and the unknowns to all others. This gave consistently 0.0 lesions with the components used in virus reconstitution, except when the nucleic acid was applied at 200 μg./ml., twenty times the amount present at the highest assay level of the reconstituted virus (0.3 lesions per half-leaf).

[10] Kindly supplied by Dr. S. S. Cohen and Dr. V. Cosentino.

[11] R. C. Williams, R. C. Backus, and R. L. Steere, *J. Am. Chem. Soc.*, **73**, 2062, 1951.

---

*ENZYME FORMATION IN PROTOPLASTS OF BACILLUS MEGATERIUM**

BY O. E. LANDMAN AND S. SPIEGELMAN

DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF ILLINOIS, URBANA, ILLINOIS

Communicated by M. M. Rhoades, July 22, 1955

INTRODUCTION

Results of the last two years[1, 2] have led rather compellingly to the conclusion that the induced synthesis of enzyme occurs via a mechanism which condenses amino acids on a template. The possibility that amino acids are "activated" prior to adsorption on the template seems likely from recent preliminary reports.[3, 4] In any event, no evidence for a stable intermediate, smaller than the fully formed enzyme, has yet emerged. If one accepts the template hypothesis, it is evident that further understanding of the mechanism of enzyme formation will not be achieved through attempts at analysis of intermediate stages of protein synthesis, since none exist. This view forces concentration on experiments designed to elucidate the nature and functioning of the template. Data obtained[2, 5] with intact cells synthesizing enzyme suggest that the template is RNA. However, the distance between the observations and the derived conclusions in such experiments is too great for certainty. The need is clear for a subcellular fraction possessing enzyme-forming ability and amenable to more directly interpretable dissection of its components and their function. The recent work of Gale and Folkes[6, 7] on ruptured cell preparations of *Staphylococcus aureus*, and of Zamecnik and Keller[8] on a liver microsome fraction, indicates that the attainment of such systems is close at hand.

Weibull's[9] observation on "protoplast" formation resulting from lysozyme treatment of *Bacillus megaterium* cells in hypertonic medium suggested that another analyzable subcellular system was available. Protoplasts are morphologically quite dissimilar from the rod-shaped cells from which they are derived, being spherical and smaller. Each rod usually yields between 2 and 3 spheres. Lester[10] and Beljanski[11] were able to show that lysozyme treated *B. megaterium* could incorporate labeled amino acids. The question remained whether protoplasts could be induced to synthesize enzyme. This potentiality has been realized. It has been found possible to devise a properly supplemented stabilizing medium, which permits protoplasts of *B. megaterium* to form beta-galactosidase at rates comparable to those of intact cells. Simultaneously, Wiame and his collaborators (personal communication) succeeded in obtaining formation of arabinokinase in lysozyme treated preparations of *Bacillus subtilis*.

It is the major purpose of the present paper to describe the methods of obtaining such preparations from *B. megaterium* and their enzyme-synthesizing properties.

MATERIALS AND METHODS

a) Strain and Conditions of Growth. The *B. megaterium* strain used is a lactose-positive mutant derived from Weibull's[9] strain KM by selective enrichment in a medium containing lactose as the primary carbon source. It has a tendency to revert, particularly in dense liquid cultures, to a lactose-negative rough variant, insensitive to lysozyme. Stability is achieved by keeping stock slants on minimal (1 per cent) lactose medium. The minimal medium has the following composition: KNO_3, 1×10^{-1} *M*; K_2SO_4, 1×10^{-3} *M*; Na_2HPO_3, 1×10^{-1} *M*; $MgSO_4$, 5×10^{-4} *M*; $MnSO_4$, 1×10^{-4} *M*; $Fe_2(SO_4)_3$, 5×10^{-5} *M*; $ZnSO_4$, 5×10^{-5} *M*; and aspartic acid, histidine, valine, cysteine, all 50 μg./ml.

It was empirically found that the growth stage of the cells employed is crucial to reproducible success in obtaining enzyme formation in protoplasts. The following procedure has consistently yielded active preparations: A suitable volume of 2 per cent Difco peptone is inoculated at a level of about $^1/_{2,000}$ that of a stationary phase culture, using cells from stock slants or young 2 per cent peptone cultures. The culture is incubated at 30° C. with shaking to provide vigorous aeration for 12–15 hours. By this time it will have reached the stationary phase and have an optical density ($O.D._{660}$) of 0.55–0.64, using filter 66 of the Klett-Summerson colorimeter and the medium as a blank. The culture is then "rejuvenated" by dilution with 2 per cent peptone to an $O.D._{660}$ of between 0.12 and 0.16 (2.3×10^7 cells/ml.) and reincubated until an $O.D._{660}$ of 0.20–0.22 is reached. It is essential that growth beyond this extent be avoided. The cells are then harvested and washed with 0.2 *M* Na_2HPO_4 adjusted to pH 7.8. They are then suspended (adjusted to an $O.D._{660}$ of 0.70) in the media described below and converted to protoplasts with the aid of lysozyme (Armour and Company) at a level of 200 μg./ml. The density of protoplasts in the usual induction experiment is such that 1 ml. contains 1 mg. of protein.

b) Enzyme Assay.—The enzyme being followed is a beta-galactosidase, which hydrolyzes the synthetic chromogenic substrate ortho-nitrophenyl-β-D-galactoside (ONPG). Its activity can therefore be readily ascertained by procedures similar to those employed[12] for the assay of *Escherichia coli* lactase.

A study of the purified *B. megaterium* enzyme and its induction has been made and will be reported separately. The properties of the enzyme are such as to lead to the following assay mixture for the attainment of maximal activity; ONPG, 1×10^{-3} *M*; Na_2HPO_4, 10^{-1} *M*, adjusted to pH 7.84; $MnCl_2$, 1×10^{-4} *M*; glucose, 0.8 *M*. The enzyme reaction is allowed to proceed at 40° C., the color due to the ortho-nitrophenol released being followed at suitable intervals by reading in a Klett-Summerson colorimeter, using filter 42*a*. Activities are reported as millimicromoles of substrate hydrolyzed per minute. Assays are always carried out on lysed preparations, since neither protoplasts nor cells exhibit their full enzyme content when intact. Dilution of enzyme leads to marked inactivation, which, however, can be avoided by including 0.1 *M* methionine in the diluent.

c) Analytical Methods.—The procedures of Ogur and Rosen[13] were employed to separate the nucleic acids and protein. DNA was estimated by the Dische[14]

reaction, and the RNA spectrophotometrically. Protein was analyzed by the Folin reagent.[15]

d) Enzymes.—The following enzymes have been employed in the present study: lipase (Nutritional Biochemical Company); 2× crystallized trypsin, crystalline ribonuclease, crystalline desoxyribonuclease (all from Worthington Biochemical Sales Company); and crystalline lysozyme (Armour and Company).

EXPERIMENTAL RESULTS

a) The Stabilizing Medium for Protoplasts.—A medium suitable for the purposes in mind must fulfill two principal requirements. Protoplasts suspended in it should remain physically intact and physiologically functional for a period sufficient to perform an induction. Further, the medium must permit the synthesis of the enzyme being followed.

The retention of beta-galactosidase was employed as an indication of physical intactness in the search for a utilizable medium. Fully induced cells, obtained by growth in a 2 per cent lactose-peptone medium, were converted into protoplasts under various conditions, and the distribution of enzyme between the protoplasts and the supernatants was determined at various intervals. Protoplasts were spun down by centrifugation in a Serval SS1 run at top speed for two minutes. This relatively high speed does not injure the protoplasts.

The validity of released beta-galactosidase as an indicator of the extent of physical destruction was justified in separate correlative experiments. These established that the proportionate loss in the enzyme from the sedimented protoplasts was equal to that observed for total protein and DNA. Further, direct microscopic counts of the remaining protoplasts correlated well with what would have been predicted from the measurements of enzyme and the other parameters noted.

Using the enzyme as the test indicator, it was found that 0.2 M sucrose and 7.5 per cent polyethylene glycol[9] failed to stabilize our protoplasts. All the enzyme activity was found in the supernatant, even after short incubation periods. On the other hand, the following compounds, at the concentrations indicated, were effective stabilizing agents: sucrose above 0.6 M, KCl and NaCl at and above 0.8 M, and Na_2HPO_4 at and above 0.5 M.

The effects of these various agents on enzyme-forming ability of intact cells were examined. On the basis of these experiments sucrose was eliminated as a possibility by virtue of its strong inhibitory effect. The data obtained suggested that the sodium phosphate buffer was the most desirable.

b) Induction Medium.—An examination was next undertaken to discover supplements necessary to achieve rates of enzyme formation in protoplasts comparable to those of intact cells held under the same conditions. As a result of these studies, the following induction medium was eventually evolved: 0.5 M Na_2HPO_4, adjusted to pH 7.84; enzymatic hydrolyzate of casein, 2 per cent; $MnCl_2$, 1×10^{-4} M; adenosine triphosphate (ATP), 1 mg./ml.; hexose diphosphate (HDP), 6 mg./ml.; lactose, 6×10^{-2} M.

Of the components listed, the hydrolyzate[7] HDP and lactose are mandatory. The omission of any one of them results in the complete absence of enzyme synthesis. The reasons for the amino acids and lactose requirements are evident, since the latter is the inducer and the former are needed for the formation of

new enzyme molecules. The HDP is apparently necessary for the energy-generating system.

In actual experiments with protoplasts the lactose is omitted during the period of protoplast formation. This avoids including in the experiment enzyme formation by cells or stages intermediate between intact cells and protoplasts. At 30° C. and a level of 200 μg/ml. of lysozyme, virtually complete conversion to protoplasts is attained in 30 minutes. The inducer and other agents are introduced at the end of this period. The progress of the lysozyme treatment can be easily followed by direct microscopic observation and count in an hemocytometer chamber. To avoid foaming during this and all subsequent incubations, aeration is continuously maintained by means of a device which rotates the tubes in a plane inclined to the horizontal.

The 0.5 *M* concentration level of the buffer is necessary for prolonged experiments with the protoplasts. It was found possible to lower the concentration to 0.3 *M* and still obtain sufficient stabilization to permit enzyme synthesis. Figure 1 compares enzyme formation in cells and protoplasts in the induction medium adjusted to 0.5 *M* and 0.3 *M* of the Na_2HPO_4. Except for length of the lag period, enzyme synthesis in intact cell is essentially the same at both concentrations. Protoplasts, on the other hand, exhibit a difference reflected by the cessation of enzyme formation in the 0.3 *M* buffer after 3.7 hours of induction. At this point it can be shown that 50 per cent or more of the enzyme formed has been released into the medium, indicating the onset of physical dissolution of the protoplasts. In the 0.5 *M* buffer enzyme synthesis continues for considerably longer periods.

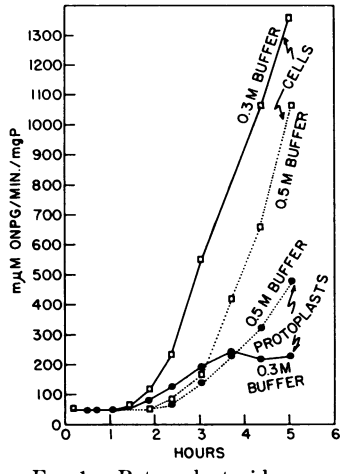

FIG. 1.—Beta-galactosidase synthesis in cells and protoplasts suspended in 0.5 *M* and 0.3 *M* induction medium. Activities are expressed in terms of mμM of substrate hydrolyzed per minute per milligram of protein.

In dealing with protoplasts, there are a few useful facts which should be mentioned. A continual energy source is absolutely essential for the maintenance of enzyme-forming ability. Manipulations in the cold, absence of HDP, and of aeration are therefore to be avoided. Complete disappearance of the ability to synthesize enzyme is followed by even brief exposures to a medium containing amino acids but no HDP. Even the omission of the amino acids leads to severe irreversible loss in enzyme-forming ability.

Finally, if it is desirable to wash and resuspend protoplasts subsequent to some treatment (e.g., with ribonuclease), resuspension should be in supernatant obtained from a freshly prepared protoplast suspension if maximal enzyme synthesis is to be observed. The nature of the responsible factor(s) here is now under investigation.[16]

c) Cells versus Protoplasts.—Qualitatively, the metabolic properties of intact cells versus protoplasts are remarkably similar, provided that the comparison is made under identical conditions. Thus the need for relatively elevated levels

of inducer and external supply of amino acids is not unique to protoplasts but is rather referable to the hypertonic medium. Surprisingly, this holds true even for the HDP requirement, as is shown in Table 1. It is evident that in 0.5 *M* buffer enzyme synthesis in intact cells is impossible if the HDP is omitted, a condition which disappears at the lower buffer levels. The utilization of HDP evidently cannot be a means of distinguishing intact cells of *B. megaterium* from protoplasts under hypertonic conditions. This is a situation markedly dissimilar from that reported[6] for *S. aureus*, in which ruptured cells can use HDP and intact cells cannot.

It is when one examines responses to various enzymes that striking differences between protoplasts and cells begin to emerge. This is clearly exhibited in Table 2 in the case of lipase and trypsin. Intact cells are completely insensitive to the enzymes whereas the synthetic ability of protoplasts is completely abolished.

These results illustrate a point worthy of the attention of those concerned with performing and interpreting experiments with subcellular fractions. Based simply on the observation recorded with lipase, one might perhaps be led to con-

TABLE 1

EFFECT OF HYPERTONIC MEDIUM ON HEXOSE-DIPHOSPHATE REQUIREMENT FOR ENZYME SYNTHESIS IN INTACT CELLS

(Enzyme Activities Expressed as mμM of ONPG Hydrolyzed per Milliliter per Minute)

Buffer Concentration	HDP (Mg./Ml.)	Enzyme Synthesized after 90 Min. Incubation	Buffer Concentration	HDP (Mg./Ml.)	Enzyme Synthesized after 90 Min. Incubation
0.5 *M*	0	0	0.05 *M*	0	966
0.5 *M*	6	294	0.05 *M*	6	1,080

TABLE 2

EFFECT OF TRYPSIN AND LIPASE ON ENZYME FORMATION IN CELLS AND PROTOPLASTS*

Enzyme Present	Cells	Protoplasts
None	1,090	620
Trypsin (100 μg./ml.)	1,140	0
Lipase (100 μg./ml.)	1,020	0

* Cells or protoplasts were suspended in inducer-free induction medium (0.5 *M*) and incubated with the indicated enzyme for 1 hour, subsequent to which inducer (0.06 *M* lactose) was added. The enzyme formed in the next 2 hours is recorded in terms of mμM ONPG hydrolyzed per milliliter.

jecture that a lipid is a key component of the enzyme-forming mechanism. However, the fact is that the loss of enzyme-synthesizing ability is a simple consequence of physical dissolution of the protoplast. After incubation with either lipase or trypsin at the levels indicated, few protoplasts can be recovered.

It is thus important in any given case to demonstrate that an observed inhibition of enzyme synthesis is not the result of a generalized destruction. This caution is also relevant to experiments involving ribonuclease (RNase) and desoxyribonuclease (DNsae). Lysis of protoplasts by RNase has been observed under certain conditions both in our own laboratory and by Brenner (personal communication). To be interpretable, experiments must be accompanied by evidence that the enzyme treatment has resulted in a selective removal of the homologous compound.

An extensive examination has been made of the effects of RNase and DNase on enzyme synthesis in intact cells and protoplasts. The details will be reported *in extenso* in a subsequent publication. We may here summarize the principal findings. RNase at levels of 1 or 2 mg./ml. does not suppress enzyme formation

in intact cells suspended in either hypertonic (0.5 *M*) or normal (0.05 *M*) induction media. Indeed, frequent stimulations are observed. Protoplasts suspended in 0.5 *M* buffer induction media gave variable responses to RNase. However, consistent inhibitions are observed if the exposure to RNase is carried out in 0.32 *M* induction medium. A typical experiment is shown in Figure 2. Here protoplasts were incubated aerobically in 0.32 *M* inducer-free induction mixture with and without 1 mg./ml. of RNase for 1 hour. They were then spun down, washed with 0.5 *M* buffer, and resuspended in 0.5 *M* induction mixture containing inducer, as is described in the last paragraph of section *b* of "Experimental Results." Samples removed after resuspension showed no destruction of protoplasts by the criteria noted above. It is evident from Figure 2 that exposure to the RNase leads to drastic inhibition of enzyme-forming ability. The apparent absence of the lag period seen in Figure 2 is due to the fact that the "zero" time sample is taken after the RNase pretreatment. The protoplasts have thus had 1.5 hours to adjust to the hypertonic medium. Analyses of protoplasts subjected to incubation with RNase show that 80–90 per cent of the RNA can be removed by the enzyme, with no significant loss of DNA or protein.

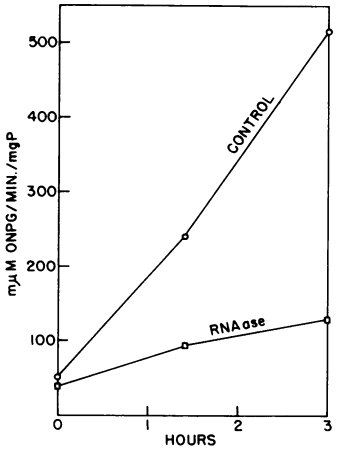

FIG. 2.—The effect of pretreatment with RNase (1 mg./ml.) on subsequent ability of protoplasts to form beta-galactosidase. Activities are expressed in terms of mμM of substrate hydrolyzed per minute per milligram of protein.

DNase did not inhibit enzyme formation in intact cells. On the contrary, in analogy with the observations of Lester[10] and Beljanski,[11] strong stimulations were frequently obtained. Two results were encountered on treating protoplasts with DNase (200 γ/ml.). One was the selective removal of DNA in amounts ranging between 20–50 per cent of the original content. The other involved a concomitant loss of about 30 per cent of the RNA, with little or no loss of protein in either case. Inhibitions of enzyme synthesis were observed only when both nucleic acids were extracted and in these cases the inhibitions were consistent and severe.

The results support the obvious and frequently suggested conclusion that RNA, derived from DNA, is a key component of the enzyme-forming mechanism. A more detailed discussion of the significance of these observations is deferred until it can be done with the aid of reconstitution experiments using the system described, and made possible by the results reported here.

SUMMARY

A procedure and stabilizing media have been described which permit the study of enzyme synthesis in protoplasts of *B. megaterium*. Enzyme-formation properties have been compared with those of intact cells under the same conditions. The protoplasts differ mainly in being susceptible to enzymatic resolution.

Treatment with either RNase or DNase can result in the selective removal of the homologous substrates. When 50 per cent or more of the RNA is thus removed in non-induced protoplasts, loss of enzyme-synthesizing capacity results.

* Aided by grants from the National Cancer Institute of the United States Public Health Service and the Office of Naval Research.

[1] H. O. Halvorson and S. Spiegelman, *J. Bacteriol.*, **64**, 207, 1952; S. Spiegelman and H. O. Halvorson, *Adaptation in Micro-organisms (Symposia of the Society for General Microbiology* [Cambridge: At the University Press, 1953]); D. S. Hogness, M. Cohn, and J. Monod, *Biochim. et biophys. acta*, **16**, 99, 1955.

[2] S. Spiegelman, H. O. Halvorson, and R. Ben-Ishai, in *Amino Acid Metabolism*, ed. McElroy and Glass (Baltimore: Johns Hopkins Press, 1955), p. 124.

[3] J. A. DeMoss and D. Novelli, *Bacteriol. Proc.*, p. 125, 1955; E. B. Keller and P. C. Zamecnik, *Federation Proc.*, **14**, 234, 1955.

[4] M. B. Hoagland, *Biochim. et biophys. acta*, **16**, 288, 1955.

[5] A. B. Pardee, these PROCEEDINGS, **40**, 263, 1954.

[6] E. F. Gale and J. P. Folkes, *Biochem. J.*, **59**, 675, 1955.

[7] E. F. Gale and J. P. Folkes, *ibid.*, p. 661.

[8] P. C. Zamecnik and E. B. Keller, *J. Biol. Chem.*, **209**, 337, 1954.

[9] C. Weibull, *J. Bacteriol.*, **66**, 688, 1953.

[10] R. R. Lester, *J. Am. Chem. Soc.*, **75**, 5448, 1953.

[11] M. Beljanski, *Biochim. et biophys. acta*, **15**, 425, 1954.

[12] J. Lederberg, *J. Bacteriol.*, **60**, 381, 1950.

[13] M. Ogur and G. Rosen, *Arch. Biochem.*, **25**, 262, 1950.

[14] Z. Dische, *Mikrochemie*, **8**, 4, 1930.

[15] O. H. Lowry, M. J. Rosebrough, A. C. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265, 1951.

[16] It has been found that replacement of Na by K in the buffer reduces the need for supernatant factor and improves enzyme formation.

---

*THE DISTRIBUTION OF PARENTAL PHOSPHORUS ATOMS AMONG BACTERIOPHAGE PROGENY**

By Gunther S. Stent and Niels K. Jerne†

VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, AND CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA

Communicated by W. M. Stanley, July 15, 1955.

Approximately half the deoxyribonucleic acid (DNA) contained in a population of T2, T4, or T6 bacteriophage particles reappears among the descendants ultimately issuing from phage-infected bacterial host cells.[1, 2] This transfer is not due to the reincarnation of entire, intact parental DNA units in progeny guise, since at least half the DNA of each of those descendant particles which harbor the transferred atoms must be of nonparental origin.[3] For an understanding of the mechanisms involved in the reproduction of the hereditary structures of the bacteriophage, it is desirable to know the distribution of the parental atoms among the progeny population, i.e., the extent to which the atomic identity of the parental DNA has been conserved or destroyed. It is the purpose of this communication to present the results of experiments which indicate that most of the transferred phosphorus atoms of the parental DNA are distributed over at least 8 but no more than 25 of the progeny. A more detailed description of these results will be presented elsewhere.

The basis of these experiments is that the bacteriophages lose their infectivity upon decay of radiophosphorus P^{32} incorporated in their DNA, the rate of inactivation being proportional to the number of P^{32} atoms per particle.[3] The fraction of