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BILIRUBIN UPTAKE *IN VITRO* BY THE RAT INTESTINAL MUCOSA

JUAN LELIO CORCHS, RAQUEL ELENA SERRANI AND EMILIO RODRIGUEZ GARAY
Departamento de Ciencias Fisiológicas, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Santa Fé 3100, Rosario (Argentina)
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SUMMARY

Flat sheets of rat jejunum incubated in the presence of unconjugated bilirubin solutions were shown to incorporate bilirubin into the tissue.

Bilirubin mucosal uptake, expressed as a function of the incubation time showed a tendency to reach a constant level within 120 min.

Solutions of bilirubin in sodium taurocholate gave an incorporation of significantly greater amounts of bilirubin than those prepared with albumin.

A structurally similar substance (biliverdin) inhibited bilirubin uptake in a way that suggested competitive inhibition. The results support the view that the mechanism of bilirubin uptake by the rat intestinal mucosa cannot be entirely explained by simple passive diffusion.

INTRODUCTION

It has been demonstrated *in vivo* that unconjugated bilirubin can be absorbed from the intestinal lumen into the blood and that it can also pass from the plasma into the intestinal lumen[1–4]. Recent experiments *in vitro* have shown that ^{14}C-labelled unconjugated bilirubin can cross the intestinal wall when placed inside normal or everted intestinal segments. The unidirectional fluxes were linear with concentration within the range from 0.01 to 0.1 mM and were approximately equal in both directions[5].

In order to obtain further insight into the mechanism by which unconjugated bilirubin is transported across the gut wall, the uptake of pigment by isolated sheets of rat jejunum was investigated using an *in vitro* tissue accumulation technique[6].

MATERIALS AND METHODS

Chemicals

Bilirubin, biliverdin, sodium taurocholate and bovine serum albumin were obtained from Sigma (St. Louis, Mo., U.S.A.). All the other reagents used were of analytical grade.

Preparation of tissue

Adult Wistar rats of both sexes were killed by cervical dislocation. The proximal

jejunum was removed and kept in Ringer–Krebs solution[7]. Segments about 1–2 cm in length were cut open to expose the mucosa. The segments were then incubated at 37 °C in a chamber provided with a reflux condenser.

Incubation conditions

Unconjugated bilirubin was dissolved in 0.10–0.15 ml 0.1 M NaOH and then added to Ringer's solution (equilibrated with O_2–CO_2 (95.5, v/v)) containing (1) bovine serum albumin to give a molar ratio of bilirubin to albumin of 2:1 (bilirubin-albumin solution), (2) 5 mM sodium taurocholate to give a molar ratio of 1:16.6 (bilirubin–taurocholate solution). Unless otherwise stated, the final concentration of bilirubin in the incubation medium was 0.3 mM. The amount of wet tissue added ranged from 100 to 150 mg for 5 ml medium. During incubation stirring was achieved using the gas mixture.

Biliverdin was dissolved in 0.1 ml of sodium carbonate solution (47.2 mM Na_2CO_3, 88.9 mM NaCl) and then mixed with the incubation medium.

At the end of the incubation period, the segments were removed, rinsed twice with cold Ringer's solution and gently blotted on filter paper.

Mucosal and serosal sheets were obtained as described by Dickens and Weil-Malherbe[9], weighed on a Mettler balance and homogenized in citrate–phosphate buffer pH 2.2 (ref. 10) to give 100 mg wet tissue per 2.5 ml buffer.

After 120 min of incubation the bilirubin concentration in the medium had decreased by 10 % of its initial value in bilirubin–albumin experiments and 20 % in the bilirubin–taurocholate experiments.

Analytical procedures

Determination of bilirubin concentration in the homogenates was carried out by a modification of Hargreaves' method[11] using a dilute diazo reagent[12].

The absorbance of the supernatant was determined at 540 nm in a DU Beckman spectrophotometer. A 91 $\pm$ 3 % ($n = 6$) recovery of the bilirubin added to normal homogenates was obtained.

In some experiments the diazotized supernatant was concentrated[13] and chromatographed on paper[14].

Total tissue water in the unsliced specimens was calculated by the difference between wet and dry weight (24 h at 100 °C). Water content was found to be constant, with a value of 0.80 ml/g wet tissue during the entire period of incubation.

The mucosal extracellular space was determined with inulin[15] and gave a value of 0.25 ml·g^{-1} wet tissue (S.E. $\pm$ 0.02) ($n = 18$).

Oxygen consumption (Q_{O_2}) was determined by Warburg's method[16] and expressed as μl O_2/mg protein per h. Protein was determined by Lowry's technique[17].

RESULTS

Bilirubin uptake by mucosal and serosal sheets

It was found that the uptake of bilirubin by the mucosal fraction was greater than that of the serosa (Fig. 1). A difference was detectable after 15 min and was significantly greater after 120 minutes incubation ($P < 0.01$).

Bilirubin uptake as a function of time. Final tissue: medium concentration ratio

The time course of bilirubin uptake by mucosal fractions, incubated in 0.3 mM bilirubin–Ringer's solution, containing either sodium taurocholate or albumin is represented in Fig. 2. It is shown that there is a greater tissue uptake of unconjugated bilirubin in the former case*. The existence of a slow component for bilirubin–albumin uptake similar to that for bilirubin–taurocholate was noted.

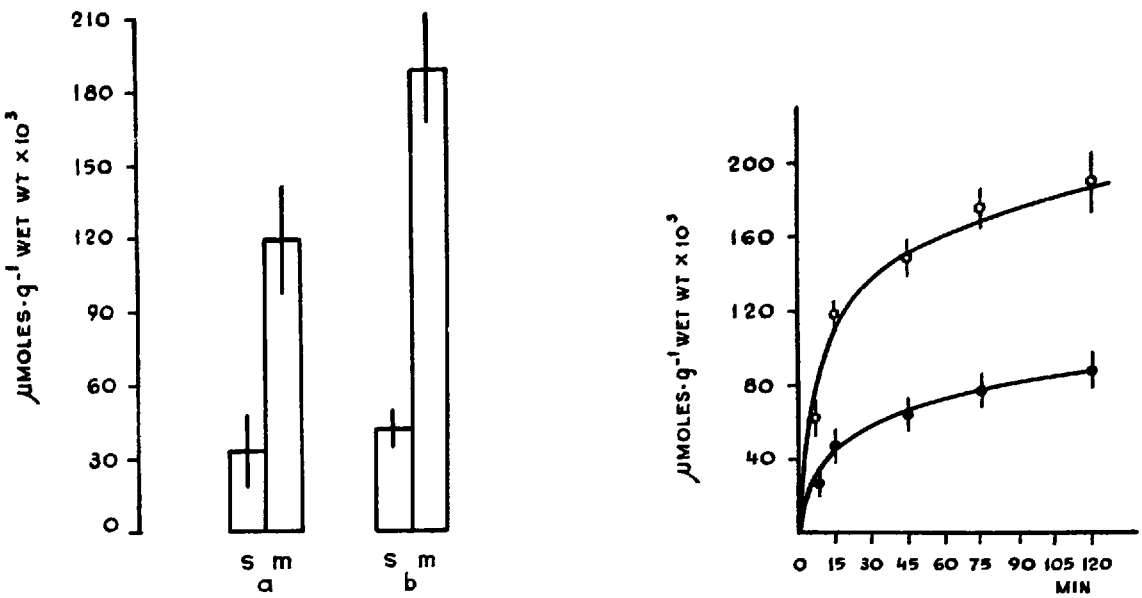

Fig. 1. Bilirubin uptake by intestinal segments: s, serosal fraction; m, mucosal fraction; a, 15 min of incubation; b, 120 min of incubation. Data expressed as mean ± S. E. for 7 experiments at each time interval. Incubation medium was bilirubin–taurocholate.

Fig. 2. Bilirubin uptake by intestinal mucosa as a function of time: ●, bilirubin–albumin; ○, bilirubin–taurocholate. Each point is the mean of ten determinations. Equations of the type $Y = C-(A\cdot e^{-\lambda_1 t} + B\cdot e^{-\lambda_2 t})$ (solid lines) fitted both sets of experimental data. C (μmoles × 10^3) represents a graphically obtained asymptotic value for each set of uptake data. Semi-logarithmic plots of the mean of differences between one corresponding C value and the individual ones (for each incubation period) as a function of time were used to obtain the fast (λ_1) and slow (λ_2) rate constants (min^{-1}) and the coefficients B (extrapolation back to $t = 0$ of the slow component of the curve) and A (difference at $t = 0$ between the original curve and the extrapolated part)[18]. The two derived equations were:

$Y = 240-(118\ e^{-0.15t} + 122\ e^{-0.007t})$

$Y = 100-(42e^{-0.15t} + 58e^{-0.012t})$

(Upper and lower solid curves, respectively).

The results for the tissue: medium concentration ratio for bilirubin[6] are shown in Fig. 3.

The final tissue:medium ratio for bilirubin dissolved in taurocholate was greater than that obtained with albumin ($P < 0.01$). In both situations the ratio was less than 1.

Effect of biliverdin on bilirubin uptake

The effect of a structural analogue, namely biliverdin, on the rate of bilirubin uptake by the intestinal mucosa was studied after an incubation period of 15 min[19]

* Sodium taurocholate solutions (5 mM) did not give any absorbance at 540 nm after adding the diazoreactive.

at a constant bilirubin concentration (0.3 mM). It was observed that from a control value (0.00 biliverdin) of 0.49 μmole·g^{-1} wet tissue·h^{-1} (S.E. $\pm$ 0.04) (n = 8), the mean rate of uptake decreased to 0.32 (S.E. $\pm$ 0.07) (n = 8), 0.21 (S.E. $\pm$ 0.02) (n = 10) and 0.19 (S.E. $\pm$ 0.04) (n = 10) at biliverdin concentrations (mM) of 0.05, 0.10 and 0.15, respectively.

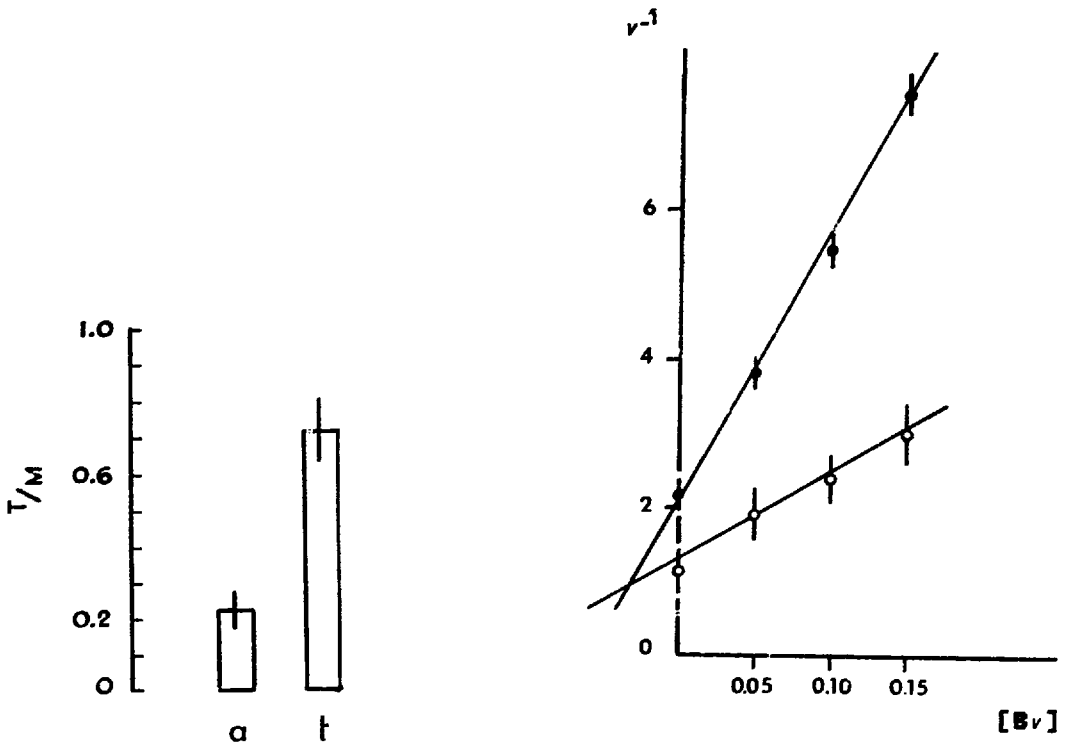

Fig. 3. Bilirubin uptake by intestinal mucosa. Tissue water: incubation medium bilirubin concentration ratio (T/M). The ratio was calculated after 120 min of incubation: a, bilirubin–albumin experiments (n = 10); t, bilirubin–taurocholate experiments (n = 10). Data expressed as mean $\pm$ S. E.

Fig. 4. The reciprocal of bilirubin uptake rate (v^{-1}) by intestinal mucosa (μmoles/g wet wt·h)$^{-1}$ plotted against biliverdin concentration (mM) in the medium (Bv). Bilirubin concentration was constant: 0.3 mM (●) or 1.1 mM (○). Each point is the mean of 7–10 determinations $\pm$ S. E. The inhibition constant (K_i) (abscissa of the intersection point of both functions) had a value of 0.035 mM. The kinetic parameters of the system (K_m, V) obtained from this same plot[27] had values of 0.55 mM and 1.25 μmoles·g^{-1} wet wt·h^{-1}, respectively.

A significant linear correlation[20] was found between the inverse of bilirubin uptake (v^{-1}) and biliverdin concentration (Bv) at a constant bilirubin concentration (0.3 or 1.1 mM) (Fig. 4). Furthermore, both lineal functions intersect at a point above the abscissa.

Sodium salycilate effect on bilirubin uptake

The addition of sodium salycilate (molar ratio of bilirubin to salicylate, 2:1) to the incubation medium containing bilirubin (0.3 mM) and albumin (molar ratio bilirubin to albumin 2:1) produced a significant increase in the bilirubin uptake by the mucosal fraction compared with the value obtained in control experiments (Table I).

TABLE I

Influence of albumin and organic anions on bilirubin uptake by intestinal mucosa. Bilirubin concentration was 0.3 mM. The molar ratio of bilirubin–albumin and bilirubin–sodium salicylate was 2:1. Concentration of sodium taurocholate was 5 mM. In parentheses are the number of experiments.

	Bilirubin uptake (μmoles·g^{-1} wet tissue wt per 45 min)	
Bilirubin + albumin	0.062 ± 0.007	(11)
Bilirubin + albumin + sodium salycilate	0.103 ± 0.010	(12)
Bilirubin + sodium taurocholate	0.148 ± 0.009	(7)

Viability of tissue preparation

Oxygen consumption of mucosal sheets was followed in a Warburg respirometer during a 1-h incubation period in the presence of bilirubin (0.3 mM), sodium taurocholate (5 mM) and Ringer's solution after a preincubation time of 30 min in the same medium. The mean value was 13.72 ± S.E. 2.1, $n = 9$ (μl O_2·mg^{-1} protein·h^{-1}) which did not differ significantly from that obtained in a control group using tissue slices incubated only in Ringer's solution (13.78, S.E. ± 1.45, $n = 10$).

DISCUSSION

In these experiments bilirubin was determined in the intestinal mucosa by a simple and reproducible method. The pigment taken up by the tissue was identified as unconjugated bilirubin because: (a) the unincubated mucosa did not contain any pigment giving a direct diazoreaction, (b) paper chromatography of diazotized homogenates (after incubation) showed only the presence of azopigment A, corresponding to unconjugated bilirubin[14].

The distribution of bilirubin (in bilirubin–taurocholate experiments) taken up at 15 and 120 min by the serosal and mucosal fractions is reported in Fig. 1. The taurocholate–bilirubin complex is mainly concentrated in the mucosa. Since serosal concentrations attained a constant level after 15 min the possibility that adsorption might be (wholly or in part) the responsible mechanism was not discarded. The relevant point, however, is that while the serosal concentration has reached a constant level during the first 15 min, the mucosal concentration is still increasing after 2 h. This supports the view that the location of the slow component of the uptake process is within the mucosal part of the gut wall.

The time course of bilirubin uptake by mucosa (Fig. 2) suggests the existence of at least two different pigment distribution spaces. This is supported by: (a) the fraction taken up by the extracellular space cannot fully account for the total uptake by tissue. If we consider the bilirubin concentration (0.3 mM) in our incubation medium and the value of 0.25 ml·g^{-1} wet tissue for extracellular space, it is easily shown that only 0.075 μmoles·g^{-1} wet tissue will reach equilibrium in this space with the incubation medium; (b) unconjugated bilirubin has been detected by histochemical methods within epithelial mammalian cells[21,22]; (c) data of bilirubin uptake as a function of time (Fig. 2) showed that the curves were composed of two exponential

terms. This suggests the existence of more than one tissue bilirubin distribution compartment[18].

In the experiments with bilirubin–taurocholate the tendency to reach a constant value with the incubation time (Fig. 2) could be explained as being due to saturation of bilirubin binding sites in the tissue. However, a similar behaviour was found in bilirubin–albumin experiments, where the equilibrium takes place with significantly lower levels of bilirubin uptake. Since we can accept that the number of sites which tightly bind bilirubin would be same in both experimental conditions, this interpretation was discarded. It seemed more reasonable to interpret the results as indicating that the amount of substrate taken up by the tissue was the expression of a net flux.

The addition of sodium salicylate to the incubation medium produced an increase in pigment uptake (Table I). This was considered to occur as a result of an increase of unbound pigment concentration owing to the displacement of bilirubin from its binding to albumin[23]. In the experiments where sodium taurocholate was used the increased bilirubin uptake seemed to be due to a similar factor[24], since in the presence of that salt, bilirubin is completely in the unbound form. However, the possibility of an increase in brush border membrane permeability due to taurocholate cannot be excluded[25].

The rate of bilirubin uptake by tissue was influenced by the presence of a structurally related substance such as biliverdin[26]. Although from this plot (Fig. 4) we cannot differentiate between competitive and mixed inhibition, the close structural similarity between substrate (bilirubin) and inhibitor (biliverdin) makes the existence of competitive inhibition the most likely explanation[27].

Uphill transport capacity (final tissue: medium concentration ratio, > 1) was not observed in any of the experiments (Fig. 3). This suggests the presence of only passive mechanisms of bilirubin uptake.

Unidirectional fluxes of unconjugated bilirubin measured with the aid of [^{14}C]-bilirubin[5] had a value very much greater than the fluxes measured in this work by a chemical procedure, which could represent a net flux. This discrepancy (and the biliverdin effect on bilirubin uptake) would suggest the existence of a bilirubin transfer mechanism due to facilitated diffusion[28].

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