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TRANSPORT, DISTRIBUTION SPACE AND INTRACELLULAR CONCENTRATION OF THE ANTI-INFLAMMATORY DRUG NIFLUMIC ACID IN THE PERFUSED RAT LIVER

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Abstract—Transport and distribution space of niflumic acid in the perfused rat liver were investigated employing the multiple-indicator dilution technique with constant infusion of the drug (step input). Niflumic acid permeated the cell membrane in both directions at very high rates and its distribution in the cellular space was flow-limited; at least at 37°, the rates of influx and efflux could not be measured. Dissociation of the niflumic acid–albumin complex also occurred at very high rates. The apparent space of distribution of niflumic acid in the liver depended on the concentration of the drug and varied between 4.37 (1 mM) and 43.5 (10 μM) times the water space; even with 90% extracellular binding to albumin, the apparent space of distribution of niflumic acid was 5.1 times greater than the water space. The high apparent spaces of distribution reflected the high intracellular concentrations. The ratio of intracellular bound plus free concentration to the extracellular bound plus free concentration (C_i/C_e) varied between 6.62 (1 mM portal niflumic acid) and 71.0 (10 μM portal niflumic acid). Metabolic transformation depended on the concentration of the free form. Intracellular binding is probably the major reason for the high concentration of the drug in the hepatic tissue.

Studies on biotransformation, excretion and distribution of the anti-inflammatory drug niflumic acid and related compounds [1, 2] revealed that the hepatic tissue has a high affinity for these compounds. In rats injected with [^{14}C]niflumic acid, for example, the concentration of radioactivity in the liver is always higher than in the blood, in spite of the fact that the plasma proteins bind the compound very strongly [1, 2]. Glasson *et al.* [2] have interpreted these observations as a consequence of biotransformation, which occurs in the liver. However, the function of biotransformation is to facilitate elimination, and it is equally possible that the liver is able to concentrate the unmetabolized drug. In fact, previous work in our laboratory strongly suggested that the apparent spaces of distribution of both niflumic [3, 4] and mefenamic [5] acids are greater than the water space, a phenomenon similar to that described for monohydric alcohols [6] and octanoate [7]. These aspects, however, have not been examined in detail until now, and the purpose of the present work was to investigate transport and distribution space of niflumic acid. The method employed in this work was the multiple-indicator dilution technique [8], with constant infusion of niflumic acid and [^{3}H]-water (step input) and mathematical analysis of the outflow curves, using the model proposed by Goresky *et al.* [9]. This study was greatly facilitated by our previous work, in which the binding parameters of niflumic acid to bovine serum albumin were measured [10].

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MATERIALS AND METHODS

Liver perfusion. Male albino rats (Wistar strain; 200–250 g) received a standard laboratory diet (Purina) and water *ad lib.* prior to the surgical removal of the liver under pentobarbital anesthesia (50 mg/kg). The perfusion technique described by Scholz *et al.* [11] was used. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer, pH 7.4, saturated with an oxygen:carbon dioxide mixture (95:5%) and containing between 5 and 50 μM fatty acid-free bovine serum albumin. The fluid was pumped through a temperature-regulated (37°) membrane oxygenator prior to entering the liver via a cannula inserted in the portal vein. Viability of the liver was judged from oxygen consumption which was monitored continuously by means of a platinum electrode. Infusion of niflumic acid and [^{3}H]-water was initiated after oxygen consumption was stabilized. This occurred, in general, 20–25 min after isolation of the liver.

Indicator-dilution experiments. Indicator-dilution experiments were performed employing the constant infusion method (step input) [8]. In all experiments [^{3}H]water and niflumic acid were infused simultaneously into the liver prior to entering the portal vein. During the first 90 sec after the onset of the infusion, the perfusate was fractionated in 0.5 to 2.0-sec fractions by means of a specially designed fraction collector [12]. After the first 90 sec, samples were taken manually in intervals ranging from 15 to 120 sec. Aliquots of these samples were used for the assay of [^{3}H]water and for the extraction and assay of niflumic acid.

Analytical. Niflumic acid was extracted from the perfusate samples with an equal volume of ethyl

acetate and assayed spectrophotometrically at 287 nm (extinction coefficient = 21 $mM^{-1} cm^{-1}$ in ethyl acetate). After the addition of ethyl acetate, the mixture was shaken vigorously for 30 sec. After standing for 1 hr, an aliquot of the upper phase was pipetted carefully into a quartz cuvette, and the absorbance at 287 nm was measured. An identical procedure was adopted for samples from the inflowing perfusion fluid (perfusion fluid before passing through the liver). Approximately 85% of niflumic acid is extracted by ethyl acetate at pH 7.4. Albumin and glucuronide conjugates, the latter accounting for 90% of the niflumic acid metabolites [2], are not extracted under these conditions.

Materials. The perfusion apparatus was built in the workshops of the University of Maringá. Fatty acid-free bovine serum albumin, niflumic acid and all enzymes and coenzymes used in the metabolite assays were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The reagent grade chemicals were from Merck (Darmstadt, FRG), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

Calculations. The dilution curves of niflumic acid were analyzed by means of the space-distributed and variable transit-time model of Goresky and coworkers [6, 9]. This model describes exchange and metabolic transformation of substances in the liver. For the case of flow-limited distribution in the whole liver space, the model of Goresky yields the following equation for a step input (constant infusion) into the liver [13] (see Appendix):

$$Q_{nif}(t) = \int_{t_0}^{t} \frac{1}{1+\Phi} Q'_{water}\left[\frac{(t-t_0)}{1+\Phi} + t_0\right] \cdot \exp[-(k_3)(t-t_0)]\, dt \quad (1)$$

In equation (1), $(1 + \Phi)$ is the ratio of the apparent distribution space of niflumic acid to the distribution space of labeled water. When Φ is zero, the distribution space of niflumic acid is equal to the water space. The parameter t_0 is the transit time in the large vessels and in the catheters between the liver and the collecting system. The term $\frac{1}{1+\Phi} Q'_{water}\left[\frac{(t-t_0)}{(1+\Phi)} + t_0\right]$ is the first derivative of the [^{3}H]water curve at time $[(t - t_0)/(1 + \Phi) + t_0]$ and divided by the factor $(1 + \Phi)$. This transformed water curve is the appropriate reference curve for niflumic acid, i.e. a hypothetical substance that occupies the same space as niflumic acid but does not suffer metabolic transformation (see Appendix). The measurable biotransformation rate parameter, k_3, is a composite rate constant, a function of the kinetic constants of the enzymic systems involved in biotransformation (K_m and V_{max}), of the intracellular concentration and of the intracellular binding degree. It has the dimensions of $mL \cdot sec^{-1} \cdot (mL$ total intracellular space accessible to niflumic acid$)^{-1}$.

In the case of flow-limited distribution, the rate constants for exchange between the extra- and intracellular spaces are high and cannot be determined [6, 9]. Their ratio, however, can be calculated. An expression relating the composite rate constants for influx (k_1) and efflux (k_2) to Φ can be derived from the known relations between transit times, distribution spaces, space ratios and rate constants [8, 12, 14, 15] (see Appendix):

$$\frac{k_1}{k_2} = 1 + \Phi + \Phi/\Theta' \quad (2)$$

In equation (2), Θ' is the ratio of the intracellular to extracellular water spaces [12, 14, 15].

When the extracellular concentration (C_e) is known, the intracellular concentration (C_i), at steady-state conditions, can be calculated from the relation [7, 16]:

$$C_i = \frac{k_1}{k_2 + k_3} C_e \quad (3)$$

For the flow-limited case, however, $k_2 \gg k_3$, and equation (3) becomes:

$$C_i = (k_1/k_2) \cdot C_e = (1 + \Phi + \Phi/\Theta') \cdot C_e \quad (4)$$

C_i calculated according to equation (4) refers to the whole intracellular water space and represents a mean over all intracellular compartments, including the bound and unbound forms [7, 16].

The parameters Φ, k_3 and t_0 were determined by fitting equation (1) to the experimental niflumic acid curves employing a non-linear least-squares procedure [17]. An iterative procedure, which generates the appropriate reference curve, was used. Preliminary estimates of t_0, Φ and k_3 were introduced, and the iteration was continued until the sum of squares of the differences between the experimental and calculated niflumic acid curves (standard error of the estimate) was minimal. Interpolation between the experimental points and calculation of the first derivative of the [^{3}H]water curve were performed by means of a spline function [17]. The trapezoid rule was used for numerical integration [17]. The bound and free niflumic acid concentrations were calculated employing the algorithm of Abumrad *et al.* [18] with the dissociation constants measured by Kelmer-Bracht *et al.* [10]. Analysis of variance and other statistical evaluations were made employing the Primer program version 1.0 (McGraw-Hill, 1988).

RESULTS

Outflow profiles of niflumic acid in response to step inputs. Figures 1 and 2 show a series of representative outflow profiles of niflumic acid obtained under several conditions. In the series of Fig. 1, albumin was absent from the perfusion fluid and the portal niflumic acid concentration was varied between 10 and 1000 μM in several liver perfusion experiments. Tritiated water was always infused simultaneously, but for clarity only the curve obtained with 1000 μM niflumic acid is shown in Fig. 1. All curves were normalized, i.e. the venous concentrations were divided by the portal one. The [^{3}H]water curve rapidly approached unity, indicating the absence of metabolic transformation. In the range between 10 and 1000 μM, niflumic acid was delayed in relation to [^{3}H]water, reflecting a higher, real or apparent, distribution space. At 10 μM, niflumic acid appeared in the venous perfusate at

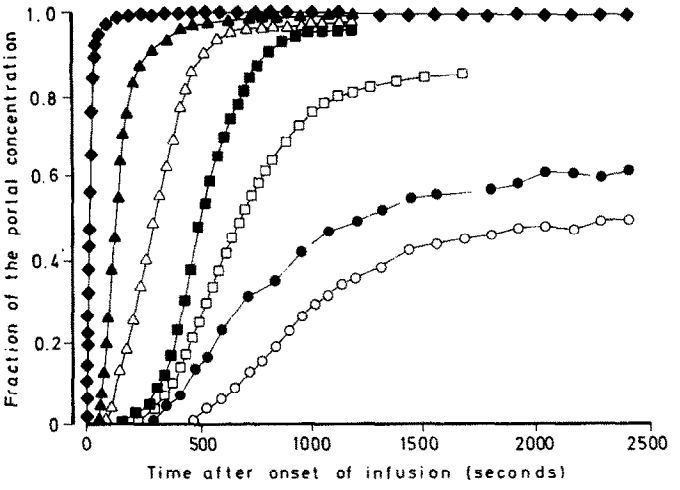

Fig. 1. Indicator-dilution experiments with various portal concentrations of niflumic acid in the absence of albumin. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4). Niflumic acid, at various concentrations, and [^{3}H]water (2.5 μCi/min) were infused simultaneously. The venous perfusate was fractionated in 0.5- to 120-sec fractions. [^{3}H]Water was determined in a β-counter. Niflumic acid was extracted with ethyl acetate and determined spectrophotometrically at 287 nm. The venous/portal concentration ratios of each substance were plotted versus the time after onset of the infusion. The portal niflumic acid concentrations were: 1 mM (▲—▲); 200 μM (△—△); 100 μM (■—■); 50 μM (□—□); 20 μM (●—●); and 10 μM (○—○). For clarity, only the [^{3}H]water curve obtained with 1 mM niflumic acid is shown (◆—◆).

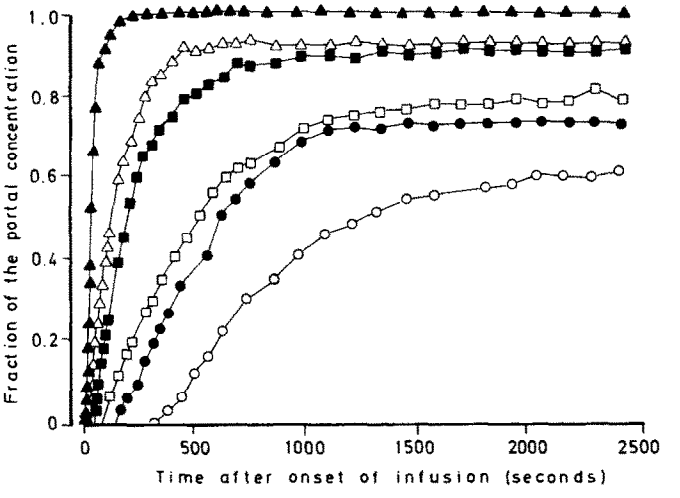

Fig. 2. Indicator-dilution experiments with variable total niflumic acid and albumin concentrations. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing albumin at various concentrations up to 50 μM. Niflumic acid (between 20 and 176.6 μM) and [^{3}H]water (2.5 μCi/min) were infused simultaneously. The concentrations of albumin and niflumic acid in the portal vein were varied, but the portal concentration of unbound niflumic acid was always 20 μM. The venous perfusate was collected in 0.5- to 120-sec fractions. [^{3}H]Water was determined in a β-counter. Niflumic acid was extracted with ethyl acetate and determined spectrophotometrically at 287 nm. The venous/portal concentration ratios were represented versus the time after the onset of infusion. The portal concentrations of albumin and niflumic acid were: absence of albumin + 20 μM niflumic acid (○—○); 5 μM albumin + 35.7 μM niflumic acid (●—●); 10 μM albumin + 51.3 μM niflumic acid (□—□); 20 μM albumin + 82.7 μM niflumic acid (■—■); and 50 μM albumin + 176.7 μM niflumic acid (△—△). For clarity, only the [^{3}H]water curve obtained with 176.6 μM niflumic acid is shown (▲—▲).

480 sec after the onset of infusion, whereas [^{3}H]water appeared at 2–3 sec. As the portal concentration was raised, however, the delay of niflumic acid diminished progressively. At low portal concentrations, the venous/portal fraction of niflumic acid approaches asymptotically steady-state values well below unity, a fact that reflected metabolic transformation. As the concentration was raised, the transformed fraction under steady-state conditions decreased. Indeed, for portal concentrations above 100 μM, the transformed fraction was very low and could not be determined with confidence.

Since albumin was absent in the series of experiments in Fig. 1, only free (i.e. unbound) niflumic acid was present in the extracellular space. In the series of experiments shown in Fig. 2, the total niflumic acid concentrations were varied from 20 μM to 176.7 μM, but the portal free niflumic acid concentration was maintained at 20 μM. This was accomplished by simultaneous increases of the albumin concentrations (up to 50 μM). The dissociation constants, necessary to calculate the correct concentrations of ligand and protein, were determined previously [10]. Figure 2 reveals that the distribution space of niflumic acid decreased as the fraction of the bound form increased. The transformed fraction also decreased. All niflumic acid curves in Fig. 2 were situated at the right of the [^{3}H]water curve even at 50 μM albumin and 176.7 μM niflumic acid, where the binding degree was as high as 90%.

Model analysis of the outflow profiles of niflumic acid. Little information can be obtained from the dilution curves without model analysis. When analyzed according to the model of Goresky and coworkers [6, 9], the forms of the outflow profiles of niflumic acid strongly suggested flow-limited distribution, a situation in which the exchange rates tend to infinity and cannot be determined. Flow-limited distribution was suggested by the fact that the niflumic acid curve was always delayed and entirely displaced to the right in relation to the [^{3}H]-water curve. It is well known that [^{3}H]water undergoes flow-limited distribution in the liver [6, 12]. Dilution curves of substances, such as palmitate, whose distribution is not flow-limited, are situated, especially in the initial upslope, at the left of the labeled water curve [16]. If the distribution of niflumic acid is really flow-limited, the experimental dilution curves should conform to equation (1). To verify this, equation (1) was fitted to all experimental curves using the numerical procedures described in Materials and Methods. In general, a good agreement was found between theory and experiment. Figure 3 shows an example and illustrates some aspects of the calculations. The experimental data shown in Fig. 3 were obtained with 5 μM albumin and 35.5 μM niflumic acid in the portal perfusate. In Fig. 3A, the experimental points (●) are compared with the theoretical curve (——), calculated with the optimized values for Φ, k_3 and t_0. The calculated curve describes reasonably well the experimental curve, the determination coefficient being equal to 0.997. If k_3 is set equal to zero, the curve calculated with the optimized values for Φ and t_0 represents the curve of the appropriate reference for niflumic acid, as defined in Materials and Methods (see also the Appendix). As revealed by Fig 3A, this curve (– – – –) was very close to the niflumic acid curve at the initial upslope, but it departed progressively and tended asymptomatically to unity. Figure 3B shows the [^{3}H]water curve and the first points of the niflumic acid curve on an expanded time scale. The continuous line joining the [^{3}H]water experimental points was calculated with the optimized t_0 value, but with both Φ and k_3 equal to zero. The agreement between the experimental points and the computed curve in Fig. 3B evidences the correctness of the numerical procedures.

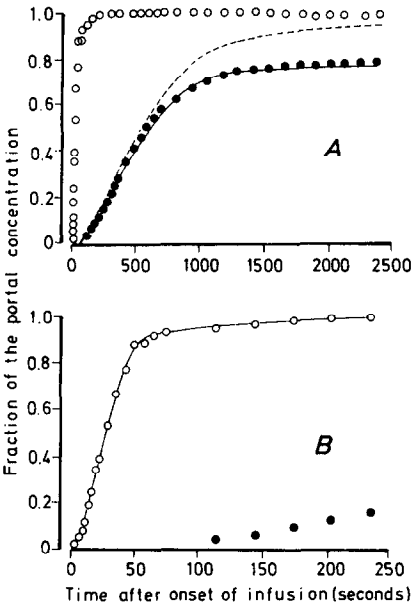

Fig. 3. Fitting of equation (1) to the experimental niflumic acid curves. The experimental conditions were 35.5 μM niflumic acid and 5 μM albumin. The experimental points of the [^{3}H]water curve (○—○) and of the niflumic acid curve (●—●) are shown. The continuous line in panel A (——) was calculated with optimized values of the parameters: $t_0 = 3.578$ sec; $k_3 = 3.21 \cdot 10^{-4}$ mL·sec^{-1}·mL^{-1}; and $\Phi = 22.38$. The standard error of the estimate was 0.0126 and the determination coefficient was equal to 0.997. The broken line in panel A (– – – –) was calculated with the same values of the parameters, but with $k_3 = 0$. In panel B the same [^{3}H]water curve as in panel A is represented on an expanded time scale. The continuous curve in panel B was calculated with Φ and k_3 equal to zero and with $t_0 = 3.578$ sec.

Distribution spaces and rates of biotransformation. In Figs. 4 and 5, the parameters obtained by fitting equation (1) to the experimental curves, $(1 + \Phi)$ and k_3, were plotted against the portal niflumic acid concentration. Figure 4 shows data from a series of experiments in which the portal niflumic acid concentration was varied in the absence of albumin (Fig. 1); and Fig. 5 illustrates a series of experiments performed with the constant portal concentration of free niflumic acid (Fig. 2). In addition to the space ratio $(1 + \Phi)$ and k_3, Figs. 4 and 5 also show the

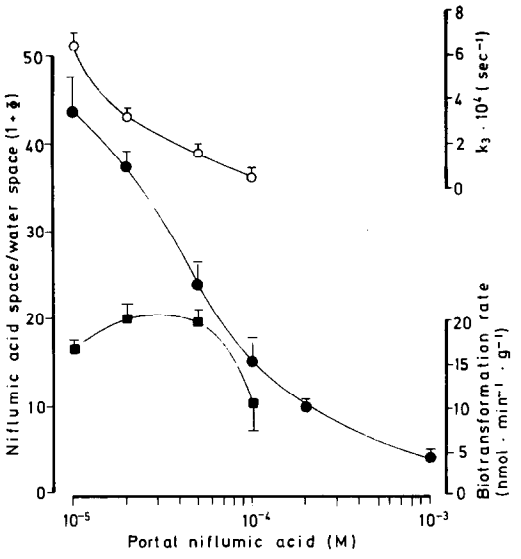

Fig. 4. Derived parameters of indicator-dilution experiments with variable portal niflumic acid concentrations, without albumin in the perfusion fluid. The parameters Φ and k_3 were obtained by means of least-squares fits of equation (1) to the experimental data. The rates of biotransformation were calculated from the portal venous differences under steady-state conditions, the flow rate, and the wet weight of the liver. Key: $(1 + \Phi)$, (●—●); k_3 (○—○); and rates of biotransformation (■—■). Each point is the mean of 3–7 experiments. The vertical bars represent standard errors.

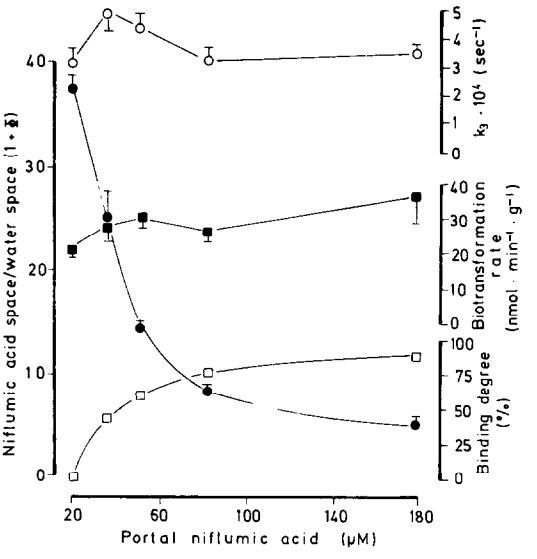

Fig. 5. Derived parameters from indicator dilution experiments performed with variable niflumic acid and albumin concentrations, but with a constant free concentration of the drug. The parameters Φ and k_3 were obtained by means of least-squares fits of equation (1) to the experimental data. The rates of biotransformation were calculated from the portal venous differences under steady-state conditions, the flow rate, and the wet weight of the liver. The binding degree was calculated using the dissociation constants determined by Kelmer-Bracht *et al.* [10]. Key: $(1 + \Phi)$ (●—●); k_3 (○—○); rates of biotransformation (■—■); and degree of binding of niflumic acid in the portal perfusate (□—□). Each data point is the mean of 3–5 experiments. The vertical bars represent standard errors.

rates of biotransformation, calculated from the portal-venous concentration differences under steady-state conditions and expressed as nmol·min^{-1}·g^{-1}. All data points are the means of 3–7 liver perfusion experiments.

In the absence of circulating albumin, the niflumic acid to water space ratio $(1 + \Phi)$ was very high at low concentrations (Fig. 4). At 10 μM portal niflumic acid, it was equal to 43.5 ± 4.7 (N = 5). However, it decreased progressively with the increasing concentrations. At 1 mM, it was reduced to 4.37 ± 0.6 (N = 3). The rate constant for biotransformation also decreased with the concentration. For concentrations above 100 μM it could not be determined with confidence. Even at 100 μM the SEM was nearly as high as the value itself. The decreasing values of the rate constant were expected as a consequence of saturation of the enzymic systems catalyzing metabolic transformation [19]. This was indeed the case, as revealed by the rates of biotransformation also represented in Fig. 4. Nevertheless, the concentration dependence shows that, in addition to saturation, a kind of substrate-inhibition was also present: between 50 and 100 μM the rate of biotransformation decreased. The maximum rate of biotransformation, measured at 50 μM, was equal to 19.6 ± 1.5 nmol·min^{-1}·g^{-1} (N = 4). This substrate-inhibition phenomenon was probably due to the metabolic effects of the drug, which impairs oxidative phosphorylation [3, 4].

Figure 5 shows the parameters calculated from the constant free niflumic acid series in addition to the binding degree of the drug in the perfusion fluid. The space ratio $(1 + \Phi)$ decreased progressively as the binding degree increased from zero to 90%. With 90% binding, however, it was still equal to 5.1 ± 0.7 (N = 3). The rate of biotransformation changed very little. There was an ascending tendency, but it lacked statistical significance, as revealed by an analysis of variance (P = 0.096). The rate constants for biotransformation also presented small variations, but these differences were not statistically significant (P = 0.075).

Extracellular versus intracellular concentrations. According to equation (4), which is valid for the flow-limited condition, the intracellular concentration (C_i) can be calculated provided that Φ, Θ' and the extracellular concentration (C_e) are known. Rigorously, the extracellular concentration decreases along the sinusoids unless metabolism is negligibly small, as for example at 1 mM portal niflumic acid. When a concentration gradient along the sinusoids exists, as for example at 10 μM portal niflumic acid, only a mean value can be calculated for C_e. Evidently, if one employs mean values for C_e in the calculations, the resulting C_i values are also mean intracellular concentrations. This can lead to deviations, the

errors being more pronounced when the portal venous differences are high. Goresky *et al.* [13] have proposed a logarithmic mean for C_e, which is valid as long as extraction is moderate. It should be added that, if calculated according to equation (4), C_i will refer to the whole intracellular water space. This does not mean, however, that the drug is only present as a free solute in the aqueous medium of the cell.

To see how C_i varies with C_e, the former was calculated from the various Φ and C_e values. For the parameter Θ', the mean value of 1.5, as determined for the hemoglobin-free perfused rat liver by previous work in our laboratory, was adopted [15, 16]. Figure 6A shows the results, C_e being represented on a logarithmic scale. When no albumin was present in the perfusion fluid, C_i increased with C_e. At one extreme, when C_e was 6.8 μM, C_i was 483 ± 54 μM. This means a large, true or apparent, concentration gradient, C_i/C_e being equal to 71.0. At the other extreme, with C_e equal to 1.0 mM, C_i was 6.6 ± 1.0 mM and the C_i/C_e ratio was reduced to 6.6. This implies that C_i saturates with increasing C_e, a phenomenon revealed more clearly in the linear representation of Fig. 6C. For the series of experiments in which the portal free niflumic acid concentration was maintained constant (○—○ in Fig. 6A), on the other hand, C_i was practically the same for the various C_e values, the mean value being equal to 1179 ± 58 μM. This finding is expected on thermodynamic grounds, provided that, according to the current view, there is no direct interaction of the protein-bound drug with the membrane. It should be added that, rigorously speaking, the constant portal concentrations of free niflumic acid do not mean constant free concentrations over the entire sinusoidal bed because the metabolic transformation produces a gradient along the sinusoids. This gradient, however, is not very pronounced, as revealed by Fig. 6B. In this graph, the mean value of the free extracellular concentration (C_{ef}), calculated according to Goresky *et al.* [13] from the portal and venous free niflumic acid concentrations and assuming binding equilibrium with albumin, is plotted against the portal total niflumic acid concentration. The portal free niflumic acid concentration was equal to 20 μM, as already mentioned for this experimental series. The mean concentration (C_{ef}), however, deviated from this value, the discrepancy being higher at the lowest concentration, as revealed by Fig. 6B. The difference between the extremes, however, amounted to only 12%. This is a small difference, which could not be detected in the calculated C_i values because the expected changes were smaller than the experimental errors.

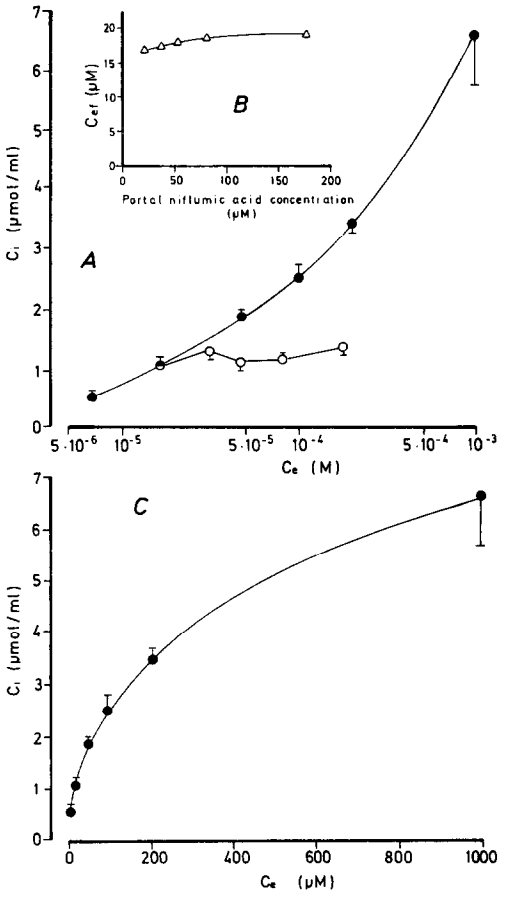

Fig. 6. Intracellular niflumic acid concentration (C_i) versus the extracellular concentration (C_e, A and C) and mean free extracellular concentration (C_{ef}) versus the portal total concentration (B). C_i was calculated employing equation (4). C_e is the logarithmic mean of the portal venous niflumic acid concentrations, calculated according to Goresky *et al.* [13]. C_e is represented on a logarithmic scale in panel A and on a linear scale in panel C. Two series of experiments are shown: (1) the series in which the niflumic acid concentration was varied in the absence of albumin (●—●; A and C); and (2) the series in which niflumic acid and albumin were varied simultaneously without alteration of the portal free niflumic acid concentration (○—○; A). For the latter experimental series, in panel B, the mean free extracellular concentration (C_{ef}) is plotted against the portal total (free + unbound) niflumic acid concentration. Each data point is the mean of 3–7 experiments. The vertical bars represent standard errors.

DISCUSSION

The main results obtained in this work can be summarized as follows: (1) the behavior of niflumic acid in the liver could be described adequately by the space-distributed variable transit-time model of Goresky; (2) niflumic acid permeated the cell membrane in both directions at very high rates, and its distribution in the cellular space was flow-limited; at least at 37°, the rates of influx or efflux could not be measured; (3) dissociation of the albumin–niflumic acid complex also occurred at very high rates; (4) the apparent space of distribution of niflumic acid in the liver depended on the concentration of the drug and was as high as 43.5 times the water space at 10 μM portal niflumic acid; even with 90% extracellular binding the niflumic acid space was 5.1 times greater than the water space; (5) the high apparent spaces of niflumic acid reflected the high intracellular pools; the intracellular

to extracellular concentration ratio (C_i/C_e) was as high as 71.0 at 10 μM portal niflumic acid; and (6) metabolic transformation seemed to correlate with the concentration of free drug.

It was possible to analyze the behavior of niflumic acid in terms of the model of Goresky *et al.* [9]. That this model provides a good description of metabolism and transport has been shown by studies performed with several substances—hydrophilic [12, 14, 15] as well as lipophilic ones [6, 7, 16, 20]. The present study differs, however, from the previous reports in that a step input (constant infusion) was used rather than an impulse input (single injection). Labeled niflumic acid is not currently available, and recoveries are more difficult to determine in the single injection method when a substance is measured spectrophotometrically because many dilution steps are necessary. Moreover, in the case of bulk infusions, the measured parameters can be related to the portal concentrations instead of being analyzed in terms of the dose injected. Theoretically, the experimental procedure adopted in this work presents no difficulties, because it is clear that the answer of the liver to a constant infusion (step input) corresponds to the time integral of the answer to a single injection [8].

Bulk infusion instead of tracer infusion under previously established steady-state conditions, however, is likely to produce some distortions in the measured parameters. One should recall that the parameters measured in this work, Φ and k_3, are functions of the concentration of niflumic acid. Although the portal concentration was essentially constant in each liver perfusion experiment, the venous concentration varied with time until steady-state conditions were achieved. Consequently, a concentration gradient, varying with time, was present along the sinusoidal beds. For this reason, the optimized Φ and k_3 values represent a mean value between the portal venous steepest (start of the infusion) and smallest (end of the infusion) concentration gradient. The effect of these concentration gradients, however, was not very pronounced. Otherwise, equation (1), which presupposes that Φ and k_3 do not vary with time, would not fit to the experimental data. In general, however, the agreement between equation (1) and the experimental data is comparable to that illustrated by Fig. 3. The determination coefficients were always between 0.95 and 0.99. The effect of concentration gradients can be reduced, but not eliminated completely, by using tracer infusions under previously established steady-state conditions. Unfortunately, as already mentioned, labeled forms of niflumic acid and its analogs are not currently available.

A question arises concerning the reason for the high intracellular pools of niflumic acid. In the case of the monohydric alcohols, their higher apparent spaces of distribution were almost reduced to the water space when ethanol was infused, and Goresky *et al.* [6] proposed a shared enzymic space, resulting from binding of the alcohols to alcohol dehydrogenase in the intracellular medium. In the case of niflumic acid, there are no experimental data that could give a definitive answer to this question. As a working hypothesis one can assume, however, that niflumic acid behaves like many other lipophilic substances and that it binds to intracellular proteins (Z protein, for example) [21]. Furthermore, it is equally possible that niflumic acid binds to intracellular membranes [22]. This becomes more plausible if one remembers that niflumic acid acts as an uncoupler of oxidative phosphorylation, and it is well known that membranous structures usually have a high affinity for substances that act as uncouplers [23]. The possibility that the high intracellular concentrations are the consequence of active transport is unlikely. Since exchange is very fast, it would be very difficult for the cell to maintain such an active transport system because its activity would have to exceed the maximal activity of the respiratory chain.

One should recall at this point the observations of Glasson *et al.* [2] concerning the high concentration of radioactivity derived from [^{14}C]niflumic acid in the livers of rats injected with this substance. Glasson *et al.* [2] interpreted this observation as a consequence of the metabolic transformation of niflumic acid, which occurs in the hepatic tissue. It is clear that biotransformation, by generating multiple forms, could be contributing to the increased concentration of radioactivity in the rat liver. Taking our results into account, however, it is highly probable that a considerable part of the radioactivity found by Glasson *et al.* [2] in the hepatic tissue actually represented the unmetabolized drug. High affinity for niflumic acid is not an exclusive property of the liver. As shown by Dorfmann *et al.* [24], the concentration ratio of synovial tissue to plasma in adult humans is equal to 1.25, a high value if one considers binding to albumin in the extracellular space. In the nervous tissue, on the contrary, the concentration of the drug is much lower than in the plasma [2].

A further question is whether niflumic acid can be regarded as a model substance for other non-steroidal anti-inflammatory drugs. Structurally, niflumic acid is very similar to a series of anti-inflammatory drugs [1]. The long list includes mefenamic acid, meclofenamic acid, etofenamic acid, and flufenamic acid and many others [1]. At least for one of these compounds experimental evidence indicates that it behaves like niflumic acid. Outflow profiles of mefenamic acid have been measured routinely during experiments in which the metabolic effects of the drug were under investigation [5]. Although the measured outflow profiles probably include both mefenamic acid and the resulting metabolites, there is no doubt that mefenamic acid also has a very high apparent space compared with the water space and that it undergoes flow-limited distribution. It is possible that these characteristics are common features of all fenamic acids.

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APPENDIX

Derivation of equations (1) *and* (2)

Consider a substance that undergoes flow-limited distribution into a given space in the liver and that is irreversibly removed from or metabolically transformed in the space. It has been demonstrated that the response of the liver to an impulse input (single injection) of such a substance can be described by the following equation [6, 9, 13, 25]:

$$Q'(t) = f(t) \cdot \exp[-k_3(t - t_0)] \qquad (1A)$$

In equation (1A), t represents the time after injection, t_0 the transit time in the large vessels, and k_3 the removal rate constant. The function $f(t)$ represents the frequency distribution of the transit times in the small vessels. This frequency distribution function can be obtained experimentally by injecting simultaneously a reference substance that occupies the same space as the substance under study. In the case of sulfobromophtalein, which is irreversibly removed from the extracellular space, the adequate reference is a substance that distributes into the extracellular space (albumin, for example [25]). For ethanol, the adequate reference is tritiated water, which undergoes flow-limited distribution into the whole aqueous space of the liver [6].

Equation (1A) describes the response to an impulse input. In the case of a step input (constant infusion), the response is the time integral of equation (1A) [8, 9]:

$$Q(t) = \int_{t_0}^{t} f(t) \cdot \exp[-k_3(t - t_0)]\, dt \qquad (2A)$$

The frequency distribution function $f(t)$ can be obtained by taking the first derivative of the outflow curve of a substance that distributes into the same space and that was infused simultaneously. This may prove impossible, especially when the distribution space of the substance depends on its concentration, as happens with niflumic acid. An adequate reference, however, can be generated from the outflow curve of another indicator that undergoes flow-limited distribution. It has been shown that the outflow profiles resulting from the single injection of substances that distribute in a flow-limited manner in the liver are related to each other by a single space ratio [9]. If $(1 + \Phi)$ is this space ratio, than the outflow profile of labeled water resulting from single injection (impulse input), or the first derivative of the outflow curve resulting from a step input ($Q'_{water}[t]$), can be transformed as follows [9]:

$$Q'_n(t) = f(t) = \frac{1}{1 + \Phi} Q'_{water}\left[\frac{(t - t_0)}{(1 + \Phi)} + t_0\right] \qquad (3A)$$

The transformed water curve, $Q'_n(t)$, can be obtained by changing both the time scale (corrected for t_0) and the amplitude by the same factor $(1 + \Phi)$. Substitution of $f(t)$ in equation (2A) yields equation (1).

The transformed water curve $Q'_n(t)$, corresponds to the curve of a hypothetical substance that crosses the cell membrane in the same way as niflumic acid, occupies the same space as niflumic acid, but does not suffer metabolic transformation. Thus, the factor $(1 + \Phi)$ is the ratio of the apparent space of distribution of niflumic acid to the water space in the liver. The space ratio $(1 + \Phi)$ can also be defined in terms of the mean transit times of labeled water ($\bar{t}_w$) and of the transformed reference ($\bar{t}_n$) [8]:

$$\frac{\bar{t}_n}{\bar{t}_w} = 1 + \Phi \qquad (4A)$$

A similar relation between mean transit times can be used to define the ratio of intracellular to extracellular spaces in the liver, Θ' [12, 14, 15]:

$$\frac{\bar{t}_w - \bar{t}_r}{\bar{t}_r} = \Theta' \qquad (5A)$$

In equation (5A), $\bar{t}_r$ is the transit time of a reference for the extracellular space. Furthermore, the following relation between the rate constants for influx (k_1) and efflux (k_2) and the mean transit time has been demonstrated to be valid [12, 14, 15]:

$$\frac{k_1}{k_2} = \frac{\bar{t}_n - \bar{t}_r}{\bar{t}_w - \bar{t}_r} \qquad (6A)$$

Solving equation (5A) for $\bar{t}_r$, substituting into equation (6A), and rearranging yields:

$$\frac{k_1}{k_2} = \frac{(1 + \Theta')\bar{t}_n}{\Theta'\bar{t}_w} - \frac{1}{\Theta'} \qquad (7A)$$

Finally, introducing $(1 + \Phi)$ for $\bar{t}_n/\bar{t}_w$, according to equation (4A), and rearranging leads to equation (2):

$$\frac{k_1}{k_2} = 1 + \Phi + \Phi/\Theta' \qquad (2)$$

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