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PEROXIDASE ACTIVITY IN RELATION TO IODIDE, 17β-OESTRADIOL AND THIOUREYLENE DRUG UPTAKE IN HUMAN POLYMORPHONEUTROPHILS

M. M. FERGUSON, *W. D. ALEXANDER,† J. M. C. CONNELL,† A. G. LAPPIN,‡ D. C. MCCRUDEN,† R. MACLURE,* R. J. MAIRS* and A. YOUNGER†

* Department of Oral Medicine and Pathology, Dental Hospital and School, Glasgow;
† Department of Medicine, Western Infirmary, Glasgow; ‡ Department of Chemistry, University of Glasgow, Glasgow, U.K.

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Abstract—In polymorphoneutrophils (PMNs) phagocytosis is accompanied by an increase in peroxidase activity. Accumulation of iodide, thioureylene antithyroid drugs and 17β-oestradiol also occurs during the process. There is no evidence of an active iodide transport system in the PMNs as pertechnetate is not concentrated and neither ouabain nor perchlorate abolishes iodide accumulation. The uptakes of ^{125}I, [^{35}S]PTU and [^{3}H]-17β-oestradiol were compared in phagocytosing PMNs and the effects of various compounds examined. In addition, chemiluminescence generation from luminol by PMNs and by horseradish peroxidase was studied. This indicated that uptake of all three compounds could be associated with activation of the peroxidase system, and inhibition of this enzyme system caused a reduction in their accumulation.

Phagocytosis of micro-organisms by polymorphoneutrophils (PMNs) is accompanied by a marked increase in their metabolic activity. Stimulation of the myeloperoxidase system in PMNs is associated with killing of the ingested micro-organisms: processes increased in this system are oxygen uptake, metabolism of glucose via the hexose monophosphate shunt and hydrogen peroxide production.

The PMN uptake of both iodide and 17β-oestradiol is increased by phagocytosis and this accumulation requires activation of the myeloperoxidase system [1, 2]. Autoradiographic studies have shown that the 17β-oestradiol is bound to granules in the cytoplasm of rat PMNs [3]. The uptake of thioureylene antithyroid drugs, such as methimazole (MMI) and propylthiouracil (PTU), is similarly increased in phagocytosing PMNs [4].

In most tissues wherein there is accumulation of iodide, a membrane-associated, active anion transport mechanism operates [5]. The thioureylene drugs are accumulated in both the thyroid gland [6, 7] and the ducts of salivary glands [8], but it would appear that the anion transport mechanism is not directly responsible for this [9]. Whereas the mode of action of the thioureylene antithyroid drugs has been linked generally to the peroxidase system [10], there is no clear consensus as to the specific mechanism.

Peroxidases have been isolated from a number of animal and vegetable tissues: that obtained from the root of the horseradish has been most extensively investigated and is known to exist in the form of several isoenzymes with different physico-chemical and kinetic properties [11]. Horseradish peroxidase contains protohaemin IX as the prosthetic group and this exists functionally as the ferryl ion in its oxidized state. In addition, cysteine has been identified in peroxidase and it has been postulated that both iodide and the thioureylene drugs may interact with the sulphydryl groups [12].

The early studies of Miller *et al.* [13] examining the reducing power, or redox potential, of thioureylene compounds have apparently not been pursued although this must be one important factor for compounds interacting with peroxidase.

It was the purpose of the present study to determine to what extent myeloperoxidase activity in the human polymorphoneutrophil is responsible for the uptake of iodide, 17β-oestradiol and the thioureylene antithyroid drug PTU. No attempt was made in this investigation to differentiate between the accumulated radioactive drug and its products although phagocytosing PMNs do metabolize thioureylene drugs (G. G. Skellern, M. M. Ferguson and R. Mairs, unpublished observation).

MATERIALS AND METHODS

Chemicals. The following radioactive isotopes were obtained from the Radiochemical Centre (Amersham, Bucks., U.K.) Na^{125}I (carrier-free), [^{35}S]PTU (46 mCi/mmole), [^{3}H]-17β-oestradiol (101 Ci/mmole), [^{14}C]progesterone (50 mCi/mmole), [^{14}C]testosterone (50 mCi/mmole), ^{99m}Tc-pertechnetate (50 mCi/ml), [^{203}Hg]mercuric acetate (2 mCi/mg Hg). All other chemicals were of the highest grade available and obtained from the following sources: ouabain, 17β-oestradiol, zymosan, methimazole, 6*n*-propyl-2-thiouracil, 2,4-dinitrophenol,

p-chloromercuribenzoic acid, 2-deoxyglucose, luminol (Sigma Chemical Co.); sodium iodide, potassium perchlorate, sodium fluoride (BDH); 3,4-dihydroxybenzoic acid (Aldrich Chemical Co.); tetra-sodium diethylstilboestrol diphosphate (W. B. Pharmaceuticals Ltd.). The sodium salts of 3,4-dihydroxybenzoic acid and *p*-chloromercuribenzoic acid were made by dissolving the solid in 0.2 N sodium hydroxide and adjusting the pH of the solution to pH 80 with 0.5 M sodium dihydrogen phosphate.

Cell preparation. Venous blood was withdrawn from healthy volunteers and mixed immediately with preservative-free heparin. The total leukocytes were obtained by selectively sedimenting the erythrocytes with Plasmagel (Laboratoire Roger Bellon) and the mononuclear cells were then separated from the PMNs by density centrifugation on Ficoll-Paque (Pharmacia). The residual erythrocytes were lysed in hypotonic saline and the PMNs finally suspended in Gey's solution (Gibco Biocult), additionally buffered with 0.02 M HEPES (pH 7.4). The average yield from 50 ml blood was 4.0×10^7 PMNs. These were at least 95% viable at the start of incubations as determined by trypan blue exclusion. Isotopes and drugs were also dissolved in Gey's solution.

Phagocytosis. Zymosan particles were opsonized by incubation at 37° for 30 min with fresh autologous plasma. The zymosan was then suspended in Gey's solution and a final zymosan concentration of 1 mg/ml was used to stimulate phagocytosis by the PMNs.

In order to quantitate phagocytosis, zymosan particles were labelled by suspending in a solution of [^{203}Hg]mercuric acetate for 12 hr at 20°. A constant specific activity of 0.1 μCi/mg zymosan was obtained after repeated washes in Gey's solution to remove unbound radioactivity. After opsonization this value was unaltered. Control media and media containing 10^{-3} M methimazole (MMI) or 10^{-3} M propylthiouracil (PTU) were examined. Following incubation for 15 min at 37° with the PMNs, at a concentration 5×10^6 cells/ml, ^{203}Hg-labelled zymosan was separated from the cells by differential density centrifugation after layering the suspension onto a solution of 6.6% (w/v) Ficoll and 23% (w/v) sodium iothalamate (Conray 480-May and Baker). The cell concentration was then adjusted to 1×10^6 cells/ml and the γ-emissions from the cells were counted using a sodium iodide scintillation detector coupled via a pulse-height discriminator to a digital ratemeter (Ecko Type M5183A).

The effect of the test chemicals on phagocytosis was further examined qualitatively by labelling zymosan with fluorescein prior to opsonization. The ingested particles were visualized using a Leitz SM-Lux microscope with a Ploemapak incident UV light system and FITC filter block.

Radio-isotope uptake by cells—system A. Two PMN incubation systems were employed. First, in order to examine cell:supernatant ratios for ^{99m}Tc-pertechnetate (0.5 mCi), [^{125}I]iodide (0.25 mCi) and [^{35}S]propylthiouracil (PTU) (0.05 mCi), 30×10^6 cells in 1 ml were incubated at 37° for 1 hr after adding the opsonized zymosan. The activities of the isotopes in the cell suspension and then of the supernatant of the suspension were measured. Cells were removed by centrifugation at 400 *g* for10 min. The PMN volume of the suspension was calculated based on a cell count and assuming a uniform cell volume (1×10^6 PMNs = volume of 0.7 μl), as this was found to be more reliable than attempting to measure packed columns of PMNs in capillary tubes. Using this system, the effect of ouabain, perchlorate, 2,4-dinitrophenol (DNP), MMI, PTU and iodide was examined.

Radio-isotope uptake by cells—system B. The second technique used a PMN concentration of 2×10^6 cells/ml. After incubation at 37° in microtitre plates in a total volume of 500 μl, the cells were harvested on glass fibre discs, washed with phosphate-buffered saline, dried and counted. This second system, employing the lower PMN concentrations, was found to be more sensitive although it did not provide cell:supernatant ratios. The uptakes of [^{125}I]iodide, [^{35}S]PTU and [^{3}H]-17β-oestradiol were examined in the presence of ouabain, perchlorate, MMI, iodide, tetrasodium diethylstilboestrol diphosphate (fosfestrol), 1,10-phenanthroline, fluoride, *p*-chloromercuribenzoate (*p*CMBA), 2-deoxyglucose (2-DOG) and 2-DOG plus glucose. In addition, the uptake of [^{14}C]-progesterone and [^{14}C]testosterone was measured in non-phagocytosing and phagocytosing PMNs. The statistical significance of the results was determined using Student's *t*-test or linear regression where appropriate.

Chemiluminescence. Following dark adaptation of the cells for 30 min chemiluminescence by the PMNs was measured by incubating 5×10^6 cells in 175 μl phosphate-buffered saline (pH 7.4) at 37° together with 25 μl luminol (10^{-5} M). The test chemicals (25 μl) were added, all to give a final concentration of 10^{-4} M, and phagocytosis was initiated by the addition of 25 μl of a 10 mg/ml solution of opsonized zymosan. Chemiluminescence was measured for 100 sec using a Packard Pico-lite luminometer. The results are expressed as a percentage of the control in which phosphate-buffered saline was substituted for test chemical.

The effect of the compounds upon peroxidase activity was determined by measuring the generation of chemiluminescence by a cell-free incubation system consisting of the following components: 100 μl horseradish peroxidase-type I (Sigma Chemical Co.) (40 mU), 100 μl glucose oxidase-type II from *Aspergillus niger* (Sigma Chemical Co.) (0.12 U), 25 μl luminol (1.2×10^{-3} M) and 25 μl MMI, PTU or 3,4-dihydroxybenzoate (3,4-DHBA). Phosphate-buffered saline (25 μl) was added to controls instead of test compound. The generation of hydrogen peroxide was initiated by the addition of 50 μl glucose solution (60 mM) and chemiluminescence was measured at 37° over a period of 100 sec.

Cyclic voltametry. The ease of oxidation of horseradish peroxidase, MMI, PTU and 17β-oestradiol was determined by measuring the anodic peak potential with cyclic voltametry using a carbon paste working electrode. The test compounds were dissolved in 0.1 M phosphate buffer (pH 7.4) and maintained at 25°. The range was established by reaction with substances of known oxidation potential, i.e. hydrogen peroxide, iodide and ferricyanide.

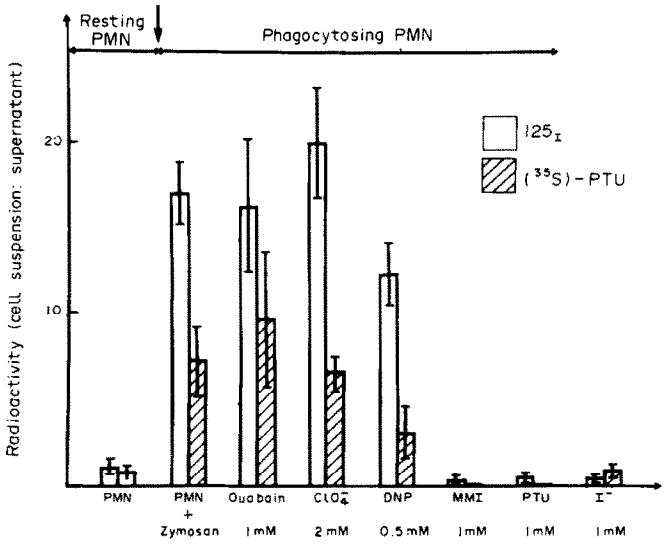

Fig. 1. Effects of various drugs on uptake of ^{125}I and (^{35}S)-PTU by human PMN ($\bar{x} \pm$ S.E.M.).

RESULTS

The fluorescein-labelled zymosan was seen to be phagocytosed by control and MMI-treated PMNs, with the ingested particles situated intracellularly in all cases. Using the [^{203}Hg]mercuric acetate-labelled zymosan no significant decrease was noted in the amount of zymosan phagocytosed by control and drug-treated PMNs, with values of 102 and 103% being obtained for MMI and PTU, respectively, in relation to controls.

Neither resting nor phagocytosing PMNs accumulated ^{99m}Tc-pertechnetate after incubation for up to 1 hr using system A, whereas there was clearly uptake of [^{125}I]iodide and [^{35}S]PTU in phagocytosing PMNs, with mean cell : supernatant ratios of 16.8 and 7.4, respectively.

In the first uptake system (A) there was a significant reduction in the cell : supernatant ratios of [^{125}I] iodide and [^{35}S]PTU when the PMNs were first treated for 10 min with 2,4-dinitrophenol, MMI, PTU and iodide prior to adding opsonized zymosan (Fig. 1). No decrease in isotope uptake was noted with either ouabain or perchlorate in this system. In the case of iodide, however, it must be appreciated that by adding cold iodide the specific activity of the radio-isotope is decreased.

When the lower cell concentration of 2×10^6 cells/ml in system B was employed, the ratio of (phagocytosing PMNs + test chemical) : (phagocytosing PMNs alone) was calculated. No difference was found in the uptake of [^{14}C]progesterone or [^{14}C]-testosterone between phagocytosing PMNs and non-phagocytosing PMNs. The effects of the various chemicals tested on the accumulation of [^{125}I]iodide, [^{35}S]PTU and [^{3}H]-17β-oestradiol are listed in Table 1. These results are expressed as a ratio, with phagocytosing PMNs in the absence of test chemical being calculated to give a value of unity. The effects of increasing concentrations of MMI, fosfestrol and

Table 1. Effects of various compounds on the accumulation of [^{125}I]iodide, [^{35}S]PTU and [^{3}H]-17β-oestradiol ($x \pm$ S.E.M.)

	Iodide	PTU	Oestradiol
PMNs + zymosan	1.00 ± 0	1.00 ± 0	1.00 ± 0
PMNs alone	0.13 ± 0.03***	0.25 ± 0.07***	0.53 ± 0.04***
Ouabain (10^{-3} M)	0.88 ± 0.09 NS	0.99 ± 0.06 NS	0.94 ± 0.06 NS
Perchlorate (2×10^{-3} M)	0.86 ± 0.03*	0.87 ± 0.04*	0.98 ± 0.14 NS
Methimazole (10^{-3} M)	0.02 ± 0.01***	0.06 ± 0.02***	0.19 ± 0.07***
Iodide (10^{-3} M)	0.01 ± 0.10***	0.79 ± 0.12 NS	0.27 ± 0.09***
Fosfestrol (10^{-3} M)	0.56 ± 0.13***	0.83 ± 0.11 NS	0.79 ± 0.11*
Phenanthroline (10^{-3} M)	0.34 ± 0.07***	0.60 ± 0.08***	0.66 ± 0.09**
Fluoride (10^{-3} M)	0.67 ± 0.06***	0.75 ± 0.14**	0.95 ± 0.05 NS
*p*CMBA (10^{-3} M)	0.32 ± 0.17**	0.47 (1 expt)	0.19 ± 0.16***
2-DOG (5×10^{-3} M)	0.10 ± 0.05***	0.13 ± 0.01***	0.39 ± 0.07***
2-DOG + glucose (5×10^{-3} M)	0.64 ± 0.24***	0.98 ± 0.03***	0.89 ± 0.13***

Results are expressed as a ratio in relation to phagocytosing PMNs in the absence of test chemical. The results of 2-DOG + glucose are compared statistically to 2-DOG alone.

PMNs, Polymorphoneutrophils; *p*CMBA, *p*-chloromercuribenzoate; 2-DOG, 2-deoxyglucose; PTU, propylthiouracil; NS, not significant.

* $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

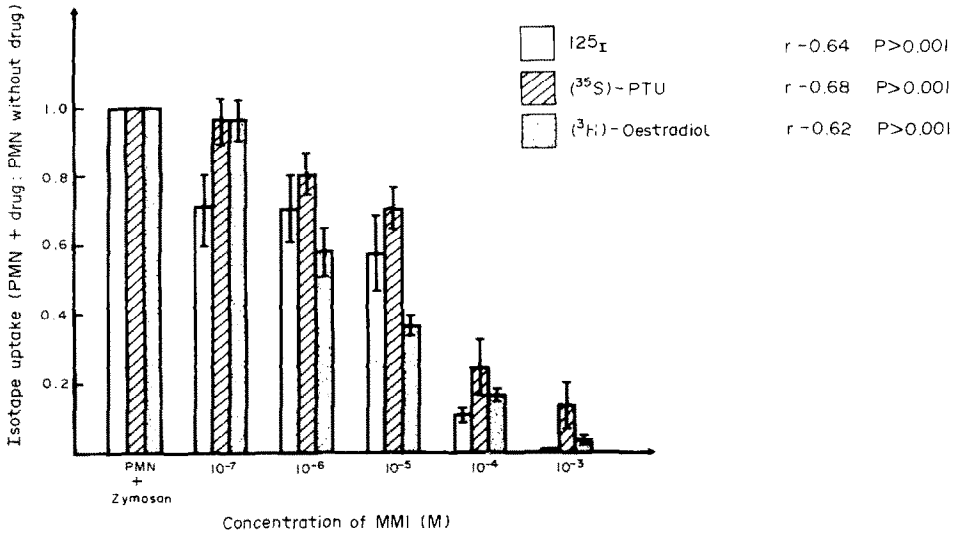

Fig. 2. Effects of various concentrations of MMI on accumulation of ^{125}I, (^{35}S)-PTU, and (^{3}H)-oestradiol in phagocytosing PMN ($\bar{x} \pm$ S.E.M.).

fluoride are shown in Figs. 2, 3 and 4, where the diminished accumulation of all three radio-labelled compounds was seen to be concentration-dependent.

In the luminometer, myeloperoxidase activity in the PMNs was initiated by phagocytosis of zymosan particles. MMI, PTU, fosfestrol, 1,10-phenanthroline, *p*CMBA, dinitrophenol, fluoride and 2-deoxyglucose all suppressed this activity to a variable extent, whereas ouabain, perchlorate and iodide had no effect on the chemiluminescence, both in intact cells and the cell-free enzyme preparation (Table 2).

The test chemicals, at a concentration of 10^{-3} M, which would undergo oxidation were ranked in order of decreasing oxidation potential: H_2O_2 (1360 mV) > peroxidase > iodide (540 mV) > PTU > MMI > fosfestrol > 17β-oestradiol > ferricyanide (430 mV). This indicated that oxidation, by myeloperoxidase, of fosfestrol, iodide, PTU, MMI and 17β-oestradiol was theoretically possible.

DISCUSSION

The cell model used in this study was the PMN in which the peroxidase system was stimulated by phagocytosis of opsonized zymosan particles. It was essential at the outset to establish whether inhibition of the myeloperosidase system or inhibition of phagocytosis was occurring. The results with both fluorescein-labelled and ^{203}Hg-tagged zymosan particles indicate that the effects noted with the thioureylene drugs could not be attributed to diminished phagocytosis.

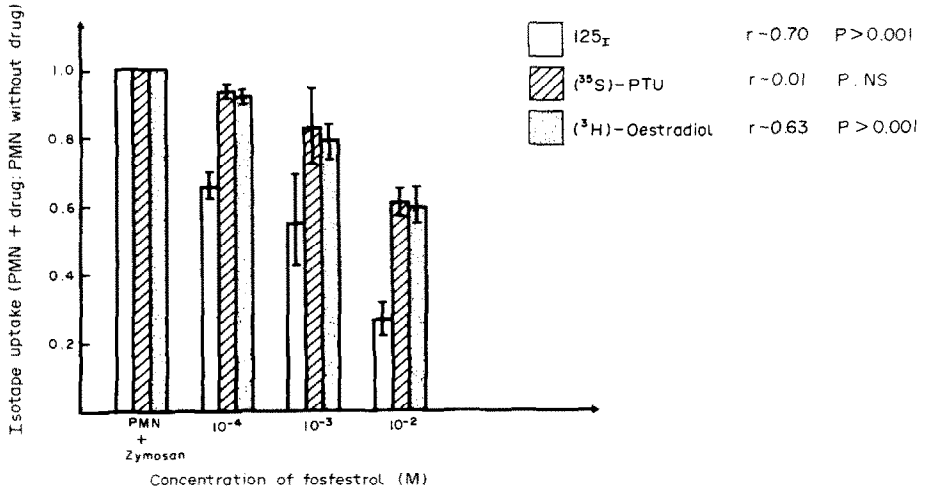

Fig. 3. Effects of various concentrations of fosfestrol on accumulation of ^{125}I, (^{35}S)-PTU, and (^{3}H)-oestradiol in phagocytosing PMN ($\bar{x} \pm$ S.E.M.).

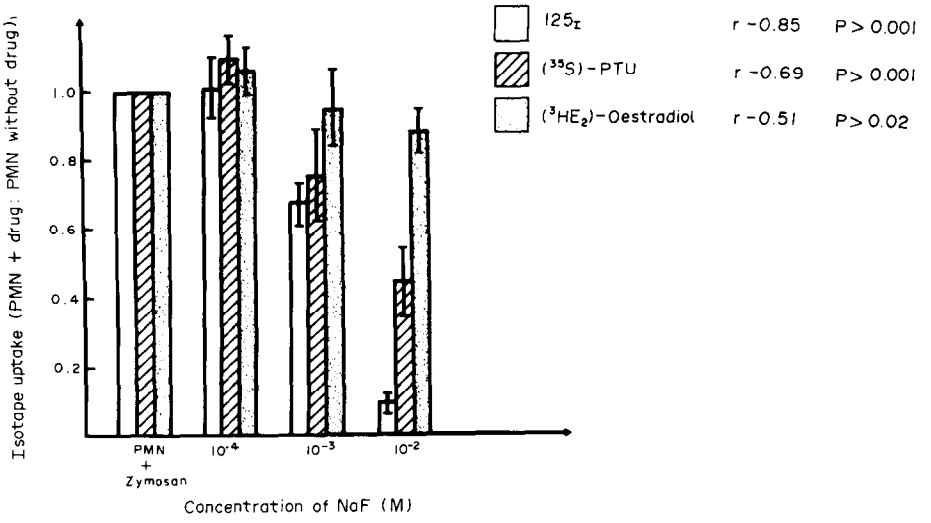

Fig. 4. Effects of various concentrations of NaF on accumulation of ^{125}I, (^{35}S)-PTU, and (^{3}H)-oestradiolin phagocytosing PMN ($\bar{x} \pm$ S.E.M.).

All iodide-concentrating tissues previously studied have been found to possess an active iodide transport mechanism which requires Na^+/K^+ ATPase activity. Anions, such as pertechnetate, and perchlorate, of comparable volume and shape to that of iodide characteristically compete for this transport system [5]. In the present investigation there was no accumulation of ^{99m}Tc-pertechnetate in either the resting PMNs or phagocytosing PMNs which was in marked contrast to [^{125}I]iodide. Furthermore, the accumulation of iodide in the phagocytosing PMNs is independent of Na^+/K^+ ATPase activity since a specific inhibitor of this enzyme, ouabain, had no effect.

ATP, produced by oxidative phosphorylation, is necessary for the iodide transport mechanism in the thyroid and when this is uncoupled by 2, 4-dinitrophenol the system is inhibited [14]. In contrast to the report of Lam and Lindsay [15], the results of this study showed a small decrease in the cell : supernatant ratio of both [^{125}I]iodide and [^{35}S]PTU in the presence of 0.5×10^{-3} M 2,4-dinitrophenol. However, it is not clear as to the mechanism of this decrease: PMNs have few mitochondria and hence oxidative phosphorylation is of limited importance in these cells. Dinitrophenol may also have an inhibitory action upon components of the peroxidase system due to its ability to complex with iron.

In the absence of an active iodide transport mechanism the small decreased ($0.05 < P < 0.1$) uptake of iodide and PTU by PMNs found with perchlorate raises the possibility that this might influence the peroxidase system, as a comparable reduction in iodination has been described with 10^{-3} M perchlorate using a cell-free enzyme system [16]. However, it was impossible to detect any decrease in luminol chemiluminescence with perchlorate using either intact PMNs or horseradish peroxidase. Any possible mode of action of this anion upon peroxidase remains unclear. With an oxidation potential of 170 mV, perchlorate cannot oxidize peroxidase or iodide; when administered it is excreted unchanged as the perchlorate.

The reason for the apparent discrepancy between

Table 2. The effect of compounds on the generation of chemiluminescence by phagocytosing PMNs and by a peroxidase-containing cell-free preparation

	Chemiluminescence (% of control)	
	PMNs	Cell-free peroxidase
Ouabain	98.2	104.0
Perchlorate	97.8	96.8
MMI	61.2	0.5
PTU	78.5	2.4
Iodide	100.9	100.5
Fosfestrol	76.1	21.5
1,10-Phenanthroline	31.0	28.4
Fluoride	94.0	60.4
*p*CMBA	11.0	20.3
DNP	67.2	66.7
2-DOG	79.3	0.0

Values are expressed as percentage of the control, wherein the drug was replaced by PBS. Results are the means of triplicate determinations.

PMNs, Polymorphoneutrophils; MMI, methimazole; PTU, propylthiouracil; *p*CMBA, *p*-chloromercuribenzoate; DNP, 2,4-dinitrophenol; 2-DOG, 2-deoxyglucose.

the effect of iodide on the PMN uptake of the radioactive chemicals and its lack of effect on chemiluminescence is not obvious. It is theoretically possible that iodide may function in two ways depending on the oxidation potential (E_o) of the other reductant present. In the case where E_o of the other reductant is greater than iodide, then iodide may act as a competitor for peroxidase. However, if the E_o of the other reductant is less then it is possible that iodide might function as an electron bridge and even facilitate oxidation of the other compound. The E_o for iodide is well established although the precise values for luminol and thioureylene drugs are not clear. In addition, they might be anticipated to exhibit complex behaviour under oxidizing conditions in a way analogous to thiourea [17].

The present experimental system employed both intact PMNs and a cell-free enzyme system, i.e. horseradish peroxidase. Using whole cells, conclusions cannot be drawn from the use of enzyme inhibitors due to their lack of specificity, although a number of properties of peroxidase are known and the present results are consistent with relating iodide, PTU and 17β-oestradiol accumulation directly to myeloperoxidase activity. The protohaemin groups of this enzyme would be attacked by 1,10-phenanthroline and fluoride, sulphydryl groups inactivated by *p*CMBA, hydrogen peroxide generation blocked by 2-deoxyglucose and peroxidase competitively inhibited by MMI and fosfestrol. The findings using the more specific glucose oxidase/horseradish peroxidase system are in agreement with the above.

The myeloperoxidase system is activated as a consequence of phagocytosis, and the ingestion of zymosan particles continues in the presence of PTU and MMI. Phagocytosis also stimulates the accumulation of iodide, PTU and 17β-oestradiol: inhibition of the myeloperoxidase system causes a reduction in the accumulation of these three substances. Hence their accumulation is apparently dependent upon peroxidase activity.

Peroxidase has an oxidation potential of approximately 1000 mV, for the compound I to compound II couple. Therefore, this enzyme is capable of oxidizing iodide, fosfestrol, PTU, MMI and 17-oestradiol, all of which have oxidation potentials in the region 450–600 mV. This suggests that the action of thioureylene antithyroid drugs is related to their oxidation potential and that these compounds can compete with iodide for interaction with peroxidase. The 17β-oestradiol is also a substrate for peroxidase [18–20] and can be bound to tyrosine similarly to iodide [21].

The role of iodide in peroxidase systems other than thyroid remains to be elucidated although iodide facilitates the bactericidal action by being incorporated into tyrosine residues of the bacterial cell wall. The additional involvement of oestradiol in peroxidase activity is also obscure but raises the possibility that this steroid sex hormone may influence PMN function despite the absence of a specific oestrogen receptor. Monocytes and NK-lymphocytes which also contain myeloperoxidase may be affected in a similar manner.

The specific accumulation of 17β-oestradiol, in contrast to progesterone and testosterone, is consistent with the uptake of steroids being related to the presence of an aromatic A-ring which is available for oxidation. A similar structure is present in the synthetic oestrogen fosfestrol, which is the watersoluble phosphate salt of diethylstilboestrol.

17β-Oestradiol is reported to be at least in part bound to protein in the PMNs [2], but this appears to be related to the peroxidase system rather than to a conventional steroid receptor as no oestrogen receptors are present in the human PMNs: it is possible that oestrogens may play a direct part in the regulation of peroxidase systems.

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