METHODS IN MICROBIOLOGY

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METHODS in MICROBIOLOGY

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PREFACE

Volume 5 of "Methods in Microbiology" is concerned with the microbial cell—methods of observing it, of studying its properties and behaviour, of analysing it chemically and immunologically, and of purifying and characterizing its various "organelles" and macro-molecular components. Wherever possible, the emphasis has been placed on quantitative methods.

We have tried to cover relatively new techniques such as reflectance spectrophotometry, isoelectric focusing and polyacrylamide gel electrophoresis which appear to us to have considerable future potential in microbiology in addition to more generally used techniques such as those for cell disintegration and hybridization of nucleic acids which are not fully described in a concise form elsewhere.

As with earlier Volumes in the Series we have left the treatment of the different topics largely to the individual authors, restricting our editorial activity to ensuring consistency and avoiding overlaps and gaps between the contributions.

As contributions accumulated it became obvious that there was too much material for a single Volume and the content was divided. Volume 5A contains Chapters concerned with the direct observation or study of whole cells or organelles while Volume 5B is concerned with the disintegration of cells, their chemical analysis and the techniques used to separate and characterize their components.

Our thanks are due to the pleasant way in which our authors have co-operated with us and particularly to those who agreed to update their contributions when delay in the publication process made it necessary.

J. R. NORRIS
D. W. RIBBONS

April, 1971

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CHAPTER I

The Disintegration of Micro-organisms

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I. INTRODUCTION

A. History

Although enzymes released from micro-organisms have long played a part in industrial process such as retting and tanning, the deliberate development of methods for extracting microbial enzymes marks the beginning of the modern phase not only of microbiology but also of biochemistry.

The experiments of Buchner (1897) which led to the isolation of cell free fermenting extracts of yeast are well known and often referred to as one of the basic archetypal experiments of modern biochemistry ranking with that of Lavoisier in chemistry. It is often forgotten however that it was in the main the criticisms of this work which led to its further extension. For instance, many critics were loath to believe that the relatively low rates of fermentation achieved by the extract could in any way represent the massive effects of a few live yeast cells, and attempts to rectify this later

led to the discovery of the role of phosphates and of coenzymes (cf. Harden and Young, 1906). Attempts to improve the yield of the intermediate enzymes in glycolysis led to the development of many individual methods of enzyme preparations particularly by Lebedev and others.

In addition to extracting enzymes from disintegrated cells much early work was concerned with the extraction of immunologically active material. Methods evolved around 1900 (cf. MacFayden and Rowland, 1903) included grinding in liquid air in ball mills etc., which were later applied to microbial enzymology. It is somewhat remarkable that the more elegant methods today used for extracting enzymes are little used in immunology.

From 1900 for the next 30 to 40 years microbial biochemistry was largely concerned with the characterization of metabolic pathways and the enzymes which catalyzed them. This is reflected in the increasing sophistication of methods for enzyme extraction. Most of these methods are aimed at increasing speed of extraction, maintaining a low temperature in order to increase the yield of the more unstable and delicate enzymes from the most refractory organisms: these include for example the enzymes of oxidative phosphorylation from Gram-positive cocci, nocardia and mycobacteria, a process which still leaves much to be desired.

In recent years, the art of enzyme extraction has centred less upon the extraction of enzymes *per se* but rather on studies of the relationship between structure and function and thus the localization of enzymes, first between so-called "soluble" and "bound fraction", and later in identifiable intracellular organelles. This has led to a reappraisal and modification of a wide range of available methods most of which had been developed previously. An additional need for this reappraisal is the growing use of extraction methods in the study of enzyme regulation and control. Interpretation of these phenomena based on the quantitation of enzyme activities in extracts often pays far too little attention to possible changes in enzyme properties and kinetics introduced by the very act of extraction.

In reflecting on these later developments, the late Dr. Majory Stephenson's analogue of Microbial Biochemistry as a person observing a house and noting food delivery and analysing dustbin contents is brought to mind. The further step of bombing the house and interpretation of the relationship between architecture, function of the inhabitants and their regulation and control would extend this analogue to our present studies, especially if the interpretation was made with almost a complete disregard for the random effect of the explosion.

The following material is an attempt to rationalize the position as far as is possible by describing the available methods for disintegrating microbes for preparing biologically active fractions. Some brief advice on using the techniques themselves is also given. In addition a summary is

made of the theory of the methods, their mode of action as well as some guidance as to choice of method and the assessment of efficacy.

B. Methods of assessment

1. *Cell disintegration.*

(a) Direct measurement of cell disintegration especially when one of the chemical methods of extraction is used, can sometimes be dispensed with and the criteria of yield of the desired enzyme activity be relied on. Nevertheless, in any comparative work and where control or location is studied by means of a physical method of disruption or with spheroplasts, some method of assessing disintegration is essential. This may include classical methods of direct counting either of numbers or mass (this Series, Vol. 1) but these often have to be adapted to take account of the effects of material released from the cells. This is especially so where for instance DNA or other polymeric material interferes with dilution prior to counting. Some methods, e.g. Coulter counter are rendered almost useless by such material as well as by the presence of cell envelopes or other large particles. In these cases an approximate estimation of the degree of disintegration may be made by proportional counts of stained smears. Here advantage may be taken of differences between whole and damaged cells: for instance damaged Gram-positive bacteria often become Gram-negative, damaged yeast cells take up basic stains more densely than undamaged cells. In the electron microscope the absence of electron dense material in cells examined in unstained drops and the shape of cells in shadowed specimens is diagnostic and can often be used quantitatively: total counts can be made in shadowed specimens if a known number of polystyrene beads (1–2 μm dia.) are mixed thoroughly with the control and disintegrated cells prior to preparing grids by the blot-dry or spraying method.

(b) *Indirect.* Estimation of cell disintegration by measuring the release of typical cell components can be made quantitatively more precise than is direct counting. However there is some difficulty in interpretation and in obtaining a normal zero time and 100% standards. For instance the measurement of the release of soluble protein is often used. This depends partly on the degree of disruption but also on the method used to separate soluble protein from insoluble debris. In the case of yeast we have adopted the standard method of separating cell debris by centrifugation for 10 min at 6000 *g* and estimating protein in the supernatant by the method of Lowry *et al.*, (1951) or with Biuret reagent (Layne, 1957).

These conditions are not entirely arbitrary but were arrived at by methods described in (a) where it was found that when ca. 100% of the yeast appeared as empty cell walls by staining and by electron microscopy,

ca. 80% of the protein appeared in solution. Further comminution of the separated cell walls resulted in a slow release of protein into solution together with increasing amounts of cell wall carbohydrates. Under these conditions it was assumed that a value of 80% protein in solution represented ca. 100% cell disintegration. In adapting this method to bacteria, account must be taken of the higher centrifugal forces needed to remove both whole and partially disintegrated cells and higher concentration of DNA in bacteria and its tendency to precipitate with proteins especially as the pH is reduced below 6·0 must also be taken into account. It is essential to ensure that sufficient buffering capacity is present to counter the fall in pH which results in almost all bacterial suspensions upon disintegration. The use of DNA'ase and the addition of extra alkali, e.g. Na or $KHCO_3$ after disintegration counter these effects.

Other chemical estimations, e.g. 260 nm/280 nm ratio, DNA and RNA, have also been used but it must be emphasized that in each case further information especially by methods in (a) are necessary before degrees of cell disintegration can be estimated. It is worthwhile to recall too, in connection with studies on the intracellular location of enzymes and other components, that the study of the time constants of their release from cells may yield extremely valuable information (Hughes, 1961; Marr and Cota Robles, 1957).

2. *Measurement of enzyme activity*

The measurement of enzyme activity in crude cell or partially purified cell-free extracts particularly from bacteria presents pitfalls which appear not to be generally appreciated. These arise in the first place from the fact that the assay method is often one which is based on the kinetics of the purified enzyme and that it is assumed that these apply in cell-free extracts and intact cells with which they are often compared. There are however now sufficient cases where such assumptions are known to be wrong. Two such may be quoted from our own work. The first concerns the properties of the glutamate decarboxylase and glutaminase of *Clostridium welchii* whose K_m, pH curve and solubility altered considerably not only during the growth of the organism but also during purification in cell-free extracts. This was due to the presence of an unidentified competitive inhibitor which appeared in the cell towards the end of the log phase (Hughes, 1950). The other occurred in studies on polyphosphate synthesis in corynebacteria where crude cell-free extracts were found to be completely inactive until polynucleotides were removed by streptomycin and manganese treatment: the increase in specific enzyme activity suggested that changes in the turnover value of the enzyme might be occurring during purification (Muhhammed, 1959).

It is clear therefore that apart from questions of permeability which arise when comparing whole cells and extracts, changes in the kinetics of enzymes and their stability in cell-free extracts may play an important role in interpretation of questions of regulation and control based on a comparison of enzymic activity. A check of the apparent enzyme yield, kinetics and stability at all stages of isolation and purification therefore is essential, although this is rarely done. A comparison of these properties in extracts prepared by different methods of disintegration and extraction are also of great value in assessing the effects of the method of disintegration.

C. Enzyme location

The criteria already discussed for comparing enzyme activities in whole cells and cell free extracts become of paramount importance in studies on enzyme location by the analytical cytological method (Marr, 1960). Additional techniques are needed and these are briefly mentioned here as far as each is affected by the method of cell disruption.

1. *Quantitative criteria*

For the most part the location of a particular site of enzymic activity in a cell organelle has relied simply on the finding that a major part of the enzymic activity found in the intact cell is located in that fraction of a cell-free extract (Hogeboom, Kuff and Schneider, 1957). It will be evident that the amount of the enzymic activities found in any fraction depends largely on the criteria already discussed and on checking the stability both of the organelle and enzyme in the fractionation procedure. An additional factor is the production of artefacts especially due to the formation of vesicles from disrupted membranes or the absorption of more soluble enzymes on insoluble proteins which may or may not be produced by the method of disruption. For instance prolonged sonication may precipitate initially soluble enzymes (e.g. pyruvate oxidase from lactobacilli, Hughes unpublished).

2. *Morphological criteria*

Morphological studies with microbes inevitably involve the electron microscope with its attendant advantages and disadvantages. The limit of resolution (ca. 0·2 μm) of classical histochemical methods does not assist here (cf. Marr, 1960; Holt and Sullivan, 1958) but newer methods involving radioautography at high resolution (Reith *et al.*, 1967) and the use of ferritin labelled antibodies (this Series, Vol. 5a) may improve this approach and assist interpretation of results obtained on isolated intracellular fractions. Few studies at high resolution of the processes of microbial disintegration

have been made and associated with the intracellular location of enzymes. But recent work on the relationship between mesosomes and the protoplasmic membrane indicate how useful such an approach may be.

II. PHYSICAL METHODS OF BREAKAGE

A. Mechanism of mechanical cell disintegration

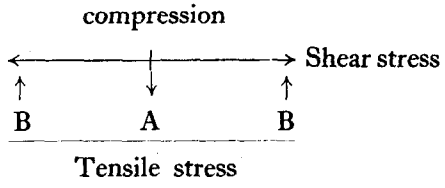
1. *Crushing and grinding*

The methods used for crushing and grinding dry microbial powders are similar to those used widely in industry and elsewhere but with the important difference that the particle size, especially of bacteria, is very much smaller than that usually dealt with. This imposes several problems not least of which is that the machines must be made to high precision and that this must be maintained in the face of constant wear. One other important difficulty is that the phenomenon of caking associated with fine powders (10–1 μm diam.) is met with almost immediately and not in the later stages of the process. However it is probably unnecessary to comminute each microbial cell into finer fragments because the aim of grinding is to facilitate enzyme extraction. Changes in permeability due to the introduction of cracks through the envelope is probably all that is necessary. This is an important consideration because both the power requirements and elaboration of equipment increase exponentially with the degree of fineness of the product. Dry grinders and crushers include ball and vibratory mills, roller mills, plate mills and powder attrition mills. No general theory is applicable to these. Three main theoretical treatments, those of Bond, Kick and Rittinger differ mainly in their definition of the measurement of comminution (cf. Marshall, 1966). The equation (1) expresses an overall balance,

$$\frac{dE}{dX} = -C\frac{1}{X}N \qquad (1)$$

where E = net energy required/weight/unit of comminution. X = size factor N = an exponent, if we neglect differences in definition of size factor of feed and product. It follows that the energy requirements and the grinding index C, depend on the properties of the material and the degree of comminution required. The solid properties of dry or frozen microbial cells as yet escape any precise definition. Nevertheless it is likely that they will behave as a crystalline or semicrystalline material when frozen and an elastic or viscoelastic solid when dried. The comminution process proceeds by the introduction of cracks at points of strain introduced by imposing

either a tensile or shear stress in each particle even in beater systems such as hammer mills and ball mills where the initial stress is compressive. This is shown diagrammatically where the compressive stress is transmitted in much the same way as in a beam test—in this model an additional bending moment will also be produced in flexible material, but this is weak compared to the other stresses.



Estimations of the force required to break bonds in ideal crystals have been made (Joffe, 1928) and show that the energy required (ca. 400 Kgcal/ml) is of the order to melt an ionic crystal. No such estimations, even approximate, can be made of the more complex structures dealt with here, but it is likely that malformations and imperfections such as reduce considerably their tensile strength will arise when microbes are dried or frozen. This reduces the energy requirements for fracture which will however remain quite large due to their small size (3–5 μm). Energy is expended producing stresses below that level resulting in cracks or breakages and this energy appears as heat and must be taken into account when working with labile material such as enzymes. Since the degree of fineness is comparatively great, surfaces in the mill such as the liners, balls or abrasives, appear rough in comparison: these too suffer attrition often quite rapidly and it is quite common to find a high degree of metal contamination in these systems. This is particularly undesirable when the metals contain toxic elements such as nickel or copper as for instance in stainless steels. There are no reports of machines made from relatively non toxic material such as titanium or the newer impact resistant ceramics.

2. *Wet milling*

Many mills used for dry grinding and crushing can also be used for wet milling microbes. Others designed specifically for this use include, the Booth Green Mill, Muys Mill, Kalnitsky Mill, Mickle, Nossal, Braun shakers. The common feature of all these devices is that breakage is probably produced directly and predominantly by hydraulic or liquid shear rather than by an initial compressional stress as in dry milling. These methods are thus analogous in action to the Chaikoff and French presses as well as to that of ultrasonics. Fluid Energy Mills are also hydraulic shear systems, but have not yet been used in microbiology.