiological character of the reported nuclear incorporation of DNA (36) also follows from the fact that strong cytoplasmic uptake of ferritin (42) or histones (43) by intact tumor cells is not accompanied by any detectable nuclear localization, whereas marked nuclear staining with fluorescein-labeled histones is seen after exposure of cells to acid media.[2]

Besides binding abnormal amounts of protein and vital dyes, a damaged cell also can be expected to show an abnormal leakage of its own cellular proteins. Data have been published on the release of soluble protein by ascites tumor cells incubated with or without glucose in the presence or absence of oxygen (35). The data show that several of the conditions that produce abnormal albumin fixation in our experiments produce increased leakage of protein in the experiments of others (35). Such leakage again can be explained on the basis of a pathological change of membrane permeability and, hence, contributes independently to the strength of our argument.

Hypertonicity and Other Infection-Enhancing Procedures

The same correlation between increased albumin fixation and cell damage is evident when cells are exposed to hypertonic media, to dehydration, and to high concentrations of polycations. As stated above, these three manipulations have been used by virologists to enhance the susceptibility of cultured cells to infectious RNA of viral origin. No definitive mechanism has been proposed yet to explain the infection-enhancing effect of procedures so different in nature (7, 46). Several authors have considered the possibility of a direct action on the stability of nucleic acid molecules (25, 32) or cellular ribonuclease (14, 25). One way to evaluate the merit of these two suggestions was to study, under similar conditions, the uptake of a macromolecule other than a nucleic acid. Because such enhancing conditions not only promote infectivity, but also lead to an enhanced albumin fixation, it can be concluded that they involve the cell rather than the macromolecule under consideration. This action on the cell could be demonstrated directly, in dye exclusion tests, by an abnormal cell permeability to dyes, occurring simultaneously and in close quantitative correlation with the increased albumin fixation. It is likely that specific factors of cellular competence are involved in the production of RNA-infection and that the uptake of albumin and infectious RNA are not comparable in every respect. The analogies between them, however, are worth pointing out. They suggest that RNA infection is initiated by a nonspecific process of macromolecular uptake. The comparison also draws attention to a major difference between these two processes, namely the absence of a distinct optimum in the case of albumin fixation. It should be noted that conditions which produce optimal enhancement, such as NaCl at 4-fold osmolarity (14), correspond in our experiments to the region of transition from a viable to a nonviable cell population. This observation supports the view expressed by Koch et al. (31, 32), Holland et al. (25) and Ellem and Colter (19) that the sharp decrease in the yield of infection centers, seen after severe exposures, is due to a loss of cell viability and to a failure of the damaged cells to support viral infection. The existence of such a well-defined optimum of enhancement, invariably followed by a drop in the yield of infection, appears to be characteristic of all the enhancing procedures that have so far been described (19, 25, 31, 32, 46, 47). We suggest, therefore, that the optimal enhancing conditions can be defined as those producing the maximal degree of cell damage still compatible with cell life. Accordingly, maximal enhancement of RNA infection would be due to a form of severe but reversible cell damage, elicited nonspecifically by a variety of adverse conditions.

It must be noted that the damage produced by polycations is preceded by a striking, physiological effect. Certain basic proteins and polyamino acids in concentrations as low as 0.01 to 0.1 μg/ml can trigger significant stimulations of albumin uptake without altering the integrity of the cell (43).[1] This effect has been attributed to increased pinocytosis, a view that is now supported by electron microscopic data.[3] At high

[2] I am indebted to Doctors R. Hancock and H. Amos for their communication of this unpublished result.

[3] Revel, J.-P., and H. J.-P. Ryser. Data in preparation.

concentrations, however, the same compounds undoubtedly lead to cell damage, as shown in previous studies (4, 33, 43) and as demonstrated above.

The concentrations at which this second effect occurs depend upon both the compound and the cell line under study and vary further with incubation conditions. For instance, Ehrlich ascites carcinoma cells grown in mice and studied in vitro generally appear to be much more delicate and sensitive than sarcoma 180 monolayers studied in Eagle's medium, and the presence of increasing serum concentration in this medium further decreases the susceptibility of the cells to the effect of polycations. This is illustrated by the fact that 20 μg of poly-L-ornithine per ml of serum-free saline markedly impair the ability of Ehrlich cells to exclude vital dyes (Table I), whereas the same concentration does not affect the integrity of sarcoma cells kept in 1% Eagle's medium (43). These considerations are essential for interpreting the cellular effects of polycations. Obviously, these compounds, in spite of their interesting effects at low concentrations, may, at higher concentrations, act in the same general way as hypertonic saline and produce reversible as well as permanent cell damage. This conclusion is consistent with recent data on the enhancing effect of polycations which show that the dose-effect curves of the most powerful polycations display a characteristic maximum followed by a drop in the yield of infections (31). It is worth noting that maximal enhancement was produced in the study of Koch et al. (31) by 20 μg/ml poly-L-ornithine, a concentration which produces considerable cell damage to Ehrlich cells incubated in comparable conditions (Table I)

The exact mechanisms by which cell damage might enhance RNA infections are not known. It is useful to recognize that such an effect does not require an increased uptake, but might be due to factors favoring the biological expression of ingested macromolecules. Under normal conditions, ingested macromolecules are segregated in membrane-bounded vesicles, the phagolysosomes (49), where they undergo digestion by lysosomal enzymes. It is reasonable to postulate that cell damage modifies this trapping process by creating new paths of uptake and new patterns of segregation, and by interfering with intracellular digestion. This view is consistent with the morphological picture of cells undergoing damage. Electron microscopic studies of the early effects of anoxia have revealed the occurrence of focal ruptures of the plasma membrane (3) and the formation of large vacuoles in the cell periphery (3, 38). This vacuolization is considered a common cellular response to a variety of adverse conditions (5) and as an early and at first reversible indicator of cellular injury (3). Furthermore, tumor cells incubated in the presence of ferritin during the occurrence of cell damage develop large peripheral vacuoles containing an appreciable number of marker molecules (42).[4] Similar observations have been made in anoxic liver tissue after injection of colloidal mercury as a marker (38). These large cytopathic uptake vacuoles could therefore account for an increased or an abnormal penetration of nucleic acids into cells exposed for a short time to adverse conditions.

Whether these uptake vacuoles result from the fusion of small pinocytotic vesicles is not established. However, the uptake process can be distinguished from what usually is called pinocytosis by the following criteria: (*a*) unlike pinocytosis, it is a pathological response to a variety of unphysiological conditions; (*b*) the uptake vacuoles are larger in size than pinocytotic vesicles; (*c*) vacuolization can occur without concurrent increase of pinocytosis. The last characteristic is illustrated by exposures to hypertonic solutions or acid pH, which produce large uptake vacuoles but no apparent increase in the number of pinocytotic vesicles (42).[4]

Such morphological differences can be expected to influence the fate of ingested macromolecules in several ways. Points of rupture of the vacuolar membrane, similar to those described by Trump and Janigan in cells exposed to sucrose (51), may allow macromolecules to reach the cytoplasm and thus to escape digestion. Also, the digestive functions of uptake vacuoles might be inadequate compared to those of ordinary phagolysosomes, either because of an insufficient enzyme content or because of suboptimal reaction conditions in the vacuolar medium. Methods developed recently for studying the function of lysosomes should allow the testing of these postulates, and make it possible to clarify the role of reversible cell damage in the enhanced biological expression of ingested macromolecules.

The mechanism of vacuolization just described, although it may be basic to the phenomenon of RNA infection, is probably of minor importance

[4] Caulfield, J. B., and H. J.-P. Ryser. Unpublished data.

in explaining the massive albumin fixation to irreversibly damaged or dead cells. In contrast to what is observed in the enhancement of infection, there is no transient optimum of albumin fixation during exposures of increasing severity. This indicates that viability is not required for albumin fixation and that albumin fixation is, in fact, increasing with cell damage. In our exposures to hypertonic NaCl, for example, the ^{131}I-activity leveled off only at what appeared to be a stage of complete disruption of the cell population. Similar results have been obtained by Ellem and Colter (19) in their measurements of the cellular fixation of ^{32}P-RNA by cells exposed to 0.9 M NaCl.

Electron microscopic studies have demonstrated that debris or bodies of tumor cells killed by exposure to hypertonic saline or other agents can absorb enormous amounts of ferritin (10), colloidal gold (42), or thorotrast.[5] Strong adsorption of fluorescein-labeled proteins to other cells damaged by other means, has been reported (17, 26, 30). These observations can account for the massive albumin-^{131}I fixation measured in our experiments in conjunction with all forms of cell damage.

One purpose of this study was to distinguish among three different forms of fixation encountered in the interaction of mammalian cells and foreign macromolecules. It was mentioned in the introduction that, under most physiological growth conditions, only small amounts of albumin are taken up by cells in culture, an observation that is consistent with the low yield of RNA infection recorded by virologists under such conditions. This physiological uptake can be stimulated, without concomitant cell damage, by very low concentrations of basic polyamino acids (0.01–10 μg/ml) and is believed to occur by pinocytosis and micropinocytosis (43).[3] The present publication, on the other hand, shows that the procedures used to enhance RNA-infection also enhance the fixation of protein and the penetration of vital dyes, and, therefore, must be regarded as causing some damage to the cell population. Although the exact nature of this reversible damage is still unknown, it appears to be associated with the formation of cytopathic uptake vacuoles, which are different in structure from pinocytotic vesicles. Finally, a third form of macromolecular fixation was described, which occurs in the course of irreversible damage and cell death. This fixation, which quantitatively exceeds the other two, is due to a phenomenon of massive adsorption to intracellular sites that are made accessible to foreign macromolecules by the disruption of the cell membrane. It is suggested that polycations such as histones or basic polyamino acids are able to initiate any one of these three processes, depending on their concentration and duration of contact with the cells.

This report also was designed to draw attention to the errors of interpretation that may result from ignoring these distinctions. It underlines, in particular, the necessity of providing adequate evidence of cellular integrity, together with any data on the physiological uptake of macromolecules. When proper precautions are taken, this macromolecular transport process is amenable to kinetic and metabolic studies (40, 43)[1] and appears to be a promising subject for further physiological and pharmacological investigation.

The author is deeply indebted to Dr. J. C. Aub, in whose laboratory this study was begun. Thanks are due to Dr. M. L. Karnovsky for comments on the manuscript and to Mrs. Margaret Heather, Miss Karin Scholl, and Miss Mary Fuday for technical assistance.

This investigation was supported by research grant GM-08482 from the U. S. Public Health Service. Dr. Ryser is a recipient of a Lederle Medical Faculty Award 1964–7.

Received for publication 1 August 1966.

[5] Revel, J.-P. Personal communication.

BIBLIOGRAPHY

1. Alexander, H. E., G. Koch, M. Mountain, and O. van Damme. 1958. Infectivity of ribonucleic acid from polio virus in human cell monolayers. *J. Exptl. Med.* **108**:493.
2. Amos, H. 1961. Protamine enhancement of RNA-uptake by cultured chick cells. *Biochem. Biophys. Res. Commun.* **5**:1.
3. Bassi, M., and A. Bernelli-Zazze 1964.ra. Ultrastructural cytoplasmic change of liver cells after reversible and irreversible ischemia. *Exptl. Mol. Pathol.* **3**:332.
4. Becker, F. F., and H. Green. 1960. The effect of protamines and histones on the nucleic acids of ascites tumor cells. *Exptl. Cell Res.* **19**:261.
5. Bessis, M. 1964. Studies on cell agony and death:

an attempt at classification. *Ciba Found. Symp., Cell Injury*. 287.

6. BICKIS, D. J., and J. H. QUASTEL. 1963. Effects of metabolic inhibitors on energic metabolism of Ehrlich ascites carcinoma cells. *Nature*. **205**:44.
7. BORRISS, E. 1965. Wechselbeziehungen zwischen ^{32}P-markierten Ribonucleinsäuren und suspendierten Gewebekulturzellen. III. Einfluss von Versen, Mg^{++} und Protamin. *Z. Naturforsch.* **20**:752.
8. BUSCH, H., E. FUJIWARA, and D. C. FIRSZT. 1961. Studies on the metabolism of radioactive albumin in tumor-bearing rats. *Cancer Res.* **21**:271.
9. BUSCH, H., S. SIMBONIS, D. ANDERSON, and H. S. N. GREENE. 1956. Studies on the metabolism of plasma proteins in tumor-bearing rats. II. Labeling of intracellular particulates of tissues by radioactive albumin and globulin. *Yale J. Biol. Med.* **29**:105.
10. CAULFIELD, J. B. 1963. Studies on protein uptake by isolated tumor cells. *Lab. Invest.* **12**:1018.
11. CHAPMAN-ANDRESEN, C. 1962. Studies on pinocytosis in amoeba. *Compt. Rend. Trav. Lab. Carlsberg*. **33**:73.
12. CHOI, Y. C., and E. R. M. KAY. 1963. Host tumor relationships and metabolism of serum proteins by Ehrlich ascites tumor cells. *Federation Proc.* **22**:433.
13. CHOI, Y. C., and E. R. M. KAY. 1964. The *in vitro* incorporation of proteins by cells of the Ehrlich-Lettré ascites carcinoma. III. Studies of the uptake of hemoglobin and its derivatives. *Federation Proc.* **23**:317.
14. COLTER, J. S., and K. A. O. ELLEM. 1961. Interaction of viral nucleic acids with mammalian cells. *Federation Proc.* **20**:650.
15. EASTON, J. M., B. GOLDBERG, and H. GREEN. 1962. Demonstration of surface antigens and pinocytosis in mammalian cells with ferritin-antibody conjugates. *J. Cell Biol.* **12**:437.
16. EASTY, G. C. 1964. The uptake of fluorescent labelled proteins by normal and tumor tissues *in vivo*. *Brit. J. Cancer*. **18**:368.
17. EASTY, G. C., M. M. YARNELL, and R. D. ANDREWS. 1964. The uptake of proteins by normal and tumor cells *in vitro*. *Brit. J. Cancer*. **18**:354.
18. ELLEM, K. A. O., and J. S. COLTER. 1960. The interaction of infectious ribonucleic acid with a mammalian cell line. II. Kinetics of the formation of infectious centers. *Virology*. **12**:511.
19. ELLEM, K. A. O., and J. S. COLTER. 1961. The interaction of infectious ribonucleic acids with mammalian cell lines. III. Comparison of infectious and RNA-uptake in HeLa cell-Polio RNA and L cell-Mengo RNA systems. *Virology*. **15**:113.
20. EYBL, V., and H. RYSER. 1964. The acute toxicity of crystallized ferritin, cadmium-free ferritin and $CdCl_2$ on Ehrlich ascites carcinoma cells. *Arch. Exptl. Pathol. Pharmakol.* **248**:153.
21. FARQUHAR, M. G., and G. E. PALADE. 1961. Glomerular permeability. II. Ferritin transfer across the capillary wall in nephrotic rats. *J. Exptl. Med.* **114**:699.
22. GOLDACRE, R. O., and B. SYLVEN. 1962. On the access of blood-borne dyes to various tumor regions. *Brit. J. Cancer*. **16**:306.
23. GRAHAM, R. C., JR., and M. J. KARNOVSKY. 1965. The early stages of absorption of injected horseradish peroxidase in the proximal tubule of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
24. HOLLAND, J. J., L. C. MCLARREN, and J. T. SYVERTON. 1959. Mammalian cell virus relationship. IV. Infection of naturally insusceptible cells with enterovirus ribonucleic acid. *J. Exptl. Med.* **110**:65.
25. HOLLAND, J. J., B. H. MOYER, L. C. MCLARREN, and J. T. SYVERTON. 1960. Enteroviral ribonucleic acid. I. Recovery from virus and assimilation by cells. *J. Exptl. Med.* **112**:821.
26. HOLTZER, H., and S. HOLTZER. 1960. The *in vitro* uptake of fluorescein-labeled plasma proteins. *Compt. Rend. Trav. Lab. Carlsberg*. **31**:373.
27. ISLIKER, H., J. C. CEROTTINI, J. C. JATON, and G. MAGNENAT. 1964. Specific and non-specific fixation of plasma proteins in tumors. *In* Chemotherapy of Cancer. P. A. Plattner, editor. Elsevier Publishing Co., Amsterdam, Netherlands. 278.
28. KALTENBACH, J. P., M. H. KALTENBACH, and L. B. LYONS. 1958. Nigrosin as a dye for differentiating live and dead ascites cells. *Exptl. Cell Res.* **15**:112.
29. KARNOVSKY, M. J. 1965. Vesicular transport of exogenous peroxidase across capillary endothelium into the T-system of muscle. *J. Cell Biol.* **27**:49A.
30. KENT, J. P. 1966. Intracellular plasma protein: a manifestation of cell injury in myocardial ischemia. *Nature*. **210**:1279.
31. KOCH, G., N. QUINTRELL, and J. M. BISHOP. 1966. An agar cell-suspension plaque-assay for isolated viral RNA. *Biochem. Biophys. Res. Commun.* **24**:304.
32. KOCH, G., S. KOENIG, and H. E. ALEXANDER. 1960. Quantitative studies on the infectivity

of ribonucleic acid from partially purified and highly purified polio virus preparations. *Virology*. **10**:329.

33. KORNGUTH, S. E., H. A. STAHMANN, and J. W. ANDERSON. 1961. Effect of polylysine on the cytology of Ehrlich ascites tumor cells. *Exptl. Cell Res.* **24**:484.
34. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265.
35. MACDONALD, K. 1959. The release of soluble proteins by ascites tumor cells. *Biochim. Biophys. Acta.* **36**:543.
36. MEIZEL, S., and E. R. M. KAY. 1965. Studies of the incorporation *in vitro* of DNA by cells of the Ehrlich-Lettré ascites carcinoma. *Biochim. Biophys. Acta.* **103**:431; and also Kay, E. R. M. 1966. Incorporation of DNA by cells of the Ehrlich-Lettré ascites carcinoma. *Trans. N. Y. Acad. Sci.* **28**:726.
37. OREN, R., A. E. FARNHAM, K. SAITO, E. MILOFSKY, and M. L. KARNOVSKY. 1963. Metabolic patterns in three types of phagocytizing cells. *J. Cell Biol.* **17**:487.
38. OUDEA, P. R. 1963. Anoxic changes of liver cells. *Lab. Invest.* **12**:386.
39. ROOF, B. S., and J. C. AUB. 1960. Isolated cells: normal and tumor. I. Preparation and protein synthesis of thymus and Yoshida ascites cells of the rat. *Cancer Res.* **20**:1426.
40. RYSER, H. J.-P. 1963. The measurement of I^{131}-serum albumin uptake by tumor cells in tissue culture. *Lab. Invest.* **12**:1009.
41. RYSER, H., J. C. AUB, and J. B. CAULFIELD. 1962. Studies on protein uptake by isolated tumor cells. II. Quantitative data on the adsorption and uptake of I^{131}-serum albumin by Ehrlich ascites tumor cells. *J. Cell Biol.* **15**:437.
42. RYSER, H., J. B. CAULFIELD, and J. C. AUB. 1962. Studies on protein uptake by isolated tumor cells. I. Electron microscopic evidence of ferritin uptake by Ehrlich ascites tumor cells. *J. Cell Biol.* **14**:255.
43. RYSER, H. J.-P. and R. HANCOCK. 1965. Histones and basic polyamino acids stimulate the uptake of albumin by tumor cells in culture. *Science*.**150**:501.
44. SBARRA, A. J., and M. L. KARNOVSKY. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leucocytes. *J. Biol. Chem.* **234**:1355.
45. SCHREK, R. 1936. A method for counting the viable cells in normal and in malignant cell suspensions. *Am. J. Cancer.* **28**:389.
46. SMULL, C. E., and E. H. LUDWIG. 1965. Infectivity of polio virus and its nucleic acid for dehydrated HeLa cell monolayers. *J. Bacteriol.* **89**:52.
47. SMULL, C. E., and E. H. LUDWIG. 1962. Enhancement of plaque-forming capacity of polio virus RNA with basic proteins. *J. Bacteriol.* **84**:1035.
48. SMULL, C. E., M. F. MALLETTE, and E. H. LUDWIG. 1961. The use of basic proteins to increase the infectivity of enterovirus ribonucleic acid. *Biochem. Biophys. Res. Commun.* **5**:247.
49. STRAUS, W. 1964. Occurrence of phagosomes and phagolysosomes in different segments of the nephron in relation to the reabsorption, transport, digestion and extrusion of intravenously injected horseradish peroxidase. *J. Cell Biol.* **21**:295.
50. STRAUS, W. 1958. Colorimetric analysis with N,N-dimethyl-p-phenylene-diamine of the uptake of intravenously injected horseradish peroxidase by various tissues of the rat. *J. Biophys. Biochem. Cytol.* **4**:541.
51. TRUMP, B. F., and D. T. JANIGAN. 1963. The pathogenesis of cytologic vacuolization in sucrose nephrosis: an electron microscopic and histochemical study. *Lab. Invest.* **11**:395.