Biochem. J. (1987) 243, 803–808 (Printed in Great Britain)

Measurement of allantoin and uric acid in human body fluids

A potential index of free-radical reactions *in vivo*?

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Free-radical attack upon uric acid generates allantoin [Ames, Cathcart, Schwiers & Hochstein (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6858–6862]. Methods are described for the accurate measurement of uric acid and allantoin in human body fluids. The concentrations of uric acid and allantoin in human serum and synovial fluid are reported. It is suggested that measurement of changes in allantoin concentration may be a useful index of free-radical reactions taking place *in vivo*.

INTRODUCTION

Uric acid is generated in the human body by the oxidation of purines, but no enzyme is present to oxidize it further. Hence human blood plasma contains uric acid at concentrations approaching 500 μM [1]. It has been suggested that one function of uric acid in human body fluids is to act as an antioxidant [1,2]. In agreement with this proposal, experiments *in vitro* have shown that uric acid protects erythrocytes against damage by singlet O_2 or t-butyl hydroperoxide [1], inhibits lipid peroxidation [3–5], decreases oxidation of haemoglobin by nitrite [6], inhibits oxidative degradation of hyaluronic acid [7], scavenges the myeloperoxidase-derived oxidant hypochlorous acid [8,9], protects against free-radical damage to DNA [10], inhibits ozone-induced degradation of nucleic acid bases [11] and binds transition-metal ions in complexes that are poorly active in promoting free-radical reactions [12–14].

If uric acid is really acting as an antioxidant *in vivo*, it ought to be possible to detect and measure its oxidation product (allantoin [1]) in human body fluids and animal tissues after situations of oxidative stress, i.e. oxidation of uric acid might be a 'marker' *in vivo* [15] for the extent of free-radical reactions. In the present paper we describe h.p.l.c. methods for the analysis of uric acid and its oxidation product allantoin [1] in human body fluids. These methods have been used to measure the concentrations of uric acid and allantoin in body fluids from healthy subjects and from patients with active rheumatoid disease, since free-radical reactions are thought to play an important role in the pathology of inflammatory joint disease [16].

MATERIALS AND METHODS

Reagents

H.p.l.c.-grade solvents were obtained from BDH Chemicals. Uric acid (sodium salt) and allantoin were from Sigma Chemical Co. The h.p.l.c. equipment was as described in references [15] and [17], with the addition of a variable-wavelength u.v. detector (Applied Chromatography Systems).

Sample preparation

Human blood was collected into heparinized tubes. Plasma was separated from erythrocytes by centrifugation at 2500 *g* for 10 min, transferred to a fresh tube and stored at −20 °C until required. Synovial-fluid samples drawn for authentic medical reasons from patients with active rheumatoid arthritis, according to the criteria of the American Rheumatism Association, were centrifuged to remove particulate matter, transferred to fresh tubes and also stored frozen until required. Blood samples drawn from healthy human volunteers, not age-matched to the rheumatoid patients, were used as controls.

Before analysis, plasma and synovial-fluid samples were thawed at room temperature and then ultrafiltered through Amicon Centrifree micro partition devices. The devices were centrifuged at 1800 *g* for 1 h at room temperature. The clear ultrafiltrates were stored frozen at −20 °C until ready for analysis.

Uric acid standards

Stock solutions of uric acid (sodium salt) were prepared by dissolving and mixing 9.5 mg of the compound in 750 ml of h.p.l.c.-grade water, and HCl was added to a final concentration of 2 mM, to give a final uric acid concentration of 66.7 μM. Standards for h.p.l.c. analysis were prepared by appropriate dilutions of this solution with 20 mM-HCl.

Preliminary experiments showed that addition of NaOH to enhance the solubility of uric acid (yielding solutions of concentration of up to 500 μM) was inappropriate for the following reasons. (i) H.p.l.c. chromatograms of standard solutions prepared by this latter method were found to yield uric acid peaks of significantly lower intensity (by as much as 35%) than those of the same concentration prepared as above, indicating that a significant amount of the uric acid had been oxidized or degraded during exposure to NaOH. (ii) Peaks corresponding to oxonic acid and allantoin (both oxidation products of uric acid) were present when standard solutions prepared with the use of NaOH were chromatographed on an Anachem S5 ODS-2 h.p.l.c. column with a mobile phase of very low or no methanol content and a low flow rate to favour separation of these

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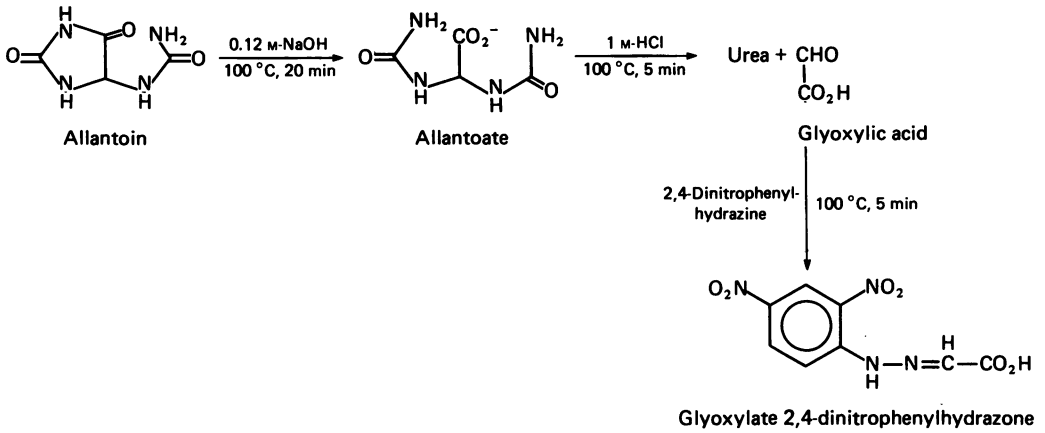

Scheme 1. Formation of glyoxylate 2,4-dinitrophenylhydrazone from allantoin

polar products. Standards of uric acid prepared by the method described above were found to be stable for at least 1 week when stored at 4 °C.

Uric acid analysis method

Samples (25 μl) of body-fluid ultrafiltrate were diluted to 500 μl with 20 mM-HCl for h.p.l.c. analysis. Then 20 μl volumes of diluted ultrafiltrate were directly injected on to a Spherisorb 5 ODS column. The mobile phase was 88% (v/v) 30 mM-sodium citrate/27.7 mM-sodium acetate buffer, pH 4.75, and 12% (v/v) methanol at a flow rate of 1.0 ml/min, continuously sparged with He during elution. Uric acid was detected either electrochemically, at an oxidation potential of +0.76V, or by its u.v. absorbance at 292 or 224 nm. The retention time of uric acid on this system was 5.12±0.07 min (mean±S.D.). Fig. 1 shows typical chromatograms for a synovial-fluid sample ultrafiltrate and a series of standards, obtained with the use of electrochemical detection at +0.76V.

Allantoin analysis method

A 20 μl sample of body-fluid ultrafiltrate was injected on to an Anachem S5 ODS-2 column with a mobile phase of 96.7% (v/v) 30 mM-sodium citrate/27.7 mM-sodium acetate buffer, pH 4.75, and 3.3% (v/v) methanol at a flow rate of 0.9 ml/min. Under these conditions typical retention times for allantoin and uric acid (detected at 224 nm) were 3.80 and 11.72 min respectively. (Allantoin is very polar and on a reversed-phase h.p.l.c. column it is difficult to obtain resolution from other serum or synovial-fluid ultrafiltrate constituents that have a similar polarity.) A fraction covering the retention-time range 3.3–5.2 min, where allantoin is known to be eluted, was collected, and then evaporated to dryness at 40 °C under a stream of N_2. The polar-fraction residue was reconstituted in 100 μl of 0.12 M-NaOH and then heated in a boiling-water bath for 20 min to hydrolyse allantoin to allantoate (Scheme 1). After the initial hydrolysis, the sample was treated with 100 μl of 1 M-HCl followed by 10 μl of a 3.00 mM solution of 2,4-dinitrophenylhydrazine in 1 M-HCl, and heating at 100 °C was continued for 5 min. This latter step hydrolyses allantoate (allantoic acid in HCl solution) to glyoxylic acid, which reacts with 2,4-dinitrophenylhydrazine to form its 2,4-dinitrophenylhydrazone. Calibration experiments showed that the molar recovery of added allantoin as glyoxylate phenylhydrazone was 98–100%.

After the derivative samples had been allowed to cool, a 20 μl volume was injected on to an Anachem S5 ODS-2 column. The mobile phase was 70% (v/v) 30 mM-sodium citrate/27.7 mM-sodium acetate buffer, pH 4.75, and 30% (v/v) methanol at a flow rate of 1.2 ml/min, continuously sparged with He during elution. Glyoxylate 2,4-dinitrophenylhydrazone was detected either by its u.v. absorbance at 360 nm or electrochemically at +0.76V. Typical retention times for the glyoxylate 2,4-dinitrophenylhydrazone and for 2,4-dinitrophenylhydrazine were 10.60 and 17.68 min respectively. A typical chromatogram obtained with the use of the u.v. detector (360 nm) is shown in Fig. 4.

The chemical modification procedure generates two diastereoisomers, syn-glyoxylate 2,4-dinitrophenylhydrazone and the anti isomer. However, the reversed-phase chromatographic method described here does not resolve these isomers.

RESULTS

Analysis of uric acid

Several h.p.l.c. methods for uric acid determination have already been described in the literature [18,19, and references cited therein], but they have employed uric acid standards made up in alkali, or else the method of making up the standard is not specified. However, exposure of uric acid to NaOH, a procedure frequently used to make up stock solutions [18], was found to cause its degradation (see the Materials and methods section). A commercial uric acid standard solution was also found to be degraded (only 75% of the uric acid expected).

Fig. 1 shows that the method used by us allowed easy detection and quantification of uric acid in ultrafiltrates of human plasma and synovial fluid. The retention time of uric acid was 5.12±0.07 min (mean±S.D., $n = 20$). The uric acid could be measured either electrochemically [18] (oxidation potential +0.76 V) or by its u.v. absorbance at 292 or 224 nm. Since the uric acid content of human body fluids is very high, a low-sensitivity setting of the electrochemical detector can be used, so that peaks corresponding to other electroactive components, present at much lower concentrations, are not visible. U.v.-absorbance detection does yield other peaks

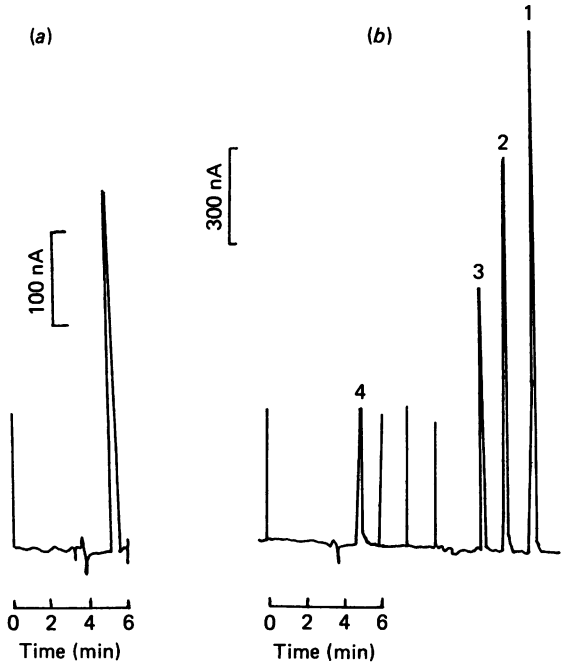

Fig. 1. H.p.l.c. electrochemical measurement of uric acid in human body fluids

Part (*a*) shows the uric acid peak obtained from an ultrafiltrate of knee-joint synovial fluid from a rheumatoid patient. Part (*b*) shows a series of standards: 1, 50 μM-uric acid; 2, 37.5 μM-uric acid; 3, 25 μM-uric acid; 4, 12.5 μM-uric acid. Electrochemical detector potential was +0.76 V. The sharp vertical lines are injection spikes.

in the chromatogram, but none of them interferes with the measurement of uric acid. The response of both electrochemical and u.v.-absorbance detectors is linear over a wide concentration range. It was found that uric acid is stable for at least 4 weeks in ultrafiltrates of synovial fluid or plasma stored at −20 °C.

Identification of the putative uric acid peak in the chromatograms was based on three pieces of evidence. Firstly, the eluent composition was varied by including different proportions of methanol, from 8 to 15% (v/v), producing large changes in retention times. However, the uric acid peaks in plasma and synovial-fluid ultrafiltrates gave retention times that were always identical with those given by an authentic uric acid sample. Secondly, the electrochemical behaviour of the peak was studied. The oxidation potential of the electrochemical detector was varied in the range 0.26–0.76 V. The peak height, plotted as a function of potential, shows the same behaviour for ultrafiltrates of body fluids and for an authentic sample of uric acid (Fig. 2). Thirdly, the u.v.-absorption spectrum (wavelength range 224–320 nm) of the putative uric acid peak was obtained. Fig. 3 shows that the spectra obtained for both plasma or synovial-fluid ultrafiltrates and for a 45 μM-uric acid standard match very closely.

Hence we conclude that this h.p.l.c. method provides an accurate identification and measurement of uric acid in serum or synovial-fluid ultrafiltrates. Table 1 summarizes some of the results obtained. Serum uric acid in healthy control subjects was in the range 258–621 μM, in broad agreement with previously published values as determined by enzymic (urate oxidase) methods [1,2,20].

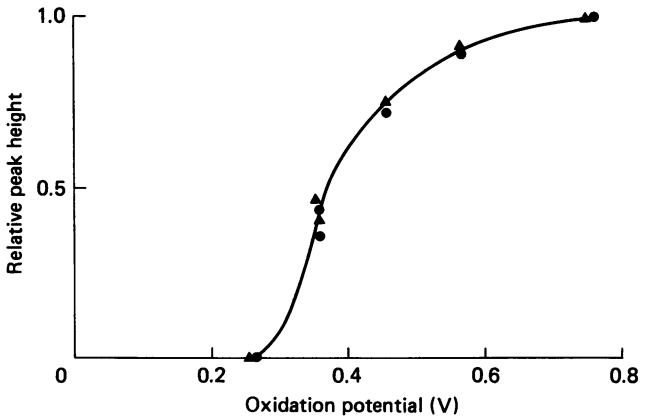

Fig. 2. Electrochemical properties of the 'uric acid peak' (Fig. 1) and authentic uric acid

The oxidation potential of the electrochemical detector was varied, and the peak height, expressed as a ratio to that at +0.76 V, is plotted as a function of potential both for a uric acid standard (●) and for an ultrafiltrate of serum from a healthy control subject (▲). Identical results were obtained with rheumatoid serum or synovial fluid.

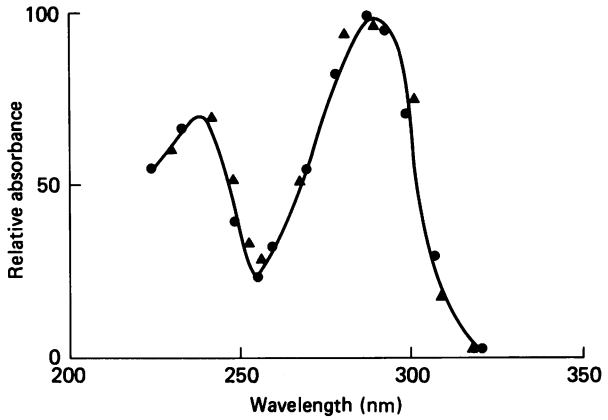

Fig. 3. U.v.-absorption spectrum of the 'uric acid peak' and authentic uric acid

The wavelength of the u.v.-absorbance detector was varied, and the peak height (relative to the $\lambda_{max.}$ at 292 nm, called 100) is plotted as a function of wavelength both for a uric acid standard (●) and for an ultrafiltrate of rheumatoid synovial fluid (▲), diluted 1/10 with eluent. Identical results were obtained with rheumatoid or control serum samples.

Serum uric acid in patients with active rheumatoid arthritis fell within the same range, but uric acid concentrations in the small number of knee-joint synovial fluids studied were significantly lower than those in serum (Table 1).

Analysis of allantoin

Allantoin has been reported [1] as a major oxidation product generated when uric acid is exposed to systems generating oxygen radicals. Allantoin is a highly polar molecule, and there are several constituents in biological fluids with a similar high polarity [21]. Hence after chromatography on an S5 ODS-2 (Anachem) column, as

Table 1. Concentrations of allantoin and uric acid in human body fluids

Fluid studied	[Uric acid] (μM) Range	[Uric acid] (μM) Mean ± S.D.	[Allantoin] (μM) Range	[Allantoin] (μM) Mean ± S.D.	[Allantoin] (% of [uric acid]) Mean ± S.D.
Serum, healthy control subjects ($n = 7$; 4 males, 3 females)	258–621	432 ± 121	14.1–25.4	18.6 ± 3.8	4.7 ± 1.6
Serum, rheumatoid patients ($n = 4$; 2 males, 2 females)	273–485	375 ± 102	20.3–45.2	36.1 ± 6.3†	10.5 ± 3.8†
Synovial fluid, rheumatoid patients ($n = 9$; 1 male, 8 females	123–351	205 ± 92*	7.2–31.3	20.9 ± 7.3	11.8 ± 5.6†

* Significantly less than serum concentrations ($P < 0.001$).
† Significantly raised when compared with normal serum samples ($P < 0.05$).

described in the Materials and methods section, a fraction covering the retention times 3.3–5.2 min, in which allantoin is eluted but uric acid is not, was collected and subjected to an alkali/acid hydrolysis procedure, which degrades allantoin to glyoxylate almost quantitatively (> 0.98 mol of glyoxylate phenylhydrazone produced/mol of allantoin). Glyoxylate was treated with 2,4-dinitrophenylhydrazine, and the resulting hydrazone was identified by h.p.l.c. (Scheme 1). This degradative procedure could not be applied directly to body-fluid samples, since the alkaline conditions employed in the first stage were found to cause a direct breakdown of uric acid into allantoin and other products. Evidence for this is presented in Fig. 4.

Glyoxylate dinitrophenylhydrazone could be quantified electrochemically (+0.76 V oxidation potential) or by its absorbance at 360 nm. Fig. 5 shows a typical

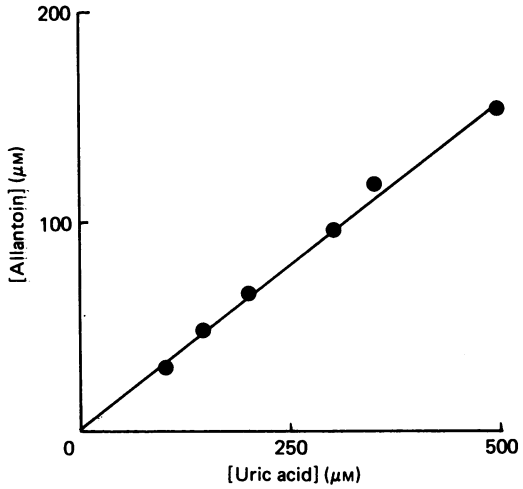

Fig. 4. Alkaline degradation of uric acid and the formation of allantoin

Exposure of uric acid to alkali causes its degradation (see the text). The data in this Figure show that allantoin is produced during this degradation, and thus uric acid must be separated from allantoin in body fluids before the double-hydrolysis procedure is performed (Scheme 1). A 200 μl sample containing the uric acid concentration shown was treated with 20 μl of 1.2 M-NaOH and heated at 100 °C for 20 min. Next 100 μl of 1 M-HCl was added, then 40 μl of 2,4-dinitrophenylhydrazine (14.2 mg/20 ml) in 1 M-HCl, followed by heating at 100 °C for 4 min. It may be seen that uric acid itself could be degraded to produce the allantoin concentrations given. Other experiments showed that the alkaline stage of the double-hydrolysis procedure was responsible for the degradation.

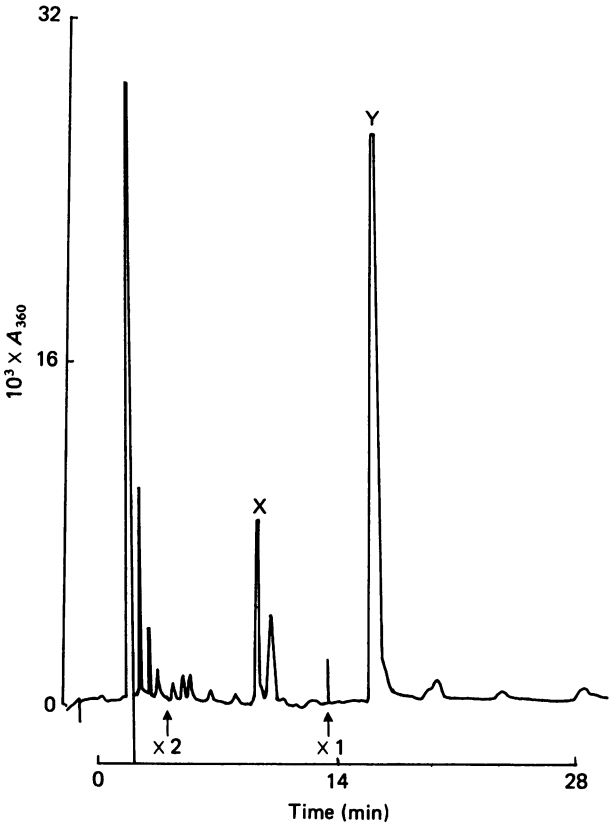

Fig. 5. Separation of glyoxylate 2,4-dinitrophenylhydrazone after treatment of human body fluids in accordance with Scheme 1

An ultrafiltrate of rheumatoid serum was chromatographed and the polar fraction was treated as in Scheme 1 (for full details see the Materials and methods section). Peak X is glyoxylate 2,4-dinitrophenylhydrazone and peak Y is 2,4-dinitrophenylhydrazine. Other peaks in the chromatogram are the dinitrophenylhydrazones of other oxo acids present in the polar fraction. Similar results were obtained with normal serum or rheumatoid synovial fluid. Arrows represent changes in detector sensitivity.

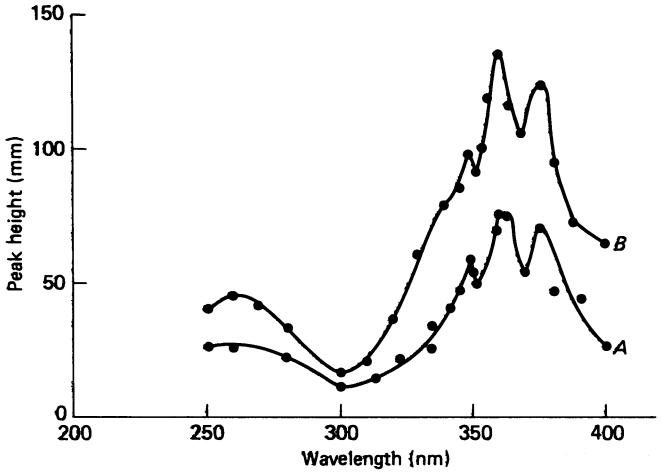

Fig. 6. U.v.-absorption spectrum of the 'glyoxylate 2,4-dinitrophenylhydrazone peak' after treatment of human body fluids in accordance with Scheme 1, compared with that of authentic glyoxylate 2,4-dinitrophenylhydrazone

The wavelength of the u.v.-absorbance detector was varied, and the peak height of the putative glyoxylate 2,4-dinitrophenylhydrazone peak (Fig. 5) is plotted as a function of wavelength. Spectrum *A*, ultrafiltrate of synovial fluid; spectrum *B*, authentic glyoxylate 2,4-dinitrophenylhydrazone.

chromatogram of a sample derived from rheumatoid serum. Allantoin was quantified by comparing the peak heights of glyoxylate dinitrophenylhydrazone with values from a standard curve obtained by treating a series of allantoin solutions (usually 2.5–60 μM) in the same way as samples (Scheme 1). Peak height was directly proportional to allantoin concentration up to at least 30 μM. The putative glyoxylate dinitrophenylhydrazone peak was identified by techniques similar to those described for uric acid (Figs. 2 and 3). For example, Fig. 6 shows that the absorption spectrum of the peak observed after formation of the dinitrophenylhydrazone derivatives and chromatography of synovial-fluid or serum ultrafiltrates was identical with that produced from an authentic sample of allantoin carried through the same procedures. It was also identical with that of an authentic sample of glyoxylate dinitrophenylhydrazone. Normal human serum contains some glyoxylate, but this appeared to be separated from allantoin during the first chromatographic stage, in that incubation of the column fraction derived from this first stage with dinitrophenylhydrazine without performing the double-hydrolysis procedure that degrades allantoin (Scheme 1) produced insignificant (< 1 μM) amounts of glyoxylate dinitrophenylhydrazone.

Table 1 shows that the allantoin content of serum from healthy human volunteers was in the range 14.1–25.4 μM. In serum from patients with active rheumatoid disease, values were significantly higher. Synovial fluid from rheumatoid patients also contained allantoin in very variable concentrations (7.2–26.3 μM). However, the allantoin content of either serum or synovial fluid of rheumatoid patients, expressed as a percentage of the uric acid concentration, was significantly raised in both fluids as compared with serum from normal subjects (Table 1).

DISCUSSION

In the present paper, we have described accurate and sensitive methods for the measurement of uric acid and allantoin in human body fluids, and drawn attention to the care necessary in making up standard solutions of uric acid. Serum uric acid concentration is not significantly changed in rheumatoid patients, but allantoin concentrations are significantly raised. The uric acid concentration in synovial fluid from the knee joints of rheumatoid patients is lower than in serum, although it must be stressed that only a small number of samples has been analysed. In both serum and synovial fluid, the allantoin/uric acid concentration ratio is raised in rheumatoid patients as compared with normal controls. Since allantoin can be generated by free-radical attack upon uric acid [1], these results are consistent with the proposal that increased free-radical reactions take place *in vivo* in human rheumatoid disease [16,22].

Of course, the observation of increased allantoin concentrations in rheumatoid body fluids does not actually prove that free-radical formation *in vivo* has been increased. Comparison of changes in allantoin concentrations with changes in other potential 'markers' of free-radical reactions *in vivo* [15,23] would be instructive.

We are grateful to the Arthritis and Rheumatism Council and the Wellcome Trust for financial support. B.H. is a Lister Institute Research Fellow. We thank Dr. David Blake for the provision of rheumatoid fluid samples.

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Received 15 September 1986/5 November 1986; accepted 20 January 1987