

Pterin-6-aldehyde, a cancer cell catabolite: Identification and application in diagnosis and treatment of human cancer

(urinary metabolite/folate degradation/tissue culture)

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ABSTRACT Active folic acid degradation with the formation of pterin-6-aldehyde is a previously undescribed characteristic of cancer cells in tissue culture. Neither normal adult epithelial and fibroblastic cells nor human amniotic cells nor mouse embryonic fibroblasts degrade folic acid to a measurable degree.

Twenty-nine patients whose diagnoses were not revealed until after the test of their first morning urine for pterin-6-aldehyde was completed were studied for the presence or absence of pterin-6-aldehyde by thin-layer chromatography. Pterin-6-aldehyde was found in the urine at about 300 nmol/ml or greater only in those 13 patients with a tissue diagnosis of cancer. When the cancer was totally resected, the pterin-6-aldehyde was no longer found in the urine postoperatively. Pterin-6-aldehyde is not found in the urine of healthy patients at this level of detection unless their diets are supplemented with folic acid.

Active human degradation of folate *in vivo* is not thought to occur (1) because the total folate content (10 mg) of a 70-kg man is maintained by a daily intake of only 50 μ g of folate (2). Urinary folate degradation products are attributed to photodecomposition and oxidation of excreted folate and bioppterin. Although pterin-6-aldehyde, pterin-6-carboxylate, and pterin are fluorescent compounds derived by the photodecomposition of folate, only pterin-6-carboxylate and pterin, but not pterin-6-aldehyde, have been found in human urine (3-8). Bioppterin, a guanosine and not a folate derivative, and its oxidation products (xanthopterin, isoxanthopterin, and leucoppterin), are also found in human urine (9, 10).

A sluggishly active folate cleavage enzyme has been demonstrated in crude liver extracts of animals (11, 12), and an inert folate cleavage enzyme, activated by HCl, has been reported in human red blood cells (13). Cleavage of folic acid occurs between C-9 and N-10, immediately yielding pterin-6-aldehyde and *p*-aminobenzoylglutamate. When [³H]folic acid was injected into men, [³H]pteridines were found in the urine (14). Moreover, when either we or our gouty patients consumed 50-100 mg of folic acid, the next first morning's freshly voided urine, which was protected from light and oxidation, contained not only folic acid but considerable quantities of pterin-6-aldehyde, pterin-6-carboxylate, and pterin, indicating, we believe, active folate degradation *in vivo*.

Herein, we describe the excretion and identification of pterin-6-aldehyde in the culture media of malignant cells, but its absence in the media of adult normal cells, embryonic cells, and amniotic cells. We also report the demonstration by thin-layer chromatography of pterin-6-aldehyde in the urine of patients with cancer, in concentrations greater than 300 nmol/ml, and its absence in adequately studied patients without malignancies.

Abbreviation: HBrF, human breast fibroblasts.

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MATERIALS AND METHODS

Tissue Culture. We used McCoy's 5a (modified) medium without Bactopeptone and added 15% dialyzed fetal calf serum and gentamicin (50 μ g/ml). In the reconstructed medium we varied the folic acid from 10 μ g/liter to 10 mg/liter and the vitamin B₁₂ from 0.5 μ g/liter to 2 mg/liter. In some experiments we added N⁵-methyltetrahydrofolate in place of folic acid. All cultures were shown to be concurrently PPLO (pleuropneumonia-like organism)-negative by culture for 3 weeks on GIBCO broth and pour plates. The cell types studied are listed in Table 1.

The fluorescent substance was isolated from two sets of experiments. In the first set the cells were grown in Falcon plastic 75 cm² flasks containing 10 ml of medium for 2-3 days, depending on the cell doubling time. The medium was discarded and replaced by fresh medium, which was allowed to remain in the flask for the ensuing 36-72 hr in order to ensure collection during logarithmic phase growth of the cells. Controls consisted of simultaneously incubated media without cells. All determinations were done in quadruplicate. In the second set each cell type was grown to near confluency, when the media were replaced. Cell growth was continued for 5 days, at which time media were collected. Isopropanol was added to the medium to a final concentration of 70% (vol/vol). The solution was stirred at 4° overnight. The precipitated proteins were removed by centrifugation, and isopropanol was removed by extracting three times with six volumes of toluene. The lyophilized aqueous phase was dissolved in 0.75 ml of distilled water and 50-100 μ l of this extract was applied to Whatman 3MM paper. The chromatogram was developed in the dark using our system I, *t*-butanol: ammonium acetate (0.1 M) (70:30, vol/vol).

A larger quantity of the fluorescent compound was obtained by inoculating nine roller bottles each containing 100 ml of medium with 25 \times 10⁶ neoplastic cells. These were allowed to grow for 6 days. The 900 ml of medium were collected and treated as described above. After lyophilization, the residue was redissolved in 100 ml of distilled water and applied to a Dowex 50-X 8 (100-200 mesh 5 \times 8 cm) column. The column was washed with 10 volumes (1000 ml) of deionized water and then stripped of positively charged substances with four volumes of 0.1 M NH₄OH. The NH₃ was evaporated by bubbling a stream of N₂ through the eluate overnight in the dark at room temperature. The aqueous extract was protected from light and lyophilized. The residue was redissolved in 10 ml of distilled water and further purified on a phosphocellulose column (capacity 0.8 meq/g, Sigma) from which the fluorescent material was eluted with distilled water and again lyophilized. The residue was dissolved in 1 ml of distilled water, again applied to Whatman 3MM paper, and developed with solvent system I. The fluorescent band with the characteristic R_F of 0.35-0.45 was eluted from the paper. A similar nonfluorescent area of the

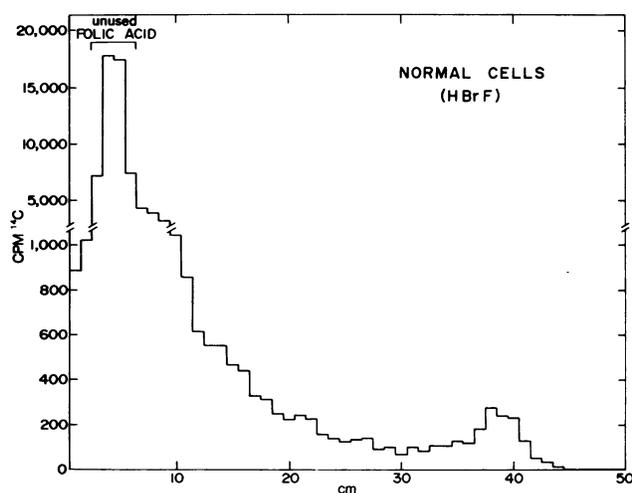


FIG. 1. The chromatogram of the medium containing [2-¹⁴C]-folate in which human breast fibroblasts (HBrF) were grown for 6 days. Cells (2×10^6) were plated in a T-75 Falcon flask and grown to almost confluency in regular McCoy's medium. The medium was then replaced with 15 ml of fresh medium containing 0.75 μ Ci of [2-¹⁴C]-folate (Amersham-Searle Corp.), and the cells were grown in this medium for 6 more days. At the end of the sixth day, the medium was collected and treated as described in *Materials and Methods*. The chromatogram was then examined under UV light in a Chromatovue[®] box (Ultra-Violet Products, Inc., San Gabriel, Calif.) for detection of absorbing and fluorescent spots and finally sectioned into 1-cm rectangles. Radioactivity was determined in a toluene-based scintillation fluid with a Packard liquid scintillation spectrometer.

paper was also eluted. Both were eluted with the same volume of water; the nonfluorescent paper eluate served as a blank for UV absorption and fluorescent spectral studies.

To determine if the fluorescent substance was derived from folic acid, we also prepared tissue cultures of the various cell types in Falcon plastic flasks with 25 cm² surface area containing 4 ml of McCoy's medium to which 0.11 μ Ci of [2-¹⁴C]folic acid had been added. The added [¹⁴C]folic acid had a specific activity of 54 μ Ci/mmol. The fluorescent compound was isolated from the medium after 5 days of cell growth, and the distribution of ¹⁴C on the chromatograph was measured. This procedure was carried out with malignant cells and with normal adult fibroblasts.

Pterin-6-aldehyde was prepared from folic acid by the method of Lowry *et al.* (5) or Waller *et al.* (15). Pterin-6-carboxylate and pterin were purchased from Sigma Chemical Co. All three compounds were used as standards in three thin-layer chromatographic systems. These consisted of Woelm silica gel plates run in two different solvent systems in one dimension: (system II) 1-propanol:H₂O:NH₄OH (66:32:2, vol/vol) and (system III) CH₃CN:isopropanol:NH₄OH:H₂O (70:10:10:10, vol/vol), and a two-dimensional system (system IV) run on Brinkmann Cel MN 300 UV-254 plates. The developing system consisted of 1-butanol-saturated water, which was then saturated with H₃BO₃ and brought to pH 7.0, with NH₄OH:1-butanol:isopropanol:NH₄OH:methanol (40:10:50:10:10, vol/vol) run in the first dimension. The developing solution for the second dimension was CH₃CN:10% citric acid made pH 9.0 with NH₄OH (60:40).

Human Urine. For the detection of fluorescent substances in the first morning urine, the specimens were kept frozen until analyzed. To 3 ml of thawed urine was added 7.0 ml of isopropanol, and the mixture was stirred, then allowed to stand at 4° for 2 hr prior to centrifuging for 30 min at room temperature at 2000 \times g. The isopropanol was removed by toluene

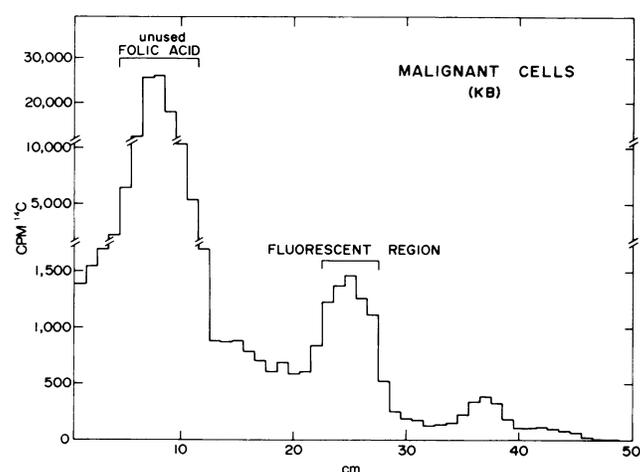


FIG. 2. The chromatogram of the medium containing [2-¹⁴C]-folate in which KB cells were grown for 6 days. KB cells (2×10^6) were plated in a T-75 Falcon flask and grown coincidentally under exactly the same conditions and in the presence of the same amount of radioactive folate as used for the HBrF cell culture. The medium collected after the 6 days growth period in the presence of [2-¹⁴C]-folate was also treated and chromatographed in the same way as done for the medium from the HBrF cell culture.

extraction and the aqueous phase was lyophilized as before. The residue was dissolved in 0.5 ml of 0.05 M NH₄OH. Eight microliters of each specimen are applied to a Brinkmann Cel MN 300 UV-254 plate. The two-dimensional developing system described above (system IV) was routinely used; by this method the lower limit of detection was about 10 nmol of pterin-6-aldehyde.

Xanthine oxidase (from buttermilk) (xanthine:oxygen oxidoreductase, EC 1.2.3.2) was obtained from Sigma Chemical Company and was used without further purification. The standard assay of Kalckar (16), which follows production of uric acid by measuring the increase of A_{290 nm}, was used.

RESULTS

Folic Acid Degradation in Normal and Malignant Cell Cultures. Figs. 1 and 2 show the paper chromatograms of separations (system I) of the growth medium containing [2-¹⁴C]folic acid collected from normal cells (human breast fibroblasts, HBrF, Fig. 1) and malignant cells during their logarithmic phase of growth (KB, Fig. 2). The chromatograms were observed for blue fluorescence under short wave (280 nm) UV light. The fluorescent region was marked, then the paper was serially sectioned into 1-cm rectangles and the ¹⁴C in each section was determined. There was neither observable blue fluorescence nor radioactivity in the region (R_F 0.35–0.45) of the chromatogram from human breast fibroblasts. There was no observable blue fluorescent substance seen on chromatograms of the growth medium from any normal, embryonic, or amniotic cells (Table 1).

In Fig. 2 there is considerable ¹⁴C in the region of the fluorescent substance (R_F 0.35–0.45) that cochromatographs with known synthetic pterin-6-aldehyde (5). In the KB cell culture, as much as 7% of the folic acid was converted to the fluorescent substance in 6 days, while in other experiments with KB cells (data not shown) as much as 25% of the ³H from [9,3',5'-³H]-folic acid was found in this region. From each of the neoplastic cell types used in this investigation (Table 1) we consistently observed a blue fluorescent substance (R_F 0.35–0.45) on paper chromatography (system I) of the growth medium. Present results indicate that the blue fluorescent substance can be de-

Table 1. Occurrence of a fluorescent substance in growth medium of cells in culture

Cell type	Origin	Fluorescent substance*
Human KB	Nasopharyngeal carcinoma	+
Human HT-29	Colon carcinoma	+
Human T-24	Bladder carcinoma	+
Human P ₂	Prostatic carcinoma	+
Human P ₃	Prostatic carcinoma	+
Human J111	Monocytic leukemia	+
Human HeLa	Uterine cervical carcinoma	+
Human HBrF	Normal breast fibroblasts	-
Human HPF	Normal prostatic fibroblasts	-
Human FL	Amniotic cells	-
Rat W-256	Breast carcinosarcoma	+
Rat L3-14A	Liver epithelial cells	-
Rat normal	Thymus fibroblasts	-
Rat R.E.F.	Embryonic fibroblasts	-
Mouse L1210	Lymphatic leukemia	+
MF8-16	Normal mouse fibroblasts	-

For details of cell culture and extraction procedures see *Materials and Methods*.

* + Indicates clearly detectable blue fluorescent substance on paper chromatography, system I (R_F 0.35–0.45). Our limit of detection of pterin-6-aldehyde is about 10 nmol.

tected chromatographically from the growth medium containing fewer than 10^6 malignant cells.

Fig. 3 shows the accumulation of radioactivity, derived from $[2-^{14}C]$ folic acid, in the region chromatographically coincident with the blue fluorescent substance at 48-hr intervals for 8 days in identical growth media with malignant (KB) and normal (HBrF) cells. It is clear that there is negligible accumulation of radioactivity in the fluorescent region in chromatograms of normal cells, while considerable and increasing accumulation is seen with malignant cells.

Characterization of the Fluorescent Substance. A provisional identification of the blue fluorescent substance was made on the basis of its UV absorption spectrum (at pH 13), which is identical with that of pterin-6-aldehyde (15), and its fluorescent spectrum (excitation 355 nm, fluorescence 450 nm, pH 6.5, determined on a Spex Fluorolog fluorometer).

The unlabeled blue fluorescent substance from all of the above malignant cell types (Table 1), whether in the logarithmic phase of growth or after confluence, displayed an identical chromatographic mobility on paper (system I) and in the three thin-layer systems (II, III, and IV) with known synthetic pterin-6-aldehyde. In addition, radioactive blue fluorescent material obtained from KB cell cultures as well as HT-29 cell cultures cochromatographed in system I with pterin-6-aldehyde.

The blue fluorescent substance, accumulated in large cell cultures (KB cells), was eluted from paper chromatograms (system I) or from thin-layer plates (system IV) and its concentration was estimated from the published extinction coefficient for pterin-6-aldehyde (278 nm, $\epsilon_{mM} = 15.5$, pH 8.0) (17). According to Lowry *et al.* (5, 6), of all the known pterin derivatives, including folic acid, pterin-6-aldehyde is the only potent inhibitor of xanthine oxidase ($K_i = 10^{-9}$ M). The results in Table 2 show that 1.8×10^{-7} M synthetic pterin-6-aldehyde inhibited the xanthine oxidase reaction by 75%, while allopurinol, a standard synthetic xanthine oxidase inhibitor, at 10^{-6} M was 60% inhibitory. The fluorescent substance eluted from

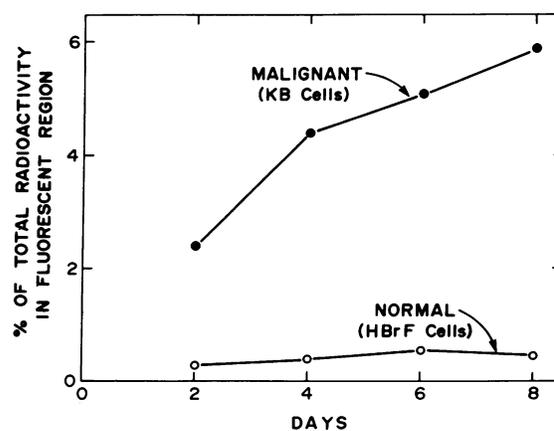


FIG. 3. Accumulation in the growth medium of a folic acid catabolite from malignant cells. Two sets of T-25 Falcon flasks were seeded, one set with KB cells, the other with HBrF, both at a density of 3×10^6 cells per flask. The cells were grown for the entire length of the experiment in McCoy's medium modified by the addition of $0.11 \mu\text{Ci}$ of $[2-^{14}C]$ folate per flask. At the times indicated, the medium from one flask of each set was collected and treated as described in *Materials and Methods*. After lyophilization, the residue was redissolved in $200 \mu\text{l}$ of distilled water; $100 \mu\text{l}$ of this solution was streaked across a 3.5-cm wide strip of Whatman 3MM paper, which was chromatographed with solvent system I and examined under UV light. Radioactivity was determined as described in *Materials and Methods*. Radiochromatograms were constructed for each flask; the amount of radioactivity present under the peak that corresponded with a blue fluorescent band visible under UV light (and which always had an R_F between 0.35 and 0.45) was expressed as percentages of the counts present in the entire chromatogram. The percentages so calculated for each flask were plotted against the time at which the medium was collected. A growth curve was determined on the cells remaining in the flasks after the medium was removed. (Note: When the medium from HBrF flasks was chromatographed with solvent system I, no fluorescence occurred in the region corresponding to R_F 0.35–0.45, but a small amount of radioactivity could be detected in that same region which was not more than that encountered in control extractions from growth medium lacking cells.)

chromatographs (system I) at 1.1×10^{-7} M inhibited the xanthine oxidase reaction by 53%.

Pterin-6-aldehyde Excretion in Patients with and without Proven Cancer. As early as 1959 we had observed the presence of a fluorescent blue substance in the chromatographed urines of rats with W-256 tumors and of patients with cancer which disappeared when the cancers were successfully eliminated

Table 2. Effect of inhibitors of buttermilk xanthine oxidase activity

Inhibitor (μM)	Substrate, hypoxanthine (μM)	% Inhibition
Allopurinol	17	60
Pterin-6-aldehyde (synthetic)	17	75
Blue fluorescent substance	17	53

Xanthine oxidase activity was measured by the method of Kalckar (16). Xanthine oxidase activity was followed by measuring the formation of urate at 290 nm. Hypoxanthine was present at a concentration $17 \mu\text{M}$ in 0.05 M potassium phosphate buffer, pH 7.5. The percent inhibition of urate formation by allopurinol ($1.0 \mu\text{M}$), synthetic pterin-6-aldehyde ($0.18 \mu\text{M}$), and the blue fluorescent substance ($0.11 \mu\text{M}$) is shown in the table. A control eluate from the chromatogram (system I) was not inhibitory.

Table 3. Occurrence of pterin-6-aldehyde in the urine of random patients

Case no.	Clinical diagnosis of cancer	Chromatographic demonstration of urinary pterin-6-aldehyde		Clinical resume and pathological diagnosis*
		Preop.	Postop.	
1	+	+		40-yr-old ♀, intra-abdominal carcinomatosis
	+		+	3 days
2	+	+		69-yr-old ♀, carcinoma of colon with metastases
	+		+	3 days
3	-	-		49-yr-old ♀, benign cystadenoma of the ovary
	-		-	3 days
4	-	-		36-yr-old ♀, chronic thyroiditis
	-		-	2 days
5	+	+		57-yr-old ♂, widespread lymphoma staging laparotomy
6	-	-		51-yr-old ♂, arthritic knee; knee joint replacement
	-		-	1 day
7	+	+		20-yr-old ♂, widespread lymphoma staging laparotomy
	+		+	1 day
8	-	-		20-yr-old ♂, osteomyelitis of mandible; plastic correction
	-		-	2 days
9	-	-		71-yr-old ♀, orthopedic surgical procedure; no malignancy
10	+	-		47-yr-old ♂, chronic glossitis; no carcinoma
	+		-	2 days
11	+	+		60-yr-old ♀, meningeoepithelioma
	-		-	5 days
12	+	+		60-yr-old ♂, hypernephroma, "sole metastasis" resected
	-?		+	5 days
13	-	-		72-yr-old ♂, osteoarthritis of cervical spine; arthrodesis
14	+	+		66-yr-old ♀, cerebellar hemangioblastoma
15	+	+		63-yr-old ♀, recurrent carcinoma of urinary bladder
	-		+	2 months after fulguration
16	-	-		33-yr-old ♀, endometriosis
	-		-	3 days
17	-	-		17-yr-old ♂, kidney transplant recipient
18	-	-		42-yr-old ♀, kidney donor
19	+?	+		71-yr-old ♀, meningioma invading T ₇ and T ₈ vertebra
	-?		+	7 days
20	+	+		48-yr-old ♀, carcinoma limited to pancreatic head; modified Whipple's; total excision of carcinoma; lymph nodes
	-		-	negative for carcinoma
			-	3 days
21	+	+		72-yr-old ♀, cecum carcinoma; no metastases at operation
	-		+	6 days; 16 days, postop.; pleural effusion
22	-	-		43-yr-old ♀, uterine fibroids
23	-	-		23-yr-old ♀, mitral stenosis valvotomy
24	+	+		45-yr-old ♂, colon carcinoma; with metastases
	+		+	1 day
25	-	-		22-yr-old ♂, fibrous dysplasia of iliac crest
	-		-	1 day
26	+	+		66-yr-old ♂, carcinoma of liver; laparotomy
	+		+	3 days
27	-	-		19-yr-old ♀, abortion
28	-	-		73-yr-old ♂, aortic stenosis; valve replaced
29	-	-		21-yr-old ♀, healthy; no disease

The extraction and chromatographic procedures are described in *Materials and Methods*.

* UCLA Hospital Medical Records.

either surgically or by x-ray therapy. Our chromatographic identification of pterin-6-aldehyde in the growth medium from malignant cells and the fact that this substance was not reported in human urine under ordinary circumstances renewed our interest in this prior observation. Pterin-6-aldehyde excretion by malignant cells into tissue culture media was, as described above, a unique characteristic of neoplastic cells from among those tested. Accordingly, the urines of 29 random patients on routine diets without added folic acid who were undergoing a surgical procedure were obtained preoperatively and, as frequently as possible, postoperatively. Neither the clinical nor the pathological diagnosis was known to us until after our analysis was performed.

Whenever these patients' urines could be examined postoperatively, we were able to determine whether the neoplasm had or had not been totally removed. Only after our analyses were completed were our results compared with those expressed by the surgeon's and pathologist's reports. These results are shown in Table 3. Of the 29 subjects, 13 had a tissue established diagnosis of cancer. In all 13 subjects, we found pterin-6-aldehyde in the urine but no folic acid. In none of the other subjects did we find pterin-6-aldehyde in the urine. In the patients whom we were able to study postoperatively, we were able to identify every patient in whom it was clear that the neoplasm had not been totally removed. Patients with benign tumors (ovarian cystadenoma, uterine fibroids, and thyroiditis) revealed no urinary pterin-6-aldehyde.

We intend to expand our initial observations and collect specimens from patients with acute infectious, chronic granulomatous and other disease states before being certain that the presence of pterin-6-aldehyde without folic acid in the urine is pathognomonic of a malignant disease.

DISCUSSION

Our results indicate that cleavage of folic acid and the concomitant formation and excretion of pterin-6-aldehyde into the growth medium appears to be a unique characteristic of cancer cells in tissue culture (Table 1). All other mammalian cell types tested, including adult normal fibroblasts and epithelial cells, amniotic cells, and embryonic cells, apparently did not catabolize folic acid to a significant degree. Although it is unlikely that all the malignant strains tested produced the pterin-6-aldehyde at the same rate, we presently have no quantitative estimates relating production to cell growth. The particular advantage of this metabolic pathway to cancer cells is not at all obvious and is currently under investigation. Because we had demonstrated the excretion of pterin-6-aldehyde by cancer cells in tissue culture, we wondered if cancers in patients would excrete pterin-6-aldehyde and whether it could be detected in the urine. We therefore obtained the urines of 29 hospitalized patients. Our analysis of the urine was made without knowledge of the diagnosis.

Of those we examined, pterin-6-aldehyde was found only

in the urines of patients bearing diagnosed cancer. Not only were we able to corroborate the clinical and pathological diagnoses without prior knowledge, but strikingly, in those cases where the malignancy was not successfully removed, our analyses were also valid. To date pterin-6-aldehyde has not been found in the urines of patients without cancer unless they are ingesting relatively large quantities of folates, as will be published elsewhere. It is important to note that in the latter case not only do we find pterin-6-aldehyde, but also folic acid in our two-dimensional chromatographic system (system IV). It is clear that a very much larger sample of patients must be carefully studied and followed for a long interval of time to ascertain the diagnostic and prognostic significance of the occurrence of pterin-6-aldehyde in the urine.

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