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INHIBITION OF SUPEROXIDE ANION PRODUCTION IN MACROPHAGES BY ANTI-INFLAMMATORY DRUGS

YOSHIHIKO ŌYANAGUI

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd. 1-6, 2-Chome, Kashima, Yodogawa-ku, Osaka 532, Japan

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Abstract—The inhibition by anti-inflammatory drugs of the production of superoxide anions ($\cdot O_2^-$) by isolated guinea pig macrophages was studied spectrophotometrically using NADH and lactate dehydrogenase. ID_{50} values were: 4×10^{-7}M (diclophenac sodium), 1×10^{-6}M (oxyphenbutazone), 1×10^{-5}M (indomethacin), 4×10^{-5}M (phenylbutazone), 7×10^{-5}M (mefenamic acid), 8×10^{-5} M (flufenamic acid), 8×10^{-5}M (colchicine), 3×10^{-4}M (aspirin), 3×10^{-4}M (benzydamine), 10^{-3}M < (dexamethasone) and 10^{-3}M < (gold sodium thiomalate). They seemed to block the cell membrane-associated mechanism to produce superoxide anions, since most of them did not abolish the generation of superoxide anions from the xanthine oxidase plus hypoxanthine system. Cytochalasin B, pyrogallol, ascorbate, NEM, L-epinephrine and chlorpromazine also inhibited, the production of superoxide anion, but many non anti-inflammatory drugs were ineffective. This technique was evaluated as a screening method *in vitro* for nonsteroidal anti-inflammatory drugs.

It has been proposed that the inhibition of prostaglandin (PG) synthetase by anti-inflammatory drugs could be used as a screening method, but the experimental procedures are difficult and it is impossible to relate the results to anti-inflammatory potency *in vivo*. The technical difficulties were ameliorated by Takeguchi *et al.* [1] who introduced a spectrophotometric assay. Using bovine seminal vesicle microsomes (BSVM) they showed that during the oxidative cyclization of arachidonic acid into PGs, L-epinephrine was also oxidized to adrenochrome at pH 8.3 and this oxidation was inhibited by indomethacin ($ID_{50} = 2 \times 10^{-6}$M) or by mefenamic acid ($ID_{50} = 2 \times 10^{-5}$M). However it is impossible to correlate the drug effect *in vivo* with the result obtained *in vitro* at non-physiological pH by tissues which have little relation to inflammation such as BSVM. The effect of a drug depends on the source of PG synthetase; The half inhibitory dose (ID_{50}) of indomethacin was 34 μM with BSVM and 0.17 μM with dog spleen microsomes [2]. In spite of the fact that leucocytes and macrophages are essential cells to the development of inflammation, the effect of drugs on the PG synthetase of these cells has not been investigated except by McCall *et al.* [3]. They observed that phagocytosing rabbit polymorphonuclear (PMN) leucocytes showed increased PG synthesis which was inhibited by 28 μM indomethacin.

The oxidation of arachidonic acid to PGs requires superoxide anions [4], and I have demonstrated that the prostaglandin phase of rat carrageenan foot-oedema was completely suppressed by repeated intravenous (i.v.) injections of superoxide dismutase (SOD : EC 1.15.1.1) [5]. Moreover, using agranulocyte rats, the role of macrophages appeared to be more important than that of leucocytes in carrageenan foot-oedema.

The aim of this investigation was to establish a simple spectrophotometric assay to quantitate the superoxide anions ($\cdot O_2^-$) generated from isolated macrophages or from a xanthine oxidase (XOD) system. The common method adopting cytochrome *c* to detect the superoxide anions, was not employed. Changes in absorbance at 550 nm, by macrophages were not sufficiently large to assay the effects of the drugs and purified protein derivatives (PPD) and phytohemagglutinin (PHA), which were intended to be used in future, caused interference. Other methods were unsuitable, because they require alkaline conditions where macrophages cannot survive. The only method suitable for the present purpose was that of Chan *et al.* [6] adopting NADH and lactate dehydrogenase (LDH).

MATERIALS AND METHODS

Macrophages. Male Harthlay guinea pigs (250–350g) were injected i.p. with 30 ml liquid paraffin oil and sacrificed 3 days later. Cells were washed from the peritoneal cavity with 100 ml fresh medium (0.93 g/l Eagle MEM "Nissui 3" for cell culture without phenol red and 0.292 g L-glutamic acid, adjusted to pH 6.5 with 10% sodium bicarbonate). Cells from 10 to 20 animals were centrifuged separately (10 min 500 *g*) at room temperature and washed once with the same medium. Precipitated cells were pooled and suspended in MEM medium. Preparations contaminated with many red cells were discarded. More than 90 percent of the cells were viable macrophages.

Assay of superoxide anion production by macrophages. The decrease of NADH absorption at 340 nm

Abbreviations used: BSVM = bovine seminal vesicle microsomes; PG = prostaglandin; XOD = xanthine oxidase; LDH = lactate dehydrogenase; NEM = *N*-ethylmaleimide; MEM = minimum essential medium for cell culture; DMF = *N*,*N*'-dimethylformamide; SOD = superoxide dismutase.

was recorded continuously with a Shimadzu Multipurpose MPS-5000 spectrophotometer at 37°. Unless indicated, the 3-ml cuvette contained 125 mM sodium phosphate buffer (pH 6.5), 0.08 mM EDTA, 1.2 U LDH, 320 μM NADH, 2.3×10^6 macrophages/ml and various concentrations of drugs. The reaction was started by the addition of NADH. Samples containing drugs but no macrophages were used to obtain basal values of NADH oxidation. DMF, final concentration 0.1%, was used to dissolve the drugs, and included in control samples. The percent inhibition was calculated from the following equation:

$$\left[1 - \frac{\Delta A\,(\text{macrophage drug sample}) - \Delta A\,(\text{drug basal})}{\Delta A\,(\text{macrophage control}) - \Delta A\,(\text{medium basal})}\right] \times 100$$

where ΔA is difference of absorption at 340 nm. before and after the reaction.

Assay of superoxide anion production by XOD. The medium and conditions were the same as in the macrophage assay, except for the addition of 0.1 U/ml XOD and 80 μM hypoxanthine instead of macrophages. The use of xanthine (80 μM) as substrate for XOD resulted in a little lower NADH oxidation and its solubility in water was inferior to hypoxanthine. The drug inhibition was calculated as in the macrophage assay.

Assay of LDH activity. LDH activity was measured by modifications of the methods of Chan *et al.* [6] and Novoa *et al.* [7] using 125 mM sodium phosphate buffer, 0.08 mM EDTA, 0.16 mM NADH and 2.4×10^{-4} U/3 ml LDH with 0.1 mM sodium pyruvate as substrate. NADH oxidation was linear for the initial 30 sec. at 37°. Sodium oxamate, a specific inhibitor of LDH, suppressed the oxidation by 97 percent at 10^{-2}M, 94 percent at 10^{-3}M and 73 percent at 10^{-4}M. Inhibitory effects of drugs were determined at 30 sec.

Chemicals. LDH (from rabbit muscle, suspended in 3.2 M ammonium sulfate, 550 U/mg), β-NADH (lyophilized disodium salt from yeast, Grade II), XOD (from cow milk, suspended in 3.2 M ammonium sulfate, 0.4 U/mg) and bovine liver catalase (Catalase I, crystal suspension in thymol saturated water, 50,000 U/mg) were the products of Boehringer Co. SOD (lyophilized powder) and bovine blood catalase (lyophilized powder, 3,000 U/mg) were purchased from Sigma Chemical Co. SOD (800 μg/ml, pH 7.0) was inactivated by boiling at 100° for 20 min.

The drugs were obtained from the following sources: diclofenac sodium (GP 45840, Ciba-Geigy), colchicine (Merck), dexamethasone (Decadron, Japan Merck, 4 mg/ml phosphate ester for injection), gold sodium thiomalate (Shiosol, Shionogi Pharm. Co. 10 mg/ml for injection), allopurinol (Zyloric, Wellcome-Tanabe, 100 mg/tablet), cytochalasin A (Aldrich Chem. Co.), cytochalasin B (Serva Feinbiochemica), 1,3-Diphenylisobenzofuran (Aldrich Chem. Co.), D_2O (Merck) and oxysuran (synthesized in this laboratory). All the other chemicals were analytical grade preparations obtained from the usual commercial sources.

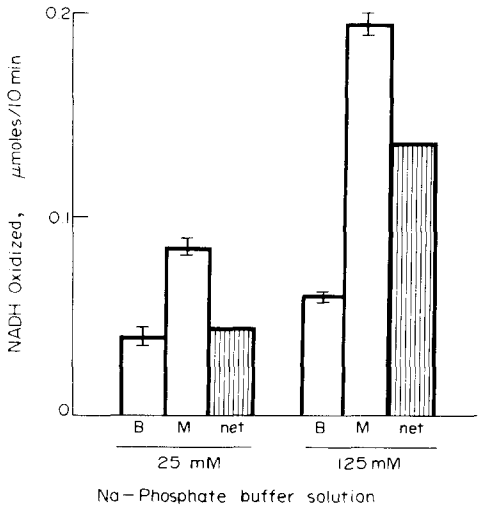

Fig. 1. Concentration effect of sodium phosphate buffer on the production of superoxide anions by macrophages. Medium contained sodium phosphate buffer, 0.08 mM EDTA (pH 6.5), 1.2 U/3 ml LDH and 320 μM NADH. Incubation was started by the addition of NADH and continued for 10 min at 37°. M: 2.9×10^6 macrophages/ml MEM medium. B: MEM medium only. Net NADH oxidized = M − B. Vertical lines represent the standard errors (S.E.M.) of the mean of three experiments.

RESULTS

Effect of sodium phosphate concentration. The production of superoxide anions from isolated macrophages or from XOD plus hypoxanthine was measured in various concentrations of sodium phosphate buffer. It was found that the oxidation of NADH by macrophages was greater at high concentrations of sodium phosphate (Fig. 1). The use of concentrations above 125 mM was not practical because of the solubility. The amount of NADH oxidized could be regarded as the level of superoxide production, because SOD suppressed completely the NADH oxidation (explained later).

The augmentation of net NADH oxidation in 125 mM buffer was attributed to phosphate anions because the addition of NaCl up to 200 mM in 25 mM buffer solution had no effect. ADP (1×10^{-3}M 5×10^{-3}M) and ATP (1×10^{-5}M 1×10^{-2}M) also increased the net NADH oxidation to the degree of that by 125 mM sodium phosphates. Addition of concentrated drugs adjusted between pH 5.0 and pH 8.0 in advance, did not influence the final pH of medium containing 125 mM sodium phosphate buffer. The concentration of sodium phosphate had no effect on the production of superoxide anions from the XOD system.

Time dependency. Figure 2 shows the time dependency of NADH oxidation by macrophages. Ten min incubation with about 2.3×10^6 macrophages/ml was used to test the inhibitory activity of drugs. Figure 3 shows the time dependency with different concentrations of substrate in the XOD system. XOD con-

centrations were tested up to 0.3 U/ml finding always linearity with the amounts of superoxide anions produced.

Effects of pH and temperature. Basal NADH oxidation changed according to the medium pH. Figure 4 shows the net maximum NADH oxidation was at pH 6.5 with living macrophages. For the XOD assay, pH 7.4 was proposed [8] but we used pH 6.5 for comparison with the macrophage assay. Incubations which were carried out at 20°, 30° and 40° with pH 6.5 medium, demonstrated that the production of superoxide anions was temperature-dependent in both assay methods.

Effect of LDH concentration. In the macrophage assay the LDH concentration–NADH oxidation relationship was linear up to 2.4 U/3ml of LDH, and 1.2 U/3ml LDH was regarded as sufficient for practical use (Fig. 5). The same concentration of LDH was adopted for the XOD assay to facilitate the comparison of results obtained from two assay methods.

Effect of SOD. It was demonstrated that NADH was oxidized only by superoxide anions from macrophages. SOD dose-inhibition curves of the macrophage and XOD assays corresponded well (Fig. 6). SOD suppressed even MEM basal NADH oxidation at higher doses. Heat-inactivated SOD had little effect on NADH oxidation.

Effect of DMF. The use of the solvent DMF facilitated the assay of many water-insoluble drugs. Figure 7 shows the effect of DMF on the production of superoxide anions. At a final concentration of 0·1% DMF had a negligible effect on the production of superoxide anions from macrophages and augmentation of the DMF concentration did not influence the inhibition by the anti-inflammatory drug diclofenac sodium. DMF(0.1–1.0%) increased by about 10 percent, the NADH oxidation induced by the XOD system.

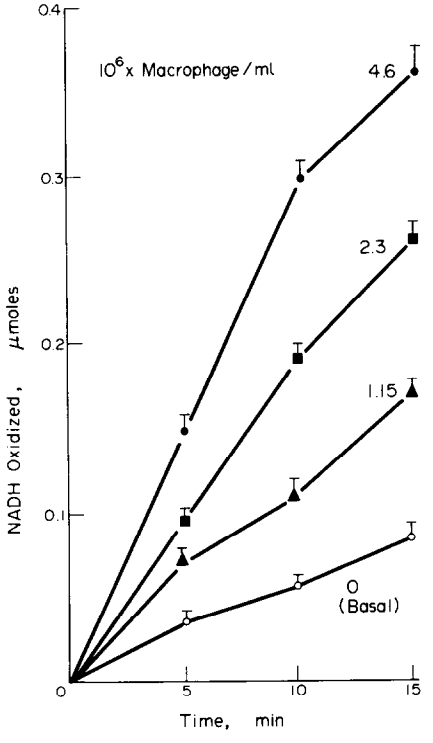

Fig. 2. Effect of macrophage concentration on superoxide anion production. Medium contained 125 mM sodium phosphate buffer (pH 6.5), 0.08 mM EDTA, 1.2 U/3 ml LDH and 320 μM NADH. Incubation was started by the addition of NADH at 37°. Final macrophage numbers/ml were 4.6 × 10^6 (●); 2.3 × 10^6 (■) and 1.15 × 10^6 (▲). The same vol (0.2 ml) of MEM medium was added in the control sample (○). Vertical lines represent S.E.M. of mean of three experiments.

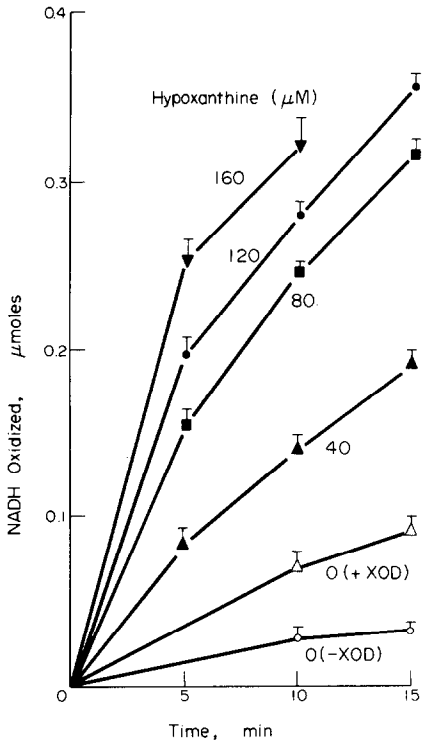

Fig. 3. Effect of hypoxanthine concentration on superoxide anion production in the XOD system. Medium and conditions are as in Fig. 2. XOD (0.1 U/ml) and hypoxanthine were mixed just before NADH addition. Hypoxanthine 160 μM (▼), 120 μM (●), 80 μM (■), 40 μM(▲), no hypoxanthine with XOD (△) no hypoxanthine or XOD (○).

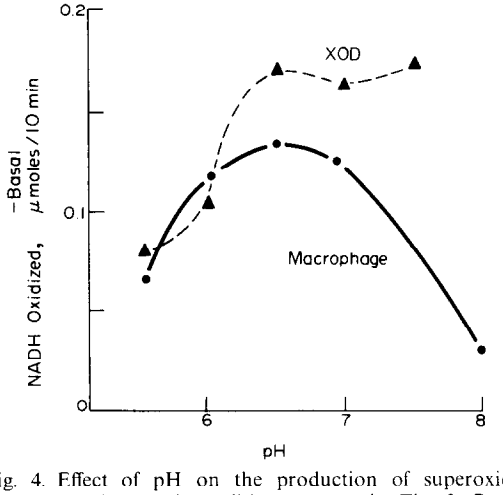

Fig. 4. Effect of pH on the production of superoxide anions. Medium and conditions are as in Fig. 2. Basal values were substracted. 2.7 × 10^6 macrophages/ml (●), 0.1 U/ml XOD +80 μM hypoxanthine (▲). Averages of two experiments.

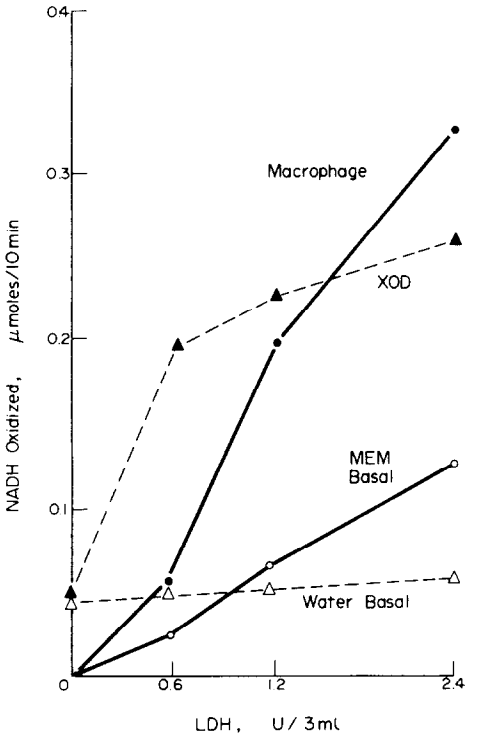

Fig. 5. Effect of LDH concentration on superoxide anion production. Medium and conditions are as in Fig. 2. except the LDH concentration. 3.3×10^6 macrophages/ml (●), 0.1 U/ml XOD + 80 μM hypoxanthine (▲), MEM medium only (○), control without LDH (△). Averages of two experiments.

Effect of macrophage concentrations on drug effects. Diclofenac sodium (10^{-6}M) and aspirin (2.5×10^{-4}M) were almost equally inhibitory at all macrophage concentrations (Fig. 8). However, this was not the case with all the drugs; the inhibitory

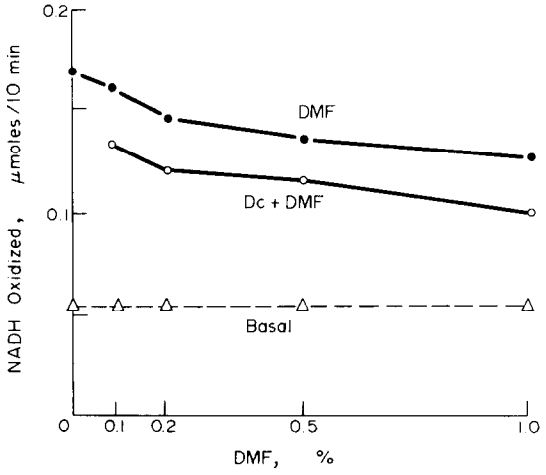

Fig. 7. Effect of DMF on superoxide anion production. Medium and conditions are as in Fig. 2. 2.0×10^6 macrophages/ml. DMF (●), DMF + 10^{-7} M diclofenac sodium (○), DMF without macrophages (△). Averages of two experiments.

activity of SOD was a little weak at 3.4×10^6M macrophages/ml. Figure 9 shows the NADH oxidizing capacities of different macrophage preparations. Macrophage preparations possessing the capacity to oxidize 0.20–0.27 μmoles NADH per 10 min with $2–3 \times 10^6$ macrophages/ml, were used routinely to test the drug action.

Inhibitions by anti-inflammatory drugs. Eleven anti-inflammatory drugs were tested on the macrophage assay (Fig. 10 and Table 1). Diclofenac sodium was the most potent inhibitor of the production of superoxide anions by macrophages, followed by oxyphenbutazone and indomethacin. Dexamethasone and gold sodium thiomalate were ineffective. Except for these two drugs all anti-inflammatory drugs tested had ID_{50} values less than 2.5×10^{-4}M.

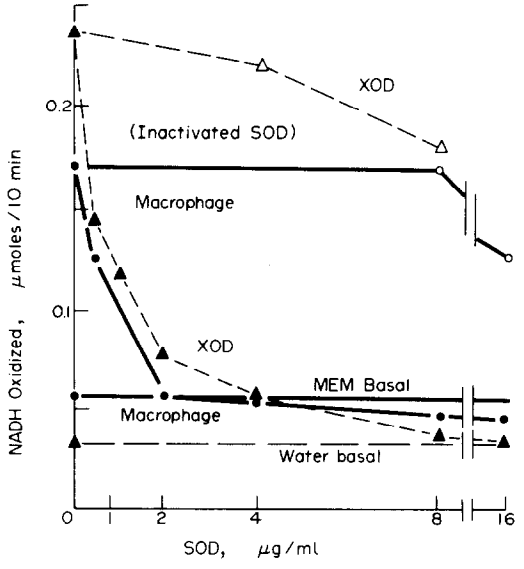

Fig. 6. Effect of SOD on superoxide anion production. Medium and conditions are as in Fig. 2. 2.8×10^6 macrophages/ml with native (●) and inactivated SOD (○). 0.1 U/ml XOD +80 μM hypoxanthine with native (▲) and inactivated SOD (△). Averages of two experiments.

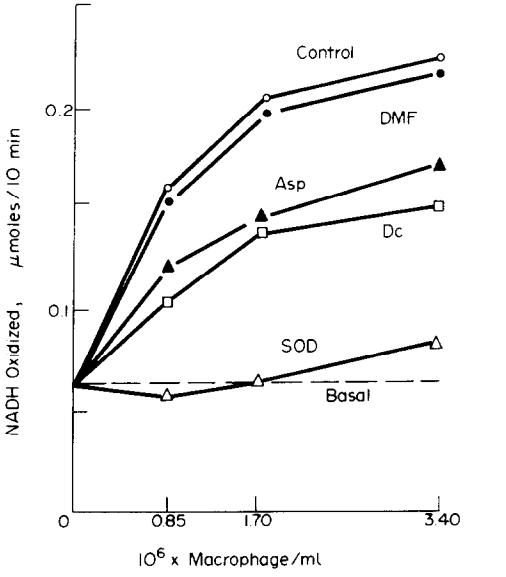

Fig. 8. Effect of macrophage concentration on drug inhibitions. Medium and conditions are as in Fig. 2. Control (○), 0.1% DMF (●), 2.5×10^{-4}M aspirin in 0.1% DMF (▲), 10^{-6}M diclofenac sodium in 0.1% DMF (□) and 4 μg/ml SOD (△).

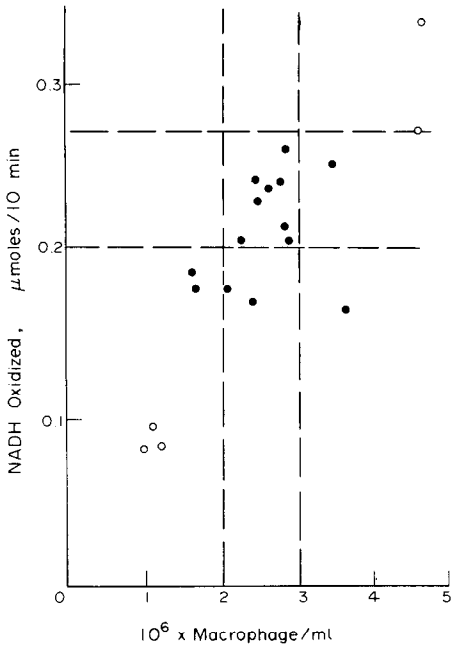

Fig. 9. Relationship between macrophage concentration and superoxide anion production. Medium and conditions are as in Fig. 2. Separately prepared macrophages (●) and diluted samples from some of them (○) were plotted. NADH oxidized [nmoles/10^6 macrophages/10 min] value using all points was 85.5 ± 4.5 (mean ± S.E.M.) and that calculated from 8 points within the dotted lines was 90.5 ± 2.5.

A drug which decreased the production of superoxide anions in the XOD assay system, can be interpreted as an inhibitor of XOD enzyme activity or a scavenger of superoxide anions. Diclofenac sodium like many other anti-inflammatory drugs had no effect on NADH oxidation induced by XOD plus hypoxanthine. This means that diclofenac sodium is not a simple scavenger of superoxide anions, but it blocks the mechanism which produces superoxide anions in macrophages. On the other hand, colchicine acted as a scavenger or XOD inhibitor of superoxide anions, because the inhibition in the XOD assay paralleled that in the macrophage assay. Indomethacin, mefenamic acid and flufenamic acid possessed both characteristics, but the inhibition of superoxide anion production was dominant. There were still differences of 4- to 30-fold in the ID_{50} in both assay methods concerning these drugs.

None of the anti-inflammatory drugs inhibited LDH activity. Oxamate (2×10^{-4}M), a specific inhibitor of LDH, suppressed by 50 per cent the NADH oxidations in both the macrophage and XOD assays.

Table 1. ID_{50} of anti-inflammatory drugs

Drugs		Inhibition ID_{50} (M)* Macrophage superoxide production	XOD superoxide production	LDH activity
Diclofenac sodium	D†	4×10^{-7}	10^{-3} <	10^{-3} <
Oxyphenbutazone	D	1×10^{-6}	10^{-3} <	10^{-3} <
Indomethacin	D	1×10^{-5}	3×10^{-4}	10^{-3} <
Phenylbutazone	D	4×10^{-5}	10^{-3} <	10^{-3} <
Mefenamic acid	D	7×10^{-5}	1×10^{-3}	10^{-3} <
Flufenamic acid	D	8×10^{-5}	3×10^{-4}	10^{-3} <
Colchicine	D	8×10^{-5}	8×10^{-5}	10^{-4} <‡
Aspirin	D	1.5×10^{-4}	10^{-3} <	10^{-3} <
Benzydamine	W	2×10^{-4}	10^{-3} <	10^{-3} <
Dexamethasone	W	10^{-3} <	10^{-3} <	10^{-3} <
Gold sodium thiomalate	W	10^{-3} <	10^{-3} <	10^{-3} <

* For calculations, see Materials and Methods. ID_{50} values were determined from at least three different doses, each being the average of three experiments.

† Solvent used for dissolving drugs, D: DMF, final concn. 0.1%, W: water.

‡ Maximum solubility.

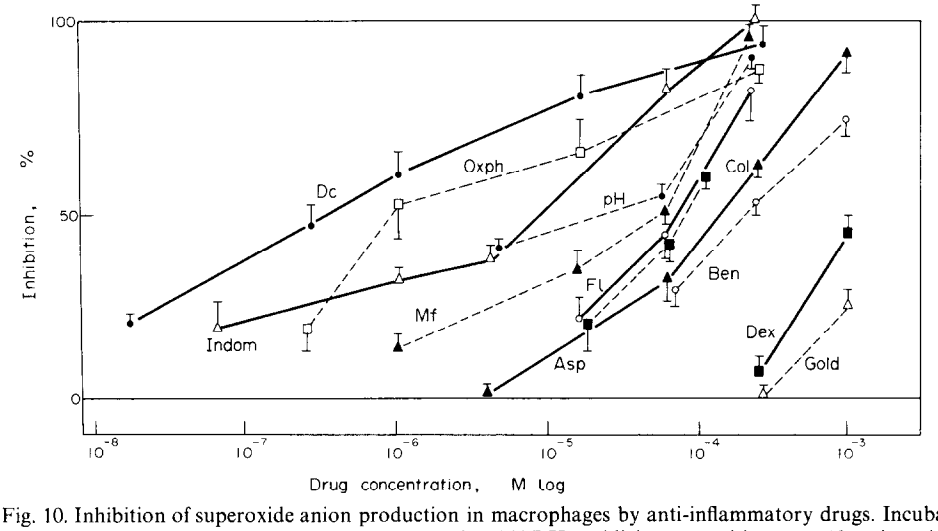

Fig. 10. Inhibition of superoxide anion production in macrophages by anti-inflammatory drugs. Incubations were for 10 min at 37° (0.20–0.27 μmoles NADH oxidizing capacities per 10 min with 2.0–3.0 × 10^6 cells). DMF (0.1% final concentration) was used to dissolve diclofenac sodium (●—), oxyphenbutazone (□---), indomethacin (△—), phenylbutazone (●---), mefenamic acid (▲---), flufenamic acid (○—), colchicine (■---) and aspirin (▲—). Benzydamine (○---), dexamethasone (■—) and gold sodium thiomalate (△---) were water soluble. Drug dilutions were by serial four fold dilution beginning at 10^{-3}M. Vertical lines represent S.E.M. of the mean of three experiments.

Table 2. ID_{50} of non anti-inflammatory agents which inhibit the production of macrophage superoxide anions

Drugs		Inhibition ID_{50} (M)* Macrophage superoxide production	XOD superoxide production	LDH activity
Cytochalasin B	D†	6×10^{-8}	$6.4 \times 10^{-5}<$	$6.4 \times 10^{-5}<$
Pyrogallol	W	3×10^{-6}	1×10^{-4}‡	$10^{-3}<$
Ascorbate	W	1×10^{-5}	2×10^{-4}	$10^{-3}<$
NEM	W	5×10^{-5}	$10^{-3}<$	$10^{-3}<$
L-Epinephrine	W	8×10^{-5}	$10^{-3}<$	$10^{-3}<$
Chlorpromazine	W	2×10^{-4}	4×10^{-4}	$10^{-3}<$
Luminol	D	2×10^{-4}	2×10^{-5}	8×10^{-4}
Oxamate §	W	4×10^{-4}	2×10^{-4}	2×10^{-5}
Allopurinol §	W	$10^{-3}<$	3×10^{-6}	

* For calculations, see Materials and Methods and Table 1.

† Solvent used, see Table 1.

For pyrogallol, ascorbate and L-epinephrine, the reactions were started within 5 min after dissolving the drugs.

‡ Dose inhibition curve of pyrogallol was not linear and inhibition was maximum at 10^{-4}M.

§ Oxamate, a specific inhibitor of LDH, was used to determine the degree of effect on the macrophage assay and XOD assay. Allopurinol which is an inhibitor of XOD reaction, was used for comparison.

These results showed that the inhibitions by drugs in the macrophage assay and XOD assay, were not due to the suppresion of LDH activities. The inhibition by oxamate in the macrophage assay was a little weaker than in the XOD assay. Some of the oxamate might be absorbed or inactivated in macrophages.

Inhibitions by non anti-inflammatory agents. Cytochalasin B was a potent inhibitor of superoxide anion production in the macrophage assay but had no effect in the XOD assay (Table 2). This was an expected result, because this agent has been reported to modulate the organization of the plasma membrane and may influence the activity of membrane enzymes [9]. The superoxide anion producing system in macrophages might be membrane-associated enzyme(s). Pyrogallol, which traps the superoxide anions in alkaline solution [10], was not as inhibitory in the XOD assay at pH 6.5. Maximum inhibition was obtained near 10^{-4}M (50 percent) and the inhibition at 10^{-3}M and 10^{-6}M was 20 percent, so that this agent also acts on superoxide anion producing sites of macrophage. Ascorbate, known as a scavenger [11], was also inhibitory in the XOD assay. NEM and L-epinephrine acted as blockers of superoxide anion production in macrophages. The inhibition by L-epinephrine (10^{-6}–10^{-3}M) in the XOD assay, decreased at higher concentrations. Lack of scavenger role of this agent was not expected and further investigations were needed. Chlorpromazine known as a hydroxyl radical scavenger [12], showed about the same inhibition in both the macrophage and XOD assays. There was a 20-fold difference between the ID_{50} of LDH inhibitor oxamate in the macrophage assay and that in LDH activity assay. Allopurinol known as an inhibitor of the XOD reaction, had no effect in the macrophage assay, suggesting that the production of superoxide anions in macrophages does not depend on the XOD reaction.

Hydroxyl radical (·OH) scavengers, D-mannitol, sodium benzoate, KI and ethanol [13, 14], the oxygen scavenger, 1,3-diphenylisobenzoate [15] and Boehringer liver catalase (50–1,000 U/ml) were ineffective (Table 3). Sigma blood catalase showed inhibitions (40 percent with 20 U/ml and 60 percent with 100 U/ml) which might be due to the contamination with SOD as reported by Halliwell [16]. Neither the mitochondrial electron transport inhibitor sodium azide nor the glycolytic inhibitors monoiodoacetic acid and NaF suppressed superoxide anion production. Rifampicin and Daunomycin were about 30 percent inhibi-

Table 3. Agents not inhibiting the production of macrophage superoxide anions at 10^{-3}M*

Free radical scavengers and catalase:
D-mannitol, sodium benzoate, KI, ethanol, 1,3-diphenyl isobenzofuran, Boehringer liver catalase (1,000 U/ml)
SH-reagents:
Reduced glutathione, dithiothreitol
Energy metabolism inhibitors:
Sodium azide, monoiodoacetate, sodium fluoride†
Antibiotics:
Tetracycline HCl‡, chloramphenicol semisuccinate, rifampicin, cephasolin sodium, penicillin G-potassium, streptomycin sulfate
Anti-tumor agents:
mitomycin C, daunomycin HCl, bleomycin HCl, cytosine arabinoside, 5-fluorouracil, cyclophosphatamide, L-asparaginase
Miscellaneous agents:
nitroglycerin (coronary vasodilator), propranolol, theophylline, phenoxybenzamine (α-blocking agent), D_2O, cytochalasin A.

* Averages of three experiment at 10^{-3}M or indicated concentration of agents, were below ID_{50}. Many of the agents were also tested at 2.5×10^{-4}M and 6.4×10^{-5}M. DMF was used as a solvent if necessary.

† About 20 percent increase of superoxide anion production was observed between 1.6×10^{-5}M and 10^{-3}M.

‡ Tetracycline above 10^{-6}M without macrophages, enhanced NADH oxidation, so the result was not clearly determined.

Table 4. ID_{50} of metal salts

Metal salts	Inhibition ID_{50} (M)* Macrophage superoxide production	XOD superoxide production	LDH activity
$MnCl_2$	4×10^{-6}	3×10^{-4}	$10^{-3} <$
$HgCl_2$	1×10^{-5}	3×10^{-4}	4×10^{-3}
$Cu(CH_3COO)_2$	3×10^{-5}	7×10^{-5}	1×10^{-3}
$FeCl_2$†	4×10^{-5}	$10^{-3} <$	$10^{-3} <$
$FeCl_3$	2×10^{-4}	$10^{-3} <$	$10^{-3} <$
$NiCl_2$	2×10^{-4}	$10^{-3} <$	$10^{-3} <$
$CoCl_2$	6×10^{-4}	1×10^{-3}	$10^{-3} <$
$(NH_4)_6Mo_7O_{24}$	$10^{-3} <$	$10^{-3} <$	——
$BaCl_2$	$10^{-3} <$	$10^{-3} <$	——
$CaCl_2$	$10^{-3} <$	$10^{-3} <$	——
$CdCl_2$	$10^{-3} <$	$10^{-3} <$	——
$PbCl_2$	$10^{-3} <$	$10^{-3} <$	——

* For calculations, see Materials and Methods and Table 1. DMF was not used to dissolve drugs.

† The reaction was started within 5 min after dissolving $FeCl_2$.

tory between 10^{-6}M and 10^{-3}M but never attained 50 percent inhibition. Theophylline and nitroglycerin showed some inhibition (about 50 percent at 10^{-3}M, 30 percent at 2.5×10^{-4}M).

Table 4 shows the inhibitory effect of metal salts. Mn^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Co^{2+} blocked the production of superoxide anions from macrophages. Cu^{2+} seemed to react mainly as a scavenger of superoxide anions. Cu^{2+} and Hg^{2+} showed some inhibition of LDH enzyme activity.

DISCUSSION

In rat carrageenan foot-oedema, it is clear that superoxide anions participate in the swelling of the prostaglandin phase [5]. In this study paraffin oil-induced peritoneal macrophages were demonstrated to produce superoxide anions. Nonsteroidal anti-inflammatory drugs inhibited significantly the production of superoxide anions from macrophages at physiological pH. ID_{50} values were a little higher than those obtained with PG synthetase assay using guinea pig lung homogenate or dog spleen microsomes [17]. There are no reports on PG synthetase from macrophages for comparison with the results obtained here. A 30 percent inhibition by diclofenac sodium (5×10^{-8}M), oxyphenbutazone (5×10^{-7}M), indomethacin (7×10^{-7}M) and mefenamic acid (8×10^{-6}M), must be appreciated, because dose–inhibition curves of anti-inflammatory drugs in the macrophage assay were rather flat compared with the PG synthetase assay. Dexamethasone had no effect. Steroids inhibit the emigration of macrophages or leucocytes and decrease the total amount of PGs at inflamed sites. Cytochalasin B was tested *in vivo* with carrageenan foot-oedema according to our previous report [5]. Two i.v. injections of 240 μg/kg cytochalasin B (six experiments) suppressed 21 ± 3 (S.E.) percent of foot swelling and 20 ± 3 percent of weight increase at 4 hr. Cytochalasin B suppressed only the prostaglandin phase (2–4 hr.). L-Epinephrine and chlorpromazine are known to possess anti-inflammatory activity. They are not used as anti-inflammatory drugs because of other undesirable properties. Ascorbate might be a good anti-inflammatory drug if it is a stable and lipophilic compound. The inhibitory effect of NEM is not clear, but the mechanism which produces superoxide anions in macrophages may contain a SH inhibitor-sensitive site. Another SH inhibitor $HgCl_2$ also inhibited the production of superoxide anions but reduced glutathione and dithiothreitol had little effect.

When a new compound with possible tested anti-inflammatory activity is found, it is worth examining its effect in the macrophage assay at a concentration of 2.5×10^{-4}M and below. It is preferable to check also the lipophilicity of the compound as Skidmore *et al.* [18] mentioned. Many indomethacin type anti-inflammatory drugs, are lipophilic and stable in aqueous solution containing 0.1% DMF.

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