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RADIO-ISOTOPIC DETERMINATION OF SUBNANOMOLAR AMOUNTS OF PEROXIDE

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A new radio-isotopic method for determination of peroxide in subnanomolar concentrations has been described. The method is based on the reactivity of peroxide with α-Ketoglutaric acid containing [I-^{14}C]-α-Ketoglutaric acid, and measurement of the resulting $^{14}CO_2$ by radioactivity. The recovery of standard peroxide, alone or mixed with some biologically derived samples, following this technique was found to be 97 ± 2.7%. The method may therefore be useful for determination of peroxides in active biological samples.

KEY WORDS: Peroxide, a-ketoglutaric acid, superoxide, pyruvate

INTRODUCTION

The formation of superoxide and peroxides is a ubiquitous phenomenon of aerobic biology. Both are known to be generated in bacteria, plants, and animals.[1] The superoxide rapidly dismutates to the relatively more stable peroxide. However, the quantitation of the latter at levels encountered in most biological samples is still a difficult problem. This report deals with the development of a simple one step radio-isotopic method of high sensitivity, based on the following reaction:

$$R\text{-}CO\text{-}COOH + H_2O_2 \rightarrow RCOOH + H_2O + CO_2$$

This reaction using pyruvate as the keto acid has been described earlier.[2] The significance of this reaction in prevention against oxygen toxicity to bacteria and animal tissues has been partly realized.[3,4] The present studies were undertaken to find out if the above reaction can also be used for the measurement of peroxide. This was achieved by the use of an [I-^{14}C]-α-Ketoacid in the above reaction and subsequent measurement of the radioactivity in the CO_2 formed. [I-^{14}C]-α-Ketoglutaric acid was allowed to react with nanomolar amounts of the peroxide in a physiological buffer, and the correlation between the CO_2 formed and the peroxide content of the reaction mixture determined. A stoichiometric relation was found to exist. It is therefore hoped that this method may have the potential of being used for quantitation of peroxide at subnanomolar concentrations.

MATERIALS

All the chemicals used in this study are routinely available. [I-^{14}C]-α-Ketoglutaric acid was obtained from Amersham International. No special apparatus other than an incubator and a liquid scintillation counter was required. The reaction between the peroxide and the Ketoacid was carried out in a glass test tube 8.5 cm × 1.8 cm, containing a side arm 2 cm × 0.5 cm. The side arm was situated at a height of about

2.5 cm from the bottom of the tube. The dimensions of the tube and its side arm are variable. The tubes were home made. Rubber stoppers were used for the side arm as well as the main opening of the tube. The CO_2 trap consisted of approx. 6 cm × 0.5 cm filter paper, fluted and packed in a 0.5 ml plastic vial and suspended through a stainless steel loop forced through the main stopper. 200 μl of 1 M hyamine hydroxide in methanol was soaked into the filter paper.

METHOD

Commercial hydrogen peroxide was first diluted in Tyrode buffer to approximately 10 mM. Its actual concentration was then determined spectrophotometrically as well as idiometrically.[4]

Spectrophotometric method:

Four test tubes were labelled and reagents added as follows:
A: Buffer 3.0 ml
B: 2.9 ml H_2O_2 + 0.1 ml Buffer
C: Buffer 2.9 ml + 0.1 ml Catalase
D: 2.9 ml H_2O_2 + 0.1 ml Catalase.

Catalase was prepared by dissolving 10 mg of bovine liver catalase (Sigma C-10) in 20 ml Tyrode buffer. The contents of the tubes were mixed and left at room temperature for 15 minutes, following which the tube D was centrifuged (1200 × g) at 4°C for 5 minutes to get rid of the bubbles produced during the reaction. The contents were then transferred to matched quartz cuvettes and absorbance read at 240 nm. Δ OD was determined and H_2O_2 Concentration calculated as follows:

$$\Delta \text{ OD} = (\text{B-A}) - (\text{D-C})$$

$$[H_2O_2] = \frac{\Delta \text{ OD} \times 1000 \times 3}{43.6 \times 2.9} \text{ mM},$$

where 43.6 is the molar extinction coefficient of H_2O_2.

Idiometric standardization was carried out as follows:

0.5 ml portions of the diluted peroxide were mixed with 0.5 ml of 0.5 M Na-acetate buffer pH 3.5, 0.1 ml of freshly prepared 2% KI, 0.1 ml of ammonium molybdate 20 mg %, and 0.2 ml of 1% starch in saturated NaCl. Sodium thiosulphate (0.5 mM) was added from a digital burette, sensitive up to 0.01 ml, until the discharge of the blue color. The time course of the reaction under conditions stipulated to be used for radioactive measurements was also determined idiometrically using the above procedure.

Spectrophotometric measurement of the time course under the reaction conditions chosen for radioactive measurements was rather difficult because of the high absorbance of α-Ketoglutarate at 240 nm at the concentrations desirable. However, the validity of the reaction could be ascertained by using a low concentration of α-Ketoglutarate and a high concentration of peroxide and monitoring OD_{240}. This could be

accomplished by mixing 2 ml of 20 mM H_2O_2 and 0.2 ml of 25 mM α-Ketoglutarate, thereby maintaining the levels under measurable limits.

Radioactive Determination of Peroxide

H_2O_2 standardized as described above was diluted in Tyrode to the extent of 1 nanomole/100 μl. 50 to 200 μl of this solution was introduced into the bottom of the test tubes mentioned before. The volumes in all the tubes were then made up to 200 μl by adding appropriate volumes of Tyrode. No additional Tyrode had to be added in the tube containing 200 μl of the sample. The blank tube contained 200 μl of Tyrode alone. Additional blanks were prepared by adding 10 μl of catalase (1 mg/2 ml Tyrode) or sodium pyruvate (0.1 M) to the peroxide-containing tubes and incubating them for 10 minutes before further processing. 100 μl of a 2.0 mM solution of α-Ketoglutaric acid prepared in Tyrode and pulsed with the radioactive analogue ([1-^{14}C]-α-Ketoglutaric acid ≈ 0.01 μCi/100 μl) was then added to the individual tubes. The side arms of the tubes were stoppered before any additions were made. As soon as α-Ketoglutarate was added to the tube, it was closed with the CO_2-trap-containing stopper. The contents of the tubes were then incubated for 45 to 60 minutes at 37°C. After this, they were cooled to room temperature in about 5 to 10 minutes. 100 μl of a 20% solution of trichloroacetic acid was then introduced through the side arm using a 1 cc tuberculin syringe and a 27 gauge needle. The withdrawal of the needle was as gentle as possible. While introducing the reactant in the tubes, care was taken to introduce them directly into the bottom, without touching the sides. The usual Eppendorf automatic pipetters with long ends were found very useful in this regard. After introduction of the acid, the tubes were reincubated for about an hour at 37°C to facilitate the transfer of CO_2 from the reaction mixture to the CO_2 trap. The CO_2 traps were then taken out and transferred directly to a vial containing 10 ml of the liquid scintillation mixture. 5 ml of absolute methanol was then added, the vials capped, contents mixed and radioactivity determined in a Beckman Scintillation Counter.

RESULTS

Decarboxylation of α-keto acids by peroxides is a known reaction[2]. Detailed studies, however, have so far been done only with pyruvic acid. In the present context of measuring the radioactivity of the CO_2 produced by decarboxylation, the use of pyruvate was not considered appropriate, since the acid itself is sufficiently volatile. α-Ketoglutaric acid was therefore the compound of choice. It is solid at ordinary temperature and has high melting and boiling points. Information on the reactivity of this acid with peroxide is however not yet available in literature. It was therefore necessary to ascertain the validity of the proposed reaction and determine the time frame of its completion, before utilizing it for the intended purpose. Figure 1 describes the concentration of H_2O_2 in terms of 0.5 mM thiosulphate required to react with the iodine liberated from the iodide-peroxide reaction.

$$2I^- + 2H^+ + H_2O_2 \rightarrow 2\,H_2O + I_2$$

$$2\,Na_2S_2O_3 + I_2 \rightarrow 2\,NaI + Na_2S_4O_6$$

The thiosulphate values plotted as the ordinate thus directly represent the concen-

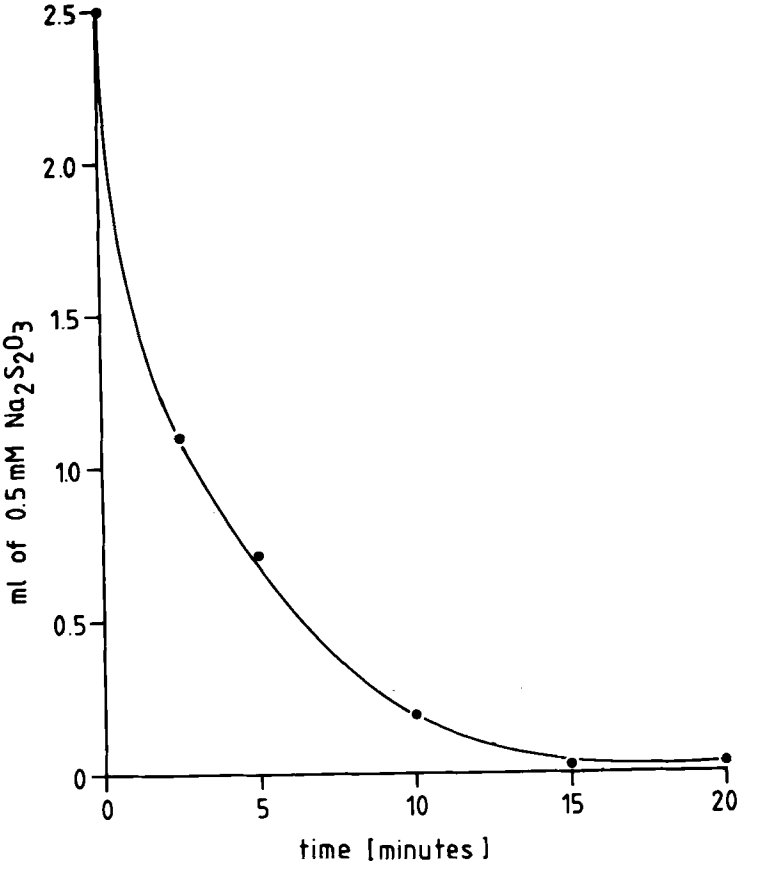

FIGURE 1 Reaction of H_2O_2 with α-Ketoglutaric acid: 1 ml of 12.5 mM H_2O_2 was mixed with 9 ml of 10 mM α-Ketoglutaric acid. 0.5 ml portions of this mixture were taken out at indicated intervals of time, mixed quickly with 0.5 ml of Na-acetate Buffer pH 3.5, 0.1 ml of 2% KI, 0.1 ml of 0.02% ammonium molybdate and 0.1 ml of 1% starch prepared in saturated NaCl. Thiosulphate was added until the discharge of the blue color. The zero value was obtained by titration of peroxide diluted without α-Ketoglutarate. No difference in the results was obtained if the pH was changed from 7.5 to 8.5. Tyrode could be substituted for phosphate buffer.

tration of H_2O_2 in the reaction mixture (1 ml thiosulphate = 0.25 micromole H_2O_2). Initially, at zero time, the peroxide to keto acid concentration ratio was ≈ 1:10. As shown in the graph the concentration decreases as a function of time. Thus, although the reaction between the ketoacid and the peroxide is rather slow, the reaction is completed within reasonable time for analytical purposes, the pseudo first order rate constant being $\approx 4.2 \times 10^{-3}$ sec^{-1} (Figure 2). Therefore the experiment does provide validity to the reaction proposed to be used. That the disappearance of peroxide takes place at the expense of α-ketoglutarate was apparent from the spectrophotometric measurements described in Figure 3. At zero time the concentration of H_2O_2 was 18.0 mM. α-Ketoglutarate concentration was 2.27 mM. By about 8 minutes the H_2O_2 concentration decreased to 15.71 mM, the decrease being equal to the amount of

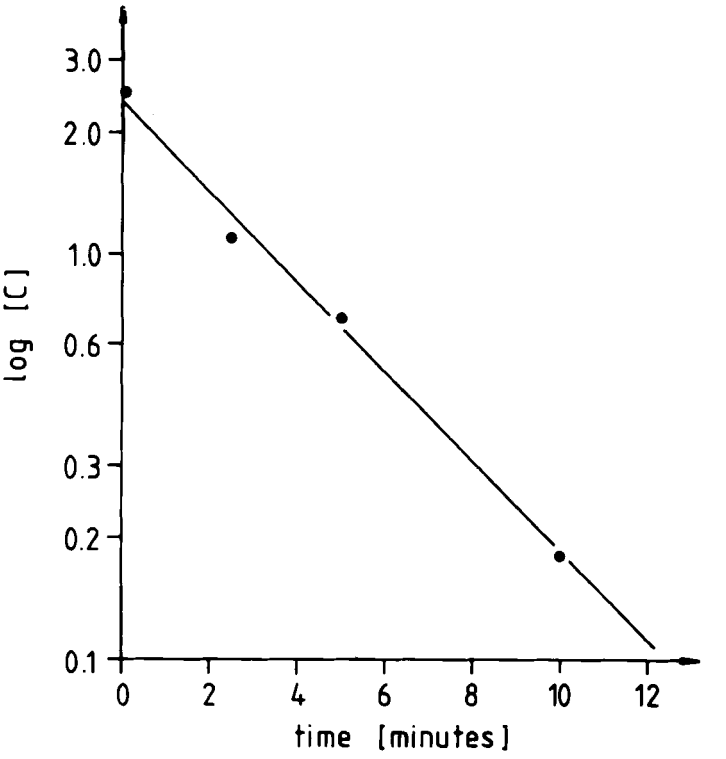

FIGURE 2 Kinetic analysis of the data in figure 1: Log C values are derived from the thiosulphate values directly. The data fit approximately to that of the pseudo first order rate equation.

TABLE 1
Recovery of H_2O_2 as CO_2

Added in reaction Mixture (nanomoles)	CO_2 collected (nanomoles)	Recovery %
0.5	0.51 ± 0.03	101
1.0	0.94 ± 0.04	94
1.5	1.48 ± 0.008	97
2.0	1.90 ± 0.14	95
Mean recovery (+ SD)		97 ± 2.7

H_2O_2 standardized idiometrically and diluted such that 100 μl contained 1 nanomole was used. Volumes of H_2O_2 solution ranging from 50 to 200 μl were added in Tyrode buffer, to a total volume of 200 μl. Initial dilution of H_2O_2 was also carried on in Tyrode buffer. Thus when 200 μl of the peroxide solution was used, no additional buffer was required. Peroxide content was determined as described in the text. Specific activity of α-ketoglutarate ranged from 600 to 2400 dpm in different experiments. The results are expressed as mean + S.D. of four experiments, conducted on different days.

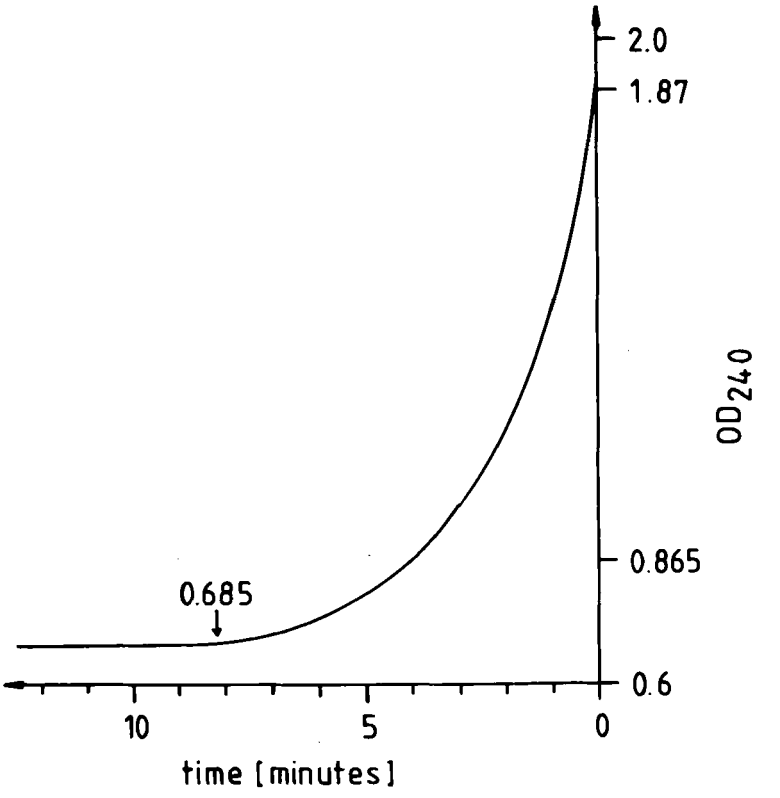

FIGURE 3 Spectrophotometric assessment of the reactivity of α-Ketoglutaric acid with hydrogen peroxide: 2 ml of 20 mM H_2O_2 was introduced into the cuvette and OD_{240} measured. 0.2 ml of 25 Mm α-ketoglutarate was then added and the rise in OD noted immediately. This rise represented absorption of α-Ketoglutaric acid. The reaction was then followed till it ceased. The decrease in OD lower than 0.786 represents H_2O_2 that reacted with the α-ketoacid. The decrease in OD from 1.87 to 0.786 represents complete disappearance of α-Ketoglutaric acid. Initial H_2O_2 = 19.6 mM, OD_{240} = 0.856. On dilution with α-KG OD of H_2O_2 = 2/2.20 × 0.856 = 0.780 = 18.00 mM; Final OD = 0.685 = 15.71 mM; H_2O_2 consumed = 18.00 − 15.71 = 2.29 mM. α-ketoglutarate added = 0.2/2.2 × 25 = 2.27 mM. [H_2O_2 disappeared]/[ketoacid disappeared] = 2.29/2.27 = 1.01 mM. The reaction of H_2O_2 with the ketoacid is therefore 1:1.

α-Ketoglutarate added. The results of the spectrophotometric measurements thus agree with those of the idiometric measurements. The two experiments indicated, in addition, that the time for the reaction to reach near completion seldom exceeds an hour, the time selected for experiments in this investigation. The results of radio-isotopic experiments, demonstrating the stoichiometry between the CO_2 formed in the reaction and the peroxide content, have been sumarized in Figure 4. The linearity between the CO_2 formed, as indicated by the radioactivity of the CO_2 collected (DPM), and the peroxide amount used in the reaction mixture is quite striking. Measurement of CO_2 radioactivity thus provides a convenient means of estimating peroxide in sub-nanomolar quantities.

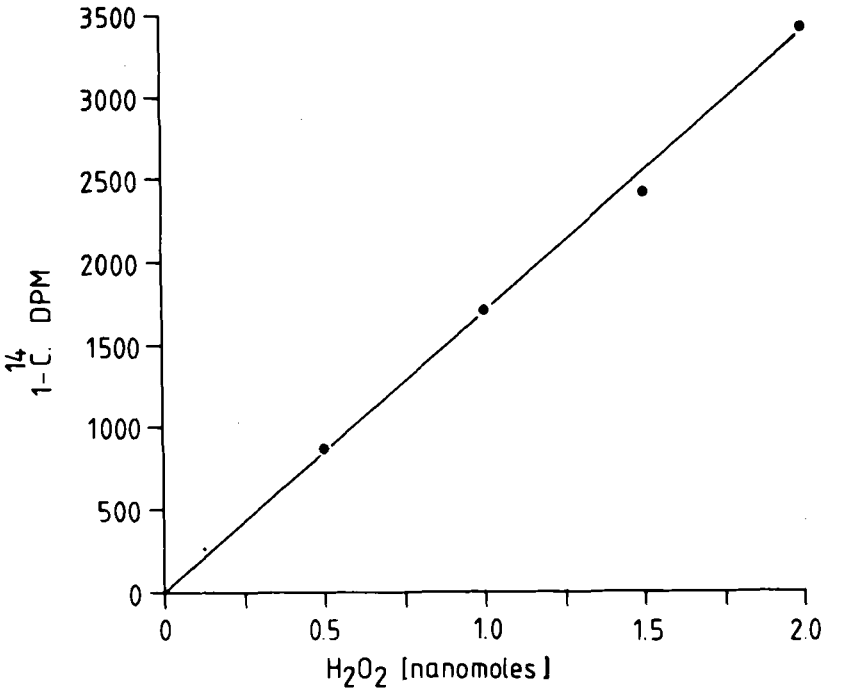

FIGURE 4 Relationship between peroxide content and the amount of carbon dioxide liberated: the procedure has been described in the text. The specific activity of α-ketoglutaric acid in the reaction mixture was 1926/nanomole. Incubation Time was 45 minutes.

Such measurements for peroxide have not been reported earlier. When radioactivity data was transformed into concentration data, using the specific acitivity of α-Ketoglutaric acid, the amount of carbon dioxide liberated was found to be quantitatively related to the peroxide content. Thus the estimation of peroxide in an unknown sample can be done by reference to a standard curve generated from known

TABLE II
Estimation of Peroxide in Olive Oil

Reactants	DPM (14CO2)	(e − b)
A. H_2O_2 + Catalase (b)	926	
B. A − Catalase (e)	10436	9510
C. olive oil + H_2O_2 + Catalase (b)	911	
D. C − catalase (e)	13704	12793
E. olive oil + Catalase (b)	969	
F. E − Catalase (e)	4950	3981
D − F = 12793 − 3981 = 8812		
Recovery of H_2O_2 from oil = 8812/9510 × 100 = 93%		

100 μl of oil samples with or without H_2O_2 and with or without catalase were mixed with 200 μl of Tyrode and 100 μl of α-Ketoglutaric acid. CO_2 was collected and radioactivity determined as described in the text. A and B represent control experiments with H_2O_2 alone.

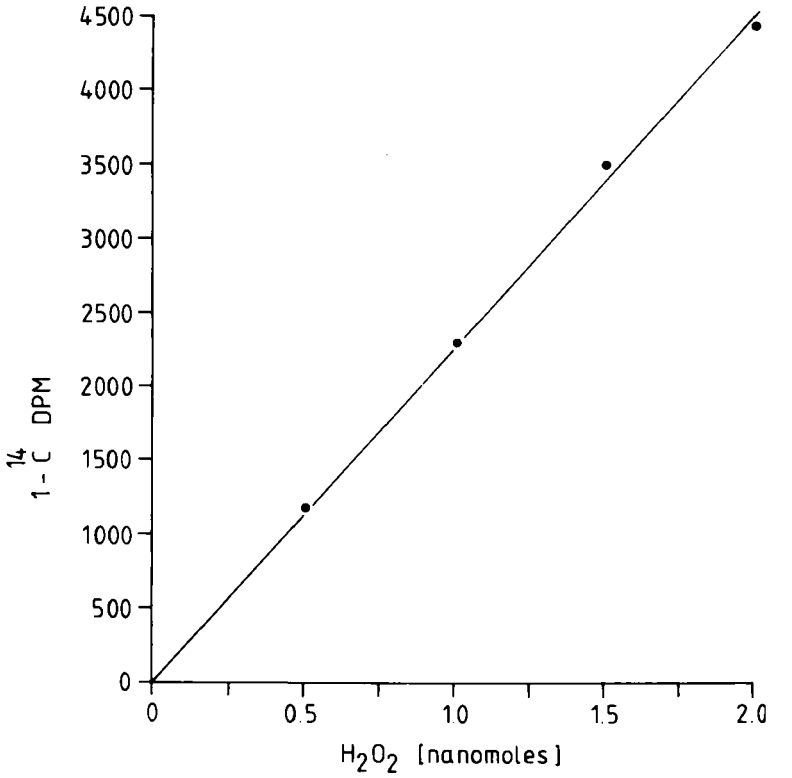

FIGURE 5 Relationship between peroxide and CO_2 formed: Evaluation of pyruvate as blank reagent: Experimental procedure has been described in the text, except that at each point in the graph a blank experiment was run, after the sample was treated with 10 μl of 0.1 M sodium pyruvate for 10 minutes. The CO_2 recovered from this sample was subtracted from the CO_2 content recovered from the experiment run without the pyruvate. Specific activity = 2500 DPM/nanomole.

amounts of peroxide as well as from the specific activity of the keto acid used. The two results must match if the recoveries are good. The recovery data have been summarized in Table I. It was again found to be quantitative within the limits of experimental error. As low as 500 picomoles of peroxide could be estimated by the technique described.

TABLE III
Peroxide content of olive oil

Sample No	Concentration
1	28.5
2	26.9
3	26.3
4	24.0
	26.5 ± 1.6

Two samples of olive oil (Aquila-GmbH&Co., Hamburg, Germany) were procured and analyzed twice at an interval of 10 days. While in the laboratory, they were stored in the refrigerator.

TABLE IV
Peroxide content in milk samples

Sample No	Concentration
	(μM)
1	18.2
2	27.0
3	26.0
4	14.5
5	14.0
6	8.0
7	13.4
8	7.0
Mean	16.01 $\pm$ 7

10 ml of milk samples were mixed with 10 mg each of 3-amino-1, 2, 4-triazole (AT) and iodoacetic acid (IA). The milk samples were selected randomly from the stores. 100 μl of the AT and IA treated samples or H_2O_2 were mixed with 200 μl of Tyrode buffer and 100 μl of α-ketoglutaric acid pulsed with the radioactive analogue. CO_2 was collected, radioactivity determined, and peroxide content calculated as described in the text.

Subsequent studies were undertaken to rule out the effect on non specific decarboxylation of α-Ketoglutaric acid. In these experiments the procedure described above was conducted on peroxide samples as such, as well as after their pretreatment with Catalase or pyruvate (blanks) as described previously. The CO_2 content attributable to peroxide was obtained by the difference in the DPMS. Use of pyruvate in the blank experiments was considered desirable in view of the unsuitability of catalase that may arise in samples containing metabolic inhibitors or specific inhibitors of catalase. The relationship between the CO_2 liberated by peroxide and α-Ketoglutarate interaction, after subtracting the blank value, obtained in presence of pyruvate, has been described in Figure 5. Again, the relationship was linear and stoichiometric. Conducting such blank experiments are desirable in biological as well as in non-biological samples, since samples do contain small amounts of nonperoxide decarboxylating activity. In addition, the keto acids themselves contain traces of lower molecular weight substances to contribute to the blank. If one gets unusually high blanks, the α-ketoglutaric acid is not of good quality. Studies on the use of this method for determination of peroxide in inactive and active biological samples are in progress. Examples of the use of this method for inactive samples such as olive oil and milk have been given here just as preliminary results. As can be seen from an exploratory experiment summarized in Table II, using catalase in the blank experiments, the peroxide content of the oil could be satisfactorily determined. This was very apparent from the experiments wherein H_2O_2 was used as an internal standard. Its additive value to the oil sample matched with the value when it was used alone. While there was no attempt to ascertain the extent of peroxidation in oil, it was interesting to find that the determination could be accomplished using this method. Some results of oil analysis are described in Table III; the average value being 26.5 μM. The peroxide in the oil perhaps represents only lipid peroxide and not hydrogen peroxide. The suitability of catalase in the blank experiments was thus surprising. Another biological sample tested was milk (Table IV). The mean peroxide concentration in this case was 16 $\pm$ 7 μM. The value in such biological samples would undoubtedly vary with variation in the source, nutritional status, the environment

and the storage conditions etc. The ultimate objective is to determine the peroxide content of live samples under steady state. The present experiments served merely to prove the principals of the technique proposed and its potential applications.

DISCUSSION

The main objective of this investigation was to develop a relatively simple radioisotopic method for peroxide determination. No such method exists at the present time. Since this oxygen metabolite is usually present in minute qualities in biological materials or under physiological steady state, development of a simple method as the one described in this communication appears useful. For example, it might be useful in studying the mechanism of oxidative stress under organ/tissue culture conditions or under pathological conditions *in vivo*. Additionally it should be helpful in determining minute amounts of peroxides in the early stages of oxidative degradation of certain nutrients. In the technique described no extractive procedure is involved. Thus peroxide degradation that accompanies homogenization and filtration procedures is completely avoided. Treatment of samples with metabolic and catalase inhibitors makes the technique more useful for biological application.

Theoretically most α-Ketoacids with -CO-^{14}COOH can be used, but nonvolatile acids should be preferred. Use of catalase in blank experiments makes the method very specific. In case of samples required to be treated with metabolic and catalase inhibitors, as may happen while conducting in vivo studies, blank experiments can be performed with pyruvate which decomposes peroxide effectively.

In this study peroxide has been satisfactorily measured up to 500 picomoles. The sensitivity, however, can be enhanced further by increasing the specific activity of α-Ketoglutarate. The method may therefore prove useful for peroxide determination at even lower levels if required. The method as described, however, seems to cover the peroxide level prevalent under most physiological conditions and can be carried out with relative simplicity and ease.

Since α-ketoglutarates may react with several hydroperoxides, the method may perhaps have a wider application than originally thought. Whether the peroxide measured is hydrogen peroxide or other hydroperoxides will depend on the history of the sample. In the case of aqueous samples the predominant peroxide will be the hydrogen peroxide. Likewise in the case of a lipid sample, the method will determine chiefly the lipid peroxide content. In samples of mixed nature, the method will be valuable for over all peroxide assesment. Additional techniques will thus have to be used to determine the contribution of individual peroxides to the total peroxide level in samples of mixed nature. The total peroxide alone however is a useful physiological index.

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