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EXCRETION OF HYDROGEN PEROXIDE IN HUMAN URINE

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The excretion of hydrogen peroxide in human urine has been demonstrated for the first time. This was accomplished by a new radioactive method developed on the basis of decarboxylation of alpha-ketoglutaric acid by H_2O_2. Urine samples were incubated with alpha ketoglutarate pulsed with iwts 1-^{14}C-analogue, and CO_2 formed by decarboxylation was determined by radioactivity measurements. Blanks were prepared by pre-incubation of the samples with catalase. Both male and female subjects were studied. On an average the concentration of H_2O_2 in urine was approximately 100 $\pm$ 60 μM (10^{-4} M). Peroxide excretion was found to be unexpectedly high and might thus be useful for clinical diagnosis and therapy in diseases purported to be related to oxidative stress.

KEY WORDS: Hydrogen peroxide, human urine, alpha-ketoglutaric acid

INTRODUCTION

The bulk of oxygen utilized by man and other animals is biochemically reduced to carbon dioxide and water. This reduction is fundamental to aerobic physiology. A small amount of oxygen, however, is also converted to superoxide[1] and hydrogen peroxide.[2] The latter are supposedly involved in the pathophysiology of several ageing and acute diseases.[1–6] However, despite a wide interest in the subject of oxygen toxicity, the topic has yet stayed out of the realm of general diagnosis. The levels of any of the active oxygen species remains unknown so far, either in blood or in urine, the two body fluids used universally for determining the metabolic and physiologic status of the subjects involved. The lack of such information may perhaps have been due to the presence of these entities in amounts supposedly defeating common laboratory analytical techniques. It may also have been due to their inherent instability and the nonspecificity with which they react with several reagents. A highly sensitive radioisotopic method of peroxide determination in aqueous and semi-aqueous media has recently been developed.[7] In this method, the peroxide is allowed to react with alpha-ketoglutaric acid mixed with its radioactive analogue (1—^{14}C-alpha-ketoglutaric acid) and the CO_2 produced by decarboxylation measured by liquid scintillation counting. The method is sensitive down to subnanomolar concentrations. The sensitivity of the method coupled with the wide interest in specific measurement of active oxygen species in body fluids prompted us to investigate if H_2O_2, one of the active species of oxygen might be excreted in human urine. Studies reported herein demonstrate, for the first time, that human urine indeed contains hydrogen peroxide in rather high concentrations. The results, therefore, open up the possibility of conducting further correlative studies on peroxide production and

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excretion under several physiological and pathological conditions relevant to oxygen utilization and its toxicity.

MATERIALS AND METHODS

All the chemicals used in this study are routinely available. Alpha-ketoglutaric acid (di-sodium salt) was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. [1-^{14}C]-alpha-ketoglutaric acid (di-sodium salt) was obtained from New England Nuclear Company, Boston, Massachusetts, U.S.A. Urine was obtained freshly from laboratory workers and diluted 1 × 20 in Tyrode buffer. The reaction was carried out in a home-made glass test tube (8.5 cm × 1.5 cm) with a side arm (2 cm × 0.5 cm) situated 2.5 cm from the bottom. Commercial hydrogen peroxide was standardized iodometrically as described before[7] and diluted to a final concentration of 10 μM using Tyrode buffer. An alpha-ketoglutarate reagent was prepared by adding 100 μl of stock [1-^{14}C]-alpha-ketoglutarate (disodium salt, specific activity 59.4 mCi/mmol) to 950 μl of a solution of its non-radioactive analogue in physiological saline. The final concentration of alpha-ketoglutarate in the reagent was 1.74 mM. The specific activity was maintained at approximately 1000–2000 dpm/nmol in different experiments. Before any addition of reagents in the reaction tube, the side arm was closed air-tight with a flexible rubber stopper. The main stopper of the tube was equipped with a plastic or stainless steel hanger ending in a small cup. The cup contained a piece of a fluted filter paper (5 cm × 0.5 cm), soaked with 200 μl of 1 M hyamine hydroxide in methanol to act as a CO_2 trap.

The suitability of the proposed method for determination of the urinary peroxide was verified by running recovery experiments. The recovery experiments were run using sets of five test tubes numbered 1 to 5. 100 μl of the standard H_2O_2 were added to the first four test tubes. 100 μl of the diluted urine samples were added to the last three test tubes (3 to 5). The contents of tubes 1 and 3 were incubated with 25 μl of catalase (6000 I.U. Sigma catalogue #4138) for 10 minutes, to serve as blanks. The final volume of the reaction mixture was made up to 0.4 ml by addition of Tyrode. The tubes were then stoppered with CO_2 traps and incubated at 37° for 30 minutes. Subsequently, the tubes were cooled to room temperature and 200 μl of a 20% (w/v) trichloroacetic acid solution injected through the stopper in the side tube. The tubes were then incubated for 45 minutes at 37°C with gentle shaking on a rotary platform. The tubes were then cooled to room temperature, and the CO_2 traps transferred to liquid scintillation vials containing 10 mL of a scintillation cocktail (Beckman, Ready Solve HP). 200 μl of glacial acetic acid was then added, the vials stoppered, and the contents mixed. The radioactivity in the mixture was then determined by scintillation counting in a Beckman instrument. The recovery of H_2O_2 added to the urine sample was then calculated as described in Table 1. As described therein, the recovery of H_2O_2 added to urine sample was quantitative. It was, therefore, decided to use the method for final determination of the peroxide in the urine samples obtained from the laboratory personnel. In experiments where more than one urine sample was examined for its peroxide content, it was not found necessary to run catalase-incubated blanks for each one of them. The blank values were similar for all of them. Also, the urine blanks were similar to the blanks obtained with pure H_2O_2 treated with catalase. However, at least one urine blank and one H_2O_2 blank were run in each group of experiments. H_2O_2 content in the urine sample was calculated by dividing with the

TABLE I

	Reaction mix	DPMs in CO_2 collected
1.	H_2O_2 + Catalase	143
2.	H_2O_2	1255
3.	True H_2O_2 Counts (2–1)	1112
4.	H_2O_2 + Urine + Catalase	150
5.	H_2O_2 + Urine	2191
6.	True H_2O_2 + Urine Count (5–4)	2041
7.	Urine + Catalase	150
8.	Urine	1001
9.	True Urine Counts (8–7)	851
10.	Counts of H_2O_2 recovered from Urine	2041 – 851 = 1190
		Counts Expected = 1112

Example of Recovery Experiment:
H_2O_2, H_2O_2 + urine and urine were incubated with alpha-ketoglutarate reagent and the CO_2 liberated was determined by liquid scintillation counting as described in the text. The blanks were prepared by incubating the appropriate samples with Catalase (6000 I U) before addition of alpha-ketoglutarate.

specific activity the net dpm (sample–blank) in the CO_2 trapped, and multiplying the result with the dilution factor. It could also be calculated by using the counts derived from the standard H_2O_2 as a reference. The two results were similar. However, an H_2O_2 standard was run with all experiments as a check for the accuracy of the procedure.

RESULTS

In initial experiments, urine samples as such and after treatment with catalase were incubated with alpha-ketoglutarate pulsed with the radiolabeled analogue and CO_2 determined. The CO_2 yield of the samples untreated with catalase was substantially greater than that of the samples treated with catalase. The difference represents the peroxide content. Subsequently, recovery experiments were conducted wherein a standard amount of H_2O_2 was added to a urine sample. The total radioactivity obtained from such a mixed sample was then compared with that obtained from an equivalent volume of unmixed urine, as well as with that derived from the peroxide processed alone. Table I describes an example of such an experiment. The results of several such experiments have been summarized in Table II. The recovery of peroxide added was observed to be quantitative. The observed difference between the CO_2 released by the samples which were not treated with catalase and the samples which were treated with catalase thus reflects the peroxide content unambiguously.

After ascertaining that recovery of H_2O_2 added in the urine samples was satisfactory, the procedure as described was used to determine the actual peroxide content of the urine samples obtained freshly from healthy individuals in the laboratory. The age of the persons varied between 20 and 35 years. Since the purpose of this study was merely to find out if H_2O_2 is excreted by human beings in their urine, in any significant amount, no other parameters such as total daily excretion and nutritional status were verified. One might assume that they would be fairly uniform in the group studied. The sample was collected between 10:00 and 11:00 am and used within 10–15 minutes after collection. The results of analyses have been described in Table III and Table IV.

TABLE II

Exp #	H_2O_2 Counts	H_2O_2 + Urine	Urine	H_2O_2 Counts Recovered	% Recovered
1	754	1464	774	690	92
2	754	1464	737	727	96
3	398	531	140	391	98
4	481	1594	1040	554	115
5	1061	1275	445	830	78
6	1061	1794	721	1073	101
7	1061	1105	138	967	91
8	1112	2041	851	1190	107
9	752	1242	360	739	98
10	752	1247	353	751	100
11	965	1270	352	918	95
12	965	1270	360	910	94
13	965	1302	564	738	76
					n = 13
					x = 95.5 ± 10.4

Recovery of H_2O_2 Added to Urine Samples:

100 μL of the standard H_2O_2 or 100 μL of the urine sample alone or mixed with 100 μL standard H_2O_2 were incubated with 100 μL of the alpha-1-^{14}C-ketoglutarate reagent in a total volume of 0.4 mL. CO_2 was collected as described in the text. The blanks consisted of the samples prepared as above and preincubated with 25 μL of catalase (6000 I.U.) prior to the addition of alpha-ketoglutarate. The counts represent the difference of the counts between samples without and with catalase treatment. 1 and 2 are representative duplicates.

TABLE III

Peroxide content of the urine samples from male subjects (20–30 years)

Sample Number	Peroxide level μM
1	76
2	110
3	199
4	126
5	249
6	103
7	78
8	64
9	63
10	56
11	46
	Mean ± S.D. = 106.4 ± 64.0

The average concentration in the urine of male subjects was 106 ± 65 μM. In the females it was 89 ± 54 μM. The chemistry of the urine samples was normal in terms of pH, glucose, blood, ketone bodies, and proteins as found by clinical urine testing tapes. (Ames Labstix, Mile Inc., Elkhart, IN 46515). Accuracy of the tapes was verified using aqueous solutions prepared freshly in the laboratory.

TABLE IV
Peroxide content of the urine samples from female subjects (25–35 years)

Sample Number	Peroxide level μM
1	137
2	119
3	139
4	170
5	87
6	55
7	37
8	28
9	26
	Mean $\pm$ S.D. = 88.6 $\pm$ 54.5

DISCUSSION

The primary objective of this investigation was to determine if there is any urinary excretion of hydrogen peroxide. No previous studies exist in this regard. Such a study would be useful, perhaps in correlating the onset and progress of certain ageing or acute diseases with oxidative stress. The lack of previous studies in this regard may be due to the commonly-held notion that H_2O_2 is present only in trace amounts, if at all. With this anticipation we explored the possibility of determining it with Varma's[7] radioisotopic method applicable for nanomolar quantities of the peroxide. When this method was applied, substantial quantities of H_2O_2 (of the order of 10^{-4} M) were found to be present in all the samples of urine tested. Therefore, since the fluid can be obtained relatively easily without any contamination with cellular debris containing enzymes, the determination of H_2O_2 may ultimately become helpful in assessing the oxidative status of a person. However, statistically-planned larger studies must be undertaken before such measurements can become clinically useful. The procedure described here is simple and can be used for such larger initial studies. The only expensive equipment is a liquid scintillation counter, available in most laboratories.

The presence of peroxide at rather high concentration in human urine as observed in this study led us to investigate the possibility of its determination also by the nonradioactive conventional techniques. Three such techniques were tried; Iodometric, Polarographic, and Enzyme coupled Spectrophotometric. These techniques are known to be sensitive, and were found to be so, at the levels of H_2O_2 observed herein, when present in simple aqueous solutions. However, none of these techniques could be used for urine analysis. No iodine was liberated when KI was added to urine samples, as indicated by the failure of a starch iodide test. The addition of an iodine solution (5 mg % in 2% [w/v] KI) to urine samples indicated that urine has substantial endogenous capacity to react with iodine. In subsequent experiments, polarographic measurements of oxygen liberated by treating urine with catalase gave less than 10% of the expected recovery. Apparently catalase, when added to urine, decomposes it through several peroxidatic reactions utilizing co-substrates other than H_2O_2. Attempts to measure H_2O_2 in urine by adding *ortho*-dianisidine in the presence of peroxidase also failed for similar reasons, although it was quite sensitive to a similar level of H_2O_2 in pure aqueous solution. The complexity of urine composition makes

it difficult to pin-point the various interfering reactions. The nonenzymatic decarboxylation of the alpha-ketoglutaric acid by the peroxide was not found to be affected by urinary constituents and could be monitored quantitatively. This is quite apparent from the high recoveries obtained in all the experiments. The use of catalase in blank experiments renders the method very specific. The biological mechanism contributing to the presence of peroxide in urine in such substantial amounts and the site of its origin remain as yet speculative. Studies in this direction are in progress. The purpose of this particular study was to demonstrate whether or not H_2O_2 is excreted by the human urinary system. That has been found to be true. The values are so substantial and measurable that they would be clinically useful.

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