It was of great interest to observe also that there was a very high degree of specificity with regards to the structure of the quinone. As can be observed in Table 2, not only did other common electron carriers such as DPN, FAD, cytochrome c, and ascorbate fail to give the by-pass of the amytal inhibition, but likewise closely related benzoquinones and naphthoquinones were considerably less active. Since it has been shown that these quinones may accept electrons from the DT diaphorase [2] the specificity must be in the interaction of the reduced quinone with the respiratory chain.

This amytal-insensitive system shows the ability to couple respiration to the esterification of phosphate. The P/O ratios observed vary depending upon the substrate used from approximately 0.9 with β-hydroxybutyrate to 1.3 with glutamate. It is difficult to determine from these values whether one or two sites of respiratory chain phosphorylation are involved. However, the low values may be partly due to a slight uncoupling activity of the vitamin K_3 as determined with succinate as substrate. The phosphorylation was completely sensitive to 10^{-4} M DNP.

The question of the antimycin A sensitivity of this respiration has been further investigated. It is felt that it must be due to an unavailability of the cytochromes of the respiratory chain on the oxidizing side of the antimycin A-sensitive site, such as, for instance, cytochrome c, which has been shown [2] to be rapidly reduced in isolated systems by reduced pyridine nucleotide in the presence of DT diaphorase and vitamin K_3. The addition of purified cytochrome c to the mitochondrial system gives a several fold stimulation of the antimycin A-insensitive respiration. This respiration is also sensitive to KCN and dicumarol.

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Oxidative Phosphorylation with Endogenous Mitochondrial Substrates

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During recent studies of oxidative phosphorylation [1,2], we observed that carefully isolated rat liver mitochondria, when incubated in media containing the usual cofactors of oxidative phosphorylation, catalyzed a substantial uptake of inorganic orthophosphate in the absence of any added exogenous substrate. In Table 1 are shown representative data that illustrate the magnitude of this activity as well as the excellent stoichiometry that was obtained when the endogenous values were subtracted from those observed with added substrate. Independent experiments revealed that the internal substrates were not readily exhausted in short term incubations at 30°. In view of the large endogenous activity found with these preparations, in contrast to what has been observed previously [3-5], it was necessary to establish by anion exchange chromatography, and other tests, that the endogenous phosphate uptake was not an artefact of the assay procedure and that it led to an aerobic synthesis of ATP.

Experiments with inhibitors indicated the following: a) the endogenous phosphate uptake is associated with an *oxidative* phosphorylation since it was completely abolished by anaerobiosis, cyanide, azide, antimycin A, dinitrophenol and other uncoupling agents; b) tricarboxcylic acid cycle intermediates are not sole contributors to the endogenous activity since only partial inhibition was obtained with malonate and fluoroacetate; c) the internal substrates are oxidized *via* pyridine nucleotides. This was demonstrated by the finding that amytal completely prevented the endogenous phosphate uptake (*cf.* Ref.[6]).

Previously we had observed [2] that a high rate of endogenous activity was characteristic of mitochondria which were stable to prolonged storage at 4°. Indeed, when mitochondria from tissues other than liver were assessed both for their endogenous activity and for their stability of oxidative phosphorylation with added substrate, a positive correlation was found; *i. e.* those preparations which exhibited the

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Table 1. Endogenous phosphorylation. Each flask contained: 80 μmoles of glycylglycine buffer, pH 7.4; 20 or 30 μmoles of orthophosphate, pH 7.4; 5 μmoles of ADP; 5 μmoles of DPN; 0.03 μmoles of cytochrome c; 50 μmoles of glucose; 0.5 mg of hexokinase; 10 μmoles of $MgCl_2$, and 0.5 ml of mitochondria in a final volume of 2 ml. 50 μmoles of DL-β-hydroxybutyrate were added only where indicated. Incubated in air for 20 min at 30°. Other details given in Ref.[2].

Added substrate	Oxygen uptake μatoms/mg N	Acetoacetate formed μmoles/mg N	Phosphate uptake μmoles/mg N	P/O
None	3.1	0	5.7	1.8
β-Hydroxybutyrate	8.5	5.3	22.0	2.6
Substrate minus endogenous	5.4	5.3	16.3	3.0

highest rate of endogenous phosphorylation were most stable to storage at 4°. Furthermore, certain pretreatments of liver mitochondria, such as freezing and thawing, or preincubation with low concentrations of phosphate which, among other detrimental changes (*cf.* Ref.[7]), abolished the endogenous activity, also decreased the stability of the stored preparations. The implication of these findings with regard to a possible correlation between endogenous phosphorylation and mitochondrial stability will be discussed.

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Particulate and Soluble D-Lactic Cytochrome c Reductase of Yeast

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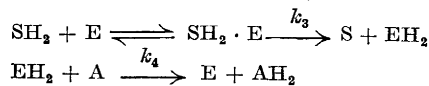
The main fraction of the D-lactic cytochrome c reductase activity (D-LDH) of aerobic yeast was associated with respiratory particles. By means of the extraction of lipids with *n*-butanol, acetone, and ether, 40 % of the enzyme was solubilized. The solubilized enzyme has been purified one thousand fold. The difference spectrum of oxidized/reduced enzyme had absorption maximum at 450 mμ. At pH 3, flavin fluorescence appeared. The flavin concentration, calculated from the spectral difference, agreed well with that obtained from fluorescence intensity. In contrast to the flavohaemoprotein L-lactic cytochrome c reductase (L-LDH), there was no haem associated with the D-enzyme, and the D-enzyme did not reduce the haem of L-LDH. The turnover number of D-LDH was 20 000 min^{-1} $mole^{-1}$, compared with 15 000 for L-LDH.

Particulate as well as solubilized D-LDH were absolutely specific for D-lactate. A preparation of D-LDH, which was obtained directly in a soluble form in the cell homogenate[1], has erroneously been determined to have relative stereospecificity [2]. The apparent Michaelis constant of D-lactate for D-LDH was under certain conditions one thousand fold lower than that of L-lactate for L-LDH.

The oxidation of D-lactate to pyruvate went to completion with excess of cytochrome c. At a relatively high buffer concentration (μ 0.08), the initial rate of reaction has been determined for a series of cytochrome *c* concentrations, each with a series of D-lactate concentrations. The results can be summarized as follows: The apparent Michaelis constant of D-lactate was proportional to the cytochrome c concentration, and the rate, obtained with excess of lactate, was proportional to the cytochrome c concentration. These criteria, which were the same for soluble and particulate enzyme, are fullfilled in the following reaction scheme (where E = enzyme, SH_2 = lactate, and A = cytochrome c):



$$SH_2 + E \rightleftharpoons SH_2 \cdot E \xrightarrow{k_3} S + EH_2$$
$$EH_2 + A \xrightarrow{k_4} E + AH_2$$

In this scheme, $k_3 \gg k_4 \times A$