BBA 45926

STUDIES OF THE EFFICIENCY OF OXIDATIVE PHOSPHORYLATION IN INTACT *ESCHERICHIA COLI* B

WALTER P. HEMPFLING

Johnson Research Foundation, The School of Medicine, University of Pennsylvania, Philadelphia, Pa., and Department of Biology, University of Rochester, River Campus Station, Rochester, N.Y. (U.S.A.)*

(Received January 13th, 1970)

SUMMARY

A method is described for the assay of oxidative phosphorylation using intact *Escherichia coli* B and based upon the initiation of respiration linked to phosphorylation by the addition of O_2 to anaerobic bacteria. Control experiments show that the quenching time of reagents used to terminate reactions is negligible. Three different methods of measuring the amount of inorganic phosphate (P_i) esterified closely agree, and the ratio of P_i esterified to intracellular reduced pyridine nucleotide oxidized, corrected for small amounts of other electron donors, is close to 3. The esterification of P_i is almost completely abolished in the presence of 2,4-dibromophenol, an uncoupler of oxidative phosphorylation. Indirect evidence for the separation in time of electron transport and phosphate esterification is presented. Previous attempts by others to determine the efficiency of bacterial oxidative phosphorylation are discussed and compared with the present method of direct assay.

INTRODUCTION

The observed efficiency of oxidative phosphorylation by subcellular preparations from bacteria is considerably less than that of mitochondria oxidizing identical substrates[1]. It is possible that this is due to damage sustained by respiratory assemblies during preparation. However, it is also possible that the efficiency of oxidative phosphorylation is intrinsically low in bacteria. A method of assay of oxidative phosphorylation utilizing intact bacteria would be more likely to measure the true phosphorylation efficiency of bacterial terminal electron transport. Such a method is described in this communication. By taking advantage of the rapidity of terminal electron transport the process of oxidative phosphorylation is isolated kinetically, rather than physically, from contiguous interfering reactions. The results provide the first direct demonstration that the oxidation of endogenous NADH in intact *Escherichia coli* B is accompanied by oxidative phosphorylation as efficient as that carried out by mitochondria from higher organisms.

* Present address.

MATERIALS AND METHODS

Preparation of resting cells

E. coli B was maintained on nutrient agar slants and cultivated for experimental purposes in Difco Tryptic Soy Broth at 37° on a rotary shaker (1 l of medium in a 2-l erlenmeyer flask). Cells were harvested 16–18 h after inoculation with a loopful of cells from a 24-h broth culture. After centrifugation in the cold the cells were washed once in 50 mM potassium phosphate buffer (pH 7.4) and then aerated in the same buffer (0.2 growth volume) for 1 h at room temperature. The use of phosphate facilitated the diminution of endogenous respiration. The cell suspension was centrifuged in the cold and then resuspended and washed twice in 50 mM Tris–HCl buffer (pH 7.2). Finally, cells were suspended in Tris buffer to a concentration of 15–25 mg dry wt. per ml and placed on ice until the beginning of the experiment (within 1 h after completing the preparation of resting cells).

Assay of oxidative phosphorylation

A series of 12-ml Pyrex centrifuge tubes was prepared, each containing 1.0 ml of the bacterial suspension, and immersed in a water bath maintained at 28°. Each tube was flushed vigorously with He or N_2 to ensure the absence of O_2. After anaerobic conditions were achieved (within 1 min after the flushing process began) the cell suspensions were allowed to stand for 10 min under N_2 or He to permit maximal reduction of endogenous NAD^+ by endogenous substrate. The temperature of the cell suspensions under these conditions was 24–25°. To initiate electron transport, 1.0 ml of air-saturated 50 mM Tris buffer was rapidly injected, and then 1.0 ml of a terminating reagent (ethanol–KOH or $HClO_4$) was added by rapid injection at a known time after the addition of O_2. Injections were performed using 2.5-ml syringes with 18-gauge hypodermic needles, and the period of exposure to O_2 was determined by a second person operating a 10-sec stop watch. The aim is to make reduced pyridine nucleotides and terminal respiratory carriers the sole electron donors oxidized at significant rates during the short time period examined (up to 10 sec after O_2 addition). The method requires that endogenous electron donors be present in amounts sufficient to reduce internal NAD^+, $NADP^+$ and terminal respiratory components, but insufficient to serve as primary electron donors during and just after the anaerobic to aerobic transition. The phosphorylation efficiency, or $P/2e^-$ ratio, is then the amount of P_i esterified divided by the amount of reduced pyridine nucleotide oxidized, corrected for the comparatively small amounts of reducing equivalents from flavoprotein and cytochrome b_1.

Preparation of extracts for metabolite assay

Following termination of reactions with 1.0 ml of 1.5 M $HClO_4$ the acid-treated suspension was allowed to stand at room temperature for 10 min and then denatured material was removed by centrifugation in the cold. 2.0 ml of the resulting supernatant solution were withdrawn and brought to pH 6.3–6.8 by the dropwise addition of a previously determined amount of triethanolamine–KOH solution (1 M triethanolamine–2 M KOH). Vigorous agitation of each sample while neutralizing assured complete mixing. The neutralized extracts were centrifuged in the cold to

remove $KClO_4$ and then assayed immediately or stored at $-20°$ until assays were performed, usually within 48 h after the experiment had been completed.

NADH and NADPH were extracted from another set of bacterial suspensions by injecting 1.0 ml of 1.4 M KOH in 85 % ethanol (a mixture of 1 part of 10 M KOH with 6 parts of absolute ethanol) to terminate reactions. The viscous mixtures were placed on ice for 5–15 min and then immersed in water maintained at $50° (\pm 2°)$ for exactly 10 min. At the end of the heating period the samples were again placed on ice and then brought to pH 7.8–8.2 by dropwise addition of a solution of triethanolamine–HCl saturated at room temperature. Continuous and vigorous mixing during neutralization was essential for good recoveries. The suspension was then allowed to stand on ice for at least 15 min before removing denatured material by centrifugation in the cold.

Metabolite and cofactor assay

Adenine nucleotides and NAD^+ were assayed as described previously[2]. NADH was assayed by using pyruvate and lactate dehydrogenase; NADPH was assayed in the same cuvette by using α-ketoglutarate, NH_4^+ and glutamate dehydrogenase[3]. Recovery of added adenine and pyridine nucleotides was in excess of 90 %, but comparable recoveries of NADH and NADPH could be made only if assayed within 2 h after extraction.

The preparations of NADH used in the estimation of ADP and AMP were freed of contaminating AMP by treatment with alkaline phosphatase, using the method developed by Höfer (see ref. 3).

Inorganic phosphate (P_i) was estimated in neutralized $HClO_4$ extracts within 24 h after extraction by the method of WÄHLER AND WOLLENBERGER[4].

All enzymes and substrates used in the assays were of the best grades obtainable from the Sigma Chemical Co., St. Louis, Mo. and Calbiochem, Los Angeles, Calif., U.S.A.

Spectrophotometric studies

Oxidation of respiratory components in intact cells was measured during the first few seconds after O_2 addition to anaerobic cells by using a dual-wavelength spectrophotometer and a stopped-flow mixing device (see ref. 25). In such experiments a suspension of *E. coli* B, treated as described, was flushed with He for 5 min at room temperature. After mixing the anaerobic bacterial suspension with an equal volume of aerobic Tris buffer by means of the stopped-flow apparatus the resulting changes of absorbance at specific wavelength pairs were recorded using a storage oscilloscope. The duration of mixing was approx. 15 msec and the light path was 1 cm.

A Phoenix Precision Instruments dual-wavelength scanning spectrophotometer was used for the measurement of the amounts of respiratory components in intact cells, and to measure NADH oxidation by pyruvate in the presence of lactate dehydrogenase as a means of testing the timing procedure for the sequential injection experiments.

RESULTS

Measurement of the period of exposure to O_2

In order to determine whether the method used for initiating and terminating reactions was capable of resolving times of exposure to O_2 of as little as 0.5 sec the

oxidation of NADH by pyruvate was initiated by the addition of lactate dehydrogenase by two methods. The time-course of NADH oxidation was followed with a dual-wavelength spectrophotometer and fast-response recorder, which enabled the observer to begin measurement of the absorbance change within 1 sec after adding lactate dehydrogenase, and, in a parallel series of determinations, the rate of NAD^+ appearance was measured by injecting amounts of lactate dehydrogenase to give concentrations identical to those used in the spectrophotometric rate determinations and then terminating the reaction by the rapid addition of $HClO_4$ (final concn. 0.5 M). The results, shown in Fig. 1, demonstrate that the sequential injection procedure is capable of establishing reasonably accurate rates of reaction over at least a 2-sec interval after addition of the enzyme. The rate of NADH oxidation was 8.6 $\mu M \cdot sec^{-1}$ (average of three determinations) while the rate of NAD^+ formation, measured by sequential injection, was 7.8 $\mu M \cdot sec^{-1}$. The lower rate obtained by the latter method is not considered significant when differences in temperature of reaction are considered (26° in the sequential injection estimations and 29° in the spectrophotometric determinations).

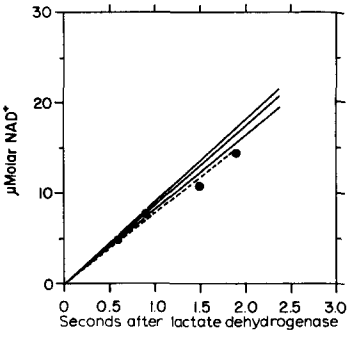


Fig. 1. Comparison of the measurement of rates of NADH oxidation with pyruvate–lactate dehydrogenase by spectrophotometric means and the sequential injection method. Initial conditions in the cuvet (spectrophotometer): 0.1 mM NADH and 5 mM sodium pyruvate in 2.0 ml of 100 mM Tris–HCl buffer (pH 7.5). Reaction initiated by rapidly stirring in 10 μl of Sigma lactate dehydrogenase, activity 100 units·ml^{-1}. Increase of NAD^+ (———) calculated from decrease of absorbance (340 *minus* 374 nm), and extrapolated to zero time from 0.9 to 1.1 sec after mixing. Initial conditions in tubes used in the sequential injection method: 0.2 mM NADH and 10 mM sodium pyruvate in 1.0 ml of 100 mM Tris–HCl buffer (pH 7.5). Reaction initiated by rapidly injecting 1.0 ml of lactate dehydrogenase, activity 1.0 unit·ml^{-1}, and terminated at the times shown by injecting 1.0 ml of 1.5 M $HClO_4$. Increase of NAD^+ (●----●) determined by enzymatic assay.

Quenching time of $HClO_4$

Although the method used to determine times of exposure to O_2 was reasonable accurate, it was possible that a significant delay in quenching could be introduced by the use of intact bacteria. To determine if the quenching time of $HClO_4$ was significant and could therefore introduce an error, anaerobic and aerobic $HClO_4$ solutions were injected into separate but identical suspensions of anaerobic cells and the amounts of ATP, ADP, AMP, and NAD^+ were measured. As shown in Table I, no significant differences were detected between the two solutions of $HClO_4$, indicating that $HClO_4$ quenched oxidative processes linked to phosphorylation before significant oxidation of NADH could occur dependent upon the O_2 present in aerobic $HClO_4$. It was

concluded that the quenching time of $HClO_4$, even though unknown, was negligibly small in these experiments. The concentrations of acid-stable metabolites and cofactors present in anaerobic cell suspensions ("zero time" values) were therefore measured in extracts obtained by injecting aerobic $HClO_4$ before injecting aerobic Tris buffer.

TABLE I

INSIGNIFICANCE OF THE QUENCHING TIME OF $HClO_4$

10.6 mg dry wt. of *E. coli* B per ml after mixing with $HClO_4$, which had been kept anaerobic by flushing with He or aerobic by equilibration with air. Other conditions in text.

Nucleotide	*Amount of nucleotide (nmoles/ml)*	
	Aerobic $HClO_4$	*Anaerobic $HClO_4$*
ATP	6.8	6.6
ADP	17.3	17.9
AMP	27.6	27.4
NAD^+	47.5	46.6

Cellular content of adenine and pyridine nucleotides

The sum of NAD^+ *plus* NADH recovered from *E. coli* B using the two extraction procedures described was a linear function of cell concentration up to at least 16 mg dry wt. per ml in a volume of 2 ml. The average value of NAD^+ *plus* NADH was 6 ± 1 μmoles per g dry wt. The observation that the sum of NAD^+ *plus* NADH is a constant reproducible value is consistent with the conclusion that the quenching time of ethanol–KOH is negligibly short, and it is also ignored.

The sum of AMP, ADP and ATP averaged 4.4 μmoles per g dry wt. with less than 15 % variation from preparation to preparation.

Oxidation of components of the respiratory chain in intact cells

The approximate amounts of reduced carriers in starved, anaerobic *E. coli* B prepared in the manner described are as follows: NADH, 1.6–2.2 μmoles$\cdot g^{-1}$; reduced flavoprotein, 0.6–0.9 μmole$\cdot g^{-1}$; and ferrocytochrome b_1, 0.30–0.35 μmole$\cdot g^{-1}$.

Fig. 2 shows the time-course of oxidation of these components following mixing of aerobic buffer with anaerobic cells in the stopped-flow apparatus. In this experiment the calculated maximal rate of activity of the respiratory chain is 31 electrons$\cdot sec^{-1}$ per cytochrome b_1 heme. At 1.5 sec after mixing the following amounts of each carrier have been oxidized: reduced pyridine nucleotide, 7.3 μM; reduced flavoprotein, 2.1 μM; and ferrocytochrome b_1, 1.9 μM. This is in accordance with several other experiments.

Evaluation of error introduced by oxidation of endogenous substrate

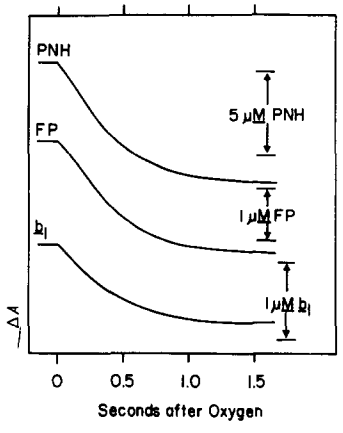
Since only the disappearance of NADH and NADPH is used as the measure of electron flow in the sequential injection experiments some estimate must be made of the rate of pyridine nucleotide reduction during the period of exposure to O_2. Unfortunately this cannot be done directly, since the endogenous electron donors available for the reduction of pyridine nucleotide are unknown. Hence, several approaches to an indirect estimation of the magnitude of error have been made:

(1) the rate of steady-state O_2 consumption by the starved cell preparations (measured with the Clark electrode) is always less than 0.5 % of the rate of endogenous NADH oxidation occurring upon the anaerobic to aerobic transition; (2) the rate of reduction of endogenous pyridine nucleotide following exhaustion of O_2 in the absence of added external substrate does not exceed 2 % of the rate of endogenous NADH oxidation occurring upon the anaerobic to aerobic transition; (3) no more than 1.8 μmoles of O_2 per g dry wt. is consumed when aliquots of starved, anaerobic bacteria are mixed with aerobic buffer (O_2 consumption is more than 90 % complete by 3 sec after mixing as measured with a collodion-covered Pt–Ag electrode); (4) the values found for $P/2e^-$ appear to be minimal (*i.e.* unwashed and inadequately starved cell preparations uniformly show $P/2e^-$ ratios well in excess of those reported here).

Based on these results, it is concluded that the oxidation of endogenous substrate does not significantly contribute to electron flow in these short-term experiments.

Esterification of P_i

The overall apparent $P/2e^-$ ratio can be estimated by measuring the amount of P_i which disappears following O_2 addition to anaerobic bacterial suspensions. The time-course of changes of P_i, ATP, NADH and NADPH is shown in Fig. 3. At 2 sec after O_2 addition 15.5 nmoles of NADH have been oxidized, NADPH has not changed, and 53 nmoles of P_i have been consumed, corresponding to a $P/2e^-$ ratio of 3.4. The formation of ATP is not the sole means of P_i esterification, since only 22 nmoles of ATP are produced. A discrepancy exists between the time of cessation of ATP



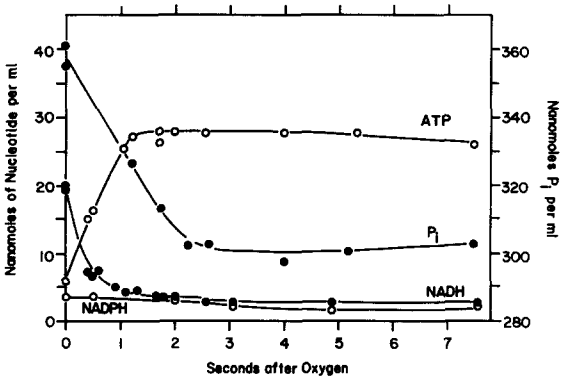


Fig. 2. Oxidation of respiratory components after mixing anaerobic *E. coli* B with aerobic buffer. 3.3 mg dry wt. of bacteria per ml (final concentration after mixing equal volumes). Flow approx. 15 ml·sec^{-1}. Amounts of oxidation of each component determined as follows: reduced pyridine nucleotide at 340 *minus* 374 nm (ref. 23) ($\varepsilon_{mM} = 4.7$); reduced flavoprotein at 510 *minus* 465 nm (ref. 23) ($\varepsilon_{mM} = 11$); and ferrocytochrome b_1 at 560 *minus* 540 nm (ref. 24) ($\varepsilon_{mM} = 16$). Other conditions as described in text. Abbreviations: PNH, reduced pyridine nucleotide; FP, reduced flavoprotein; b_1, ferrocytochrome b_1.

Fig. 3. Esterification of P_i and associated nucleotide changes after mixing anaerobic *E. coli* B with aerobic buffer. 11.7 mg dry wt. of bacteria per ml after mixing. Total P_i concentration shown. Other conditions as described in text.

increase and the time of cessation of P_i consumption. The slow release of P_i following cessation of the burst of respiration and the constancy of ATP indicates that no correction need be applied for ATPase activity during the period examined. It is possible that turnover of P_i may be appreciable, but in the measurement of net phosphorylation turnover is not considered. A marked decrease of the $P/2e^-$ ratio is observed if more than 100 mM K^+ is included in the cell suspension (W. P. HEMPFLING, unpublished observations).

Since NADPH was not oxidized within 2 sec after O_2 addition no measurements of that compound were made in succeeding experiments.

Changes of adenine nucleotide concentrations

The discrepancy discovered between the amount of P_i disappearing and the amount of ATP formed prompted a further experiment in which changes of AMP, ADP and ATP were measured following the initiation of electron transport by O_2 addition. Representative results are shown in Fig. 4. During the first 0.5 sec the concentration of ATP increases, that of AMP decreases, while that of ADP remains relatively constant. These initial changes are explained by assuming that adenylate kinase is active and operates to reattain equilibrium simultaneously with the phosphorylation of ADP, which appears to be the sole acid-stable P_i acceptor in oxidative phosphorylation[6]. ADP decreases significantly only after nearly half of the available AMP has been consumed. Electron transport ceases after about 1.5 sec, in substantive agreement with the results obtained using the stopped-flow technique (Fig. 2). No further changes of adenine nucleotide concentration are observed up to at least 5 sec after O_2 addition.

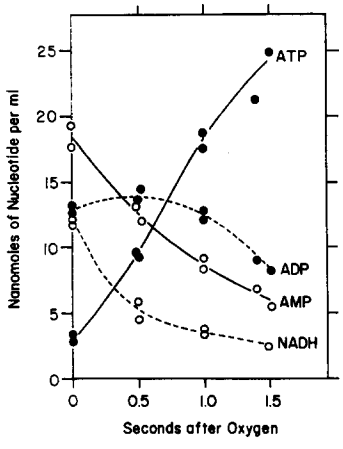


Fig. 4. Changes of adenine nucleotides and NADH after mixing anaerobic *E. coli* B with aerobic buffer. 6.8 mg dry wt. of bacteria per ml after mixing. Other conditions as described in text.

Calculation of the $P/2e^-$ ratio from changes of adenine and pyridine nucleotides

The amount of phosphate esterified can be calculated from the changes of ADP and ATP or from the changes of AMP and ATP. Both methods of calculation should agree within experimental error, and the value thus obtained should be identical to

the P/$2e^-$ ratio obtained by measuring P_i disappearance directly. To estimate P/$2e^-$ by measuring ADP and ATP changes (Method 1) the following expression is used:

$$(2\,\Delta\text{ATP} + \Delta\text{ADP})/-\Delta\text{NADH} = \text{P}/2e^- \quad (1)$$

The second method of calculation of P/$2e^-$ is predicated on the assumption that the following reaction, catalyzed by adenylate kinase, is responsible for the disappearance of AMP:

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\ \text{ADP} \quad (2)$$

The disappearance of 1 mole of AMP is therefore accompanied by the disappearance of 1 mole of ATP and the appearance of 2 moles of ADP. Hence, the value of P/$2e^-$ (Method 2) is given by:

$$\Delta\text{ATP} + (-\Delta\text{AMP})/-\Delta\text{NADH} = \text{P}/2e^- \quad (3)$$

Method 2 has been chosen as the more suitable of the two presented since it takes advantage of the doubled sensitivity of assay of AMP through adenylate kinase, pyruvate kinase and lactate dehydrogenase[3] and since it tends to yield more conservative values for the amount of P_i esterified. After 1.5 sec in the experiment shown in Fig. 4, P/$2e^-$ = 3.8 by Method 1 and P/$2e^-$ = 3.5 by Method 2.

Reproducibility of apparent P/$2e^-$

Because of the number of possible sources of variation of results it was deemed most appropriate to express the overall P/$2e^-$ ratio obtained for several different cell preparations in order to evaluate the reproducibility of results. Data are given in Table II. Wide variations occur from preparation to preparation in the amounts of NADH oxidized but the amounts of phosphate esterified per unit of NADH oxidized are relatively constant at P/$2e^-$ = 3.5 ± 0.3 (mean ± S.E.).

Effect of 2,4-dibromophenol

As shown in Table III, the addition of 300 μM 2,4-dibromophenol to the cell suspension prior to oxygenation abolishes the changes of adenine nucleotides but does not affect the oxidation of NADH. Therefore the net esterification of P_i measured

TABLE II

REPRODUCIBILITY OF THE APPARENT P/$2e^-$ VALUE

The methods used for measurement of the amount of P_i esterified and NADH oxidized are given in parentheses: (2) ΔATP + ($-\Delta$AMP); (3) decrease of P_i; (4) decrease of NADH; (5) increase of NAD^+.

Expt. No.	*P_i esterified* (μmoles·g^{-1})	*NADH oxidized* (μmoles·g^{-1})	*Apparent P/$2e^-$*
1	4.53 (3)	1.32 (4)	3.4
2	5.00 (2)	1.43 (4)	3.5
3	4.82 (2)	1.34 (5)	3.6
4	3.08 (3)	0.86 (4)	3.6
5	4.40 (3)	1.25 (4)	3.5
6	3.13 (3)	1.04 (4)	3.0
7	5.33 (3)	1.44 (4)	3.7

in these experiments is due only to oxidative phosphorylation, although the possibility that P_i turns over in the presence of dibromophenol is not eliminated.

TABLE III

EFFECT OF 2,4-DIBROMOPHENOL ON NUCLEOTIDE CHANGES AFTER MIXING ANAEROBIC *E. coli* B, SUSPENDED IN A SOLUTION CONTAINING 300 μM 2,4-DIBROMPOHENOL, WITH AEROBIC BUFFER

7.1 mg dry wt. of bacteria per ml after mixing. NADH was calculated from the differences of amounts of NAD^+ present in each sample compared to a fully aerobic bacterial suspension at the same cell concentration (44.1 nmoles NAD^+ per ml).

Nucleotide	*Nucleotide concn. (μM) at specified seconds after O_2*		
	0	*0.9*	*2.4*
ATP	3.8	3.4	3.9
AMP	17.6	18.0	17.0
NADH	11.7	4.1	2.6

TABLE IV

CONTRIBUTION OF REDUCING EQUIVALENTS DERIVED FROM REDUCED FLAVOPROTEIN AND FERROCYTOCHROME b_1 TO APPARENT $P/2e^-$ VALUE

Apparent $P/2e^-$, as measured by the sequential injection method, is the quotient of P_i esterified and reduced pyridine nucleotide oxidized; corrected $P/2e^-$ takes into account the reducing equivalents arising from other electron donors in the respiratory system. Data derived from the experiment shown in Fig. 2.

Electron donor	*Oxidized (μM)*	*$P/2e^-$*	*P_i esterified (calculated) (μM)*
Reduced pyridine nucleotide	7.3	3	21.9
Reduced flavoprotein	2.1	2	4.2
Ferrocytochrome b_1	1.0	1	0.5
Total P_i esterified:			26.6
Total electron pairs passing through the equivalent of three phosphorylation sites: $(7.3 + 1.4 + 0.2) =$			8.9
Apparent $P/2e^- = 26.6/7.3 =$		3.6	
Corrected $P/2e^- = 26.6/8.9 =$		3.0	

Correction of apparent $P/2e^-$

A consistent error has been made in the calculation of $P/2e^-$ in the foregoing description of results, since no account was taken of reducing equivalents arising from reduced flavoprotein and ferrocytochrome b_1. It is possible to estimate the magnitude of this error by the use of the data shown in Fig. 2 and the appropriate calculation is made in Table IV. Assumptions are made that electron pairs passed from flavoprotein to O_2 proceed through electron transport steps linked with two sites of phosphorylation, and that single electrons passed from cytochrome b_1 to O_2 go through steps linked with one site of phosphorylation per pair of reducing equivalents. Based on these assumptions failure to consider the oxidation of reduced flavoprotein and ferrocytochrome b_1 leads to an overestimate in the apparent $P/2e^-$ value. In the example derived from Fig. 2 and shown in Table IV the apparent $P/2e^-$ ratio deter-

mined with that cell preparation would be some 20 % in excess of the true $P/2e^-$ value. Variations in the flavoprotein content of different cell preparations suggest that the range of correction necessary is some 15–25 %. Hence an apparent $P/2e^-$ ratio of 3.5 leads to a corrected value of 2.6–3.0.

It is concluded that three sites of oxidative phosphorylation exist in the terminal respiratory sequence of *E. coli* B.

DISCUSSION

Measurement of the efficiency of bacterial oxidative phosphorylation

The preparation of subcellular fractions from bacteria with respiratory and phosphorylating activities is relatively simple[6]. Such preparations are useful for the study of the effects of inhibitors, uncouplers and natural cofactors and metabolites on respiratory processes, as well as for the dissection of the apparatus coupling phosphorylation to respiration[7,8]. Unfortunately, heterotrophic bacteria have consistently given rise to preparations which exhibit $P/2e^-$ ratios considerably less than those possessed by mitochondrial preparations oxidizing similar substrates. It seems likely that these lower efficiencies are due to damage sustained by respiratory assemblies during preparation rather than to an intrinsically lower efficiency of bacterial oxidative phosphorylation, and that, at best, only a semiquantitative estimate of oxidative phosphorylation efficiency is obtained by the use of subcellular preparations.

If low $P/2e^-$ values are the result of damage to respiratory assemblies during preparation of subcellular fractions an obvious way of circumventing this error would be to design a method of assay of oxidative phosphorylation which did not depend upon the comminution of cells. Several attempts at such an assay have been made previously. ZAITSEVA *et al.*[9] reported a maximal P/O ratio of 1.5 in *Azotobacter vinelandii* in a modified Burk's medium based on the rates of $^{32}P_i$ esterification and of O_2 consumption. The possibility of energy expenditure due to ion transport and substrate entry was ignored, and no information was obtained about the time required for the equilibration of external $^{32}P_i$ with intracellular P_i. In similar experiments with *E. coli* B we have found that the 15-sec period of exposure of cells to isotope used by ZAITSEVA *et al.* is inadequate to assure complete equilibration. Hence the P/O value reported is probably appreciably lower than the true value.

The measurement of changes of intracellular acid-soluble nucleotides[10] or net P_i esterification dependent upon the respiration of intact cells following initiation of electron transport is probably more accurate than the $^{32}P_i$ uptake method, since there is no dependence upon isotopic equilibration. The assumption that the measurement of ATP alone accounts for all P_i esterification, however, is a serious shortcoming of such experiments. Microorganisms contain appreciable adenylate kinase activity, and ATP measurements purporting to account for all P_i must be accompanied by data showing that AMP does not vary. For example, GIBSON AND MORITA[11] have shown that AMP does not change following exposure of *Chromatium* strain D to light; under those circumstances it is reasonable to conclude that the observed increases of ATP represent the net esterification of P_i. It is best, however, to show that disappearance of P_i and changes of adenine nucleotides agree quantitatively, as has been done in the present study.

A third approach to the measurement of the efficiency of bacterial oxidative

phosphorylation is through molar growth yield studies. The demonstration by BAUCHOP AND ELSDEN[12] that Y_{ATP} of a number of anaerobically-grown microorganisms is relatively constant at about 10.5 g dry wt. per mole ATP has prompted a number of studies of the growth yield per mole of O_2 or NO_3^- consumed as a measure of the $P/2e^-$ ratio under growing conditions. The elegant experiments of STOUTHAMER and co-workers[13,14] have been shown that about 30 g (dry wt.) of *Aerobacter aerogenes* is formed per pair of reducing equivalents passed to O_2 or NO_3^- during growth at the expense of glucose. Objections have been raised that the "aerobic" value of Y_{ATP} may differ significantly from the "anaerobic" value[15]; however, the results reported in the present communication support the conclusion of HADJIPETROU *et al.*[13] that 3 moles of ATP are formed per pair of electrons transferred to O_2. It is also concluded, due to the similarity of results, that Y_{ATP}, at least in coliform bacteria, is the same whether the cells are grown anaerobically or aerobically (see also ref. 16) and that the three sites of oxidative phosphorylation present in *E. coli* B (a coliform bacterium closely related to *A. aerogenes*) function in the growing cell.

Possible sources of error in the present method

Although some effort has been made to eliminate major sources of error as they apply to the present method of assay of oxidative phosphorylation some difficulties remain to be solved. For example, other electron donors not measured in this study may serve as sources of reducing equivalents which are passed through phosphorylation sites under other conditions of cell growth, preparation or incubation. A pigment[17] absorbing light maximally at 503 nm in the anaerobic state, is present in *E. coli* B grown in minimal medium[27] and may be oxidized at a rate sufficient to cause error in the estimation of the $P/2e^-$ value. This pigment is present in only very small quantities in organisms grown in tryptic soy broth and has therefore been neglected in these studies.

Benzo- and naphthoquinones, shown by KASHKET AND BRODIE[18] to participate in electron transport by particles from *E. coli*, do not appear to be oxidized during the 2-sec transition period after O_2 addition to anaerobic cells. Attempts to measure spectrophotometrically the oxidation of endogenous quinone upon the addition of O_2 have shown that ultraviolet absorbance rises only slowly, and that no changes of absorbance in the quinone regions of the spectrum occur within the first few seconds. Therefore a further error in estimation of the number of electrons passed to O_2 due to oxidation of reduced benzo- and naphthoquinones during the time period studied is unlikely. However, should the period of observation be lengthened for some reason, quinone oxidation would have to be taken into account.

The levels of constituents of the tricarboxylic acid cycle have not been measured in the cells employed, as the controls seem to indicate that they do not serve as major sources of reducing equivalents or of ATP generation by substrate-level phosphorylation. Growth of cells under different conditions may significantly alter the enzyme composition, thereby affecting the pool sizes of such compounds in "starved" cells.

Energy-linked changes (*e.g.* energy-dependent ion transport and volume changes) will contribute to an underestimate of the true $P/2e^-$ ratio. K^+ (refs. 2 and 19), especially, and probably P_i (ref. 20) are taken up in an energy-dependent manner so as to intercept intermediates of oxidative phosphorylation or to bring about the direct hydrolysis of ATP. In accordance with this point, the previous report[21] of a $P/2e^-$ ratio

less than 1 during NO_3^- reduction by intact *E. coli* B is undoubtedly an underestimate due to the inadvertent use of KNO_3 in those experiments.

Metabolites linked to substrate-level phosphorylation may change in such a way as to bring about an overestimate of the $P/2e^-$ ratio. In the present cell preparations the amounts of the components of the glucose 6-phosphate and fructose 1,6-diphosphate homeokinetic groups[2] are negligible, while the constituents of the 3-phosphoglyceric acid homeokinetic group, although present in appreciable amount, change only slowly after oxygenation. The ability of 2,4-dibromophenol to prevent essentially all P_i esterification while permitting NADH oxidation to proceed shows that substrate-level phosphorylation does not contribute to the observed $P/2e^-$ measurement.

Although most preparations of cells treated in the manner described contain sufficient endogenous material to bring about adequate NAD^+ reduction, in some cases starvation proceeds too far. Under those circumstances the addition of small amounts of ethanol (1–2 mM) to the anerobic cell suspensions brings about slow NADH formation which is complete (about 2 μmoles NADH per g dry wt.) within 5 min. The products of this slow ethanol oxidation have no noticeable effect on the events subsequent to O_2 addition. The experiments reported here do not include those in which ethanol was necessary for adequate NAD^+ reduction.

The rapidity of the oxidation reactions observed tempt one to reduce the temperature at which the experiments are carried out in order to make kinetic analysis more accurate[10]. ZAITSEVA *et al.*[9] report that uncoupling of phosphorylation from respiration in Azotobacter occurs in chilled samples. This phenomenon also occurs in *E. coli* B using the present method when the cell suspensions are mixed with O_2 at 5° (W. P. HEMPFLING, unpublished observations). Until a complete account is obtained of the effects of temperature on bacterial oxidative phosphorylation indiscriminant use of lowered temperatures to slow reaction rates should be avoided.

Variation of the $P/2e^-$ ratio with time after O_2 addition

The $P/2e^-$ ratio, in all experiments so far carried out, increases with time up to about 1.5–2.0 sec after O_2 addition. That pattern is shown in Fig. 5 for the experiments

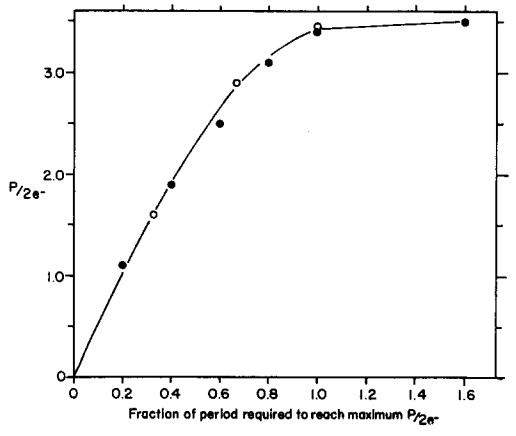


Fig. 5. Change of $P/2e^-$ ratio after mixing anaerobic *E. coli* B with aerobic buffer. Normalized comparison of the experiments shown in Figs. 3 (●) and 4 (○).

of Figs. 3 and 4. It is possible that such a phenomenon could be due to a transient accumulation of intermediates of oxidative phosphorylation or to the formation of an ionic gradient[26] across membrane barriers associated with respiratory assemblies during a fraction of the first second after initiation of electron transport. Estimates of the amount of intermediate or gradient generated equivalent to the esterification of P_i can be made based on the amount of reduced pyridine nucleotide oxidized and the deficit of phosphate esterification below the maximal $P/2e^-$ ratio observed at 1.5–2.0 sec. These calculations show that the hypothetical intermediate rises throughout the first 500 msec, reaches a maximum at 500–750 msec, and then falls. The amounts are appreciable: 1.5–2.5 μmoles per g dry wt. at 500–750 msec after oxygenation. PINCHOT[7] has reported the existence of an intermediate of oxidative phosphorylation in extracts of *Alcaligenes faecalis*, but only further investigation will determine if this has any relationship to the phenomena reported here.

Applications of the method

The results reported in this communication demonstrate directly for the first time that oxidative phosphorylation in a heterotrophic bacterium proceeds with efficiency equal to that of mitochondria from higher organisms. These data do not support the idea that all heterotrophic bacteria are capable of equally efficient oxidative phosphorylation, since the ability to conserve energy will vary according to the enzymatic apparatus available to a given organism.

The addition of an uncoupler of oxidative phosphorylation does not increase the rate of endogenous NADH oxidation, although phosphorylation is abolished. Nevertheless, during steady-state formate oxidation by *E. coli* B an increase of some 50–100 % of the rate of respiration is observed following addition of 2,4-dibromophenol (W. P. HEMPFLING, unpublished observations), indicating that some form of respiratory control dependent upon oxidative phosphorylation does exist under steady-state conditions. A rapid relaxation method, such as the sequential injection procedure described here, does not appear to be suitable for the solution of the problem of respiratory control in bacteria. However, the measurement of intracellular nucleotide concentrations[22] in the presence and absence of uncouplers under steady-state conditions may be appropriate.

Application of the methods of microbial genetics to the problems of oxidative phosphorylation has so far been restricted to eucaryotic microorganisms. Given the ability to assay the number of phosphorylation sites in a bacterial respiratory system, the search for mutants deficient in one or more of those phosphorylation sites, using organisms capable of genetic recombination, becomes feasible. Furthermore, the study of the adaptive characteristics of the energy-conservation system from the point of view of P_i esterification can be examined in systems perhaps quite different from the mitochondrion in organization and development.

ACKNOWLEDGMENTS

I wish to thank Miss B. Mae Taylor, M.T. (A.S.C.P.) and Mr. George E. Biron for able technical assistance. Thanks are further due to Dr. Jane Gibson and Dr. Berton C. Pressman for counsel and encouragement, and Dr. R. W. Estabrook for the use of laboratory facilities. This work was supported by Public Health Service

Grant No. GM 12202 and by a grant from the National Science Foundation, No. GB-5938. The author was supported by a Scholarship from the Pennsylvania Plan to Develop Scientists in Medical Research during part of his tenure at the University of Pennsylvania.

REFERENCES

1 N. S. GEL'MAN, M. A. LUKOYANOVA AND D. N. OSTROVSKII, *Respiration and Phosphorylation of Bacteria*, Plenum Press, New York, 1967, p. 16.
2 W. P. HEMPFLING, M. HÖFER, E. J. HARRIS AND B. C. PRESSMAN, *Biochim. Biophys. Acta*, 141 (1967) 391.
3 R. W. ESTABROOK, J. R. WILLIAMSON, R. FRENKEL AND P. K. MAITRA, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 474.
4 B. E. WÄHLER AND A. WOLLENBERGER, *Biochem. Z.*, 329 (1958) 508.
5 R. D. HILL AND P. D. BOYER, *J. Biol. Chem.*, 242 (1967) 4320.
6 A. F. BRODIE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 6, Academic Press, New York, 1964, p. 284.
7 G. PINCHOT, *Perspectives Biol. Med.*, 8 (1965) 180.
8 S. ISHIKAWA AND A. LEHNINGER, *J. Biol. Chem.*, 237 (1962) 2401.
9 G. N. ZAITSEVA, N. K. SYONG AND A. N. BELOZERSKII, *Biokhimya*, 28 (1963) 172.
10 J. RAMIREZ AND L. SMITH, *Biochim. Biophys. Acta*, 153 (1968) 466.
11 J. GIBSON AND S. MORITA, *J. Bacteriol.*, 93 (1967) 1544.
12 T. BAUCHOP AND S. R. ELSDEN, *J. Gen. Microbiol.*, 23 (1960) 457.
13 L. HADJIPETROU, J. GERRITS, F. A. G. TEULINGS AND A. H. STOUTHAMER, *J. Gen. Microbiol.*, 36 (1964) 139.
14 L. HADJIPETROU AND A. H. STOUTHAMER, *J. Gen. Microbiol.*, 38 (1965) 29.
15 E. HERNANDEZ AND M. JOHNSON, *J. Bacteriol.*, 94 (1967) 996.
16 V. KORMANČÍKAVÁ, L. KOVÁČ AND M. VIDOVÁ, *Biochim. Biophys. Acta*, 180 (1969) 9.
17 A. LINDENMAYER AND L. SMITH, *Biochim. Biophys. Acta*, 93 (1964) 445.
18 E. R. KASHKET AND A. F. BRODIE, *J. Biol. Chem.*, 238 (1963) 2564.
19 S. G. SCHULTZ AND A. K. SOLOMON, *J. Gen. Physiol.*, 45 (1961) 355.
20 P. MITCHELL AND J. M. MOYLE, *J. Gen. Microbiol.*, 9 (1953) 257.
21 W. P. HEMPFLING, S. STEINBERG AND R. W. ESTABROOK, *Abstr. 6th Intern. Congr. Biochem., New York*, (1964) 779.
22 A. WORCEL, D. GOLDMAN AND W. CLELAND, *J. Biol. Chem.*, 240 (1965) 3399.
23 B. CHANCE AND B. HAGIHARA, *Proc. 5th Intern. Congr. Biochem. Moscow, 1963*, Vol. 5, Pergamon Press, New York, 1963, p. 3.
24 S. S. DEEB AND L. P. HAGER, *J. Biol. Chem.*, 239 (1964) 1024.
25 B. CHANCE, in A. WEISSBERGER, *Techniques of Organic Chemistry*, Vol. 8, Interscience, New York, 2nd. Ed., 1963, p. 728.
26 P. MITCHELL, *Nature*, 191 (1961) 48.
27 K. OLDEN AND W. P. HEMPFLING, *Bacteriol. Proc.*, 1970, in the press.