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

I. Oxidative Activities with Soluble Substrates

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ABSTRACT

This paper describes experiments conducted with membranous and soluble fractions obtained from *Escherichia coli* that had been grown on succinate, malate, or enriched glucose media. Oxidase and dehydrogenase activities were studied with the following substrates: nicotinamide adenine dinucleotide, reduced form (NADH), nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), succinate, malate, isocitrate, glutamate, pyruvate, and α-ketoglutarate. Respiration was virtually insensitive to poisons that are commonly used to inhibit mitochondrial systems, namely, rotenone, antimycin, and azide. Succinate dehydrogenase and NADH, NADPH, and succinate oxidases were primarily membrane-bound whereas malate, isocitrate, and NADH dehydrogenases were predominantly soluble. It was observed that *E. coli* malate dehydrogenase could be assayed with the dye 2,6-dichlorophenol indophenol, but that porcine malate dehydrogenase activity could not be assayed, even in the presence of *E. coli* extracts. The characteristics of *E. coli* NADH dehydrogenase were shown to be markedly different from those of a mammalian enzyme. The enzyme activities for oxidation of Krebs cycle intermediates (malate, succinate, isocitrate) did not appear to be under coordinate genetic control.

INTRODUCTION

The processes of respiration and attendant phosphorylation are extremely complex and involve many enzymes and cofactors arranged in fixed spatial frames on or in a membrane matrix. The process of protein synthesis is also extremely complex and involves many enzymes and cofactors. Although the cytological location of the major sites of in vivo protein synthesis has not been so thoroughly studied, there is a compelling and growing body of evidence which points to the involvement of membranes in this process (1).

The respiratory chain of *E. coli* has not been extensively studied and the information concerning its energy-producing potential, sensitivities, and components is limited. It is the purpose of this series of investigations to study the respiratory and

protein synthesizing capabilities of *E. coli* membrane-envelope fragments. An ultimate goal is to determine if the ability of a cell to organize complex enzymatic sequences in membranes includes the linking together of different, but interdependent, processes. The specific question we are attempting to answer is: Could energy for protein synthesis at a membrane surface be provided by membrane-associated high energy intermediates produced during respiration?

In *E. coli* only one membrane system has been recognized, namely the plasma membrane. Previous work from this laboratory has shown the existence of plasma membrane-bound ribosomes in this organism and has provided evidence in favor of their active role in in vivo protein synthesis (2–4). Evidence that anaerobic protein synthesis is sensitive to uncouplers of oxidative phosphorylation has also been obtained in yeast and *E. coli* (5–7), and this evidence provides indirect support for the possibility of the interdependence of these processes. Examples have been described in which high energy intermediates formed during respiration could be used directly for various work processes such as ion accumulation and reversed electron flow (8–10).

The plasma membrane of *E. coli* is the innermost layer of a complex envelope structure which consists of several contiguous layers (1). From the outside to the inside surfaces the following are found: a lipoprotein layer, a lipopolysaccharide layer, a globular protein layer, a rigid mucopeptide layer, and, finally, the plasma membrane which is in contact with the cytoplasm and nucleoplasm. In order to cause minimal distortion of the surface normally in contact with the cytoplasm, we have tried to isolate the plasma membrane by gentle techniques. In pursuit of this objective we have not tried to separate the plasma membrane from the external layers; therefore, the proper designation of these fractions is "membrane-envelope."

In this paper we examine the respiratory capabilities with different soluble substrates. Successive papers of this series will deal with respiratory capabilities with higher fatty acids, the identification and sequencing of the components of the electron transport chain, and characteristics of an in vitro membrane-envelope, protein-synthesizing system.

METHODS

Maintenance and Characteristics of the Organism

E. coli W-6 was kindly supplied by Dr. Martin Lubin of Harvard University Medical School. The strain was isolated from *E. coli* W by a penicillin selection technique and is an auxotroph unable to synthesize proline (11, 12). The cells were grown overnight at 23° with vigorous shaking in Lubin's supplemented medium A which contained 7 grams K_2HPO_4, 0.5 gram $Na_3C_6H_8O_7 \cdot 2H_2O$ (citrate), 0.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g $(NH_4)_2SO_4$, 0.5 g proline and 2 g glucose per liter at pH 7.0. 2¼ ml of this suspension were added to sterile vials containing 0.25 ml glycerol and mixed. These suspensions, stored at −13°, served as sources of inocula for other incubations to be described here. To prepare inocula for use in the succinate preparations, the following procedure was used: An inoculum (0.25 ml) of thawed glycerol-frozen cells as described above was added to 50 ml of Lubin's supplemented medium A containing 2 mg/ml of succinate in place of glucose. The culture was grown overnight and stored frozen in glycerol as already described. To prepare an inoculum of malate-grown cells the same procedure was used with 2 mg/ml of malate replacing the succinate and with the frozen succinate cells serving as an inoculum.

Preparation of Spheroplasts

The general procedure is illustrated in Table I for making spheroplasts from cells grown in a defined salts medium with either succinate or malate as sole energy source,[1] and from cells grown on a glucose, Penn Assay Broth (PAB), enriched medium. The defined growth medium used for "succinate" and "malate" cells was Lubin's supplemented medium A containing either 2 g per liter of succinic acid or 2.3 g per liter of malic acid as the energy source. The medium used for the "glucose-PAB" cells was simply Difco Antibiotic Medium #3 (Difco Laboratories, Inc., Detroit). To ensure an adequate surface for oxygenation the cultures were grown in Erlenmeyer flasks having capacities of 5 times the volume of the medium. Cells were grown at 37° with vigorous shaking. After overnight growth the "succinate" or "malate" cultures were transferred to fresh 37° medium in 20 liter carboys and returned to vigorous shaking on a New Brunswick heavy duty model TR 3 shaker at 37° in order to induce logarithmic growth. The overnight culture of "glucose-PAB" cells was transferred to fresh 37° medium in a

[1] Although Lubin's medium A contains citrate, we have established that the citrate is not at all utilized as a carbon source by this organism.

TABLE I

Details of Spheroplast Production

This table provides data to supplement the general description of procedures discussed in the text

	Succinate cells	Malate cells	Glucose-PAB cells
A. *Starter culture*			
1) ml inoculum	0.75	1.0	2.5
2) ml medium	600	800	700
B. *Overnight growth (vigorous shaking at 37°)*			
Klett reading next morning with #66 filter	206	187*	236
C. *Induction of log growth*			
1) dilute with indicated volume of fresh medium	2400 ml	3200 ml	2100 ml
2) vigorously shake at 37° for indicated time	3 hr	2 hr	35 min
3) Klett reading change during this period (Start → Finish)	55 → 130	46 → 134	83 → 120
D. *Spheroplast formation*			
1) for glucose cells see text	—	—	—
2) for succinate and malate cells.			
a) add to culture:			
sucrose	216 g	288 g	—
yeast extract	18 g	24 g	
1 N NaOH	36 ml	46.5 ml	
10% (w/v) $MgSO_4$	30 ml	40 ml	
penicillin "G" (1.59×10^6 units/g)	1.44 g	1.92 g	
b) continued vigorous agitation at 37°	2 hr	2 hr	—
E. *Klett reading change during this period*	98 → 120	103 → 145	—

* Overnight growth was only to a reading of 19. 5 hr of further growing time was required next morning.

6 liter Erlenmeyer flask and returned to shaking at 37°.

Spheroplast formation was initiated by making additions to the rapidly growing cells in the carboys as shown in Table I. In order to initiate spheroplast formation in the case of the glucose-PAB cells, the culture was transferred to a 20-liter carboy that contained the following ingredients at room temperature:

1) Sufficient Difco Antibiotic medium for 9 liters (157.5 g) in a volume of 6 liters.

2) 3 liters 60% sucrose

3) 2.7 g proline

4) 100 ml of 10% $MgSO_4$

5) 2.2 g penicillin "G" (final concentration 300 units/ml).

The Carboy was allowed to stand with no shaking at 37°. It was found that when the medium was prewarmed to 37°, the resulting acellular preparation was less dependable for subsequent protein synthesis studies, i.e., either the preparation was inactive or the activity quickly disappeared on storage at −13°. Spheroplast formation was monitored by phase contrast microscopy and was stopped when approximately 90% of the cells were in the form of spheroplasts. Cells were harvested in a Sharples continuous centrifuge and were washed by gently suspending in "storage medium" which contained 9% glycerol (w/v), 0.05 M Tris, and 10^{-3} M $MgCl_2$ at pH 7.4. Resuspension at this and other stages was accomplished by placing small quantities of the sediment in 40 ml plastic Sorvall Centrifuge tubes which contained up to 20 ml of medium and by gently "pumping" with a loose fitting glass or Teflon ball on a rod. The total volume of storage medium used at this step was 800 to 1000 ml for the succinate and malate cells and 2800 ml for the glucose-PAB cells. This suspension was centrifuged at 21,000 *g* for 15 min in a Sorvall model RC2B refrigerated centrifuge. The pellet was resuspended in storage medium and portions were stored in liquid nitrogen. At this stage the volume was 50 ml for the succinate and malate cells and 100 ml for the glucose-PAB cells.

TABLE II

Fractionation of Tissue Suspensions

This table provides data to supplement the general description of procedures discussed in the text

	Succinate cells	Malate cells	Glucose cells
O.D.$_{430}$ before sonication	8.5	15.5	5.4
O.D.$_{430}$ after sonication	5.7	10.6	3.5
Volume of suspension fractionated	5 ml	10 ml	10 ml
mg protein/ml	9.8	27.5	7.3
Fractionation			
Centrifuge 10 min at 3500 *g*			
Pellet (P_1)			
Supernatant fraction S_1			
Concentration mg protein/ml	8.9	26.6*	7.1
Dilute S_1 with 9 vol of "suspending medium" and centrifuge 10 min at 3500 *g*			
Pellet P_1': protein % of S_1	6%	3%	3%
mg protein/ml	0.55	1.0	0.40
Supernatant fraction S_1': protein % of S_1	93%	97%	93%
Centrifuge S_1' 15 min at 20000 *g*			
Pellet P_2: protein % of S_1	27%	14%	14%
mg protein/ml	2.4	2.1	0.82
Supernatant fraction S_2: protein % of S_1	66%	75%	67%
Centrifuge S_2 60 min at 105,000 *g*			
Pellet P_3 : protein % of S_1	24%	18%	10%
mg protein/ml	2.7	3.7	1.1
Supernatant fraction S_3 : protein % of S_1	40%	60%	55%
Concentrated S_3			
Centrifuge undiluted S_1 directly for 60 min at 105,000 *g*-			
protein conc. mg/ml	5.3	20.5	4.0

* The reasons why more protein is present in the "malate" cells than the corresponding succinate cells are: 1) 4 liters of "malate cells" were grown compared to 3 liters of succinate cells. 2) During spheroplasting the optical density in the "malate" culture increased whereas that in the succinate culture decreased.

Preparation of the Fractions (Table II)

The frozen suspension was thawed in warm water and a 25% vol of "C" salts medium was added. The "C" medium contained: 6 g Na_2HPO_4, 3 g NaCl, 110.9 mg Na_2SO_4, 3.86 g (4×10^{-4} M) $MgCl_2 \cdot 6H_2O$ per liter. The suspension was placed in a small beaker in ice and a stream of nitrogen gas was played over the surface. A small amount of Dow Corning AF antifoam agent (Dow Corning Corp., Midland, Mich.) was placed high on the inside surface of the beaker. It was found that sonication caused a change in optical density with a maximum at 430 mμ, and this reading, determined with a Beckman Model DU Spectrophotometer, was used to monitor the progress of the sonication treatment. A Branson sonifier model S-75 was used at power setting of 8 for two successive 20 sec treatments. The optical density decreased during this treatment to about 66% of the starting value. This suspension was centrifuged at 3500 *g* for 10 min to remove whole cells and large fragments (P_1) and to produce supernatant fraction S_1 which represents the complete acellular preparation. Subsequent stages of centrifugation to obtain pellets p_1', P_2, and P_3, are shown in Table II. These pellets were resuspended in "suspending medium" and stored in small portions in liquid nitrogen. "Suspending medium" was prepared by mixing "storage" and "C" salts media in equal parts. A concentrated soluble fraction was obtained by centrifuging undiluted S_1 for 60 min at 105,000 *g*. This fraction "S_3" was then stored in small portions in liquid nitrogen. Protein concentrations for the various

fractions were determined by the method of Lowry et al. (13) and are shown in Table II.

Enzyme Assays

OXYGEN UPTAKE STUDIES: A model #4004 Clark oxygen electrode purchased from Yellow Springs Instrument Co. was used in all studies. The basic electronic circuit has been previously described (14); however, in accord with a suggestion made by Dr. W. W. Kielley, the indicated sensitivity control was replaced by a 10 turn, 50,000 ohm Helipot. Either a 1 ml or a 2 ml glass cuvette was used and the electrode fitted with a rubber "O" ring sealed the vessel. A slanted side arm port with an opening 1 mm in diameter and 15 mm in length was used for making additions to the vessel. Such insertions were made with micro syringes. The vessel was maintained at 30° and the contents were stirred continuously with a magnetic flea during the assay. The electrode response was calibrated for the system by the oxidation of known quantities of NADH according to the method of Chappel (15). The buffer was 0.011 M histidine in 0.002 M $MgSO_4$ titrated to pH 6.5 with H_2SO_4 (16). This buffer was chosen on the basis of several considerations. A low tonicity was desired to minimize problems of permeability of NADH and possibly other substrates into membrane vesicles, such as has been encountered with mammalian mitochondria. A nonphosphate buffer was desired because if high energy intermediates formed during respiration could be used directly for work, the unavailability of phosphate might favor such processes over possible adenylate phosphorylation mechanisms. NADH oxidase and succinate dehydrogenase, two activities considered fundamental in electron transport systems, were found to exhibit optimal activities in this buffer system. With the additions necessary for the spectrophotometric assay the pH of the reaction mixture was 6.8 and the buffering capacity was sufficient to limit the rise in pH due to the addition of 1 μmole of KOH to 0.1 pH unit.

In all cases the appropriate *E. coli* fraction in aliquots from 5 to 100 μl, plus buffer to 1 ml final vol, was used to establish endogenous rates which usually amounted to about 0.5 μg atoms oxygen/10 min/10 mg protein. Reactions were started by injecting substrate in a volume of 10 μl, and the new rate of oxygen uptake was followed for several minutes. Reported values have been corrected for endogenous rates. The final concentrations for NADH and NADPH were 1 mM. All other substrates (neutralized before use) were present at a 5 mM concentration. Malate and isocitrate oxidations were markedly stimulated by the addition of 50 μl of a cofactor mixture which contained adenosine triphosphate (ATP) at 0.2 M, nicotinamide adenine dinucleotide (NAD) at 0.08 M, nicotinamide adenine dinucleotide phosphate (NADP) at 0.08 M, and nicotinamide at 0.4 M, all neutralized to pH 7.0. Similarly, pyruvate and α-ketogluterate oxidation responded to 50 μl of another cofactor mixture which contained DL-α-lipoic acid at 0.1 mM, cocarboxylase at 0.8 mM, CoA at 2.6 mM, cysteine at 0.128 M, NAD at 0.04 M, ATP at 0.04 M and guanosine diphosphate (GDP) at 0.02 M. The reported rates with each of these substrates were obtained in the presence of the appropriate cofactor mixture. For any particular fraction and a given substrate the same rate of oxygen uptake per unit weight of protein was observed with different amounts of enzyme, and for 1 or 2 ml of reaction volumes whenever these parameters were varied.

SPECTROPHOTOMETRIC ASSAYS: Enzyme assays were based on the rate of change of optical density at 340 mμ when pyridine nucleotides were the electron donor or acceptor, on the rate of change at 600 mμ when 2,6-dichlorophenol indophenol (DCPIP) was employed as electron acceptor, or on the rate of change at 425 mμ when ferricyanide was used. The millimolar extinction coefficient was taken as 6.22 for reduced pyridine nucleotides (17), 20 for DCPIP (18), and 1.0 for ferricyanide (19).

The complete reaction mixture consisted of 1 to 50 μl of appropriate suspension, additions as indicated in Table III, and buffer to a final volume of 1 ml. Assays were performed with a Beckman Model DU spectrophotometer with Gilford Model 2000 accessories or with a Cary Model 16K automatic recording spectrophotometer. The initial rates were established for the complete mixture minus an essential component such as substrate, cofactor, or enzyme. After the final addition the new rate was established and background corrections were made as required. The following is a list of additions frequently used but not all additions were made in every case (see Table III): 1) 0.1 ml of neutralized substrate to give a final concentration of 5 mM except for NADH or NADPH which because of their high molar extinction coefficients were usually present at 0.4 mM; 2) 30 μl of HCN freshly generated within 3 hr prior to use by mixing equal volumes of 0.8 N KCN and 0.8 N H_2SO_4; 3) in the cases of malate, isocitrate, and glutamate dehydrogenases, 50 μl of the same cofactor mixture described in the oxygen uptake section was used; 4) when DCPIP was the electron acceptor, 0.1 ml of a 0.15% solution in 0.05 M K_2SO_4 was added, except in the case of NADH dehydrogenase when 0.3 to 0.4 ml was used. All reactions were run at 30°.

Specific enzyme activity is expressed as μmoles of substrate oxidized or reduced per 10 min per 10 mg protein or as μg atoms of oxygen taken up per 10 min per 10 mg protein (20).

TABLE III

Special Features of Spectrophotometric Assays

Further details concerning the additions can be found in the text

Enzyme	Wave length of assay	Substrate	Cofactor that caused maximal stimulation	12 mM HCN X = present	2,6-Dichlorophenol Indophenol (DCPIP) X = present
	mμ				
NADH oxidase	340	NADH	None	None	None
NADH dehydrogenase (λ600)	600	NADH	None	X	X
NADH dehydrogenase (λ425)	425	NADH	None	X	None*
Succinate dehydrogenase	600	Succinate	None	X	X
Malate dehydrogenase (λ340)	340	Oxalacetate‡	NADH‡	X	None
Malate dehydrogenase (λ600)	600	Malate	Cofactor mix or NAD	X	X
Isocitrate dehydrogenase (λ340)	340	Isocitrate	Cofactor mix or NADP	X	None
Isocitrate dehydrogenase (λ600)	600	Isocitrate	Cofactor mix or NADP	X	X
Glutamate dehydrogenase (λ340)	340	α-ketoglutarate + 0.05 M NH_4Cl	Cofactor mix or NADH	X	None
Glutamate dehydrogenase (λ600)	600	glutamate	Cofactor mix or NAD	X	X
NADPH oxidase	340	NADPH	None	None	None

* Neutralized potassium ferricyanide (1mM) was present.

‡ It is important to note that upon storage both oxalacetate and NADH may develop inhibitors for this assay. In such circumstances fresh solutions will again elicit full activity from the enzyme system.

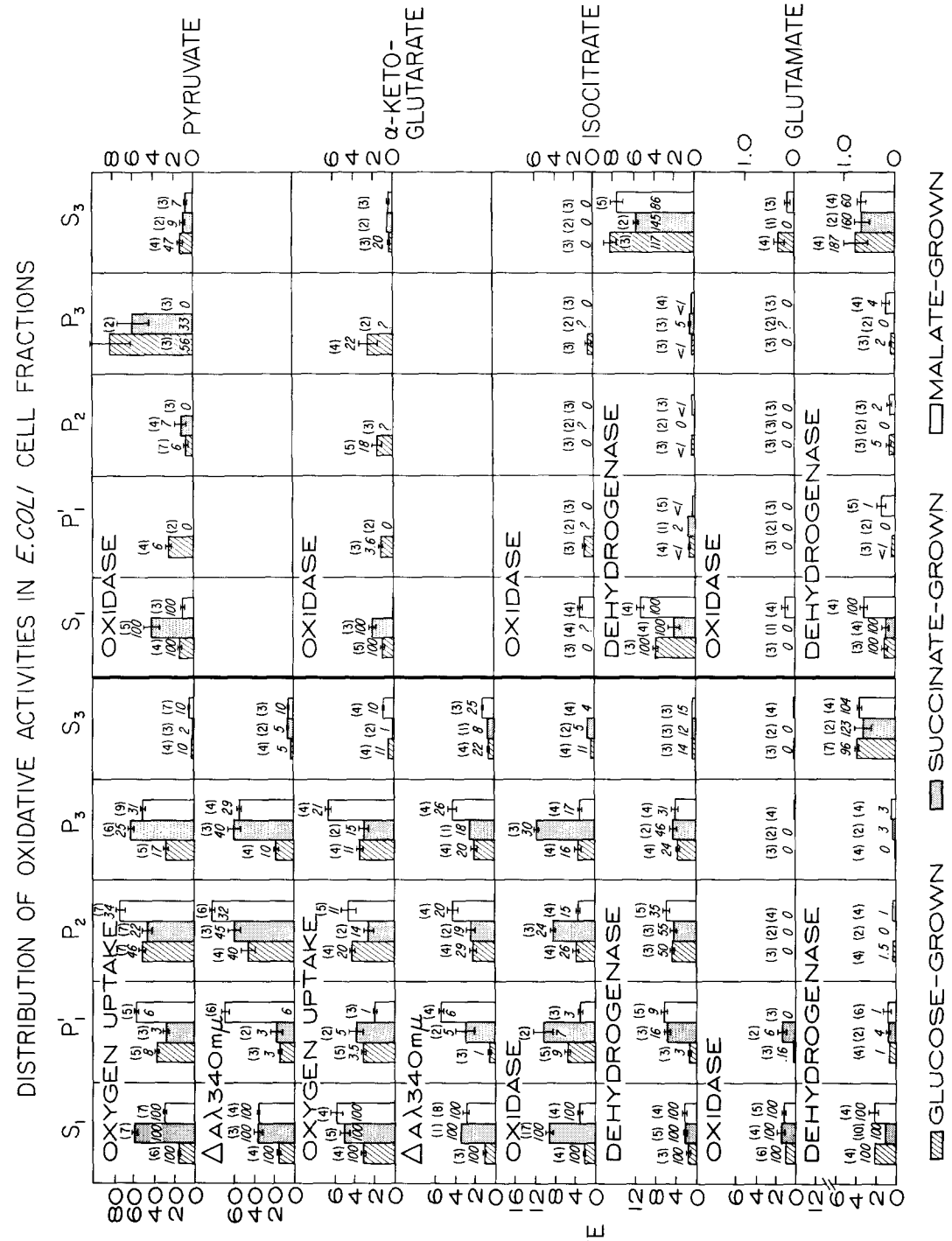
COMMERCIAL ENZYMES: Malate dehydrogenase and isocitrate dehydrogenase were pork heart preparations purchased from Sigma Chemical Co., St. Louis, Mo. Glutamate dehydrogenase, isolated from bovine liver, was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

General Characteristics of Respiratory Activities

RESPIRATORY CONTROL: Bacteria in general (21) and *E. coli* in particular (20, 22) do not exhibit

FIGURE 1 Distribution of oxidative activities among cell fractions from *E. coli* grown on different energy sources. The "striped" bars indicate growth of cells in glucose-Penn Assay Broth, the "stippled" bars indicate growth in succinate, and the clear bars indicate growth in malate. The heights of the bars depict specific activities as indicated by the ordinate scale. The unit for specific activity is micromoles of substrate oxidized per 10 min per 10 mg of protein. Numbers in the bars indicate per cent distribution of total enzyme activity based on the amount of enzyme in fraction S_1 as 100%. Table II shows details for obtaining the various fractions indicated at the top of the vertical columns. The number of determinations for each activity is shown in parenthesis above the bars, and the standard error of the mean is indicated by the "I" at the top of each bar. Note the difference of scale for the ordinates in the various assays shown. The specific details for each enzyme determination are given in the Methods section and Table III. For malate and isocitrate dehydrogenases the methods based on dichlorophenol indophenol reduction were used. NADH and NADPH oxidases were measured by oxygen uptake and by the change in optical density at 340 mμ. Substrate concentration was 1 mM in the oxygen uptake assay and 0.4 mM in the spectrophotometric assay.


DISTRIBUTION OF OXIDATIVE ACTIVITIES IN E.COLI CELL FRACTIONS
NADH
NADPH
SUCCINATE
MALATE
OXYGEN UPTAKE
ΔAλ340mμ
OXIDASE
DEHYDROGENASE
PYRUVATE
α-KETO-GLUTARATE
ISOCITRATE
GLUTAMATE
S1
P'1
P2
P3
S3
GLUCOSE-GROWN
SUCCINATE-GROWN
MALATE-GROWN

respiratory control. The low tonicity medium used in this work would not be expected to preserve such control if it were initially present. The rates of oxygen uptake observed with the substrates NADH, NADPH, succinate, pyruvate and α-ketoglutarate in the S_1, P_2 and P_3 fractions obtained from succinate-grown cells were not stimulated by the addition of 1 μmole ADP in the presence of added phosphate (0.02 M). In fact such addition usually depressed oxygen uptake by about 30%. Similarly, the addition of 10^{-4} M 2,4-dinitrophenol did not stimulate oxygen uptake. These results show that the respiratory rates measured in the present work were not limited by the absence of phosphate acceptor. The rates of oxidation of NADH and succinate were not stimulated by the addition of 20 μmoles of phosphate and so the absence of phosphate in the medium does not seem to affect these respiratory indexes. On the other hand, phosphate did stimulate oxygen uptake when the substrate was either pyruvate or α-ketoglutarate.

ACTION OF INHIBITORS AND UNCOUPLERS: Slater has summarized the effects of various inhibitors and uncouplers on mammalian mitochondrial respiration (23). That information provides a means for comparing the relative sensitivies of the respiratory chains in higher organisms and *E. coli*. Unless otherwise noted, S_1 from succinate-grown cells was tested and oxygen uptake was measured.

1. Rotenone: 0.03 μmole per gram protein is sufficient to inhibit the oxidation of NAD-linked substrates by rat liver mitochondria (23). In the present studies we have found no effect on the rate of NADH oxidation in the range of 0.068 to 13.6 μmoles per gram protein.

2. Antimycin: Inhibitions with rat heart mitochondria are usually obtained with 400 μg per gram of protein, whereas for rat liver 100 μg per gram protein is sufficient. In the present studies it was found that succinate oxidation was not adversely affected by antimycin in the range of 540 to 10,800 μg/g protein.

3. Cyanide: NADH oxidase of Keilen and Hartree heart muscle preparations is inhibited 93.9% by 0.33 mM cyanide, 93.3% by 1 mM cyanide, and 99.6% by 10 mM cyanide (23). With the S_1 fraction from malate-grown cells, it was found that NADH oxidase was inhibited 82% by 4 mM cyanide, 88% by 8 mM cyanide, 90% by 12 mM cyanide, and 94% by 20 mM cyanide. Succinate oxidation in this system was inhibited 17% by 4.8 mM cyanide, 76% by 8 mM cyanide, 90% by 12 mM cyanide, and 97% by 20 mM cyanide.

4. Azide: Chance and Williams reported that 3.9×10^{-4} M azide inhibited the respiration of guinea pig mitochondria 80% with succinate as substrate (24). Azide in the range of 1 to 5×10^{-4} M showed no inhibitory effect on either NADH or succinate oxidation with S_1 fractions from succinate-grown cells. Higher concentrations were tried with S_1 fractions from malate-grown cells, and it was found that 1×10^{-2} M azide did not inhibit NADH oxidation but did inhibit succinate oxidation by 25%.

5. Oligomycin: 0.085 to 0.170 mg per gram of rat liver mitochondria will cause 70 to 100% inhibition of respiration coupled to phosphorylation, depending on the substrate. No inhibition of oxygen uptake was found in the range of 0.27–5.4 mg per gram protein with either NADH or succinate as substrate in the studies with *E. coli* cell fractions. This finding is not unexpected in view of the apparent lack of respiratory control.

6. Chloramphenicol: Freeman and Haldar (25) have reported that at 10^{-3} M this antibiotic inhibits NADH oxidation by beef heart mitochondria by 50% and that 6×10^{-3} M will almost completely abolish the oxidation. On the other hand, succinate oxidation by rat liver mitochondria was almost insensitive to a 6×10^{-3} M chloramphenicol concentration at the same time that α-ketoglutarate oxidation was virtually eliminated. The authors argue for a specific effect at the level of NADH dehydrogenase. We have found that NADH oxidation was inhibited 25% at 1×10^{-3} M and 43% at 3×10^{-3} M but succinate oxidation was not affected at the higher concentration.

7. Bovine serum albumin: The unusual finding was made that defatted serum albumin at a concentration of 0.01 mM completely inhibits succinoxidase, inhibits succinate dehydrogenase only 10%, and has no effect on NADH oxidation.

OXIDATIVE ACTIVITIES AS INFLUENCED BY GROWTH HISTORY: Fig. 1. illustrates the levels of various oxidative enzyme activities found in the initial sonicated suspensions (S_1) obtained from cells grown in succinate, malate, or glucose-PAB medium and in the particulate and soluble fractions obtained from these suspensions. Several interesting features become apparent upon examination of this figure: a. Some activities appear to be almost completely associated with particulate fractions. In this group are included: NADH oxidase, succinate dehydrogenase, succinoxidase, NADPH oxidase, pyruvate oxidase, and α-ketoglutarate oxidase.

b. Some activities are found exclusively in the soluble fraction. In this group are included malate dehydrogenase, isocitrate dehydrogenase, and glutamate dehydrogenase.

c. If the "Glucose-PAB" cells are taken as a standard of reference, it can be seen that growth in succinate caused a marked increase in succinoxidase and NADH oxidase, but caused no corresponding increase for enzymes concerned with the oxidation of other Krebs cycle intermediates, namely malate and isocitrate.

d. Although growth in succinate markedly increased the levels of enzymes capable of oxidizing NADH and succinate, growth in malate caused no such increase in the levels of enzymes involved in the oxidation of malate itself or of succinate, and isocitrate.

e. The P_3 fraction, which is normally considered to be a ribosomal fraction, is rich in content of oxidative enzymes. This is particularly evident in the case of succinate-grown cells where this fraction exhibited the highest specific activity for the oxidations of NADH and succinate.

f. Although malate served as an excellent carbon and energy source for growth under conditions of vigorous aeration, enzymes for oxidizing malate by oxygen seem to be virtually inactive in the acellular fractions.

Several of these findings seem contrary to what might be expected and were pursued further. The clarification obtained from these studies comprises the main part of the remainder of this report.

On the Presence of High Levels of Oxidative Activity in the P_3 Fraction

An aliquot of "succinate" P_3, containing 2.12 mg protein, 13.1 units[2] of NADH oxidase and 2.44 units[2] of succinoxidase in a volume of 0.8 ml was placed on top of 26 ml of a 5–20% sucrose gradient in a Spinco centrifuge tube. The tube was centrifuged for 3 hr at 55000 *g* in a SW 25.1 head in a Spinco Model L ultracentrifuge. The tube was emptied through a needle puncture at the bottom and 0.7 ml fractions were collected. The distribution of material absorbing at 260 mμ wavelength is shown in Fig. 2. The free ribosome peak is seen in fractions 28–38. A pellet containing 1 mg protein was also obtained. Each fraction was assayed for succinoxidase and NADH oxidase activity but none was found other than in the pellet. The pellet contained 59% of the applied NADH oxidase and 21% of the original succinoxidase. The less than full recovery may be attributed to loss of activity due to the high sucrose concentration and the freezing and thawing of the pellet material before assay.

A sample of pellet material was taken for electron microscopy and a representative micrograph

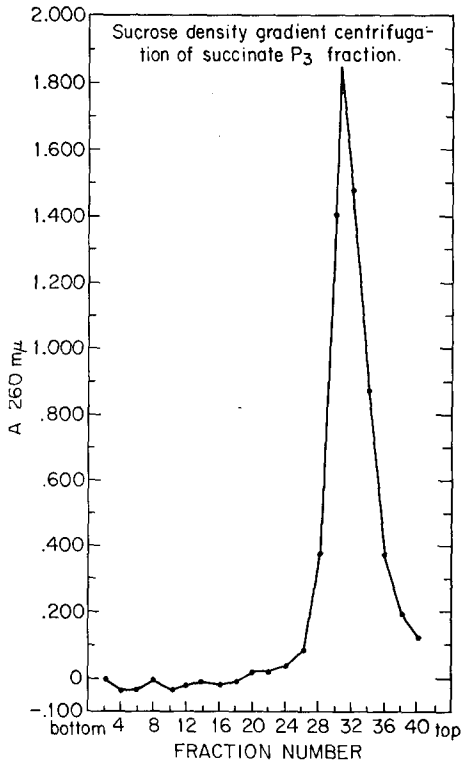


FIGURE 2 Sucrose density centrifugation of Succinate-P_3 fraction to separate free ribosomes from membrane vesicles.

An aliquot of 0.8 ml of succinate P_3 fraction containing 2.12 mg protein, 13.1 units[2] of NADH oxidase and 2.44 units[2] succinoxidase was placed on top of 26 ml of a 5–20% sucrose gradient and centrifuged for 3 hr at 55,000 *g* in a Spinco Model L ultracentrifuge. The tube was emptied by a needle puncture at the bottom and 0.7 ml fractions were collected. The bottom is represented by fraction #1 and the top by fraction #40. The peak in tube 31 represents free ribosomes. Oxidase activity was recovered only from the pellet. An electron micrograph of a portion of this pellet material is shown in Fig. 3.

[2] Specific Activity unit equals μmoles of substrate oxidized or reduced per 10 min per 10 mg protein.

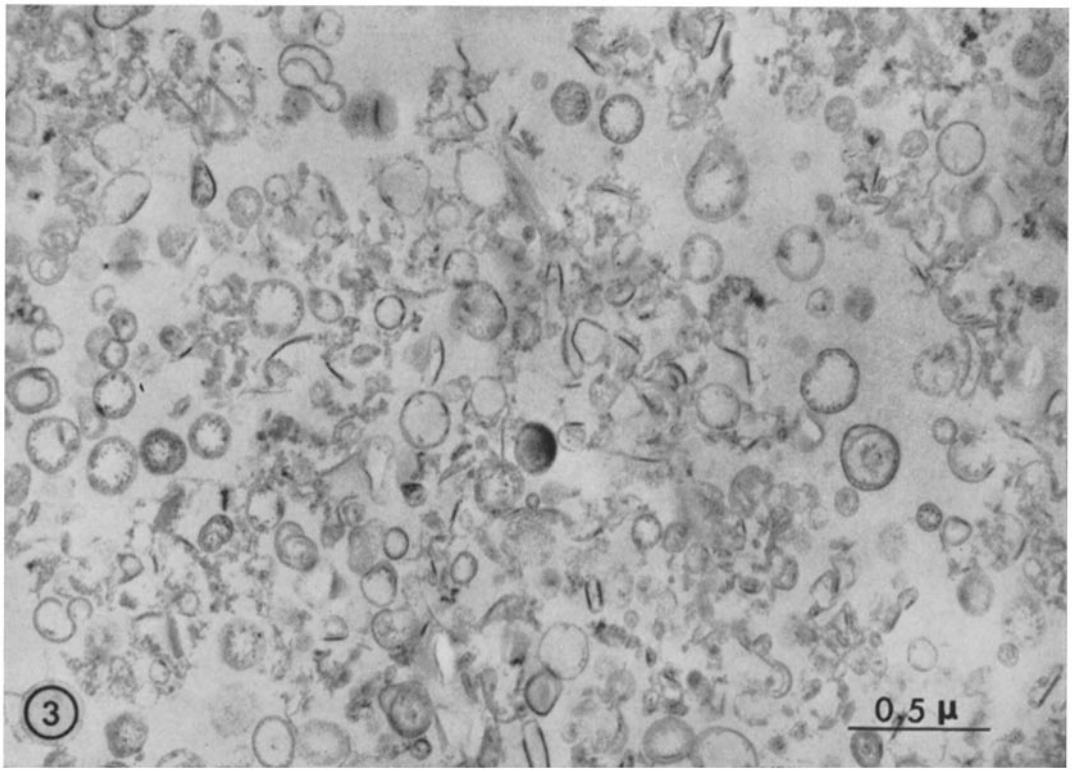


FIGURE 3 The pellet obtained in the experiment shown in Fig. 2 was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer pH 6.8 followed by postfixation in 1% osmium tetroxide, dehydrated in graded solutions of ethanol, and then embedded in Epon. The black bar represents 0.5 µ. × 36,000. This micrograph was taken by Dr. Blair Bowers of the National Heart Institute.

is shown in Fig. 3. The pellet is seen to consist of small vesicles and cell fragments. The presence of this material in the ribosome (P_3) fraction explains the high oxidase activity.

COFACTOR REQUIREMENTS: To the extent that malate or isocitrate oxidation by oxygen was observed in this work, it was dependent upon the addition of the cofactor mixture containing NAD, NADP, ATP, and nicotinamide. Marked stimulation of dehydrogenation of malate, isocitrate and glutamate also resulted upon the addition of this cofactor mixture. Table IV shows that for malate dehydrogenase activity assayed either by malate oxidation (DCPIP–600 mµ) or by oxalacetate reduction (NADH–340 mµ), NAD was far superior to NADP. The coenzyme specificity was less pronounced when the dehydrogenases were measured by DCPIP reduction than when they were measured by coenzyme reduction. However, in the DCPIP assay, addition of NADP did not enhance the rate of DCPIP reduction by malate dehydrogenase when 0.4 mM NAD was already present. Similarly, NAD addition did not enhance dye reduction by isocitrate dehydrogenase when 0.4 mM NADP was already present.

In view of this lack of additivity of coenzyme stimulated activities for each dehydrogenase in the DCPIP assay and the almost complete coenzyme specificity exhibited in the assay measuring reduced coenzyme directly, it would appear that an NAD-specific malate dehydrogenase and an NADP-specific isocitrate dehydrogenase were present.

A possible explanation for the partial stimulatory effect of the "other" coenzyme in the DCPIP assay is as follows: A small amount of endogenous coenzyme is continuously cycled between substrate and dye. If rapid hydrolysis of the coenzyme can also occur when the solution is warmed, the rate of dye reduction may be limited by the low

TABLE IV

Cofactor Requirements

Additions	GS_3* Specific Activity§	GS_3* % ± S.E.M.‖	GS_3* No. of Exp.	MS_3‡ Specific Activity§	MS_3‡ %± S.E.M.‖	MS_3‡ No. of Exp.
	Malate dehydrogenase (DCPIP–600 mμ)					
None		6.7±3.4	3		38±6	4
+cofactor mixture¶	6.2±0.3	100	3	5.1+0.4	100	4
+NADP		24±1.5	3		48±6	4
+NAD		85±11	3		130±21	4
+ATP		5.3±1.2	3		9±0	2
+Nicotinamide		10±6	3		36±5	4
	Malate dehydrogenase (NADH–340 mμ)					
+NADPH		3.8±6	3		4.8±1.1	3
+NADH	38–88**	100	3	51–132**	100	3
	Isocitrate dehydrogenase (DCPIP–600 mμ)					
None		6.7±1.8	3		10±4	6
+cofactor mixture¶	13±.4	100	3	10.4±4	100	6
+NADP		134±7	3		139±7	6
+NAD		17±1	3		57±11	6
+ATP		6.3±1.2	3		5.3±1.7	6
+Nicotinamide		6.5±0.5	2		44±10	6
	Isocitrate dehydrogenase (NADP–340 mμ)					
+NADP	16.0±1	100	3	15.7±2	100	3
+NAD		3.7±0.9	3		<1±<1	3

* S_3 fraction from glucose-PAB cells.

‡ S_3 fraction from malate-grown cells.

§ Micromoles substrate oxidized or reduced per 10 min per 10 mg protein.

‖ % activity compared to activity in the presence of the cofactor mixture or the preferred coenzyme ± standard error of the mean.

¶ Cofactor mix: 10 mM ATP, 4 mM NAD, 4 mM NADP, 20 mM nicotinamide. When tested individually, the same concentration of each cofactor was used.

** As discussed in the text, the assay malate dehydrogenase (NADH–λ340), can be influenced by tissue concentration and the freshness of the oxalacetate and NADH solutions. The relative efficiency of utilization of NADPH in this assay, however, seemed to be unaffected by these variations.

level of appropriate coenzyme. The addition of a relatively large amount of the "other" coenzyme may protect the endogenous cofactor from loss and thereby maintain a higher rate of dye reduction than would be observed in the absence of such addition.

FURTHER STUDIES ON MALATE OXIDATION: Table V (column 3) shows that porcine heart malate dehydrogenase will not catalyze the reduction of DCPIP by malate plus NAD. Porcine malate dehydrogenase in the presence of S_3 fraction from malate-grown *E. coli*, exhibits nearly full activity when assayed by the reduction of oxalacetate by NADH (column 2) but almost no activity when assayed with DCPIP (column 4). *E. coli* malate dehydrogenase can be assayed both by

TABLE V

Relative Abilities of Mammalian and E. coli Dehydrogenases to React with Coenzymes and 2,6-dichlorophenol Indophenol

Compond Assayed Spectrophotometrically	Coenzyme		2,6-dichlorophenol indophenol (DCPIP)	
	1	2	3	4
Enzyme	Alone	+"S_3"	Alone	+"S_3"
1. Malate dehydrogenase (NAD)	Oxalacetate + NADH*		Malate + NAD + DCPIP (ox)*	
	Malate + NAD		Oxalacetate + NAD + DCPIP (red)	
Porcine (heart)‡	100§	86	0	8
E. coli malate "S_3"	100	—	100	—
2. Isocitrate dehydrogenase (NADP)	Isocitrate + NADP*		Isocitrate + NADP + DCPIP (ox)*	
	Oxalsuccinate + NADPH		Isocitrate + NADP + DCPIP (red)	
Porcine (heart)‡	100	110	13	46
E. coli malate "S_3"	100	—	100	—
3. Glutamic dehydrogenase (NAD)	α-ketoglutarate + NH_4 + NADH*		Glutamate + NAD + DCPIP (ox)*	
	Glutamate + NAD		α-ketoglutarate + NH_4 + NAD + DCPIP (red)	
Bovine (liver) ‖	100	70	0	?
E. coli malate "S_3"	0	—	100	—

* The equations represent the assay systems. Reactants are on top lines and products are on lower lines.

‡ Sigma Chemical Co., St. Louis. Mo.

§ Equal enzyme activities for purified enzyme and *E. coli* fraction S_3 as measured by the oxidation of reduced coenzyme are arbitrarily set at 100%. The reactivity with dye of this amount of *E. coli* S_3 enzyme is also set as 100%. The percents in columns 2 and 4 show what part of the full activity (in the particular assay) was obtained when an equal (100%) quantity of purified enzyme was added to the *E. coli* S_3 fraction.

‖ Calbiochem, Los Angeles, Calif..

reduction of oxalacetate by NADH and by oxidation of malate by DCPIP via NAD.

Why can the *E. coli* malate dehydrogenase react with DCPIP whereas the porcine malate dehydrogenase cannot react even when it is assayed in the *E. coli* extract? The obvious possibility that *E. coli* malate dehydrogenase may react directly with either DCPIP or NAD seems unlikely because the reaction with dye is greatly stimulated over endogenous levels by the addition of NAD (Table IV). The transfer of electrons from NADH to DCPIP requires some other factor such as an NADH dehydrogenase or other substance that can be reduced by NADH and oxidized by DCPIP. The factor may be free from the bacterial malate dehydrogenase or bound to it in the form of a complex. The fact that porcine malate dehydrogenase when added to the soluble bacterial extract does not react with the dye argues for the existence of a complex *unless* the free factor is already saturated by malate dehydrogenase or otherwise is not available to the NADH produced by the porcine enzyme.

When 0.4 mM NADH was added to a reaction system containing malate, NAD, DCPIP, and the *E. coli* extract, the rate of dye reduction was increased 550%. Therefore, the "factor" which can link *free* NADH to dye was not saturated. In fact, with the concentration of DCPIP employed in these studies, this bacterial extract contained sufficient NADH:DCPIP reductase activity (NADH dehydrogenase) to reduce dye at the rate of 40 μmoles per 10 min per 10 mg protein. Dye reduction with bacterial malate dehydrogenase

TABLE VI

*Comparative Assays for Three Redox Enzymes**

All activities are expressed as μmoles of substrate converted per 10 min per 10 mg protein

System	Oxalacetate/Malate		α-Ketoglutarate/Glutamate		Oxalsuccinate/Isocitrate	
Electron Donor:	NADH	Malate	NADH	Glutamate	Isocitrate	Isocitrate
Electron Acceptor:	Oxalacetate‡	DCPIP	α-ketoglutarate	DCPIP	NADP	DCPIP
Enzyme Source						
MS_1	200	2.1	0	0.6	8	5.4
MS_3	440	3.6	0–1	0.6	15	7.7
MP_2	1	0.20	0	0.1	0	0.1
MP_3	3	0.4	0	0.2	0	0.2
GS_1	120	2.2	0	0.2	6	3.9
GS_3	220	3.8	0–1	0.8	14	8.3
GP_2	1	0.2	0	0.1	0	0.2
GP_3	3.5	0.1	0	0.1	0	0.1

* Conditions for assays described in methods section.

‡ This assay was influenced by freezing and thawing and the concentration of the tissue fractions as discussed in the text. The activities listed here represent the higher values obtained in a preparation that had been subjected to freeze and thaw treatment.

proceeded at a rate of only 2 μmoles per 10 min per 10 mg protein. If free NADH is an intermediate in this reaction, the limiting rate in the overall process is the rate of formation and of release of NADH. If the porcine enzyme has a higher affinity for the reduced coenzyme than does the bacterial enzyme, the subsequent slower release of NADH from the dehydrogenase would explain the lack of reactivity of the porcine enzyme-generated NADH with the dye. A possibly similar situation has recently been explored in *Mycobacterium phlei* (26) where it was shown that the ability of the bacterial malate dehydrogenase to cause DCPIP reduction was explainable in terms of two separate and soluble enzymes (i.e., malate dehydrogenase and NADH dehydrogenase).

On the other hand, a complex involving *E. coli* malate dehydrogenase and "factor" could also explain the observations. Such a complex would permit more efficient utilization of the reducing equivalents generated by the bacterial enzyme (than by the porcine enzyme) for dye reduction. Although this alternative is more complicated than the former, it can not be rejected at this point.

A qualitatively similar situation exists for the isocitrate system. There was too little activity for glutamate dehydrogenase in S_3, assayed by reduction of α-ketoglutarate in the presence of ammonia, to establish a reliable equivalence of enzyme amounts for the purified enzyme and S_3. In the absence of such information it was not possible to see what per cent of the glutamate dehydrogenase added to S_3 could be assayed with DCPIP.

FURTHER CONFIRMATION OF THE ABSENCE OF MALATE DEHYDROGENASE ACTIVITY IN PARTICULATE FRACTIONS: Since the ability of malate dehydrogenase to be assayed by DCPIP appears to depend upon the participation of another factor, the possibility remained that malate dehydrogenase activity was present in the particulate fractions but that "factor" was either missing or less efficient in coupling malate dehydrogenase activity to DCPIP reduction. This complication was averted by assaying for dehydrogenase activity at 340 mμ in the absence of dye. This was also done for isocitrate and α-ketoglutarate dehydrogenases.

Table VI shows that the distributions of activities obtained by this method were essentially the same as those obtained by the technique based on DCPIP reduction. That is, these activities were found exclusively in the soluble fractions. The much higher values obtained for malate dehydrogenase activity by the oxalacetate reduction assay than by the malate oxidation assay reflect the equilibrium constant for this reaction which favors malate.

INFLUENCE OF CONCENTRATION OF CELL FRACTION ON DEHYDROGENASE ACTIVITIES: Specific activities remained essentially constant over a wide range of tissue concentrations for

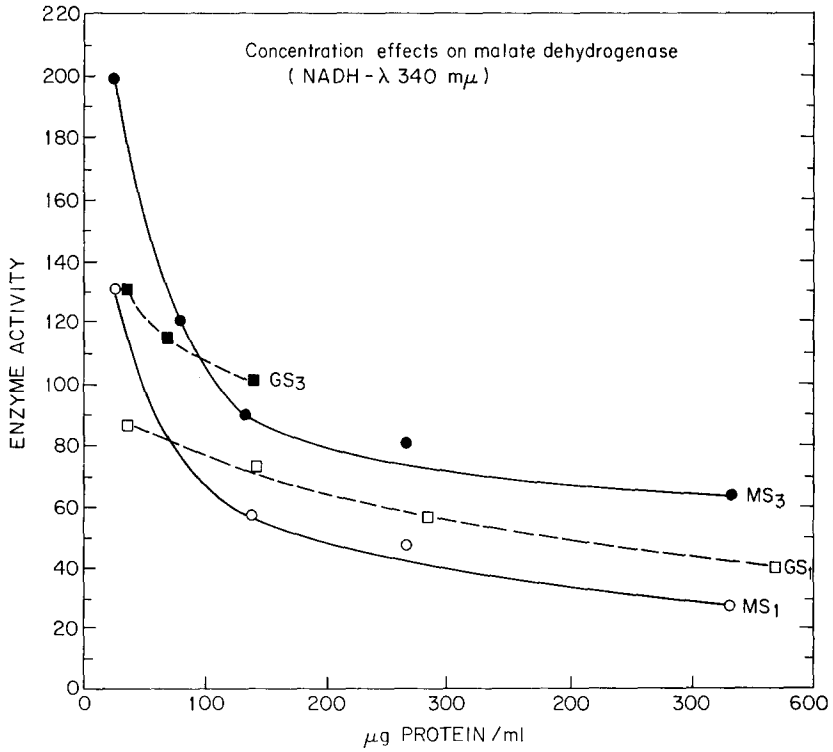


FIGURE 4 Change in activity of malate dehydrogenase (NADH-340 mμ) as a function of tissue concentration. The units for the ordinate are μmoles of NADH oxidized (or of oxalacetate reduced) per 10 min per 10 mg of protein. "MS_1" and "GS_1" represent the S_1 fractions from malate- and glucose-grown cells, respectively. "MS_3" and "GS_3" represent the S_3 fractions from the respective cells.

malate dehydrogenase (DCPIP: 600 mμ), and isocitrate dehydrogenase (NADP: 340 mμ); and they were comparable to the values shown in Table VI. On the other hand, malate dehydrogenase (NADH: 340 mμ) showed erratic behavior. With most preparations the apparent specific activity decreased with increasing tissue concentration as shown in Fig. 4. When a preparation was reused after having been returned to liquid nitrogen for storage, it sometimes happened that the specific activity was significantly higher and that the dehydrogenase activity became less sensitive to changes in tissue concentration. If an enzyme complex does exist for malate dehydrogenase it may be restricted in its ability to accept oxalacetate and NADH. Dilution or freezing and thawing, then, could cause a conformational change that facilitates reaction with oxalacetate and NADH. We have found that a preparation of MS_3 with an activity of 100 units[2] at a concentration of 0.04 mg protein/ml could be raised to an activity of 360 units[2] by diluting to 0.002 mg protein/ml or to an activity of 310 units by freezing in liquid nitrogen and thawing three times.

Commercial pig heart malate dehydrogenase showed no concentration effects when assayed by the change in absorbance at 340 mμ.

NADH DEHYDROGENASE AND NADH OXIDASE: Minakami et al. (19) have called attention to the sensitivity of mammalian mitochondrial NADH oxidase and dehydrogenase to NADH concentration in the range of 0.1 to 1.2 mM. Maximal activity was obtained at about 0.15 mM, and further addition of substrate caused inhibition. They also showed that the reaction was markedly dependent upon the concentration of the dye 2,6-dichlorophenol indophenol so that even at high dye concentrations ($A_{600m\mu}$ = 3.0) the dehydrogenase was still not saturated. They cautioned against using data obtained in this system for the evaluation of kinetic constants and suggested that ferricyanide was a preferred electron acceptor to be

TABLE VII
Distribution and Activities of NADH-Oxidizing Enzymes

Numbers represent μmoles of NADH oxidized per 10 minutes per 10 mg protein. NADH concentration was 1.5 mM for ferricyanide reduction, 0.4 mM for DCPIP reduction, 0.4 mM for the spectrophotometric oxidase determination, and 1.0 mM for the oxygen uptake studies. Starting absorbance was 3.8 optical density units for DCPIP. The Cary 16K spectrophotometer was used and the extinction coefficient at this high DCPIP concentration was the same as observed at all lower concentrations

	Dehydrogenase		Oxidase	
Cell fraction	Ferricyanide	2,6-Dichlorophenol Indophenol	Spectrophotometric	Oxygen uptake
MS_1	685	120	30	36
MP_2	102	59	73	82
MP_3	71	53	51	55
MS_3	920	87	5.1	5.9
GS_1	770	98	15.6	15.3
GP_2	47	26	51	46
GP_3	52	45	27	19
GS_3	1335	150	2.7	1.3

used for assaying NADH dehydrogenase. We have found that the assay of our NADH oxidase at 0.4 mM NADH in the spectrophotometric technique and at 1.0 mM NADH in the oxygen electrode technique gave similar values (Fig. 1 and Table VII). In the ferricyanide assay of dehydrogenase with MS_3 we have found that the activity at 0.2 mM NADH was significantly lower than at 0.4 and at 0.8 mM NADH. Therefore, the *E. coli* NADH oxidizing enzymes show no evidence of the substrate inhibition described by Minakami et al. for the mammalian system. In contrast to the mammalian system, the dehydrogenase activity was not increased by doubling the ferricyanide concentration from 1 mM to 2 mM. A phosphate buffer, pH 7.4, was used by Minakami et al. We have found that the nonenzymatic reduction of ferricyanide by NADH was markedly enhanced in the presence of phosphate as compared to that observed in the nonphosphate, histidine buffer.

We have confirmed that 2,6-dichlorophenol indophenol is less satisfactory than ferricyanide as an electron acceptor in the NADH dehydrogenase assay because of its smaller affinity for the electrons removed by the dehydrogenase and because of the difficulty of saturating the system at workable concentrations of the dye. The data in Table VII, which were obtained at starting absorbancies of 3.8 at 600 mμ for DCPIP, are indicative of the difference in distributions of NADH dehydrogenase and oxidase among particulate and soluble fractions rather than of the full potential for NADH dehydrogenation. The relatively high concentration of NADH dehydrogenase activity in the soluble fraction was shown by the ferricyanide-dehydrogenase assay.

PH REQUIREMENT FOR MALATE OXIDATION: Malate oxidation is unique in many respects. Although the rate of respiration with malate as substrate was quite low in the pH range 7 to 7.5, a dramatic increase in both the rate of dehydrogenation (as measured with DCPIP) and in the rate of oxygen uptake occurred at pH 8.5 in an S_1 fraction from glucose-grown cells. These results indicate that when the cells are growing oxidatively on malate a high internal pH may be maintained.

DISCUSSION

Oxidative activities for various fractions of *E. coli* have been previously reported. Because growth histories and fractionation procedures are most often different, direct comparisons are usually not possible. However, in Table VIII some comparative values are listed from recent papers by Gray et al. (27) and Kashket and Brodie (20) and from our own work. The relative activities and distributions obtained for the various enzymes listed are comparable. This suggests that our data are representative of *E. coli* in general rather than of the particular strain we have studied.

A general description of these activities based on the present work (Fig. 1) and supported by some reports from other laboratories can be made. *E. coli* acellular preparations are capable of very rapid NADH oxidation at rates up to ten times greater than that for succinate oxidation. Oxidative enzymes for NADH, NADPH, and succinate are firmly bound to the components of the membrane-envelope fractions. A very active soluble NADH dehydrogenase is also present (Table VII). The marked difference in distribution for NADH dehydrogenase and oxidase activities (Table VII) need not suggest a soluble dehydrogenase that is distinct from the bound form. The NADH oxidase distribution most likely reflects the location of

TABLE VIII

Comparisons of Oxidative Activities in Different Studies with E. coli Cell Fractions

Reference		(26)	This paper	(20)	This paper
Strain		K-12	W-6	W	W-6
Carbon Source		Glucose-amino acids	Glucose-PAB	Malate or* Succinate	Malate or Succinate
Temp.		25°	30°	30°	30°
pH		?	6.8	7.4	6.8
Enzyme	Comparable cell fraction				
NADH	P_2	81‡§	51		
oxidase	P_3	Discarded	27	8.7‖	62¶
	S_3	1.6	2.7		
Succinate	P_2			29.4**	8.3¶
oxidase	P_3	—	—	2.17‖	11.5¶
	S_3				
Malate	P_2			2.2**	0‡‡
oxidase	P_3	—	—	0	0.2
	S_3				
Succinate	P_2	15.4	4.6		
dehydrogenase	P_3	Discarded	3.4	—	—
	S_3	Negligible	0.3		
Malate	P_2	Negligible	1		
dehydrogenase	P_3	Discarded	3	—	—
	S_3	120	440§§		
Isocitrate	P_2	Negligible	0		
dehydrogenase	P_3	Discarded	0	—	—
	S_3	14.3	15		
Glutamic	P_2				
dehydrogenase	P_3				
	S_3	0.6‖‖	1.0	—	—

* Either malate or succinate was used as carbon source, but the published paper did not identify which data was obtained with which carbon source.

‡ All numbers represent μmoles of substrate changed per 10 min per 10 mg protein.

§ When the cells were grown without vigorous aeration this value was 10.5 instead of 81.

‖ S_3 fraction was present.

¶ From succinate-grown cells.

** Part of P_2 was discarded. This is actually a mixture of part P_2 and part P_3.

‡‡ From malate-grown cells.

§§ This system is dependent on concentration of S_3. See Fig. 5 and text.

‖‖ Fraction not identified but is assumed to be analogous to S_3.

cytochromes. The presence of very high NADH dehydrogenase activity separate from the cytochromes raises several important questions. What is the natural acceptor for hydrogen removed so efficiently from the soluble pool of NADH? When NADH is released from soluble dehydrogenases is there a competition between the soluble NADH dehydrogenase and the membrane-bound oxidase which under our conditions of assay was only 2 to 5% as efficient as the soluble system?

Previous reports of malate oxidase in *E. coli* have focused attention on vesicular-membrane structures that require some essential soluble component (20, 28). Our studies suggest that the membranous components (at least of W-6) are totally incapable of malate oxidation unless the soluble NAD-dependent malate dehydrogenase is added. Similarly, the capacity for isocitrate oxidation depends on the presence of a soluble NADP-dependent isocitrate dehydrogenase. Malate oxida-

tion presents some other unique and unresolved questions. Kashket and Brodie (20) concluded that vitamin K rather than ubiquinone is involved in the oxidation of malate. Cox et al. (28), on the other hand, have concluded that malate oxidation primarily uses ubiquinone and that vitamin K may function in some minor route of oxidation.

Growth of the organisms in either succinate or malate did not lead to a coordinate increase in the activities of the measured Krebs cycle oxidative enzymes (Fig. 1). This observation suggests that in *E. coli* these enzymes may not be under coordinate genetic control. This observation together with the fact that some of the Krebs cycle dehydrogenases are physically separated from each other (malate and isocitrate compared to succinate) and from the sites of NADH oxidase activity suggests that possible differences in function exist for these enzymes in mammalian and *E. coli* cells.

The differences between the characteristics of the respiratory chains in *E. coli* and higher organisms are quite striking. The reported cytochromes are entirely different (21). P/O ratios observed in *E. coli* are extremely low (20, 21). Respiratory control appears to be absent and inhibitors which are capable of blocking respiration in mammalian mitochondria have virtually no effect on the operation of the "respiratory chain" of this organism (21).

The finding of high oxidase activity in a conventional ribosomal fraction, although at first surprising, is not new. The small particle fraction obtained by Kashket and Brodie (20) has properties similar to those of a ribonucleoprotein (RNP) fraction studied by Tissières (29). Tissières showed that the NADH oxidation activity of such a preparation sedimented at a faster rate than did the RNP particles themselves. Furthermore, he showed that the cytochrome-containing proteins of this RNP preparation could be separated from the RNP particles by density gradient centrifugation. We have accomplished a similar fractionation with respect to the oxidase activity and have presented electron micrographs showing the membrane-vesicular nature of this material.

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