STUDIES ON THE EVOLUTION OF NUCLEOPROTEIN FRACTIONS OF THE CYTOPLASM DURING THE GROWTH OF A CULTURE OF *POLYTOMELLA COECA*

I. RIBONUCLEIC ACID CONTENT OF CELLS AND GROWTH RATE

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The hypothesis of a relationship between ribonucleic acid (RNA) concentration and the rate of protein synthesis (BRACHET, CASPERSSON) has been frequently drawn upon to explain the variations in RNA content of the cells of various microorganisms as a function of the stage of growth of the culture in which they are studied[1,2,3,4,5].

Recently CALDWELL, MACKOR, AND HINSHELWOOD[6] have shown, from a comparison of mutants of a yeast with different rates of growth or of cells from the same strain cultivated under different conditions, that there existed a linear relationship between the RNA content per mg organic nitrogen and the reciprocal of the mean generation time.

As will be pointed out below, the very elegant result obtained by these authors from a study of cultures which were most likely in the exponential phase of growth, is by no means verified if one compares the cells of a single culture of the colourless flagellate, *Polytomella coeca*, collected during different stages of its development. The complex nature of the relationship observed under these conditions appeared to depend, most likely, on the fact that the RNA is tied to lipido-protein particles of various sizes and with varying RNA contents, the relative proportions of which may differ according to the stage of evolution of the culture. These particles play different physiological roles as is proved by their markedly heterogeneous enzymatic constitution and their varying turnover rate of RNA[7,8,9,10,11,12]; and it would therefore not be at all surprising that their relative concentrations be markedly different during the latent phase, the exponential phase of growth, and at the point where the medium has been exhausted of certain of its constituents. BRACHET AND CHANTRENNE[13,14] have already shown that modifications of this type are produced during the embryonic development of the chick. Finally, a study on baker's yeast, unfortunately interrupted by the war[2], had provided some evidence in favour of the same idea. However, the quantitative extraction and fractionation of yeast cytoplasm, like that of bacteria, is made practically impossible by the mechanical resistance of their membranes. In order, therefore, to continue our studies, we have made use of a much more suitable material, the *Polytomella coeca*, a colourless flagellate of the group *Volvocales*, which possesses a very fine and fragile

References p. 133.

membrane and which can be cultivated as easily as the most common bacteria and yeasts[15].

The first part of our work, devoted to the study of various nucleoprotein fractions of *Polytomella* and their behaviour during the evolution of a culture, as well as the parallel modifications in the rate of synthesis of proteins, is outlined in this report.

MATERIAL AND METHODS

The strain *Polytomella coeca* utilized was provided by Dr A. LWOFF. The cultures were grown in flasks of 4 to 20 litres in the following medium, adjusted to pH 5: ethanol 3 ml; sodium acetate, 1 g; magnesium sulphate 0.1 g; ammonium sulphate 1 g; aneurine 10^{-4} *M*, 1 ml; Fe^{+++} in the form of citrate 1.82 mg/litre of bi-distilled water[15]. The growth is followed by measurements of "extinction" with a Coleman 14 spectrophotometer, the number of cells per unit volume being determined by means of a calibration curve. Direct cell count was obtained by spreading a known volume of culture, fixed with ZENKER liquid, on a graticule and counting the total number of cells contained in the volume.

The cells are collected quantitatively by means of a Sharpless centrifuge at 15,000 r.p.m., which leaves them perfectly intact. They are washed in the same centrifuge by the rapid passage of several litres of liquid.

The determination of protein N was carried out by means of the MARKHAM apparatus[16] following mineralization of the cells, which had been previously treated with 10% trichloracetic acid and delipidated by the method of SCHNEIDER[17].

The estimation of RNA was carried out by the orcinol method of BARRENSCHEEN AND PEHAM[18] on a 5% perchloric acid extract[19]. It is important that the perchloric acid extraction be carried out only after complete elimination, by repeated washings with trichloracetic acid and alcohol, of substances which react with orcinol to give abnormal coloration. The results obtained by this method were controlled on the entire cells as well as on the various cytoplasmic fractions by a study of the absorption spectra of the perchloric extracts in the Beckman spectrophotometer. The absorption spectra observed corresponded exactly with those given by purified RNA.

The fractionations of the cytoplasmic extracts were carried out in refrigerated centrifuges. During centrifugations at 40,000 r.p.m. for which an air centrifuge was used, the temperature did not exceed that of the laboratory by more than 2 or 3° C.

The lipid-soluble P was estimated by the method of SCHNEIDER[17], cytochrome oxidase by the method of UMBREIT[20] in the presence of a sufficient concentration of cytochrome as to make the reaction rate independent of it.

RESULTS

A. *Notes on the cytochemistry of the Polytomella cells*

Mammalian tissue, and in particular hepatic tissue, has constituted the essential material of modern cytochemical research. In order to make use of the information furnished by the study of the *Polytomella*, which synthesize starch in abundance and are closely related to chlorophyll organisms, it is necessary that we be able to compare them with what we know of mammalian tissues. We shall therefore list at the beginning of this study the few observations made on *Polytomella* cells which may be utilized for such a comparison. Unless specified, our description concerns cells in the exponential phase of growth.

The cells divide every 5 to 7 hours; protein synthesis is thus rapid and it is found, as one would expect, that the proportion of RNA to protein is as high as 6 to 10%.

To this high RNA content there is a corresponding high basophilia. On smears obtained after fixation with Zenker liquid, toluidine blue in particular is fixed in large quantities throughout the cytoplasm, but more than anywhere else, in the half of the cell bearing the flagellae.

Since the cells of *Polytomella* contain large quantities of metaphosphate (J. WIAME,

References p. 133.

private communication), part of the basophilia observed could be due to this substance. As is shown in Table I, the proportion of metaphosphate "tied to the proteins"[21] and which the acid fixative utilized probably does not extract, is however small. The major portion of toluidine blue fixed is therefore indicative of the presence of RNA and not of metaphosphate. It may be added that at the stage of evolution of the cultures considered here, the metachromatic reaction characteristic of metaphosphate does not appear in the cells fixed with ZENKER liquid and colored with toluidine blue.

TABLE I

DISTRIBUTION OF P IN VARIOUS FRACTIONS SEPARATED FROM CELLS IN EXPONENTIAL PHASE OF GROWTH

	P in mg	% of total P
Total P	207.7	100
Inorganic P (orthophosphate)	4.6	2.2
Free metaphosphate (soluble in 10% TCA)	46.4	22.4
Protein bound metaphosphate	1.9	0.9
Ribonucleic acid	39.4	19.0

The nucleus, which is quite small, does not fix toluidine blue except at the small central nucleolus. It can therefore be concluded that the quantity of RNA contained in the nucleus is insignificant in comparison to that present in the cytoplasm.

We attempted without success to show the presence of desoxyribonucleic acid in the nucleus, even during division, by the method of FEULGEN. Despite the fact that wide variations in hydrolysis periods were utilized, we were unable to detect any convincing reaction, aside from a vague pink coloration in the nucleolus or at its periphery. It is therefore considered in what follows that the concentration of desoxyribonucleic acid in the cell is also negligible.

The *Polytomella* cells are suspended in a volume of liquid equal to about 10 times the volume of the cells. Their contents are then homogenized by simply passing the suspension through the orifice of a syringe applied tightly against the bottom of a lusteroid tube. Microscopic examination is employed to control the efficiency of homogenization, which can be carried far enough so that all cell fragments have disappeared. At this point, the very fine cell membranes are no longer visible. Centrifugation of the suspension for 10 minutes at 2,300 r.p.m. results in the appearance at the bottom of the tubes of a pellet of grains of starch on top of which is a thick layer of large granules. The supernatant liquid has the appearance of a suspension of very fine particles.

After their separation from the starch grains, the large granules are readily resuspended in the rest of the homogenate. The resulting suspension is submitted to a series of successive centrifugations* of increasing speeds. The supernatant liquid from the last centrifugation is saved. The various fractions obtained represent thus all the material present in the treated cells, a result which cannot be obtained with cells, like those of the liver, the mechanical dispersion of which is always incomplete.

Numerous authors have reported that the results of this type of fractionation differ

* The precise conditions under which the centrifugations were carried out will be outlined in the second part of this work.

References p. 133.

according to the nature of the suspension liquid utilized. The ideal case appears to be that in which the dispersion of the cells modifies as little as possible the conditions under which the cytoplasmic particles are located in the normal cell from the point of view of p_H, concentration of various cations, etc. We have therefore chosen as the dispersion liquid the physiological solution of CHAMBERS[22] (KCl 0.52%, NaCl 0.175%, sodium citrate 0.25%) which, when injected into the interior of protist cells, apparently does not modify the physical properties of cytoplasm. The only modification made in this solution consisted of buffering it at p_H 7 with $M/200$ phosphate buffer.

RNA, protein N, liposoluble P and cytochrome oxidase of the various fractions were analyzed (*cf.* Tables II and III). The results obtained are markedly similar to those furnished by the fractionation of cytoplasmic particles of the liver (*cf.* especially Reference[9]). As in the case of the liver, the RNA content of the granules increases from the largest to the smallest, the highest content (34% of the protein content in the smallest granules separated out) being however considerably higher in the case of *Polytomella*. The liposoluble P content of the granules is strikingly similar to that obtained in the case of the liver and, as in this organ, is highest in the granules of intermediate size. Finally, 80% of the cytochrome oxidase of the *Polytomella* extract is found in the pellet obtained by centrifugation at 10,000 r.p.m. for 10 minutes. Only the largest particles therefore contain this characteristic enzyme, as in the case of mammalian tissue.

TABLE II

RESULTS OF A FRACTIONATION OF CELLS COLLECTED DURING THE EXPONENTIAL PHASE OF GROWTH AND DISPERSED IN THE CHAMBERS SOLUTION

Fractions	γ RNA in 1 l of culture	γ protein N in 1 l of culture	γ RNA per 100 γ proteins
Pellet 10 min 2.300 r.p.m.	83	411	3.24
Pellet 10 min 10,000 r.p.m.	262	634	6.60
Pellet 10 min 40,000 r.p.m.	141	192	11.7
Pellet 60 min 40,000 r.p.m.	433	203	34.1
Supernatant 60 min 40,000 r.p.m.	450	1128	6.4

It may be added that the phosphorylase found by LWOFF in *Polytomella* is present, according to this author, in granules which are visible in the microscope.

A fact which merits some attention is the quantitative importance of the protein N contained in the granules. According to the time at which the *Polytomella* cells are collected, the protein N sedimented at 40,000 r.p.m. in 1 hour represents 55 to 80% of the total protein N. The nuclei and membranes represent only a tiny portion of the pellet obtained. It appears therefore somewhat difficult to neglect the existence of this fraction, of rather complex chemical constitution, in whatever study one may undertake on the growth of the *Polytomella* cells.

A second particularity of *Polytomella* shared, it is true, by all cells in very active division studied up to the present, is the high proportion of RNA remaining in the

References p. 133.

TABLE III

RESULTS OF A FRACTIONATION OF CELLS COLLECTED DURING THE EXPONENTIAL PHASE OF GROWTH AND DISPERSED IN $M/200$ PHOSPHATE BUFFER, p_H 7.3

Fractions	liposoluble P in 1 l of culture	γ protein N in 1 l of culture	γ liposoluble P per 1 mg protein N
Pellet 10 min 10,000 r.p.m.	81	1865	43.5
Pellet 10 min 40,000 r.p.m.	13.5	104	130
Pellet 60 min 40,000 r.p.m.	8.75	129	67.8
Supernatant 60 min 40,000 r.p.m.	6.25	469	13.3

supernatant fluid following centrifugation for 60 minutes at 40,000 r.p.m. As SZAFARZ[23] has recently shown in our laboratory by an electrophoretic study of *Polytomella* extracts centrifuged at 40,000 r.p.m. for 1 hour, all the RNA present in the supernatant liquid is combined with other substances, most likely proteins, with which it forms compounds of mobility considerably inferior to that of nucleic acid itself. It is therefore quite possible that it may be included in "nucleoproteins" with sedimentation speeds smaller than that of the granules, but which may nevertheless show a similar chemical composition, and be in particular associated with phospholipids.

We shall not discuss this question which will be dealt with in the succeeding study. It should be noted however that the liposoluble P content of the supernatant liquid (*cf.* Table III) corresponds exactly to what we would expect to find if we were to admit that this fraction of liposoluble P is entirely part of granules having the same composition as the smallest which we have been able to isolate (at 40,000 r.p.m. for 1 hour).

There remain two additional points worth mentioning. In most cases, we homogenized the *Polytomella* cells in a $M/200$ phosphate buffer solution at p_H 7.2. The fractionation results obtained under these conditions did not turn out to be different from those obtained by fractionation in the CHAMBERS solution described above. On the other hand, we did not succeed in locating the *Polytomella* nuclei after homogenizing the cells. Whether they remain whole or are dispersed in fragments, the RNA which they contain should not constitute more than a very small part of one of the cytoplasmic fractions studied.

B. *Study of the evolution undergone by Polytomella cells during culture growth*

From the very start of our observations on *Polytomella*, it became evident that their RNA content could vary considerably according to the physiological conditions under which they were placed, and does not show a constant and simple relationship either with the quantity of protein present or with the rate of synthesis of the latter.

If, *e.g.*, we compare the quantity of RNA per unit volume of culture and the number of cells roughly measured by the "extinction", in two cultures, one of which is aerated by means of a strong current of air bubbles and the other by a slow current, it is found that for the same number of cells, the quantity of RNA is twice as great in the feebly aerated culture where the growth is slowest.

References p. 133.

Similar results are obtained by comparison of a culture in a Roux bottle and one in a vigorously aerated flask, studied at maximal growth, at equal times following seeding, and at neighbouring extinction coefficients. The vigorously aerated culture contains, per unit volume, 6 times as much protein as the Roux bottle culture; but, for the same weight of protein, 2.5 times less RNA.

It also appeared that the different physiological conditions under which the cells may have been placed had very different effects on the various nucleoprotein fractions of cytoplasm. If, by centrifugation for 10 minutes at 40,000 r.p.m., we separate the cytoplasmic nucleoproteins of two samples of cells, the first taken at the point at which growth slows down in a completely nutritive medium, the other during slow growth in a medium poor in Fe^{+++}, we find that the ratio of the RNA of the supernatant liquid to the RNA of the pellet may attain in the first case the value of 8, while in the second case it may drop to as low as 0.12[24].

Thus the RNA content of the cells and its distribution between the fractions varies greatly according to the physiological conditions under which the cells are placed. It is then to be expected that similar modifications will appear during the evolution of a culture, since the various stages of this evolution reflect the transformations undergone by the medium as a result of the metabolic activity of the cells.

When a *Polytomella* culture in a complete medium is seeded under the conditions indicated above, the speed with which the number of cells increases begins to rise slowly. During this first stage, the protein content of the cells increases more rapidly than their number. We were unable to study sufficiently accurately the behaviour of the RNA at this point, the quantities available for analysis being too small. No sooner does the logarithmic phase of growth commence than the number of cells (determined photometrically) increases at the same rate as the quantity of protein present per unit volume of culture. When growth slows down, this parallelism between the quantity of protein present and the number of cells is maintained, so long as all the cells retain their mobility and none are lysed. As soon as lysis begins, quantitative examination becomes impossible and measurements are discontinued. This stage of the culture evolution, so difficult to determine in cultures of yeast or bacteria, is very noticeable in the case of *Polytomella*. As soon as motility ceases, the cell membrane breaks, and grains of starch appear in the culture medium.

Fig. 1 shows the evolution of the rate of protein synthesis (k) during the exponential phase of growth and during its slowing down phase. If Q is the protein content of 100 ml of culture and T the "mean generation time" of the proteins:

$$k = \frac{1}{Q} \cdot \frac{dQ}{dt} = 2.3026 \frac{d \log_{10} Q}{dt} = \frac{1}{T} \cdot \ln 2$$

CALDWELL, MACKOR, AND HINSHELWOOD[6] having recently established, in the case of yeast, the existence of a linear relationship between the reciprocal of the "mean generation time" and the quantity of RNA per mg of N, we could expect that in our experiments, the rate of protein synthesis k might be proportional at any given time to this same quantity.

However the quantity of RNA per mg protein N increases during the exponential phase where k is constant and continues to increase when the value of k diminishes. The quantity of RNA per mg N subsequently decreases and finally approaches its initial value, when the value of k is practically zero.

References p. 133.

Thus, in our case, there is no relation between the rate of increase of proteins at the various stages of growth and the quantity of RNA per mg protein N (Fig. 1).

A more complete analysis of the behaviour of the RNA would enable us to clarify the nature of this disagreement. If, at the various stages of evolution of the culture, we homogenize the cells in phosphate buffer and separate the nucleoproteins into two fractions (pellet and supernatant liquid) by centrifugation for 10 minutes at 40,000 r.p.m., we find (Fig. 2) that the RNA content of the two fractions rises exponentially during the exponential increase of the protein N. It continues to increase with the same speed for 6 or 7 hours after the probable moment of cessation of exponential increase

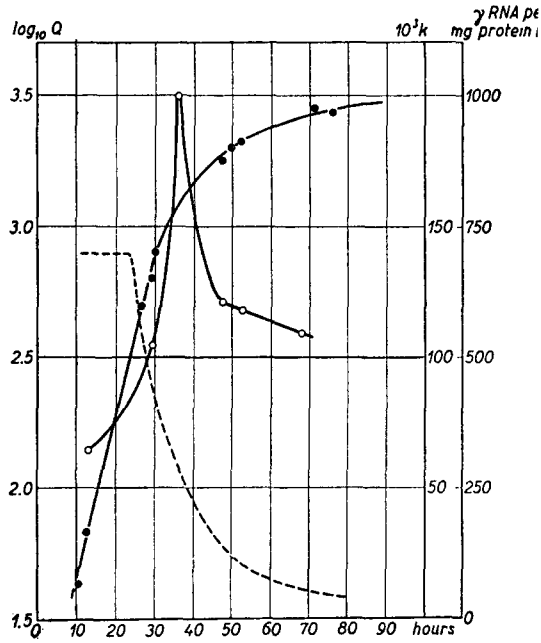

Fig. 1. Rate of increase of protein nitrogen and of ribonucleic acid per mg protein nitrogen during the evolution of a culture

—●——●—: $\log_{10} Q$ (γ protein N per 100 ml of culture)

—o——o—: γ RNA per mg protein N

Dotted curve: rate of increase of protein N

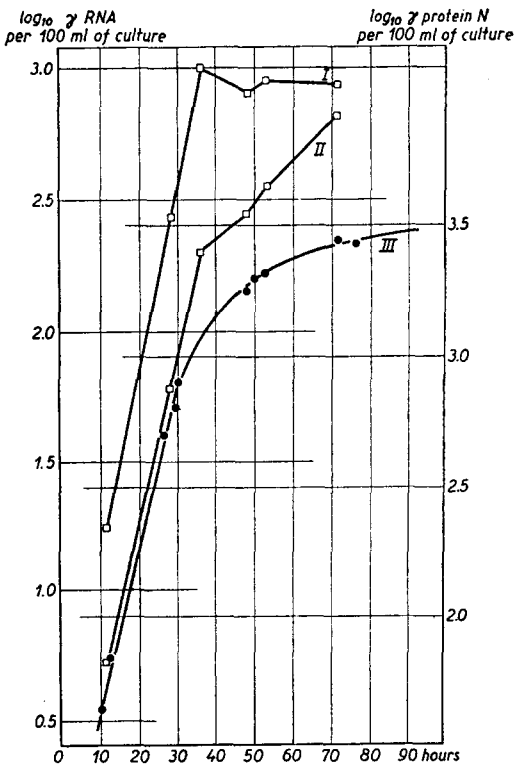

Fig. 2. Quantities of RNA of the supernatant liquid and the pellet separated by centrifugation for 10 minutes at 40,000 r.p.m., at various stages of the evolution of a culture (quantities per 100 ml of culture)

Curve I: $\log_{10}$ RNA of supernatant
Curve II: $\log_{10}$ RNA of pellet
Curve III: $\log_{10}$ protein N

of protein N. After this, the behaviour of the two fractions becomes quite different. While the quantity of RNA of the supernatant liquid decreases rapidly, the RNA content of the pellet continues to increase slowly up to the beginning of autolysis of the culture. In addition, the rate of increase of the RNA of the supernatant liquid during its exponential phase is slightly higher than that of protein N during the same phase. The prolongation of the phase of exponential increase of RNA beyond the end of the phase of exponential increase of protein N and the fact that the RNA of the supernatant liquid increases slightly more rapidly than the protein N during the exponential phase of growth lead to the same result, *viz.*, the increase of the quantity of RNA per mg of

References p. 133.

protein N up to a maximum (Fig. 1) where this quantity is triple that which it was at the beginning of the exponential phase of increase of the protein N.

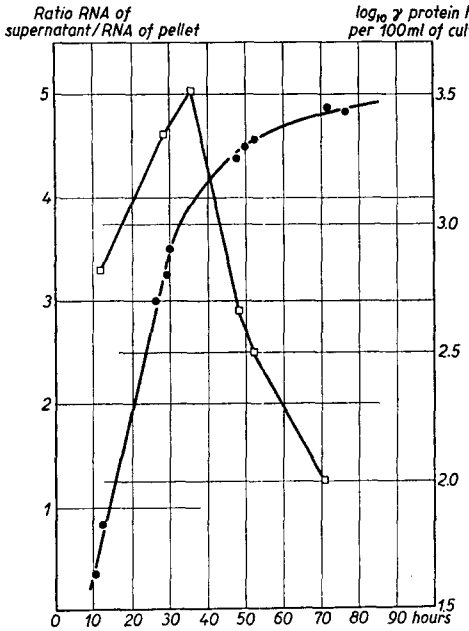

Fig. 3. Ratio of RNA of the supernatant to RNA of the pellet, separated by centrifugation for 10 minutes at 40,000 r.p.m., at various stages of the evolution of a culture

—●——●—: $\log_{11}$ protein N

—□——□—: ratio RNA of supernatant to RNA of pellet

In addition, the growth of the RNA of the supernatant being more rapid than that of the RNA of the pellet during the exponential phase of this growth, and the RNA of the supernatant subsequently decreasing while the RNA of the pellet continues to increase, the ratio existing at each instant between the RNA of the two fractions (Fig. 3) necessarily undergoes large variations, its maximum value being attained several hours after the end of the exponential phase of increase of the protein N.

DISCUSSION

A complete discussion of these results will not be attempted here. This cannot properly be done prior to their comparison with the results obtained by a study of the rate of synthesis of RNA by means of ^{32}P labelled phosphate, to be presented in the second part of this work.

The various results presented in the graphs are expressed as quantities of substance per unit volume of culture. To make them clearer, it is necessary to picture what happens in the individual cell. This can be done without difficulty since, under our experimental conditions, the number of cells per unit volume of culture is approximately proportional to the quantity of protein N found. Our representation of the evolution of the cells will be even more precise if we consider that the RNA measured is, in fact, part of lipidoprotein granules. We can therefore interpret our results in the following manner:

1. During the exponential phase of growth, the increase in the number of the smallest particles is slightly faster than cellular multiplication; that of the larger particles takes place with the same speed.

2. During a period of several hours following the exponential phase of growth, the increase in the number of particles of all sizes continues to take place with the same rate as previously, while the rate of cellular multiplication decreases appreciably.

3. Following this short period, there takes place a rapid degradation of the smaller particles, while the number of the larger ones continues to increase at a rate superior to that of cell multiplication.

In our case, the factor that stops the exponential growth of the culture appears to be the decrease of oxygen concentration in the medium, as is shown by the fact that an increase in the rate of air bubbling when the rate of cell multiplication diminishes causes a noticeable increase in the latter. Whatever may be the nature of this limiting factor, we may in any event record the fact that it exerts a rather unequal influence

References p. 133.

on the various constituents of the cell, and that, in particular, the smaller ribonucleoprotein particles have, during the slowing down phase of growth, a remarkable independence with respect to the cells of which they form a part.

SUMMARY

1. The chemical composition of the cytoplasmic ribonucleoprotein particles of the flagellate, *Polytomella coeca*, varies according totheir speed of sedimentation and resembles that of the ribonucleoprotein particles of mammalian liver.

2. A comparison of the *Polytomella* cells during the various stages of growth of a culture shows that there is no linear relationship between the quantity of ribonucleic acid per mg protein nitrogen and the rate of protein synthesis.

3. The increase in ribonucleic acid content of the various nucleoprotein fractions takes place at a rate which may differ (1) from the rate of multiplication of the cells, (2) from one fraction to another.

RÉSUMÉ

1. Les particules ribonucléoprotéiques du cytoplasme d'un flagellate, *Polytomella coeca*, ont une constitution chimique variable suivant leur vitesse de sédimentation et rappellant celle des particules ribonucléoprotéiques du foie de mammifère.

2. Aucune relation linéaire n'apparaît entre la quantité d'acide ribonucléique par mg d'azote protéique et la vitesse de synthèse des protéines si l'on compare des cellules de *Polytomella* aux diverses étapes de la croissance d'une culture.

3. L'augmentation de la teneur en acide ribonucléique des diverses fractions nucléoprotéiques s'effectue à une vitesse qui peut être différente de la vitesse de multiplication des cellules et différente d'une fraction à l'autre.

ZUSAMMENFASSUNG

1. Die chemische Zusammensetzung der Ribonukleoproteinteilchen des Cytoplasmas des Geisseltierchens, *Polytomella coeca*, ändert sich nach ihrer Sedimentationsgeschwindigkeit und ist der der Ribonukleoproteinteilchen der Säugetierleber ähnlich.

2. Ein Vergleich der *Polytomella*-Zellen während verschiedener Wachstumsperioden einer Kultur zeigt, dass kein lineares Verhältnis zwischen der Ribonukleinsäuremenge pro mg Proteinstickstoff und der Geschwindigkeit der Proteinsynthese besteht.

3. Die Zunahme des Ribonukleinsäuregehaltes der verschiedenen Nukleoproteinfraktionen geschieht mit einer Geschwindigkeit, die 1 von der Vermehrungsgeschwindigkeit der Zellen, und 2 von einer Fraktion zur anderen verschieden sein kann.

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