ENZYME LOCALIZATION IN *BACILLUS MEGATERIUM*

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Weibull (1953*a*) has shown that after osmotic lysis of protoplasts of *Bacillus megaterium*, prepared by controlled treatment with lysozyme, only two microscopically detectable structures remain; membrane-like bodies termed "ghosts," and granules. The ghosts were found to sediment as a dark yellow fraction by centrifugation at 10,000 G (Weibull, 1953*b*) and to contain almost all the pigmented material and cytochromes of the whole cell. The latter observation is in agreement with the particulate nature (particles sedimenting between 22,000 and 100,000 G) of the cytochrome complement of *Pseudomonas fluorescens* (Stanier *et al.*, 1953; Wood, 1955), *Escherichia coli* (Asnis *et al.*, 1956), and *Acetobacter suboxydans* (Smith, 1954). In subsequent experiments Weibull (1956*a*) has obtained evidence that the ghost is the cytoplasmic membrane, perhaps in a denatured form.

A brief treatment of the yellow ghost fraction in the sonic oscillator destroys the ghost structure and leads to the formation of granules which could be sedimented at 105,000 G (Weibull, 1953*b*). The similarity in chemical and physical properties between these granules and the particulate fractions isolated after the mechanical disruption of several different types of bacteria have led Stanier (1954) to propose that "the bacterial cytochrome system, along with some other enzymes, may be a built-in part of the cell membrane, and not, as in higher organisms, a mitochondrial component."

The present work supports this concept by furnishing evidence for the localization of enzymes responsible for succinate, α-ketoglutarate, lactate, and malate oxidation in the ghost of *B. megaterium*.

MATERIALS AND METHODS

B. megaterium strain KM (Northrop, 1951) was grown at 36 C on a medium containing glucose, 1.0 per cent; DL-asparagine, 1.0 per cent; NH_4Cl, 0.5 per cent; K_2HPO_4, 0.3 per cent; KH_2PO_4, 0.1 per cent; Na_2SO_4, 0.1 per cent; $MgSO_4 \cdot 7H_2O$, 0.01 per cent; and 8×10^{-5} M ferric citrate (final pH, 7.0). Cells were grown with vigorous aeration on a rotary shaker and harvested by centrifugation at the end of the phase of exponential growth.

For the preparation of protoplasts, cells were suspended in a medium consisting of 0.04 M potassium phosphate buffer pH 7.0, 10 per cent (w/v) polyethylene glycol (mol wt, 3000–3700), 2×10^{-3} M $MgSO_4$, and approximately 0.04 per cent lysozyme (final concentration of approximately 11 mg bacterial dry weight per ml suspending medium). Protoplast formation was complete after 10–15 min incubation at room temperature.

The following procedure was employed for the preparation of a total lysate (TL) used in dye reduction experiments. Protoplasts were collected by centrifugation at 13,000 G for 5 min and the supernatant containing lysozyme and cell wall lysate was decanted. A suspending medium consisting of 0.04 M potassium phosphate buffer pH 7.0 and 2×10^{-3} M $MgSO_4$ was used at this stage and in all subsequent operations. The protoplast pellet was lysed by the addition of a small quantity of suspending medium and of deoxyribonuclease. The latter constituent was added to decrease the viscosity of the lysate. Osmotic lysis and the formation of a gel was almost instantaneous. However, a homogeneous suspension was obtained after 3–5 min agitation at room temperature. The lysate was diluted with the suspending medium to a final concentra-

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tion of approximately 5 mg bacterial protein and 0.05 μg deoxyribonuclease per ml. All subsequent operations were carried out at 0–5 C.

For fractionation, the total lysate was subjected to the minimal centrifugal force necessary to sediment all the ghosts. An aliquot of the lysate was centrifuged for 10 min at 24,000 G. The supernatant was removed by decantation and centrifuged for an additional 10 min at 24,000 G. The yellow ghost rich pellets were combined, washed once with the suspending medium by centrifugation at 24,000 G for 10 min, and were resuspended in a final volume such as to have the same ghost concentration as the total lysate (approximately 0.7 mg protein per ml).

The supernatant resulting from the two successive centrifugations of total lysate contained some small pieces of ghost as seen in the phase contrast microscope. Therefore, it was centrifuged for an additional 10 min at 24,000 G and the negligible yellow pellet was discarded. The supernatant was adjusted to contain 4.0 mg of protein per ml, which corresponds to its concentration in the total lysate. Using the symbols suggested by Alexander and Wilson (1955) the ghost fraction (P_1) and the supernatant (S_1) represent a 24*p* 2 × 10 and a 24*s* 3 × 10 fraction, respectively.

Manometric experiments were performed at 36 C in air. The procedure used for the preparation of TL, S_1, and P_1 was essentially the same as that used in dye reduction experiments, with the exception that a more concentrated total lysate was prepared (approximately 15 mg bacterial protein per ml). The S_1 and P_1 fractions contained approximately 12.0 and 2.5 mg protein per ml, respectively. Enzyme activity was determined during the period of linear oxygen uptake.

Experiments on the rate of reduction of 2,6-dichlorophenol indophenol were performed with a model DU Beckman spectrophotometer at 600 mμ. Readings were taken at 1 min intervals for 10 min and enzyme activity was determined during the period of linear dye reduction.

Protein was determined by the biuret reaction.

High speed centrifugations were performed with the "spinco" preparative centrifuge, rotor no. 40, at 40,000 rpm.

The substrates used were commercial samples of analytical grade. With the exception of Na_3 citrate and Na pyruvate, all acidic substrates were neutralized with KOH. Polyethylene glycol ("carbowax" 4000) was obtained from the Union Carbide and Carbon Corporation. Armour crystalline lysozyme and General Biochemicals Inc. crystalline deoxyribonuclease were used in all experiments. Adenosine triphosphate (disodium, crystalline) and coenzyme A were products of Pabst Laboratories. Diphosphopyridine nucleotide (95 per cent pure) was obtained from Boehringer Inc., triphosphopyridine nucleotide (95 per cent pure) from the Sigma Chemical Co., cocarboxylase from Schwartz Laboratories, and cytochrome C from Nutritional Biochemicals Corp.

RESULTS

Analysis of fractions. The supernatant and ghost fractions represented approximately 85 per cent and 15 per cent respectively, of the protein of the total lysate.

In agreement with the work of Weibull (1953*a*), examination of the total lysate and ghost fraction with the phase contrast microscope revealed the presence of ghosts and granules. Many of the granules are presumably a polymer of β-hydroxybutyrate (Lemoigne *et al.*, 1944). A considerable number of granules were attached to ghosts, but the majority were free in the suspending medium. The supernatant fraction was relatively free of visible structures, containing a few granules, and small, almost circular bodies of approximately the same contrast as ghosts.

When ghosts are viewed through a thin layer of agar (1 mm), they show weaker contrast but more detailed internal structure. It is thus possible to see many ghosts with round holes. The dimensions of these correspond to the small weakly contrasting bodies of the supernatant, and indicate the presence of some ghost fragments in this fraction.

Localization of enzymes based on dye reduction. The average results of several experiments on the rate of 2,6-dichlorophenol indophenol reduction in the presence of various substrates are presented in table 1. In preliminary experiments with total lysate in the presence of either succinate or lactate, the addition of KCN resulted in an increase in the rate of reduction of 15 and 85 per cent, respectively. Therefore, KCN was routinely added in all subsequent dye reduction experiments. The order in which the fractions were tested was of importance when dealing with labile enzyme systems, like that responsible for

TABLE 1

Localization of enzymatic activities by dye reduction

Enzyme concentration in the total lysate (TL), ghost fraction (P_1), and supernatant (S_1) was determined by the rate of dye reduction in the presence of nonlimiting concentrations of the appropriate substrates. The fractions were analyzed in the following order: TL, S_1, P_1, and TL. The values for TL listed in the table are therefore an average of 2 determinations. The data represent a mean value of at least 2 experiments.

The cuvettes contained either 1.0 mg protein (TL) or 0.8 mg protein (S_1) or 0.15 mg protein (P_1), plus 150 μmoles potassium phosphate buffer pH 7.0, 30 μmoles KCN (freshly neutralized), 15 μmoles $MgSO_4$, and 0.17 μmoles 2,6-dichlorophenol indophenol. Substrates added: 50 μmoles of fumarate, pyruvate, succinate, *cis*-aconitate, or citrate; 100 μmoles of α-ketoglutarate, glucose, or L-glutamate; 300 μmoles L-malate; 600 μmoles DL-lactate. Final volume: 3 ml. All constituents incubated for 5 min at 25 C before adding cold enzyme fraction. Readings taken at 1 min intervals at 600 mμ against cuvette containing all constituents except substrate and dye. Correction made for endogenous incubation.

Substrate	Units Activity†			Specific Activity*			Per Cent of Total Lysate	
	TL	S_1	P_1	TL	S_1	P_1	S_1	P_1
Succinate	19.5	0.5	17.5	19.5	0.7	127	2.5	89.7
α-Ketoglutarate	11.0	1.5	11.5	11.0	2.0	81	13.7	105
DL-Lactate	159	2.0	54.0	163	3.0	348	1.3	34.0
Pyruvate	99.0	90.0	1.0	97.0	115	7.0	91.0	1.0
Glucose	174	169	15.0	171	218	110	97.2	8.6
Citrate	21.3	19.0	0.0	21.4	25.2	0.0	89.0	0.0
cis-Aconitate	32.5	27.0	0.0	32.0	34.5	0.0	83.0	0.0
Fumarate	89.5	14.0	20.5	91.0	19.0	143	15.6	22.9
L-Malate	190	115	95	186	146	700	60.5	50.0
L-Glutamate	8.9	5.3	1.6	9.1	7.0	11.0	59.5	17.4

* Units activity per mg protein.

† One unit of activity equals a change in optical density of 0.001 per min.

lactate oxidation. To account for this lability, the total lysate was assayed twice; once before and once after the supernatant and ghost fractions. All fractions were kept in an ice bath prior to analysis.

On the basis of specific activity and percentage recovery of activity (table 1), it is apparent that the enzymes responsible for succinate and α-ketoglutarate oxidation are localized in the ghost fraction. Likewise, the enzyme systems responsible for pyruvate, citrate, and *cis*-aconitate oxidation are localized in the supernatant.

Based on specific activity, the system for lactate oxidation appears to be associated with the ghost fraction. The recovery of only 34 per cent of the activity in this fraction may be explained by the instability of the system. Experiments with a reconstituted system have shown that the lactate oxidizing activity of the ghost fraction is more labile than that of the total lysate.

The results indicate that the supernatant is responsible for glucose oxidation. The rather high specific activity of the ghost fraction may indicate contamination by supernatant enzymes. The L-malate oxidizing system is associated with both fractions, although the specific activity of the ghost fraction is almost five times that of the supernatant.

For fumarate, the specific activity of the ghost fraction is higher than both the total lysate and supernatant, but, as in *Azotobacter vinelandii* (Alexander and Wilson, 1956), recovery of activity is very low. This recovery may not be explained on the basis of a labile enzyme system. With respect to L-glutamate oxidation, the concentration in the ghost fraction is high, but the majority of activity is recovered in the supernatant.

The enzyme activity of a reconstituted system (ghost fraction plus supernatant) was tested in each experiment. The values obtained were essentially the same as that for the average total lysate in the presence of either fumarate, α-ketoglutarate, pyruvate, L-malate, or glucose. In

experiments with the remaining substrates, the reconstituted system was consistently less active than the average total lysate by the following amounts: succinate (18 per cent), DL-lactate (64 per cent), *cis*-aconitate (18 per cent), citrate (26 per cent), and L-glutamate (29 per cent).

Purification of ghosts. The enzyme systems responsible for succinate, lactate, and α-ketoglutarate oxidation are localized in the ghost fraction (P_1). This fraction also accounts for approximately 50 per cent of the L-malate oxidation of the lysate. If these activities are indeed associated with ghosts, there should be no decrease in the respective specific activities after repeated washing and purification of the ghosts.

The results of such an experiment are shown in table 2. It was found that if P_1 is centrifuged over a layer of 2 per cent agar, the granules become firmly imbedded in agar, while the ghosts form a loosely packed upper pellet. By gentle agitation with a small quantity of suspending medium, a suspension of ghosts almost free of granules may be obtained.

TABLE 2

Effect of purification of ghosts on enzymatic activity

Total lysate (TL), ghost fraction (P_1), and supernatant (S_1) were prepared as indicated under Materials and Methods. P_1 was centrifuged over 2 per cent agar at approximately 3,000 G for 10 min, the supernatant removed, and the ghosts in P_2 resuspended by gentle agitation with 0.04 M potassium phosphate buffer pH 7.0 containing 2×10^{-3} M $MgSO_4$. The large granules penetrate and adhere to the agar, thus permitting a separation of large granules and ghosts. P_2 was centrifuged over 2 per cent agar at approximately 3,000 G for 10 min, the supernatant removed, and P_3 was obtained by gentle agitation with the suspending medium. P_3 was centrifuged at 5,000 G for 5 min, the supernatant removed, and P_4 was resuspended.

The procedure for enzyme assay was essentially the same as that described in table 1. The fractions were assayed in the following order: P_4, P_1, P_3, S_1, TL.

Substrate	Specific Activity*					Concentration†	
	TL	P_1	S_1	P_3	P_4	P_3	P_4
DL-Lactate	254	858	25	819	740	0.95	0.86
Succinate	16	79	0.5	91	92	1.14	1.16
L-Malate	146	551	98	522	560	0.95	1.02
α-Ketoglutarate	7	47	0.0	39	51	0.83	1.07

* Units activity per mg protein.

† Ratio of specific activity in the fraction to that of P_1.

With respect to succinate, α-ketoglutarate, and L-malate oxidation, the specific activity of the purified ghost fraction (P_4) is at least as great as that of P_1 (table 2). With respect to lactate oxidation, the specific activity of P_4 is 86 per cent of that of P_1. Since the over-all accuracy of the method is ± 15 per cent, one may conclude that there is no change in the capacity of washed and purified ghosts to oxidize DL-lactate.

Since the specific activity is based on protein, the absence of a significant increase in ghost specific activity as a result of purification, indicates that the majority of granules removed are nonprotein. This agrees with the low protein content reported for the granules of *B. megaterium* (Weibull, 1953*b*). The results suggest that the enzymatic activity of the ghost suspension is not due to a classical adsorption of either soluble enzymes or particles by the ghosts and that the ghosts are the sole site of succinate, lactate, and α-ketoglutarate oxidation.

Experiments on oxygen uptake. In preliminary experiments with the total lysate, the addition of either Mg^{++} or cofactors resulted in a 25 per cent stimulation in the rate of oxygen uptake in the presence of either succinate or citrate, while the addition of both constituents resulted in a 50 per cent stimulation. Therefore, Mg^{++} and cofactors were added in subsequent experiments on the distribution of activity between TL, S_1, and P_1.

The average results of several experiments on oxygen uptake are shown in table 3. It is apparent that all fractions are capable of consuming oxygen. The results in general agree with those obtained by dye reduction experiments, but the separation of activities between S_1 and P_1 is not as clearly defined.

With the exception of α-ketoglutarate, the specific activity of the ghost fraction is at least twice that of the total lysate for the activities localized in the ghost fraction by dye reduction experiments (succinate, DL-lactate). Based on percentage recovery of activity, and in agreement with the results of dye reduction experiments, the enzyme systems responsible for pyruvate, citrate, and glucose oxidation are localized in the supernatant. L-Malate oxidation is associated

TABLE 3

Localization of enzymatic activities by oxygen uptake

Each Warburg vessel contained approximately 14.6 mg protein (TL) or 11.9 mg protein (S_1) or 2.3 mg protein (P_1), plus 56 μmoles potassium phosphate buffer pH 7.0, 12 μmoles $MgSO_4$, 0.8 mg adenosine triphosphate, 0.8 mg diphosphopyridine nucleotide, 0.16 mg triphosphopyridine nucleotide, 0.04 mg cocarboxylase, 0.04 mg coenzyme A, and 0.02 mg cytochrome C. Substrates added: 50 μmoles of fumarate, pyruvate, citrate, L-glutamate, L-malate, or α-ketoglutarate; 200 μmoles of succinate, DL-lactate, or glucose. The center well contained 0.2 ml 5 N KOH. Final volume: 2 ml at 36 C. Correction made for endogenous incubation.

Substrate	Units Activity†				Specific Activity‡				Per Cent of TL	
	TL	S_1	P_1	R*	TL	S_1	P_1	R*	S_1	P_1
Succinate	40.5	21.5	14.5	79.0	2.8	1.9	6.5	5.7	53.7	35.8
α-Ketoglutarate	55	26	4	58	3.6	2.2	1.6	4.0	47.4	7.3
DL-Lactate	144	30	82	139	9.4	2.5	35.0	9.4	20.8	57.1
Pyruvate	123	63	5	94	8.1	5.2	2.1	6.4	51.2	4.1
Glucose	176	128	5.5	175	12.3	10.9	2.5	12.5	72.9	3.1
Citrate	182	118	4	149	12.8	10.2	2.0	11.0	64.8	2.2
Fumarate	189	155	92	194	13.3	13.6	41.5	14.2	82.0	48.6
L-Malate	179	110	158	186	11.8	9.2	61.6	12.8	61.5	88.4
L-Glutamate	32	7	0.0	32	2.1	0.6	0.0	2.2	21.9	0.0

* R = reconstituted system.

† One unit of activity equals uptake of 1 μL oxygen per 20 min.

‡ Units activity per mg protein.

with both fractions. In disagreement with the previous experiments, the system for α-ketoglutarate oxidation appears to be present in the supernatant.

The stoichiometry of L-malate and fumarate oxidation by the ghost fraction in the absence of cofactors was investigated. A value of 0.44 was obtained for the ratio of μmoles of oxygen taken up to μmoles of either L-malate or fumarate used. This value is close to a ratio of 0.5 and indicates the oxidation of both substrates to the oxalacetate or pyruvate stage. This is in agreement with the finding that pyruvate oxidation is a property of the supernatant.

The results of an experiment on the effect of high speed centrifugation of the supernatant on the oxygen consumption of this fraction are presented in table 4. Centrifugation at 105,000 G for 60 min resulted in the formation of a thin, white, gelatinous pellet (P_2), representing approximately 18 per cent of the protein of S_1. The L-malate and fumerate oxidizing activity of the supernatant (S_2) was found to be 20 per cent less than that of S_1, while the citrate and DL-lactate activities remained unchanged. No enzymatic activity was found associated with P_2. In a subsequent experiment with L-malate, the S_2

TABLE 4

Effect of high speed centrifugation on oxygen uptake of S_1

A supernatant (S_1) fraction, prepared as described in Materials and Methods, was centrifuged at 105,000 G for 60 min. The supernatant (S_2) and the pellet (P_2) were adjusted to the original volume of supernatant (S_1) centrifuged.

Each Warburg vessel contained approximately 8.3 mg protein (S_1) or 6.9 mg protein (S_2) or 1.5 mg protein (P_2), plus 40 μmoles potassium phosphate buffer pH 7.0, and 5 μmoles $MgSO_4$. Substrates added: 50 μmoles of L-malate, fumarate, or citrate; 200 μmoles DL-lactate. The center well contained 0.2 ml 5 N KOH. Final volume: 1.3 ml at 36 C. Correction made for endogenous incubation.

Substrate	Units Activity*			Percentage Recovery of Activity of S_1 in S_2
	S_1	S_2	P_2	
L-Malate	67	53	0	79
Fumarate	44	35	0	80
Citrate	22	24	0	109
DL-Lactate	10	10	0	100

* One unit of activity equals uptake of 1 μL oxygen per 20 min.

fraction obtained by a high speed centrifugation for 120 min contained 75 per cent of the activity of S_1. The results clearly indicate the presence of a nonsedimentable auto-oxidizable enzyme system.

DISCUSSION

For enzyme localization, we have employed the criteria of Alexander and Wilson (1955). Based on dye reduction and manometric experiments, the system responsible for citrate and *cis*-aconitate oxidation is localized in the supernatant. Since the oxidation of these two substrates would require the function of aconitase and isocitric dehydrogenase, both enzymes are localized in the supernatant.

In most cases, the total recovery of activity in manometric experiments is lower than in those involving dye reduction. This is to be expected because of the increase in complexity of the system, and is comparable to the synergistic effect on respiration, obtained by coupling particles and supernatant of *A. vinelandii* (Alexander, 1956). The recovery of 130–150 per cent of the activity for L-malate and fumarate in manometric experiments may be explained by a partial disintegration of a particulate system, as in *Hydrogenomonas facilis* (Atkinson and McFadden, 1954). The treatment of 15*p*20 particles of this organism in a sonic oscillator leads to the formation of 81*p*20 particles with increased hydrogenase activity.

α-Ketoglutarate dehydrogenase is localized in the ghost on the basis of dye reduction experiments. However, in manometric experiments, 47 per cent of the activity is recovered in the supernatant and 7 per cent in the ghost fraction. This difference may be explained by the presence in the supernatant of a factor or factors functioning between the dehydrogenase and oxygen. The activity of the supernatant is, therefore, a consequence of ghost contamination of this fraction. Since as much as 47 per cent of the activity of the total lysate is recovered, it is probable that the supernatant factor is rate limiting.

The results agree with the particulate succinic dehydrogenase described for several bacteria (Alexander, 1956), the particulate lactic and α-ketoglutaric dehydrogenases of *Serratia marcescens* (Linnane and Still, 1955), the nonsedimenting α-ketoglutaric oxidase of *M. tuberculosis* (Millman and Youmans, 1955), and the presence of both a particulate and nonsedimenting malic dehydrogenase in *S. marcescens* (Linnane and Still, 1955). Although several enzymatic activities have been found associated with particles of different size, to the authors' knowledge this work is the first association of enzymatic activity with a large bacterial structure like the ghost. The nonsedimenting nature of aconitase and isocitric dehydrogenase is in accord with results obtained with several bacteria (Alexander, 1956) and animal cells (Schneider and Hogeboom, 1956). The finding of a nonsedimenting system for glucose oxidation is in contrast to the particulate glucose oxidase of *A. suboxydans* (Widmer *et al.*, 1956) and *P. fluorescens* (Wood, 1955).

Both protoplasts and whole cells of *B. megaterium* have similar permeability properties with respect to small molecules like sucrose, urethane, and phosphate, indicating that the permeability barrier of the cell is retained in the protoplast (Weibull, 1956*a*). After osmotic lysis of protoplasts in the presence of Mg^{++}, very close to one ghost is obtained per protoplast (Wiebull, 1956*b*). These findings, plus the similarity in size distribution of ghosts and protoplasts, suggest that the ghost is the cytoplasmic membrane.

The experiments of Newton (1956) have shown that if *B. magaterium* is treated with a fluorescent derivative of polymyxin, and then with lysozyme, the fluorescent derivative is associated with the protoplast membrane. The rupture of protoplasts by sonic oscillation results in the formation of 100,000 G particles, which retain up to 90 per cent of the absorbed derivative. These findings demonstrate the fragility of the protoplast membrane and are in accord with the findings of Mitchell and Moyle (1956), that the membrane readily disintegrates into small particles; and the results of Weibull (1953*b*) on the fragmentation of ghosts after a brief treatment in the sonic oscillator. The fragility of the protoplast membrane could explain the localization of the same enzymatic activities both in the ghost of *B. megaterium* and the submicroscopic granules of other bacteria. It suggests that the active particles described by many authors may be a built in part of the protoplast membrane, instead of discrete structures existing in the cytoplasm.

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SUMMARY

Protoplasts of *Bacillus megaterium* have been subjected to osmotic lysis and separated into a ghost fraction and supernatant by differential centrifugation. Experiments based on dye reduction show that the enzyme systems responsible for all the succinate, DL-lactate, and α-ketoglutarate oxidation, and 50 per cent of the L-malate oxidation of the total lysate are localized in the ghost fraction. Experiments on repeated washing and purification of the ghosts indicate that these activities are indeed associated with this structure. The supernatant accounts for all the glucose, pyruvate, citrate, and *cis*-aconitate oxidation of the total lysate, and 50 per cent of the L-malate oxidation. Experiments based on a high speed centrifugation of the supernatant, indicate the presence in this fraction, of an autooxidizable enzyme system capable of coupling with L-malate, citrate, fumarate, and DL-lactate dehydrogenation.

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