phosphatase activities required added Mg++ and were inhibited by atebrine, chlorpromazine and azide to about the same extent as the triphosphatases.

The above findings seem to indicate that freshly prepared rat liver microsomes exhibit a nucleoside triphosphatase activity which resembles that of structurally damaged rat liver mitochondria in regards to behavior toward certain activators and inhibitors, lacking nucleoside specificity, and firm association with the membrane structure. In addition, microsomes seem to contain a "latent" nucleoside diphosphatase activity, which can be rendered manifest by disruption of the microsomal membranes. This enzyme resembles the nucleoside triphosphatase in its behavior toward certain activators and inhibitors, but differs from the latter in possessing a more restricted array of nucleoside specificity, as well as in being more loosely associated with the microsomes.

An enzyme with a similar array of specificity has been known for some time [10-13], but its association with microsomes and its latent character have not been reported.

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By-Pass of the Amytal-Sensitive Site of the Respiratory Chain in Mitochondria by Means of Vitamin K_3

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A soluble diaphorase (DT diaphorase) from liver has been isolated which can couple the oxidation of extramitochondrial reduced pyridine nucleotide through vitamin K_3 to the respiratory chain of mitochondria [1]. The cellular distribution of this enzyme has been shown[2], and significant amounts can be extracted from isolated mitochondria [3].

The presence of this enzyme in intact mitochondria and its interaction with intramitochondrially-generated reduced pyridine nucleotide is demonstrated in this paper by the bypassing of the point of amytal inhibition in the respiratory chain with the addition of vitamin K_3.

Mitochondria freshly prepared from rat liver as previously described [4] were incubated in a isotonic, buffered medium containing substrate, phosphate, Mg++, adenosine triphosphate, hexokinase and glucose. In these conditions the pyridine nucleotide-linked oxidation of substrates showed the usual high rates and strong sensitivity to amytal as is shown for glutamate in Table 1. The addition of low concentrations of vitamin K_3 (5×10^{-6} M) to the amytal-inhibited system gave a complete recovery of the maximal respiration. It can also be seen from Table 1 that this respiration was highly sensitive to antimycin A, KCN, and dicumarol. The high degree of inhibition obtained by low concentrations of dicumarol strongly implies the role of DT diaphorase, as the high sensitivity of this enzyme to dicumarol is considered to be very characteristic[2].

It was found that the concentration the of vitamin K_3 was important in this reaction as concentrations under 5×10^{-6} M were limiting in the oxidation of glutamate in these conditions, while higher concentrations were often inhibitory. This inhibition seemed to be related to a loss of pyridine nucleotide and could be protected at least partially by added nucleotide or nicotinamide.

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Table 1. Effect of vitamin K_3 and various inhibitors on the respiration of mitochondria in the presence of amytal.

Additions	μatoms oxygen
none	5.49
vitamin K_3	5.57
dicumarol	5.32
amytal	0.22
amytal + vitamin K_3	5.25
amytal + vitamin K_3 + antimycin A	1.57
amytal + vitamin K_3 + KCN	1.18
amytal + vitamin K_3 + dicumarol	1.13

The complete system contained per Warburg vessel: 10 μmoles glutamate, 20 μmoles Tris buffer (pH 7.4), 20 μmoles orthophosphate (pH 7.4), 4 μmoles $MgCl_2$, 2 μmoles adenosine triphosphate, 24 μmoles glucose, an excess of yeast hexokinase, 50 μmoles sucrose, and mitochondria from 200 mg rat liver. The amounts of the additions were as follows: 5×10^{-3} μmoles vitamin K_3, 10^{-2} μmoles dicumarol, 1.0 μmoles amytal, 1 μg antimycin A, and 1.0 μmoles KCN. Final volume, 1.0 ml. Temperature, 30°C. Reading begun after 6 min. thermoequilibration. Time measured, 20 min.

Table 2. Effect of various oxidation-reduction compounds on the respiration of mitochondria in the presence of amytal.

Additions	μatoms oxygen Expt. 1	Expt. 2	Expt. 3
none	4.47	7.00	5.15
amytal	0.73	0.33	1.12
amytal + 10^{-5} M vitamin K_3	5.75	8.23	6.05
amytal + 10^{-3} M DPN	0.79		
» + 10^{-3} M FAD	0.93		
» + 10^{-3} M FMN	0.89		
» + 6×10^{-5} M cytochrome c	0.55		
amytal + 10^{-5} M ascorbate	0.88		
» + 10^{-5} M ferricyanide		0.38	
» + 10^{-5} M silicomolybdate		0.35	
amytal + 10^{-5} M vitamin K_1		0.46	
» + 10^{-5} M vitamin K_2		0.43	
amytal + 10^{-5} M 1,2-naphthoquinone			1.13
» + 10^{-5} M 1,4-naphthoquinone			1.37
» + 10^{-5} M 2-hydroxy-1,4-naphthoquinone			1.59
» + 10^{-5} M 2-methyl-3-hydroxy-1,4-naphthoquinone			2.80
» + 10^{-5} M p-benzoquinone			0.88
» + 10^{-5} M 2-methylbenzoquinone			0.79
» + 10^{-5} M 2,6-dimethyl-benzoquinone			0.74
» + 10^{-5} M coenzyme Q_0			0.72

Conditions were the same as Table 1. Quinones were added in 10 μl ethanol. Time measured, 20 min.

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 bypass 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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