CLOSTRIDIUM ACIDI-URIDI AND CLOSTRIDIUM CYLINDROSPORUM, ORGANISMS FERMENTING URIC ACID AND SOME OTHER PURINES

H. A. BARKER AND J. V. BECK

Division of Plant Nutrition, University of California, Berkeley

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Although the decomposition of uric acid and other purines by microorganisms in the presence of oxygen has been the subject of numerous investigations, the corresponding anaerobic process has received surprizingly little attention. The only study of this subject to our knowledge is that of Liebert (1909) who showed that when glass-stoppered bottles are completely filled with a medium consisting of 1 per cent uric acid and 0.1 per cent K_2HPO_4 dissolved in tap water, and are inoculated with garden soil or canal mud, the uric acid is rapidly converted into ammonia, carbon dioxide and acetic acid. The organism responsible for this conversion was described as an actively motile, obligately anaerobic spore-forming rod and was given the name of *Bacillus acidi-urici*. The present investigation was undertaken to learn more about this type of organism. The specific objectives were to isolate a number of uric-acid-fermenting anaerobes, to determine their characteristics and systematic position and to establish the most favorable conditions for their growth. A more detailed study of the biochemistry of purine fermentations by these organisms is presented elsewhere (Barker and Beck, 1941).

Enrichment and isolation. Preliminary experiments showed that excellent enrichment cultures of uric-acid-fermenting bacteria can readily be obtained from soil inocula by using a modification of Liebert's medium. The modified medium contains uric acid 0.3–0.75 per cent, yeast autolyzate 0.5 vol. per cent, K_2HPO_4 0.03 per cent, $MgSO_4 \cdot 7H_2O$ 0.01 per cent, phenol-red indicator solution 0.1 vol. per cent NaOH to pH 7.2–7.6 and boiled-out distilled

water. In preparing the medium the uric acid should be suspended in almost the full volume of liquid and then neutralized at the boiling temperature. Small glass-stoppered bottles are completely filled with the medium and, after being inoculated with about 0.5 gm. of soil, are closed and incubated at 25–37°C.

Growth generally occurs within 24–48 hours and is accompanied by an increase in alkalinity. If the medium initially contains more than 0.5 per cent urate a heavy precipitate of ammonium urate forms soon after decomposition begins, but this gradually disappears later. A moderately heavy bacterial slime also frequently develops. The formation of gas bubbles was never observed in enrichment cultures.

On microscopic examination, active enrichment cultures are always found to contain large numbers of medium-sized, actively motile, spore-forming rods which are remarkably uniform in appearance. The scarcity of extraneous anaerobes is doubtless due to the low content of organic matter other than uric acid in the enrichment medium and to the moderately high alkalinity (pH 8.0–8.5) which develops soon after fermentation begins.

In spite of the microscopic homogeneity of the first enrichment cultures, one or two transfers were made before undertaking isolations. The medium used for transfers was the same as for the initial enrichments except that it was supplemented with 0.01 per cent $Na_2S \cdot 9H_2O$.

In one set of enrichment cultures uric acid was replaced by guanine and adenine, respectively. With guanine, large numbers of bacteria morphologically similar to those in urate cultures developed within 48 hours. With adenine, on the contrary, no noteworthy growth or decomposition occurred even after several weeks incubation at 37°C.

Pure cultures were isolated from the uric-acid enrichment cultures by means of the shake culture method. This method was found to be preferable to the use of anaerobic plates because of the great sensitivity of the organisms towards oxygen. In deep agar tubes protected by a pyrogallol-K_2CO_3 seal, or even without any seal, colonies invariably develop readily, while anaerobic plates frequently give completely negative results. The isolation me-

dium differed from the enrichment medium only in having 2 per cent agar, 0.2 per cent uric acid and 0.01 per cent $Na_2S \cdot 9H_2O$. The latter was added as a sterile 1 per cent solution after autoclaving.

By the end of 24–36 hours incubation at 35°C. small colonies of the uric-acid-decomposing bacteria may be seen surrounded by localized zones of precipitated ammonium urate. These zones gradually become larger as growth proceeds while at the same time the precipitate commences to redissolve immediately around the colony. Extension of the clear zones usually continues until all the precipitated ammonium urate disappears again and the medium resumes its original appearance. By this time the colonies have ceased to grow and, when well isolated, have attained a diameter of 1–2 mm. They are whitish in color and are compact or somewhat coarsely lobed with irregular edges.

By repeated reisolation from shake cultures, some thirteen strains of uric-acid-fermenting bacteria were obtained in pure culture. All were spore-formers. Numerous attempts to obtain non-spore-forming anaerobic uric-acid-decomposing organisms were invariably unsuccessful; non-spore-forming organisms, when isolated, always proved to be unable to attack uric acid.

The purity of isolated cultures was tested by microscopic examination and by inoculating heavily into tubes of iron-milk (Spray) and semisolid agar containing 5 vol. per cent yeast autolyzate, 1 per cent tryptone and 0.5 per cent glucose. As will be shown later the uric-acid-fermenting organisms are unable to develop to an appreciable extent in either of these media. Contaminants are therefore easily detected.

Permanent or storage cultures were made by stab inoculation into the medium used for isolation. Once spores have formed, such cultures will remain viable for months or years. Working cultures were kept in the same medium with 0.1 per cent agar. When inoculated at the bottom immediately after autoclaving these media do not require an anaerobic seal.

Natural occurrence. The limited data at present available indicate that bacteria capable of decomposing uric acid under anaerobic conditions are widely distributed in soils. So far, active

enrichment cultures have been obtained from 0.05–0.5 gm. samples of ten soils from different sections of California. These include heavily manured garden soils, soils from experimental plots that have received no fertilizer for over 25 years, acid forest soils, alkali desert soils, neutral field soils and soils that have been in storage for many years. Also cultures have been obtained from a number of samples of San Francisco bay mud and from a

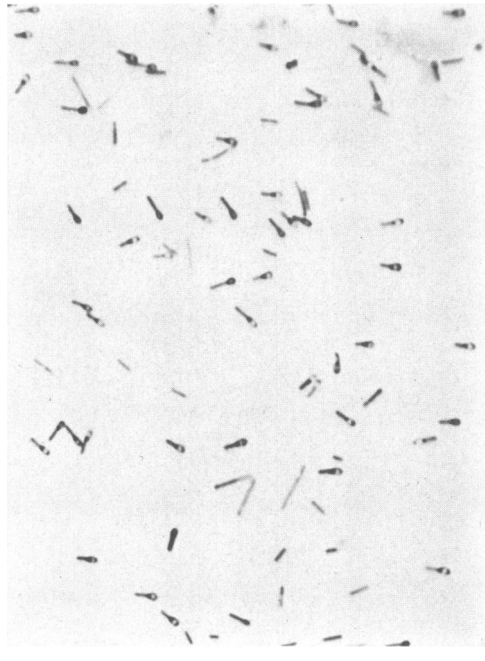

FIG. 1. STRAIN 9A. 24-HOUR CULTURE IN 0.2 PER CENT URATE LIQUID MEDIUM Showing vegetative and sporulating cells. Gram stain. × 1060

very sandy soil from near Provo, Utah. No soil tested has ever failed to give the organisms.

Fecal material of the yellow-shafted flicker (*Colaptes auratus*), collected so as to minimize outside contamination, was also found to contain anaerobic uric-acid bacteria. This observation is of interest in view of the possibility that the uric acid which constitutes the main nitrogenous end product of avian metabolism may be decomposed mainly by bacteria of this type.

Morphology. All the uric acid-fermenting bacteria isolated or observed in enrichment cultures were medium-sized, actively motile, spore-forming rods. Of the thirteen isolated strains, twelve (nos. 2, 3, 5, 5a, 9a, 10, 10a, 11, 12, 42, S1 and SJ1) are very similar in respect to the details of their morphology (fig. 1). The vegetative cells are straight rods varying from 2.5 to 4 microns

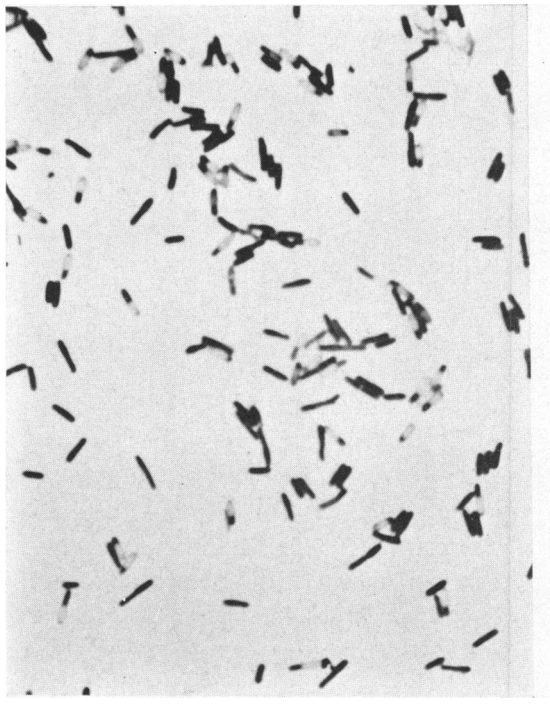

FIG. 2. STRAIN HCl. 24-HOUR CULTURE IN 0.2 PER CENT URIC ACID LIQUID MEDIUM

Showing vegetative and sporulating cells. Gentian violet. × 1060

in length and from 0.5 to 0.7 micron in width. The spores are oval (average size 1.1 x 0.9 micron) and are located terminally where they cause a distinct enlargement of the rod. Motility is by means of about ten peritrichous flagella.

Strain HCl (fig. 2) differs markedly from the others. Its vegetative cells are conspicuously larger (4 to 7 x 1.0 micron) and

the spores are much longer (1.7 to 3.0 x 1.0 to 1.1 microns) so as to be roughly cylindrical in shape. The spores are variably located in the cell (terminal, subterminal or central) and cause little or no swelling. The flagellation of this organism also is peritrichous.

All strains form spores abundantly under favorable conditions within 12–20 hours after inoculation. However, under unfavorable conditions of temperature or pH (see below), the usually straight, short, single cells are transformed to bent filaments which may reach 30 to 40 microns in length and which have little tendency to form spores even after several days incubation. When normal sporulation occurs the organisms soon lose their motility and physiological activity; autolysis of the residue of the vegetative cell quickly sets in.

When gram stains (Hucker method) were made with cells from young cultures all strains except 2 and 9a were found to be gram-negative. Strains 2 and 9a are sometimes weakly gram-positive in both one and two day cultures, though on other occasions they also are completely gram-negative.

Deep agar colonies have already been described. Surface colonies on anaerobic plates are variable in appearance, the variability depending mainly on moisture conditions and to a lesser extent upon the strain. Some colonies are small (1–2 mm.), opaque, white, raised, round with smooth edge, concentric markings and rubbery consistency. Other colonies are very thin, transparent, soft and spreading so as to almost cover the plate. The edges of these latter often have long finger-like projections which may reach 0.5 to 2 cm. in length and about 1 mm. in width, the projections being only 1–2 mm. distant from each other. Colonies of this type are easily overlooked and are best seen by reflected light. Several other intermediate colony types were also observed.

Cultural characteristics. The ability of the isolated strains to grow in the following media has been tested: (1) distilled water; uric acid 0.2 per cent; yeast autolyzate 0.5 vol. per cent; agar 0.1 per cent; K_2HPO_4 0.03 per cent; $MgSO_4 \cdot 7H_2O$ 0.01 per cent; $CaSO_4$ 0.25 vol. per cent of a saturated solution; $FeSO_4 \cdot 7H_2O$

0.0002 per cent; NaOH to pH 7.2–7.4; $Na_2S \cdot 9H_2O$ 1 vol. per cent of a 1 per cent solution. The sulfide solution was autoclaved separately and added just before inoculating. (2) Like (1) but with urate replaced by 1 per cent glucose. (3) Like (1) but with both 0.2 per cent urate and 1 glucose. (4) Like (1) but with 1 per cent peptone. (5) 1 per cent tryptone or (6) 30 vol. per cent yeast autolyzate instead of urate. (7) Iron-milk prepared according to Spray. All media were incubated at 35°C. in $\frac{5}{8}$-inch test tubes filled to a depth of about 8 cm. The tubes were inoculated at the bottom with 0.1–0.2 ml. of a young culture in urate medium. No anaerobic seal was used.

All thirteen strains grew excellently in media (1) and (3) containing urate but quantitative determinations of glucose in medium (3) by the method of Hassid showed that after five days incubation no glucose had been decomposed. As would be expected from this result, no growth occurred in medium (2) containing glucose as the sole energy source. The iron-milk cultures were also completely negative. In media (4), (5) and (6), containing peptone, tryptone, and yeast autolyzate, respectively, macroscopically visible growth did not occur with any strain even after five days incubation. However, microscopic examination revealed a few actively motile cells in tubes inoculated with strains 2, 9a, 10, 11 and 42. In view of the complex nature of these media it seems probable that the limited growth was due to the presence of small quantities of purines.

The different abilities of various strains to decompose the organic nitrogenous compounds of yeast autolyzate and tryptone both in the presence and absence of uric acid was further studied in an experiment in which determinations were made of ammonia production. Semisolid agar media were used. The data are given in table 1.

It can be seen that those strains capable of growing to a limited extent in yeast autolyzate and tryptone liberated ammonia from these materials. The quantity of "extra" ammonia is equivalent to about 5 per cent of the tryptone and 2 per cent of the yeast autolyzate nitrogen and is roughly the same in the presence as in the absence of urate. This indicates that the organic nitrogen

compounds from which ammonia is derived are directly fermented rather than decomposed by an oxidation-reduction reaction with uric acid.

Uric acid is not the only purine which these organisms attack readily and upon which they grow. This was shown by inoculating two representative strains, 5a and 9a, into the usual semisolid medium containing 0.1 per cent guanine, xanthine, hypoxanthine

TABLE 1

Production of ammonia from yeast autolyzate and tryptone by C. acidi-urici

All data refer to ammonia produced in excess of the amount equivalent to the urate nitrogen. Negative values indicate production of ammonia in amounts less than that equivalent to urate. All cultures were incubated 5 days at 35°C.

STRAIN	MEDIA							
	30 per cent y. aut.*		20 per cent y. aut. + 0.2 per cent urate		1 per cent tryptone†		1 per cent tryptone + 0.2 per cent urate	
	Mgm. NH_3-N/ml.	Per cent of y. aut-N	Mgm. NH_3-N/ml.	Per cent of y. aut-N	Mgm. NH_3-N/ml.	Per cent of trypt.-N	Mgm. NH_3-N/ml.	Per cent of trypt.-N
2	0.04	2.6	0.03	2.9	0.06	4.9	0.03	2.4
3	0.01	0.6	−0.02	0.0	0.00	0.0	−0.01	0.0
5	0.00	0.0	0.00	0.0	0.00	0.0	−0.02	0.0
5a	0.00	0.0	−0.02	0.0	0.00	0.0	−0.04	0.0
9a	0.04	2.6	0.01	1.0	0.06	4.0	0.05	4.0
10	0.04	2.6	0.02	1.9	0.09	7.4	0.05	4.0
10a	0.00	0.0	−0.03	0.0	0.00	0.0	−0.03	0.0
11	0.04	2.6	0.01	1.0	0.06	4.9	0.05	4.0
12	0.00	0.0	−0.02	0.0	0.00	0.0	−0.01	0.0
42	0.04	2.6	0.01	1.0	0.07	5.7	0.05	4.0
S1	0.00	0.0	0.00	0.0	0.00	0.0	−0.03	0.0
SJ1	0.00	0.0	−0.01	0.0	0.00	0.0	−0.04	0.0
HCl	0.00	0.0	−0.10	0.0	0.00	0.0	−0.10	0.0

* Contains 1.56 mgm. N/ml.

† Contains 1.22 mgm. N/ml.

or adenine instead of urate. Good growth of both strains was obtained in 24–30 hours at 35° with guanine and xanthine. With hypoxanthine growth also occurred but was appreciably slower, while with adenine no development was evident even after a week. A more detailed account of decomposition of these various purines will be given in a subsequent paper (Barker and Beck, 1941). It will also be shown that the principal products are ammonia, carbon dioxide and acetic acid.

Classification. On the basis of the above described morphological and cultural characteristics it is possible to undertake the identification and classification of the uric acid-fermenting bacteria.

Since all strains studied are obligately anaerobic, spore-forming rods, with peritrichous flagella, it is obvious that they may be placed in the genus *Clostridium.*

The specific identification of the organism is somewhat more difficult. So far as we have been able to ascertain it cannot be identified with any generally recognized species of *Clostridium* (Spray (1936, 1939), Prévot (1938), McCoy and McClung (1939)) because of its inability to develop normally upon either carbohydrates or protein hydrolysates. For example, of the 51 species listed by Spray (1939), only one, *C. werneri,* appears on casual examination to be even somewhat similar to our organism. But *C. werneri* ferments cellulose whereas our organism does not. The only previously described bacterium which is obviously very closely related to, if not identical with our organism is Liebert's *Bacillus acidi-urici.* We have therefore decided to accept his specific name and call this type of organism *Clostridium acidi-urici* (Liebert) Barker and Beck.

The above name is intended to apply more particularly to the twelve strains morphologically similar to strain 9a. Strain HCl differs so markedly from the others in respect to the size of the vegetative cells and the size, shape and position of the spores, that it seems best to consider this organism as a separate species for which we propose the name *Clostridium cylindrosporum.*

FACTORS INFLUENCING GROWTH

In order to learn how to obtain rapid, abundant and consistent growth of *C. acidi-urici* an investigation was made of the response of the organism to a number of environmental and nutritional factors. These included yeast autolyzate, reducing agents, pH and temperature.

Yeast autolyzate.[1] Low concentrations of yeast autolyzate or

[1] Yeast autolyzate is prepared by allowing a suspension of 1 pound of baker's yeast in 500 ml. of tap water to autolyze at 50°C. for 24 hours. The suspension

similar material increase the growth rate of all strains. This stimulation is also apparent with enrichment cultures. In a first enrichment culture, inoculated with one or more grams of soil, yeast autolyzate may have little effect on growth, evidently due to the presence of an adequate supply of growth stimulants in the soil. But transfers from original enrichment cultures develop slowly or not at all unless yeast autolyzate is added.

In studying the influence of yeast autolyzate on pure cultures an effort was made to answer two questions. (1) Is yeast autolyzate essential for the growth of these organisms? (2) If so, what quantity is required for rapid and abundant growth?

Because of limitations of space, the experiments dealing with these questions cannot be presented here. The results dealing with the necessity of yeast autolyzate may be summarized, however, by stating that of the two strains studied one (3) could be shown to require this substance, while the other (9a) grew very poorly without it. It seems probable that by rigidly excluding growth-factor contamination, the development of all strains could be shown to depend upon such materials.

As to the quantity of yeast autolyzate required for rapid and abundant growth, it appears that 0.5 per cent is almost as good as 10 per cent and is considerably better than any lower concentration. The most desirable concentration is 1–2 vol. per cent. As much as 30 vol. per cent yeast autolyzate has no inhibitory action on growth.

Reducing agents. *C. acidi-urici* is an obligate anaerobe which does not grow in the presence of oxygen. But even when oxygen is excluded by the customary methods, the organism frequently fails to develop unless the medium is supplied with a small amount of some reducing agent. For this purpose either thioglycollic acid or hydrogen sulfide is satisfactory at a concentration of 0.01 to 0.02 per cent. The influence of various concentrations of sulfide on growth is illustrated in table 2.

pH. To study the influence of pH on growth the usual semi-

is then boiled to remove carbon dioxide, neutralized to pH 6.8–7.0 with 1 N NaOH, filtered (preferably after adding Filteraid) and sterilized in small flasks. The nitrogen content varies from 0.5–1.0 per cent.

solid medium containing 0.5 per cent yeast autolyzate and 0.2 per cent urate was used except that the K_2HPO_4 content was increased to 1 per cent to minimize pH changes resulting from urate decomposition. The medium was sterilized at pH 7.6 and then separate portions were adjusted to other pH values by addition of sterile solutions of NaOH or HCl. After inoculation (0.1 ml.) the tubes were sealed with rubber stoppers to prevent drying, but no anaerobic seal was used. pH was measured with a glass electrode and ammonia was estimated by distillation at the beginning and after 25 and 96 hours incubation at 37°C.

Table 3 gives the data obtained after 25 hours incubation. It can be seen that most rapid development occurs in media of initial

TABLE 2

Effect of sodium sulfide concentration on growth

Semisolid urate medium in unsealed tubes with 0.1 ml. inoculum

TUBE	$Na_2S \cdot 9H_2O$	GROWTH AS INDICATED BY TURBIDITY			
		26 hours	54 hours	78 hours	100 hours
	per cent				
1	0.000	−	−	−	+++
2	0.001	−	+	++	+++
3	0.006	−	++	+++	+++
4	0.012	+++	+++	+++	+++

pH 7.6 to 8.1, though growth is possible over a considerably wider range. After 96 hours incubation the urate was completely decomposed in all media. In other experiments the lower pH limit for growth was found to be about pH 6.5. Table 3 also shows the pH changes that result from urate decomposition even in a well buffered medium.

Temperature. The influence of temperature on growth and urate decomposition by strain 10a is illustrated in figure 3. The percentage decomposition is based on ammonia determinations by the conductivity method. All points are averages of analyses on three replicate semisolid agar cultures in the usual medium. Very similar results were obtained with strains 3 and 12.

Figure 3 shows that the temperature range of 19° to 37°C. is

suitable for the organism. Undoubtedly it can also grow at lower temperatures than those studied. The optimal temperature evidently lies between 31° and 37°C.

TABLE 3

Effect of pH on growth and decomposition of urate by strain 9a

INITIAL pH	AFTER 25 HOURS INCUBATION		INITIAL pH	AFTER 25 HOURS INCUBATION	
	pH	Per cent decomposition		pH	Per cent decomposition
6.75	6.61	0	7.70	8.15	81
6.99	6.97	32	7.82	8.30	81
7.12	7.15	42	8.15	8.35	96
7.35	7.52	63	9.10	8.71	18
7.60	7.98	85	9.50	9.45	0

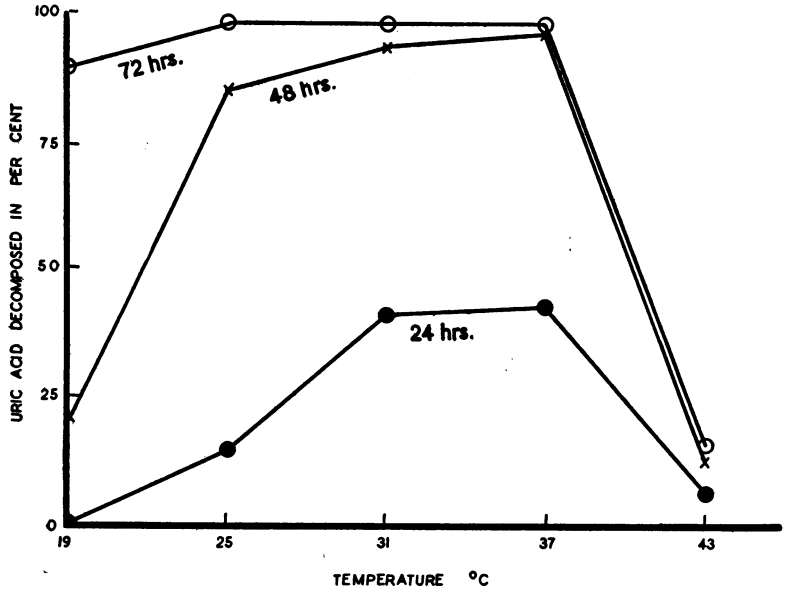

FIG. 3. THE INFLUENCE OF TEMPERATURE ON THE DECOMPOSITION OF URIC ACID BY GROWING CULTURES OF STRAIN 10A

The results of these studies of environmental and nutritional factors have been used in choosing the media and cultural conditions already described in the section on cultural characteristics.

CONDUCTIVITY METHOD OF AMMONIA ANALYSIS

Several of the experiments dealing with the influence of environmental factors on growth and urate decomposition involved large numbers of ammonia determinations. In order to save time, it was decided to replace the ordinary distillation method of ammonia analysis by a somewhat less accurate but far simpler and more rapid method based on the change in electrical conductivity resulting from the conversion of sodium urate to ammonia, carbon dioxide and acetic acid.

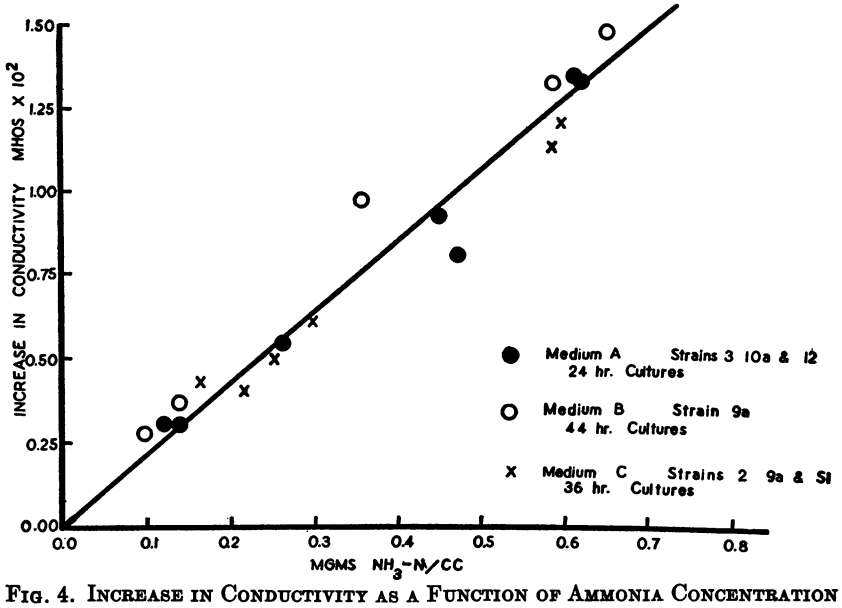

FIG. 4. INCREASE IN CONDUCTIVITY AS A FUNCTION OF AMMONIA CONCENTRATION IN URIC ACID CONTAINING MEDIA FERMENTED BY C. ACIDI-URICI

The conductivity measurements were made at room temperature using a small conductivity cell of about 5 ml. capacity and a wheatstone bridge. Measurements could be made at the rate of 40 to 50 per hour. The method was standardized by comparing the increase in conductivity with the ammonia content of the medium as determined by distillation and titration. Typical results for several media and for several strains of *C. acidi-urici* are given in figure 4. The media differ only in respect to yeast autolyzate (0.1–0.5 vol. per cent) and K_2HPO_4 (0.01–0.2 per

cent). For most subsequent experiments medium A, containing 0.2 per cent uric acid, 0.2 vol. per cent yeast autolyzate, 0.01 per cent K_2HPO_4, 0.005 per cent $MgSO_4 \cdot 7H_2O$, 0.1 per cent agar and 0.01 per cent thioglycollic acid, pH 7.6–7.8, was used.

It can be seen in figure 4 that the change in conductivity is almost directly proportional to the ammonia concentration. The slope of the line drawn through the experimental points is 4.6×10^{-3} mgm NH_3-N/ml./Mho. This value was used to calculate ammonia from conductivity data in subsequent experiments.

SUMMARY

1. The anaerobic decomposition of uric acid and some other purines is brought about by a highly specialized spore-forming bacterium of which a number of strains have been isolated from soil.

2. Since these organisms differ markedly from any hitherto recognized species of *Clostridium*, they are placed in the new species, *Clostridium acidi-urici* (Liebert) Barker and Beck, and *Clostridium cylindrosporum*.

3. The influence of various environmental and nutritional factors upon the growth of these organisms has been investigated and satisfactory culture media are described.

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