

# INTRACELLULAR LOCATION OF CAROTENOID PIGMENTS AND SOME RESPIRATORY ENZYMES IN *SARCINA LUTEA*<sup>1</sup>

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The study of the analytical cytology of the bacterial cell was initiated with the work of Schachman *et al.* (1952), which revealed the presence of a variety of particles in extracts of many microorganisms. Since that time, numerous workers have isolated and characterized some of these particles. This work has been thoroughly reviewed by Alexander (1956). More recent studies (Cota-Robles *et al.*, 1958; Tissieres and Watson, 1958) suggest that these various particles may be divided into two broad categories. The first includes large particles with which are associated a variety of enzymatic activities, often respiratory in nature. The second includes small particles, rich in ribonucleoprotein. The chromatophores of photosynthetic bacteria represent perhaps a third and distinct type.

Until recently, little could be said about the origin or intracellular location of any of these kinds of particles; in particular it was unclear whether particles of the first kind, the "respiratory" particles, existed in the particulate state within the cell, or whether they were artifacts of cell breakage. Weibull (1953), on the basis of experiments with protoplasts of *Bacillus megaterium*, suggested that the entire cytochrome content of the cell is associated with the cell membrane. Stanier (1954) made a more precise and general statement of this hypothesis, and suggested that the particles found in extracts of mechanically disrupted cells, with which are associated respiratory enzymes, are derived from the membrane by comminution. More recently, Marr and Cota-Robles (1957), Storck and Wachsman (1957), and Mitchell and Moyle (1956) have presented evidence supporting the

hypothesis of Weibull and Stanier. The present paper presents results of experiments on *Sarcina lutea* designed to determine the intracellular location of certain cell components, including the characteristic carotenoid pigments. The results show that the "respiratory particles" are derived from the plasma membrane, and moreover indicate that the carotenoid pigments of this chemotrophic bacterium are associated with the plasma membrane.

## MATERIAL AND METHODS

*Biological material.* A strain of *S. lutea* obtained through the courtesy of Dr. G. Stanier was used. A 10- to 12-hr stock culture grown in double-strength nutrient broth (16 g nutrient broth per L) was used to inoculate 250 to 350 ml of the same medium in 1-L Erlenmeyer flasks to give an initial cell density of approximately  $10^5$  cells per ml. After incubation on a rotary shaker (300 rpm) at 34 C for 10 to 12 hr, the cells were harvested by centrifugation, washed once with 0.05 M phosphate buffer (pH 7.4), and resuspended in the same buffer. Preliminary experiments showed that cells grown under these conditions were in the exponential phase of growth at the time of harvesting.

Viable cell counts were performed by spreading 0.1 ml of cell suspensions, appropriately diluted, on nutrient agar plates. The colonies were counted after 48 hr incubation at 34 C.

Bacterial mass was estimated from the optical density at 650 m $\mu$ , using a standard curve relating bacterial dry weight to optical density at this wave length. The carotenoid pigments do not absorb light appreciably at this wave length.

*Preparation and fractionation of cell-free extracts.* Cells were ruptured by grinding a wet cell paste with levigated alumina (2.5 g alumina per g of wet cells). The ground paste was extracted with 0.05 M phosphate buffer (pH 7.4), and centrifuged for 10 min at 5000  $\times$  G to remove the

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bulk of the alumina and whole cells; the supernatant was centrifuged for 5 min at  $23,000 \times G$  to remove traces of alumina and unbroken cells. This supernatant (TE) was centrifuged at  $23,000 \times G$  for 90 min, and the pellet ( $P_1$ ) was resuspended in phosphate buffer. The supernatant ( $S_1$ ) was centrifuged at  $104,000 \times G$  for 120 min in a Spinco preparative ultracentrifuge, yielding a supernatant fluid ( $S_2$ ) and a pellet ( $P_2$ ), which was resuspended in phosphate buffer.

We have used 0.05 M phosphate buffer (pH 7.4) throughout, since preliminary experiments showed that this buffer caused a minimum "leakage" of carotene from the various particle fractions.

*Sonic disruption.* Forty ml of a suspension of cells (40 mg dry weight per ml) in phosphate buffer were treated in a Raytheon 10 kc sonic oscillator operating at full electrical power. The temperature was maintained at 5 C during the treatment. For kinetic studies, samples were removed at various times and the volume in the oscillator was kept at 40 ml by the addition of buffer; the further treatment is described under results. For the purpose of fractionation, extracts were prepared by a 25-min treatment in the sonic oscillator, centrifuged at  $5000 \times G$  for 15 min, and the resulting supernatant (TE) was treated as described above.

*Enzyme assays.* All assays were conducted at 34 C. In each case, the results are expressed as micromoles of substrate reacting per hour. Specific activity is given as either micromoles per hour per mg protein or  $\mu$ moles per hr per  $\mu$ g carotenoid pigment ( $Q_{O_2}$  (carotene)). All spectrophotometric assays were conducted in a Beckman model DU spectrophotometer using 1 cm cells.

1. Adenosine deaminase was assayed by measuring the decrease in optical density at 265  $m\mu$  with time. The cell contained in a final volume of 3.0 ml, 24  $\mu$ moles adenosine, 0.1 to 0.2 ml extract, and 270  $\mu$ moles tris(hydroxymethyl)aminomethane(Tris)-HCl buffer (pH 8.3) (Cota-Robles *et al.*, 1958). The reaction was started by the addition of extract.

2. Cytochrome *c* oxidase activity was determined by following the decrease in optical density at 550  $m\mu$  with reduced mammalian cytochrome *c* as substrate. The cell contained 30  $\mu$ moles potassium phosphate (pH 7.0), 0.10  $\mu$ moles of reduced mammalian cytochrome *c*,

and 0.05 to 0.2 ml cell extract in a volume of 3.0 ml. The reaction was started by the addition of the cytochrome. The cytochrome was reduced according to the method of Smith (1954a) who showed that cytochrome *c* peroxidase does not interfere with the assay when the cytochrome is reduced in this way. Potassium cyanide ( $10^{-6}$  M) inhibited the cytochrome oxidase activity 50 per cent.

3. The rate of reduction of potassium ferricyanide with succinate as substrate was taken as a measure of succinic dehydrogenase activity. The reduction was determined by following the decrease in optical density at 400  $m\mu$  against time. The cell contained potassium phosphate (pH 7.0), 300  $\mu$ moles;  $MgSO_4$ , 50  $\mu$ moles; KCN, 1  $\mu$ mole; sodium succinate, 250  $\mu$ moles; potassium ferricyanide, 50  $\mu$ moles; and 0.1 to 0.2 ml of extract in a final volume of 3.0 ml. A similar system without succinate was used to determine endogenous dye reduction, and this value was subtracted from that obtained with the complete system. The reaction was started by the addition of the potassium ferricyanide.

4. Reduced diphosphopyridine nucleotide (DPNH) oxidase activity was estimated by following the decrease in optical density at 340  $m\mu$  with DPNH as the substrate. The cell contained, in a final volume of 3.0 ml, DPNH, 0.15  $\mu$ moles;  $MgSO_4$ , 50  $\mu$ moles; potassium phosphate (pH 7.0), 300  $\mu$ moles; and 0.1 to 0.2 ml cell extract. The reaction was begun with the addition of the DPNH.

5. Succinoxidase was determined manometrically. The flasks contained, in a final volume of 2.2 ml, potassium phosphate (pH 7.0), 300  $\mu$ moles;  $MgSO_4$ , 50  $\mu$ moles; and 0.5 to 1.5 ml of extract or whole cells in the main compartment, 250  $\mu$ moles of sodium succinate in the side arm, and 0.2 ml of 20 per cent KOH in the center well. The assay was carried out at 34 C in an atmosphere of air. In this case the activity is expressed as  $\mu$ L  $O_2$  per hr.

Preliminary manometric experiments showed that this strain of *S. lutea* had a high rate of endogenous respiration; this is similar to the findings of Dawes and Holms (1958) with this organism. To reduce the endogenous respiration the cells were suspended in a volume of 0.05 M phosphate buffer equal to the volume of nutrient broth in which they had been grown, and shaken

TABLE 1  
Absorption maxima of total pigment extract and individual pigments

Absorption Maxima (m $\mu$ )		
Pigment A <sup>a</sup>	Pigment B <sup>a</sup>	Total pigment extract <sup>b</sup>
413, 436, 468	413, 436, 468	413, 436, 468

<sup>a</sup> Pigments A and B were obtained by the chromatographic separation of the total pigment extract on a Ca(OH)<sub>2</sub> column developed with a mixture of 1 volume of absolute methanol to 1 volume of petroleum ether.

<sup>b</sup> Total extract obtained as described under Materials and Methods.

at 34 C for 3 hr. After this time, they were harvested and resuspended in 0.05 M phosphate buffer. This treatment reduced the endogenous respiration 75 per cent. "Endogenous reduced" cells were used for all manometric experiments.

For experiments on the inhibition of whole cells with cyanide, the inhibitor was added from the side arm at the same time as the substrate. It has been found that this procedure is necessary to demonstrate the cyanide sensitivity of the respiration of *Sarcina*. If the cyanide is added to the cell suspension before the addition of the substrate there is no inhibition, presumably because the cyanide is bound by compounds such as oxaloacetate and pyruvate (Warburg, 1949).

**Chemical determinations.** 1. The following method was used for the extraction and estimation of the carotenoid pigments. To a 1-ml sample of a cell suspension was added 2.5 ml of 10 per cent trichloroacetic acid. After centrifugation, the residue was extracted twice with a total volume of 5 to 10 ml of hot absolute methanol, and made up to either 5 or 10 ml.

Preliminary experiments showed that methanol extracts of our strain of *S. lutea* contain two carotenoid pigments; a xanthophyll and a carotene. These pigments are similar to those described by Chargaff and Dierych (1932) in another strain of *S. lutea*. The spectra of both of the isolated pigments in methanol and of the total methanol extract prepared as described above are identical (table 1). Thus the optical density at the major maximum (436 m $\mu$ ) may be taken as a measure of the total pigment content. This is expressed in milligrams assuming that the specific extinction coefficients of the

*Sarcina* pigments are the same as that of  $\beta$ -carotene ( $E_1^{1\% \text{ cm}} = 2500$ ; Miller, 1937).

2. Phospholipid was estimated by determining the phosphate in the hot methanol extract. No additional phosphate was extracted by further extraction with methanol or with methanol-ether. The phosphate content was determined by the method of Dryer *et al.* (1957).

3. Protein was determined by the biuret method (Weichselbaum, 1946), and by the method of Lowry *et al.* (1951).

**Sources of chemicals.** Crystalline lysozyme was obtained from Nutritional Biochemicals Corporation. DPNH (92 per cent pure) and mammalian cytochrome *c* (horse heart, 90 per cent pure) were purchased from the Sigma Chemical Company. All other reagents were reagent grade.

## RESULTS

**Recovery in extracts of the enzymatic activities of whole cells.** Before beginning the study of the cellular location of enzymes found in extracts, we thought it necessary to determine to what extent these enzymes reflected the metabolic activity of the whole cell. We shall consider the importance of this point in more detail in the discussion.

The enzymes which we are concerned with are succinoxidase, succinic dehydrogenase, cytochrome *c* oxidase, and DPNH oxidase; tables 2 and 3 show the results of a series of experiments to determine the recovery of each of these enzymes in sonic extracts. Table 2 shows that whole cells and extracts take up equal amounts of oxygen per mole of succinate; therefore, the

TABLE 2  
Recovery of succinoxidase activity

	Whole Cells	Sonic Extract
$\mu\text{L O}_2$ per mole succinate.....	33.6	33.6
$\mu\text{L O}_2$ per hr per $\mu\text{g}$ pigment (Q <sub>O<sub>2</sub></sub> (carotene)).....	77.5	36.8
Recovery (per cent).....	(100)	45.9

Oxygen uptake was measured as described in Materials and Methods. "Endogenous-diminished" cells were used. Values reported here are corrected for the remaining endogenous respiration. Each vessel contained an amount of whole cells (1 mg dry weight) equivalent to 0.28  $\mu\text{g}$  carotene, or of extract (5.5 mg protein) equivalent to 4  $\mu\text{g}$  carotene.

rate of oxygen consumption with excess succinate ( $Q_{O_2}$  (carotene)) can be used directly to compare the rates of succinic acid utilization by whole cells and by extracts. Table 2 also shows that the  $Q_{O_2}$  (carotene) of sonic extracts is about one half that of whole cells.

In table 3, the activities of succinic dehydrogenase, cytochrome *c* oxidase, and DPNH oxidase are compared with the rate of utilization of succinic acid, calculated from the data of table 2 ( $\mu\text{L O}_2$  per hr divided by  $\mu\text{L O}_2$  per  $\mu\text{mole}$  succinate). Succinic dehydrogenase is quantitatively recovered. The rate of DPNH oxidation is about five times greater than the rate of succinic acid utilization. This is not unexpected since each of the two or three DPN-linked oxidations involved in the complete oxidation of succinate will have a rate at least equal to the rate of succinate utilization. It seems probable, then, that a substantial fraction of the DPNH oxidase of the whole cell is recovered in the extracts.

The rate of cytochrome *c* oxidation in extracts is only 10 per cent of that of succinate oxidation in whole cells. This low recovery, which probably results from the use of mammalian cytochrome *c* as substrate in the assay, makes it impossible to say if the measured cytochrome oxidase in extracts is sufficient to account for the whole cell oxidation of succinate. Further evidence to show that the activity measured in the extract is significant in terms of whole cell respiration was obtained by the use of known cytochrome

TABLE 3

*Relationship of respiratory enzyme activity in sonic extracts to the succinoxidase activity of whole cells*

Enzyme Activity	(1) Substrate /hr/ $\mu\text{g}$ Pigment	(2) Equivalent $Q_{O_2}$ (Carotene)	(3) Fraction of Whole Cell $Q_{O_2}$ (Carotene)
	$\mu\text{mole}$		%
Succinic dehydrogenase.....	2.6	87.5	113
Cytochrome oxidase.....	0.45	7.9	10.2
DPNH oxidase.....	12.3	415	535

Enzymes were assayed as described in Materials and Methods. Extract was made from "endogenous-diminished" cells. Value of column (3) was calculated using the data of table 2.

TABLE 4  
*Inhibition of succinoxidase activity*

Inhibitor	Whole Cells	Sonic Extract
	%	%
Potassium cyanide, $10^{-3}$ M.....	41	34
Sodium azide, $10^{-4}$ M.....	95	96

Succinoxidase activity was measured manometrically as described in Materials and Methods, except that in the experiment with azide, both the inhibited suspension and the control were at pH 6.0. Each vessel contained an amount of whole cells (1.58 mg dry weight) equal to  $0.33 \mu\text{g}$  of carotene, or of extract (5.8 mg protein) equivalent to  $4.6 \mu\text{g}$  carotene. The whole cells were "endogenous-diminished" and the extract was made from similar cells.

oxidase inhibitors. Table 4 presents the results of an experiment in which the inhibition by azide and cyanide of oxidation of succinate by whole cells and sonic extracts are compared. It is seen that the degree of inhibition is similar in each case.

It can be concluded that the oxidation of succinate is proceeding via a cytochrome system in both whole cells and extracts, corroborating the conclusion of Smith (1954b).

*Particulate nature of the carotenoid pigments.* The carotenoid pigments of some nonphotosynthetic bacteria occur as particulate protein complexes (Saperstein and Starr, 1955). The first step in our attempt to localize the pigments in the cells of *S. lutea* was to determine if this were true for this organism. Fractionation of the extract obtained by alumina grinding was carried out as described in Materials and Methods. Each of the various fractions was assayed for carotene, protein, adenosine deaminase, succinic dehydrogenase, and cytochrome oxidase. A parallel fractionation of an extract prepared by sonic oscillation of the cell suspension for 25 min was carried out. The results of the two experiments are shown in table 5. These experiments show clearly that the carotenoid pigments of *S. lutea* are associated with particles in cell-free extracts.

*Nonassociation of the carotenoid pigments with the cell wall.* It was possible that the pigment-bearing particles obtained in cell-free extracts are derived from the cell wall. Two kinds of experiments were used to examine this possibility.

In the first, purified cell wall material was

TABLE 5

*Distribution of cytochrome oxidase, succinic dehydrogenase, adenosine deaminase activities, and carotene and protein in fractions of extracts of alumina ground and sonically disrupted cells*

Component	Sonic Extract									Alumina Extract										
	Amt of component				Amt Related to TE (%)			Specific activity <sup>a</sup> (% of TE)			Amt of component				Amt related to TE			Specific activity <sup>a</sup> (% of TE)		
	TE	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>	TE	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>
Adenosine deaminase <sup>b</sup>	7.8	0.05	0.02	7.8	0.5	0.2	100	0.04	0.02	1.34	1.7	0	0	1.7	0	0	100	0	0	1.34
Cytochrome oxidase <sup>b</sup>	2.61	1.07	0.53	1.03	41	20.5	39.5	3.42	1.64	0.53	1.32	0.85	0.36	0.09	64.5	27.5	6.5	6.8	1.77	0.09
Succinic dehydrogenase <sup>b</sup>	4.9	2.1	0.9	1.9	43	18	39	3.58	1.44	0.53	2.1	1.4	0.6	0.14	67	27.5	6.5	7.05	1.77	0.09
Carotene <sup>c</sup>	5.9	2.4	1.2	2.3	41	20	39	3.42	1.60	0.53	1.83	1.22	0.49	0.13	67	27	7	7.05	1.74	0.09
Protein <sup>d</sup>	7.4	0.89	0.95	5.5	12	12.5	74.5	—	—	—	2.29	0.22	0.34	1.73	9.5	15.5	75	—	—	—

See Materials and Methods for definition of fractions.

<sup>a</sup> Amount per mg protein.

<sup>b</sup> Expressed as  $\mu$ moles substrate reaching per hr per ml of fraction.

<sup>c</sup> Expressed as  $\mu$ g carotene per ml of fraction.

<sup>d</sup> Expressed as mg protein per ml of fraction.

prepared by the method of Salton (1953). It was examined visually and spectrophotometrically and was found to be devoid of pigment.

The second approach was to study the distribution of the pigment in preparations obtained by the action of lysozyme on whole cells. Two identical cell suspensions in Tris buffer (pH 7.4) were treated as follows: to suspension A, sucrose was added to a final concentration of 1 M and lysozyme to a final concentration of 50  $\mu$ g per ml; to suspension B, only lysozyme was added to the same final concentration; a third suspension (C) served as an untreated control. The suspensions were incubated for 3 hr at 34 C, then centrifuged at 5000  $\times$  G for 15 min and the pigment content of each of the pellets was determined. Parallel experiments with cell suspensions treated with lysozyme showed that in the absence of sucrose the optical density fell to 10 per cent of the initial value in this time: the optical density of a suspension protected by 1 M sucrose fell to about 40 per cent of its initial value, and when this suspension was diluted with water the turbidity decreased very much more than when it was diluted with 1 M sucrose. Analysis of the pellets from the three suspensions showed that the pigment content of protoplasts (tube A) was 97 per cent of that of the untreated cells (tube C), and that the pigment content of lysed protoplasts (ghosts) from tube B was 92 per cent of the untreated cells.

*Cellular localization of the carotenoid pigments and enzymes.* The experiments so far reported

demonstrate that in extracts of mechanically disrupted cells, the carotenoid pigments of *S. lutea* are associated with particles which are nearly completely sedimented by centrifugation at 23,000  $\times$  G for 90 min, and which are not derived from the cell wall. We cannot decide, on the basis of these experiments, between two possible cellular sources of the particles found in extracts: (a) the particles appear in the extract as a result of release from the cells of pre-existing particles, or (b) the particles arise by comminution of a larger structure during the disruption. Until recently a similar ambiguity was present in all the work on the analytical cytology of the bacterial cell.

Marr and Cota-Robles (1957) have developed a method which gives promise of being able to eliminate this ambiguity. These workers studied the kinetics of the decrease of the viable count and turbidity of suspensions of *Azotobacter agilis* (*A. vinelandii*) exposed to sonic waves. They showed that the decrease of viable count and turbidity are both results of a single physical process which leads to rupture of the cell envelope (with death of the cell) and the further disintegration of the envelope to particles too small to scatter visible light. They also showed that both turbidity and viable count decrease exponentially and that the rate constant for the decrease of turbidity is only 40 per cent of that for the decrease of viable count. They concluded that the lethal rupture of a cell involves the disintegration of enough of the cell envelope to

reduce its light scattering power 60 per cent. The loss of such a large portion of the envelope will certainly result in the release of the entire content of the cell into the medium. The remaining empty cell envelope (or hull) continues to be comminuted to very small particles.

The extract from sonic treatment may be divided into two fractions by centrifugation at a force just sufficient to sediment intact cells and large fragments of the cell envelope. The pellet will be composed of these two structures, and the supernatant will contain the material released from the ruptured cells, and also the small particles derived from the envelope by comminution. If samples of the suspension are removed at various times during the sonic treatment and centrifuged, the kinetics of the disappearance of a component from the pellet can be determined. If during the course of sonic treatment a component disappears from this pellet at a rate equal to the rate of decrease of the viable count, we may conclude that all of this component in each cell is released when the cell is ruptured, and may infer that this component is contained in the cytoplasm. On the other hand, if a component disappears from this pellet at a rate equal to the rate of decrease of turbidity, we may conclude that the component is released into the medium only after the comminution of the cell envelope. These conclusions are valid whether the component being studied is soluble or particulate in nature.

With these considerations in mind, the following experiments were undertaken with *S. lutea*. Cell suspensions of this organism were treated in the sonic oscillator as described under Materials and Methods. Samples were removed at various times. The optical density at 650  $m\mu$  was determined and a portion was diluted for viable cell count. The remainder was centrifuged at 5000  $\times$  G for 10 min in the cold to remove whole cells and large fragments of the hull. The supernate was retained, and the pellet was suspended in water. The pellet was assayed for carotenoid pigments and phospholipid. The supernate was assayed for cytochrome *c* oxidase, succinic dehydrogenase, DPNH oxidase, and adenosine deaminase.

The results of these experiments are shown in figures 1 and 2. Following Marr and Cota-Robles (1957), we have expressed the results as the log per cent of each component still sedimentable at 5000  $\times$  G at each time, taking the amount

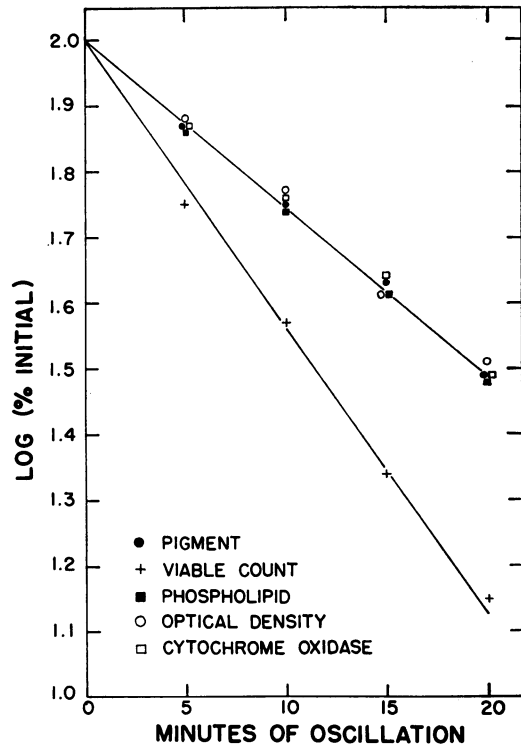


Figure 1. Rate of release of pigment, phospholipid, and cytochrome oxidase. Pigment and phospholipid were estimated in the residue after centrifuging at 5000  $\times$  G for 10 min. The logarithm of the per cent of the initial substance still sedimentable is plotted.

Cytochrome oxidase was assayed in the supernatant. The enzyme still sedimentable was estimated by subtracting the amount released from a constant such that the value at 20 min coincides with the value for the optical density. The logarithm of the per cent of the initial substance still sedimentable is plotted.

sedimentable at zero time as 100 per cent. The sample removed 5 min after the start of the sonic treatment has been used as the zero time sample, since preliminary experiments had shown that during the first 5 min of treatment, the optical density and viable count both increased, because the cuboidal packets characteristic of *Sarcina* were disaggregating. This is in agreement with the findings of Kinsloe *et al.* (1954) with *Micrococcus varians*. After 5 min, optical density and viable count both decreased exponentially.

It can clearly be seen from the figures that the rate of release of pigment, phospholipid, cytochrome oxidase, succinic dehydrogenase and

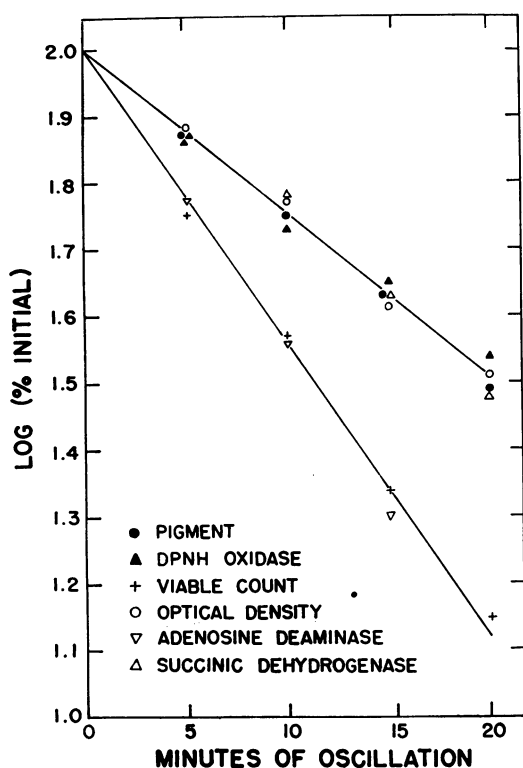


Figure 2. Rate of release of succinic dehydrogenase, DPNH oxidase, and adenosine deaminase by sonic treatment. Succinic dehydrogenase and DPNH oxidase were assayed in the supernatant after centrifugation of the sample for 10 min at  $5000 \times G$ . The enzyme still sedimentable was estimated by subtracting the amount released from a constant such that the value at 20 min coincides for the value of the optical density. The logarithm of the per cent of the initial substance still sedimentable is plotted.

Adenosine deaminase was assayed in the supernatant and the amount sedimented was calculated by subtracting from the total enzyme released, the amount released after 20 min of treatment being taken as total.

DPNH oxidase are all equal to the rate of decrease in optical density; hence these enzymes are all released into the supernatant only by comminution of the cell envelope. On the other hand the rate of release of adenosine deaminase and the rate of decrease of the viable count are equal, indicating that this enzyme is released from the cell by the rupture of the envelope.

#### DISCUSSION

*Particulate nature of the carotenoid pigments.* Saperstein and Starr (1955) showed that the

pigments of corynebacteria, mycobacteria, and micrococci occur as easily sedimentable protein complexes. The carotenoid pigments of *Micrococcus lysodeikticus* have been found to be associated with particulate matter prepared by ammonium sulfate precipitation of lysed cells (Jackson and Lawton, 1958). The results shown in table 5 demonstrate that this is also true of the carotenes of *S. lutea* and further support the suggestion of Saperstein and Starr that the association of carotenoids with proteins may be widespread in bacteria, and not limited to the photosynthetic bacteria.

*Significance of enzymes studied to the metabolism of the intact cell.* Alexander and Wilson (1955) have proposed the use of three criteria in the localization of enzymes in soluble or particulate fractions of extracts of bacteria: (a) recovery of both enzyme and cell mass in the isolated fractions must approach 100 per cent of that found in the original extract, (b) a high percentage of the total activity must reside in the fraction to which the function is being attributed, and (c) the concentration of the enzyme in the isolated fraction must be equal to or greater than in the initial cell-free preparation. These criteria suffice to assess the validity of a proposed attribution of an enzyme to a particular fraction of a cell-free extract, but are not in themselves sufficient to eliminate the possibility that the enzyme in question represents only a portion of the corresponding metabolic activity of the whole cell. For this purpose, we propose two additional criteria: (a) that the activity of a given enzyme in the initial cell-free extract represents a substantial fraction of the corresponding activity in the intact cell, and (b) that if there is more than one enzyme associated with the same activity, that the ratio of the activities of the enzymes in the extract be the same as the ratio of the corresponding activities in the intact cell.

Two examples may make our point more clear. Alexander (1956) reported that the succinic dehydrogenase activity of extracts of *A. agilis* (*A. vinelandii*) is only 5 per cent of that of the intact cells. It is not clear how important the recovered enzyme is in the oxidation of succinate by the intact cells; in the extreme case, it may represent only 5 per cent of the total cellular succinic dehydrogenase activity, the remainder of the activity being associated with an enzyme that was not recovered. Ernster and Navazio (1956)

have studied the distribution of the two isocitric dehydrogenases in the soluble and mitochondrial fractions of rat liver homogenates. The triphosphopyridine nucleotide (TPN)-linked dehydrogenase is soluble; the DPN linked enzyme is found in the mitochondria. In homogenates, the activity of the TPN-linked dehydrogenase is much greater than that of the DPN-linked enzyme. A less careful study would have shown only that a large portion of the isocitric dehydrogenase of liver is soluble, and the remainder mitochondrial, without revealing that this distribution is a reflection of the individual distributions of two different enzymes.

It has already been pointed out that in the experiments reported here the amounts of succinic dehydrogenase, succinoxidase and DPNH oxidase in extracts are in good agreement with the amounts to be expected from the metabolic activities of the whole cells. In the case of cytochrome *c* oxidase the recovery is low but the data of table 4 show that this activity is probably a valid measure of the cytochrome oxidase of intact cells.

*Particulate nature of components of succinoxidase system.* We chose to determine the intracellular location of the succinoxidase system since it has been extensively studied in both bacterial and mammalian cells, and has been shown to reside on, or to be part of the mitochondrial membrane of animal cells (Siekvitz and Watson, 1956; Ball and Barnett, 1957). There are many examples in the literature of the particulate nature of bacterial

TABLE 6

*Relative distribution of cytochrome oxidase, succinic dehydrogenase, and carotenoids in parallel fractions of alumina ground and sonically disrupted cells*

Component	Fraction			
	TE	P <sub>1</sub>	P <sub>2</sub>	S <sub>2</sub>
Cytochrome oxidase . . . . .	1.00	1.99	1.08	0.17
Succinic dehydrogenase . . . . .	1.00	1.98	1.22	0.17
Carotenoids . . . . .	1.00	2.06	1.09	0.17

The data of table 4 have been recalculated to show for each component the ratios of the per cent specific activity in the extract prepared by alumina grinding to the per cent specific activity in the extract prepared by sonic disruption. The ratio in the TE was taken as unity.

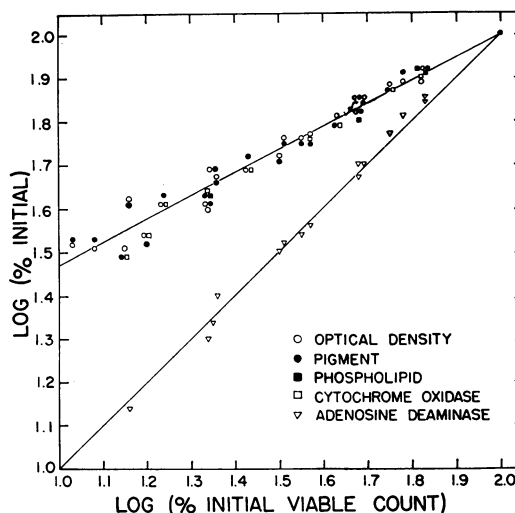


Figure 3. Rate of release of pigment, phospholipid, cytochrome oxidase, and adenosine deaminase as functions of the decrease of the viable count. The logarithm of the per cent still sedimentable at each time is plotted against the logarithm of the initial viable count at that time.

respiratory enzymes, including succinic dehydrogenase, DPNH oxidase, and cytochrome oxidase; this has been adequately reviewed by Alexander (1956). Our results, presented in table 5, are another example of the particulate nature of the succinoxidase system.

A comparison of the fractionation of extracts of alumina ground and sonically disrupted cells suggests that the particles bearing carotene, succinic dehydrogenase, and cytochrome oxidase are the same. This is seen from an examination of table 6, in which we have recalculated the data of table 5 to show the relative distribution of these three constituents in parallel fractions of the two kinds of extracts. It is seen that although the amounts of each component are markedly different in parallel fractions of the two extracts, the relative distributions of each component in different fractions is the same. The relative distributions in different extracts will, of course, depend on the particular experimental conditions, especially on the extent of comminution of the particles in the two extracts.

*Cellular location of the pigments and enzymes.* Several scattered observations suggest that the carotenoid pigments of chemotrophic bacteria are associated with the cell membrane. Salton (1956) found that the cell walls of the pigmented



organisms *S. lutea*, *M. lysodeikticus*, and *Staphylococcus aureus* are completely devoid of pigment. We have confirmed this observation. Recently, it has been noted (Gilbey *et al.*, 1958) that "ghosts" of *M. lysodeikticus* prepared by lysis of protoplasts contained a carotenoid pigment.

An association of respiratory enzymes with the plasma membrane is suggested by the work of Weibull (1953) on protoplasts of *B. megaterium*, and by the more detailed work of Storck and Wachsmann (1957) on the same organism. Mitchell and Moyle (1956), have suggested that a large fraction of the cytochromes and of the succinic dehydrogenase are associated with the plasma membrane of *S. aureus*.

This association is more definitely shown by the elegant work of Marr and Cota-Robles (1957). Using their methods and criteria, we have shown that the pigment, phospholipid, cytochrome oxidase, DPNH oxidase, and succinic dehydrogenase are associated with the cell envelope (figures 1 and 2). The fact that with two very different organisms, release of phospholipid parallels the decrease in turbidity, whereas the release of a soluble enzyme parallels the decrease of viable count, would indicate that this method may be applied generally.

In figure 3, we have plotted the decrease (as log per cent initial) of several of the variables studied versus the decrease of viable count (as log per cent initial). A log-log plot compensates for any day-to-day variations in time rates of decrease, and allows the direct comparison of numerous experiments. In this plot, any variable which has the same rate of decrease as the viable count will have a slope equal to one. A component with a rate of decrease less than the rate of viable count decrease will have a slope of less than one.

Figure 3 contains the results of five independent experiments in which cytochrome oxidase, carotenoids, adenosine deaminase, and turbidity were measured. This figure includes experiments run with cells in the stationary phase of growth (incubated 24 hr) as well as those in the logarithmic phase. It is seen that only the curve for adenosine deaminase, the soluble enzyme, has a slope of 1. The slope of the line through all the other points is 0.53, and the maximal deviation is 0.16. A replotting in the same way of the data of Marr and Cota-Robles (1957) shows that the mean slope of hydrogenase, cytochromes and

turbidity is 0.48, with a maximal deviation of 0.24.

In conclusion, our experiments show that the carotenoid pigments, cytochrome oxidase, phospholipid, DPNH oxidase, and succinic dehydrogenase are associated with the cell envelope. Since we have definitely shown that the carotenoids are not in the cell wall, we conclude that the pigment is associated with the cell membrane. Our results also support the hypothesis of Stanier (1954), Weibull (1953) and Mitchell and Moyle (1956) that the respiratory enzymes are intimately associated with the cell membrane, and do not exist as intracellular mitochondriallike particles.

#### SUMMARY

The carotenoid pigments of *Sarcina lutea* have been found to occur in cell-free extracts as easily sedimentable particulate protein complexes.

Experiments using lysozyme to remove the cell wall show that the pigment is not associated with this structure. By studying the rates of release of cell components in the sonic oscillator, it has been possible to show that the pigment and the enzymes of the succinic oxidase system are associated with the cell membrane, and do not occur as particles within the cytoplasm. Findings that indicate that these components may be present on the same particle were also presented. It was shown that the activities of these enzymes as studied in cell-free extracts were a substantial percentage of the activity of these enzymes in whole cells.

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