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TOXICITY OF TETRACYCLINES IN RAT-SMALL-INTESTINAL EPITHELIUM AND LIVER

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Abstract—Rats, treated for 3 days or longer with high concentrations of oxytetracycline (OTC) suffered from a severe damage of structure and function of the small intestinal epithelium and showed an excessive accumulation of fat in the liver. In an attempt to explain these toxic effects, several possible modes of action of this drug were investigated. No direct correlation was found between the strong inhibitory effect on mitochondrial protein synthesis, leading to drastically lowered mitochondrial cytochrome levels in the rapidly dividing small intestinal epithelial cells, and a disturbance of mitochondrial energy production. After chloramphenicol treatment, producing a similar decrease of cytochrome contents, no epithelial cell damage was observed. At a stage of the OTC-treatment in which minimal cytochrome levels were reached but still no structural damage of the epithelium was observed, the energy-stores in the epithelial cells and the rate of oxidative phosphorylation in isolated intestinal mitochondria appeared to be unaffected. After 3–4 days, when morphological alterations of the villus were clearly observed histologically, the rate of proliferation of the crypt cell population was not appreciably affected, suggesting again a lack of influence on energy metabolism in the crypt cells. During short incubations of isolated villous cells, the cellular energy charge and the rate of aminoacid incorporation into protein was strongly decreased after anaerobiosis and addition of KCN, oligomycin or 2,4-DNP, conditions which impair the mitochondrial energetic function. However, incubation with 100–500 μg/ml of several tetracyclines, of which doxycycline showed the strongest effect, led to a significant inhibition of cytoplasmic protein synthesis without affecting the rate of glycolysis and respiration or the energy charge in the epithelial cells. These observations tend to support the view that the toxic effects of OTC are mainly based on its interaction with cytoplasmic protein synthesis under conditions of drug accumulation in blood and tissues. The lowering of energy charge in liver and intestine after prolonged treatment may be interpreted as secondary effects of this action, namely of fatty infiltration in the liver and structural damage of the villous epithelium respectively.

A NUMBER of antibiotics which inhibit protein synthesis in bacterial 70 S ribosomes also interfere with the mitochondrial protein synthesis in eucaryotic cells such as yeast, Neurospora crassa and mammalian systems.[1–3] The consequences of this inhibitory action for mitochondrial structure and function can be studied most successfully in tissues with a rapid and continuous *de novo* synthesis of mitochondrial components. In our previous work, the small intestinal epithelium of adult rats had been used as a model system because of its unequalled high cellular turnover rate.[4] Treatment of rats with chloramphenicol (CAP) or oxytetracycline (OTC) during about 48 hr led to drastically decreased levels of the mitchondrial cytochromes aa_3 and b in the intestinal villous and crypt cells without significantly affecting the contents of a variety of other mitochondrial- and extramitochondrial enzymes.[4,5] Prolongation of the OTC treatment beyond 2 days caused a considerable loss of body weight, lethargy, diarrhea and severe structural damage of the intestinal epithelium, resulting in a

lowered protein yield of the villous cells after isolation. No similar toxic effects were observed with CAP even after 96 hr of treatment although both antibiotics evoked an equal decrease of cytochrome levels.[4-6] These results suggested that the toxicity of OTC was not based on its interference with mitochondrial protein synthesis but that the antibiotic had another site of action in mammalian cells, appearing after accumulation of the drug during prolonged treatment at serum concentrations above 50 μg/ml.[4] High-concentration effects of CAP, e.g. a direct inhibition of cellular respiration,[7,8] were not expected because the intramuscular administration guaranteed serum levels not surpassing 50 μg/ml during the whole course of the experiment.[4]

In the present study, more information was obtained about the energy metabolism of the intestinal mitochondria *in vivo* and *in vitro* under conditions of greatly depressed cytochrome levels (about 2 days after the start of the injection series) and after prolonged treatment with OTC when villous cell loss occurred. In addition, attemps were made to localize other targets of OTC in the intestinal cells. The villous and crypt cell preparations proved to be useful models for a study of the action of tetracycline antibiotics at subcellular levels in mammalian systems.

EXPERIMENTAL

Animals. Male, specific pathogen free, Wistar rats, weighing 200–230 g, were used throughout. They were maintained on a normal laboratory diet and water *ad lib.* Rats were sacrificed by cervical dislocation.

Chemicals. Enzymes and cofactors were purchased from Boehringer and Sons (Mannheim, W. Germany), 2,4-dinitrophenol and oligomycin from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.), fatty acid poor bovine serum albumin from Pentex (Kaukakee, Ill., U.S.A.) and TMPD (*N*, *N*, *N'*, *N'*-tetramethyl 1,4-phenylenediaminedihydrochloride) from Fluka, A. G. (Buchs, Switzerland). All other chemicals were analytical grade.

Antibiotics. Chloramphenicol (sodium succinate) was obtained from Nogepha, Amsterdam, Holland. Oxytetracycline (Terramycin), obtained from Chas. Pfizer & Co., Inc., New York and Tetracycline (Achromycin), purchased from R.I.T., Genval, Belgium, were used in the form of their hydrochlorides. Doxycycline (Vibromycin), obtained from Pfizer, was used as the hyclate salt (hydrochloride, hemihydrate, hemi-ethanolate).

Administration of the antibiotics in vivo. Serum levels of D(−)-threo chloramphenicol (CAP) between 10 and 50 μg/ml were continuously maintained by intramuscular injection of 280 mg CAP-succinate/kg body wt every 4 hr. Oxytetracycline was injected intramuscularly over 8 hr at a dose of 300 mg/kg body wt per 24 hr. After 48 hr of treatment, serum levels fluctuated between 50 and 120 μg/ml. Further details are reported elsewhere.

Assays. The conditions for the determination of protein and alkaline phosphatase (EC 3.1.3.1) are reported by Iemhoff *et al.*[9] Esterase activity was measured as described by Higgins and Lapides.[10]

Preparation of epithelial cell suspensions, homogenates and mitochondrial fractions. Villous- and crypt cells originating from the whole length of the small intestine were harvested separately according to the high-frequency vibration technique of Harrison and Webster[4,11] in a medium containing 0·01 M Tris–HCl buffer, 0·13 M NaCl and

5 mM EDTA (final pH 7·6). Cells and cell sheets were collected by centrifugation for 15 sec at 800 g_{max}. Homogenates of villous cells were prepared in a medium, containing 0·25 M sucrose, 20 mM Tris–HCl buffer, 1 mM EDTA and I mg/ml bovine serum albumin (final pH 7·4), by treatment of the suspension for 60 sec with a small Polytron homogenizer (type PT_{10}, Kinematica, Luzern, Switzerland) set at position 4·5 (8000 rev/min). Subfractionation of the homogenate was carried out according to the scheme, extensively described by Hülsmann *et al.*[12] The gentle homogenization procedure used gave a small yield of mitochondria. Their quality however, was better than of those obtained by earlier methods[13] where higher yields were obtained.

Preparation of rat liver mitochondria. Rat liver mitochondria were isolated according to Schneider[14] in a medium containing 0·25 M sucrose, 0·01 M Tris–HCl and 1 mM EDTA (pH 7·4). After removing the fluffy layer on top of the mitochondrial pellet by washing with sucrose, the mitochondria were washed four times by resuspension in the isolation medium and resedimenting at 6500 *g* for 10 min. The final pellet was suspended in the isolation medium to a final concentration of about 30 mg protein/ml.

Polarographic experiments. Oxygen uptake was recorded polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co.) at 30° in an air-saturated medium in a final volume of 1·6 ml. Villous cells were incubated in a Ca^{2+}-free Krebs Ringer bicarbonate buffer, pH = 7·4. The incubation medium for cell homogenates and mitochondrial preparations contained 250 mM sucrose, 20 mM Tris–HCl, 1 mg/ml bovine serum albumin, 5 mM potassium phosphate and 1 mM EDTA. The final pH was 7·2. The measurements were started after addition of 1–4 mg of cellular or mitochondrial protein. For calculation of P/O ratios and respiratory control indices (RCI) from the stimulation of respiration rates caused by the addition of 200 nmoles ADP, the method of Chance and Williams[15] was applied, except in the experiments described in Table 4 with intestinal mitochondria. In that case, the basic reaction medium in the polarographic vessel was supplemented with 2 mM $MgCl_2$, 16 mM glucose, 10 IU of dialyzed hexokinase (EC 2.7.1.1) and 200 nmoles ADP. After determination of the oxygen uptake following the addition of the mitochondria in the presence or absence of OTC, the reaction was terminated by addition of $HClO_4$ (final concentration 4 per cent). After deproteinization and neutralization with KOH, the samples were assayed for hexosemonophosphate. Further details are reported elsewhere.[16]

Determination of adenine nucleotides and creatine phosphate in intestinal segments, epithelial cells and liver. Rats were anaesthetized with Nembutal. After opening their abdomen through a midline incision and cannulation of the lumen at the proximal part of the duodenum, the terminal ileum was tied off and cut near the ligature. The lumen contents of the small intestine were carefully removed by rinsing with saline (temperature: 38°). The jejunal part of the intestine was quickly frozen by means of a Wollenberg clamp, precooled in liquid nitrogen. The frozen tissue was pulverized in a porcelain mortar, kept cold with liquid nitrogen, mixed with 5% $HClO_4$, thawed and centrifuged. The pellet was used for the determination of DNA.[17] After filtering the supernatant through "Millipore" filter (pore size 0·4 μm) it was neutralized with KOH and centrifuged. ATP,[18] ADP,[19] AMP[19] and creatinephosphate[20] (CP) were determined enzymically within 3 hr from the start of the operation.

Freeze-clamping of the right lobe of the liver was performed after elevation by

means of a loose ligature conducted through the organ with a needle. Determinations of DNA and adenine nucleotides were carried out as described for intestine.

Levels of adenine nucleotides and DNA in freshly isolated villous cells were determined after gentle shaking of 1·5 ml of a cell suspension (15–20 mg protein) in a Ca^{2+}-free Krebs–phosphate buffer, containing 10 mM glucose, for 20 min in polyethylene vials at 37°. The cells were rapidly disrupted by the addition of 1·5 ml 10% $HClO_4$, immediately followed by an ultrasonic desintegration procedure. Hereafter the same procedure was followed as described for freeze-clamped samples.

Glucose metabolism in isolated intestinal villous cells and human erythrocytes. Glucose utilization was measured in 2 ml of a Ca^{2+}-free Krebs Ringer–phosphate buffer (pH 7·4) containing 10 mM [1−^{14}C]glucose (20 μCi/m-mole). Villous cell suspensions were incubated in polyethylene vials for 30 min at 37°, as described in the previous section. The cells were preincubated with the drugs for 10 min before the addition of glucose. Glucose consumption, lactate production and the production of CO_2, originating from the 1-position of glucose, were measured according to Iemhoff *et al.*[9] Human erythrocytes were collected from heparinized blood samples and washed three times with 8 volumes of saline. The top layer and buffy coat were discarded and the packed cells were suspended in an equal volume of the incubation medium. For measurements of glucose metabolism, the same procedure was applied as described for villous cells.

Incorporation of ^{14}C labeled amino acids into protein of isolated villous and crypt cells. Isolated villous and crypt cells were suspended in a Ca^{2+}-free Krebs Ringer–phosphate buffer, pH 7·4, fortified with a mixture of L-amino acids of the same concentration and composition as used in the Basal Medium, described by Eagle *et al.*[21] Incubations of 1·1 ml aliquots (protein content 5-20 mg) were carried out in polyethylene vials at 37° as described before. After preincubation in the presence of the antibiotics for 5 min, the incorporation of labeled amino acids was started by addition of 0·25 μCi[^{14}C]amino acid mixture (sp. act. 52 mCi/mAtC; code CFB.25, Radiochemical Centre, Amersham) and was stopped after 15 min by the addition of 6 ml ice-cold 5% trichloroacetic acid (TCA). The precipitate was centrifuged and resuspended in TCA (5% w/v) by ultrasonic homogenization. After standing at 90° for 30 min the suspension was centrifuged again and the pellet was washed once with TCA and once with 5 ml ethanol–diethyl ether (2:1, v/v). The final pellet was taken up in 0·5 ml NCS solubilizer (Amersham, Searle) and sonified. This solution was transferred to counting vials, 10 ml toluene liquifluor was added and counting was performed in a liquid scintillation counter with automatic standardization.

Measurement of ^{3}H thymidine incorporation into DNA of villous and crypt cells after in vivo *administration of the label.* Control rats and rats treated for 80 and 60 hr with OTC, were injected intraperitoneally with [^{3}H]-thymidine ([^{3}H]methyl-thymidine, specific activity 37·8 Ci/m-mole, Radiochemical Centre, Amersham). Treatment with OTC was continued for 20 and 40 hr respectively. The animals were then sacrificed and their guts were removed. A duodenal segment (5 cm below the pylorus) was processed for autoradiography. According to Harrison and Webster,[11] different villous cell fractions were harvested during the vibration procedure, as judged from the specific activity of alkaline phosphatase and esterase (Table 1). The first fraction (vibration time 0–10 min, amplitude 0·2 cm) mainly contained cells from the apical areas of the villi and the third fraction (18–27 min) appeared to be rich in basal

TABLE 1. DISTRIBUTION OF LABELED DNA IN CRYPT AND VILLOUS CELL FRACTIONS 20 AND 40 hr AFTER [^{3}H]METHYL-THYMIDINE LABELING

Cell fraction	Total protein (mg)		mg protein / mg DNA		Esterase (nmoles × min^{-1} × mg^{-1} protein)		Alkaline phosphatase (nmoles × min^{-1} × mg^{-1} protein)		Specific incorporation (10^{-3} × dis/min × μg^{-1} DNA)			
									20 hr†		40 hr†	
	Control	OTC*	Control	OTC	Control	OTC	Control	OTC	Control	OTC	Control	OTC
Villous fraction 1	29	18	16·9	16·7	2900	3110	390	175	0·4	0·6	1·4	1·2
Villous fraction 2	37	12	16·4	15·1	2600	2780	165	83	1·1	1·0	1·3	1·3
Villous fraction 3	24	15	15·2	13·9	1960	2040	141	55	1·4	1·3	1·0	1·2
Crypt fraction	30	27	9·7	10·5	1020	1010	32	29	1·3	1·4	1·1	1·0
Total fractions	120	72										

* Period of OTC-treatment: 100 hr.

† Period after pulse-labeling with [^{3}H]thymidine.

For details concerning the isolation of cells, the antibiotic treatment and the determinations see Experimental. Each value represents an average of six animals, except for the calculations of specific incorporation, where values derived from three animals were used.

villous cells. After isolation of the crypt cells by means of dilatation and vibration, the fractions were centrifuged and the cells resuspended in isolation medium, from which samples were drawn for the assay of protein and enzymes. The cells were then precipitated with ice-cold TCA (5% w/v) and centrifuged again. The precipitates were washed four times with 10 ml 5% TCA and twice with 10 ml ethanol–diethylether (2:1, v/v) and were finally taken up in 5% TCA. Part of this suspension was used for determination of DNA.[17] The other part was centrifuged again. The final pellet was taken up in 0·5 ml NCS and counted, as described for the protein samples in the previous section.

Autoradiography. Duodenal segments were fixed in neutral formalin. Paraffin sections were cut at 6 μm and coated with Ilford K-2 emulsion. After an exposure time of 4 weeks the sections were poststained with hematoxylin and eosin. The average distance covered by labeled cells was determined from micrographs of perfect longitudinal cut crypts and villi. For each animal 10–15 sections were examined and 20–25 crypts and villi were measured.

RESULTS AND DISCUSSION

Turnover rate of the intestinal cell population after OTC-treatment. As shown in previous reports,[4,5] after treatment of rats for 48 hr with OTC or CAP a minimal value of cytochrome aa_3 content in the epithelial cells was reached corresponding with about 25–30 per cent of the control value, whereas cytochrome b showed a residual activity of about 40 per cent. No further decrease was observed after prolonged treatment. The residual activities of the cytochromes may be explained by an incomplete inhibition of mitochondrial protein synthesis by OTC or CAP under the *in vivo* conditions or, alternatively, by a strong reduction of the turnover rate of the intestinal cell population. According to Firkin and Linnane,[8] long-term treatment of Hela cell cultures with CAP caused an 80 per cent decrease of cytochrome aa_3 content resulting in a loss of dividing activity. Since a similar inhibition of the proliferation of intestinal crypt cells should provide a reasonable explanation for the structural alterations of the villous epithelium after prolonged treatment with OTC, this hypothesis was tested by comparing the proliferative activity of crypt cells from normal rats with those from OTC rats during a period between 60 and 100 hr after the first injection of the antibiotic. For that purpose, the incorporation of [^{3}H]thymidine in the crypt cells and the migration of the label from the crypt to the villous top was measured as described in the Experimental section.

Table 1 shows that 20 and 40 hr after the injection of [^{3}H]thymidine the specific activity of DNA in the three villous fractions and in the crypt of the OTC-rats does not differ significantly from the control. Incorporation of [^{3}H]thymidine into the DNA of the crypt cells did not seem to be impaired by the antibiotic treatment for 60 or 80 hr. The data also suggest that the migration of label to the villus top had not been delayed by OTC-treatment for 100 hr. However, because of the OTC-induced morphological changes, especially of the villus, examined histologically,[6] and the incomplete separation of cells from different regions of the villus, indicated by the presence of label in villous fraction 1 20 hr after the pulse, autoradiography was considered as a more accurate technique to trace a possible change in turnover time after prolonged antibiotic treatment.

Table 1 also shows that the protein/DNA ratio in the fractions was not significantly changed, indicating that the diminished yield of villous cell protein (cf. also Ref. 4) was accompanied by a loss of DNA, which is most easily explained by an increased extrusion of villous cells during the antibiotic treatment. The loss of villous cells contributes to the sharp decrease in villus length, observed histologically (Fig. 1). Finally, the specific activity of the marker enzyme alkaline phosphatase, in contrast with esterase, was decreased after OTC-treatment for 100 hr (Table 1). A similar specific depression of this enzyme has been observed following *in vivo* administration of cycloheximide, a potent inhibitor of protein synthesis, at concentrations not inhibiting cell migration.[22]

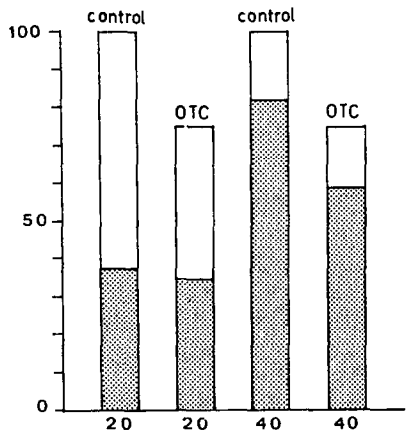

FIG. 1. Abscissa: hours after [^{3}H]thymidine pulse. Ordinate: average distance of migration (arbitrary units of length) of [^{3}H]thymidine-labeled cells (shaded) on the duodenal villi (open). 0 = crypt bottom, 100 = villus length of control rats. Period of OTC-treatment = 100 hr.

Autoradiographic results, given in Fig. 1, show that the distance travelled by the label 20 hr after the [^{3}H]thymidine pulse was not significantly changed by OTC-treatment. In both cases, the boundary between labeled and unlabeled cells was located 8-12 cell positions above the villus-crypt junction. Apparently OTC-treatment did not drastically alter the rate of proliferation of the crypt cells. After another 20 hr the label was at about the same space from the villus top in control and OTC rats. This means, however, that the migration rate of the villous cells was reduced, because the villus length had decreased markedly (Fig. 1). This retardation may be explained by a partial loss of cells during their migration to the villus top, in addition to a reduction of cell size, due to a general dehydration of the animal, as is occasionally observed during treatment with high doses of OTC.[23]

Influence of OTC and CAP administration in vivo *on respiration and energy production of intestinal mitochondria* in vitro. From the lack of influence of the OTC-administration on the proliferative activity of the crypt cells it might be concluded that the energy production in these cells had not been drastically reduced by the antibiotic. However, this does not exclude a diminished mitochondrial energy production, because, as in beating heart cells,[24] the glycolytic capacity of the intestinal tissue might conpensate for such a decrease. A deficiency of oxidative phosphorylation may result from two factors: a lowered rate of electron transport, induced by critical levels of the affected cytochromes and/or a partially uncoupled state of the mito-

chondria, as might arise under conditions of a decreased rate of synthesis of some of the coupling factors (cf. Refs. 8, 24, 25). These two possibilities were investigated in experiments with isolated villous cells, homogenates and mitochondrial fractions derived from 48 hr OTC- or CAP-treated rats, when minimal levels of the cytochromes aa_3 and b were obtained. The results are presented in Table 2.

Villous cell suspensions in Krebs–phosphate buffer maintained a high rate of endogenous respiration for at least 3 hr after the isolation. A KCN-sensitivity of 90–95 per cent was found. In most of the control preparations the Q_{O_2} value was not influenced to an appreciable extent by the addition of glucose, pyruvate and cofactors such as ADP and NAD^+. Succinate, an amino acid mixture or 2,4-dinitrophenol gave a slight and variable stimulation. After gentle homogenization of a cell suspension in sucrose medium in the presence of ADP, P_i and bovine serum albumin, the respiration decreased to a low level, but could be greatly enhanced by the addition of the substrates indicated in Table 2. No stimulation by cytochrome *c* or NADH was observed. Polarographic measurements of oxidative phosphorylation in homogenates and mitochondrial preparations were only successful in a Mg^{2+}-free sucrose-medium in the presence of 1 mM EDTA in order to suppress the hydrolysis of ATP and ADP by the Zn^{2+}-dependent enzyme alkaline phosphatase.[26] Addition of 1·5–3 mM Mg^{2+} to the medium caused a similar stimulation of state 4 respiration as could be induced by the uncoupler 2,4-DNP (results not shown). According to preliminary experiments, Mg^{2+} stimulates the mitochondrial-bound hexokinase in the preparations. The glucose arises from the sucrase activity present in contaminating brushborder fragments. Furthermore, the preparations contain a specific oligomycin-sensitive Mg^{2+}-ATPase, which has been noticed also in mitochondrial preparations of heart- and skeletal muscle.[27,28] Under our conditions we obtained P:O and RCI values, which exceeded those measured by Clark and Sherrat[29] in mitochondrial preparations from guinea-pig mucosa. These authors described a defective oxidative phosphorylation in small intestinal mitochondria isolated from the rat. As shown in Table 2, the only significant differences in maximal Q_{O_2}, observed between control and 48 hr OTC- or CAP-treated rats, appeared with sonicated preparations in the presence of cytochrome *c* and TMPD plus ascorbate as substrate (sonication caused a ten-fold stimulation of respiration). In that case, the decrease of Q_{O_2} to about 35 per cent of the control value, agrees with the strongly reduced activity of cytochrome *c* oxidase mentioned before.[4,5] However, since the lowered level of TMPD-ascorbate oxidation always exceeded the respiration rates found with the other substrates, the electron transport process via cytochrome aa_3 need not be rate-limiting for the overall oxidation with these compounds, neither in the control, nor in the treated rats. The results of the measurements of state 3 oxidation rates, P:O ratios and RCI values with succinate and NAD^+-linked substrates, summarized in Table 2, indicate that neither an influence on the rates of electron transport, nor on the phosphorylating efficiency could be detected in the cytochrome-depleted mitochondria. Similar results for heart mitochondria were obtained by Hallman[3] after treatment of neonatal rats for 4 days with CAP. Furthermore, Firkin and Linnane[30] failed to detect an effect of CAP on the respiration and P:O ratios of mitochondria from regenerating liver. Apparently, even in isolated mitochondria lacking 60–70 per cent of their content of cytochrome b and aa_3, state 3 oxidation rates are not limited by the turnover rates of these cytochromes. However, these experiments do not entirely exclude the possibility that under

TABLE 2. RESPIRATION RATES AND OXIDATIVE PHOSPHORYLATION IN VILLOUS CELLS AND HOMOGENATES FROM CONTROL RATS AND RATS TREATED FOR 48 hr WITH OTC OR CAP

Substrate added	Cells		Total homogenates					
	Q_{O_2}		Q_{O_2}		P:O		RCI	
	Control $n = 8$	Treated $n = 16$	Control $n = 8$	Treated $n = 6$	Control $n = 8$	Treated $n = 6$	Control $n = 8$	Treated $n = 6$
—	19·2 ±0·7	17·5 ±1·0	3·1 ±0·4	3·6 ±0·5				
Glucose (30 mM)	20·5 ±1·2	17·9 ±0·9						
Succinate (5 mM)	24·0 ±1·5	20·5 ±1·7	31·2 ±2·7	27·8 ±2·7	2·14 ±0·12	2·05 ±0·13	2·47 ±0·16	2·21 ±0·41
Glutamate (5 mM)			15·2 ±1·8	15·7 ±2·0	3·20 ±0·14	3·09 ±0·17	3·15 ±0·13	3·03 ±0·16
3-DL-Hydroxybutyrate (5 mM)			15·3 ±0·9	14·1 ±1·2	2·84 ±0·10	2·80 ±0·11	2·24 ±0·15	2·16 ±0·16
Pyruvate (10 mM) plus L-malate (2 mM)			15·1 ±1·3	13·6 ±1·3	3·02 ±0·13	2·94 ±0·18	2·21 ±0·12	2·35 ±0·14
IMPD (0·5 mM) plus ascorbate (10 mM) plus cytochrome *c*			106 ±6·2	44·6 ±8·1 $P < 0·01$				

The Q_{O_2} values, measured polarographically as described in Experimental, are expressed as nmoles O_2 consumed/min/mg protein followed by their standard errors. P-values were calculated using the Student *t*-test. The Q_{O_2} of homogenates is given for state 3 respiration rates. Final substrate concentrations are indicated in parenthesis. All substrates were added in the form of their sodium salt. Calculations of respiration rates with TMPD plus ascorbate, measured with sonicated preparations, were corrected for auto-oxidation. Because the results with CAP- and OTC-rats showed no significant differences, all data obtained after both kinds of treatment, were pooled for the calculation of mean values and standard errors. With preparations of isolated mitochondria (results not shown), P: O-ratios and RCI-values were essentially similar to those measured with total homogenates, the Q_{O_2}-values increased about twice.

certain *in vivo* conditions higher rates of respiration are required, which cannot be sustained by the low levels of the affected cytochromes (cf. Ref. 31). Therefore we felt that additional information concerning the energy metabolism in the intestine *in vivo* was needed.

Levels of adenine nucleotides and creatine phosphate in small intestinal tissue and liver of the rat in vivo *after OTC- or CAP-treatment.* A depression of oxidative phosphorylation *in vivo* was expected to evoke similar alterations as occur under hypoxic conditions, e.g. a decrease of the energy stores in the epithelial cells, concomitant with an increased rate of glycolysis.[32] Such changes can only be measured accurately with the aid of the freeze-clamping technique, because a preliminary isolation of epithelial cells leads to a rapid and irreversible fall in ATP content of the tissue.[9] However, the contribution of the muscle layer to the energy reserves in the freeze-clamped samples cannot be measured directly. Assuming an equal nucleotide content per mg of dry matter in intestinal epithelial cells and muscle of control rats, about 40 per cent of the estimated nucleotides is derived from the muscle layer.[33] An unequal distribution of creatine phosphate (CP), between epithelial cells and muscle cells is unlikely, as we have found that about 60 per cent of the total activity of creatine kinase in small intestinal tissue was recovered in isolated villous- and crypt cells.* Therefore, a specific influence of the antibiotic treatment on the energy metabolism of the epithelial cells should manifest itself in a significant change in the content of adenine nucleotides, as well as of CP in total intestinal tissue. For comparison, levels of nucleotides were measured in liver of rats subjected to the same OTC regime.

Table 3 shows that intramuscular treatment of the rats for 2 days with OTC or for 3 days with CAP had no significant influence on the levels of ATP, ADP, AMP and CP in intestine and liver, as compared with saline-injected rats. This indicates that, in accordance with the *in vitro* results with isolated mitochondria, the cytochrome-poor mitochondria in intestine kept their normal ATP-generating capacity. However, after 3 days of OTC-treatment, the energy charge of the adenylate system in liver and intestine, proposed by Atkinson[34] as an important metabolic regulatory parameter, and the CP-content of intestine showed a significant decrease. Histochemical investigations showed an excessive accumulation of fat in the liver (unpublished results), in agreement with many earlier reports about the effects of high doses of OTC or tetracycline (TC) on liver,[35–37] and a structural damage of the intestinal villus,[6] in which no accumulation of fat was found.

Both observations may explain the substantial fall of phosphate potential in these organs or, alternatively, both phenomena are the consequences of a decreased ATP-content of the cells. In the latter case, the energy deficiency is most probably caused by a direct effect of OTC, accumulated in liver and intestine, on energy providing processes, e.g. oxidative phosphorylation or glycolysis. This hypothesis was further tested using isolated intestinal cells and mitochondria from intestine and liver as model systems.

Influence of OTC on glucose utilization in intestinal epithelial cells and erythrocytes. According to a recent report of Carević and Čerlek,[38] erythrocytes of patients treated with OTC (1 g/day for 2 days) and human erythrocytes incubated in the presence of 100 μg/ml of this antibiotic, showed a strongly reduced rate of glucose utilization. However, our results apparently conflict with this result. Under similar conditions

* H. R. de Jonge, unpublished results.

TABLE 3. LEVELS OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE IN JEJUNUM AND LIVER OF THE RAT *in vivo*

Drug	Dose/injection (mg/kg body wt)	Number of injections per 24 hr	Duration of treatment (hr)	Metabolite concentrations (nmoles/mg DNA)* ATP I	ATP L	ADP I	ADP L	AMP I	AMP L	CP I	Energy charge*,† I	Energy charge*,† L	ATP* ADP + AMP I	ATP* ADP + AMP L
Saline ($n = 5$)	— (I.M.)	3–6‡	48–72‡	230 ±9	1470 ±52	41 ±2	356 ±18	9 ±2	42 ±5	220 ±15	0·89 ±0·02	0·88 ±0·01	4·56 ±0·30	3·66 ±0·20
OTC ($n = 4$)	100 (I.M.)	3	48	223 ±14	1312 ±39	44 ±3	370 ±21	7 ±2	39 ±7	210 ±21	0·89 ±0·03	0·87 ±0·01	4·48 ±0·25	3·21 ±0·24
OTC ($n = 4$)	100 (I.M.)	3	72	167 ±10 P<0·05	810 ±62 P<0·05	70 ±6 P<0·05	758 ±40 P<0·05	16 ±4 P<0·05	86 ±11 P<0·05	132 ±9 P<0·05	0·80 ±0·02 P<0·05	0·72 ±0·02 P<0·05	2·00 ±0·29 P<0·05	0·96 ±0·17 P<0·05
CAP ($n = 3$)	200 (I.M.)	6	48	250 ±12		45 ±5		12 ±5		234 ±19	0·88 ±0·03		4·40 ±0·33	
CAP ($n = 3$)	200 (I.M.)	6	72	233 ±8		38 ±6		10 ±4		250 ±16	0·89 ±0·03		4·85 ±0·30	

* Values are given as means ±S.E.

† The energy charge of the adenylate system is defined as the ratio (ATP + ½ADP)/(ATP + ADP + AMP).[34]

‡ No significant differences were found between saline-injected rats after different methods of treatment. Therefore, all values were pooled for calculation of mean values and standard errors.

Abbreviations used: I = intestine, L = liver, I.M. = intramuscular, n = number of rats, P = probability value, calculated by the Student t-test.

in vitro, incubation with 100–300 μg/ml of oxytetracycline, tetracycline or doxycycline for 30 min did not affect glucose consumption of intestinal epithelial cells (mean value: 15·8 nmoles $\times$ min^{-1} $\times$ mg^{-1} protein glucose used) or human erythrocytes (2·07 μmoles $\times$ hr^{-1} $\times$ ml^{-1} packed cells). Also the lactate production (27·5 and 3·9 respectively) and the production of CO_2 originating from the 1-position of glucose (2·4 nmoles $\times$ min^{-1} $\times$ mg^{-1} protein for villous cells) were not appreciably affected. If an extremely high dose of OTC (400 mg/kg body wt) was given to rats by means of a stomach tube or intraperitoneal injection, essentially the same results (not shown) were obtained with villous cells isolated 4 hr later. Since a direct action of the tetracyclines on glycolysis could not be confirmed by our experiments, we tend to believe that the changes in carbohydrate metabolism observed after tetracycline-treatment[39,40] mainly result from an impaired function of the fatty liver.

Influence of oxytetracycline on oxidative phosphorylation in vitro. As suggested by Linnane *et al.*,[41,42] considering mitochondrial ribosomes as an integral part of the mitochondrial membrane, inhibitors of mitochondrial protein synthesis may interact with other membrane functions, e.g. oxidative phosphorylation and electron transport. Indeed, it has been established that high concentrations of CAP,[7,8] carbomycin, oleandomycin, paromycin[41] and mikamycin[43] may interfere with respiration. Brody *et al.*[44] described an inhibitory action of 5×10^{-4} M OTC (300 μg/ml) on oxidative phosphorylation and octanoate oxidation by isolated Mg^{2+}-poor mitochondria from rat liver and brain. This inhibition could be reversed in the presence of excess Mg^{2+}-ions.

Reinvestigation of these effects (Table 4, Fig. 2) revealed a rather complex inter-

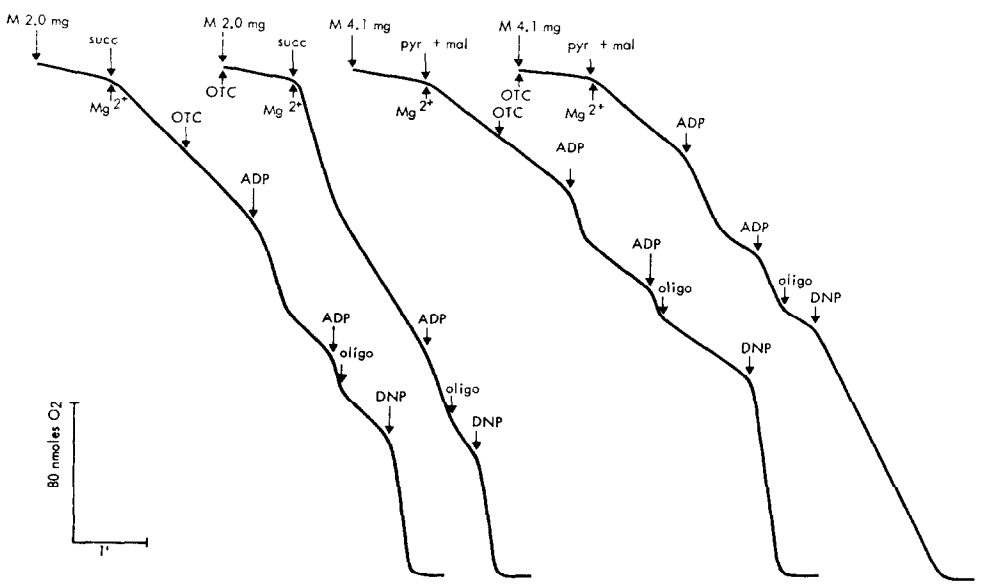

FIG. 2. Effect of oxytetracycline on mitochondrial respiration. Oxygen uptake was determined polarographically at 30° in a medium containing 250 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, 5 mM potassium phosphate buffer and 1 mg/ml BSA, pH 7·2. Additions: M = liver mitochondria (mg of protein); 2 mM Mg^{2+} as $MgCl_2$; 200 μg/ml OTC (0·33 mM); 200 nmoles ADP; 10 μg oligomycin; 10^{-4} M 2, 4-dinitrophenol; substrate concentrations: see Table 4.

TABLE 4. EFFECT OF OXYTETRACYCLINE ON OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA ISOLATED FROM RAT SMALL INTESTINAL EPITHELIUM AND LIVER

		Intestinal mitochondria					Liver mitochondria							
		Mg^{2+} omitted			2mM Mg^{2+}		2 mM Mg^{2+}							
Substrate	OTC (μg/ml)	Q_{O_2} (state 4)	Q_{O_2} (state 3)	Q_{O_2} (uncoupled)*	P:O† A‡	P:O† B§	Q_{O_2} (state 4) A	Q_{O_2} (state 4) B	Q_{O_2} (state 3) A	Q_{O_2} (state 3) B	Q_{O_2} (uncoupled) A	Q_{O_2} (uncoupled) B	P:O A	P:O B
Succinate (5 mM)	0	28	70	75	2·3		21		117		124		2·1	
	200	41	48	76	1·2	2·2	50¶	19	65	115	118	120	0·6	1·9
Pyruvate (10 mM)	0	11	35	37	3·0		7		44		46		2.9	
+ L-malate (2 mM)	200	13	17	26	1·4	2·6	9	7	20	39	27	40	1·4	2·4

Q_{O_2} values are expressed as nmoles O_2 consumed/min/mg of protein. The polarographic experiments were carried out six times with different mitochondrial preparations. All results were quite reproducible.

* The uncoupled state was induced by 10^{-4} M dinitrophenol (DNP) added to ADP supplemented mitochondria.

† P:O ratios were determined using the glucose hexokinase assay described in Experimental. No inhibition of HK-activity was observed under these conditions (200 μg/ml OTC, 10 I.U. HK).

‡ Columns A represent P:O or Q_{O_2} values measured if OTC was added 1 min prior to Mg^{2+}.

§ Columns B refer to the experiments in which preincubation with 2 mM $MgCl_2$ for 1 min preceded the addition of OTC.

¶ Higher values were obtained during a transitory period immediately after the addition of succinate (Fig. 2).

action of the antibiotic with the respiratory chain of Mg^{2+}-depleted mitochondria from rat liver and intestine, isolated in sucrose–Tris–EDTA medium. At concentrations of 150–200 μg/ml (175–250 nmoles/mg of protein), OTC caused a stimulation of succinate respiration in the energy-controlled state 4,[15] but only slightly affected state 4 oxidation rates with NADH-linked substrates (glutamate, pyruvate plus malate). Furthermore, an inhibitory effect of OTC on state 3 respiration was observed with all substrates tested. Addition of the uncoupler DNP released the inhibition of succinate oxidation but only partly restored the respiration with NADH-linked substrates. Concomitantly, P:O ratios, measured with the glucose-hexokinase assay, showed a strong decrease if OTC was added prior to the addition of Mg^{2+}-ions (Table 4, column A). Where this influence on the phosphorylation process could not be released by subsequent addition of excess magnesium (2–8 mM), it could be prevented to a large extent by preincubation of the mitochondria with 2–3 mM Mg^{2+} (Table 4, column B).

The interaction of the drug with succinate respiration may be summarized as a uncoupling effect, resulting in a lowering of the P:O ratio and stimulation of controlled respiration, superimposed on a partial oligomycin-like inhibition of the process of energy transfer, implying a depression of state 3 respiration rates. The electron transfer between succinate and oxygen appeared unobstructed, since DNP was able to release this depression. If NADH-linked substrates are offered, the uncoupling effect of OTC, traced by a strong decline of the P:O ratio, does not manifest itself clearly in a stimulated rate of state 4 respiration. This may be explained by an additional influence of the antibiotic on the rate of transfer of reducing equivalents from these substrates to cytochrome b, suggested as well by a decrease of the Q_{O_2} values in the uncoupled state.

The behaviour of Mg^{2+}-poor mitochondria in the presence of OTC and other tetracyclines is similar, in certain aspects, to the detrimental action of 25–100 mM Na^+ on oxidative phosphorylation, described by Gómez-Puyou *et al.*[45] for liver mitochondria, incubated in a Mg^{2+}-free sucrose–EDTA medium. Here too, the respiratory control could be restored by including Mg^{2+} in the incubation mixture. This similarity and the insensitivity of the tetracycline action to subsequent addition of Mg^{2+} suggest that tetracyclines exercise a rather aspecific and readily irreversible influence on the structure of the Mg^{2+}-depleted mitochondrial innermembrane, where the respiratory chain and the enzymes involved in β-oxidation of fatty acids are localized. An explanation of this action, merely based on the Mg^{2+}-chelating properties of tetracyclines, as proposed by Brody *et al.*[44] does not agree with the low binding constant of the Mg^{2+}-tetracycline complex[46] and the inability of excess Mg^{2+} to cancel the action of these drugs.

Whereas a direct inhibition of oxidative phosphorylation by high concentrations of tetracyclines is well established by these *in vitro* experiments, a similar action *in vivo* can be expected only if the concentration of Mg^{2+}-ions in the mitochondria of the cells is very low and if tetracyclines accumulate in this region. However, radiolabeling techniques showed that tetracycline is selectively bound to the microsomal fraction of liver, kidney and muscle cells of the rat.[47] Stronger arguments against a disturbance of oxidative phosphorylation by these antibiotics in the intact cell could be derived from measurements of respiration rates and adenine nucleotide levels of isolated villous cells.

TABLE 5. ADENINE NUCLEOTIDE LEVELS IN ISOLATED VILLOUS CELLS

Additions	Concn.	Σ (ATP + ADP + AMP)	Adenine nucleotide levels (nmoles/mg DNA)			ATP + ½ADP	ATP
			ATP	ADP	AMP	ATP + ADP + AMP	ADP + AMP
—		260	99	83	78	0·54	0·61
Nitrogen*		254	24	90	140	0·27	0·10
2,4-DNP	0·2 mM	243	12	80	151	0·21	0·05
Oligomycin	35 μg/ml	258	22	100	136	0·28	0·09
KCN	0·7 mM	239	10	59	170	0·17	0·04
OTC	500 μg/ml	281	105	106	70	0·56	0·60
TC	500 μg/ml	254	95	71	88	0·51	0·60
Doxy	300 μg/ml	268	103	98	67	0·57	0·62

Incubations were carried out as described in Experimental. Mean values are shown, calculated from three different experiments. For further details see also Table 2.

* Anaerobiosis was maintained by a stream of nitrogen gas through two needles in rubber-capped incubation vials during the whole course of the incubation.

The Q_{O_2} of villous cells, incubated for 10 min in a glucose containing Krebs-Ringer–bicarbonate buffer, did not change after addition of 100–300 μg/ml OTC, TC or doxycycline (results not shown).

Table 5 shows that the total nucleotide content per mg of DNA did not differ significantly from the value obtained with freeze-clamped samples of jejunum (Table 3). This confirms the intact state of the isolated villous cells (cf. also Ref. 12). The energy charge in these cells appeared to be highly dependent on a proper functioning of the intestinal mitochondria. Incubation of villous cells in a glucose-containing medium under anaerobic conditions or in the presence of the uncoupler 2,4-DNP, oligomycin, an inhibitor of oxidative phosphorylation, or KCN, led to a remarkable lowering of the cellular energy charge. However, high concentrations of OTC, TC or doxycycline had no effect on this parameter, although penetration into the cells was indicated by the influence of these antibiotics, especially of doxycycline, on the aminoacid incorporation into cellular protein under comparable experimental conditions (see Table 6).

TABLE 6. INHIBITORY EFFECT OF TETRACYCLINES ON THE INCORPORATION OF [^{14}C]-LABELED AMINO ACIDS INTO PROTEIN OF ISOLATED SMALL INTESTINAL VILLOUS- AND CRYPT CELLS

Additions	Time of incubation (min)	Incorporation activity (dis/min × mg^{-1}protein)		Percentage of inhibition of extramitochondrial protein synthesis	
		Villous cells	Crypt cells	Villous cells	Crypt cells
None	7·5	260	1299		
None	15	482	2575		
CAP (50 μg/ml)	15	469	2510		
Puromycin (300 μg/ml)	15	37	126	92 (89–96)	95 (92–98)
OTC (100 μg/ml)	15	419	2270	10 (8–12)	11 (8–13)
OTC (300 μg/ml)	15	355	1832	24 (23–26)	27 (24–31)
TC (100 μg/ml)	15	389	2083	17 (14–19)	17 (13–19)
TC (300 μg/ml)	15	290	1581	38 (34–41)	37 (35–38)
Doxy (100 μg/ml)	15	368	1810	21 (19–22)	28 (25–30)
Doxy (300 μg/ml)	15	184	890	60 (50–70)	65 (60–70)
KCN (0·7 mM)	15	45	237	90 (88–93)	90 (86–94)
Nitrogen*	15	56	291	88 (85–91)	88 (82–93)
Oligomycin (35 μg/ml)	15	97	529	80 (77–84)	79 (76–83)
2,4-DNP (0·2 mM)	15	83	424	83 (80–86)	84 (79–89)

Each value of the incorporation activity represents the average of four determinations. The incorporation activities after 7·5 or 15 min were diminished with the small values found at zero min incubation. The extent of inhibition of cytoplasmic protein synthesis was obtained after correction for the diminished incorporation activity, caused by the action of 50 μg/ml CAP on mitochondrial- and bacterial protein synthesis.

* See the legend of Table 5.

Inhibition of cytoplasmic protein synthesis by tetracyclines. Experiments with cell free systems from rat liver[48] and yeast,[49] with intestinal slices[50] or rings[51] and *in vivo* studies of incorporation of labeled amino acids into various organs of the rat[52] indicate that tetracyclines at high concentrations not only inhibit bacterial or mitochondrial protein synthesis, but in addition act on the 80 S-ribosomes of eucaryotic cells. Therefore, cytoplasmic protein synthesis is also partially inhibited. The results

of our studies with isolated villous and crypt cells, presented in Table 6, are in agreement with this concept. The incorporation of labeled aminoacids into villous and crypt cell protein, followed during an incubation period of 7·5–15 min, showed a linear rate. The capacity of the crypt cells for protein synthesis, expressed on a protein base, surpassed that of the villous cells about five times, probably reflecting the *in vivo* situation.[53] During these short-term incubations with a mixture of labeled amino acids the extent of inhibition of cytoplasmic protein synthesis by 100–300 μg/ml of the antibiotics varied from 10–25 per cent for OTC to 28–65 per cent for doxycycline. Puromycin, a strong inhibitor of cytoplasmic- and mitochondrial protein synthesis, inhibited the incorporation by about 95 per cent. Although it was found that the rate of protein synthesis in isolated intestinal cells was very sensitive to an impairment of oxidative phosphorylation induced by KCN or anaerobiosis (Tables 5 and 6), even under conditions of a high aerobic glycolysis, this could not explain the inhibitory effects of the tetracyclines, because the adenine nucleotide levels in the isolated villous cells were unaltered under similar experimental conditions (Table 5). According to Ling and Morin,[51] inhibition of amino acid transport by these drugs may only be expected as an effect, possibly secondary to the inhibition of synthesis of a transport protein possessing a rapid turnover rate. It is unlikely that this action interfered with our studies in which very short incubation periods were used.

The much stronger inhibitory action of doxycycline as compared with OTC or TC may be due to its higher lipophilicity,[54] which possibly enables it to penetrate the intestinal cell much easier than the other tetracyclines. Despite the potential danger of the use of doxycycline suggested by our incorporation studies, this antibiotic is specially recommended for patients with renal insufficiency. However, as shown by the work of Schach von Wittenau *et al.*,[54] it has little chance to become toxic because of the low maintenance dose and the enhanced elimination of this compound from the blood by diffusion into the intestinal lumen, which prevents its accumulation in blood and tissues.

In the liver the action of high concentrations of OTC on cytoplasmic protein synthesis may be responsible for a disturbance of lipoprotein synthesis and/or transport resulting in fatty infiltration, also observed after puromycin treatment[55] which might cause the decrease of the energy charge in this organ after 3 days of antibiotic treatment.

In intestine, the unchanged rate of proliferation of the epithelial cells in the OTC rats does not necessarily conflict with a partial inhibitory action of OTC on overall protein synthesis, because a degree of inhibition, much less than 80 per cent, does not significantly affect the number of proliferating crypt cells.[56] However, in the villus region it may influence the synthesis of some protein components of the brushborder, e.g. alkaline phosphatase,[22] transport proteins[51] or glycoproteins of the glycocalix. The latter might increase the lability of cell membranes, suggested by the increased loss of soluble enzymes from the cells during the isolation procedure,[4] and induce a loosening of cells from the connective tissue. Also chelating of calcium by the tetracyclines might play a role in the displacement of the villous cells (cf. Ref. 11). Moreover, an additional effect of the drug on glycoprotein synthesis and release by the mucus-producing Goblet cells is conceivable in the light of a recent report of Tucker and Webster,[57] indicating an impaired transport and secretion of pancreatic proteins after administration of high doses of tetracycline. In our view the decrease of energy

charge in the intestinal tissue might be a consequence of the structural damage of the epithelium, resulting from the proposed actions of OTC mentioned above. Finally, inhibition of protein synthesis in other organs such as liver, kidney and pancreas may severely impair their function and exert secundary effects on the gastrointestinal morphology (e.g. dehydration[23]) or function (e.g. decreased absorption of fat[58]). Also, alterations of the intestinal microflora may affect absorption of some nutritients, such as iron.[59]

In conclusion it may be stated that the toxicity of OTC under our experimental conditions is most probably based on a primary action of this antibiotic on cytoplasmic protein synthesis rather than on an interaction of the drug with cellular energy metabolism either directly, or indirectly via depletion of mitochondrial cytochromes in rapidly proliferating tissues.

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