Gasometric Determination of Hydrogen Peroxide. (17322)

J. Bennett Clark, Orville Wyss, Felix Haas, and Wilson S. Stone
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From the Laboratories of Bacteriology and Genetics, The University of Texas, Austin, Texas.

We[1] have reported the determination of small amounts of peroxide in bacterial culture media by measurement of the oxygen evolution in a Warburg flask when a catalase preparation is added to the peroxide-containing substrate. These determinations were made because the mutagenic properties of irradiated broth have been linked with its peroxide concentration.[2,3] Further work on methods of peroxide determination has shown that the generally accepted overall equation for the breakdown of hydrogen peroxide by the action of catalase,

$$2H_2O_2 \longrightarrow 2H_2O + O_2,$$

does not express the quantitative relationships that occur in the reaction as we employed it. When excess catalase was used to give the rapid completion of the reaction desired for quantitative estimation, the resulting gas output always exceeded the theoretical computed from the above equation.

Methods. Known concentrations of hydrogen peroxide were made by dilution of an 82% stock solution, which was checked by iodometric titration, acid permanganate titration, and the colorimetric method of Bonét-Maury.[4] This colorimetric method utilizes a titanium sulfate reagent which gives a yellow color in the presence of H_2O_2. A standard curve was prepared by using known concentrations of peroxide in both aqueous and amino acid substrates, and duplicate tubes were found to always agree within ± 0.1%. The catalase preparations used were catalase "Sarret" (furnished by Vitazyme Laboratories) and a blood preparation extracted according to the method of Sevag.[5] Oxygen evolution was measured in the Warburg respirometer or, when large concentrations of peroxide were used, in a gas burette. The substrate was either water or the amino acid components of the standard synthetic medium for *Micrococcus pyogenes* prepared at 10 times the concentration used for growing the organisms.

Experimental. Aliquots of the amino acid solution were irradiated by a quartz ultraviolet light at various temperatures for 20 and 40 minutes and the resulting peroxide concentrations determined by the colorimetric method of Bonét-Maury and by the catalase gasometric method. Table I shows the discrepancies between these two methods. Since the colorimetric method is based on a standard curve made with known concentrations of peroxide and consistently gave accurate results when employed on known samples, it would appear that the gasometric results were in error.

As a further check of the assumed inaccuracy of the gasometric method, known concentrations of H_2O_2 were added to water or to the amino acid substrate, and the peroxide concentration indicated by oxygen evolution was calculated. Table II records some typical results.

As a check on experimental methods, known

TABLE I.
H_2O_2 Content of Irradiated Amino Acid Solution.

Irradiation		ppm H_2O_2	
Temp.	Time, min.	Colorimetric	Gasometric
2.5°	20	4.5	6.8
2.5°	40	7.2	16.3
23°	20	9.0	18.1
23°	40	16.0	31.4
40°	20	9.5	19.8
40°	40	14.2	26.2
58°	20	9.2	19.8

1 Wyss, O., Clark, J. B., Haas, F., and Stone, W. S., *J. Bact.*, 1948, **56**, 51.

2 Stone, W. S., Wyss, O., and Haas, F., *Proc. Nat. Acad. Sci. U.S.*, 1947, **33**, 59.

3 Wyss, O., Stone, W. S., and Clark, J. B., *J. Bact.*, 1947, **54**, 767.

4 Bonét-Maury, P., *Compt. rend.*, 1944, **218**, 117.

5 Sevag, M. G., and Mauvèg, L., *Biochem. Z.*, 1936, **288**, 41.

TABLE II.
Recovery of Hydrogen Peroxide by Gasometric Method.

Substrate	ppm H_2O_2 Added	ppm H_2O_2 by O_2 evolution
Water	8.1	17.6
Amino acids	8.3	19.7
Water	16.8	28.6
Amino acids	16.8	28.5
Water	16,400	26,100
Water	32,800	52,400

concentrations of H_2O_2 were decomposed by acid permanganate and the oxygen evolution measured. This reaction releases one mol of O_2 per mol of peroxide and the concentration indicated by O_2 evolution always checked very closely with the initial known concentration. Thus the contradictory results are inherent in the catalase-peroxide reaction and not in the experimental method. The gas evolved by the catalase reaction was analyzed and found to be 100% oxygen. As the concentration of enzyme was decreased the total gas evolution decreased to the expected amount, one mol for each 2 mols of H_2O_2 decomposed. Further decrease in the enzyme concentration, so that no active catalase could be detected after gas evolution ceased, gave low values for the peroxide content as would be expected from the incomplete reaction.

Discussion. The mechanisms postulated for the reaction between hydrogen peroxide and catalase have been discussed by Stern,[6,7] and Sumner,[8] and others. Most of the work done with catalase has been based on the rate of reaction and not the total gas evolved.[9,10] It is obvious from the work by Molland[11] that the reaction is complex and difficult to describe by any simple set of equations. If the mechanism postulated by Keilin and Hartree[10] is correct,

$$4Fe^{+++} + 2H_2O_2 \longrightarrow 4Fe^{++} + 4H^+ + 2O_2$$
$$4Fe^{++} + 4H^+ + O_2 \longrightarrow 4Fe^{+++} + 2H_2O$$
$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

it would appear that under the conditions of our measurements (a well buffered substrate with excess catalase) the second step does not occur. Or if the mechanism postulated by Chance[12] is correct,

$$\text{Cat (OH)}_4 + H_2O_2 \leftrightharpoons [\text{(Cat(OH)}_3\text{(OOH)I} \rightleftharpoons \text{Cat(OH)}_3\text{(OOH)II}]^* + H_2O$$
$$\text{Cat (OH)}_3\text{(OOH)I}^* + H_2O_2 \rightleftharpoons \text{Cat (OH)}_4 + H_2O + O_2$$
$$2H_2O_2 \leftrightharpoons 2H_2O + O_2$$

the reaction does not go to completion under the conditions of our experiments, but stops at one of the intermediate complexes which spontaneously breaks down. It is presumed that the catalase is inactivated in yielding the 1:1 ratio of peroxide to oxygen.

Summary. Determinations of the peroxide content of irradiated broth using catalase in a Warburg respirometer were found to be inaccurate. In the presence of excess catalase the oxygen evolved approaches a value of twice that indicated by the accepted general equation for enzymatic decomposition of H_2O_2; if too little enzyme is used the reaction is slow and incomplete. The method is unsatisfactory for the quantitative determination of peroxide.

6 Stern, K. G., 1942, A Symposium on Respiratory Enzymes, pp. 74-103, University of Wisconsin Press.

7 Oppenheimer, C., and Stern, K. G., 1939, Biological Oxidation, The Hague.

8 Sumner, J. B., *Adv. Enzymol.*, 1941, **1**, 163.

9 Northrop, J. H., *J. Gen. Physiol.*, 1924, **7**, 373.

10 Keilin, O., and Hartree, E. F., *Proc. Roy. Soc. B.*, 1937, **124**, 397.

11 Molland, J., *Acta Path. et Microbiol. Scand.*, 1947, **66**, 9.

* Intermediate complexes formed.

12 Chance, B., *Nature*, 1948, **161**, 914.

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