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RESPIRATION AND PROTEIN SYNTHESIS IN *ESCHERICHIA COLI* MEMBRANE-ENVELOPE FRAGMENTS

III. Electron Microscopy and Analysis of the Cytochromes

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ABSTRACT

The membranous nature of pellets obtained from broken *Escherichia coli* spheroplasts by successive centrifugation at 3500 *g* (P_1), 20,000 *g* (P_2), and 105,000 *g* (P_3), has been established by electron microscopy. Spectrophotometric analysis has shown that about 90% of the cytochromes are concentrated in the particulate fractions. The crude ribosomal pellet (P_3) contained as much of the total cytochromes as did the pellet obtained at 20,000 *g* (P_2). The high cytochrome content of P_3 is consistent with its high oxidative activity (1) and the presence of membrane vesicles in this fraction. Analysis at 77°K intensified the optical extinction of all the cytochrome absorption bands, but the degree of intensification was not uniform for each fraction nor for each band within a given fraction. Carbon monoxide had little or no inhibiting effect on NADH oxidation. Reduced plus carbon monoxide difference spectra yielded artifactual absorption bands in the wave length regions where reduced vs. oxidized absorption bands normally occur. Succinate and NADH, either together or separately, reduced nearly all of the cytochromes, indicating that the cytochrome portion of the electron-transport chain is shared by both substrates. A tentative formulation of the electron-transport chain is presented.

INTRODUCTION

The importance of membranes in complex biological processes is becoming increasingly more evident (2). In many microorganisms such as *Escherichia coli*, there appears to be essentially only one intracytoplasmic membrane system.[1] This membrane system is involved in at least two major interdependent processes vital to cell growth, namely protein synthesis and respiration. In this series of investigations we are trying to elucidate the biochemical, structural, and functional nature of *E. coli* membranes and the processes of respiration and protein synthesis in membrane fragments. Ultimately we would like to know if energy of the respiratory chain can be used directly for protein synthesis in the membrane without the required participation of ATP.

In this paper we present electron micrographs

[1] Although Pontefract et al. have recently described mesosomes in *E. coli* 1 γ (3), the general presence of membrane-attached mesomes in *E. coli* has not been established.

of the particulate fractions derived from *E. coli* and an analysis of the cytochromes and their distribution among the fractions. A tentative formulation of the electron-transport chain is presented.

MATERIALS AND METHODS

Preparation of E. Coli Fractions (1)

E. coli W-6, a proline auxotroph, was grown on proline-supplemented Difco Antibiotic Medium #3 (Difco Labs, Detroit, Mich.), and spheroplasts were produced from an early log phase culture by growth in the presence of penicillin. The spheroplasts were recovered by centrifugation, washed, stored in liquid nitrogen, and thawed as previously described (1). The suspension was then subjected to two 20-sec bursts of ultrasonic irradiation with a Branson sonifier (Branson Instruments Co., Stamford, Conn.) at 0°C, and fractions P_1, S_1, P_1', P_2, P_3, and S_3 were prepared (1). For cytochrome analysis all pellets were resuspended in 0.1 M Tris at pH 7.2. Protein concentration was determined by the method of Lowry et al. (4). A summary description of the fractions follows: Sonicate—total homogenate having a protein concentration of 12 mg/ml. P_1—pellet obtained from sonicate by centrifuging for 10 min at 3500 *g*, resuspended to a concentration of 7.3 mg protein/ml and accounting for 12% of the total protein of the sonicate. S_1—supernatant fraction obtained during the preparation of P_1. Protein concentration was 10.5 mg/ml, and 86% of the total protein was contained in this fraction. P_1'—pellet obtained by centrifuging a 1:10 dilution of S_1 for 10 min at 3500 *g*. It was resuspended to a concentration of 3.9 mg protein/ml and accounted for 2.9% of the protein of the sonicate. P_2—pellet obtained by centrifuging the supernatant fraction of P_1' for 15 min at 20,000 *g*. It was resuspended to a concentration of 5.8 mg protein/ml and accounted for 9.5% of the protein of the sonicate. P_3—pellet obtained by centrifuging supernatant fraction of P_2 for 60 min at 105,000 *g*. It was resuspended to a concentration of 7.3 mg protein/ml and accounted for 12% of the protein of the sonicate. S_3—supernatant fraction obtained by centrifuging undiluted sonicate for 60 min at 105,000 *g* containing 8 mg protein/ml and accounting for 66% of the protein of the sonicate.

Instrumentation and Procedures

An Aminco-Chance Dual-Wavelength Split-Beam Recording Spectrophotometer (American Instrument Co., Inc. Silver Spring, Md.) was used. The instrument was equipped with an end-on photomultiplier tube with a 1.73 in. diameter photo cathode, EMI-9558QC, having an S-20 spectral response and fitted with a quartz window, a set of two gratings (one for the sample beam and one for the reference beam) with 600 grooves/mm blazed for 500 nm, and a tungsten-iodide light source.

For low temperature spectra a sample holder was used which contained in fixed positions two 1 ml capacity cells with lucite windows and a 2 mm light path. The holder was designed to fit snugly into a 665 ml capacity Dewar flask having an inside diameter of 2¾ in. and an inside height of 7½ in. One such Dewar flask was used outside the instrument for preparing samples, and another (unsilvered-clear glass) Dewar flask was used in the instrument.

All samples were analyzed in a medium containing equal volumes of glycerol and 0.1 M Tris at pH 7.2. The liquid samples contained in the cells of the sample holder were rapidly frozen by immersion in liquid nitrogen for several minutes. When removed from liquid nitrogen they were in the form of an optically clear ice which rapidly developed cracks. After several minutes at room temperature, devitrification was evidenced by a milky appearance which started at the cell periphery and gradually closed in until the samples appeared homogenous and nearly opaque. At this point the sample holder was plunged back into the liquid nitrogen and left immersed until the rapid boiling subsided. The sample holder was then placed in the Dewar flask in the sample compartment of the instrument which contained liquid nitrogen to a level just below the sample and reference cells.

Between successive spectra run on the same preparation, the frozen samples were thawed by placing the sample holder in a bath of distilled water at room temperature to a level just below the opening of the cells. The content of each cell was withdrawn, treated as required, and returned. For each preparation an oxidized vs. oxidized spectrum was recorded after vigorously shaking the suspension in air for 1 min. This base line was adjusted by use of the recorder's compensation trimpots to produce a line as nearly horizontal as possible. With these same trimpot settings additional spectra were recorded in the following manner: The contents of both cells were removed and placed in separate tubes. One was treated with dithionite in order to reduce its cytochromes, and the other was simply mixed with a stirring rod. The untreated sample was returned to the reference cell, the treated sample to the sample cell, and (after devitrification) a reduced vs. oxidized spectrum was recorded. The contents of both cells were then removed, pooled, and treated with more dithionite. A part of this suspension was placed in the reference cell and the rest was bubbled with carbon monoxide for 3 min in the dark and then placed in the sample cell. A reduced plus carbon monoxide vs. reduced spectrum was recorded. Low temperature spectra were generally recorded with a slit width of 0.5 mm.

For room temperature spectra, 3.5 ml samples were used in 5 ml capacity quartz cuvettes having an optical path of 1 cm. The order of analyses for a given preparation was: (*a*) oxidized vs. oxidized with adjustment of base line compensation trimpots as required, (*b*) reduced vs. oxidized, (*c*) reduced vs. reduced with readjustment of base line compensation trimpots, (*d*) reduced plus carbon monoxide vs. reduced. For the reduced versus oxidized spectrum, dithionite crystals were added directly to the sample in the cuvette, and both the sample and (untreated) reference contents were mixed with plastic stirring rods. For the reduced vs. reduced spectrum, the sample and reference suspensions were removed, pooled, reduced with dithionite, and then returned to the cells. For the reduced plus carbon monoxide vs. reduced spectrum, the reduced suspension was bubbled with carbon monoxide in the dark for 3 min. The medium used at room temperature was 0.1 M Tris at pH 7.2 and the slit width was 0.5 mm.

Identification and Assay of Cytochromes

Optical densities were measured as peak to trough differences. Peak and troughs were located as follows: Soret 1 (Cytochrome $b_1(\gamma)$)—427 nm and 410 nm at 77°K, 430 nm and 409 nm at 296°K; Soret 2—437 nm and 456 nm at 77°K, 442 nm and 462 nm at 296°K; Cytochrome b_1 (α)—558 nm and 538 nm at 77°K, 560 nm and 540 nm at 296°K; Cytochrome a_1 (α)—591 nm and 603 nm at 77°K, 595 nm and 585 nm at 296°K; Cytochrome a_2 (α)—626 nm and 647 nm at 77°K, 634 nm and 652 nm at 296°K. The identity of the alpha bands and the Soret band of Cytochrome b_1 was taken from the literature (5–7). Although the location of the Soret 2 band corresponds to that reported for the Soret band of cytochrome a_1 (5) we would prefer to not designate it as such until this cytochrome has been purified and the identity firmly established.

Quantitation of the cytochrome content in terms of micromoles per gram of cell fraction protein could readily be done in the case of cytochrome b_1 which has been crystallized from *E. coli* (7), but not with cytochrome a_1 which has not been purified.

Cytochrome a_2 (cytochrome oxidase) isolated from *Pseudomonas aeruginosa* has been intensively studied (8–10), and spectral parameters obtained with this enzyme have been used for quantitation of cytochrome a_2 in *Azotobacter vinelandii* (11) and in *Haemophilus parainfluenzae* (12). Oxidized and reduced spectra have been published for highly purified cytochrome oxidase (8), crystalline cytochrome oxidase (9), and reconstituted cytochrome oxidase made from purified heme a_2 and a protein moiety (10). Nevetheless, we have chosen not to use these data for the quantitation of cytochrome a_2 content of *E. coli* because of the following considerations: (*a*) Although difference spectra for the three preparations cited above are similar to each other, the peak for cytochrome $a_2(\alpha)$ occurs at 624 nm in the partially purified preparation, at 607 nm in the crystalline preparation, and at 630 nm in the reconstructed enzyme. (*b*) The shape of the difference spectrum for the cytochrome $a(\alpha)$ absorption band is not the same in all three preparations. (*c*) The bands which occur at 420 nm and 520 nm in the *Pseudomonas aeruginosa* preparations are not seen in the fractions obtained from *E. coli*. (*d*) Cytochrome a_2 from *Pseudomonas aeruginosa* contains both heme a_2 and a *c*-type heme (13), whereas cytochrome *c* seems to be absent from *E. coli* membranes[2] (reference 15 and this paper).

Electron Microscopy of Isolated Fractions

"Sonicate," P_1, P_1', and P_2, which had been stored in liquid nitrogen in suspending medium (1), were thawed and centrifuged for 20 min at 90,000 *g* at about 4°C. P_3 was stored in liquid nitrogen as a frozen pellet. For embedding purposes all pellets were treated according to the Ryter-Kellenberger procedure (16), which includes resuspension in 2% (w/v) agar, fixation in osmium tetroxide plus tryptone, posttreatment in uranyl acetate, dehydration in a graded series of acetone, and finally embedding in Vestopal W. The Ryter-Kellenberger acetate-Veronal buffer (pH 6.0) contained 0.01 M $MgCl_2$.

Sections were cut with glass knives on an LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.). No poststaning of sections was applied.

Electron micrographs were taken with either a Philips EM 200 or an EM 300 electron microscope operating at 80 kv.

RESULTS

Electron Microscopic Observations

Morphological characterization of fractions was performed by means of thin sectioning. The main components observed were ribosome-like material and cell envelope (cell wall plus cell membrane (17)) fragments. Nucleoplasmic structures (DNA fibrils) were not identified with certainty. In the envelope fragments, cell wall and membranes are not easily distinguishable from each other because, although the peptidoglycan (mucopeptide) layer has been affected by the penicillin treatment, both the plasma membrane and cell wall remnants (globular protein, lipopolysaccharides, and lipoproteins) reveal triple-

[2] A soluble cytochrome C-552 is present in certain strains of *E. coli* grown anaerobically in the presence of nitrate (14).

layered profiles (18–20). Nevertheless, some difference can be noticed. Large broken cell wall fragments generally tend to curl inwards, whereas membranes seem to form vesicles after disruption (19). However, the image obtained does not always permit a clear-cut identification of the observed triple-layered structures with respect to membrane or cell wall components.

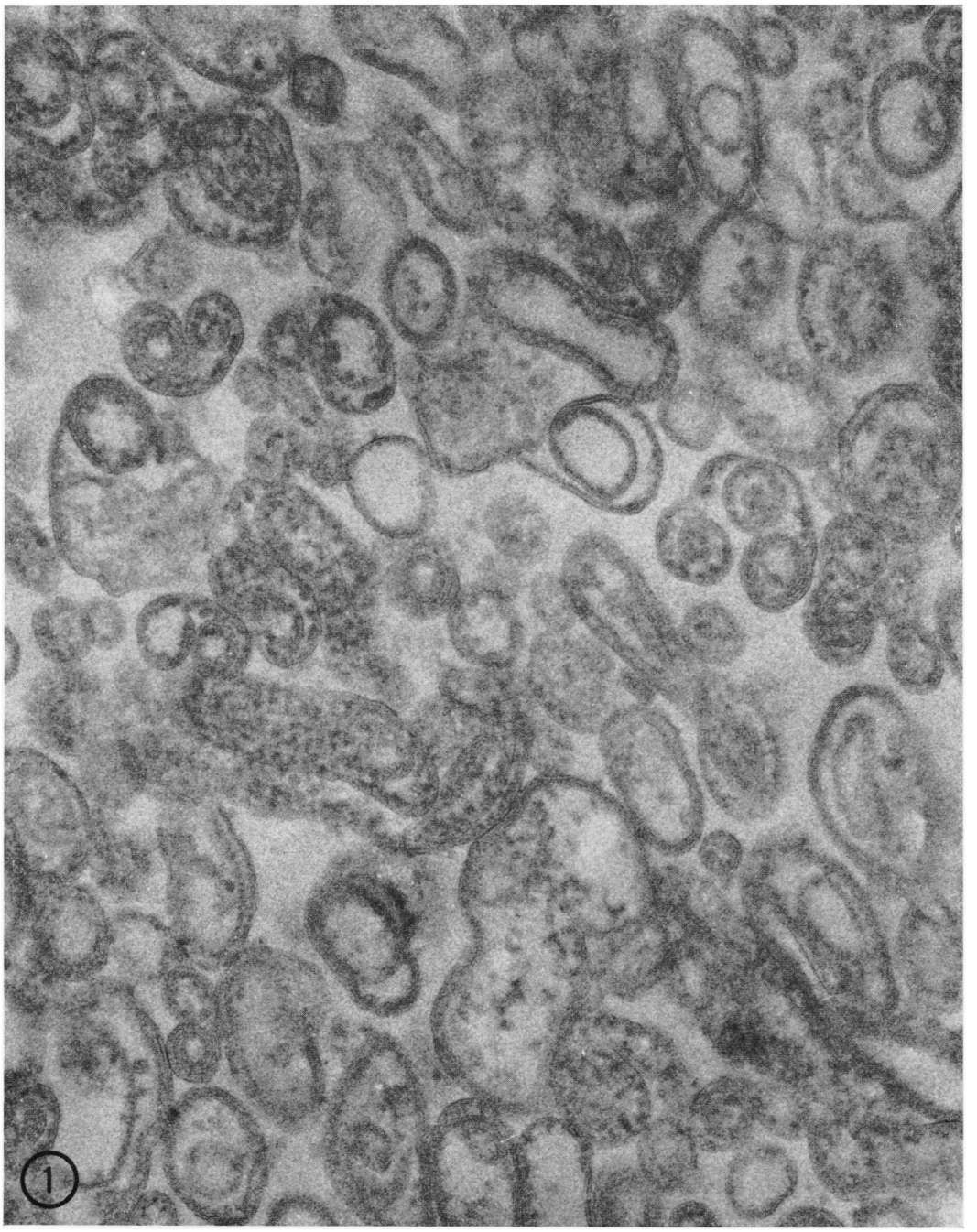


FIGURE 1 Sonicated spheroplasts. The preparation shows envelope fragments of variable size and ribosome-like material. × 120,000.

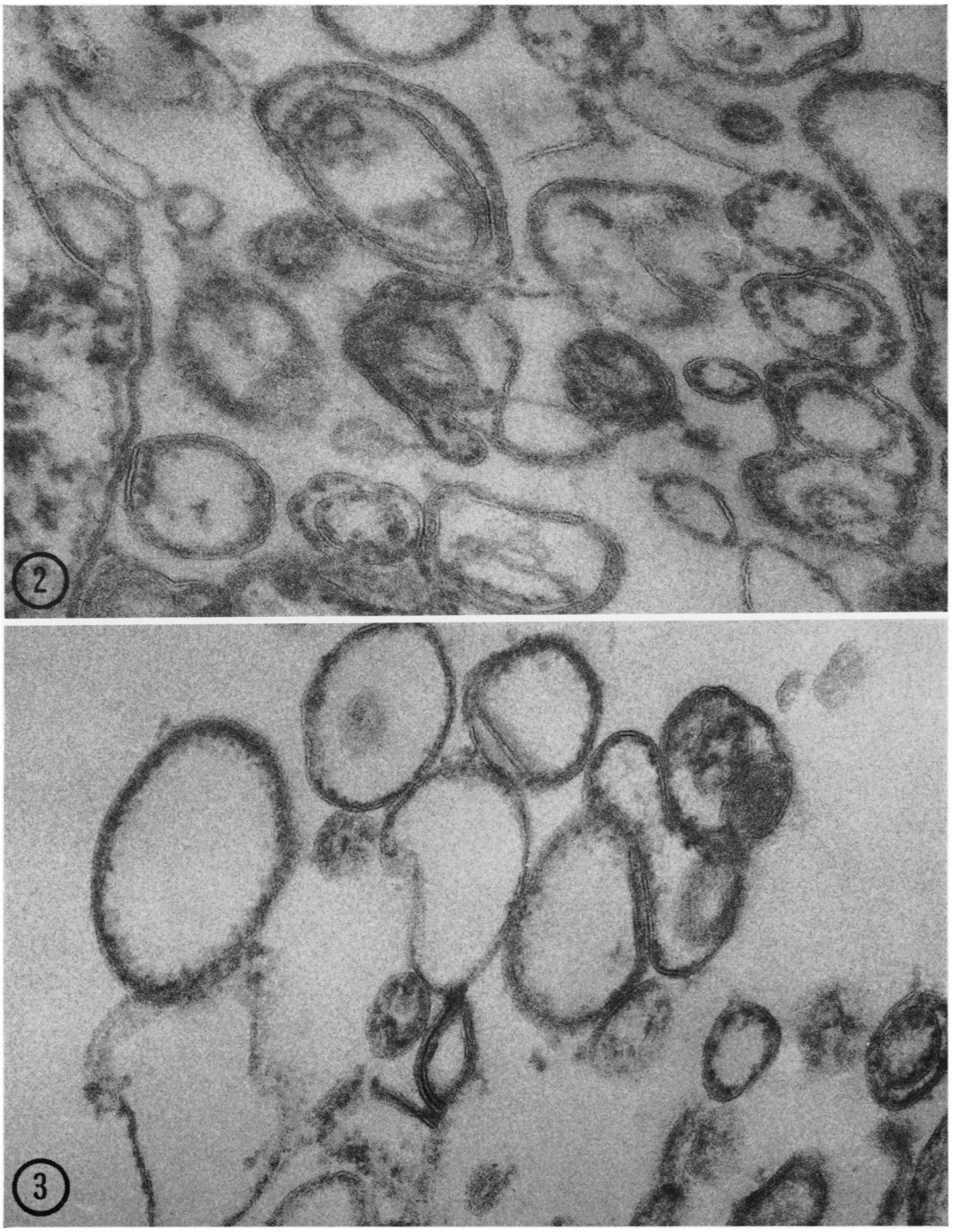


FIGURE 2 Pellet fraction P_1, obtained from "sonicate" (Fig. 1) by centrifuging at 3500 *g* for 10 min. Large envelope fragments and cytoplasmic material are present. × 120,000.

FIGURE 3 Pellet fraction P_1', obtained from diluted supernatant of P_1 by centrifuging at 3500 *g* for 10 min. Similar appearance as P_1 (Fig. 2), although there seems to be less cytoplasmic material present in fraction P_1'. × 120,000.

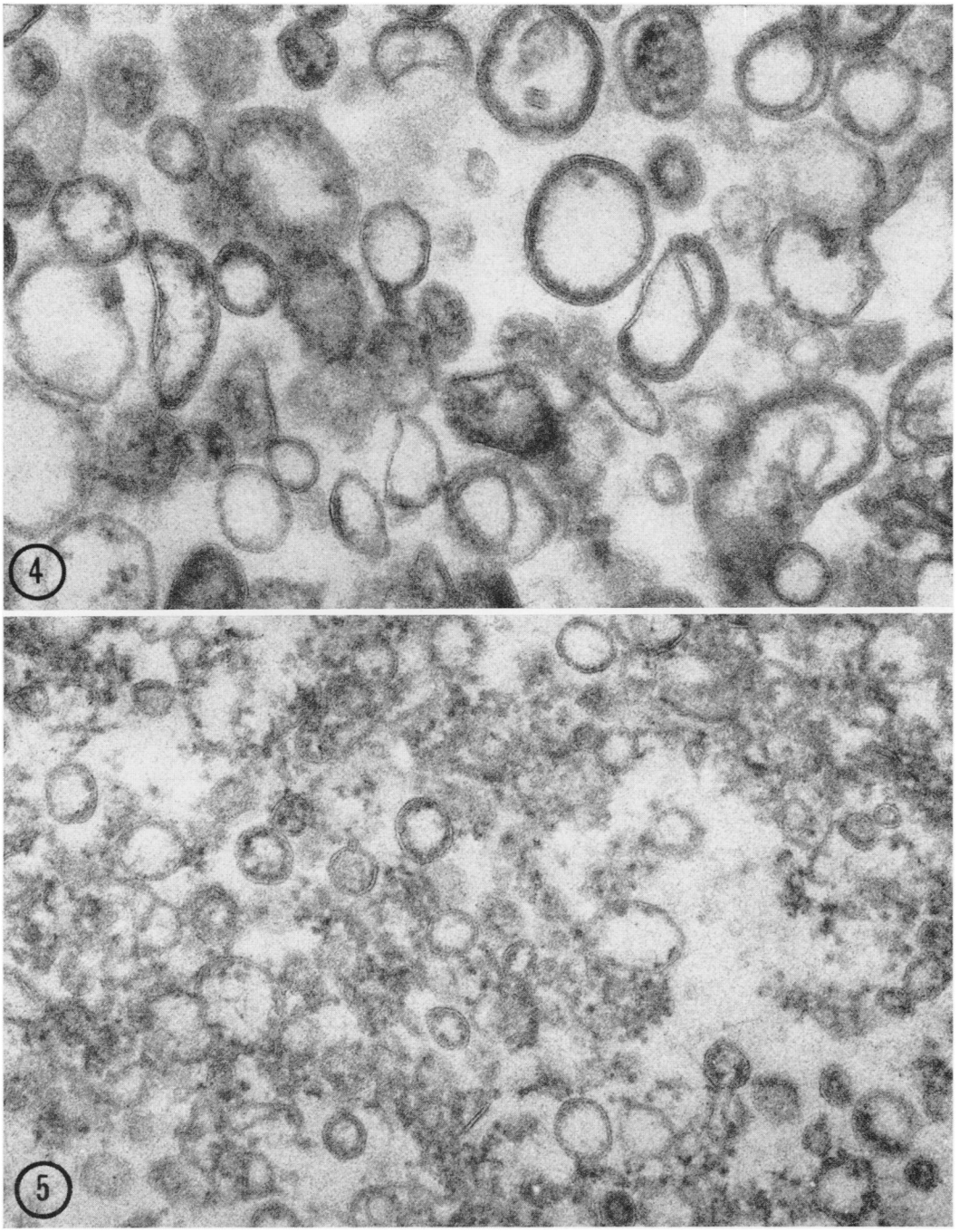


FIGURE 4 Pellet fraction P_2, obtained from supernatant of P_1' by centrifuging for 15 min at 20,000 *g*. Predominantly vesicle-like elements can be observed. × 120,000.

FIGURE 5 Pellet fraction P_3, obtained from supernatant of P_2 by centrifuging for 60 min at 105,000 *g*. The fraction shows small vesicles and ribosomal clusters. × 120,000.

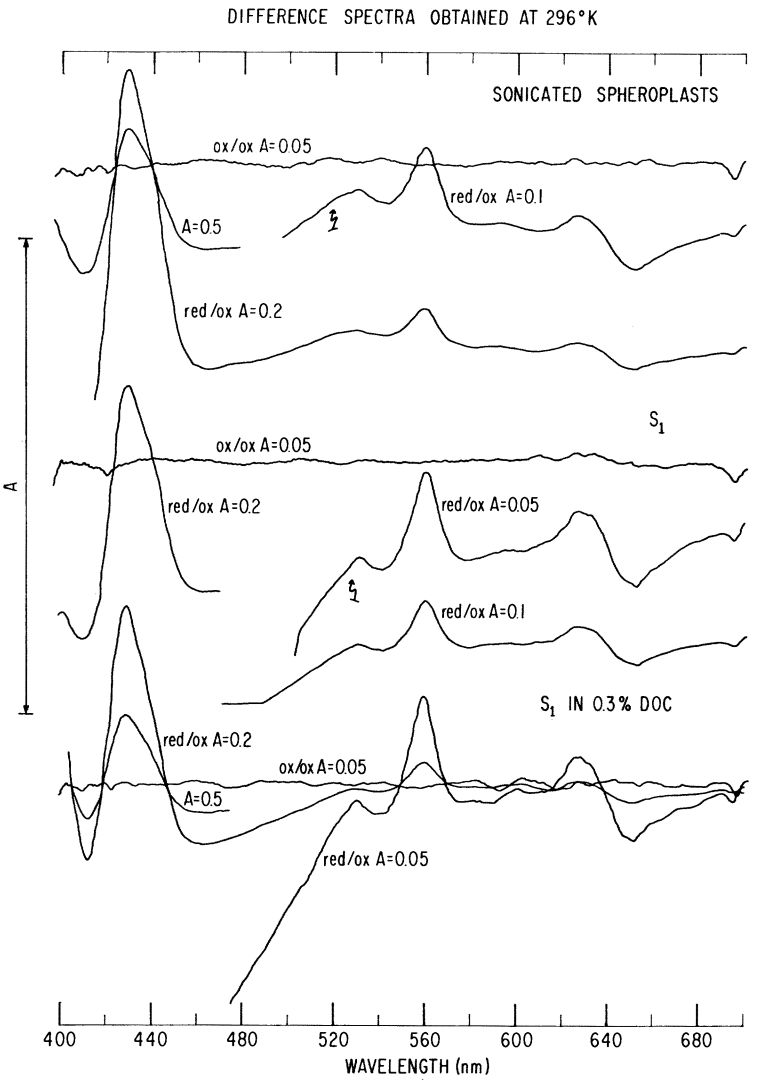


FIGURE 6 Sensitivity is indicated for each trace by the value assigned to "A" in optical density units. Thus A = 0.5 means that the span shown in the left-hand margin represents 0.5 optical density units[3]. The redox state of the suspensions in the sample and reference cuvettes is indicated by expression red or ox/red or ox where the first term (red = reduced, ox = oxidized) represents the contents of the sample cuvette, and the second term the contents of the reference cuvette. The bent arrow shows that a particular trace was moved from an off-scale position nearer to the ox/ox baseline. Descriptions of the *E. coli* cell fractions are given in the Methods section. Protein concentrations were 5.2 mg/ml for sonicated spheroplasts, 3.5 mg/ml for S_1, and 4.5 mg/ml for S_1 in 0.3% DOC (deoxycholate).

The sonicate (Fig. 1) contains cell envelope fragments of variable size, frequently with presumed ribosomes attached. Pellet fractions P_1 (Fig. 2) and P_1' (Fig. 3) contain predominantly large envelope fragments. In the case of P_1, however, slightly more cytoplasmic material seems to be present. The pellet fraction P_2 is shown in Fig. 4. This fraction contains relatively more small membranous components than are found in

[3] Duplicate tracings at different sensitivity settings were obtained as a check on the location and size of small peaks and to determine whether the baseline had shifted during the period of obtaining the spectrum. This fact is shown by the intersection of the difference spectra obtained with various sensitivity settings at the base line level of zero net absorption.

P_1 (Fig. 2) or P_1' (Fig. 3). Many vesicle-like elements are to be detected but few ribosomes. Much cytoplasmic material (ribosome-like clusters) is, on the other hand, to be found in pellet fraction P_3 (Fig. 5). In addition, numerous small vesicles are present.

Cytochrome Analysis

Many spectra were obtained for each fraction at room temperature and at 77°K. Fig. 6 is representative of the room temperature spectra. A broad Soret band is seen with a peak of 430 nm (cytochrome $b_1(\gamma)$), and a shoulder is indicated at 442 nm. The β band of cytochrome b_1 is seen at 530 nm, and the α band at 560 nm. A weak α band for cytochrome a_1 is present at 595 nm, and a stronger α band for cytochrome a_2 is seen at 634 nm. These spectra may be compared with those obtained at 77°K and shown in Fig. 7. The Soret band is clearly seen to contain two components, one with a peak at 427 nm and the other at approximately 437 nm. The β and α

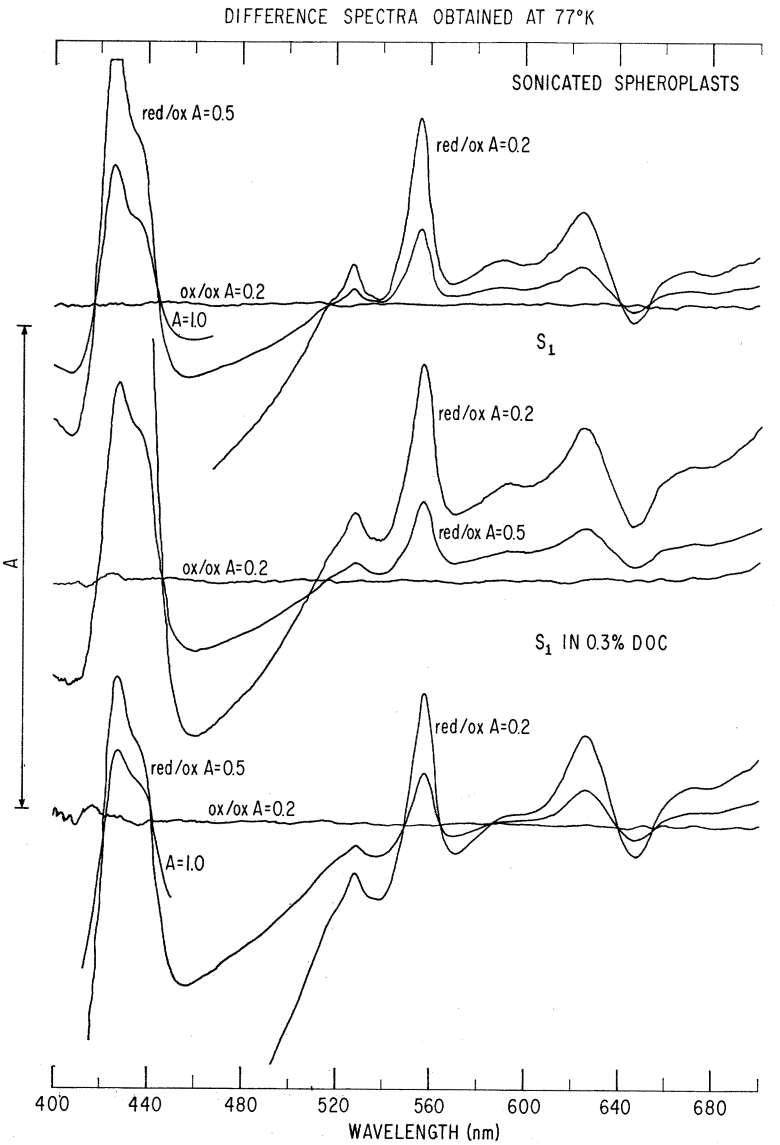


FIGURE 7 Designations are the same as for Fig. 6. Protein concentrations were 3.7 mg/ml for sonicated spheroplasts, 3.3 mg/ml for S_1, and 3.3 mg/ml for S_1 in 0.3% DOC.

bands of cytochrome b_1 occur at 528 nm and 558 nm, respectively. The α band of cytochrome a_1 is clearly resolved at 591 nm, and cytochrome a_2 shows a peak at 626 nm and a sharp trough at 647 nm. The effect of low temperature and the devitrified state on these spectra is twofold. First, there is a slight shift of the bands towards the blue end of the spectrum with a concomitant sharpening and improvement of resolution. Secondly, there is a marked enhancement of apparent extinction coefficients (21).

In principal, therefore, it would seem advisable to perform cytochrome analyses at low temperature. There are other factors, however, which tend to offset the obvious advantages of working at low temperature for quantitative analysis. The enhancement of extinction obtained at low temperature depends upon the characteristics of the microcrystals of ice formed in the devitrified state. These characteristics in turn are dependent upon the precise nature of the suspending medium (22) and upon the conditions of freezing in liquid nitrogen and subsequent warming to obtain devitrification. Not only is there a possibility for variation among different samples, but within the same sample individual absorption bands appear to be affected differently by the same conditions of preparation for low temperature analysis.

In Table I it can be seen that, in general, Soret bands are less amplified than α bands, and that particulate fractions generally show less amplification of absorption bands than is found for supernatant fractions. The presence of soluble components in S_1 appears to enhance the absorbancy at 77°K of cytochromes contained in the particulate components. This is evident in the data of Table II which show an invariably poor recovery for cytochromes when the particulate components are analyzed separately at 77°K. Comparison of these data with those obtained at 296°K shows that the relative content of cytochromes in the particulate fractions was undervalued in the data obtained at lower temperature whereas the relative content of cytochromes in S_3 was overvalued. Recoveries greater than 100% usually obtained in S_1 plus P_1 fractions may indicate a depressant effect exerted by the components of P_1 on the optical absorbancy properties of the other components of the sonicated spheroplasts. It can also be seen (Table III) that the ratio of absorbancies of the cytochrome b_1 Soret band to that of cytochrome b_1 (α) is less in the 77°K spectra than in the corresponding 296°K spectra. This effect has been observed before (21–23).

TABLE I

Enhancement of Cytochrome Extinction Coefficients by Low Temperature (77°K)

	Sonicate	P_1	S_1	P_2	P_3	S_3
Soret 1 (Cytochrome b_1)	19.9*	20.8	17.5	18.5	11.8	30.0
Soret 2	26.7	15.3	26.4	21.1	11.1	31.3
Cytochrome b_1	43.0	26.9	40.8	25.0	12.7	76.5
Cytochrome a_1	21.4	—	28.0	31.0	—	—
Cytochrome a_2	29.0	57.3	43.8	15.3	12.6	
Cytochrome a_2'	62.0	20.8	46.0	26.2	11.4	

* Numbers denote relative optical density at 77°K compared to optical density at 296°K for the same concentration solution in the same length light path (i.e. mg/ml per cm light path). For a_2' peak and trough wavelengths were taken as 625 and 602 mm at 77°K and at 627 and 610 mm at 296°K.

Because of the nonuniform effects of low temperature on individual cytochrome absorption bands, the apparent extent of concentration of the absorbing substances in the different fractions is quite different at low temperature and room temperature.

Carbon Monoxide Spectra

For all low temperature spectra, the oxidized vs. oxidized spectrum was set as a horizontal base line with voltages applied as needed to compensate for any inequalities in the light coming from the reference and sample beams. In order to avoid the necessity of an additional cycle of thawing, refreezing, and devitrification to recheck

TABLE II

*Per Cent Distribution of Cytochromes among Fractions**

Fraction	Soret 1 $b_1(\gamma)$	Soret 2	b_1 (α)	$a_1(\alpha)$	$a_2(\alpha)$
Conducted at 77°K:					
P_1	10.4	9.9	9.9	—	9.6
S_1	84.0	95.	103	111	118
% Recovery	94	105	113	111	128
P_1'	7.4	6.4	5.3	6.7	5.4
P_2	31.4	31.0	26.9	31.3	22.3
P_3	21.2	17.3	14.6	33.7	15.3
S_3	15.4	15	14.9	—	11.3
	75	70	62	72	54
% Recovery‡	89	74	60	65	46
Conducted at 296°K:					
P_1	10	17.4	15.8	0	4.4
S_1	96	96	108	85	71
% Recovery	106	113	124	15	75
P_1'§					
P_2	33.9	39.2	46.3	21.5	38.5
P_3	35.8	41.9	49.3	8.6	31.8
S_3	10.2	12.8	10.0	0	3.7
	80	94	106	30	74
% Recovery‡	83	98	98	35‖	102

* The total optical absorbance in the sonicate is taken as 100%.

‡ Compared to S_1 which is the parent fraction for these components.

§ There was insufficient P_1' fraction for analysis at 296°K. Therefore the % recovery at 296°K would actually be somewhat greater than indicated.

‖ The magnitude of absorption of $a_1(\alpha)$ is too small for accurate measurement, especially in the fractions assayed at 296°K.

the base line with a reduced vs. reduced spectrum, it was assumed that the original voltage adjustments would still serve to balance the reference and sample beams as long as the same sample and reference cells were used. Accordingly, a reduced plus carbon monoxide vs. reduced spectrum was run directly after recording the reduced vs. oxidized spectrum. A typical spectrum is shown in Fig. 8 *a* (curve 3). Maxima occur at 430, 530, 537, 560, and 637 nm. These locations of maxima and minima and the appearance of the spectrum suggest the following possible identifications for carbon monoxide complexes: cytochrome *o* with a shoulder at 412 and peaks at 530 and 560 nm which correspond to peaks at 417, 536, and 568 nm reported for room temperature spectra (5); cytochrome a_1 with peaks at 430 and 537 nm corresponding to peaks at 427–428, 548, and 585–592 nm obtained at room temperature; and cytochrome a_2 with a peak at 637 nm corresponding to a single peak obtained at room temperature in the same location (5).

For the room temperature analyses, reduced vs. reduced control spectra were routinely performed. This led to the recognition of a pronounced optical artifact. When the base line was adjusted for a pair of oxidized samples (curve 1 in Fig. 8 *b*), a subsequent scan with a pair of reduced samples yielded the spectrum shown in curve 3 of Fig. 8 *b*. Instead of obtaining a relatively flat base line as expected, pronounced maxima and minima were obtained. The general appearance and location of several of these "absorption bands" correspond to those obtained at 77°K in the presence of

TABLE III

Relative Concentration of Cytochromes in Different Fractions

Fraction	Soret 1 $b_1(\gamma)$	Soret 2	$b_1(\alpha)$	$a_1(\alpha)$	$a_2(\alpha)$	Ratio $b_1(\gamma)/b_1(\alpha)$
Conducted at 77°K (2 mm light path):						
Sonicate	115.7 ± 13.4* (3)	68.9 ± 5.9 (3)	19.8 ± 1.0 (3)	0.82 ± 0 (3)	10.9 ± 1.0 (3)	5.8
P_1	100.5 ± 8.5 (2)	57.0 ± 0 (1)	16.3 ± 0.7 (2)	1.7 ± 0 (1)	8.7 ± 1.0 (2)	6.4
S_1	113.0 ± 20 (2)	76.0 ± 6.7 (3)	23.7 ± 1.5 (5)	1.07 ± 0.46 (4)	14.9 ± 1.5 (5)	4.8
P_1'	293 ± 70 (3)	152 ± 36 (3)	36.3 ± 11.3 (3)	2.0 ± 0.23 (3)	20.1 ± 5.8 (3)	8.1
P_2	382 ± 20 (3)	225 ± 20 (3)	56.0 ± 6.5 (3)	2.7 ± 0.55 (3)	25.5 ± 1.5 (3)	6.8
P_3	205 ± 22 (3)	99 ± 11 (3)	24.0 ± 1.5 (3)	2.3 ± 1.5 (3)	13.8 ± 2.2 (3)	8.5
S_3	27.0 ± 1.2 (3)	15.6 ± 1.9 (3)	4.5 ± 0.58 (3)	0.58 ± 0 (1)	1.86 ± 0.44 (2)	6.0
Conducted at 296°K (1 cm light path):						
Sonicate	29.0 ± 0 (2)	12.9 ± 0.2 (2)	2.3 ± 0 (0.15)‡ (2)	0.19 ± 0 (2)	2.06 ± 0.14 (2)	12.6
P_1	24.2 ± 0.2 (2)	18.7 ± 0.2 (2)	3.0 ± 0.1 (0.19) (2)	—‖ (2)	0.76 ± 0.12 (2)	8.1
S_1	32.4 ± 1.2 (3)	14.4 ± 0.2 (4)	2.9 ± 0 (0.19) (4)	0.19 ± 0.04 (4)	1.7 ± 0 (4)	11.2
P_1'§						
P_2	103 ± 1.5 (2)	53.2 ± 0 (2)	11.2 ± 0.1 (0.72) (2)	0.44 ± 0.09 (2)	8.4 ± 0 (2)	9.2
P_3	86 ± 0.5 (2)	45.0 ± 0 (2)	9.5 ± 0.5 (0.61) (2)	0.14 ± 0 (2)	5.5 ± 0.3 (2)	9.1
S_3	4.5 ± 0.25 (2)	2.5 ± 0 (2)	0.35 ± 0 (0.02) (2)	—‖ (2)	0.11 ± 0.11 (2)	12.8

* All numbers represent optical density units per gram of protein for the appropriate light path (i.e. 2 mm at 77°K and 1 cm at 296°K) ± standard error of the mean. Integers in parentheses represent number of experiments.

‡ Numbers in parentheses in this column are μmoles cytochrome b_1 per gram of protein using ΔE 560-540 nm (ϵ = 15,600) calculated from the data in (7).

§ There was insufficient P_1' for analysis at 296°K.

‖ These peaks were too small to be measured.

carbon monoxide. Readjustment of the base line compensation trimpots helped to flatten the curve, but inflections still remained (curve 4). The reduced plus carbon monoxide vs. reduced spectrum obtained after readjustment of the trimpot settings (curve 5) was almost identical to the trimpot-corrected reduced vs. reduced control (curve 4) in the 460–600 nm and 600–780 nm regions. The traces in Fig. 8 *c*, which were obtained with P_2 at room temperature, further emphasize these features. A comparison of the trimpot-corrected reduced vs. reduced spectrum (curve 3) with the subsequently run reduced plus carbon monoxide vs. reduced spectrum (curve 4) shows that the maxima and minima occurring in the region of 450–620 nm are not really representative of carbon monoxide cytochrome complexes. This same conclusion must apply to the corresponding absorption bands obtained at 77°K and described above. When the deviations in base line occurring in the control curve 3 are subtracted from the carbon monoxide trace (curve 4), a resultant spectrum is obtained (dotted line) which shows maxima at 414 and 437 nm and minima at 428

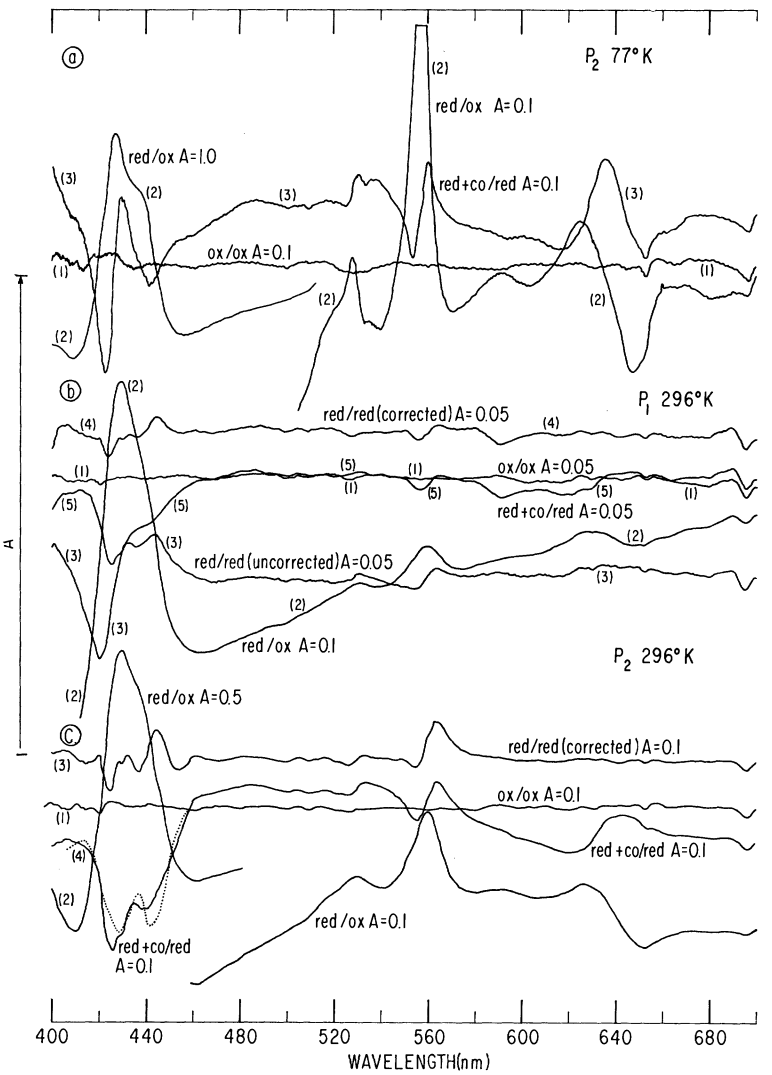


FIGURE 8 Designations are the same as in Fig. 6. For "red + CO" samples, carbon monoxide was bubbled through the reduced sample for 3 min in the dark. Protein concentrations were 1.2 mg/ml for P_2 at 77°K, 3.1 mg/ml for P_1 at 296°K, and 2.9 mg/ml for P_2 at 296°K.

and 442 nm. These may represent cytochrome *o* and cytochrome a_1 carbon monoxide complexes. The broad peak occurring between 638 and 647 nm is also real and probably represents the cytochrome a_2 carbon monoxide complex.

It was also observed that the NADH oxidase activity (determined spectrophotometrically (1)) of the S_1 fraction was not particularly sensitive to inhibition by carbon monoxide treatment. Bubbling with carbon monoxide for 5, 10, and 15 min, respectively, caused 7, 21, and 23% inhibition of oxygen uptake. Exposure to daylight for 30 min did not restore activity. The observed inhibition may have been partly due to protein denaturation caused by the bubbling.

Reduction of Cytochromes by NADH and Succinate

Taking the extent of reduction caused by dithionite as 100%, the per cent of reduction caused by NADH and succinate was determined (Table IV). It was found that all or nearly all of the cytochromes could be reduced by either

NADH or succinate. The combined presence of both substrates did not lead to any greater reduction than when either was present alone.

The absorption bands generated by either substrate could be caused to disappear by shaking in air for 1 min and to reappear upon allowing the suspension to stand undisturbed for a few minutes. The data in Table IV also show that cyanide inhibits the reduction of cytochrome a_2. When more air was admitted to the hydrocyanic acid (HCN)-treated suspension, cytochrome a_2 was more readily oxidized than were the other cytochromes.

Indicated Distribution of Flavoproteins

Chance and Williams (24) quantitated the flavoprotein content of rat liver mitochondria by use of the wavelength pair 465 and 510 nm and a molar extinction coefficient of 11,500. It is not known that these parameters would apply to the *in situ* flavoproteins of *E. coli*. However, an approximate estimate of flavoprotein distribution may be obtained by comparing the absorbancies at these two wavelengths for the 296°K spectra and the (apparently) corresponding wavelengths of 458 and 505 nm for the low temperature spectra. When this is done for the various cell fractions, it is seen (Table V) that in contrast to the situation for cytochromes, an appreciable concentration of flavoproteins is present in the soluble fraction (S_3). This is consistent with the high levels of dehydrogenase activity for NADH and malate in the soluble fraction. (1)

The conditions for analysis at 77°K seem also to enhance absorbancy for flavoprotein to a different degree in the different fractions.

TABLE IV

Per Cent Reduction of Cytochromes by NADH and Succinate

Substrate	NADH			Succinate alone	NADH + succinate	
	Alone	+HCN	+HCN +air		Alone	+HCN +air
Soret 1 (cytochrome $b_1(\gamma)$)	95	78	64	81	80	
Soret 2	100	80	61	87	91	
Cytochrome b_1	85	71	60	65	83	50
Cytochrome a_1	100	86	114	86	100	86
Cytochrome a_2	100	38	8	97	85	12

The particulate components of the sonicate (pellet obtained at 105,000 *g* in 60 min) were present at a concentration of 2.2 mg protein per ml in 0.1 M Tris at pH 7.2 in open cuvettes at room temperature. Reduction obtained with dithionite was taken as 100%. The figures represent maximum reduction obtained in the presence of either a small amount of solid NADH or 5.7 mM succinate. HCN was generated just before use by mixing 0.8 N KCN and 0.8 N H_2SO_4, and its final concentration in the incubation mixture was 12 mM.

TABLE V

Distribution of Flavoproteins among Fractions

	Sonicate	P_1	S_1	P_2	P_3	S_3	P_1'
A/g							
77°K—2 mm light path	13.5*	13.9	17.6	36.0	23.9	11.1	28.8
296°K—1 cm light path	1.9	3.1	2.7	10.1	7.4	3.3	
Relative absorbance 77°K/296°K	35.5‡	22.4	32.6	16.3	16.1	16.8	
% Distribution							
77°K	100	12	112	26	21	55	6.2
296°K				24§	22§	54§	

* A/g is given in optical density units per gram for the appropriate light path (i.e. 2 mm at 77°K and 1 cm at 296°K).

‡ Relative absorbance has been calculated for the same light path—A/g for 1 cm light path.

§ The distribution at 296°K was calculated on the bases of the sum of P_2, P_3, and S_3 as 100%.

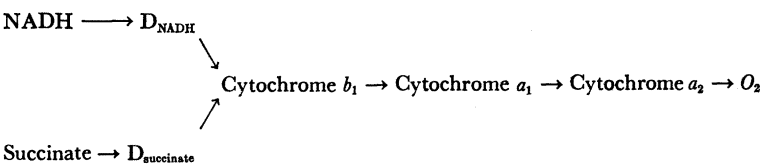
DISCUSSION

We have recently reported that oxidase activity for succinate and NADH, in *E. coli* W-6, is concentrated in particulate fractions derived from this organism (1). In the current work the membranous vesicular nature of these fractions is documented by electron microscopy. We further establish that the cytochromes are also concentrated in the particulate fractions, particularly P_2 and P_3. Although P_3 (105,000 *g* pellet from 20,000 *g* supernatant fraction) is traditionally considered as a ribosome fraction, our studies of oxidase activity (1), cytochrome distribution, and electron microscopy point to the relatively high membrane content of this fraction.

Low temperature (77°K) spectroscopy has permitted a very substantial increase in the sensitivity of the spectrophotometric analysis of cytochromes. However, we have found great variability in the magnitude of this increase from fraction to fraction and from one absorption band within a fraction to another. It is important to stress that the undependability of quantitative low temperature cytochrome analysis illustrated in these studies applies to tissue extracts in a glycerol water medium. Wilson in his very careful study (22) has shown that aqueous sucrose is in general superior to aqueous glycerol for quantitative analysis. However, that technique was not tested with several different cell fractions at 77°K and 296°K as was done here. It seems advisable at this time to approach the quantitative application of low temperature cytochrome analysis with some caution. The difference in effects of low temperature conditions on extinction coefficients of Soret and α absorption bands has been observed previously (21–23).

A potentially troublesome optical artifact was observed when reduced carbon monoxide spectra were studied. Several "absorption bands" appeared in the difference spectra for reduced plus carbon monoxide vs. reduced samples. Many of these *apparent* absorption bands were also present when reduced vs. reduced controls were run. The principle underlying the technique for obtaining difference spectra is that when light of the same wave length and intensity is passed through two solutions, any small differences in optical properties of the two solutions will be detected and greatly magnified. In the Aminco-Chance spectrophotometer the light beams follow different paths and are diffracted by different gratings. If the wavelengths of the two beams are out of synchrony by even a fraction of 1 nm, then in regions where reduced cytochrome absorption bands normally occur, (and consequently where there is a greater change of absorption with wavelength), a difference spectrum may be generated. Although the baseline adjustment trimpots are useful for equalizing irregularities which occur at particular spaced points along the scan, the type of band which is generated by this anomaly is so sharp that it frequently occurs between the available adjustment locations and therefore is not easily removed. Very pronounced absorption band artifacts of this nature were also found when reduced vs. reduced horse heart cytochrome *c* was analyzed (not shown). It is therefore quite important in the use of this kind of spectrophotometer to verify absorption bands obtained in the reduced plus carbon monoxide vs. reduced spectra by comparing each spectrum with the appropriate reduced versus reduced control. We have found that oxygen uptake by our preparation derived from *E. coli* W6 was not particularly sensitive to inhibition by treatment with carbon monoxide, although evidence was obtained for the formation of some authentic carbon monoxide complexes.

The particulate fractions obtained from *E. coli* contain NADH and succinate dehydrogenases, cytochrome b_1, cytochrome a_1, and cytochrome a_2. In trying to formulate the nature of the electron-transport chain(s?), we would logically place the dehydrogenases at the beginning and, drawing upon our knowledge of mitochondrial systems, would consider the following basic sequence to be most likely:



In an earlier study with intact cells of *E. coli* B, Castor and Chance obtained carbon monoxide action spectra which indicated that cytochrome *o*

is the major oxidase for log phase cells (25). For stationary phase cells, cytochrome a_2 or cytochrome *o* could function as the cytochrome oxidase. The nature of the cells, conditions of the experiment and the substrate oxidized were different than those reported here. On the basis of our results, we find no evidence for a major role of cytochrome *o* in NADH oxidase activity. The role of cytochrome *o* clearly requires further study. It is probable that iron-sulphur proteins also participate directly in the chain (26). We have observed electron paramagnetic resonance signals in the region of g = 1.94 which appeared upon addition of either succinate or NADH to a suspension of membrane fragments (R. W. Hendler and H. Kon. Unpublished observations). Further studies are in progress to determine the role of iron-sulphur proteins in the *E. coli* electron-transport chain.

Kashket and Brodie have suggested that ubiquinone is an obligatory electron-carrier between succinate dehydrogenase and cytochrome b_1 and that vitamin K is the intermediate between NADH dehydrogenase and cytochrome b_1 (27). This conclusion, however, has been questioned by Cox et al. (28). Since the role of coenzyme Q type compounds in respiration is still unsettled, and since we have no data on this point, we have not placed them in the scheme at this time.

Our present findings that cyanide inhibited the reduction of cytochrome a_2 much more than that of cytochrome a_1 or cytochrome b_1 are consistent with the above scheme. We have recently reported, however, that succinate and NADH oxidases have remarkably different sensitivities to azide, chloramphenicol, and bovine serum albumin (1, 29). For example, defatted bovine serum albumin could completely inhibit succinoxidase activity while exerting a minimal (10%) inhibition on succinate dehydrogenase activity, and no effect on NADH oxidase. We considered, therefore, the possibility of two separate chains, one for NADH and one for succinate. Our present findings, that either NADH or succinate can reduce all or nearly all of the cytochromes in a combined particulate fraction, are clearly not consistent with this alternative and, in fact, tend to establish the idea that one set of cytochromes is available for the oxidation by oxygen of both substrates. It seems most likely at present that if nonheme iron functions between succinate dehydrogenase and the cytochrome chain, this portion of the succinate oxidase assembly may be the site of albumin action. Further studies will be necessary to resolve the question.

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