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Biotin uptake by isolated rat intestinal cells

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Isolated intestinal mucosa cells of rats were used to investigate the intestinal transport of biotin. This method utilizing a double-label isotope technique showed that uptake could not be saturated, even in a wide range of biotin concentrations (0.01–2 μM). A metabolic inhibitor (antimycin A) did not prevent cell uptake of biotin. The transport mechanism was independent of temperature (Q_{10} = 1.04). When excess biotin was added to the incubation medium, there was no efflux of the vitamin from intestinal cells. The results also showed that the cells did not concentrate the vitamin, regardless of its concentration in the incubation medium. The mechanism of biotin uptake by rat cells at physiological concentrations is thus a passive diffusion phenomenon.

Introduction

The mechanisms involved in the intestinal absorption of biotin have not been investigated extensively and are still controversial. Prior work with everted intestinal sacs of rats in vitro suggested that the absorption process occurred via simple passive diffusion [1,2], but a saturable mechanism (K_m = 1.0 mM, V_m = 22 ± 2 nmol/min per ml tissue water) was shown in hamsters [3]. These methods determined absorption with relatively unreliable microbiological assays [1] or employed excessively high biotin concentrations (1 μM–1 mM). On the basis of the daily dietary requirements of rats, estimated at 0.5–3 μg/day [1], the calculated intestinal concentration is 0.6 μM [4].

In order to further elucidate the mechanism of intestinal biotin transport, we used isolated rat intestinal cells. This technique has been shown to be useful for demonstrating the saturable transport of riboflavin [5], whereas the everted-sacs technique showed the same process to be one of passive diffusion [6].

In comparison to other intestinal preparations, this technique also offers the advantage of enabling intracellular passage of the substrate to be easily followed, thereby avoiding the problem of unstirred layers.

Materials and Methods

Chemicals. [8,9-^{3}H]Biotin (35 Ci/mmol), [*carboxyl*-^{14}C]dextran (1.24 mCi/g) and ^3H_2O were obtained from New England Nuclear (Boston, U.S.A.). The scintillation liquid (RIA-LUMA) was purchased from Lumac (Schaesberg, The Netherlands).

Silicon oil was from Wacker Chemie (Munich, F.R.G.). Unlabeled biotin and all other reagents were the purest grade available and were obtained from Sigma (St. Louis, U.S.A.).

Solutions. Solution A (pH 7.3) comprised 96 mM NaCl/1.5 mM KCl/5.6 mM KH_2PO_4/27 mM sodium citrate.

Solution B (pH 7.3) comprised 140 mM NaCl/16 mM Na_2HPO_4/1.5 mM EDTA/0.5 mM dithiothreitol.

Hanks' medium composed of 3 mM phosphate buffer (pH 7.3)/136 mM NaCl/0.6 mM $CaCl_2$/5.2 mM KCl/0.8 mM $MgSO_4$/5 mM glucose.

Solutions of labeled biotin were prepared by isotopic dilution.

Isolation of intestinal cells. The methods of Weiser [7] and of Hegazy et al. [8] were used to isolate intestinal cells from male Wistar rats. The animals were fed standard chow, and weighed between 200 and 250 g. After killing by cervical dislocation, the entire small intestine was removed and the luminal contents were rinsed with oxygenated medium A at 37°C.

After eliminating the first 5 cm of the duodenum and the last 10 cm of the jejunum, the preparation was filled with medium A and submersed in the same medium for 10 min at 37°C with oxygenation. It was then emptied, filled with solution B and incubated for 3 min at 37°C under oxygenation, after which it was gently palpated with the fingers for 2 min. The contents containing mucosal cells were filtered through 0.25 mm mesh gauze and recovered in 100 ml of Hanks' medium at room temperature. This treatment with solution B was repeated three times.

The cells were washed twice with Hanks' medium at 22°C and recovered by centrifuging at $70 \times g$ for 2 min. Cell viability was checked with the Trypan blue dye exclusion method [9]. The cells were then suspended in Hanks' medium to obtain a protein concentration in the order of 1 mg/ml. The enterocytes were used within 2 h of removal of the intestine. Light microscopy of the suspension showed single cells, as well of clumps containing between 2 and 10 cells. The majority of the isolated cells was composed of villus-tip cells, recognized by their oblong shape and conspicuous brush border [10].

The protein content was determined with the method of Lowry et al. [11].

Determination of biotin uptake. Isolated enterocytes (about 2 mg cellular protein) were incubated in 2 ml of Hanks' solution (pH 7.3, 37°C) with gentle stirring. [*carboxyl*-^{14}C]Dextran (1 μCi/ml) was added to measure the volume of adherent fluid. After a 15 min incubation, 10 μl of labeled biotin was added to final concentrations of 0.01–2 μM.

200-μl aliquots of the suspension were removed at 1, 3, 6, 10 and 20 min. The cells were separated from the medium by a rapid centrifugation (8400 $\times g$) through 100 μl of silicon oil ($d = 1.022$) layered on top of 100 μl of 25% trichloroacetic acid. The supernatant and oily layer were eliminated by vacuum aspiration, and 80 μl of the acid phase were transferred to 3 ml of scintillation liquid. Intracellular radioactivity was determined with an LKB-1215 Rackbeta liquid scintillation spectrometer in conditions for determining double-labeled samples.

The same procedure was repeated at 0°C with 0.5 μM biotin. The metabolic inhibitor (antimycin A at 10 μg/ml) was added 15 min before the labeled biotin (0.5 μM).

The aqueous cellular volume was determined by replacing the labeled biotin with ^3H_2O (2.5 μCi/ml).

Statistics. Curves characterizing the intracellular uptake of biotin were calculated with a computer program designed in the laboratory. The program furnished the parameters of a nonlinear function with the least-squares method. The linear-regression analysis of initial rate of biotin uptake as a function of concentration was also determined with the least-squares method. Averages were expressed as mean ± S.E. The statistical significance of the temperature and inhibitor experiments was evaluated by a variance analysis [12].

Results

Cellular aqueous volume and adherent fluid

Cellular aqueous volume was determined to be 10.2 ± 2.3 μl/mg protein ($n = 25$). The volume of adherent fluid was determined at 10.3 ± 0.7 μl/mg of protein ($n = 138$).

Kinetics of intracellular uptake of biotin

As stated in the Introduction, it was of particular interest to examine biotin transport in the range of physiological concentrations. The range adopted was between 0.01 an 2 μM, the lower value being imposed by the sensitivity of the radiochemical method used.

Fig. 1 shows the time-courses of intracellular biotin for different concentrations of vitamin. Uptake reached practically a maximum after 6 min,

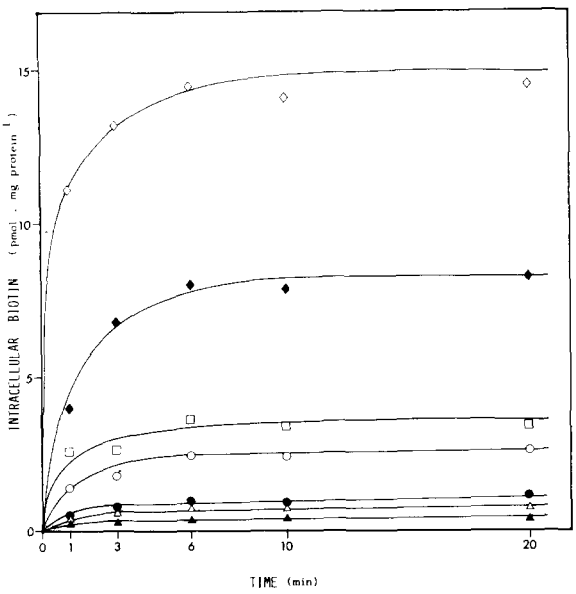

Fig. 1. Time-course of biotin uptake by isolated intestinal cells. Cells (2 mg protein) from the small intestine were preincubated for 15 min with stirring in 2 ml of Hanks' medium (pH 7.3 at 37°C) containing [*carboxyl*-^{14}C]dextran. At zero time, 10 μl of [8,9-^{3}H]biotin was added: final concentration 0.01 μM (▲), 0.05 μM (△), 0.1 μM (●), 0.33 μM (○), 0.5 μM (□), 1 μM (◆), and 2 μM (◇). 200-μl aliquots were withdrawn at the times shown to determine uptake as described in Materials and Methods. Values are means of five experiments.

regardless of concentration. It can also be seen that the equilibrium between internal and external biotin was reached within 10 min of incubation.

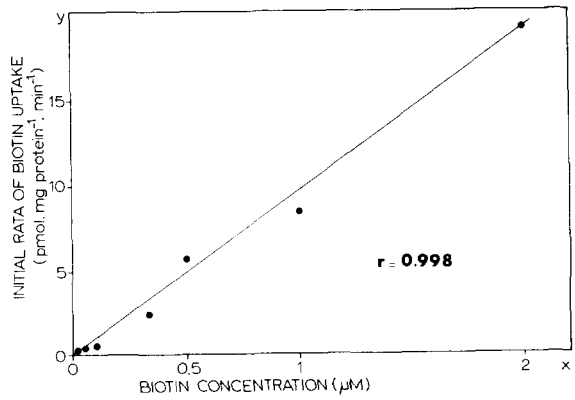

Fig. 2. Relationship between initial rate of biotin uptake (y) and biotin concentration in the incubation medium (x). Values are the means of five experiments; r is the correlation coefficient of the least-squares regression line ($y = 9.399x - 0.096$). y is expressed in pmol/mg protein per min and x as μM biotin.

TABLE I

EFFECT OF TEMPERATURE AND METABOLIC INHIBITOR ON BIOTIN UPTAKE

Experiments were performed as described in Fig. 1 at 37°C and 0°C. Labeled biotin (0.5 μM) was then added and the incubation was continued for 20 min. In the inhibitor experiment, antimycin A (10 μg/ml) was added 15 min before labeled biotin (0.5 μM). Results are means ± S.E. of seven experiments.

Biotin (μM)	Biotin uptake (pmol/mg protein per 20 min)		
	37°C	0°C	antimycin A
0.5	3.26 ± 0.58	2.84 ± 0.55	2.88 ± 0.56

Based on the cellular aqueous volume, it was possible to calculate the intracellular biotin concentration at equilibrium for each solution used. The mean intracellular/extracellular concentration ratio was 0.86 ± 0.20, showing that there was no intracellular accumulation of biotin inside intestinal cells.

Fig. 2 shows the initial rates of uptake (y) as a function of biotin concentration (x). Uptake was linear with respect to biotin concentration ($y = 9.399x - 0.096$, $r = 0.998$).

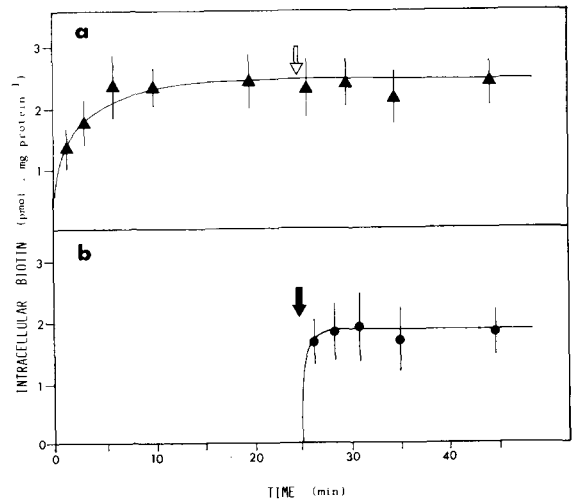

Fig. 3. Time-course of biotin uptake by isolated intestinal cells. Cells (2 mg protein from the small intestine were preincubated for 15 min with stirring in 2 ml of Hanks' medium (pH 7.3 at 37°C) containing [*carboxyl*-^{14}C]dextran. (a) (▲) After incubation with [8,9-^{3}H]biotin (0.5 μM), unlabeled biotin (5.44 μM) was added (⇓). (b) (●) After incubation with unlabeled biotin (0.5 μM), [8,9-^{3}H]biotin (0.5 μM) was added (↓). Values are means ± S.E. of five experiments. For details see Materials and Methods.

Effect of temperature and metabolic inhibitor

In addition to the 37°C incubation, biotin uptake was also examined at 0°C. In order to detect any possible energy-dependent process, antimycin A was also added to the medium, at a dose which completely blocks mitochondrial respiration. Uptake measured after 20 min (Table I) showed that the process was unaffected by either the temperature decrease or the presence of the inhibitor ($F = 1.007$, $P = 0.385$ for 2 and 18 degrees of freedom).

Counterflow experiments

After a 25 min incubation with labeled biotin (0.5 μM), the addition of an excess of unlabeled vitamin (5.44 μM) did not lead to an efflux of the vitamin from cells (Fig. 3a). Furthermore, Fig. 3b shows that a prior 25 min incubation with 0.5 μM unlabeled biotin did not change the transport of the labeled vitamin (0.5 μM) when it was added.

Discussion

The method of isolated cells used in this work led to a direct approach to the study of biotin absorption, in comparison to prior studies with everted sacs [1–4]. The present method furnishes data on the intracellular accumulation and initial rates of absorption by the mucosal cells of the gut [8,13].

Systematic double-labeling enabled us to correct biotin uptake in each experiment, since the volume of adherent fluid varied from 7 to 13 μl/mg of protein. The mean volumes of adherent and intracellular water determined were of the same order of magnitude as those reported in guinea pigs [8]. The difference may be due to the different methods used to assay protein (biuret method vs. the method of Lowry et al.).

The results obtained here are consistent with passive diffusion being the mechanism of biotin transport in isolated cells of the rat intestine. Several points are noteworthy. (i) There was no accumulation of biotin in the cells, regardless of the concentration of the vitamin tested. (ii) The intial rate of entry was proportional to external concentration in the incubation medium. (iii) The extrapolation of the regression line to the ordinate (Fig. 2) showed that it passed practically through the origin ($y = -0.0962$ pmol/mg protein per min, $P > 0.66$ for the null hypothesis that the regression line passed through the origin). It is thus highly improbable that there are saturation kinetics at concentrations lower than 0.01 μM.

It was impossible to detect a saturation of biotin uptake in a wide range of concentration. This suggests the absence of carrier-mediated transport. In addition, it is known that temperature changes in the medium affect chemical and enzymatic reactions to a greater degree than purely physical processes, e.g., simple diffusion. On the basis of this fact, it is possible to differentiate chemical and physical processes by determining the Q_{10} value according to the following equation [14]:

$$\log Q_{10} = \frac{10}{T_2 - T_1} \times \log \frac{P_2}{P_1}$$

where P_1 and P_2 are the apparent permeability coefficients (uptake/media concentration) at temperatures T_1 and T_2. Q_{10} values between 1.03 and 1.30 indicate a physical process, while values in a range between 2 and 4 suggest a process in which chemical reactions are involved [15].

Temperature had a negligible effect on biotin uptake ($Q_{10} = 1.04$), thus showing that the mechanism involved was purely physical.

In addition, experiments with antimycin A confirmed that the entry of the vitamin into cells is not an active process requiring a cellular energy supply.

Finally, the addition of an excess of biotin after reaching equilibrium did not cause efflux of the vitamin, thereby confirming that passage of the vitamin occurs by simple diffusion.

This result cannot be attributed to the use of isolated cells, since recent work using similar experimental designs has shown that biotin transport in *Escherichia coli* [16] and isolated rat liver cells [17] is an energy-dependent process.

In conclusion, our findings confirm prior results [1–4] obtained in vitro with everted rat intestinal sacs.

The passive transport of biotin in small intestinal cells could partially explain why biotin deficiency due to intestinal malabsorption is an extremely rare event.

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