Thymine glycol and thymidine glycol in human and rat urine: A possible assay for oxidative DNA damage
(cancer/aging/metabolic rate/reactive oxygen species/DNA repair)

RICHARD CATHCART, ELIZABETH SCHWIERS, ROBERT L. SAUL, AND BRUCE N. AMES
Department of Biochemistry, University of California, Berkeley, CA 94720
Contributed by Bruce N. Ames, May 15, 1984

ABSTRACT Thymine glycol is a DNA damage product of ionizing radiation and other oxidative mutagens. In an attempt to find a noninvasive assay for oxidative DNA damage in individuals, we have developed an HPLC assay for free thymine glycol and thymidine glycol in urine. Our results indicate that humans excrete about 32 nmol of the two glycols per day. Rats, which have a higher specific metabolic rate and a shorter life span, excrete about 15 times more thymine glycol plus thymidine glycol per kg of body weight than do humans. We present evidence that thymine glycol and thymidine glycol are likely to be derived from repair of oxidized DNA, rather than from alternative sources such as the diet or bacterial flora. This noninvasive assay of DNA oxidation products may allow the direct testing of current theories which relate oxidative metabolism to the processes of aging and cancer in man.

Oxygen radicals and other reactive oxygen species are generated in vivo as a consequence of normal metabolism (1–4). The oxidation of certain cellular components by these oxygen species could contribute to aging and age-dependent diseases such as cancer. Oxygen radicals have been shown to produce base damage and strand breaks in DNA (1, 3), as well as to initiate the process of lipid peroxidation (1, 3). The latter results in the formation of lipid hydroperoxides, which, in the presence of cellular iron-containing compounds, can also break down to yield oxygen radicals (1). Numerous defense mechanisms within the cell have evolved to limit the levels of reactive oxygen species and the damage they induce (1–5). Among the cellular defenses are the enzymes superoxide dismutase, catalase, and glutathione peroxidase (1–4), as well as antioxidants such as β-carotene (1–4), the tocopherols (6), and uric acid (7). However, low levels of reactive oxygen species can escape these cellular defenses (8) and produce damage to DNA, protein, and unsaturated fats. One product that is formed in DNA in vitro as a consequence of chemical oxidation or ionizing radiation (an oxidative mutagen) is thymine glycol (5,6-dihydroxydihydrothymine) (9, 10). Mammalian DNA repair systems are known to excise this lesion from DNA in vitro (5, 11–17). We report here the identification, in both human and rat urine, of the cis isomer of thymine glycol (5,6-dihydroxydihydrothymine) and its deoxyribonucleoside thymidine glycol (5,6-dihydroxydihydrothymidine) and we propose that these bases are derived from repair of oxidized DNA.

MATERIALS AND METHODS

Preparation of cis-[3H]Thymine Glycol and cis-[3H]Thymidine Glycol. [methyl-3H]Thymine and [methyl-3H]thymidine (Schwarz/Mann), approximately 26 Ci/mmol (1 Ci = 37 GBq), were oxidized to the cis-glycols by potassium permanganate (9). After removal of the unreacted permanganate with Na2S2O3, the supernatant was adjusted to 1 M NH4OAc (pH 8.8) and loaded onto a 0.5 × 2 cm boronate affinity column (Affi-Gel 601, Bio-Rad), equilibrated with the same buffer. The column was washed with 1 M NH4OAc (pH 8.8) (buffer A), the cis-glycol fraction was removed by elution with 0.1 M HOAc, and the eluate was concentrated by lyophillization. [3H]Thymine glycol and [3H]thymidine glycol were further purified by reversed-phase HPLC, using a 5-µm C18 column (4.6 mm × 25 cm; Supelco, Bellefonte, PA) with water as the mobile phase. All water used for buffers or HPLC was purified with a Milli-Q system (Millipore). Nonradioactive thymine glycol and thymidine glycol were synthesized and purified in an analogous manner.

Purification of Thymine Glycol and Thymidine Glycol from Urine. [3H]Thymine glycol and [3H]thymidine glycol (200,000 dpm each; 4.5 pmol) were added as internal standards to a 20-ml urine sample. Twenty milliliters of 2 M NH4OAc (pH 8.8) was added and the mixture was allowed to stand overnight at 4°C. After centrifugation, the supernatant was filtered through a Nalgene type S filter unit (0.2 µm; Nalgene). The filtrate was slowly applied to a 1.0 × 10 cm Amicon PBA-60 boronate affinity column (Amicon), equilibrated with buffer A, then washed with 30 ml of buffer A, which reduced the absorbance at 254 nm of the eluate to 0.1–0.3. The column was further washed with 15 ml of water and then eluted with 25 ml of 0.1 M HOAc to give the boronate-binding fraction. This fraction, which contained 75–80% of the radioactive internal standards, was lyophilized and resuspended in 1 ml of H2O.

Fraction 1, containing thymine glycol and thymidine glycol, was purified by semipreparative HPLC, using a 5-µm C18 column (10 mm × 25 cm; Supelco) which was run isocratically in H2O at 3 ml/min. Fractions of 2 ml were collected. Two peaks of radioactivity, corresponding to thymine glycol (7 min) and thymidine glycol (16 min) were pooled separately and concentrated by lyophillization (Savant centrifugal lyophillizer). Each fraction 1 was further purified using the same column at 2.0 ml/min for thymine glycol and 2.5 ml/min for thymidine glycol with H2O as a mobile phase. Fractions of 0.5 ml were collected, radioactivity was determined, and fractions were pooled, to yield thymine glycol fraction 2 and thymidine glycol fraction 2. These fractions were lyophillized to dryness in a small test tube. The yields of radioactivity from the internal standards after the HPLC purification steps and lyophillization were 55–65%.

Conversion of Thymine Glycol and Thymidine Glycol to Thymine and Quantitation of Thymine. Because thymine glycol and thymidine glycol have negligible UV molar absorbivities, our analytical determination used the conversion of these compounds to thymine, which could be analyzed by HPLC with UV detection. Both thymine glycol and thymidine glycol were converted to thymine by a modification of the HCl reduction method of Baudisch and Davidson (18) with a yield of 65–70%. Thymidine is not a major product of thymidine glycol reduction because, under these conditions, it is hydrolyzed to thymine. HI solution (0.1 ml; Fisher, 55%, without preservative) was added to each sample of lyophi-
lized fraction 2, which was then stoppered and heated at 100°C for 35 min. Reaction mixtures were then cooled and neutralized with 0.18 ml of 4 M NaOH and 0.35 ml of 1 M unbuffered NH₄OAc.

Partial purification of the thymine from the HI reaction was achieved by using a 5-µm C18 column (4.6 mm × 25 cm; Supelco) with a mobile phase of 0.10 M NH₄OAc (pH 6.0) and a flow rate of 2.0 ml/min. The chromatographic region corresponding to thymine (7.8–8.0 min) was collected and lyophilized (fraction 3). The purified thymine fraction was resuspended in 0.4 ml of H₂O, and 50 µl of this was assayed for radioactivity. Recovery of [³H]thymine was usually 25–35% based on the amount of labeled glycol originally added to the urine. An additional HPLC step was carried out to quantify the thymine in fraction 3 using a 5-µm C18 column (4.6 mm × 25 cm; Supelco), equilibrated with H₂O at 35°C and a flow rate of 1 ml/min. The detector used in this case was a Kratos model 773 (Kratos, Westwood, NJ) set at 264.5 nm and 0.001 absorbance unit full-scale output, and the thymine peak area was integrated using a Nelson analytical model 4416 data system. The overall recovery of radioactivity from the internal standard was used to convert this thymine measurement to the thymine glycol or thymidine glycol level in the original urine sample. For the analysis of 24-hr urine samples, values were expressed as nmol of glycol/kg of body weight per day. For single-collection urine samples, values were normalized to creatinine in the original urine as determined with a creatinine analysis kit (Sigma). Controls (20 ml of water instead of urine) were processed through all the steps of the procedure including addition of the radioactive internal standards. The amount of thymine obtained from these controls was negligible. Approximately 2 weeks are required for one technician to process 12 urine samples through the entire procedure.

Analysis of Rat Urine. Twenty-four-hour urine samples from both germ-free and conventional-flora male Sprague–Dawley rats (150–200 g) were obtained from Charles River Biotechnical Services. Conventional-flora rats (male, Sprague–Dawley, 150–200 g) were also housed in our own laboratory in metabolic cages (Nalgene) and given laboratory chow (5015C, Ralston Purina, Richmond, IN) and water ad lib. Each 24-hour rat urine sample was diluted to 25 ml with water, and a 10-ml portion was analyzed for thymine glycol and thymidine glycol as described for human urine.

RESULTS

Validation of the Analytical Method. Purification of thymine glycol and thymidine glycol from urine was effected using phenyl boronate affinity chromatography to select cis-glycols followed by two successive HPLC steps. Because thymine glycol and thymidine glycol have negligible UV molar absorptivities, they were then converted to thymine (by HI reduction) to allow HPLC/UV detection. The validity of our analytical method was supported by (i) evidence that the detected UV absorbance is from thymine and (ii) evidence that this thymine is derived from thymine glycol or thymidine glycol.

(i) Evidence that the detected UV absorbance is from thymine. Authentic thymine glycol and thymidine glycol yield thymine in 65–70% yield on HI reduction. The presumptive thymine purified in fraction 3 from HI reduction of several samples of individual rat thymine glycol fraction 2 was pooled and chromatographed. The detector used in this case was a Hewlett-Packard 1040A photodiode array system that can obtain a full UV spectrum "on the fly" and was programmed to acquire a spectrum of each peak on the upslope, apex, and downslope of the peak. As shown in Fig. 1, rat thymine glycol fraction 3 contained a peak that co-migrated with authentic thymine on a C18 reversed-phase column.

(ii) Evidence that the thymine is derived from urinary thymine glycol and thymidine glycol. After either the fraction 1 or the fraction 2 step, HPLC fractions immediately adjacent to the [³H]thymine glycol or [³H]thymidine glycol internal standard-containing chromatographic regions were treated with HI. For these reactions, no detectable thymine was produced. The only fractions yielding thymine after HI treatment were those coincident with the sharp, well-separated HPLC peaks of radioactivity from the internal standards.

Source of Urinary Thymine Glycol and Thymidine Glycol in the Rat. Our hypothesis is that thymine glycol and thymidine glycol in the urine are derived from repair of oxidized DNA in the tissues. Several experiments in rats provided evidence against alternative sources such as the diet or bacterial flora. Rats were administered 10⁶ dpm [³H]thymine glycol or [³H]thymidine glycol by intravenous injection or gastric intubation. When given intravenously, the radioactivity from both compounds was recovered in the urine in greater than 75%
yield within 24 hr of injection. Less than 5% of the radioactivity was recovered in the feces. Subsequent chromatography of the radioactivity recovered from the urine showed that neither compound had been appreciably metabolized. In particular, [3H]thymidine glycol was not hydrolyzed to [3H]-thymine glycol. However, when given by gastric intubation, 80% of the [3H]thymidine glycol was recovered intact in the feces within 3 days, with only 5% of the radiolabel recovered intact in the urine. This means that, if thymidine glycol were present in the diet or formed in the gut, only a very small fraction of it would be absorbed and contribute to its presence in the urine. The fraction of [3H]thymidine glycol that was actually absorbed from the gut in this experiment was probably closer to zero, because the 5% recovery in urine could have arisen via in vitro extraction of fecal [3H]thymidine glycol from the walls of the metabolic cage by the rat urine.

When [3H]thymidine glycol was administered to rats by gastric intubation, 44% was recovered intact in the urine within 24 hr. This result identifies a potential problem because, if thymidine glycol is present in the diet or formed in the gut, it would be absorbed from the gut and be recovered as urinary thymine glycol. To assess this possibility, rats were fed a nucleic acid-free diet (glucose and water) for a period of 4 days. Both thymine glycol and thymidine glycol continued to be excreted by the glucose-fed rats on all 4 days. The levels of thymidine glycol were not diminished by the glucose diet (Table 1). The levels of thymine glycol, however, were about 40% lower than those for rats eating the regular chow diet (Table 1), suggesting that a component of urinary thymine glycol is directly or indirectly related to diet (see Discussion). Because the pharmacokinetic studies had shown that orally administered thymine glycol and thymidine glycol are rapidly excreted, the continued output of these compounds after 4 days on a nucleic acid-free diet showed that thymidine glycol, and most of the thymine glycol, were not of dietary origin. It should be noted that the glucose-fed rats lost an average of 10 g of body weight during the 4 days of glucose feeding, compared with Chow-fed controls, which gained about 20 g body weight during the same period.

No significant difference was observed in the levels of urinary thymine glycol and thymidine glycol between normal and germ-free rats (Table 1). This rules out a microbial source of urinary thymine glycol and thymidine glycol.

Thymine Glycol and Thymidine Glycol Levels in Human Urine. Four different urine samples were obtained from one normal healthy male over a period of 3 months. Each of these samples was analyzed five times for thymine glycol and thymidine glycol (Table 2). Very little variation with time was seen in the level of either glycol for this particular individual. Urine was then obtained from five normal healthy male and female volunteers of various ages on 2 different days. Only a morning sample was obtained on one day but, on day 2, 20 ml of the morning sample was segregated from a 24-hr specimen. The results shown in Fig. 2A demonstrate that thymine glycol levels vary considerably among individuals and among samples from a given individual. In contrast to the thymine glycol variability, levels of thymidine glycol among samples and among individuals were relatively constant (Fig. 2B). Further information on the variation among samples from a single individual was obtained from analysis of a total of 13 separate urine samples from person D. The average levels in urine samples from this donor were 4.6 ± 3.9 pmol of thymine glycol/mmol of creatinine and 0.4 ± 0.2 pmol of thymidine glycol/mmol of creatinine (mean ± SD). To date, we have analyzed urine from nine different individuals, and the averages of their average levels were 2.4 ± 2.2 and 0.6 ± 0.3 pmol/mmol of creatinine for thymine

Table 1. Summary of rat studies

<table>
<thead>
<tr>
<th></th>
<th>Level, nmol/kg per day</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rats per group</td>
<td>Thymine glycol</td>
<td>Thymidine glycol</td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>10</td>
<td>5.5 ± 1.7</td>
<td>1.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Germ-free rats</td>
<td>3</td>
<td>7.8 ± 0.4</td>
<td>1.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Glucose-fed rats</td>
<td>3</td>
<td>3.3 ± 1.0</td>
<td>1.5 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Each value is mean ± SD.

Table 2. Multiple analyses of thymine glycol and thymidine glycol in urine samples from one individual

<table>
<thead>
<tr>
<th></th>
<th>Thymine glycol</th>
<th>Thymidine glycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>Amount, pmol/μmol of creatinine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/1/83</td>
<td>1.8 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>5/1/83</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>5/3/83</td>
<td>2.5 ± 0.3</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>5/4/83</td>
<td>1.8 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.9 ± 0.4</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

Four urine samples were collected from a 54-year-old white male. The samples were obtained on arising in the morning except for the first sample which was obtained in the afternoon. Each value is the mean ± SEM of five separate determinations.

FIG. 2. Variation in human thymine glycol (A) and thymidine glycol (B). Bars: ♂, day 1, morning; ♀, day 2, morning; ♂, day 2, 24-hr sample. Each day 1, morning urine level, is the mean of four separate determinations, with a SEM per sample of less than 15%. Day 2 levels are results of single determinations. The 24-hr sample of thymidine glycol, person B, was lost.
glycol and thymidine glycol, respectively. The thymine glycol levels in human urine samples clustered about what appears to be a "basal" level of about 1 pmol/μmol of creatinine with occasional urine samples containing up to 20 pmol/μmol of creatinine. The variability in thymine glycol levels may be directly or indirectly related to diet (see Discussion).

Comparison of Thymine Glycol and Thymidine Glycol Levels Between Rats and Humans. A comparison of thymine glycol and thymidine glycol excreted/kg of body weight per day between rats and humans is given in Table 3. For the purpose of this comparison, the average values in human urine of 2.4 and 0.6 pmol/μmol of creatinine for thymine glycol and thymidine glycol, respectively, were converted to units of nmol/kg/day by multiplication by the conversion factor 165 μmol of creatinine/kg per day (20). On a body-weight basis, the rat excretes approximately 14 times as much thymine glycol and 17 times as much thymidine glycol as the human (Table 3). Because of the possible influence of diet on both human and rat thymine glycol output, we also compared the estimated "basal" level of thymine glycol for humans (1 pmol/μmol of creatinine or 0.165 nmol/kg per day) with the thymine glycol level for glucose-fed rats (3.3 nmol/kg per day, Table 1). In this comparison, rats excrete about 20 times more thymine glycol than humans on a body-weight basis.

**DISCUSSION**

Urinary Thymine Glycol and Thymidine Glycol Appear to Come from Repair of Tissue DNA. We have demonstrated that human urine and rat urine contain both thymine glycol and thymidine glycol. We propose that urinary thymine glycol and thymidine glycol are derived from the repair of oxidatively damaged DNA. This hypothesis is supported by the following evidence: (i) both in vitro and in vivo, thymine residues in DNA are converted to the glycol by chemical oxidation and ionizing radiation (9, 10, 14, 21, 22); (ii) repair enzymes exist that are specific for the excision of this lesion from the DNA (5, 11-17). The existence of such an enzyme in itself suggests that its substrate, the thymine glycol residue, exists in nature, and that thymine glycol residues in DNA are biologically undesirable to the cell; (iii) our pharmacokinetic studies in the rat showed that, once excised from DNA in the tissues, both glycols are eliminated within 24 hr from the blood to the urine without further metabolism; (iv) urinary thymine glycol in rats is not of dietary origin because it is not absorbed from the gut, though thymine glycol can be; (v) most thymine glycol is not of dietary origin, because thymine glycol continues to be excreted when rats are fed a nucleic acid-free diet; (vi) both glycols are not of microbial origin, as shown by the germ-free rat study; and (vii) in the cell, the thymine moiety resides almost exclusively in DNA* so that the glycols are more likely to be indicators of damage to DNA, rather than damage to RNA, free thymine, or thymine mononucleotides.

We have not yet determined whether a component of the rat's urinary thymine glycol is of dietary origin. The glucose-fed rats excreted 40% less thymine glycol than the chow-fed rats, and a direct explanation for this observation is that some thymine glycol is present in the chow or is formed in the gut. An alternative explanation is that dietary factors (e.g., total caloric intake) indirectly influence thymine glycol

* A rat liver cell contains 9.31 pg of DNA (23), which is 5.6 × 10^{12} daltons of DNA. Assuming 309 daltons per DNA residue and that 26.4% of the residues are thymidine residues (24) gives 4.8 × 10^{10} thymine residues in DNA per cell. This is more than 2000 times the number of dTTP molecules present, which is 0.4 pmol/μg of DNA (25) or 2.2 × 10^4 molecules per cell. RNA contains almost no thymidine.

---

**Table 3. Comparison of thymine glycol and thymidine glycol in humans and rats**

<table>
<thead>
<tr>
<th>Urine</th>
<th>Thymine Glycol</th>
<th>Thymidine Glycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>0.39 ± 0.36</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Rat</td>
<td>5.5 ± 1.7</td>
<td>1.7 ± 0.8</td>
</tr>
<tr>
<td>Rat vs. human</td>
<td>14</td>
<td>17</td>
</tr>
</tbody>
</table>

Values for humans are mean ± SD of nine individuals. Values for rats are mean ± SD of 10 animals.

levels by affecting metabolism in ways that alter the rate of damage to tissue DNA. The weight loss by the glucose-fed rats is evidence that the metabolic state of these animals was altered, possibly leading to less thymine glycol formation. In humans, the amount of thymidine glycol excreted showed little variation in level, while that of thymine glycol was quite variable in certain individuals, over a basal level of about 1 pmol/μmol of creatinine (Fig. 2). It remains to be determined whether the variability in human urinary thymine glycol is caused by dietary thymine glycol, dietary influences on DNA damage, or factors independent of diet.

Among different urine samples from the same individual, we found that thymine glycol and thymidine glycol variation was uncorrelated. This suggests that the two glycols are derived from different sources. One possible explanation is that thymine glycol is derived from repair of nuclear DNA while thymidine glycol is derived from nucleolytic degradation of mitochondrial DNA, since at present there are no reported thymine glycol glycosylases present in that organelle. Although mitochondrial DNA is less than 1% of the total DNA of the cell (26), mitochondrial DNA may be a major source of DNA oxidation products because 90% of the oxidative metabolism of the cell occurs in the mitochondria (27). Further studies are needed to identify the origin of urinary thymine glycol and thymidine glycol.

The Flux of Oxidative DNA Damage in Humans. From Table 3, the total amount of thymine glycol and thymidine glycol excreted by humans was 0.49 nmol/kg per day. For a 65-kg man, this total is about 32 nmol/day, which is approximately 1.9 × 10^{16} thymine glycol residues excreted per individual per day. We do not know the relative degree to which different cell types produce thymine glycol and thymidine glycol but, for demonstration purposes, we have calculated the total of these produced by an "average" cell by dividing the total urinary output of these glycols by the number of cells in the human body (6 × 10^{12}, ref. 28) to give an average of 320 oxidized thymine residues per cell per day. Thus, a considerable amount of cellular DNA damage occurs and is repaired every day. It should be noted that cis-thymine glycol is only one of several possible products of oxidative DNA damage (29) and so this estimate of oxidative damage may be low. A mammalian glycosylase specific for another DNA oxidation product, 5-hydroxymethyluracil, has recently been reported (5). Preliminary findings from our laboratory indicate that 5-hydroxymethyluracil is present in human urine and in rat urine at levels comparable with those of thymine glycol and thymidine glycol (unpublished observation).

Oxidative DNA Damage and Longevity. A rat has a much higher metabolic rate than a human, and metabolic rate is inversely correlated with longevity (30). Rat's urinary DNA damage could be one major contributor to aging (4, 30, 31). We suggest that the higher rate of oxygen metabolism per mass of body tissue in the rat leads to a greater flux of oxygen radicals and an enhanced rate of thymine glycol formation in DNA. A higher level of oxidative DNA damage in the rat may be partly responsible for the correspondingly higher age-specific cancer rate for the rat, as well as its shorter life.
span compared with the human. Analysis of urinary thymine glycol and thymidine glycol in other species should be carried out to further test this hypothesis.

Biochemical Epidemiology. Urine is the likely repository for the family of DNA damage products excised from DNA. It should be possible to identify those mutagens that produce DNA damage in individual humans by examining the by-products of this damage in urine. The lesions produced by several substances have already been identified in animals by administering radiolabeled mutagens or high doses of unlabeled mutagens to the animals and examining the DNA damage products recovered in urine (32, 33). As reported here, modern analytical technology is now powerful enough to analyze particular DNA damage products in human urine at the levels at which they occur through natural exposure. We believe that this approach might help to circumvent the difficult problem of extrapolating carcinogenesis results from rodents to humans and also to define the spectrum of substances that damage DNA. This approach may also prove to be useful for testing current theories about proposed “anticarcinogens” such as selenium, β-carotene, vitamin C, and vitamin E (4). We hope that measurement of the oxidative DNA damage products thymine glycol and thymidine glycol will enable us to optimize the intake levels of anticarcinogens in individuals.

We wish to thank Edie Yamasaki, Fred Jacobson, Mahmoud Saleh, and Robin Morgan for help on earlier aspects of this study. This work was supported by Department of Energy Contract DE-AT03-76EV70156, by a grant from the National Foundation for Cancer Research, and by National Institute of Environmental Health Sciences Center Grant ES01896. R.C. was supported in part by National Institutes of Health and American Heart Association postdoctoral fellowships.