LOCALIZATION OF ENZYMES IN THE MICROBIAL CELL

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A wealth of literature exists on the morphology and cytological relationships of the internal structures of the microbial cell, but the organization of the subcellular constituents necessary to catalyze the many biochemical processes required for microbial activity and proliferation has eluded precise characterization. The microscopically visible inclusion bodies have been variously regarded as fat globules, volutin, glycogen, gonidia, intracellular parasites, chromatinic bodies, or the equivalent of a true nucleus. In no instance, however, has a specific metabolic activity been attributed to these inclusions, nor have any data been presented relating a possible physiological differentiation of the microbial cell with known cytological entities. On the basis of *a priori* reasoning alone, it is highly improbable in a system as complex as the cell that there be no organization above the molecular level, especially in view of the numerous simultaneously occurring processes, some of which appear to be entirely opposing. For example, it has been demonstrated frequently that many substances such as diphosphopyridine nucleotide (DPN) or adenosine triphosphate (ATP) can be synthesized, utilized in some metabolic process, and likewise be destroyed, all by different groups of enzymes. That some structural orientation within the cell is necessary is obvious, but the requisite cellular organization has not been investigated until recently. It remained for the animal physiologist first to associate metabolism with definite cell constituents.

The initial step in these studies on animal cells was the introduction of a technique for the separation of particulate constituents by differential centrifugation. This method is based upon the principle that granules of varying masses and densities will sediment at different velocities in a centrifugal field so that it becomes possible to separate intracellular bodies by centrifugation. The procedure was developed by Claude, who used it to determine the physiological contributions of the cellular components of mammalian liver. Further extensions and refinements with animal cells have been introduced by Hogeboom, Schneider and their co-workers (47), and the approach more recently has been extended to plant tissue with equally fruitful results (37). These two excellent reviews should be consulted for orientation in the field of enzymatic cytochemistry of multicellular organisms.

In general, four separate fractions have been isolated and characterized: nuclear, mitochondrial, microsome, and soluble. The most completely studied of these structures, the mitochondrion, has been aptly termed the powerhouse of the cell since it supplies most of the energy for cell metabolism by virtue of its ability to carry out oxidative phosphorylation. The major known function of the soluble material is its role in catalyzing the anaerobic breakdown of glucose to lactic acid. The nucleus and microsomes are as yet poorly described; the former is characterized by the presence of deoxypentose nucleic acid (DNA) and the enzymes responsible for the synthesis of DPN, the latter by their high content of ribonucleic acid (RNA) and the presence of esterase and glucose-6-phosphatase (47). Although enzymatic cytochemistry is yet in its formative stages, already considerable knowledge exists regarding subcellular physiology in multicellular organisms.

In bacteriology, however, there is but little information regarding the correlation of cell structure and function. Some scattered observations have been made in the past regarding the site of action of an enzyme, but only in the last few years has there been any serious attempt to study the cytochemistry of the microbial cell. The ultimate aim of such studies is the correlation of cytological with the biochemical disciplines to give a picture of the intracellular organization of the microorganism.

TECHNIQUES FOR CYTOCHEMICAL STUDY

Three general approaches have been used for research in enzymatic cytochemistry, each with certain inherent advantages and shortcomings. These include: (a) cytological observations, utilizing certain key indicator staining reactions on the viable organism; (b) centrifugal separation of

structures within the intact cell, a method developed and applied mainly by Holter and his coworkers (48); and (c) the homogenate technique involving differential centrifugation of constituents isolated from ruptured cells. The procedure of separation *in vivo* requires the use of centrifugal force to stratify cellular contents followed by direct enzyme analysis of the material removed by microdissection. It is of limited applicability to cells of the size commonly encountered in microbiology and has only been used with amebae (48) and, in conjunction with staining procedures, in *Spirillum volutans* (53). Therefore, the discussion in this review will be limited to the other two methods of study.

Cytological Studies

The cytological analysis of the physiological properties of the microbial granules received its initial impetus from the work of Mudd and his collaborators (72, 73) at the University of Pennsylvania on the structures which exhibit localized stained areas following exposure to certain O/R dyes. The reduction of various tetrazoles, particularly triphenyltetrazolium chloride (TTC), to the insoluble formazan, the accumulation of indophenol blue following exposure of the cell to the Nadi reagents (α-naphthol and dimethyl-*p*-phenylenediamine), and the occurrence of the sequence of color changes with Janus green B that is so typical of the animal mitochondrion, were all observed to be associated with granular loci in cells of *Mycobacterium tuberculosis* and *Mycobacterium thamnopheos* (73). The granules exhibiting these reactions were opaque to the electron beam and, upon intense electron bombardment, melted or volatilized. On the basis of the reaction of the internal bodies with these three O/R indicators, as well as some ancillary cytological observations, it was suggested that these particles were bacterial mitochondria. In a subsequent paper, similar inclusion bodies in *Escherichia coli*, *Bacillus megaterium*, *Micrococcus cryophilus* and *Saccharomyces cerevisiae* were reported to catalyze identical indicator reactions (72). In *E. coli* and *M. cryophilus*, the three processes were localized in electron-dense intracellular structures. The electron opaque material of the former, 0.2–0.4 μ in diameter, interestingly gave the typical sequence of color changes in either the intact bacterium or in bacteriophage lysates, although the particles in the extracts showed slower rates of change than those within the unruptured cell.

Various strains of *Salmonella typhosa* accumulated the end products associated with the oxidation-reduction activity on these three test indicators (26). In this organism and in several of the others giving positive cytological reactions, the same granules stained with Sudan black B. These loci did not coincide with the nuclear areas of the bacteria since staining occurred at different sites in the cell when either the DeLamater or Feulgen nuclear stains were used. These cytological studies have been extended to a large number of other microorganisms, including bacteria, yeasts, fungi, and even protozoa. The rate of reduction of the various tetrazoles was stimulated in the presence of exogenous substrates in contrast to the slow endogenous reduction; the effective substances include many of the intermediates of the tricarboxylic acid (TCA) cycle (17, 75).

In cells of *Spirillum volutans* centrifuged at 400,000 $\times$ G, granules concentrated at the centrifugal end of the organism with but little distortion of the cell. Some of these structures became prominent following exposure to Janus green B. Nevertheless, King and Beams (53) hesitated to conclude that these bodies were mitochondria because of the lack of specificity of the stain. In addition to Janus green B, the Nadi reagent and the tetrazoles, potassium tellurite also has been used to differentiate constituents of the bacterial cell. More than 40 years ago, King and Davis (54) reported on the deposition of black, reduced tellurium compounds in cells of *Escherichia coli*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Salmonella typhosa*, *Brucella abortus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Clostridium tetani* and *Saccharomyces cerevisiae*. Similar observations were made by Wachstein (112), who also used the neotetrazolium chloride as a vital indicator.

The results and conclusions of the Pennsylvania group regarding the cytological and morphological aspects of the structures which they propose to call bacterial mitochondria are completely summarized in a series of reviews by Mudd (68–71). These "mitochondria" have been defined (69) as "cytoplasmic granules, dense by electron microscopic and phase microscopic examination, possessing limiting membranes, and possessing coordinated systems of oxidative re-

ductive enzymes; they contain phospholipid and at least in certain cases metaphosphate." Such electron-dense granules are spherical to ellipsoidal in shape and are volatilized in the strong electron beam.

Other cytologists, however, have disagreed with interpretations of this school of thought. In *Caulobacter vibrioides*, for example, two types of intracellular bodies occur, only one of which, the electron-dense granule, volatilizes during electron microscopy. This structure does not show the mitochondrial indicator reactions and is believed to be volutin on the basis of its decrease in size or complete elimination in phosphorus-deficient cultures, its metachromatic reaction, and its melting upon electron bombardment (15, 42). The electron-opaque structures which also volatilized were identical to the metachromatic or volutin particles in several other bacteria, including *Mycobacterium phlei* and *Aerobacter aerogenes* (36, 98, 126). In the latter two, moreover, entirely different techniques for cytological examination were used, one involving examination of identical fields in the light and electron microscopes, the other being carried out in conjunction with extensive chemical analyses.

Considerable controversy has been aroused regarding the validity of the interpretations based upon these cytochemical indicators. In the fungus *Allomyces*, for example, the sites of TTC reduction did not coincide with the structures considered to be mitochondria; rather, the most rapid reduction occurred in the fat droplets (83). The formazan produced from TTC was found to accumulate in the intracellular lipid droplets in cells of animal tissue as well (110, 130). In their study of the intracellular reduction of TTC by the eggs of *Psammechinus*, Gonse and Yotsuyanagi (38) report that, although it is in fact the mitochondria that are responsible for the reduction of the tetrazole, the mitochondria fail to show formazan staining in the presence of lipid bodies, the latter inclusions alone showing the red color. If the bacterial equivalent of a mitochondrion is below the limit of resolution of the light microscope, as may indeed be true, then only the lipids would give a positive reaction. Another objection to the use of TTC has been raised by Weibull (115), who demonstrated that the formazan produced at many sites in *B. megaterium* increases in amount until only one or two areas of accumulation are observed, these secondary loci not corresponding to those initially detected. That such large bodies of formazan deposition do occur by coalescence rather than being indicative of true intracellular reductive activity is supported by the observation that solutions of chemically reduced TTC contained bodies identical to the secondary areas in intact *B. megaterium*. The size of the accumulation zone in the microorganism increases with longer exposure periods (61, 74) and gives extragranular and even extracellular staining (71, 98). Considerable doubt exists, therefore, of the usefulness of TTC as a cytochemical tool for the determination of regions of metabolic activity.

The suitability of the other stains used in the identification of the bacterial granules as mitochondria also has been the subject of some debate. Stafford (100) points out that indophenol produced in the Nadi reaction is lipid-soluble, the blue substance even having been used as a fat stain. Results in agreement with this observation have been reported in studies with *C. vibrioides* where the locus of reduction of the tetrazoles and Janus green B and the oxidation of the Nadi reagent corresponded to structures described as cell-sap vacuoles. The electron-opaque granules in this bacterium never showed this type of O/R activity (42). In *A. aerogenes* also, those bodies which stained with tetrazoles and Janus green B did not correspond to the electron dense material (98). Cells of *C. vibrioides* or isolated pea seedling particles exhibited the same sites of deposition of chemically reduced TTC or Janus green B and autooxidized Nadi reagent as when the change occurred by enzymatic activity of the organism (40, 42, 100). Thus, the granules can adsorb the dyes even in the absence of any enzymatic process; therefore, it seems that the localization of the enzymes associated with these changes is not necessarily at the point at which the color change is seen to take place. The lack of a clear-cut specificity of Janus green B for mitochondria of plant cells has already been reviewed (78, 100). One must, therefore, interpret the observations made with these substances with caution, taking into account the artifacts and possible pitfalls involved in the use of cytological methods in studies of enzymatic cytochemistry. This is especially important in organisms which contain structures, especially lipid material, which will adsorb or accumulate the reaction product.

This point is well illustrated in studies applying

the Gomori histochemical technique for demonstration of the intracellular distribution of phosphatase activity. The phosphate released by the enzyme is precipitated by a metal salt, and the region of such precipitation is taken as the site of that metabolic transformation. This method has been used to localize alkaline and acid phosphatases in the protozoa *Colpidium campylum* and *Tetrahymena gelii* (29, 104) as well as in a large group of bacteria (8, 91). In cells from young *E. coli* cultures, dark cytoplasmic granules produced during the Gomori procedure were noted, similar to those bodies which show the accumulation of the red formazan during TTC staining. The Gomori stain was also found to accumulate in the cytoplasmic granules in cells of *Bacillus subtilis*, *Bacillus brevis*, *Bacillus cereus* var. *mycoides*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa* and *Serratia marcescens*, such structures corresponding to the bacterial "mitochondria" (91). One would conclude from these observations that the alkaline phosphatase of these cells is in the particles. Cytochemically, however, such results are open to considerable doubt. For example, Wachstein and Pisano (113) have demonstrated that the locus of precipitation does not necessarily depend upon any enzymatic activity in microorganisms since the same distribution occurred even in the absence of the substrate. Apparently a nonspecific adsorption of the precipitating material occurs so that the Gomori phosphatase technique is of only doubtful cytochemical validity. Bayliss and collaborators (8), also suggest caution in interpreting the sites of such localization on this same ground, that the distribution of the precipitate may not exactly parallel the distribution of the enzyme. Experimentally, direct assay of alkaline phosphatase in cell fractions of *Azotobacter vinelandii* indicates that the apparent distribution observed microscopically does not reflect the phosphatase localization (3).

In summary, evidently a great need remains for the reinvestigation and reevaluation of cytochemical staining reagents, especially in microbial cells where the specificity never has been clearly established. Three qualifications are necessary for a useful cytochemical indicator: (a) the color change should be specific for a single enzymatic reaction; (b) adsorption of the dye should be specific, not relying, for example, on solubility in lipoidal constituents; and (c) the color reagent should be adsorbed at the site of the enzymatic transformation (100). Even so, the cytological approach falls short of answering the need for a method in studies of the subcellular organization of the microorganism. The small size of the cell, the restricted number of enzyme reactions which can be determined specifically by microscopic means, and the inability to provide quantitative data limit the usefulness of the techniques. Changes observed in the light microscope cannot reveal the locus of action of an enzyme which is associated with submicroscopic granules that are known to be present in many and probably all bacterial species. An approach better suited for quantitative cytochemical investigations would be one in which metabolic transformations and chemical components can be determined directly, with no question as to the specificity of the assay.

Differential Centrifugation of Cell Extracts: The Homogenate Technique

A more objective method which, however, also has certain inherent limitations, is that used so extensively with mammalian liver cells, the homogenate technique. It provides a direct determination of the physical, chemical and physiological properties of structures separated and isolated by differential centrifugation of cell extracts. This approach has the advantage not only of providing the quantitative data necessary for cytochemical research but is also adaptable to many enzymes, with the specific metabolic change being determined rather than some secondary reaction. The use of differential centrifugation in the preparation of metabolically active constituents of the bacterial cell dates from the studies of Stephenson (102), who in 1928 reported on the localization of lactic dehydrogenase and several other enzymes in *Escherichia coli*.

In studying enzyme distribution by this method, an assumption is made that the structures isolated after rupture of the cell are a "reasonably" accurate representation of the same material in the intact microorganism. The tenuousness of such an assumption is obvious, but since this approach is the only one available at present that provides quantitative data, it is necessary to understand the artifacts introduced by cellular rupture, accounting for them when possible or even eliminating their importance by appropriate criteria.

One serious pitfall in these studies is the possibility that cell breakage may lead to a concomitant rupture of the granules with either a comminution in size or a complete loss of soluble

constituents from within the particles. In *Azotobacter vinelandii*, alumina grinding led to recovery of effectively all of the succinic and malic oxidases in the particles, while cell lysis by use of the sonic oscillator resulted in an inability to sediment much of this activity (2). Particle fragmentation by sonic vibrations has also been reported for *Hydrogenomonas facilis* (6) and *E. coli* (35). Breakage by means of rapid agitation of the cell paste with glass beads also may be unsatisfactory since this too can lead to extensive disruption of the granules (80). Thus, neither sonic oscillation nor the use of glass can be highly recommended in studies of intracellular enzyme distribution. Although lysozyme possesses the advantage for bacterial disruption of not requiring any drastic physical treatment, it does lead to the formation of ghosts and protoplasts (116), which may even retain the ability to grow and divide (62). Consequently, the value of lysozyme in localization studies also may be questioned. There is a pressing need, then, for some method of cell breakage which will lead either to no granular disorientation or to a minimum thereof. It has been our preference to use alumina grinding (3) with the azotobacter since this was found to give maximal recovery of catalytic activity in the granules, although no claim is made that this is the final technique of choice.

When the bacterium is ruptured, the intracellular components are released into an alien environment, one that might change both their morphological and biochemical properties. This alteration may be overcome to a great extent by the use of an appropriate menstruum. Since studies with animal tissue have established that use of sugar solutions gives reproducible and cytochemically valid results, it has been our practice with the azotobacter to isolate the fractions only in sucrose or lactose solutions. These fractions are as active metabolically as the original microorganism except for succinic dehydrogenase. Only a small percentage of this enzyme's activity in the unruptured azotobacter was recovered, regardless of the method of cell breakage or the composition of the extracting liquid. This residual succinic dehydrogenase is very stable, however, little change in activity occurring in extracts kept frozen for more than a year. An enzyme stability in microbial fractions analogous to that of animal tissues also has been reported in *Saccharomyces cerevisiae*, where the use of lactose in the suspending fluid led to higher recoveries of aconitase than in the absence of the sugar (45). In *Serratia marcescens*, too, an effect of the composition of the menstruum on the enzymatic constitution of the isolated units has been reported (56). If a carbohydrate is used in the suspending fluid, it should not be one that can be utilized by the organism, as is frequently true of sucrose.

In a suitable menstruum, it is essential to have constituents that will have no deleterious effect, yet will provide particles that represent those in the intact organism morphologically and biochemically. Solutions of electrolytes cause agglutination of *A. vinelandii* particles with resultant difficulty in the separation and differentiation of structural types (3, 23). In the absence of electrolyte in the extraction fluid, however, it has been possible to demonstrate a biochemical heterogeneity among the granules of *A. vinelandii*. In fact, media of high ionic strength have even been included as part of the isolation procedure to precipitate particles which remain in solution following the initial low-speed centrifugation (44). The rate of sedimentation of particles in a nonelectrolyte suspension is appreciably lower, so that higher centrifugal fields are necessary to precipitate the same substituents.

To provide a firm foundation for establishing cytochemically valid results, criteria have been proposed for the determination of the intracellular site of enzymatic activity in the microbial cell (3). These are based upon certain qualifications originally proposed for liver cell distribution studies by Hogeboom and Schneider (47). To establish quantitative validity, the sum of the enzymatic activities and cellular mass found in the isolated fractions must be equivalent to the amount found in the unfractionated cell extract. This requirement serves to indicate interactions among protoplasmic components and may also aid in ascertaining the reliability of the enzyme determination, since the absence of some cofactor or the assay of a complex system would be reflected in a low recovery. Second, the fraction containing the structure with which the enzyme is associated should have a large percentage of the activity. The sources of error in the homogenate technique are sufficiently prominent that the finding of a small amount should be considered of dubious cytochemical significance. Third, the active fraction should contain a concentration of enzyme greater than or equal to that in the extract, taken as 1.0, where concentration is de-

fined as the ratio of the specific activity in the fraction to that in CE (cell extract). This requirement tends to minimize some of the adsorption and rupture effects and also may indicate the presence of enzymes such as the catalase of *A. vinelandii* (3), localized throughout the cell, which would have been overlooked in fractions comprising only a small portion of the cell mass.

The discussion in succeeding sections will include only localization data that are quantitative since there are no means of assessing the role of an enzyme where the results are presented qualitatively. It cannot be overemphasized that fractions giving a positive test for the presence of some substance do not necessarily represent the cytological site for that material. There are many examples to illustrate how entirely contradictory conclusions have been reached on the basis of nonquantitative determinations or incomplete recoveries, the malic dehydrogenase being a common offender (5, 17).

The assay of complex systems presents another source of difficulty arising in localization studies. If O_2 uptake is used as an indicator of the presence of an enzyme, a deficiency in any step between substrate and oxygen would lead to low recovery and possibly erroneous conclusions. Only fractions containing the complete electron transport mechanism will show gas consumption, regardless of the site of maximal physiological activity. For example, incomplete recoveries in reactions of the tricarboxylic acid (TCA) cycle measured by O_2 utilization have frequently been recorded in bacterial preparations (67, 109), and similar observations have been made in preliminary attempts to localize the D-mandelic acid oxidizing system of *Pseudomonas fluorescens* (44). With the latter, 10 per cent of the activity of CE was found in the particles, none in the supernatant fraction. Subsequent studies, however, demonstrated that the enzyme, mandelic racemase, was exclusively a function of the supernatant. Another complex system is exemplified by the hydrogenlyase of *E. coli*, for which the recombination of particles with nongranular cell material led to a fourfold increase in reaction rate (105). This "synergistic" effect is probably the result of the recombination of components separated by the centrifugation but necessary to function together in the catalysis of the complex hydrogenlyase reaction. Clearly, then, it is essential in establishing the intracellular locale of an enzyme to assay only the enzymatic reaction in question under conditions where the rate-limiting component is the concentration of the catalyst itself.

It is especially important at this point in the cytochemical study of bacteria for a well-defined fractionation procedure to be proposed which will permit isolation of constituents in a pure form for physiological analysis, one that will eliminate the present multiplicity of methods with the resultant difficulties in ascertaining similarities in functions of the structures in various microorganisms. Despite the potential sources of difficulty in this technique of correlating cytology and metabolism, it still can provide fruitful dividends in the amount of information that can be revealed.

PHYSICAL AND CHEMICAL PROPERTIES OF ISOLATED CONSTITUENTS

The symbols suggested by Alexander and Wilson (3) will be used here to describe isolated fractions. This system designates structures on the basis of the centrifugal force and time used to sediment them from suspension. Thus, the 144*p*60 fraction consists of those particles (*p*) which are sedimented at 144,000 × G in 60 minutes, the prefix indicating the force in 1,000 × G and the suffix referring to the time in minutes. In surveying the array of fractionation procedures employed, it seems necessary to extend this nomenclature to supernatant fractions. The 144*s*60 is then the material remaining in solution after centrifugation for 60 minutes at 144,000 × G. The supernatant presumably corresponds to the nongranular cell constituents, the ground plasm. Caution must be maintained, however, in ascribing non-sedimentable constituents to the ground plasmic portion of the cell, since any enzyme liberated from lysed particles would be recovered in this supernatant. Fractions have been reported in the literature with no presentation of the time of centrifugation. These constituents will be designated without the suffix; for example, 15*p*, 144*s*, etc. The symbol for the supernatant in this terminology should not be confused with that used in analytical centrifugation for which the term 40 S refers to the macromolecular component sedimenting at 40 Svedberg units, where 1 S is 10^{-13} cm per sec per unit field, whereas 40*s* is the supernatant of a centrifugation at 40,000 × G.

Cytological investigations of bacterial inclusions have been concerned primarily with those bodies visible in the light microscope. The pres-

ence in the cell of structures visible only by electron microscopy has been known since at least as early as 1941, when Sevag and co-workers (94) demonstrated such submicroscopic structures in extracts of *Streptococcus pyogenes*. Since that time, similar granules have been described in a wide range of bacteria (table 1); these bodies generally are found to have a diameter of 10–50 mμ. It would be helpful if one could interrelate the centrifugation procedure with the presence of a single particle of a known diameter and sedimentation rate, but no such fractionation technique is yet available. However, it is known that only components possessing sedimentation constants of 20 S or greater are spun to the bottom of the centrifuge tube by the preparative centrifuges now in use, so that the submicroscopic granules of size 10–50 mμ correspond to the 20 to 40 S bodies (12, 109). Using a technique called adhesion partitioning, Backus (7) has observed spherical granules of diameter 30–60 mμ in partitioned *Escherichia coli* cells. All other determinations of the morphological characteristics of the "submicroscopic" granules have been carried out on cell lysates.

The analytical centrifuge is a very helpful tool in studies of components of small size which cannot be readily differentiated by ordinary laboratory procedures. With this instrument, enumeration and characterization of the submicroscopic structures are possible. Sedimentation patterns of microbial extracts reveal the presence of several distinct macromolecular components (table 2). These include a fast-moving constituent with a sedimentation constant of *ca.* 40; one, or possibly two, slower moving peaks at approximately 20 and 29 S; a sharp spike in the vicinity of 8 S; and a broad, slowly moving band in the ultra-centrifugal patterns with a sedimentation constant of about 5 S. Macromolecular components which sediment more rapidly have been found in *Streptococcus pyogenes* associated with particles containing a green pigment (94), and in *Pseudomonas fluorescens, Saccharomyces cerevisiae, Rhodospirillum rubrum* and an unidentified blue-green alga (90). The particles in the latter two organisms contain the photosynthetic pigments. The spiked component exhibiting the inflection at approximately 8 S can be eliminated by the addition of desoxyribonuclease to the extracts, indicating that this constituent consists of DNA (90, 95, 114). Material associated with this peak would not be sedimented by the forces available for preparative centrifugations; thus, deoxypentose nucleic acid would be expected to be recovered in the supernatant.

TABLE 1

Size of submicroscopic granules

Organism	Fraction	Diameter	Reference
		mμ	
Aerobacter aerogenes	110*p*90	15	109
Azotobacter vinelandii	144*p*	15–25	3
Escherichia coli	Cell	30–60	7
	105*p*90	20–50	12
	Lysate	10–15	57
Micrococcus pyogenes	15*p*	10–50	66
Mycobacterium tuberculosis	145*p*180	50	129
Pseudomonas fluorescens	22*p*60	10–100	101
Rhodospirillum rubrum	Chromatophores	60	90
Streptococcus pyogenes	90*p*60	21, 46	94

The 40 S constituent is abundant in bacterial extracts and makes up the major portion of the cellular granules. The purified 40 S particle observed in electron micrographs is spherical and has a diameter of approximately 15 mμ (90, 109). The slowly moving band with a sedimentation value of *ca.* 5 S probably represents a heterogeneous mixture of soluble substances found in the ground plasm.

That the isolated submicroscopic particles are indeed discrete cellular entities is supported by the sedimentation patterns, which show separate peaks rather than the undifferentiated broad band with no inflections which might be expected if the granular material represented only a heterogeneous preparation of parts randomly broken from the main cell body. In addition, the macromolecular constituents reported for bacteria remain the same even with various methods of cell breakage, from a mild lysozyme stripping of the surface to the most drastic techniques of rupture. For example, the results summarized in table 2 demonstrate that cell lysis by nine different agencies does not alter the major components of microbial extracts. Also, the values obtained for the peaks in the ultra-centrifugal patterns of such a wide morphological and physiological diversity of microorganisms possess a remarkable reproducibility.

TABLE 2

Sedimentation constants

Organism	Method of Rupture	Sedimentation Constants*					Reference
		Large *p*	*ca* 40 S	*ca* 20–29 S	*ca* 8 S	*ca* 5 S	
Aerobacter aerogenes	Glass		40	22	9		109
	Press		40	29, 20	12, 8	5	13, 14
Azotobacter vinelandii	Alumina		39	25	9.0†	3.8	2
Bacillus megaterium	Alumina		42	31		3.9	114
	Lysozyme		41	26	15–16†‡	2.8–3.9	114
	Sonic		43	27		4.4	114
Blue-green alga	Alumina	300	41	30		4.7	90
Clostridium kluyverii	Drying		40	26	9.7†	6.0	90
Escherichia coli	Alumina		40	29, 20	8†	5	12
	Alumina		32–38	20–25	8.5†‡	5	90
	Mill		39	25, 21	8.7†‡	4.9	95, 96
	Phage		45	29		4.4	90
	Sonic		36–45	23–29		4.6–5.8	90
Micrococcus pyogenes	Alumina		39	27	10.0†	3.2	90
Proteus vulgaris	Alumina		35	23		4.1	90
Pseudomonas fluorescens	Alumina		34–38	22–25	7.9†	3.5–5.3	90
	Pressure	58	44	29		4.0	90
	Sonic	58	44	29		6.5	90
Rhodospirillum rubrum							
Dark-grown	Alumina	55	42	29		4.6	90
Light-grown	Alumina	190	38	29	9.4†	6.4	90
Saccharomyces cerevisiae	Alumina	70	42			6.3	90

* 1 S = 1 Svedberg unit = 10^{-13} cm/sec/unit field.

† Reported as a spiked peak.

‡ Contains deoxypentose nucleic acid.

During fractionation of the extract, lipid material rises centripetally in the high-speed centrifugation with the consequent recovery of this substance at the surface of the final supernatant (2, 45, 65). The cytological relationship or the biochemical function of this constituent is yet unknown, although the ease with which it can be differentiated from other portions of the homogenate make it likely that such investigations will be undertaken.

The composition of the cellular particles and the distribution of certain biochemical substances within the microbial cell are important in understanding the cytological and physiological contributions of the isolated structures. Investigations of the chemical constitution of the fractions have demonstrated a surprising degree of similarity among various bacterial genera. Most of the cellular mass measured as dry weight, protein, or nitrogen is recovered in the final supernatant, generally more than 50 per cent being in this one fraction alone (3, 44, 55, 66, 90). The presence of most of the cell nitrogen in the ground substance imposes certain restrictions upon the maximum value for the concentration coefficient of an enzyme localized in this portion of the organism. In a cell containing two-thirds of its nitrogen in the ground plasm, the maximum value for the coefficient would be 100/66.7 = 1.5, assuming quantitative recovery of the function in the one fraction. Particulate enzymes will, however, show appreciably higher values. Acid-soluble phosphorus compounds are also predominantly found in this same region of the cell (12, 66).

Data demonstrating the amounts of various chemical components recovered in the particles are summarized in table 3. The bacterial granules are especially rich in phospholipid; in *E. coli*, for example, 67 per cent of the phospholipid of CE was bound to these bodies. A similar observation has been reported for *Micrococcus pyogenes* var. *aureus* (22). There is some controversy regarding the distribution of RNA in the preparations. In

TABLE 3

Chemical composition of cell granule fraction

Organism	Fraction	Per Cent Cell Extract				Reference
		Protein	Ribonucleic acid	Acid-soluble P	Phospho-lipid P	
Azotobacter vinelandii	7*p*30	15.5*	16.3			3
	60*p* + 144*p*	15.3*	16.4			3
Escherichia coli	41*p*15		6.1	7.2	46	12
	105*p*90		25.9	13.2	31	12
	144*p*60	22	51			55
	160*p*60	33	86			90
Micrococcus pyogenes	15*p*		2.6	1.6	30	66
Pseudomonas fluorescens	160*p*60	26	89			90

* Data expressed as total nitrogen.

their studies of the macromolecular organization of bacteria, Schachman and collaborators (90) found essentially all of the RNA in the granules of *P. fluorescens* and *E. coli*. Reports of other workers differ in that, although the particles are rich in this nucleic acid, a large proportion was always found in the cell's ground plasm. In *Azotobacter vinelandii*, moreover, the RNA is apparently localized throughout the organism, parallel to the distribution of cell-nitrogen (3).

Analysis of the purified submicroscopic granules indicates that they contain 9–10 per cent nitrogen and approximately 20–25 per cent lipid. The 90*p*60 components of *Streptococcus pyogenes* consist of 0.82–1.4 per cent phosphorus and 0.4–0.8 per cent purine-nitrogen, while the 15*p* granules of *Micrococcus pyogenes* are reported as having 2.4 per cent RNA, 6.9 per cent α-amino nitrogen and 41 per cent protein. These inclusion bodies contained either insignificant traces of deoxyribonucleic acid or none (66, 81, 94).

Several investigators have attempted to isolate a particle corresponding to the bacterial nucleus or to some nuclear equivalent. In studies of *A. vinelandii*, *E. coli*, *M. pyogenes*, and *P. fluorescens*, 80–90 per cent of the DNA of CE was found in the supernatant fraction alone (3, 12, 55, 66, 90). Fernell and King (30), carrying out similar analyses on seven bacterial genera, also found that almost all of the DNA was in the fluid remaining above the particle pellet. This failure to recover DNA in a bound form may reflect a primitively organized nucleus or a structure with no limiting membrane or, more probably, may arise from the presence of a fragile nuclear membrane which is ruptured at the time of cell lysis. Considerable difficulty has also been experienced in isolating animal cell nuclei (47). For further cytological as well as metabolic studies, it is essential to find a method whereby nuclei can be isolated in a purified form. Until the cytochemical organization of the bacterial nucleus is established on firmer grounds, one must be hesitant in concluding that enzymes localized exclusively in the supernatant are properties of the ground cytoplasm.

In the preparation of bacterial extracts, it is standard practice to include only the supernatant of relatively high speed centrifugations. The possibility of eliminating certain intracellular structures with the "debris" is illustrated in certain chemical analyses performed on intact cells of *E. coli* and on the extract prepared therefrom. The extract contained approximately half of the protein and RNA but possessed 80 per cent of the DNA, *i.e.*, half of the protein and RNA but only one-fifth of the DNA was lost (55). This points to the presence in the whole cells of a structure, spun out with the unbroken cells, that is rich in RNA but has little or none of the deoxypentosenucleic acid. Similarly, microscopic examination of most of these extracts reveals the absence of the inclusions seen in light micrographs of probably all bacterial species. The lysate prepared is, therefore, an incomplete representation of the cell substance and probably will be found to lack many of the metabolic properties of the intact microorganism.

A fraction consisting of particles visible by light microscopy has been prepared by sedimentation in low gravitational fields of lysozyme-lysates of *Bacillus stearothermophilus* (17, 33, 34, 63). This

"red fraction" could reduce TTC with the accumulation of the formazan and contained malic dehydrogenase, adenosinetriphosphatase, apyrase, aldolase, the absorption maximum for cytochrome c as well as cytochrome oxidase measured by *p*-phenylenediamine or hydroquinone. These bodies also could catalyze the aerobic oxidation of various intermediates of the TCA cycle. The validity of the investigations of these constituents of *B. stearothermophilus* has been questioned (101, 114), since some of the bodies stained with nuclear dyes and thus the preparation may have represented a heterogeneous mixture of subcellular units (*cf.* 34). Additionally, the limitations in the use of lysozyme as a means of cell rupture for cytochemical investigations have already been discussed.

Large particles comparable in size to those found within the unbroken bacterium have, however, been isolated by other procedures. Structures of diameter 0.2 μ have been sedimented from *Mycobacterium tuberculosis* lysates obtained by the abrasive action of the ball mill (129). Extracts of *A. vinelandii* prepared by sonic oscillation, alumina grinding, or rapid agitation with small glass beads contain structures which, following purification, were found to be 0.2–0.4 μ in diameter and dense to the electron beam, similar to those in the intact azotobacter. This fraction is pink to red in color and is observed as a distinct layer between the firmer pellet and the supernatant produced at low gravitational forces (3). It was originally designated the 25*p*30 particle but is more aptly described as the 7*p*30 component.

LOCALIZATION OF THE ELECTRON TRANSPORT SYSTEM

The presence of the cytochrome respiratory pigments is readily ascertained by examination of the absorption spectrum, but there exists as yet no quantitative means of determining precisely the amount of the electron carrier. Absorption maxima of various cytochromes have been demonstrated in suspensions of chemically or enzymatically reduced particles isolated from several microbial species, including cytochromes a_1, a_2 and b_1 in the 15*p* of *Aerobacter aerogenes* (107) and the 20*p* of *Proteus vulgaris* (67), a_2 and b_1 in the 20*p*60 of *Escherichia coli* (35), b_1 and c in particles of *Azotobacter vinelandii*, and the classical a, b and c in granules prepared from *Saccharomyces cerevisiae* (19). In the bacteria examined, there was never any evidence of a cytochrome a–a_3 peak in the region of 600–610 mμ in particles, supernatant or initial extract, although the terminal oxidase may have been highly active—*e. g.*, even with a $Q_{O_2}(N)$ of 14,000 by azotobacter granules. Particles in the fungus *Neurospora crassa* also contain a cytochrome c band (108). The concentration of the c peak in azotobacter has been estimated qualitatively from the difference spectra of various fractions. Most of the respiratory pigment was observed in the particles with little or none in 144*s* (4). The slight bands observed in the supernatant were probably not physiologically significant, since the slightest trace of residual absorbing material in this transparent fluid would produce a pronounced inflection in the visible spectrum. The red color of the pellet spun to the bottom of the centrifuge tube probably is a result of the presence of the cytochromes in these aerobes.

Chemical reduction of the particles results in a marked bleaching in the 450 mμ region of the spectrum, indicating the presence of flavin compounds (2, 101). A cold trichloracetic acid extract of the particles also shows the occurrence, particularly in the small granules, of abundant flavins characterized by absorption maxima at 380 and 440 mμ.

Because of its ability to oxidize *p*-phenylenediamine, cytochrome oxidase may be assayed by this reaction, although a determination using the natural substrate, bacterial cytochrome c, would be preferable. The mammalian cytochrome c is nonfunctional in the *p*-phenylenediamine oxidase system of *A. vinelandii* and other bacteria. Although there is some question regarding the specificity of this oxidation, the similarity in its localization to that of the DPNH (reduced diphosphopyridine nucleotide) oxidase and succinoxidase systems, both cytochrome-linked processes, lends support to its usefulness. This is also borne out by the observations of Militzer *et al.* (63) regarding the similarities in cyanide inhibition of the *p*-phenylenediamine oxidase of bacteria and the classical mammalian cytochrome oxidase. Quantitative localizations have been reported in two fungal species, *Neurospora tetrasperma* and the cellulolytic *Myrothecium verrucaria*, the yeast *S. cerevisiae*, and also in *A. vinelandii* (table 4). In each organism, the terminal oxidase is completely localized in the intracellular granules, from 80 to more than 100 per cent of the original activity being recovered

TABLE 4

Localization of systems for electron transport and oxidative phosphorylation

Component	Organism	Fraction	% Cell Extract*	Concentration†	Reference
Adenosine triphosphate synthesis	*Escherichia coli*	41*p*15		0.01	12
		105*p*90		6.85	12
Cytochrome oxidase	*Azotobacter vinelandii*	7*p*30	37.5	2.70	4
		60*p* + 144*p*	30.3	2.10	4
	Myrothecium verrucaria	25*p*60	88		24
	Neurospora tetrasperma	16*p*60		2.27	21
	Saccharomyces cerevisiae	31*p*60	107	3.03	45
Diphosphopyridine nucleotide-cytochrome reductase	*Saccharomyces cerevisiae*	30*p*60	86		97
Reduced diphosphopyridine-nucleotide oxidase	*Azotobacter vinelandii*	7*p*30	23.0	1.33	4
		60*p* + 144*p*	49.4	3.37	4
Succinoxidase	*Aerobacter aerogenes*	110*p*90		5.25	109
	Azotobacter vinelandii	7*p*30	16.4	1.18	3
		60*p* + 144*p*	62.6	4.32	3
	Escherichia coli	41*p*15		4.3	12
		105*p*90		1.6	12

* Per cent of the activity of the cell extract that is recovered in the fraction.

† Ratio of specific activity in fraction to that in the cell extract.

in fractions containing these bodies. This organization is analogous to that in cells of higher organisms. Qualitative evidence supporting such a distribution has been reported with particles of other microorganisms (6, 49, 56, 101, 108) and, at least in several instances, the consumption of O_2 by the oxidase is inhibited by cyanide.

A second means whereby the intracellular distribution of the electron-carrier mechanism can be determined is by the study of the enzyme or complex catalyzing the oxidation of reduced pyridine nucleotides. The recovery of essentially all the DPN-cytochrome c reductase and DPNH oxidase in the particles of the azotobacter and yeast, together with the high concentrations in these structures, establishes that these enzymes too are a metabolic function of the granules. The DPNH oxidase has also been reported by Cota-Robles and Marr (23) to be associated with the 100*p* substituents of *A. vinelandii*, while both the DPNH and TPNH oxidases of *E. coli* are bound to 102*p*40 granules (5).

Ancillary evidence for the association of hydrogen transport with the sedimentable structures exists in the presence of essentially all of the succinoxidase, judged by either percentage or concentration criteria. The ability of microbial particles to consume O_2 during the oxidation of a wide variety of other organic acids has been reported frequently in the bacteriological literature. The system catalyzing the oxidation of malate, the "malic oxidase," is of especial interest because it presumably requires a pyridine nucleotide, flavins, and cytochromes in the transfer of electrons from substrate to the terminal acceptor. This system in *A. vinelandii* and *P. fluorescens* is recovered almost completely in the particles, with only insignificant traces being found with the ground plasm constituents (4, 101). The concentration in the microbial particles of cytochrome and flavin pigments, cytochrome oxidase, complexes involved in the oxidation of reduced pyridine nucleotide coenzymes, and the overall system involved in the aerobic oxidation of various organic acids indicates that the transport mechanism of the microorganism is one of the biochemical properties of this structure.

INTRACELLULAR DISTRIBUTION OF ENZYMES CATALYZING THE TRICARBOXYLIC ACID CYCLE

Studies of the site of activity of enzymes concerned in the TCA cycle, based upon O_2 uptake measurements, have led to few definitive cytochemical results because of the requirement for the particles in order to observe a detectable gas exchange. All isolated fractions from *Aerobacter aerogenes*, for example, gave no, or at best only slight, O_2 utilization on pyruvate, citrate,

α-ketoglutarate or fumarate (109). Direct enzyme determinations, however, have established the distribution of most of the enzymes and have provided explanations for the anomalous manometric data.

All subsequent investigations on the localization of succinic dehydrogenase in the microbe agree with the report of Stephenson (102) who, almost 30 years ago, demonstrated that the dehydrogenase found in *Escherichia coli* extracts was associated with particulate substances. More recent reports, summarized in table 5, confirm that the granules are the sole loci of the enzyme, since they contain almost 90 per cent of the amount present in CE as well as a concentration greater than 30 times that in the nonsedimentable cell substance. The distribution is thus analogous to the entire succinoxidase system, which contains both the substrate enzyme and the carrier mechanism.

Particles isolated from cells of *Pseudomonas fluorescens*, *E. coli* or *A. aerogenes* do not take up O_2 in the presence of fumaric acid as substrate although the appropriate carriers are present (5, 101, 109). On the other hand, if a nonphysiological electron acceptor such as methylene blue is included in the system, as has been done with *Mycobacterium tuberculosis* and *Serratia marcescens*, fumarate is rapidly oxidized by the supernatants of high-speed centrifugation (56, 65). In *S. marcescens*, the ratio of the specific activities of supernatant to particles is 3.65. This indicates that fumarase is situated in the ground plasm, a distribution that has been confirmed by specific determinations of the one-step reaction as indicated in table 5 (*cf.* 80). The results are not as well defined for the malic dehydrogenase. In both *P. fluorescens* and *Azotobacter vinelandii*, most of the oxidative activity of the cell extract on malic acid was recovered in the particles, the submicroscopic bodies of the latter exhibiting a concentration of

TABLE 5

Localization of enzymes catalyzing reactions of the tricarboxylic acid cycle

Enzyme	Organism	Fraction	% Cell Extract	Concentration	Reference
Aconitase	*Azotobacter vinelandii*	144*s*90	61.0	0.86	4
	Saccharomyces cerevisiae	10*s*30	102	1.05	80
	Saccharomyces cerevisiae	31*s*60	55	0.87	45
Citrate dehydrogenation	*Mycobacterium tuberculosis*	37*s*15		2.17	64
	Serratia marcescens	25*s*/25*p*		6.40*	56
Fumarase	*Azotobacter vinelandii*	144*s*90	35.3	0.50	4
	Saccharomyces cerevisiae	10*s*30	93.9	1.16	80
	Saccharomyces cerevisiae	31*s*60	93	1.47	45
Fumarate dehydrogenation	*Serratia marcescens*	25*s*/25*p*		3.65*	56
Isocitric dehydrogenase	*Saccharomyces cerevisiae*	31*s*60	92	1.45	45
	Azotobacter vinelandii	144*s*90	93.7	1.41	4
	Serratia marcescens	25*s*/25*p*		9.86*	56
α-ketoglutaric decarboxylase	*Azotobacter vinelandii*	144*s*90	91.7	1.30	4
α-ketoglutaric dehydrogenase	*Serratia marcescens*	25*s*/25*p*		0.031*	56
Malic dehydrogenase	*Saccharomyces cerevisiae*	30*p*60	20		97
		30*s*60	80		97
	Serratia marcescens	25*s*/25*p*		1.06*	56
"Malic oxidase"	*Azotobacter vinelandii*	7*p*30	8.8	0.63	4
		60*p* + 144*p*	63.8	4.42	4
Succinic dehydrogenase	*Azotobacter vinelandii*	7*p*30	36.0	2.08	4
		60*p* + 144*p*	53.0	3.62	4
	Saccharomyces cerevisiae	30*p*60	85		97
	Saccharomyces cerevisiae	60*p*10	All		19
	Serratia marcescens	25*s*/25*p*		0.030*	56

* Activity for cell extract not given in original paper. Value is calculated on basis of ratio of specific activities of supernatant (25*s*) to particles (25*p*), after correction for endogenous reduction rate (25*s*/25*p*).

4.42 for this "malic oxidase" (4, 101). Direct measurements of the dehydrogenase itself, however, indicate a somewhat different localization. Both particles and ground plasm derived from *S. marcescens* contain appreciable dehydrogenase activity as measured by dye reduction. In this organism, the specific activities of the two fractions were approximately equivalent, leading one to propose that the malic dehydrogenase is localized in both granules and nonparticulate areas of the cell. Data suggesting a similar distribution for the enzyme in yeast have also been reported (table 5).

The intracellular sites of three other enzymes involved in catalyzing reactions of the TCA cycle have been studied: aconitase and isocitric dehydrogenase carrying out the interconversions of the tricarboxylic acids, and the α-ketoglutaric metabolizing enzyme. The available data indicate clearly that at least two are properties of the nonsedimentable constituents of bacteria and yeast. The dehydrogenation of citric acid by *S. marcescens* and *M. tuberculosis*, a reaction sequence requiring aconitase and isocitric dehydrogenase, occurs almost exclusively in the supernatant of high-speed centrifugation of CE. Direct measurements of the two enzymes give results which clearly demonstrate that their activity is in the ground substance. Recoveries of aconitase were frequently incomplete despite repeated trials, intimating some degree of instability. Such lability is typical of aconitase, especially in dilute solutions, regardless of the tissue from which it is isolated. It is of interest that the localization of the isocitric dehydrogenase is the same for yeast and azotobacter, although the former is DPN-linked whereas the latter is specific for TPN. The dehydrogenase is apparently more stable since excellent recoveries have been obtained in azotobacter extracts, more than 90 per cent in 144*s*90 alone. Despite the prevailing opinion to the contrary, these enzymes are not usually associated with the granules of animal cells since most of the activity is recovered in the soluble fraction (47). Neither the 102*p*40 particles of *E. coli* nor the 110*p*90 structures of *A. aerogenes* could produce significant oxidation of α-ketoglutaric acid (5, 109), while the 144*s*90 supernatant of *M. tuberculosis*, in the presence of methylene blue, maintained an appreciable rate of O_2 uptake (65). These findings agree with the quantitative estimates of the decarboxylative activity for this substrate in *A. vinelandii* extracts, which also show the activity is nonsedimentable. In contrast, however, are the data of Linnane and Still (56), who concluded that the α-ketoglutaric dehydrogenase of *S. marcescens* is firmly bound to the bacterial particles. These contradictory results require further investigation with a more standardized procedure for enzyme assay.

In a more recent communication, the enzymes aconitase, isocitric dehydrogenase, α-ketoglutaric dehydrogenase, fumarase and malic dehydrogenase of *M. tuberculosis* have been localized quantitatively. The greatest percentage in each instance was found in the 144*s* fraction (63a).

There is an interesting similarity in the cytochemical centers of activity in the metabolism of α-ketoglutarate and pyruvate. The studies of Moyed and O'Kane (67) clearly show that two components are necessary for the operation of the pyruvic oxidase in *P. vulgaris*, particles sedimentable at 20,000 $\times$ G and the supernatant. The latter contains the pyruvic dehydrogenase which reduces 2,6-dichlorophenolindophenol and also liberates CO_2 in the presence of cocarboxylase while the system required to link the dehydrogenase to O_2 was only in the granules. In *Saccharomyces cerevisiae* also, all of the pyruvic decarboxylase is included in the nonsedimented constituents (19).

On the basis of the localizations observed, certain predictions are possible whose validity can be determined directly by manometric experiments upon isolated and recombined fractions. Using the Warburg respirometer, we have tested the apparent intracellular distribution of these physiological activities important in terminal carbon metabolism of *A. vinelandii*, with the following results.

(a) Although all members of the TCA cycle are oxidized to completion by CE fortified with appropriate coenzymes, the particles showed essentially no O_2 uptake on pyruvate, acetate (with or without a "sparker"), citrate, α-ketoglutarate or isocitrate even in the presence of various cofactors. This confirms the separation by differential centrifugation of the substrate enzymes (aconitase, isocitric dehydrogenase and the α-ketoglutaric dehydrogenase) from the O_2-activation complex. Similar observations have been reported for *E. coli* and *A. aerogenes* (5, 105, 109). The keto acid likewise was not decarboxylated by a suspension of the isolated granules.

(b) Particles actively oxidize both succinate

and malate, the former at a much slower rate. Coenzyme additions did not stimulate the velocity of either reaction. Washed granules of *P. fluorescens* can also oxidize these two substrates (101). The oxidation of L-malate by particle suspensions proceeds only one step, *i.e.*, to the oxalacetate level. This is evident from the ratio of μmol O_2/μmol malate which never was greater than 0.50 even upon addition of metals and coenzymes. In contrast, CE oxidizes malate to completion. In analogous fashion, the rate of O_2 uptake by the particles on succinate decreases rapidly as the fumarate level is approached, although, because of the slow rate of reaction, the effect is never as clear-cut as with malate.

(c) Particles of the azotobacter prepared free of soluble material show little O_2 uptake on fumarate although it is difficult to eliminate all of the oxidation because of its highly active fumarase. This result, together with the observations on the one-step succinate oxidation, points to a soluble fumarase. Granules of *P. fluorescens* also fail to take up O_2 in the presence of fumarate (101).

(d) A synergistic effect is evident upon coupling 144*s* with particles during the oxidation of citrate, acetate, or α-ketoglutarate. Either fraction alone showed little to no activity on these intermediates, but a combination of the two gives a disproportionate increase in the rate—an oxidation velocity three to four times greater than that of fractions treated separately. Swim and Gest (105) report an identical synergism during the oxidation of citric and α-ketoglutaric acids by *E. coli* extracts. They suggest that the effect arises from the coupling of soluble enzymes with the particle-bound electron transport systems necessary for O_2 uptake.

These manometric observations confirm the distribution data and serve to support the spatial mechanism suggested by the enzyme assays for the subcellular organization of reactions of the TCA cycle. It thus seems clear that the catalysis of this biochemical sequence requires the simultaneous participation of different structures within the microbial cell, both the granular and supernatant constituents being necessary.

By the techniques presently available, it is not possible to determine whether TCA cycle enzymes recovered in the supernatant originate in the ground cytoplasm or are rendered soluble during cell disruption. Nevertheless, the data at hand indicate unmistakably that a marked difference in structural association exists between most of the TCA enzymes and the succinic dehydrogenase or electron carrier mechanism. Since it is always tempting to conceive of physiological sequences contained on a single structure, one can postulate that the active particle contains a fluid core surrounded by the more rigid substance containing the flavin-cytochrome chain.

BIOCHEMICAL HETEROGENEITY OF CELL PARTICLES

It has not yet been possible to present a definite enzymatic differentiation between the large and the small granules, *i.e.*, to find an enzyme in one type which is absent from the other. Even in animal tissues, few enzymes have been found in the microsomes that are clearly missing in the mitochondria (47). In *Azotobacter vinelandii*, however, a quantitative difference does exist between the two. With four enzymes or enzyme systems, succinic dehydrogenase and the succinic, malic and DPNH oxidases, the concentration coefficients and per cent recoveries have been consistently and significantly higher in the submicroscopic 60*p* and 144*p* inclusions than in the 7*p*30 granules visible in the light microscope (tables 4 and 5). These functions are from 1.7 to 7 times more concentrated on a nitrogen basis in the smaller bodies of the organism, with coefficients ranging from 3.4 to 4.4. At least some of the activity in the large granules may arise from incomplete removal of the smaller structures, since electron photomicrographs of the 7*p*30 fraction show the presence of material morphologically similar to the submicroscopic bodies. This suggests that the entire electron transport system as well as the succinic dehydrogenase may be associated with intracellular structures with an approximate diameter of 15–25 mμ.

Abundant evidence suggests that this granule is biochemically a complex structure. Not only are the enzymes carrying out electron transport and the oxidation of several organic acids associated with it, but also the velocity of such substrate oxidation is not stimulated by the addition of various coenzymes (2, 5), indicating that the requisite cofactors, cytochromes, and flavins are bound to the particle in a physiologically available form. Since the submicroscopic structure can function in the transport of electrons from substrate to O_2 at a rate comparable with that of the intact microorganism and can do so in the absence of added enzymes or coenzymes, it appears that not only are the components of

the system bound to the particles, but also the steric placement of all the individual reactants in the granules is so arranged as to facilitate maximal metabolic activity. The organization may be conceived as a continuous shuttle of electrons from substrate through the necessary intermediates to the final electron acceptor, O_2. Each successive product formed in the reaction sequence must be at a site where the subsequent enzyme can affect the next change. Such complexity suggests a cell organelle that functions in the metabolism of the organism as a basic and integral unit.

Several types of evidence indicate a physical heterogeneity among the granules found in the submicroscopic particle fraction. In azotobacter extracts, for example, prepared and fractionated in the complete absence of electrolytes, the pellet consisting of the particles requiring the greatest gravitational forces for sedimentation contains white as well as red components (2). Sevag and co-workers (94) report size differences in the particles of *Streptococcus pyogenes*, electron micrographs revealing two types with mean diameters of 21 and 46 mμ.

Ultracentrifugal analysis has indicated that there are several particle types in the centrifuged pellet differentiable by their sedimentation rates but as yet incapable of being separated completely because of their similarity in size and density. If a mixture of these particles is subjected to several centrifugations at a force less than that necessary to clear completely the solution of the particles, the sedimented material removed, and the supernatant recentrifuged, then a physiological property present in the larger of these granules, assuming uniform density, should be the most concentrated in the first precipitate (A) and the concentration should decrease in each subsequent pellet (B, then C). Conversely, if the property were associated with the smaller granule, then the concentration of that property should increase in each subsequent precipitate spun to the bottom of the tube.

The distribution data for *A. vinelandii* demonstrate that the oxidative activity is concentrated in the small particles. A suspension of these, minus the 7*p*30 fraction, was sedimented as described in the preceding paragraph in the absence of electrolyte that might cause agglutination and poor differentiation. From the results presented in table 6, it is evident that a differentiation of small particle metabolic types occurs. The fraction with the most rapidly sedimenting constituents possesses the highest concentration coefficients and the greatest oxidative capabilities, whereas the fraction sedimenting the most slowly has the lowest biochemical activity.

Among the macromolecular components found upon analytical centrifugation, the 40 S component is the probable active structure. Therefore, as a first approximation, it is proposed that the 40 S particle is carrying much if not all of the oxidative activity associated with the flavin-cytochrome electron chain and the dehydrogenase for succinic acid. It is yet to be determined whether the oxidative ability found with the other particle fractions results from occluded 40 S granules.

Purified 40 S particles have been demonstrated previously (90) to contain the enzymes ribonuclease, catalase, apyrase, formic dehydrogenase and the succinic dehydrogenase. Even more significant, however, is the localization of the system catalyzing the accumulation of energy in the form of ATP during O_2 uptake. The sole site of such oxidative phosphorylation in *Escherichia coli* is in the 105*p*90 granules (table 4), *i.e.*, the fraction containing structures of size 20–50 mμ which correspond to the 20, 29, and 40 S components. The results concerned with electron transport, the oxidation of succinic acid, and the system involved in the formation of ATP during aerobic oxidations suggest that the submicroscopic particle is the structure in the

TABLE 6

Differentiation of small particles with respect to oxidative activity

Fraction	Malic Oxidase		Succinoxidase		Cytochrome Oxidase	
	% Cell extract	Concentration	% Cell extract	Concentration	% Cell extract	Concentration
Initial	(100)	(1.0)	(100)	(1.0)	(100)	(1.0)
A	40.0	4.0	42.4	6.3	53.2	7.9
B	22.3	2.4	43.2	5.3	35.9	4.4
C	2.5	0.33	5.5	1.7	4.2	1.3
s	0.1	0.01	0.0	0.0	1.4	0.0
% Recovery	64.9		91.1		94.7	

Fractions prepared in 0.25 M sucrose. A is 60*p*60, B is 144*p*60, C is 144*p*K60, and *s* is the supernatant, as defined by Alexander and Wilson (3).

microorganism which is analogous in function to the mitochondrion of multicellular organisms. As has been already discussed, the limitations of cytochemical staining reagents and the wide morphological diversity of microorganisms make it essential that determination of physiological similarities among structures of different organisms be based on quantitative, biochemical technique and the analogies, on parallelisms in biochemical properties rather than by similarities in staining or morphology. The most consistent and characteristic biochemical properties associated with the mitochondria of higher plants and animals are the presence of the enzymes succinic dehydrogenase and cytochrome oxidase and the system for electron transport and oxidative phosphorylation. The finding of these in the small granules lends credence to their similarity to the "powerhouse" organelle of other tissue. Final decision regarding this analogy must await more complete studies of the distribution of enzymes in all fractions of the cell.

METABOLIC SPECTRUM OF THE PARTICULATE STRUCTURES

The localization of several other enzymes in the particles of microbial cells has been studied in a number of laboratories although the cell extract usually did not contain the intracellular bodies visible by light microscopy (table 7). Hydrogenase, the enzyme which activates molecular H_2, has been the subject of several such investigations. Essentially all of the enzymatic activity is associated with the cell particles of species of *Azotobacter*, with recoveries of more than 90 per cent recorded for 144*p*90. The enzyme in both *Hydrogenomonas facilis* and *Escherichia coli* measured by the reduction of methylene blue or ferricyanide was also associated with the sedimentable constituents (6, 35). Since the sites for the H_2 activation and for electron transport coincide, the expectation that isolated particles will completely oxidize H_2 using molecular O_2 as the terminal acceptor has been demonstrated with the hydrogen bacterium; moreover, the per cent of the activity of CE recovered by measurement of either dye reduction or by this Knallgas reaction was identical. The formic dehydrogenase of two tested bacterial species, *E. coli* and *Serratia marcescens*, is also bound to the granular bodies, as evidenced by high concentration coefficients and by the recovery percentage (31, 35, 56, 103). Quantitative data demonstrating the localization of several other dehydrogenases in the intracellular granules are presented in table 7. Some qualitative evidence has been gathered for the presence in the particles of adenosine triphosphatase, apyrase, cysteine desulfhydrase, glucose dehydrogenase, nitratase and ribonuclease (5, 12, 16, 26a, 34, 90), but the interpretation of these observations must await further study.

In addition, the bacterial particles possess certain nonenzymatic properties of interest in

TABLE 7

Enzymes associated with cell granules

Dehydrogenase	Organism	Fraction	% Cell Extract	Concentration	Reference
Formic	*Escherichia coli*	3 rpm – *p*30†		2.10	31
	Serratia marcescens	25*p*20	All		56
Glucose-6-phosphate	*Pseudomonas fluorescens*	*p*	All		127
α-glycerophosphate	*Serratia marcescens*	25*p*/25*s*		4.99*	56
	Saccharomyces cerevisiae	30*p*60	99		97
Hydrogenase (H_2)	*Azotobacter agile*	144*p*90	95	10.0	39
	Azotobacter vinelandii	144*p*90	91	3.51	39
	Hydrogenomonas facilis	15*p*20	72		6
Lactic	*Serratia marcescens*	25*p*/25*s*		19.4*	56
L-mandelic	*Pseudomonas fluorescens*	22*p*60	72	4.09	44
		Fine *p*	18	1.73	44

* Rate for cell extract is not reported in original paper. Value is calculated from ratio of specific activities of particles (25*p*) to supernatant (25*s*) after correcting for endogenous (25*p*/25*s*).

† Particulate fraction sedimented by a 30-min centrifugation at 3.000 rpm.

chemotherapeutics. For example, the 20p30 particles of *Micrococcus pyogenes* var. *aureus* bind penicillin at a rate 7 to 12 times that of the intact staphylococcus (22). Millman and Youmans (65) demonstrated that, among the isolated protoplasmic constituents derived from the tubercle bacillus, only the particles (microscopic and submicroscopic) possess immunogenic activity for tuberculosis in mice while the supernatant and lipid material were inactive.

Many of the enzymes of the granule fraction are apparently strongly bound to the particle since they are neither released by autolysis (102), by extraction with buffers at various pH values, or by digestion with trypsin or papain (31); nor are they solubilized by weak acids or bases, freezing and thawing, or treatment with salt or alcoholic solutions (49). Some of the dehydrogenases are also not released from the particles even when exposed to violent mechanical agitation (56).

These microbial constituents can, however, be readily ruptured and comminuted into smaller fragments which sediment at appreciably lower rates. For example, even with an exposure of *Azotobacter vinelandii* to the 10-kc sonic oscillator for a period as short as 3 minutes considerable disintegration of the granules took place, since as much as 20–35 per cent of the succinic, malic and cytochrome (p-phenylenediamine) oxidase could not be sedimented at 144,000 × G. Repaske (82) noted that all of the azotobacter succinic dehydrogenase was in particles sedimentable at 144,00 × G when the organism was ruptured by grinding with alumina or by use of the Booth-Green mill, but 50–70 per cent was not in the residue following high-speed centrifugation if the cells were ruptured in the sonic vibrator. When the material was subdivided according to its position in the tube, it was observed that the specific activity and per cent of the enzyme increased with increasing depth. This result indicates that the succinic dehydrogenase was not rendered soluble, but rather that the sonic treatment has produced smaller fragments that sediment more slowly. In agreement with this view is the limited purification of the succinate system achieved, *i. e.*, the comminuted unit was refractive to further disorientation, lysis and "solubilization." Particle disruption has also been reported for *E. coli*, for which either exposure to sonic radiation or the use of desoxycholate is effective (35).

This disruptive effect has led to the "unmasking" of enzymatic activity in the granules. When isolated particles of *H. facilis* were exposed to the oscillator, the amount of recovered hydrogenase increased, a gain of up to 86 per cent (6). This effect may result from increased enzyme availability resulting from destruction of the particle membrane or by a partial breakage of the intracellular granules with more ready access of enzyme to substrate. Nossal (80) has presented results suggesting that "masked" aconitase and fumarase may occur in *Saccharomyces cerevisiae*, since the sum of the activities of aconitase and fumarase in the isolated fractions was consistently higher than that found in the original extract. Similar effects have been reported frequently in animal tissue where the mitochondrion presents an intracellular barrier.

The metabolic function of the large granules remains completely obscure since no enzyme has yet been found exclusively in the fraction. Cytological evidence, however, strongly suggests that the electron dense granules of diameter approximately 0.1–0.5 μ give the metachromatic reaction typical of the classical volutin (36, 42, 98, 126). Consideration of the possible physiological role of the structures exhibiting the metachromatic staining reaction thus seems warranted.

The pioneering studies of Wiame (118–121) demonstrate that the property of metachromacy associated with the volutin granules is a result of the presence of a polymerized phosphorus-containing molecule in the form of metaphosphate. Yeast rich in this polyphosphate stains metachromatically and, even when the polymer is extracted from the organism, the isolated material gives the same test with toluidine blue. Pure solutions of hexametaphosphate also exhibit metachromacy; the color reaction is accompanied by a shift in the absorption maximum from 630 to 530 mμ. Since nucleic acids and adenosine triphosphate, as well as ortho-, pyro- and triphosphates, showed no spectral shift, Wiame concluded that the metachromatic color change was quite specific for metaphosphates among the biochemical P compounds. When *Corynebacterium diphtheriae* was treated with N HCl, the metachromatic granules were removed as was the chemical substance characterized as metaphosphate (28). Similarly, if the diphtheria bacillus was grown in an environment where no such structures were formed, then

the cells were devoid of metaphosphates. Cells of *Aerobacter aerogenes*, grown in media deficient in P, K or energy source, contained no volutin granules by microscopic examination; analysis of the culture demonstrated that the chemical substance that showed the corresponding change was the acid-insoluble metaphosphate. Conversely, during the formation of volutin, the compound increasing most markedly was the trichloracetic acid-insoluble metaphosphate (98). This extensive cytochemical evidence forces the conclusion that metaphosphate must be an important constituent of these volutin or metachromatic granules in microbial cells.

The properties of electron density and melting of these larger granules in the electron beam have been correlated with the presence of metaphosphates in these structures in *Mycobacterium avium*. Mixtures of sodium metaphosphate and calcium ribonucleate also show volatilization upon intense electron bombardment (88). The volutin granules of *A. aerogenes* demonstrated by chemical means to contain metaphosphate also were more electron-opaque than the remainder of the bacterium and disintegrated under high-electron intensity (98). Metaphosphates have been found in the following microorganisms by chemical analysis: *A. aerogenes* (98), *Aspergillus niger* (60), the alga *Chlorella pyrenoidosa* (99), *C. diphtheriae* (28), the photosynthetic flagellate *Euglena* (1), two species of *Mycobacterium* (88, 124), and *S. cerevisiae* (118). The granules visible in the intact azotobacter by light microscopy, as well as the isolated 7*p*30 fraction containing analogous electron-opaque bodies, also demonstrate positive metachromatic staining.

Two types of metaphosphates occur in microbial cells—one soluble in cold trichloracetic acid, the other soluble only in the hot acid. These differ not only in their solubility characteristics, but also in their physiological reactivity. The insoluble polyphosphate is the more active form, rapidly exchanging with P^{32}-labeled orthophosphate, the interconversion of the orthophosphate with the complex insoluble P compound being reversible. During the formation of volutin, it is the insoluble and never the soluble phosphate that parallels the cytological alteration (51, 98, 122). Metaphosphate synthesis from orthophosphate by yeast and bacteria requires the presence of a source of energy, such as glucose or a TCA cycle intermediate, as well as potassium ions and nitrogen (88a, 92, 98). The change in metaphosphate level during the growth cycle occurs only in the insoluble polyphosphate while the acid-soluble compound exists in a steady state concentration during cell proliferation (52). More than 80 per cent of the orthophosphate which disappears from the reaction mixture during alcohol oxidation by cell extracts of bakers' yeast can be recovered as metaphosphate, the same material giving a typical metachromatic reaction with toluidine blue (128). Yeast extracts also can synthesize metaphosphate from ATP. Enzymes catalyzing the conversion of the polymer to orthophosphate are common in the microbial realm and have been the subject of considerable investigation (50).

Speculation regarding the role of this substance in microbial metabolism has suggested that the metaphosphate-containing metachromatic granules serve as an energy reserve or storage structure for the cell, particularly since the P bond in the molecule is energy-rich, and can supply energy for various synthetic processes (25, 118). Such a function would be analogous to that of arginine and creatine phosphates of multicellular organisms. This view is supported by reports that azide and dinitrophenol prevent the formation of both metaphosphate and the electron-dense particles with little effect on fermentation or O_2 uptake (98, 121), an effect typical of the uncoupling of phosphorylation. The role of the polyphosphate in cell synthesis is also advanced by the demonstration in yeast that the formation of protein and nucleic acid apparently occurs at the expense of this energy-rich compound (20, 122). Experiments under defined conditions indicate the presence in yeast of an enzyme, metaphosphate-ADP transphosphatase, by virtue of which P^{32}-metaphosphate can act as a P donor to convert ADP to ATP. Adenosine monophosphate, however, would not act as a phosphate acceptor (46). Similar data have been reported for *Mycobacterium smegmatis*, the extracts being able to use the metaphosphate for the phosphorylation of glycerol in the presence of ATP or ADP, but not with the monophosphate. Apparently, the metaphosphate phosphorylates the diphosphate to give ATP, which then is utilized in the conversion of glycerol to glycerophosphate (125). Here, then, is a probable biochemical property of the larger cell granules:

the electron-opaque, metaphosphate-containing volutin granules may serve as the mechanism for the storage of energy liberated during oxidative reactions, with ATP acting as the intermediate. When energy is required for other cell processes, it would be released, again through the intermediacy of the nucleotide.

ENZYMES LOCALIZED IN OTHER CELL CONSTITUENTS

Several biochemically unrelated enzymes are localized in the ground plasm of the microbial cell. For example, all of the 6-phosphogluconic dehydrogenase, measured by DPN or TPN reduction (127), and the benzoylformic carboxylase (44) of *Pseudomonas fluorescens* are localized in the supernatant fraction as is adenosine deaminase in *Azotobacter vinelandii* (3). The alkaline phosphatase of *A. vinelandii* (3) and the pyruvic decarboxylase of yeast (19) exhibit a similar intracellular distribution. The localization of the dipeptidase in the ameba has been ascertained, using an ingenious *in vivo* approach: the protozoan is centrifuged so that the cytoplasmic constituents become layered within the cell, the stratified portions are removed with a microneedle, and the dipeptidase assayed in the isolated portions. The results indicate that this enzyme is in the nongranular region of the ameba (48).

With certain enzymes studied in different microorganisms, the intracellular distributions are not identical. For example, all of the peroxidase of yeast is found in the 60*p*10 granules (19), whereas the enzyme in the *Neurospora tetrasperma* mycelium is entirely recovered in 16*s*60 (21). Likewise, 80–95 per cent of the lactic dehydrogenase of *Saccharomyces cerevisiae* is found in the supernatant fraction (19, 97), but the particles carry the greatest concentration of the dehydrogenase in *Serratia marcescens* (table 7). These differences may arise from the use of different fractionation procedures or may be the result of an enzyme whose distribution pattern differs among diverse groups of organisms.

Another type of subcellular distribution is possible, one in which the enzyme is found throughout the cell with no single structure bearing all of the activity. An example of this type of organization might be microbial catalase. Although Chantrenne (19) has reported that most of H_2O_2 decomposing capacity of *S. cerevisiae* resides in the supernatant, purified 40 S particles, as well as the 150*p*120 granules of *A. vinelandii*, also exhibit this activity (16, 90). Quantitative estimates of catalase in the latter suggest that the enzyme is found in all isolated fractions roughly in proportion to their nitrogen content, since the concentration coefficients are all approximately 1.0 (3). Determinations of the azotobacter catalase performed subsequent to those already reported give coefficients even closer to the 1.0 level of CE. Analogous distributions have been found for this enzyme in tobacco leaf and animal tissue cells. Histochemical tests point to a possible similar localization of the lipases of a number of bacterial species (8). Considering this type of intracellular organization of physiological activity, one might reinvestigate the localization of the malic and alcohol dehydrogenases, since 20 per cent of each is in the sedimentable components of *S. cerevisiae* and the remaining 80 per cent in the 30*s*60 supernatant (97).

Studies of the localization of enzymes associated with the cell surface are not yet amenable to the techniques discussed here, and an entirely different cytochemical approach has been used, indirect but nevertheless capable of providing certain biochemical and cytological information. As early as 1932, Wilkes and Palmer (123) studied the pH activity curves of invertase in intact *S. cerevisiae* and in cell extracts. A change in the reaction of the medium had an identical effect on the cell-free enzyme and on the catalyst in the intact yeast. This led these investigators to propose that the site of invertase was in the outer portion of the organism where it would be readily accessible to the external environment. Myrbäck and Vasseur (76) used a similar analysis to demonstrate that lactase and trehalase of yeast were found in the external region of the cell surface. Later work with the P^{32} exchange reactions of *Candida albicans* pointed to the role of the surface in phosphate transport (79).

Rothstein and his associates have carried out extensive investigations on the relationship of the yeast cell surface to its metabolism and have obtained significant and interesting results (27, 84–87). In their early studies, they found that bakers' yeast could convert ATP to inorganic phosphate and adenylic acid, and the products could be recovered quantitatively in the external medium. After 90 per cent hydrolysis of such P^{32}-labeled ATP, all the isotope was

in the extracellular fluid, and none was within the cell itself. Since the adenosinetriphosphatase was not extracellular, it was concluded that the enzyme functioned at the surface of the microorganism (85). Similarly, if glucose-6-phosphate or glucose-1-phosphate were added to a suspension of the microorganisms, the amount of glucose disappearing from solution was equivalent to the amount of orthophosphate appearing; or if the sugar-phosphorus was tagged, all of the label remained in the medium. The most likely explanation of these findings is that the appropriate phosphatases are at the surface, although such enzymes apparently have no role in glucose decomposition since glycolysis proceeds even when these phosphatases are poisoned with molybdate. Their function might be in the removal of phosphate from phosphorylated hexoses, converting them to utilizable sugar residues which can enter the cell to be metabolized therein (86). Volk (111) reached a similar conclusion with regard to the surface-localization of enzymes dephosphorylating some intermediates in glycolysis on the basis of studies of the effect of inhibitors on fermentation by *Propionibacterium pentosaceum*. The α-glycerophosphatase as well as enzymes dephosphorylating adenosine diphosphate, triphosphate, inorganic pyrophosphate, and phenylphosphate are also reported to be situated at the cell surface of yeast (85, 93).

More recent investigations of the intracellular locale of invertase have confirmed the earlier reports. Under conditions where fermentation was inhibited, the ratio of extracellular to intracellular sugar produced during the complete hydrolysis of sucrose to the monosaccharides by bakers' yeast invertase was 215:1. This effect indicates a surface-bound invertase and is not the result of the occurrence of an extracellular catalysis, since the medium contained none of the hydrolytic activity (27). A similar localization seems to exist for the enzyme in spores of the cellulolytic fungus *Myrothecium verrucaria* (58). Other enzymes reported to be associated with the microbial cell surface are the ascorbic acid oxidase of *M. verrucaria* spores (59) and the adaptive sarcosine oxidase of *Pseudomonas aeruginosa* (10).

There is also an apparent function of the cell surface in the glycolytic pathway. After extensive studies on the effect of uranium on the metabolism of hexoses by yeast, Rothstein (87) proposed that the initial steps in glycolysis, presumably the phosphorylation of the hexose, occur at the surface of the microorganism. In a subsequent communication, Rothstein and Demis (84) analysed the stimulatory effect of potassium on the fermentation of hexoses by yeast cells and concluded that the stimulation could not be explained by the passage of potassium into or out of the cell, nor did the effect depend upon intracellular potassium (84). The idea was advanced, therefore, that some part of the metabolism of glucose must occur at the cell surface where the potassium can exert its effect. Since phosphohexokinase is known to require this cation, it may be this enzyme that is functioning at the outer portion of the organism.

Because of these reports of the association of several enzymes with the microbial surface, study of this component of the cell by the techniques of differential centrifugation will present a significant addition to our knowledge of subcellular physiology, especially since this cytochemical method can provide data by which comparisons of enzymatic function among various structures can be made. Although some of this activity may be bound to the cell membrane, for which no isolation procedure has yet been devised, other physiological functions are undoubtedly localized in the cell wall, which can be prepared from lysates in good yield. The bacterial walls sediment at very low gravitational force and are usually white in color when precipitated at the bottom of the centrifuge tube (3, 41, 66, 89, 117). In the azotobacter, this white fraction appears between the residual intact cells and the pink layer containing the large particles. Because of the large quantity of white abrasive which obscures the wall layer, it cannot be seen in extracts prepared by alumina grinding. Since the walls sediment so rapidly, they are not included in cell extracts as usually prepared. Until cell-free preparations are modified to include all parts of the organism, especially the wall and large particles, it is necessary to be cautious in proposing metabolic pathways on the basis of data derived solely from cell lysates. For example, if the phosphohexokinase presumably associated with the yeast surface is entirely a function of the wall material, then the CE as now devised would be devoid of this activity, and a possibly erroneous conclusion would be advanced with respect to the pathway

of glucose breakdown. In addition to the cell wall, another protoplasmic constituent whose metabolic properties have not yet been investigated is the bacterial flagellum, although progress has been made on the isolation of this structure (117).

Bowen and Dagley (13, 14) have utilized centrifugal techniques in their study of the association of enzymatic function with macromolecular components of enteric bacteria. They found that the oxalacetic decarboxylase and citridesmolase of *Aerobacter aerogenes*, as well as the citridesmolase and glutamic and arginine decarboxylases of *Escherichia coli*, were associated with the 12 S constituent. Similar studies of the known components found in the small particle fraction would clarify the biochemical heterogeneity of these structures.

Only a few reactions involved in cell synthetic processes have been localized. Particles containing the chlorophyll and carotenoid pigments have been isolated from the blue-green alga *Synechococcus cedorum*; the resultant supernatant contained none of the chlorophyll but did possess the phycocyanins (18). These chlorophyll-bearing granules had a diameter of *ca.* 220 mμ and could be seen in the intact alga and in the CE. Intact cells of a green sulfur bacterium, *Chlorobium limicola*, possess similar granules (11). Extracts of photoautotrophically-grown *Rhodospirillum rubrum* contain a large component with a sedimentation constant of 190 S which carries all of the bacteriochlorophyll and carotenoids found in CE and in the unruptured autotroph (90). Measurements of the diameter of these structures by examination of electron micrographs give a value of 60 mμ after application of corrections for the apparent flattening resulting from desiccation. Probably 5,000 to 6,000 of these particles occur in each cell, and they comprise about 15 per cent of the nitrogen of the intact *R. rubrum*. These structures, which are probably the sites of the initial photochemical reaction in the photosynthetic process, have been designated as chromatophores. The chromatophores are not found in extracts of the bacterium if the culture is grown under conditions where no photosynthesis occurs. Chloroplast fragments have been isolated from the alga *Spirogyra* which can catalyze photosynthesis and phosphorylation in the presence of light (106).

Only one example of the association of a non-photochemical synthetic reaction with isolated structures of bacteria is presently available. In their study of protein synthesis by extracts of *Micrococcus pyogenes* var. *aureus*, Gale and Folkes (32) prepared a fraction by low-speed centrifugation which consisted of "broken cell envelopes each containing a small round mass of electron-dense material in the centre," probably large particles contained within the staphylococcal wall. This fraction is capable of incorporating glutamic acid into protein in the presence of ATP and hexosediphosphate. The rate and extent of incorporation were increased by the addition of a mixture of amino acids or bacterial nucleic acids. This fraction can also synthesize catalase, β-galactosidase, enzymes involved in the production of acid from glucose, and ribonucleic acid, as measured by the uptake of C^{14}-uracil. These pioneering investigations should lead to the study and localization of other enzymes catalyzing reactions required for cell synthesis and proliferation.

Although enzymatic cytochemistry requires the study of single enzymes, it is important to realize that numerous and continual interactions exist between enzymes situated on different structures in the growing and metabolizing microorganism. See, for example, the earlier discussion of the requisite systems for the functioning of the TCA cycle (pp.77–80). An interaction also occurs in denitrification by *Pseudomonas stutzeri*. Washed 15*p*20 particles were unable to denitrify whereas the supernatant of this fraction could liberate N_2 from nitrite but only for a short time interval. A combination of the two, however, produced gas for more than 2 hours; thus, both of these constituents are needed for optimal N_2 production in the reduction of nitrite (77). The biochemical association of the various structural units of the cell is nevertheless a field as yet almost wholly unexplored. One interesting problem arising from our present limited knowledge is the spatial and enzymatic mechanism whereby the high energy P "bonds" formed in the submicroscopic structures are transported from the site of formation to the site of storage, presumably the large electron-opaque granules.

In a series of three papers, Gunsalus, Gunsalus and Stanier (43, 44, 101) have presented an outstanding study of the association of a specific metabolic process, the conversion of mandelic acid to benzoic acid, with defined morphological

entities of the bacterium. The breakdown of D-mandelic acid by *P. fluorescens* requires the presence of mandelic acid racemase producing the L- from the D-mandelic acid, the L-mandelic dehydrogenase which leads to the formation of benzoylformic acid, a decarboxylating enzyme to yield benzaldehyde, and a pyridine nucleotide-linked benzaldehyde dehydrogenase giving benzoic acid. To localize the enzymes involved, a cell extract of the pseudomonad was fractionated into two particle fractions and a nonparticulate supernatant. Three of these enzymes were localized in the ground plasm material while only the L-mandelic dehydrogenase was bound to the granules.

An urgent need remains for the discovery of enzymes or chemical constituents which are completely associated with a single structural unit of the cell. Such components will aid in the establishment of a well-defined method of differentiation of isolated fractions, one independent of any centrifugal or morphological properties of the protoplasmic substance. Preliminary studies with azotobacter indicate that the isocitric dehydrogenase of the ground plasm or the succinic dehydrogenase of the granules may be convenient initial tracers, although neither the latter nor any other enzyme so far localized will clearly differentiate various morphological types of intracellular granular inclusions.

From the cytological point of view, it is desirable to have some knowledge regarding the position, arrangement, and morphological characteristics of the submicroscopic granules within the unruptured cell. No method is presently available to the cytologist whereby this information can be obtained, although the technique of adhesion partitioning (7) seems to offer some promise. Several reports lead one to suspect that the submicroscopic particles may be situated in proximity to the surface of the cell. In *Bacillus megaterium*, for example, loci in the cell periphery, near the membrane, give positive reactions with the Nadi reagent and Janus green B (9). Because of the similarity in chemical composition of the cell walls and particles of diameter 10–50 mμ isolated from *M. pyogenes*, Mitchell and Moyle (66) have suggested that these particles may form a continuous layer immediately within the bacterial wall. Similar sites for these bodies in bacteria have been proposed by Weibull (114), Cooper (22), and Cota-Robles and Marr (23a).

The bacterial cell is thus cytologically and physiologically differentiated into subcellular structures, representing a high degree of biochemical organization both at the microscopic and submicroscopic level. The separate structures carry out different enzymatic functions, but apparently an interrelationship exists between these bodies. Bacteriology still lacks precise knowledge of the physiological organization of the cell. Such information can help not only the cytologist to explain the nature of the intracellular bodies, but also the physiologist, to whom it reveals the multi-enzyme reaction sequences associated with different portions of the bacterial structure. In addition, there is a great need for the fusion of these two disciplines into one which will express microbial metabolism in terms of cellular organization.

The similarities in localizations reported herein, especially for the catalysts functioning in reactions of the tricarboxylic acid cycle and the transport of electrons, indicate a remarkable degree of uniformity in the intracellular distribution of enzymes in the cells of microorganisms. A comparison of the enzymatic cytochemistry of the microorganism with that of cells of higher plants and animals indicates here too a close resemblance in the subcellular physiology of the unicellular and multicellular organism, although certain decided differences do indeed exist. Such a similarity lends credence to the doctrine of comparative physiology, viewed here in a spatial and anatomical sense, *i.e.*, a possible basic pattern for the subcellular organization of enzymatic activity.

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