

## THE OXIDATION OF SODIUM LACTATE BY HYDROGEN PEROXIDE.

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(Received for publication, March 1, 1924.)

The consideration of the manner in which various metabolites are removed from the living cell by the process of oxidation presents a problem which, if it is to be considered quantitatively, necessitates a detailed knowledge of the course of comparable reactions *in vitro*. It is, therefore, desirable to consider such reactions from many points of view before attempting to analyze the more complicated reactions that are associated with the living tissue.

Starting with the admirable researches of Fletcher (1) increasing importance has been attached to variations in the concentration of lactic acid in the animal body. There is, however, very little known about the manner in which lactic acid disappears after its production during muscular contraction. The conditions regarding this phase of muscle physiology have recently been reviewed by Shaffer (2), who points out that there are four ways in which lactic acid may disappear in the animal body. There is the possibility that there may be a coupled reaction, part of the lactic acid being oxidized to provide energy to reconvert the remainder to glucose. Again, there may be a conversion of all the lactic acid to glucose, the oxidation of some other metabolite producing this energy. The third possibility is that part of each molecule of the lactic acid is oxidized with a production of acetaldehyde, which in turn is converted into glucose. Finally, there is the possibility that the lactic acid is entirely burned.

In view of these manifold possibilities a knowledge of the changes *in vitro* is desirable. Such reactions were first studied, in the case of lactic acid, by Fenton (3) who found that there was a slow oxidation of lactic acid by  $H_2O_2$ . Later the problem was taken up by Dakin (4) who investigated it in much greater detail. In both researches attention was directed toward the various products of the reaction rather than to the manner in which the reaction took place.

It has been shown by the writer, in a previous paper (5), that it is possible to follow the course of the oxidation of an organic acid by means of determinations of the rate of production of  $\text{CO}_2$ , as carried out in the apparatus designed by Osterhout for this purpose. The work reported here is an attempt to trace by means of this method the mechanism of the oxidation of sodium lactate by  $\text{H}_2\text{O}_2$ .

The modification of the Osterhout apparatus devised by Irwin (6) was used in these experiments. It was arranged in a brass frame in such a way that it could be lowered into a constant temperature bath. The illumination of the tubes was provided by a 150 watt Daylight lamp placed opposite a window in the bath, so that by means of a mirror and a tube observations could be made without disturbing the apparatus. It was found that this method of reading the indicator tubes is much easier than when the tubes are suspended in air since in the bath there are no high lights and the tubes appear perfectly flat. The temperature maintained in these experiments was  $25^\circ\text{C} \pm 0.1^\circ\text{C}$ .

Early in the work the desirability of calibrating the apparatus became apparent. The method described by Parker (7) is not adaptable to the Irwin type of apparatus owing to the impossibility of letting in  $\text{CO}_2$ , but an application of the mass law was made which proved to be useful.<sup>1</sup> In order to apply this method of calibration it is necessary to assume that there is an even distribution of  $\text{CO}_2$  throughout the apparatus at any given time. If this assumption is made, then it is possible to compute the milligrams of  $\text{CO}_2$  needed to change the colors in the indicator tube between the standards which in this case had the values pH 7.78 and 7.36. It is also necessary that the indicator of the apparatus contain a solution of  $\text{NaHCO}_3$  of known concentration. The method is as follows:

$$\frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]} = k_a \quad (1)$$

in which  $k_a$  = the dissociation constant of  $\text{H}_2\text{CO}_3$ .

$$[\text{HCO}_3^-] = [\text{NaHCO}_3]_T \quad (2)$$

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<sup>1</sup> The writer wishes to express his indebtedness to Dr. E. J. Cohn for the suggestion of this method and for his assistance in its development.

in which  $\gamma$  is the dissociation constant of  $\text{NaHCO}_3$ .

$$[\text{H}_2\text{CO}_3] = [\text{CO}_2] k \quad (3)$$

in which  $k$  is the absorption coefficient of  $\text{CO}_2$ .

From 1, 2, and 3

$$\frac{[\text{H}]}{[\text{CO}_2] k} = \frac{k_a}{\gamma} \quad (4)$$

or

$$\frac{[\text{H}]}{[\text{CO}_2]} = \frac{k_a}{\gamma} = \frac{k}{\text{NaHCO}_3} \quad (5)$$

Equation (5) was used to calculate the concentration of  $\text{CO}_2$  at pH 7.78 and 7.36. The value of  $k_a$  used was the one given by Kendall,  $3.5 \times 10^{-7}$ ; and the value of  $\gamma$ , since the concentration of the  $\text{NaHCO}_3$  was  $\text{M}/10,000$ , was taken as 1. The absorption coefficient of  $\text{CO}_2$  was also taken as 1.

For pH 7.78 ( $[\text{H}] = 0.167 \times 10^{-7}$ )

$$\frac{0.167 \times 10^{-7}}{[\text{CO}_2]} = \frac{3.5 \times 10^{-7}}{0.0000841}$$

$$[\text{CO}_2] = 0.004 \text{ mg. in 10 cc.}$$

For pH 7.36 ( $[\text{H}] = 0.44 \times 10^{-7}$ )

$$\frac{0.44 \times 10^{-7}}{[\text{CO}_2]} = \frac{3.5 \times 10^{-7}}{0.0000841}$$

$$[\text{CO}_2] = 0.0106 \text{ mg. in 10 cc.}$$

Hence

$$0.0106 - 0.004 = 0.0066 \text{ mg. CO}_2 \text{ to change 10 cc. from pH 7.78 to 7.36.}$$

The volume of the apparatus involved was found to be 90 cc., therefore the number of milligrams of  $\text{CO}_2$  necessary to change the indicator between the standards is 0.0594 mg.

Knowing the amount of  $\text{CO}_2$  needed for a unit change of the indicator it is a very simple matter to evaluate  $\frac{\Delta \text{CO}_2}{\Delta T}$  in which  $T$  is unit

time. From this the average velocity of the reaction can be determined, which in turn forms the basis of the integral curve.

The sodium lactate used in these experiments was *i*-lactic acid to which NaOH was added until the reaction of the solution was pH 6.9; the concentration of the lactate was  $m/50$ . The  $H_2O_2$  was used in molar solution and the reaction was adjusted to the same point as the lactate. It is necessary that care be taken not to use alkaline solutions in the reaction flask of the Osterhout apparatus since any buffer action at this point tends to vitiate the results.

In all the experiments reported the ratio of the peroxide to the lactate is the same, *i.e.* 25 cc. of the lactate to 10 cc. of the peroxide.

The first experiments were on the reaction between sodium lactate and  $H_2O_2$  without a catalyst. Fig. 1 shows the points found experimentally for the rate of production of  $CO_2$ . It will be seen that the rate falls off from the start in such a manner that at the end of 2 hours it is very slow. At this point the readings were stopped since, when the time of change of the indicator tube is over 15 minutes, the accuracy of the method is impaired.

The total amount of  $CO_2$  produced in the reaction is given in Fig. 2, this curve being the integral of the rate curve in Fig. 1. There is a slight lag in the curve at the start, the importance of which will be pointed out in conjunction with other experiments.

The next step in this series of experiments was the determination of the amount of  $H_2O_2$  consumed in this reaction. The method used was that of Kingzett, which is more applicable for a solution such as is dealt with here than the permanganate method, since the former is not influenced by the presence of organic matter. It consists in determining the amount of iodine released by the peroxide from KI in the presence of  $H_2SO_4$ .

A mixture of the reagents was made up as in the experiments on the production of  $CO_2$ , but in larger quantities. A current of air free from  $CO_2$  was passed through the solution and samples were taken from time to time. The results of these titrations are given in Fig. 3, Curve A. Instead of a gradual diminution of the peroxide present it was found that the  $H_2O_2$  (or its equivalent in active oxygen) at first rises and then falls off, indicating that an increase in the oxidative energy of the reaction has taken place. There is but one apparent

explanation for this result; some product or constituent of the reaction is activating the oxygen of the air passed through the reaction mixture. The result of this process is the formation of a peroxide-like substance.

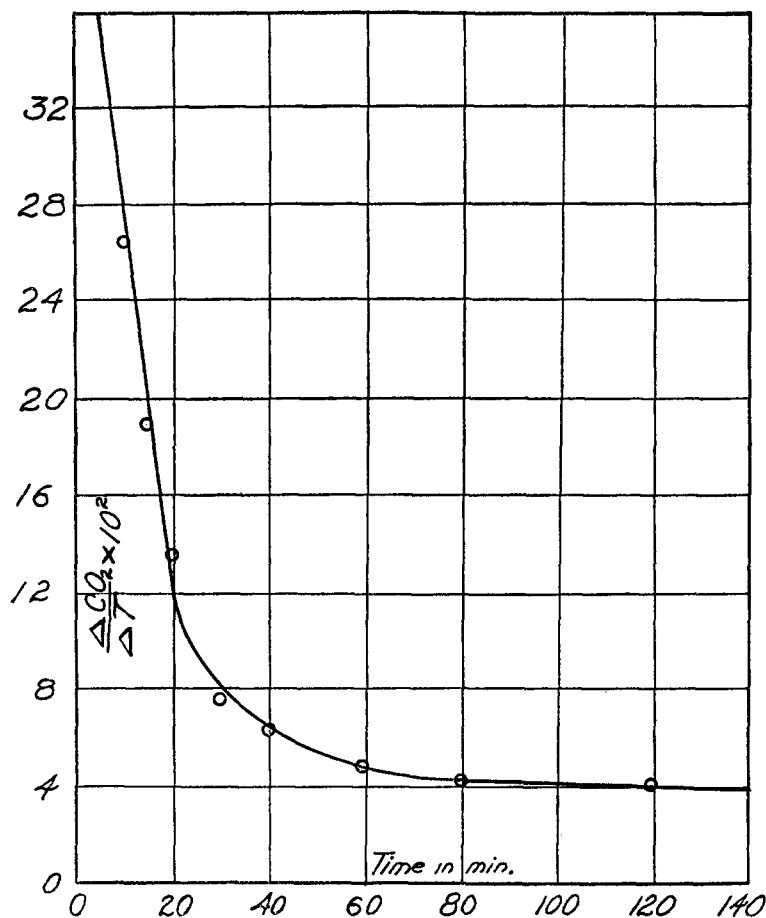


FIG. 1. The rate of production of  $\text{CO}_2$  from sodium lactate and  $\text{H}_2\text{O}_2$ . The points represent the average of ten experiments.

If this explanation is correct the increase in oxidative activity that is found in the presence of air should not appear when the reaction is carried out in an atmosphere that is free from oxygen. For this reason experiments were made in which nitrogen instead of air was

passed through the solution, the results being given in Curve *B* of Fig. 3. There is no increase in this case; on the contrary the oxidative activity of the solution decreases from the start.

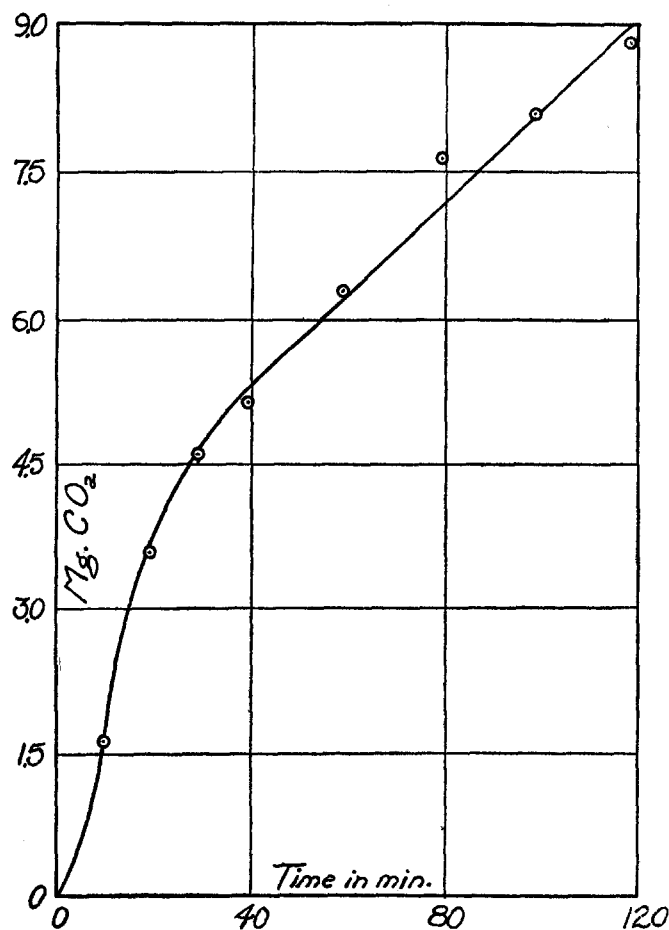


FIG. 2. The total CO<sub>2</sub> produced during the reaction between the lactate and the peroxide. (From Fig. 1.)

The question at once arises as to what constituent of the reaction brings about the increased activity denoted by the rise in Curve *A* of Fig. 3. Dakin (4) points out that among the products of the reaction between ammonium lactate and H<sub>2</sub>O<sub>2</sub> is acetaldehyde. This

compound is known to be particularly active in the formation of a peroxide, acetyl peroxide, when allowed to react with oxygen.<sup>2</sup>

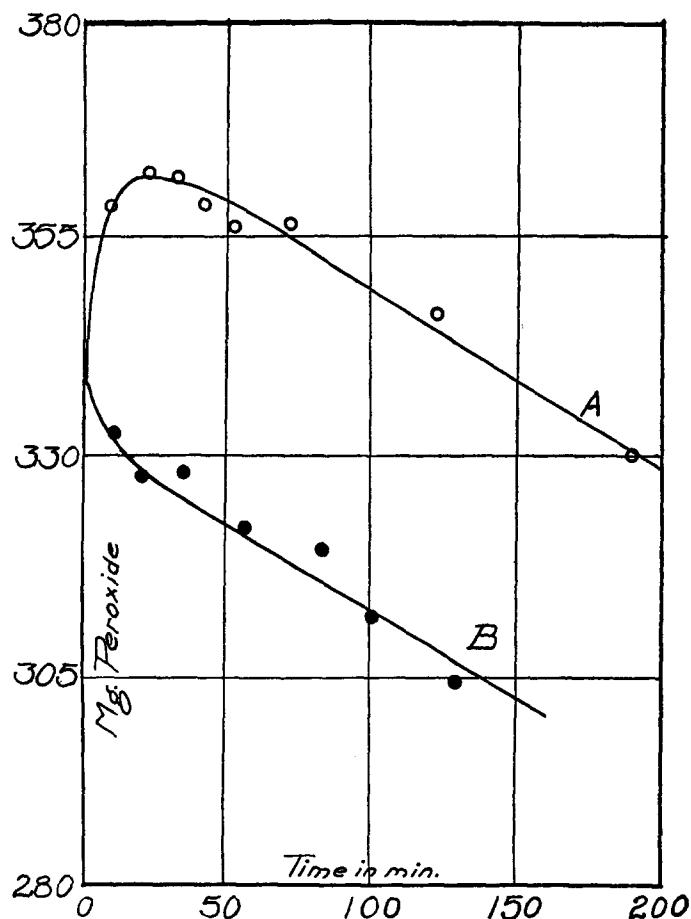


FIG. 3. The changes in the oxidative activity of the peroxide-lactate reaction. For convenience the changes in the oxidative activity of the solution have been plotted in terms of  $H_2O_2$ . Curve A is the reaction in the presence of air free from  $CO_2$ ; Curve B is the reaction in an atmosphere of nitrogen.

<sup>2</sup> The writer has found that the formation of a substance which acts like peroxide in a solution containing acetaldehyde and oxygen, proceeds more rapidly when  $H_2O_2$  is added.

The formation of such peroxides has been discussed in detail by Baeyer and Villiger (8) who have shown that aldehydes readily form compounds of this nature which, in turn, react with other oxidizable constituents of the system. Thus, in the case of benzaldehyde, benzoylhydrogen peroxide is formed, which reacts in the absence of other substances with a molecule of benzaldehyde to form three molecules of benzoic acid. If, therefore, a secondary reaction takes place in the mixture we are discussing there may be a production of acetic acid, but if this reaction cannot go on, owing to a lack of oxygen, then the only products will be acetaldehyde and carbonates due to the primary reaction. It has been noted that when the reaction between  $\text{H}_2\text{O}_2$  and sodium lactate goes on in an atmosphere of nitrogen there is no increase in the oxidative activity of the solution as is found when there is an abundance of oxygen. There is, nevertheless, a consumption of oxygen (although no  $\text{CO}_2$  is liberated), indicating that the reaction between the  $\text{H}_2\text{O}_2$  and the lactate goes on to a certain degree under this condition. The only alternative would be to suppose that the  $\text{H}_2\text{O}_2$  spontaneously decomposes which does not seem probable in a neutral solution.

If we examine the solution to determine the amount of lactic acid used in the reaction there should be no apparent change, since the method of examination (Clausen's (9)) will not differentiate between acetaldehyde and lactic acid.

In order to test this the reagents were mixed as in the previous experiments, and at the end of  $3\frac{1}{2}$  hours in an atmosphere of nitrogen the amount of lactic acid was determined. It was found that the amount of bisulfite bound was equivalent to 220 cc. of the iodine solution used for titrating, while control experiments of equivalent amounts of lactic acid used 225 cc. The difference of 2.27 per cent is within the experimental error. This would seem to indicate that the reaction does not go beyond the stage of acetaldehyde unless free oxygen is present. These results are quite in accord with those of Dakin, who found that the first distillation gave practically nothing but aldehyde.

Since we know the amount of oxygen consumed in the reaction it should be possible to calculate how much acetaldehyde is formed from time to time when no atmospheric oxygen is present and, from this,



the probable amount of organic peroxide formed in the secondary reaction, if the acetaldehyde alone is concerned with the formation of this peroxide and the reaction goes on as already outlined. When such a calculation is made, however, it is found impossible to correlate the amount of oxygen activated in the presence of air with the amount of aldehyde that is formed during the reaction in nitrogen. It is plausible to consider that the increase in the oxidative activity of the solution in the presence of air is due to the reaction of acetyl peroxide with sodium lactate rather than with acetaldehyde as we have heretofore considered. This reaction might well take place very rapidly and result in the conversion of practically all of the lactate to aldehyde in a short time. Such an hypothesis, although not susceptible of proof, is quite attractive since it explains the increase in oxidative activity at the start. If the secondary reaction goes slowly, as it well may, then the slow production of  $\text{CO}_2$  will be accounted for, since the  $\text{CO}_2$  released by the primary reaction with the lactate would be bound immediately by the base liberated at the same time. There can be no liberation of  $\text{CO}_2$  from the solution until the reaction between acetyl peroxide and some other constituent has taken place with a production of acetic acid from the peroxide. The fact that the liberation of  $\text{CO}_2$  is dependent on the secondary reaction accounts for the shape of the curve in Fig. 2.

That there are carbonates in the solution makes possible the consideration that perhaps there are percarbonates formed during the reaction. A test was made for the presence of percarbonates by adding a solution of  $\text{AgNO}_3$  to a sample of the reaction mixture. The dense precipitate was centrifugalized and washed. It was then treated with  $\text{KI}$  and  $\text{H}_2\text{SO}_4$  and shaken with a small amount of chloroform. There was no perceptible coloration of the globule of chloroform, hence it is assumed that there is no percarbonate formed during the reaction.

Certain experiments were carried out to determine the effect of iron on the amount of  $\text{CO}_2$  produced in this system and also its effect on the secondary reactions taking place. The salt used was  $\text{Fe}_2\text{Cl}_6$  in a concentration of  $\text{M}/10$ , and the experiments were conducted in two series. In the first group the iron was added to the peroxide before the mixture was added to the lactate solution, and in the second

group the iron was added to the lactate before the peroxide. This variation in the type of experiment was made in order to determine if there was any difference to be noted when the iron was in a highly oxidized state and when it was in the reduced condition. The experiments were carried out in the same manner as those of the first series with the exception that to each mixture there was added 0.05 cc. of iron solution. The results of the first group of experiments are given in Fig. 4, Curve *A*. This is the integral curve of the reading on the Osterhout apparatus and, by a comparison with the curve found when no iron is present (Fig. 4, Curve *B*), there is noted a decided increase in the amount of  $\text{CO}_2$  produced.

The results of the second group of experiments of this series are given in Fig. 5, Curve *A*. There is a decided lag in the curve at the start, but following this period there is a considerable production of  $\text{CO}_2$  so that in the end the amount produced is practically the same as when the iron is not present (Fig. 5, Curve *B*).

Comparison of the two curves on the effect of iron shows that the condition of this element is of importance in the reaction, especially at the start. This is far more strikingly shown in the following experiments on the disappearance of  $\text{H}_2\text{O}_2$ .

The procedure in these tests was the same as in the preceding determinations of  $\text{H}_2\text{O}_2$ . The iron was added in amounts equivalent to those used in the experiments on the production of  $\text{CO}_2$ . The results are given in Fig. 6. Curve *A* shows the amount of peroxide used up in the reaction if the iron is added at the same time as the peroxide. There is not, as will be observed, any great difference between this reaction and the one that takes place when no iron is present (*cf.* Fig. 3). As might be expected there is a greater utilization of the peroxide, but this is all. Curve *B* summarizes the second type of experiment with iron. In this case the amount of  $\text{H}_2\text{O}_2$  (or its equivalent) instead of increasing at the start decreases; this decrease is later followed by an increase. The cause of the decrease is readily determined by an observation of the variations in acidity that occur during the reaction. When the iron is added to the sodium lactate solution there is a production of acid so that the pH falls from nearly neutral to below 5.0. This is due to the reduction of the ferric salt to the ferrous with a production of acid. When the  $\text{H}_2\text{O}_2$  is added

to the solution the iron is again oxidized and the acidity comes back to the original point. These changes in acidity coincide in point of time with the changes that are noted in regard to oxidative activity.

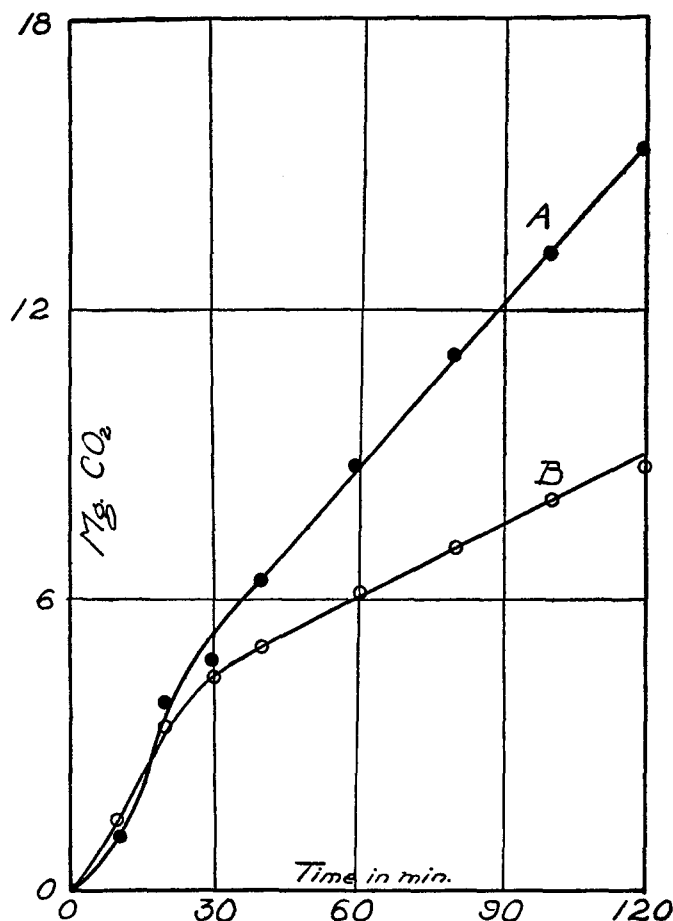


FIG. 4. The effect of iron on the amount of CO<sub>2</sub> produced during the reaction between sodium lactate and H<sub>2</sub>O<sub>2</sub> when the iron and the peroxide are mixed before addition to the lactate. Curve A is the reaction with iron; Curve B is the reaction without iron.

The fact that iron itself is reduced would indicate that the action of this metal in the process of catalyzing oxidations is the oxidation of the iron itself, as has been suggested by Baudisch (10), and by Haber

(11) (*cf.* Mellor (12)). These authors have considered that there is in all probability a formation of a complex between the actor, which in our case is the peroxide, and the inductor, the iron. Baudisch

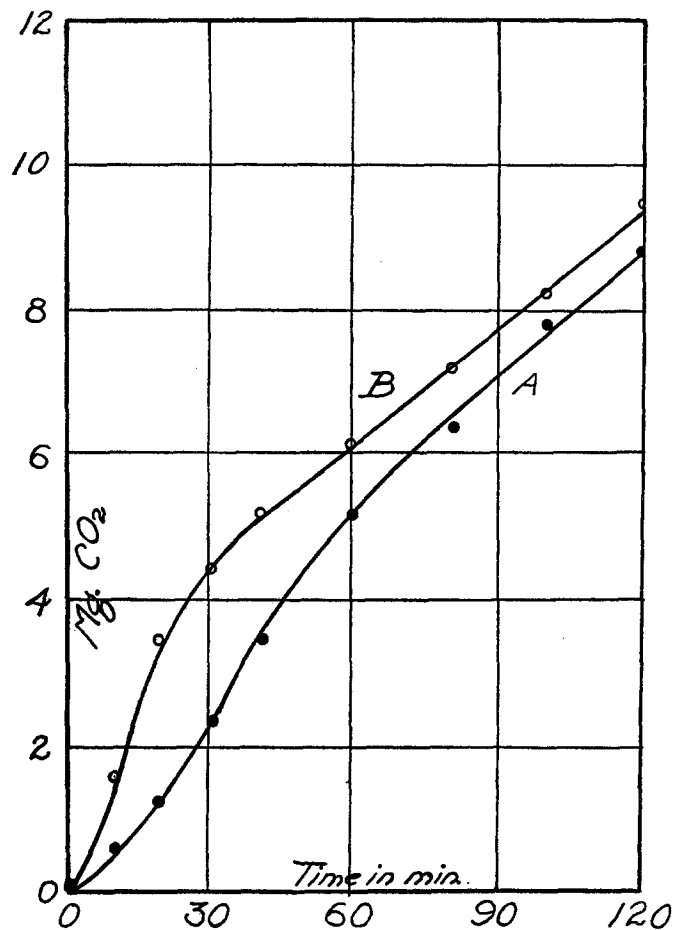


FIG. 5. The effect of iron on the amount of  $\text{CO}_2$  produced when the iron is added to the lactate before the addition of the peroxide. Curve *A* is the reaction when iron is present; Curve *B* is the reaction when iron is not present.

developed a theory that, on the basis of Werner's coordination hypothesis, it is possible to consider that the iron salt may form the nucleus of a coordination compound. This hypothetical compound

may be conceived to be of high oxidative energy which is available for the oxidation of other compounds. In the experiments that we are discussing it is quite possible that the iron, when it is added to the

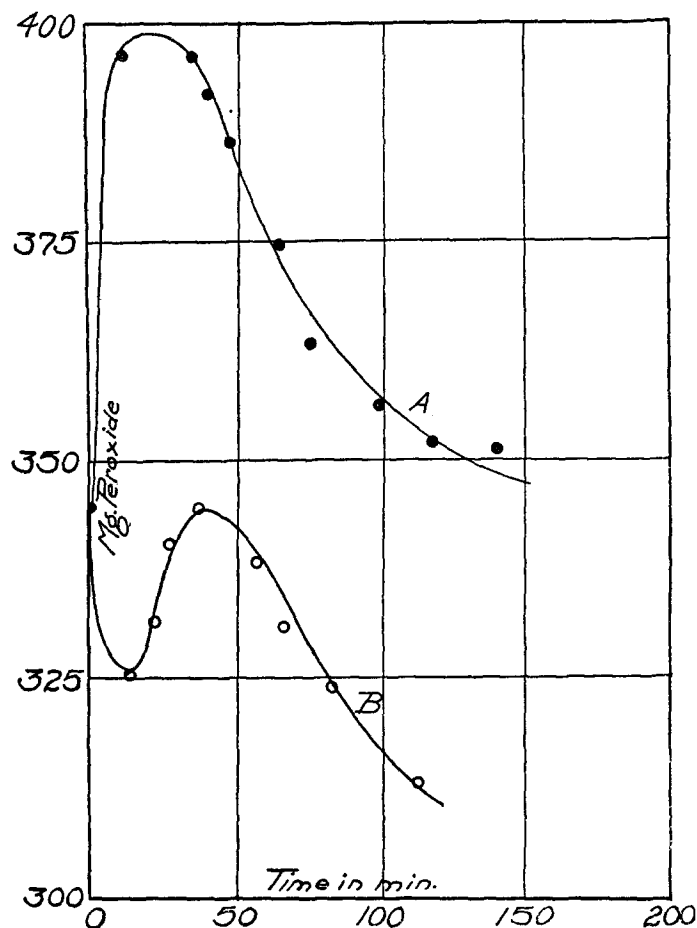


FIG. 6. The effect of iron in different states on the oxidative activity of the reaction. Curve *A* is the result with oxidized iron; Curve *B* is the result with reduced iron.

peroxide, forms just such a compound as Baudisch pictures. This would account for the activity that is shown by the increased production of CO<sub>2</sub>. On the other hand, when the iron is added to the lactate

solution there is an immediate reduction which, as has been mentioned, results in a production of acid. Such a condition may well be unfavorable for the production of the complex which can be considered necessary for manifestation of the catalytic activity of the iron. Not only is the activity of the inductor inhibited but the action of the peroxide on the lactate solution is in part prevented, so that the  $\text{CO}_2$  produced is below the amount when no iron is used.

There is another factor, recently pointed out by Reiner (13), that may play an important part in the reaction between  $\text{H}_2\text{O}_2$  and sodium lactate. From a series of studies on the oxidation of acetaldehyde by  $\text{H}_2\text{O}_2$  in the presence of various catalysts, this author comes to the conclusion that the reaction between  $\text{H}_2\text{O}_2$  and acetaldehyde—a reaction which is very slow—is increased by the action of catalysts which, in turn, are accelerated by the acetic acid that is produced in the reaction. In the reactions reported here there is, as has been mentioned, a production of acetic acid as the product of the second reaction between acetyl peroxide and either acetaldehyde or lactate. This may act in the manner that Reiner describes in the reactions he has studied. On the other hand, the experimental data do not indicate that there is any factor playing such a part other than the acetyl peroxide. There is no indication in the curves of a third accelerating substance.

The possibility of acetaldehyde acting to carry on the reaction has been considered by Palladin and Sabinin (14). From their results on the effect of killed yeast on lactic acid, these authors came to the conclusion that after a short period of activity of the yeast there would be a certain amount of acetaldehyde produced which would be sufficient to carry on the reaction. They are of the opinion that the action of the aldehyde is one of dehydrogenation but there is no explanation of the manner of this reaction. Another observation by these authors, which is of interest in connection with other statements made here, is the fact that the acetaldehyde is not converted into alcohol in their experiments, which they explain as being due to the large excess of hydrogen acceptor present in the reaction mixture. In our experiments upon the oxidation of lactic acid there is always an excess of  $\text{H}_2\text{O}_2$  which, on the basis of Palladin's explanation, would account for the fact that we do not find any oxidation beyond the stage of acetaldehyde when the reaction is carried out in an atmosphere of nitrogen.

## SUMMARY.

By means of a modification of the technique of the Osterhout apparatus it is possible to follow the production of  $\text{CO}_2$  from sodium lactate when acted upon by  $\text{H}_2\text{O}_2$ .

The results of this process indicate that the reaction is not a simple one but is of an autocatalytic type. This conclusion is borne out by the fact that the determinations of  $\text{H}_2\text{O}_2$  during the reaction show an increased amount of peroxide during the earlier stages of the reaction. This is considered to be due to the formation of a peroxide by the oxidation of the acetaldehyde (formed by the interaction of  $\text{H}_2\text{O}_2$  and sodium lactate) with the oxygen of the air. When the reaction is carried out in an atmosphere of nitrogen no increase is observed.

Further experiments in nitrogen tend to show that acetaldehyde is the end-product of the action of  $\text{H}_2\text{O}_2$  alone.

The effect of  $\text{FeCl}_3$  upon the reaction depends upon the previous treatment of the iron salt. If the iron solution is added to the  $\text{H}_2\text{O}_2$  before mixing with the lactate there is an increased amount of  $\text{CO}_2$ . If, however, the iron is added to the lactate before the addition of the peroxide, the action tends to inhibit the production of  $\text{CO}_2$ .

The reaction of  $\text{H}_2\text{O}_2$  with sodium lactate is comparable to the action of killed yeast and methylene blue as determined by Palladin and his coworkers.

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