mitochondria.[2] Precise localization of these phosphorylations along the respiratory chain has been difficult to approach experimentally. However, it has been established that the oxidation of ascorbate to dehydroascorbate via the cytochrome system of isolated mitochondria is accompanied by a coupled phosphorylation.[3,4,5] Since ascorbate reduces ferricytochrome c non-enzymatically, it has been tentatively concluded that the phosphorylation observed is actually coupled to the oxidation of ferrocytochrome c by oxygen via cytochrome oxidase. However, participation of ascorbate in an as yet unknown enzymatic phosphorylation could not be rigorously excluded in those tests. We wish to report a direct demonstration of phosphorylation coupled to electron transport between ferrocytochrome c and oxygen which does not involve the presence of ascorbate or any other non-enzymatic reductant of cytochrome c in the test system.

Ferrocytochrome c, prepared by reduction of ferricytochrome c by hydrogen (palladium catalyst), was incubated with rat liver mitochondria, orthophosphate labeled with P^{32}, ADP, Mg^{++}, tris-(hydroxymethyl)-aminomethane buffer (tris) *p*H 7.4, ethylenediaminetetraacetate (Versene), and antimycin A. Enzymatic oxidation of ferrocytochrome c proceeded at the expense of dissolved oxygen and was followed spectrophotometrically at 550 mμ, using reported extinction coefficients.[6] The phosphate uptake was measured by determining P^{32} in the organic phosphate fraction after removal of phosphomolybdic acid by solvent extraction.[7] The P:O ratio was calculated as millimicromoles of orthophosphate taken up per 2 millimicromoles of ferrocytochrome c oxidized. Antimycin A was added to inhibit reduction of ferricytochrome c by endogenous substrates[8]; apparently it did not inhibit oxidation of ferrocytochrome c or the coupled phosphorylation significantly in the concentration used (0.02 γ/ml.).

The table shows the experimental details and results of four experiments.

It is seen that phosphate uptake accompanied oxidation of ferrocytochrome c, with observed P:O ratios from 0.44 to 0.86. These values are in agreement with the P:O ratios earlier obtained with ascorbic acid as reductant[3] which approached 1.0. Omission of ferrocytochrome c or its substitution by ferricytochrome c produced in most cases insignificant "base-line" phosphorylation. Both the oxidation of ferrocytochrome c and the coupled phosphorylation were completely inhibited by 5×10^{-4} *M* cyanide, indicating the participation of cytochrome oxidase. The presence of 1×10^{-4}*M* 2,4-dinitrophenol completely inhibited phosphate uptake and increased the rate of oxidation of ferrocytochrome c. It is also seen that the P:O ratio is relatively constant with time up to about 65% oxidation of the ferrocytochrome c.

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TABLE I

PHOSPHORYLATION COUPLED TO OXIDATION OF FERROCYTOCHROME C

Final concentrations in reaction media (total volume 1.0 ml.): 8.5×10^{-4} *M* orthophosphate labeled with P^{32} ($1.0\text{–}1.5 \times 10^{6}$ c.p.m./μμP.), 0.0015*M* ADP, 0.020 *M* TRIS *p*H 7.4, 0.0010 *M* Versene, 0.02γ antimycin A per ml., and 1.65×10^{-5} *M* cytochrome c. The reaction was started by adding the mitochondria derived from 0.5 mg. wet weight rat liver suspended in 0.20 ml. of 0.075 *M* sucrose + 0.001 *M* versene; incubation for 2 to 15 min. at 23–26°.

	$-\frac{1}{2}\Delta Fe^{II}$ cyt. c	$-\Delta PO_4$, millimicromoles	P:O
Fe^{II} cyt. c	3.1	1.9	0.61
Fe^{II} cyt. c + 5×10^{-4}*M* KCN	−0.1	0.13	
Fe^{II} cyt. c + 1×10^{-4}*M* DNP	5.1	0.05	0.01
Fe^{III} cyt. c		0.18	
Fe^{III} cyt. c + 5×10^{-4}*M* KCN	−0.4	0.05	
No cyt. c		0.10	
Fe^{II} cyt. c	2.9	2.5	0.86
Fe^{III} cyt. c		0.48	
Fe^{II} cyt. c	1.0	0.68	0.68
Fe^{III} cyt. c		0.05	
Fe^{II} cyt. c, 2 min.	1.0	0.53	0.53
Fe^{II} cyt. c, 15 min.	4.3	1.9	0.44
Fe^{III} cyt. c, 2 min.		0.07	
Fe^{III} cyt. c, 15 min.		0.18	

It may be concluded that one of the three phosphorylations occurring during passage of a pair of electrons from DPNH to oxygen is coupled to the oxidation of ferrocytochrome c by oxygen, probably via cytochromes a and a_3. This phosphorylation is completely compatible with known thermodynamic data on the respiratory chain.

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MOLECULAR COMPLEX FORMATION IN FREE RADICAL REACTIONS

Sir:

In the course of a study of the action of inhibitors in the initiated oxidation of hydrocarbons, we were led to the conclusion that the behavior of inhibitors could well be accounted for by this sequence of reactions

$$RO_2\cdot + In \rightleftharpoons [In \longrightarrow RO_2\cdot] \quad (1)$$

$$[In \longrightarrow RO_2\cdot] + RO_2\cdot \longrightarrow \text{products} \quad (2)$$

Two critical tests of this hypothesis have now been applied and the results seem sufficiently definitive to warrant their release at this time. The commonly accepted mechanism of inhibitor action by aromatic amines and phenols involves the removal of a labile hydrogen in the first step of the reaction.

$$\begin{matrix} ArOH \\ \text{or} \\ ArNHR \end{matrix} + RO_2\cdot \longrightarrow RO_2H + \begin{matrix} ArO \\ \text{or} \\ ArNR \end{matrix} \quad (3)$$

Since such a reaction would not be expected to be highly exothermic, it would be anticipated that the rate of the reaction and therefore the inhibitor

efficiency should be decreased by deuteration of the inhibitors. It is noteworthy that isotope effects of a considerable magnitude have been observed in the chain carrying step of air oxidation[1] and in the degradative chain transfer reaction in allyl acetate polymerization.[2] It has been found that N-D-N-methylaniline and N-D-diphenylamine give oxidation curves, with cumene and tetralin as substrates in chlorobenzene solution, which are congruent with those observed in the presence of the corresponding undeuterated compounds. An illustrative example is shown in Fig. 1.

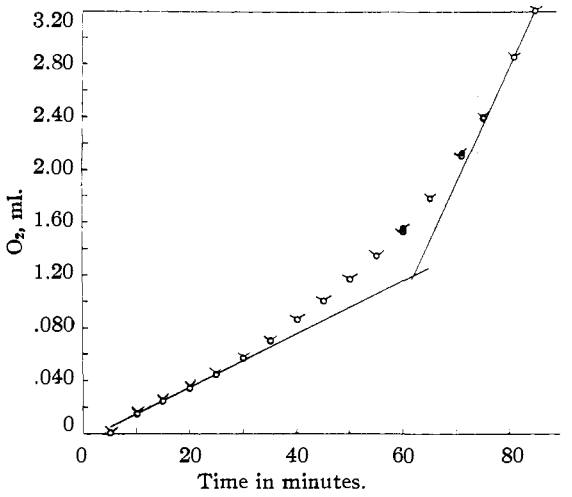
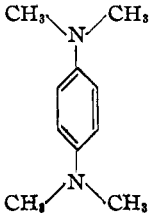


Fig. 1.—Oxidation of cumene (2.4*M*) in chlorobenzene at 62.5° inhibited by $C_6H_5NHCH_3$ (O) and $C_6H_5NDCH_3$ (O), initiated by $1.01 \times 10^{-2}M$ azoisobutyronitrile; inhibitor concentrations, 3.33×10^{-3} *M*.

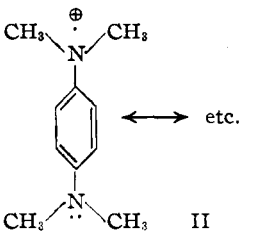
As a specific test for complex formation we have studied the action of tetramethyl-*p*-phenylenediamine (I).



I

Despite the fact that this compound contains no "labile" hydrogen it is a powerful inhibitor and stops two oxidation chains[3] in both nitromethane and chlorobenzene solution with tetralin as a substrate. Furthermore, in nitromethane the purple color of the Wurster cation II is developed during the early part of the inhibition period and is subsequently dissipated.

In an especially dramatic experiment water was included as a second phase in a repetition of the



II

oxidation in chlorobenzene. During the early part of the reaction *the color of the Wurster cation appeared in the aqueous phase.* The ion was positively characterized by measurement of its visible absorption spectrum. The inhibition continued but with a very low efficiency and eventually the dye disappeared from the solution. These observations indicate that hydrolysis of the complex occurs according to equation 4 and the result represents

$$In\text{-}RO_2\cdot + H_2O \rightleftharpoons In\cdot^+ + RO_2H + OH^- \quad (4)$$

the detection of gross amounts of an intermediate species which has neither undergone hydrogen abstraction nor attachment of $RO_2\cdot$ to a specific carbon atom of the inhibitor.

It is very evident that these formulations permit wide extension in the field of radical chemistry in solution. Examples too numerous to cite present themselves. A few especially significant related problems or observations are, (1) the postulated complex formation in the chain transfer reaction of growing polystyrene radicals in bromobenzene solution,[5] (2) the stereospecific free radical addition of hydrogen bromide to 1-cyclohexenyl bromide,[6] and (3) the general problem of the mechanism of aromatic substitution by free radicals.[7]

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(2) P. D. Bartlett and F. A. Tate, THIS JOURNAL, **75**, 91 (1953).

(3) The stoichiometric assignment is made on the basis of the length of the induction period. This is made possible by the use of appropriate efficiency factors for the production of radicals from azo-bis-isobutyronitrile by two independent methods which will be reported at an early date.[4]

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THE CONVERSION OF CHOLESTEROL TO PREGNENOLONE IN BOVINE ADRENAL HOMOGENATES[1]

Sir:

The transformation of cholesterol to 17-hydroxycorticosterone and corticosterone has been demonstrated in the isolated perfused bovine adrenal.[2] Based upon *in vitro* perfusion studies, the sequence of reactions shown in Fig. 1 was postulated to account for cholesterol conversion to corticoids.[3]

(1) Aided in part by the Research and Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-255.

(2) O. Hechter, *et al.*, *Recent Progress in Hormone Research*, **6**, 215 (1951).

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