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

HIGH AMPLITUDE PSEUDO-ENERGIZED MITOCHONDRIAL SWELLING AND MEMBRANE PERMEABILITY CHANGES INDUCED BY VARIOUS NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—The action of various non-steroidal anti-inflammatory drugs (NSAID) that are also potent uncoupling agents for oxidative phosphorylation has been studied by measuring light scattering by rat liver mitochondria in different media. These drugs all induce a pseudo-energized high amplitude swelling, except gold salts and salicylates. The amplitude of this swelling is not very dependent on the concentration of the drugs, but the rapidity of the swelling is closely concentration-dependent. Swelling can be induced even with gold salts if *p*-chloromercuribenzoate (*p*-CMB) or Mersalyl is present in the medium, and the rate of swelling initiated by the other NSAID is considerably enhanced. Iodoacetamide and *N*-ethylmaleimide could not replace *p*-CMB or Mersalyl in this respect. This difference is probably related to the fact that when the first two thiol inhibitors bind to the mitochondrial membrane, they increase the anionic charges on this membrane. These data also show the importance of thiol groups for the NSAID-induced swelling. This swelling is pseudo-energized (i.e. appears without any substrate), which suggests that the phenomenon is related only to the physico-chemical changes induced in the membrane by the drugs. This phenomenon is critically affected by the ionic composition of the medium, and a monovalent cationic as well as an anionic selectivity has been found for it. This selectivity can be explained by way of electrostatic forces and gives information on the structure of the membrane, mainly the possible presence of highly polarizable groups. These results suggest that the overall effect of NSAID on mitochondrial metabolism might be related to their activities on ion transport across the mitochondrial membrane.

THE UNCOUPLING activity of the non-steroidal anti-inflammatory drugs (NSAID) has been known for a long time,[1,2] and the role of this phenomenon has been discussed by many authors[3,4] in seeking to explain the anti-inflammatory activity of these compounds. Adams and Cobb[5] were the first to suggest that this uncoupling activity is one of the most important biochemical properties of these drugs.

It is known that the normal respiration of isolated mitochondria in suspension is associated with low amplitude swelling–shrinking cycles.[6] On the other hand, many investigators have studied the high amplitude changes in volume induced in isolated mitochondria by many media or agents. These changes often are only partially reversible. Raaflaub[7,8] studied these changes by measuring the turbidity of mitochondrial suspensions as a function of light scattering. He first suggested the existence of at least two types of high amplitude mitochondrial swelling: a passive one, in

which mitochondria behave like simple osmometers (e.g. in mannitol suspension), and an active change requiring respiration or the presence of high energy compounds. More recently a pseudo-energized[9] type was described, in which swelling was promoted by inducing, with various compounds, a physico-chemical change in the membrane. Tapley[10] showed that agents capable of uncoupling phosphorylation also had specific effects on swelling, some initiating the swelling, others inhibiting it.

Energized or pseudo-energized swelling of mitochondria is greatly stimulated by swelling agents. Inorganic phosphates, calcium, cadmium, ferrous ions, thyroxine and thyroactive compounds, free fatty acids, reduced and oxidized glutathione, insulin, oxytocin, vasopressin and somatotrophin are examples of various kinds of swelling agents.[11] Swelling induced by calcium or free fatty acids has been related to their uncoupling activity.[12] The swelling activity of thyroxine has been related by several authors to the physiological[13] activity of this hormone and to some possible pathological situations in the thyroid gland.[14] The swelling activity of oxytocin, vasopressin and insulin hormones has been related to the presence of a disulfide linkage in these molecules,[15,16] but some authors ascribe the effects to calcium and zinc contamination.[17,18] The effects of ferrous ion and glutathione are probably due to the induction of lipid peroxidation.[19,20] More recently, some new antibiotics, reviewed by Lardy *et al.*[21,22] were found to alter the mitochondrial permeability to different ions with a specific ionic selectivity for each of them: valinomycin,[23] gramicidins,[24] thyrocidins,[24] monazomycin,[25] the macrotetralide actins[26] and alamethicin.[27] They are also able to initate considerable mitochondrial swelling in appropriate media.

To correlate these findings, Blondin[9] *et al.* after Azzi and Azzone,[28] introduced the concept of pseudo-energized swelling. They demonstrated that the swelling induced by these antibiotics and also by cadmium, calcium and zinc (but only after an energy-requiring translocation into the mitochondrion has taken place for the two latter divalent cations) does not require the presence of any substrate in the mitochondrial suspension, if the ionic composition (cationic as well as anionic) of the medium has some specific characteristics. He defined the pseudo-energized swelling as the result of an ion-induced modification of the mitochondrial membrane leading to the accumulation of additional water inside the rearranged membrane (especially the cristae). Most of the swelling agents act on this system as carriers, bringing the ions to the site where they can induce these membrane changes. This concept has been criticized recently. The type of membrane rearrangement has been abandoned, but the fact that a physicochemical change in membrane properties is the cause of the mitochondrial swelling is nevertheless widely accepted.

MATERIALS AND METHODS

Preparation of mitochondria. Livers from Wistar albino rats of about 200 g (fasted 24 hr before the experiment, killed by cervical disruption and then bled) were chilled immediately after removal by immersion in sucrose, 0·25 M, with 2 mM EDTA (ethylene diamine tetracetate). The liver was then cut with scissors into pieces of approximately 0·5 cm in thickness and weighed. After four thorough washes with 0·25 M sucrose + 2 mM EDTA, the pieces of liver were divided into two equal parts and transferred into two Potter–Elvehjem homogenizers with Teflon pestles containing 30 ml sucrose, 0·25 M, + EDTA, 2 mM. The tissue was homogenized for 1 min with a rotating speed of about 720 rev/min and the temperature was maintained

around 0° by working in the cold room with an ice container. The homogenate was filtered through gauze and then centrifuged at 900 *g* for 5 min (2700 rev/min) in the rotor SS-34 of the Sorvall RC-2B refrigerated centrifuge. After the first centrifugation, the supernatant was centrifuged again at 4500 *g* for 10 min (6250 rev/min) in the same rotor. The mitochondrial pellet, after discarding the fluffy layer, was gently resuspended by hand homogenization with a Teflon pestle in 10 ml of cold 0·25 M sucrose + 2 mM EDTA and centrifuged for 10 min in the same rotor at 12,500 *g* (10,100 rev/min). The final mitochondrial pellet was rinsed with cold 0·25 M sucrose + 2 mM EDTA and then resuspended in a volume of 2 ml of the same solution as a stock suspension of mitochondria containing about 45 mg protein/ml. Mitochondrial protein was estimated by the Folin method.[29] All the solutions for mitochondrial preparations were at pH 7·4.

Pharmacological studies. Suspensions of mitochondria were made in 3 ml of 0·15 M chloride solutions of the five alkali cations (Li^+, Na^+, K^+, Rb^+, Cs^+) and in the four halide salts of potassium (F^-, Cl^-, Br^-, I^-) buffered at pH 7·4 with 30 mM *N*-Tris (hydroxymethyl) methyl 2-amino ethane sulfonic acid (TES), a zwitterionic buffer which does not cause mitochondrial swelling, as indicated by light scattering measurements (see Fig. 1). The mitochondrial protein concentration was 150 μg/ml. Reduction in light scattering was measured with a DU-2 Beckmann spectrophotometer connected to a Gilford recorder, at 520 nm, using rectangular glass cuvettes of 10 mm light path. The experiments were initiated by adding the drugs dissolved in ethanol or dimethylsulfoxide (DMSO). The drugs were added in small amounts (max., 50 μl) of stock solution by way of a small plastic spoon with little holes. This amount of DMSO or ethanol does not affect the swelling *per se* (see Fig. 1). The suspension of mitochondria was allowed to stabilize for 4 min before adding the drugs. The results were continuously recorded for 10 min from 5 sec after the addition of the drug, at least five times for each type of incubation.

As mentioned in the Results, most of our experiments were conducted without any added substrates in the medium, but in some cases we did add to the medium a respiratory substrate, sodium succinate, 5 mM, + rotenone, 12 μg, to avoid the consumption of any other endogenous substrate. This combination of succinate and rotenone did not affect *per se* the mitochondrial swelling (see Fig. 1).

Reagents. The NSAID were generous gifts from different drug companies: Biorex Labs (London); Geigy; Parke Davis & Co.; Imperial Chemical Industries (G.B.); Merck Sharp & Dohme; Upsa (Agens, France); Schering; Continental Pharma (Brussels, Belgium); Merck & Company; Seresci (Brussels, Belgium); Boots (Nottingham, England). Valinomycin and iodoacetamide were obtained from Calbiochem (Los Angeles, Calif.); rotenone and *N*-ethylmaleimide were obtained from Aldrich Chemical Co. (Milwaukee, Wisc.); the sodium salts of Mersalyl and *p*-chloromercuribenzoate were products of Sigma Chemical Co. (St. Louis, Mo.). *N*-Tris (hydroxymethyl) methyl 2-amino ethane sulfonic acid and the other zwitterionic buffers were Schwarz Mann products (Van Nuys, Calif.). The alkali chlorides and the halide salts of potassium were of the purest grade commercially available.

RESULTS

Effect of NSAID on mitochondrial swelling. Twenty-three various NSAID were tested on rat liver mitochondria (150 μg). At various concentrations (Table 1), each

TABLE 1. TWENTY-THREE NON-STEROIDAL ANTI-INFLAMMATORY DRUGS TESTED ON RAT LIVER MITOCHONDRIA

Four Arylalkanoic acids
1. (*p*-Isobutylphenyl) acetic acid (ibufenac), 0·4 mM
2. 2-(4-Isobutylphenyl) propionic acid (ibuprofen), 0·4 mM
3. Indomethacin, 0·4 mM
4. Fenclozic acid (Myalex), 2·5 mM

Five Anthranilic acid derivatives (fenamates)
5. Flufenamic acid, 0·15 mM
6. Mefenamic acid, 0·6 mM
7. Meclofenamic acid, 0·3 mM
8. 2-(2-Methyl-3-chloro anilino) nicotinic acid (Clonixin), 0·3 mM
9. Niflumic acid (Nifluril; Inflaryl), 0·3 mM

Five Pyrazolone derivatives
10. Phenylbutazone, 0·3 mM
11. Piperazine salt of phenylbutazone (pyrazinobutazone), 0·3 mM
12. Trimethazone, 0·3 mM
13. Oxyphenbutazone (Tanderil), 0·3 mM
14. Sulfinpyrazone (Anturan), 3·5 mM

One Phenoxyacetic derivative
15. Clamidoxic acid, 0·8 mM

Two Terpenoids
16. 18β-Glycyrrhetinic acid, 0·3 mM
17. Hemisuccinate of 18β-glycyrrhetinic acid (carbenoxolone; Biogastrone), 0·3 mM

Two Gold salts
18. Aurothiomalate (Myochrysin), 1·6 mM
19. Aurothiosulfate (Sanochrysin), 1·6 mM

Four Salicylates
20. Sodium salicylate, 3 mM
21. Aspirin, 3 mM
22. Salicylamide, 3 mM
23. Hexahydrosalicylic acid, 3 mM

of them induced swelling, except the gold salts and the salicylate derivatives, in 3 ml of 0·15 M KCl buffered medium (TES, 30 mM, pH 7·4) as recorded by decrease in light scattering measured at 520 nm (Fig. 1). For the two gold salts and four salicylate derivatives, various concentrations were tested and, even with the very high non-pharmacological levels listed in Table 1, no effect on swelling has been observed. No differences in the swelling induced by the other 17 compounds was observed when: (1) a respiratory substrate (sodium succinate, 5 mM) with rotenone (12 μg) was present in the medium, or (2) no substrate was present in the medium. This strongly suggests that the swelling observed was of the pseudo-energized type.

Concentration response in 0·15 mM *KCl buffered with* 30 mM *TES*, pH 7·4. Six of the NSAID were tested to find relations between the concentration and the swelling. As shown in Fig. 2, the velocity of the phenomenon is closely related to the concentrations of drugs present in the medium for flufenamic acid (0·07 to 2·4 mM), indomethacin (0·4 to 3·2 mM), mefenamic acid (0·3 to 3 mM), phenylbutazone (0·3 to 2·4 mM), oxyphenbutazone (0·3 to 2·4 mM) and ibuprofen (0·3 to 2·4 mM).

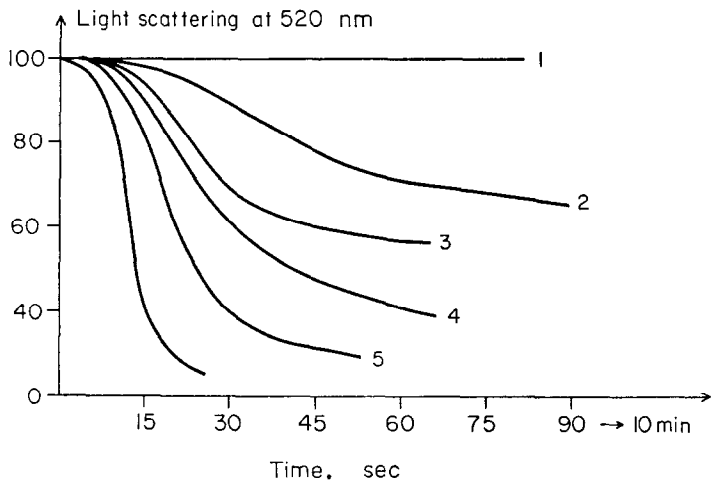

FIG. 1. Some examples of pseudo-energized mitochondrial swelling induced by NSAID. Medium: 3 ml KCl 0·15 M, and TES, 30 mM, pH 7·4. Mitochondria, 150 μg protein. No substrate in the medium. Time 0 = +5 sec. (1) Basic line = no changes in light scattering (with or without TES buffer). A similar line was also recorded when ethanol (50 μl), dimethylsulfoxide (50 μl) or rotenone (12 μg) was added to the medium. The four salicylate derivatives and the two gold salts listed in Table 1 do not affect this basic line. Swelling induced by: (2) flufenamic acid, 0·15 mM; (3) 2-(4-isobutylphenyl) propionic acid, 0·4 mM; (4) indomethacin, 0·4 mM; (5) phenylbutazone, 0·3 mM; (6) niflumic acid, 0·3 mM. Identical curves were recorded when a respiratory substrate (sodium succinate, 5 mM, + rotenone, 12 μg) was present in the medium.

The total amount of swelling seems to be perhaps also related to the concentration. After 10 min, the swelling seems to almost cease and the actual decrease in light scattering is rather less with small concentrations of drugs than with higher concentrations. The recording of swelling for periods longer than half an hour does not

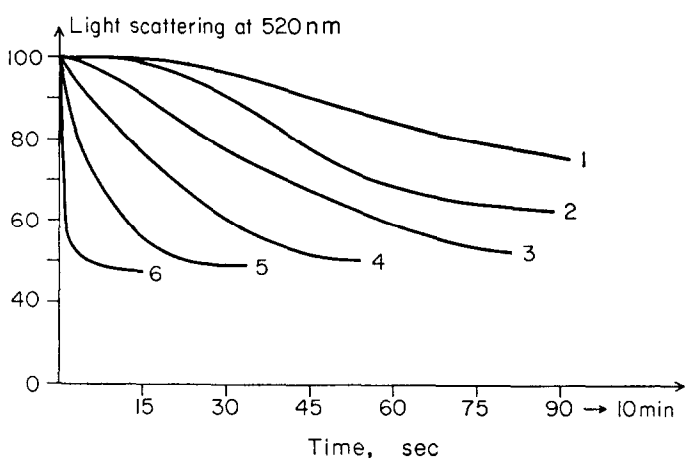

FIG. 2. An example of concentration response in mitochondrial-induced swelling. Medium: 3 ml KCl, 0·15 M, and TES, 30 mM, pH 7·4. Mitochondria, 150 μg protein. No substrate. Time 0 = +5 sec. Flufenamic acid: (1) 0·07 mM, (2) 0·15 mM, (3) 0·3 mM, (4) 0·6 mM, (5) 1 mM, (6) 2 mM.

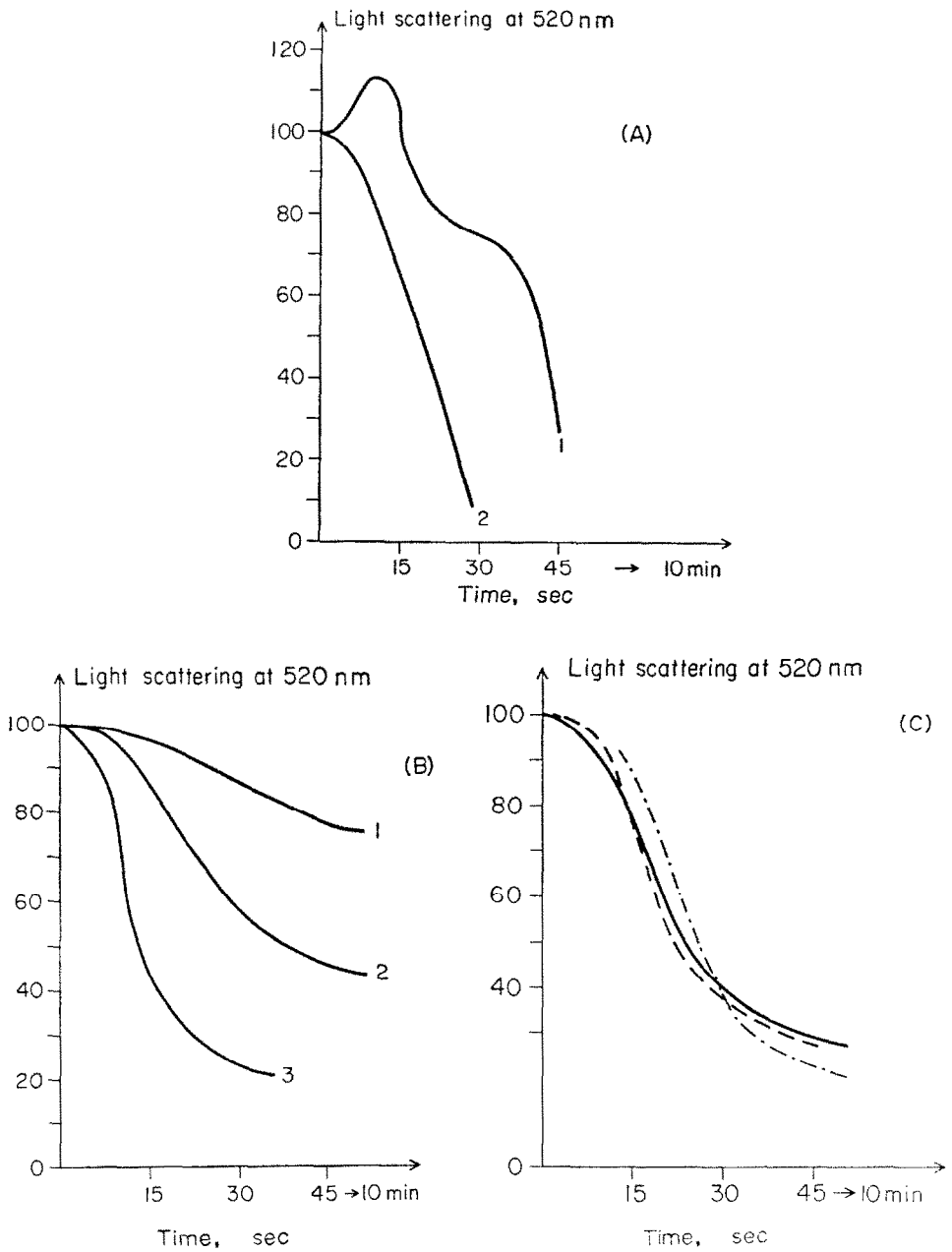

FIG. 3. Effect of pH changes on NSAID-induced swelling. A. Medium 3 ml KCl, not buffered. No substrate. Mitochondria, 150 μg protein. Time 0 = +5 sec. (1) HCl added to bring the pH to 2: shrinking followed by swelling. (2) K_2CO_3 added to bring the pH to 10 = swelling. B. (1) Indomethacin, 0·4 mM, pH 5·6; (2) indomethacin, 0·4 mM, pH 7·4; (3) indomethacin, 0·4 mM, pH 8·4. Medium: KCl, 0·15 M, buffered with various zwitterionic chloride buffers. Mitochondria, 150 μg protein. No substrate. Time 0 = +5 sec. C. Indomethacin, 1 mM, pH 5·6; – – –; indomethacin, 0·4 mM, pH 7·4, ——; indomethacin, 0·15 mM, pH 8·4, –·–·–. Medium: KCl, 0·15 M, buffered with various zwitterionic chloride buffers. Mitochondria, 150 μg protein. No substrate. Time 0 = +5 sec. In B and C, there is no spontaneous change in light scattering due to pH itself before adding the drug.

show any difference than this recorded after 10 min, and the results for such a long period of incubation are probably meaningless.

Influence of pH. pH by itself can influence mitochondrial swelling. At pH 2 induced by HCl, shrinking of mitochondria followed by rapid lytic swelling is observed (Fig. 3A). At pH 10 (or more) induced by K_2CO_3, spontaneous lytic swelling is observed (Fig. 3A). However, in the range of pH between 5·6 and 8·4 adjusted by various zwitterionic buffers (with no effect by themselves on swelling), mitochondria appear to be stable with no spontaneous swelling.

The pH effect has been studied for flufenamic acid, ibuprofen, phenylbutazone and indomethacin. At the same concentration (Table 1), the swelling increases with a change from low to high pH (Fig. 3B). This pH dependence can also be detected by the fact that it is necessary to increase the NSAID concentration when decreasing the pH of the medium to record the same swelling (Fig. 3C).

Influence of ionic composition of the medium on swelling. Ionic composition has a strong influence on swelling induced by the NSAID, as shown for the following drugs: indomethacin (0·4 mM), phenylbutazone (0·3 mM), flufenamic acid (0·3 mM) and 2-(4-isobutylphenyl) propionic acid (0·4 mM).

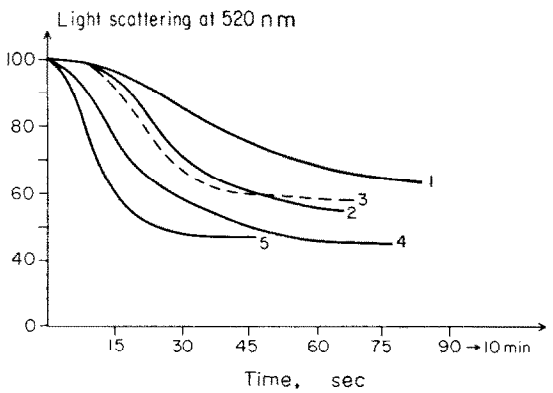

FIG. 4. Influence of alkali cation on NSAID-induced mitochondrial swelling. Mitochondria, 150 μg protein. No substrate. Time 0 = +5 sec. In each case, buffer was TES, 30 mM, pH 7·4. 2-(4-Isobutylphenyl) propionic acid, 0·4 mM. (1) NaCl, 0·15 M; (2) KCl, 0·15 M; (3) LiCl, 0·15 M; (4) CsCl, 0·15 M; (5) RbCl, 0·15 M. Ion selectivity $Rb^+ > Cs^+ > Li^+ = K^+ > Na^+$ (for ibuprofen).

The effect of alkali cations on swelling was studied by inducing mitochondrial swelling with the same concentration of drug in LiCl, NaCl, KCl, RbCl or CsCl, all at 0·15 M, buffered in each case by 30 mM TES, pH 7·4. The results, shown for one drug, demonstrate a similar qualitative ionic selectivity for the four different drugs; some quantitative differences were observed, however, for each of them. These differences do not affect the ionic sequence observed, but do suggest that each of these drugs has its own alkali selectivity even if they show a similar general pattern of selectivity, $Rb^+ \geqslant Cs^+ > Li^+ \geqslant K^+ > Na^+$, which could be due to an expansion of the membrane, making some groups more available on it (Fig. 4).

A similar analysis for the same four drugs was performed with the halide anions. The swelling was induced in KI, KBr, KCl or KF, all at 0·15 M, buffered in each case by 30 mM TES, pH 7·4 (Fig. 5). A spontaneous swelling was observed in the KI

medium, as reported by others in previous studies. But this spontaneous iodide-induced swelling was tremendously increased when one of the four drugs was added to the medium. With the three other halide anions, no spontaneous swelling was recorded; but here too, as for the cation series, a similar pattern of selectivity, probably due to the availability of new membrane groups, was found for the four drugs studied, with quantitative variations also from one drug to the other. This halide sequence is $I^- > Br^- > Cl^- > F^-$.

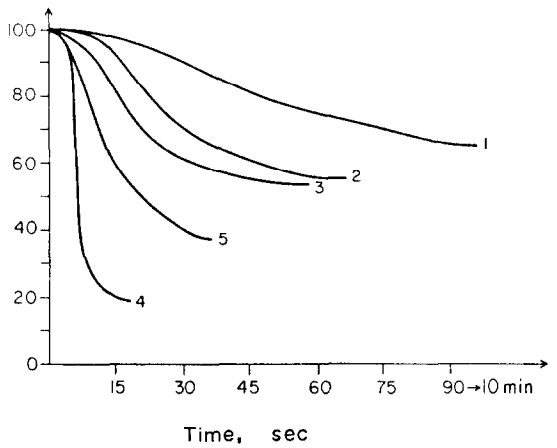

FIG. 5. Effect of halide anions on mitochondrial swelling. Mitochondria, 150 μg protein. No substrate. Time 0 = +5 sec. In each case, buffer was TES, 30 mM, pH 7·4. 2-(4-Isobutylphenyl) propionic acid, 0·4 mM. (1) KF, 0·15 M; (2) KCl, 0·15 M; (3) KBr, 0·15 M; (4) KI, 0·15 M; (5) spontaneous swelling of mitochondria in KI, 0·15 M.

Irreversibility of the swelling induced by NSAID. Energized swelling of mitochondria is usually reversed by adding to the medium at the end of the swelling phase ATP and manganese or magnesium ions, or both. We have tried to reverse the drug-induced swelling and to induce a shrinking by ATP with Mn^{2+} or Mg^{2+}, or both, after we had induced mitochondrial swelling with each of the 17 NSAID (Fig. 1). In no case were we able to reverse this drug action and induce mitochondrial shrinking. This finding provides another argument for considering that this drug-related swelling is pseudo-energized.

Effect of some thiol inhibitors on NSAID-induced mitochondrial swelling. Certain thiol inhibitors like salyrgan (Mersalyl) are able to prevent or inhibit the stimulation of mitochondrial state 4 respiration by NSAID.[30,31] Mersalyl at 0·035 mM and *p*-CMB (which has the same effect on mitochondrial respiration) at 0·05 mM seem to induce very little swelling by themselves (Fig. 6A), but they considerably enhanced the magnitude of swelling if added to the medium before or after each of the NSAID studied (Fig. 6B). Furthermore, they promoted swelling in the presence of gold salts, 1·6 mM (Fig. 6C), which are otherwise incapable of inducing swelling at these pharmacological levels. On the other hand, *N*-ethylmaleimide (0·05 mM) and iodoacetamide (0·05 mM), two other thiol inhibitors, had no effect at all on swelling.

These differences in the action of various thiol inhibitors are discussed later; but the effect of Mersalyl and *p*-CMB on the NSAID-induced swelling suggests that some sulphydryl groups may be involved in this phenomenon.

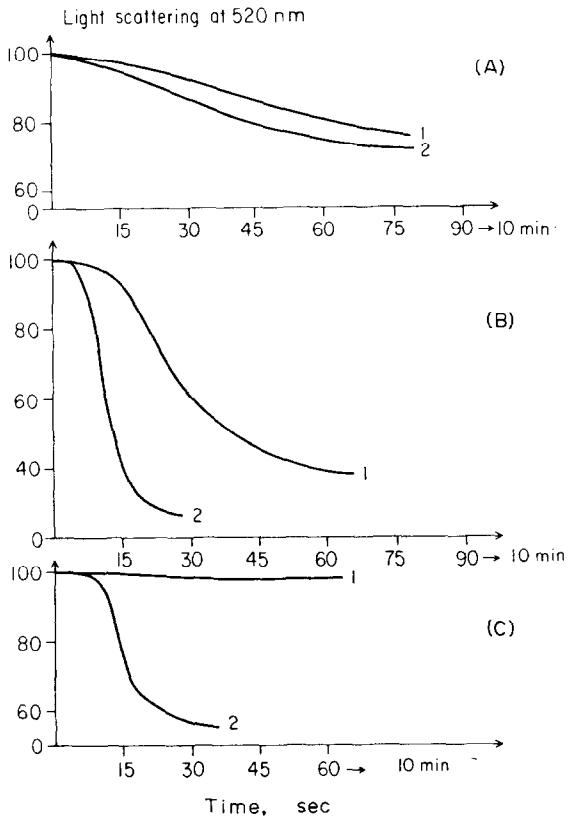

FIG. 6. Effect of thiol inhibitors on mitochondrial swelling. Mitochondria, 150 μg protein. No substrate. Time 0 = +5 sec. Buffer was TES, 30 mM, pH 7·4. KCl, 0·15 M, 3 ml. A. (1) Mersalyl, 0·035 mM; (2) *p*-chloromercuribenzoate, 0·05 mM. B. (1) Indomethacin, 0·4 mM; (2) Mersalyl, 0·035 mM, + indomethacin, 0·4 mM. C. (1) Aurothiomalate, 1·6 mM; (2) Mersalyl, 0·035 mM, + aurothiomalate, 1·6 mM.

Swelling of kidney mitochondria. All the previous studies were carried out using rat liver mitochondria. We tried to repeat all these experiments on kidney mitochondria. These were prepared by exactly the same method as liver mitochondria, except that we used kidney cortex (after having carefully removed the capsule and the medulla). The results were similar in each case to those found with liver mitochondria, except that the actual swelling recorded was rather less in magnitude for the same amount of mitochondrial protein with the same concentration of drug (Fig. 7), all other experimental conditions being similar.

DISCUSSION

The NSAID that we have tested in the present studies are able to induce mitochondrial swelling at concentrations that may be pharmacologically attained. They uncouple oxidative phosphorylation[3,31] at nearly all of these concentrations. Two important exceptions seem to be salicylate and its analogues,[32] and the gold salts,[33] which are known to be good uncoupling agents but failed to induce mitochondrial

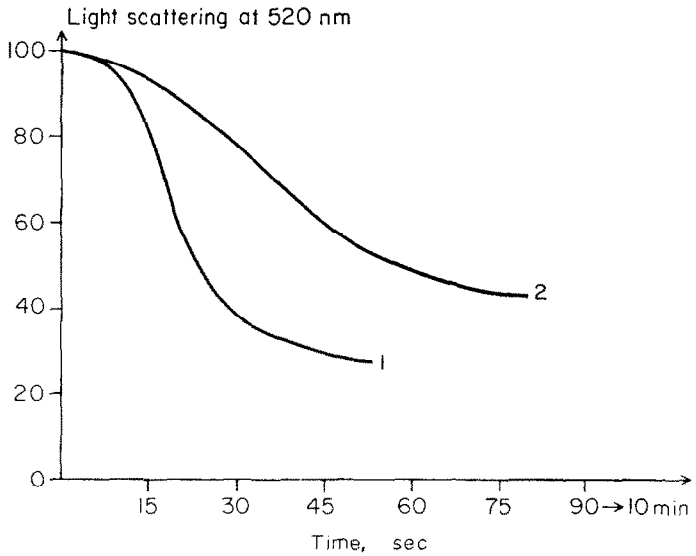

FIG. 7. Difference between NSAID-induced swelling in liver and kidney mitochondria. KCl, 0·15 M, 3 ml and 30 mM TES, pH 7·4. No substrate. Time 0 = +5 sec. Drug = phenylbutazone, 0·3 mM. (1) Liver mitochondria, 150 μg protein; (2) kidney mitochondria, 150 μg protein.

swelling in our experiments. This "test" can thus establish some differences in the effects, and presumably mechanisms of action, on mitochondria of these NSAID.

On the other hand, the uncoupling action as studied by previous authors,[3,31] required various respiratory substrates such as succinate or α-ketoglutarate.

The presence or absence of these substrates in the medium does not affect the swelling induced by NSAID, and even the presence of rotenone, which inhibits all the endogenous substrates except succinate,[34] has no apparent effect on the induced swelling.

We are therefore considering swelling of the pseudo-energized type[9], almost certainly related to important physicochemical changes induced in the mitochondrial membrane by the drugs. All attempts to reverse the swelling by adding ATP (with or without Mg^{2+} or Mn^{2+}, or both) were unsuccessful and the phenomenon seems thus to be irreversible.[35]

This phenomenon appears to be concentration-dependent, at least as regards the rapidity of the swelling, and perhaps also the magnitude of the swelling, as shown with at least six drugs.

The swelling is related to various constituents of the incubation medium. The concentrations of protons and of hydroxyl anions seem to be very important, as shown by a marked pH dependence of the phenomenon. Considerable swelling in an alkaline medium with very little swelling in an acid medium was obtained for the same drug concentration, which may be related to a possible role played by the NSAID in the proton translocation across the membrane. These findings are in accord with the chemiosmotic hypothesis of Mitchell[36] for coupling activity, with some studies of the H^+ transport across artificial bilayers,[37,38] and with our own results on the effects of NSAID on intramitochondrial pH as studied by bromothymol blue-labelled mitochondria.[39]

Also of interest is the fact that ions other than protons or hydroxyl anions can effect NSAID-induced swelling. By varying the alkali cations of the incubation medium with a constant anion (chloride), a preferential cationic sequence has been found which (at least qualitatively) seems to be the same for the four drugs studied (flufenamic acid, phenylbutazone, indomethacin, ibuprofen): $Rb^+ \geqslant Cs^+ > Li^+ \geqslant K^+ > Na^+$.

By studying the selectivity of artificial phospholipid bilayers and of glasses used for ion specific electrodes, Eisenman[40,41] showed that only 11 sequences, of 120 possible (5!), can exist if we make the assumption that only the following mechanisms are involved: (1) equilibrium cation specificity depends upon the free energy difference between ion/membrane and ion/water interactions; (2) free energies of interactions involving the alkali cations depend largely upon electrostatic forces; (3) in most of the systems studied, the electrostatic forces are Coulombic forces (i.e. forces between two nonpolarizable point-charges varying as the inverse square of the distance, d^{-2}).

When we consider a membrane with negatively charged sites, the cation preferred will be the cation which experiences the greatest decrease in free energy when this membrane site becomes its nearest neighbour rather than water.

For two different cations (a and b) the affinity will be regulated by the equation $(\Delta Fa_{site} - \Delta Fb_{site})^{(1)} - (\Delta Fa_{water} - \Delta Fb_{water})^{(2)}$. If the site has a very high electrical field, (2) can be neglected; if the site has a very low electrical field, (1) can be neglected. In the first case, the affinity will decrease with increasing ionic radius of the ions: $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$, which is the sequence XI of Eisenman.[41] In the second case, the affinity will be related to the free energy of hydration of the ions and will decrease in the opposite way: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$, which is the sequence I described by Eisenman.[41] The other sequences are located between these two, and depend on the importance of the membrane charges and their electrical field.

We can relate our sequence to the sequence II of Eisenman:[41] $Rb^+ > Cs^+ > K^+ > Na^+ > Li^+$. This indicates that the presence of negative charges in the membrane does not much affect the activity of NSAID in inducing mitochondrial swelling, which must be thus associated to anionic sites with a weak electrical field.

But in our sequence, as in many other biological and nonbiological systems,[42,43] there is a displacement of Li^+, the smallest ion, which is due to the fact that the assumption of Eisenman[41] is not completely true. We must suppose, as explained by Eisenman himself and others,[44,45] the presence of highly polarizable sites (the field of which varies greatly depending upon the site's immediate molecular environment), inside the membrane for which the induced forces are non-Coulombic and vary with the inverse of d^5, instead of the inverse of the square of the distance (which is still more important than some analogue forces existing in water and varying with d^{-3} and d^{-4}). The difference for a small ion such as Li^+ minus the attraction for a large ion such as Cs^+ will be therefore much more marked for these polarizable sites and may explain the displacement of Li^+ in the sequence.

The fact that Li^+ shifts two places in our series, instead of one, suggests that many polarizable sites of the membrane, such as carboxyl groups, are involved in the mitochondrial swelling induced by NSAID.

The halide series we found to be: $I^- > Br^- > Cl^- > F^-$, which is related to the free solution mobility of these anions, and suggests (on the basis of a theory similar to that built for alkali cations, only 7 sequences described out of $4! = 24$) that

cationic sites with a weak electrical field must be associated with the phenomenon of NSAID-induced mitochondrial swelling as well as anionic sites with weak electrical field.

We have recorded a spontaneous swelling in potassium iodide, 0·15 M, and I^- in smaller concentrations can induce swelling by itself in other media.[46] This swelling too seems to be of the pseudo-energized type and the NSAID tremendously enhance this spontaneous I^- swelling. The significance of this I^- effect is still not well understood, but could perhaps be related to some effects observed with iodide on artificial bilayers.[47,48]

In a previous work,[31] we have shown that Mersalyl can inhibit the uncoupling activity induced by NSAID, which implies a role for membrane thiol groups in this action of the drugs on mitochondrial respiration.

It seems that SH groups are also involved in the NSAID-induced swelling, but instead of inhibiting the swelling, Mersalyl and also *p*-CMB (which has been described to have by itself a small swelling activity,[10,49,50] which we also found for Mersalyl in our own experiments) considerably enhance the effects of NSAID on mitochondrial swelling. On the other hand, *N*-ethylmaleimide (NEM) and iodoacetamide, two other thiol inhibitors, have no influence on this swelling. One explanation can be that Mersalyl and *p*-CMB, by reacting with thiol groups of the membrane, increase the negative charge of this membrane while NEM and iodoacetamide do not modify in any manner the charges of the membrane, as shown by Lynn and Brown[51] with large anion-induced swelling. This swelling-promoting effect can even be seen with Mersalyl in combination with drugs like some gold salts, which alone never induce swelling at pharmacological concentrations.

Most of the effects that we have seen with NSAID on rat liver mitochondria have been reproduced on rat kidney mitochondria. The only difference in our experiments was that for the same concentration and the same amount of mitochondria (as measured by the Folin protein determination[29]) the magnitude of the swelling is smaller with the kidney mitochondria. These data confirm some other results comparing kidney mitochondria and liver mitochondria[52] in different swelling experimental conditions.

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