CLXVII. THE BACTERIAL PURIFICATION OF GAS-WORKS LIQUORS

II. THE BIOLOGICAL OXIDATION OF AMMONIUM THIOCYANATE

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ONE of the most widespread liquid trade wastes is what is commonly known as "gas liquor", being waste liquor produced in gas works. It usually consists of a dilute solution of ammonium salts, phenols and other "tar acids", salts of thiocyanic and thiosulphuric acids and small quantities of other substances. The most obvious way of treating any liquid trade waste is to admit it to the sewers and allow it to be purified or otherwise disposed of with the sewage. An investigation has therefore been carried out into the response of gas liquor to processes of sewage purification, and in the course of this work the behaviour of the individual substances contained in the liquor has been studied. Amongst these substances is ammonium (occasionally calcium) thiocyanate, the biological oxidation of which is the subject of this paper.

In a previous communication [Happold & Key, 1932] the removal of phenol and of catechol derivatives from gas liquor was described and it was shown that a gram negative vibrio (0-1) which possessed an indophenol oxidase system was capable of utilizing phenol as a source of carbon and was responsible for its breakdown.

In the study of phenol destruction on the bacteria beds it had been observed that the continuous action of the phenol on sewage effluents facilitated the isolation of vibrio 0-1 by exerting a selective bactericidal action on the rest of the flora. A similar result was expected in these further studies.

The investigation has as before been approached from two separate angles, (i) the study of the conditions of breakdown on the bacteria beds where a large concentration of adapted bacterial life is readily available, and (ii) the study of the bacterial life present in the effluents from these beds and of the capacity of individual micro-organisms to oxidize ammonium thiocyanate. The two sections are divided for the purpose of this report.

STUDIES ON THE BEDS

The general method of testing has been to prepare several units of sewage purification systems, either bacteria beds or activated sludge units, ascertain that these are all in the same condition and produce the same effluents from the same sewage, and then to feed sewage to one unit, and the same sewage to which have been added substances contained in gas liquor to other units. A comparison of the effluents produced gave information concerning the effect of the constituents of the liquors.

For the purpose of investigating the behaviour of ammonium thiocyanate a bacteria bed was used which, with an average sewage, gave a purified effluent of oxygen absorption (o.a.) (4 hr. in contact with excess acid KMnO_4 solution at 80°F.) of about 0.6 pt./100,000. When thiocyanate was added to the sewage to give a concentration in the sewage of 2.4 pt. CNS/100,000 the o.a. of the effluent during the first day had increased to 2.1 pt./100,000, indicating that at least 75% of the thiocyanate had passed unchanged through the bed. On the second day of such treatment the o.a. of the effluent had fallen to 1.5 pt./100,000 and after 6 days had diminished to its original 0.6 pt./100,000 and no trace of thiocyanate remained in the effluent. Apparently during this period the purification system was becoming accustomed to thiocyanate, developing bacteria which could assimilate the substance, and when this had been accomplished complete purification had occurred. When the amount of thiocyanate in the sewage was increased to 4.8 pt./100,000 a similar deterioration of the effluent occurred, followed by complete recovery in a few days. A further temporary deterioration occurred when the dose of thiocyanate was doubled again, but later when the dose was redoubled to give 20 pt. CNS/100,000 no adverse effect was noticed, the bacteria having apparently developed sufficiently to deal with this increase.

Later a repeat of this test was carried out and it was found that by gradually increasing the dose of thiocyanate a concentration of at least 120 pt./100,000 could be completely destroyed during the passage through the bed. This should be contrasted with the fact that originally the bed was unable to deal with one-fiftieth of this amount.

It was also found that activated sludge behaved towards thiocyanate in a very similar manner and it was thought that the phenomenon deserved further chemical and bacteriological study.

Accordingly, a special activated sludge was cultivated to deal with comparatively large quantities of thiocyanate in sewage, the cultivation taking several weeks. The method consisted of aerating a sewage containing 2 pt. CNS/100,000 with an ordinary activated sludge for 24 hr., afterwards "conditioning" the sludge by a further 24 hr. aeration, repeating the process with fresh sewage, and so on. When the whole of the thiocyanate was being dealt with in the first 24 hr. the dose was increased. This procedure was continued until 40 pt. CNS/100,000 were being oxidized in 24 hr. A test was then carried out on the rate of disappearance of the substance.

Rate of oxidation of ammonium thiocyanate

Test 1. The sewage used in the test had an o.a. of 6.7, and the initial concentration of ammonium thiocyanate was 41 pt. CNS/100,000. Table I shows

Table I. *Rate of oxidation of ammonium thiocyanate by activated sludge*

Time of aeration hr.	Conc. CNS pt./100,000	Average rate of disappearance of CNS between readings pt./100,000/hr.
Initial	41	0
1	41	0
2	41	1
3	40	2
5.5	35	2
7	32	2
9	28	3.5
13	14	—
23	Trace	—

how the concentration diminished during the aeration period and also the hourly rate of disappearance between the observations. The thiocyanate concentration was estimated by the usual silver nitrate method, it having been shown that the presence of sewage did not invalidate the titration.

It will be seen that the rate of oxidation of thiocyanate actually increased as its concentration diminished, being negligibly small at first and becoming quite rapid later. Several explanations of this observation suggested themselves: one was that the oxidation of the thiocyanate did not take place until the sewage had been to a large extent purified. A test was therefore made on this point.

Test 2. In Table II are shown the amounts of CNS remaining in (a) sewage, and (b) water after various periods of aeration in the presence of equal amounts of the same sludge.

Table II. *Oxidation of thiocyanate in sewage and water*

Time of aeration hr.	(a) CNS in sewage pt./100,000	(b) CNS in water pt./100,000
Initial	43	44
1	42	43
2	42	43
3	42	42
5	39	40
7	36	37
9	32	32
13.5	19	21
24	0	0

There is no significant difference between the last two columns, showing that the oxidation of thiocyanate was uninfluenced by that of the sewage. The presence of sewage does not therefore account for the negligibly small rate of disappearance of thiocyanate during the early stages of the tests.

Test 3. For this test the sludge was divided into two equal parts. One part was aerated with water for 24 hr.; the second was aerated with water containing 55 pt. CNS/100,000 for the same period, at the end of which the CNS had been reduced to 2 pt./100,000. To each mixture were then added 60 pt. CNS/100,000 and aeration continued, the concentrations after various times being shown in Table III.

Table III

Time hr.	(a) CNS in sludge previously aerated with water pt./100,000	(b) CNS in sludge previously aerated with CNS sol. pt./100,000
0	60	62
3	59	55
7	50	42

The second sludge was obviously much more active than the first, particularly during the first 3 hr. It appears that aerating in the absence of oxidizable matter, a process which would "activate" the sludge with respect to sewage, actually "deactivates" it in relation to thiocyanate, and that some time must elapse before activity is regained. Now in Tests 1 and 2 the sludge had been aerated in the absence of thiocyanate before the commencement of the runs in order to "activate" it. This had actually resulted in temporary "inactivation", causing the oxidation to proceed very slowly at first, although becoming more rapid later.

It was also shown that allowing the sludge to stand out of contact with air, though in the presence of thiocyanate, temporarily destroyed its activity in the same way as did aerating in the absence of thiocyanate.

Effect of concentration

Test 4. The sludge was divided into three equal parts and these were aerated in the presence of different concentrations of thiocyanate, with the results which are expressed in Table IV.

Table IV. *The effect of concentration on the oxidation of thiocyanate*

	A	B	C
Initial conc., pt./100,000	33	67	131
Amounts gone, pt./100,000:			
After 3 hr.	3	2	1
After 6 hr.	6	7	6
After 9 hr.	12	12	9
After 12 hr.	19	20	14
After 13.5 hr.	23	29	20
After 24 hr.	All	All	40

Little difference was shown in the rates of oxidation in the first two cases, the influence of concentration being almost negligible. In the third case, where the concentration was considerably greater than that to which the sludge was accustomed, the oxidation proceeded rather more slowly.

These observations have been confirmed in Test 5, in which the most active sludge yet obtained was used. 30 % of sludge, settled for 1 hr., would completely destroy 200 pt. CNS/100,000 in 12 hr. It was found that with less than 300 pt. CNS/100,000 present the rate of reaction was almost independent of concentration. With larger concentrations, however, the rate was greatly reduced, being only one-tenth as great at 562 pt./100,000 as it was at 282 pt./100,000. The limiting concentration above which oxidation is retarded depends on the condition of the sludge.

Oxidation products

It was found that the products of oxidation of ammonium thiocyanate were not further oxidized by acid potassium permanganate, and that they did not alter the alkalinity of the solution. The only reaction which satisfied these conditions appeared to be

by which the thiocyanate is converted into ammonium sulphate. Determinations of ammonia and sulphate were therefore made during the oxidation of a solution containing 286 pt. CNS/100,000 the results being shown in Table V.

Table V

	After 24 hr. pt./100,000	After 48 hr. pt./100,000
Decrease in CNS	132	286
Increase in NH_3	41	81
Increase in SO_4	231	488
NH_3 increase calculated from CNS decrease	39	84
SO_4 increase calculated from CNS decrease	218	473

In both cases the calculated values are very close to the observed, indicating that the above equation is a quantitative expression of the oxidation process.

Character of activated sludge

The sludge was similar in appearance to ordinary activated sludge except that it was usually lighter in colour, being grey rather than brown, and that it was composed of larger floccules. Under the microscope the sludge had a normal appearance; protozoa were plentiful, though *Beggiatoa*, sulphur-containing organisms, were absent.

THE ISOLATION OF THE THIOCYANATE SPLITTING ORGANISM

During the time these experiments were being made the bacteriology of the sewage fed to the beds or treated with activated sludge, and that of the effluents produced was carefully studied. Viable counts were made on (1) meat-extract agar, (2) meat-extract agar containing 0.05% ammonium thiocyanate, and (3) on a synthetic agar containing thiocyanate. Tap water was used for this synthetic agar, which was a mistake since it gave the impression that organisms were growing on a medium in which NH_4CNS was the sole source of C + N, whilst, as shown by Bigger [1937], many tap waters (and Leeds is no exception) contain nutrient material for *B. coli* and probably for other organisms. At first the samples were incubated at 37°, but later samples were also incubated at 22°. Detailed results are not given, but the following significant facts emerged.

(1) The proportion of viable organisms present in the effluents from the thiocyanate beds which possessed an indophenol oxidase system increased from 5–10% of the total present to 80–95% as the dose of thiocyanate increased.

(2) 90% of the viable bacteria present in the later effluents from sewage containing large quantities of thiocyanate were identical with a strain (CNS 3) which had been observed in steadily increasing proportions as the amount of thiocyanate oxidation increased.

Over 50 strains of bacteria were subcultured into media containing thiocyanate and these were tested both with and without aeration. The results were negative.

It was next shown that the flagellates present in the effluents from the thiocyanate beds could be excluded without impairing the function of the effluent to break down thiocyanate.

During this time some natural selection of strains had been going on in the beds and a further series of studies was commenced with six and then with three separate species. Separate cultural studies with these gave negative results, but a bacterial sludge prepared from a mixture of the three species, one of which was CNS 3 and one probably Friedländer's bacillus, did cause the breakdown of thiocyanate. The cultural conditions for the operation of this sludge in the previously mentioned synthetic medium were determined to be as follows.

(1) Sludges which had almost completed the breakdown of thiocyanate did not reveal the same lag on reinoculation into media containing CNS, as did inoculations from non-thiocyanate media.

(2) A light inoculum took much longer to initiate CNS breakdown than did a heavy one.

(3) The breakdown was not observed at 37°.

(4) Bouillon added to the synthetic medium retarded, but KNO_3 accelerated, the rate of CNS attack.

With these facts in mind, the three species were tried in synthetic media containing 0.5% KNO_3 ; the organism CNS 3 removed the thiocyanate in 9 days.

Since then cultures of this organism have repeatedly caused the breakdown of CNS and all subcultures of the organism have grown true to type.

No utilization of thiocyanate in bouillon thiocyanate media has been detected after 2 months' culture. When thiocyanate is the sole constituent added to sterile tap water (0.02%) there may be little detectable utilization for a period up to 3 weeks' time, but once utilization has commenced it is usually complete in 48 hr. Work which is in progress in conjunction with Dr W. C. Evans indicates that the rate of utilization of NH_4CNS by the organism is profoundly influenced by the inorganic ions present in the medium.

We suggest that the organism CNS 3 should be called *B. thiocyan oxidans*.

The organism is a slender Gram-negative motile bacillus growing best at about 22° on nutrient agar. It commences to pigment on the second day and the colour gradually intensifies to a canary yellow; the pigment diffuses throughout the medium. The bacillus possesses no saccharolytic properties and does not liquify gelatin.

It has also been confirmed in this work that the decomposition product of ammonium thiocyanate is ammonium sulphate.

DISCUSSION

Beyond the economic interest which is attached to this breakdown of ammonium thiocyanate by the organism *B. thiocyan oxidans* there is an academic interest because of the probable existence of an adaptive enzyme [see Karstrom, 1930; Knight, 1936] system—a thiocyanase system—in cultures grown with thiocyanates as sole source of carbon and nitrogen.

The results obtained with the sewage on the beds indicated (1) that the utilization is bacterial in origin, since the rate of action increases with time, and (2) that the organism has to adapt itself to its unusual substrate. This probable explanation is further supported by the cultural work with the single organism.

One expects "adaptive" enzymes to be concerned with chemical reactions which provide abundant energy for the cell, and in this respect, the reaction

fulfils these requirements.

To the best of our knowledge there is no previous reference to the bacterial breakdown of thiocyanate and indeed, the CNS ion has generally been referred to as one of the more toxic anions for bacteria. Eisenberg [1919], quoted by Stephenson [1930], gives the following order of toxicity of the anions for a large number of bacterial species:

The series is very similar to the Hofmeister series for the precipitation of the proteins of egg white by their sodium salts. Recent work [Lockemann & Ulrick, 1936] has also stressed the disinfectant action of thiocyanate solutions.

In the present study it would appear that whereas *B. thiocyan oxidans* obtains its energy more readily from sources like bouillon, it can in absence of these attack the thiocyanate molecule.

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