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UPTAKE OF RIFAMPICIN BY ISOLATED RAT LIVER CELLS. INTERACTION WITH SULFOBROMOPHTHALEIN UPTAKE AND EVIDENCE FOR SEPARATE CARRIERS

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Abstract—The mechanism of rifampicin uptake and its relation with that of BSP were studied in isolated rat liver cells. The cells take up rifampicin by a saturable process independent of metabolic energy since antimycin A and potassium cyanide were without effect. The analysis of the uptake revealed two systems which both follow Michaelis–Menten kinetics: the first, with high affinity, displayed an apparent K_m of 0.13 ± 0.08 mM and an apparent V_{max} of 2.3 ± 1.2 nmoles/min/mg protein; the second, with much lower affinity, had an apparent K_m of 1.02 ± 0.46 mM and a V_{max} of 16.0 ± 5.9 nmoles/min/mg protein. Whereas rifampicin exhibited an apparently competitive inhibition on BSP uptake, the uptake of rifampicin was not inhibited by BSP. Moreover, no reciprocal counterflow was observed between these two drugs. These results suggest that rifampicin enters the liver cells by a carrier-mediated process independent of that previously reported for BSP uptake. It is concluded that several carriers are involved in the uptake of anionic drugs by rat liver cells.

Rifampicin administration *in vivo* rapidly causes a large decrease in the initial plasma disappearance rate of sulfobromophthalein (K_1 BSP) and a slight hyperbilirubinemia in man [1]. The absence of binding of rifampicin [2] and of the related rifamycin SV [3] to cytoplasmic acceptor protein, although the latter displays high affinity for BSP, suggests that an interaction on these proteins is not involved. The impairment of BSP uptake is probably located at the membrane level as suggested previously as an explanation for the diminution of K_1 BSP by rifamycin SV in the rat [3]. A competition for the same carrier protein is conceivable since BSP and rifampicin are both organic anions at neutral pH. Four main pathways are postulated for transport of organic compounds in the hepatocyte: one for bile salts, one for organic anions, one for cations and one for uncharged compounds such as ouabain [4]. The influence of protein binding on the uptake of BSP has been well documented [5–8]; the mechanism of rifampicin uptake by liver cells, however, remains still unknown. In the present work we attempt to characterize the process of rifampicin uptake by isolated liver cells, and to study the relation with the mechanism of BSP uptake. Evidence will be given that rifampicin uptake by liver cells is carrier-mediated. The inhibition of BSP uptake by rifampicin, also observed *in vitro*, cannot be explained by a competition for the same carrier. This implies that more than one carrier protein is required for the uptake of organic anions other than bile salts by liver cells.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats weighing 250–300 g (Charles River Inc., Elbeuf, France) were maintained on standard rat chow and water until killed.

Chemicals. [^{35}S]Sulfobromophthalein sodium, [^{14}C]carboxyldextran and the counting scintillation medium (ACS®) were purchased from Amersham Radiochemical Centre and tritiated water from the Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. [$^{14}C_{38}$]- or [$C_{43}{}^{3}H_3$]-labeled and cold rifampicin were gifts of Lepetit Research Laboratory, Milan, Italy. Antimycin A was obtained from the Sigma Chemical Co., St Louis, MO, U.S.A., and unlabeled sulfobromophthalein was purchased from Fluka A.G., Buchs, Switzerland. All other reagents were of analytical grade.

Isolation of liver cells. Isolated liver cells were prepared in accordance with Seglen [9] with the slight modifications described by Le Cam *et al.* [10]. The cells were washed twice in the incubation medium (120 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 20 mM Tris-(hydroxymethyl)-aminomethan gassed with a mixture of O_2 (95%) and CO_2 (5%) and adjusted at pH 7.4 with orthophosphoric acid). The cell suspension was stored at 4° in a culture flask at a concentration of about 40 mg protein/ml as measured by the biuret method [11], before use; the maximal period of storage was 3 hr. Vitality of the cells was assessed as follows: 85–95 per cent of the cells had a highly refractive membrane under light microscopy and excluded Trypan Blue (0.4%, w/v); the intracellular K^+ concentration determined according to Howard *et al.* [12] was 130–160 m-equiv/l after isolation. We verified that it remained unchanged for the following 3 hr under these storage conditions.

Uptake experiments. The incubations were performed on aliquots of the cell suspension diluted to 1 mg protein/ml. After equilibration at 37° in the medium (0.8 ml) for about 5 min, unless otherwise indicated, uptake was initiated by addition of labeled rifampicin, dissolved in dimethylsulfoxide (DMSO) 50%, or labeled BSP dissolved in the incubation medium, and stopped by a 10 sec centrifugation in an Eppendorf 3200 microcentrifuge. In pilot experiments, we verified that 0.17 M DMSO had no effect on rifam-

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picin uptake (rifampicin concentrations being 0.3 or 1 mM).

The supernatant was discarded, the inside of the tube wiped, the pellet dissolved in 4% Na cholate and the radioactivity counted in ACS, using an Intertechnique spectrometer with external standardization. The total radioactivity of the pellet was corrected for the extracellular radioactivity computed from the supernatant radioactivity and the [^{14}C]carboxyldextran diffusion space. This space was measured on each preparation; its mean ±S.D., calculated from 19 experiments, was 0.7 ± 0.2 μl/mg protein. Thus, rifampicin in this space never exceeded 8% of its amount within the whole pellet. The short time incubations (less than 1 min) were performed directly in microtubes (capacity 1 ml). The longer ones took place in a 25 ml Erlenmeyer flask shaken during incubation, aliquots of which (800 μl) were transferred at given times to microtubes and centrifuged. When unlabeled rifampicin or BSP were tested as inhibitors, they were added 5 sec before the labeled substrate. Antimycin A and KCN were preincubated 10 min with the cells.

Countertransport studies. The cells (1 mg/ml) were loaded in an Erlenmeyer flask for 5 min at 37°, in 8 ml of the incubation medium containing the labeled substrate. The cold substrate to be tested was then added and aliquots (800 μl) were transferred at given times to microtubes and spun down. The pellet was treated as indicated above for uptake studies and the radioactivity measured.

Measurement of the intracellular volume. The hepatocytes were incubated with [^{3}H]H_2O and [^{14}C]carboxyldextran for 2 min as in the uptake experiments. The diffusion space of [^{3}H]H_2O minus that of [^{14}C]carboxyldextran allowed an estimation of the aqueous cell volume [13].

RESULTS

Time course of rifampicin uptake. The rifampicin content of isolated liver cells increased linearly during the first minute at 37° and reached equilibrium at about 5 min (Fig. 1). Extrapolation of the linear part of the curve to time zero gives a positive intercept. Such an intercept represents rapid adsorption on the cell membrane, as previously shown for BSP [7] and bile salts [14]. The effect of temperature also supports this view, since at 0° there remains no detectable uptake of rifampicin whereas 60 per cent of the adsorption is still present. The initial velocity of the uptake was determined from the slope of the linear part of the time course curve from measurements made at 10, 20 and 30 sec following the addition of rifampicin. Each point was the mean of a triplicate determination which always agreed within 8 per cent. The linearity of the uptake during this 30-sec period indicates that the positive intercept results only from adsorption of rifampicin on the outside of the cells, which took place before the first sample was taken. In pilot experiments we verified that rifampicin uptake was linearly related to amount of cell protein.

The adsorption data of rifampicin on the cells were analyzed by a Scatchard plot (Fig. 2) which suggested binding to 2 sites: the first had a dissociation constant of 60 μM and a capacity of 0.7 nmole/mg protein; the second was of lower affinity, with a dissociation constant of 1.6 mM and a capacity of 11 nmoles/mg protein.

Irrespective of probable intracellular binding of rifampicin to various organelles (see below for discussion) the total apparent concentrations of this drug in the cells at equilibrium were 0.55, 1.9 and 7.2 mM for respective concentrations of 0.04, 0.2 and 1 mM in the medium. This was calculated on the basis of 2 μl of intracellular volume/mg protein. These figures indicate a concentration factor of 14, 10 and 7 for total drug, respectively.

Influx of rifampicin as a function of extracellular concentration. Figure 3 shows the initial velocity (mean of five experiments) of rifampicin uptake at various external concentrations in a double reciprocal plot. A break in the vicinity of 30 μM divides the plot into two parts. Extrapolation of the first slope on the y and x axes gives a maximal velocity of 1 nmole/min/mg protein and an apparent Michaelis constant of 50 μM, whereas with the second slope a maximal velocity of 18 nmoles/min/mg protein and an apparent Michaelis constant of 1.4 mM were obtained. The K_m values calculated from experiments with hepatocytes obtained from five separate animals were 0.13 ± 0.08 mM (S.D.) and

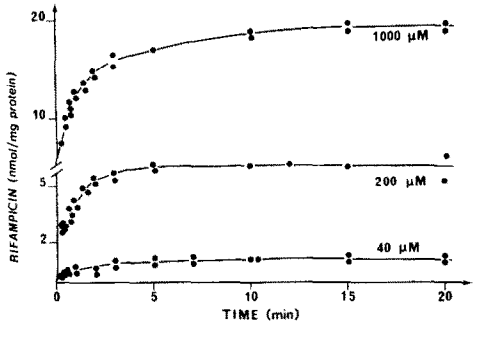

Fig. 1. Time course of rifampicin uptake by isolated rat liver cells at three different concentrations of the antibiotic (40, 200 and 1000 μM). Experimental conditions are indicated under Materials and Methods. Adsorption and initial uptake velocity were determined from zero intercept and slope of the linear part of the curve, respectively.

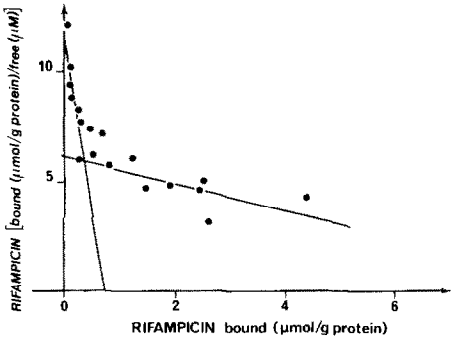

Fig. 2. Scatchard plot of rifampicin adsorption on liver cells. Rifampicin bound was determined as indicated in the text. The concentration range was 7 μM–1 mM. Each point is the average of 3–18 determinations. There are two sets of binding sites. Dissociation constants are obtained from the slopes of the lines: $K_1 = 60 \times 10^{-6}$ M and $K_2 = 1.6 \times 10^{-3}$ M and the number of binding sites from the intercepts on the abcissa: $n_1 = 0.7$ nmole/mg protein, $n_2 = 11$ nmoles/mg protein.

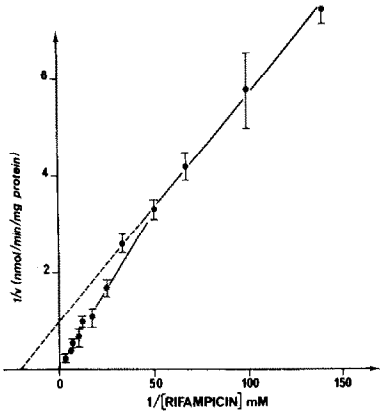

Fig. 3. Lineweaver–Burk plot of rifampicin uptake by liver cells. Initial velocities were determined as indicated in the text. The plot appears divided into two linear parts. From the intercepts on the axes we calculated two apparent Michaelis constants (K_{m_1}: 50 μM; K_{m_2}: 1.4 mM) and two maximal velocities (V_1: 1 nmole/min/mg protein. V_2: 18 nmoles/min/mg protein). The vertical bars are S.E.M. (5 determinations).

1.02 ± 0.46 mM. The V_{max} for the high and low affinity systems were 2.3 ± 1.2 and 16.0 ± 5.9 nmoles/min/mg protein respectively. The large standard deviation might be due in part to biological variations and also, for the high affinity process, to the limited sensitivity of the determination method in the range of low concentrations. The constants for the low affinity process could not be determined in the optimal conditions since the low solubility of rifampicin in the incubation medium did not permit the investigation of concentrations higher than 1 mM.

Effects of metabolic inhibitors. To discriminate between facilitated diffusion and active transport we tried to ascertain whether the rifampicin uptake was energy-dependent. The effect of antimycin A (up to 15 μg/ml), KCN (up to 10 mM) and ouabain (up to 10 mM) was investigated: none of these compounds had any effect on rifampicin uptake by isolated liver cells. These metabolic inhibitors failed to provoke any significant effect on the adsorption of rifampicin on the cells.

Effect of temperature on uptake and adsorption. We measured the initial uptake velocity of rifampicin at five temperatures between 10 and 37°, at an external concentration of 300 μM. Plotting the logarithm of the velocity against the reciprocal absolute temperature according to Arrhenius yielded a straight line; its slope indicated an activation energy of 22 kcal/mole. When the same procedure was applied to the adsorption data, a heat of reaction of 3 kcal/mole was obtained.

Relationship between rifampicin and BSP uptake. BSP was without effect on rifampicin uptake even if 20 μM of BSP (3 times the K_m of BSP uptake) were added, together with 20 μM of rifampicin (about 0.2 K_m of rifampicin uptake) (Fig. 4). In contrast, rifampicin clearly inhibited BSP uptake into the cells (Fig. 5). The double-reciprocal plot showed that 300 μM rifampicin did not change the maximal velocity of the BSP uptake, but increased the K_m 3-fold. This plot allowed us to calculate a V_{max} and a K_m of 2.3 nmoles/min/mg protein and 6 μM, respectively. These kinetic constants of BSP uptake are quite similar to those reported by other authors [7]. To characterize

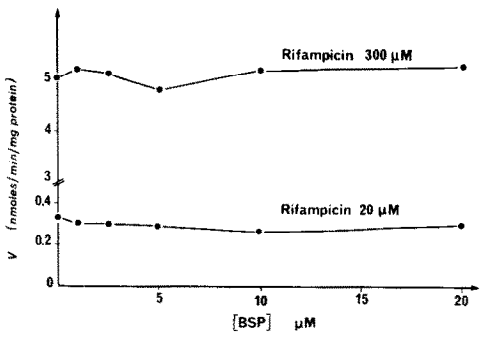

Fig. 4. Initial velocity of rifampicin uptake by liver cells as a function of BSP concentration in the medium. The rifampicin concentrations used are indicated on the graph.

further the inhibition of BSP uptake by rifampicin, we examined the results according to the Dixon representation shown in Fig. 6. The resulting plots are linear for the low range of rifampicin concentration (less than 100 μM); this part of the plots allowed us to calculate an inhibition constant of 60 μM. For higher concentrations the plots present a downward bend, thus revealing an inhibition which is only apparently competitive.

In addition, we observed that the addition of cold rifampicin to the medium containing cells pre-equilibrated with labeled BSP did not induce an efflux of radioactivity out of the cells (Fig. 7), nor did cold BSP induce any counter transport of labeled rifampicin. Under the same experimental conditions we verified that cold rifampicin or cold BSP induced an efflux of labeled rifampicin or BSP respectively (data not shown).

DISCUSSION

The isolated liver cells take up rifampicin rapidly, resulting in an internal concentration about 10 times higher than the external concentration. The concentration gradient may be only apparent because a large part of the rifampicin inside the cell is probably bound to proteins. The independence of rifampicin uptake from metabolic energy, as suggested by its insensitivity to KCN and antimycin A, supports the idea of a passive uptake mechanism. These metabolic inhibitors markedly diminished the uptake of taurocholate by isolated liver cells, even at a concentration much lower than

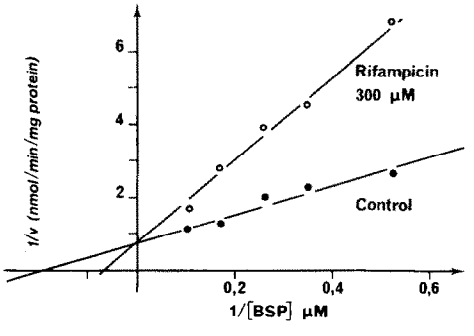

Fig. 5. Lineweaver–Burk plot of BSP uptake by liver cells in the presence of rifampicin. The reciprocal initial velocities were plotted against the reciprocal BSP concentrations.

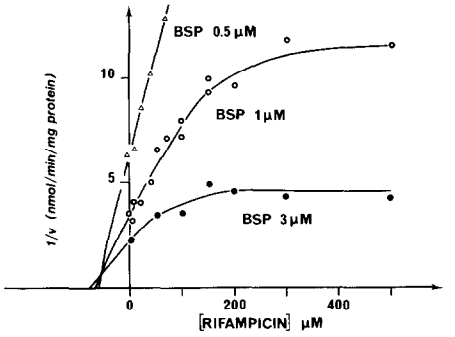

Fig. 6. Dixon plot of BSP uptake by liver cells in the presence of rifampicin. The reciprocal initial velocities were plotted against rifampicin concentrations. Concentrations of BSP were 0.5, 1 and 3 μM. The results were obtained from two experiments.

those used in the present work [15]. The kinetic analysis of the uptake indicates that the initial velocity is concentration-dependent and saturable. From the data obtained from five different preparations and plotted according to Lineweaver and Burk, it becomes apparent that two systems saturable with the substrate concentration are involved in rifampicin uptake. The saturability indicates carrier-mediated processes. The first system is a high affinity one with an apparent K_m of about 50 μM and a maximal velocity of 1 nmole/min/mg protein. The second system has much lower affinity for rifampicin (apparent K_m 1.4 mM and maximal velocity 18 nmoles/min/mg protein). It is of interest that the K_m of the former carrier is in the range of the concentrations of rifampicin achieved in the serum when the antibiotic is given *in vivo*. The second slope, however, which is observed at high rifampicin concentrations, could in fact result only from a non-specific membrane effect mimicking a second affinity system.

The Scatchard plot of the adsorption data reveals the existence of two sites. The values of the dissociation constant are in a range similar to that of the Michaelis constant of the uptake systems. This supports the view that the initial extracellular binding is probably the first step in the uptake of rifampicin. This step occurs very rapidly and cannot be the rate-limiting factor of the uptake process. The values found for activation energy of the adsorption and uptake processes support this idea. An activation energy of 22 kcal/mole is well in the range of those of carrier-mediated transport [16] and in this study it concerns the catalytic step of the uptake since the fixation of the substrate on the protein is very rapid and, therefore, cannot be the limiting factor.

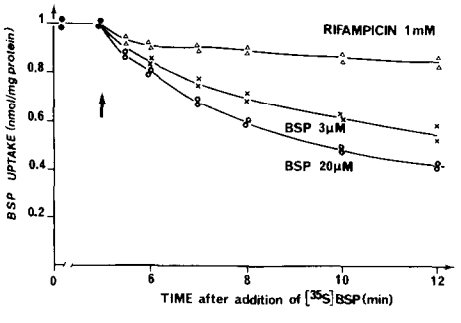

Fig. 7. Effect of adding 3 or 20 μM unlabeled BSP or 1 mM unlabeled rifampicin on the amount of labeled BSP in cells preloaded with this hot substrate (counter-transport study). Experimental conditions are indicated in the text. The arrow indicates the time of addition of the cold substrate.

The involvement of a carrier protein in the uptake of bile acids and other organic anions has been previously proposed for taurocholate [15, 17, 18], cholate [14], indocyanine green (ICG) [19], and BSP [5–7, 19]. More recently a protein which displays a high affinity for BSP has been isolated from rat liver plasma membrane [8]. Therefore the question arises whether all the organic anions, other than bile salts, are taken up by the same carrier protein. Several reports support this hypothesis: it was shown that ICG, BSP and bilirubin exhibit mutual competition for uptake [19] and that rifamycin SV, a compound closely related to rifampicin, competitively inhibits the uptake of ICG by rat liver *in vivo* [20]. It was also observed that ICG competitively inhibits BSP uptake in isolated liver cells; rose bengal, another anionic compound, however, was without effect [7].

The intervention of a common carrier for organic anion uptake by liver cells seems to be a good explanation for the delayed plasma disappearance rate of BSP after rifampicin administration [1, 3]. However, our results are in disagreement with such an hypothesis. The observed inhibition caused by rifampicin on BSP uptake could not be really competitive in spite of an increased K_m and an unchanged maximal velocity, since BSP was without effect on rifampicin uptake. The results plotted according to Dixon allow us to characterize an inhibition which is only apparently competitive. The mutual counter-transport study of BSP and rifampicin failed to demonstrate any efflux of radioactive substrate from preloaded cells by addition of an excess of the other drug, which confirms the absence of really competitive inhibition. A common carrier-mediated uptake therefore cannot be postulated since two necessary conditions, mutual competitive inhibition and reciprocal counterflow, are not met. The observed inhibition of BSP uptake by rifampicin may be similar to that of dibromosulphthalein uptake by the bile salts [21]. Our data suggest that the inhibition could result from rifampicin fixation on a membrane site different from that of BSP, causing modification of the membrane and especially a decrease in the affinity for BSP without changing the maximal velocity of the uptake. Such an effect in a Lineweaver–Burk plot mimicks competition, but the fixation of the substrate and of the inhibitor does not occur at the same site. The inhibition gives the appearance of an allosteric process; rifampicin acts as an effector of type K on BSP uptake. The calculated inhibition constant represents the concentration of rifampicin necessary to increase the K_m of BSP uptake 2-fold. A non-specific interaction between rifampicin and liver cell membrane could be postulated since it was recently reported that rifampicin also inhibits non-competitively the uptake of two bile salts, cholate and taurocholate [22].

The results of the present work provide evidence that more than one carrier is involved in the uptake of anionic drugs by liver cells. Several transport mechanisms for the transfer of anionic compounds from liver into bile have also been postulated [23, 24]. The existence of several pathways for the transfer of anionic

compounds from plasma to bile means that drugs administered simultaneously do not necessarily interact for their elimination. The definition of families of related compounds which show a common pathway for hepatic uptake and/or biliary elimination would indeed be helpful to predict possible interactions in the hepatic disposition of therapeutic agents and toxic compounds.

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