Biochimica et Biophysica Acta, 501 (1978) 405—414

BBA 47452

OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA ISOLATED FROM SMALL INTESTINAL EPITHELIUM OF THIAMPHENICOL-TREATED RATS AND CONTROL RATS

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(Received August 9th, 1977)

Summary

Treatment of rats with thiamphenicol in a dose of 125 mg/kg per day for 60—64 h causes specific inhibition of mitochondrial protein synthesis, leading to a drastic decrease of the cytochrome *c* oxidase activity in intestinal epithelium. At the same time the mitochondrial ATPase activity becomes resistant to inhibition by oligomycin. Experiments with isolated intestinal mitochondria revealed that respiration in state 3 is diminished by 55% with succinate (5 mM) and by 40% with pyruvate (10 mM) plus L-malate (2 mM) as the substrates, both as compared to intestinal mitochondria isolated from control rats. P : O ratios as well as respiratory control indices are comparable in the two groups of animals. Uncoupled respiration is inhibited by 35% with succinate as the substrate, while the succinate cytochrome *c* reductase activity remains unaltered. No inhibition of uncoupled respiration with pyruvate plus L-malate as the substrates was observed. The ATP-P_i exchange activity in the mitochondria from the treated animals is diminished by about 75%. It is concluded that in the mitochondria of the treated animals the inhibition of the coupled respiration (state 3) is caused by the limitation of the ATP-generating capacity and that electron transport is rate limiting only with the rapidly oxidized substrates such as succinate, if respiration is uncoupled.

Introduction

Mitochondria fulfill all requirements to perform their own translation process, that can be distinguished from cytoplasmic protein synthesis by its sensitivity towards a number of inhibitors (for a review see ref. 1). Chloramphenicol for instance is a specific inhibitor of mitochondrial protein synthesis, whereas cycloheximide blocks only the cytoplasmic process. In vivo treatment

of animals with chloramphenicol or oxytetracycline leads to a decrease of the amount of cytochromes *b* and aa_3 in various organs [2—4], whereas the ATPase activity becomes resistant to inhibition by oligomycin [4]. This suggests that in animals one or more subunits of these enzymes are made in the mitochondria. In the lower eukaryotes *Saccharomyces cerevisiae* and *Neurospora crassa* a number of mitochondrial translation products have been identified. It could be shown in yeast that three subunits of the cytochrome *c* oxidase [5], four subunits of the mitochondrial ATPase [6] and one subunit of the bc_1 complex [7] are made on mitochondrial ribosomes. In *N. crassa* only two subunits of the ATPase are of mitochondrial origin [8], whereas also one, respectively, three subunits of the $b \cdot c_1$ complex [9] and cytochrome *c* oxidase [10] are mitochondrial translation products. Most other mitochondrial proteins as well as the further subunits of the enzyme complexes mentioned above are products of the cytoplasmic protein synthesis.

There are indications that chloramphenicol treatment of animals [11,12] or animal cells in culture [13,14] can lead to impairment of energy-dependent processes, due to the low activity of the affected respiratory chain enzymes. Inhibition of state 3 respiration was indeed observed in liver mitochondria from weanling mice after oral administration of chloramphenicol with the diet for 9—11 days [15] and in mitochondria isolated from regenerating rat liver after intraperitoneal treatment with chloramphenicol [16]. In both studies no inhibition of uncoupled respiration with succinate was noticed, although the cytochrome *c* oxidase activity was reduced to 20% [15] and 50% [16] of the control values. A number of attempts, however, to show an effect on oxidative phosphorylation in mitochondria isolated from tissues with a cytochrome aa_3 content of 30—40% of control values failed, indicating that the affected enzymes are not rate limiting under the experimental conditions used [2,17].

In this paper we describe the effects of in vivo treatment of rats with the chloramphenicol analogue thiamphenicol on the respiratory and phosphorylation capacity of mitochondria from intestinal epithelium. Because of its high turnover rate (48 h in the rat), the intestinal epithelial cell is quite suitable to investigate how inhibition of mitochondrial protein synthesis impairs the bioenergetic functions of the mitochondria and possibly as a consequence the energy-dependent processes in the whole organ.

Materials and Methods

Animals. Male Wistar rats weighing 220—330 g were used. They were maintained on a normal laboratory chow and obtained water ad libitum but were fasted overnight before the experiments.

Antibiotic and mode of administration. Thiamphenicol glycinate (Urfamycine®) was generously supplied by Zambon via Inpharzam Amsterdam, The Netherlands. Dialysis bags, containing 1.1—1.65 ml (0.5 ml per 100 g body weight) of a solution of thiamphenicol in saline were placed subcutaneously in the neck of the animals under light ether narcosis [3]. The bags were replaced twice a day. The duration of the treatment was 60—64 h. Control rats were treated with saline in the same way.

Preparation of cell suspensions. Rats were killed by decapitation and subsequent bleeding. The small intestine, from the ligament of Treitz to about

10 cm proximal of the ileocecal sphincter was rapidly excised and put in ice-cold isolation medium (0.154 M NaCl, 10 mM Tris · HCl and 5 mM EDTA, pH 7.4). Luminal contents were washed out with isolation medium. Intestinal cells were separated from underlying tissue using the vibration technique of Webster and Harrison [18], as described by Gijzel et al. [4]. Only villus cells were isolated. After 25 min of vibration the cells were collected by centrifugation at 1000 × *g* for 20 s, and the pellet suspended in about 15 ml homogenization medium containing 0.3 M mannitol, 10 mM Tris · HCl, 5 mM EDTA, pH 7.4.

Preparation of mitochondria. For the isolation of mitochondria from small-intestinal epithelial cells a procedure developed by van den Berg [19] was used. The cells were disrupted by means of nitrogen cavitation after a 30 min equilibration period under a pressure of 750 lb/inch^2 in a modified Parr bomb. (Parr Instrument Co., Moline, Ill., U.S.A.). After adjusting the volume to 40 ml and adding one drop of antifoam (Dow Corning Corp., Midland, U.S.A.) the disrupted cells were further homogenized by a few strokes in a loosely fitting glass-teflon Potter-Elvehjem homogenizer, followed by centrifugation for 5 min at 750 × *g*. The pellet was resuspended and recentrifuged three times with 10 ml homogenization medium. The combined supernatants were centrifuged for 10 min at 10 000 × *g*. The mitochondria were washed once and finally suspended in 3—6 ml homogenization medium.

Preparation of submitochondrial particles. Mitochondria were frozen in portions of 1 ml at −80°C during about 1 week. After thawing the preparations were sonicated twice for 15 s at 0°C using a Branson sonifier with microtip operating at step 2. The suspensions were centrifuged for 2 h at 40 000 × *g* and the pellets suspended in 1 ml water.

Enzyme assays. Succinate cytochrome *c* reductase (system) activity was determined spectrophotometrically at 30°C by following the reduction of added horse heart cytochrome *c* at 550 nm [20]. The incubation mixture contained: 75 mM sodium phosphate, 10 mM succinate, 1 mM KCN and 0.1 mM cytochrome *c*. The pH was 7.4. The reaction was started by the addition of the enzyme preparation that was sonicated at 0°C during 15 s.

Cytochrome *c* oxidase (EC 1.9.3.1) was assayed spectrophotometrically at 20°C [21]. Prior to incubation the enzyme was activated in 1% digitonine at 0°C during 15 min. Adenylate kinase (EC 2.7.4.3) was measured as described by Schnaitman and Greenawalt [22]. The enzyme activity was released by incubation of the preparation in 0.5% Lubrol WX (Sigma) at 0°C for 15 min. Glutamate dehydrogenase (EC 1.4.1.3) was determined according to Iemhoff and Hülsmann [23]. Lubrol-activated samples were used. ATPase (EC 3.6.1.3) activity in submitochondrial particles was measured at 37°C and pH 7.8 in a total volume of 0.5 ml, containing: 50 mM Tris/carbonate, 4 mM $MgSO_4$, 5 mM phosphoenolpyruvate, three units pyruvate kinase, 5 μl ethanol and 40—90 μg protein of the enzyme preparations. Before starting the reaction by adding ATP (4 mM), the samples were preincubated for 10 min in the presence of the inhibitors oligomycin (2 μg/ml) or F_1 inhibitor (30 μg protein/ml). P_i was determined according to the method of Fiske and Subbarow, following the modification of Sumner [24]. F_1 inhibitor was isolated from bovine heart as described by Pullman and Monroy [25], with omission of the purification on a DEAE-cellulose column.

Respiration and oxidative phosphorylation. Oxygen consumption was measured at 30°C with a Clark type of oxygen electrode. The incubation medium contained: 250 mM sucrose, 20 mM Tris · HCl, 1 mg/ml bovine serum albumin, 5 mM potassium phosphate and 1 mM EDTA, pH 7.2 (cf. ref. 17); 0.3—1.2 mg of mitochondrial protein were added to a final volume of 1.6 ml. The respiratory parameters P : O ratio, RCI, state 3 and uncoupled respiration were calculated as described by Estabrook [26]. State 3 respiration was induced by adding 250 nmol ADP, uncoupled respiration was measured in the presence of 10^{-4} M dinitrophenol.

ATP-P_i exchange in mitochondria was assayed at 37°C and pH 7.0 in a medium containing: 0.3 M sucrose, 25 mM sodium phosphate, 1 mg/ml bovine serum albumin, 5 mM KCN, 5 mM Tris/sulfate and 10 mM ATP. $^{32}P_i$ was added to get a specific radioactivity of 2 Ci/mol. The reaction was started by addition of about 0.3 mg protein in a final incubation volume of 0.5 ml. Incorporation of $^{32}P_i$ into organic phosphate was determined after using the extraction procedure of Lindberg and Ernster as described by Pullman [27].

Protein determination. Protein was determined with the biuret method [28]. Protein content of submitochondrial particles was determined according to Lowry et al. [29].

Results

Table I shows the extent to which the cytochrome *c* oxidase activity is declined in the intestinal epithelium during the treatment. The activities of all other enzymes tested as well as the total protein yield remain constant, indicating that cytoplasmic protein synthesis was not also inhibited, at least if one assumes that the turnover of the intestinal cell population continues at the same rate. From the data given in Table I it can also be concluded that the pur-

TABLE I

PROTEIN YIELD AND ENZYME ACTIVITIES IN HOMOGENATES AND MITOCHONDRIA OF INTESTINAL EPITHELIAL CELLS FROM CONTROL AND THIAMPHENICOL-TREATED RATS

Protein yield is given in mg protein per 100 g body weight. Enzyme activities of adenylate kinase, succinate cytochrome *c* reductase and glutamate dehydrogenase are expressed as nmol substrate utilized/min per mg protein. Cytochrome *c* oxidase activity is expressed as the first-order reaction rate constant in min^{-1} per mg protein. Results are given as mean values ± S.E. The number of experiments is given in parentheses.

		Control animals	Thiamphenicol-treated animals
Protein yield	Homogenate	69.6 ± 1.7 (6)	70.5 ± 5.4 (4)
	Mitochondria	9.2 ± 0.5 (6)	8.5 ± 1.3 (4)
Adenylate kinase	Homogenate	305 ± 55 (4)	323 ± 48 (4)
	Mitochondria	783 ± 63 (4)	838 ± 43 (4)
Glutamate dehydrogenase	Homogenate	69.7 ± 2.3 (3)	62.5 ± 6.5 (3)
	Mitochondria	175 ± 6 (4)	224 ± 4 (4)
Succinate cytochrome *c* reductase	Homogenate	28.2 ± 5.5 (4)	38.8 ± 8.1 (4)
	Mitochondria	128 ± 13 (4)	138 ± 12 (4)
Succinate cytochrome *c* oxidase	Homogenate	39.4 ± 3.7 (5)	9.6 ± 0.9 (4)
	Mitochondria	77.6 ± 4.5 (5)	27.4 ± 3.7 (4)

ity of the mitochondria isolated from the two groups of animals is the same because the specific activities of the enzymes adenylate kinase, succinate cytochrome *c* reductase and glutamate dehydrogenase are comparable. Moreover, the decrease of cytochrome *c* oxidase activity in the mitochondria of the treated animals with regard to the activity of this enzyme in the control mitochondria is about the same as in the corresponding homogenates.

The ATPase activity is decreased by about 30% in submitochondrial particles of the treated animals (Table II). The enzyme, however, becomes resistant to inhibition by oligomycine during the treatment: only 32% is sensitive to the inhibitor at a concentration which gives maximal inhibition, while in the control preparations 80% of the total enzyme activity could be inhibited. The affinity for the F_1 inhibitor, however, is about the same in both preparations. Comparable results were already obtained by Gijzel et al. [4], who studied the effect of oxytetracycline treatment on intestinal epithelium of the rat. Possibly part of the oligomycin- and F_1 inhibitor-insensitive activity can be ascribed to aspecific phosphatase activity due to brush border contamination of the isolated mitochondria [17,30]. From the inhibition by the F_1 inhibitor it can be concluded that this contamination, if present, must be of the same order in both preparations.

Now, the question arises if a decline in the cytochrome *c* oxidase activity and altered properties and lowered activity of the ATPase leads to an inhibition of either respiration or oxidative phosphorylation or both. The results are shown in Table III.

With pyruvate plus malate as the substrates state 3 respiration in the mitochondria of the thiamphenicol-treated rats is decreased by 40% as compared to the controls. No significant difference in the uncoupled respiration was observed, indicating that the inhibition of state 3 respiration cannot be ascribed to a limiting capacity of the electron transport chain. P : O ratios and respiratory control indices do not change drastically. Thus phosphorylation remains coupled to the electron transport also after thiamphenicol treatment. With succinate as the substrate both coupled and uncoupled respiration are inhibited in the mitochondria of the thiamphenicol-treated rats. However, the ratio of un-

TABLE II

SPECIFIC ACTIVITY OF ATPase AND INHIBITION BY F_1 INHIBITOR AND OLIGOMYCIN IN SUBMITOCHONDRIAL PARTICLES OF INTESTINAL EPITHELIUM FROM CONTROL AND THIAMPHENICOL-TREATED RATS

ATPase activity is expressed as μmol ATP hydrolyzed per min per mg protein. Results are given as mean values ± S.E. of four experiments.

Addition	Control animals		Thiamphenicol-treated animals	
	Specific activity	Inhibiton (%)	Specific activity	Inhibition (%)
None	5.27 ± 0.37		3.59 ± 0.41	
Oligomycin (2 μg/ml)	1.08 ± 0.16	80 ± 2.7	2.48 ± 0.45	32 ± 5.8
F_1 inhibitor (30 μg protein/ml)	1.33 ± 0.13	75 ± 2.2	1.01 ± 0.08	71 ± 2.3
Oligomycin + F_1 inhibitor	0.76 ± 0.12	86 ± 1.9	0.82 ± 0.07	77 ± 2.3

TABLE III

OXIDATIVE PHOSPHORYLATION IN INTESTINAL EPITHELIAL MITOCHONDRIA FROM CONTROL AND THIAMPHENICOL-TREATED ANIMALS

Respiration rates are expressed as nmol O_2 consumed per min per mg protein. Results are given as mean value ± S.E. The number of experiments are given in parentheses. P, probability value calculated with the Student's t-test.

	State 3 respiration	Uncoupled respiration	RCI	P:O ratio	Uncoupled respiration / State 3 respiration
Succinate (5 mM)					
Control (9)	137 ± 12	139 ± 12	2.4 ± 0.08	1.9 ± 0.04	1.0 ± 0.02
Thiamphenicol (6)	64 ± 7.6 $P < 0.001$	89 ± 9.4 $P < 0.05$	2.2 ± 0.10 n.s.	1.8 ± 0.08 n.s.	1.4 ± 0.04 $P < 0.001$
Pyruvate (10 mM) plus malate (2 mM)					
Control (9)	67 ± 6.3	81 ± 8.6	3.0 ± 0.25	2.8 ± 0.10	1.2 ± 0.06
Thiamphenicol (6)	40 ± 4.8 $P < 0.05$	62 ± 8.9 n.s.	2.3 ± 0.19 n.s.	2.5 ± 0.18 n.s.	1.5 ± 0.05 $P < 0.005$

n.s., not significant.

coupled to state 3 respiration of 1.4 (in control rats 1.0) shows that also with succinate as the substrate electron transport is not rate limiting in the decreased state 3 respiration.

Presumably the inhibition of the uncoupled respiration must be ascribed to the decreased activity of cytochrome *c* oxidase. This conclusion is based on the observation already mentioned that the succinate cytochrome *c* reductase activity in both the mitochondrial preparations is the same (Table I). Although the activities of this enzyme are less than can be expected from the respiration rates with succinate as a substrate in both preparations (Table III), the observed equality suggests that the bc_1 complex, which is also partially dependent on mitochondrial protein synthesis [31], is not rate limiting in uncoupled respiration in the mitochondria of the treated animals.

The possibility that the effects of thiamphenicol treatment on respiration are due to a direct effect of the drug on some respiratory parameters, rather than to the effects of its inhibiting action on mitochondrial protein synthesis, could be ruled out because in the presence of extremely high concentrations of thiamphenicol (up to 500 μg/ml; data not shown) no changes could be detected on any of the respiratory parameters mentioned in Table III.

For rat-liver mitochondria it is assumed that ATP synthesis is not the rate-limiting step in state 3 respiration owing to the sigmoidal dose vs. response curve which is obtained when state 3 respiration is measured with increasing amounts of oligomycin [32]. It was interesting, therefore, to compare titration curves in normal mitochondria and those deficient in oligomycin-sensitive ATPase by the thiamphenicol treatment. The results of these experiments as shown in Fig. 1 call for two comments. In the first place it appears that state 3 respiration is completely sensitive to oligomycin. So only the oligomycin-sensitive ATPase is involved in coupled respiration also in the mitochondria of the

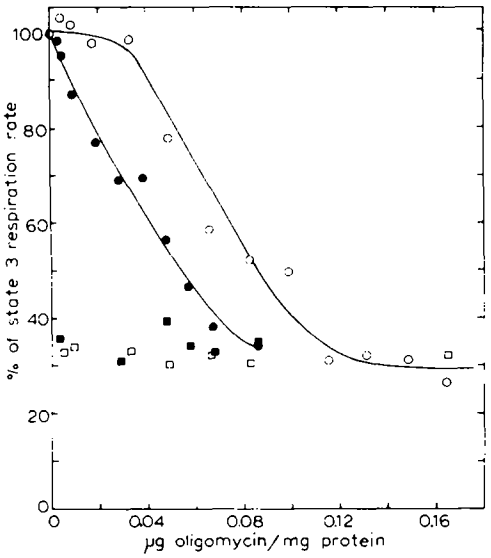

Fig. 1. Comparison of the oligomycin inhibition curves for state 3 respiration in intestinal epithelial mitochondria of thiamphenicol-treated rats (●———●) and mitochondria from control animals (○———○). Mitochondria were incubated in a standard medium with 5 mM succinate (final volume 1.6 ml) and brought in state 4 by addition of 150 nmol ADP. Then state 3 was induced by adding 1500 nmol ADP. After 1—2 min the oligomycin solution was added in a volume of 3 μl. The respiration rate was measured 4 min after the addition of oligomycin. This was repeated for every oligomycin concentration tested. 100% (uninhibited) respiration rates were for the mitochondria from the treated animals 68 nmol O_2 consumed/min per mg protein and for the control mitochondria 180 nmol O_2 consumed/min per mg protein. □, state 4 respiration of the control mitochondria; ■, state 4 respiration of the mitochondria from the thiamphenicol-treated rats.

treated animals. In the second place it should be noticed that the lag phase observed in the dose vs. response curve of the control mitochondria is absent in the mitochondria of the thiamphenicol-treated rats. This is interpreted to mean that the oligomycin-sensitive ATPase has become rate limiting in the decreased state 3 respiration. Fischer [16] also observed a somewhat increased sensitivity of state 3 respiration to inhibition by rutamycin in regenerating liver mitochondria from chloramphenicol-treated rats.

TABLE IV

ATP-P_i EXCHANGE IN INTESTINAL EPITHELIAL MITOCHONDRIA FROM CONTROL AND THIAMPHENICOL-TREATED ANIMALS

Exchange activity is expressed as nmol P_i exchanged per min per mg protein. Results are given as mean values ± S.E. The numbers of experiments are given in parentheses.

Addition	Control animals (7)		Thiamphenicol-treated animals (5)	
	Specific activity	Inhibition (%)	Specific activity	Inhibition (%)
None	83.5 ± 15.3		20.3 ± 3.7	
Oligomycin (2 μg/ml)	2.6 ± 0.9	97	2.0 ± 0.4	90
Dinitrophenol (10^{-4} M)	1.6 ± 0.3	98	1.9 ± 0.5	91

Further evidence for the diminished capacity of intestinal mitochondria from thiamphenicol-treated animals with respect to oxidative phosphorylation is obtained from the observation that the ATP-P_i exchange is drastically decreased (Table IV). The ATP-P_i exchange reaction is completely inhibited by the uncoupler dinitrophenol as well as by oligomycin in the mitochondria of both groups of animals. The fact that oligomycin inhibits the reaction in the mitochondria from the treated rats means that only the oligomycin-sensitive ATPase participates in the exchange reaction. This conclusion has also been drawn by Wagner and Rafael [15].

Discussion

In our study concerning the effects of treatment with inhibitors of mitochondrial protein synthesis in animals we used the antibiotic thiamphenicol rather than chloramphenicol. Thiamphenicol has the advantage of only scarcely being detoxified by glucuronidation in the liver [33]. For this reason serum levels can be maintained high enough to completely inhibit mitochondrial protein synthesis. In our laboratory serum levels have been determined in rats, after a single dose of thiamphenicol of 250 mg/kg body weight, using the subcutaneous administration in dialysis bags (see Materials and Methods). Shortly after application serum levels of about 150 μg/ml are reached but there is a rapid and gradual decrease until after 12 h concentration of 5—10 μg/ml are obtained (Blom, A. and Kroon, A.M., unpublished).

In mouse liver chloramphenicol treatment leads to the formation of megamitochondria [34] depleted of inner membranes. Depletion of inner membranes has also been described for HeLa cells [35] grown in the presence of chloramphenicol. After treatment of rats with thiamphenicol no megamitochondria were observed in electron micrographs of intestinal epithelial tissue, fixed by glutaraldehyde by means of vascular perfusion. The amount of inner membrane, however, is indeed reduced also in this case [36].

The yield and purity of the mitochondria from thiamphenicol-treated rats and control rats are the same (Table I). So, in spite of the above-mentioned morphological alterations, the behaviour of the mitochondria was not altered in differential centrifugation.

Subcellular fractionation of intestinal epithelial homogenates from rats is hindered by the presence of mucus, which causes aggregation of subcellular structures [37]. In order to obtain a good yield of mitochondria it is necessary to wash the low spin pellet many times. Possibly as a consequence the quality of the mitochondria recedes and the RCI is lower than usually found for liver mitochondria. ATP hydrolysis by a number of enzymes possibly present in the mitochondrial preparation and subsequent lowering of the RCI could be prevented in part by omitting Mg^{2+} from the standard incubation medium [17].

Comparison of the respiratory properties of mitochondria of control and thiamphenicol-treated rats revealed that coupled respiration in the latter is decreased, most pronounced with succinate as the substrate. These results seem to be in conflict with the work of de Jonge [17], who performed similar experiments with intestinal epithelial mitochondria from chloramphenicol- and oxytetracycline-treated rats. An explanation for the discrepancy might be that

a temporary restoration of mitochondrial protein synthesis occurs, particularly during the treatment with chloramphenicol. The latter drug is rapidly detoxified and excreted by the liver [33] so that in the periods of low serum levels protein synthesis may resume and restore the affected enzyme to levels just high enough to maintain normal respiration and phosphorylation rates. Our results, however, are in accordance with those of Fischer [16] and of Wagner and Rafael [15]. The fact that treatment with an inhibitor of mitochondrial protein synthesis renders the ATPase insensitive to inhibition by oligomycin and induces a status of impaired coupled respiration and ATP-P_i exchange, are in good agreement with the assumption that one or more subunits of the enzyme complexes involved are made on mitochondrial ribosomes and that these subunits play an essential role in the energy-transferring process. Except for the oligomycin-sensitive ATPase the total ATPase activity (F_1 inhibitor sensitive) also decreases in submitochondrial particles. It is difficult to say whether this decrease reflects the activity in whole mitochondria or that the cold lability of oligomycin-insensitive ATPase [38] is responsible for the lowered activity. Anyway, the lowering of 30% of total ATPase activity cannot be responsible for the decrease with 55% of the state 3 respiration with succinate as substrate nor for the reduction of the ATP-P_i exchange with 75%.

The final aim of this study is to investigate whether and to what extent treatment of animals with antimicrobial agents which are also inhibitors of mitochondrial protein synthesis can lead to impairment of energy-dependent processes and functions of the intestine. The results presented here suggest that this possibility indeed exists. The observation already mentioned that the total amount of epithelial protein was not decreased after 60 h of treatment suggests that the residual ATP-synthesizing capacity of the cells is sufficient to drive the process of cytoplasmic protein synthesis, at least if cell turnover remains unaffected. Whether also enough energy can be produced or not under circumstances in which a higher supply of ATP is necessary (active transport) is presently under investigation.

Acknowledgements

The authors wish to thank Dr. G.P.B. Kraan for his valuable suggestions and Professor Dr. F.J. Loomeijer for his interest and reading of the manuscript.

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