



Uncoupling effects of diclofenac and aspirin in the perfused liver and isolated hepatic mitochondria of rat

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Abstract

Gluconeogenesis, glycolysis and glycogenolysis were studied in rat perfused liver following the infusion of various concentrations of diclofenac and aspirin, two non-steroidal anti-inflammatory drugs (NSAIDs). Glucose synthesis was measured in livers isolated from 48-h fasted rats perfused with Krebs-Henseleit bicarbonate buffer containing L-lactate (2 mM) and pyruvate (0.1 mM) as precursors. Both diclofenac (0.01–0.1 mM) and aspirin (1–10 mM) had an inhibitory effect on gluconeogenesis (GNG). The inhibition was dose-dependent and reversible. For the estimation of glycogenolysis and glycolysis, the rates of glucose release and of lactate and pyruvate production were measured in livers of well-fed rats perfused with substrate-free buffer. Infusion of diclofenac (0.1 mM) or aspirin (5 mM) strongly stimulated glycogenolysis and glycolysis (GGL/GL). In general, an increased oxygen consumption by the liver tissue was also noted in both types of experiments, as deduced from the continuous monitoring of oxygen concentration changes in the effluent. Such a pattern of response can be attributed to the uncoupling effects of the two drugs on oxidative phosphorylation. Measurements of respiration rates and membrane potential in isolated liver mitochondria submitted to various concentrations of diclofenac and aspirin confirms this assumption. Thus, 0.01 to 0.2 mM diclofenac stimulates state-4 respiration and slightly inhibits state 3, decreasing the respiratory control ratio, while the membrane potential is decreased or collapsed (depending on the drug concentration). Similar effects are recorded for aspirin at higher concentrations (0.2–5 mM), even though state 3 is not affected in this case. Arguments are presented that the concentrations of the drugs used largely correspond to the pharmacological doses employed in antipyretic and anti-inflammatory treatments. Therefore, a greater consideration should be given to the uncoupling effect, at least from the toxicological viewpoint.

Keywords: Diclofenac; Aspirin; Non-steroidal anti-inflammatory drug; Glucose metabolism; Oxidative phosphorylation; Liver; Mitochondrion

1. Introduction

Abbreviations: NSAID(s), non-steroidal anti-inflammatory drug(s); EDTA, ethylenediamine tetraacetate; Hepes, N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Pi, inorganic phosphate; diS-C2-(5), 3,3'-diethylthiadicarbocyanine iodide; ACR, acceptor control ratio; RCR, respiratory control ratio.

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According to a hypothesis proposed by Vane [1], it has been accepted for almost 20 years that non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac and aspirin, act by inhibiting the synthesis of specific prostaglandins. However, certain experimental discrepancies between the extent of the inhibi-

tion of prostaglandin biosynthesis and the anti-inflammatory efficiency (see Refs. [2–4]) as well as other observations determined Weissmann's group [2,3] to advance a new hypothesis, based on the capacity of NSAIDs to disrupt a series of membrane-dependent processes, including signal transduction at the level of plasmalemma.

In a preliminary study dedicated to the effects of diclofenac on the metabolism of the perfused liver of rat, we noticed a rather strong effect of this drug on both gluconeogenesis (inhibition) and glycogenolysis (stimulation), at concentrations calculated by us to be similar to those used in anti-inflammatory treatments. Such a pattern of response, associated with an increased tissue oxygen consumption, suggested as a logical explanation an uncoupling effect on oxidative phosphorylation, which was also confirmed by the literature data for other NSAIDs [5–11].

However, the uncoupling effect, which is in general accompanied by heat production and Ca^{2+} release from mitochondria (see, for example, Ref. [12]) is apparently at variance with the anti-inflammatory action, regardless of the hypothesis considered for the explanation of this action. These intriguing observations prompted us to look more deeply into the problem, extending the study to aspirin (the most common NSAID) and to isolated mitochondria, for a more direct evaluation of the uncoupling effect.

Although the effect of aspirin on oxygen consumption of liver slices [11] and isolated mitochondria [6,7,10] is a rather old observation, diclofenac has not been studied from this point of view and neither aspirin nor diclofenac have been employed in the perfused liver. In fact, there is only one conspicuous study of this type, dedicated to the effect of mefenamic acid (also an NSAID) on glucose metabolism in the perfused liver of rat [9].

In our preliminary studies [13,14] we have also combined the above-mentioned approach with recordings of mitochondrial swelling and ultrastructural aspects of the perfused liver and isolated mitochondria of both rat and guinea pig. The present paper presents our final and most important results regarding the effects of diclofenac and aspirin on glucose metabolism in the perfused liver of rat and on oxidative phosphorylation of isolated hepatic mitochondria.

2. Materials and methods

2.1. Liver perfusion and related methodology

Male albino rats of an inbred Wistar line (200–250 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and after the removal of viscera the liver was perfused *in situ*, as previously described [15,16]. The liver was cannulated via the hepatic portal vein (for influx) and the supradiaphragmatic portion of the inferior vena cava (for efflux). A non-recirculating (i.e., through-flow) hemoglobin-free perfusion was used. The perfusion medium, Krebs-Henseleit bicarbonate buffer (pH 7.3–7.4), was pumped into the liver with the aid of a peristaltic pump, at a rate of 32.5–33.0 ml/min. The flow rate was constant for the same liver during the entire period of perfusion. The perfusion medium was continuously gassed with $\text{O}_2:\text{CO}_2$ (19:1) and oxygenated by a rotatory disc oxygenator. The glucose precursors (i.e., lactate and pyruvate) and the drugs were dissolved in the perfusion medium and pumped into an in-line infusion chamber with the aid of an infusion pump, so as to enter the liver at a constant rate (0.2–0.4 ml/min) and to give the desired final concentration in the perfusion medium. When necessary, the compounds to be presented to the liver were brought to pH 7.4 with NaOH or HCl.

Drug concentrations administered in the perfusion were in the usual range employed in such studies. Thus, diclofenac was varied from 0.01 to 0.1 mM, whereas aspirin from 1 to 10 mM. These figures parallel the normal clinical doses used in inflammation treatments, i.e., 150 mg/day for diclofenac [17] or 5–8 g/day for aspirin [4].

For a complete depletion of the hepatic glycogen reserve, 48-h-fasted rats were used in GNG studies. In this case, 2 mM lactate and 0.1 mM pyruvate (final concentrations in the perfusion medium) were employed as glucose precursors (substrates). The effects on GGL and GL were studied on livers obtained from well-fed rats perfused with medium without substrate. The intensity of glycogenolysis was estimated by measuring glucose liberation from endogenous glycogen, whereas the intensity of glycolysis

was estimated through the lactate and pyruvate originating from glucose catabolism.

Changes of oxygen concentration in the effluent were monitored continuously using an in-line oxygen electrode attached to a potentiometric recorder. A value of 2.07 μ atoms of oxygen/ml perfusion medium was used as a basis for calculating oxygen concentration. This was expressed as an oxygen quotient (QO_2), in matoms oxygen/100 g body weight and hour. This way of expression is needed because the oxygen concentration in the effluent depends not only on the rate of oxygen consumption by the tissue but also on the perfusion rate. At a constant oxygen consumption by liver, the concentration of oxygen in the effluent will be higher at higher perfusion rates, when more oxygen passes through the system in the time unit.

Effluent samples were collected at 3-min intervals and assayed spectrophotometrically for glucose, lactate and pyruvate, immediately after the perfusion end, using the appropriate Biochemica Combination kits, depending on the experiment.

2.2. Isolation of mitochondria and the measurement of respiration-dependent parameters

Mitochondria were isolated from the livers of freshly decapitated animals, essentially according to Ref. [18]. The isolation medium consisted of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and 0.1 mM EDTA, while the final washing and suspending media lacked the chelating agent.

Respiration rates were measured polarographically at 25°C, in a 0.5-ml cell, with a Clark oxygen electrode (Yellow Springs), in a medium consisting of 175 mM sucrose, 50 mM KCl, 10 mM phosphate buffer, 20 mM Tris (pH 7.4), 0.5 mM EDTA and 2 mM $MgSO_4$. Glutamate (10 mM) plus malate (5 mM) or succinate (10 mM) (potassium salts) were used as respiratory substrates. Mitochondria (1 mg/ml with succinate or 2 mg/ml with glutamate) were injected through the stopper capillary and the oxygraphic traces recorded in this way represented the basal respiration (basal state or state 2; see Ref. [12] for nomenclature). After 1–2 min, 0.1–0.2 mM ADP was injected, resulting in the so-called state 3 respiration, characterised by a higher rate of oxygen consumption. When ADP was exhausted (i.e., phos-

phorylated to ATP), the respiration rate decreased again, resulting in state 4 (similar to the basal state). The ratio between the state 3 and the basal state is known as the acceptor control ratio (ACR), whereas the ratio between state 3 and state 4 is known as the respiratory control ratio (RCR). They have similar values and significances, both representing important indices of mitochondrial integrity and phosphorylation ability [12].

Diclofenac or aspirin were usually injected at state 4, but in some cases they were added at state 3 or even at the basal state, from a buffered stock solu-

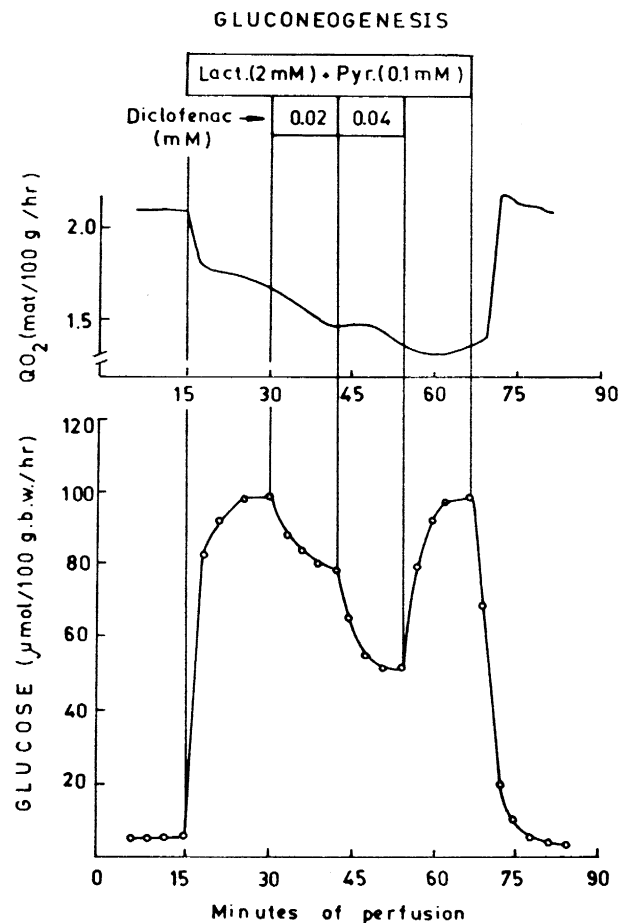


Fig. 1. Effect of successive additions of low concentrations of diclofenac on glucose synthesis and oxygen evolution in the perfused rat liver. The conditions are as described in Section 2. QO_2 , depicted in the ordinate, represents the oxygen level recorded by the electrode immersed into the hepatic effluent, correlated to animal weight and flow rate. This coefficient is a negative reflection of specific oxygen consumption by the liver tissue.

tion, so as to obtain the desired final concentration in the oxygraph cell. In fact, all the concentrations mentioned above or which will appear later in this presentation refer to final concentrations in the reaction mixture.

Membrane potential generated by succinate respiration and the kinetic behaviour of this potential following the addition of ADP and the tested drugs were monitored by spectrophotometric recording of the absorbance changes (at 660 nm) of the membrane potential-sensitive probe diS-C₂-(5), in a Specord-M-40 spectrophotometer (Carl Zeiss), based on previously described principles [19,20] and methodology [21]. It should be mentioned, however, that it was not our intention to measure absolute values of membrane potential but to use the absorbance changes in a

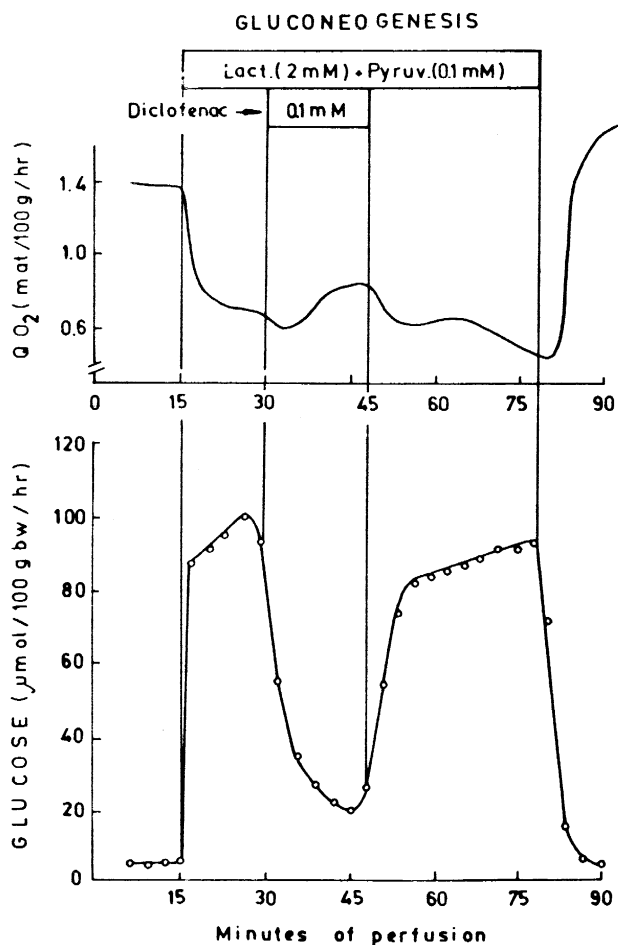


Fig. 2. Strong effect of a high concentration of diclofenac (0.1 mM) on glucose synthesis and oxygen evolution in the perfused rat liver.

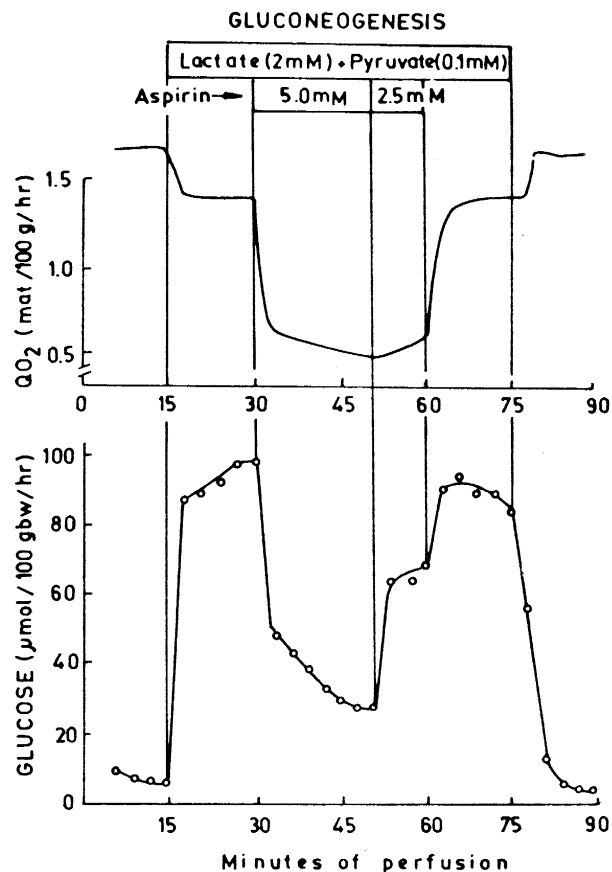


Fig. 3. Effect of increasing concentrations of aspirin on gluconeogenesis in the perfused rat liver.

qualitative manner, mainly for kinetic observations. The basic suspending medium consisted of: 100 mM sucrose, 80 mM KCl, 5 mM Tris-Hepes (pH 7.3), 5 mM KP_i , 2 mM $MgCl_2$ and 0.5 mM EDTA. Specific details of this type of experiments can be found in the corresponding figure legends.

2.3. Chemicals

Biochemica Test-Combination kits for glucose (GOD-Perid), lactate and pyruvate and all enzymes and coenzymes used for assays were from Boehringer-Mannheim GmbH. Tris and pyruvate were from Merck (Darmstadt) and lactate from Riedel-de-Haën (Hanover). ADP, rotenone and oligomycin were purchased from Sigma and diS-C₂-(5) from Eastman-Kodak. Diclofenac and aspirin were from the Romanian Drug Enterprises (Bucharest). All other

chemicals were of the highest purity commercially available.

3. Results

3.1. Effects of diclofenac and aspirin on hepatic gluconeogenesis

As can be seen in Fig. 1, the first 15 min of perfusion with Krebs-Henseleit bicarbonate medium alone are characterised by a very low content of glucose in the effluent. The addition of lactate and pyruvate in the perfusion medium induces a very rapid increase of glucose synthesis, which remains

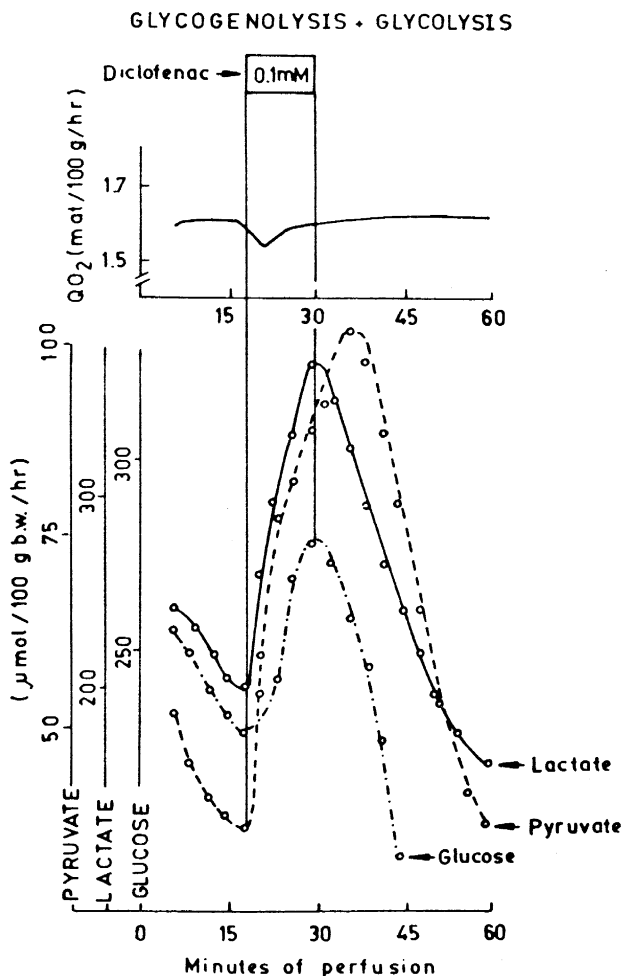


Fig. 4. Effect of a high concentration of diclofenac (0.1 mM) on glycogenolysis, glycolysis and oxygen evolution in the perfused rat liver.

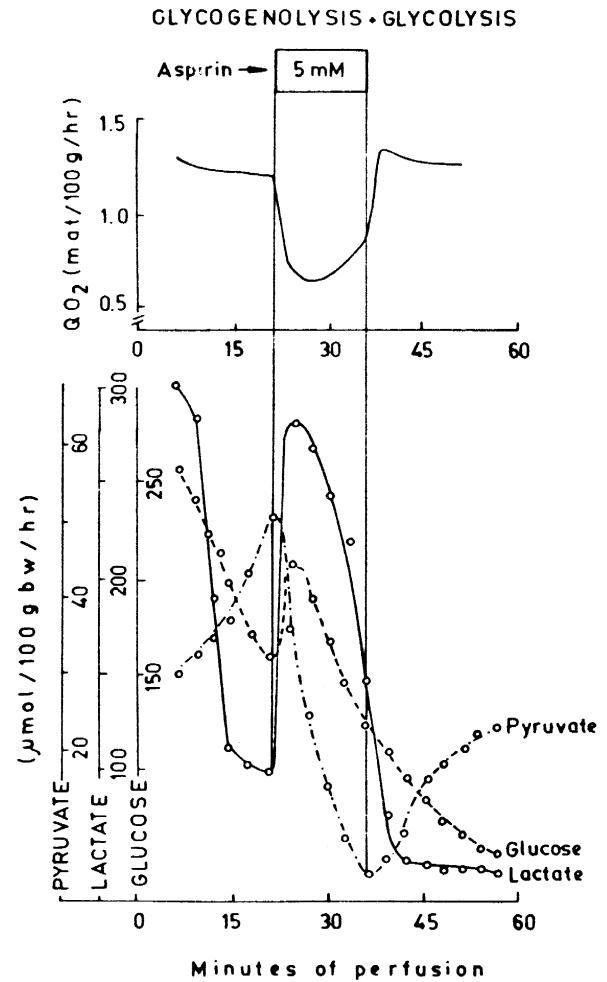


Fig. 5. Effect of a high concentration of aspirin (5 mM) on glycogenolysis, glycolysis and oxygen evolution in the perfused rat liver.

constant over the entire period of substrate administration, if no other addition is made. However, diclofenac administration (0.02–0.1 mM) determines a decrease of GNG (Figs. 1 and 2). The inhibition of glucose synthesis is proportional to diclofenac concentration and lasts as long as the drug is present in the perfusion medium. The interruption of diclofenac administration restores the normal glucose synthesis. It is worth noting that oxygen quotient in the effluent also decreases in the presence of diclofenac (see especially Fig. 1), meaning an increased oxygen consumption by the liver tissue. However, at 0.1 mM drug (Fig. 2), where GNG is 80% inhibited, after an initial slight increase, the trend of oxygen consumption reverses.

As in the case of diclofenac, the presence of aspirin (1–10 mM) in the perfusion medium induces the inhibition of GNG and the decrease of oxygen quotient (Fig. 3). Similarly, the inhibition is proportional to the drug concentration and it is reversible.

3.2. Effects of diclofenac and aspirin on hepatic glycogenolysis and glycolysis

As can be seen from Fig. 4, the administration of diclofenac (0.1 mM) in the perfusion medium determines an increase of both GGL and GL. A slight decrease of oxygen quotient can also be observed. The effects are reversible for all the parameters tested.

Aspirin (5 mM) also produces an increase of both GGL and GL (Fig. 5). The visible decrease of oxygen quotient in the effluent indicates a strong increase of oxygen consumption by the liver tissue during aspirin administration. The effects are, again, reversible.

3.3. The effects of diclofenac and aspirin on respiration

The effects of various concentrations of *diclofenac* on the respiratory parameters of rat liver mitochondria are presented in Fig. 6.

Fig. 6 shows several oxygraphic traces recorded in the presence of glutamate plus malate. In trace a, after about 1 min of basal respiration (following the addition of mitochondria), the respiration is stimulated by injection of ADP, attaining 89.9 ng atoms O/min/mg protein (state 3). After ADP exhaustion, the respiration rate decreases to 18.6 ng atoms O/min/mg protein (state 4). The ratio between the two states (RCR) is 4.83. Diclofenac added now on state 4 stimulates the respiration proportionally to its concentration. The highest concentration used (0.2 mM) stimulates the respiration by 166% ($49.6/18.6 = 2.66$). When diclofenac is added on state 3 (trace b), a small inhibition occurs (about 25% at 0.1 mM). If oligomycin (which inhibits the utilisation of ADP/ATP) is added on state 3, the resulting low respiration rate can be increased (77%) by the consecutive addition of 0.1 mM diclofenac (trace c). This phenomenon is in general similar to (but less extensive than) that produced by 0.1 mM dinitrophenol (DNP), a classical uncoupling agent (see trace d as an illustration of the removal of oligomycin inhibition by DNP). Similar effects could be observed on succinate-dependent respiration (see Ref. [14]).

High concentrations of *aspirin* (0.2–5 mM) also have uncoupling effects. This is demonstrated by the

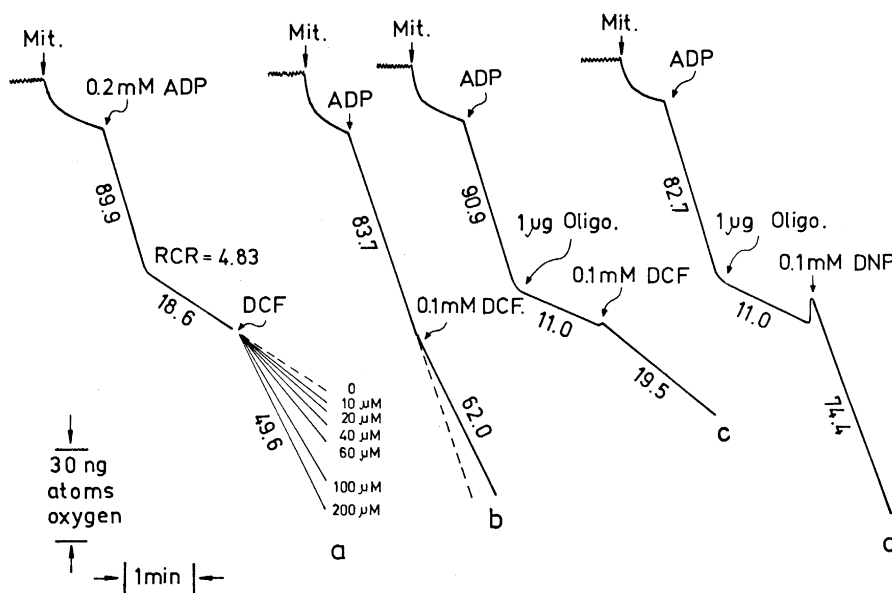


Fig. 6. Effects of the addition of diclofenac on different respiration states of rat liver mitochondria. 1 mg of mitochondrial protein (Mit) is suspended in the 0.5-ml oxygraph cell under conditions described in Section 2 for glutamate respiration. This is followed by the addition of 0.2 mM ADP and different drugs, as indicated by arrows: DCF = diclofenac, Oligo = oligomycin, DNP = 2,4-dinitrophenol (see text for other details).

recordings shown in Fig. 7. Trace a of Fig. 7 represents the control. Trace b in Fig. 7 shows that aspirin added on state 4 stimulates the respiration proportionally to its concentration. The respiration is also stimulated if aspirin (2 mM) is added directly on the basal state (trace c). However, the same concentration of aspirin has no effect on state 3 (trace d). Added after oligomycin, 5 mM aspirin partly removes the inhibition produced by that drug (trace e). As in the case of diclofenac, succinate-dependent respiration is also affected (see Ref. [14]).

3.4. Effects on membrane potential

Since uncouplers affect membrane permeability, especially for protons (see, for example, Ref. [12]), we expect that diclofenac and aspirin decrease membrane potential difference generated by mitochondrial respiration. As can be seen in Fig. 8, this phenomenon does indeed occur. However, the actual concentration of diclofenac necessary for a total collapse of membrane potential differs from one individ-

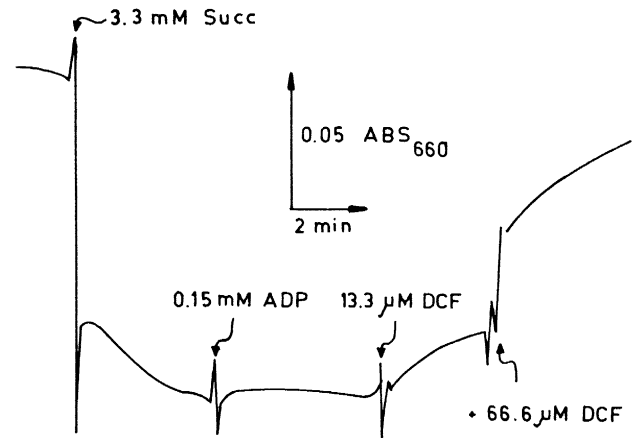


Fig. 8. Effect of diclofenac on membrane potential developed by succinate respiration in rat liver mitochondria. The suspending medium (1.5 ml in each cuvette) consists of 100 mM sucrose, 80 mM KCl, 5 mM Tris-Hepes (pH 7.3), 5 mM KP_i , 2 mM $MgCl_2$, 0.5 mM EDTA and $6.6 \mu M$ rotenone. 1 mg of mitochondrial protein is added in each cuvette and $2.5 \mu M$ diS-C₂-(5) to the sample, the baseline being controlled by a zero adjusting programme. The additions indicated in the figure are made concomitantly into both cuvettes.

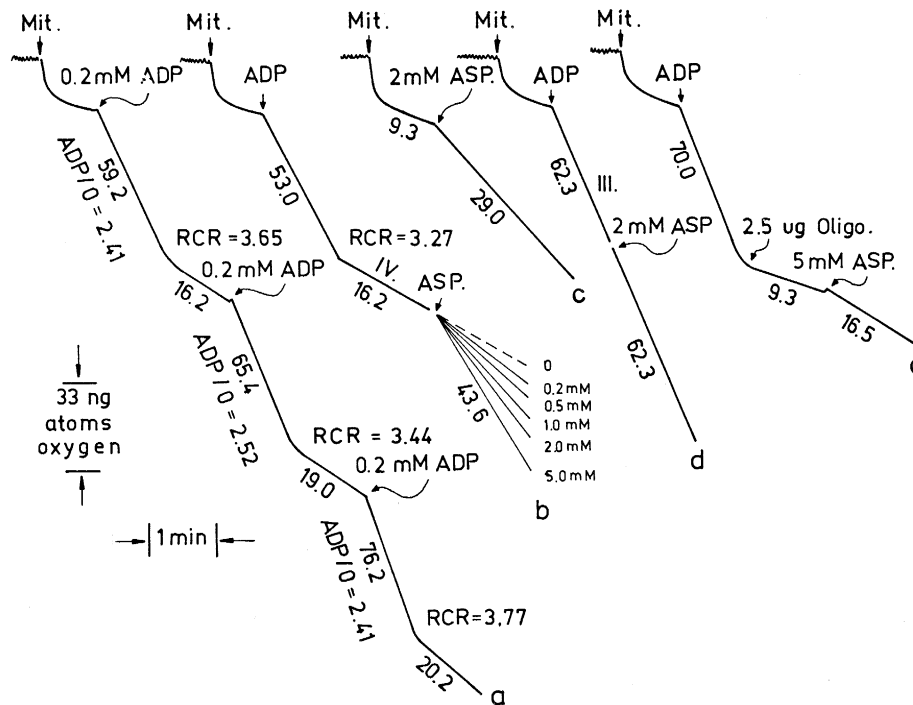


Fig. 7. Effects of the addition of aspirin on different respiration states of rat liver mitochondria. Conditions are identical to those described in Fig. 6.

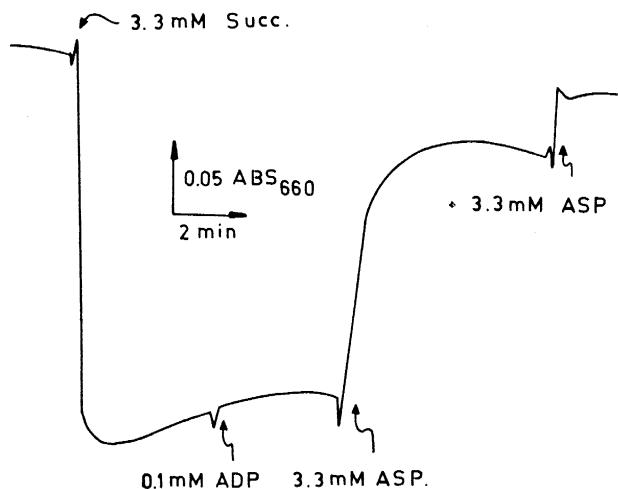


Fig. 9. Effect of aspirin on membrane potential developed in rat liver mitochondria. Conditions are identical to those in Fig. 8.

ual preparation to another. The trace presented in Fig. 8 results from a sensitive liver preparation, where less than 0.1 mM diclofenac collapses the membrane potential. For another preparation (not shown here), we had to use approx. 0.25 mM drug in order to obtain the full collapse of the membrane potential.

A high concentration of aspirin (at least 5 mM) also produces the collapse of the membrane potential generated by succinate respiration in rat liver mitochondria (Fig. 9). As can be seen, however, 3.3 mM aspirin is not enough to collapse the membrane potential irreversibly, a slight tendency of recovery being observed. The results are in total agreement with those obtained by the oxygraphic method.

4. Discussion

From the results presented above, the effects of diclofenac and aspirin on carbohydrate metabolism can be summarised in terms of a stimulation of glycogenolysis/glycolysis and an inhibition of gluconeogenesis. As is well known, glycolysis is the only source of energy in the eukaryotic cell under anaerobic conditions or when oxidative phosphorylation is uncoupled. By releasing the pressure exerted on the electron flow, uncoupling stimulates the respiration in a futile cycle. For thermodynamic reasons, under these conditions, glycogenolysis and glycolysis are

also expected to be stimulated and produce the necessary ATP for the cell. Thus, the stimulating effects of the two drugs on GGL/GL could be the result of an uncoupling process. In fact, our data obtained on isolated mitochondria provide more direct evidence for the uncoupling phenomenon. This evidence can be summarised as follows: (1) stimulation of state 2 (the basal state) and of state 4 of the respiration, with a consequent decrease of ACR and RCR, respectively; (2) partial removal of the respiratory inhibition produced by oligomycin; (3) decrease and even collapse of the membrane potential.

All these effects, also supported by swelling studies and electronmicroscopic observations presented elsewhere [14], are characteristic of uncouplers and can be easily explained in terms of the chemiosmotic theory of energy conversion in mitochondria [12,22]. In fact, by their chemical structure, diclofenac, aspirin and other NSAIDs are usually monocarboxylic acids with one or two aromatic rings. Such structures are more or less hydrophobic, with different degrees of membrane solubility (see Ref. [9]), having a structure resembling that of typical uncoupling agents, i.e., compounds capable of carrying protons across lipid membranes [23]. The resemblance of diclofenac and aspirin with dinitrophenol (a classical uncoupler) can be seen in Fig. 10.

By virtue of this resemblance, the uncoupling effects of NSAIDs are not only possible but expected. We have observed that even other (more or less) hydrophobic agents with a smaller degree of similarity, such as different local anesthetics and analgesics are capable of producing uncoupling effects [21]. The biochemical results presented here are, in fact, in agreement with those of Kimmelmeier and Bracht [9]

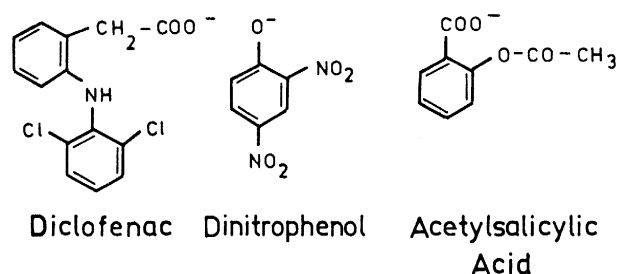


Fig. 10. Similarity between the two NSAIDs used and a classical uncoupler (DNP).

obtained with mefenamic acid in rat perfused liver. Also, Brass and Garrity [8], working on isolated hepatocytes, find that certain NSAIDs (such as meclofenamate, ibuprofen and indomethacin) intensify glycolysis. Itinose et al. [24] observe the stimulation of glycogenolysis and glycolysis and the inhibition of gluconeogenesis by the analgesic drug acetaminophen.

Considering the relatively hydrophobic properties of NSAIDs and the observation that they start uncoupling at rather low nominal concentration, it is to be expected that these drugs accumulate at a higher ratio in the membranes than in the rest of the cell. Thus, uncoupling is more likely produced not only by specific proton transport (as in the case of the true uncouplers), but also through membrane disordering (at least at higher concentrations). The observation that at high concentrations diclofenac starts inhibiting state 3 respiration indicates that the integral membrane proteins, such as the respiratory complexes, can also bind these drugs. The fact that diclofenac or aspirin added to rat liver mitochondria after oligomycin stimulate respiration much less than DNP may also reflect a direct effect of these drugs on the mitochondrial ATPase or at least on an ATP utilising process. However, different uncoupling efficiencies of the two drugs should also be considered in this respect.

In the case of the local anesthetics, a good correlation could be established between the degree of hydrophobicity and the uncoupling potency of a series of tertiary amines [21].

Although it is often stated or implied (e.g., Refs. [8,9]) that uncoupling occurs at concentrations higher than those considered pharmacologically relevant, our results (as well as the analysis of the literature data) suggest a different situation. Thus, there is a certain parallelism between the concentrations needed for uncoupling and for the analgesic or the anti-inflammatory effects of NSAIDs. According to the data presented in Ref. [4], the peak plasma concentration of aspirin in patients treated for inflammation is 2 mM, whereas the tissue concentration is even higher (12 mM), which means that aspirin exerts its anti-inflammatory effects at concentrations at which its uncoupling action is very clear. Of course, it may be argued that the real concentration in the liver tissue could increase appreciably due to the binding which

would occur during the perfusion (when the drug is infused at a constant rate). Although a certain degree of binding is likely to occur, as shown for diclofenac by clearance studies [25], there are two arguments against a strong and extensive binding. Since the effects are dose-dependent, a large increase in the bound drug should lead to a large time-dependent increase in the effects. At least for aspirin, our data show that this is not the case, the main effect being produced immediately after the addition of the drug, although, in the case of diclofenac, a slight time-dependent additive effect can be observed. The fast reversibility of the effects also argue against a strong and extensive binding, even in the case of diclofenac. Thus, most likely, uncoupling occurs at concentrations close to the pharmacological doses. Therefore, we think that more attention should be given to this phenomenon, at least from the toxicological viewpoint.

Based on the available information, one could probably argue that the uncoupling effect has some importance in the analgesic action of NSAIDs and similar drugs. The anti-inflammatory action, however, can hardly be reconciled with the uncoupling effect, which has two important consequences: Ca^{2+} release from mitochondria and heat liberation [12].

Calcium is implicated as a second messenger in the control of many cellular events (see, for example, Ref. [26]), including the aggregation of the inflammation cells and the release of the inflammatory factors [2,3]. Therefore, an increase of Ca^{2+} in the cytosol would not stop the inflammation but stimulate this process. For glucose metabolism, however, Ca^{2+} release may represent one of the mechanism by which uncoupling can influence it. More specifically, glycogen phosphorylase (the key enzyme in glycogen breakdown), which is stimulated by increased calcium concentration [27] will intensify GGL and GL. Also, under the conditions of high levels of cytosolic calcium, the reaccumulation of this ion by mitochondria or its extrusion from the cell, which are energy-dependent processes of the highest priority, can easily compete with GNG and thus inhibit it.

As regards the heat dissipation, its consequences for the specific action of NSAIDs are very debatable. Logically, heat should increase the inflammation process. Nevertheless, experience shows that cold (and not heat) is among the triggering factors of acute

inflammation. Thus, despite an apparent incompatibility, uncoupling may have a certain significance for the action of NSAIDs.

It is clear, however, that such a complicated process as the anti-inflammatory action involves much more components of the cell than the lipid phase of the membrane (which mediates the uncoupling phenomenon). So far, the limited understanding of the metabolic regulation (especially, that of energy control) of such intricate processes does not provide a clear cause–effect relationship. Even the effect of NSAIDs on carbohydrate metabolism, which must be a secondary one, may be much more complicated than we described. For example, it has been shown relatively recently that, in the perfused liver, certain prostaglandins (of the type involved in the inflammation process) are able to stimulate both glycogenolysis [28] and gluconeogenesis [29,30]. However, since the concentration of prostaglandins in our study was not measured, it is difficult to assess the relevance of such observations for the uncoupling phenomenon, proposed by others and further documented by us as the main mechanism by which NSAIDs affect glucose metabolism.

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