THE ACTION OF RIBONUCLEASE ON NEOPLASTIC GROWTH

III. STUDIES BY INTERFERENCE MICROSCOPY

by

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INTRODUCTION

In previous papers, observations of the *in vitro* and *in vivo* effects of ribonuclease on neoplastic growth have been described[1,2,3,4]. The *in vitro* action of ribonuclease has been followed by chemical methods and expressed in terms of changes in RNA/DNA, free nucleotide/DNA and protein/DNA ratios.

Since the interference microscope provided a method of determining the mass of individual cells, it was considered that it might be of interest to apply this technique to the problem of ribonuclease action. The method would permit one for the first time to study the quantitative response of isolated living cells to various treatments and to correlate the results obtained by chemical methods with data obtained on isolated living cells.

METHODS OF MEASUREMENT

Interference microscopy

The use of an interference microscope enables the mass of a small object on a slide to be determined, if the refractive index of the material is known. The specific protein increments, α (change in refractive index produced by the addition of 1 g to 100 ml), of most proteins are very similar ($\sim$0.00185, ADAIR AND ROBINSON[5]) and a linear relationship holds up to quite high concentrations (BARER[6]). The values for nucleic acids are very similar (BARER AND TKACZYK[7]). Somewhat lower values are obtained for carbohydrates and lipids. But the mass of cellular material is largely protein *e.g.* a typical analysis of Landschütz ascites cells gives protein 46.5 mg, RNA 5.2 mg, DNA 2.6 mg, free nucleotides 1.5 mg, per ml ascitic fluid. Cell mass can therefore be determined with considerable accuracy by this method, as has been shown by DAVIES AND WILKINS[8,9], BARER[6], and by MELLORS, KUPFER AND HOLLANDER[10]. With the Baker interference microscope (SMITH[11]) using a shearing system, effectively two beams are used. The first beam passes through a clear portion of the slide beside the specimen. The measuring beam passes through the specimen. If the specimen and reference region have equal thicknesses and n_r is the refractive index of the clear medium while n_s is that of the specimen, the path difference between the two beams $= (n_s - n_r)t$. This is equal to the number of wavelengths retardation $\times$ the wavelength (λ = 5461 A for mercury green).

With the Smith system, the analyser is first rotated until the medium appears black and the reading taken. The reading is then recorded when the specimen appears black. 180° rotation is equivalent to 1 wavelength and for $x°$ measured in a clockwise direction $5.461 \cdot 10^{-5} \cdot x/180 =$

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References p. 537.

$(n_s - n_t)t$, where t is the thickness in cm. In those cases where the specimens have a thickness which differs from that of the medium $5.461 \cdot 10^{-5} \cdot x/180 = n_s t_s - n_r t_r$ and for different specimens $5.461 \cdot 10^{-5} \cdot x_1/180 - 5.461 \cdot 10^{-5} \cdot x_2/180 = n_{r_1} t_{r_1} - n_{s_2} t_{s_2}$. The quotient $n_s t_s/100\,\alpha$ is equal to the mass per unit area of the specimen. The total mass of the specimen will equal $n_{s_1} t_{s_1}/100\,\alpha \times$ area of specimen. Changes in mass of the specimen can therefore be recorded over a period of time, if the retardation and area are measured.

Ascites tumour cells, in a free suspension, tend to assume a spherical form as shown in Fig. 1b. Provided that the nuclear and cytoplasmic density do not differ greatly a measurement through the diameter of the cell enables the mass to be determined. In practice the cells tend to rest on the surface of the glass in the way shown in Fig. 1c. The total mass is difficult to determine but changes of mass over a period of time can be recorded, provided that the cell diameter does not change markedly during the course of the measurements. The diameter is measured with a micrometer eyepiece, in two mutually perpendicular directions. The arithmetic mean may be used, or alternatively the area calculated for an ellipse as $\pi\, a\, b$, where a and b are the major and minor axes. The cells, as a rule, show slight deviations from a regular shape. However, the general accuracy of the observations is considerably increased, if the cells are slightly compressed between the slide and coverslip as shown in Fig. 1d. In this case the centre of the call is flattened until the nucleus effectively fills the whole thickness of the layer, while the cytoplasm remains as an annulus round the exterior of the nucleus. In this case a uniform extinction is obtained over a reasonable proportion of the area of the cell.

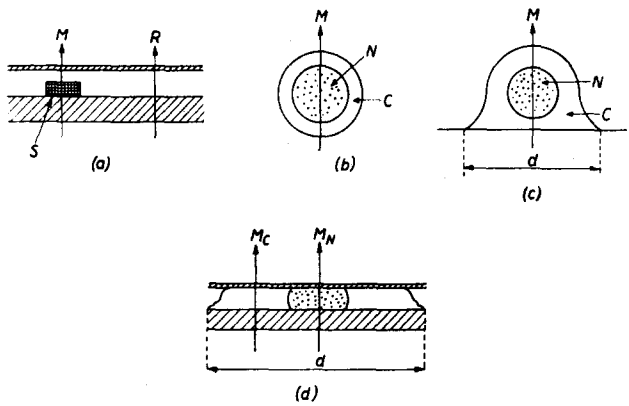

Fig. 1. (a) Method of measurement with Smith (Baker) shearing type of interference microscope. S = specimen; M = measuring beam; R = reference beam. (b) Measuring beam passing through centre of spherical cell. N = nucleus; C = cytoplasm. (c) Measuring beam passing through centre of cell spread on a flat surface. d = diameter of cell image. (d) Measuring beam passing through cell which is compressed between slide and coverslip. Mn = measuring beam through nucleus; Mc = measuring beam through cytoplasm.

Readings can then be taken both on the nucleus and the cytoplasm. If the mean diameters of the nucleus (d_n) and whole cell (d) are determined:

$$\text{Mass of nucleus} = \frac{n_n t}{100\,\alpha} \times \frac{\pi\, d_n^2}{4}$$

$$\text{Mass of cytoplasm} = \frac{n_c t}{100\,\alpha} \times \frac{\pi\,(d^2 - d_n^2)}{4}$$

In order that measurements of this kind can have significance, it is essential that the cells under observation should be in a healthy state. Two requirements must be met:

(1) The cells must have an adequate supply of nutrient material in the medium,

(2) They must have an ample supply of oxygen.

When freshly withdrawn from the peritoneal cavity, the ascitic fluid is densely packed with cells, which rapidly exhaust the medium on a slide. The suspension is therefore centrifuged on a hand centrifuge and the clear supernatant fluid withdrawn. A very small number of cells are then drawn up and suspended in the fluid. The large volume of fluid per cell provides an adequate food supply. An adequate supply of oxygen is ensured by first placing a small piece of microscope

References p. 537.

coverslip (M) on the slide (Fig. 2). A drop of cell suspension is placed on M and the whole is covered with a coverslip of normal size. By careful control of the size of the drop of cell suspension, the thickness of the liquid layer can be quite accurately regulated. The coverslip is sealed with the usual molten mixture of wax and vaseline. The large airspace A ensures that an adequate supply of oxygen reaches the cells; ascites cells mounted in this way have been kept alive for twenty-four hours on the microscope stage, in a flattened condition.

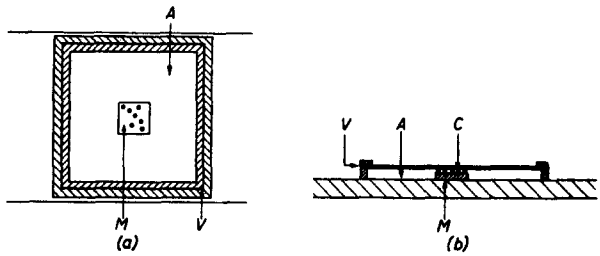

Fig. 2. Method of mounting tumour cells in a healthy condition. M = small square of microscope coverslip; C = cell suspension; A = air space; V = wax.

Biochemical method

The ascitic fluid withdrawn from the animal was placed as quickly as possible in incubation tubes containing a known amount of ribonuclease. The enzyme was, in most cases, dissolved in as little saline as possible, or even in ascitic fluid. The contents of the tube were then carefully mixed. In most cases the tubes were gently shaken throughout the incubation.

At given times, a sample was withdrawn from the tube and centrifuged at once at 3,500 r.p.m. for 3 min. The supernatant fluid was separated and stored.

Both cells and supernatant fluid were treated according to SCHNEIDER[12] and different fractions were obtained: acid-soluble (free nucleotides), TCA extract (DNA and RNA) proteins. The acid-soluble part was analysed by the ultra-violet spectrophotometric method, using the difference $E_{200}-E_{300}$ as a measure of the free nucleotide content.

The TCA extract was analysed according to SCHNEIDER[12] for RNA by using the modification of LUSENA[13], for DNA by using the usual technique.

The proteins were determined by the application of MEHL's[14] method, after digestion of the TCA residue by *N* KOH.

EXPERIMENTAL MATERIALS

The experiments with ribonuclease are divided into three sections. In the first are included those using Ehrlich ascites cells and the "quick-growing" Landschütz Ascites strain[15]; these two strains seemed to react in a comparable way to the enzyme treatment, both visually and from the point of view of mass measurements. After successive weekly re-implantations over a period of *ca.* 2 months the Landschütz strain became much less active. The tumour growth rate was slower, the median survival time gradually increased from 7 days to 16 days[15], and the tumour cells when viewed under the interference microscope were much less active and less capable of being slightly flattened without permanent damage to the cell membrane. In the second section are given results with the "slow-growing" Landschütz strain, and in section three are the results obtained by incubating these inactive cells at *ca.* 36° C, which greatly increased their activity. In the case of the active Landschütz and Ehrlich cells most of the measurements were made at room temperature, as the cells moved so actively at 36° C that measurements were difficult to record.

In the earlier experiments, Worthington or G.B.I. ribonuclease was used, the initial concentration being 10 mg/ml saline. In the later experiments, ribonuclease prepared from beef pancreas and purified by chromatography was kindly supplied by Mr. AVIS, the concentration in this case being 6 mg/ml saline. A second sample from this source was used at a concentration of 10 mg/ml.

EXPERIMENTAL RESULTS

In the control experiments, a drop of cell suspension was mixed with a drop of saline and the volume of the fluid adjusted before covering with a coverslip and sealing

References p. 537.

in the usual way. In the experiments where the cells were treated with ribonuclease, the enzyme was dissolved in saline, a drop of this solution was added to a drop of the cell suspension, and the preparation sealed as above.

Ehrlich and "active" Landschütz cells

The table below gives the general picture of the results obtained. In the case of cells treated with enzyme the most usual effect was to obtain a maximum in the mass

TABLE I

Strain E = Ehrlich L = Landschütz	*Age of implant (days)*	*Change* % per cell*	*t(min)*	*Biochemical data*		
				RNA/DNA	*Protein/DNA*	*t(min)*
E	3	—18	60			
		M 13	35			
L	3	0	60			
		0	60			
L	3 (+ 15 day fluid)	M 62	60	M 50	M 70	60
L	5 (+ 13 day fluid)	M 27	75			
L	5 (+ 5 day fluid)	0	60			
E	6	M 7	16			
		M 11	30			
L	6	—10	60	—15	—10	60
		0	60			
E	6	—25	60			
		0	60			
E	6	M 20	25			
		M 9	30			
L	10	— 6	60	M 25	M 15	60
		0	60			
L	10	0	60			
				Same mouse		
L	10	M 25	45	11-day	M 25	60
		M 13	50	M 33		
L	12	—12	60	M 9	M 13	60
		M 10	55			

* The symbol "M" before the percentage change indicates that a maximum was obtained at the time stated. The % increase was calculated between the reading at zero time, obtained by extrapolation, and the reading at the time of the maximum.

A positive (negative) sign indicates that there was a progressive increase (decrease) in mass, the % change being calculated between the reading at zero time and the reading at 60 min.

Summary

Total No. of cells 22; Maxima 10; Decrease 5; No definite effect 7.
Mean value of mass maximum (10 readings) = 20%.

References p. 537.

readings, this maximum occurring at times which varied from 16 min to 75 min after the initial mixing of the cell suspension and the enzyme solution. This was followed by a slow decrease in the mass of the cell. In extreme cases the cell was finally completely disrupted, after periods varying from 1 h onwards. In the later stages of enzyme treatment the formation of regions of high mass in the nucleus of the cell was also observed.

Following the results obtained by chemical methods and previously described[15], the greatest increase in mass was obtained in the case of tumour cells from a young implant, suspended in ascitic fluid from an old implant. Graphs of mass against time were plotted in all cases, and the results obtained with twenty two sets of measurements are summarised in Table I. The most striking example of a mass maximum, obtained by both methods of measurement, is shown in the curve of Fig. 3.

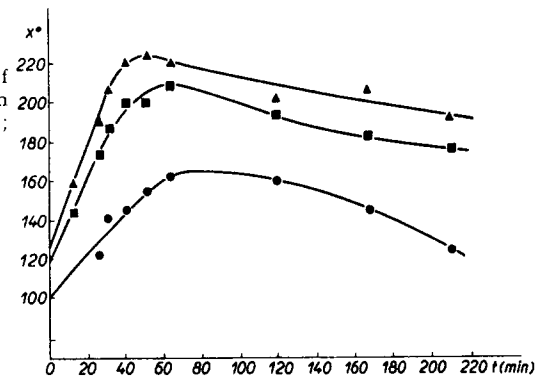

Fig. 3. Changes in mass with time of ascites tumour cell under treatment with ribonuclease. Cytoplasm●; Nuclear sap■; Nuclear inclusions▲.

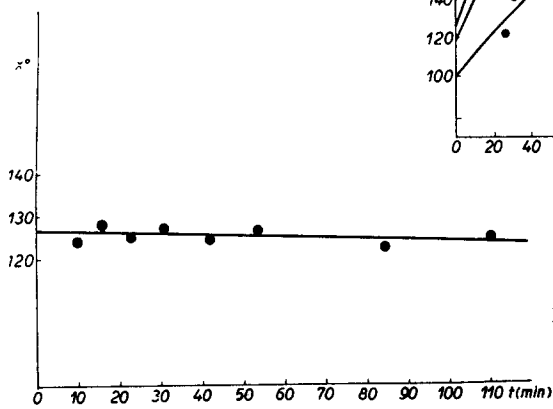

Fig. 4. Mass readings against time for ascites tumour cell (Control experiment).

Slow-growing Landschütz tumour cells

The cells in this case were mostly very inactive, being rounded up, and putting out few pseudopodia. The enzyme treatment appeared to have very little effect, either visually or in the mass readings (see Table II).

Slow-growing Landschütz cells incubated at about 36°

The cells were much more active when incubated, and the general effect was again a maximum in the mass readings as in Section I. The accuracy of the measurements was less than in the first group, owing to cell movement.

Control experiments

These were carried out with Ehrlich and Landschütz tumour cells at room temperature, and with the slow-growing Landschütz cells at 36°. In most cases, no effect or a slight decrease (as in Fig. 4) were observed.

References p. 537.

TABLE II

Strain	Age of implant (days)	% Change in mass	t(min)	Biochemical measurements		
				% Change in RNA/DNA	% Change in protein/DNA	t(min)
L	3 (+ tyrode soln)	M 20	35			
		0	40			
L	6	0	55			
		— 6	60			
L	6 (+ 17 day fluid)	0	60	0	0	60
		0	60			
L	11	— 8	45	— 6	— 3	60
		+ 14	50			

Summary: No. of cells 8; No. of maxima 1; Increase 1; Decrease 2; No definite effect 4.
Value of maximum 20.

TABLE III

Strain	Age of implant (days)	% Change per cell	t(min)
L	7	M 13	35
		M 11	30
L	8	M 19	50
		0	
		M 23	35

Summary: No. of readings 5; Maxima 4; Decrease —; No definite effect 1.
Mean value of mass maximum = 17 %.

TABLE IV

Strain	Age of implant (days)	% Change in mass	t(min)
L	3	0	60
		— 12	60
E	5	0	60
		0	60
E	6	M 8	60
		0	60
L	6	0	60
		M 5	60
E	7	0	60
		0	60
Incubated at *ca.* 36° L	5	— 8	60
		— 18	60
L	7	— 10	60
		— 10	60

Summary: No. of cells 14; Maxima 2; Decrease 5; No effect 7.
Mean value of mass maximum = 7 %.

Visual observation of cell behaviour

In the active cells described in Section I, the appearance of the cells was as shown in Fig. 5c, *i.e.* there was considerable membrane activity. By ciné filming of the ascites cells at 38° C, it was observed that the cell membrane was in continuous undulating motion. This was accompanied by the putting out of long branching pseudopodia, and was observed to be associated with the active intake of droplets of the medium (Fig. 5d). In the slow-growing type cells of Section II, there was very much less membrane activity—the cells were more rounded and pseudopodia put out were fewer in number and much smaller (Fig. 5a).

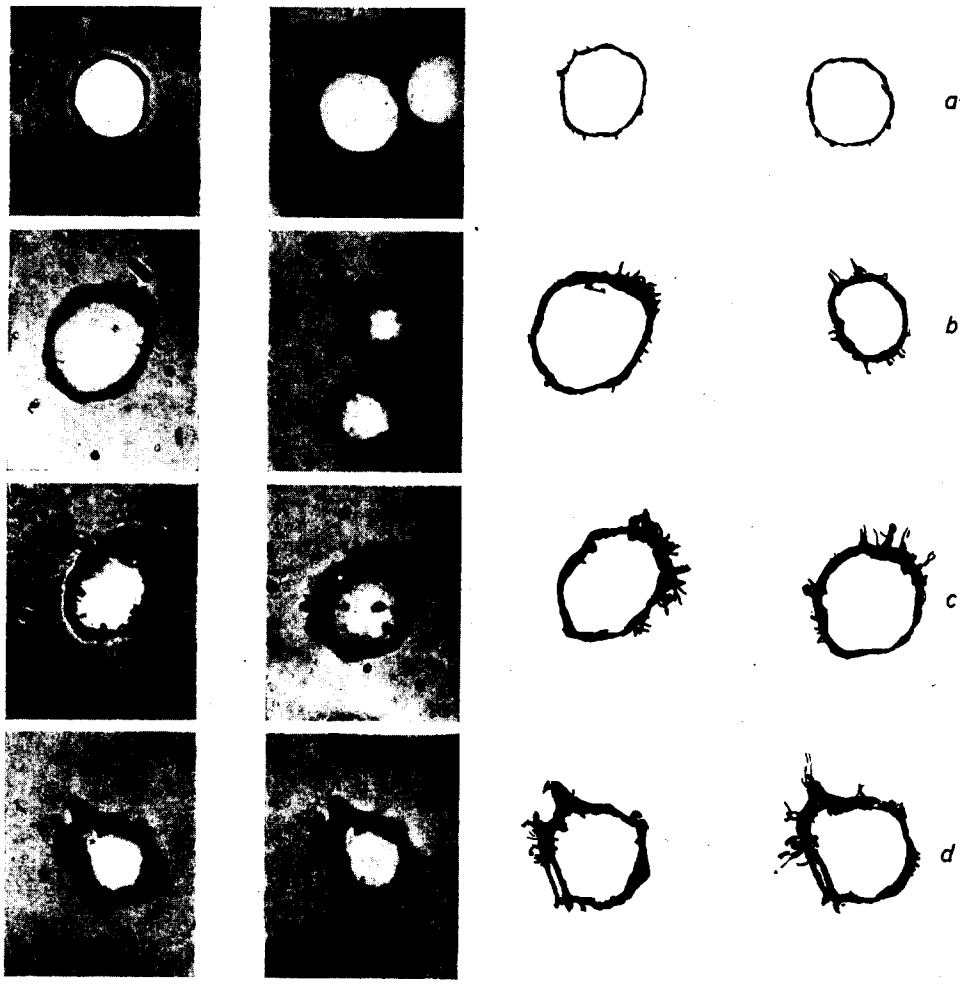

Fig. 5 and Plate. Interference microscope photographs of ascites tumour cells. Contrast set to bring out cell membrane and cell borders. (a) Slow-growing Landschütz cells at room temperature, showing very small degree of membrane activity (100 × shearing objective). (b) Slow-growing Landschütz cells at 38.5° C, showing increased membrane activity (100 × shearing objective). (c) Ehrlich Ascites cells at room temperature, showing a degree of activity comparable to that of the Landschütz cells at 38.5° C (100 × shearing objective). (d) Ehrlich Ascites cells at 38.5° C. Movements are too rapid for still pictures. Two shots of the same cell from time-lapse ciné film are shown. Drop of medium taken up by pinocytosis can be seen.

References p. 537.

The visual effect of ribonuclease was initially to cause a rounding up of the cell and the disappearance of the thin branching pseudopodia normally observed. This was followed later by the spreading out of the cell membrane in a thin halo round the cell, and the formation of many balloon-shaped pseudopodia, accompanied by a high degree of cellular movement. These rounded pseudopodia spread out rapidly from the cell membrane and then suddenly snapped back.

It was observed that the effect of the enzyme on the cells, as shown by observation and mass measurements, was always greater when the cells were originally in an active state and showed much membrane activity. In the case of the slow-growing suspensions where little membrane activity could be seen, the enzyme produced little or no effect.

DISCUSSION

Mass changes in living cells

In the experimental section, it was mentioned that the ascites tumour cells are normally in a highly active state associated with undulating motion of the cell membrane and uptake of drops of medium (pinocytosis). In an actively growing tumour, the cells are taking up material from the medium at a greater rate than the rate of loss. The mass of the cell will increase continuously until it reaches a value which is double that of the original daughter cell and will then divide. Cells normally divide at intervals of 1–2 days. Within the period of one hour an increase in mass of not more than 4% would be expected to occur even under ideal conditions. With a deficient medium, a temperature lower than 30° C, or with unhealthy cells the expected increase would be even smaller. The control experiments using the interference microscope indicate that this is the case; in only two out of fourteen experiments were slight increases in mass observed and these were about the limits expected for normal growth. Biochemical measurements indicate no significant increase of ribosenucleic acid or protein during this period.

In any heterogeneous collection of cells, as in an ascites tumour, there will be a proportion which are in an unhealthy state. The rate of uptake of material will be less than the rate of loss; the mass of the cell will slowly decrease until cytolysis and death finally ensue. A certain proportion of control experiments (~35%) with the interference microscope indicate this effect; biochemical controls also (~40%) show a decrease in ribose nucleic acid and protein.

The stimulating effect of ribonuclease

A time-lapse ciné film indicates that in the presence of ribonuclease, the cells are at first stimulated. There is a retraction of the very fine pseudopodia but an increase in the rate of movement of the cell membrane. The mass readings indicate that in the case of fast-growing cells between 5 and 11 days old, or with younger cells in old fluid, a maximum in the mass readings is observed within one hour, the magnitude of this increase being beyond the range expected for normal growth. The biochemical data also indicate an increase in the RNA/DNA and protein/DNA ratios in a high proportion of similar experiments. In a few cases there is a steady decrease of mass, as occurs also in the case of young cells treated with ribonuclease. These presumably correspond to those cases in control experiments where a gradual disintegration of

References p. 537.

the cells is taking place. They also give rise to a decrease in RNA/DNA and Protein/DNA ratio. Even in those cases which show the stimulation, the increase in mass is also followed by a slow decrease during which time the cellular structure is continuously breaking down.

It may be suggested that an increase in the RNA/DNA ratio does not necessarily indicate an increase in the amount of ribose nucleic acid. But the interference microscope measurements indicate that treatment with ribonuclease leads to an increase of nuclear density; at the end of an experiment dense masses can be seen in the nucleus. This does not suggest that there is a decrease of chromosomal DNA, as a result of the treatment. LEDOUX[16] *et al.* have found in the case of frogs' eggs that ribonuclease treatment leads to the formation of basophilic regions in the nucleus. KAUFMANN AND DAS[17] have observed similar effects in nuclei in onion root tips.

KAUFMANN AND DAS have also observed the stimulating effect of ribonuclease. Differentiating cells that have attained their final degree of polyploidy by a process of endomitotic replication of chromonemata can be induced to divide by this treatment. *In vitro* studies by HEPPEL[18] have indicated that the presence of ribonuclease stimulated the synthesis of polynucleotides. The present series of observations are therefore in agreement with the hypothesis of BRACHET[20] and CASPERSSON[21] on the correlation between RNA and protein synthesis. It must be pointed out however that the increase in protein/DNA ratio does not necessarily indicate that new protein has been synthesized. Such a change could arise from an increased absorption of protein from the medium. In the case of ribose nucleic acid, the evidence suggests that true synthesis has occurred as has already been discussed[15]. But the new material is of no ultimate benefit to the cell because the stimulation is followed by loss and by eventual breakdown of structure. The growth inhibition produced by ribonuclease (LEDOUX[3]) may in fact arise from the abnormal stimulation.

Mechanism of penetration

The degree of cellular response to ribonuclease depends upon the activity of the cells. Those cells which show little membrane activity or pinocytosis, as for example the slow-growing Landschütz at 18° C, are apparently unaffected by the enzyme and remain stable for long periods. Active cells, showing a high degree of membrane activity and pinocytosis show marked visual response and rapid changes of mass. The biochemical studies give similar results—marked changes in RNA/DNA ratio and protein/DNA ratio only in the case of cells which are known to be in an active state. These results suggest that the uptake of ribonuclease by the cell is an active process involving membrane activity and pinocytosis.

In this connection it may be of interest to mention some interesting observations of GOLDACRE[22] on *Amoeba proteus*. In the normal culture medium, the amoeba puts out pseudopodia and encloses food particles by phagocytosis; pinocytosis does not occur. If an amoeba is placed in a solution of ribonuclease of similar concentration to that used in our experiments, the streaming process is reduced. Many small pseudopodia are put out and pinocytosis takes place. Such a phenomenon has been observed in the case of certain other proteins by HOLTER[23].

It would appear that the use of large protein molecules may have an inherent advantage for therapeutic purposes, because they will be taken up most readily by cells such as those of fast-growing tumours which are actively transporting the medium.

References p. 537.

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SUMMARY

The action of ribonuclease, *in vitro*, on a number of suspensions of living ascites cells, in different physiological conditions, has been followed and measured by interference microscopy.

The results show that ribonuclease can produce an important initial increase in the cellular mass (biochemical estimations, made in parallel, indicate that the RNA/DNA and protein/DNA ratios increase during this first phase of the enzyme action) together with a large speeding up of membrane movements and the appearance of cellular deformations.

This stimulation is followed by a loss of mass and, sometimes, by a breakdown of cellular structure. This could be in relation with the anti-tumour properties of ribonuclease.

The effect of the enzyme depends on the initial state of the cells and the results obtained suggest the penetration of ribonuclease to be an active process depending on membrane activity and pinocytosis.

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