ANAEROBES IN SEWAGE

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DESPITE THE FACT that anaerobes are widely distributed in nature, are found in large numbers in the intestinal tract of man and other animals, and have been isolated from soil in many parts of the world, there are few references to their isolation from sewage. Practically all the studies that have been made of sewage treatment have concerned aerobes, either their rôle in the purification of sewage or their reduction in numbers.

Sewage purification by such processes as the Imhoff tank and septic tank is probably, however, of anaerobic nature, and in other processes also anaerobes may play an important part.

The study of the anaerobes in sewage, as presented in this paper, is divided into three parts:

1. The historical review of the literature from the reports of Klein who reported anaerobes in the sewage of London, to the present time, including other sources which indicate their presence.
2. The description of the methods used for determining the numbers of anaerobes present and the identification and isolation of these anaerobes.
3. The study itself which deals with the sources of the samples, the number and species of anaerobes found in the several steps of sewage treatment by the activated sludge process, and a special study of the occurrence of *B. coli* and *Cl. welchii*.

Historical

Cl. welchii

Klein[1] reported finding *B. enteritidis sporogenes,* now usually identified as *Cl. welchii,* in samples of sewage from several sources in the Thames River at Richmond and in mud deposits in the river's mouth.

Klein and Houston[2] found no definite parallelism between the total number of bacteria and the number of either *B. coli* or *B. enteritidis sporogenes,* nor similarly between the number of *B. coli* and spores of *B. enteritidis sporogenes.* They asserted that sewage may contain 500,000 *B. coli* and 1,000 spores of *B. enteritidis sporogenes* per c.c. or may contain much smaller numbers.

Houston[3] examined the public water supplies of London and reported that in 155 samples of a filtered water supply, 3 samples yielded spores of *B. enteritidis sporogenes* when 10 c.c. of the sample were used for cultural purposes. Houston[4] also examined Tunbridge deep well water, using 1,000 c.c. for examination, and obtained negative results for *B. enteritidis sporogenes* in 2 samples examined. In his examination of water of Loch Laggan in Scotland, 81 of 85 samples yielded no *B. enteritidis sporogenes* in 10 c.c. of water. The burn and river samples gave 3 positive results for *B. enteritidis sporogenes* out of 26 samples examined when 10 c.c. amounts were used. In the examination of samples of water from Loch Ericht, out of 100 samples examined for spores of *B. enteritidis sporogenes* only 1 sample yielded a positive result.

These are the first, and until now have remained the most extensive, investigations on the presence of *Cl. welchii* in sewage and water. They cover a number of years and some hundreds of samples.

The method used probably did not lead to isolation of this organism in pure culture. This method consisted in inoculating sterile milk, previously boiled and cooled, with definite amounts of sewage or water. The inoculated milk was then heated for thirty minutes at 80° C., and incubated at 37° C. If stormy fermentation took place some of the whey was injected intravenously into a rabbit. If certain symptoms appeared in the rabbit and the rabbit died as the result of this injection the conclusion was reached that the presence of *Cl. welchii* (*B. enteritidis sporogenes*) had been demonstrated.

Certainly pure cultures of *Cl. welchii* were not obtained in this manner, and from the characteristics Klein ascribed to this organism he undoubtedly worked with mixed cultures of *Cl. welchii* and some putrefactive anaerobe.

Simonds[5] cites the following persons who reported *Cl. welchii* from either water or sewage: Gehrman from sewage; Schattenfroh and Grassberger from spring water; Henseval from the Belgian coast; Hatchel and Freas from water. Simonds found *Cl. welchii* in 2 c.c. of water of Galveston Bay which receives sewage from the city as well as from ships at the dock. He found that samples taken several miles from the outlet of the channel and one-half mile from the baths gave negative results with 20 c.c. Creel[6] found an anaerobe in drinking water on trains which resembles *Cl. welchii* and is his anaerobe "B." Cumming[7] in an investigation of the Potomac River watershed isolated a lactose splitting anaerobe in many places along the Potomac River. He stated that similar organisms have been found in the waters at Grand Forks, N. D., at Cumberland, Md., and at Baltimore, Md., after the water had been treated with hypochlorite. Larner[8] reported *Cl. welchii* in the chlorinated water supply of Montclair, N. J.

CL. SPOROGENES

There are a few references which state that this organism has been found in sewage or in water. Klein[9] found *B. cadaveris sporogenes* in manure, street dust, sewage and similar filth, that is to say, this organism occurs in almost the same material as *Cl. welchii*. Fuller[10] cites Johnson finding *Cl. sporogenes* in sewage at Columbus, Ohio.

CL. TETANI

There are no available references to the finding of *Cl. tetani* in sewage, although this organism is isolated from sources which indicate it might be present in sewage. Noble[11] in a review of the literature on the presence of *Cl. tetani* in nature, cites: Lortet finding *Cl. tetani* in mud dredged from the bottom of Lake Geneva and from the water of the Dead Sea; Ringling reporting *Cl. tetani* in the bilge water of ships. Noble stated that if found in soils it is in soil that has been fertilized with animal feces or in dust or dirt from streets. Tulloch[12] reported that in examination of feces of men returned from overseas, out of 21 examined 7 were positive for *Cl. tetani*. Of 35 fecal specimens from civilians 5 were positive for *Cl. tetani*. Tenbroeck and Bauer[13] have recently found spores in the feces of hospital patients in China.

VIBRION SEPTIQUE

There is no available literature which notes the presence of this anaerobe in either water or sewage. It is said to be present in the intestines of men and animals and in the soil of cultivated lands.[14]

CL. BOTULINUM

Tanner and Dack[15] isolated *Cl. botulinum* from 1 sample of sewage examined. Dubovsky and Meyer[16] did not find *Cl. botulinum* in 8 samples of sewage examined. They also obtained negative results in the examination of soil from a sewage farm[17] and from sewage discharge berths.

Tanner and Dack[18] examined 10 samples of feces and reported 2 of these were positive for *Cl. botulinum*. Recently Kahn[19] did not find *Cl. botu-*

linum in feces from 65 people. K. F. Meyer[20] reported negative results in the examination of 88 samples of feces from healthy people.

MISCELLANEOUS

Klein[9] found in addition to *Cl. welchii* and *Cl. sporogenes* a third anaerobe from sewage. This anaerobe is "Bacillus butyricus of Botkin" which he claims is more or less associated with filth of various kinds. He also made a study of these three organisms which he isolated from sewage and gave them various characteristics which are given in his above report.

L. M. Horovitz[21] stated that he found twenty-five species of anaerobes in river water from Petrograd after filtration and treatment with ozone. He grouped them under eight varieties, of which seven are given in the abstract from which this reference is taken. These groups are: *Amylobacter butyricus Duclaux, B. gruberi No. 2, B. solidus Luderitz, B. ernsti, B. saccharobutyricus Klecki, Gran. saccharobutyricus immobilis non liquifaciens* and *Cl. pasteuranan.*

Amylobacter butyricus Duclaux, B. gruberi No. 2, B. saccharobutyricus Klecki and *Cl. pasteuranan* are synonyms for *Cl. butyricum.*[14] *Granulo saccharobutyricus liquifaciens immobilis* is none other than *Cl. welchii.*[22]

These references give an idea of the number of names one organism can accumulate and how confusing much of the literature is in regard to anaerobes.

Raab[23] recently isolated an anaerobe from the filtered water supply of Minneapolis which ferments lactose and which he claims is not *Cl. welchii.* He gives the cultural characteristics in some detail and calls attention to the fact that anaerobic spore-forming lactose fermenters found in water supplies may not necessarily be *Cl. welchii.*

Kinnicutt, Winslow and Pratt[24] state: "It is strange that obligate anaerobes have never been found in numbers either in feces or in sewage."

Courmont[25] in a study of the bacterial flora of activated sludge effluent found no obligatory anaerobes present.

Metcalf and Eddy[26] cite Rideal (Samuel Rideal, Sewage and the Bacterial Purification of Sewage, 1906, p. 72) as listing the following obligatory anaerobes present in sewage: *Bacillus amylobacter butyricus* (*Cl. butyricum*), *B. enteritidis sporogenes* (*Cl. welchii*), *B. cadaveris sporogenes* (*Cl. sporogenes*), *Spirillum rugula* and *Spirillum amyliferum.*

Kinnicutt, Winslow and Pratt[24] stated that cellulose fermentation is carried out by specific anaerobic spore-forming rods (Omelianski).

How many of these identifications are reliable is a question. Levine[27] makes this statement: "The terms *Cl. welchii* and *Cl. sporogenes* as employed by water works operators and analysts, designate not a specific organism but the whole group of anaerobic lactose fermenters."

The literature may be summarized as follows: (1) *Cl. welchii* is undoubtedly present in foul sewage in considerable numbers. (2) No other anaerobe has been reported a sufficient number of times or has been isolated by methods which would indicate its common occurrence in sewage. (3) Other anaerobes have been reported from sources that would indicate their occasional presence in sewage. (4) All references to the presence of anaerobes have related to foul sewage or sewage polluted water. No study has been made of the anaerobes in sewage during the course of its treatment by the different methods now in use.

METHODS

Since directions for isolating specific anaerobes from such a mixture as sewage are not easily available, the methods used are described in detail.

CL. WELCHII

For the isolation of *Cl. welchii* the following method was used:

Whole sterile milk, previously boiled and cooled, was inoculated with various amounts of sewage and heated to 80° C. for 20 minutes. The tube was then sealed with sterile white vaseline, cooled and incubated at 37° C. for 24 hours. If typical stormy fermentation occurred, transfers were made by means of sterile Pasteur pipettes to another tube of sterile, previously-cooled and boiled milk. This tube was sealed with sterile white vaseline and cooled and incubated as before. This above process was carried out several times, and then transfers were made to tubes of liver agar, which had been melted and cooled to about 60° C. These tubes were immediately cooled, after inoculation, in cold water and then incubated at 37° C. At the end of 24 hours colonies were picked by means of sterile Pasteur pipettes and transferred to tubes of milk. This process of picking from liver agar cultures to milk and transferring to liver agar cultures was repeated several times. At the end of this part of the process several colonies were picked from the liver agar cultures and transferred to tubes of beef heart medium. The beef heart cultures were incubated for 2 days at 37° C., and then tested for the presence of aerobes by means of transfers to agar slants.

If motility tests and Gram stain showed the beef heart culture to contain a non-motile Gram positive organism with the characteristics of *Cl. welchii,* transfers were made to dextrose, lactose, saccharose, salicin and glycerin, and also to milk and gelatin.

If the colony isolated from liver agar and transferred to beef heart medium gave the cultural reactions of *Cl. welchii* after incubation at 37° C., a rabbit was injected* with ½ c.c. of the beef heart culture. This beef heart culture was first centrifuged and the injection made intravenously. The culture was regarded as positive in the samples tested by this method when the typical bloated appearance and the condition of the internal organs corresponded to the description given of rabbits treated in this manner.

No attempt was made to isolate *Cl. welchii* from every tube showing stormy fermentation of milk. Generally an attempt was made to isolate *Cl. welchii* from three tubes of every set of samples. The tubes chosen were always from the highest dilution of the sample showing stormy fermentation of milk.

CL. TETANI

For the isolation of *Cl. tetani* the method used by Tenbroeck and Bauer [13] was followed:

Various amounts of sewage which had been heated to 80° C. for 20 minutes were transferred to tubes of sugar-free broth containing sterile pieces of rabbit kidney or spleen. The tubes were sealed with sterile white vaseline and incubated for 4 days at 37° C. At the end of the period of incubation, stain and motility tests were made from the sediment in the bottom of these broth tubes. If characteristic motile spore-bearing anaerobes were found, some of the sediment was heated to 80° C. for 20 minutes and transferred by means of a sterile Pasteur pipette to a fresh tube of sugar-free broth and sterile rabbit tissue, sealed with sterile white vaseline and incubated for 4 days at 37° C. This process was repeated several times at intervals of 4 days, until an apparently pure culture was obtained. A small amount of this culture was transferred by means of a sterile Pasteur pipette to a series of liver agar shake cultures. At the end of 48 hours typical tetanus-like colonies were isolated from these shake cultures and transferred to a new series of shake cultures. This process was repeated until an apparently pure culture of *Cl. tetani* was obtained. Typical *Cl. tetani* colonies were then transferred to tubes of sugar-free broth and rabbit tissue. These tubes were sealed with sterile white vaseline and incubated for 10 days at 37° C.

At the end of this time some of the broth was transferred to a centrifuge tube and centrifuged for 1 hour at a high rate of speed. White mice were inoculated subcutaneously with this fluid, two receiving 0.001 c.c.; two 0.01 c.c.; two 0.01 c.c. plus approximately one unit of tetanus antitoxin; and two 0.1 c.c. plus approximately one unit of antitoxin.

If the mice receiving 0.001 c.c. of the culture died and the mice receiving 0.1 c.c. of the culture plus one unit of antitoxin lived, then it was considered that the presence of *Cl. tetani* had been demonstrated.

CL. BOTULINUM

The method used in this laboratory for the demonstration of *Cl. botulinum* in soils was followed in attempting to demonstrate its presence in sewage.

* This was not carried out with every culture.

This consists in heating the sewage to 80° C. for 1 hour, cooling and adding to tubes or flasks of beef heart medium. These flasks or tubes are then sealed with sterile white vaseline and incubated at 37° C. for 10 days. At the end of this time some of the fluid of the culture is taken off, centrifuged at high speed for 1 hour, and ¼ c.c. of the supernatant liquid is injected intraperitoneally into a white mouse. If death of the mouse occurs in 48 hours the following procedure is carried out: 1 mouse is injected with ¼ c.c. of the culture plus ¼ c.c. of the standardized Type "A" *Cl. botulinum* antitoxin; 1 mouse is injected with ¼ c.c. of the culture plus ¼ c.c. of the standardized Type "B" antitoxin and a third mouse is given ¼ c.c. of the culture. If 1 mouse survived after injection of this culture plus antitoxin and the other 2 died, providing 1 of the mice that died did not receive antitoxin, the above test was repeated, and if this second set of injections gave the same result, then it was regarded that the presence of *Cl. botulinum* had been demonstrated and was of the type represented by the mouse which had been injected with the culture and antitoxin and had lived.

VIBRION SEPTIQUE

Vibrion septique was isolated by the method suggested by the Medical Research Committee.[28]

Various amounts of the sample of sewage were added to nutrient salicin broth, previously cooled and boiled, and heated to 80° C. for 20 minutes. The tubes were then sealed with sterile white vaseline, cooled, and incubated at 37° C. for 24 to 48 hours. At the end of the period of incubation a second set of tubes of salicin broth, previously boiled and cooled, were inoculated with a small amount of fluid from the first set of tubes and then treated in the same manner. After several transfers from salicin broth to salicin broth, providing gas was produced in each case after incubation, transfers were made to salicin agar shakes. Colonies were picked from the salicin agar after incubation at 37° C. for 48 hours, and transferred to salicin broth.

This was repeated several times until an apparently pure culture of an anaerobe was present. Transfers were then made to beef heart medium. This medium was incubated at 37° C. for 48 hours. Transfers were then made to agar slants and if no aerobic growth was visible after 2 days at 37° C. transfers were made from the beef heart culture to the following media: Glucose broth, lactose broth, saccharose broth, salicin broth, gelatin, milk and beef heart media. If these cultural tests showed the possible presence of *Vibrion septique* the beef heart cultures were incubated for 10 days at 37° C. The fluid of the beef heart culture was then centrifuged and 1/10 c.c. of the supernatant liquid was injected intraperitoneally into mice. If the cultural and the pathogenic reactions were the same as those of *Vibrion septique,* the organism isolated was called *Cl. septique.*

CL. SPOROGENES

Cl. sporogenes was a difficult organism to isolate. There is no one medium that could be used to demonstrate its presence nor any one medium in which it would quickly outnumber other organisms present. *Cl. welchii* proved to be the one organism hard to eliminate.

The following method was used to isolate *Cl. sporogenes* from sewage:

The sewage was first heated to 80° C. for 30 minutes and then definite amounts were added to tubes of beef heart medium. These tubes were incubated for 2 days at 37° C., and transfers made to tubes of melted gelatin. The tubes of gelatin were cooled, sealed with sterile white vaseline, and incubated at 37° C. for 3 days. At the end of the period of incubation, transfers were made to another set of gelatin tubes. This second set of gelatin tubes were treated the same as the first set. If these tubes showed liquefaction of the gelatin after they were incubated at 37° C. and placed in ice water, transfers were made to liver agar shake cultures. These liver agar shake cultures were incubated at 37° C. At the end of 48 hours of incubation typical wooly colonies, if present, were picked and transferred to gelatin. If these tubes of gelatin showed liquefaction after incubation and being placed in ice water, transfers were made to liver agar shake cultures as before. After incubation at 37° C. for 48 hours colonies were picked and transferred to tubes of recently boiled and cooled milk. If these tubes showed proteolysis after incubation at 37° C. it was assumed that *Cl. sporogenes* might be present. Transfers were then made to liver agar shake cultures from tubes showing proteolysis. At the end of 2 days incubation at 37° C. colonies were picked and transferred to milk. This procedure was repeated until an apparently pure

culture of a proteolytic anaerobe was obtained. Transfers were then made to beef heart medium and incubated at 37° C.

Transfers were then made to the following media: glucose broth, lactose broth, maltose broth, milk, gelatin and beef heart. If the cultural reactions were those of *Cl. sporogenes*, it was assumed that the organism isolated by this method was *Cl. sporogenes*.

The presence of *B. coli* was determined by the methods given in "Standard Methods for the Examination of Water and Sewage." [29]

The samples of sewage were collected in sterile wide mouth glass stoppered bottles. The samples were always examined on the day collected. Dilutions of the sample were always with sterile 0.85 per cent NaCl. Dilutions were in most cases in tenths. The samples were heated so that the presence of spores rather than vegetative cells was demonstrated. The number of organisms reported are the reciprocal of the highest dilution of the sample of sewage in which the organism was found.

All media were incubated and tested for sterility before being used. Sugar broths were prepared by adding sterilized 10 per cent solutions to the sterile broth under aseptic conditions.

EXPERIMENTAL DATA

An attempt was made to determine the numbers of *Cl. welchii, Cl. tetani* and *Cl. botulinum* present in several types of sewage samples from different sources. These sources include foul sewage (9 samples), Imhoff tank sludge (1 sample), activated sludge (1 sample), dried activated sludge (1 sample), effluent from activated sludge tanks (2 samples) and tannery waste sludge (2 samples).

In the samples collected *Cl. welchii* was found in all save 1 sample, *Cl. tetani* was found in 13 samples, and *Cl. botulinum B.* in 1, a tannery waste sludge. The amounts of the samples examined ranged from 50 c.c. to 1 c.c. depending on source and the organism being sought. The higher amounts were generally used when examining foul sewage and sewage effluents for *Cl. botulinum B.*

It was further thought desirable to study the fate of anaerobes during the course of treatment by the activated sludge process. This process is considered to be chiefly aerobic and very little, if anything, is known of the rôle played in the process by anaerobes.

Samples were collected at several steps of the process from the entrance of the sewage to the plant until it left either as effluent or sludge. The samples collected were: foul sewage (4 samples), effluents from settling tank (5 samples), wet sludge (4 samples), pressed sludge (3 samples) and dried sludge (5 samples). These samples were collected at the Maywood Activated Sludge Plant, Maywood, Illinois.

A study was made of the comparative number of certain organisms, namely, *Cl. welchii, Cl. tetani, Cl. sporogenes* and *Vibrion septique,* and these were found to be present as follows:

Foul Sewage

Cl. welchii	100 per c.c.
Cl. tetani	1 per 10 c.c.
Cl. sporogenes	1 per 10 c.c.
Vibrion septique	0 in 10 c.c.

Wet Sludge

Cl. welchii	10 to 10,000 per g.
Cl. tetani	0 to 10 per g.
Cl. sporogenes	10 to 10,000 per g.
Vibrion septique	0 to 10 per g.

Pressed Sludge

Cl. welchii	500 to 50,000 per g.
Cl. tetani	1 to 10 per g.
Cl. sporogenes	100 to 1,000 per g.
Vibrion septique	0 to 100 per g.

Heated Sludge

Cl. welchii	10 to 50,000 per g.
Cl. tetani	0 to 10 per g.
Cl. sporogenes	10 to 2,000 per g.
Vibrion septique	1 to 10 per g.

Effluent

Cl. welchii	5 to 10 per c.c.
Cl. tetani	None in 1 c.c.
Cl. sporogenes	0 to 10 per c.c.
Vibrion septique	None in 1 c.c.

It is apparent that *Cl. welchii* is by far the most numerous anaerobe of those

studied and that *Cl. sporogenes* is less numerous with *Vibrion septique* and *Cl. tetani* present in much smaller numbers and not to be considered as being very common members of the anaerobic flora of sewage.

The third part of the study dealt with the relative numbers of *B. coli* and *Cl. welchii* in the several steps of the activated sludge process.

These organisms represent two distinct types of bacteria commonly found in sewage and are probably the two most numerous members of their respective types present. *B. coli* represents the non-spore-forming Gram negative anaerobe and *Cl. welchii* represents the spore-forming anaerobes. Since they are the two types of organisms that are considered in the examination of water for its sanitary quality, it was considered advisable to study them particularly.

Samples for this study, also collected at the Maywood Activated Sludge Plant, were taken as follows: foul sewage (10 samples), aeration tank (7 samples), settling tanks (7 samples), wet sludge (9 samples), effluents (9 samples), pressed sludge (9 samples), and dried sludge (9 samples). Organisms were found in these samples as follows:

Foul Sewage

B. coli.....10,000 to 1,000,000 per c.c.
Cl. welchii..... 10 to 1,000 per c.c.

Aeration Tanks

B. coli.....10,000 to 1,000,000 per g.
Cl. welchii..... 10,000 to 100,000 per g.

Sludges

B. coli.....100,000 to 10,000,000 per g.
Cl. welchii..... 10,000,000 per g.

Settling Tanks

B. coli.....1,000 to 100,000 per c.c.
Cl. welchii..... 1 to 1,000 per c.c.

Dried Sludge

B. coli..... 0
Cl. welchii.....1,000 to 1,000,000 per g.

It was evident that during the course of the treatment *Cl. welchii* increased in relative numbers over *B. coli* in every step of the process. Both showed marked increase in number in the aeration tanks and wet sludges. Both showed marked decrease in the numbers in the effluents as compared with the sludges and aeration tank.

In none of the samples collected was there any evidence of large numbers of spore-forming aerobes present. The few that were present were eliminated by dilution of the sample, and the purification of the anaerobe under examination was then the problem of eliminating the undesirable anaerobes.

SUMMARY AND CONCLUSIONS

Anaerobes are common members of the bacterial flora of sewage, although they may not be so numerous as the aerobes. The anaerobes found most commonly present in foul sewage are those found in the largest numbers in the intestinal tract of man. Of those studied *Cl. welchii* is the most numerous, with *Cl. sporogenes* in smaller numbers, and *Cl. tetani* and *Vibrion septique* still more rare and not demonstrable in every sample of sewage. *Cl. botulinum B.* was found once (tannery waste) in 39 samples of sewage.

During the course of treatment of sewage by the activated sludge process any actual reduction of anaerobes cannot be considered to be demonstrated. The numerical difference between influent and effluent may be considerable, but if the numbers present in the sludge are taken into account this reduction is only apparent. Sludge contains more anaerobic organisms than foul sewage.

Comparing influent and effluent the reduction in numbers of *B. coli* and *Cl. welchii* by the activated sludge process is marked, based on the numbers in raw sewage and the effluent from the settling tanks. *B. coli* is reduced in numbers to a greater extent than is *Cl. welchii*. The relation in numbers between *B. coli* and *Cl. welchii* during the course of treatment is

interesting. *Cl. welchii* is increased in its relative numbers during the whole course of the treatment.

The sludge when pressed contains large numbers of both aerobic and anaerobic organisms. When this sludge is dried in an oven large numbers of *Cl. welchii* survive, and the non-spore-forming aerobes do not survive.

Conclusions are that:

1. Anaerobes are present in sewage, but in smaller numbers than aerobes.

2. The most common anaerobes present in sewage are those species found in the intestinal tract of man.

3. In the samples examined *Cl. welchii* was the most common anaerobe found. *Cl. sporogenes* was found in less abundance. *Cl. tetani* and *Vibrion septique* were found in small numbers. *Cl. botulinum* was found but once.

4. Treatment by the activated sludge process does not actually reduce the numbers of anaerobes to any extent. The sludge from an activated sludge plant contained more anaerobes than the foul sewage entering the plant.

5. *Cl. welchii* survived this process better than *B. coli*.

6. Dried and pressed sludge may contain many anaerobes, mostly of the *Cl. welchii* group.

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