Biochemical Pharmacology, Vol. 36, No. 4, pp. 489–493, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

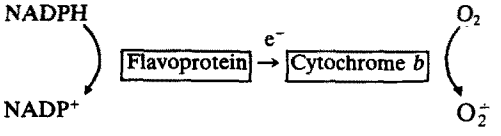
THE INHIBITORY EFFECTS OF SOME IODONIUM COMPOUNDS ON THE SUPEROXIDE GENERATING SYSTEM OF NEUTROPHILS AND THEIR FAILURE TO INHIBIT DIAPHORASE ACTIVITY

ANDREW R. CROSS
Department of Biochemistry, Medical School, University Walk, Bristol, BS8 1TD, U.K.

(*Received* 19 *May* 1986; *accepted* 4 *September* 1986)

Abstract—I have recently reported the inhibition of the neutrophil superoxide generating oxidase by very low concentrations of diphenylene iodonium (A. R. Cross and O. T. G. Jones, *Biochem. J.* **237**, 111, 1986). Here I report on the sensitivity of the oxidase to two other iodonium compounds, iodonium thiophen and iodonium biphenyl. In addition, the lack of inhibition of dye reductase activity in a solubilized preparation of the oxidase is described suggesting that the superoxide forming enzyme system of neutrophils does not possess an intrinsic dye reductase activity.

The professional phagocytes (polymorphonuclear leukocytes (PMN), monocytes, macrophages and eosinophils) possess a membrane-bound enzyme system which can be activated to produce toxic oxygen radicals in response to a wide variety of stimuli. The purpose of this enzyme system is to protect the organism against microbial or parasitic infection and malignancy by directing the radicals against the target cell. Whilst the beneficial effects of these oxygen radicals are well known, it has become clear that inappropriate production of oxygen radicals can have severe deleterious effects in disease, including rheumatoid arthritis and crystal induced arthropathies [1]: Crohns disease, acute respiratory distress syndrome and reperfusion injury [2]: and carcinogenesis [3]. The enzyme system consists of at least two components, a FAD containing flavoprotein and a unique low potential *b*-type cytochrome (cytochrome b_{-245}). When activated the system reduces molecular oxygen to the superoxide anion radical at the expense of NADPH. We believe this superoxide generating oxidase is organized as shown in the scheme:



There is some controversy over whether the oxidase is capable of reacting at significant rates with any artificial electron acceptors (i.e. whether it exhibits any diaphorase activity). The first workers to investigate this phenomenon closely found no diaphorase activity with four artificial electron acceptors (cytochrome *c*, DCPIP, ferricyanide or methylene blue [4]). Subsequently several groups have ascribed diaphorase activity to the superoxide generating enzyme on the grounds of coincidence of subcellular localization, absence in resting cells or cells from a patient with chronic granulomatous disease (in which the oxidase is absent) and inhibition of oxygen uptake in the presence of artificial acceptors [5, 6]; similar K_m for NADPH and FAD, and similar sensitivity to the flavin analogue, quinacrine [7]. However, others have described a number of diaphorases in neutrophils which can be purified free of the superoxide generating activity, including a NADH cytochrome *c* reductase which increases 2-fold in activity following stimulation of the cells [8–11]. A thorough investigation of diaphorase activity in guinea-pig neutrophils by Bellavite *et al.* [12] found no increase in diaphorase activity in stimulated cells, the K_m for NAD(P)H for oxygen and dye reduction were different, there was no inhibition of oxygen uptake in the presence of artificial electron acceptors and the partially purified oxidase did not reduce dye acceptors even in anaerobiosis.

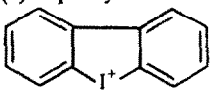
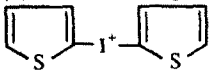
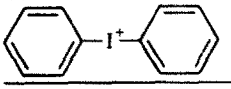
I have recently described an inhibitor of the neutrophil oxidase which reacts to form a covalent complex with a polypeptide at the level of, or prior to, the flavoprotein component of the complex [13]. This compound, diphenylene iodonium, inhibits the oxidase in a time dependent manner, at concentrations approaching 1:1 stoichiometry with the FAD content of the preparation. In view of the potential anti-inflammatory properties of compounds which inhibit the radical-generating system of neutrophils, here I report on the inhibitory effects of two analogues of diphenylene iodonium which are less toxic to mitochondria. In addition the effect of diphenylene iodonium on the diaphorase activity of the solubilized superoxide generating oxidase of porcine neutrophils is described.

MATERIALS AND METHODS

Iodonium diphenyl and iodonium dithiophen were generous gifts from Dr F. Earley (Department of Biochemistry, University of Southampton). Diphenylene iodonium sulphate was synthesised from 2-aminobiphenyl as described by Gatley and Sherratt [14] and Collette *et al.* [15]. The structures are shown in Table 1.

Preparation of soluble oxidase. Solubilized superoxide generating oxidase was prepared from

Table 1. Inhibition of the superoxide generating oxidase by iodonium compounds

	Concentration required to inhibit 50% activity of: Oxidase (μM)	Mitochondria* (μM)
(a) Diphenylene iodonium 	0.060	0.23
(b) Iodonium thiophen 	0.067	2.5
(c) Iodonium biphenyl 	1.6	88

* Data from [21].

pig neutrophils as described previously [16, 17] and stored in 1-ml aliquots in liquid nitrogen.

Determination of O_2^- production. NADPH-dependent superoxide production was determined by the superoxide dismutase sensitive rate of reduction of cytochrome *c* as described previously [18]. The incubation mixture consisted of 2 mM NADPH, 100 μM horse heart cytochrome *c* (Sigma type III) in 1 ml 50 mM triethanolamine hydrochloride pH 8.0 at 20°. The difference in the rate of cytochrome *c* reduction determined in the absence and presence of 100 μg superoxide dismutase (3000 units mg^{-1}) was used to calculate the rate of superoxide production, assuming 1 mol. superoxide reduces 1 mol. cytochrome *c*.

Measurement of dye reductase activities. NADPH (0.5 mM) and NADH (0.5 mM) dependent dye reductase activities were determined in a dual wavelength spectrophotometer at 20° in the presence or absence of superoxide dismutase (300 U ml^{-1}) in 50 mM triethanolamine hydrochloride pH 8.0 using the following acceptors and wavelength pairs; 100 μM horse heart cytochrome *c* (19.9 mM^{-1} cm^{-1}, 550–540 nm) [19]; 50 mM DCPIP (21.8 mM^{-1} cm^{-1} at pH 8.0, 600–522 nm) [20], and 60 μM potassium ferricyanide (1.04 mM^{-1} cm^{-1}, 405–500 nm). In each case the reaction was initiated by the addition of the enzyme.

Inhibition studies. Inhibition of the solubilised O_2^- generating oxidase was assayed either by adding small (< 10 μl) quantities of iodonium diphenyl, iodonium thiophen or diphenylene iodonium dissolved in 50% ethanol to the O_2^- assay mixture or by incubation of small (<10 μl) quantities of inhibitors with 1 ml aliquots of enzyme for 2 hr or 24 hr on ice before assay. Addition of 10 μl of 50% ethanol alone had no effect on superoxide production. None of the iodonium compounds had any effect on the rate of superoxide production by xanthine/xanthine oxidase in the cytochrome *c* assay at the highest concentration tested (100 μM).

Determination of flavin and cytochrome b_{-245} content. The flavin and cytochrome contents were assayed in oxidised-minus-reduced difference spectra using $\Delta\varepsilon_{450-500} = 11.3\ mM^{-1}\cdot cm^{-1}$ and $\Delta\varepsilon_{559-540} = 21.6\ mM^{-1}\cdot cm^{-1}$ respectively [16].

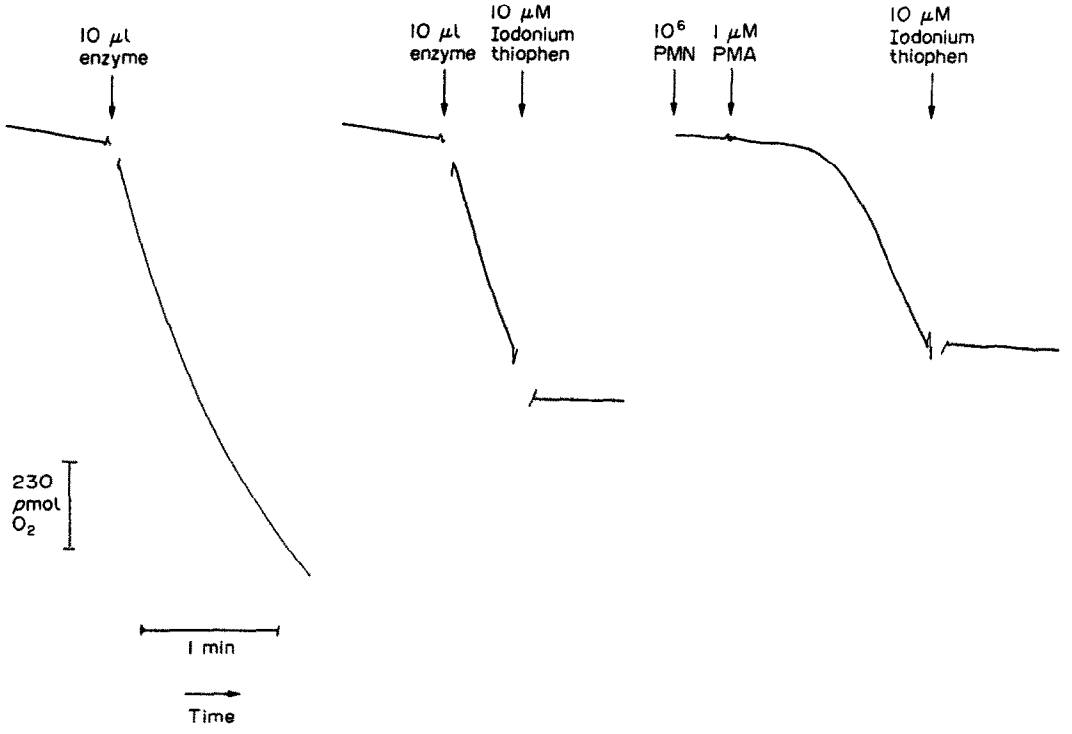


Fig. 1. The effect of iodonium compounds on superoxide production by solubilised oxidase and intact neutrophils. Superoxide production was determined by the superoxide dismutase sensitive rate of reduction of cytochrome *c* as described in Materials and Methods.

RESULTS

Inhibition of $O_2^{\cdot}$ production from the solubilised enzyme by diphenylene iodonium

The profile of inhibition by iodonium thiophen was virtually identical to the inhibitory properties of diphenylenene iodonium found previously [13]. Iodonium thiophen caused rapid inhibition of enzyme activity when added to the buffer during the superoxide assay. At concentrations above 10 μM inhibition was apparently complete within the mixing time (approx 8 sec, Fig. 1); at lower concentrations the inhibition became increasingly time dependent (Fig. 2). With respect to this time dependence, the inhibition of the neutrophil oxidase closely resembles the inhibition of NADH-ubiquinone reductase by diphenylene iodonium [21]. Owing to the spontaneous decay of enzyme activity ($t_{1/2}$ = 36 sec during enzyme turnover in the $O_2^{\cdot}$ assay) measurement of enzyme inhibition at very low concentrations of the inhibitor becomes increasingly difficult and therefore the enzyme was preincubated on ice for 1 hr in the presence of low concentrations of iodonium thiophen. After incubation of the enzyme with inhibitor for 24 hr (at 0°) inactivation of the enzyme had occurred before addition to the assay. Under these conditions 31% inhibition of the oxidase activity is observed at concentrations of iodonium thiophen at which the stoichiometry of the inhibitor:FAD content of the enzyme is 1:1. (Fig 3). Addition of 100-fold excess of iodonium thiophen does not cause total inhibition, approximately 6% of the activity remaining at this concentration. At higher concentrations (300–600-fold excess) no residual activity could be detected.

Addition of 50 μM iodonium thiophen to phorbol myristate acetate-stimulated intact neutrophils (Fig. 1) caused marked inhibition of superoxide gen-

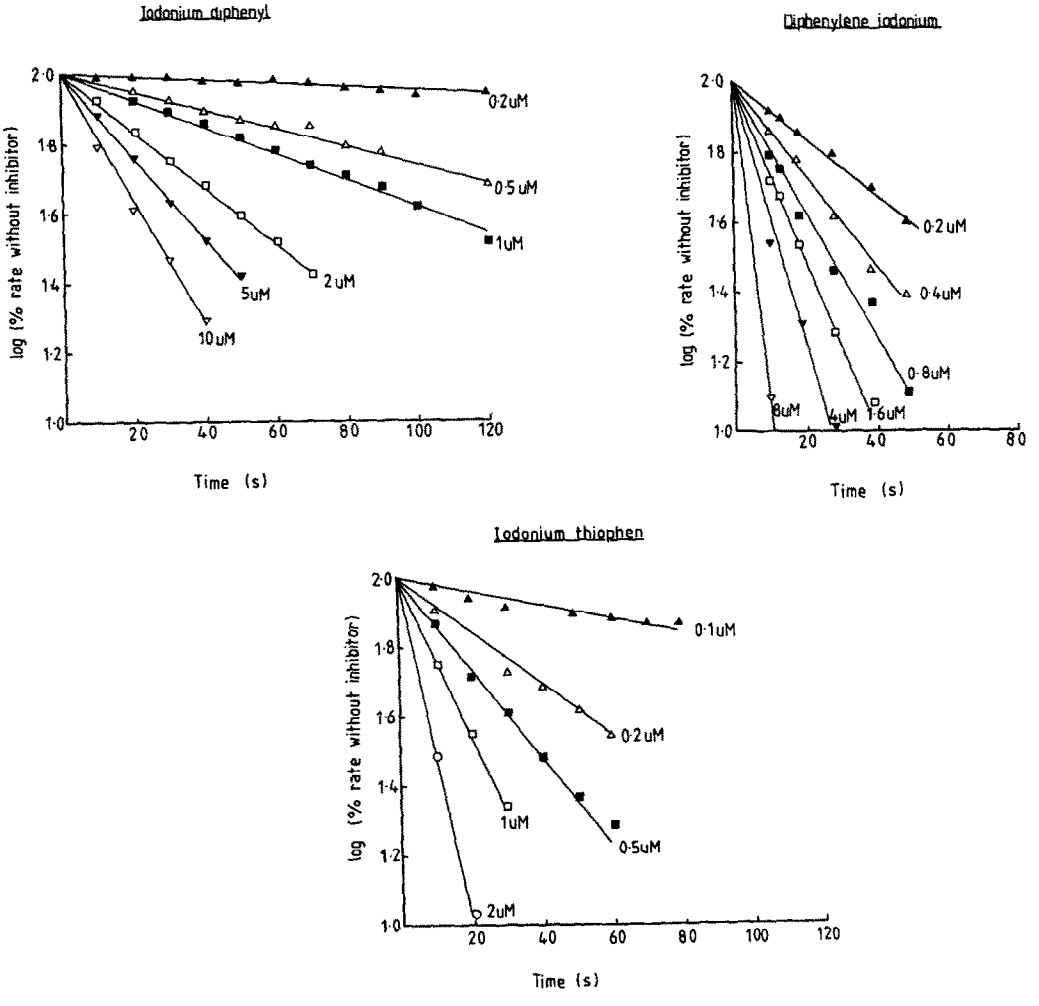


Fig. 2. The time dependence of the inhibition of the solubilised superoxide generating oxidase by iodonium compounds. Superoxide production was determined by the superoxide dismutase sensitive rate of reduction of cytochrome *c* as described in Materials and Methods. Inhibitor was added to the assay mixture at the required concentration and the reaction initiated by the addition of the solubilised oxidase. The activities are expressed as the log of the percentage of the initial rate in the absence of the inhibitor, corrected for the spontaneous decay of activity. The cytochrome b_{-245} and flavin concentrations were 1.87 nM and 2.44 nM respectively. The protein concentration was 4.8 μg ml^{-1}.

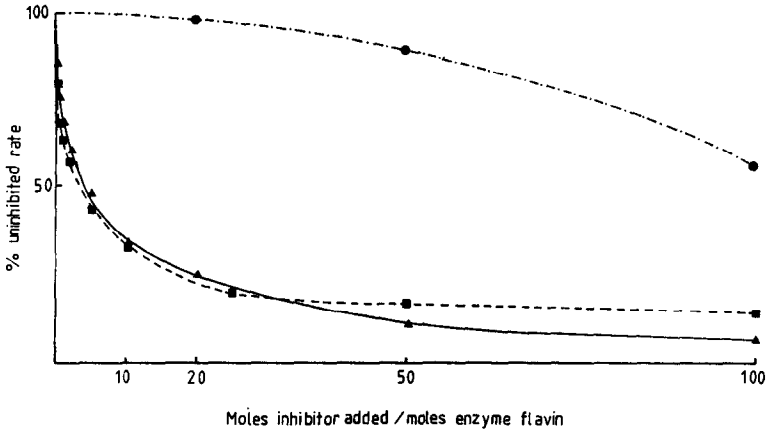


Fig. 3. The effect of a 24 hr incubation of the superoxide generating oxidase with iodonium thiophen, diphenylene iodonium and iodonium biphenyl. Aliquots of the superoxide generating oxidase were incubated for 24 hr on ice and assayed for residual oxidase activity as described in the Materials and Methods section. The cytochrome b_{-245} and flavin contents were 347 μmol ml^{-1} and 354 pmol ml^{-1} respectively. The protein concentration was 0.8 mg ml^{-1}. ▲, iodonium thiophen; ■, diphenylene iodonium; ●, iodonium biphenyl.

eration indicating that the cells are permeable to the inhibitor (in keeping with the lipid solubility of iodonium compounds) or its site of action is exposed to the external medium.

Iodonium diphenyl was a much less effective inhibitor than either iodonium thiophen or diphenylene iodonium, a 100-fold excess causing only 44% inhibition (Fig. 3).

Effect of diphenylene iodonium on dye reductase activities

As can be seen in Table 2 there is some diaphorase activity exhibited towards all three acceptors in the solubilized preparation with NADPH as the electron donor and towards DCPIP and ferricyanide with NADH as donor. In the presence of 4 μM diphenylene iodonium (which inhibited 99% of the superoxide forming capacity after 1 min) there was no inhibition of any of the dye reductase activities.

DISCUSSION

In many respects the inhibition of the neutrophil oxidase by diphenylene iodonium and iodonium thiophen is strikingly similar to the inhibition of NADH ubiquinone reductase by diphenylene iodonium described by Ragan and Bloxham [21]. In particular, the disproportionately large increase in inhibitor concentration required to inhibit the residual 30% of activity (Fig. 3) and the time dependence of inhibition at low concentrations of inhibitor (Fig. 2) suggest very similar mechanisms of inhibition. The neutrophil superoxide generating system does differ in being more sensitive to iodonium diphenyl and iodonium thiophen than is the mitochondrial enzyme however (Table 1). These two compounds have virtually the same activity toward the neutrophil oxidase, but the mitochondrial enzyme is 10-fold less sensitive to iodonium thiophen. Iodonium biphenyl is a much less effective inhibitor of either enzyme. It has previously been shown that diphenylene iodonium forms a covalent adduct with a component of the oxidase. In view of the probable involvement of the neutrophil superoxide generating system in inflammatory processes and its extreme sensitivity to iodonium compounds, it is possible that compounds of this type (without the undesirable hypoglycemic

Table 2. The effect of diphenylene iodonium on superoxide generation and dye reductase activities by solubilized neutrophil membranes

	Activities (nmol. min^{-1}. ml^{-1}) to:					
	Cytochrome *c*		DCPIP		Ferricyanide	
	−SOD*	+SOD†	−SOD	+SOD	−SOD	+SOD
NADPH	247	7.1	11.8	9.2	n.d.	37.5
+diphenylene iodonium	2.3	7.0	0	10	n.d.	36.4
NADH	41	<0.1	n.d.	8.4	n.d.	352
+diphenylene iodonium	0	<0.1	n.d.	7.4	n.d.	352

* Rate in the absence of SOD minus the rate in its presence (i.e. the rate due to superoxide alone).
† Rate in the presence of SOD.
n.d. = not determined.

properties of diphenylene iodonium [22]) may prove to have useful anti-inflammatory properties.

The lack of inhibition of dye reductase activities by diphenylene iodonium under conditions where superoxide production is 99% inhibited strongly suggests the dye reductase activities are not associated with the superoxide generating system itself. It could be reasoned that the electron transport system could be inhibited by these compounds after the point at which the dyes are reduced but since it has been shown that diphenylene iodonium acts at the level of, or prior to, the flavoprotein [13] this does not appear to be the case. It can be concluded therefore that the neutrophil superoxide generating system does not possess intrinsic dye reductase activity under normal circumstances in agreement with [12] and that the diaphorase activity is a contaminant of oxidase preparations. One explanation for the contradictory findings in the literature may be the findings of Gabig and Lefker [23] who found little dye reductase activity associated with the solubilized enzyme, but after separating the flavoprotein moiety from the cytochrome b_{-245}, significant dye reductase activity appears to be associated with the flavoprotein.

Acknowledgements—I would like to acknowledge Mr C. Yea, Professor O. T. G. Jones and the Medical Research Council for assistance in the preparation of solubilized enzyme, and the Arthritis and Rheumatism Council for financial support.

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