to glucose addition; *i.e.*, those systems in which NAD^+ is accumulating appear to be more prone to oscillate. It is suggested that this increase in the NAD^+ level is an early reflection of substrate limitation.

Fig. 2 shows another experiment in which NAD^+, ATP, and fructose 1,6-diphosphate were measured. These data show that the oscillations are not limited to the pyridine nucleotide. We were not able to show oscillations in O_2 consumption using the Gilson vibrating platinum electrode and more dilute cell suspensions.

When phase-plane plots[5] of fructose 1,6-diphosphate *vs.* ATP concentration are made from the data of Fig. 2, it is found that ATP is out of phase with fructose 1,6-diphosphate throughout the experiment. This is consistent with the concept that fructose 1,6-diphosphate levels change in response to changes in the activity of phosphofructokinase which in turn is controlled by the level of the various adenine nucleotides[14]. Furthermore, these changes appear to be readily and continuously reversed.

We are presently investigating the responses of additional intermediates and are trying to elucidate more definitively the conditions necessary for oscillations.

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The inhibitory effect of uncouplers of oxidative phosphorylation on mitochondrial respiration

It has been known for some time that excessive concentrations of uncouplers of oxidative phosphorylation will inhibit mitochondrial respiration. For the case of

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TTFB, tetrachlorotrifluoromethylbenzimidazole; TMPD, tetramethyl-*p*-phenylenediamine; ETP, electron-transport particle.

substituted nitrophenols a hypothesis was proposed by HÜLSMANN[1] and HEMKER[2] that explained the inhibitory effect on the basis of binding of the uncoupler with an unknown factor involved in the energy-conserving reactions of mitochondria. This mechanism could explain differences in inhibitory power between equally strong uncouplers. However, the observation by WENNER[3] that the inhibition could be overcome by higher substrate concentration did not seem compatible with this mechanism. Later work by WILSON[4] has shown that the inhibition of succinate oxidation is kinetically of the competitive type between substrate and uncoupler, *i.e.* the uncoupler increases the K_m for the substrate. The present study was undertaken to locate more precisely the site of inhibition. Although the inhibitory effect does extend to uncoupler-activated ATPase[2], only experiments on mitochondrial respiration will be reported here.

TABLE I

UNCOUPLING AND INHIBITORY ACTIVITY OF VARIOUS AGENTS

In a volume of 3 ml were incubated at room temperature and pH 7.4: 50 mM KCl, 50 mM Tris chloride, 50 mM sucrose, 10 mM P_i, 5 mM $MgCl_2$, 1 mM EDTA, 1 μg/ml rotenone, 4 mM succinate and 2.3 mg/ml rat-liver mitochondria. The rate of oxygen uptake was determined after addition of varying amounts of the indicated agents. In the case of gramicidin B the medium consisted of: 250 mM sucrose, 20 mM Tris chloride, 5 mM NaCl, 3 mM succinate and 2.2 mg/ml mitochondria.

Compound	*Concentration (M) required for*	
	Maximal respiration	*50% inhibited respiration*
2,4-Dinitrophenol	$3 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$
Dicumarol	$5 \cdot 10^{-6}$	$1.5 \cdot 10^{-5}$
FCCP	$3 \cdot 10^{-7}$	$4 \cdot 10^{-5}$
TTFB	$7 \cdot 10^{-7}$	$7 \cdot 10^{-6}$
Gramicidin B	$4.1 \cdot 10^{-7}$	$9 \cdot 10^{-7}$

The inhibition of respiration is not limited to uncoupling agents in the classical sense, like 2,4-dinitrophenol, dicumarol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) or tetrachlorotrifluoromethylbenzimidazole (TTFB) but is also observed with excessive concentrations of several agents inducing ion transport, like gramicidin B (see Table I). In the latter case the inhibited state can be reached either by titrating into the solution excess sodium at a fixed gramicidin level or by adding excess gramicidin at a fixed sodium concentration.

The inhibition is observed with most of the substrates tested, *viz.* succinate, α-oxoglutarate, malate, glutamate, β-hydroxybutyrate (and NADH in submitochondrial particles), the exceptional substrates being tetramethyl-*p*-phenylenediamine (TMPD) (*plus* ascorbate) and choline. The degree of inhibition in each case is dependent on the substrate concentration[4].

Inhibition of respiration by the classical uncoupling agents is observed in mitochondria of different sources as well as in several non-phosphorylating submitochondrial particles, for instance heart-muscle preparation[2] and electron-transfer particle (ETP)[5]. In submitochondrial particles no inhibitory effect by agents inducing ion transport has yet been observed.

Both in intact mitochondria and in submitochondrial particles the inhibition is readily reversible, for instance by the addition of serum albumin which binds the uncouplers[6]. With intact mitochondria the phosphorylating system is still completely intact after the treatment with uncoupler and serum albumin since a cycle of enhanced respiration can be shown upon addition of a limited amount of ADP (ref. 7).

The inhibitory effect of any combination of uncouplers or agents inducing ion transport is additive, *i.e.* the addition of so-called "optimal" concentrations of two or more uncouplers together will result in a submaximal rate of respiration. An extreme example of this may be found in intact mitochondria, where even the lowest concentration of uncoupler will inhibit respiration in the presence of ADP and P_i (ref. 4) (Fig. 1).

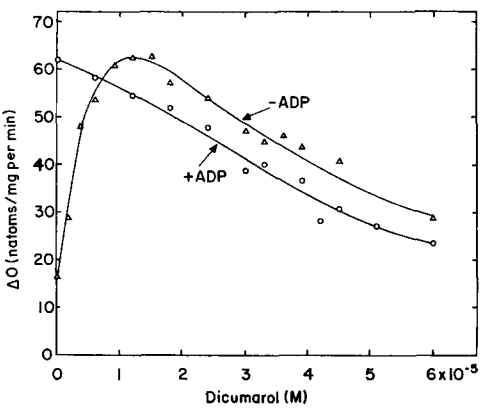

Fig. 1. Oxygen uptake was determined with a Clark oxygen electrode at room temperature and pH 7.4 in a medium containing: 50 mM sucrose, 50 mM Tris chloride, 50 mM KCl, 10 mM P_i, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 μg/ml rotenone, 4 mg/ml rat-liver mitochondria and 3 mM succinate. The rate was measured at different dicumarol concentrations in the presence or absence of 1 mM ADP.

The degree of reduction of the cytochromes in the inhibited state was measured by recording difference spectra at liquid-nitrogen temperature between maximally respiring and partially inhibited mitochondria. In this way it was found that under conditions of inhibition there was always a complete oxidation of all cytochromes (Table II; see also ref. 7). By direct analysis in acid and alkali extracts it was also established that the inhibited state was accompanied by an almost complete oxidation of the endogenous nicotinamide nucleotides of the mitochondria, regardless of the substrate present. Therefore, inhibition of respiration by excessive concentrations of uncouplers is characterized by a virtually complete oxidation of all carriers in the respiratory chain. This observation militates against localization of the inhibitory effect anywhere in the respiratory chain or in the energy-conserving reactions. In that case we would expect a reduction of at least some of the carriers in the respiratory chain as is the case in State 4 (*cf.* ref. 7).

Both the competitive nature of the inhibition and the highly oxidized state of the respiratory chain in the inhibited state point to an influence of the uncoupler at the level of the interaction of the substrate with the primary dehydrogenase. This is also borne out by the fact that the ferrocyanide reduction by succinate in ETP is

inhibited by uncouplers. Although some other dehydrogenases are known to be directly inhibited by uncouplers[8] this can not be the sole reason for the respiratory inhibition in intact mitochondria, since addition of ADP (which by itself is not inhibitory) lowers the concentration of uncoupler required to give half-maximal inhibition (Fig. 1).

TABLE II

PERCENTAGE REDUCTION OF ELECTRON CARRIERS IN THE UNCOUPLED AND THE INHIBITED STATE

In a volume of 3 ml the following were incubated at room temperature and pH 7.4: 50 mM Tris chloride, 50 mM KCl, 50 mM sucrose, 10 mM P_i, 4.2 mM glutamate, 4.2 mM malate, 4.7 mg/ml rat-liver mitochondria. After determining the steady-state rate of oxygen uptake, samples were taken either for chemical determination of nicotinamide nucleotides or for measurement of cytochrome spectra at liquid-nitrogen temperature. The reduction of cytochromes obtained upon anaerobiosis is taken as 100%. Data given are percentages of this value.

Dicumarol (*M*)	*Rate of oxygen uptake* (*natoms/mg protein per min*)	*Reduction* (%)				
		cyt aa_3	*cyt c*	*cyt b*	*NAD*	*NADP*
10^{-5}	47	0	17	33	40	98
10^{-4}	24	0	0	0	0	50

It seems very likely, then, that the inhibition is due to lack of penetration of the substrates through a barrier or membrane, a conclusion also reached by HARRIS, HÖFER AND PRESSMAN[9] on the basis of respiratory experiments. In this respect it may be significant that the oxidation of the only positively charged substrates TMPD and choline is not inhibited. Further direct evidence for this hypothesis comes from recent experiments in which it was shown that the accumulation of substrate anions by mitochondria is indeed inhibited by relatively high concentrations of uncouplers[10]. The picture emerges that regulation of substrate metabolism in intact mitochondria can be exerted through the availability of energy for penetration; decreasing the available energy below a certain level will result in inhibition of substrate entry and thereby of substrate utilization.

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