Biochemical Pharmacology, Vol. 24, pp. 267–275. Pergamon Press, 1975. Printed in Great Britain.

INTERACTIONS BETWEEN NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND BIOLOGICAL MEMBRANES—III

EFFECT OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON BOUND MITOCHONDRIAL BROMOTHYMOL BLUE AND POSSIBLE INTRAMITOCHONDRIAL pH VARIATIONS INDUCED BY THESE DRUGS

JEAN-PIERRE FAMAEY,* MICHAEL W. WHITEHOUSE† and WILLIAM C. DICK‡
UCLA School of Medicine, Department of Medicine, Los Angeles, Calif. 90024, U.S.A.

(*Received* 23 *October* 1972; *accepted* 31 *May* 1974)

Abstract—Ten nonsteroidal anti-inflammatory drugs (NSAID), which are known to affect mitochondrial membrane properties, were examined for their ability to induce variations of absorbance in isolated mitochondria preloaded with bromothymol blue (BTB). By studying optical density curves at 618 nm and at the isosbestic point of BTB, an index, ICC_{BTB}, was defined, which reflects BTB color changes. The NSAID, and some alcohols related to them, increase this index. This change is related to the drug concentration as well as to the ionic composition of the incubation medium. Such BTB color variations in mitochondria could be due to alkalinization of the matrix space which occurs simultaneously with reorientation of the BTB molecules at their mitochondrial location. At concentrations which affect many other functions in various biological membranes, NSAID may alter the mitochondrial membrane permeability and promote the penetration of alkali cations into the mitochondria. The ionic movements cause the interior of the mitochondria to become alkaline, through a parallel excretion of protons or a concomitant uptake of hydroxyl ions, or both, while a simultaneous water influx causes mitochondrial swelling.

Nonsteroidal anti-inflammatory drugs (NSAID) have been shown to uncouple mitochondrial oxidative phosphorylation [1–3]. The properties of the uncoupling activity of valinomycin, a potent increaser of mitochondrial cation permeability, are very similar to those of NSAID uncoupling effects [4, 5]. Most of the NSAID, as well as valinomycin, induce mitochondrial swelling *in vitro* [6, 7]. Some of the NSAID at higher concentrations are even able to induce cell swelling *in vitro* [8]. The relations between uncoupling and swelling effects have been discussed by several authors [9–11]. Some uncoupling agents are also swelling agents, which is the case for NSAID [7], but others have no swelling effects or even inhibit the swelling induced by other compounds [9, 12, 13].

It has been shown that swelling agents, with or without uncoupling effects, induce pH changes within mitochondrial spaces [14]. Techniques for measuring these pH changes have been described. One method involves measurements of H^+ and OH^- in the extramitochondrial and mitochondrial phases by titrimetric methods [15, 16]. Using 5,5′-dimethyl 2,4-oxazolidine dione (DMO) distribution measurements, another method has been described for calculating intracellular pH in intact muscle [17]. The technique of DMO measurements has been adapted to isolated mitochondria, but this technique, though precise, is very complicated [18]. It has not yet been possible to introduce a minute electrode into mitochondria to measure their internal pH, although this has been achieved for muscle fibers [19–21] and even for smaller cells like those of the alga, *Nitella* [22].

A more simple and rapid technique is the use of pH chromophoric indicators, such as bromothymol blue, 3,3′-dibromothymolsulfonphthalein (BTB), with a pH range from 6·0 to 7·6 and a p*K*a = 7·0, which "label" the mitochondria with no described interferences with normal mitochondria metabolism [23]. Bromocresol purple, 5,5′-dibromo-*O*-cresolsulfonphthalein (BCP), with a pH range from 5·2 to 6·8 and a p*K*a = 6·3, was used by the same authors [23] as a good chromophoric indicator of changes in extramitochondrial pH, and they confirmed its suitability for this purpose by direct pH measurements using glass electrodes. There is a good correlation between experimental findings obtained with the BTB technique and those obtained

* Present address: Laboratory of Pharmacology, Rheumatological Unit, School of Medicine, Free University of Brussels, Brussels, Belgium.

† Present address: Department of Experimental Pathology, John Curtin School of Medical Research, Canberra, A.C.T., Australia.

‡ Present address: Center for Rheumatic Disease, Baird Street, Glasgow, Scotland.

with the DMO technique when the intramitochondrial pH changes are induced by calcium [24]. This divalent cation may induce an intramitochondrial alkalinization.

Recently, criticisms were made of the first interpretations of the BTB method. It appears that only a part of the bound BTB is able to react to pH changes within the mitochondria, and this part varies from one metabolic state to another, due to changes in spatial orientation of the chromophoric sites of the BTB molecules into the mitochondrial membrane [25, 26]. These changes may be related to the development of different electrical potentials across the membrane for each metabolic state [26]. Even at the same metabolic state, changes of absorbance have been noticed with BTB from one mitochondrial incubation medium to another as a result of similar changes in spatial orientation of the indicator molecule [27, 28].

When color variations can be ascribed to intramitochondrial pH changes, they only reflect the pH changes of the "BTB space" which is probably the inner membrane or the matrix space [27, 29]. The purpose of the present paper is to study the effect of various NSAID on BTB-labeled mitochondria, since NSAID promote an iso-osmotic swelling. This swelling is related to changes in mitochondrial membrane properties induced by these drugs, which have been described to alter many biological and nonbiological membranes [30–36].

The effects of NSAID on changes of absorbance of BTB-labeled mitochondria have been analyzed. The relationships of such changes is BTB repartition and spatial orientation as well as to possible pH changes within the mitochondria have been discussed.

MATERIALS AND METHODS

Preparation of mitochondria. Unlabeled mitochondria were prepared according to the technique described in the first paper of this series [7]. After the final centrifugation at 12,500 *g*, the mitochondrial pellet was resuspended in cold sucrose–EDTA with BTB (4 nmoles/mg protein) and then centrifuged again at 12,500 *g* for 10 min in the same rotor. The final pellet was rinsed with cold sucrose–EDTA and then resuspended in 2 ml of 0·25 M cold sucrose, to yield a stock suspension of mitochondria containing about 45 mg protein/ml.

Mitochondrial protein was estimated by the method of Lowry *et al.* [37]. Kidney mitochondria were prepared essentially in the same manner from kidney cortex, after careful removal of the capsule and medulla. All solutions used to isolate mitochondria were at pH 7·4. The BCP-labeled mitochondria were prepared by the same technique with 9 nmoles/mg protein of BCP in the sucrose–EDTA medium.

Pharmacological studies. Suspensions of mitochondria were made in 3 ml of 0·15 M solutions of various alkali halides buffered at pH 7·4 with 30 mM *N*-Tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), a zwitterion buffer which does not react by itself with BTB and has no effect on liver and kidney mitochondria, as indicated by light scattering measurements [7].

The mitochondrial protein concentration in these suspensions was approximately 150 μg/ml. Light scattering was measured in a DU-2 Beckman spectrophotometer connected to a Gilford recorder. Measurements were done simultaneously at the isosbestic point of BTB (515 nm) and at 618 nm, using rectangular glass cuvettes of 10-mm light path, for normal as well as for BTB-loaded mitochondria.

The NSAID (listed in Table 1) were incubated with normal and BTB-loaded mitochondria from rat liver and kidney. With normal mitochondria, after addition of drug, the decrease in light scattering recorded at 618 nm was smaller than the decrease recorded at 515 nm. The difference between the optical density (O.D.) recorded at these two wavelengths defines a variable *A*:

$$A = (\text{O.D.}^{0}_{618} - \text{O.D.}^{0}_{515}).$$

A is essentially dependent on swelling, as demonstrated by several authors who found a good correlation between decreases in light scattering and swelling of isolated mitochondria [38–40]. The difference between the O.D. obtained at 618 nm and at the isosbestic point, after addition of drug, with BTB-loaded mitochondria, gave us a variable *B*:

$$B = (\text{O.D.}^{\text{BTB}}_{618} - \text{O.D.}^{\text{BTB}}_{515})$$

which depends not only on mitochondrial swelling, but also on changes in absorbance at 618 nm due to BTB color variations. By subtracting *A* from *B*, we define an index (see Fig. 1):

$$B - A = \text{ICC}_{\text{BTB}} \text{ (Induced Color Changes of BTB)}$$

which is only a function of changes in BTB color. A similar index, ICC_{BCP}, was calculated for BCP by plotting the difference between readings made at 583 nm and at the isosbestic point.

The first authors who described this technique used 700 nm as reference [23]. More recently, Shane and Routh [41] have suggested the use of the isosbestic point to provide a reference point for spectro-photometric measurements. We selected this reference point because, at this wavelength (515 nm for BTB), the absorbance related to BTB color is independent of pH variation, and the only recorded changes in optical density after addition of drug are due to mitochondrial swelling. The fact that changes in light scattering due to mitochondrial swelling are better observed in the range of 510–530 nm than at any other wavelength was another reason for such a choice [10, 33, 39, 40].

The experiments were initiated by adding the drugs dissolved in ethanol or dimethylsulfoxide (DMSO). The drugs were added in small amounts of stock solution (maximum 50 μl) and the drop was rapidly mixed with the incubation medium by using a small plastic spoon with little holes. This amount of ethanol or

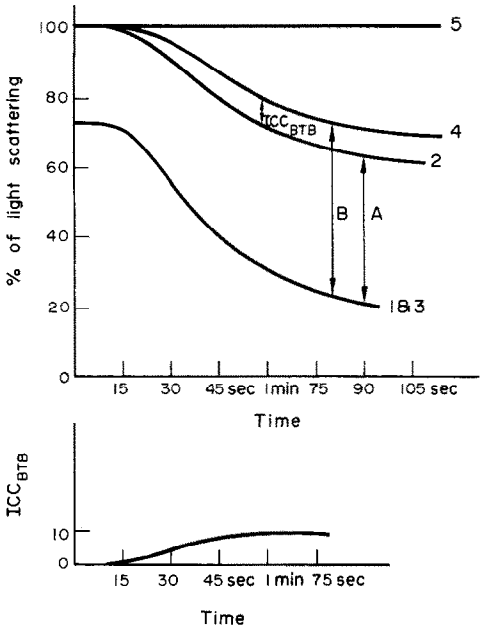

Fig. 1. Determination of ICC_{BTB} index. Liver mitochondria, 150 μg protein; no substrate; time 0 = +5 sec. Medium = 3 ml KCl, 0·15 M, with 30 mM TES, pH 7·4 in the presence of Ibufenac 0·4 mM. Upper panel: swelling of mitochondria expressed as light scattering. 1 = Unloaded mitochondria at the isosbestic point; 2 = unloaded mitochondria at 618 nm; 3 = BTB-loaded mitochondria at the isosbestic point; 4 = BTB-loaded mitochondria at 618 nm; and 5 = unloaded or BTB-loaded mitochondria at 618 nm in the presence of DMSO or ethanol (50 μl) alone. Lower panel: ICC_{BTB} (induced color changes of BTB) is expressed as differential percentage of light scattering with the same scale units used for swelling measurements.

DMSO did not cause any mitochondrial swelling or BTB color changes *per se* (Fig. 1).

To permit the decrease of absorbance in state 4 BTB-labeled mitochondria, resulting from the transfer from a sucrose medium to an isotonic KCl medium [28], as well as to avoid any basic swelling, the mitochondria were allowed to stabilize in suspension for 4 min, after which time no more variations, at any wavelength, were recorded. The results were continuously recorded for 10 min, from 5 sec after addition of the drug. Since the NSAID all induced pseudo-energized mitochondrial swelling [7] (i.e. appearing: (a) in the absence of any osmotic pressure changes in the external medium, (b) without any respiratory substrate in the incubation medium, and (c) thus only related to mitochondrial membrane permeability changes to soluble cations and anions which become able to penetrate into the mitochondria, increasing the internal osmotic pressure and causing entry of water [42, 43]), no respiratory substrates were added to the media. The respiratory states of the mitochondria were defined according to Chance and Williams [44].

Table 1. Nonsteroidal anti-inflammatory drugs (NSAID) tested

NSAID	Concn (mM)
Arylalkanoic acids	
Ibufenac [(*p*-isobutylphenyl) acetic acid]	0·4
Ibuprofen [2-(4-isobutylphenyl)propionic acid]	0·4
Indomethacin	0·4
Pyrazolones	
Phenylbutazone	0·3
Its piperazine salt (pyrazinobutazone)	0·3
Oxyphenbutazone (Tanderil®)	0·3
Anthranilic acid derivatives	
Flufenamic acid	0·3
Mefenamic acid	0·3
Niflumic acid	0·3
Clonixic acid (Clonixin®)*	0·3

* 2-(2′-Methyl-3′-chloro aniline) nicotinic acid.

Reagents. NSAID and corresponding alcohols were generous gifts from various drug companies: Boots (Nottingham, England), Geigy, Parke Davis & Co., Upsa (Agens, France), Schering, Seresci (Brussels, Belgium), Merck Sharp & Dhome; bromothymol blue was obtained from Eastman Kodak Co. (Rochester, N.Y.), bromocresol purple from Baker Chemical Co. (Phillipsburg, N.J.), *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid from Schwarz–Mann (Van Nuys, Calif.) and ADP from Calbiochem (Los Angeles, Calif.). All the other chemicals were of the purest grade commercially available.

RESULTS

The NSAID listed in Table 1 were incubated with normal and BTB-loaded mitochondria from rat liver and kidney. The same amount of mitochondria was used with each drug, as determined by measurements

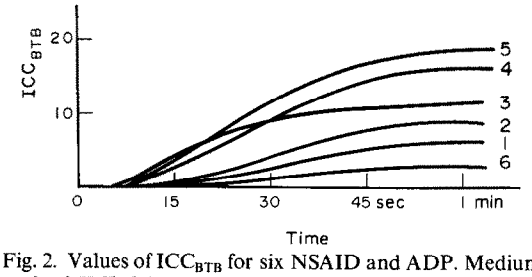

Fig. 2. Values of ICC_{BTB} for six NSAID and ADP. Medium = 3 ml KCl, 0·15 M, with 30 mM TES, pH 7·4, time 0 = +5 sec. Liver mitochondria, 150 μg protein. 1 = Mefenamic acid, 0·3 mM; 2 = ibuprofen, 0·4 mM; 3 = indomethacin, 0·4 mM; 4 = phenylbutazone, 0·3 mM; 5 = niflumic acid, 0·3 mM, or clonixic acid, 0·3 mM (similar curves); and 6 = absence of O_2 in presence of 125 μM ADP. ICC_{BTB} (induced color changes of BTB) is expressed as differential percentage of light scattering with the same scale units used for swelling measurements. (100% is light scattering at time 0 in the absence of drug).

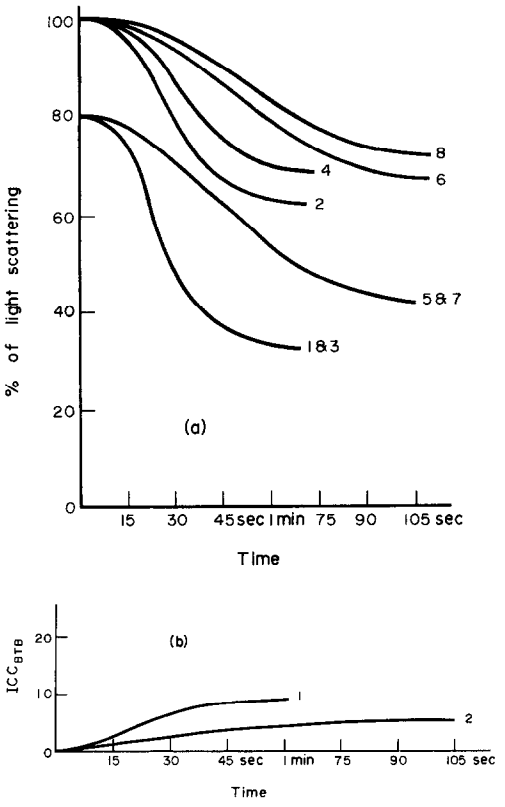

Fig. 3. Difference between rat liver and kidney mitochondria light scattering and ICC_{BTB}. Mitochondria, 150 μg protein; no substrate; time 0 = +5 sec. Medium = 3 ml KCl, 0·15 M, with 30 mM TES, pH 7·4, pyrazinobutazone, 0·3 mM. (a) Swelling of mitochondria expressed as light scattering. Light scattering of unloaded mitochondria (recorded for 10 min): 1 = Isosbestic point; 2 = 618 nm. Light scattering of BTB-loaded liver mitochondria (recorded for 10 min): 3 = isosbestic point; 4 = 618 nm. Light scattering of unloaded kidney mitochondria (recorded for 10 min): 5 = isosbestic point; 6 = 618 nm. Light scattering of BTB-loaded kidney mitochondria (recorded for 10 min): 7 = isosbestic point; and 8 = 618 nm. (b) ICC_{BTB} of rat mitochondria: 1 = liver, 2 = kidney. ICC_{BTB} is expressed as differential percentage of light scattering in the same scale units used for swelling measurements. (100% is light scattering at time 0 in the absence of drug).

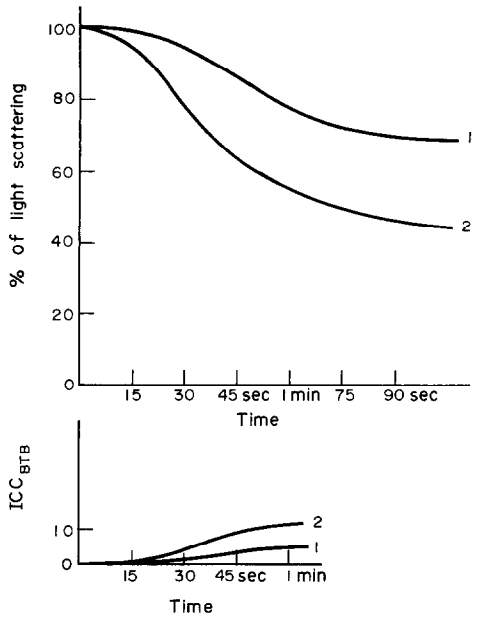

Fig. 4. Difference between anti-inflammatory acid and the corresponding alcohol. Liver mitochondria, 150 μg protein; no substrate; time 0 = +5 sec. Medium = 3 ml KCl, 0·15 M, with 30 mM TES, pH 7·4. 1 = Light scattering (= swelling) at the isosbestic point and ICC_{BTB} induced by flufenamic acid, 0·3 mM (recorded for 10 min). 2 = Light scattering (= swelling) at the isosbestic point and ICC_{BTB} induced by flufenamyl alcohol, 0·1 mM (recorded for 10 min). ICC_{BTB} is expressed as differential percentage of light scattering in the same scale units used for swelling measurements. (100% is light scattering at time 0 in the absence of drug).

of mitochondrial proteins. Changes in light scattering induced by these drugs were recorded at the isosbestic point and at 618 nm. The index ICC_{BTB} versus time was plotted for each (Fig. 2). An increase of the index is due to an increase in the blue phenolic anion within the mitochondria (recorded at 618 nm), reflecting alkalinization of the medium in contact with the BTB chromophoric groups.

Drug-induced changes in light scattering. Each of these drugs induced changes in light scattering of liver and kidney mitochondria suspensions in 0·15 M KCl with 30 mM TES, pH 7·4 (Fig. 3). These changes are

Table 2. Alcohols tested and corresponding acids

Acids	Alcohols
Flufenamic acid (0·3 mM)	Flufenamyl alcohol* (0·1 mM)
Ibufenac [(*p*-isobutylphenyl) acetic acid] (0·4 mM)	(*p*-Isobutylphenyl) ethanol (0·2 mM)
Ibuprofen [2-(4-isobutylphenyl)propionic acid] (0·4 mM)	2-(4-Isobutylphenyl) propanol (0·2 mM)

* *N*-(α,α,α-trifluoro-*m*-tolyl)-2-aminobenzyl alcohol.

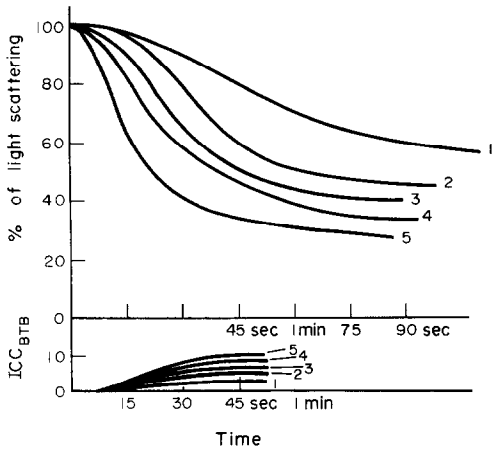

Fig. 5. Effect of ionic composition of the medium on the ICC_{BTB} index. Liver mitochondria, 150 μg protein; no substrate; time 0 = +5 sec. Flufenamic acid, 0·3 mM; all the medium (3 ml) buffered with 30 mM TES, pH 7·4. Light scattering (=swelling) at isosbestic point and ICC_{BTB} in various alkali chlorides (recorded for 10 min). 1 = Na^+; 2 = K^+; 3 = Li^+; 4 = Cs^+; 5 = Rb^+. ICC_{BTB} is expressed as differential percentage of light scattering in the same scale units used for swelling measurements. (100% is light scattering at time 0 in the absence of drug).

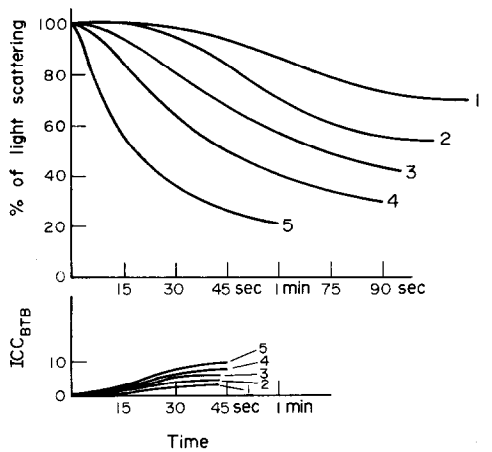

Fig. 7. Effect of drug concentration on the ICC_{BTB} index. Liver mitochondria, 150 μg protein; no substrate; time 0 = +5 sec. Medium = 3 ml KCl, 0·15 M, with 30 mM TES, pH 7·4. Light scattering (=swelling) at isosbestic point and ICC_{BTB} with various doses of flufenamic acid (recorded for 10 min). 1 = 0·07 mM; 2 = 0·15 mM; 3 = 0·3 mM; 4 = 0·6 mM; 5 = 1·2 mM. ICC_{BTB} is expressed as differential percentage of light scattering in the same scale units used for swelling measurements. (100% is light scattering at time 0 in the absence of drugs).

dependent on the drug concentration and on the pH and ionic composition of the incubation medium [7]. They are less marked with kidney than with liver mitochondria and are not reversible in the presence of added ATP (even after addition of Mg^{2+} or Mn^{2+} cations) [7].

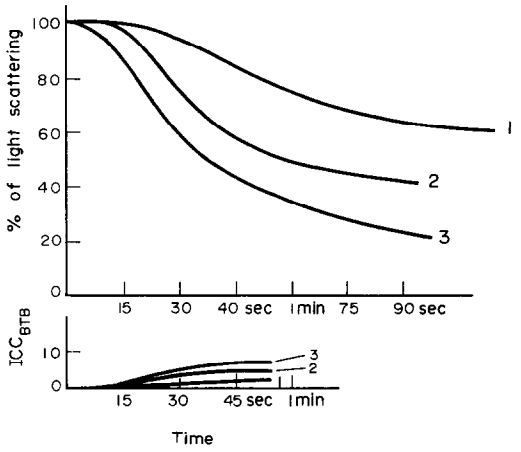

Fig. 6. Effect of ionic composition of the medium on the ICC_{BTB} index. Liver mitochondria, 150 μg protein; no substrate; time 0 = +5 sec. Flufenamic acid, 0·3 mM; all the medium (3 ml) buffered with 30 mM TES, pH 7·4. Light scattering (=swelling) at isosbestic point and ICC_{BTB} in various potassium halides (recorded for 10 min). 1 = F^-; 2 = Cl^-; 3 = Br^-. ICC_{BTB} is expressed as differential percentage of light scattering in the same scale units used for swelling measurements. (100% is light scattering at time 0 in the absence of drug).

BTB color changes. At any moment, when recorded simultaneously under the same conditions (incubation medium, drug concentration), the decrease in light scattering is less marked at 618 nm than at the isosbestic point, for liver as well as for kidney mitochondria. This is true for normal and BTB-loaded mitochondria (Fig. 3), and must be due to a difference in the absorption spectrum of mitochondria at these two wavelengths.

In Fig. 2, the increase of the index reflects an increase in blue color (i.e. of the BTB phenolic anion), which could mean: (a) a change in spatial orientation of the BTB molecule chromophoric sites, (b) an alkalinization of the BTB space, or (c) the two phenomena occurring simultaneously (see Discussion). Some of the alcohols related to the NSAID also induced similar changes in light scattering *in vitro*, due to mitochondrial swelling and an increase in BTB blue color (Fig. 4, Table 2). The concentration of alcohol needed to induce swelling or promote BTB color changes is smaller than that of the corresponding acid.

It was important to know what happened to the external (buffered) pH during the experiments. This was determined by plotting the ICC_{BCP} index, which was equivalent to zero. The swelling curves at 583 nm were similar for BCP-loaded and for unloaded mitochondria. This confirms that the zwitterion buffer was adequate to maintain the external pH at least above 6·8 (a pH which has no effect *per se* on mitochondrial swelling) [7] by neutralizing both the added drug and the protons extruded by the mitochondria in response to the drug action on the mitochondrial membrane. A similar control experiment was also performed with

pH glass electrodes, confirming that there were no external pH variations and, at the same time, confirming that the BCP method was appropriate for such measurements, as postulated by others [23].

Differences between the drugs. Significant differences in activity have been found for at least five of the drugs studied. The most potent drugs for inducing mitochondrial swelling [7] are also the most potent in inducing ICC_{BTB} changes. In order of increasing activity in affecting the ICC_{BTB} and causing mitochondrial swelling, these drugs are mefenamic acid < ibuprofen < indomethacin < phenylbutazone < niflumic acid (as shown in Fig. 2).

Effect of varying the ionic composition of the medium. The effect of changing the alkali cation or the halide anion in the external medium was studied in detail with two drugs: ibuprofen (0·4 mM) and phenylbutazone (0·3 mM). Figure 5 shows the results obtained by changing the alkali cations (with phenylbutazone), i.e. by incubating mitochondria in LiCl, NaCl, KCl, RbCl or CsCl at 0·15 M each and maintaining the external pH at 7·4. These results were carefully checked by using BCP in parallel experiments and by measurements with glass electrodes.

It has been shown in a previous paper [7] that a selective cation sequence exists relating the principal cation of the medium and the degree of mitochondrial swelling initiated by any given swelling agent. We found that a similar cation sequence (at least for these two drugs) exists when studying ICC_{BTB} changes: $Rb^+ \geqslant Cs^+ > Li^+ \geqslant K^+ > Na^+$. Excluding the iodide ion, which is known to induce swelling (and probably color changes) by itself [45], the relative order of the halide anions was found to be $Br^- > Cl^- > F^-$ (Fig. 6) in producing swelling and changes in ICC_{BTB}.

Effect of drug concentration. Here too, the relationship between the degree of swelling induced by the drugs and the magnitude of the ICC_{BTB} changes was apparent. By increasing the concentration of phenylbutazone from 0·3 to 1·2 mM or of flufenamic acid from 0·07 to 1·2 mM, a dose-related increase in the rate of mitochondrial swelling was found which could be correlated with the rapidity at which BTB-space alkalinization occurred (Fig. 7).

Differences between kidney and liver mitochondria. With the same dose of drug and the same quantity of mitochondrial protein (all the variables, e.g. medium composition, being the same), the swelling was greater with liver mitochondria than with kidney mitochondria. This was apparently also true for the ICC_{BTB} changes induced by NSAID (Fig. 3b). Liver mitochondria always responded to a given drug concentration with a greater degree of BTB-space alkalinization than did the kidney mitochondria.

DISCUSSION

It is clear that the NSAID used in the present study induce an increase of ICC_{BTB}. This is due to an increase in the blue BTB phenolic anion, which can only occur if the chromophoric group of the molecule is in contact with an alkaline medium.

The mechanisms associated with BTB color changes are more complicated than was assumed previously, and it appears necessary to speak about a BTB mitochondrial space instead of the intramitochondrial space when referring to pH variations associated with BTB color changes within mitochondria. Chance *et al.* [29] and Chance alone [27] suggested that BTB is located in the inner mitochondrial membrane of the matrix space. On the other hand, related probably to molecular orientation in space, under anaerobic conditions only one-third of the bound BTB is able to react to pH changes within the mitochondria, whereas two-thirds respond rapidly to addition of acids and alkali to the suspending medium [25, 26]. Thus in metabolic state 5 (as defined by Chance and Williams [44]), the BTB changes observed depend upon both proton changes and the relative buffering capacities of the space outside and inside the mitochondrion. In state 4 (when ADP is lacking), almost all the BTB is able to react immediately with extramitochondrial acids and alkali, which must be due to a molecular orientation different from that in state 5. It appears from these data that there must be changes in color of BTB due to spatial reorientation of the molecules of BTB when changing from metabolic state 5 to controlled or resting state 4 through aerobic steady state 3, probably related to the development of an electrical potential across the membrane. This results in an increase of absorbance when a sucrose medium is used and a decrease of absorbance when a KCl medium is used [28].

Having started with state 4 mitochondria and having worked in aerobic conditions with drugs which are swelling agents as well as uncoupling agents, interpretation of our results in terms of pH changes within mitochondria presents some difficulties. The acid drugs, added to incubation media, appeared to be neutralized by the zwitterion buffer since extramitochondrial pH never fell under 6·8 as determined by ICC_{BCP} measurements and never varied as measured by titration with glass electrodes. On the other hand, they induced membrane permeability changes to ions with concomitant mitochondrial swelling [6, 7]. Under similar experimental conditions, they also revealed free mitochondrial membrane sulfhydryl groups [46]. Various authors have emphasized the fact that NSAID interfere with biological membranes [30–35], including mitochondrial [6, 7], and even with nonbiological membranes [36, 47]. Under these conditions, NSAID also alter membrane properties without modifying the pH of the incubation medium. So it appears that only the excess of acidic drugs is neutralized by the external buffer, and most of the added NSAID reacts with the mitochondrial membrane by fixation on specific groups such as sulfhydryl [4, 5, 7, 46, 48, 49].

If, as postulated by Mitchell *et al.* [25], in aerobic states almost all the bound mitochondrial BTB reacts immediately with added acids or alkali, no variations of color should be observed in our experiments. But a

change in spatial orientation of the BTB molecules into the mitochondrial membrane, induced by these acidic drugs reacting with it, would explain the BTB color variations that we observed. Such a spatial reorganization of one-third of the BTB molecules in a KCl medium, when going from state 4 to state 5, results in an increase of absorbance [28]. In our specific aerobic state, in the presence of added NSAID, we postulate that, by changing its spatial orientation, a part (probably related to NSAID concentration) of the bound BTB becomes able to react with the only medium where the pH situation could be compatible with an ICC_{BTB} variation, i.e. the medium of "the intramitocondrial space".

Since NSAID are acid drugs (although similar and even more potent results were found with the corresponding alcohols) with a p*Ka* between 4 and 6, a decrease in BTB blue color would be expected. In fact, a BTB blueing is observed, indicating a pH shift to the basic side. Such data suggest either that these acids, non-ionized, penetrate the mitochondrial membrane, and the protons they carry through the membrane are neutralized by the alkaline medium within the mitochondrial "interieur milieu", or, more probably, that the drugs are excluded from the internal mitochondrial compartment by strong fixation to various membrane-reacting sites, as suggested previously by us [4, 5, 7].

Nevertheless, this ICC_{BTB} increase is not easily compatible with an increase of absorbance due solely to a simple spatial reorganization of the BTB molecules, when compared to color changes observed by us in mitochondrial preparations changing from state 4 to state 5 by simultaneous O_2 deprivation and ADP addition. This kind of change, despite the lack of dilution due to the absence of swelling in this condition, is not so important. The NSAID-induced ICC_{BTB} increase must be explained by an alkalinization of the matrix space occurring simultaneously with the BTB spatial reorganization.

It is known that physiological membrane changes are related to the swelling properties of the drugs [42, 43]. The NSAID associated with some external sites of the intramitochondrial membrane (and there is some evidence to implicate sulfhydryl groups as very important sites in the relationship between NSAID and the mitochondrial membrane [4, 5, 7, 46–49]) may alter membrane permeability to alkali cations and halide anions according to selectivity sequences that are dependent both on the kind of drug and on the membrane structure. This property is the main cause of the swelling phenomenon observed *in vitro* and reported in a previous paper [7].

Each cation or anion has its own characteristics in regulating biophysical properties of biological systems [50]. In our system, each ion regulates the amplitude of swelling by penetrating to a different degree under the stimulus of the same drug at the same concentration. The general pattern of NSAID-induced swelling has been shown to be qualitatively (if not quantitatively) the same with each NSAID: $Rb^+ \geqslant Cs^+ > Li^+ \geqslant K^+ > Na^+$ and $(I^-) > Br^- > Cl^- > F^-$. According to the ion selectivity theory of Einsenman [51, 52], these patterns of selectivity could be related to the presence of sites with weak electrical fields and polarizable sites like carboxyl groups (i.e. with a field strength that varies greatly, depending upon the immediate molecular environment [7, 8, 53]). This selectivity was found to be the same for the NSAID-induced BTB color changes.

For each cation penetrating the mitochondria, there must be either another cation with the same charge exchanged for it or the concomitant uptake of an anion with an equivalent negative charge to maintain ionic balance. The mitochondrial membrane is much more permeable to alkali cations than to halide anions, which are low-permeant anions compared to phosphate or acetate ions [54, 55]. Therefore, even if relatively few anions do penetrate into the mitochondria, there will be, in any case, a net increase of cations which can only be compensated by an expulsion of protons (which may, or may not, be associated with concomitant uptake of hydroxyl ions) with consequent alkalinization within the mitochondria, probably within the matrix space. It is clear that (according to the ionic selectivity pattern) in the presence of the same amount of drug, more Rb^+ than Na^+ will penetrate the mitochondria, and therefore more protons will be expelled (or more hydroxyl ions will penetrate) into a rubidium-rich medium than into a saline medium. In theory, by replacing one anion with a second more permeant anion (Cl^- with Br^-, for example), less proton expulsion (or less sequestration of hydroxyl ions) is needed for maintaining internal ionic balance (and the converse, when the second anion is less permeant). This is true if exchanging the permeant anion has no effect as such on cation permeability, which seems not to be the case in our experiments. Replacement of Cl^- with Br^- or F^- does not reduce (in the case of Br^-) or increase (in the case of F^-) the intensity of the matrix pH change but acts, in fact, just the reverse. This may be due to the fact that these latter halide ions act much more by increasing (with Br^-) or decreasing (with F^-) cationic permeability *per se* than by neutralizing a proton efflux or substituting for hydroxyl ions in a corresponding anion influx.

Thus, the anions may act by two opposite mechanisms on the mitochondrial membrane drug response: (1) they may alter the cationic permeability, or (2) they could replace the induced proton expulsion (or hydroxyl sequestration) by themselves penetrating the organelles. In fact, the first phenomenon seems so dominant that it completely masks the second one. However, with more potent permeant anions like acetate or phosphate, which fail to mediate BTB alkalinization in response to calcium ions [23, 24], the second mechanism is dominant.

In summary, the NSAID that we have studied seem to have two properties revealed by the variations in BTB color that they induce in prelabeled mitochondria. First, the NSAID apparently have an effect on the

spatial orientation of the BTB molecules in the BTB space, i.e. probably the matrix space. Second, they seem to induce membrane permeability changes which are responsible for a cation influx (and to a lesser degree an anion influx as well) from the external medium, accompanied by water which causes the swelling. Simultaneously, to maintain an electrical and ionic balance within mitochondria, an efflux of protons (and most probably also an influx of hydroxyl ions) is induced which causes an intramitochondrial alkalinization. Swelling and intramitochondrial pH variations are closely related: with a constant neutral external pH, parameters which affect the drug-induced swelling (e.g. ionic composition of the medium, tissue origin of the mitochondria) similarly affect the drug-induced BTB color changes.

Criticism may be raised against the levels of the drugs used in the present study, which are a little higher than those observed in plasma of patients treated with the same drugs. The concentrations were chosen for several reasons. (1) The concentrations selected were those at which oxidative phosphorylation is uncoupled; we have found no real correlations between this effect on pH and the ability of drugs to uncouple oxidative phosphorylation. All NSAID used in these experiments are uncouplers [3–5], but we failed to reproduce the same results with 2,4-dinitriphenol (0·05 to 0·2 mM), a more classical uncoupling agent.* The concentrations were selected also (2) to be sufficient to saturate other specific drug-binding sites on lysosomes and other non-mitochondrial organelles, and (3) to ensure rapid responses by the mitochondria (swelling, BTB blueing) and to obviate effects of long incubation (spontaneous lysis). Swelling and BTB blueing were observed with lower doses of all these drugs (e.g. flufenamic acid, phenylbutazone), but the time period for the development of these changes was considerably extended.

Many reports describe the NSAID effects on various membranes [30–36]. They stabilize erythrocytes against osmotic shock at drug levels at least comparable to, or higher than, those used in the present study [30, 31], which indicates that the same drug concentrations render an intracellular membrane leaky and an extracellular membrane steady. At high levels, these drugs labilize lysosomal membranes. Therefore, it would seem wiser at the present time to conclude that NSAID may affect various membranes differently, depending on the concentrations of drugs available and on the natural composition and function of the membrane, rather than to seek a simplistic generalization concerning the drug–membrane interaction.

Acknowledgements—J.P.F. was the recipient of Post-doctoral fellowships from ULCA and Université Libre de Bruxelles; W.C.D. had a traveling fellowship from the Fulbright Foundation.

The authors thank Mrs. A. Robbrecht for typing the manuscript. We are most grateful to Dr. L. Fenoy (Wilmington, Delaware) for the gift of flufenamyl alcohol, to Dr. C. M. Pearson for continual support and encouragement, and to the United States Public Health Service for financial support (Grant No. GM 15759).

* Unpublished data.

REFERENCES

1. T. M. Brody, *J. Pharmac. exp. Ther.* **117,** 39 (1956).
2. S. S. Adams and R. Cobb, *Nature, Lond.* **131,** 773 (1958).
3. M. W. Whitehouse, in *Progress in Drug Research* (Ed. E. Jucker), Vol. 8, p. 321. Birkhäuser, Basel and Stuttgart (1965).
4. J. P. Famaey and J. Mockel, *Biochem. Pharmac.* **22,** 1487 (1973).
5. J. P. Famaey and J. Mockel, *Fifth Int. Congr. Pharmac.*, abstr. 393. San Francisco (July 1972).
6. N. Nakanishi, H. Imamura and V. Ikegami, *J. pharm. Soc. Japan* **91,** 702 (1971).
7. J. P. Famaey, *Biochem. Pharmac.* **22,** 2693 (1973).
8. J. P. Famaey and M. W. Whitehouse, *Biochem. Pharmac.* **22,** 2707 (1973).
9. J. B. Chappell and G. D. Greville, *Biochem. Soc. Symp.* **23,** 39 (1963).
10. A. L. Lehninger, *Physiol. Rev.* **42,** 467 (1962).
11. R. Cereijo-Santaló, in *Membranes and Ion Transport* (Ed. E. E. Bittar), Vol. 2, p. 229. Wiley Interscience, London (1970).
12. J. B. Chappell and G. D. Greville, *Nature, Lond.* **183,** 1737 (1959).
13. P. Emmelot, *Nature, Lond.* **188,** 1197 (1960).
14. A. L. Lehninger, E. Carafoli and C. S. Rossi, *Adv. Enzymol.* **29,** 259 (1967).
15. C. S. Rossi, J. Bielawski and A. L. Lehninger, *J. biol. Chem.* **241,** 1919 (1966).
16. A. R. L. Gear, C. S. Rossi, B. Reynafarje and A. L. Lehninger, *J. biol. Chem.* **242,** 3403 (1967).
17. W. J. Waddel and T. C. Butler, *J. clin. Invest.* **38,** 720 (1959).
18. S. Addanki, F. D. Cahill and J. F. Sotos, *Science, N.Y.* **155,** 1678 (1967).
19. Z. A. Sorokina, *Cytology, USSR* **3,** 48 (1961).
20. M. Lavallee, *Circulat. Res.* **15,** 185 (1964).
21. N. W. Carter, F. C. Rector, Jr., D. S. Campion and D. W. Seldin, *J. clin. Invest.* **46,** 920 (1967).
22. G. A. Kurella, in *Glass Microelectrodes* (Eds. M. Lavallee, O. Schanne and N. C. Herbert), p. 182. Wiley, New York (1969).
23. B. Chance and L. Mela, *J. biol. Chem.* **241,** 4588 (1966).
24. A. L. Lehninger, *Ann. N.Y. Acad. Sci.* **147,** 816 (1969).
25. P. Mitchell, J. Moyle and L. Smith, *Eur. J. Biochem.* **4,** 9 (1968).
26. L. Smith, *Ann. N.Y. Acad. Sci.* **147,** 856 (1969).
27. B. Chance, in *Biochemistry of Mitochondria* (Eds. E. C. Slater, Z. Kaniuga and L. Wojtczak), p. 93. Academic Press, London (1967).
28. G. F. Azzone, G. Piemonte and S. Massari, *Eur. J. Biochem.* **6,** 207 (1968).
29. B. Chance, L. Mela and E. J. Harris, *Fedn Proc.* **27,** 902 (1968).
30. A. D. Inglot and A. Wolna, *Biochem. Pharmac.* **17,** 269 (1968).
31. Y. Mizushima, S. Sakai and M. Yamaura, *Biochem. Pharmac.* **19,** 227 (1970).

32. G. Weissmann, in *A Symposium on the Interaction of Drugs and Subcellular Components in Animal Cells* (Ed. P. N. Campbell), p. 203. Little, Brown, Boston (1968).
33. G. Weissmann, G. Sessa and S. Weissmann, *Biochem. Pharmac.* **15,** 1537 (1966).
34. J. L. Barker and H. Levitan, *Biochim. biophys. Acta* **274,** 638 (1972).
35. H. Levitan and J. L. Barker, *Nature New Biol.* **239,** 55 (1972).
36. S. MacLaughin, *Nature, Lond.* **243,** 234 (1973).
37. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
38. H. Tedeschi and D. L. Harris, *Biochim. biophys. Acta* **28,** 392 (1958).
39. L. Packer, *J. biol. Chem.* **235,** 242 (1960).
40. A. L. Koch, *Biochim. biophys. Acta* **51,** 429 (1961).
41. N. A. Shane and J. I. Routh, *Analyt. Chem.* **39,** 414 (1967).
42. G. Blondin, W. J. Vail and D. E. Green, *Archs Biochem. Biophys.* **129,** 158 (1968).
43. A. Azzi and G. F. Azzone, *Biochim. biophys. Acta* **131,** 468 (1967).
44. B. Chance and G. R. Williams, *Adv. Enzym.* **17,** 67 (1956).
45. J. E. Rall, J. Roche, R. Michel, O. Michel and S. Varrone, *Biochim. biophys. Acta* **62,** 622 (1962).
46. J. P. Famaey and M. W. Whitehouse, *Sixth Pan-American Congress on Rheumatic Diseases.* Toronto, Canada (June 1974).
47. V. S. Markin, L. I. Kristalik, E. A. Liberman and V. P. Topaly, *Biofizika* **14,** 256 (1969).
48. A. Fluharty and D. Sanadi, *Proc. natn. Acad. Sci. U.S.A.* **46,** 608 (1960).
49. P. G. Heytler, *Biochemistry, N.Y.* **2,** 357 (1963).
50. J. M. Diamond and E. M. Wright, *A. Rev. Physiol.* **31,** 581 (1969).
51. G. Eisenman, *Biophys. J.* **2,** part 2, 259 (1962).
52. G. Eisenman, in *Ion Selective Electrodes* (Ed. R. A. Durst), Special Publ. 314. National Bureau of Standards, Washington, D.C. (1969).
53. G. Einsenman, *Proc. Twenty-third Int. Congr. Physiol. Sci.*, p. 489. Excerpta Med. Found., Amsterdam (1965).
54. A. L. Lehninger, in *The Mitochondrian, Molecular Basis of Structure and Function*, p. 157. W. A. Benjamin, Amsterdam (1965).
55. J. B. Chappel, P. J. F. Henderson, J. D. MacGivan and B. H. Robinson, in *A Symposium on the Interaction of Drugs and Subcellular Components in Animal Cells* (Ed. P. N. Campbell), p. 71. Little, Brown, Boston (1968).