Summary

The divinylacetylene described in a previous paper reacts with thio-*p*-cresol to form a crystalline derivative whose structure as di-(*p*-tolylthio)-1,6-hexine-3 is demonstrated by its oxidation to the known β-*p*-tolylsulfonepropionic acid. The following transformation products of the new derivative are described: di-(*p*-tolylthio)-1,6-dibromo-3,4-hexene-3, di-(*p*-tolylsulfone)-1,6-hexandione-3,4, di-(*p*-tolylsulfone)-1,6-hexine-3, di-(*p*-tolylsulfone)-1,6-dibromo-3,4-hexene-3, di-(*p*-tolyl-sulfone)-1,6-hexanone-3.

WILMINGTON, DELAWARE RECEIVED SEPTEMBER 29, 1932
PUBLISHED MAY 6, 1933

[CONTRIBUTION FROM THE STREAM POLLUTION LABORATORY, UNITED STATES PUBLIC HEALTH SERVICE]

The Catalysis of Air Oxidations by Iron Salts, Phosphates and Pyrophosphates[1]

BY EMERY J. THERIAULT, C. T. BUTTERFIELD AND P. D. MCNAMEE

It is clear that the case for the rôle of iron, copper, manganese and other suggested carriers of oxygen in biological oxidations could be considerably strengthened if catalysts containing these elements were to be prepared and made to perform *in vitro* the same type of oxidations which are apparently accomplished by living cells. Such a model of biocatalytic oxidation should satisfy the essential conditions that the *P*H value, the salt concentration and the temperature of the liquid medium fall within the normal physiological range. Thus the oxidations accomplished at the *P*H values reached by sodium hydroxide, calcium hydroxide and even by sodium carbonate, while of considerable interest in themselves, are of a different order from those under consideration. Likewise the oxidations accomplished by the more energetic oxidizing agents such as potassium permanganate in alkaline solution or by Fehling's solution, are of remote interest to the present discussion. Within these limitations, the iron phosphate model of biocatalytic oxidation proposed by Spoehr[2] still remains as an outstanding example.

In several respects the claims made by Spoehr for his catalytic agent are truly remarkable. The iron pyrophosphate mixture itself is readily prepared from inorganic materials and its action on a variety of organic materials is supposedly pronounced at *P*H values ranging from 6.8 to 8.8 even at body temperatures. The conclusions reached by Spoehr were apparently confirmed by Degering and Upson[3] and by Degering,[4] although

(1) Presented at the New Orleans Meeting of the American Chemical Society, April, 1932.
(2) Spoehr, THIS JOURNAL, **46**, 1494–1502 (1924).
(3) Degering and Upson, *J. Biol. Chem.*, **94**, 423 (1931).
(4) Degering, *ibid.*, **95**, 409 (1932).

the lowest temperature used by these experimenters (48°) is relatively high. On the other hand it is disquieting to note that Meyerhof and Matsuoka[5] have actually used pyrophosphates for the inhibition of oxidations induced by iron, in a manner analogous to the action of the cyanides. It is difficult to reconcile the reasonably extensive data presented by Spoehr and his associates on the catalytic properties of the iron pyrophosphates with the inferences drawn by Warburg[6] from the findings of Meyerhof and Matsuoka regarding the inhibitory influence of the pyrophosphates. Lohman,[7] for instance, has reported the presence of pyrophosphates as normal constituents of bacterial cells.

In view of the importance in the general field of biological oxidations of such a catalytic agent as described by Spoehr and of certain obvious possibilities of application to sanitary problems, it has appeared advisable to test the efficacy of the iron pyrophosphates under conditions approximating those encountered under natural conditions, using various precautions which are not stated explicitly in the literature of the subject. In particular, it has appeared necessary to ensure the strict sterility of the experimental solutions.

Procedure

In general the procedure adopted consisted in following the course of the oxidation by a direct measurement of the absorption of molecular oxygen instead of by an analysis of one of the reaction products, such as carbon dioxide.

In experiments with the undiluted catalyst, use was made of the apparatus described by Theriault and McNamee[8] for the aeration of liquids in a closed system, the absorption of oxygen being followed by examinations of the enclosed air. The precision of the apparatus is well within 5 mg. of oxygen, so that minor changes in the oxygen content of the system are readily followed.

For the reason that the salt concentration in the buffered catalytic mixture is relatively high in comparison with the mineral salt content of polluted waters, or the tonicity of body fluids, numerous experiments were also made in which the salt concentration was reduced to one-tenth of the maximum by dilution with sterile distilled water. These diluted mixtures accordingly contained 0.01 mole of sodium pyrophosphate and 0.0024 mole of iron per liter, together with 0.03 mole of buffer salts. The concentration of dextrose in these experiments was also reduced to a figure of 5 to 20 mg. per liter to correspond with the load of organic pollution ordinarily encountered in polluted streams. The salt concentration in these experiments was still somewhat comparable with the lowest salt concentrations used by Spoehr and Smith.[9] The concentration of dextrose, however, was of a much lower order of magnitude.

The procedure used in experiments with the diluted catalyst mixtures followed closely along the lines recommended by Theriault[10] for the determination of the oxygen demand of polluted waters. Prior to a test the sterile water used for dilution purposes together with the sterile stock solutions of dextrose, catalyst and buffer salts, were stored

(5) Meyerhof and Matsuoka, *Biochem. Z.*, **150**, 1–11 (1924).
(6) Warburg, *Science*, **61**, 575 (1925).
(7) Lohman, *Biochem. Z.*, **203**, 164–171 (1928).
(8) Theriault and McNamee, *Ind. Eng. Chem.*, **22**, 1330 (1930).
(9) Spoehr and Smith, THIS JOURNAL, **48**, 236–248 (1926).
(10) Theriault, Supplement No. 90 to Public Health Reports, 1931.

in cotton-plugged containers in a constant temperature room until equilibrium had been established between atmospheric and dissolved oxygen at the desired temperature of incubation. Thus, in experiments at 20 and at 37°, the initial dissolved oxygen content of the distilled water was, respectively, 9.0 and 6.7 mg. per liter. For a test a large volume of the diluted mixture was prepared and, after thorough mixing, it was siphoned into 300-ml. bottles with suitable precautions against bacterial contamination in case it was desired to test the efficacy of the catalyst. Control tests with the addition of bacteria and of plankton were also made.

The loss of water by evaporation from the incubated sub-samples was prevented by inverting the bottles in pans of water. This procedure is also very effective in preventing the reaeration of the sub-samples. Diffusion effects are demonstrably negligible in respect to the interchange of dextrose and also of the catalyst mixture, at least when properly fitted, glass-stoppered bottles are used. Nevertheless, to avoid any possible complication from the slow diffusion of traces of the usual disinfectants such as phenol, chlorine or mercuric chloride, no special precautions were taken to ensure the continued sterility of the water used as a seal. It was inevitable, therefore, that bacteria should eventually work their way into the sterile sub-samples. It should be noted, however, that proper allowance for the accidental contamination of the sub-samples from this and other causes can readily be made on the basis of the bacteriological examinations which paralleled the oxygen demand tests. In any event, the contamination of the sub-samples was seldom observed prior to the fifth day of incubation.

Determination of Dissolved Oxygen.—Considerable difficulty was experienced in the earlier experiments with the diluted catalyst in obtaining accurate values for the dissolved oxygen of the solutions when the usual Winkler procedure was used. The difficulty was definitely traced to the presence of unusual amounts of phosphates which act to depress the P_H value of the samples, thereby greatly increasing the required period of absorption. In the absence of ferrous salts, accurate results can be obtained by starting the determination with the addition of 4 ml. of manganous sulfate solution (480 g. of $MnSO_4 \cdot 4H_2O$ per liter) instead of the customary 1 or 2 ml. portion. On shaking, a copious precipitate of manganous phosphate is obtained. The test is then continued by the addition of 2 ml. of alkaline iodide mixture (500 g. of sodium hydroxide and 150 g. of potassium iodide per liter). A period of contact of fully ten minutes is then allowed, with occasional shaking, before adding 2 ml. of concentrated sulfuric acid (sp. gr. 1.84) to dissolve the precipitate. The titration of the liberated iodine is then completed within fifteen minutes. Owing to the protective action of the phosphates, no special precautions are required to guard against interference by ferric salts.

The direct determination of dissolved oxygen was avoided in experiments where the undiluted catalyst was aerated in a closed system, the loss of oxygen being followed by examinations of the enclosed air.

Preparation of the Catalyst.—The catalyst was prepared by dissolving 6.49 g. of $FeCl_3 \cdot 6H_2O$ or 6.67 g. of $FeSO_4 \cdot 7H_2O$ in 800 ml. of 0.125 M $Na_4P_2O_7$. The precipitate of iron pyrophosphate first formed dissolved readily in the added excess of sodium pyrophosphate to give a very clear solution.[11] This concentrated solution was then diluted to one liter with phosphate solutions buffered at the desired P_H value and with glucose solutions of appropriate strength. The catalyst prepared in this manner was 0.1 M in respect to $Na_4P_2O_7$ and 0.024 M in respect to Fe. The concentration of buffer salts was generally adjusted to give 0.3 M KH_2PO_4 plus NaOH when the P_H value of 7.2 was

(11) On the basis of diffusion experiments, Spoehr and Smith, THIS JOURNAL, **48**, 237–238 (1926), conclude that "the iron is in a complex molecule which is molecularly dispersed." In our own experiments the practical absence of positively charged colloidal forms of iron was indicated by the fact that 8000 ml. of the solution could be filtered through a 1 × 3 in. Berkefeld filter without clogging or appreciable diminution in the rate of filtration. Direct tests on the filtrate likewise indicate that virtually all of the catalyst mixture will pass through a Berkefeld filter.

selected. In other experiments an equivalent amount of Na_2HPO_4 was used. These concentrations are in close agreement with those used in some experiments by Spoehr (Ref. 2, p. 1497; 150 ml. of water, 6.7 g. of $Na_4P_2O_7 \cdot 10H_2O$, 1 g. of $FeSO_4 \cdot 7H_2O$ and 17 g. of $Na_2HPO_4 \cdot 12H_2O$).

It is evident from Spoehr's work that the concentration of the buffer salts may be varied considerably without inhibiting the activity of the catalyst. Thus in a given experiment (Ref. 2, p. 1499) at P_H 7.2 with 0.125 *M* KH_2PO_4 the amount of carbon dioxide formed was 0.6662 g. in 216 hours. At P_H 6.8 with 0.25 *M* KH_2PO_4 the carbon dioxide production still amounted to 0.5344 g. At P_H 8.8, when the dipotassium phosphate was omitted altogether, the carbon dioxide production was 0.4690 g. The temperature in these experiments was 38°. Spoehr (Ref. 2, pp. 1499–1500) concludes that "the highest rate of oxidation does not correspond to the greatest alkalinity, but rather is attained in a very slightly alkaline solution." The P_H value of 7.2 may accordingly be regarded as most favorable to the activity of the catalyst. It should be noted, however, that this particular P_H value is also favorable to the growth of numerous varieties of bacteria.

There are some indications that the catalyst prepared from the ferrous salts may be somewhat more active than when the ferric salts are used. Thus in a given experiment by Spoehr (Ref. 2, p. 1498), "The carbon dioxide formed in the case of the ferrous sulfate totaled 0.5600 g. and in that of the ferric sulfate 0.5392 g." This difference in activity appears to be negligibly small. Spoehr and Smith (Ref. 9, pp. 239 and 241) state that some substances, such as formic acid, which are oxidized by the ferro compound, are not affected by the ferri derivative. Dextrose, however, does not appear to be in this category. As a matter of analytical convenience in the direct determination of dissolved oxygen, the ferric salts were accordingly used in most of the work presented in this paper. Numerous experiments, however, were made with the ferrous salts.

On the basis of the results reported by Kiehl and Hansen,[12] it is reasonable to assume that the rate of hydration of sodium pyrophosphate, to form disodium phosphate, would be very slow at room temperatures and at P_H values of 7.2 or over. Mellor[13] indicates that aqueous solutions of the alkali pyrophosphates are stable even at boiling temperatures.

Reagents.—The usual C. P. or "Analyzed" variety of reagents was used without recrystallization. No difference in activity was observed when the catalyst was prepared with any one of several brands of sodium pyrophosphate or of iron salts. The composition of the sodium pyrophosphate was checked by titration with hydrochloric acid using thymol blue as an indicator. The iron compounds were likewise checked by titration of reduced solutions with potassium permanganate and by oxygen absorption tests.

In accordance with the reaction $4Fe^{++} + O_2 = 4Fe^{+++} + 2O^{=}$ one gram of Fe^{++} should require 0.143 g. of oxygen for complete oxidation to the ferric state. On this basis one gram of $FeSO_4 \cdot 7H_2O$, corresponding to 0.201 g. of Fe^{++}, should likewise require 28.80 mg. or 20.15 cc. of oxygen under standard conditions. The applicability of the above equation was checked by a direct measurement of the disappearance of atmospheric oxygen when the catalyst prepared from ferrous sulfate was aerated in a closed system. In four concordant series of observations the observed loss was 20.34 ± 0.22 cc. per gram of $FeSO_4 \cdot 7H_2O$, in good agreement with the theoretical value of 20.15 cc. This compares with experimental values of 27.5 and 27.6 cc. per gram of $FeSO_4 \cdot 7H_2O$ in two experiments reported by Smith and Spoehr.[14] The reason for the discrepancy is not clear, although it may be connected with some peculiarity of the aeration apparatus

(12) Kiehl and Hansen, THIS JOURNAL, **48**, 2802 (1926).
(13) Mellor, "Comprehensive Treatise," Vol. VIII, p. 975.
(14) Smith and Spoehr, THIS JOURNAL, **48**, 111 (1926).

used by these experimenters. They also give 27.4 cc. as the calculated value for the oxygen absorbed by one gram of $FeSO_4 \cdot 7H_2O$.

Sterilization of the Solutions.—It was considered inadvisable to heat the concentrated catalyst solution to 100°. This solution was accordingly sterilized by filtration through a Berkefeld filter, Grade W, prior to the addition of the buffer salts and of the dextrose. The buffer solutions were sterilized by autoclaving for fifteen minutes after a steam pressure of fifteen pounds had been reached. All bottles, siphons and pipets were likewise sterilized in an autoclave. The stock solutions of dextrose were sterilized by free steam in an Arnold sterilizer for twenty minutes on three successive days. It was necessary to sterilize the glucose solutions intermittently because of the decomposition which occurs even at P_H 8 when these solutions are heated to 100°.

Results with the Undiluted Catalyst.—The results obtained in a series of observations at 20° with the undiluted ferripyrophosphate catalyst have been plotted in Fig. 1. The aeration method of procedure was used in these experiments and the addition of phosphate was delayed until the fifth day. The concentration of dextrose was one gram per liter to correspond with the carbonaceous matter content of activated sludge and similar materials.

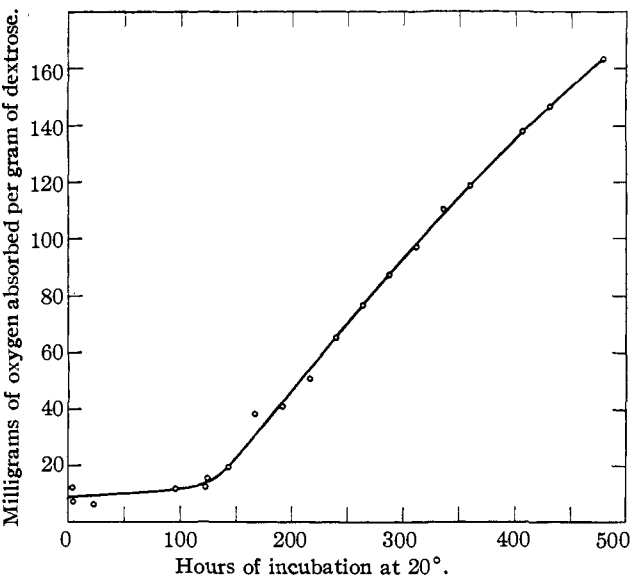

Fig. 1.—The oxidation of dextrose in the presence of ferripyrophosphate catalyst and disodium phosphate.

As shown in Fig. 1, the oxygen demand values during the first ninety-six hours of incubation fluctuated between the extremes of 6.3 and 12 mg. per liter, the general average being 9.4 for the first four observations. In part this decrease must be credited to a constant error owing to a purely physical uptake of oxygen for the establishment of equilibrium when the aeration method is used. No correction has been applied for this apparent loss of

oxygen, which is negligibly small in relation to an expected maximum oxygen demand of 1067 mg. from one gram of dextrose. Bacteriological findings over this preliminary period of incubation were strictly negative.

The experiment was continued with the addition on the fifth day of 0.285 mole of Na_2HPO_4 as the dry salt previously dehydrated at 110° for one hour. Bacterial contamination, nevertheless, was accidentally introduced at some point in this manipulation, presumably as a spore-forming organism resistant to a temperature of 110°. This organism did not grow on ordinary agar plates at P_H 7.2. It did grow very well on a medium containing 10% by volume of the catalytic mixture. As the P_H value of the catalytic mixture was practically unchanged on the addition of the disodium phosphate, it is necessary to ascribe the oxidation which occurred beyond the fifth day (see Fig. 1) to bacterial action rather than to an hydroxyl-ion effect. It should be noted, however, that the experiment was conducted at 20°. At much higher temperatures the decomposition of dextrose would be greatly accelerated by a P_H value of 9.3.

The maximum observed oxygen depletion was 163 milligrams per gram of dextrose after 480 hours. The oxidation had therefore proceeded to the extent of about 15% when the experiment was discontinued. This result may be compared with an observed oxidation of 19% in 456 hours in an experiment at 38° by Spoehr (Ref. 2, pp. 1497–1498) in which the concentration of dextrose was about 20 grams per liter. In either case the velocity constants deducible from these data are of the order of magnitude reported by Symons and Buswell[15] for the biochemical oxidation of purified organic materials by bacteria in the absence of plankton.

Results with the Diluted Catalyst.—Typical oxygen demand curves obtained with the diluted catalyst mixtures buffered at P_H 7.2 are presented in Figs. 2, 3 and 4. The corresponding bacterial results are presented in Tables I, II and III. Excepting the controls, the concentration of dextrose was 10 mg. per liter in all of these experiments, to correspond with the concentration of organic matter in highly polluted streams. The temperature of incubation in the various experiments ranged from 20 to 50°.

Observations at 20°.—The results obtained at 20° are plotted in Fig. 2. In Series 3 use was made of a control mixture containing the buffered catalyst under sterile conditions but without the addition of dextrose. The oxygen demand values ranged from 0.06 to 0.45 mg. of dissolved oxygen per liter. When allowance is made for an experimental error of about 0.1 mg., it is seen that there was still an apparent loss of oxygen of the order of a few tenths of a milligram per liter.

In Series 4 (Fig. 2) the buffered catalyst mixture contained 10 mg. of dextrose per liter, and sterile precautions were observed in preparing the

(15) Symons and Buswell, *Ind. Eng. Chem.*, **24**, 462 (1932).

TABLE I

BACTERIAL RESULTS AT 20°

Period of incubation, hours	Series 4	Series 5 Bacteria per cc.	Series 6
0	0	1,540	1,540
20	0	.....	.....
24	.....	2,700,000	820,000
48	1,200	15,900,000	12,100,000
72	12,000	6,310,000	6,010,000
	0	5,370,000	5,660,000
96	5,500	3,460,000	1,230,000
	1,970	.....	.....
120	1,240,000	.....	.....
	1,310,000	.....	.....
144	.....	1,590,000	220,000
168	17,000.000	.....	.....
192	.....	990,000	198,000
216	1,320,000	.....	.....
	2,840,000	.....	.....
240	.....	850,000	250,000

samples for incubation. For the first four days the loss of dissolved oxygen in this experiment was within the experimental error. Thereafter the oxygen demand increased sharply, reaching a maximum of about 6 milligrams per liter in nine days. This sudden increase, however, was paralleled

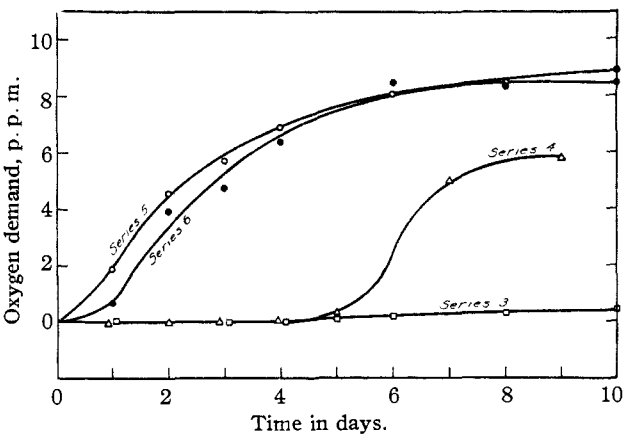

Fig. 2.—Oxidation of glucose at 20°: Series 3, □; Series 4, △; Series 5, ○; Series 6, ●.

by a corresponding increase in the bacterial count which, as shown in Table I, reached a maximum value of 17 millions per cc. on the seventh day. There is no indication in this experiment that the catalyst itself was primarily effective in inducing the oxidation of the dextrose.

In Series 5 (Fig. 2) the procedure was varied by adding a relatively light inoculum of bacteria and plankton (*Colpidium*) along with the buffered

catalyst mixture and glucose. As shown in Fig. 2, there was a lag of several hours before a steady rate of oxidation was established, corresponding to the lag in the onset of bacterial growth. The maximum oxygen demand in this experiment was 8.92 on the tenth day when the experiment was discontinued.

In accordance with the reaction

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$$

the theoretical requirement for the complete oxidation of 10 mg. of dextrose to carbon dioxide and water is 10.67 mg. of oxygen. It is evident that complete oxidation was approached in this experiment. It should be noted, however, that the inoculation contained both bacteria and plankton. In the absence of plankton the rate of oxidation is greatly retarded and the percentage of completed oxidation may likewise be greatly reduced. Thus in experiments by Butterfield, Purdy and Theriault,[16] with pure cultures of *Bact. aerogenes*, the oxidation of dextrose generally ceased when 50 to 60% of the theoretical oxygen requirement had been satisfied. The nature of the inoculum evidently should be considered in estimating the probable extent of a deoxygenation.

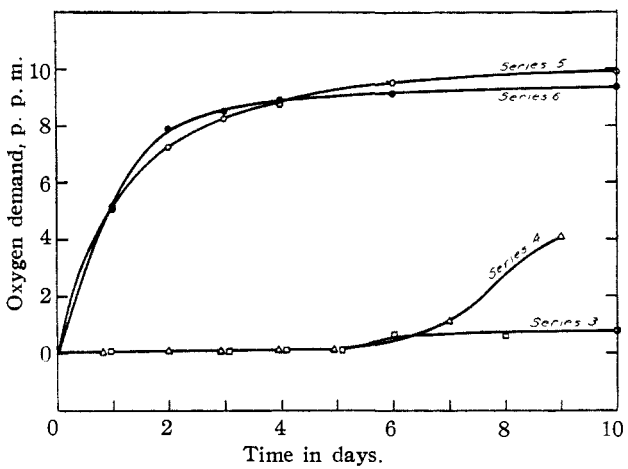

Fig. 3.—Oxidation of glucose at 37°: Series 3, □; Series 4, △; Series 5, ○; Series 6, ●.

As a control on these experiments, a series of observations was made on a buffered mixture inoculated with bacteria and plankton but with the omission of the catalyst mixture. These results (Series 6) have also been plotted in Fig. 2. In general the deoxygenation curve for this series of observations conforms to the pattern obtained in Series 5. In Series 6, however, the lag was more pronounced, so that the deoxygenation curve for this experiment should be shifted back about one day in comparing the

(16) Butterfield, Purdy and Theriault, *Public Health Reports*, **46**, 393–426 (1931).

results with those obtained in Series 5. The bacteriological results given in Table I confirm this peculiarity of the deoxygenation curves. Thus in Series 5 a bacterial count of 2,700,000 per cc. was reached on the first day. The corresponding count in Series 6 was only 820,000 per cc. It should be noted, however, that during the logarithmic phase of growth the bacterial count may readily double in twenty or thirty minutes. These results suggest the possibility that the catalyst mixture may be effective in stimulating bacterial growth, although this tendency has not been confirmed in other experiments.

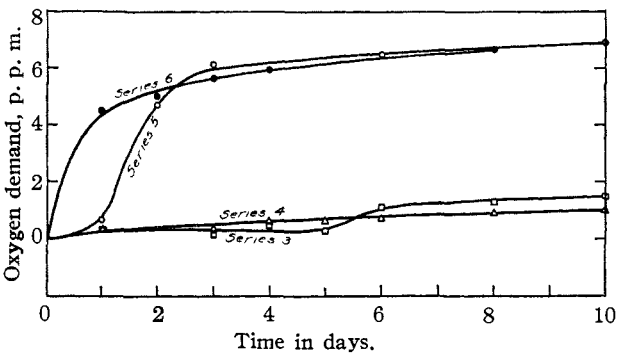

Fig. 4.—Oxidation of glucose at 50°: Series 3, □; Series 4, △; Series 5, ○; Series 6, ●.

Observations at 37°.—In general, the results at 37° were in good agreement with those obtained at 20°. This is shown by the oxygen demand data plotted in Fig. 3 and by the bacterial results presented in Table II.

TABLE II

BACTERIAL RESULTS AT 37°

Period of incubation, hours	Series 4	Series 5 Bacteria per cc.	Series 6
0	0	3,800	3,800
20	0	.....	.....
24	.....	15,100,000	5,500,000
27	.....	13,100,000	4,200,000
48	.....	3,300,000	610,000
72	1,200	1,360,000	290,000
	37,000	.....	.....
96	41,000	899,000	268,000
120	1,860	.....	.....
144	.....	880,000	260,000
168	88,000	.....	.....
216	250	.....	.....
	150,000	.....	.....
240	.....	800,000	220,000

In Series 3 at 37° (Fig. 3) a control mixture containing the buffered catalyst without the addition of dextrose was incubated for ten days. As

in the corresponding series of observations at 20°, there was a measurable loss of oxygen beyond the expected experimental error even though dextrose was absent. On the tenth day this loss had reached a figure of about 0.80 mg. per liter, whereas in the corresponding experiment at 20° the apparent loss was only 0.43. This apparent loss of oxygen should probably be ascribed to a slight displacement during incubation of the ferro-ferri equilibrium from its original position. Ferrous salts to the extent of 1 milligram per liter as iron will cause an apparent loss of 0.14 milligram of dissolved oxygen, unless the permanganate modification of the Winkler method for dissolved oxygen is used. The diluted catalyst mixture used in these experiments contained 133 mg. per liter of ferric iron. The apparent loss of oxygen is to be regarded as a blank on the procedure which, to avoid other analytical difficulties, was necessarily adopted in these experiments. No correction has been applied to any of the data given in this paper for this apparent loss of oxygen.

In Series 4 at 37° (Fig. 3) the solutions contained 10 mg. of dextrose per liter in addition to the buffered catalyst. As in the corresponding experiment at 20°, an attempt was made to maintain sterile conditions in preparing the experimental solutions. As shown in Table II, a bacterial count of less than 50,000 per cc. was maintained for the first five days. The subsequent increase in the bacterial count was much less marked than in the corresponding experiment at 20°. The oxygen demand curve likewise did not show as pronounced an increase as at 20°. In comparing these results it should be borne in mind that bacterial contamination in these experiments was purely fortuitous so that an exact correspondence in bacterial counts is not to be expected. It is also to be considered that the bacterial counts represent only those organisms which would grow on standard agar. The bacterial count therefore is to be taken simply as an index of contamination and not as a quantitative measure of oxygen absorption.

In Series 5 and 6 at 37° (Fig. 3), a buffered solution containing 10 mg. of dextrose per liter was incubated with and without the addition of the catalyst mixture. In each case, however, the samples were inoculated with bacteria and plankton as in Series 5 and 6 at 20°. The deoxygenation curves at 37° (Fig. 3) show a marked similarity and this result is confirmed by the bacteriological observations recorded in Table II.

Observations at 50°.—The results obtained at 50° confirm the trends manifested in experiments at 20 and 37°. As shown in Fig. 4, the apparent loss of oxygen from the sterile solutions containing the buffered catalyst (Series 3 and 4) was practically the same whether dextrose was absent as in Series 3 or present as in Series 4. Thus, after ten days of incubation at 50° the apparent loss of oxygen was 1.49 and 1.25 mg. per liter, respectively, in Series 3 and 4. The difference between these two values is consistent with known limits of experimental error. In comparison with the corresponding

observations at 20 and 37° it is evident that, with increasing temperatures, there is a progressively greater displacement of the ferro-ferri equilibrium toward the ferrous condition. It should also be noted that the bacterial counts remained very low in Series 4.

TABLE III

BACTERIAL RESULTS AT 50°

Period of incubation, hours	Series 4	Series 5 Bacteria per cc.	Series 6
0	0	840	840
20	0	.....	.....
24	....	1,000	1.060,000
	....	1,900	.....
27	....	.....	1,610,000
48	....	1,500	865,000
	....	18,200	.....
72	0	158,000	224,000
96	0	86,000	.....
120	0	.....	.....
	20	.....	.....
144	170	20,000	.....
192	21,000	1.600	5,000
240	36,000	1,500	.....
	31,000	.....	.....

In Series 5 and 6 at 50° (Fig. 3) the inoculation consisted of both bacteria and plankton as in the case of the corresponding series of observations at 20 and 37°. The plankton, however, failed to grow at 50°, so that the oxidation of the dextrose was effected only by the bacteria. This circumstance offers a very satisfactory explanation for the decreased percentage of oxidation observed in these experiments in comparison with the more complete oxidation observed at 20 and 37° in Series 5 and 6. Mention should also be made of the fact that the lag in Series 5 which contained the catalyst is much more pronounced than in Series 6 from which the catalyst mixture was omitted. This is a reversal of the condition observed in the corresponding experiments at 20°. It is probably fair to conclude that, in either case, the lag was due to the inherent variability of bacterial growth, especially when a comparatively light inoculum is used.

Discussion

Spoehr (Ref. 2, p. 1500) notes a pronounced lag in carbon dioxide formation during the oxidation of dextrose at 37° and cites a similar observation by Witzemann[17] in the catalytic oxidation of dextrose with hydrogen peroxide and disodium phosphate. Spoehr and Smith (Ref. 11, p. 239) state that "no definite theory can as yet be advanced to account for this lag, but it is probable that intermediate products . . . are being formed." In our experiments the lag which was observed in most experiments was

(17) Witzemann, *J. Biol. Chem.*, **45**, 1-22 (1920).

clearly due to the delayed growth of bacteria when a light inoculum was added at the start or when reliance was placed on chance contamination of the samples.

As no attempt was made in these studies at the strict duplication of published experimental conditions, it would be hazardous to institute too close a comparison between the results presented in this paper and the findings of Spoehr and his associates. This is certainly true of the results obtained by Degering and Upson at 98°, and by Spoehr and Smith at 63.5°, although a luxuriant growth of thermophilic microörganisms may be obtained at the latter temperature. In the absence of specific information regarding bacteriological precautions, reasonable doubt may nevertheless be expressed regarding the validity of the claims made by Spoehr for the catalytic activity of the iron pyrophosphate complex at 38° and by Degering and Upson in similar experiments at 48°.

The objection regarding the possible inclusion of living cells in the appraisal of proposed models of biocatalytic oxidation is not peculiar to the work of Spoehr. It recurs persistently throughout the extensive literature of oxidations of glucose and other organic substances *in vitro*. Thus Dube and Dhar,[18] in their work on the induced oxidation of glucose in presence of insulin, carefully state that "in every experiment a fresh solution of insulin was taken, as it was observed during the experiments that the solution putrefies on keeping." As they make no further mention of bacteriological precautions, it is perhaps not unreasonable to infer that their results may have been complicated by uncontrolled bacterial fermentations, especially in their work with the bicarbonate buffers. Negative results were obtained by Spoehr and Smith (Ref. 11, p. 247) under similar conditions.

Numerous other instances might be cited where an apparent neglect of the biological factor introduces a reasonable degree of uncertainty in otherwise rigidly controlled investigations. The presence of supposedly antiseptic agents, such as hydrogen peroxide, or the use of relatively high temperatures (50 to 60°) is by no means an adequate safeguard against bacterial contamination. At much higher temperatures, however, the oxidation of glucose and related compounds may be complicated by purely thermal effects. Likewise it should be hazardous to ascribe an oxidation to hydroxyl-ion catalysis when the P_H value of a medium ranges from 9 to 10, as many organisms thrive under those extreme conditions.

Discounting known thermal, bacterial or enzymatic, and hydroxyl-ion effects, it appears doubtful whether the purely catalytic oxidation of such a fairly stable compound as dextrose has ever been accomplished *in vitro*. In fact, the necessity for assuming the presence in biological oxidations of a highly specialized intracellular catalyst largely disappears if the view is adopted, along the lines of Clark's[19] reinterpretation of the Wieland

(18) Dube and Dhar, *J. Phys. Chem.*, **36**, 444 (1932).

(19) Clark and co-workers, Hygienic Laboratory Bulletin No. 151, pp. 335, *et seq.*

hypothesis, that the products of bacterial metabolism emerge from the cell as highly reactive reduction products which may then combine with dissolved oxygen, if this element happens to be present, or with other easily reducible compounds. In this sense, the various respiratory pigments based on complex organic combinations with iron, copper, manganese, zinc, etc., would serve simply as reservoirs for the maintenance of an abundant supply of available oxygen beyond the restricted solubility of dissolved oxygen. With a view to simulating bacterial and other metabolisms, it may be more logical to look for a catalyst for the primary reduction process rather than for the secondary oxidation of the reduction products.

Such a view of bacterial decompositions is strengthened by the fact that the specific rate of oxidation of organic matter through microbial agencies is independent of the concentration of available oxygen. It has also been shown that this specific rate of oxidation is much the same whether oxygen is supplied as dissolved oxygen or as nitrates.[20] It would seem unnecessary in this instance to assume the intermediate action of a specialized oxygen carrier which also acts as a respiratory catalyst or ferment. There is evidence also that the temperature coefficient is of the same order of magnitude in aerobic processes for the so-called stabilization of organic matters as in the strictly anaerobic processes of digestion by gasification. It is suggestive that the underlying process is basically the same in either case.

Summary

1. The efficacy of the iron pyrophosphates as catalysts for the air oxidation of dextrose has been tested at ordinary temperatures and at P_H values within the physiological range.

2. Negative results were invariably obtained in the range from 20 to 50° whenever bacteria were excluded from the solutions.

3. It does not appear that the iron pyrophosphate mixtures are effective in stimulating the oxidations accomplished by bacteria alone or by symbiotic growths of bacteria and plankton.

4. Within the inherent variability of bacterial growth, it is safe to say that numerous varieties of bacteria exist which are quite indifferent to the presence of iron pyrophosphates.

5. The customary bacteriological precautions should obviously be observed in studies of catalytic oxidations within the normal range of microbial growth.

6. The view is expressed that assumptions regarding the presence of specialized intracellular catalysts may be unnecessary in the interpretation of the known facts regarding bacterial oxidations.

CINCINNATI, OHIO

RECEIVED OCTOBER 8, 1932
PUBLISHED MAY 6, 1933

(20) *Cf.* Theriault, Public Health Bulletin No. 173, for a review.