Localization of Enzymes in *Bacillus megaterium*, Strain M

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SUMMARY: Protoplasts were prepared from cells of *Bacillus megaterium*, strain M, by lysozyme treatment in the presence of sucrose. The protoplasts were shocked osmotically and the lysate thus obtained was fractionated into membranous 'ghosts' and soluble protoplasm by means of differential centrifugations. The distribution of various enzymes between these two fractions was studied. The bulk of the cytochromes, the succinic dehydrogenase and the diphosphopyridine nucleotide oxidase of the lysate was recovered in the 'ghost' fraction. On the other hand, the soluble protoplasm contained most of the isocitric dehydrogenase, the catalase, the hexokinase and the acid phosphatase of the lysed bacteria. Considerable amounts of malic dehydrogenase, lactic dehydrogenase and fumarase were found in both the 'ghosts' and the soluble protoplasm. None of the enzymes studied was localized in the ribonucleic acid-containing particles of the bacterial protoplasm.

Many of the enzymes of animal cells are localized in subcellular structures such as mitochondria and microsomes. It is doubtful whether a similar state of affairs prevails in bacterial cells. Numerous investigations (reviewed by Alexander, 1956) have made it clear that, in extracts obtained by a mechanical or sonic disintegration of bacterial cells, several enzymes are associated with the sedimentable particles. Without additional evidence it cannot be settled whether these particles exist as such in the intact cells or whether they represent the remnants of larger, subcellular structures. In some cases at least, however, the 'differential release technique' devised by Marr & Cota-Robles (1957) may be useful for discriminating between these two possibilities.

Some of the hazards of the mechanical and sonic disintegration procedures are avoided when the bacterial cells are degraded enzymically or autolytically. Thus bacterial 'ghosts' (probably mainly cytoplasmic membranes) have been isolated and characterized after treating *Bacillus megaterium*, strains KM and M, with lysozyme (Weibull, 1953*a, b*; Vennes & Gerhardt, 1956; Weibull, 1956; Storck & Wachsmann, 1957; Weibull & Bergström, 1958). 'Ghosts' of *Micrococcus lysodeikticus* have been studied in the same way (Gilby, Few & McQuillen, 1958). Mitchell & Moyle (1956*a, b*) used an autolytic procedure for the isolation of a plasma-membrane fraction from *Staphylococcus aureus*. Spiegelman, Aronson & Fitz-James (1958) reported the isolation of bacterial nuclei by means of the enzymic degradation of the cytoplasmic membrane.

The present study deals with the distribution of certain enzymes between the 'ghosts' and the rest of the protoplasm of *Bacillus megaterium*, strain M. This study thus links up closely with the investigation of Storck & Wachsmann (1957). These workers studied the KM strain of the same organism. However,

it was considered of some interest to compare the enzymic properties of two strains of the same species. Since, moreover, the chemical properties of the 'ghosts' of the M strain have been studied in some detail (Weibull, 1957; Weibull & Bergström, 1958), it has been possible to bring into relation the enzymic and chemical properties of one and the same subcellular structure.

METHODS

Organism, growth conditions and harvesting. *Bacillus megaterium*, strain M (Baumann-Grace & Tomcsik, 1957) was grown in the medium described by Gladstone & Fildes (1940). Fernbach flasks having a volume of 2–8 l. and containing 250–1000 ml. medium were inoculated with the organism and incubated for 16 hr. at 30° on a rotary shaker (speed of shaker 100 rev./min.). The bacteria, then being in the stationary growth phase, were harvested by centrifugation and suspended in 0.02M-phosphate buffer (pH 7). The concentration of the bacteria in the suspension was made about 4 times that in the growth medium at harvesting.

Preparation of bacterial fractions. To each 225 ml. of bacterial suspension, prepared as described above and brought to room temperature, 75 ml. 2M-sucrose and 6 ml. 1% (w/v) lysozyme were added. Protoplast formation was completed within about 30 min. The protoplasts were centrifuged for 20 min. at 20,000 g (Spinco model L preparative ultracentrifuge with the no. 30 rotor) and were then resuspended in 100 ml. of cold (4°) distilled water containing 0.2 mg. crystalline deoxyribonuclease. Because of the released deoxyribonucleic acid, the lysate thus obtained was at the beginning rather viscous, but after *c.* 15 min. showed the viscosity of a normal aqueous solution. A sample of the lysate (L) was withdrawn and the remainder was subjected to the fractionation procedure outlined in Fig. 1. During this fractionation the temperature of the bacterial preparations was kept below 5°.

Thus three kinds of bacterial preparations were obtained: total lysate (L), 'ghosts' (G) and soluble protoplasm (S). The volumes of these preparations were adjusted so that each of them contained (per ml.) material from the same amount (*c.* 15 mg.) of bacteria. A reconstituted lysate (S + G) was obtained by mixing equal amounts of S and G. Compared with the original lysate, this reconstituted system thus contained the equivalent of half the amount of bacterial dry matter/ml. This was taken into account when calculating the figures in Tables 3 and 4. The bacterial preparations were used immediately for enzymic assays.

Enzyme assays. All assays except the hexokinase and phosphatase determinations were carried out at room temperature (20–22°). The enzymic activities of the various bacterial fractions were determined in the shortest possible time, each enzyme being assayed in turn. Prior to the determinations, the enzyme solutions were kept in an ice bath. Samples (1 ml.) were allowed to warm up for 1 min. at room temperature before being used in the tests.

The method for assaying succinic, malic, lactic, isocitric and formic dehydrogenases was essentially that used by Storek & Wachsman (1957). Dichloro-

phenol indophenol was, however, replaced by potassium ferricyanide as the hydrogen acceptor. The reaction mixture contained: 0.3 ml. 0.5 M-phosphate; 0.1 ml. 0.15 M-MgSO₄; 0.2 ml. 0.15 M-KCN; 0.2 ml. 0.008 M-K₃Fe(CN)₆; 1 ml. substrate; 1 ml. enzyme solution; water to 3.0 ml. The final pH value of the mixture was 7.0. The following solutions were used as substrates: 0.3 M-sodium DL-lactate; 0.1 M-sodium allo + DL-isocitrate; 0.05 M-sodium succinate, formate, DL-malate. The reduction of K₃Fe(CN)₆ was followed with the Beckman DU spectrophotometer at 400 m μ in 1 cm. cuvettes. Readings were taken at 1 min. intervals for 10 min. Corrections were made for endogenous reduction of the ferricyanide.

Fig. 1. Centrifugation procedure for the preparation of bacterial fractions.

Fumarase was determined according to the method devised by Racker (1950). The spectrophotometric readings were, however, taken at 305 m μ instead of at 300 m μ since some of the enzyme solutions exhibited a rather strong absorption at the latter wavelength. The reaction mixture contained: 0.5 ml. 0.2 M-phosphate (pH 7.3); 1 ml. 0.05 M neutralized sodium fumarate; 0.5–1.0 ml. enzyme solution; water to 3.0 ml. Readings were taken at 30 sec. intervals for 5 min.

Diphosphopyridine nucleotide (DPNH) oxidase was assayed by measuring the decrease in absorption of the reaction mixture at 340 m μ (Haas, Horecker & Hogness, 1940). The reaction mixture contained: 1.0 ml. 0.05 M-phosphate (pH 6.8); 0.5 ml. 0.001 M-DPNH (obtained from the Sigma Chemical Co.); 0.025–0.050 ml. enzyme solution; water to 3.0 ml. Readings were taken at 30 sec. intervals for 5 min.

Catalase was determined by the permanganate titration method devised by Bonnichsen, Chance & Theorell (1947).

Acid phosphatase was assayed with *p*-nitrophenylphosphate as substrate (Bessey, Lowry & Brock, 1946). The determinations were carried out as described by Boman (1955); 0.100 ml. of enzyme solution was used, however, instead of 0.010 ml., and 0.025 ml. 0.15 M-MgSO₄ was added to the reaction mixture as an activating agent. The determinations were carried out at 37°. The pH value of the reaction mixture was 6.0.

Hexokinase was determined by the method described by McDonald (1955). The addition of sodium fluoride did not affect the results. The determinations were carried out at 5°.

With the exception of the decomposition of hydrogen peroxide by catalase, the enzymic reactions studied generally proceeded at constant rates during the whole incubation time.

Chemical analyses. Ribonucleic acid (RNA) was determined according to Schneider (1945), and protein by the biuret method (Gornal, Bardawill & David, 1949; Weibull & Bergström, 1958). Cytochromes were estimated by light absorption measurements. A minute amount of sodium dithionite was added to the suspension or solution to be investigated and the absorption between 500 and 650 m μ was determined with the Beckman model DU spectrophotometer. The amount of cytochromes in the samples was estimated from the height of the peaks at 552, 558 and 600 m μ (when present). A correction for unspecific absorption was made according to Weibull (1948). Material which exhibited strong unspecific absorption was suspended in 70% (w/v) glycerol instead of water.

The dry weights of bacteria were measured after drying samples at 100° for 24 hr.

Electron microscopy. The electron micrographs were taken with an RCA model EMU-2D electron microscope. The specimens were fixed with osmium tetroxide vapour for 2 hr. at room temperature. The spraying technique devised by Backus & Williams (1950) was used for applying the specimens to the grids. Before fixing and spraying, the specimens were dialysed for 12 hr. against distilled water.

RESULTS

Microscopical morphology of the bacterial extracts

Only one structural element, spherical bodies of low contrast and having a diameter of *c.* 1 μ , was observed in the total lysate and in fraction G ('ghost' fraction) when specimens were viewed under the phase-contrast microscope. In some of these bodies (the 'ghosts'), a few granules were seen. No structures or bodies were seen in fraction S (soluble protoplasm).

Plates 1 and 2, figs. 1–4, show the electron microscopical appearance of sprayed and dried drops of the bacterial fractions. Two kinds of structures can be seen in the total lysate (fig. 1): (i) approximately circular membranes evidently representing 'ghosts'; (ii) small granules forming a coherent mass at the edge of the drops. Practically no small granules are present outside the membranes in fig. 2 ('ghost' fraction) but some granular elements can be seen inside these membranes (cf. Weibull & Bergström, 1958). The membranes are

totally missing in fig. 3 (soluble protoplasm). Plate 2, fig. 4, shows fraction S (soluble protoplasm) at a higher magnification and a higher dilution. Both free and aggregated particles can be seen. The smallest particles have a diameter of 100–200 Å. and may thus correspond to the '40 S particles' described by Schachman, Pardee & Stanier (1952) and others. No well-defined elements having larger dimensions than these particles are present in the specimen of soluble protoplasm shown in Pl. 2, fig. 4. Inspection of a large number of similar drops gave the same result.

Table 1. *RNA content of 'ghosts' of Bacillus megaterium, strain M*

Each figure represents an average value obtained from three batches of bacteria.

No. of washings of the 'ghosts'	RNA in dried 'ghosts' (%)	RNA in 'ghosts' as % of total RNA
1	1.62	4.6
4	0.82	2.3

Chemical characteristics of the bacterial extracts

Table 1 shows the RNA content of fraction G ('ghost' fraction).

It appears that repeated washing of the 'ghost' fraction decreased its RNA content to some extent. It can also be concluded that at least 95% of the bacterial RNA was present in the soluble protoplasm of *Bacillus megaterium* strain M, since no nucleic acids have been found in the cell wall of this organism (Weibull & Bergström, 1958).

In order to estimate the specific activities of the bacterial enzymes, i.e. activities/mg. protein, the protein content of extracts obtained from three batches of bacteria was determined. According to these analyses, the 'ghost' fraction contained on an average 13% and fraction S (soluble protoplasm) 87% of the protein of the total lysate.

Schachman *et al.* (1952) showed that the bulk of the bacterial RNA in many bacteria is found in particles having a diameter of *c.* 150 Å. and a sedimentation constant of 20–40 S. In order to ascertain to what extent the RNA of *Bacillus megaterium*, strain M, is associated with such particles, fraction S was centrifuged for 2 hr. at 103,000 *g* in a Spinco centrifuge with the no. 40.2 rotor. The supernatant fluid was transferred to fresh tubes and again centrifuged for 2 hr. at 103,000 *g*. Three additional centrifugations of this kind were performed and the supernatants were analysed for RNA. Fig. 2 gives the results. It can be seen that about 15% of the RNA cannot be sedimented at 103,000 *g*. Thus, this part of the bacterial RNA is not associated with particles having a sedimentation constant of 20–40 S.

In order to ascertain whether any sedimentable cytochromes were present in fraction S (soluble protoplasm), this fraction was centrifuged for 20 min. at 103,000 *g*. The sediment was suspended in a small volume of 70% (w/v) glycerol. After adding a minute amount of dithionite the suspension was investigated spectrophotometrically. A peak was observed in the 550–560 *mμ* range. By comparing the height of this peak with that exhibited by the 'ghost'

fraction under similar conditions, it appeared that the cytochromes present in the sedimentable part of the S fraction corresponded to about 5% of the cytochromes of the 'ghost' fraction. When the supernatant fluid of the centrifuged S fraction was investigated spectrophotometrically after a tenfold concentration by pervaporation, no peak at all could be detected in the 500–650 $m\mu$ range. It could thus be concluded that the 'ghost' fraction contained about 95% of the cytochromes of the bacterial cells.

Fig. 2. RNA content of the soluble protoplasm of *Bacillus megaterium*, strain M, before and after centrifugations at 103,000 g. Between each centrifugation, the supernatant fluids were transferred to fresh tubes. Relative values are given, the RNA content of the protoplasm before the first centrifugation being put equal to 100.

Enzymic studies

Table 2 shows the results of a series of enzyme determinations carried out on a batch of lysed protoplasts. It can be seen that, for each enzyme, the measured activities were proportional to the amount of enzyme solution used in the tests. Similar results were obtained when fraction G ('ghost' fraction), fraction S (soluble protoplasm) and a reconstituted lysate were tested.

Table 3 shows the amounts of the enzymes studied which were found in the bacterial preparations. Six batches of bacteria were analysed. The activities are expressed as μ mole (in the case of catalase mmole) of substrate split, reduced, oxidized or hydrated/min. and/l. of an incubation mixture containing bacterial material equivalent to 1.0 g. (dry wt.) whole bacteria. These figures could easily be calculated from the experimental results since the measured activities were proportional to the amount of enzyme solution used in the tests (see Table 2).

The figures collected in Table 3 shows that the amount of each bacterial enzyme varies considerably from one batch of bacteria to another.

The scope of the present investigation was primarily to study the presence or absence of various enzymes in certain fractions of the bacterial cells. The

Table 2. *The relationship between the enzymic activity of samples of a bacterial lysate (lysed protoplasts of Bacillus megaterium, strain M) and the volume of the samples*

Each figure represents one determination. Relative values are given, the activity of each enzyme in a 1 ml. sample being put equal to 100. The $\pm$ sign indicates 95 % fiducial limits.

Enzyme tested	Enzymic activity in sample of		
	1 ml.	0.5 ml.	0.25 ml.
Succinic dehydrogenase	100	51.1	23.2
Malic dehydrogenase	100	46.0	23.8
Lactic dehydrogenase	100	46.8	26.7
Isocitric dehydrogenase	100	53.9	23.7
Fumarase	100	53.1	26.9
DPNH oxidase	100	44.0	24.0
Catalase	100	52.3	27.8
Hexokinase	100	52.0	—
Acid phosphatase	100	49.1	24.0
Average	100	49.8 $\pm$ 2.7	25.0 $\pm$ 1.5

Table 3. *Enzymic activities exhibited by six different batches of Bacillus megaterium, strain M*

The minimum and maximum values found are given in the table. The enzymic activities are expressed as μ mole (in the case of catalase, mmole) of substrate split, reduced, oxidized or hydrated per min. and per l. of an incubation mixture containing material equivalent to 1 g. dry wt. bacteria. In the case of the catalase-hydrogen peroxide reaction, the initial decomposition rate was used for calculating the enzymic activity. All other reactions proceeded generally at constant rates during the incubation time.

Enzyme tested	Total lysate	Fraction G (‘ghost’ fraction)	Fraction S (soluble protoplasm)	Reconstituted lysate
Succinic dehydrogenase	0.80–2.03	1.24–3.06	< 0.02–0.23	0.67–2.75
Malic dehydrogenase	3.19–5.55	0.80–2.80	0.61–0.81	1.31–3.15
Lactic dehydrogenase	0.87–2.03	0.41–0.92	0.56–1.21	0.93–2.10
Isocitric dehydrogenase	0.62–0.90	< 0.02–0.12	0.73–1.06	0.03–0.37
Formic dehydrogenase	< 0.02	< 0.02	< 0.02	< 0.02
DPNH oxidase	5.58–25.2	19.6–46.5	< 0.2	10.6–37.2
Fumarase	145–376	33.6–194	138–348	105–326
Catalase	4.85–17.0	0.06–0.22	4.60–18.2	5.30–15.2
Hexokinase	3.56–10.9	0.40–0.53	3.00–10.2	4.56–14.2
Acid phosphatase	0.78–1.65	0.01–0.06	0.79–1.97	0.59–1.48

situation in this respect is most clearly demonstrated when the relative amounts of each enzyme in the fractions studied are compared with one another. For this purpose, the enzyme content of each bacterial fraction was calculated, giving the amount of each enzyme in the total bacterial lysate a value of 100. Table 4 shows the results; each figure is the average of determinations carried out on six batches of bacteria. Three of the investigated ‘ghosts’ fractions were washed once, the remainder four times (see Fig. 1). No significant differences between the enzymic activities of these two types of ghosts were observed.

It can be seen from the figures in Table 4 that less than 10 % of the succinic dehydrogenase and the DPNH oxidase of the lysed bacteria were found in

fraction S (soluble protoplasm). The bulk of these enzymes were found in the 'ghost' fraction. On the other hand, less than 10 % of the isocitric dehydrogenase, the catalase, the hexokinase and the acid phosphatase were found in the 'ghost' fraction. Considerable amounts of lactic and malic dehydrogenase and of fumarase were present in both fraction S and the 'ghost' fraction. The recoveries of malic and isocitric dehydrogenase in the reconstituted lysate were low, whereas the DPNH-oxidase activity was considerably higher in this system than in the original lysate. The DPNH oxidase activity of the 'ghosts' was also remarkably high.

Table 4. *Relative amounts of enzymes in cell extracts prepared from Bacillus megaterium, strain M*

The average values obtained from six batches of bacteria are given. The activity of each enzyme in the total lysate is put equal to 100. The bacterial material present in each extract was derived from the same amount of bacteria. The $\pm$ sign indicates 95 % fiducial limits.

Enzyme	Total lysate	Fraction G ('ghost' fraction)	Fraction S (soluble protoplasm)	Reconstituted lysate
Succinic dehydrogenase	100	145.3 $\pm$ 21.1	4.5 $\pm$ 4.7	136.8 $\pm$ 18.9
Malic dehydrogenase	100	32.6 $\pm$ 16.3	15.7 $\pm$ 2.9	49.7 $\pm$ 14.5
Lactic dehydrogenase	100	41.2 $\pm$ 10.2	57.2 $\pm$ 11.2	102.8 $\pm$ 15.4
Isocitric dehydrogenase	100	3.5 $\pm$ 6.4	112.0 $\pm$ 19.4	27.1 $\pm$ 15.3
Fumarase	100	32.2 $\pm$ 11.8	101.8 $\pm$ 17.9	112.8 $\pm$ 22.1
DPNH oxidase	100	261.5 $\pm$ 61.0	< 1	209.0 $\pm$ 76.0
Catalase	100	1.3 $\pm$ 0.7	100.2 $\pm$ 11.9	92.0 $\pm$ 3.6
Hexokinase	100	5.5 $\pm$ 2.0	87.5 $\pm$ 8.7	143.4 $\pm$ 22.7
Acid phosphatase	100	3.0 $\pm$ 1.6	99.6 $\pm$ 16.1	83.6 $\pm$ 8.9

As mentioned in the preceding section, the protein content of the 'ghosts' was 0.13 and that of fraction S (soluble protoplasm) 0.87 when the protein content of the total lysate was put equal to 1.00. These values were used for calculating the concentration of the enzymes, i.e. the enzymic activities/g. protein. Table 5 shows the results. The specific activity of each enzyme in the total lysate was put equal to 1.00.

It was shown above that about 85 % of the bacterial RNA was sedimented at 103,000 g. It was considered of interest to establish whether some of the enzymes found in the S fraction (soluble protoplasm) could be sedimented in the same field. For this purpose samples of the S fraction were centrifuged for 2 $\times$ 2 hr. at 103,000 g transferring the supernatant fluids to fresh tubes between the centrifugations. The RNA content and the enzymic activities of the supernatant fluid were then determined. The results are given in Table 6 from which it can be seen that, on the average, about 85 % of the various enzymic activities remained in the supernatant fluid whereas about 78 % of the RNA was sedimented. Since about 15 % of the total RNA was not sedimented even after repeated centrifugations (see Fig. 2), it can be concluded that more than 90 % of the sedimentable RNA present in the bacteria can be removed from the protoplasm without affecting seriously the activities of the enzymes studied.

Table 5. *Concentration of enzymes (enzymic activities/g. protein) in fractions prepared from Bacillus megaterium, strain M*

The average values obtained from six batches of bacteria are given. The concentration of each enzyme in the total lysate is put equal to 1.00. The $\pm$ sign indicates 95 % fiducial limits.

Enzyme	Total lysate	Fraction G ('ghost' fraction)	Fraction S (soluble protoplasm)
Succinic dehydrogenase	1.00	11.2 $\pm$ 1.6	0.05 $\pm$ 0.05
Malic dehydrogenase	1.00	2.51 $\pm$ 1.25	0.18 $\pm$ 0.03
Lactic dehydrogenase	1.00	3.17 $\pm$ 0.79	0.66 $\pm$ 0.13
Isocitric dehydrogenase	1.00	0.27 $\pm$ 0.49	1.29 $\pm$ 0.22
Fumarase	1.00	2.48 $\pm$ 0.91	1.17 $\pm$ 0.21
DPNH oxidase	1.00	20.1 $\pm$ 4.7	< 0.01
Catalase	1.00	0.10 $\pm$ 0.05	1.15 $\pm$ 0.14
Hexokinase	1.00	0.42 $\pm$ 0.15	1.01 $\pm$ 0.10
Acid phosphatase	1.00	0.23 $\pm$ 0.12	1.14 $\pm$ 0.18

Table 6. *RNA content and enzymic activities of the soluble protoplasm of Bacillus megaterium, strain M, before and after centrifugation at 103,000 g for 2 $\times$ 2 hr.*

Between the centrifugations, the supernatant fluids were transferred to fresh tubes. Relative values are given, the enzymic activities and the RNA content before the first centrifugation being put equal to 100. The $\pm$ sign indicates 95 % fiducial limits.

Enzyme	Activity after centrifugation, % of initial values
Malic dehydrogenase	74.4 $\pm$ 8.4
Isocitric dehydrogenase	91.5 $\pm$ 11.4
Fumarase	83.0 $\pm$ 6.5
Hexokinase	76.8 $\pm$ 9.3
Acid phosphatase	88.8 $\pm$ 9.0
Catalase	103.8 $\pm$ 18.4
RNA	22.6 $\pm$ 1.6

DISCUSSION

The aim of the present investigation was primarily to locate certain enzymes or more precisely certain enzymically-active proteins in *Bacillus megaterium*, strain M. The method used, fractionation of lysed organisms by differential centrifugation and determination of enzymic activities in the fractions, is, like other methods used for the same purpose, subject to several sources of error. First, the subcellular structures to be investigated may be more or less degraded during the lysis, secondary aggregates may be formed and adsorption phenomena may take place. Secondly, the lysis and the fractionation procedure may involve the denaturation of enzyme proteins. Thirdly, inhibitory and activating agents may influence the enzymes studied to different degrees in the various bacterial fractions. This is especially likely when the enzymes are not purified before their activities are measured.

To avoid misinterpretations of the experimental results, we have taken into

consideration the criteria proposed by Hogeboom, Schneider & Striebich (1953) and by Alexander & Wilson (1955). These criteria can be formulated as follows (cf. Alexander & Wilson, 1955). First, for each enzyme, the sum of the activities of the isolated fractions should approach 100 % of that found in the original extract. Secondly, a large percentage of the total activity should reside in the fraction to which the enzymic function is being attributed. Thirdly, the concentrations of the various enzymes (i.e. enzyme activities/g. protein) in the isolated fractions should be similar to or higher than those in the total lysate.

When the first two of these criteria are taken into account, the figures of Table 4 evidently strongly suggest that the succinic dehydrogenase and the DPNH oxidase of *Bacillus megaterium*, strain M, are located in the bacterial 'ghosts'. The isocitric dehydrogenase, the catalase, the hexokinase and the acid phosphatase, on the other hand, seem to be situated in the soluble protoplasm. No certain conclusions can be drawn about the location of the malic and lactic dehydrogenases and the fumarase.

When the figures in Table 5 are taken into account, then the third criterion can be used for testing the conclusions drawn in the preceding paragraph. In the main, these conclusions are not affected. It is evident, however, that the concentrations of the malic and lactic dehydrogenases are appreciably higher in the proteinaceous part of the 'ghosts' than in the same part of the soluble protoplasm. The concentration of the malic dehydrogenase in the soluble protoplasm is remarkably low. However, the total recovery of the malic dehydrogenase is also low (see Table 4).

The activities of the DPNH oxidase and the succinic dehydrogenase are appreciably higher in the sum of the fractions than in the total lysate. This might be due to an 'unmasking' effect (Alexander, 1956). It is more difficult to explain the low activities of the malic and the isocitric dehydrogenases in the reconstituted lysate.

A comparison between our results and those obtained by Storck & Wachsman (1957) suggests that the enzymic organization of cells of the KM strain of *Bacillus megaterium* is rather similar to that of the M strain. A difference may be indicated by the fact that Storck & Wachsman found lactic dehydrogenase mainly in the 'ghosts' from the KM strain, while we found this enzyme in the 'ghosts' and in the soluble protoplasm of the M strain. Both investigations indicate, however, a high specific activity for the 'ghosts'.

With respect to other bacterial species, the present investigation confirms earlier results concerning the location of the cytochrome system, the DPNH oxidase, the succinic dehydrogenase and the isocitric dehydrogenase (Alexander, 1956; Mitchell & Moyle, 1956*b*; Cota-Robles, Marr & Nilson, 1958). Mitchell & Moyle (1956*b*) found malic and lactic dehydrogenases mainly in the plasma-membrane fraction of *Staphylococcus aureus*. Linnane & Still (1955), however, found the same enzymes both in the particulate and soluble fractions of the protoplasm of *Serratia marcescens*. According to Alexander (1956) fumarase is situated mainly in the soluble protoplasm of several bacterial species.

Our findings concerning the location of catalase agree with the experiments of Few, Fraser & Gilby (1957). These workers found that the catalase of intact *Micrococcus lysodeikticus* cells was confined to the interior of the organisms since the activity of this enzyme was independent of the pH value of the surrounding medium. On the other hand, Alexander & Wilson (1955) reported that catalase was present in all fractions of the protoplasm of *Azotobacter vinelandii* which they examined.

Contrary to our results, Mitchell & Moyle (1956*b*) found acid phosphatase mainly in the plasma-membrane fraction of *Staphylococcus aureus*. To our knowledge, no investigations have been made on the distribution of hexokinase in bacteria. According to our experiments, the hexokinase, like the acid phosphatase, is probably not present in the 'ghosts' and hence not in the cytoplasmic membrane of *Bacillus megaterium*, strain M. This would imply that these enzymes are not directly involved in the transportation of glucose and phosphatase across the permeability barrier of the cells (cf. Rothstein, 1954; Mitchell, 1957).

The enzymic properties of those particles of the bacterial cytoplasm which have a sedimentation constant of about 40 S and which contain the bulk of the bacterial RNA have been discussed repeatedly (Schachman *et al.* 1952; Bradfield, 1956; Alexander, 1956). None of the enzymes studied by us was found to be associated with such particles, since these enzymes were located either in the bacterial 'ghosts', which contain only 2–4 % of the bacterial RNA, or in the soluble protoplasm, from which more than 90 % of the sedimentable RNA could be removed without seriously affecting the enzymic activities. In this connexion it should be mentioned that, according to a recent report (Cota-Robles *et al.* 1958), no enzymes except a ribonuclease were found in the RNA-containing particles of *Azotobacter agilis*.

If one assumes that at least some enzymes exist exclusively in certain sub-cellular structures of the bacterial cell, then the purity of the fractions isolated by us could be estimated from the enzymic determinations. Thus fraction S (soluble protoplasm) may contain about 5 % of 'ghost' material (according to the electron microscopical findings in a highly fragmented form) since 5 % of the total succinic dehydrogenase of the lysed cells was found in this fraction. The same conclusion can be drawn from the spectrophotometric cytochrome determinations. Conversely, about 5 % of the soluble protoplasm may be adsorbed on the 'ghosts', judging from the amounts of isocitric dehydrogenase, catalase, hexokinase and acid phosphatase found in the 'ghost' fraction.

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C. WEIBULL, H. BECKMAN & L. BERGSTRÖM—ENZYMES IN *BACILLUS MEGATERIUM*.
PLATE 1

(Facing p. 530)

C. WEIBULL, H. BECKMAN & L. BERGSTRÖM—ENZYMES IN *BACILLUS MEGATERIUM*.
PLATE 2

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EXPLANATION OF PLATES

PLATES 1-2

Electron micrographs of sprayed drops of bacterial extracts obtained by shocking osmotically protoplasts of *Bacillus megaterium*, strain M. The extracts were dialysed for 12 hr. against distilled water and fixed for 2 hr. at room temperature with osmium tetroxide vapour.

PLATE 1

Fig. 1. Total lysate. $\times 3000$.

Fig. 2. Fraction G ('ghost' fraction). $\times 3000$.

PLATE 2

Fig. 3. Fraction S (soluble protoplasm). $\times 3000$.

Fig. 4. Fraction S (diluted 1/100). $\times 32,000$.

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