EVIDENCE FOR THE INVOLVEMENT OF IRON IN THE ADP-ACTIVATED PEROXIDATION OF LIPIDS IN MICROSOMES AND MITOCHONDRIA

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We have recently demonstrated a TPNH-linked peroxidation of lipids in rat liver microsomes (Hochstein and Ernster, 1963a,b). The reaction was greatly stimulated by ADP and to a lesser extent by other nucleoside pyrophosphates and by inorganic pyrophosphate. A similar, though nonenzymically induced, peroxidation of lipids was found to occur with ascorbate instead of TPNH, in this case in both microsomes and mitochondria. The stimulating effect of the pyrophosphates was tentatively interpreted as being due to the formation of an iron chelate, the peroxide of which might act as an initiator of the observed lipid peroxidation. In this paper we wish to report on some technical complications, that have arisen in the course of our continued studies, whose resolution now has led to a direct demonstration of the involvement of iron in the pyrophosphate-activated peroxidation of lipids in microsomes and mitochondria.

As was reported previously (Hochstein and Ernster, 1963a,b), incubation of rat liver microsomes at 22-25°C in the presence of 0.3 mM TPNH and 4 mM ADP resulted in an O_2 uptake of 80-100 mμmoles/min./mg protein, accompanied by the formation of 4-5 mμmoles malonaldehyde/min./mg protein. These rates were observed fairly consistently over a period of more than six months until, from one day to the next, they dropped to about one-tenth of the original values. This drop coincided with a change in the batch of ADP used (from

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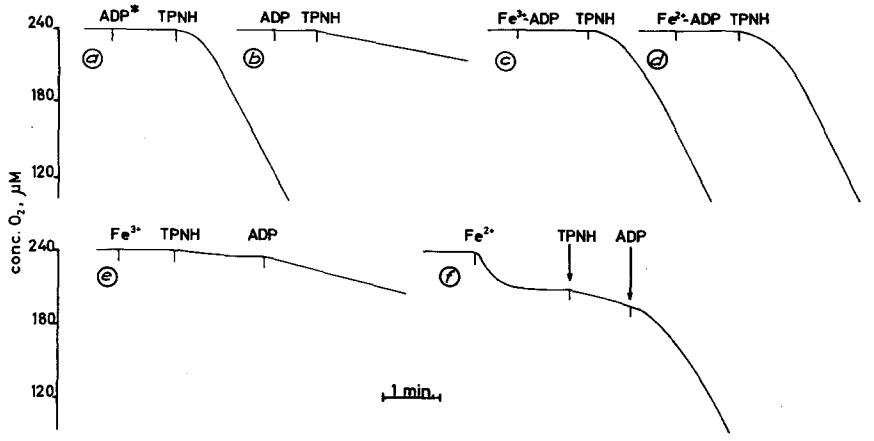

Fig. 1. Effects of ADP and Fe on the TPNH-linked peroxidation of lipids in rat liver microsomes. The assay system contained 3.6 mg microsomal protein, 25 mM Tris buffer, pH 7.5, 150 mM KCl, 0.3 mM TPNH, 4 mM ADP (Lot No. 33B-822-1, or, when marked with asterisk, Lot No. 32B-700-3; cf. Table I), and, when indicated, 12 μM Fe^{2+} or Fe^{3+}, in a final volume of 1.5 ml. O_2 uptake was measured with a Clark oxygen electrode at 24°C.

one with Lot No. 32B-700-3 to a new batch with Lot No. 33B-822-1; both from Sigma Chemical Co.). When the new sample of ADP was mixed with 0.1-0.5 mole % Fe^{2+} or Fe^{3+} and allowed to stand at room temperature for about one hour, it duplicated the effect of the old batch in promoting a high rate of O_2 uptake (Fig. 1). When Fe^{3+} was added to the microsomes directly, it did not improve their response to ADP; when Fe^{2+} was added directly, it did elicit a good ADP effect, but it also caused a transient O_2 uptake on its own, i.e., without TPNH and ADP, a phenomenon which was not encountered in the earlier experiments. All of these findings strongly suggested that the previously observed effect of ADP probably was due to a contamination of the old batch of ADP with complex-bound iron. This explanation could be verified when, a month later, a sample of ADP of Lot No. 32B-700-3 was received. Analysis of Fe with the bathophenantroline reagent (Diehl and Smith, 1960) revealed the presence of 364 mμg Fe/mg of 97 % pure $Na_2ADP.3H_2O$, i.e., appr. 0.3 mole % Fe. Some Fe was also found in the "inactive" ADP preparation, Lot No. 33B-822-1, as well as in a number of other commercial samples of nucleotides (Table I), but these impurities were relatively small. In all cases the Fe was present

entirely or predominantly as Fe^{2+}. It may also be of interest to mention that, in the case of the nucleoside di- and triphosphates, it was necessary to treat these with 1 M HCl for 10 min. in boiling water bath before the addition of the bathophenantroline reagent in order to obtain maximal color development; this probably is due to a strong binding of the Fe to the pyrophosphate moiety of the nucleotides.

Table I. Fe content of some commercial samples of nucleotides.

Sample	Lot No.	mμg Fe/mg	Sample	Lot No.	mμg Fe/mg
AMP	A10B-069	17	IMP	69-709-1	32
ADP	32B-700-3	364	IDP	73B-7380	4
"	33B-822-1	36	ITP	64-270	17
ATP	41B-665	2	"	42B-732	39
"	48-93	16	UDP	32B-761-1	87
"	53B-7110	16	"	43B-816	26
"	112B-749	58	UTP	22B-767	37
CMP	129-754-2	33	DPN	93B-7080	3
CTP	43B-757-11	74	"	93B-7300	2
"	23B-658	36	DPNH	122B-604	51
GMP	119-758	1	TPN	51B-685	58
GDP	73B-7130	0	TPNH	73B-7330	27
GTP	23B-777	34			
"	82B-794	49	$Na_4P_2O_7 \cdot 10H_2O$		2

Fig. 2 shows the effect of varying concentrations of ADP and Fe^{2+} on the TPNH-induced O_2 uptake of microsomes. ADP and Fe^{2+}, in the desired proportions, were mixed in advance and the mixtures were allowed to stand at room temperature until their addition to the microsomes no longer caused the transient O_2 uptake typical of Fe^{2+} (cf. Fig. 1). At a concentration of Fe^{2+} of 12 μM (i.e., that corresponding to the concentration of Fe^{2+} present in the earlier experiments when using 4 mM Fe-contaminated ADP), 1 mM ADP gave maximal O_2 uptake (Fig. 2a); this rate could be increased further by about 20 % when the concentration of Fe^{2+} was raised to 40 μM (Fig. 2b).

In Fig. 3, a comparison is made of the effects of various nucleoside diphosphates, as well as of ATP, AMP, and inorganic pyrophosphate, all tested

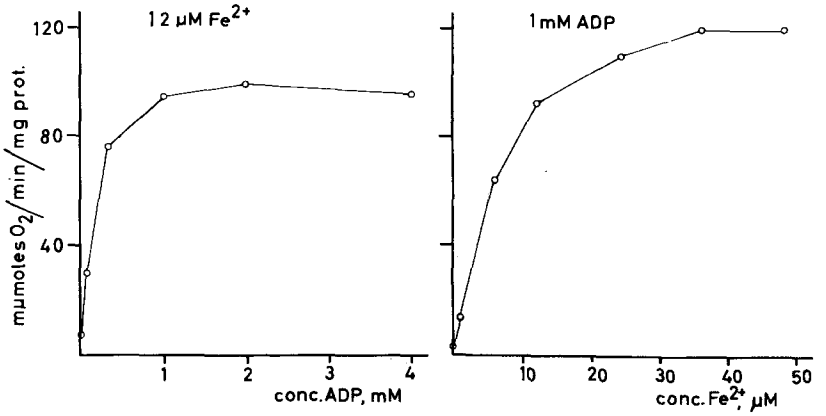

Fig. 2. Effects of varying concentrations of ADP and Fe^{2+} on the TPNH-linked peroxidation of lipids in rat liver microsomes. Conditions as in Fig. 1.

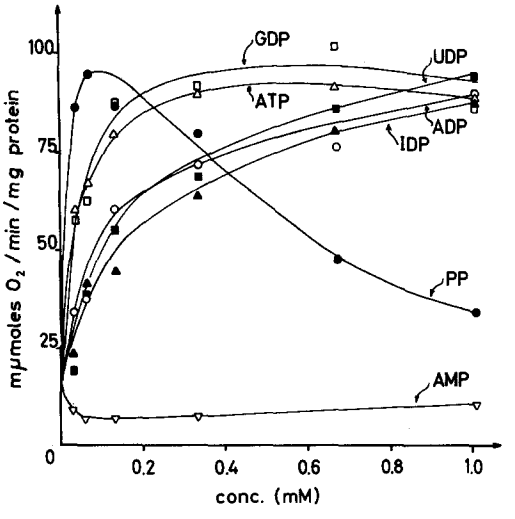

Fig. 3. Effect of various nucleotides and of inorganic pyrophosphate on the TPNH-linked peroxidation of lipid in rat liver microsomes. Fe^{2+}, 12 μM, was added together with the nucleotides or the inorganic pyrophosphate. Other conditions as in Fig. 1.

in varying concentrations together with a constant concentration of Fe^{2+}, 12 μM. In agreement with previous results (Hochstein and Ernster, 1963a,b), all nucleoside diphosphates, as well as ATP, gave increasing activity with increasing concentration, whereas inorganic pyrophosphate was active at low and less so at high concentrations, and AMP was inactive. In contrast to earlier reports, however, the same maximal activity was obtained with all the nucleo-

side pyrophosphates and with inorganic pyrophosphate, and the nucleotide concentrations required for maximal activity were lower than those found previously; also, ATP and GDP were the most efficient among the nucleoside pyrophosphates tested, and not, as previously found, ADP. Clearly, these discrepancies may be ascribed to the presence of uncontrolled amounts of iron in the earlier experiments. In all the series shown in Figs. 2 and 3, the O_2 uptake was accompanied by an accumulation of malonaldehyde as could be ascertained by the thiobarbituric acid reaction. The molar ratio, O_2/malonaldehyde, was, as before, about 20.

Table II. Effect of Fe^{2+}, Fe^{3+}, their ADP complexes, and of Cu^{2+} on lipid peroxidation in microsomes and mitochondria.

Additions: TPNH or DPNH, 0.3 mM; ascorbate, 1 mM; ADP, 1 mM; Fe^{2+}, Fe^{3+}, or Cu^{2+}, 12 μM. Other conditions as in Fig. 1.

Additions	mμmoles O_2/min./mg protein					
	MICROSOMES			MITOCHONDRIA		
	TPNH	Ascorbate	DPNH	TPNH	Ascorbate	DPNH
None	1	5		0		
ADP	11	15		0	4	
Fe^{2+}	8	148			12	
Fe^{2+}-ADP	74	224	2	0	113	1
Fe^{3+}	1	23			16	
Fe^{3+}-ADP	72	219	2	0	112	1
Cu^{2+}	1	23				
Cu^{2+}, ADP	1	23				

Data presented in Table II illustrate the effects of TPNH, ascorbate, and DPNH on the O_2 uptake of microsomes and mitochondria as measured in the presence of Fe^{2+} or Fe^{3+} alone or as the corresponding ADP complexes as well as of Cu^{2+}. In microsomes, both TPNH and ascorbate induced high rates of O_2 uptake when Fe^{2+}-ADP or Fe^{3+}-ADP was present, whereas only ascorbate was active in the case of mitochondria; DPNH was inactive in both cases. These

results are analogous to those previously obtained with the Fe-contaminated ADP. When Fe^{2+} alone was added, a transient O_2 uptake was observed with the microsomes (cf. Fig. 1), but not with the mitochondria. Subsequent addition of TPNH caused a low rate of O_2 uptake in the microsomes, and virtually none in the mitochondria. Ascorbate, on the other hand, induced a relatively high rate of O_2 uptake in the microsomes (yet significantly lower than in the presence of Fe^{2+}-ADP), and a relatively low rate in the mitochondria. When Fe^{3+} alone was present, TPNH induced practically no, and ascorbate only a relatively slow O_2 uptake in both microsomes and mitochondria. These findings will be elaborated on further in a forthcoming communication (Ernster and Nordenbrand, 1964). Finally, Cu^{2+} alone acted similarly to Fe^{3+} alone, but in combination with ADP it did not duplicate the effect of Fe^{3+}-ADP.

From these results it may be concluded that the activation of microsomal and mitochondrial lipid peroxidation by ADP and other pyrophosphates involves the participation of iron.

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