BACTERIAL PROTOPLASTS

I. PROTEIN AND NUCLEIC ACID METABOLISM IN PROTOPLASTS OF *BACILLUS MEGATERIUM*

by

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Exciting advances are being made in our knowledge of the structure of bacteria—cell walls, flagella, nuclei, fine-structured membranes, "mitochondria", all these and others are being prepared, stained, sectioned and electron-micrographed in increasingly convincing and beautiful fashion. On the other hand, the functions and capabilities of sub-cellular fractions are less well understood and continue to be studied. "Particles" of various shapes and sizes, prepared in a variety of ways, are being found to possess singular properties—certain enzyme systems and pigments, for instance, are found exclusively in certain "particles". Ultimately our understanding of the bacterial cell as an integrated unit will depend upon knowledge of the relationship between structure and function.

There are many ways in which bacteria can be taken apart (HUGO[1]) and most of them have been used both by cytologists and biochemists. One of the more favoured methods at present is supersonic disintegration. GALE AND FOLKES[2] have reported on the incorporation of labelled amino-acids and the synthesis of proteins by staphylococci disrupted supersonically and then extracted in various ways. It is difficult to know however, just what bacterial structures are involved and the material used represents only a small fraction of the original dry weight of the cells. It may well be that these limitations are inherent in mechanical methods of breakage. An alternative method with certain advantages would be controlled enzymic degradation of intact bacterial cells. Unfortunately, few enzyme systems are known which can be used on living bacteria and only a few bacterial species are susceptible to these. At the present time lysozyme (FLEMING[3]) appears to be the most useful.

LESTER[4] has shown that lysozyme-treated *Micrococcus lysodeikticus* retains some ability to incorpotate leucine into the protein fraction and that this can be enhanced by additional presence of deoxyribonuclease (DNA-ase) or abolished by ribonuclease (RNA-ase). Similar findings were reported by BELJANSKI[5] who used glycine in place of leucine. One important feature emerging from this work was that activity was dependent upon the lysozyme treatment being carried out in the presence of sucrose. In neither case was the enzymically prepared material characterised so far as its structural relationship to intact cells was concerned.

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Lysozyme has long been known to cause lysis of certain species of bacteria but recently SALTON[6] has shown that the isolated cell walls of *Bacillus megaterium* and *Sarcina lutea* as well as *M. lysodeikticus* are "substrates" for this enzyme. Moreover WEIBULL[7] has used lysozyme to digest living cells of *B. megaterium* in the presence of sucrose and has obtained two, three or four spherical protoplasts from each intact cell when the cell wall and cross septa have dissolved. The protoplasts can be prepared in excellent yield, comprise about 80% of the dry weight of the original cells, and respire glucose at the same rate as intact organisms. They are, however, extremely fragile and while they retain some of the physiological characteristics of cells, they have not been shown to form colonies or even to divide. Similar preparations have been obtained by the writer from *M. lysodeikticus* and *S. lutea*. It seems possible that the material used by LESTER[4] and BELJANSKI[5] contained comparable structures.

Protoplasts, formed by controlled degradation of intact, living cells, would seem to be ideal for preliminary studies of the relation between structure and function at a level one step below that of the intact cell. This is the first of a series of papers in which various aspects of the biochemistry and physiology of protoplasts are discussed. Here it is shown that the rates of protein and nucleic acid metabolism are comparable with those in intact, growing cells. In the following paper[8] in collaboration with SALTON, the development of bacteriophages in protoplasts is described. A further paper will deal with adaptive enzyme synthesis.

METHODS

Organism and growth conditions

Bacillus megaterium KM (kindly provided by Dr. M. R. J. SALTON) was trained to grow in a synthetic medium containing basal salts solution "C" — NH_4Cl, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 3 g; Mg^{+2}, 10 mg (as $MgCl_2$); S, 25 mg (as Na_2SO_4) and water to 1000 ml; glucose, sterilised separately, was added to give a final concentration of 0.5% w/v (MCQUILLEN AND ROBERTS[9]). This medium is referred to as C/G. The organism was grown overnight at 30° C in tubes containing 50 ml of aerated medium. After several sub-cultures, the mean generation time during the exponential phase of growth was 60–90 min and the dry weight of organisms had reached nearly 1 mg/ml when growth ceased. (These properties were unchanged after plating out on 2% peptone-agar). The cells were harvested from the medium C/G and washed with C medium before use.

Preparation of protoplasts

Cells were suspended in C or C/G containing 7.5% w/v sucrose (C/S or C/G/S). Crystalline lysozyme (Armour) was added (100 μg/ml) and after 10–30 min at room temperature, conversion of the bacilli to spherical protoplasts was complete as determined by inspection under the phase contrast microscope. Suspensions could be diluted with sucrose-containing media and were sometimes centrifuged and carefully resuspended in C/G/S, but reduction in the osmotic pressure of the suspending medium, vigorous aeration, or shaking, all caused breakage of the protoplasts, liberation of viscous cell contents including deoxyribonucleic acid (DNA), and appearance of "ghosts" and particles.

Metabolic experiments

B. megaterium has a strictly aerobic metabolism and it was necessary to devise an aeration procedure which would cause minimum damage to the delicate protoplasts. The most satisfactory method was found to be the use of stoppered test-tubes half filled with the appropriate suspension of cells or protoplasts in a medium containing the substances being studied. The tubes were gently rocked on a specially constructed rocking platform. In some cases 0.01% w/v gelatin was added since there was some evidence that this gave additional protection to the protoplasts.

Isotopically-labelled substrates

$CH_2NH_2{}^{14}COOH$ (0.9 mC/m-mole and 2.7 mC/m-mole); $CH_3{}^{14}COONa$ (2.65 mC/m-mole) and randomly-labelled D-glucose (2.25 mC/m-mole) were obtained from the Radiochemical Centre, Amersham, England. Randomly-labelled uracil and thymine (each approx. 2.7 mC/m-mole) were worked up in this laboratory from residues obtained from the same source.

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Chemical fractionation of cells and protoplasts

The methods used were similar to those described by McQUILLEN AND ROBERTS[9]. Samples from incubation mixtures were centrifuged and the pellet extracted with 5% trichloracetic acid (TCA) in the ice-chest for 30 min and then, after spinning down again, washed once with cold 5% TCA. Lipid was extracted by 75% aqueous ethanol for 30 min at 40–50° C followed by 1/1 ether/75% aqueous ethanol for 15 min at 37° C. Nucleic acids were then removed by 5% TCA extraction for 15 min at 95° C. The protein residue was washed with acidified ethanol and then ether. In a few experiments the initial cold TCA extraction was carried out by adding an equal volume of 10% TCA directly to the sample.

Protein fractions were dissolved in 0.1 *N* NaOH or hydrolysed with 6 *N* HCl in sealed tubes for 16 hours at 105° C, dried down and redissolved in water. Samples for counting were transferred directly to polythene planchets unless they contained TCA in which case they were first washed four times with an equal volume of ether. Counts were made on samples less than 1 mg/sq cm after drying.

Chromatography

Protein hydrolysates were run two-dimensionally on Whatman No. 1 paper using *sec*-butanol/formic acid/water followed by phenol/NH_3/water[9]. Nucleic acid was hydrolysed in 0.1 *N* HCl at 100° C for 1 hour and after removal of the acid, the hydrolysate was chromatographed one-dimensionally in 70% *tert*-butanol in 0.8 *N* HCl. Radio-autographs were prepared on Ilford X-ray film (Industrial G) and ultra-violet light photographs on Ilford Reflex Contact Document Paper.

EXPERIMENTS AND RESULTS

The ability of protoplasts to carry out synthetic reactions was tested by adding isotopically-labelled glycine, acetate and glucose to comparable suspensions of intact cells and protoplasts and, after rocking at 30° C for 1 hour, fractionating the preparations as described above. Table I shows that glycine and glucose carbon was incorporated into both protein and nucleic acid and that acetate contributed carbon to lipid and protein fractions (a small amount of activity was also found in the nucleic acid: this would be expected if acetate is a partial precursor of aspartic acid and this of pyrimidines as is the case in *Escherichia coli* (McQUILLEN AND ROBERTS[9]).

TABLE I

FIXATION OF GLYCINE, ACETATE AND GLUCOSE BY PROTOPLASTS

Tracer		*Lipid c.p.m.*	*Nucleic acid c.p.m.*	*Protein c.p.m.*
Glycine	KM	—	2110	9490
	P	—	1020	6890
Acetate	KM	1125	420	8280
	P	1925	100	6860
Glucose	KM	—	240	1500
	P	—	180	590

Each tube contained 4 mg of cells (KM) or protoplasts (P) in 10 ml C/S. ^{14}C-glycine (7.5 μg, 45,000 c.p.m.) and ^{12}C-glucose (100 mg) were added to one pair; ^{14}C-acetate (31 μg, 165,000 c.p.m.) and ^{12}C-glucose (100 mg) to another pair; and ^{14}C-glucose (1 mg, 83,000 c.p.m.) to the third pair of tubes. Results are expressed as c.p.m. fixed after 1 hour at 30° C.

These results suggested that glycine would be a useful tracer since incorporation into nucleic acid as well as protein implied probable synthesis of purines and conversion of these into nucleic acid, *i.e. de novo* synthesis, whereas GALE AND FOLKES[10] have shown

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that uptake of a single amino-acid into protein can apparently occur in the absence of net protein synthesis (see DISCUSSION). Most of the subsequent work was therefore carried out using labelled glycine so that incorporation of radio-activity into both protein and nucleic acid could readily be followed.

By preparing radio-autographs of chromatograms of protein hydrolysates it was confirmed that protoplasts as well as intact cells converted tracer glycine to protein glycine. Similarly radio-autographs and ultra-violet light photographs of nucleic acid hydrolysate chromatograms showed that the radio-activity incorporated from glycine was localised in adenine and guanine suggesting the presence of conventional pathways of purine synthesis.

Effects of relative concentrations of organisms and glycine

Since *B. megaterium* can grow with glucose as sole carbon source it is evident that glycine can be synthesised by this organism. The extent of incorporation of tracer glycine might thus depend on the relative amounts of exogenous and endogenous glycine. The effects of varying the relative concentrations of glycine and organism were studied. It was found that increasing the exogenous glycine concentration from 0.5 to 4 μg/ml resulted in nearly 4 times more activity being taken up by protoplasts in the same incubation period. Similar effects were observed when the amount of organism was altered. Table II expresses the glycine fixation in terms of μg/mg of organism and it can be seen that in a 3 hour incubation, protoplasts incorporated glycine equivalent to 1% of their own dry weight under certain conditions, while intact cells fixed almost double this amount. That such high levels of incorporation were into both protein and nucleic acid fractions is shown by the data in Table III. Since the incubation system in these experiments was satisfactory for growth of the whole cells, the capabilities of the intact organisms were perhaps not surprising but it was gratifying to find that the fragile protoplasts were almost equally competent.

TABLE II

EFFECT OF SUSPENSION DENSITY ON GLYCINE UPTAKE

Dry weight of organism/ml μg	*Glycine fixed* μg/mg *of organism*	
	KM	*P*
800	0.23	0.19
160	1.9	1.4
16	17	10

Intact cells (KM) and protoplasts (P) incubated in C/G/S for 3 hours at 30° C in the presence of labelled glycine (1 μg/ml).

Time course of glycine incorporation

In order to check more rigorously the relative ability of protoplasts, a three-hour experiment was set up with intact cells (KM), protoplasts (P and S), and lysed protoplasts (L). The latter was a suspension of protoplasts which had been shaken vigorously to cause breakage and served as a control since intact cells which had escaped lysozyme action would still be present and active. The four preparations were suspended in C/G/S containing a mixture of 18 amino-acids including glycine, each at a final concentration

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TABLE III

INCORPORATION OF GLYCINE INTO PROTEIN AND NUCLEIC ACID

Dry weight of organism μg/ml		2000	2000	200	200
Glycine concentration μg/ml		3.75	41.2	3.75	41.2
KM	Protein	0.45*	2.2	4.7	8.6
	Nucleic acid	0.10	0.48	4.1	5.5
	Total	0.55	2.7	8.8	14.1
P	Protein	0.37	1.3	2.7	3.9
	Nucleic acid	0.10	0.32	2.1	2.8
	Total	0.47	1.6	4.8	6.7

Intact cells (KM) and protoplasts (P) incubated in C/G/S for 30 min at 30° C in the presence of labelled glycine.

* Results are expressed as μg glycine fixed into protein and nucleic acid fractions per mg of organism.

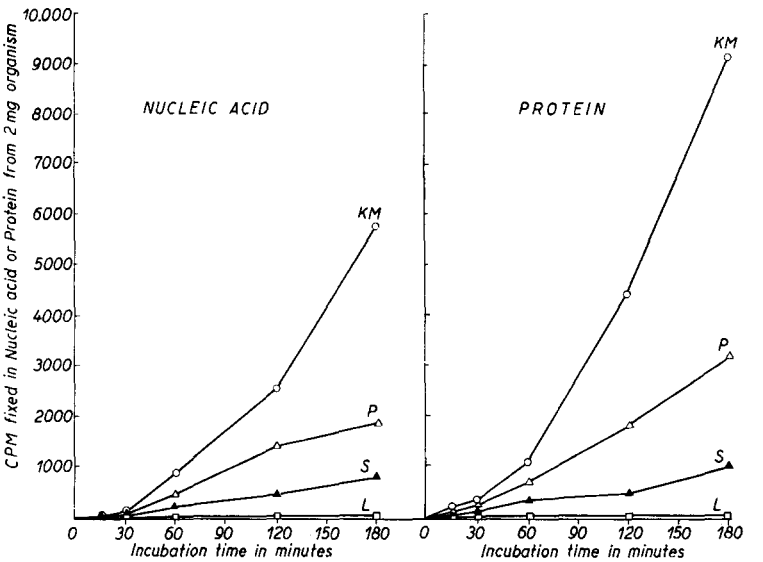

Fig. 1. Time course of incorporation of glycine into protein and nucleic acid. Four tubes each contained 2 mg of organism in 10 ml of C/G/S supplemented with 18 amino-acids (each 50 μg/ml). 28 μg ^{14}C-glycine (34,000 c.p.m.) was added making 528 μg total glycine per tube. Incubation temperature 30° C. KM – intact cells; P – rocked protoplasts; S – stationary protoplasts; L – lysed protoplasts.

of 50 μg/ml (McQUILLEN AND SALTON[11]). Tracer glycine was also added to each tube and KM, P and L were rocked in the usual way to aerate the suspensions, while the second tube of unbroken protoplasts, S, was left stationary. Samples were removed from each tube at intervals and fractionated into nucleic acid and protein. Fig. 1 shows the results obtained.

The lysed protoplasts incorporated virtually no radio-activity at all; the stationary protoplasts became active in both nucleic acid and protein but at a much lower level than in the rocked protoplasts. There are two comments relevant to the latter finding —firstly, there was of necessity some disturbance of S during manipulations for sampling, *etc.*, and secondly, the removal of samples (5 ml, 2 ml, 1 ml at times 15, 30 and

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60 min from a total of 10 ml) left only a shallow layer of suspension during the latter part of the experiment when much of the fixation occurred. Semi-aerobic conditions might thus have prevailed.

The incorporation of glycine carbon into the protein and nucleic acid of the intact organisms proceeded well and at an increasing rate, as would be expected if the cells were growing. The rocked protoplasts were about 1/3 as efficient at fixing glycine. The rate of appearance of radio-activity in protoplast protein increased somewhat with time but the same cannot be said for the nucleic acid. Whether this is significant is not known.

Each tube contained the equivalent of 2 mg dry weight of *B. megaterium* and 528 μg of glycine (34,000 c.p.m.). After 3 hours the protein of the intact cells contained 9100 c.p.m. *i.e.* 9100/34,000 $\times$ 528 μg = 140 μg of glycine had been incorporated into the protein. This amounts to 7% of the total dry weight. The corresponding figure for the protoplasts was 2.5%. In view of the fragility of the protoplasts and the difficulty maintaining them in aerated suspension for any length of time, this performance was considered satisfactory.

Inhibition of glycine incorporation

o-Dinitrophenol inhibited the uptake of radio-activity from glycine into both protein and nucleic acid of protoplasts to about the same extent (Fig. 2A). Uranyl chloride did not reduce uptake into the protein of either cells or protoplasts (occasionally there was a small stimulation) nor did it inhibit the fixation in nucleic acid of whole organisms. It was markedly inhibitory, however, to the incorporation of activity into protoplast nucleic acid (Fig. 2 B).

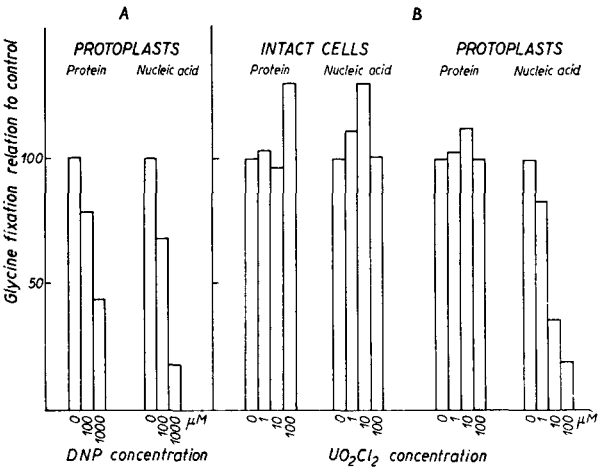

Fig. 2. Inhibition of glycine incorporation. Intact cells and protoplasts (each 0.5 mg/ml) in C/G/S containing labelled glycine (1 μg/ml) incubated for 2 hours at 30° C. Incorporation into protein and nucleic acid fractions expressed relative to 100 for control in the absence of inhibitor. A. – *o*-dinitrophenol; B. – uranyl chloride.

Incorporation of pyrimidine

Randomly-labelled uracil and thymine were also added to preparations of intact cells and protoplasts. Out of 17,800 c.p.m. of uracil added, intact cells fixed 8100 c.p.m

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into the nucleic acid fraction in 100 min at 30° C; protoplasts fixed 4920 c.p.m. The activity in each case was found in both uridylic and cytidylic acids of hydrolysates. It was not established whether thymine was also synthesised from uracil. When labelled thymine was used in similar systems no radio-activity was fixed either by intact cells or by protoplasts.

DISCUSSION

Protoplasts formed by lysozyme treatment of *B. megaterium* have been elegantly demonstrated by WEIBULL[7]. MITCHELL[12] has pointed out that FISCHER[13] described similar structures as far back as 1900. Apparently identical bodies have been seen in cultures of *B. megaterium* lysing under the influence of bacteriophage[8]. Further, the margins of old colonies of the same organism plated on peptone-agar have on occasion yielded preparations containing protoplast-like structures[14]. It seems that protoplasts may occur in the natural course of events and may have some as yet unknown significance. Certainly they are proving to be as interesting from a biochemical point of view as they are cytologically.

The main difficulty in studying the behaviour of these sub-cellular forms is their extreme sensitivity to various physical treatments. Lysis, when it occurs, is very rapid and results in the dense protoplasts becoming pale "ghosts" (still containing granules) and liberating viscous DNA and other cell contents into the medium. However, suspensions of protoplasts in sucrose media remain intact for some days at room temperature and for weeks at 4° C—there is no rapid autolysis. The stimulatory action of DNA-ase found by LESTER[4] and BELJANSKI[5] with lysozyme-treated *M. lysodeikticus* may be related to action on the DNA released from bursting organisms rather than on protoplasts as such, if indeed these were formed in their experiments; the action of RNA-ase in abolishing activity has not been adequately explained (but see GALE AND FOLKES[2] on the action of RNA-ase on disrupted staphylococci). At first sight it appears unlikely that the enzyme can penetrate the protoplast membrane since substances of much smaller molecular weight do not leak out and there is evidence of the existence of an osmotic barrier round the protoplast (MITCHELL[12]).

All the experiments described in this paper were carried out on protoplast preparations of *B. megaterium* in which the contribution by residual whole cells was negligible (*c.f.* Fig. 1). The rates of incorporation of radio-activity from glucose, acetate, glycine and uracil into any or all of the lipid, nucleic acid and protein fractions were comparable with those in intact growing cells; the extent of fixation of glycine in some experiments was equivalent to about 5% of the dry weight of the preparation. These findings suggest that fixation of ^{14}C in these experiments represents net synthesis in the sub-cellular system as well as in the whole organisms. Although GALE AND FOLKES[10] have shown that ^{14}C-labelled amino acids can be incorporated into protein of *Staphylococcus aureus* in the absence of net protein synthesis, the demonstration that protoplasts can maintain the growth of bacteriophages[8] and can form adaptive enzymes[14] further support the suggestion that protein and nucleic acid syntheses occurred in the present study.

The only closely related work known to the author is that of LESTER[4] AND BELJANSKI[5] mentioned above. These workers studied respectively the incorporation of radio-active leucine and glycine into the protein fraction of *M. lysodeikticus* treated with lysozyme in the presence of sucrose. LESTER[4] obtained a fixation of about 0.0025 μ-mole

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of leucine per mg of protein in 2 hours at 37° C in the presence of DNA-ase which enhanced activity. BELJANSKI'S[5] results calculate out at about 0.004 μ-mole of glycine per mg protein in 2 hours at 37° C. The data in the present paper show that markedly higher activities can be obtained with the *B. megaterium* system. For example, protoplasts fixed 3.9 μg glycine into the protein fraction of 1 mg of organism in 30 min at 30° C. Assuming a protein content of 50% w/w, this amounts to 0.1 μ-mole glycine fixed per mg protein (Table III). In another experiment (Fig. 1), in 3 hours at 30° C, protoplasts fixed 2.5% of their own dry weight of glycine into protein *i.e.* about 0.67 μ-mole per mg protein. The rate at which glycine radio-activity appeared in the nucleic acid fraction was less than in protein but protoplasts were in each case comparable in activity with intact organisms.

The ratio of glycine/organism plays a part in determining the level of fixation—over a considerable range, reducing the amount of organism or increasing the glycine concentration results in an enhanced fixation per unit weight of organism (Tables II and III). This may be due to necessity for a high concentration of glycine in order to saturate the fixation system and/or to a "competition" between endogenous and exogenous glycine. Similar effects have been observed with *Escherichia coli* using tracer acetate (MCQUILLEN AND ROBERTS[9]) and labelled amino-acids[15].

One difference between the incorporation of glycine carbon into nucleic acid and into protein of protoplasts is the sensitivity of the former but not the latter to inhibition by uranyl chloride. No explanation can be offered at present for this difference and the same situation does not obtain with intact cells where neither process is sensitive. It is known that bacterial cell walls often react strongly with this reagent (MCQUILLEN[16]). All the incorporation reactions tested were inhibited by dinitrophenol (*cf.* BELJANSKI[5]).

Glycine fixation into both protein and nucleic acid of protoplasts can continue for at least three hours without diminution in rate—if anything there is an increase which suggests continued synthesis of those systems responsible for incorporation. Altogether the results are both qualitatively and quantitatively closely parallel to those obtained with intact, growing cells and it might well be concluded that protoplasts are capable of growth. There remains the enigma of why such preparations of protoplasts cannot divide and form colonies. It may be that something is occurring which is analogous to the "unbalanced growth" of *E. coli* recently described by COHEN AND BARNER[17]. Alternatively it may be that removal of the cell wall prevents the synthesis of some essential substance or structure. Further investigations of these points are being undertaken.

SUMMARY

1. A simple experimental system has been devised for the study of biochemical and physiological properties of protoplasts formed by treatment of *Bacillus megaterium* with lysozyme.

2. The uptake of the radio-active tracers, glucose, acetate, glycine, uracil and thymine, into protein and nucleic acid fractions of the sub-cellular system has been compared with that in intact cells.

3. ^{14}C from carboxyl-labelled glycine appears in protein glycine and also in adenine and guanine of the nucleic acid; uracil is converted to uridylic and cytidylic acid residues of the nucleic acid; thymine is not incorporated by either preparation.

4. The rates of incorporation of ^{14}C from tracers by protoplasts are comparable with those by intact growing cells.

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RÉSUMÉ

1. Un dispositif expérimental simple pour l'étude des propriétés biochimiques et physiologiques des protoplastes obtenus par traitement de *Bacillus megaterium* avec du lysozyme a été mis au point.

2. L'incorporation de traceurs radioactifs, glucose, acétate, glycocolle, uracyle et thymine, dans les fractions protéiques et nucléiques du système subcellulaire a été comparée à celle observée avec les cellules intactes.

3. Le ^{14}C du glycocolle marqué dans son carboxyle apparaît dans le glycocolle protéique et également dans l'adénine et la guanine des acides nucléiques; l'uracyle est transformé en résidus uridyliques et cytidiliques des acides nucléiques; la thymine n'est incorporée par aucune des deux préparations.

4. Les vitesses d'incorporation du ^{14}C à partir des traceurs par les protoplastes sont comparables à celles observées dans le cas des cellules intactes en croissance.

ZUSAMMENFASSUNG

1. Ein einfaches experimentelles System wurde erfunden, um das Studium der biochemischen und physiologischen Eigenschaften der durch Lysozym-Behandlung von *Bacillus megaterium* entstandenen Protoplasten zu ermöglichen.

2. Die Einverleibung von radioaktiven markierten Substanzen, Glukose, Azetat, Glykokoll, Urazil und Thymin, in die Protein- und Nukleinsäurefraktionen des subzellularen Systems wurde mit derjenigen von unversehrten Zellen verglichen.

3. ^{14}C aus in der Karboxylgruppe markiertem Glykokoll erscheint im Proteinglykokoll, sowie im Adenin und Guanin der Nukleinsäure; Urazil wird in Uridyl- und Cytidylsäurereste der Nukleinsäure verwandelt; Thymin wird von keinem dieser Präparate einverleibt.

4. Die Geschwindigkeit der Einverleibung des durch markierte Substanzen in Protoplaste eingeführten ^{14}C ist derjenigen von unversehrten, im Wachsen begriffenen Zellen ähnlich.

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