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# THYMIDINE CONCENTRATIONS IN SERUM AND URINE OF DIFFERENT ANIMAL SPECIES AND MAN

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**Abstract**—Thymidine concentrations were determined in the serum and urine of different animal species and man with a sensitive microbiological method, using Lactobacillus arabinosus ATCC 8014. In contrast to man, dog and other higher animals, where little or no thymidine could be found, considerable amounts (0.1–1 μg/ml) were present in serum and urine from rat and mouse. In mice thymidine concentrations were seen to be enhanced by infections. Some tissue values of rats were determined and the influence of the sample preparation on the amount of thymidine found was investigated.

During the microbiological determination of sulfonamides, trimethoprim or related compounds in serum and urine, we had previously found (unpublished results) that unlike with man, difficulties arose with samples from several animal species. Using Bacillus subtilis or Bacillus pumilus as test organisms in a microbiological large agar plate assay, hazy zones of inhibition or even largely regrown inhibition zones around the cylinders were observed.

Thymidine (TdR), which is known to antagonize both the antibacterial activity of these drugs [1] and their bactericidal effect [2] was found to be responsible in this case as well. As only few data are available on TdR concentrations in animals and man, the serum, urine and some tissues of several animal species were assayed for TdR, using a microbiological method with Lactobacillus arabinosus ATCC 8014 as the test organism.

## MATERIALS AND METHODS

*Chemicals.* Thymidine and thymine were obtained from Merck (Darmstadt). Microanalysis and NMR-spectroscopy showed that these compounds have a purity of >99 per cent. All other chemicals were of analytical grade. DNA from Herring, alkaline phosphatase from calf intestine and Deoxyribonuclease II from bovine spleen were obtained from Fluka (Buchs, SG).

*Microbiological assay.* The method originally described by Landsford *et al.* [3] was used with some modifications [4]. The assay was based on the reversal by TdR of the growth inhibition by trimethoprim (final concentration 0.5 μg/ml). This assay was also adapted to an agar diffusion assay, carried out on large plates (23 × 23 cm, NUNC-BIO assay plate No. 1015).

100 ml of the single strength modified synthetic medium described [4], plus 1.5 g agar (Difco) are autoclaved for 15 min at 120°, cooled down to 45° and inoculated with *L. arabinosus* ATCC 8014. The inoculum is prepared from an overnight culture in 10 ml Lactobacilli AOAC Broth (Difco). The suspension is centrifuged once, washed with 0.9% saline and diluted 1:10. One ml of this suspension is used as inoculum (gives about $10^6$ cells/ml). The inoculated agar is poured onto the plates, which are refrigerated for between 30 min and 2 hr after the agar has solidified. The trimethoprim concentration in this assay is 0.5 μg/ml.

Another variation of this assay was used as well. Bacto niacin medium (Difco) also proved to be suitable for determination of TdR after addition of L-asparagine: 200 mg/l, meso-inosit: 2 mg/l, nicotinic acid: 300 μg/l, arginine: 100 mg/l, sodium formate: 20 mg/l, thymine: 100 mg/l, folic acid: 20 mg/l, agar: 1.5 g/l.

Trimethoprim was added to a final concentration of 0.263 μg/ml. After autoclaving, 100 ml of this medium was inoculated with 0.8 ml of the 10-fold diluted and washed preculture. This modification proved to be somewhat more sensitive, probably due to the lower concentration of the inhibitor.

Standard solutions of TdR are prepared in either water, phosphate buffer pH 7.0 or in 2.5 or 5% trichloroacetate (TCA). Each cylinder is filled with 0.2 ml applying a random scheme, using either three or six cylinders for the same concentration.

After incubation for 16 hr at 37° distinct growth zones around the cylinders are observed and measured (Fig. 1). Plotting the growth zone diameter against the logarithm of the TdR concentration gives straight lines over a wide range, the lowest concentration determined being 12 ng/ml (Fig. 2). For the determination of unknown concentrations of TdR, a random scheme and a computer program, based on determination with four points, was used [5].

*Sample preparation.* Samples of many complex fluids and microbiological testing media could be assayed without any difficulty either in tubes or on agar plates. Difficulties, however, arose with protein-containing samples, such as sera or tissue homogenates. Heat treatment of sera (30′ at 56°), to destroy complement, which inhibits growth of the test organism, proved not to be sufficient as greatly varying results were obtained. On agar plates. these samples formed precipitates around the cylinders, interfering with correct reading of the growth zones,

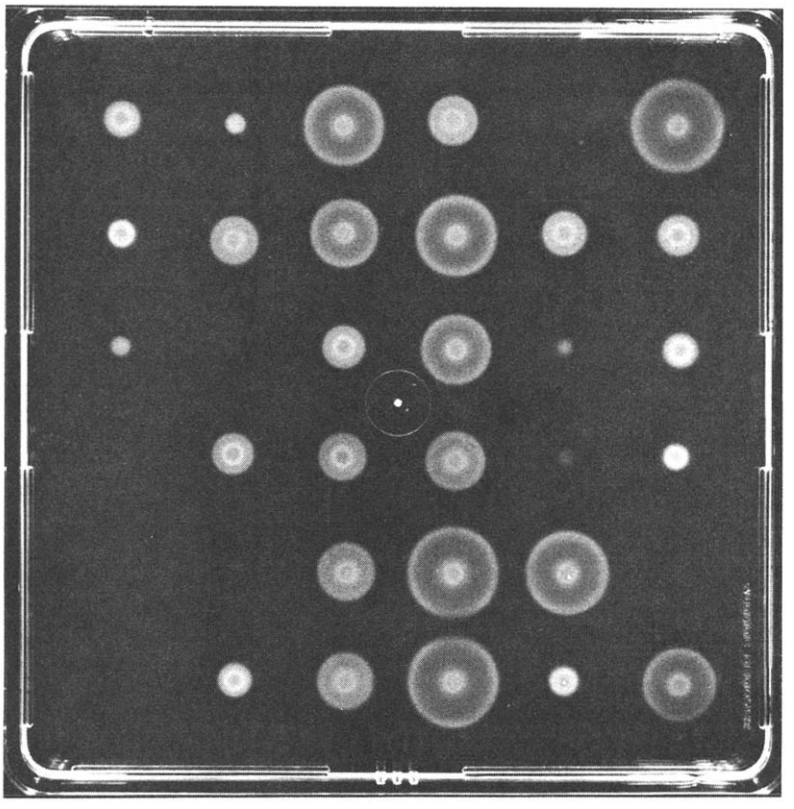

Fig. 1. Determination of thymidine on large plates with *L. arabinosus* ATCC 8014.

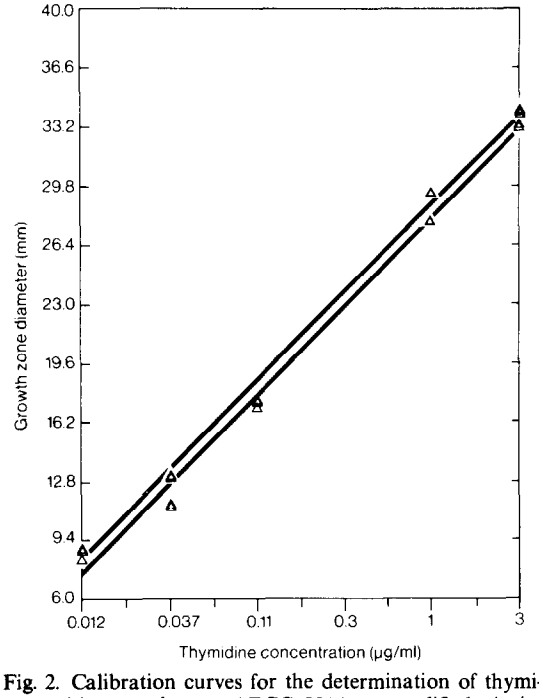


Fig. 2. Calibration curves for the determination of thymidine with *L. arabinosus* ATCC 8014 on modified niacin-assay medium. Experiments run at 2 different days. Thymidine standard in phosphate buffer pH 7.0 upper curve: $Y = 28.784 + 4.648 \cdot \ln x$; lower curve: $Y = 27.976 + 4.652 \cdot \ln x$.

especially at low TdR concentrations. Protein removal by precipitation with TCA resulted in a 2–3-fold dilution of the sample after neutralization. Small samples were difficult to handle. TCA in the applied concentration of 2.5 or 5%, after neutralization with NaOH, did not, however, interfere with the assay. Growth zones were slightly larger and corresponding standard solutions had to be used. TCA precipitation was therefore only used during homogenization of tissues.

Blood, serum and urine were ultrafiltered through 'Centriflow' filter cones (Amicon), which retain proteins with molecular weights >50,000. The clear ultrafiltrate could be used either in the tube or agar plate assay without any problems. In order to obtain an estimate of the percentage of free or protein bound TdR, known amounts of TdR were added to sera of mice, rats, rabbits, dogs, monkey and man before ultrafiltration, and also to the ultrafiltrate. Identical regression lines were obtained, indicating that virtually all TdR is unbound and passes freely into the filtrate. The same result was acquired adding TdR to 10% bovine serum albumin before and after ultrafiltration. Recovery of known amounts of added TdR is shown in Table 1 for two cases.

Urine from cats and dogs was collected by catheterization, whereas urine from other animals was collected in "metabolic cages".

## RESULTS

*Thymidine content in the serum and urine of different animal species.* Samples (serum and urine) were ultrafiltered and assayed for TdR in the agar plate assays. In some instances control experiments were run in

Table 1. Recovery of thymidine with the *L. arabinosus*-agar plate assay on modified niacin-assay medium. Computerized four-point assay with a standard deviation within 3 per cent

| Medium | TdR added µg/ml | TdR recovered µg/ml | TdR recovered % |
|---|---|---|---|
| Human serum ultrafiltered | 1.5 | 1.439 | 96 |
| | 0.375 | 0.376 | 100 |
| | 0.188 | 0.175 | 93 |
| | 0.094 | 0.079 | 84 |
| | 0.047 | 0.054 | 115 |
| | 0.0234 | 0.023 | 98 |
| Dog urine ultrafiltered | 1.5 | 1.508 | 100 |
| | 0.75 | 0.672 | 90 |
| | 0.375 | 0.373 | 100 |
| | 0.187 | 0.185 | 99 |
| | 0.094 | 0.098 | 104 |
| | 0.047 | 0.051 | 108 |

tubes and good accordance was found between values determined either on agar plates or in test tubes.

Besides samples from healthy animals commercially available desiccated samples or blood conserves were assayed. Samples from animals with different experimental infections, used as a routine in our laboratories, were also investigated. These samples were obtained from animals in the moribund state. Control experiments showed that no TdR has been transferred with the bacterial suspension used for infection. Results are summarized in Tables 2 and 3.

Particularly high concentrations were found for mouse and rat, whereas TdR concentrations of most other species are at the fringe of the method's sensitivity. Infection seems to increase these values.

*Thymidine contents in tissues.* In early experiments tissues were homogenized in the 'Brown MSK-Homogenisator' in 0.9% saline and were processed immediately. The homogenate was centrifuged, ultrafiltered and analyzed. By this method extraordinary high TdR

Table 2. Thymidine contents in ultrafiltered serum and urine from different species. Standard deviation within 5 per cent

| Species (source) | Serum µg/ml | Urine µg/ml |
|---|---|---|
| Mouse, pool of 40 animals | 0.166 | 0.728 |
| pool of 17 animals | 0.163 | 0.410 |
| Rat, pool of 40 animals | 0.266 | 0.741 |
| pool of 9 animals | 0.146 | ND* |
| mean of 10 animals | 0.207 (0.142–0.318) | 1.163 (0.077–3.888) |
| Rabbit, mean of 7 animals | 0.042 (0.014–0.078) | 0.234 (0.161–0.263) |
| Rhesus monkey | 0.056 | ND* |
| Dog, mean of 6 animals | 0.023 (0.012–0.054) | 0.031 (0.016–0.059) |
| Man, (desiccated serum, Difco) | 0.017 | — |
| (blood conserve) | 0.021 | — |
| volunteer | ~0.010 | <0.010 |
| Calf | 0.117 | — |
| Beef (blood, Oxoid) | 0.064 | — |
| Sheep (blood, Oxoid) | 0.077 | — |
| Cat (desiccated serum, Difco) | <0.020 | — |
| | — | <0.020 |
| Deer (desic. serum, Difco) | <0.020 | — |
| Goat (desic. serum, Difco) | <0.020 | — |
| Chicken (desic. serum, Difco) | 0.029 | — |
| Horse (laked horse blood, Difco) | <0.028 | — |

* ND = not determined

Table 3. Thymidine contents in the serum and urine of infected mice. Mean of 4 animals (S.D. 5 per cent)

| Infective organism | TdR-concentration serum µg/ml | TdR-concentration urine µg/ml |
|---|---|---|
| *E. coli* 1346 | 0.249 | 1.421 |
| *Salmonella typhimurium* | 0.329 | 1.870 |
| *Pneumococcus* | 0.152 | 2.564 |
| *Pr. mirabilis* | 0.432 | 3.354 |
| *Pr. vulgaris* | 0.743 | 3.727 |
| Trachoma Virus | 0.078 | 0.891 |
| Sendai Virus | 0.063 | 0.776 |
| Herpes Virus | 0.907 | 1.271 |
| Influenza Virus (Asia) | 0.449 | 0.521 |
| Columbia-SK-Virus | 1.021 | 1.342 |
| Uninfected animals | 0.164 | 0.455 |

Table 4. Thymidine content in some tissues and influence of sample preparation. The tissues were homogenized and ultrafiltered

| Organ | Sample preparation | TdR content μg/g tissue |
|---|---|---|
| Rat liver | homogenized in 0.9% NaCl | 4.28 |
| lung | homogenized in 0.9% NaCl | 0.484 |
| kidney | homogenized in 0.9% NaCl | 32.11 |
| Rat liver | homog. in the presence of 5% TCA | <0.050 |
| lung | homog. in the presence of 5% TCA | 0.150 |
| kidney | homog. in the presence of 5% TCA | 0.114 |
| Dog feces | suspended and diluted in phosphate buffer | 2.625/2.790 |
| | suspended and diluted in 5% TCA | 0.138/0.084 |

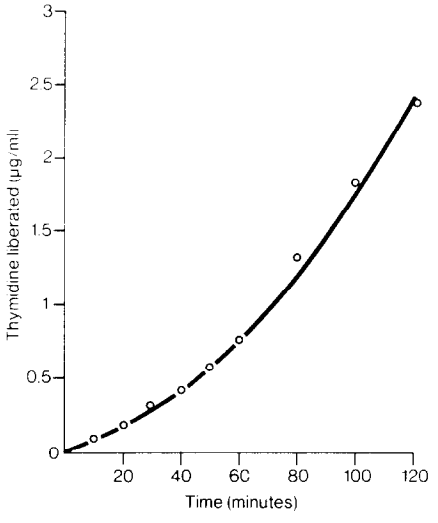


Fig. 3. Liberation of thymidine from DNA by alkaline phosphatase. DNA (1 mg/ml) in Tris–HCl-buffer pH 8.0 treated with 0.1 unit/ml alkaline phosphatase (0.1 mg/ml protein).

values were obtained. These high TdR contents in tissue homogenates interfered greatly with the microbiological determination of trimethoprim or related compounds.

It was, however, assumed that these high TdR levels do not represent actual values, but are due to liberation of TdR from DNA-material during homogenization. Homogenization was therefore carried out in the presence of 5% TCA. Under these conditions low levels of TdR in various tissues were found. 5% TCA was seen not to change the TdR content determined in the same sample which was incubated with TCA for up to 5 hr. The TdR concentrations determined in some rat tissues, and the influence of the sample preparation, are shown in Table 4.

That it is TdR, liberated from DNA by DNAses and phosphatases, which is responsible for high TdR levels found in tissues (unless TCA-protection is applied), was demonstrated in the following experiment: A DNA solution (1 mg/ml in Tris–HCl-buffer pH 8.0) was treated with alkaline phosphatase. Incubation at 37° for varying periods showed an increase in TdR with time (Fig. 3). DNA alone and phosphatase, which were run as controls, did not permit growth of the test organism. Whether the cleavage of DNA was due to a phosphodiesterase activity of the phosphatase preparation used or due to a DNAse impurity was not investigated. A similar experiment using DNAse gave the same result.

## DISCUSSION

The microbiological methods described have allowed the reliable and reproducible determination of TdR concentrations down to 12 ng/ml in the plate assay or to a few ng in the tube assay. The sensitivity is therefore sufficient to estimate those low concentrations which begin to inhibit the activity of antifolate drugs. Ultrafiltration proved to be the simplest method for removal of protein in blood, serum, or plasma, which interferes with the assay.

Surprisingly great differences were found in the TdR content in the serum and urine of various animal species. The concentration in human serum and urine was found to be very low and at the fringe of the method's sensitivity. The figures given in Table 2 for man may represent the upper limits, since recovery experiments (Table 1) did not reveal any TdR in serum. Considerable amounts are, however, present in mice and rats. The figures given for urine of rats and rabbits may be influenced to some extent by fecal contamination, which is difficult to avoid, though care was taken and special metabolic cages were used. In the feces of dogs considerable amounts of TdR may be found though most of it does not represent free TdR.

It seems interesting that in mice with experimental infections the TdR values in serum and urine were generally found to be considerably increased. This may be explained by destructive processes resulting in the generation of purulent material and especially the liberation of TdR from nucleic acids. It has been shown in rats that other destructive processes—like those provoked by x-irradiation—resulted in increased levels of pyrimidines, especially TdR, in the urine [6].

For rat blood 0.85 μg/ml TdR were reported by Gross and Rabinowitz [7]. This value is higher than that reported here, probably due to the method of determination. There are few reports in literature on TdR levels to permit a comparison, however, the values determined for mouse serum are in accordance with values recently reported by Hughes *et al.* [8], who used a radioimmunoassay for the determination. As it is assumed that the values obtained represent steady state values, high turnover rates are required

to maintain a constant level in spite of very short halflifes, demonstrated with exogenously administered TdR in many animals [9–12] and especially in man [13, 14]. The present investigation of the few rat organs make it likely that TdR levels in different organs are different, though it is not clear whether the TdR found is localized intra- or extra-cellularly. Concerning the microbiological determination of antifolate drugs it proved mandatory to prevent the liberation of TdR from DNA during the process of homogenization and sample preparation. As in some animals the TdR content in serum and urine may interfere with the assay of antifolates it may be advisable to use an assay medium containing horse blood, which may split TdR to thymine by the action of TdR phosphorylase [15].

It was recently shown that minute amounts of TdR prevent a bactericidal action of antifolates in *E. coli* [2], preventing cell destruction as long as TdR is present [16]. In the light of the TdR concentrations determined here, it can hardly be assumed that antifolate drugs act bactericidally in mice and rats. This is in accordance with results (unpublished) which failed to show a bactericidal effect of TMP or Co-trimoxazole in rat or mouse blood.

Unlike these two rodent species, the TdR concentrations found in man seem to be sufficiently low to permit the assumption that these drugs have a bactericidal effect which was recently shown to occur in human blood and urine [2, 17].

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