CCXVII. THE RÔLE OF ENZYMES IN ACTIVATED SLUDGE AND SEWAGE OXIDATIONS.

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IN a previous paper [Wooldridge & Standfast, 1933] it has been shown that the oxygen absorption of sewage from aqueous solution saturated with air generally depends upon the presence of bacteria, although these bacteria need not necessarily be alive provided that certain of their oxidation enzymes are still active. Experiments with activated sludge, using the Thunberg-methylene blue technique [Wooldridge, 1933], indicated that sewage sludges possessed active oxidation-reduction enzymes, which appeared to be of importance in the various processes of purification of sewage. In 1932 [Wooldridge & Standfast, 1932] a short note summarizing the results of these experiments was published and it was tentatively concluded "that the most important factor in sewage purification is a series of catalysed oxidation-reduction reactions determined by bacterial enzymes present in either living or dead bacterial cells or liberated by them into the fluid of the reaction system". In the papers of this series, but particularly in the present and subsequent ones, evidence in support of this view is given. This evidence has been obtained by determining the absorption of oxygen by sewage or sludge or both by direct measurement in a Barcroft respirometer under different conditions, the basic conditions simulating, on a small scale, those applying to the activated sludge process of sewage purification.

The effect of sterilization. When sewage is shaken with air in a Barcroft respirometer a rapid absorption of oxygen is observed, the extent of this absorption depending largely upon the so-called "strength" of the sewage [Wooldridge & Standfast, 1936, 1]. If the sewage is previously sterilized either by heating it for 30 min. at 120° or by passage through a Seitz bacterial filter no oxygen will be absorbed provided that precautions are taken to keep the system sterile throughout the experiment. Similar results are obtained with activated sludge; thus the rapid oxidation of this material is entirely inhibited by previously autoclaving the sludge. Further, a sterile mixture of sludge and sewage exhibits no oxygen uptake whether the mixture itself is autoclaved or whether the constituents are sterilized separately and subsequently mixed.

This loss of ability of sewage systems to absorb oxygen when they are rendered sterile can be demonstrated equally well by treatment with disinfectants. For this purpose such oxidizing disinfectants as permanganate, iodine, hydrogen peroxide *etc.* are to be avoided and chemically inert reagents such as ether, toluene, chloroform *etc.* are used. In the experiments recorded in this paper, the bulk of the disinfectant, after its period of action on the sewage, was generally removed, first by separation of the aqueous and non-aqueous layers and then by removal of the remaining disinfectant from the treated sewage by gentle aeration at low pressure. Tests for bacterial sterility were carried out on all the treated sewages and in these early experiments (*cf.* Tables VIII and IX) it was invariably

Table I. *The inability of sterile sewage or sterile sludge to absorb oxygen.*

The sewage (C.S.) used in these experiments was filtered through filter paper and then allowed to stand overnight alone or in contact with the disinfectants given below. The autoclaved (C.S. 120°) and the Seitz-filtered (C.S. sf.) sewage were prepared from the untreated sewage by autoclaving at 120° for 30 min. or by passage through a Seitz filter respectively. The respirometers were shaken at 24°. In each right-hand cup were placed 3 ml. of the sewage mixture and in the left-hand cup 3 ml. of water that had been treated similarly to the sewage. Volatile reagents were removed as far as possible by evacuation. As usual carbon dioxide was absorbed by potash. The figures give the oxygen absorption in μ l. at N.T.P. in 24 hours; the method of sterilization, if any, is given at the head of the columns.

C.S. untreated 240	C.S. 120° 2	C.S. sf. 0.5	10% ether 4	10% chloroform 4.5	1% toluene 1.5
10% phenol 4.5	2% formaldehyde 3	Untreated sludge 610	Sludge 120° 2	C.S. 120° + Sl. 120° 3	

found that when the sewage was sterile no oxygen absorption (within experimental error) took place (Table I). It would appear that little, if any, purely chemical oxidation took place with the sewages examined, for destruction of all oxidizable substances by every method of sterilization adopted is unlikely.

Effect of addition of non-sterile sewage upon oxygen absorption by sterile sewage. That the oxidizable material of a sewage has not been destroyed or removed to any great extent by the process of sterilization is readily shown by adding a small amount of non-sterile sewage to an autoclaved or a Seitz-filtered sewage, when a steady uptake of oxygen subsequently develops. The addition of the "seeding" of non-sterile sewage appears to initiate the process of oxidation, probably as the result of the bacteria added in the seeding. Sterilization possibly induces some change in the constitution of the original sewage, for the rates of absorption of oxygen by seeded sewages, sterilized by different means, sometimes differ significantly (Table II).

Table II. *Reactivation of sterile sewage by the addition of non-sterile sewage.*

The right-hand cups of the respirometers contained 1 ml. of the sterile sewage mentioned below. All cups contained 1 ml. of the sterile buffer and except for the sterile controls 0.1 ml. of a non-sterile crude sewage was placed in the side arms and tipped into the main bulk of the cups after preliminary shaking for 1 hour. The contents of each cup were made up to 3 ml. with sterile tap-water. Carbon dioxide was absorbed in all cups by potash. The figures represent the oxygen uptake in μ l. at N.T.P. The temperature of the experiments was 22°.

Sewage	Sewage seeding	2	18	24	42	48	66	72	90	138 hr.
C.S. sf.	0	0	0	0	0	0	0	0	0	0
C.S. sf.	+	17	21	29	59	67	80	84	91	101
C.S. 120°	0	0	0	0	0	0	0	0	0	0
C.S. 120°	+	9.2	13	19	48	54	70	72	76	86

Oxidation of sterile sewage in the presence of sludge. It is to be expected that the rate of oxidation of sterile sewage upon the addition of a small inoculum of crude sewage would be slow, as the amount of bacteria or enzyme thus added is small. Sterile sewage, however, may be oxidized rapidly by the addition of activated sludge. If equal amounts of washed sludge are placed in the cups of a Barcroft respirometer and a quantity of sterile sewage is added to the sludge in one cup then a greater absorption of oxygen takes place in this cup than in the other. This difference, which is readily followed manometrically, is, in fact, a

Table III. *Oxidation of sterile sewage in presence of sludge.*

In these experiments all cups except those of the controls contained 1 ml. of sludge, 1 ml. of phosphate buffer pH 7.4 and 0.5 ml. of water. Each left-hand cup had 0.5 ml. of water and each right-hand cup 0.5 ml. of the sterile sewage, the C.S. sf. and C.S. 120° being placed in the side-arm and subsequently tipped into the main cup after preliminary shaking for 20 min. The sterile control respirometers had 1 ml. of water in their cups in place of the sludge. C.S. sf., C.S. 120° and Sl. 120° = Seitz filtered sewage, autoclaved sewage and autoclaved sludge respectively. Carbon dioxide was absorbed by potash. The figures represent the oxygen absorbed in μ l. at N.T.P. by the sewage in the presence of sludge.

Sewage added (right-hand cup)	Presence of sludge (in both cups)	5 hr.				
		1	2	3	4	
0.5 ml. non-sterile C.S.	+	50	90	114	122	123
0.5 ml. C.S. sf.	+	49	83	105	113	119
0.5 ml. C.S. 120°	+	53	67	75	77	79
0.5 ml. C.S. sf.	0	0	0	0	0	0
0.5 ml. C.S. 120°	0	0	0	0	0	0
0.5 ml. sludge 120°	+	19	36	54	72	92
0.5 ml. sludge 120°	0	0	0	0	0	0
0.5 ml. sludge 120° + 0.5 C.S. sf.	0	0	0	0	0	0

measure of the rate of oxidation of the "sterile" sewage in the presence of the sludge. The results given in Table III show this oxidizing activity of sludge towards sterile sewage. Autoclaved sludge does not absorb oxygen but, like sterile sewage, it still contains constituents which can be readily oxidized by enzymically active sludge. A mixture of autoclaved sludge with a Seitz-filtered sewage shows no oxygen absorption (Table III). From the experiments described above it appears that the greater proportion, if not the whole, of the oxygen-absorbing power of sewage and sludge depends upon the presence of living material or of agents, e.g. enzymes present in the sewage or sludge, that are equally easily destroyed.

Organisms as a source of enzymes in sewage oxidations. Activated sludge must be considered as a living system comprising many forms of life, the chief of which are normally bacteria and protozoa. As the oxidation of sewage probably depends upon the presence of living organisms or their enzymes, the ability of a number of bacterial suspensions (some prepared from organisms isolated from sewage, others from laboratory stocks) to oxidize sterile sewage has been examined (Table IV). Most bacteria, when present in large numbers, in pure or mixed populations effect oxidation of sterile sewage to some extent. Some bacteria, e.g. laboratory strains of *Staph. aureus* and *Strep. faecalis*, brought about very little oxidation of the sewage. The results of Table IV are not strictly comparable, as neither the sewages nor the density of the bacterial suspensions were standardized, although the bacterial suspensions usually contained approximately 5×10^9 cells per ml. The most active organisms were *Bact. alkaligenes*, *Proteus vulgaris*, *Pseudomonas pyocyanea* and *Ps. fluorescens*; *Bact. coli* appeared to be less active. These bacterial suspensions were similarly able to bring about the oxidation of autoclaved sludge. The question of the importance of protozoa in sewage oxidations is frequently discussed and opinions are divided as to whether they are useful or deleterious in processes of sewage purification. Certain protozoa, absolutely free from bacteria, have been examined to see whether they are able to effect oxidation of sewage. For this purpose Miss Robertson of the Lister Institute kindly supplied two strains of bacteria-free protozoa, viz.

Table IV. *Oxidation of sterile sewage or sludge by bacteria or protozoa.*

The sewages used in the experiments below were not identical throughout and therefore the rate of oxidation in the presence of any one organism cannot be compared strictly with that obtained by other organisms; large differences are probably significant as the suspensions were of approximately the same strength as determined by turbidity standards ($\cong 5 \times 10^6$ standard of *Bact. coli*). The organisms used consisted of pure cultures either isolated from sewage or obtained from laboratory cultures, except when otherwise stated. The bacteria were grown on nutrient agar for 24 hours at 22° or 37°, were washed off with sterile quarter-strength Ringer solution, washed twice and resuspended in this Ringer solution. The protozoa were grown in special bacteria-free media and were kept free from these organisms. These suspensions were used much thicker than those of bacteria. Sludge 120°, C.S. 120°, and C.S. sf. signify respectively autoclaved sludge, autoclaved sewage and sewage after passage through a Seitz bacterial filter. All cups of the Barcroft respirometers contained 0.5 ml. of sterile phosphate buffer, pH 7.4, and 1 ml. of the organism used except in the control experiments 24, 25 and 26 (no organism) and experiments 21 and 22 (only 0.5 ml. so that 1 ml. of mixed organism could be used in experiment 23). 1.5 ml. of sterile sewage or autoclaved sludge suspension were added to the right-hand cups and the total volumes were made up in both cups to 3 ml. with sterile water. CO₂ was absorbed by KOH. The protozoal cups were examined for bacterial sterility at the end of the experiments and none showed evidence of bacterial contamination. The figures represent the oxygen absorption in μ l. at N.T.P.

Organism; present in both cups	Sewage preparation in right cup	1	2	3	4	5 hr.
Bacteria from sewage.						
1. Mixed bacteria grown direct from sewage	C.S. 120°	32	57	73	84	93
2. Organism No. 1	C.S. sf.	23	35	45	49	55
3. Organism No. 2	C.S. sf.	32	53	69	76	80
4. Organism No. 9	C.S. sf.	21	34	44	52	59
5. Organism No. 14	C.S. sf.	49	87	108	112	114
6. Organism No. 16	C.S. sf.	24	33	39	43	49
Bacteria from other sources.						
7. <i>Pseudomonas pyocyanea</i>	Sludge 120°	155	267	333	417	—
8. <i>Ps. pyocyanea</i>	C.S. 120°	83	116	145	169	—
9. <i>Ps. fluorescens</i>	Sludge 120°	35	80	119	—	178
10. <i>Ps. fluorescens</i>	C.S. 120°	23	57	105	—	189
11. <i>Ps. fluorescens</i>	C.S. sf.	15	45	87	—	170
12. <i>Chr. prodigiosum</i>	C.S. 120°	21	42	58	68	—
13. <i>Bact. coli</i>	C.S. 120°	16	28	39	49	—
14. <i>Bact. aerogenes</i>	C.S. sf.	28	52	80	103	127
15. <i>Proteus vulgaris</i>	C.S. sf.	54	96	137	—	215
16. <i>Staph. aureus</i>	C.S. sf.	1.5	2.1	3	3.6	4
17. <i>Bact. alkaligenes</i>	C.S. sf.	60	112	161	—	257
18. <i>Strep. faecalis</i>	C.S. sf.	6.5	13	19	20	20
Protozoa (bacteria free).						
19. <i>Polytoma uvella</i>	C.S. sf.	35	49	58	69	—
20. <i>Euglena gracilis</i>	C.S. sf.	11.5	24	38	51	—
Bacteria + Protozoa.						
21. <i>Ps. fluorescens</i>	C.S. sf.	56.6	95	123	—	204
22. <i>Polytoma</i>	C.S. sf.	71.4	91	104	—	122
23. <i>Ps. fluorescens</i> + <i>Polytoma</i>	C.S. sf.	119	165	209	—	257
Controls (typical).						
24. —	Sludge 120°	0	0	0	0	0
25. —	C.S. 120°	0	0	0	0	0
26. —	C.S. sf.	0	0	0	0	0

Polytoma uvella and *Euglena gracilis*, which could be cultivated in the absence of bacteria. Suspensions of washed protozoa in quarter strength Ringer solution were prepared and their ability to promote oxidation of sterile sewage was examined in the Barcroft apparatus. Although the rate of oxidation of sterile sewage by bacteria-free protozoa appears to be comparatively poor, it nevertheless is definite but less than that of most of the bacteria examined. Table IV contains the results obtained with the most active preparations used; the turbidity of these suspensions was considerably greater than that of the bacterial suspensions examined. (Protozoal suspensions usually had a turbidity equivalent to that of suspensions of *Bact. coli* containing $20-50 \times 10^9$ organisms per ml.) The ability of *Polytoma* to cause oxidation of sterile sewage, although feeble, can be shown by the anaerobic methylene blue technique [*v.* Wooldridge, 1933]. Thus a suspension of *Polytoma* that took 46 min. to reduce 1 ml. of a 1/5000 solution of methylene blue at pH 7.4 was able to effect the same reduction in 25 min. in the presence of 2 ml. of a Seitz-filtered sewage: the methylene blue was not reduced by the sterile sewage alone.

Table V. *A comparison of the rates at which various organisms cause oxidation of sterile sewage.*

In these experiments the Barcroft respirometer had equal quantities of organism in the cups; sterile sewage was added to the right cup only. The volume of the liquid was made up to 3 ml. with 0.5 ml. sterile buffer, pH 7.4, and sterile tap-water. The thickest suspensions of both the sludge and the organisms were in Ringer solution and were of such a strength that on drying on a boiling water-bath and in a hot air-oven at 98°, the dry weight obtained on cooling in a desiccator was 0.02 g. per ml. suspension. The figures represent the uptake of oxygen in μ l. at N.T.P. by the sewage. CO₂ was absorbed by KOH.

Organism suspension	Dry wt.							
	per ml.	1	2	3	4	6	9	22 hr.
Sludge	mg.							
	20	80	116	141	156	—	—	—
	2	30	60	95	115	—	—	—
<i>Ps. fluorescens</i>	0.2	9	20	29	38	—	—	—
	20	82	93	100	106	—	—	—
	2	26	61	78	82	—	—	—
	0.2	10	20	35	41	—	—	—
<i>Bact. coli</i>	0.02	7	10	12	14	18	25	172
	20	20	29	33	37	—	—	—
	2	11	19	26	33	—	—	—
	0.2	6	10	17	22	—	—	—
<i>Chr. prodigiosum</i>	0.02	5.5	9	11	11.5	12.5	14	78
	20	29	39	45	49	—	—	—
	2	14	27	39	45	—	—	—
	0.2	2	5	9	13	—	—	—
Staphylococcus	20	3	4	5	6	—	—	—
	2	2	3	3	4	—	—	—
	0.2	0	0	0	0	—	—	—
<i>Polytoma uvella</i>	20	14	19	20	21	—	—	—
	2	3	4	5	7	—	—	—

A direct comparison of the oxidative powers of a few of these organisms towards the same sterile sewage was made by using suspensions containing equal amounts of the various cells as measured by the dry weight of the suspensions (Table V). The activity of sludge was most nearly approached by that of *Pseudomonas fluorescens*: *Bact. coli* and *Chromobacterium prodigiosum* were less active and a laboratory strain of a staphylococcus showed very little oxidizing

activity. *Polytoma* showed an oxidizing power somewhat greater than that of the staphylococcus, but less than that of *Bact. coli*. The weakest suspensions of *Bact. coli* and *Pseudomonas fluorescens* show little difference in the first 4 hours but after that, probably because of a greater ability of the latter organism to proliferate in the sewage, the difference between their oxidizing powers becomes greater. Previous results, obtained with the test for biochemical oxygen demand in 5 days, have tended to exaggerate somewhat these differences between different organisms, for no oxygen was absorbed from solution when *Bact. coli* was added to the sterile sewage to an extent of 0.02 mg. per ml. whilst the corresponding addition of *Ps. fluorescens* caused the oxygen to be almost completely removed (> 16 precipitate 10^5). This difference was no doubt due to the fact that *Ps. fluorescens* grew well during the experimental period of 5 days, thereby producing a large amount of active enzymes, whilst *Bact. coli* died off without causing absorption of a measurable quantity of oxygen [v. Wooldridge & Standfast, 1933].

Butterfield *et al.* [1931], working with a dilute peptone-sugar medium in place of a sewage, brought forward evidence to show that protozoa, e.g. *Colpidium*, by keeping down the numbers of bacteria stimulate the bacteria to further growth and thus increase the rate of absorption of oxygen so that the value obtained for the mixture of bacteria and protozoa is greater than that of the sum of the two acting separately. The effect of mixing pure suspensions of *Ps. fluorescens* and *Polytoma uvella* has been examined with the Barcroft apparatus but no increased oxidation of the sewage as a result of the presence of the protozoa with the bacteria at least during the few hours of this experiment (Table IV, expts. 21, 22 and 23) was found. It is nevertheless possible that the mixed population would prove to be more active when it had settled down into equilibrium.

Addition of urethane to oxidizing sewage and sludge. The previous experiments appear to indicate that sewage or sludge is not oxidized by air in the absence of living organisms, especially bacteria. Many bacteria [Quastel & Whetham, 1925; Quastel & Wooldridge, 1925] possess enzymes, such as dehydrogenases, that will effect the oxidation of a variety of substances under suitable aerobic or even anaerobic conditions. Wooldridge [1933] has shown that sewage sludges possess some of these enzymes and that sewage usually contains constituents in solution that are able to act as substrates for these enzymes. The results given in Tables VI and VII testify that, as with oxidations by bacterial dehydrogenations, sewage oxidations as observed in the Barcroft apparatus are entirely inhibited by the presence of 5% urethane.

Addition of cyanide to oxidizing sewage and sludge. Wooldridge [1933] showed that activated sludge possesses indophenol oxidase besides various dehydrogenases and suggested that both types of enzyme were important in sewage oxidations. If this is so then, as the activity of indophenol oxidase is completely inhibited by $M/1000$ KCN, this concentration of cyanide should arrest the absorption of oxygen by sewage. Experiment [Table VII] indicates that the uptake of oxygen by crude sewage or by sludge is greatly inhibited by this and greater concentrations of cyanide, particularly for the first few hours. Gradually, however, the inhibiting effect appears to wear off, probably as a result of changes which the cyanide itself undergoes. The inhibiting effect of cyanide can be shown quite readily by placing solid KCN in the side-arm of the Barcroft cup, when apparently sufficient HCN diffuses over into the fluid to inhibit the oxidation. It is perhaps surprising that the effect is so soon demonstrable, for it is apparent as soon as the manometers are closed. In this way a continuous supply

Table VI. *The effects of cyanide and of urethane upon the oxidation of crude sewage.*

The right cup of the Barcroft respirometer contained 2 ml. crude sewage, 0.5 ml. phosphate buffer pH 7.4 and 0.5 ml. of water or of the inhibitor, the strength of which was such that its final concentration was as stated below. The cyanide solutions were previously neutralized with HCl to pH 7.4. The contents of the left cup were the same except that the sewage was replaced by water. The solid KCN was placed in the side-arm of the cups and was not in direct contact with the fluid at all. KOH papers were present to absorb CO₂. The figures represent the absorption of oxygen in μ l. at N.T.P. Viable counts were done on the contents of the cup of the crude sewage and the solid KCN experiments at the end of the experiment.

	3 hr.	5 hr.	7 hr.	11 hr.	21 hr.	27 hr.	Viable count bacteria
Crude sewage alone	130	235	291	366	451	545	15×10^6
C.S. + solid KCN in side-arm	36	36	37	38	41	42	0.9×10^6
C.S. + <i>M</i> /200 KCN	10	12	14	18	125	310	—
C.S. + <i>M</i> /500 KCN	15	15	21	26	175	321	—
C.S. + <i>M</i> /1000 KCN	15	21	25	56	345	410	—
C.S. + <i>M</i> /10,000 KCN	60	165	274	350	440	520	—
C.S. + 5% Urethane	—	—	3	—	5	5	—

Table VII. *The effects of cyanide and of urethane on the oxidation of activated sludge.*

1 ml. of a suspension of washed sludge was placed in each right-hand cup of the respirometers. 0.5 ml. of buffer, pH 7.4, was put into each cup and the contents were made up to 7 ml. with the addition of water or inhibitor solution so that the strength of the latter was as stated in the table. The solid KCN was placed in the side-arms out of contact with the fluid. The figures give the oxygen uptake in μ l. at N.T.P. CO₂ was absorbed as usual by KOH. Final bacterial counts were made on the suspensions of sludge in the control and solid KCN experiments, and both gave figures of approximately 1 million per ml. Experimental temperature, 21°.

	1 hr.	2 hr.	3 hr.	5 hr.	7 hr.	21 hr.	Viable count bacteria
Sludge alone	33	52	70	103	136	338	3×10^6
Sludge + solid KCN in side-arm	6	13	18	26	33	70	3×10^6
Sludge + <i>M</i> /10 KCN	7	11	12	17	20	—	—
Sludge + <i>M</i> /100 KCN	11	18	26	38	45	—	—
Sludge + <i>M</i> /1000 KCN	15	29	38	64	86	—	—
Sludge + <i>M</i> /10,000 KCN	22	32	52	79	103	—	—
Sludge + 5% urethane	—	—	—	—	1.3	3	—

of cyanide is available and the inhibiting effect continues for long periods. In order to discover whether this inhibition is due to sterilization of the medium, bacterial counts were made at the end of the experiments on the crude sewage, and on the sewage and sludge mixture with solid KCN in the side-arms. It was found that although the number of viable bacteria was reduced in the case of sewage and cyanide to about a sixteenth of those present when the cyanide was absent yet there were still approximately a million viable organisms per ml. whilst with sludge the fall in numbers seemed inappreciable. The viable count on the sludge mixture must be accepted with reserve as any change in the number of viable organisms actually enmeshed in, or adsorbed on, the sludge would not be determined. In both experiments, however, the number of viable organisms present at the end was sufficient to make it appear unlikely that the effect of the cyanide is solely or even largely a bactericidal one.

Bacterial viability and oxygen uptake of sewage and sludge. It has previously been reported [Wooldridge & Standfast, 1933] that sterile sewage, incubated

under the conditions of the test for biochemical oxygen demand, will not absorb dissolved oxygen but that it will do so if heavily inoculated with a suspension of *Ps. fluorescens* previously treated with formalin so that many of the bacterial oxidizing enzymes remain active although the organism has lost the power of proliferation. Attempts to extend this work to a demonstration of oxygen absorption in the Barcroft apparatus have been only partially successful. The results given in Table VIII illustrate that formaldehyde-sterilized bacteria are capable of inducing the oxidation of sterile sewage; the oxygen uptake is small, viz. about 20 μ l. per ml. of sewage in 5 hours, but significantly different from the controls. Sterilization of the sewage by other disinfectants always resulted in

Table VIII. *The oxidation of sterile sewage by formaldehyde-treated bacterial suspensions.*

The suspensions used had an opacity $\equiv 10 \times 10^7$ cells of *Bact. coli* per ml. The suspension was treated overnight at room temperature with the concentration of formaldehyde given below. All right-hand cups contained 1 ml. of autoclaved sewage and 1 ml. of phosphate buffer (sterile) was placed in all cups. 1 ml. of the treated bacterial suspension was added to each cup of the respirometer as given below. Other experimental details as in previous tables. Each experiment was made in quadruplicate and the mean results are given below. The replicates differed little one from another. The sterility of the mixtures in the cups was tested at the beginning and end of the experiment.

Organism	HCHO concn. %	μ l. O ₂ absorbed at N.T.P. in hours					Viable bacteria	
		1	2	3	4	5	Begin- ning	End
<i>Ps. fluorescens</i>	0.36	9.3	14	17.4	20.4	22.2	0	0
"	0.15	10.1	14.3	18.6	23.1	26.3	0	0
"	0.05	17.1	26.1	33.3	34.2	34.8	+	+
None	0	6	7	7.5	8	8	0	0
Mixed sewage	1.1	9	13	14.7	16.1	16.9	0	0
"	0.8	11.4	16	18	20.1	21.2	0	0
"	0.55	11.4	15.8	18.2	20.5	21.3	0	+
None	0	3	3.8	4.8	5	5	0	0

Table IX. *Oxygen absorption by sewage sterilized by irradiation with ultraviolet light.*

The sewage was sterilized by irradiation with ultraviolet light for 75 min. at a distance of 4 in. The irradiation was given in three periods of 25 min. with 10 min. intervals. The temperature of the sewage, which was gently stirred during irradiation, did not rise above 42°. The right-hand cups of the respirometers contained 3 ml. of the sewage mentioned below and the left-hand cups contained 3 ml. of tap-water. CO₂ was absorbed by KOH. All cups were sterilized at the beginning of the experiment, and 0.5 ml. of the contents of the right-hand cups was added to 5 ml. of broth at the end of the experiment in order to test for viable organisms. The results given are the mean of the readings of 5 replicate respirometers.

Sewage	ml. oxygen absorbed in 3 hours	Growth on inocu- lation into broth
Crude	37.6 (s.e. = 1.25)	+
Irradiated with ultraviolet light	27.5 (s.e. = 2.08)	+
Autoclaved	0.5 (s.e. = 3.48)	+

destruction of the ability to absorb oxygen. Irradiation with ultraviolet light sterilizes and ultimately destroys the power of sewage to oxidize in the presence of air, but if the irradiation be carefully controlled the sewage may be rendered sterile while still able to absorb oxygen. It is clear from the results given in Table IX, where each experiment was made in quintuplet, that the sterile

irradiated sewage absorbed oxygen almost as rapidly as did the crude sewage. Cook & Stephenson [1928] showed that bacteria killed by irradiation with ultra-violet light may retain the activity of oxidizing enzymes when they are able to absorb oxygen in the presence of suitable substrates, and so the uptake of oxygen by irradiated sewage is probably due to the fact that although the bacteria have been killed all their oxidizing enzymes have not been destroyed.

Identical suspensions of bacteria were irradiated with ultraviolet light for variable periods of time in order to obtain suspensions with the same total number of cells but with different numbers of viable organisms. The abilities of these treated suspensions to induce oxidation of sterile sewage were determined by the Barcroft apparatus, and the results compared with the viable counts obtained by examining the contents of the cups at the beginning and at the end of the experiments, the counts being made by the agar roll tube method [Wilson, 1922]. The results are given in Table X. The number of viable organisms did

Table X. *Bacterial viability and sewage oxidations.*

The suspension of washed *Ps. fluorescens* used had a turbidity equivalent to that of a *Bact. coli* suspension of 30×10^6 cells per ml. 5 ml. volumes of this suspension were exposed to irradiation by ultra-violet light in quartz bulbs 4 in. away from the lamp for the times mentioned below. The temperature of the bulbs was kept low by submitting them continuously to a current of cold air. 0.5 ml. of the bacterial suspension, when used, was placed in each cup of the manometer, and 2 ml. of the Seitz-filtered sewage were placed in all right-hand cups. 0.5 ml. sterile buffer, pH 7.4, was in all cups. CO_2 was absorbed by KOH. All experiments were made in quadruplicate and checked by the method recommended by Corbet and Wooldridge [1936].

Exposure to U.V.L. min.	V.C. in cup		Oxygen uptake in $\mu\text{l.}$ after			
	Original	Final	30 min.	60 min.	90 min.	120 min.
0	5635×10^6	3760×10^6	41	63	73	86
5	1250×10^6	340×10^6	44	70	82	87
10	125×10^6	47×10^6	29	64	72	77
15	20×10^6	7×10^6	7	31	59	80
0	0 (Sterile sewage only)	0	3.5	4	4.6	5

not increase, but, in fact, decreased somewhat during the course of the experiment, and hence bacterial proliferation is an unlikely explanation of the results obtained. The viable organisms in the initial counts varied from 5635×10^6 down to 20×10^6 per ml. yet at the end of 2 hours the oxygen absorption was the same; even the initial rate of oxidation shows no direct correspondence with the viable count as is seen from the fact that the oxygen uptake in 30 min. with 5635×10^6 organisms was only 6 times that with 20×10^6 organisms although the ratio of the viable organisms present was 282 to 1. This experiment clearly indicates that the oxidation of sterile sewage can be induced by bacterial cells that are not regarded as being alive by ordinary standards of proliferation.

DISCUSSION.

No attempt has been made, in this series of papers, to study the clarification stage of the activated sludge process of sewage disposal but much work has been carried out upon the second stage of oxidation. In effect this process is a complex of oxidations of the numerous constituents found in sewages, and practically the whole of these oxidations would seem to depend upon biological factors. Sterile sewage and sterile sludge generally undergo no oxidation but when micro-organisms such as certain bacteria or protozoa are added the oxidation will take place.

Different organisms are not necessarily equally effective and no doubt the activity of any particular organism will depend upon the nature of the sewage, i.e. whether it contains substances which can act as substrates for the particular enzymes available, and also upon the condition of the oxidative enzymes of the organism. Wooldridge *et al.* [1936] have shown that the activity of these bacterial enzymes may vary considerably with varying conditions. The protozoa examined exhibit less activity than that of the bacteria, but even if the protozoa, under the conditions of the activated sludge process, were more active than appears from these experiments, their activity, in comparison with that of the very much more numerous bacteria usually present, must in practice be of secondary importance. The results have not demonstrated increased activity resulting from mixing protozoa and bacteria together but it may be that these experiments, which were only allowed to proceed for 5 hours, were too short to show the effects reported by Butterfield *et al.* [1931].

It has been shown previously [Wooldridge, 1933; Wooldridge & Standfast, 1932 and 1933] that sewage and sludge possess such enzymes of oxidation as indophenol oxidase and dehydrogenases, and it was suggested that these enzymes are mainly bacterial in origin. Previously Keilin [1929] indicated an important co-ordinating mechanism by which many cells possessing both these systems can utilize molecular oxygen in their cellular oxidations. The co-ordinating mechanism is an "oxygen carrier" and is a substance, e.g. cytochrome, which can exist in both an oxidized and a reduced form, the two being readily interconvertible. Molecular oxygen is activated by the indophenol oxidase and the activated oxygen reacts with the reduced form of the carrier. The resulting oxidized form of the carrier diffuses away from the oxidase and may reach a dehydrogenase, where it will react with the activated substrate of the dehydrogenase, reverting again to the reduced form of the carrier. In this way the molecular oxygen is used indirectly to oxidize the substrate of the dehydrogenase. The view that this or a similar mechanism operates in sewage oxidations is supported by the experiments which show that either cyanide or urethane inhibit these oxidations, for cyanide inhibits the oxidase but not the dehydrogenases while the urethane inhibits dehydrogenases but not indophenol oxidase.

The view that enzymes, as distinct from the whole living organism, are responsible for many of these oxidations is supported by these inhibition experiments, and also by the experiments with formaldehyde-treated organisms, with crude sewage carefully sterilized by irradiation with ultraviolet light, and with bacterial suspensions irradiated with ultraviolet light which show that the oxidation depends more upon the presence of active enzymes than upon the number of viable organisms present. Although these experiments clearly indicate that bacterial enzymes, not associated with living organisms, can be effective in causing oxidation of sewage and sludge the enzymes of living and especially of actively proliferating organisms will also be active and, mass for mass, are likely to be more active than those present in dead cells. With the activated sludge process however the amount of enzymically active material associated with the preponderating numbers of dead cells making up the bulk of the sludge is probably very great and hence the oxidizing activity of this material is likely to be of considerable importance in the oxidations effected by activated sludge.

SUMMARY AND CONCLUSIONS.

1. The work described in this paper supports the view previously put forward that the most important factor in biological oxidations of sewage is a series of catalysed oxidation-reduction reactions determined by bacterial enzymes present in either living or dead bacterial cells or liberated by them into the fluid of the reacting system.

2. Crude sewage, sterilized by filtering through a Seitz bacterial filter, by heat or by various "disinfectants", does not usually absorb oxygen when shaken in a Barcroft microrespirometer (cf. 5 and 6 below). Autoclaved sludge does not absorb oxygen, even on vigorous aeration.

3. Addition of a seeding of crude sewage, a suspension of washed sludge, a suspension of washed bacterial cells (of several species) or a suspension of bacteria-free protozoal cells induces the oxidation of sterile sewage or sterile sludge. Some species of bacteria effect the oxidation of sterile sewage rapidly but with others the rate is comparatively slow. Two species of protozoa have been examined and these are less active than most bacteria. A mixed suspension of *Ps. fluorescens* and *Polytoma uvella* caused no more rapid oxidation of the sterile sewage than the sum of the rates obtained when the bacterium and the protozoon were allowed to act separately.

4. Urethane (5%) inhibits oxidation of sewage and sludge. Cyanide ($M/200$ to $M/10,000$ KCN, neutralized, or the vapour from solid KCN) inhibits the uptake of oxygen by crude sewage and by sludge but the inhibition is not complete and usually lessens with time, presumably due to the alteration of the cyanide.

5. Bacteria carefully treated with formaldehyde, although unable to proliferate on the usual nutrient media, are nevertheless capable of inducing a definite though slow oxidation of sterile sewage.

6. Sewage sterilized by irradiation with ultraviolet light will still absorb oxygen on shaking with air provided the irradiation is not too severe.

7. Several originally identical suspensions of *Ps. fluorescens*, irradiated with ultraviolet light for different periods of time so that the number of viable cells differed considerably from suspension to suspension, oxidized sterile sewage at rates largely independent of the proportion of viable cells present.

8. Oxidation of the constituents of sewage and of sludge depends generally upon the presence of certain oxidative enzymes (dehydrogenases and oxidases) of micro-organisms. These enzymes may be effective whether the organisms are alive or dead, provided that the method of killing has not destroyed the enzymes. Although protozoa possess enzymes that can oxidize the constituents of sewage, it is concluded that the greater proportion of the oxidation is brought about by bacterial enzymes, the bacteria being both far more numerous and generally much more active.

9. Oxidations of sewage depend mainly on the presence of bacterial enzymes, which themselves may be associated with either living or dead cells. No direct comparison has been made between the enzymic activities of living and dead cells but it is probable that the activity of the living proliferating cell for certain oxidations is greater than that of the dead cell. The dead cell is important in oxidations by activated sludge as the sludge is likely to contain a large proportion of dead bacterial cells many of which are probably enzymically active.

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REFERENCES.

- Butterfield, Purdy & Theriault (1931). *Industr. Engng Chem.* **23**, 213.
Cook & Stephenson (1928). *Biochem. J.* **22**, 1368.
Corbet & Wooldridge (1936). *Biochem. J.* **30**, 132.
Keilin (1929). *Proc. roy. Soc. B* **104**, 210.
Quastel & Whetham (1925). *Biochem. J.* **19**, 520, 645.
—— & Wooldridge (1925). *Biochem. J.* **19**, 652.
Wilson (1922). *J. Bact.* **7**, 405.
Wooldridge (1933). *Biochem. J.* **27**, 193.
—— Knox & Glass (1936). *Biochem. J.* **30**, 926.
—— & Standfast (1932). *Nature*, **130**, 664.
—— ——— (1933). *Biochem. J.* **27**, 183.
—— ——— (1936). *Biochem. J.* **30**, 141.