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Tissue distribution and effects on mitochondrial protein synthesis of tetracyclines after prolonged continuous intravenous administration to rats

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Impairment of mitochondrial protein synthesis by mitochondriotropic antibiotics like the tetracyclines and chloramphenicol occurs at the same relatively low concentration as necessary in serum of man for efficient antibacterial action during therapy (1–5 μg/ml serum [1]). Complete inhibition of mitochondrial protein synthesis results at every cell division in a 50 per cent diminution of the activity of cytochrome *c* oxidase and ATP synthetase. This may lead to interference with energy-dependent processes of cells and tissues, especially in rapidly dividing tissues. Evidence for impaired cell proliferation has been put forward in experiments with mammalian cells cultured in the presence of low levels of tetracyclines [2] or chloramphenicol [3]. The beating of rat-heart cells, cultured in a tetracycline- or chloramphenicol-containing medium becomes more dependent on glycolysis than in control cells [4]. These effects have been shown to be the result of a decreased synthesis and not of direct interference of the inhibitors with the enzymes [5]. Inhibition of cytoplasmic protein synthesis by tetracyclines is also known, but only at a much higher concentration (>50 μg/ml) [6, 7].

Prolonged *in vivo* administration of tetracyclines (i.e., during a number of cell divisions) can, therefore, result in two kinds of effect. At low concentrations, energy dependent functions will be progressively impaired; this can finally result in inhibition of cell division, notably in rapidly dividing tissues. High concentrations of tetracyclines will have a direct cytotoxic effect by blocking all protein synthesis.

Experiments aimed to investigate these two possible effects of tetracyclines *in vivo* are presented here. A continuous infusion technique enabled us to maintain a constant serum concentration, and to study the tissue distribution and effects of tetracyclines during prolonged treatment.

Materials and Methods

Animals and reagents. Male Wistar rats, weighing about 200 g, were used in all experiments. Oxytetracycline (OTC)—*sub forma* HCl—was supplied by Gist-Brocades N.V. (Delft). Doxycycline (DC)—*sub forma* HCL—was a gift of Pfizer & Co (Brussels). All other chemicals were of analytical grade.

Continuous infusion procedure. The method was developed based on a procedure described by Löbbecke *et al.* [8]. A catheter was placed in the vena jugularis and passed subcutaneously to the head of the animals. At this point it was connected to a metal pipe. This was fixed on the skull and connected with polyethylene tube to an infusion pump. This latter tube was interupted twice. Once to include a swivel to obtain optimal freedom of movement for the experimental animals; once for a T-junction to deairate the system. By means of the infusion pump a constant amount of solution was administered at a rate of 0.1 ml/hr. Infusion solutions contained 0.15 M NaCl in case of control experiments or varying amounts of DC or OTC dissolved in 0.15 M NaCl. 50 U/l of heparin to prevent clotting, was added in all cases.

The pH of the infusion was lowered with HCl to 2.5 to make the solutions stable for periods up to 4 days [9]. Only twice a week, therefore, fresh solutions had to be prepared.

Collection of specimens. Blood was obtained from the tail vein. At the end of the infusion period organs were removed quickly to determine the antibiotic and protein content and various enzyme activities. The organs were made as blood-free as possible by perfusing them with saline. 20% (w/v) homogenates were made in 0.15 M NaCl and stored at −20° for various times.

Analytical assays. The determination of doxycyline (DC) and oxytetracycline (OTC) in serum and tissue, was carried out within 24 hr. The fluorometric method has been described in detail before [9].

The cytochrome *c* oxidase activity was determined with the method of Cooperstein and Lazarow as modified by Borst *et al.* [10]. Cytochrome spectra, α-glycosidase activity, and thymidine kinase activity were measured with the methods described previously [5, 11]. Protein determinations were performed with a modified Lowry method [12].

Results

Maintenance of antibiotic levels in rat sera. One of the difficulties in studying the *in vivo* effects of prolonged inhibition of mitochondrial protein synthesis by e.g. tetracyclines is the maintenance of relatively low serum concentrations without temporary overloads. Concentrations of tetracyclines above 50 μg/ml, occurring either permanent or incidentally may have a direct inhibitory effect on cytoplasmic protein synthesis [6, 7], while levels below about 2 μg/ml will inhibit even mitochondrial protein synthesis insufficiently [13]. An attractive, though technically complicated method available to achieve constant levels is a continuous infusion technique. The mobility of the rats is limited minimally, while serum levels are the most constant in comparison to all other methods described [14].

Within the range of doses up to 40 mg/kg/day there was a linear relationship between the amount of OTC or DC administered and the level reached in the serum. Moreover, the serum levels remained constant during the whole period of treatment; the deviation at a fixed dose was less than 1 μg/ml over a period of 8 days. At any given dose, the OTC level in serum was 3.3 times higher than the DC level. DC penetrates better into the tissues, an observation also made by others [15, 16].

Tissue-distribution of DC and OTC. After treating the animals with different doses of DC or OTC during 7 days, the ratio between the serum- and tissue concentrations of the antibiotics are, with the exception of liver and kidney, only varying between very small ranges. The antibiotic content of several tissues can be calculated, therefore, once serum concentrations are known. As can be seen from Table 1, tissue concentrations of DC are always higher than those of OTC at the same administered dose. This reflects the better penetration of DC into tissues.

The higher tetracycline concentrations in the kidney may be ascribed to antibiotic present in the excretory products,

Table 1. Tissue distribution of oxytetracycline or doxycycline after seven days of continuous infusion with different doses of the tetracyclines*

	OTC (mean ± S.D.)	DC (mean ± S.D.)
Gut	0.6 ± 0.03	4.3 ± 0.75
Heart	2.1 ± 0.21	7.3 ± 0.63
Liver	4.2 ± 3.42	7.4 ± 5.21
Lung	2.5 ± 0.17	15.0 ± 1.08
Spleen	0.7 ± 0.12	3.3 ± 0.21
Kidney	6.0 ± 1.04	21.6 ± 11.99
Bloodcells	0.1 ± 0.01	0.4 ± 0.33

* Tissue levels are expressed as the ratio between tissue concentration (μg/g wet weight) and serum concentration (μg/ml serum). Triplicate measurements were done on two separate samples of tissues of each of at least five different rats.

which are according to Blanchard *et al.* [16] high and very variable. In contrast to the stability found in non-excretory organs, there were large variations in the ratios for liver and kidney (see Table 1). In one animal usually a high ratio for liver went together with a low ratio for kidney and *vice versa*. There is an apparent balance between these two main excretory systems, an observation which has been made before for doxycycline by Blanchard *et al.* [16].

The lowest antibiotic concentrations are measured in blood cells. This observation does agree with our previous conclusion that at least the red cells are impermeable for tetracycline chelates [7].

Effects of different doses DC and OTC after 7 days of continuous infusion. Animals were infused with 8, 27 or 40 mg/kg/day of DC or OTC. The control rats (infused with 0.15 M NaCl) and the animals receiving 8 and 27 mg/kg/day OTC or 8 mg/kg/day DC grew equally well during the 7 days of treatment and showed a normal weight gain of about 25 g. The animals receiving 27 mg/kg/day DC showed a lower gain in body weight (about 10 g), while animals receiving 40 mg/kg/day OTC or DC remained on the same body weight. From Table 2 it can be seen that in animals showing less or no weight gain tetracycline concentrations in several tissues are high enough to expect also partial inhibition of cytoplasmic protein synthesis [6, 7], which may explain the different growth behaviour. Table 2 also shows the effect of several tetracycline concentrations on mitochondrial protein synthesis, measured as the cytochrome *c* oxidase per mg of protein. The extent of the decrease of activity in the organs probably reflects the difference in turnover time of the mitochondria involved. Above concentrations of about 50 μg/g the decline of cytochrome *c* oxidase activity is lower. This can be explained by assuming that at this concentration cytoplasmic protein synthesis is also impaired, which will result in a lower total protein content of the tissues. Because the cytochrome *c* oxidase activity is expressed per mg protein, the calculated activity will be thus relatively higher. The inhibitions were not reflected in the wet weights of the organs. Most likely this criterium is too rough.

Only the spleen showed a clear difference also in wet weight between control and antibiotic treated animals. The latter showed a normal and the former, infused with 0.15 M NaCl only, an enlarged spleen. This difference was recorded at antibiotic concentrations far below 50 μg/g and needs further clarification.

The observation that the *specific* activity of the cytochrome *c* oxidase activity is lowered already indicates that overall protein synthesis is not affected, at least not to the same degree as mitochondrial protein synthesis. The observation that the animals do gain weight normally at the lower doses of OTC and DC is in line with this conclusion. None the less we tested the amount and activities of some enzymes that are synthesized in the cytoplasm. The data are shown in Table 3. The measurements were restricted

Table 2. Cytochrome *c* oxidase activity of several tissues after seven days of continuous infusion with 8, 27 or 40 mg/kg/day of oxytetracycline or doxycycline*

	OTC tissue level (μg/g)	Cyt. *c* oxidase activity	DC tissue level (μg/g)	Cyt. *c* oxidase activity
	2.4	2.8 (77)	3.9	2.6 (73)
Gut	8.2	2.6 (73)	21.0	2.3 (64)
	12.0	2.5 (68)	35.0	2.3 (65)
	8.2	25.3 (55)	6.3	26.2 (57)
Heart	25.1	23.9 (52)	36.1	29.0 (63)
	40.0	26.7 (58)	60.0	40.9 (89)
	8.0	30.8 (58)	7.0	30.7 (58)
Liver	24.0	28.7 (54)	12.0	27.0 (51)
	64.0	34.5 (65)	41.0	28.1 (53)
	9.8	3.6 (51)	14.8	3.6 (51)
Lung	32.1	3.8 (54)	68.0	5.0 (70)
	54.0	4.2 (58)	113.0	5.3 (75)
	3.0	4.2 (62)	4.0	3.5 (51)
Spleen	9.9	4.2 (61)	13.5	3.9 (58)
	16.1	3.7 (55)	27.0	3.1 (46)
	21.0	15.6 (40)	13.1	16.4 (42)
Kidney	80.0	19.1 (49)	76.0	13.3 (34)
	130.0	26.5 (68)	168.0	28.1 (72)

* Serum levels were under these conditions 4, 15 and 21 μg/ml respectively for OTC and 1, 4 and 8 μg/ml respectively for DC. Cytochrome *c* oxidase activities are calculated as the first-order reaction rate constants in min^{-1} per mg protein. Between the brackets the cytochrome *c* oxidase activities are expressed as percentages of the control values. The values given are the mean of triplicate measurements in two separate samples of the tissues of each of at least two different rats.

Table 3. Effect of treatment with oxytetracycline on various parameters of mitochondrial and cytoplasmic protein synthesis*

Tissue	Parameter	Control animals	OTC-treated animals
Liver	cytochrome *c* oxidase act.	100	59
	cytochrome aa_3	0.20	0.13
	cytochrome *c*	0.32	0.39
Heart	cytochrome *c* oxidase act.	100	51
	cytochrome aa_3	0.39	0.26
	cytochrome *c*	0.45	0.48
Intestinal epithelium	cytochrome *c* oxidase act.	100	70
	cytochrome aa_3	0.06	0.04
	cytochrome *c*	0.14	0.13
	thymidine kinase act.	9.3	9.4
	α-glucosidase act.	0.44	0.44

* Animals were treated with 8 mg/kg/day of oxytetracycline by continuous i.v. infusion for 7 days. The preparation of the homogenates, the mitochondria and intestinal epithelial cells is described under methods and previously [45, 11]. Cytochrome *c* oxidase is expressed as percent of control values. The cytochromes aa_3 and *c* are given as nmoles per mg mitochondrial protein in the experiments with liver and heart and per mg epithelial cell protein for the intestinal tissue. Thymidine kinase is expressed in pmoles/mg cell protein/min; α-glucosidase in μmoles/mg cell protein/min. All values are the mean of three separate experiments. The standard error was less than 4% in all cases.

to liver, heart and intestinal epithelium. They were not routinely done in all distribution studies. The data show that not only the activity of cytochrome *c* oxidase is decreased, but that also spectrally the amount has fallen. For the cytoplasmic parameters no inhibitions are observed. Note that the spectral data for liver and heart were obtained with isolated mitochondria.

Discussion

Our studies show that the steady state distribution of OTC and DC over various organs and tissues of the rat during continuous i.v. infusion is heterogeneous and, moreover, different for the two tetracyclines. The distribution depends on the properties of the tetracyclines such as pK and degree of chelation and on the affinity between the drug and cellular constituents. In this respect the degree of lipid solubility is probably very important. In man [1, 17, 18] and dogs [19] large differences in pharmacokinetics of OTC and DC have been described.

Of the total amount of OTC in serum 30 per cent is protein bound, of DC 80–90 per cent. This difference is also reflected in serum-half-lives; the serum half-life of DC (14–17 hr) is about twice the serum half-life of OTC (7–9 hr). There exists also a large difference in lipophilicity. DC is far more lipophilic than OTC; the partition coefficient in *n*-octanol and water at pH 7.5 of the former is 0.60, of the latter only 0.025 [20]. In mice [21] and rats [16] a serum half-life of DC of 7 hr is reported, indicating a weaker binding to serumproteins or a different way of excretion of DC as compared to the situation in man and dogs.

If the distribution were homogeneous and the serum half-life were 8 hr, one expects a serum concentration of 10 μg/ml infusing 15 mg tetracycline per kg/day. The serum values actually reached at this dose are, however, 8.3 μg OTC/ml and 2.5 μg DC/ml, showing that the distribution is not homogeneous. As already mentioned above, more DC then OTC has to be administered to obtain the same serum levels of both tetracyclines. In man [1] and dogs [22] a reverse situation is found.

This discrepancy can be explained by the weaker binding of DC to serum proteins in rats than in man or dogs [23]. After i.v. administration of the same amount of DC or OTC, DC will diffuse to the tissues much faster because of its more lipophilic properties. However, the amount of DC freely available for the tissues is limited in man and dogs because of the higher degree of binding to proteins. In rats DC is less protein-bound and, therefore, more DC diffuses into the tissues as compared to OTC in rats as well as compared to the situation for DC and OTC in man and dogs. From Table 2 it can be seen that tissue levels of DC are indeed higher than those of OTC at the same administered dose, with the exception of the kidney. The latter phenomenon can probably be explained by the removal of the larger part of DC via non-renal routes [22].

The tissue to serum concentration ratio (Table 1) found in our study is in general higher than reported by others [15, 16]. This may be due to the effect of prolonged continuous i.v. infusion. In fact, we are dealing here with a real steady state distribution, whereas most distribution studies are done a few hours after administration of a single dose. Methodological differences may explain the differences further. In general, the less precise microbiological assay is used for this purpose. The tissue concentrations found in our studies are comparable to the values measured for the tetracycline distribution by a HPLC method [24].

From Table 1 and 2 it follows that at a dose of 8 mg/kg/day OTC or DC all tissue concentrations remain far below the level at which other effects than inhibition of mitochondrial proteinsynthesis can be expected. At this dose serum concentrations are 1 μg DC/ml and 4 μg OTC/ml. For therapeutic purposes in man 100–200 mg DC/day and 500–1000 mg OTC/day are used, leading to serum levels of about 3–4 μg/ml [1]. The current therapeutic doses of OTC or DC will, therefore, only impair mitochondrial protein synthesis, assuming that the tissue distribution of DC and OTC in rats is comparable to the situation in man. An indirect effect on cytoplasmic protein synthesis and energy-dependent functions of cells and tissues can be expected at serum concentrations of 1 μg DC/ml of 4 μg OTC/ml if the treatment lasts so long that a number of cell cycles pass. It should be recalled that the *specific* activity of cytochrome *c* oxidase is clearly depressed.

A treatment during 7 days may, therefore, have effects on rapidly proliferating tissues such as intestinal epithelium. Several morphological changes are seen in the intestinal tract cells and there is a marked reduction in cytochrome *c* oxidase and ATP synthetase capacity of the intestinal mitochondria [25]. Nonetheless, only marginal disturbance of intestinal functions could be found [26] under these circumstances. In our study with tetracyclines we also did not find gross adverse effects.

In general, our study supports the conviction that tetracyclines in the doses normally used in antibiotic therapy [1] inhibit mitochondrial protein synthesis and not cytoplasmic protein synthesis. The experience that tetracyclines have no serious side effects notwithstanding this inhibitory action has, of course, to be explained. Most likely a decrease of up to 50 per cent of the activity of the terminal enzyme of the respiratory chain does not lead to the situation that oxidative phosphorylation becomes rate-limiting for adequate functioning of most tissues and organs.

The steady state tissue distribution studies presented in this paper show that oxytetracycline and doxycycline in doses comparable to those used in antibiotic treatment do not accumulate in most tissues. They inhibit specifically mitochondrial protein synthesis. Only at high dose does cytoplasmic protein synthesis also seem to be impaired.

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Laboratory of Physiological Chemistry
State University
School of Medicine
Bloemsingel 10
9712 *KZ Groningen*
The Netherlands

COBY VAN DEN BOGERT
ALBERT M. KROON

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Na^+, K^+-ATPase activity and noradrenaline turnover in brown adipose tissue of rats exhibiting diet-induced thermogenesis

Recently it has been demonstrated that the hyperphagia induced by offering rats a varied and palatable 'cafeteria' diet is accompanied by large, compensatory increases in heat production that can prevent the development of obesity [1]. This diet-induced thermogenesis (DIT) is similar to the non-shivering thermogenesis (NST) exhibited by cold-adapted animals, since both involve the sympathetic noradrenergic activation of brown adipose tissue (BAT). Furthermore it can be shown that the thermogenic response of cafeteria-fed and cold-adapted rats to noradrenaline is almost entirely due to increases in BAT oxygen consumption [2, 3]. The high thermogenic capacity of BAT is due