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HYDROGEN PEROXIDE IN HUMAN BLOOD

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Blood and plasma of humans and rats were analyzed for hydrogen peroxide. The samples were analyzed after deproteinization with trichloroacetic acid, immediately after they were withdrawn from human volunteers or rats. A radio-isotopic technique based on peroxide-dependent decarboxylation of 1-^{14}C-alpha-ketoacids and consequent liberation of $^{14}CO_2$ was used. The results demonstrate the presence of micromolar levels of H_2O_2, both, in the plasma as well as in the whole blood. The values in the whole blood were substantially greater than the plasma. This was true for rats as well as humans. The presence of such significant quantities of H_2O_2 in the blood have been demonstrated for the first time. The investigation, therefore, opens a newer avenue of research on diseases purported to be related to the generation of oxygen radicals *in vivo*.

KEY WORDS: Blood, plasma, peroxide, oxidative stress

INTRODUCTION

The formation of active oxygen in the body has been suggested to participate in the genesis of several age-dependent and acute diseases.[1-7] The measurement of such species in the biological fluids, however, is difficult, primarily because of their low concentrations and transient existence. Chemically, the most stable active species is hydrogen peroxide. However, because of the presence of catalases, peroxidases, and many nonenzymatic but potent reducing agents such as ascorbate and glutathione, peroxide is also presumed to be present in very low amounts, at least in the blood. Recently, however, a simple and highly sensitive radio-isotopic technique of peroxide determination has been developed.[8] In this technique, the peroxide containing sample is allowed to react with 1-^{14}C-alpha-ketoglutarate and the $^{14}CO_2$ produced by peroxide-dependent decarboxylation is measured radio-isotopically.

We, therefore, felt it desirable to investigate the steady state existence of peroxide in blood, using this newly developed simple and specific technique. The results demonstrate that the steady state concentration of this active species of oxygen in the whole blood as well as in the plasma is indeed fairly measurable and not as low as generally thought.

MATERIALS AND METHODS

The blood sample was drawn from the subcubital vein in a 1 cc tuberculin syringe, and a measured amount transferred directly to a microfuge tube containing ice cold 5%

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trichloroacetic acid (TCA) solution in the ratio of 1:3.5 (v/v). A protein-free supernatant was obtained by centrifugation in an Eppendorf microfuge for one minute. A portion of the blood ($\approx$0.8 mL) was transferred to another microfuge tube containing 0.5 mg heparin and 1.0 mg of 3-amino-IH-1,2,4 triazole (AT) and plasma prepared by quick centrifugation. This was followed by its immediate deproteinization in TCA as described above. The deproteinized extracts of blood and plasma were neutralized with microliter volumes of ice cold 1.25 MNaOH. The time involved in sample preparation and the maintenance of ice cold conditions are considered to be of essence. The time involved between blood withdrawal and preparation of the deproteinized samples ready for analysis did not exceed 1.5 minutes for blood and 2.5 minutes for plasma. The stock peroxide reagent consisted of alpha-ketoglutarate (8.0 mM) mixed with trace amounts of its 1-^{14}C-analogue and $NaHCO_3$ (25 mM). The specific activity of the reagent was approximately 500 DPM's/nanomole.

The reactions were carried out in glass test tubes (7 cm $\times$ 1.5 cm) having a sidearm closed with a rubber stopper. The main stopper for the test tube was also rubber tight and equipped with a suspended 0.5 ml plastic cup containing a fluted filter paper ribbon (0.5 $\times$ 6 cm) soaked with 200 μL of 1 M methanolic hyamine hydroxide acting as a CO_2 trap.

50 to 100 microliters of the blood or the plasma extract were added to 100 microliters of the peroxide reagent contained in the above tubes. The tubes were then immediately stoppered with CO_2 traps and incubated at room temperature for 15 minutes. The contents were then acidified by injecting 200 μL of 20% TCA through the rubber stopper in the sidearm using a tuberculin syringe having a 28-gauge needle. The tubes were then transferred to an incubator that contained a rotary platform adjusted to 60 r.p.m. and maintained there for 60 minutes at 37°C. Subsequently they were cooled to room temperature, CO_2 traps transferred into a liquid scintillation cocktail (Beckman Ready-Solv) and radioactivity determined in a Beckman beta counter. The blanks were prepared for each sample by treating them with 25 μL of catalase (5000 IU, Sigma Chemical Co., C-10), prior to the addition of the peroxide reagent. The peroxide content was then calculated by comparing the amount of CO_2 derived from the samples and the standard H_2O_2 run simultaneously or using the specific activity data. The two match within the limits of experimental error ($< 10\%$). Standard H_2O_2 was prepared from commercially available 30% solution and standardized spectrophotometrically as described previously.[8]

Control Experiments with Catalase and Peroxidase

In order to rule out the artifactual generation of H_2O_2 during acidification, control experiments were conducted on blood samples that were treated with calalase or peroxidase before acidification. Simultaneous measurements were also made on the same blood samples without subjecting them to catalase or peroxidase treatment.

In these experiments, a 0.1 mL portion of the freshly drawn blood was transferred to 5% TCA as described earlier. Another 0.1 mL fraction was also added to 1 mL of 5 mM NaH_2PO_4 (pH 5.6) containing 3 mg of Sigma catalase (C-10) and incubated anaerobically for 30 to 45 minutes after which 0.1 mL of 58.8% TCA was added quickly to accomplish deproteinization. The protein-free extracts of the samples prepared as above were neutralized and processed for H_2O_2 determination as described above.

Control experiments were also conducted wherein the blood sample was incubated with peroxidase and dianisidine mixture prior to acidification. In these cases, 0.1 mL of blood samples were added to 1 mL of 5% TCA (control) and also to 1 mL of 5 mM phosphate buffer pH 7.5 containing 3 mg of Horse-Radish peroxidase (Sigma P-8125) and 0.2 mg of orthodianisidine HCl. The latter mixture was incubated at 37°C for 30 minutes and deproteinized by adding 100 uL of 58.8% TCA. The deproteinized extracts were then processed for H_2O_2 determination.

RESULTS AND DISCUSSION

At first, the experiments were designed to determine the reliability of the method in terms of the $^{14}CO_2$ recovered from the TCA deproteinized blood samples. These extracts following neutralization were processed for H_2O_2 determination as such, and after treatment with catalase. A simultaneous recovery experiment was also run. Results of such a representative experiment have been described in Table I. As is apparent, the radioactivity derived from blood extracts processed without treatment with catalase was much higher than the sample processed after treatment with catalase. This demonstrated the feasibility of the method. The recovery of H_2O_2 added to the deproteinized blood samples is also quantitative. It should be mentioned, however, that the blanks of the blood or plasma extracts were somewhat higher as compared to the pure H_2O_2 blanks. This indicates the presence of the traces of minor decarboxylating agents in the blood, in addition to H_2O_2. This is, however, not unexpected and running a catalase blank for each of the samples is, therefore, essential. The use of this enzyme in the blanks makes the method highly specific for peroxide.

Table II describes the peroxide levels prevalent in the blood and plasma of six normal male volunteers among the laboratory workers. No specific attention was

TABLE I
Recovery Experiment

Number	Reaction Mixture	DPM's in CO_2 Collected
1	H_2O_2 + catalase	132
2	H_2O_2	804
3	True H_2O_2 counts	672
4	H_2O_2 + sample + catalase	398
5	H_2O_2 + sample	1522
6	True H_2O_2 + sample counts (5–4)	1124
7	Sample + catalase	446
8	Sample	961
9	True sample counts (8–7)	515
10	Counts of H_2O_2 recovered (6–5)	609
	Counts Expected	672
	% Recovery	91%

Sample for analysis was prepared from the freshly drawn blood as described in the text. Deproteinized blood sample, H_2O_2, H_2O_2 + deproteinized blood sample, were incubated with alpha-ketoglutarate reagent and the CO_2 liberated was determined by liquid scintillation counting. The blanks were prepared by treating the appropriate sample with catalase (5000 I.U.) for 10 minutes before addition of the peroxide reagent. Similar recoveries were also obtained with deproteinized plasma samples.

TABLE II
Peroxide content of blood and plasma samples

Sample Number	Blood Peroxide Level (uM)	Plasma Peroxide Level (uM)	Blood/Plasma
1	288	27	10
2	114	13	11
3	136	57	42
4	577	18	3
5	178	38	21
6	436	53	12
Mean	288 ± 185	34 ± 18	16 ± 14

Blood samples were drawn and processed freshly as described in the text. Each estimation was run in duplicate. The variation between the duplicates was less than 10%.

given to dietary intake – which is considered fairly uniform. The ages of the persons were between 30–35 years. As is apparent, hydrogen peroxide was found to be present in all the samples assayed. Its concentration in the whole blood ranged from 114 to 577 μM. The values in the plasma ranged from 13 to 57 μM. These observations thus clearly indicate that the peroxide content of the whole blood as well as of plasma is highly significant, and it can be determined with relative ease using this simple technique. It is noticeable, however, that the concentration in the plasma is substantially lower than that in the whole blood suggesting that red or white blood cells are rather richer in this regard.

The presence of such measureable amounts of peroxide in the blood may look intriguing at the first glance because of the presence of catalases, peroxidases and other reducing substances. Since the samples of analyses were prepared within minutes, the amounts reflect in all probability the steady state range. The source of such high peroxide content is difficult to explain at present. It can be speculated, however, to be derived, among other reactions, from the well known hemoglobin oxidation reactions.[9]

$$\text{hemeFe}^{2+} + O_2 \rightarrow \text{hemeFe}^{2+} \text{----} O_2 \rightarrow \text{hemeFe}^{3+} + O_2^{\cdot -}$$

$$\text{Hb} + O_2 \rightarrow \text{Hb} \ldots\ldots O_2 \rightarrow \text{metHb} + O_2^{\cdot -}$$

The superoxide produced in the above reactions will be dismutated to H_2O_2 and O_2.

TABLE III
Peroxide content of blood and plasma of normal Sprague-Dawley rats

Sample Number	Blood (μM)	Plasma (μM)	Blood/Plasma
1	220	–	–
2	218	–	–
3	430	–	–
4	445	26	17
5	478	40	12
6	421	68	6.2
Mean	366 ± 116	45 ± 21	12 ± 5

Blood was drawn by cardiac puncture and processed as described in the text for human blood. Each sample was analyzed in duplicate. The variation between the duplicates was very minimum (< 5%). No plasma was analyzed in the first three samples.

TABLE IV
Effect of catalase pretreatment on blood peroxide level

Sample	Experiment Number 1	2	3	4
A. Control	1199	1075	1188	1431
B. Experimental	273	190	264	335
% H_2O_2 decomposed by catalase treatment	77	82	78	77
Mean % of decomposition	78 ± 2.4			
Mean % residual H_2O_2	22 ± 2.4			

The numbers in the table represent the DPMs corresponding to the H_2O_2 levels. 0.1 mL fractions of rat blood drawn freshly by cardiac puncture were introduced in 1 mL of 5% TCA (control), as well as in 1 mL of deaerated 5 mM NaH_2PO_4, pH 5.6 containing 3 mg of catalase (exp). The samples collected into the latter mixture were incubated anaerobically for 30–45 minutes. 100 uL of 58.8% TCA was added to deproteinize these samples. H_2O_2 was determined in the supernatants as described in the text. The anaerobicity was maintained by incubation under vacuum and continuous flushing in nitrogen.

The present results, therefore, are in conformity with the existence of the above reactions in red blood cells. In our own control experiments, we observed that the ability of catalase to remove blood peroxide is only marginal unless the conditions are made anaerobic. We also did not find any inhibition of catalase by hemoglobin. These observations are in line with the concept that the endogenous level of H_2O_2 represents the balance of the amount being formed and the amount being decomposed. The amount decomposed seems to be replaced effectively by the amount produced. It is, therefore, not essential to presume that red blood cell catalase leaves the cell devoid of peroxide – especially because of its high Km (25 mM). A dynamic equilibrium appears quite feasible.

The authenticity of the findings is proven also from control experiments wherein blood was processed for peroxide determination after treatment with catalase as well as peroxidase. As would be apparent by reference to Tables IV, anerobic treatment of samples with catalase lowered the peroxide level by approximately 80% in comparison to the controls where blood was processed directly for deproteinization,

TABLE V
Effect of peroxidase/dianisidine pretreatment on blood hydrogen peroxide level

Sample	Experiment Number 1	2	3	4*
A. Control	2319	2028	1217	1297
B. A treated with peroxidase/ dianisidine	42	2	116	91
% Decomposition of H_2O_2 by peroxidase treatment	98	100	95	93

The numbers in the Table represent the DPMs corresponding to H_2O_2 levels. 0.2 mL of rat blood freshly drawn by cardiac puncture was added immediately to either (A) 1.0 mL of 5% TCA (control) or (B) 1 mL of 5 mM phosphate buffer (pH 7.5) containing 2 mg of horse radish peroxidase and 0.1 mL of O-dianisidine (experimental). The total volume was adjusted to 1.4 mL with dH_2O. The experimental tubes were then incubated at 37° for 30 minutes, following which 0.1 mL of 58.8% TCA was added. A clear supernatant was obtained from the controls and experimentals by centrifugation in a microfuge. 0.5 mL of the supernatant was neutralized and processed for H_2O_2 determination as described in the text. *Only 0.1 mL of blood was used.

neutralization and the assay without catalase pretreatment. In blood samples treated with peroxidase (Table V) little peroxide could be detected.

This report emphasizes the presence of peroxide more in the blood cells than in the plasma. No previous report exists on the whole blood. Very low levels of peroxide, however, have been reported in the plasma.[10,11] The low plasma levels can be ascribed to the destructive effects of the techniques followed, such as storage of blood before plasma separation, long periods of centrifugation for plasma separation, exhaustive extraction of the separated plasma with noxious solvents, such as methanol and hexane, forcing the extracts through very fine bore capillary columns by high pressure solvent infusion at high pH 10, etc. Our procedure circumvented all these destructive steps. Thus, Varma's method appears to be potentially useful for further physiological studies. In previous studies we have seen the usefulness of this method in the determination of hydrogen peroxide in urine[12] as well as in aqueous humor.[13] Confirmatory evidence on the presence of peroxide in human blood has also been obtained from the analysis of blood samples derived from rats maintained under highly controlled conditions of the laboratory. As will be seen in Table III, the peroxide content in rat blood is similar to that in the human blood. A greater congruity among the levels in the rats' blood, however, appears consistent with the uniformity in weights, genetics (inbreeding) and general habitat. All animals weighed 250 $\pm$ 10 g, and are brother/sister offsprings through innumerable generations (Sprague-Dawley). It should be emphasized, also, that these studies were meant only to find out if the peroxide is present in the blood to any significant level, and if so, can it be measured reproducibly, and were not meant to establish any normal physiological levels. Such studies entailing a larger number of subjects and more rigorous control of age, sex, genetic variations, and dietary consumption of reducing nutrients are in progress.

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