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REVERSED-PHASE LIQUID CHROMATOGRAPHIC INVESTIGATION OF NUCLEOSIDES AND BASES IN MUCOSA AND MODIFIED NUCLEOSIDES IN URINES FROM PATIENTS WITH GASTROINTESTINAL CANCER

KATSUYUKI NAKANO*, KATSUHISA SHINDO and TOSHIO YASAKA
Perfect Liberty (PL) Medical Data Center, 1 Kamiyamacho, Tondabayashi, Osaka 584 (Japan)
and
HIDEKI YAMAMOTO
The Second Department of Surgery, Osaka University Medical School, Fukushima-ku, Osaka 553 (Japan)
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SUMMARY

Reversed-phase high-performance liquid chromatography (HPLC) has been used to determine the level of nucleic acid metabolites in perchloric acid extracts of gastrointestinal mucosa. By comparing the levels of these compounds in the normal portion with the levels in the neoplastic portion of mucosa resected from patients with malignant cancer, it was found that uracil was significantly elevated in the neoplastic colorectal mucosa (adenocarcinoma) of eight patients with colorectal cancer ($P < 0.01$, statistically significant with the paired t test). The mean level of uracil in neoplastic colorectal mucosa was 2.7-fold higher than that in normal mucosa. However, in neoplastic gastric mucosa, only one out of four patients with gastric cancer showed elevated uracil. In neoplastic mucosa, the levels of hypoxanthine and uridine for colorectal cancer, and inosine for gastric cancer, were also significantly higher than those in normal mucosa ($P < 0.05$, with the paired t test). The urinary modified nucleosides were prefractionated with a boronate affinity gel column, and their levels determined by the same HPLC method. No significant differences in the concentrations of pseudouridine, 1-methylguanosine, N^2-methylguanosine or N^2,N^2-dimethylguanosine were observed in pre- and post-operative urines from patients with colorectal cancer and normal urines.

INTRODUCTION

Nucleotides in biological samples have been studied using several modes of high-performance liquid chromatography (HPLC) in order to examine normal metabolic and disease processes[1–3] in the liver[4–7], heart[8–11], brain[12], granulation tissue[13], Hela cells[14] and tissue perfusates[15,16]. Less information is available on the level of nucleosides and bases in tissue, because the major metabolites of nucleic acid catabolism in tissues such as the liver and heart, or blood cells, examined so far were nucleotides and coenzymes. Until now, the endogenous compounds in acid extracts

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of gastrointestinal (GI) mucosa have not been studied by the HPLC method. The investigation of mucosal compounds is of primary importance because most GI adenocarcinomas arise originally from the mucosal tissue of the stomach or intestine. Therefore, differences in the profiles of ultraviolet (UV)-absorbing compounds in normal and neoplastic mucosa could provide important information on the nucleic acid metabolism in mucosa of patients with malignant cancer.

On the other hand, the urinary modified nucleosides derived from the enzymatic degradation of transfer RNA have been investigated as biochemical markers in cancer detection[17–20]. In more recent studies, these nucleosides have been prefractionated on a boronate affinity gel column, and then determined by reversed-phase HPLC techniques[21,22].

Recently, we used reversed-phase HPLC to examine the UV-absorbing compounds in perchloric acid extracts of the normal portion and the neoplastic portion of mucosa, resected surgically from patients with GI cancer. We also studied the change in nucleoside levels in urine samples collected before and after surgical operation on the same patient with colorectal cancer. In this paper, we describe the results complementary to the accompanying paper[23].

EXPERIMENTAL

Chromatographic conditions

The UV-absorbing compounds and nucleoside fractions in perchloric acid extracts of GI mucosa, and the urinary nucleosides of patients with colorectal cancer and of normal subjects, were analyzed under the following chromatographic conditions: HPLC instrument, Hitachi 638-30 (Hitachi, Tokyo, Japan); column, Develosil ODS-5 (5 μm, 250 × 4.6 mm I.D.; Nomura Chemicals, Nagoya, Japan); pre-column, Develosil ODS (15–30 μm, 50 × 4.0 mm I.D.); elution, linear gradient from 0.02 *M* KH_2PO_4 (pH 4.53) to 40% methanol–water (3:2, v/v) in 35 min; flow-rate, 1.2 ml/min; temperature, ambient; detection, UV at 260 and 280 nm (0.16 a.u.f.s.); sample volume, 100 μl unless stated otherwise.

Boronate gel affinity chromatography

The boronate affinity gel column technique was used to isolate nucleosides in 1 ml of perchloric acid extracts of mucosa homogenate and 0.5 ml of urine samples. The isolation procedure was a slight modification of the method developed by Gehrke *et al.*[21]. The boronate gel, Affi-gel 601 (Bio-Rad Labs., Richmond, CA, U.S.A.), was packed in the plastic column (60 × 9 mm I.D.; bed volume, 0.83 ml) and equilibrated with 0.25 *M* ammonium acetate, pH 8.8. A 1-ml volume of the mucosa extracts or 0.5 ml of urine samples (samples were adjusted to pH 9.5 with 2.5 *M* ammonium acetate) was loaded on top of the column. The column was washed with 8 ml of 0.25 *M* ammonium acetate, pH 8.8. Nucleosides were eluted with 4 ml of 0.2 *M* formic acid. The eluate was evaporated under reduced pressure and redissolved in 0.5 ml of the starting buffer for HPLC analysis.

Sample collection

The normal and neoplastic portions of mucosa from the colorectum and stomach were obtained by surgical operation on eight patients with malignant colorectal

cancer and four patients with malignant gastric cancer, respectively. Resected mucosa samples were frozen immediately after the operation and kept at −70°C until used.

Urine samples one day before and one week after the surgical operations on eight patients with malignant colorectal cancer were collected in the morning after a 12-h fasting period. Urine samples from sixteen normal subjects were obtained from the PL Osaka Health Control Center.

Sample preparation

The preparation of normal and neoplastic mucosa samples was as follows. Mucosa samples (colorectum or stomach), 1.25 g wet weight, were minced with scissors to ease the following homogenization. After the addition of 3 ml of cold water, the minced mucosa samples were homogenized using a micro Waring blender. The blender vessel was washed with 1 ml of cold water, then 5 ml of cold perchloric acid (5%, w/v) were added to the homogenized mucosa. The mixture was vortexed vigorously and allowed to stand on ice for about 30 min. Then, the mixture was centrifuged at 1500 *g* for 10 min. The supernatant was neutralized with 10 *M* potassium hydroxide (to approximately pH 5). The pH-adjusted samples were centrifuged to remove precipitable perchlorate. The resulting supernatants were stored at −20°C until HPLC analysis.

Urine samples were stored at −20°C, and centrifuged at low speed to remove the precipitable compounds before use.

Peak identification

The UV-absorbing compounds in the perchloric acid extracts of GI mucosa and in prefractionated urines were identified on the basis of the retention times, simultaneous injection of standards, peak-height ratios, UV-absorption spectra of fractionated HPLC peaks and the enzymatic peak shift, as developed by Brown and co-workers[24–26]. In addition, the nucleosides were confirmed by the HPLC separation of samples prefractionated on the boronate affinity gel column.

RESULTS

Chromatograms of extracts of mucosa and muscle

The chromatograms in Fig. 1 show the UV-absorbing compounds in perchloric acid extracts of the normal and neoplastic portions of mucosa, and of normal muscle from sigmoid colon, resected surgically from a patient with sigmoid colon cancer. Fig. 1 is typical of the profiles observed for most GI mucosa and muscle. The peaks present in the majority of GI mucosa samples are numbered.

The chromatograms of standards appear in the accompanying paper[23]. The chromatographic conditions developed by Hartwick *et al.*[27] were slightly modified to improve the separation of the early part of a chromatogram of serum profile[26] for UV-absorbing compounds. Based on all the data from the identification techniques, the endogenous compounds present in GI mucosa and muscle were identified as uracil, uric acid, hypoxanthine, xanthine, uridine, inosine and guanosine. Peak 1 in Fig. 1 was confirmed as uracil from the characteristic change (bathochromic shift) of the UV-absorption spectrum at alkaline pH, as shown in Fig. 2, as well as from the retention time, simultaneous injection of the standard and the peak-height ratio.

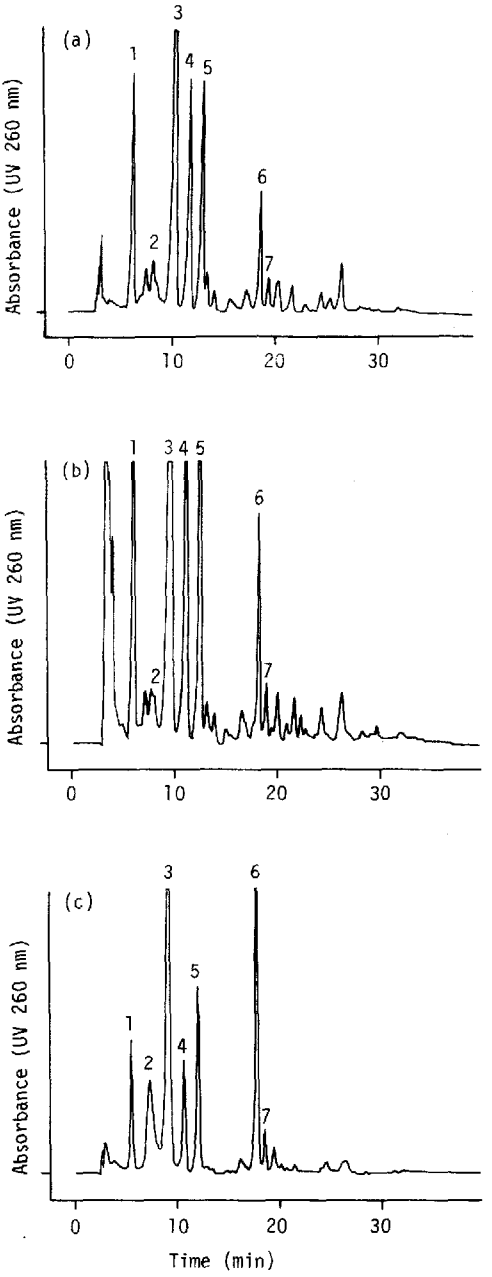

Fig. 1. Chromatograms of perchloric acid extracts of normal mucosa (a), neoplastic mucosa (b) and normal muscle (c) of the sigmoid colon from a patient with malignant sigmoid colon cancer. Injection volume: 100 μl, corresponding to 13.9 mg of wet tissue. For chromatographic conditions see text. Peaks: 1 = uracil; 2 = uric acid; 3 = hypoxanthine; 4 = xanthine; 5 = uridine; 6 = inosine; 7 = guanosine.

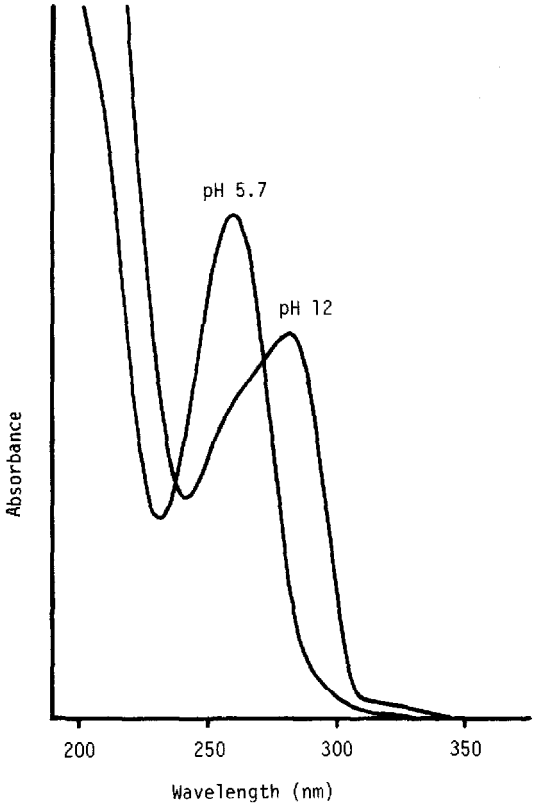

Fig. 2. UV absorption spectra of peak 1 (uracil) in Fig. 1.

The majority of these peaks corresponded to components found in serum[26] and saliva[28]. Compared with the profiles of serum and saliva, the profiles of GI mucosa and muscle showed relatively high levels of hypoxanthine, xanthine and especially uracil, and a low level of uric acid as seen in Fig. 1.

Fig. 1 also compares the chromatograms of normal mucosa with those of neoplastic mucosa and normal muscle from the same patient. A considerable increase in all the major components except uric acid was found in neoplastic mucosa.

Peak 2 in Fig. 1c represents uric acid and nucleotides. From the HPLC analysis obtained after using the boronate affinity gel column, it was found that muscle samples contained relatively large amounts of nucleotides.

Compound levels in mucosa from cancer patient

The concentrations of endogenous compounds in perchloric acid extracts of GI mucosa were determined by the HPLC method described. Fig. 3 shows the levels of these compounds in the normal and neoplastic portions of mucosa from the same patient. A significant elevation of uracil was found in neoplastic colorectal mucosa (adenocarcinoma) from eight patient with colorectal cancer ($P < 0.01$, statistically significant with the paired t test). The mean level of uracil in neoplastic colorectal mucosa was 2.7-fold higher than in normal mucosa. In the neoplastic mucosa of

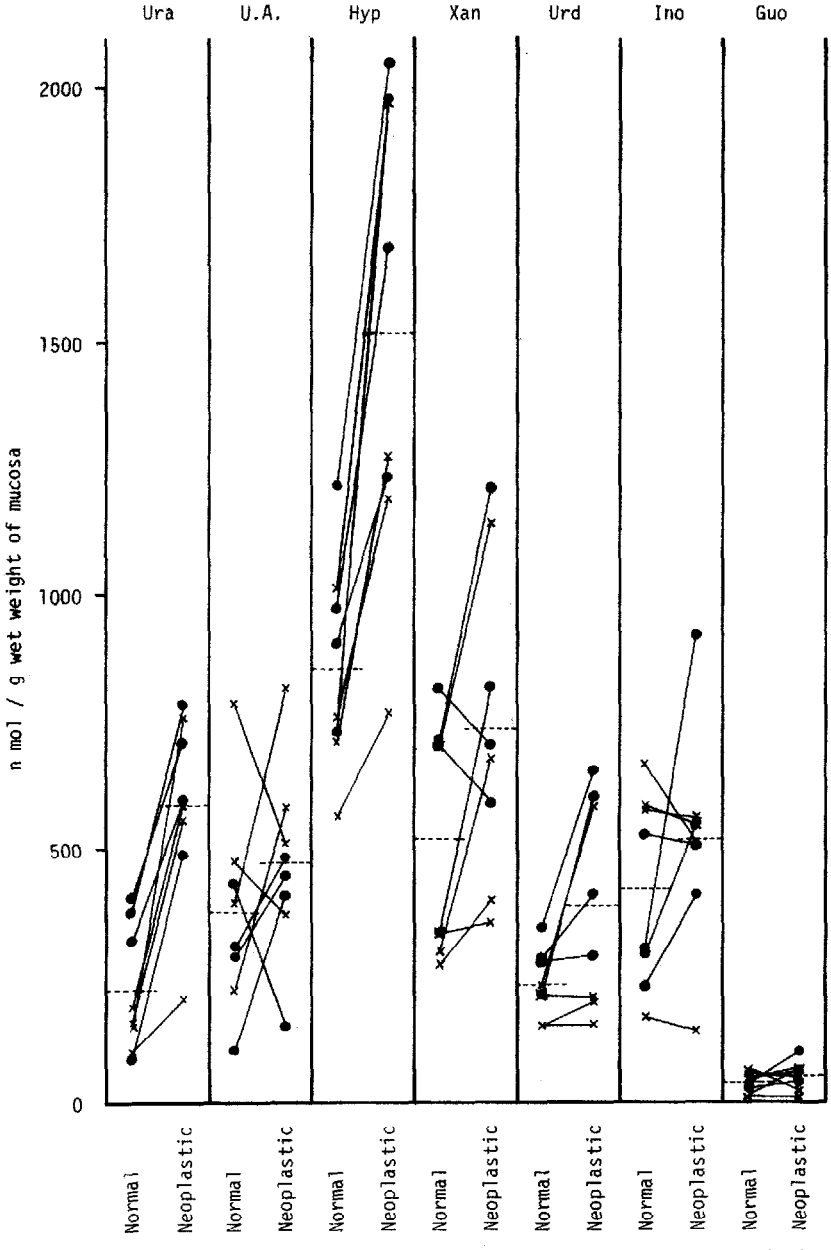

Fig. 3. The levels of compounds in perchloric acid extracts of normal and neoplastic mucosa from colorectum, resected surgically from eight patients with colorectal cancer. The broken lines are the mean levels. The dots represent males, the crosses females. Abbreviations used: Ura = uracil; U.A. = uric acid; Hyp = hypoxanthine; Xan = xanthine; Urd = uridine; Ino = inosine; Guo = guanosine.

colorectum, the levels of hypoxanthine and uridine were also significantly higher than those in normal mucosa ($P < 0.05$, with the paired t test).

However, an increase in the uracil level in neoplastic mucosa was found in only one out of four patients with gastric cancer, as shown in Fig. 4. In the neoplastic

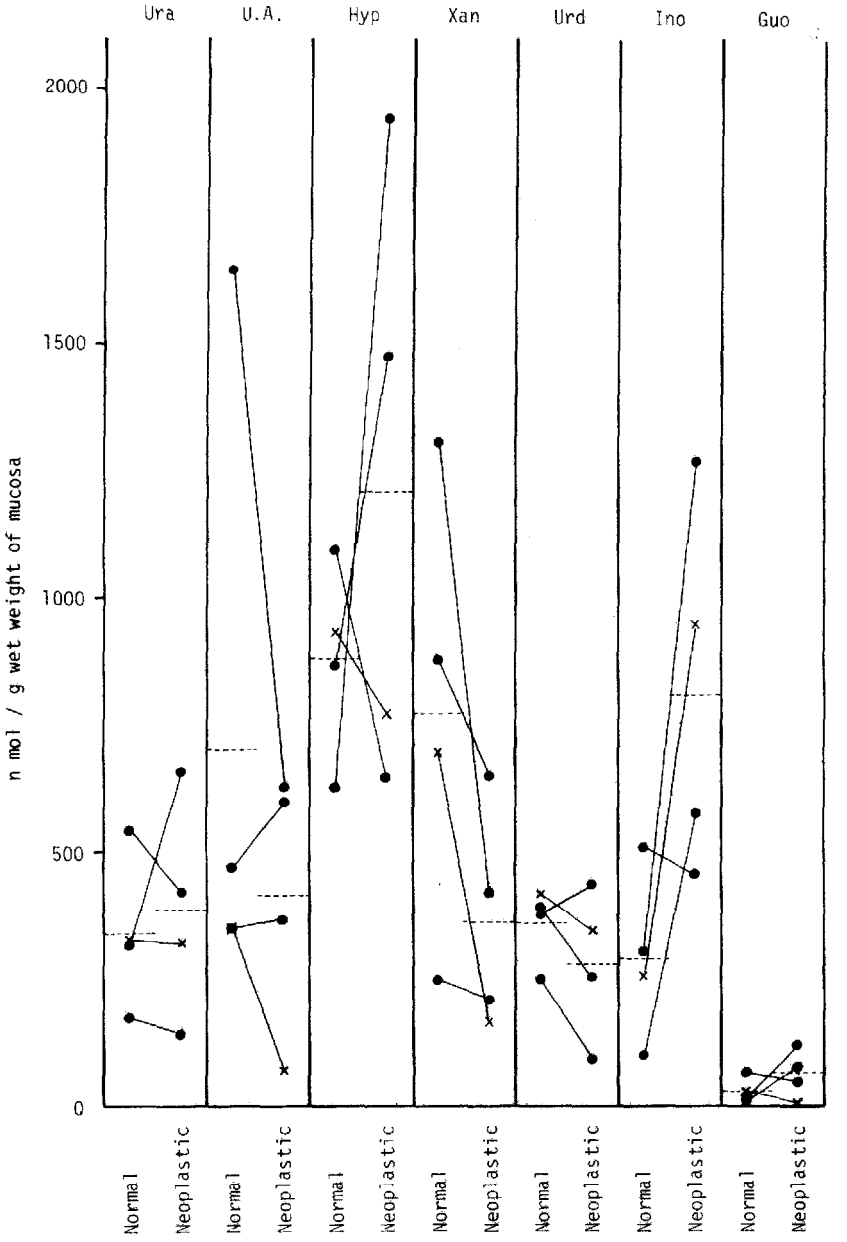

Fig. 4. The levels of compounds in perchloric acid extracts of normal and neoplastic mucosa from the stomach, resected surgically from four patients with gastric cancer. For symbols and abbreviations, see Fig. 3.

mucosa of the stomach, the level of inosine was significantly higher than that in normal mucosa according to the paired t test ($P < 0.05$).

Urinary nucleosides analysis

Urinary nucleosides were isolated by using a boronate affinity gel column. They were then separated and quantified by the HPLC procedure described. Fig. 5

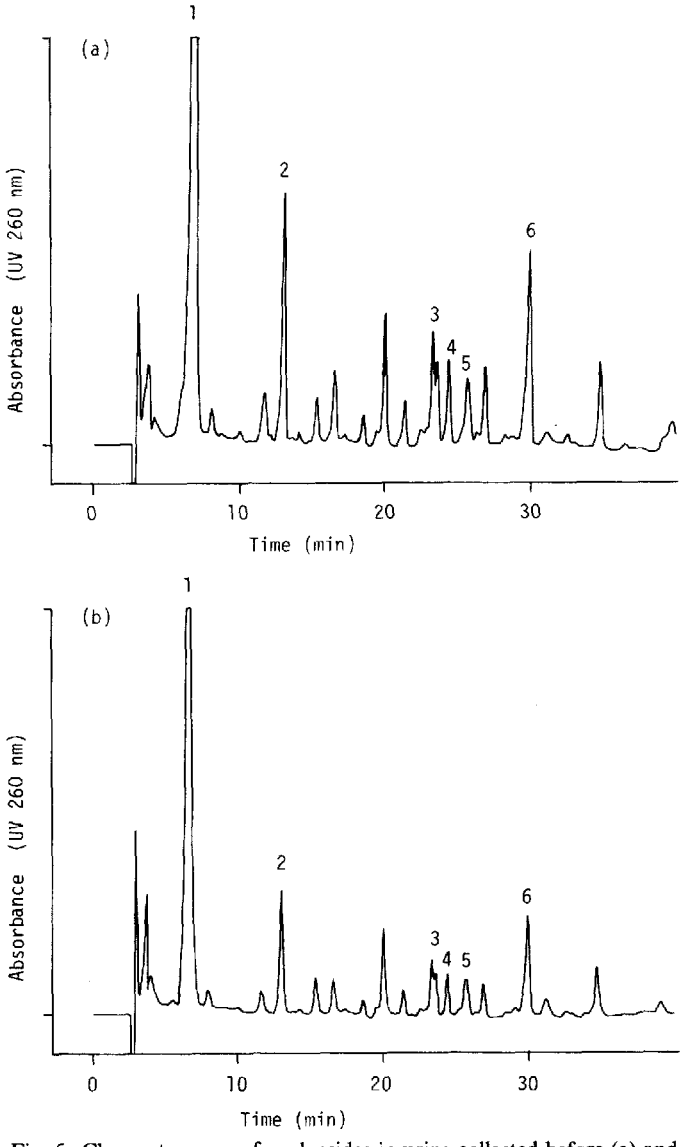

Fig. 5. Chromatograms of nucleosides in urine collected before (a) and after (b) surgical operation on a patient with malignant rectal cancer. Injection volume: 200 μl, equivalent to the same volume of urine. For chromatographic conditions see text. Peaks: 1 = pseudouridine; 2 = uridine + 1-methyladenosine; 3 = 1-methylinosine + unknown compound; 4 = 1-methylguanosine; 5 = N^2-methylguanosine; 6 = N^2,N^2-dimethylguanosine.

shows chromatograms of nucleosides in urines taken before and after a surgical operation on a patient with malignant rectal cancer.

Based on the data from the identification techniques and the results reported by Gehrke *et al.*[21,22], the nucleosides present in the majority of urine samples were identified as pseudouridine, 1-methylguanosine, N^2-methylguanosine and N^2,N^2-dimethylguanosine. The peak with the retention time of 13 min (peak 2) contained 1-methyladenosine + uridine and the peak with the retention time of 23.5 min (peak 3) was identified as 1-methylinosine together with an unknown compound.

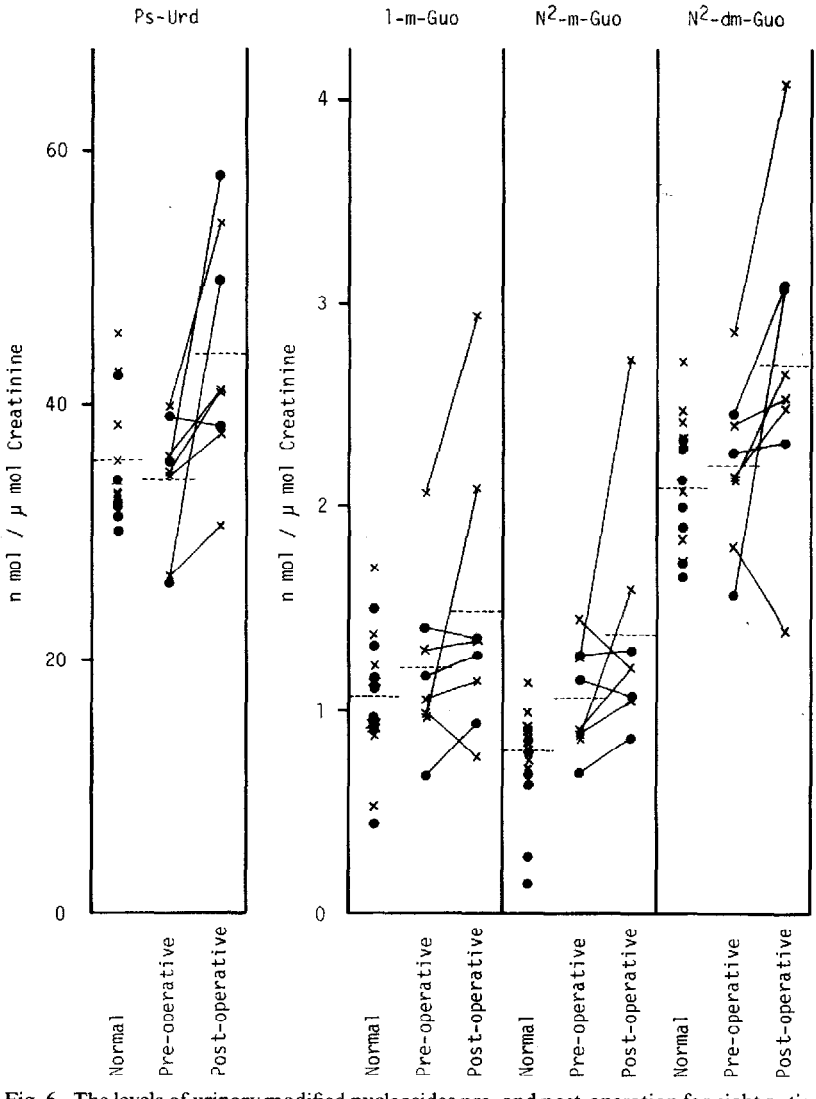

Fig. 6. The levels of urinary modified nucleosides pre- and post-operation for eight patients with colorectal cancer, and sixteen normal subjects. For symbols, see Fig. 3. Abbreviations used: Ps-Urd = pseudouridine; 1-m-Guo = 1-methylguanosine; N^2-m-Guo = N^2-methylguanosine; N^2-dm-Guo = N^2,N^2-dimethylguanosine.

Urinary nucleoside levels of cancer patients

The concentration of modified nucleosides in urine samples was determined by the HPLC method. The urinary nucleoside levels were then converted on the basis of the urinary creatinine level of each sample.

Fig. 6 shows a comparison of urinary nucleoside levels pre- and post-operation for cancer patients and normal subjects. Contrary to previous reports[17–20], the present results did not show an elevation of modified nucleosides in urines from patients with colorectal cancer.

DISCUSSION

An increase in excretion of urinary uracil in leukaemic patients has been reported by Horrigan[29] and Adams *et al.*[30]. The activity of dihydrouracil dehydrogenase (DHUDH, E.C. 1.3.1.2), which is a rate-limiting enzyme of the pyrimidine degradation system, is decreased in rat hepatoma[31,32], embryonic liver[31,32] and human leukaemia[33]. The elevated uracil level in neoplastic colorectal mucosa observed in this study may be related to the decrease in DHUDH activity.

The decrease in activity of xanthine oxidase (E.C. 1.2.3.2), the rate-limiting enzyme of inosine monophosphate (IMP) catabolism, has also been observed in hepatoma and in other tumours[31,32]. Thus, the increased level of hypoxanthine and xanthine in neoplastic mucosa we observed may be related to the decrease in activity of xanthine oxidase.

Several investigations reported elevation of the modified nucleosides in urines from cancer patients[17–20]. Very recently, it was reported that the urinary pseudouridine levels in patients with lung cancer were significantly higher than those of controls and decreased after surgical operation[34]. To confirm these findings the urinary nucleosides, prefractionated on the affinity gel column, were analyzed by the reversed-phase HPLC technique. However, our results show no elevation of modified nucleosides in the preoperative urines of patients with colorectal cancer as compared with postoperative urines and normal urines, as shown in Fig. 6.

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