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INTERACTION BETWEEN NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND BIOLOGICAL MEMBRANES—IV

EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND OF VARIOUS IONS ON THE AVAILABILITY OF SULFHYDRYL GROUPS ON LYMPHOID CELLS AND MITOCHONDRIAL MEMBRANES

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Abstract—A stimulation effect of various nonsteroidal anti-inflammatory drugs (NSAID) on a sulfhydryl–disulfide interchange reaction between serum protein sulfhydryl groups and 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) has been described. We now describe a similar reaction between 65 μM DTNB and protein sulfhydryl groups in rat, rabbit and human lymphocyte membranes and rat liver mitochondria. This reaction is greatly accelerated in the presence of ten different NSAID. The stimulation of this sulfhydryl–disulfide interchange reaction appears to be dependent on drug concentration. It is also closely related to the ionic composition of the incubation medium which, by itself, according to specific cationic and anionic sequences, influences the availability of sulfhydryl groups on membranes. The reaction of 0·6 mM carboxypyridine disulfide (CPDS), which is another molecule able to react specifically with sulfhydryl groups on membranes, is affected in a similar way by NSAID. According to the well known involvement of sulfhydryl groups in inflammatory reactions, this peculiar effect of NSAID on the availability of membrane sulfhydryl groups, especially of lymphoid cells, could be related to some of their clinical properties.

The need of thiol groups for the development of inflammatory processes has been well established [1–3]. They seem also very important for the effects of various nonsteroidal anti-inflammatory drugs (NSAID) on several biological systems *in vitro*. Factors which influence the stability of lysosomal membranes would prevent inflammatory reactions [4,5]. Anti-inflammatory gold salts, which are concentrated into these organelles [6], also inhibit various lysosomal enzymes such as acid phosphatases, glucuronidases and cathepsins, the mechanism of action presumably being the binding with sulfhydryl groups [7,8]. NSAID act as uncoupling agents on mitochondrial oxidative phosphorylation [9]. This property has been related to their anti-inflammatory actions [9,10]. Vicinal dithiols [11] as well as thiol groups adjacent to primary amino groups [12] might be involved in this subcellular activity. The role of sulfhydryl groups in this uncoupling mechanism has been recently confirmed by demonstrating that Mersalyl, a thiol inhibitor, blocks the stimulation of oxygen consumption induced by NSAID, which is restored by addition of cysteine, a thio-amino acid [13]. The same thiol inhibitor enhances the swelling effects of NSAID on isolated mitochondria [14] or cell suspensions [15].

Two potent sulfhydryl binding agents, ethacrynic acid and *N*-ethylmaleimide, inhibit the growth of experimental cotton pellet granuloma of the rat [16]. Cysteine reverses this inhibition and also antagonizes the anti-inflammatory effects of aspirin and indomethacin on similar granuloma [16].

An acceleration of the sulfhydryl–disulfide interchange reaction *in vitro* between serum protein sulfhydryl groups and 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) or Ellman's reagent [17] has been described recently under the influence of several NSAID [18]. A good correlation has been found between this reaction *in vitro* and assays *in vivo* such as the guinea pig ultraviolet erythema [19] and the rat adjuvant arthritis [20]. The increased rate of this reaction may be indicative of free sulfhydryl groups uncovered by NSAID through an induced conformational change of the protein molecules. It has also been proposed that free DTNB, which has a configuration not unlike that of several anti-inflammatory arylacid compounds, increases in concentration as its binding sites are saturated by NSAID.‡

The stimulation of a similar sulfhydryl–disulfide interchange reaction between DTNB as well as carboxypyridine disulfide (CPDS) [21], probably a more suitable agent for the study of surface sulfhydryl groups of lymphocytes [22], and membrane sulfhydryl groups of lymphoid cells and mitochondria, under the influence of various NSAID, is described in the present paper.

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‡ M. Hitchens, unpublished observations (cited by T. Y. Chen, in *A. Rep. med. Chem.* **2**, 219, 1966).

MATERIAL AND METHODS

Rabbit or rat thymocytes and lymphocytes as well as human lymphocytes were prepared as described elsewhere [14]. Cells, 6×10^8 as determined in a Coulter electronic counter, were incubated for 5 min in various isotonic alkali, alkaline earth and ammonium chlorides and potassium halides with 65 μM DTNB, buffered with 30 mM Tris-HCl, pH 7·4, in the presence or absence of NSAID. The supernatant was removed by rapid centrifugation at 350 g, filtered through Whatman GF/C papers, and the amount of yellow 5-thio-2-nitrobenzoic acid was determined at 412 nm in a DU-2 Beckman spectrophotometer using rectangular glass cuvettes of 10 mm light path. For estimating the amount of membrane and intracellular sulfhydryl groups participating in the reaction, (a) supernatants prepared in the absence of DTNB were secondarily treated with 65 μM, and (b) cells pretreated for 5 min with and without NSAID in the various media were resuspended and incubated for 5 min in Hanks' medium containing 65 μM DTNB. Cell viability assessed by Trypan blue exclusion [23] was 95 per cent after each incubation. Spontaneous cell leakage, with or without NSAID, was simultaneously determined (a) by measurements of protein leakage in the supernatants by the Lowry technique [24] and (b) by determination of the ultraviolet absorbance at 280 nm for these same supernatants prepared in the absence of DTNB in a DU-2 Beckman spectrophotometer using rectangular quartz cuvettes of 10 mm light path. In several experiments an attempt at rough quantitation of the number of sulfhydryl groups available per cell was made by determining the threshold of DTNB needed to obtain the same maximum enhancement of the sulfhydryl–disulfide interchange reaction after 5 min.

Similar experiments were conducted with rat liver mitochondria prepared as described by Famaey and Mockel [13]. Mitochondrial protein, 0·75 mg, as determined by the Lowry technique [24] was used for each incubation. The mitochondrial supernatants were separated by centrifugation at 12,500 g in a Sorvall RC-2 refrigerated centrifuge. Uncoupling and swelling effects of DTNB on these mitochondrial preparations were controlled as described in other studies [12,13].

The effect of CPDS (0·6 mM) was similarly tested on rabbit thymocytes (6×10^8 cells) by following the absorbance of 6-mercaptonicotinic acid produced by the sulfhydryl–disulfide interchange reaction at 344 nm in a DU-2 Beckman spectrophotometer using rectangular quartz cuvettes of 10 mm light path.

Reagents. Ibuprofen and ibufenac were generous gifts from Boots Drug Co. (Nottingham, England); phenylbutazone, oxyphenbutazone and sulfinpyrazone were from Geigy (Basel, Switzerland), pyrazinobutazone from Seresci (Brussels, Belgium), clonixic acid from Schering (USA), flufenamic and mefenamic acids from Parke Davis & Co. (USA), and indomethacin from Merck Sharp & Dohme (USA). Flufenamyl alcohol was obtained from Dr. L. Fenoy (Wilmington, Del.) and ibuprofen alcohol was a gift of Boots Drug Co. 5,5'-Dithiobis-2-nitrobenzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, Wisc.). The sodium salt of Mersalyl was from Sigma Chemical Co. (St. Louis, Mo.) and carboxypyridine disulfide from Newcell Biochemicals (Berkeley, Calif.). All the other chemicals were of the purest grade commercially available. Human thoracic duct lymphocytes were obtained through the courtesy of Dr. H. Paulus (Los Angeles, Calif.).

RESULTS

The sulfhydryl–disulfide interchange reaction between lymphocyte membranes (rabbit thymocytes) and DTNB (65 μM) is influenced by the ionic composition of the incubation medium according to cationic ($Li^+ < Na^+ < Cs^+ < K^+ < Rb^+$) and anionic ($Cl^- < Br^- < I^- < F^-$) sequences for alkali cations

Table 1. Influence of the ionic composition of the medium on the DTNB sulfhydryl–disulfide interchange reaction with rabbit thymocytes*

Incubation media		Supernatant of cells incubated with DTNB		DTNB + supernatant of cells incubated without DTNB	Supernatant of cells pretreated without DTNB and then incubated in Hanks' with DTNB
Alkali chlorides (0·15 M)	LiCl	132·5 ± 11	(0·2 10^8)	104·5 ± 4	126 ± 7·5
	NaCl	212·5 ± 9	(0·6 10^8)	112 ± 9	184·5 ± 8
	KCl	314 ± 13·5	(1·1 10^8)	150 ± 10·5	256 ± 12·5
	RbCl	410·5 ± 11·5	(2·5 10^8)	168 ± 5·5	399 ± 10·5
	CsCl	249 ± 10	(0·7 10^8)	128 ± 6	207 ± 6
Potassium halides (0·15 M)	ClK	314 ± 13·5		150 ± 10·5	256 ± 12·5
	BrK	361·5 ± 16		152 ± 7·5	299 ± 11
	IK	426 ± 12·5		156 ± 11	351 ± 6·5
	FK	461·5 ± 9·5		161 ± 6·5	387 ± 16·5
(0·15 M)	NH_4Cl	280·5 ± 12	(0·9 10^8)	139 ± 6	210 ± 8·5
Alkaline-earth chlorides (0·11 M)	$CaCl_2$	160 ± 5·5		110 ± 8	120 ± 6·5
	$MgCl_2$	155 ± 6·5		110 ± 7	116 ± 4

* Rabbit thymocytes (6×10^8 cells) were incubated for 5 min in 3 ml of various isotonic media at 37°, buffered with 30 mM Tris-HCl, pH 7·4. DTNB = 65 μM. Results are means of 12 measurements ± standard deviation. They are expressed in percentage of controls = 100, which are incubations of pure Hanks' medium, containing no cells, with 65 μM DTNB. Spectrophotometric determinations were made at 412 nm. Estimation of the number of membrane sulfhydryl groups available per cell = (n × 10^8).

Table 2. Estimation of cell leakage with rabbit thymocytes*

(1) Under the influence of various media				(2) Under the influence of NSAID (and corresponding alcohols) in KCl, 0·15 M			
	Media	Ultraviolet absorbance (280 nm)	Supernatant protein concn (g/l.)	Drugs	Concn (mM)	Ultraviolet absorbance (280 nm)	Supernatant protein concn (g/l.)
Alkali chlorides (0·15 M)	LiCl	345 ± 13·5	0·8	Anthranilic acid derivatives			
	NaCl	644 ± 16	1·7	Flufenamic acid	0·3	1864 ± 12	4·25
	KCl	1027 ± 7·5	2·5	Mefenamic acid	0·3	1676 ± 13·5	3·8
	RbCl	1670 ± 12	4·2	Clonixic acid	0·3	1964 ± 11	4·6
	CsCl	715·5 ± 8·5	2	Pyrazolone derivatives			
Potassium halides (0·15 M)	ClK	1027 ± 7·5	2·5	Phenylbutazone	0·3	1605 ± 11·5	3·6
	BrK	1167 ± 11	2·9	Pyrazinobutazone	0·3	1630 ± 12	3·6
	IK	1329 ± 12	3·2	Oxyphenbutazone	0·3	1572 ± 14·5	3·4
	FK	1412 ± 10·5	3·5	Sulfinpyrazone	0·3	1578 ± 9·5	3·5
(0·15 M)	NH_4Cl	890 ± 10·5	2·2	Aryl-alkanoic acid derivatives			
Alkaline-earth chlorides (0·11 M)	$MgCl_2$	430 ± 11·5	1·2	Ibuprofen	0·4	1264 ± 13	2·5
	$CaCl_2$	427 ± 10	1·1	Ibufenac	0·4	1598 ± 14·5	3·5
				Indomethacin	0·5	1860 ± 10·5	4·1
				Corresponding alcohols			
				Ibuprofen alcohol	0·4	1359 ± 16·5	2·9
				Flufenamyl alcohol	0·3	1627 ± 10·5	3·7

* Determination of cell leakage in 5-min incubates of rabbit thymocytes (6×10^8 cells): (1) in 3 ml of various isotonic media at 37°, buffered with 30 mM Tris-HCl, pH 7·4; and (2) under the influence of several NSAID in 3 ml KCl, 0·15 M, at 37°, buffered with 30 mM Tris-HCl, pH 7·4. Spectrophotometric determinations were made at 280 nm and the results are means of 12 measurements ± standard deviation. They are expressed in percentage of controls = 100, which are for (1) and (2) pure Hanks' medium containing no cells. Protein concentrations of supernatants, expressed in g/l., are means of triplicate determinations.

and halide anions (Table 1). Cell leakage, similarly affected by ions of the medium, was observed for each incubation. This leakage was not very important, as shown by determination of the supernatant proteins by the Lowry technique [24] and of the ultraviolet absorbance of the supernatant at 280 nm (Table 2). It had no effect on cell viability as estimated by trypan blue exclusion [23] (95 per cent after each incubation). Some sulfhydryl groups of intracellular origin were implicated in the sulfhydryl–disulfide interchange reactions, as established by treating these supernatants with DTNB (65 μM) (Table 1). However, the results of DTNB (65 μM) incubations in Hanks' medium with washed lymphocytes pretreated in various ion-specific media demonstrated that most of the reacting sulfhydryl groups were of membrane origin (Table 1).

Ten NSAID were tested on this reaction. They all accelerated the sulfhydryl–disulfide interchange process (Table 3) but their relative potencies were not similar. The anthranilic acid derivatives (fenamates) were the most potent in accelerating this interchange reaction. The pyrazolone derivatives were also quite active and no significant differences were found between them. But the arylalkanoic acids even at higher concentrations exhibited less activity than the

Table 3. Effects of ten different NSAID (and two corresponding alcohols) on DTNB sulfhydryl–disulfide interchange reactions in KCl medium with rabbit thymocytes*

Drugs	Concn (mM)	Supernatant of cells incubated with DTNB		DTNB + supernatant of cells incubated without DTNB	Supernatant of cells pretreated without DTNB and then incubated in Hanks' with DTNB
Anthranilic acid derivatives					
(1) Flufenamic acid	0·3	285 ± 7·5	($3·2\ 10^8$)	142 ± 4	252 ± 7·5
(2) Mefenamic acid	0·3	273 ± 8	($2·9\ 10^8$)	131 ± 3	249 ± 7
(3) Clonixin [2-(2-methyl-3-chloroanilino)nicotinic acid]	0·3	312 ± 9		152 ± 3·5	285 ± 9
Pyrazolone derivatives					
(4) Phenylbutazone	0·3	214 ± 7·5	($2·3\ 10^8$)	109 ± 5·5	202 ± 6·5
(5) Pyrazinobutazone (piperazine salt of phenylbutazone)	0·3	199 ± 6		121 ± 5·5	194 ± 6·5
(6) Oxyphenbutazone	0·3	201 ± 6·5	($2·2\ 10^8$)	115 ± 4·5	191 ± 5·5
(7) Sulfinpyrazone	0·3	197 ± 7·5		137 ± 3·5	189 ± 7
Aryl-alkanoic derivatives					
(8) Ibuprofen	0·4	141 ± 4·5	($1·6\ 10^8$)	106 ± 4	119 ± 6
(9) Ibufenac	0·4	157 ± 5·5		118 ± 3·5	136 ± 3·5
(10) Indomethacin	0·5	297 ± 7·5	($3·3\ 10^8$)	124 ± 3·5	294 ± 5
	0·4	168 ± 7·0		111 ± 4·5	152 ± 5·5
Corresponding alcohols					
(11) Ibuprofen alcohol	0·4	152 ± 4·5		111 ± 5·5	128 ± 5
(12) Flufenamyl alcohol	0·3	227·5 ± 9·5	($2·5\ 10^8$)	118 ± 2·5	218 ± 7·5

* Rabbit thymocytes (6×10^8 cells) with various NSAID were incubated for 5 min in 3 ml KCl, 0·15 M, at 37°, buffered with 30 mM Tris-HCl, pH 7·4. DTNB = 65 μM. Results are means of 12 measurements ± standard deviation. They are expressed in percentage of controls = 100, which are supernatants of thymocyte incubations in isotonic KCl in the absence of NSAID. Spectrophotometric determinations were made at 412 nm. Estimation of the number of membrane sulfhydryl groups available per cell = ($n \times 10^8$). Order of potency of the NSAID effects: (3) > (1) = (2) > (4) = (5) = (6) = (7) > (10) > (8) = (9).

Table 4. Effect of concentration and ionic composition of the medium on the NSAID stimulation of the DTNB sulfhydryl–interchange reaction with rabbit thymocytes*

Drug	Concn (mM)	Media NH_4Cl (0·15 M)	NaCl (0·15 M)		KCl (0·15 M)	$CaCl_2$ (0·11 M)
Flufenamic acid	0	100	100	(0·6 10^8)	100	100
	0·08	211 ± 8·5	123 ± 7	(0·7 10^8)	109 ± 2·5	NP
	0·15	268·5 ± 10	196 ± 11	(1·2 10^8)	143 ± 10	128 ± 6·5
	0·4	322 ± 6	203 ± 4·5	(1·2 10^8)	370·5 ± 4·5	149 ± 8·5
	0·8	324 ± 12·5	536 ± 3	(3·2 10^8)	381 ± 7·5	257 ± 10·5
	1·6	NP	548 ± 11·5	(3·2 10^8)	NP	317 ± 9

* Rabbit thymocytes (6×10^8 cells) were incubated for 5 min in 3 ml of various isotonic media at 37°, buffered with 30 mM Tris-HCl, pH 7·4, under the influence of increasing concentrations of flufenamic acid. DTNB = 65 μM. Results are means of 12 measurements ± standard deviation. For each specific medium the results are expressed in percentage of controls = 100, which are supernatants of thymocyte incubations in each isotonic medium in the absence of any drug. Spectrophotometric determinations were made at 412 nm. Estimation of the number of membrane sulfhydryl groups available per cell = (n × 10^8). NP = experiment not performed.

other NSAID tested. This acceleration appeared to be also closely dependent on the ionic composition of the medium (Table 4). At low concentrations the phenomenon was dose-related (Table 4). At high concentrations no more changes were detected in the sulfhydryl–disulfide interchange reaction, as if all membrane sulfhydryl groups were uncovered above a specific threshold for each NSAID in each specific medium. As in the absence of NSAID, the incubation of DTNB (65 μM) with supernatants or the incubation in Hanks' of DTNB (65 μM) with washed pretreated cells demonstrated that most of the involved sulfhydryl groups were of membrane origin (Table 3).

Two alcohol molecules corresponding to two NSAID (ibuprofen and flufenamic acid) appeared to be equipotent to the acidic molecules in stimulating this DTNB sulfhydryl–disulfide interchange reaction (Table 3).

In several cases the threshold concentration of DTNB needed to produce a maximum enhancement of the sulfhydryl–disulfide interchange reaction was estimated. Such an estimation gives an idea of the number of DTNB molecules needed to saturate the lymphocyte reacting groups. This number, which appeared to be dependent on the kind of ions as well as on the nature and concentration of drugs present in the medium, is a rough approximation of the number of sulfhydryl groups made available per cell.

The results obtained with rabbit thymocytes incubated with CPDS (0·6 mM) and ibuprofen, in a set of experiments similar to those performed with DTNB, confirmed the stimulating effect of NSAID on sulfhydryl–disulfide interchange reactions occurring at the membrane level (Table 5).

Mitochondria (0·75 mg mitochondrial protein) were similarly treated with DTNB (65 μM) which was also studied for its uncoupling and swelling properties on these organelles. Incubation of mitochondria, DTNB and each of the ten studied NSAID revealed that a similar sulfhydryl–disulfide interchange reaction between DTNB and mitochondrial membranes was accelerated in the presence of NSAID. This acceleration also appeared to be dependent on drug concentration and ionic composition of the medium (Table 6). The incubation of DTNB with supernatants obtained by centrifugation of mitochondria suspensions preincubated with NSAID demonstrated that very few intramitochondrial sulfhydryl groups were implicated in this reaction (Table 6).

Table 5. Effect of ibuprofen at various concentrations on the sulfhydryl–disulfide interchange reaction between CPDS and rabbit thymocytes in isotonic NH_4Cl*

Concn (mM)	Absorbance of supernatant
0	100
0·08	150 ± 6·5
0·15	162·5 ± 5
0·4	171 ± 4·5
0·8	296 ± 5·5
1·5	404 ± 4·5

* Rabbit thymocytes (6×10^8 cells) were incubated for 5 min in 3 ml NH_4Cl, 0·15 M, at 37°, buffered with 30 mM Tris–HCl, pH 7·4, under the influence of increasing concentrations of Ibuprofen. CPDS = 0·6 mM. Results are means of 12 measurements ± standard deviation. The results are expressed in percentage of controls = 100, which are supernatants of thymocyte incubations without drug in isotonic NH_4Cl. Spectrophotometric determinations were made at 344 nm.

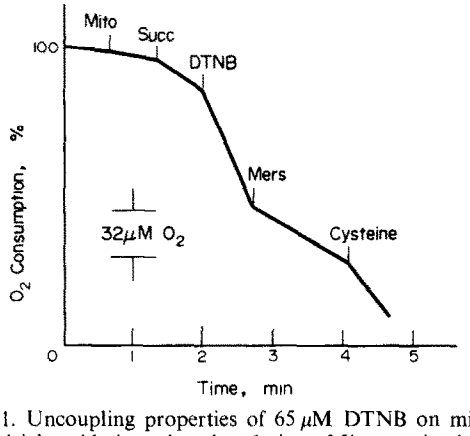

Fig. 1. Uncoupling properties of 65 μM DTNB on mitochondrial oxidative phosphorylation. Mito = mitochondria, 0·75 mg mitochondrial protein; Succ = succinate of sodium, 5 mM (respiratory substrate); Mers = Mersalyl, 35 μM. Cysteine, 0·15 mM. Medium = 2 ml with 20 mM KCl, 5 mM $MgCl_2$, 10 mM PO_4 buffer, pH 7·4, 20 mM Tris-HCl buffer, pH 7·4, and 225 mM sucrose. 100% is 200–240 μM O_2. Oxygen consumption is recorded by a Clark electrode connected to a Gilson Medico-electronics oxygraph (Madison, Wisc.)

Table 6. Effect of flufenamic acid at various concentrations on the sulfhydryl–disulfide interchange reaction between DTNB and rat liver mitochondria in isotonic KCl medium*

Concn (mM)	Supernatants of mitochondria incubated with DTNB		DTNB + supernatants of mitochondria incubated without DTNB
0	100	(100)	100
0·08	112·5 ± 9		102 ± 1·5
0·15	200 ± 9·5	(127 ± 5)	110 ± 6
0·4	413 ± 6·5		114 ± 4·5
0·8	481 ± 11	(236 ± 7·5)	143 ± 5

* Rat liver mitochondria (0·75 mg mitochondrial protein) were incubated for 5 min in 3 ml KCl, 0·15 M, at 37°, buffered with 30 mM Tris-HCl, pH 7·4, under the influence of increasing concentrations of flufenamic acid. DTNB = 65 μM. Results are means of 12 measurements ± standard deviation. The results are expressed in percentage of controls = 100, which are supernatants of rat liver mitochondria incubations without drug in isotonic KCl. Spectrophotometric determinations were made at 412 nm. Results of similar incubations in isotonic NaCl (12 measurements ± standard deviation) are given in parentheses.

At 65 μM, DTNB uncoupled the mitochondrial oxidative phosphorylation; this uncoupling effect was not modified by the presence or absence of NSAID, but was inhibited by Mersalyl and restored by cysteine (Fig. 1 and Table 7). DTNB alone or when added to NSAID did not stimulate swelling of mitochondria or lymphocytes *in vitro*, even at high concentrations.

DISCUSSION

Our experimental data provide evidence for involving membrane sulfhydryl groups in the reaction of NSAID with rat liver mitochondria and rabbit thymocytes. We also obtained similar data with rat thymocytes and lymphocytes and with human thoracic duct lymphocytes. The sulfhydryl–disulfide interchange reaction between DTNB and sulfhydryl groups, mainly of membrane origin, was accelerated in the presence of ten NSAID as well as with two corresponding alcohols, which implies that acidic function is probably not essential for such a biochemical effect, as suggested by us for other biophysical properties [26].

The 344 nm absorption peak of 6-mercaptonicotinic acid resulting from the CPDS sulfhydryl–disulfide interchange reaction with protein sulfhydryl groups could partially interfere with those of most of the classical NSAID. Therefore, only ibuprofen was tested in this reaction which was similarly accelerated by this drug.

The ionic composition of the medium has a marked influence on the number of sulfhydryl groups made available for the interchange reaction. Thus this ionic composition modulates the sulfhydryl influence of NSAID as well as other biochemical properties of these drugs [14,15].

A rough determination of the number of sulfhydryl groups was attempted in several cases for rabbit thymocytes. However, since DTNB may not react with all the surface sulfhydryl groups [27], this quantitation

Table 7. Respiratory ratios illustrating the uncoupling properties of DTNB*

Drugs	Respiratory ratios (with succinate, 5 mM)
DTNB	4·2 ± 1 (5)
Ibuprofen	4 ± 1·22 (9)
Flufenamic acid	4·3 ± 1·31 (9)
DTNB + ibuprofen	4·2 ± 1·1 (5)
Ibuprofen + DTNB	4 ± 1·22 (5)
DTNB + flufenamic acid	4·2 ± 1·1 (5)
Flufenamic acid + DTNB	4·3 ± 1·31 (5)
DTNB + Mersalyl	1·1 ± 0·2 (5)
DTNB + Mersalyl + cysteine	3·7 ± 0·32 (5)

* Respiratory ratios of DTNB and some NSAID alone and in association, and of DTNB in the presence of Mersalyl alone or with cysteine. Mitochondria = 0·75 mg mitochondrial protein. DTNB = 65 μM; ibuprofen = 0·25 mM; flufenamic acid = 0·2 mM; Mersalyl = 35 μM; cysteine = 0·15 mM. Respiratory substrate was succinate of sodium, 5 mM. Incubation medium: 2 ml with 20 mM KCl, 5 mM $MgCl_2$, 10 mM PO_4 buffer, pH 7·4; 20 mM Tris-HCl buffer, pH 7·4; and 225 mM sucrose. Respiratory ratios are defined as the ratios of respiration rates in the presence of inhibiting or uncoupling agents to that observed in state 4 as defined by Chance and Williams [25]. Number of experiments from which mean values were calculated is given in parentheses.

was probably not relevant enough for a fine estimation of the effect of NSAID or ions on availability of cell membrane sulfhydryl groups. Nevertheless, these numbers were of such magnitude that marked membrane changes had to be produced. The swelling of the lymphoid cells under the influence of the same drugs at similar doses [15] could be the reflection of these changes. This is corroborated by the fact that the drugs that had the greatest effect on lymphocyte swelling, the anthranilic acid derivatives or fenamates [15], were also the most potent drugs in accelerating this sulfhydryl–disulfide interchange reaction. Moreover, it is most probable that some changes were induced by DTNB alone, since even in the absence of NSAID the number of sulfhydryl groups available was very important.

Ionic sequences, most probably regulated by the laws of ionic selectivity described by Eisenman [28,29], were obtained. Similar ionic sequences were previously found by us for the swelling effects of NSAID on isolated mitochondria [14] and lymphocytes [15]. Analysis of these sequences on the basis of Eisenman's laws gave us information about the membrane sites implicated in these swelling mechanisms. In the present case, however, too many parameters were involved in the sulfhydryl–disulfide reaction to allow any relevant analysis of these ionic sequences (e.g., varying the medium composition influences by itself the interchange reaction, but has no effect *per se* on lymphocyte swelling).

The fact that DTNB itself behaves identically to NSAID in its uncoupling effect on mitochondrial oxidative phosphorylation and is inhibited by Mersalyl and restored by cysteine confirms that, as suggested by Hitchens,* DTNB shares common sites with NSAID because its chemical configuration is not unlike that of several arylacidic NSAID.

On the other hand, no mitochondrial or cell swelling effects were noticed with DTNB, as they are with most of the NSAID. This is not necessarily at variance with the uncoupling results, since it is known that some NSAID (e.g. salicylates) do not exhibit swelling properties [14]. It must also be pointed out that if NSAID accelerate the sulfhydryl–disulfide interchange reaction between 65 μM DTNB and mitochondrial membranes, they do not modify the uncoupling effect of such an amount of DTNB (see respiratory ratios of Table 7 [25]). This suggests that the new sulfhydryl groups uncovered by the NSAID could be different from those engaged in the uncoupling mechanism.

All these results could be of some importance for a better understanding of the biochemical pharmacology and perhaps also for the clinical properties of NSAID. At low doses, these drugs are able to stabilize various membranes, such as erythrocyte [30,31] and lysosomal membranes [32], although they labilize similar membranes at high concentration [33]. Mitochondrial [14] and lymphocyte swelling [15] under the influence of various NSAID, at concentrations similar to those used in the present study, is one example of membrane labilization by these drugs. The importance of sulfhydryl groups for this drug effect is demonstrated by the enhancing action of thiol inhibitors such as Mersalyl on the phenomenon. The relationship between NSAID-induced mitochondrial swelling and uncoupling properties of these drugs on mitochondrial oxidative phosphorylation is not well understood, but the integrity of mitochondrial membrane sulfhydryl groups seems to be required for observing this uncoupling effect [13]. Sulfhydryl groups are also implicated in the effects of gold salts on lysosomes [6–8].

From these data, it appears most probable that sulfhydryl groups of membrane proteins must have an important role to play in the development of this peculiar NSAID biphasic effect of membrane stabilization and labilization, which could explain some clinically useful properties of these drugs such as the protection of inflammatory tissues against further lytic action of lysosomal enzymes by stabilizing these organelle membranes. Whether sulfhydryl groups are favorable to stabilization or labilization is still not understood.

It is evident that this accelerating effect of NSAID is a general effect on proteins containing disulfide groups. But in the case of lymphoid cells, some of these being B-lymphocytes, the fact that various immunoglobulins, especially IgM, are present on the surface of the cells must be kept in mind [34]. Breakage of disulfide bonds of these immunocompetent molecules could contribute significantly to the results. Such an influence of NSAID on immunoglobulins could be of great importance for the development of immunological responses to various immunogens, including the unknown antigens probably implicated, as most generally postulated to-day [35], in the inflammatory reactions observed in connective tissue diseases [36].

It is evident from our data that, on lymphocyte and mitochondrial membranes, new sulfhydryl groups are made available for DTNB or CPDS sulfhydryl–disulfide interchange reaction under the influence of NSAID and related alcohol molecules which have many other biochemical properties in common with acidic NSAID [26]. This means that these drugs are probably able, by reacting with various sites of the membrane, to modify its structure, which results in an expansion of cell or organelle surface. By this general effect, sulfhydryl groups become available for various reactions, which then probably occur in the inflammatory area as well as in other areas.

In rheumatic diseases such as rheumatoid arthritis and lupus erythematosus, it has been shown that sulfhydryl erythrocyte membrane groups and serum protein sulfhydryl levels are especially low [37,38]. The serum sulfhydryl–disulfide interchange reaction is impaired in adjuvant arthritis as well as in ultraviolet guinea-pig erythema and it is restored by administration of NSAID but not of sulfhydryl donors [19,20]. This last observation is very important and not necessarily at variance with our conclusions. It pointed out, however, that simply increasing the total number of free sulfhydryl groups has no anti-inflammatory effect *per se*, while an increase of specific membrane or plasma protein sulfhydryl groups, like that observed under the influence of NSAID, could be at least related to some pharmacological effects of these drugs. Whether these are anti-inflammatory or secondary effects remains a moot point. Further studies *in vivo* with alcohol molecules

* M. Hitchens, unpublished observations (cited by T. Y. Chen, in *A. Rep. med. Chem.* **2**, 219, 1966).

related to NSAID would certainly be helpful in answering this question. The required concentration for observing this sulfhydryl–disulfide interchange reaction *in vitro* was slightly higher than those observed in the plasma of patients treated with these drugs. This could argue for relating this effect on sulfhydryl groups to clinical secondary or even toxic effects of NSAID, while other biochemical properties, such as protaglandine-synthetase inhibition by lower concentrations of these drugs [39] could explain their anti-inflammatory, antipyretic and analgesic properties [40]. Nevertheless, the existence of drug segregation mechanisms in various organelles, cells [6] or tissues [41] must be kept in mind and could account for a true pharmacological role played by this NSAID effect on sulfhydryl groups.

It can be concluded from our results that the importance of sulfhydryl groups has been confirmed for the well known effects of NSAID on biological membranes, which are probably related to secondary and even toxic effects of these drugs, but perhaps are also related to their true anti-inflammatory and analgesic properties. These findings corroborate various data from the literature which demonstrate the importance of thiol groups in the development of inflammatory processes even if some doubts persist concerning the precise biochemical role of these groups.

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