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INTERACTIONS BETWEEN NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND BIOLOGICAL MEMBRANES—II

SWELLING AND MEMBRANE PERMEABILITY CHANGES INDUCED IN SOME IMMUNOCOMPETENT CELLS BY VARIOUS NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—Non-steroidal anti-inflammatory drugs (NSAID) that are known to induce pseudo-energized high-amplitude swelling of mitochondrial preparations also induce swelling in various types of lymphocytes, namely: rat and rabbit thymocytes, rat and rabbit lymph node cells, and human and rat circulating lymphocytes obtained by thoracic duct drainage. The swelling of these immunocompetent cells was measured by light scattering. With the important exception of gold salts (no effect) and salicylates (very poor activity), almost all the NSAID that we have studied were able to induce cell swelling *in vitro*, but only at relatively high concentrations of these drugs compared to the concentrations which induced mitochondrial swelling (liver, kidney). The most dramatic effect was observed with *N*-arylanthranilates (fenamates), especially with flufenamic acid, which was used to study the mechanism of this drug action. The magnitude of the swelling is poorly related to the concentration of the drug, but the rapidity of this phenomenon seems to be a concentration-related phenomenon. If *p*-chloromercuribenzoate (*p*-CMB) or Mersalyl is present in the medium, the rate of NSAID-induced swelling is enhanced and appreciable cell swelling can even be demonstrated for drugs which otherwise (i.e. without *p*-CMB or Mersalyl) cause little cell swelling, e.g. phenylbutazone. By contrast, iodacetamide and *N*-ethylmaleimide fail to enhance the swelling. These activities seem to be related both to reactions with membrane thiol groups and modifications of the membrane charges by these compounds. The ionic composition of the medium is critical for this swelling activity and a cationic as well as an anionic selectivity was found for this phenomenon. These results suggest that one possible effect of the NSAID on leukocytes implicated in the immune and inflammatory responses may be on the permeability of the cell membrane to various ions.

THE EFFECTS of non-steroidal anti-inflammatory drugs (NSAID) on mitochondrial metabolism have been studied by several authors, especially for their uncoupling properties in oxidative phosphorylation.[1,2] More recently, the action of these drugs on isolated mitochondria has been studied using another parameter, induction of high-amplitude pseudo-energized swelling,[3,4] which reflects some drug-induced changes in the membrane permeability of mitochondria.

The swelling of ribosomes[5] and both swelling and shrinking of chloroplasts[6–9] have also been observed under various conditions, and even whole cells have been studied for their swelling properties. Ponder[10] and Dick,[11,12] a long time ago,

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reviewed the different techniques used to record water uptake by cells. Though many tissues have been studied by various methods, only suspensions of cells can be observed opacitometrically by measuring changes in light scattering, first clearly described by Orsköv.[13] The main work in this field has been carried out on red blood cells (e.g. De Venuto,[14] Lefevre[15] and Dawson and Widdas[16] in correlation with sugar transport), sea urchin's eggs (e.g. *Arbacia punctulata*,[17] *Cummingia* and *Chaetopterus* studied by Lucké *et al.*[18]) and bacterial cell suspensions (e.g. recent studies of Bernheim[19–21] on *Pseudomonas aeruginosa* and *Escherichia coli*).

Recently, Mela[22–25] studied mathematically the elastic forces involved in mitochondrial swelling and in the swelling of the egg of a sea urchin, *Stongylocentrus pupuratus*, taken as a model for whole cell swelling.

In recent years interest has been aroused in the early cellular events following the stimulation of more or less pure lymphocyte preparations by various agents including mitogens and antigens.[26,27] One of the phenomena observed during mitogenesis is cellular swelling.[26] Evidence is available about the activity of the NSAID on the metabolism of immunocompetent cells.[28] Very few reports in the past concern leukocyte osmotic and swelling properties.[29]

The present study was designed to establish whether or not intact lymphoid cells exhibit swelling and other permeability changes under the influence of NSAID, resembling the response of liver and kidney mitochondria to the same drugs,[3] and to study some of the variables involved in this apparently more general phenomenon.

MATERIAL AND METHODS

Preparation of cells. Rabbit and rat thymocytes and lymphocytes were obtained from 6- to 12-week-old New Zealand albino rabbits (2·5 to 3·0 kg) and male Wistar rats (150–250 g). Animals were sacrificed by cervical cord dislocation and the thymus and lymph nodes (lumbar and popliteal nodes from the rats, mesenteric nodes from the rabbits) were dissected in Hanks' balanced salt solution, pH 7·4. These cells were passed through a No. 40 wire gauze screen and briefly centrifuged (200 *g*) to remove debris. The cells were then washed twice in Hanks' solution, pH 7·4 by centrifugation at 350 *g* for 10 min and finally resuspended in the same Hanks' solution to give a stock suspension (approximately 2×10^8 cells/ml). The cell number was determined in a Coulter electronic counter. Cell viability (approximately 95 per cent) was routinely assessed by Trypan blue exclusion[30] before beginning each series of experiments.

Human thoracic duct cells were obtained through the courtesy of Dr. H. Paulus from two patients with "classical" rheumatoid arthritis who were undergoing thoracic duct drainage.[31] Cells were removed from thoracic duct lymph by continuous sterile centrifugation, the supernatant being returned to the patients. After that, these cells were treated exactly the same as the animal lymphoid cells and finally resuspended in Hanks' balanced solution, pH 7·4. These human cells were obtained on three occasions for each of the two patients. Rat thoracic duct lymphocytes were collected by Mrs. D. J. Whitehouse and treated like the other animal cells after being isolated from the lymph by centifugation at 350 *g*.

Pharmacological studies. Aliquots of cell suspension (0·1 ml) were diluted to 3 ml in 0·15 M solutions of either ammonium chloride or one of the five monovalent alkali metal chlorides or 0·11 M solutions of the four divalent alkaline-earth metal chlorides or 0·15 M solutions of the four halide salts of potassium. All these

salt solutions were buffered at pH 7·4 with 30 mM *N*-Tris (hydroxymethyl) methyl 2-amino ethane sulfonic acid (TES), a zwitterionic buffer which, like any other zwitterionic buffer tested, does not affect the cell volume by itself, as indicated by light scattering measurements (see Fig. 1). This buffer was selected also because it has no significant effect on lymphocyte and thymocyte metabolism (e.g. incorporation of amino acids-^{14}C or nucleosides-^{3}H into cell polymers). Reduction in light scattering was measured using a DU-2 Beckman spectrophotometer, connected to a Gilford recorder, at 510 nm, using rectangular glass cuvettes with a 10 mm light path. The experiments were initiated by adding the drugs in small volumes (max. 50 μl) from stock solutions in ethanol or dimethylformamide (DMF), by way of a small plastic spoon with little holes. These amounts of organic solvent, did not affect the swelling *per se* (Fig. 1). The suspension of cells was allowed to stabilize for 4 min before adding the drugs to obtain an adequate baseline recording of initial light scattering. The optical density of each cell suspension was then continuously recorded for 10 min, from 5 sec after adding the drug, at least five times for each type of incubation.

Reagents. The NSAID were generous gifts from various drug companies: Geigy; Parke Davis & Co.; Imperial Chemical Industries (G.B.); Merck Sharp & Dohme; Upsa (Agens, France); Riker; Schering; Continental Pharma (Brussels, Belgium); Biorex Laboratories (London, England); Merck & Co.; Seresci (Brussels, Belgium); Boots (Nottingham, England); Smith & Nephew (Harlow, England); and also from Research Institute for Pharmacology and Biochemistry (Prague, C.S.S.R.). Iodoacetamide was obtained from Calbiochem (Los Angeles, Calif.); *N*-ethylmaleimide from Aldrich Chemical Co. (Milwaukee, Wisc.); the sodium salt of Mersalyl and *p*-chloromercuribenzoate from Sigma Chemical Co. (St Louis, Mo.). The different zwitterionic buffers were Schwarz-Mann products (Van Nuys, Calif.). The alkali and alkaline-earth metal chlorides and the potassium halides were of the purest grade commercially available. Flufenamyl alcohol was obtained through the courtesy of Dr. L. Fenoy (Willmington, Del.), while alcohols corresponding to ibruprofen and ibufenac were obtained from Boots Drug Company.

RESULTS

Thirty-two NSAID and analogues listed in Table 1 were tested against rat and rabbit thymocytes and lymphocytes for their ability to induce cell swelling in various buffered media (TES, a zwitterionic buffer, 30 mM, pH 7·4) as recorded by decrease in light scattering measured at 510 nm. Human lymphocytes obtained by thoracic duct drainage were also tested by this method but only under optimal conditions observed in studies of drug actions on animal lymphoid cells. The results of these experiments are summarized in Table 2 (for animal as well as human cells), in which each value is the mean of at least five incubations (with 5×10^6 cells in 3 ml), and shown in Fig. 1.

The data shown in Table 2 and other observations revealed the following points:

(1) NSAID-induced cell swelling can be observed with rat, rabbit and human immunocompetent cells but only with some drugs under certain specific conditions.

(2) No species difference between rat, rabbit and human cells could be detected (for the same number of cells, 5×10^6 cells in 3 ml). There was also no difference between the swelling observed with thymocytes and lymphocytes from nodes or lymph.

TABLE 1. THIRTY-TWO NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND ANALOGUES TESTED

Four arylalkanoic acids
1. 2-(4-Isobutylphenyl) propionic acid (ibuprofen)
2. (*p*-Isobutylphenyl) acetic acid (ibufenac)
3. Indomethacin
4. Fenclozic acid (Myalex)

Two phenoxyacetic acids
5. Clamidoxic acid (SNR 1805)
6. 4-Allyloxy-3-chlorophenoxyacetic acid (alclofenac)

Seven anthranilic acid derivatives (*N*-arylanthranilates or fenamates) and analogues
7. Flufenamic acid
8. Mefenamic acid
9. Meclofenamic acid
10. 2-(2′-Methyl-3′-chloro anilino) nicotinic acid (Clonixin)
11. *N*-phenylanthranilic acid*
12. *O*-phenoxybenzoic acid*
13. Diphenylamine*

Six pyrazolone derivatives
14. Phenylbutazone
15. Piperazine salt of phenylbutazone/pyrazinobutazone)
16. Trimethazone
17. Oxyphenbutazone (Tanderil)
18. Sulfinpyrazone (Anturan)
19. γ-Ketophenylbutazone (Ketason)

One hydroxamic acid derivative
20. *p*-Butoxyphenylacethydroxamic acid (bufexamac)

Two terpenoids
21. 18β-glycyrrhetinic acid*
22. Hemisuccinate of 18β-glycyrrhetinic acid (carbenoxolone; Biogastrone)

Three water-soluble gold preparations
23. Aurothiomalate (Myochrysin)
24. Aurothiosulfate (Sanochrysin)
25. Aurothioglucose (Solganal)

Seven salicylates
26. Acetylsalicylic acid (aspirin)
27. Sodium salicylate
28. Salicylamide
29. γ-Resorcylic acid
30. 5-*p*-Fluorphenyl-*O*-acetylsalicylic acid (flufenisal)
31. Hexahydrosalicylic acid*
32. Gentisic acid

* Not used clinically as an NSAID.

(3) Among the 13 media examined, isotonic ammonium chloride seems to be the best for inducing cell swelling with an added NSAID.

(4) Among the drugs examined, the *N*-arylanthranilates (fenamates), especially flufenamic acid, appear to be the most potent in inducing cell swelling. Of the pyrazolone drugs, only trimethazone proved to be potent of itself. In the arylalkanoic group, ibuprofen and indomethacin are active, but at relatively high concentration. Of particular interest is the lack of activity of the gold salts and the very poor activity of the salicylates, which have also been observed for mitochondrial swelling[3] (Fig. 1).

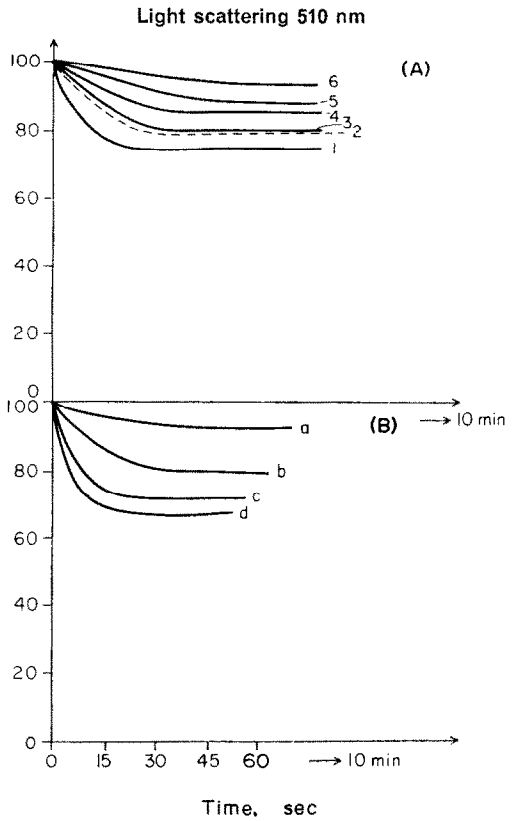

FIG. 2. Rabbit thymocytes (5×10^6 cells) in 3 ml of: (A) Various alkali chlorides buffered with 30 mM TES, pH 7·4. Flufenamic acid, 0·15 mM. (1) NH_4^+, (2) Rb^+, (3) K^+, (4) Li^+, (5) Na^+, (6) Cs^+. (B) Various halides of potassium with 30 mM TES, pH 7·4. Flufenamic acid, 0·15 mM. (a) F^-, (b) Cl^-, (c) Br^-, (d) I^-. Time 0 = + 5 sec.

is similar for those drugs which have shown activities in different media. The cation sequence (with some restrictions for NH_4^+, see Discussion) seems to be $(NH_4^+) > K^+ = Rb^+ \geqslant Li^+ \geqslant Na^+ \geqslant Cs^+$, and the anion sequence seems to $I^- \geqslant Br^- \geqslant Cl^- > F^-$. No selectivity was observed among the alkaline-earth divalent cations, since cell swelling was minimal in all media containing these ions.

Effect of drug concentration (*Fig.* 3). Flufenamic acid was tested in four different media (NH_4Cl, KCl, LiCl and NaCl) to find a relation between the concentration and the swelling activity. As shown in Fig. 3, the velocity of the phenomenon is closely related to the concentrations of drugs present in the medium (0·03 to 0·15 mM in NH_4Cl, 0·15 to 0·6 mM in KCl, 0·3 to 1·2 mM in LiCl, 0·6 to 2·4 mM in NaCl). A concentration–response relationship was also found in NH_4Cl for mefenamic acid (0·3 to 0·6 mM), clonixin (0·3 to 1·2 mM) and ibuprofen (0·8 to 2 mM).

The extent (as opposed to rapidity) of swelling seems to be possibly also related to drug concentration. After 10 min, the swelling of the cells at room temperature almost ceases and the actual decrease in light scattering (i.e. degree of swelling) is rather less with small concentrations of NSAID than with higher concentrations.

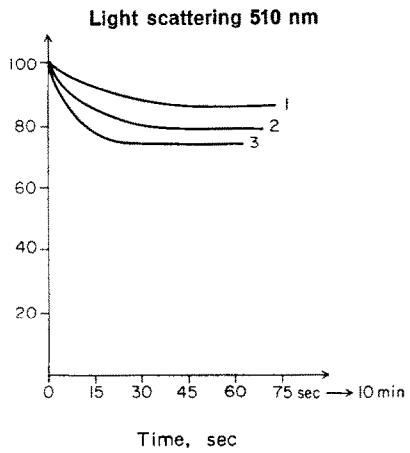

FIG. 3. Rabbit thymocytes (5×10^6 cells) in 3 ml NH_4Cl, 0·15 M, buffered with 30 mM TES, pH 7·4 Effect of various doses of flufenamic acid: (1) 0·03 mM, (2) 0·07 mM, (3) 0·15 mM. Time 0 = + 5 sec.

This was also true in several experiments in which we recorded the swelling for 1 or 2 hr and where a very slow swelling seemed to persist (1–5 per cent of the swelling recorded in the first 10 min).

Effect of pH (*Fig.* 4). In the pH range from 5·0 to 8·6 in a KCl medium (buffered with various zwitterionic buffers), no direct pH influence was observed on cell swelling. But it has been observed that with a constant concentration of flufenamic acid the amplitude of swelling increases with a change from low to high pH. This pH effect is also established by the fact that to record the same degree of swelling we needed to increase the actual concentration of flufenamic acid when decreasing

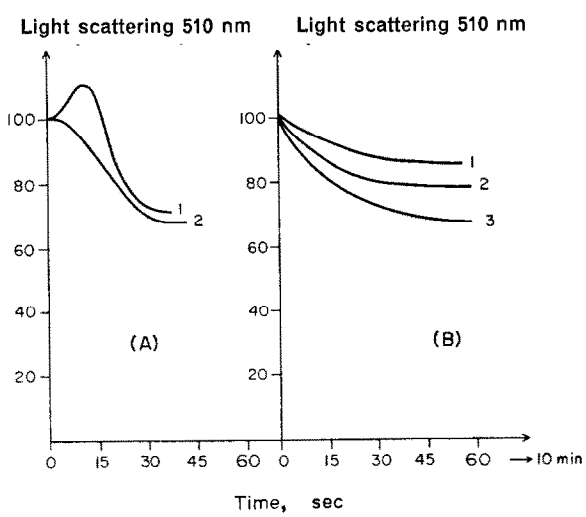

FIG. 4. Rabbit thymocytes (5×10^6 cells) in 3 ml KCl. (A) No buffer: (1) effect of pH 2 induced by HCl; (2) effect of pH 11 induced by K_2CO_3. (B) Zwitterionic buffers: (1) pH 5 + flufenamic acid, 0·15 mM; (2) pH 7·4 + flufenamic acid, 0·15 mM; (3) pH 8·6 + flufenamic acid, 0·15 mM. Time 0 = +5 sec.

the pH of the medium. At very low pH (pH 2 adjusted with HCl), shrinking of the cells followed by rapid swelling was observed, and at very high pH (pH 10 or more, adjusted with K_2CO_3), a spontaneous swelling was also recorded. These facts show that (1) pH by itself can induce some swelling activities, and (2) pH can modify the activity of swelling agents, presumably by controlling the extent of their ionization or of membrane sites' ionization.

Effect of some thiol inhibitors on NSAID-induced cell swelling (*Fig.* 5). Certain thiol inhibitors (Mersalyl, 0·035 mM, and *p*-CMB, 0·05 mM) increase mitochondrial swelling.[3] In the presence of NSAID, Mersalyl and *p*-CMB were found to amplify NSAID-induced cell swelling similarly, especially with some drugs, like phenylbutazone, which otherwise induced a very moderate swelling. This facilitating effect, however, was less dramatic than that seen when the same concentrations of *p*-CMB or Mersalyl were applied, together with NSAID, to mitochondria. By contrast, *N*-ethylmaleimide (0·05 mM) or iodoacetamide (0·05 mM) did not facilitate drug-induced cell swelling. These rather selective properties of the thiol inhibitors will be discussed later, but they suggest that some thiol groups in the membrane may be involved in this drug-related swelling phenomenon.

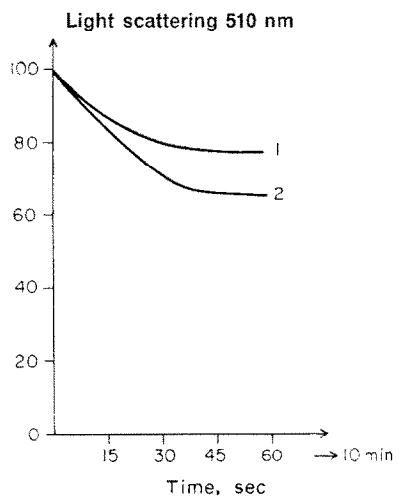

FIG. 5. Rabbit thymocytes (5×10^6 cells) in 5 ml KCl, 0·15 M buffered with 30 mM TES, pH 7·4. (1) Flufenamic acid, 0·15 mM; (2) flufenamic acid, 0·15 mM, with Mersalyl, 0·035 mM. Time 0 = +5 sec.

DISCUSSION

NSAID are known to uncouple oxidative phosphorylation[1,2,32] at concentrations attainable *in vivo* after therapeutic dosing. With the exception of gold salts and salicylates, they are also able to induce mitochondrial swelling at the same concentrations.[3,4] It now appears that most of these drugs are also able, at relatively higher concentrations, to induce swelling of whole cells, especially immunocompetent cells like lymphocytes and thymocytes, in the absence of added proteins; the fenamates,

especially flufenamic acid, seem to be the most potent in this respect. The phenomenon appears to be (as for mitochondrial swelling) concentration-dependent and closely related to the ionic composition of the incubation medium. The proton and hydroxyl concentrations of the medium are also of some importance, as shown by the considerable cell swelling recorded in alkaline medium and the slight swelling recorded in acid medium with the same amount of drug. By varying the cationic and anionic composition of the medium, we were able to find a cation selectivity for the monovalent cations and an anion selectivity for the monovalent anions. The preferential cationic sequence, $(NH_4^+) > K^+ = Rb^+ \geqslant Li^+ \geqslant Na^+ \geqslant Cs^+$, which we have found for this drug-induced swelling can be described according to the Eisenman theory[33,34] of ionic selectivity (as explained in the first paper of this series.[3]) This sequence corresponds to No. V for Eisenman with a shift of two places for Li^+. This means that anionic sites with weak as well as strong electrical fields are involved in the cationic permeability of the membrane and also suggests that many of these anionic sites are highly polarizable sites, like carboxyl groups.

The data concerning NH_4^+ have received little theoretical attention because of the resulting complexities (which is also true of other non-alkali monovalent cations like Ag^+ or Tl^+),[35] but here, forces other than Coulombic forces must be involved. The greater affinity for NH_4^+ in our sequences, compared to K^+ and Rb^+, the two more potent alkali cations, is similar to that found by other investigators working with different biological and nonbiological systems (for example, the extraction by macrotetralide actin antibiotics of various salts into organic solvents like dichloromethane).[36] Nevertheless, the possibility that NH_4^+, which is in the incubation medium in equilibrium with a noncharged form, might cross the membrane as NH_3 must be kept in mind.

On the other hand, we found that the anion selectivity for the halide series was $I^- \geqslant Br^- \geqslant Cl^- > F^-$, which, according to the ionic selectivity theory, is related only to the presence of cationic sites with weak electrical fields on the membrane.

There is thus a difference between the absolute field strength of the anionic sites (involved in the permeability and the movement of cations across the cell membrane) and the absolute field strength of the cationic sites (involved in the permeability and the movement of anions across the same cell membrane). This lack of correlation, which differs from the good correlation between the absolute field strengths of both sites in mitochondrial membranes, could be one of the explanations for the much greater swelling observed with mitochondria as compared to cells when exposed to drugs and the need for higher concentrations of these drugs to induce cell swelling in these experiments.

It seems also that thiol groups are involved in the NSAID-induced cell swelling which is shown by the enhancing effect on swelling promoted by ionic thiol inhibitors, Mersalyl and *p*-CMB. On the other hand, the relatively apolar reagents, *N*-ethylmaleimide and iodoacetamide, were unable to enhance any type of swelling (mitochondrial or intact cells). This could be explained by the fact that the first two thiol inhibitors react with membrane thiol groups and at the same time increase the negative charge of the membrane (which is, as we know, involved in swelling activities), but the latter two thiol inhibitors would not affect ionic composition of the membrane after reacting with exposed membrane thiol groups.[3,37] However, Ellman's reagent (DTNB = 5,5′-dithiobis-2-nitrobenzoic acid), which should also increase the mem-

brane anionic charges after reacting with membrane thiols, did not enhance NSAID-induced swelling.*

A justifiable criticism of the relevance of this drug-induced phenomenon of cell swelling *in vitro* to the therapeutic actions of the same drugs *in vivo* is that very high concentrations are needed to induce such swelling. The same objections have also been raised concerning the relevance of the mitochondrial swelling and uncoupling activity induced by NSAID.[38]

It is not known how the intracellular concentrations of the drugs are related to plasma levels. Drug segregation can occur into cells or organelles, providing higher concentrations of drugs than in the ambient medium. This has been demonstrated with gold salts and liver lysosomes.[39] Drug segregation mechanisms have also been demonstrated in various tissues.[40]

On the other hand, *in vivo*, drugs may have an affinity for specific carrier proteins, carrying them to their sites of action to provide higher drug concentrations at the cell receptors than are present in the plasma.[41]

Finally, in the particular case of cell swelling, the phenomenon seems clearly to be only the reflection of some more fundamental changes in the membrane permeability elicited by these drugs. The elasticity of the cell membrane as shown by Mela[22–25] is an important factor for the recording of swelling activities. If no swelling was recorded with lower concentrations of drugs, this does not necessarily imply that membrane permeability changes do not occur under the influence of the drugs, but merely suggests that the influx of ions and water molecules through the membrane was not sufficient to induce a notable expansion of the cell volume.

Such a phenomenon was very well demonstrated with mitochondria from different tissue origin; thus under the same experimental conditions, the volume of brain mitochondria increases only 2 per cent when the volume of liver mitochondria increases 200 per cent, in the presence of the same swelling agents.[42] This is only due to the specific geometrical arrangement of the cristae in brain mitochondria which diminishes the elasticity of these organelles. But, as shown by radioactive measurements, even without swelling, significant ion uptake into brain mitochondria can be observed under the influence of swelling agents.*

In conclusion, it appears that NSAID at relatively high concentrations are able to induce swelling of immunocompetent cells exposed to them *in vitro*. This phenomenon *in vitro* could be the crude reflection of a more physiological property of these drugs—their ability to alter membrane permeability for ions and small molecules—already demonstrated for mitochondrial membranes[3] and for some salicylate derivatives on artificial phospholipid bilayers.[43]

These changes in the surface of cell membranes could perhaps explain many recent reports describing the ability of NSAID to stabilize erythrocytes against osmostic shock[44–46] and, on the other hand, the changes in the internal ionic composition of the cells could perhaps explain (by a catalytic effect) some of the enzymatic inhibition or activation by these drugs that has been described in recent years.[47,48] Nevertheless, whether or not all these phenomena are of importance for therapeutic effects or for toxicological properties of these drugs remains a moot point.

* J. P. Famaey and M. W. Whitehouse, unpublished data.

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REFERENCES

1. M. W. WHITEHOUSE, *Progress in Drug Research* (Ed. E. JUCKER), Vol. 8, p. 321. Birkhäuser, Basel and Stuttgart (1965).
2. J. P. FAMAEY and J. MOCKEL, *Biochem. Pharmac.* **22,** 1487 (1973).
3. J. P. FAMAEY, *Biochem. Pharmac.* **22,** 2693 (1973).
4. N. NAKANISHI, H. IMAMURA and V. IKEGAMI, *J. Pharm. Soc. Japan* **91,** 702 (1971).
5. H. TEDESCHI, J. M. JAMES and W. ANTHONY, *J. Cell Biol.* **18,** 503 (1963).
6. R. A. DILLEY and L. P. VERNON, *Biochemistry, N.Y.* **3,** 817 (1964).
7. S. IZAWA, *Biochim. biophys. Acta* **102,** 373 (1965).
8. J. YOUNG and L. PACKER, *Biochim. biophys. Acta* **126,** 399 (1966).
9. K. YAMASHIKA, M. ITOH and K. SHIBATA, *Biochim. biophys. Acta* **162,** 610 (1968).
10. E. PONDER, in *Hemolysis and Related Phenomenon*, p. 76. Churchill, London (1948).
11. D. A. T. DICK, *Int. Rev. Cytol.* **8,** 387 (1948).
12. D. A. T. DICK, in *Cell Water*, p. 86. Butterworth, London (1966).
13. S. L. ORSKÖV, *Biochem. Z.* **279,** 241 (1935).
14. F. DE VENUTO, *Proc. Soc. exp. Biol. Med.* **129,** 106 (1968).
15. P. G. LEFEVRE, *Biochim. biophys. Acta* **120,** 395 (1966).
16. A. C. DAWSON and W. F. WIDDAS, *J. Physiol. Lond.* **172,** 107 (1964).
17. B. LUCKÉ and E. N. HARVEY, *J. cell. comp. Physiol.* **5,** 473 (1935).
18. B. LUCKÉ, H. K. HARTLINE and R. A. RICCA, *J. cell. comp. Physiol.* **14,** 237 (1939).
19. V. F. BERNHEIM, *J. gen. Microbiol.* **30,** 53 (1963).
20. V. F. BERNHEIM and L. LACK, *Can. J. Microbiol.* **17,** 323 (1971).
21. V. F. BERNHEIM, *Microbiol.* **4,** 87 (1971).
22. M. J. MELA, *Biochim. biophys. Acta* **150,** 541 (1968a).
23. M. J. MELA, *Biochim. biophys. Acta* **150,** 553 (1968b).
24. M. J. MELA, *Biochem. J.* **7,** 95 (1967).
25. M. J. MELA, *Biochem. J.* **8,** 83 (1968).
26. CH. K. NASPITZ and M. RICHTER, *Prog. Allergy* **12,** 1 (1968).
27. J. C. GOWANS and D. D. MACGREGOR, *Prog. Allergy* **9,** 1 (1965).
28. M. W. WHITEHOUSE, *Biochem. Pharmac.* (Suppl.) **17,** 293 (1968).
29. B. LUCKÉ, H. G. HEMPLING and J. MACKLER, *J. cell. comp. Physiol.* **47,** 107 (1956).
30. E. A. BOYSE, *Meth. med. Res.* **10,** 34 (1964).
31. O. WEGELIUS, V. LAINE, B. LINDSTRÖM and M. KLOCKARS, *Acta med. scand.* **187,** 539 (1970).
32. S. S. ADAMS and R. COBB, *Nature, Lond.* **181,** 773 (1958).
33. G. EISENMAN, *Biophys. J.* **2,** 259 (1962).
34. G. EISENMAN, in *Ion-Selective Electrodes* (Ed. R. A. DURST) Special Publication 314. National Bureau of Standards, Washington, D.C. (1969).
35. G. EISENMAN, *Adv. analyt. Chem. Instrum.* **4,** 213 (1965).
36. G. EISENMAN, S. CIANI and G. SZABO, *J. memb. Biol.* **1,** 346 (1969).
37. J. LYNN and BROWN, *Biochim. biophys. Acta* **110,** 445 (1965).
38. F. DICKENS, in *Salicylates, An International Symposium*, (Ed. A. ST. J. DIXON, B. K. MARTIN, M. J. H. SMITH and P. H. N. WOOD), p. 62. Churchill, London (1963).
39. G. WEISSMAN, *N. Engl. J. Med.* **286,** 141 (1972).
40. G. WILHELMI and R. PULVER, *Arzneimittel-Forsch.* **5,** 221 (1955).
41. S. J. PILIERO and C. COLOMBO, *J. clin. Pharmac.* **7,** 198 (1967).
42. D. F. TAPLEY and C. COOPER, *Nature, Lond.* **178,** 1119 (1956).
43. V. S. MARKIN, L. I. KRISTALIK, E. A. LIBERMAN and V. P. TOPALY, *Biofizika* **14,** 256 (1969).
44. J. H. BROWN, H. K. MACKEY and D. A. RIGGILO, *Proc. Soc. exp. Biol. Med.* **125,** 837 (1967).
45. A. D. INGLOT and E. WOLNA, *Biochem. Pharmac.* **17,** 269 (1968).
46. Y. MIZUSHIMA, S. SAKAI and M. YAMAURA, *Biochem. Pharmac.* **19,** 227 (1970).
47. J. R. VANE, *Nature New Biol.* **231,** 235 (1971).
48. M. WEINER and S. J. PILIERO, *A. Rev. Pharmac.* **10,** 171 (1970).