TRITIATION OF TYLOSIN AND METABOLIC STUDY IN THE RAT

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Tritiated antibiotic tylosin was prepared by aqueous exchange, purified, then stabilized under phosphate form. After oral administration to rats, the balance study showed the following distribution: urine 10%, feces 46%. Urinary and fecal metabolites were identified as tylosin and desmycosin; 65% of the urinary radioactivity was tritiated water. The elimination is biphasic. ^{3}H-Tissue accumulation and depletion have been studied; 80~90% of the recovered radioactivity was associated to tritiated water. In six tissues including blood, muscle and liver, tissue residue levels were established within 20 days at 0.1~0.3 ppm; depletion is slow.

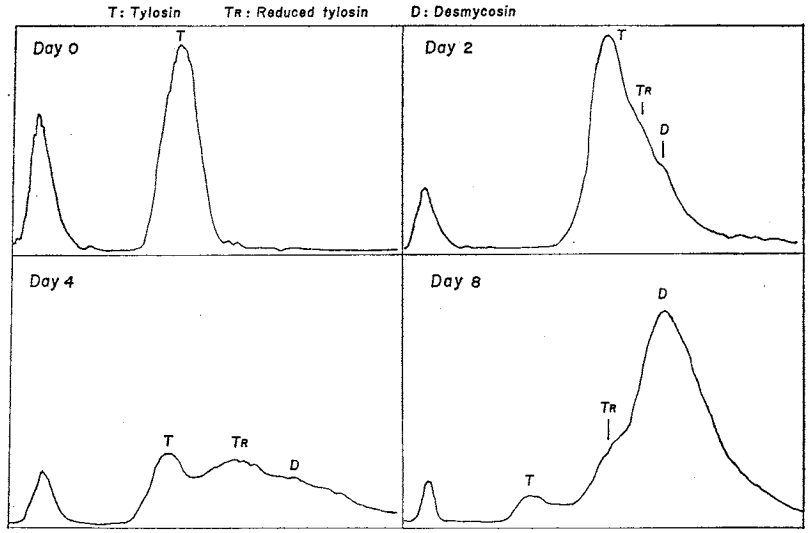
The metabolism of antibiotics in higher animals has been the subject of many studies based on microbiological determination in urine and feces as well as in tissues. These studies have permitted main elimination pathways to be determined as well as the residual quantities presenting antibiotic activity in edible tissues. However, measurement of antibiotic activity can only give a partial view of what really happens in the organism; the antibiotic could be metabolized in one or several metabolites having antibiotic properties or not. More detailed studies are few because analytical methods other than microbiological ones are lacking, and labelled molecules are difficult to obtain. Tritiated molecules of monensin[1] and mikamycin[2] have been obtained in recent trials in the latter direction. For the same reason, tritiation of tylosin (an antibiotic of the macrolide group used exclusively as an additive in animal feeds) has been commenced to study its metabolism in regard to metabolic balance and tissue residues. Only partial results over a short period have been obtained with tritiated tylosin lactate in rat[3], which show considerable individual variation. Particular attention has been given to obtaining a tritiated, high specific activity molecule which is stable and presents the physical and antibiotic characteristics of ordinary tylosin.

Preparation of Tritiated Tylosin

The WILZBACH process[4] was unsuccessful. Tylosin tartrate was labelled by aqueous exchange with tritiated water (source activity: 25 Ci, contact time: 10 days, temperature: 40°C), and labile tritium exchanged in methanol (Service des Molécules marquées, C.E.A., Saclay, France). The reaction product was then purified by column chromatography, and thin-layer chromatography. The aqueous solution was alkalinized by sodium hydroxyde to pH 9.2, and the tylosin was extracted by ethyl acetate. After the solvent was evaporated under vacuum, the residue was taken again by the chloroform-ethyl acetate mixture (2: 1) and deposited on a Merck silica gel column (diameter 15 mm, length 300 mm) equilibrated in ethyl acetate.

A first elution by the chloroform - ethyl acetate mixture (2: 1) eliminated the least polar impurities. The column was then eluted by the acetone - chloroform mixture (2: 1), and successive fractions collected were examined by thin-layer chromatography. The fractions corresponding to tylosin were regrouped and concentrated.

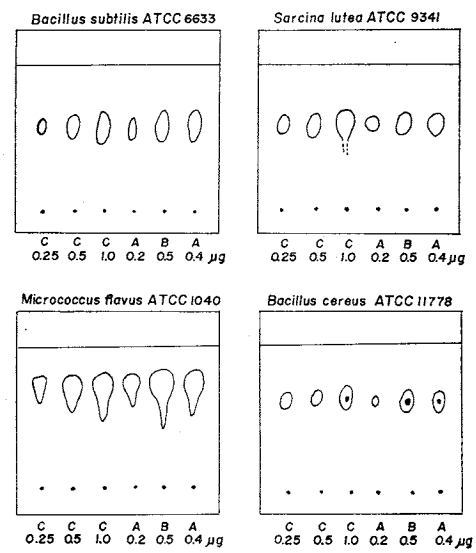
Fig. 1. Evolution of non-stabilized tritiated tylosin.



Complementary purification was done by thin-layer chromatography in the following conditions: plates of silica gel (G Merck), thickness 0.75 mm, activated 2 hours at 110°C, elution by acetone-chloroform mixture (2: 1), control spots revealed by pulverization of an aqueous solution of potassium permanganate and sodium carbonate. The silica band containing the tylosin was scraped, then eluted by methanol.

The purified tylosin was radiochemically pure but unstable. Fig. 1 shows its degradation over a period of time; after 8 days, almost all the tylosin was transformed into desmycosin. Study of this degradation suggests that tylosin tritiation occurs in the lactonic cycle because desmycosin, which results from tylosin by mycarose loss, is tritiated; its specific activity is about the same order.

Fig. 2. Bioautographs of standard and tritiated TLC-chromatographied tylosins.
A=Tylosin standard; B=Tylosin phosphate; C=^{3}H-Tylosin phosphate



The tritiated tylosin thus obtained was immediately stabilized in the form of phosphate by the following technique: the ^{3}H-tylosin was dissolved in a minimum of ethyl acetate, to which were added 4 volumes of isopropyl ether; then, drop by drop, and while agitating, a diluted solution of ortho-phosphoric acid was added into the ethyl acetate until a flaky, white precipitate appeared. The precipitate was centrifuged, rinsed several times with ethyl acetate then with ethyl ether, put into solution in 10 ml of water, filtered on a magnesia column, then a phosphate buffer at pH 6.5 was added. The radiochemical purity was checked by thin-layer chromatography (25 mm silica gel-G Merck, activated 2 hours at 110°C) with the following solvent systems:

System I : acetone - chloroform - ammoniac (2: 1: 1 drop for 20 ml)
System II : acetic acid - ethyl acetate - water (1: 2: 1)

System III: saturated aqueous solution of $MgSO_4$ - acetone (4: 1).

For 3 weeks no degradation was observed. The tritiated molecule was identified as tylosin phosphate using physico-chemical and microbiological methods. Thin-layer chromatography behavior with the three solvent systems was identical. Absorption spectra in ultra-violet were identical for the two products in solution in the buffer pH 6.5 with an absorption maximum of 293.5 nm. Specific antibiotic activity of the labelled product measured by diffusion technique in the gelose implanted with *Sarcina lutea* ATCC 9341, corresponded to that of tylosin phosphate. Bioautographs done with four different test organisms (*S. lutea* 9341, *Micrococcus flavus* ATCC 10240, *Bacillus cereus* ATCC 11778 and *B. subtilis* ATCC 6633) gave identical results for the tritiated product and the standard of tylosin (Fig. 2).

Specific activity of tritiated tylosin phosphate thus prepared was 6.5 mCi/mM.

Administration of the Drug

Balance experiment. Five Wistar male rats and 2 females weighing 500 and 250 g were placed in metabolism cages and fed *ad libitum*. They received 1.9 mg suppress of ^{3}H-tylosin phosphate in aqueous solution by stomach intubation. Urine and feces were collected separately every day for 12 days. The feces were stored at −25°C, and the urine was analyzed immediately.

Accumulation and depletion experiment. Nineteen Wistar male rats weighing 450 g each received 50 ppm of ^{3}H-tylosin phosphate in their ration, or a daily dose of 0.78 mg (4.85 μCi). Supplementation was continued for 32 days. The rats were sacrificed during this period at days 1, 3, 6, 8, 10, 13, 15, 17, 21, 25 and 32.

^{3}H-Tylosin supplementation was then stopped. The remaining rats were killed at 8, 24, 32, 48, 56, 72, and 96 hours after last administration of the labelled product.

After each sacrifice the following samples were taken: blood, liver, kidneys, lungs, muscles of hind legs, dorsal and ventral muscles, kidney and epididymal fat.

Measurement of Radioactivity

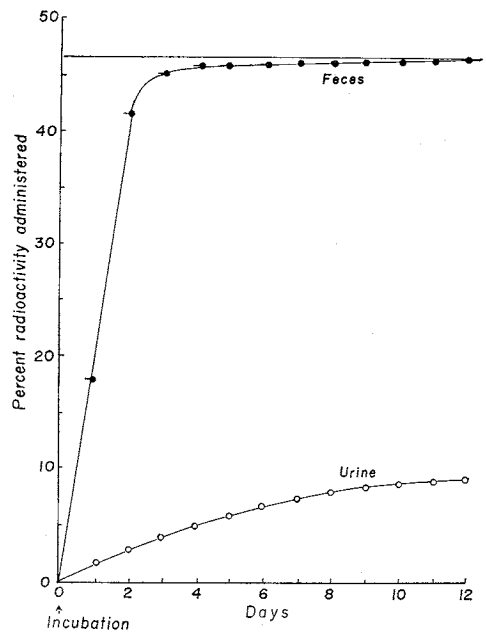
Measurements were done by liquid scintillation (Nuclear Chicago 6860 and Intertechnique SL 30) counters. Quenching was corrected by the external standarization method. Flasks were counted 12 hours after being put into the apparatus and stored away from light so as to eliminate perturbation due to luminescence phenomena.

Urine assay. Radioactivity determinations were performed on 1 and 2 ml of each sample introduced into a liquid scintillator containing Triton X100.

Feces assay. Feces collected for 1 day were dried in an oven at 100°C for 12 hours, then finely gound. Double samples of 350~500 mg were weighed in gelatin capsules and burned in an automatic combustion apparatus (OXYMAT Intertechnique) which permits the tritiated water to be collected in a scintillating mixture with a dioxane-toluene-naphthalene-butyl PBD base.

Tissue assay. Fresh tissues and organs were finely ground with a little water by Ultra Turrax

Fig. 3. Cumulative elimination of ^{3}H-tylosin in the rat after oral single dose administration of 4 mg/kg.



grinder, and four samples of 500 mg of homogenized ground material were taken in gelatin capsules to be successively burned, then counted. Remaining tissues or organs were lyophilized, and 350~500 mg were weighed in gelatin capsules then burned.

Identification of ^{3}H Metabolites in Urine and Feces

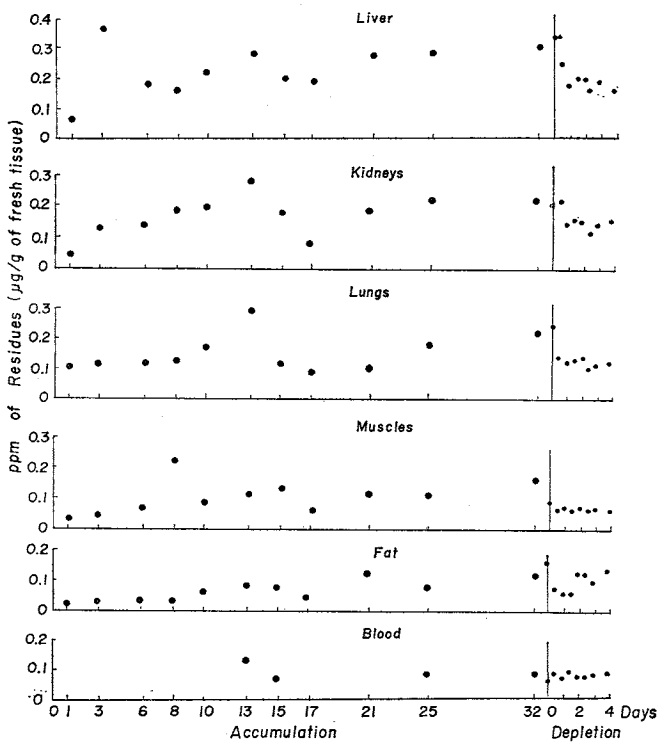
Urine and metabolic feces extracts from rats of the "balance" experiment were chromatographed on silica plate in conditions already described. Zones corresponding to tylosin and desmycosin were scraped, eluted by methanol, and the eluates counted by liquid scintillation. Chromatograms done in the same conditions were developed by bioautography (*Sarcina lutea*).

Results and Discussion

Fig. 3 shows the cumulative elimination balance of ^{3}H-tylosin. About 10% and 46% of the activity ingested are excreted respectively in urine and feces. Fecal elimination is biphasic: for the first three days it is very rapid (90%), then it continues a long time at a low level. The second phase corresponds to the elimination of the absorbed part of the tylosin which is excreted in an unchanged form and in the form of desmycosin, revealing passage by the bile pathway already reported by WORTH[3]. Sixty-five percent of urinary radioactivity and less than 1% of fecal radioactivity correspond to tritiated water. The remaining urinary activity is due exclusively to tylosin and desmycosin, confirming the work of WORTH[3].

Diagrams of Fig. 4 show the evolution of the amount of radioactive residue in different tissues and organs during prolonged administration of ^{3}H-tylosin, then when it stops. Residual amounts concern "dry" residues, 80~90% of total radioactivity being due to tritiated water, except for the blood where it represents only 25%. In spite of rather large variation, a part of which may be attributed to different individual behavior[3], it seems that radioactive amounts level off between 15 and 21 days, which are 0.3 μg/g in liver, 0.2 μg/g in kidneys and lungs, 0.15 μg/g in muscles and 0.1 μg/g in fat. When ^{3}H-tylosin administration is stopped, the amount of tissue radioactivity does not markedly decrease during the first 4 days except in liver and kidneys. Blood content remains unchanged at 0.1 μg/g, which corresponds to very slow tylosin renewal in various organs.

Fig. 4. Accumulation and depletion of labeled residues of tylosin in tissues and organs of rats receiving 100 ppm of the tritiated antibiotic in their diet during 32 days



With a classical method of determination of tylosin[5,6], with a detection limit of about 0.2 μg/g, we could not determine the presence of antibiotic residues in the same tissues and organs. It should also be noted that VALDEBOUZE *et al.*[7] and KLINE *et al.*[8] could not detect tylosin residues respectively in various tissues and organs of calf and pig given feed supplemented with 300 ppm and 500 ppm; that is, with doses very much higher than those used in the present experiment. However, these authors[9] showed residues with antibiotic activity which they could identify by bioautography with tylosin, desmycosin and reduced desmycosin in tissues of sheep given 1,000 mg of tylosin by intravenous injection. On the other hand, it is shown in the present experiment that the forms excreted in urine and feces exclusively represent tylosin and desmycosin. It may be that radioactive residues present in different tissues studied at doses equal or higher than the detection limit of the microbiological method could correspond to linked forms (to proteins, for example) constituting a slow renewal compartment.

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