of the suborder Faviina (Fig. 1) and the corals that exhibit a positive correlation between δC^{13} and δO^{18} (Fig. 2).

The isotopic differences between the ahermatypic corals (circles, Fig. 2) and the other corals may be due to the absence of associated zooxanthellae in the former. The present results do not, however, provide direct evidence bearing on the controversy as to whether zooxanthellae are used as food by their coral hosts or whether they merely stimulate coral metabolism and calcification by producing oxygen and absorbing carbon dioxide and waste products (*17*).

The results appear to have a bearing on the study of ancient reefs and reef-derived sedimentary rocks. It is confirmed that the isotopic composition of reef-derived carbonate sediments will be mainly determined by the relative proportions of carbonates supplied by different organisms; effects of depth, water temperature, and calcification rate (*11*) are relatively minor in comparison with the wide range of isotopic variability that can be attributed to vital effects of the various calcifying organisms. It is possible that the variability of isotopic composition of carbonate sediments recorded by Gross (*12*) and Friedman (*22*) is partly due to primary differences, as well as to the introduction of secondary calcite during diagenesis and lithification. A fossil reef or a reef-derived carbonate rock consisting dominantly of coral fragments should be deficient in both C^{13} and O^{18}, in comparison with carbonate sediments remote from a reef (*3*) where green algae and mollusks are dominant carbonate contributors.

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Histones and Basic Polyamino Acids Stimulate the Uptake of Albumin by Tumor Cells in Culture

Abstract. *Basic proteins and polyamino acids are taken up by mammalian cells at rates up to 3000 times greater than serum albumin. When given together with serum albumin they increase the albumin uptake by a factor that correlates with their own rate of uptake and can reach more than 50-fold. The lowest threshold of activity detected (10^{-10}M) is comparable to the activities of the most potent membrane-active agents.*

A number of electron microscope and histochemical investigations have demonstrated the penetration of intact proteins into mammalian cells. Little is known, however, about the order of magnitude, the mechanism, and the significance of this process. In a previous study (*1*) we reported that monolayers of sarcoma-180 cells, grown and tested in Eagle's medium, bound I^{131}-labeled albumin at a low rate approximating 10^5 molecules per cell per hour. We have since found that the addition of low concentrations of histones or of basic polyamino acids increases this albumin uptake 10- to 50-fold. Now we want to describe this effect, to comment on its possible mechanism, and to discuss its biological implications.

Monolayers of sarcoma-180 cells were exposed for periods of 30 seconds to 2 hours to tissue-culture medium containing human serum albumin labeled with I^{131} (*1*). After rinsing, detachment with trypsin, and washing, the cells were treated with 5 percent trichloroacetic acid and the specific radioactivity of the total cell protein was determined (*1*).

As shown in Fig. 1, calf thymus histone (*2*) stimulated the uptake of albumin up to 15-fold. Crude histone preparations of different commercial origin had comparable effects. However, different histone fractions from a single tissue gave strikingly different results. Thus, arginine-rich histones were markedly more active than crude histones, whereas the lysine-rich histone fraction was essentially inactive (*2*) (Fig. 2). Significant stimulation ($P < .005$) was produced by 1.0 μg/ml of crude histone and 0.3 μg/ml of arginine-rich histone. In comparable concentrations, protamine sulfate and poly-L-lysine stimulated albumin uptake to a lesser extent, but their threshold concentrations and the time curves of their effects were similar. All the basic polyamino acids tested (*2*) stimulated albumin uptake, the most active being poly-L-ornithine, which increased the uptake about 45-fold at a concentration of 10 μg/ml (Fig. 2). Its threshold for activity was well below 0.1 μg/ml, that is, considerably less than $5 \times 10^{-10} M$, a concentration which, under the conditions of our experiments, corresponds to about 10^4 molecules per cell. The D-lysine, DL-lysine, and L-histidine polymers and an L-lysine : tyrosine copolymer (19 : 1)

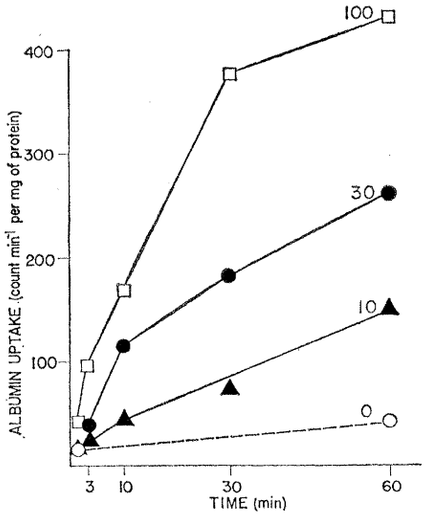

Fig. 1. Effect of 10, 30, and 100 μg/ml of calf thymus histone (*2*) on the time curve of the uptake of albumin labeled with I^{131}. The first time point of the control (lowest curve) gives an estimate of the adsorption of albumin to the cell surface. Net uptake is indicated by an increase of activity above this initial value. Whereas for the control the net uptake amounts to approximately twice the adsorption, the uptake goes to 25 times this initial labeling in the presence of the highest concentration of histone. Extrapolation to time zero suggests that all four curves cut the ordinate at roughly the same point, so that increases in final uptake due to histone cannot be accounted for by increases in initial adsorption.

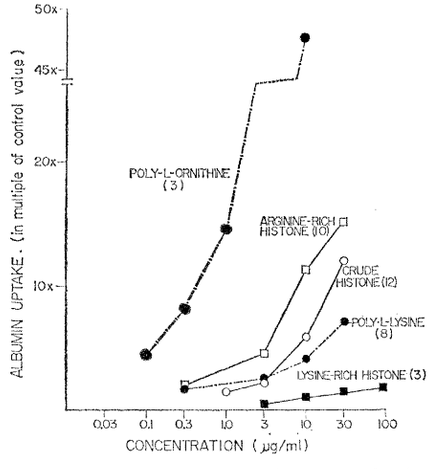

Fig. 2. Semilogarithmic plotting of the dose-effect relationship for three types of histones (solid lines) and two basic polyamino acids (broken lines) (*2*). Incubation for 2 hours at room temperature in Eagle's medium containing 1 percent horse serum. The ordinate represents the uptake of I^{131}-labeled albumin expressed as multiple of the control uptakes measured for each experiment. The curves are averages of the number of experiments indicated in parentheses. The ordinate has been telescoped between 25 $\times$ and 45 $\times$ in order to bring the highest value into the figure.

were more active than poly-L-lysine. The activity of poly-L-lysine was decreased by the introduction in the chain of an acidic amino acid; poly-L-lysine : L-glutamic acid (7 : 3) had little stimulatory effect, and the same copolymer with a ratio of 1 : 1 decreased the uptake to less than the control values. The addition of polyglutamic acid in vitro also decreased or abolished the stimulating effect of the basic polyamino acids. It appears from these comparisons that the overall basicity of the polyamino acids is a prerequisite for their stimulatory activity. However, differences within the group of basic polyamino acids—as, for instance, between poly-L-ornithine and poly-L-lysine, or between poly-D- and poly-L-lysine—can hardly be explained on the basis of overall charges and suggest that activity is determined by more subtle structural features. Different preparations of poly-L-lysine, with molecular weights varying between 15,000 and 70,000, had essentially similar activities when compared on a weight basis. Nonpeptide compounds of lower molecular weights, such as spermine, spermidine, and stilbamidine, were without effect.

Several types of control experiments were performed to determine whether damage to the cell membrane or a gross change in cellular permeability could account for the striking increases in albumin uptake produced by histones or polyamino acids in concentrations of 30 μg/ml or less. Dye exclusion tests on monolayers or on cell suspension derived from exposed monolayers failed to show any increase in staining; pulse-labeling of monolayers with I^{131}-labeled albumin at the end of an exposure to histones did not reveal any increase in albumin adsorption to the cells; both types of increase would be expected in the case of cell damage. When cells were subcultured after exposure to histones, their rate of growth was indistinguishable from that of the control cells. Furthermore, direct observation with the phase-contrast microscope of monolayers grown in tissue-culture chambers did not reveal any morphological changes that would suggest a cytotoxic effect of the tested compounds. It should be emphasized, however, that the use of concentrations higher than those recorded in Fig. 2 led to abnormal cell reactions, such as cell detachment from the glass, cell clumping, increased albumin adsorption, and impaired dye exclusion.

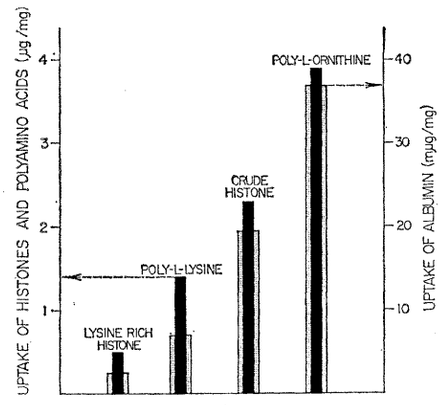

Fig. 3. Correlation between the cellular uptake of basic compounds and their ability to stimulate albumin uptake. This experiment measured the simultaneous uptake of I^{131}-labeled albumin (broad column, scale on the right) and one of four different basic compounds labeled with fluorescein (narrow column, scale on the left). The concentrations were 10 μg/ml of polyamino acids or histones (*2*) and 8 μg/ml of albumin. The average size of the cell samples was 3.4 mg of protein. Incubation for 3 hours at room temperature in Eagle's medium without horse serum. For comparison, all uptakes are expressed in micrograms and millimicrograms per milligram of cell protein.

To obtain a better understanding of the mode of action of histones and polyamino acids, we labeled them with fluorescein by the method used for preparing fluorescent antibodies and studied their binding to the cell. After exposure of cells to 3 μg/ml of labeled poly-L-ornithine, examination in the fluorescence microscope revealed distinct fluorescence limited at first (after 1 minute) to the cell outline but appearing later on (after 30 minutes) over the whole cell. Frozen sections were prepared from cell pellets embedded in gelatin. Sections 5 μ thick, going through the nuclear region, showed optically empty nuclei in contrast with markedly fluorescent cytoplasm. This fluorescence indicated that histones and polyamino acids are readily taken up into the cell cytoplasm. Measurements of the quantity of fluorescent material taken up were made by extracting the fluorescence, that is, by making the cells soluble with 0.1 percent sodium duodecyl sulfate. Such determinations showed that different basic compounds are taken up at different rates, and that these rates are correlated with the relative ability to stimulate albumin uptake. This correlation was best demonstrated in double-label experiments in which we used several compounds that differed

widely in effectiveness (Fig. 3). In these experiments the uptake of the basic compounds was more than 100 times greater than the uptake of albumin.

The striking parallelism between the uptake of fluorescent compounds and radioactive albumin represented in Fig. 3 could be most simply explained if both molecules were taken up in association. However, no trace of complex formation between albumin and poly-L-ornithine could be detected in vitro (*3*).

The stimulatory effect of histones and polyamino acids could be reduced by increasing the serum concentration in the incubation medium. Several metabolic inhibitors were tested, including sodium fluoride, dinitrophenol, potassium cyanide, and iodoacetate, but only the last had some effect. At a concentration of $10^{-4}M$, iodoacetate decreased by half the stimulation produced by 30 μg/ml of histones, but in the absence of histone the albumin uptake was entirely insensitive to iodoacetate. Also, the temperature dependence of albumin uptake was different when measured in the presence or in the absence of histones, with a Q_{10} above 2 in the presence of histones, and below 2 in their absence. These distinct metabolic characteristics reflect differences in the mechanism of these two forms of uptake and suggest that histones do not in fact increase the basic albumin uptake per se. Rather they initiate a penetration process of a different character, in the course of which albumin appears to be driven into the cell in a more passive way. This hypothesis is in agreement with the fact that albumin lags behind histones in terms of absolute uptake, even when the ratio of albumin to polymer in the medium is greater than 1.

Protamine and basic polyamino acids are known to increase phagocytosis in leukocytes in concentrations comparable to those we used (*4*). It is tempting therefore to postulate that the effects we have measured result from a comparable stimulation of membrane movements. However, we have no morphological evidence as yet pointing to an increase in pinocytosis or endocytosis; until such data are available we prefer not to exclude other possible modes of action, such as group translocation or molecular rearrangements in the membrane itself. Reversible changes in fine structure have been demonstrated in the membranes of red blood cell ghosts treated with polylysine (*5*). Moreover, histone concentrations as low as 1 μg/ml have been shown to act on a different type of membrane and to induce marked changes in mitochondrial metabolism and in mitochondrial adenosine triphosphatase activity (*6*). In view of this plural action and of the low threshold of activity, histones and basic polyamino acids must be considered to be highly potent membrane-active agents.

The clumping, detachment, and cell damage we observed at higher protamine and histone concentrations (100 μg/ml and more) are consistent with the data of Becker and Greene (*7*). It is relevant to point out in this respect that the majority of investigators studying the interaction of histones and protamine with living cells have used concentrations of 100 μg/ml and more (*7–11*). The interpretation of results obtained under such conditions must take into account the possibility of cytotoxic reactions, which may obscure the biological significance of the experimental observation; this note of caution may also apply to the current studies of the role of histones in genetic regulation (*9*).

The effect of histones and related substances on macromolecular penetration does not seem to be restricted to proteins. Protamine has been shown—albeit in different concentration ranges—to increase the amount of foreign RNA capable of functioning in fibroblasts (*11, 12*). Preliminary data from our laboratory indicate that polysaccharides such as inulin are influenced in a comparable fashion. It appears therefore that histones and polyamino acids might constitute useful tools for introducing into mammalian cells a variety of macromolecules of biological or therapeutic interest.

Finally, our data provide good evidence for the selective uptake of various proteins. Arginine-rich histones are taken up 10 times more readily than lysine-rich histones and some 1000 times more so than human serum albumin. This selectivity adds to the biological interest of the uptake process and raises the possibility that cellular histones might exert regulatory functions at extranuclear sites.

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6 August 1965

Nucleoside Phosphatases of Fetal and Maternal Blood Cells: Electron Microscope Study

Abstract. *Surface membranes of the blood cells located within the maternal blood sinuses of the rat placenta contain reaction products of nucleoside phosphatases. Fetal blood cells separated from maternal blood by the placental labyrinth show no activity during normal or prolonged gestation. Neonatal blood cells examined 34 hours after parturition show these enzyme activities.*

Recent studies of the fine structural localization of enzymes capable of hydrolyzing nucleoside phosphate esters have shown that there is enzyme activity on the surface membranes of a variety of adult blood cells (*1*). In our study, erythrocytes, leukocytes, and platelets localized within maternal blood sinuses of the chorioallantoic placenta of the rat also showed discrete nucleoside phosphatase reaction products on their surface membranes.